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Sullivan, John Michael

PATCH CLAMP STUDY OF A LARGE CONDUCTANCE, INWARD
RECTIFYING, POTASSIUM CHANNEL IN CULTURED MOUSE HIPPOCAMPAL
NEURONS

City University of New York

PH.D. 1987

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by

J. Michael Sullivan

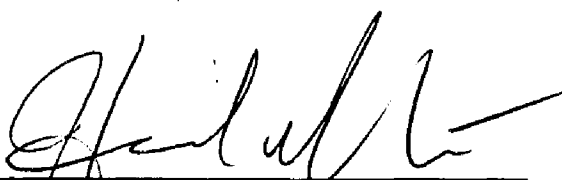
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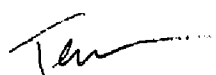
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Abstract

PATCH CLAMP STUDY OF A LARGE CONDUCTANCE, INWARD RECTIFYING, POTASSIUM CHANNEL IN CULTURED MOUSE HIPPOCAMPAL NEURONS

by

J. Michael Sullivan

Adviser: S.A. Cohen, M.D.

Patch clamp methodology was used on primary dissociated cultures of mouse embryonic hippocampus to identify large conductance, inward rectifier (IR), potassium-selective channels. IR exhibits prominent rectification above the reversal potential which changes consistently with the external K^+ concentration (K_o^+). Fast ramp voltage clamps infer that the rectification is an open channel property. Single channel (SC) I-V curves display a $g(V-V_K)$ -dependence with slope conductance dependent on the square root of K_o^+ .

IR has a voltage-dependent probability of opening ($P_v(o)$) around resting membrane potential (RMP) and as $P_v(o)$ is high at RMP, IR may contribute to the free running RMP. Depolarization increases $P_v(o)$ above RMP, while hyperpolarization decreases it (e-fold/34 mV). The decrease in $P_v(o)$ with hyperpolarization occurs largely from a decrease in the burst length (e-fold/44 mV). The requirement of three

exponentials to fit the closed time distribution and one to fit the open times suggests that a minimum of three Markovian closed and one open state(s) are necessary to describe SC kinetics. SC gating behavior is maintained when isolated into 10 nM free calcium (Ca_i^{2+}) but is lost at 1 nM, indicating that IR is voltage- and Ca_i^{2+} -dependent.

Averaged SC currents from voltage ensembles display a voltage- and time-dependent partial deactivation that is faster with increasing hyperpolarization as predicted by steady-state measurements of the voltage-dependence of burst length. Ensemble averages predicted the temporal shape of the whole-cell IR currents. Both cesium and barium block the SC currents by what may be two different mechanisms.

IR serves as a RMP-active conductance. Its potential function in signal transduction is developed by analytical use of the conductance and kinetic data to predict the I-V curve at physiological K_o^+ . This suggests that IR may function to: 1) aid in setting the RMP, 2) aid in cell repolarization after single action potentials (APs), 3) participate in attenuation of burst firing of APs, and 4) participate anomalously in the generation of prolonged depolarizations of membrane potential as K_o^+ accumulates in the perisomal restricted diffusional spaces in the intact hippocampus.

This thesis is dedicated to my father who first motivated my scientific development through his interests in geology, astronomy and science in general as well as for his teaching me that extraterrestrial life should not be ruled out as a possibility.

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LIST OF ABBREVIATIONS

A	Amperes (current)
AHP	afterhyperpolarization potential
AMFD	anomalous mole fraction dependence
4-AP	4-aminopyridine
AP	action potential
ATP	adenosine triphosphate
ATP _i	adenosine triphosphate, intracellular concentration
Ba ²⁺	barium (ion)
Ba _o ²⁺	barium (ion), extracellular concentration
BN	bubble number
Ca ²⁺	calcium (ion)
Ca _i ²⁺	calcium (ion), intracellular concentration
CaK	calcium-dependent potassium (referring to channels)
CAP	cell-attached-patch
Cl ⁻	chloride (ion)
CHN	cultured hippocampal neuron (usually implying mouse)
CNS	central nervous system
Cs ⁺	cesium (ion)
Cs _o ⁺	cesium (ion), extracellular concentration
dB	decibel
DS	dissecting solution
FBS	fetal bovine serum
g	grams
GABA	gamma amino-butyric-acid

GHK	Goldman-Hodgkin-Katz (theory)
G Ω m	gigaohm
g_x	conductance (x is a descriptor, e.g., IR, K ⁺)
G_x	same as for g_x above
H ⁺	hydrogen (ion)
H _O ⁺	hydrogen (ion), extracellular concentration
HH	Hodgkin-Huxley
HS	horse serum
5-HT	serotonin
IOP	inside-out patch
ips	inches per second
IR	inward rectifier (or inward rectification)
I-V	current-voltage (as in the conductance function)
I_x	macroscopic membrane current, isolated ionic type (x)
K ⁺	potassium (ion)
KHz	kilohertz
K _i ⁺	potassium (ion), intracellular concentration
K _O ⁺	potassium (ion), extracellular concentration
l	liter
Li ⁺	lithium (ion)
Li _O ⁺	lithium (ion), extracellular concentration
M Ω m	megaohm
ml	milliliter
mm	millimeter
mM	millimolar
ms	millisecond

mV	millivolt
nA	nanoampere
Na ⁺	sodium (ion)
Na _i ⁺	sodium (ion), intracellular concentration
Na _o ⁺	sodium (ion), extracellular concentration
nM	nanomolar
NRR	negative resistance region
nS	nanoSiemen (10 ⁻⁹ mho)
pA	picoampere
PDS	paroxysmal depolarizing shift
P(o)	probability of opening
pS	picoSiemen (10 ⁻¹² mho)
PTZ	pentylenetetrazole
P _v (o)	probability of opening (voltage-dependence implied)
Rb ⁺	rubidium (ion)
RBC	red blood cell
Rb _o ⁺	rubidium (ion), extracellular concentration
REDS	restricted extracellular diffusional space
RMP	resting membrane potential (mV)
rpm	revolutions per minute
SC	single channel
SQRT	square root
Sr ²⁺	strontium (ion)
Sr _o ²⁺	strontium (ion), extracellular concentration
Tau	time constant (ms)
TEA ⁺	tetraethylammonium (ion)

TEA_i^+	tetraethylammonium (ion), intracellular concentration
TEA_o^+	tetraethylammonium (ion), extracellular concentration
Tl^+	thallous (ion)
Tl_o^+	thallous (ion), extracellular concentration
TTX	tetrodotoxin
ug	microgram
um	micrometer
uM	micromolar
uS	microSiemen (10^{-6} mho)
V	volts
ΔV	displaced patch potential (mV)
ΔV_{rev}	reversal potential (displaced as in CAPs) (mV)
V_K	potassium equilibrium potential (mV)
V_{rev}	reversal potential (mV)
WC	whole-cell
WCR	whole-cell recording

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Chapter I

INTRODUCTION

A. The Study

Recent technological advances in biophysics have been combined with cell culture techniques to allow study of the single molecular elements of ion conduction through biological membranes. In this study the patch clamp technology has been applied to cultured hippocampal neurons (CHNs). In the resting cell membrane, which in these cells is predominantly a potassium (K^+) electrode, an inward rectifying (IR) K^+ channel was identified that has interesting and potentially important conductance (G or g) and kinetic properties. The properties of this channel and its participation in determining the resting potential may have functional implications for neuronal integration and abnormal hyperexcitable states.

B. Hippocampus

The hippocampus is one of the first and certainly the most well studied mammalian cortical area. In the early 1960's, the work of Green and his collaborators (1961a-d, 1964) characterized the morphological aspects of the hippocampus, its afferent and efferent connections, the nature of electrical activity of pyramidal cells and its relationship to the theta rhythm of the EEG as well as seizures in situ. Kandel and Spencer (1961a-c; Spencer and Kandel, 1961a,b) studied the electrophysiological properties of hippocampal pyramidal cells including the nature of cell firing, neuronal membrane time constants, the organization of synapses, the electrical input-output relationships of pyramids, the depolarizing afterpotentials, and states

of repetitive firing. Due to the relative anatomical simplicity (Lorente de No, 1934; Kandel and Spencer, 1961a; Ramon y Cajal, 1909, 1911), the well charted nature of the input-output pathways and synaptic connections (Andersen, 1975; Chronister and White, 1975), the possible involvement in memory or long-term potentiation (Barnes and McNaughton, 1980; Bliss and Lomo, 1973), the low seizure threshold (e.g., Green, 1964; Prince, 1978a, 1982; Purpura, 1964), and the effects of pyramidal cells on local as well as more peripheral neuronal excitability (MacVicar and Dudek, 1980), the hippocampus has received a great deal of attention over the last 25 years.

Because of the lamellar nature of hippocampal architecture, the input-output circuitry is largely unperturbed in thin transverse slices maintained in vitro (Skrede and Westgaard, 1971; Yamamoto, 1972). The slice has provided a powerful tool for current and voltage clamp analysis of pyramidal neurons over the past decade.

Neuronal excitability and the information processing capability of pyramidal neurons is ultimately delegated to the conductive and kinetic properties of voltage-, neurotransmitter-, and ion-sensitive channels and their spatial distributions and densities as they exist in defined geometrical boundaries that guide and impress signal flow. The hippocampal pyramidal neuron has two distinct dendritic fields (basal and apical) which communicate with a soma that initiates firing volleys along its attached axon. As most of the synapses are on the dendrites (Andersen et al., 1980; Brown and Johnston, 1983) they provide a prominent sensor or input station to the neuron. With the axon as the output device, and with its communicative parameter being frequency (of firing), the soma (with dendrites) must serve to communicate with the

output through a voltage to frequency transfer function. The selection, placement, and densities of appropriate ion channels in the somal-dendritic area, according to genetic design, is therefore critical to the information processing capability. The nature of the currents that flow through channel populations will also be profoundly affected by the nature of the boundary conditions which exist on either side of the cell membrane; in the hippocampus the nature of the restricted extracellular diffusional space (REDS) (Green, 1961a, 1964) could have significant effects on currents, ion accumulation, and abnormal firing states (Attwell et al., 1971a,b; Green, 1964; Prince and Schwartzkroin, 1978). Understanding the nature of a pyramidal neuron's ability to process information must depend on detailed knowledge about the nature of its molecular signalling elements, the ion channels.

C. Voltage and Current Clamp Studies of Pyramidal Cells

To understand the nature of excitability in hippocampus, voltage and current clamp techniques were applied to pyramidal neurons in the slice preparation. One important and well studied phenomenon has been the paroxysmal depolarizing shifts (PDS) associated with epileptiform seizure bursts. A number of hypotheses attempting to explain PDS were based on giant synaptic potentials, nonsynaptic active membrane responses arising predominantly in dendrites, as well as ephaptic phenomena (see Prince, 1978) which relates to intercellular communication that occurs through extracellular voltage gradients or accumulations of ions (i.e., K_0^+). The intrinsic bursting behavior of CA3 and inducible bursting in CA1 pyramids resembles the behavior of pyramidal neurons during an epileptic seizure. Voltage and current

clamp analysis was used to illustrate the electroresponsiveness of this phenomenon. Other than the Hodgkin-Huxley (HH) (Hodgkin and Huxley, 1952) fast sodium (Na^+) conductance there is a slow, tetrodotoxin (TTX)-insensitive Na^+ conductance (Dichter, 1983) as well as fast and slow activating calcium (Ca^{2+}) conductances that appear to take significant origin in the dendrites (Ilinas and Hess, 1976; Wong and Prince, 1978; Wong et al., 1979). Johnston et al. (1980), Brown and Griffith (1983), and Wong et al. (1979) have studied these Ca^{2+} currents which appear to arise from two distinct conductances that do not show the Ca^{2+} -induced inactivation found in other preparations (Chad and Eckert, 1984). One of the conductances may be responsible for Ca^{2+} -action potentials, useful in communicating information unattenuated from the peripheral dendritic tree to more proximal locations; this conductance has fast kinetics and is steeply voltage-dependent. The other Ca^{2+} conductance has slow kinetics and a low voltage threshold and may be active over a significant portion of the repolarization phase of a pyramidal cell's firing period. Hotson et al. (1979) have discovered an anomalous (or inward) rectification subthreshold to cell firing that appears to contain Ca^{2+} , Na^+ and probable K^+ components. It is the net behavior of the sum total of conductances operating subthreshold to cell firing that will have a modulating effect on firing frequency, the rate of achieving threshold and, for bursts of activity, the rate at which bursts are terminated.

Related to the above Ca^{2+} conductances is the Ca^{2+} -dependent K^+ (CaK) conductance that is present in pyramidal neurons. This outward rectifying K^+ conductance could be modulated by depolarization-activated Ca^{2+} currents and may act to aid in

repolarization subsequent to single action potentials (AP) (Adams et al., 1982); MacDermott and Weight, 1982) or participate to exert control over the afterhyperpolarizations (AHP) following bursts of APs (Alger and Nicoll, 1980; Brown and Griffith, 1983; Brown et al., 1982; Haas and Konnerth, 1983; Hablitz, 1981a,b; Hotson and Prince, 1980; Nicoll and Alger, 1981; Schwartzkroin and Stafstrom, 1980). This conductance has received significant attention over the last few years since it exerts a hyperpolarizing influence in the presence of a depolarizing process and may be involved in regulating the burst termination in a bursting neuron (e.g., CA3). More than one CaK conductance may participate in these phenomena. Brown and Griffith (1983) and Lancaster and Adams (1986) have voltage clamped conductances that are activated by membrane depolarization, require the presence of external Ca^{2+} (Ca_o^{2+}) and patent Ca^{2+} conductors, and have extremely slow activation kinetics, which are on the order of hundreds of milliseconds (ms). At least a portion of this conductance is blocked by norepinephrine or histamine, which appear to block inward Ca^{2+} currents (Haas and Konnerth, 1983). Wong and his collaborators (Clark and Wong, 1983; Numann et al., 1982) used the patch clamp-whole cell recording (WCR) technique to study this effect on acutely dissociated pyramidal-shaped neurons and found single channel (SC) conductances on the order of 100-110 picosiemens (pS) (Numann et al., 1982) with channels activated by intracellular Ca^{2+} (Ca_i^{2+}).

A recent model for epileptogenesis by Traub and Llinas (1979) places a significant portion of the PDS activity in the dendritic region and proposes that CaK conductances can exert dramatic attenuation on this epileptogenic process. Nevertheless, blockade of

g_{K-Ca} with intracellular EGTA injection shows that during depolarizing current injection (to stimulate a PDS) damped oscillogenic voltage phenomenon still occur and appear to be the origin of burst spikes (Hablitz, 1981b). The biophysical nature of this membrane potential oscillator could have important functional implications for a bursting system and an epileptogenic neuron. Whisler and Johnston (1978) have proposed a model for the PDS in an epileptogenic neuron in which three conductances participate to position a negative resistance region (NRR) into a stable (crossing of voltage axis with positive slope) or unstable region of the I-V plot. The conductances which might provide for a NRR below threshold are of importance to the problem of epileptogenesis; IR is an essential component to their model.

Halliwel and Adams (1982) have established the presence of two additional conductances in hippocampal pyramidal neurons that are active around resting membrane potential (RMP) but below AP firing threshold. One is a voltage-sensitive noninactivating K^+ conductance activated in the subthreshold range of membrane potential that is blocked by muscarinic agonists (or external barium (Ba^{2+})) and is under direct presynaptic control. It has been called the M conductance and has been found in other neuronal preparations (Brown et al., 1980, 1982; Brown and Adams, 1980, Brown and Constanti, 1980). The M conductance is partially active at resting potential, and when activated by depolarization exerts a strong steady-state outward rectifying influence on the neuron. Blockade of M currents with muscarinic agonists or Ba^{2+} results in linear current-voltage (I-V) plots, removes the hyperpolarizing braking action

below threshold and permits both membrane potential oscillations and repetitive firing. I_M , then, is a synaptically (acetylcholine) modulated conductance that can tune up or down the firing frequency of a neuron. It may have pacemaker modulatory effects on hippocampal neurons. A similar conductance sensitive to muscarinic agonists is found active around RMP in heart cells (nodal and ventricular) and automaticity can be induced in ventricular muscle (normally not automatic) with Ba_O^{2+} (Hiraoka et al., 1980). In hippocampal pyramidal or sympathetic neurons the currents are rather small (1 nanoamperes (nA)) possibly indicating a low copy number of the channel in the membrane or a low outward conductance; SC M currents have been recorded recently and exhibit inward rectification (IR), at least in heart muscle (Sakmann et al., 1983).

The other conductance identified in hippocampus has been called g_Q by Halliwell and Adams (1982). It appears to be a relatively nonselective K^+/Na^+ conductance that is hyperpolarization activated at potentials below -70 mV with slow monoexponential kinetics. I_Q currents have a steady-state IR property apparently because the channels close with membrane depolarization into subthreshold potential ranges; however, the I-V behavior at depolarized potentials larger than reversal potential (V_{rev}) was not adequately studied by Halliwell and Adams (1982). I_Q is blocked by external cesium (Cs_O^+) but not by Ba_O^{2+} and is at least partially active at the RMP. It is dissimilar to IR conductances in other preparations such as egg cells or skeletal muscle but is similar to the I_{h-f-K_2} pacemaker current system in heart (see Discussion). Its functional implication for excitability in hippocampus is, however, not yet clear. A recent

study (Madison et al., 1986) has identified a hyperpolarization activated slow current in the hippocampal slice that shows remarkable similarity to I_Q . However, the current is carried by chloride (Cl^-) rather than Na^+ or K^+ and may be the reason why I_Q has been shown to be anion-modulated (Segal and Barker, 1984; Mayer and Westbrook, 1983).

In addition to the inward (or anomalous) rectification found by Halliwell and Adams (1982) and earlier by Purpura et al. (1967, 1968) in hippocampal pyramidal cells, there exists another type of IR subthreshold to cell firing as shown by Hotson et al. (1979). This process is blocked by manganese or TTX and, therefore, apparently involves a Ca^{2+} and/or Na^+ conductance. Moreover the IR, defined as a slope resistance increase with depolarizing current, is enhanced by Ba_O^{2+} , which readily permeates Ca^{2+} channels and has inhibitory influences on K^+ conductances. In the presence of Ba_O^{2+} the membrane potential is oscillogenic and gives rise to bursts of repetitive discharges. Burst discharges can also be induced by other means of blocking K^+ conductances, (e.g., tetraethylammonium (TEA^+) ions) or by increasing external K^+ (K_O^+), which should shift the K^+ equilibrium potential (V_K) and possibly modulate g_K (Schwartzkroin and Prince, 1980). It appears that a K^+ conductance(s) participates in this IR in hippocampal neurons since when efforts are taken to block or shift the potential dependence of K^+ conductances, burst generation can arise (Hotson, 1982; Hotson and Prince, 1981). Such burst generation dependence on K^+ and Ca^{2+} balance is well noted in molluscan systems (Meech and Standen, 1975; Meech, 1978; Gorman et al., 1981; Gorman and Thomas, 1980).

Penicillin, a convulsant agent which does not affect IR, AHP, or Ca^{2+} spikes in hippocampus, nevertheless can induce bursting epileptogenesis presumably by decreasing inhibitory GABA-activated Cl^- conductance (Hotson, 1982). However, disinhibition of pyramidal neurons is not sufficient for bursting behavior since granule cells of hippocampus neither possess an IR nor Ca^{2+} spikes and cannot generate bursting behavior in the presence or absence of penicillin.

To summarize, at least two inward or anomalous rectifications exist in hippocampal neurons, one at deep hyperpolarized potentials (Purpura et al., 1967, 1968) and another subthreshold to firing potential (Hotson, et al., 1979). These conductances could have significant roles in setting or stabilizing the behavior of the resting membrane of the hippocampal pyramidal neuron. IR conductances in other preparations have been shown to be a major component of the steady-state I-V curve below firing threshold although their role remains unclear in the dynamic behavior of the neuron. This study was designed to probe the electrical activity of the resting membrane of the hippocampal pyramidal cells at a cellular and macromolecular level. It depends critically on two major technological advances over the past decade: primary dissociated cell culture of higher central nervous system (CNS) neurons and the patch clamp technology. We should briefly discuss these advances.

D. New Methodology

In the 1970's it became possible to maintain low density neuronal cultures of the CNS. Banker and Cowan (1977) succeeded in growing primary dissociated embryonic (16-19 days) rat hippocampus in vitro for several weeks. This neuronal population is relatively enriched for

pyramidal cells which are postmitotic at the time of dissection (Altman and Baylor, 1975; Angevine, 1964, 1975). Those neurons which survive in culture are very similar in shape and dendritic distribution to pyramidal neurons in the intact preparation, and claims have been made that cultured neurons even be distinguished on the basis of morphologic similarity to pyramidal neurons from hippocampal CA1 or CA3 regions (Banker and Cowan, 1979). According to biochemical (Seifert et al., 1983; Walker and Peacock, 1982), developmental (Bartlett and Banker, 1984a,b; Rothman and Cowan, 1981; Seifert et al., 1983), immunological (Schaffner and Moskal, 1984; Schaffner and Schnarr, 1983), and electrophysiological (Peacock, 1970; Peacock and Walker, 1983; Segal, 1983; Segal et al., 1984; Segal and Barker, 1984a,b) criteria, pyramidal neurons in culture are very similar to cells in the intact tissue. Neurons in culture are a relatively simplified and analytic system and are free of the constraints, for example, of a REDS, thus allowing much greater control over membrane solution boundary conditions than is possible in the slice preparation. Peacock et al. (1979) also succeeded in culturing primary mouse dissociated hippocampus which maintains the same functional properties (Peacock, 1979; Peacock and Walker, 1983). Perhaps the most important property of cultured neurons for the technique used in this study is that the surfaces of most cells are very clean (see e.g., Banker and Cowan, 1979). This property of the cell surface is critical to the success of the patch clamp methodology.

Ever since the invention of the voltage clamp by Marmont and Cole (1949), experimental control of voltage over extensive membrane surfaces became readily possible in cells with ideal uniform geometries

like, for example, the squid giant axon (Cole, 1961; Taylor et al., 1961). As not all systems have such idealized geometries there was incentive to develop methods for voltage clamping cell membrane regions with nonideal spatial geometries. The original version of the patch clamp developed by Neher and Lux (1969) and Frank and Tauc (1963) borrowed extensively from Strickholm's work (1961) and allowed recording of currents flowing through small membrane patches in molluscan neurons under independent voltage clamp. As the method is extracellular, and the amplifier is a virtual ground current-to-voltage converter, changes in pipette potential are seen as changes in potential across the patch of membrane under the glass pipette. The early patch voltage clamp operated to clamp over approximately a 100 micrometers (μm)² element of membrane. A criterion for use of the method is the formation of a high resistance seal between glass and cell surface such that the pipette is effectively isolated from the bath electrically. Use of the method allowed study of fast activating K^+ currents in molluscan systems (Neher, 1971) and a modification of the method was employed by Fishman (1975) to do noise analysis on small patches of membrane. Statistical estimation of SC properties derived from noise analysis (e.g., Neher and Stevens, 1977) as well as direct measurement of channels in artificial bilayers (Boheim, 1974; Busath and Szabo, 1981; Ehrenstein and Lecar, 1977) suggested that biological channels had pS conductances that underwent binary gating transitions. Each open channel should carry, therefore, picoampere (pA) order currents for millivolt (mV) driving forces. Subpicoampere noise electronics had recently become commercially available and was incorporated into the previous large patch circuitry in the mid 1970's.

Neher and Sakmann in 1976 were then able to record acetylcholine-activated ion channels using fire polished patch clamp pipettes sealed to tens of megaohm (M Ω) resistances onto enzymatically cleaned tissue cultured muscle (Neher and Sakmann, 1976). The discrete conductance fluctuations were the first observation of single biological channel function in situ. An analysis of the noise sources came in a later paper which showed that the seal resistance of pipette to membrane was a major factor in obtaining the essential low noise conditions (Neher et al., 1978). The renaissance of the patch clamp method came when Neher (1982) and Sigworth and colleagues (Hamill et al., 1981) developed methods to promote gigaohm (G Ω) seals of patch pipettes to clean cell surfaces. This order of seal resistance, isolating the patch and the channels under it both electrically and mechanically from the bath and the remainder of the cell membrane, allowed the first functional analysis of SC macromolecules. With this condition one can control transpatch voltage with a single external electrode. The G Ω seal technique has revolutionized the field of electrophysiology and neurobiology. The method has illustrated that there are many more types of channels than previously expected from classical macroscopic voltage clamp methods. The conductance and kinetic detail that comes from the technique have provided rapid strides in understanding how channels function in biological systems.

The patch clamp technique has only recently been applied to study the electrophysiological properties of the CNS neurons when used in combination with primary dissociated cell culture techniques to obtain isolated neurons with clean surfaces. A mating of primary cell culture

techniques with patch clamping has made feasible preparations which were heretofore inaccessible to adequate voltage clamp analysis (e.g., hippocampal neurons or primary dissociated heart cells). Probably one of the key contemporary issues in these studies is the discovery of new types of channels (e.g., a large conductance IR Na^+ -dependent K^+ channel in cardiac muscle (Kameyama et al., 1984)) that lend credence to the genetic diversity available to particular cells for the design of membranes capable of information transfer in a particular repertoire of codes and response capabilities. For example, a diversity of new types of IR channels are now becoming apparent through the advance of the above techniques (Mayer and Westbrook, 1983; Latorre et al., 1984; Sakmann et al., 1984; Reuter, 1984; Kameyama et al., 1984).

Using cell culture methods and conventional voltage clamp analysis a number of studies have illustrated that cultured neurons do provide advantages that may be difficult to obtain in the intact preparations. Segal (1983), Segal and Barker (1984a,b) and Segal et al. (1984), have, for example, recently used cultured rat hippocampal neurons to study agonist- and voltage-activated ionic conductances. As the electronic, synaptic, and firing behavior of these neurons are similar to the intact preparations, many if not most of the electrophysiological properties are maintained across cell dissociation and culture (Kandel and Spencer, 1961a; Schwartzkroin, 1975, 1977). Segal and his colleagues were able to illustrate that CHNs have the fast HH Na^+ conductance that is blocked by TTX. GABA-activated chloride (Cl^-) conductances and acetylcholine-activated cation conductances are also present. Recent results in our laboratory have shown that mouse CHNs have macroscopic GABA-activated Cl^- conductances that can be isolated

to the SC level (Sullivan and Cohen, unpublished observations).

However, a major focus of Segal and Barker's (1984b) recent work has been the K^+ conductances present in rat CHNs.

Segal et al. (1984) have found the I_A conductance in CHNs to be a depolarization-activated K^+ conductance that inactivates over time and is sensitive to block by 4-aminopyridine (4-AP). This conductance, which is found in other CNS neurons (e.g., Gustafsson et al., 1982, in hippocampal CA3 neurons), was originally discovered by Connor and Stevens (1971a,b) and Neher (1971) in snail neurons. The familiar TEA^+ -sensitive delayed rectifier current is present, has a substantial conductance in this preparation, and shows partial inactivation. Ca^{2+} -dependent K^+ currents were also found (Segal and Barker, unpublished observations) but have not yet been characterized. The I_Q current of Halliwell and Adams (1982) was repeated in CHNs and displays similar properties to that in the slice preparation, but in addition manifests an anion sensitivity. Muscarinic K^+ currents are only rarely found in CHNs. A conductance which was not developed in the Segal and Barker (1984b) study provides a transient inward current when activated by hyperpolarization and appears to be K^+ -selective; this current may result from the same conductance that is the focus of this study, that being an IR, large conductance, K^+ -selective channel.

E. Perspective

With knowledge from previous voltage clamp studies on macroscopic conductances in hippocampal neurons one was better able to apply the SC recording techniques to study the CHNs as an established system. This study began with the observation of IR I-V curves obtained from

large conductance channels found active in the resting membrane of mouse CHNs (Sullivan and Cohen, 1985). This IR channel has kinetics and conductance properties best studied at hyperpolarized potentials where other conductances are fortuitously silent. As mentioned before, IR conductances make substantial contributions to I-V relationships around RMP. IR has been studied in other preparations which serve as a basis of comparison to this work.

Katz (1949) originally described a conductance mechanism in K_2SO_4 -depolarized muscle which exhibited anomalous or, as it is currently called, IR. This nomenclature refers to the ability of a conductance pathway (i.e., channel) to pass inward current more readily than outward current. This particular conductance property of skeletal muscle is predominantly K^+ -selective. Its chord conductance is larger on hyperpolarization than on depolarization (hence IR), and moreover the conductance is not strictly voltage-dependent but also is influenced by the level of K_o^+ (a $g(V-V_K)$ -dependence) (Hagiwara et al., 1974; Hestrin, 1981; Leech and Stanfield, 1981; Ohmori, 1980; Stanfield et al., 1981). The K_o^+ dependence suggested that there are modulatory sites (K^+ receptors) accessible from the outside of the channel which affect the conductance or gating process (Ciani et al., 1978). Since Katz's discovery, this conductance process has been found in a variety of both excitable and nonexcitable preparations and is typically active at resting potentials and at more hyperpolarized potentials (Hille, 1984; Kandel and Tauc, 1966; Nelson and Frank, 1967; Sauve et al., 1983). Of the more well studied preparations a conductance mechanism similar to that reported herein was found in the

egg cells of starfish and tunicates during work pioneered by Hagiwara and his collaborators (Hagiwara et al., 1974).

Another "fingerprint" of the conductance mechanism in egg cells (or muscle) is shown by replacing K_O^+ with isotonic thallous ion (Tl_O^+). Tl_O^+ is more permeable than K^+ in the egg IR channel and provides a steeper conductance. However, when K_O^+ is only partially replaced with Tl_O^+ the macroscopic conductance goes through a minimum over a particular range of mole fractions of Tl^+ to K^+ (Hagiwara et al., 1977). This represents the so called anomalous mole fraction dependence (AMFD), a peculiarity of IR macroscopic conductances which provides an indication that channel permeation by a single ion is not independent of other permeant ions (Eisenman et al., 1977). IR channels from other preparations typically violate the independence principle: either more than one ion can be in the channel at one time (a long pore) or the effective permeation of one ion is influenced by the boundary condition of ion activities at the pore mouths (Neher et al., 1978b).

A multitude of studies on IR in egg cells and skeletal muscle now furnish data on permeation characteristics of other monovalent and divalent ions (e.g., rubidium (Rb^+), Na^+ , lithium (Li^+), strontium (Sr^{2+}), Ba^{2+} , Cs^+ , hydrogen (H^+), Ca^{2+}) (Hagiwara et al., 1976, 1978; Hagiwara and Yoshu, 1979; Standen et al., 1980; Standen and Stanfield, 1978a,b, 1979). Some ions which can pass (e.g., Rb^+) also have blocking effects, whereas other ions which do not pass (Cs^+ , Ba^{2+}) affect channel permeation by way of open channel block or alterations in the voltage sensitivity of gating, respectively. Ionic modulation appears critical for this channel and reports

have appeared showing effects of H^+ , Na^+ , and Ca^{2+} on the conductance process or its kinetics.

A number of studies have appeared recently concerning the nature of IR conductances in mammalian neurons, including sympathetic, thalamic, cerebellar Purkinje, olfactory cortex and hippocampal neurons. The conductance mechanisms also appear in rod photoreceptor cells, vestibular hair cells, horizontal cells, other "sensory" neurons, basophilic leukemia cells, retinal cells, pancreatic islet cells, macrophages, fibroblasts, heart muscle, nodal tissue and other preparations. Most of these studies were qualitative descriptive reports (see Discussion) of the macroscopic or SC conductance properties of IRs in the respective preparations. Thus IR appears to be an important process in many cell types. A number of hypotheses have been put forward to place IRs into an excitability context. Kandel and Tauc (1966) and Frank and Nelson (1967), working on IR in molluscan systems, proposed that it acted to short circuit the soma during hyperpolarizing voltage excursions so that synaptic transmitter release would not have been driven out of a stimulus proportional range. A similar hypothesis was later proposed by Fain and Lisman (1981) for rod photoreceptors. In egg cells, IR has been suggested to provide for prolonged depolarizations which might be associated with fertilization potentials (Hille, 1984). IRs may also contribute significantly to leakage conductances in many systems (Sakmann and Trube, 1984a,b). Finally, IRs have been thought to take a role in subthreshold excitability and membrane potential oscillations and pacemaker currents (Hotson et al., 1979). In this sense, as discussed above, IRs may play a role in hyperexcitable states such as epilepsy.

A potentially new way to view IRs will be presented in the Discussion. This study will then attempt to relate the conductance and kinetic properties of an IR channel found in CHNs to the context of normal and abnormal neuronal excitability.

Chapter II

MATERIALS AND METHODS

A. Culture of Embryonic Mouse Hippocampal Neurons

Swiss Webster mice (Buckberg Laboratory Animals, Tompkins Grove, NY) that were 14 to 16 days pregnant, were asphyxiated with CO₂ and doused in ethanol-soaked sponges for 20 minutes. The uteri were then surgically and aseptically removed and transferred to a sterile 100 mm diameter Falcon tissue culture dish (model 3001, B-D Corporation, Cockeysville, MD) filled with cold dissecting solution (DS). DS consisted of Eagle's minimal essential media (MEM, #410-1700, GIBCO Laboratories, Grand Island, NY) supplemented with 6 grams (g)/liter (l) dextrose, 2 nanomolar (nM) L-glutamine (GIBCO 320-5030), and 15 millimolar (mM) HEPES (Eastman Kodak, Rochester, NY) (all final concentrations). Each mouse, on average, contained 14 embryos. The brain was removed from each embryo and the hippocampi were subsequently dissected and cultured according to the procedure of Peacock et al. (1979). Twelve hippocampi were pooled in cold DS and then transferred with a sterile Pasteur pipette into a sterile centrifuge tube containing 2 milliliters (ml) of 0.1 milligrams (mg)/ml trypsin (Sigma, St. Louis, MO) in Earles balanced salt solution (GIBCO 310-4010) and incubated for 20 minutes at 35.5°C under 5% CO₂ (Incubator # 1820, VCR, South Plainfield, NJ). The tube was centrifuged at 2300 revolutions per minute (rpm) for three minutes in a tabletop centrifuge (Triac centrifuge, Clay Adams, B-D Corporation, Parsippany, NJ) to sediment the tissue. The supernatant was poured off and the tissue pellet immediately resuspended in a feeding media

consisting of MEM supplemented with 5% (v/v) fetal bovine serum (FBS) (GIBCO, #230-6140), 5% (v/v) horse serum (HS) (GIBCO, #230-6050), 2 mM L-glutamine, and 6 g/l dextrose. The suspension was transferred to a 35 millimeter (mm) Falcon dish and was triturated four times with a 25-gauge needle on a 5-ml syringe. Glass coverslips (18-mm circles, Carolina Biological), that were coated with collagen or poly-L-lysine, were placed inside 35-mm diameter Falcon culture dishes. Prior to plating, a bubble of feeding media (0.4 ml) was placed on each coverslip and a drop of cell suspension was added to the bubble using a sterile serological pipette. About 1/4-1/2 hippocampus was plated per dish. The cultures were incubated overnight in the 5% (v/v) CO₂, 35°C incubator, and the next day either 1.5 ml of feeding media was added to the dish or four of the coverslips were transferred to 60-mm culture dishes (Falcon #3002) in which embryonic mouse whole brain cultures minus hippocampus had been grown over the previous week (7-9 days old) (Seifert et al., 1983). These whole brain cultures had been plated at a density of one brain per dish and were grown in a media of MEM supplemented with 6 g/l dextrose, 2 mM glutamine, 2% (v/v) HS, and 20% (v/v) FBS. At the time the coverslips were transferred, the 20% FBS-containing media was removed from the whole brain cultures and was replaced by 3 ml of a serum-free, chemically-defined medium (Bottenstein, 1983; Bottenstein et al., 1979; Seifert et al., 1983) consisting of Eagles MEM supplemented with 2 mM L-glutamine, 6 g/l dextrose, 5 micrograms (ug)/ml insulin, 100 ug/ml human transferrin, 30 nM sodium selenite, 0.3 nM triiodothyronine, 20 nM hydrocortisone, and 20 nM progesterone. All cultures were grown under 5% CO₂ at 35°C, and media was changed weekly for both serum-containing and serum-free

cultures. All hormones and supplements except L-glutamine (GIBCO) and dextrose (Fisher Scientific, Fairlawn, NJ) were purchased from Sigma (St. Louis, MO). Examples of cultures grown in serum and chemically defined media are presented in Figure MM-1. Cells selected for electrophysiological analysis had pyramidal shaped somas and apical and basilar dendrites. Examples are provided in Figure MM-2. All cultures were initiated and maintained by the technical personnel in the laboratory.

B. Preparation of Coverslips for Cell Culture

Coverslips (Carolina Biological) were initially prepared by soaking in concentrated nitric acid for 12 hours, then thoroughly rinsed with tap and then deionized water, autoclaved, and finally placed in sterile 35-mm Falcon culture dishes.

1. Preparation of Collagen-Coated Coverslips (Bornstein, 1985)

Rat tails were amputated from sacrificed animals and stored in 70% ethanol for at least two weeks at -10°C to sterilize. To prepare 100 ml of liquid collagen suspension, three tails were thoroughly dissected for their collagen fibers by mechanically twisting with hemostats to remove the surrounding tissue and leaving the collagen fibers exposed. The fibers were aseptically cut off and stored in sterile, distilled, deionized water until the dissection was complete. They were then transferred to a sterile bottle containing a stirring bar and 50 ml of sterile 0.1 M acetic acid. The contents of the bottle were stirred overnight in the cold at 4°C and decanted, and an additional 50 ml of 0.1 M acetic acid was added to dissolve the collagen thoroughly. The collagen suspension was then transferred into sterile 30 ml Nalgene centrifuge tubes and centrifuged for one hour at

Figure MM-1. Neurons Grown in Serum and Chemically-Defined Media.

Primary dissociated mouse embryo hippocampus was grown in serum-containing or chemically-defined media. In serum-containing cultures, growth was relatively heavy with a background of nonneuronal cells of a variety of types. In chemically-defined media, cultures grown on polylysine are well maintained at low density with very little undergrowth. Cells at the center of each photograph illustrate characteristic morphologies of cells used for this study.



Chemically-Defined, Poly-lysine



Serum-Collagen

Figure MM-1. Neurons Grown in Serum and Chemically-Defined Media

Figure MM-2. Examples of Pyramidal Shaped Neurons.

This four-panel collage shows representatives of the types of cellular morphology used to select neurons for electrophysiological analysis. The panels (a) and (b) show pyramidal shaped neurons that have one apical and two prominent basilar dendrites. Both cells would fit into the Major-Process-Dominated class of Banker and Cowan (1979), which apparently originate from CA1 and CA3. In panels (c) and (d) a pyramidal neuron was internally dialyzed with a WCR pipette filled with Lucifer yellow (3.2 mg/100 μ l H_2O plus 1.4 M LiCl). Panel (c) is a Nomarski image before injection of Lucifer yellow. The approaching pipette was popped into the cell after GOhm sealing for about five minutes of intracellular dialysis and then gently removed to reseal the membrane. In (d) the fluorescent microscopic distribution of Lucifer yellow in the cell in (c) is seen after five to ten minutes. Note the local distribution of the dendritic arbor. The measurement bar is approximately 12 μ m long.

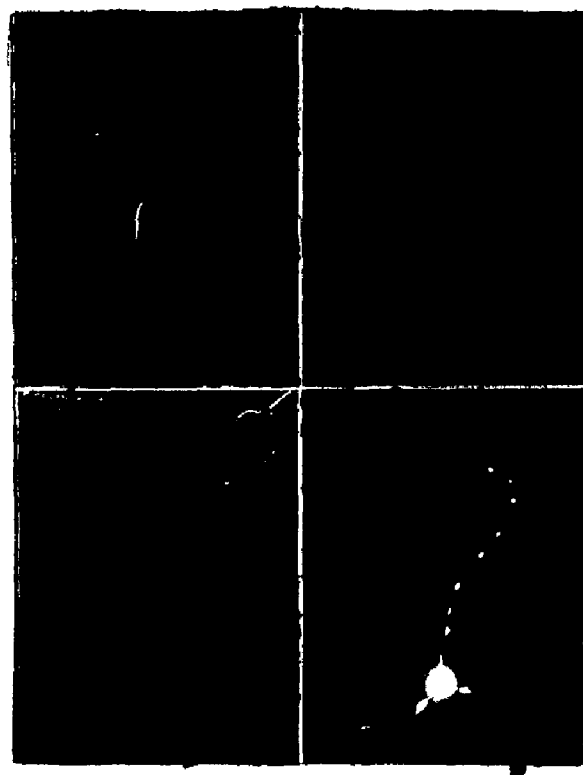


Figure MM-2. Examples of Pyramidal Shaped Neurons

10,000 rpm in a Beckman JA-20 rotor. The supernatant was removed and dialyzed overnight in a sterile dialysis apparatus in batches of 150 to 200 ml. To polymerize the collagen 85 micromolar (μM) riboflavin (in water) was added to the collagen solution 1 part to 5 under subdued lighting. Fifty microliters (μl) of the mixture was then spread over each coverslip in 35 mm Falcon dishes. The dishes were then exposed to fluorescent white light from two inches for 12 minutes to polymerize the collagen and were subsequently stored in the culture incubator until used. Sometimes the collagen-coated coverslips were stored under the ultraviolet light in the Baker Edgegard laminar flow hood (Baker Company, Sanford, ME) overnight.

2. Preparation of Poly-L-lysine-Coated Coverslips

To prepare poly-L-lysine coated coverslips (Letourneau, 1975; Yavin and Yavin, 1974), coverslips pretreated in the same manner as for collagen-coated coverslips were soaked in sterile poly-L-lysine solution (20 $\mu\text{g/ml}$ in water) for 30 minutes, allowed to dry, and then transferred to empty, sterile 35 mm Falcon culture dishes until use.

C. Solution Fabrication

Solutions were fabricated from stocks that were made with reagent grade chemicals (Sigma, St. Louis, MO; Aldrich Chemical Company, Milwaukee, WI; Baker Chemical, Phillipsburg, NJ, my home town) and double deionized water in polymethylpropylene volumetric flasks and were filter sterilized (0.22 μm , Nalgene Type S filters, Nalgene Company, Rochester, NY) and typically stored at 4°C. From these stocks appropriate volumes were mixed to formulate the solutions of choice listed in Table I. These experimental solutions, also prepared from double-deionized or double-deionized-distilled water, were filter

Table I
SOLUTION COMPOSITION (in mM)^a

Solution	Tris HCl (pH 7.2)	NaCl	KCl	KF	CaCl ₂	MgCl ₂	Hepes-KOH (pH 7.2)	EGTA-KOH (pH 7.2)	Glucose	Sorbitol
Bath										
Extracellular A	0	145	5.6 (1.4)	-	1.0	0.8	4.0	-	5.6	-
Extracellular B	137	0	5.6 (1.4)	-	1.0	1.0	2.0	-	-	50
<u>WCR Solutions</u>										
Intracellular ^b	-	-	-	140	1.0	2.0	10.0	11.0	-	-
Extracellular										
1) 10 K ⁺	-	145	10	-	0.1	1.0	4.0	-	5.6	-
2) 30 K ⁺	-	125	30	-	0.1	1.0	4.0	-	5.6	-
3) 75 K ⁺	-	80	75	-	0.1	1.0	4.0	-	5.6	-
4) 140 K ⁺	-	15	140	-	0.1	1.0	4.0	-	5.6	-
<u>CAP Solutions</u>										
140 K (pCa8)	-	-	140	-	0.135	1.0	4.0	2.2	-	-
140 K (pCa3)	-	-	145.6	-	1.0	0.1	4.0	-	-	-
75 K (pCa8)	-	-	75	-	0.135	1.0	4.0	2.2	-	-
75 K (pCa3)	-	-	75	-	1.0	1.0	4.0	-	-	-
30 K (pCa8)	-	-	30	-	0.135	1.0	4.0	2.2	-	-
30 K (pCa3)	-	-	30	-	1.0	1.0	4.0	-	-	-
300 K (pCa8)	-	-	300	-	0.135	1.0	4.0	2.2	-	-
300 K (pCa3)	-	-	300	-	1.0	1.0	4.0	-	-	-

^aSolutions brought to final pH = 7.2 using concentrated NaOH, KOH or HCl which resulted in ~ 1 mM changes in K⁺, Na⁺, or Cl⁻. pCa8 and other Ca²⁺-EGTA solutions were formulated using the Henderson-Hasselbach equation and a calculated pK_{Ca-EGTA} = 6.815 (Martell and Smith, 1974; Dresdner, R.P., personal communication).

^bDeCoursey et al., 1984.

sterilized prior to use and stored in glass bottles or in small volumes in pipette filling syringes at 4°C. The WCR solution used here is that of DeCoursey et al. (1984) and low Ca^{2+} -EGTA solutions were fabricated using a pK_{Ca} (dissociation constant) of 6.815 which was adjusted for pH (7.2), temperature (22°C) and ionic strengths (0.15 M) (K.P. Dresdner, personal communication, 1983; Dresdner and Kline, 1985; R.Y. Tsien, personal communication, 1983; Tsien and Rink, 1980).

D. Experimental Preparation

Cultures grown from three days to three weeks in poly-L-lysine- or collagen-coated coverslips were examined visually on a Nikon (Diaphot) (Nikon Inc., Garden City, NY) inverted phase contrast microscope at 200 X. Only those generously populated by characteristic pyramidally shaped neurons were studied. Cultures containing neurons judged suitable for patch clamping on the basis of low background non-neuronal cell overgrowth were removed from media dishes and placed into small 35 mm Falcon dishes containing solutions A or B (Table I); IR was found when cells were bathed in either of the two. Coverslip cultures were bathed under 2-3 mm of the above solutions for about 3-5 minutes with intermittent agitation and then transferred to two other dishes sequentially containing the same solution. This procedure proved necessary to obtain stable high GΩ seals, perhaps due to cell surface adherent soluble material that is diluted by sequential washings. Coverslips were then introduced into a specially designed chamber on the compound inverted microscope equipped with Nomarski interference contrast optics; the fluid in the chamber (about 2 mm) was typically the same solution in which the cells were washed.

Solution changes were generated in one of two ways: (1) manual changes with a Pasteur pipette under a constant aspiration sink using a chamber drain connected to a peristaltic pump (Model #7553-10, 1-100 rpm, Cole Parmer Instrument Co., Chicago, IL) or (2) fire-polished blunt-tipped pipettes (5-50 μ m) containing the solution of choice and under positive pressure from a hypodermic syringe. Neither method was entirely satisfactory: isolated patches usually broke during manual solution changes and the pipette perfusion method was often complicated with clogged lines and difficulty in alignment of perfusion pipette flow with cellular or patch targets. When drugs were added they were: (1) added before placing the patch pipette onto the cell or (2) added in concentrated small volumes through a microliter pipette during cell-attached-patch (CAP) or WCR or (3) added by the perfusion pipette method. All preparations of cultures for recording and all experiments were conducted at room temperature (20-22°C).

E. Patch Clamp Pipette Fabrication

Patch or WCR pipettes were produced in batches from two stock glasses: (1) Rochester Scientific #5068 (Rochester, NY), which is a product of Drummond glass (Broomall, PA) and may contain a Kimble soft glass (R6), a Kimble borosilicate (N51A) or a Corning borosilicate (7800); and (2) specialty glass Corning 7052 (Kovar sealing glass), a borosilicate originally suggested by Dr. James L. Rae (Rush Presbyterian Medical Center, Chicago IL., personal communication), which has excellent sealing properties to CHNs and other preparations (Rae and Levis, 1984a,b). The 7052 Corning was obtained from Garner Glass Company (Claremont, CA) and was the most frequently used glass in these experiments. An interesting difference in the compositions of

Corning 7800 and Corning 7052 is the concentration of barium oxide (0.5% vs. 2.5%); high concentrations of divalent ions in patch pipettes aid in sealing in many preparations and, perhaps, the incorporation of Ba_2^{2+} into the glass structure provides a localized charged vehicle to aid sealing where membrane and glass oppose. Rochester Scientific Glass 5068 shows batch dependence in its ability to form seals in different laboratories. As most of 5068 is N51A (Kimble) or Corning 7800, which are functionally equivalent glasses, it is interesting that recently the concentration of barium oxide was reduced in these glasses due to its leaching on storage into pharmaceutical solutions.

Glasses (O.D. 1.65 mm, I.D. 1.20 mm) were pulled by gravity into pipettes in two stages according to the Hamill et al. (1981) recipe on a Kopf 700 C microelectrode puller (David Kopf Instruments, Tujunga, CA). The filament was shaped according to Corey and Stevens (1983) and contained five turns with an outer diameter of 5 mm. The initial pull, under a constant current of 16.5 Amperes (A), was 9 or 10 mm to thin a region of the pipette to about 300 μm . The pipette was then recentered so that the center of the thinned region was in line with the top of the filament. The second pull, at 9.5 to 10.5 A, resulted in two approximately symmetric pipettes with geometries similar to those obtained at other laboratories. Steep conical tapers near the tip were more easily generated with Boralex glass than with 7052 Corning, but there is some dispute that the Hamill et al. (1981) geometry (Sakmann and Neher, 1983) is the best one for the most stable seals (James Rae, personal communication). Pipettes from both glasses had outer diameters of about 2-3 μm and internal diameters of about

1.0-2.0 μm prior to polishing (Figure MM-3). Pipettes were stored in glass jars (Frederick Haer and Company, Brunswick, ME) between manipulations or use.

Patch pipettes were then coated with Sylgard[®] elastomer 184 (Dow Corning, Midland, MI) to reduce pipette capacitance and make the pipette surface hydrophobic to reduce noise (Corey and Stevens, 1983; Hamill et al., 1981). A 9:1 ratio (w/w) of resin to curing agent was used that had been partially cured at room temperature in small plastic beakers for 3-4 hours and then stored at -20°C ; new batches of Sylgard[®] were prepared about once every other week or as needed. Pipettes were coated to within 100 μm of the tip under a dissecting stereo microscope (Olympus, Tokyo, Japan) while held in a curing jig fashioned after Corey and Stevens (1983) with the filament driven at 3 Volts (V) and 8 A (DC) (24 Watts) (Model D612T filtered DC power supply, Epsco Inc., Addison, IL). Coating and curing was performed according to Hamill et al. (1981) and Corey and Stevens (1983) except that pipettes were tilted with tips up about 20° to aid seal formation by preventing creeping of the curing agent toward the tip. Usually two to three passes through the filament were necessary to completely cure the Sylgard[®]. Pipettes were then stored in dust-free glass jars until fire polished. Patch pipettes were fire polished by a controlled current source driving a platinum wire (0.01 inches in diameter, A.M. Systems, Seattle, WA) loop that was shaped into an approximately 120° circular arc. A glass ball (7052 Corning, 300-500 μm in diameter) was formed at the midpoint of the arc. The ball was present: (1) to prevent platinum from boiling off the filament onto pipettes and preventing sealing and (2) to generate a spherically

Figure MM-3 Production of Patch Clamp Electrodes

Shown are various stages in the fabrication of patch clamp pipettes from Corning 7052 and Rochester Scientific Boralex 5068 glasses. Panel (a) illustrates raw pipettes at low magnification to illustrate tip geometry. Note the more tapered conical appearance of Boralex pipettes. Panel (b) illustrates the raw pipettes in (a) at higher magnification to show the tapers to the tip. Panel (c) illustrates the same pipettes after fire polishing to a bubble number of 3.5; inset shows tip geometry at even higher magnification. Note more blunt conical tip geometry with Boralex. Panel (d) shows Sylgard[®]-coated pipettes; (Δ) illustrates the location of the edge of coating (<100 μm from tip). Panel (e) shows a coated fire-polished Boralex pipette at low magnification to view the visible regions of the pipette. Symbols in the upper left corner of each panel indicate appropriate use of the μm ruler at the bottom of the Figure.

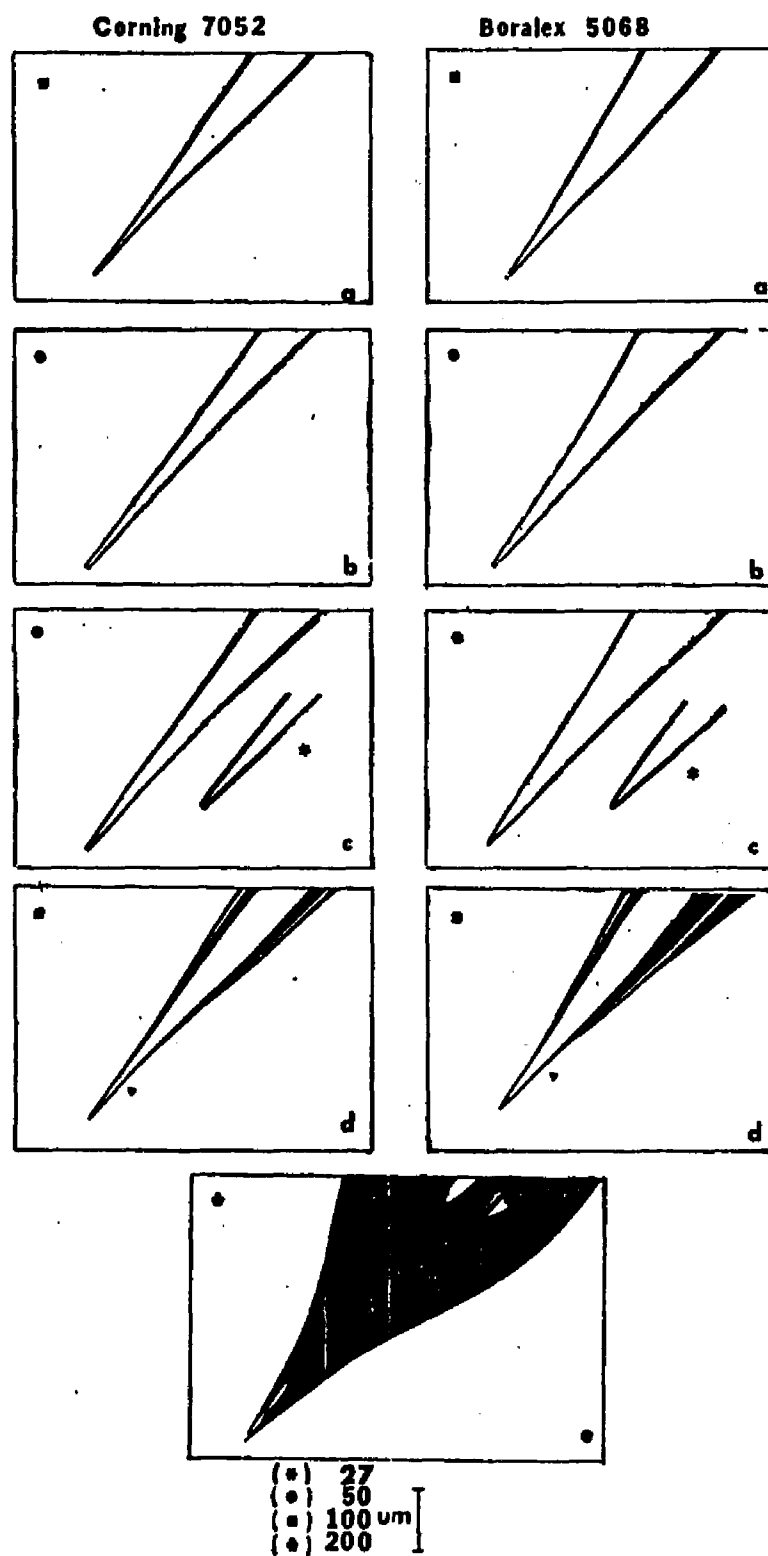


Figure MM-3. Production of Patch Clamp Electrodes

distributed heat source which allowed for symmetrical fire polishing at the rim of the pipette tip. The glass ball was changed periodically whenever sealing problems arose and empirical measures were taken to reestablish the method. The constant current source was built using a Variac[®] source (Power Stat, 3 PN 116 B, Superior Electrical Company, Bristol, CT) to drive a 6.3 V CT, 4 A (rms) filament stepdown transformer (Stancor P8648, Logansport, IN). One output of the transformer was routed in series to an ammeter (Simpson 2152 0-3 AC, Elgin, IL) and then to one side of the filament support; the other transformer output was routed directly to the opposite filament support. Constant current was maintained at about 2 A by adjusting the wiper on the Variac[®]. The filament support was mounted onto a Leitz micromanipulator positioned adjacent to a Nikon compound inverted microscope (Nikon Inc., Garden City, NY) set up for 200 X Nomarski (Plan 20 DIC lens). Pipettes were fire polished under direct visual observation by placing them into a holding jig mounted to the stage which repeatably swung the pipette into the viewing area. The filament was then brought up to the pipette under gross and fine control. No air currents were used during fire polishing. Fire polishing continued for a few seconds until the proper (visually familiar) appearance was generated with the tip 5-20 μm from the filament. WCR pipettes were not fire polished and generally were not coated with Sylgard[®].

Pipettes were tested for proper fire polishing by measuring their bubble numbers (BN) (Corey and Stevens, 1983). A six-inch piece of PE 200 tubing (Becton Dickinson Company, Parsippany, NJ) was attached to the back of the pipette and to a #16-gauge hypodermic needle mounted on a 10 ml syringe that was filled with air to the 10 ml mark. To

obtain the BN the pipette was placed under positive pressure into a small transilluminated vial of high pressure liquid chromatography grade methanol (Fisher Scientific, Fairlawn, NJ). Positive pressure on the syringe continued until threshold, when a bubble stream first appeared at the pipette tip vicinity; the BN was the number (in mls) read directly off the syringe at the point when the bubbles first appeared. From the Laplace equation: $P = 2T/r$, where T is the surface tension of air in methanol (constant), P is the applied pressure (from the syringe) and r is the radius of the smallest bubble (at threshold); r should be equivalent to the limiting radius of curvature of the pipette inner diameter and therefore a measure of the extent of polishing. Fire polishing of pipette batches was continued until BNs of 3.0 to 3.5 were obtained. Some pipettes were stored under vacuum until use, as one effect of fire polishing may be to remove hydration water from the glass. Under vacuum fire-polished pipettes could be used on the day of fabrication and with reasonably good success on the following day. Examples of production, fire polishing, and Sylgard[®] coating for 5068 and 7052 pipettes is illustrated in Figure MM-3.

Pipettes were filled just prior to use with the solutions listed in Table I by on-syringe filtering with a 0.22- μ m pore filter (Gelman, #4192, Ann Arbor, MI) connected to fine (about 200 μ m O.D.) home-made polystyrene needles. All solutions had been filter sterilized (0.22 μ m Nalgene type S filter) twice before use in the filling syringes. Pipette tips were filled by suction on immersion in the solution of choice in a 1 ml plastic beaker and backfilled with the polystyrene needle to an 8-10 mm height of fluid to just cover the silver-silver chloride electrode; air bubbles were removed by gentle tapping.

F. Electrophysiological Setup

Cells were viewed at 400 X on a Nikon (Diaphot) inverted microscope equipped with Nomarski optics (Plan 40 DIC lens) and mounted on an air table (Vibraplane, Model 1201-01-12, Kinetic Systems, Inc., Boston, MA) surrounded by an aluminum screen Faraday cage. Leitz micro-manipulators were used to position recording and perfusion pipettes. A Dagan 8900 Patch-Whole cell clamp (Dagan, Inc., Minneapolis, MN) was used as the recording instrument. A WPI (1830/1831 series, WPI, New Haven, CT) pulse generator was used to provide +5 V signals to a pulse voltage divider box (WPI-1100a) to set command potentials for the voltage clamp. A function generator (Wavetek, Model 185, San Diego, CA) was used to generate voltage ramps; its output was connected directly to the command port of the Dagan or routed to a voltage divider network prior to being applied to the Dagan. All experimental signals were monitored continuously on a Tektronix 5113 dual beam storage oscilloscope (Tektronix, Beaverton, OR) and stored on FM tape.

The Dagan 8900 patch clamp is an adapted version of an original circuit design by Dr. D.P. Corey. The circuit incorporates an ultra high impedance (100 M Ω m or 10 G Ω m), ultra low noise pA-to-mV converter which is housed in the headstage box and mounted on a Leitz manipulator in the Faraday cage. The Teflon or Lucite pipette holders were the design of E. W. Wright (Model PC-S3, Guildford, CT) and plugged directly into the headstage box Teflon BNC (UG 625 B/U or UG 1094/U). The high M Ω m feedback resistors (1%) in the headstage were Kobra resistors (K01243 series, K & M Electronics, West Springfield, MA), and the composite amplifier used a selected dual JFET U401 (Siliconix, Santa Clara, CA) with 3 milliamperes per side and an NE5534A.

differential (Signetics Corp., Sunnyvale, CA). The resistor shield and the headstage box were driven to the command potential to minimize capacitive currents. The headstage command voltage divider was $1/10$ and RC limited to 0.1 microseconds (us). The main circuit box contained the following sequential stages: (1) differential amplifier, (2) high frequency boost, (2a) feedback path: headstage gain compensation, series resistance compensation, autozero, capacitance compensation network (3 time constant (Tau), 0-5 us, 0-2 ms, 1-10 ms), current clamp facility, voltage command stage, (3) gain stage, (4) filter (2 pole 18 decibel (dB)/decade); also includes timing clock and liquid crystal display to monitor signals. The circuit was modified to allow for multiple Tau corrections in the 60Ω feedback resistors as well as to provide clock time pulses for external synchronization and a resistor ladder on the gain switch to indicate the selected gain to external output devices. A multiturn cermet pot was also included to more sensitively balance the offset on the gain amplifier. Generally the filter on the Dagan was kept in the wide band position and the output of the amplifier was connected to a unity-gain, eight-pole Bessel filter (Frequency Devices, Model LPF 902B, Haverhill, MA) that was run on nickel-cadmium batteries to remove line noise. The filter was generally set at 1-2.5 kilohertz (kHz) for patch clamping and 1 kHz for WCR and was zeroed with respect to Dagan and the tape recorder. The output of the filter was connected to a four-track Vetter Model B FM tape recorder (A.R. Vetter Company, Rebersburg, PA). The current outputs and voltage commands from the Dagan occupied two channels of the recorder designed to respond to fast transient signals (Boards FM BT-5, Vetter Company). The gain signal was routed to a third channel

and the fourth channel on the recorder was reserved for voice. The speed on the tape recorder was set at either 3-3/4 inches per second (ips) or more typically at 7-1/2 ips, which provided 3dB-down cutoffs of 1.125 kHz and 2.25 kHz, respectively; the Vetter apparently has a Chebychev-type transfer function (personal communication, Ed Nelson, Vetter representative).

The connecting electrodes were silver/silver chloride junctions that were renewed frequently. The junction in the pipette holder was protected with a piece of polyethylene tubing (PE 50, Becton Dickinson Company, Parsippany, NJ) over most of its length; the ground electrode was inserted directly into the recording chamber through a wire connected to the insulated ground screw on the headstage box.

The pipette holders had a side port for positive or negative pressure application to the pipette fluid column during experimental setup. The port was attached air tight to a 1 meter piece of flexible plastic tubing.

G. Experimental Procedure and Data Acquisition

The filled pipette was lowered into the bath under constant positive pressure and positioned over a cell selected for study on the basis of morphologic criteria. The cell was first photographed on black and white film (TRI-X, 400 ASA) using a Nikon 35 mm camera. The autosearch button was iteratively pressed to cancel junction and pipette potentials while a clock pulse potential was driven into the pipette to monitor the resistance of the electrode. For the solutions listed in Table I and the ENs used, the pipettes had resistances typically of 5-15 M Ω m. The pipette was then lowered onto the cell surface as monitored visually by a dimpling of the somal membrane as

well as a decrease in the current flowing through the pipette (higher resistance, 20-50 M Ω). Slight suction (< 10 ml H₂O) was then applied by mouth through the flexible plastic tube attached airtight to the pipette holder. Often the resistance in the seal abruptly increased as measured with the test pulse, and the current noise decreased dramatically to well below 1 pA (0.2-0.4 pA at about 2 kHz). This was indicative of formation of a G Ω m seal which ranged from 1 to greater than 30 G Ω m. In some experiments seals formed only after some time with or without followup suction, while in others, seals formed on contact of glass to the cell surface. Success of sealing to clean cells was about 60-80%. As the biophysical nature of the seal is not known (e.g., the interacting forces, the distances, the selectivities to ions) it is not clear why seals can form slowly or abruptly. Its abrupt nature in most patches may indicate that it is the result of a highly cooperative process between glass and the membrane surfaces that may involve the loss of water from the bonding region (see Hamill et al., 1981, for references). Throughout pulling of suction and formation of G Ω m seals, the offset correction button was pressed to allow continuous tracking of 0 mV potential so that there was minimal background current after seals were formed. Only seals greater than 1 G Ω m were useful for experimental analysis using voltage jumps or changes in holding potential. Once a pipette had been in contact with a cell surface it was not possible to obtain further seals and the pipette was discarded. Generally, 20-40 pipettes were used during a full working day.

Once a seal was obtained, the capacitative currents in response to the square voltage pulses through the pipette and seal were nulled using the three Tau correction circuit following the procedure of Corey (Dagan 8900 Manual notes). If CAP clamp recording was being performed, then the experimental setup was complete. If WCR was to be used (100 MOhm Resistor in the Headstage), then after the capacitative currents were nulled on the square-wave correction potential and the holding potential set in the pipettes (to calculated V_K), a brief pulse of suction was applied to rupture the membrane. Rupture was indicated by the appearance of a large capacitative current reflecting the whole-cell (WC) membrane capacitance. Series resistance control was then turned on and adjusted to correct the relaxation to as near an impulse function as possible with the loop gain (dynamic) increased to be just below oscillation. The experiment then proceeded.

If an IR channel was identified in the resting membrane, holding potentials (DC) or command potentials were then used to examine the voltage sensitivities and conductance while the output was stored on tape at 1.12 or 2.25 kHz. Experiments were terminated whenever (1) the seal failed, (2) patches became very noisy or steeplechase channels were seen and interpreted as seal breakdown (Hamill et al., 1981; Sachs, 1983; Sachs and Auerbach, 1983), (3) sudden changes in magnitudes of currents or the DC current occurred, possibly indicative of changes in membrane potential, or (4) the cell appeared visually swollen or compromised.

Patches or whole cells typically were held from about 5 minutes to over an hour. On those preparations in which inside-out patch (IOP) manipulations were performed, the pipette was simply pulled back from

the cell surface. The IOP mode was the most unstable preparation-i.e., the seals were readily lost upon isolation or the current magnitudes became rounded, apparently due to the formation of a vessicle at the tip; however, on passage through the air-solution interface to break apparent vessicles, seals were usually lost. This may indicate that other processes may operate (e.g., changes in the properties of the cytoplasmic bleb or gelling) to change the nature of the impedance of patches in such isolations.

H. Data Analysis

Data from the tape recorder were typically played back at slower speed to three devices: (1) the storage oscilloscope, (2) a Gould two-channel chart recorder (Model 220, Gould, Inc., Saddle Brook, NJ)--band-limited at about 125 Hz, or (3) a twelve-channel A-D converter sampling at 4 X Nyquist frequency to write data onto digital magnetic tape (Data General System, Dr. Fred Sachs' Laboratory, SUNY, Buffalo, NY). The oscilloscope was used directly in the storage mode to measure the amplitude of SC currents as well as to observe and record substates or channel block. Specific data of interest on substates or channel block were photographed directly from the oscilloscope screen. WC current amplitudes and time courses were played back onto the chart recorder for analysis usually at 1/4 to 1/2 real time speed (250-500 Hz) to bring the tape recorder near to the band limit of the chart recorder. For SC kinetics the data were played back at 1/8 speed (about 300 Hz) onto the chart recorder; this would not severely attenuate the channel events slower than 1-2 msec in duration and manual kinetic analysis was done with this in consideration. For some experiments open, closed, and burst duration

times were measured manually and stored in tables prior to constructing histograms.

Voltage-dependent probability of opening ($P_v(o)$) curves were generated in one of three ways: (1) analog filtering at 1 Hz and sample averaging the mean currents in ratio to the actual SC currents seen on the oscilloscope, (2) neglecting fast events (< 2 ms) and summing the total duration of bursts over the total of open and closed times, or (3) using the automated analysis (IPROC) routine of Sachs et al. (1982).

Ensemble analysis was done by playing the currents back into a Tracor signal averager (Model TN-1505, Tracor Northern, Middleton, WI) which was triggered by the tape-stored voltage command pulses. The averager was set for 9-bit resolution with 2 ms per binwidth. The stored output was played back onto the chart recorder or photographed directly from the storage scope of the instrument.

An automated analysis routine, IPROC, written by Fred Sachs, Jim Neil, and N. Barkakati (Sachs et al., 1982) at SUNY-Buffalo, was used on location to analyze data from four patches with the analog files digitized on their Data General Eclipse System mentioned above. Parameter files were set up to adjust windows on the channel amplitudes as well as the burst kinetics. From these parameter files the program accessed the data and analyzed the files automatically for SC current amplitudes, probability of opening $P(o)$, data stationarity, open- and closed-time histograms, system noise properties, substates, etc. The routine uses a pattern recognition heuristic algorithm that continually updates the estimates of channel amplitudes and baseline. The results of each file were played out onto a Tektronix Versatek printer.

Exponential histograms (density-adjusted binwidth) and Gaussian functions were fit using the International Mathematical Statistical Library routines incorporated into a program called FITS written by Jim Neil. Another program, also written by Jim Neil, called ALLOFF was used to do multiple exponential fittings of closed-time histograms. Taus fit from the exponentials were used in a program called 4 STATE, which tests for the ability of data to fit a four-state sequential reaction scheme ($C \rightleftharpoons C \rightleftharpoons C \rightleftharpoons O$) and gives the rate constants if the data converges in the fit.

Statistical Analysis System (SAS) (SAS Institute, Cary, NC) was accessed on the City University of New York IBM mainframe computer and used for linear (PROC REG) and nonlinear (PROC NLIN-Marquardt) least-squares fitting of functions to certain elements of the data set.

Chapter III

RESULTS

A. Conductance Properties of IR1. Typical IR Currents Seen in Cell-Attached Patches

Figure R-1 shows typical inward currents seen upon G Ω m sealing to the cell soma with a high K $_o^+$ concentration (75 mM) in the pipette. That these channels are not an artifact of sealing is supported by the observation that in some patches inward channel currents were seen upon pressing the pipette to the cell prior to seal formation and during formation of G Ω m seals. Under all CAP conditions, the displacement steady-state holding voltage from RMP (-52 mV, n = 8, K $_o^+$ = 5.3 mM) will be given (e.g., V = -30 mV = 30 mV hyperpolarized from RMP) since absolute transpatch voltage was not obtained after a CAP, as it was not technically feasible to gain WC access using fire-polished 7052 Corning pipettes. At 0 mV (resting potential) the channel is mostly open and shows grouping of events into bursts of openings separated by longer closed periods. Hyperpolarization of the patch (-18, -32, -51, -67 mV) progressively increases the magnitude of the open-channel current but progressively decreases the average open time. Depolarization from 0 mV promotes smaller inward currents until in the approach to the reversal potential (20-30 mV from V $_{rev}$) the proportion of current to voltage begins to decrease. The average amount of time the channel is open increases with depolarization, however. The channel then exhibits voltage-sensitive gating with the P $_v$ (o) increasing over a voltage range where the conductance is decreasing and vice versa.

Figure R-1. Example of Soma IR in CHNs

The array reports the activity of an IR channel found under CAP conditions with 75 mM KCl in the pipette. The channel was active upon sealing to the cell, and its conductive and kinetic properties were examined by changes in pipette potential. Potentials listed are voltage displacements of transpatch potential relative to the RMP. Note the substates observed at potentials below RMP ($\Delta V = 0$ mV). Cell was bathed in Solution A. ΔV_{rev} was about +32 mV, and conductance, about 50 pS.

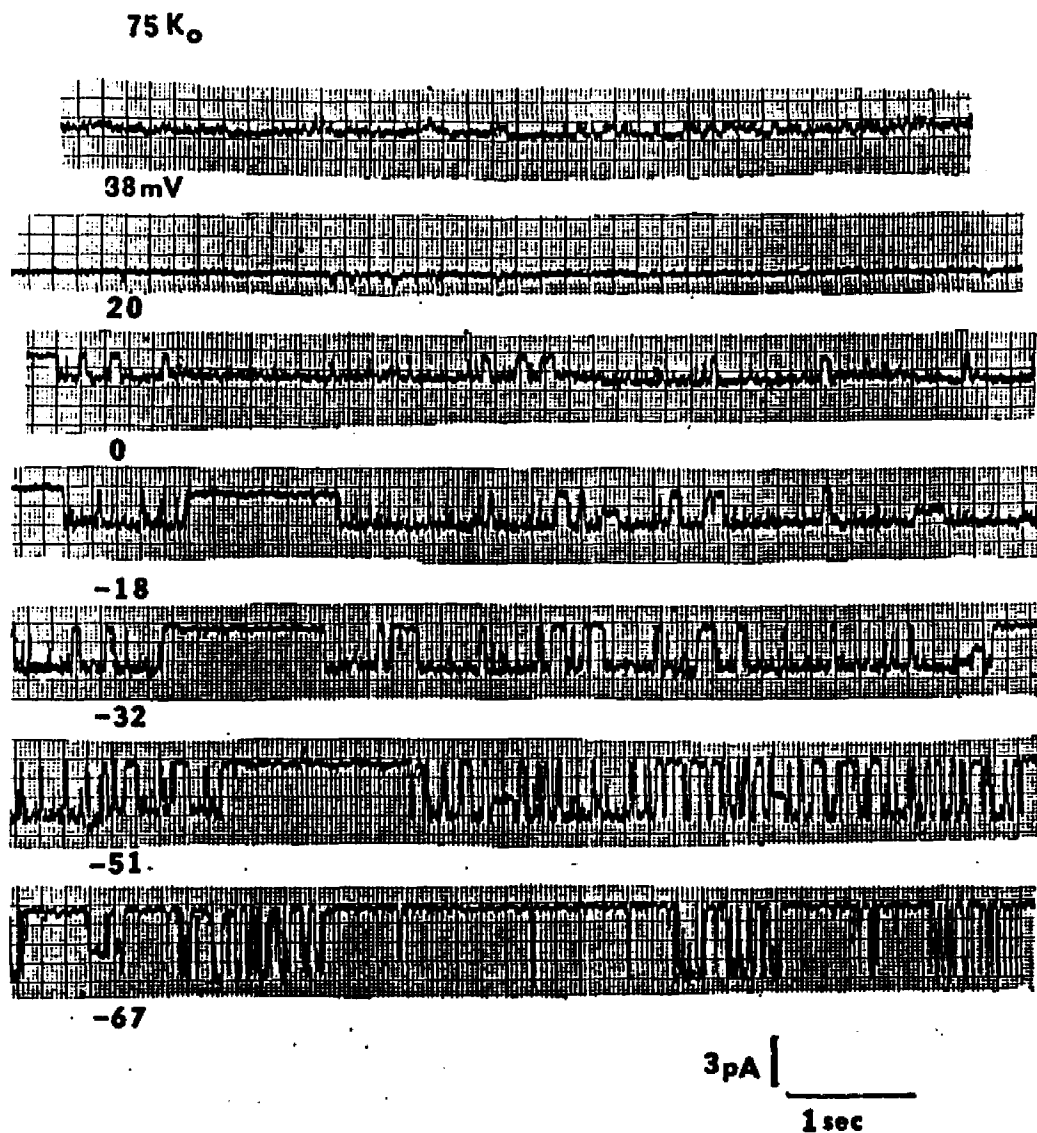


Figure R-1. Example of Somal IR in CHNs

The behavior of the channel at potentials more depolarized than the apparent ΔV_{rev} is peculiar. In many patches no outward current was seen, whereas in others outward current was consistently small with progressive depolarization such that the outward conductance was smaller than the attenuation of inward conductance below ΔV_{rev} . It is assumed that small outward currents originated from the same channel seen below ΔV_{rev} , although there is no proof for this assumption. In some patches progressive depolarization activated outward conducting channel currents with distinctly different conductive and kinetic behavior that result from other channel types present in the patch.

2. Steady-State I-V Curves for Single Channels at Different K_o^+

The conductance decreases dramatically by depolarizing patches above ΔV_{rev} with high K_o^+ indicating that the channels observed in the resting membrane had an IR characteristic. Inward (or anomalous) rectification refers to a decrease in the conductance with depolarization or, conversely, an increase in conductance with hyperpolarization. This property was initially seen with symmetric K^+ in the pipette at 140 mM so it was of interest to observe behavior at a variety of K_o^+ concentrations. Figure R-2 shows the I-V curves at four K_o^+ concentrations: 30, 75, 140 and 300 mM. These curves represent data from 43 experiments, and regression lines were fit to each data set below $\Delta V = 0$ mV. The SC currents showed similar behavior with or without Na^+ added to replace some K_o^+ or when citrate or nitrate replaced chloride. At all K_o^+ concentrations tested the SC I-V characteristic exhibits IR, but the degree of rectification appears to decrease with K_o^+ from 300 to 30 mM.

Figure R-2. IR SC I-V Curves with Different K_0^+ s

(a) Steady-state I-V curves were generated with data from 43 CAPs with different concentrations of K_0^+ in the pipette. Currents were measured by playback onto a storage oscilloscope. Potentials indicate displacement of transpatch voltage. The different experimental conditions were: (not illustrated) 5.3 mM K_0^+ ($n = 1$), (●) 30 mM K_0^+ ($n = 2$), (*) 75 K_0^+ ($n = 12$), (■) 140 K_0^+ ($n = 24$), (▲) 300 K_0^+ ($n = 4$) were the K_0^+ concentrations used.

(b) Linear regression equations were fit to the data points at potentials hyperpolarized from $\Delta V = 0$ to obtain estimates for the limiting slope conductances at each K_0^+ . These equations are:

$$5.3 \quad K_0^+: I = 0.02269 \Delta V - 0.639, \quad g = 22.69 \text{ pS}$$

$$30 \quad K_0^+: I = 0.05116 \Delta V - 1.481, \quad g = 51.16 \text{ pS}$$

$$75 \quad K_0^+: I = 0.07671 \Delta V - 2.286, \quad g = 76.71 \text{ pS}$$

$$140 \quad K_0^+: I = 0.12354 \Delta V - 5.755, \quad g = 123.54 \text{ pS}$$

$$300 \quad K_0^+: I = 0.21668 \Delta V - 8.176, \quad g = 216.68 \text{ pS}$$

All fits were significant at the $p < 0.05$ level.

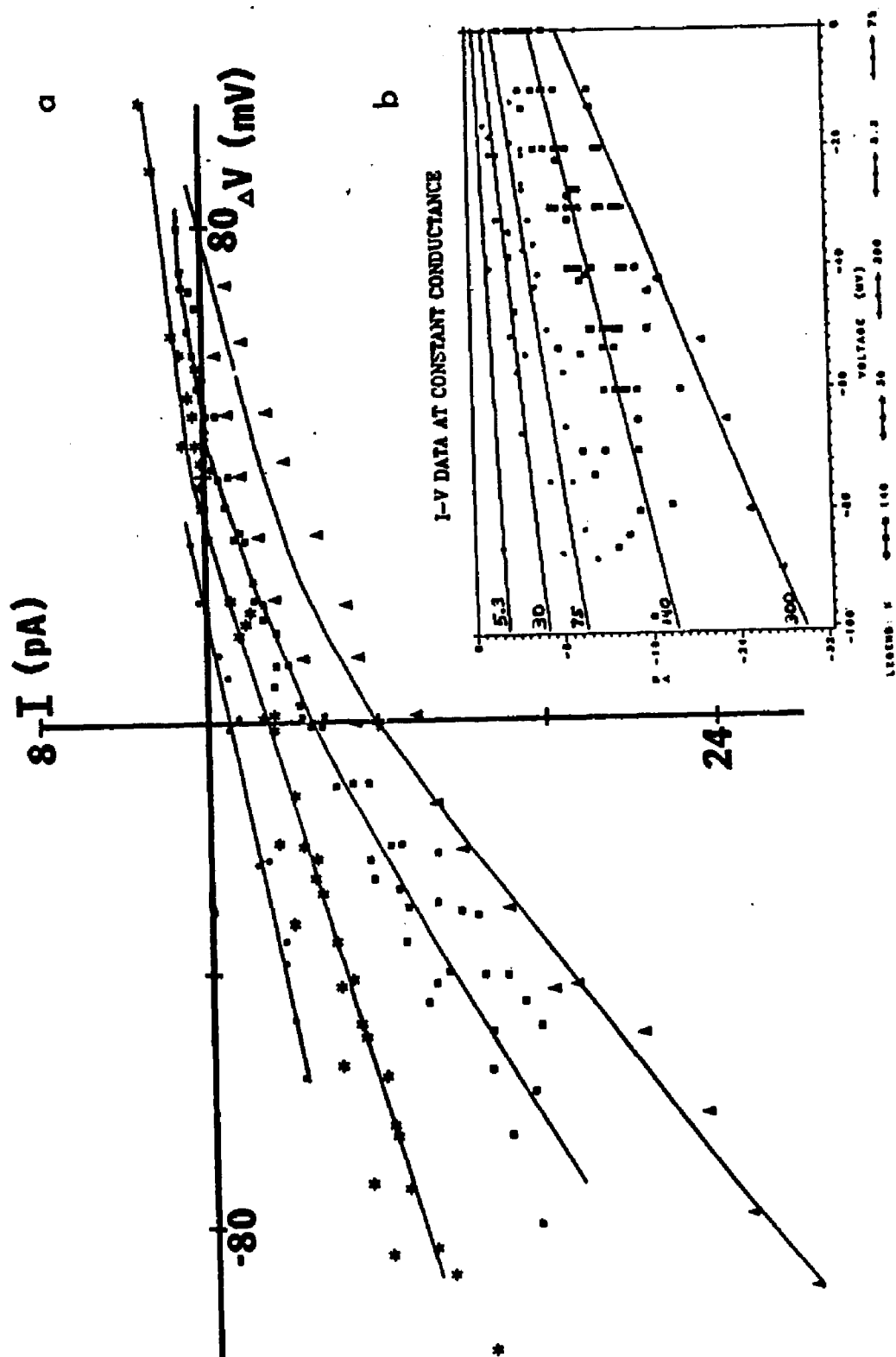


Figure R-2. IR SC I-V Curves with Different K_o^+ s

The conductance of IR, estimated from the steepest slope of the I-V curves (i.e., the constant limiting slope conductance at hyperpolarized potentials), shows a positive dependence on K_o^+ . As illustrated in Figure R-2b, fitted conductances ranged from 23 to 217 pS as K_o^+ increased from 5.3 to 300 mM. With the increase in conductance, the reversal potential, ΔV_{rev} , shifted towards depolarized values as K_o^+ was increased in the pipette. To summarize, the resting somal membrane IR channel has voltage-sensitive kinetics and a conductance that is dependent on both the voltage and V_K , the K^+ equilibrium potential (through K_o^+). Such a $g(V-V_K)$ dependence is a characteristic of IR conductances found in other excitable cell types (Hagiwara 1979; Hagiwara and Takahashi, 1974; Leech and Stanfield, 1981). A number of models have been presented to explain IR behavior and will be reviewed in the Discussion.

Potential problems arising from the use of CAPs to study the effects of voltage and K_o^+ on channel conductance will be argued now. One potential problem is the unknown absolute magnitude of the voltage and the K^+ gradients across the patches. Since on-cell patches are the most physiologically relevant method to study channels active in the resting membrane (Sakmann and Neher, 1984), this mode of analysis for characterization of resting membrane channels was used throughout. However, when IOPs were pulled off into 140 mM K^+ solutions, the current magnitudes were very similar when voltages were imposed to match those estimated in the CAPs. This result agrees with the assumption that intracellular K^+ (K_i^+) was about 140 mM, consistent with evidence from other excitable cells (Galvan et al.,

1984). The mean RMP of these CHNs (-52 mV, $N = 8$, S.A. Cohen, unpublished observation) was similar to pyramidal neurons in the intact hippocampus (Kandel and Spencer, 1961a), pyramidal neurons from acutely dissociated hippocampus (Clark and Wong, 1983; Gray et al., 1982), or CHNs in other laboratories (Peacock, 1979; Segal, 1983). IR is thus consistent with a channel active in a hippocampal pyramidal neuron at normal resting voltage. A similar channel was recently identified in acutely dissociated hippocampal pyramidal cells (Wong and Clark, 1983; Huguenard and Alger, 1985). It has been generally accepted with the patch clamp methodology that patch membrane bleb effects on voltage or ion gradients, access resistances, or diffusion limitations are minimal (Sachs and Auerbach, 1983; Sakmann and Neher, 1984).

Another potential problem in characterizing the conductance of a channel with an IR I-V characteristic and high $P_V(0)$ at depolarized potentials is that open-closed fluctuations may not be recognized at these potentials. This could give the mistaken appearance at depolarized potentials that no outward channel current was flowing except patch leakage current, thus generating an IR characteristic. Nevertheless, random process fluctuations to the closed state, even if brief, should be observed at potentials more depolarized than ΔV_{rev} if sampled for statistically sufficient time periods. The ability to detect fluctuations, however, would be dependent on the rate at which the potential changes. If voltage was changed slowly to greater than ΔV_{rev} , slow corresponding changes in $(P_V(0))$ might appear to obscure the identification of fluctuations, whereas rapid changes (of duration on the order of single open periods) to depolarized voltages

across an open channel would show the development of any outward current as the potential parameter ranges from low to high $P_V(o)$.

Fast ramp voltage clamps provide a powerful tool to resolve the previous dilemma. Ramp voltage clamps have been used in many preparations to analyze the macroscopic impedance properties of biological membranes (Fishman, 1970; Palti and Adelman, 1969). Since the rate of change of voltage is controlled and is not instantaneous, the frequency band of any time- (and voltage-) dependent process can be effectively tuned into operation. This property is not possible from step or impulse driving functions which have a white noise characteristic, where the driving signals contain all frequencies. Thus, conductances can be separated on the basis of their kinetics and voltage dependence by changing the rate and magnitude of ramp voltage clamps. This unique value of a time-dependent voltage clamp can also be exploited on the SC level (Yellen, 1982). Perhaps one of the most useful features of this method is that I-V curves can be generated very rapidly, in fact ideally in two ramps (see below).

Shown in Figure R-3 are the results of a typical ramp voltage clamp experiment with 75 mM K_o^+ , which would provide an outward gradient for K^+ . The I-V curve was determined first using slow variation of the holding potential. Then from a holding potential of $\Delta V = 0$, ramps of 18 mV/msec were randomly applied for 10 ms in the positive and negative ranges. In this particular patch the ideal situation of a single active channel was found, so complications of multiple units to the analysis can be ignored. The conductance estimated from the maximum slope was 84 pS using the holding potential method. When ramps were given to the patch at a time when the channel was closed the

Figure R-3. Fast Ramp Voltage Clamp of IR

Patches containing IR were voltage clamped by positive and negative ramps of 180 mV over 10 msec from a holding potential of $\Delta V = 0$ mV (resting potential). Shown here is a SC CAP with 75 mM KCl in the pipette. Positive and negative voltage ramps from a function generator were randomly applied to the command input. Playback identified ramps applied during an open channel duration (inset top) and when the channel was closed (inset bottom). Direct point by point subtraction of leakage from total current identified open channel specific current which was used to construct the I-V plot. Leakage conductance was about 9 pS. ($\blacktriangle$) positive ramp, ($\triangle$) negative ramp, ($\bullet$) from stable holding potentials. The cell was bathed in Solution A.

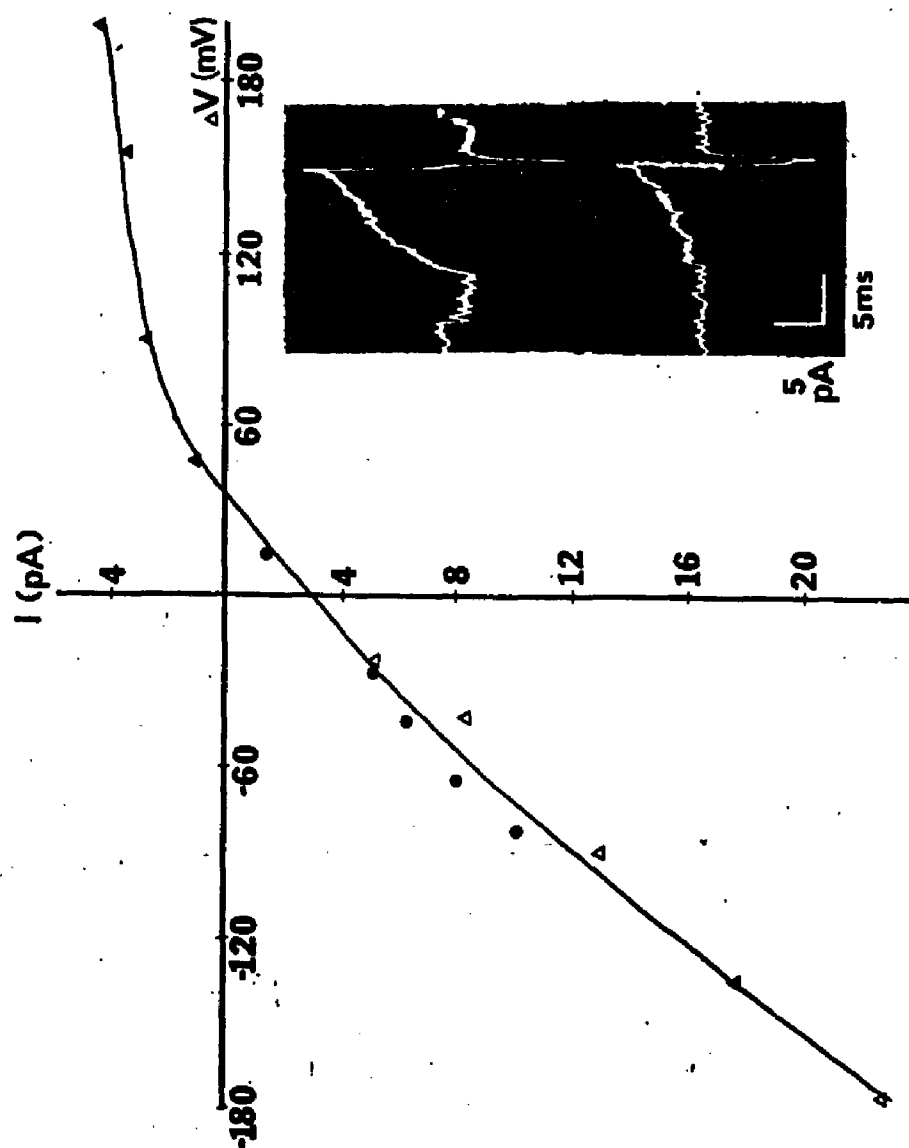


Figure R-3. Fast Ramp Voltage Clamp of IR

leakage conductance was obtained from the currents responding to voltage points along the ramp. In this particular patch the leakage conductance was about 9 pS and linear. When the channel was open over the entire ramp duration the subtraction of the leakage current from the total current allowed determination of the open-channel specific current and the respective I-V plot. An I-V plot can be established under these conditions with a minimum of two ramps, one to get leakage conductance and a second to get total conductance if the channel remains open over the relevant voltage range. Thus, the process is more rapid and efficient than the slow holding potential method. Moreover, since the command potential is changing in time, channel openings, closures, or passage through ΔV_{rev} can be clearly seen directly as inflection points or abrupt changes in the slope of the current trajectory. This avoids problems with slow changes in transpatch voltage discussed above. That is, since the leakage currents are subtracted and the ramp is fast (e.g., faster than the mean open time on average), the potential problems of losing the fluctuations at high open probability voltages and misinterpreting the leakage conductance are avoided.

The inset to Figure R-3 shows results from two selected ramps where a channel was open (upper) or closed (lower). These functions were subtracted to give the portion of the I-V curve depolarized to $\Delta V = 0$ mV. It is easily seen that the channel conductance changes dramatically above ΔV_{rev} , from 84 pS to 14 pS (16% of maximum). Thus, the IR characteristic seen in the steady-state I-V plot is confirmed with the ramp technique. Moreover, the voltage at which the inflection point occurs on the open-channel current is consistent with

extrapolation of the steady-state I-V curve to the reversal potential. In addition, the results of negative ramps are shown on the I-V plot. The estimated conductance from the steepest portion of the plot is 102 pS, similar to the result obtained from slow voltage changes.

An interesting result came from analysis of these ramp voltage clamp experiments. Many ramps conducted current to about the channel specific ΔV_{rev} and then the trajectory bent dramatically, as shown in Figure R-4. This type of behavior yielded ramp I-V plots with no outward current and was consistent with IR behavior observed in some patches with the slow holding potential method. One interpretation of these results is that conductance through the pore is affected by an internal blockage mechanism that has a certain probability of partially or totally occluding the pore in a voltage- or time-dependent manner. If such a model is representative, the nature of a blocking particle would be of significant biophysical interest (see Discussion). A fast voltage-sensitive rectification gate could also explain some of this behavior.

3. IR Reversal Potential Dependence on K_o^+

The permeability ratio is a concept familiar from theoretical work on ion conductances (e.g., Goldman, 1943; Krasne, 1978; Latorre and Miller, 1983; Eisenman and Horn, 1983) and originally the findings of Hodgkin and Katz (1949) on the squid axon. An essential characteristic for electrical excitability in biological systems is that some ion channels must have selective permeability to one group (cations, anions) of ions over another or the ability to distinguish amongst ions of the same group (e.g., Na^+ , K^+). The selectivity filter, as it is commonly called, is a channel process that is capable of recognizing

Figure R-4. Precipitous Drop in Conductance During Some Ramps

On many ramps the current did not typify the behavior in the previous example. Shown in a, b, c, f, and h are smooth current (conductance) changes during positive (c), negative (a, b, h) and leakage ramps (f). In d, e, g, i, j and k current precipitously bends (into the voltage axis) indicating a large sudden change in conductance. Conditions were a CAP with 75 mM K_0^+ . Cell was bathed in Solution A. Ramps were 18 mV/ms.

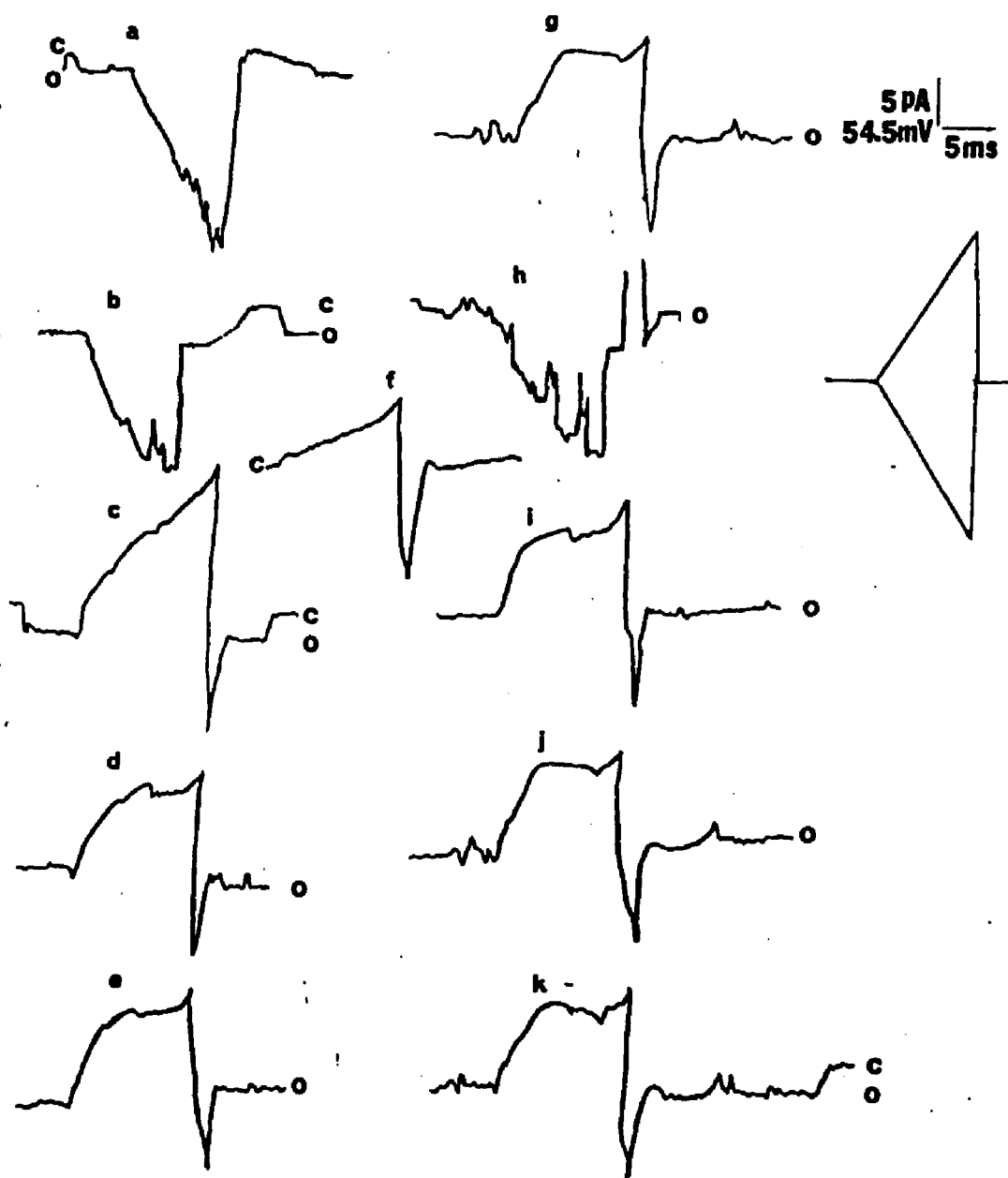


Figure R-4. Precipitous Drop in Conductance During Some Ramps

or is tuned to certain physical parameters of selectable ions in solution such as size, charge magnitude and its spatial distribution, polarizability, and hydration. Ions which randomly encounter the pore mouth (which itself could be designed for electrostatic attraction) and are able to match the selectivity filter template or "window" are then justified for passage through the channel proper. The permeability ratio is a statistical mean of the error in this recognition process (low selectivity implying greater error in matching). The selectivity criteria are determined typically on the basis of reversal potential measurements or the potential where no net current is found in the two-phase transmembrane system, which is typically assumed to be at steady-state (Finkelstein and Mauro, 1977). For a system in this condition, the application of equilibrium thermodynamics is justified, and selectivity is inferred on the basis of concentration-dependent changes in the Nernst potential for an isolated ionic conductance. Recognizing that there are possible errors in this contemporary method (Eisenman and Horn, 1983), an ion channel absolutely selective for a single monovalent ion provides Nernst reversal potentials (at 25°C) of 58 mV per log ratio of the transmembrane ion gradient.

In Figure R-5 the mean ΔV_{rev} s from experiments where KCl was varied in the CAP pipettes are plotted versus the concentration of K_0^+ . The points are best fitted with a line that has a slope of 58 mV/log K_0^+ . Within experimental error this strongly suggests that IR is a highly selective K^+ channel. Many other preparations show IR conductances which are K^+ -selective (see Discussion). The conclusion is supported by the fact that replacement of chloride (the principle anion) with nitrate or citrate does not alter the conductance

Figure R-5. IR Reversal Potential vs. K_o^+

Estimated reversal potential from numerous patches (30 mM K_o^+ , $n=2$; 75 mM K_o^+ , $n=7$; 140 mM K_o^+ , $n=17$; 300 mM K_o^+ , $n=3$) are plotted vs. the concentration of K_o^+ . ΔV_{rev} s are taken as the displacement voltages from $\Delta V = 0$ (RMP, -52 mV). Some values of ΔV_{rev} are not apparent due to overlap on the graph. A linear regression equation fit to the data is:

$$\Delta V_{rev} = 0.2172 * (K_o^+) + 18.67$$

and is represented by the solid line. Using the fitted function there is a 58 mV change in ΔV_{rev} for a $\log_{10}$ -fold change in K_o^+ . The regression fit is significant at the $p < 0.05$ level. Note plot of ΔV_{rev} vs. K_o^+ uses (O), while the predicted values from the regression fit use (X).

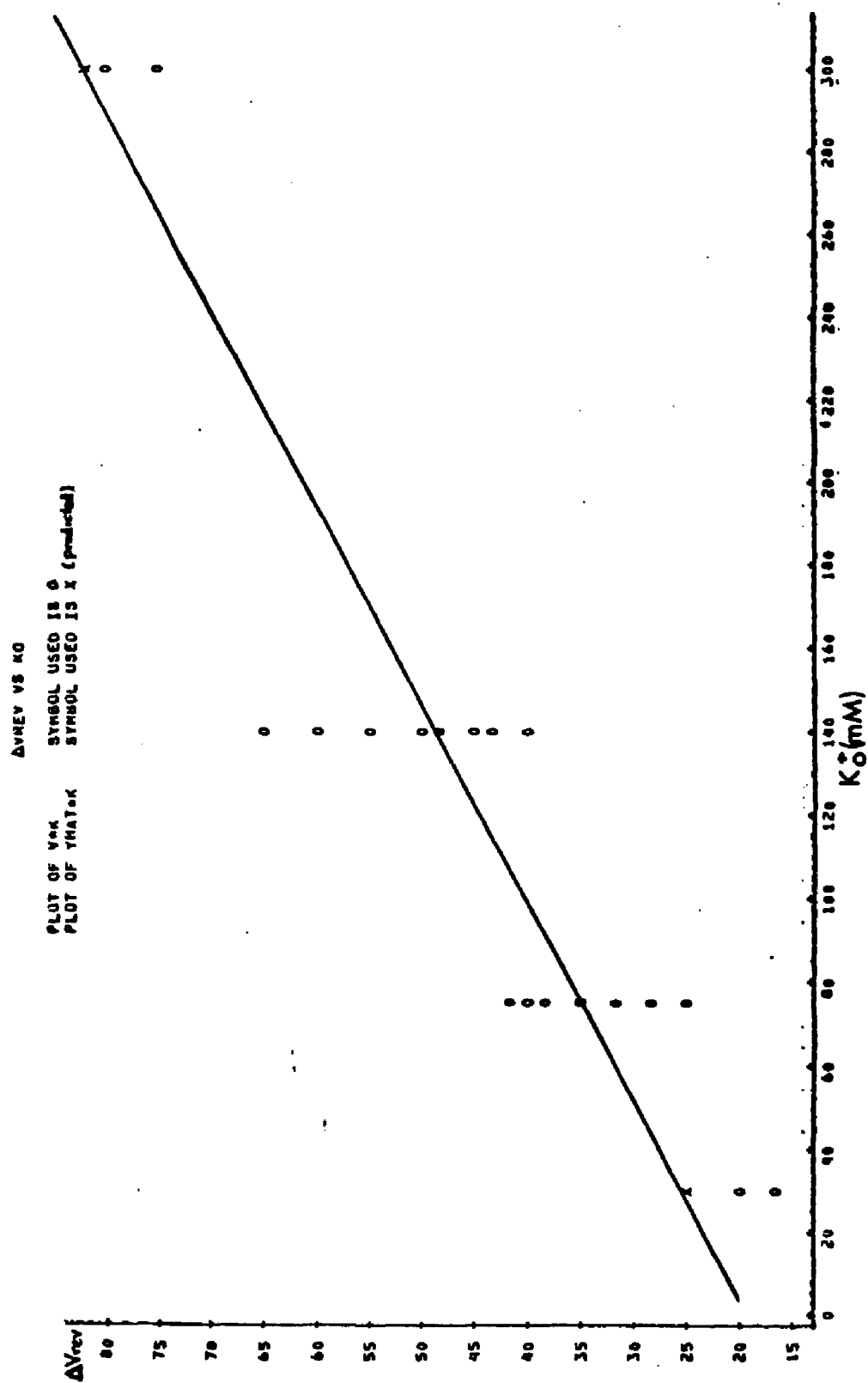


Figure R-5. IR Reversal Potential vs. K_0^+

or typical V_{rev} (data not shown). Total replacement of K_o^+ with Na_o^+ or Cs_o^+ fails to permit detection of this channel in CAPs, and partial replacement of K_o^+ with Na_o^+ provides currents consistent with the level of K^+ in the pipette.

4. Single Channel Conductance vs. K_o^+

The process of ion permeation through open channels may be subject to enzymatic or Eyring rate theory models. Many K^+ channels show concentration-dependent conductances that fit rectangular hyperbolic curves. A saturation of conductance with ion loading, as well as other tests, shows that many K^+ channels have one or more binding sites for ions during passage through the channels. IR channels in tunicate eggs and skeletal muscle are believed to be multi-ion pores (Hille and Schwarz, 1978). A unique feature of multi-ion IR channels in these systems is the dependence of macroscopic conductance on K_o^+ to the power relationship of about 0.5. The physical chemical basis of this property is related to the nature of the supposed multiple binding sites in the channel, how they interact, the relative free energy well depths, and the relation of barriers and sites to the field drops across the membrane (Hille, 1984; Hille and Schwarz, 1978).

Figures R-6a show the limiting slope conductances (i.e., at hyperpolarized potentials) of numerous patches plotted versus the square root (SQRT) of K_o^+ . This is done both without and with the theoretical restriction that $g_{IR} = 0$ when $K_o^+ = 0$ mM and by using the best estimated conductance at each K_o^+ from Figure R-2b. The data are fitted well in all cases by a linear relationship, which indicates to a first approximation that the conductance of this IR channel is proportional to the concentration of $(K_o)^{1/2}$. To

determine the precise power of this relationship one can plot the results on double logarithmic coordinates as shown in Figures R-6b. The slope of this relationship, plotted for the different constraints above, varied from 0.5 to 0.65 which is similar to the power relationship (0.62) found by Sakmann and Trube (1984a) in an IR in ventricular muscle and a 0.65 result if one performs a similar calculation on Fukushima's data (1982) on the tunicate egg.

From the conductance versus K_o^+ relationship it is possible to estimate the SC conductance under conditions of what is usually considered normal (3-5.6 mM) physiological K_o^+ . It should be noted, however, that K_o^+ may be much greater than 5.6 mM under certain conditions (see Discussion). It is assumed that the relationship of conductance to $\text{SQRT}(K_o^+)$ remains linear in extrapolation to low K_o^+ where data access was limited due to recording system noise. It is unclear whether this is justified but experiments could be performed at lower K_o^+ (e.g., one IR SC with 5.3 mM K_o^+ had a conductance of 25 pS) if noise was reduced by using a different headstage design (Rae and Levis, 1984b). Nevertheless, one can predict using the fitted equation ($g_{IR} = 12.7 * \text{SQRT}(K_o^+)$) restricting $g_{IR} = 0$ when $K_o^+ = 0$ mM that at 5.0 mM the conductance will be 28 pS, at 2 mM K_o^+ , 18 pS and at 1.0 mM K_o^+ , 12.7 pS, assuming constant K_o^+ . A direct participation of K_o^+ in the gating mechanism (Ciani et al., 1978) and its kinetics may also be observable, especially at low K_o^+ where the process of activation (electrochemical gating) may not yet be saturated.

According to the Goldman Hodgkin Katz (GHK) current equation:

$$I = (F^2/RT) * (V * P_K) * \frac{([K_o] - [K_i] * \exp(-VF/RT))}{(1 - \exp(-VF/RT))}$$

Figure R-6. SC Conductance is Dependent on the SORT of K_o^+

(a) SC conductances (G) from numerous CAPs were plotted vs the $\text{SORT}(K_o^+)$. Some values of G are not apparent due to overlap on the graph. Linear regression lines were fit to the data points without ($\blacktriangle\blacktriangle$) or with ($\blacklozenge\blacklozenge$) the restriction that $G = 0$ pS at $K_o^+ = 0$ mM. The linear regressions used to fit the data points in the SC I-V curve (Figure R-2b) at potentials hyperpolarized below $\Delta V = 0$ mV provided fitted sample limiting slope conductances at each K_o^+ which here are also plotted vs. $\text{SORT}(K_o^+)$ without ($\blacksquare\blacksquare$) the restriction that $G = 0$ when $K_o^+ = 0$ or with ($\bullet\bullet$) it.

Linear regression equations fit to the data points are:

$$(\blacktriangle\blacktriangle) \quad G = 16.54 * \text{SORT}(K_o^+) - 44.71$$

$$(\blacklozenge\blacklozenge) \quad G = 12.70 * \text{SORT}(K_o^+)$$

$$(\blacksquare\blacksquare) \quad G = 12.90 * \text{SORT}(K_o^+) - 19.51$$

$$(\bullet\bullet) \quad G = 11.29 * \text{SORT}(K_o^+)$$

All fitted regression lines ($\ast\ast$) are significant at the $p < 0.05$ level.

Figure R-6. - Continued

(b) To determine the exponent (n) of the relationship $G=(K_o^+)^n$, data were plotted on $\log_{10}G$ vs. $\log_{10} (K_o^+)$ coordinates. Data from numerous patches were plotted, as above, without ($\blacktriangle$) or with ($\bullet$) the $G = 0$ at $K_o^+ = 0$ restriction, with restricted points estimated from the functional expression ($\blacklozenge$) in (a) as K_o^+ approaches 0 mM. Data points from the I-V fits (Figure R-2b) were also plotted ($\blacksquare$) without the low K_o^+ restriction. Linear regression equations fit to the data points are:

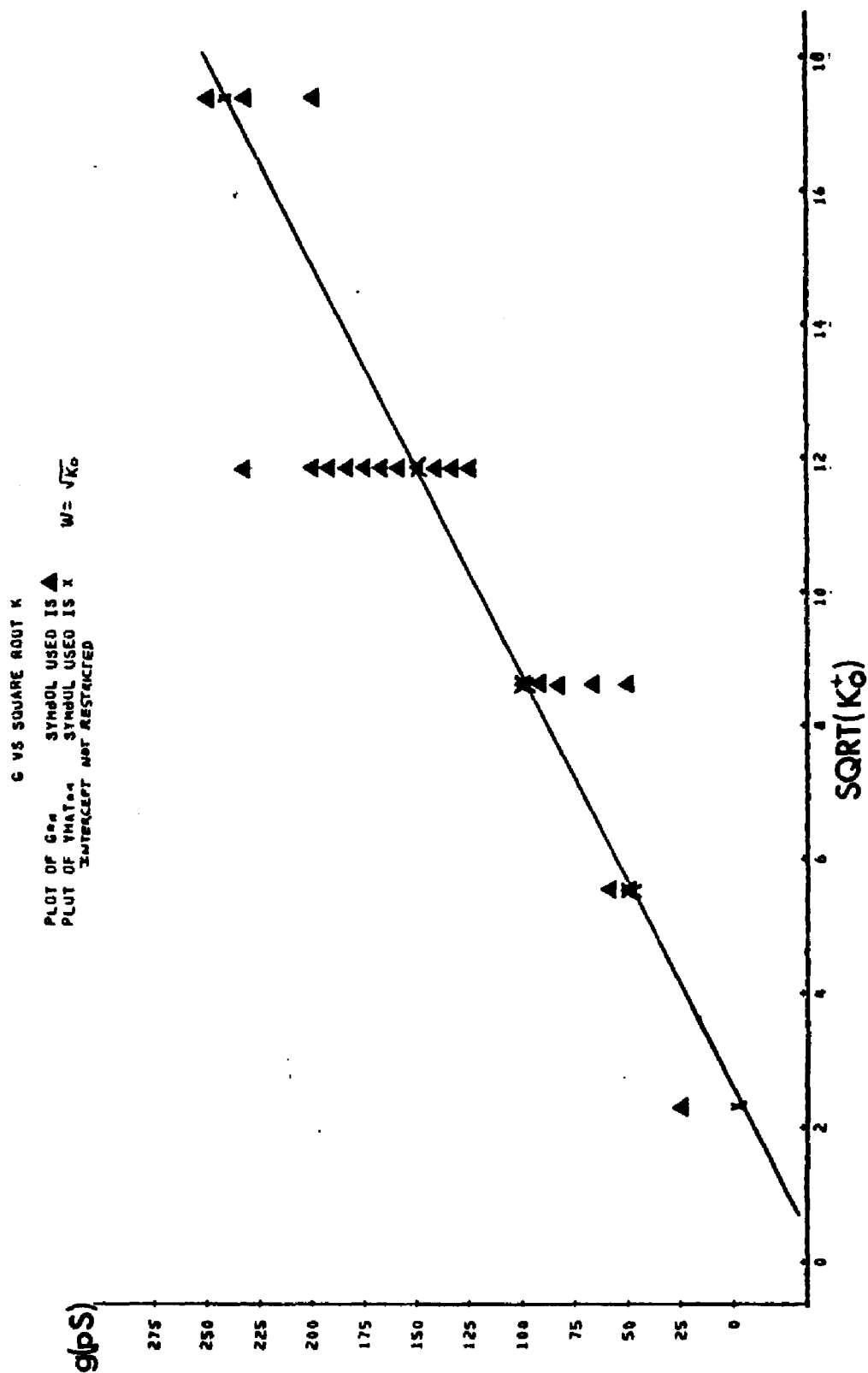
$$(\blacktriangle) \log_{10}G = 0.657*\log_{10}K_o^+ + 0.748$$

$$(\bullet) \log_{10}G = 0.507*\log_{10}K_o^+ + 1.062$$

$$(\blacksquare) \log_{10}G = 0.547*\log_{10}K_o^+ + 0.923$$

Under the various modes of analysis n varies from 0.51 to 0.66.

All regression lines are significant at the $p < 0.05$ level.



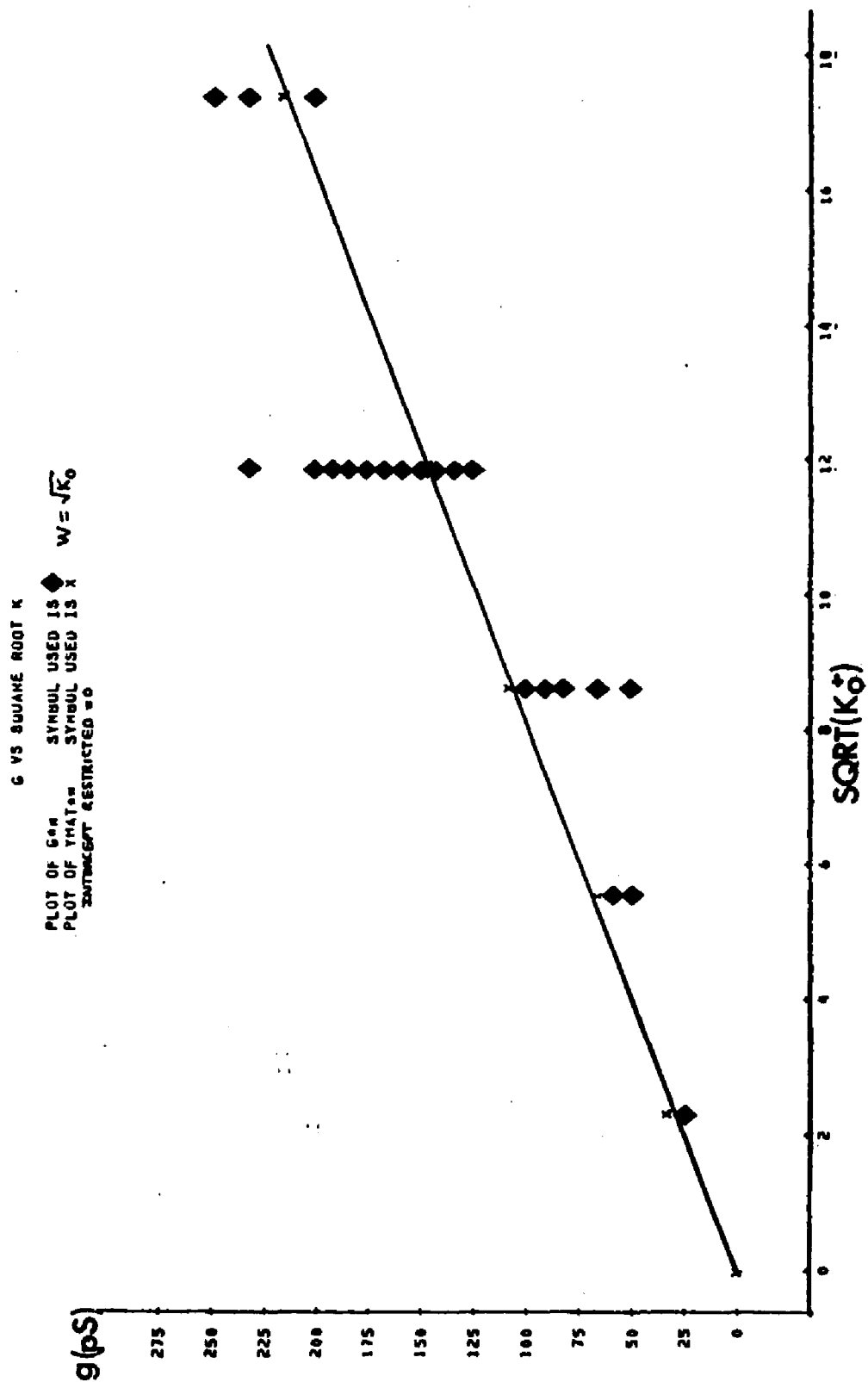


Figure R-6a. SC Conductance is Dependent on the SQRT of K_0^+

CONDUCTANCE VS K RELATIONSHIP
 PLOT OF g_{SC} SYMBOL USED IS ■
 PLOT OF Y_{HAT} SYMBOL USED IS x $w = \sqrt{K_0}$
 FITTED 9% FROM FIGURE R-2b

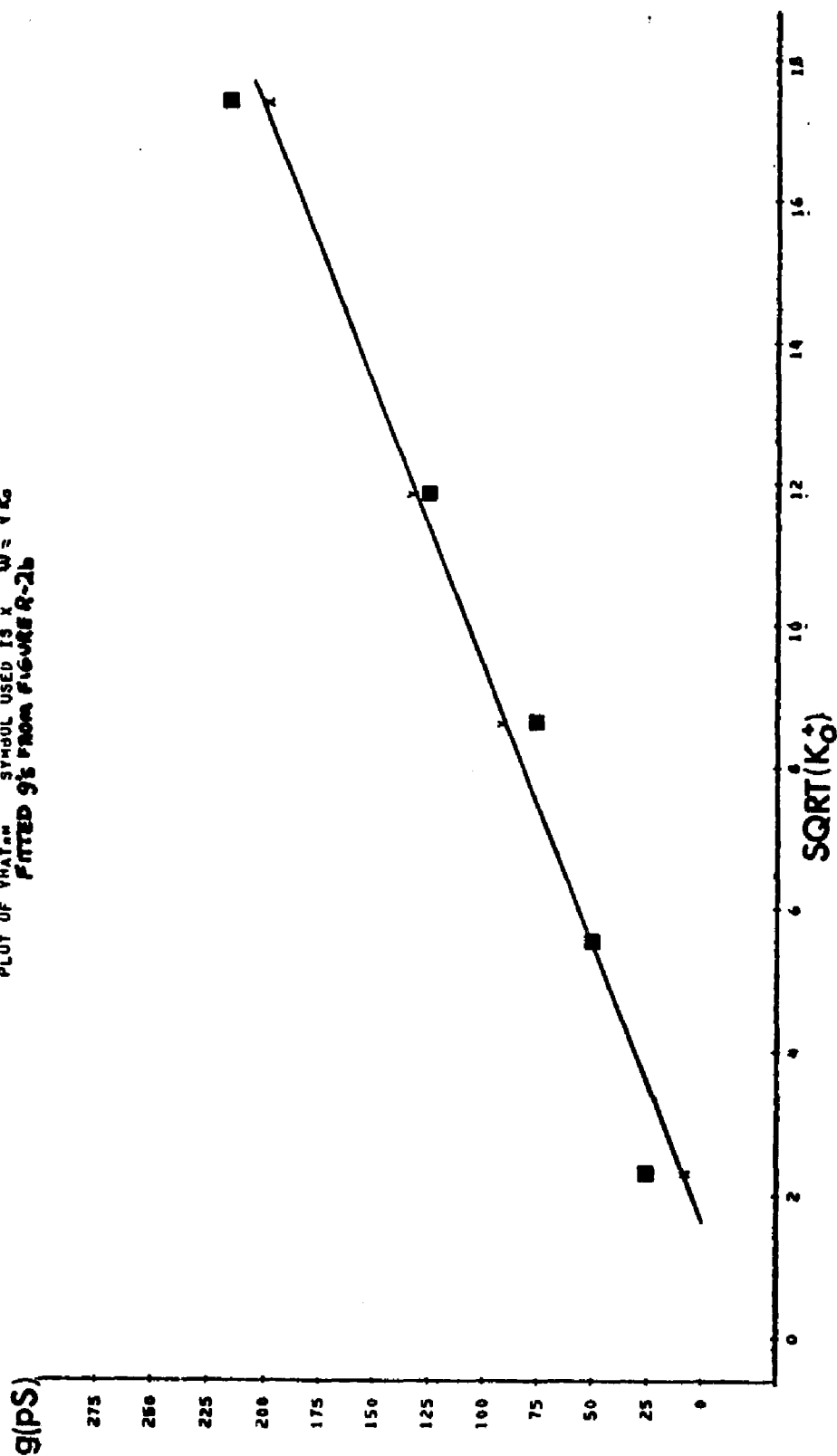


Figure R-6a. SC Conductance is Dependent on the SQRT of K_0

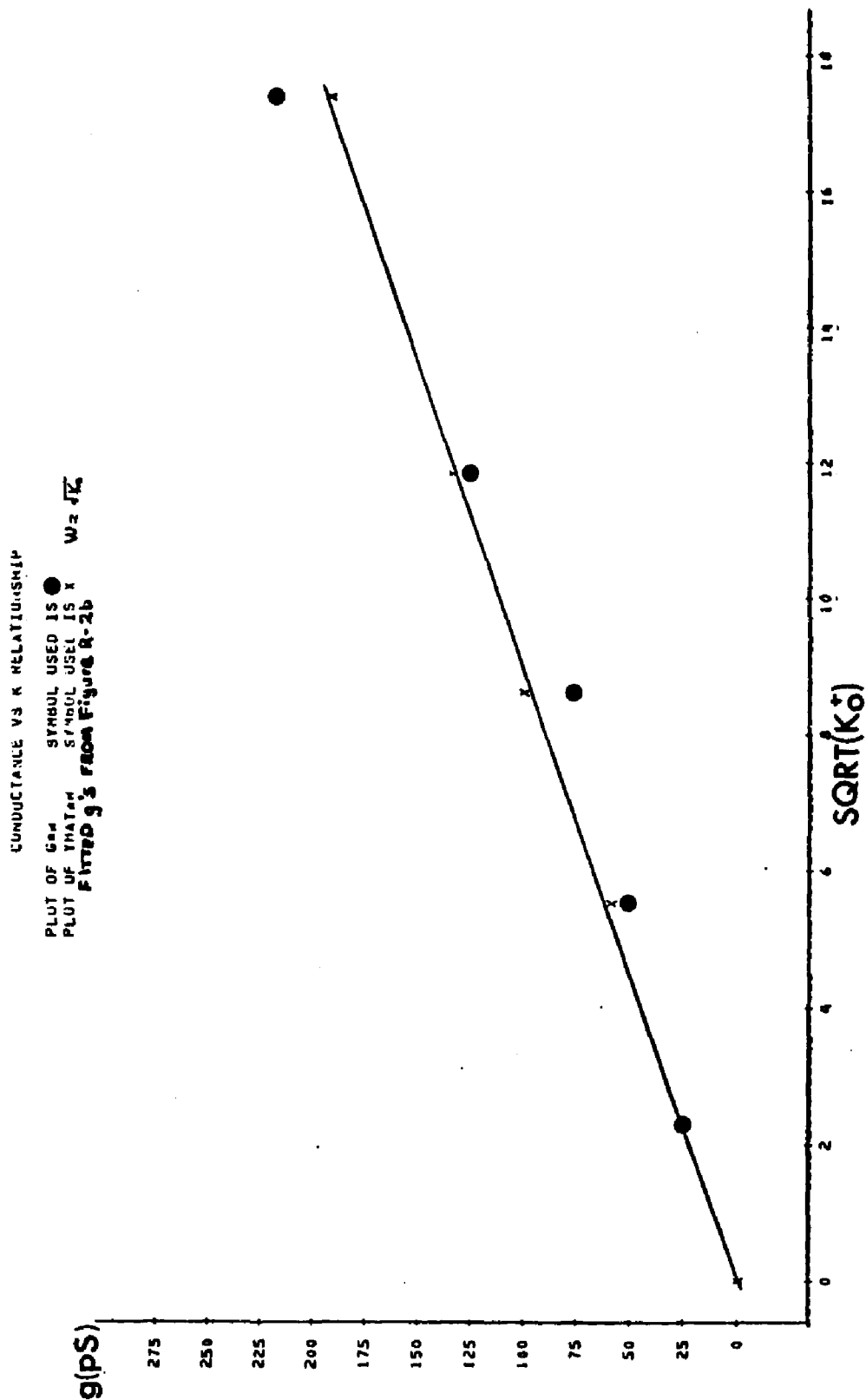


Figure R-6a. SC Conductance is Dependent on the SORT of K_0^+

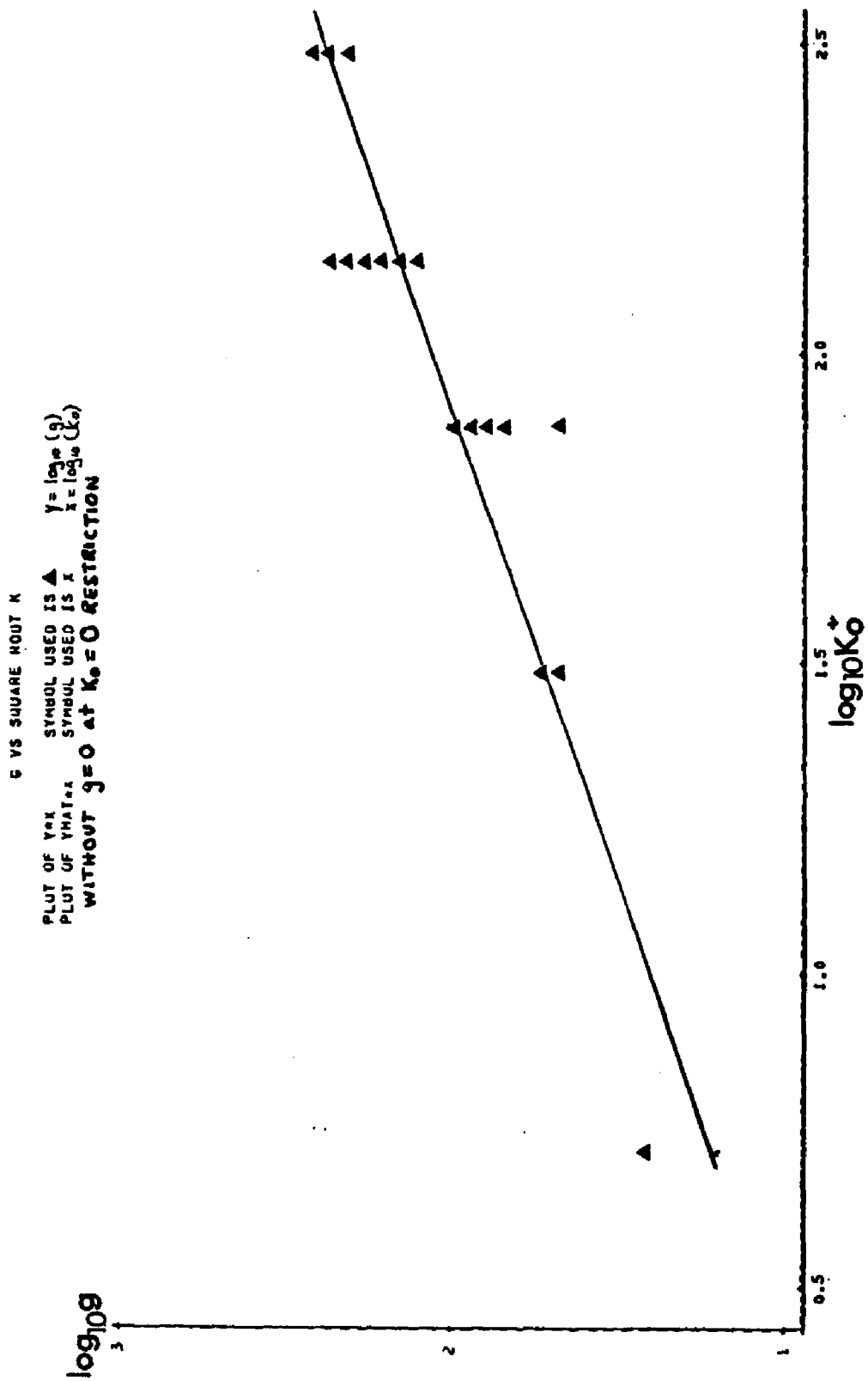


Figure R-6b. SC Conductance is Dependent on the SORT of K_0

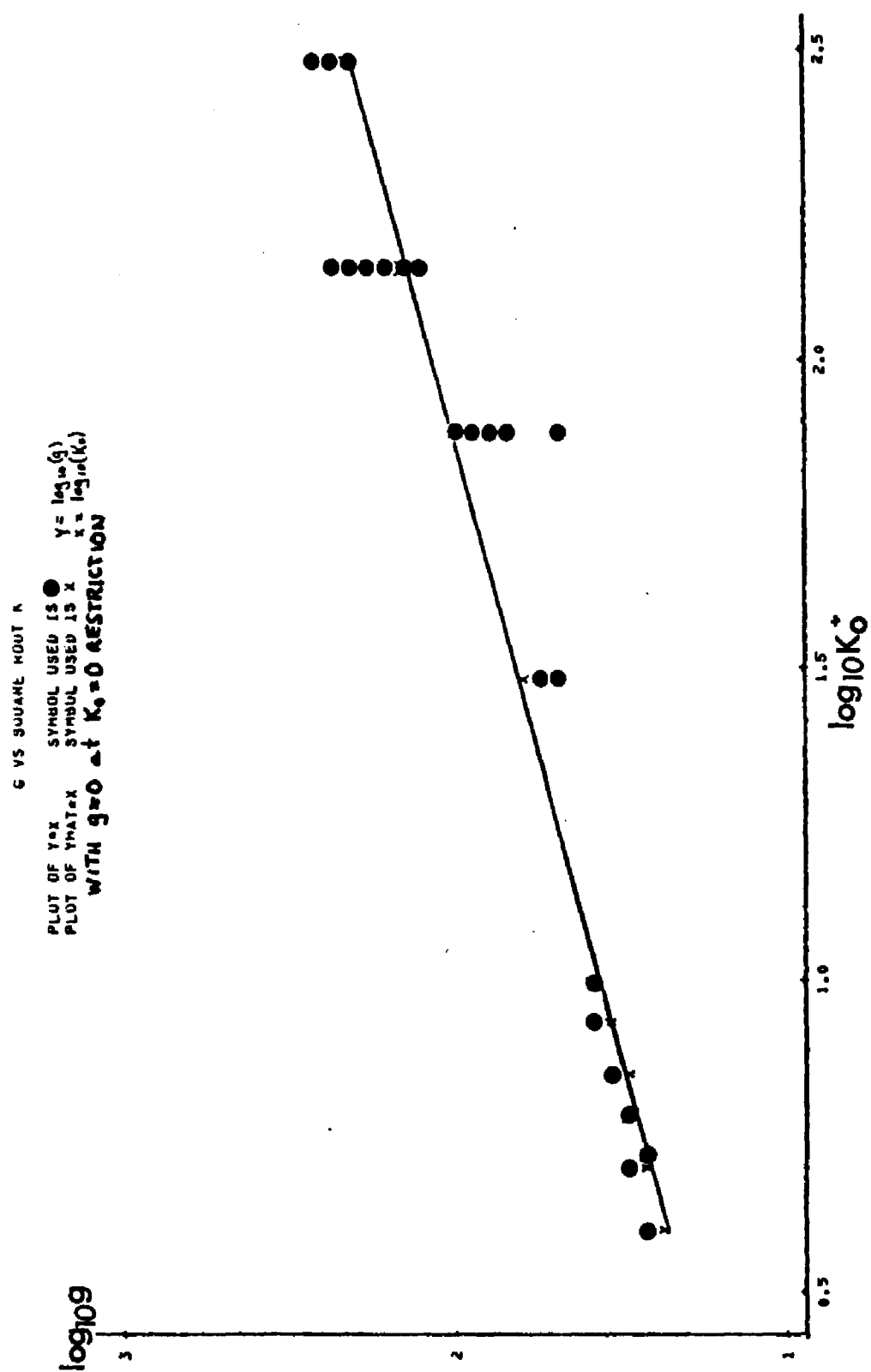


Figure R-6b. SC Conductance is Dependent on the SORT of K_0^+

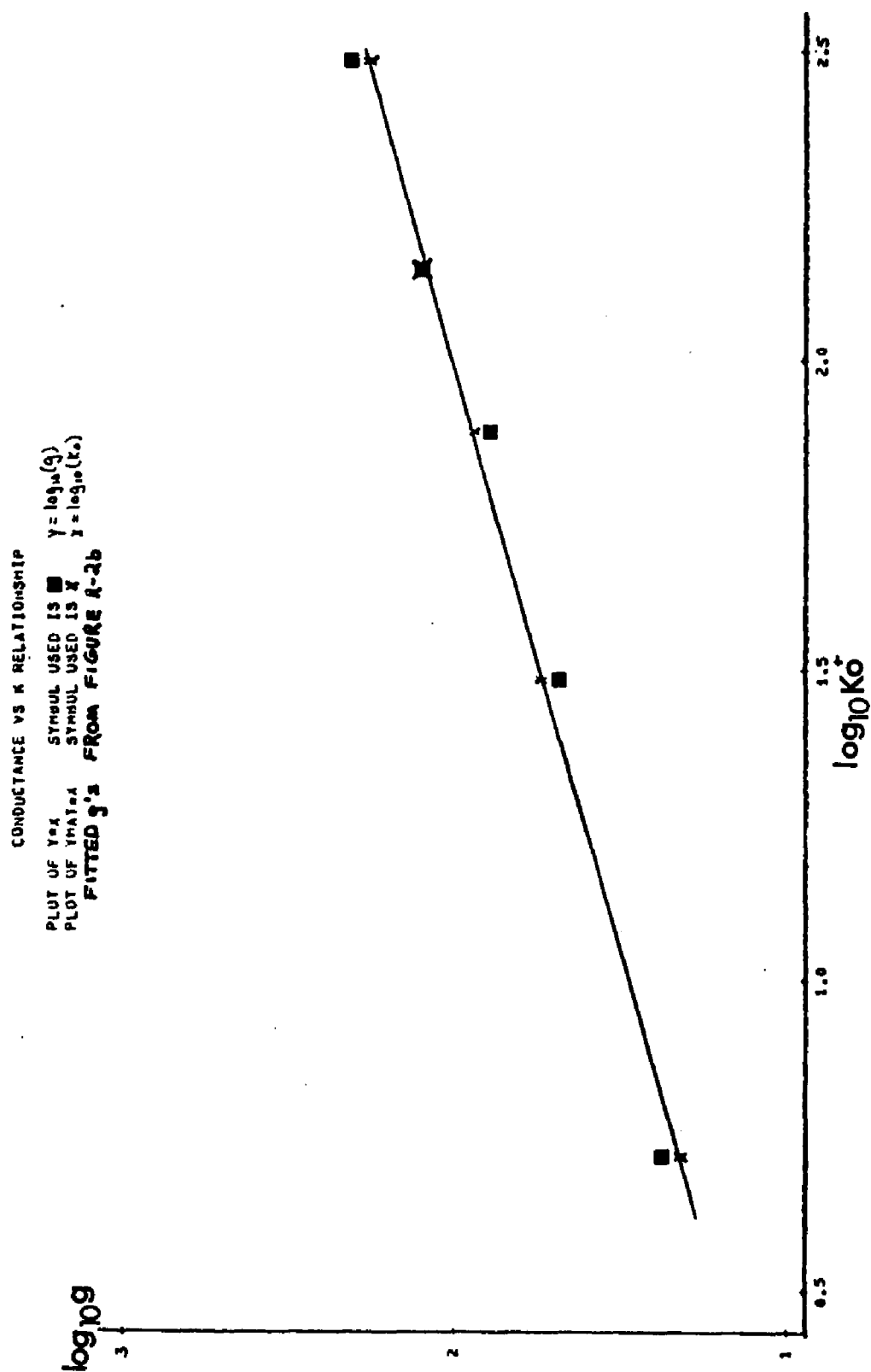


Figure R-6b. SC Conductance is Dependent on the SORT of K_0^+

(where $(V = (V - V_K))$) linear changes in K_O^+ at constant voltage should provide for linear changes in current around ΔV_{rev} if the assumptions of the constant-field equation (linear drop in potential and constant diffusion coefficient across a homogeneous membrane) hold. Inasmuch as the constant field equation does not account for IR (Hall et al., 1963; Noble, 1965) it was of interest to compare the properties of IR in CHNs to the theoretical model. From Sakmann and Trube's (1984a) equation #4 the calculated permeability constant in the above equation (P_K) at different K_O^+ s (30-300 mM) ranges from 4.7 to 5.8×10^{-14} cm³/sec, which is similar to that of other K^+ channels. Permeability is relatively independent of K_O^+ so we can compare the effects of K_O^+ on I at constant voltages ($\Delta V = 0$ to 100 mV). Figure R-7a plots mean currents estimated from the equations fit in Figure R-2b at each voltage against K_O^+ . Unlike the linear relation expected from the GHK equation, a rectangular hyperbolic curve is found for each voltage that saturates at different inward currents depending on ΔV but with a constant K_m of about 380 mM K_O^+ at each voltage. A plot of I_{max} vs. ΔV is shown in Figure 7b with a slope of 480 pS. This evidence that K^+ current through the channel saturates with increasing K_O^+ at constant ΔV and the $\text{SQRT}(K_O^+)$ dependence on conductance suggests that K^+ ion permeation is a saturating and negatively cooperative (Hill coefficient less than one) process. A multi-ion pore with multiple, energetically interactive, K^+ binding sites may be consistent with this data.

Future experiments are being designed to demonstrate the existence of a multi-ion pore. This would be true if one could show that permeability ratios are concentration-dependent or that g_{IR} and

Figure R-7. Open-Channel Currents Saturate with Increasing K_O^+ at Constant Voltage.

(a) Open-channel currents at each voltage were estimated by use of the regression fits at each K_O^+ from Figure R-2b at potentials hyperpolarized from $\Delta V = 0$ mV. At each voltage (0, -20, -40, -60, -80, -100 mV) the current at each K_O^+ concentration was taken from the fits and plotted vs K_O^+ . At each voltage channel current (I) vs. K_O^+ plots were fit to the equation:

$$I = \frac{I_{\max} * K_O^+}{K_m + K_O^+}$$

using a Marquardt nonlinear least-square method. Here $I_{\max}$ is the estimated saturation current from the rectangular hyperbolic functions and K_m is the K_O^+ at which $I = 0.5 * I_{\max}$. Both $I_{\max}$ and K_m are parameters estimated from the fits, and are indicated at each voltage in the inset box. Note that K_m changed insignificantly with voltage. All fits were significant at the $p < 0.05$ level.

(b) Plot of $I_{\max}$ determined from (a) vs. ΔV . The points are fit (by eye) to the line:

$$I_{\max}(V) = 0.48 * V - 19$$

with a maximum conductance expected from the channel at high K_O^+ (approaching 1 M) of 480 pS.

CHANNEL CURRENTS SATURATE WITH INCREASING K_0
 PLOT OF I_{max} SYMBOL IS VALUE OF V

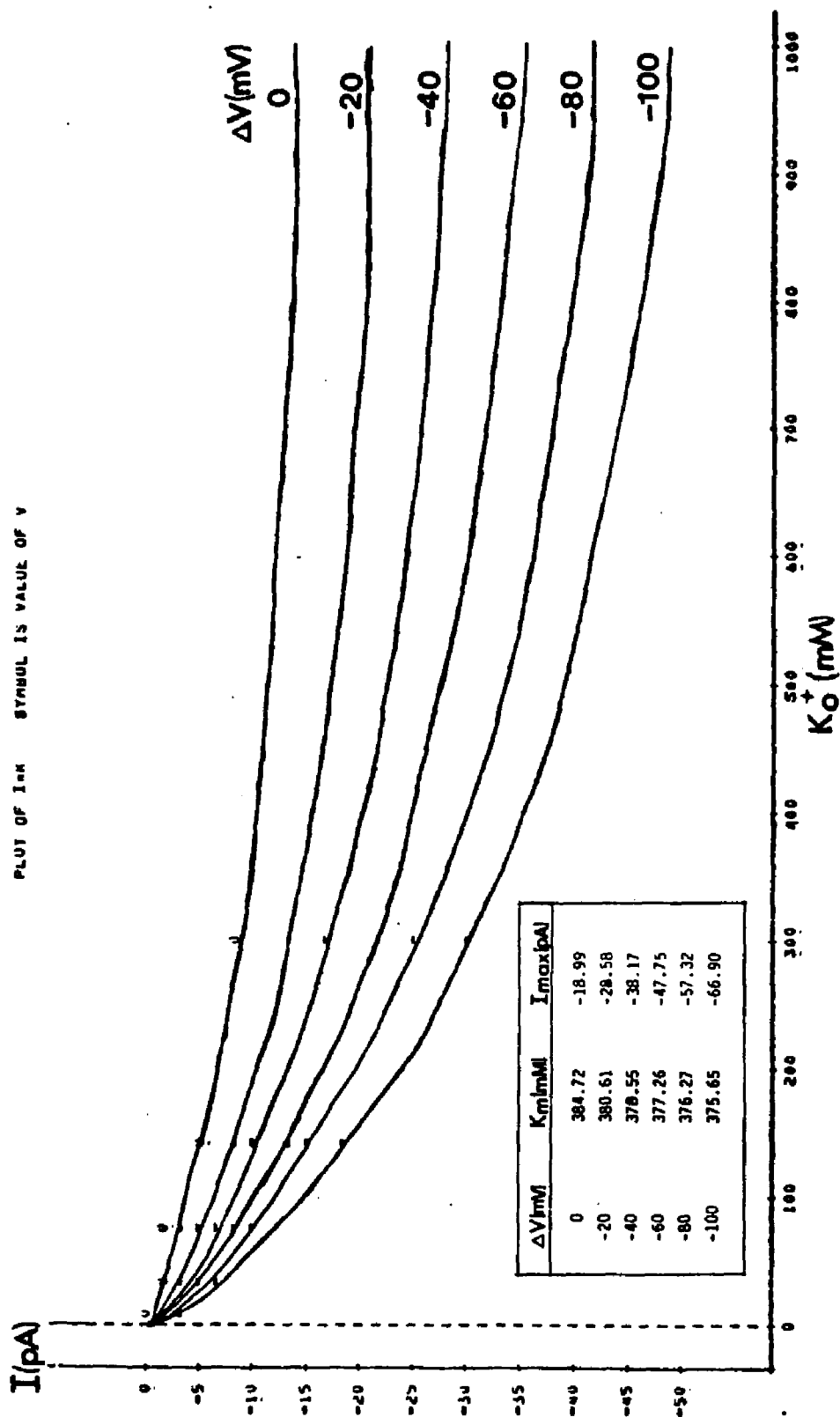


Figure R-7a. Open Channel Current Saturates at High K_0^+ at Constant Voltage

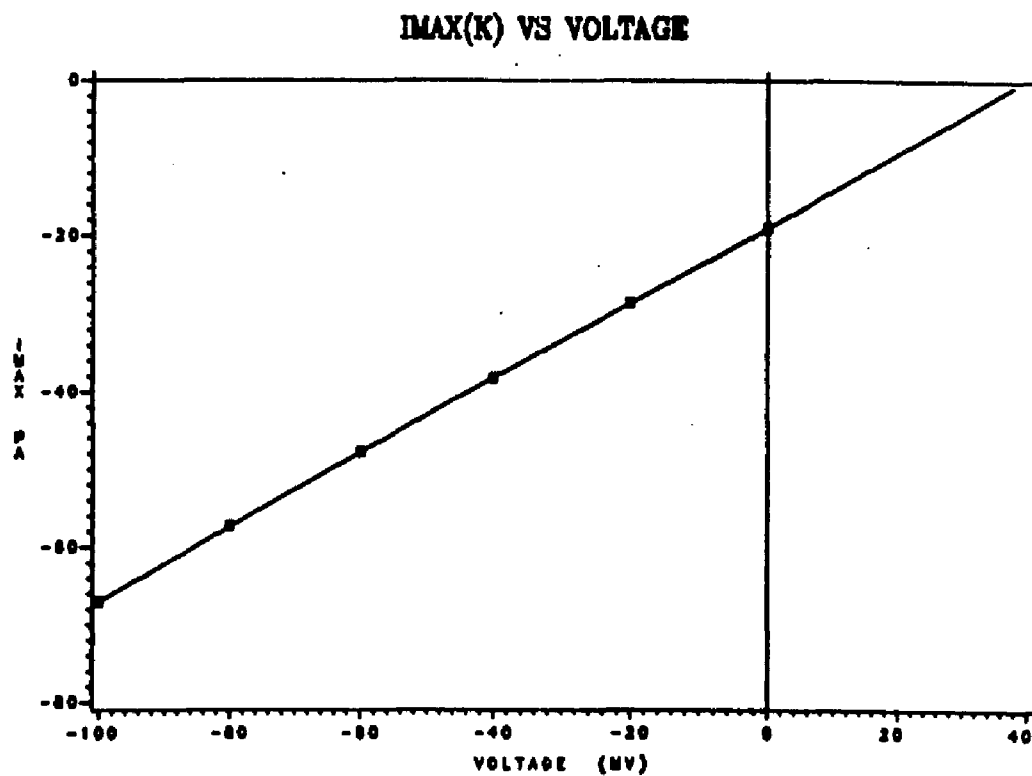


Figure R-7b. Open Channel Current Saturates at High K_o^+
at Constant Voltage

ΔV_{rev} have an AMFD in Tl_O^+/K_O^+ mixtures (Hagiwara et al., 1977; Hille and Schwarz, 1978).

5. Subconductance State Phenomenon

In the course of SC experiments it was often possible to observe partial closures from the maximum SC open-current level to a level about two-thirds of maximum. Careful scrutiny of SC patch recordings made it clear that substate events could represent an important element of the channel mechanism. To this date a minimum of four open-channel current levels have been resolved. They carry approximately 25%, 50%, and 75% of the maximum open-channel current (however, see Discussion under conductance models).

Representative traces of events are shown in Figure R-8 at a number of holding potential displacements of a SC patch with 75 mM K_O^+ . In the first trace (-57 mV) the channel has opened to the maximum level, and after a few brief unresolved flickers to a lower conductance state or the closed state, the channel jumps to the 26% current level and remains there steadily for about 1 ms before jumping to another state(s) and then finally closing. The trace below (-57 mV) shows an opening to the 67% level followed by a further increase in conductance to the maximum level. The trace at -41 mV shows an opening to the maximum (perhaps preceded by a sojourn to a level slightly less than maximum) current level, which then decreases to the 46% level for a time before increasing to the 67% level and then attaining the maximum level. Toward the end of this burst the channel drops back to submaximal (67%) level followed by an apparent transition to the closed state, then to return back to the submaximal state and finally the maximal state. The previous event was symmetric but the events

Figure R-8. Subconductance State Fluctuations Within Bursts of Channel Openings

(a) Representative Substate Transitions. Representative traces are shown from a CAP with 75 mM K_o^+ at two different holding potentials where substate occurrences were more obvious.

(b) I-V plots of Subconductance State events. The patch from (a) was used to construct I-V plots by searching for substates and measuring the current at different holding potentials; some points are averages of a number of similar amplitude transitions. Best fits of I-V curves were made by eye, and the conductances and their relative proportions are indicated in the figure. The cell was bathed in Solution A.

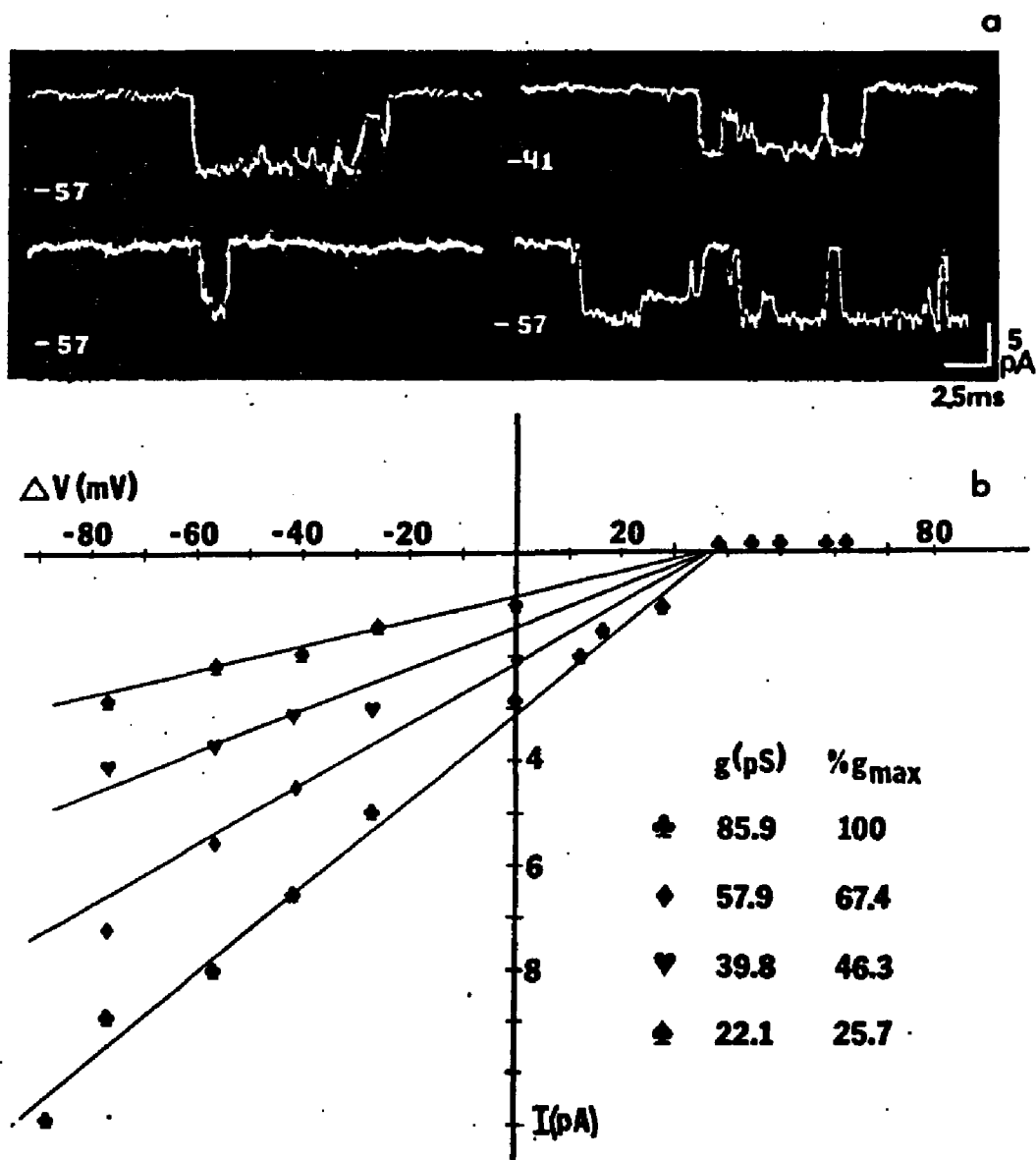


Figure R-8. Subconductance State Fluctuations Within Bursts of Channel Openings

preceding it in the burst were not. In the last trace (-57 mV) the channel burst begins with a prolonged sojourn at the maximum open level before an epoch at the 67% level which precedes an apparent brief symmetric closed transition and then a final closure. The channel then opens to the 46% level, then closes, then opens to the 100% level, drops to 66%, then attains the maximum open level again before closing. These results suggest but certainly do not statistically prove that not all transitions among states are symmetric.

The I-V curves for the states taken from a number of bursts at different holding potentials are shown in Figure R-8b. All the states appear to emulate the I-V IR property of the maximum state (which was used in the construction of all previous I-V plots). However, the full depolarized I-V curve for any substate has yet to be determined but could easily be done with the ramp voltage clamp. At 75 mM K_O^+ the maximum state was characterized by a conductance of 86 pS, the 67% state, by one of 58 pS, the 46% state, by one of 40 pS and the 26% state, by one of 22 pS. Subconductance levels appeared proportionally distributed in other patches (e.g., at 75 mM K_O^+ , $n = 2$) including patches at 140 mM K_O^+ ($n = 2$) and 300 mM K_O^+ ($n = 1$). It is interesting that the substate structure follows an approximate one-quarter:one-half:three-quarters:one relative conductance characteristic. We will show in the Discussion, however, that the substate conductances form a logarithmic series which may be indicative of a new model for IR.

A number of critical questions about the substates must be posed. One is whether they represent substates or independent events. Practically all substates seen are associated with bursts which include

the maximum conductance level. That is not to say openings do not occur to the substates: they do, but direct uncorrelated openings appear to be very rare. One can therefore treat these sublevels of current as events typically correlated with the maximum level; they alone do not appear to behave in a Poisson process manner.

Autocorrelation analysis of the events inside of bursts are required to substantiate these claims, but this will not be attempted without more data and computer assistance.

The second question relates to the probabilities of occurrence of states. Again, this parameter is not yet fully quantified. Absolute probabilities of occurrence of all states do not seem equivalent. The two most probable subconductance states appear to be the 100% level (estimate > 99% of all openings) and the 66% level (estimate < 1% of all openings) but interactions between any sublevel and maximum level appear possible. A possible state diagram for the subconductance levels is shown in Figure R-9.

Another question that can be posed is the symmetry of conditional probabilities of the transitions: is the probability of going from one state to a different state equal among all possible paths or biased along one particular path? Unsymmetric transition probabilities among substates could indicate that the principles of microscopic reversibility or detailed balance are being violated and that an open channel may not be a process at equilibrium (Lauger, 1983, 1984, 1985; Finkelstein and Peskin, 1984). This property would imply that the channel uses energy and is therefore not a passive processor of the electrochemical gradient. We will pursue this later in the Discussion. Another question is whether the magnitude of K_0^+ has

Figure R-9. Hypothetical Mechanistic Arrangement of Open Channel States in IR.

The 100% (O_4) and 66% (O_3) appear most probable, but apparent interactions among all states have been noted. The closed state shown is the short lived state which provides gaps inside of bursts.

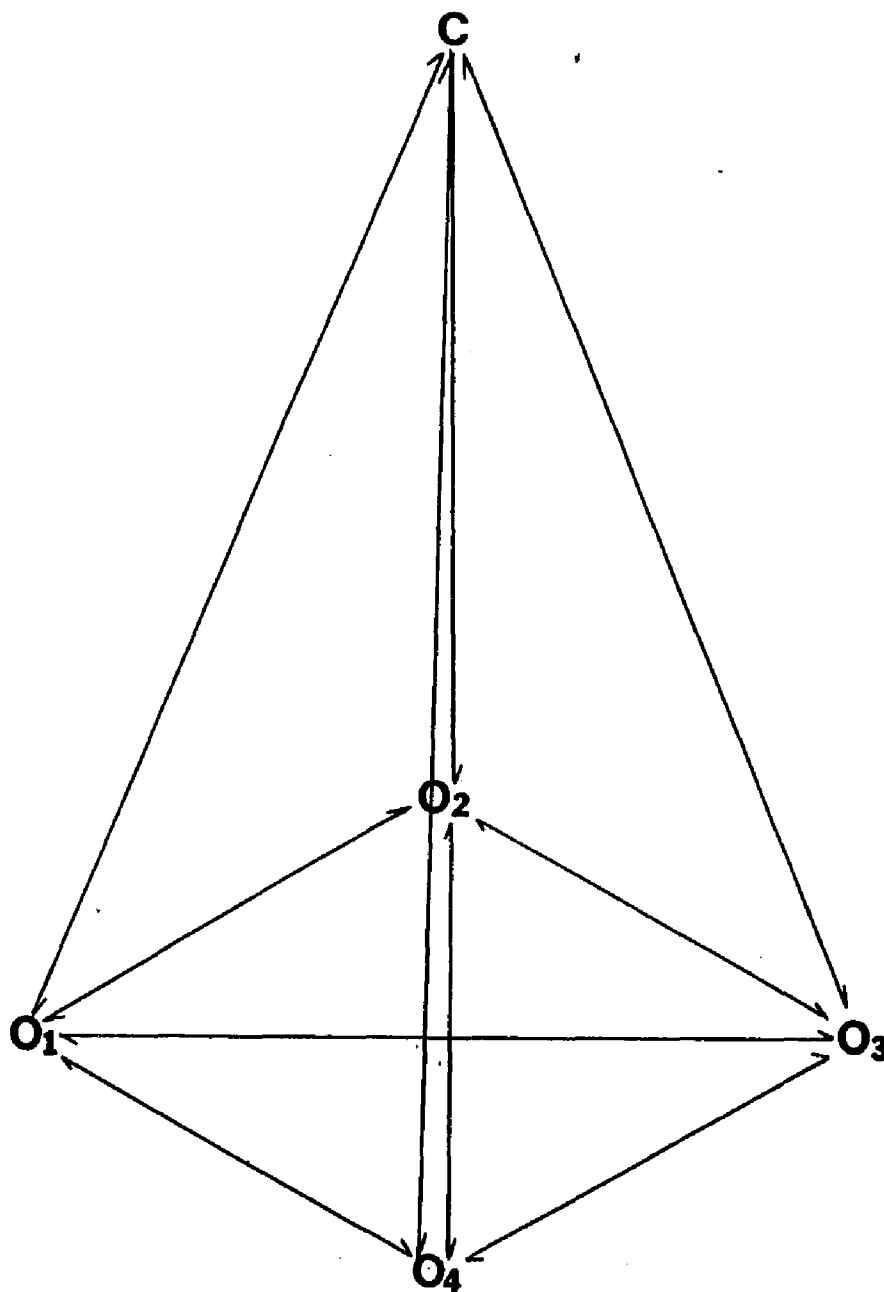


Figure R-9. Hypothetical Mechanistic Arrangement of Open Channel States in IR

any effect on the probabilities of these states or their conditional probabilities which would intimately invoke the participation of K_O^+ in the gating mechanism. There is currently some data to suggest that K_O^+ may play a role in changing the voltage dependence of the probability of opening (see Results-B1) in this channel and in IRs in other systems (Sauve et al., 1983; Kurachi, 1985).

6. Unusual Subconductance State Kinetic Behavior

On two different patches both held at -60 mV, the three quarter subconductance state occurred both randomly from the 100% level as well as in isolation for varying periods of time. The subconductance state occurred: (1) stably over a period of seconds with brief sojourns to the 100% level on one patch, or (2) randomly to the 68% level in most of one file with a prolonged subconductance fluctuation at the file end. The data were analyzed manually (patch 1) or using IPROC (patch 2).

An example of such behavior is illustrated in Figure R-10, with a continuous stretch of data from patch 1. Initially, the behavior was noticed because the currents at -60 mV were about 30% smaller than expected at this voltage and K_O^+ . However, as indicated (max) the SC currents jumped to a larger current magnitude instantaneously in one burst and remained in this approximately 30% higher conductance level for about 1 second. Noted on line 4 with the cursor is a brief transition back to the 68% level which is similar to the appearance of subconductance (three-quarter state) states in other patches. Other more brief flickers from the 100% level may be to this state as well. The channel operation remained kinetically stable at the 100% level until the activity stopped with a 2.2-second closure. When activity

Figure R-10. Subconductance States Can Be Kinetically Stable and Exhibit Periods of Higher Probability of Occurrence

Continuous data are from a 140 mM K_o^+ CAP held at -60 mV. Initially the currents originated from the 68% conductance level. The channel then latches up to the 100% conductance level (indicated by max) for about a second. From the 100% level there are brief transitions to the 68% state as indicated with the arrows. Note that the channel enters a prolonged closed (desensitized?) duration before returning to the 68% conductance level.

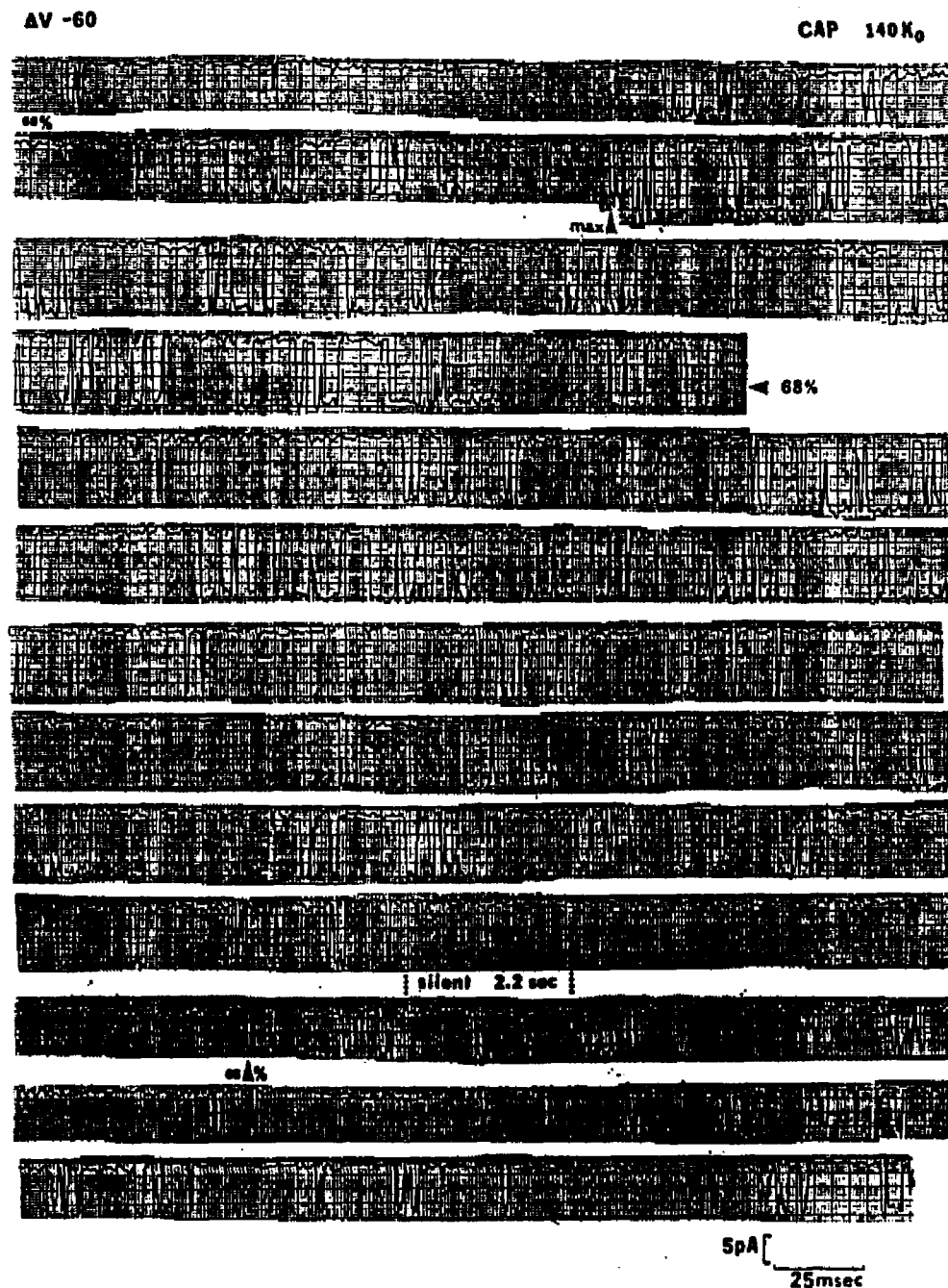


Figure R-10. Subconductance States Can Be Kinetically Stable and Exhibit Periods of Higher Probability of Occurrence

resumed it was back in the 68% state and remained so for the remainder of the file. This channel activity could indicate distinct kinetic modes of behavior and the possibility of desensitized states (e.g., channel modification) separating such modes or the conductance states themselves.

Another interesting feature of this data was that the burst durations appeared longer at the 100% conductance level than at the 68% level. Burst durations were therefore measured manually in the independent stretches of data from the 68% and 100% conductance states and plotted in histograms in Figure R-11. The distributions could be fit to single exponentials with time constants of 3.6 and 6.8 msec, respectively, for the 68% and 100% conductance levels. Thus, the 100% conductance level appears kinetically distinct from its 68% partner, as its mean burst duration is twice as long. It is unique that kinetic analysis is possible on these subconductance states. In other reported channels subconductance states have such a low probability that it has not yet been possible to do kinetic analysis of their activity.

An argument for the prolonged changes in conductance level arising from unique channel states rather than changes in membrane potential is two fold: (1) the conductance levels of pertinence have been seen in other patches where their activity has been very brief (e.g., 1 to a few ms), apparently too fast for sudden reversible changes in membrane potential, and (2) if membrane potential increases (more hyperpolarized) caused the occurrence of the 100% state, one would expect from burst analysis that hyperpolarization would decrease the burst length (see below), not increase it to two times its original length.

Figure R-11. Subconductance States Have Different Burst Duration Histograms

Data stretches of long duration from the SC in Figure R-10 were used to generate burst duration histograms using the procedure of defining the minimum closed period to be 2 ms. In both the 68% and 100% conductance levels single exponential histograms appear well fit to the data with the 100% level having a mean burst duration of 6.7 ms and the 68% level having a Tau of 3.6 ms. The patch was held at -60 mV with K_o^+ at 140 mM. Continuous presentations of the two representative conductance states are illustrated. In the prolonged 68% state, markings (Δ) indicate sojourns to the 100% state for comparison.

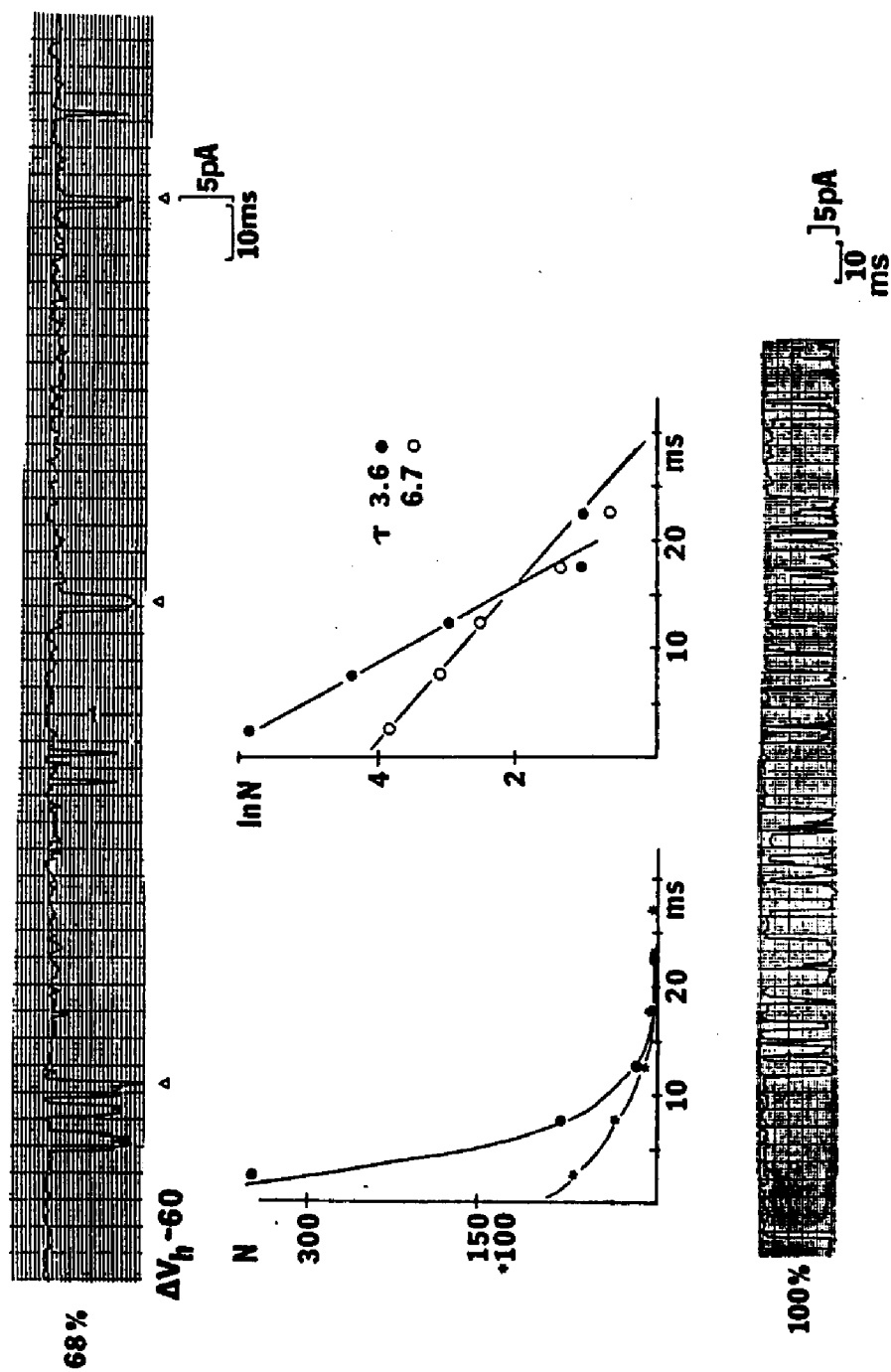


Figure R-11. Subconductance States Have Different Burst Duration Histograms

It appears that subconductance states in the IR channel are kinetically distinct and have the potential for long-lived stability.

In another experiment (140 mM K_0^+ , -60 mV) a transient but stable presentation of the submaximal conductance state was found and analyzed using IPROC. Amplitude distribution analysis in Figure R-12 showed two peaks fit by Gaussian curves with means of 8.36 ± 0.22 pA and 10.65 ± 0.28 pA. Twenty-nine percent of the events were to the subconductance level and occurred randomly throughout the record as well as stably at the end of the file as shown in Figure R-13 where the amplitude stationarity of on states is plotted over the course of the record. One can see that small numbers of events occurred to the lower conductance state during the initial part of the record and that an abrupt shift in amplitude occurs about 42 seconds into the file.

Three standard deviation windows on the amplitudes around each Gaussian mean were used to segregate the events for kinetic analysis. The resulting histograms for the ON times are shown in Figure R-14. The open times of the 100% conductance level fit a single exponential distribution function with a characteristic Tau of 5.37 ms while the subconductance state was fit by the sum of two exponentials with Taus of 0.19 msec and 3.16 msec. The analysis here is similar to that done by manual burst analysis since at -60 mV open times and burst times should be quite similar in magnitude. Comparing fitted Taus from the distributions shows that the maximum state has a Tau of 5.37 ms while that of the slower 78% state here is 3.16 ms. This result is very similar to that from the patch analyzed above except that another fast distribution (0.19 msec) appears at the lower state; this may be due to the sensitivity of machine-aided analysis. These results appear to

Figure R-12. Amplitude Distribution Functions in a Patch Where the 75% Subconductance State was Stable

In this patch (140 mM K_o^+ , -60 mV) the 75% state appeared both randomly through the course of the record and steadily over a period of time. IPROC was used to generate amplitude histograms of the events and was fitted with the sum of two Gaussian curves with means of 8.36 ± 0.22 pA and 10.65 ± 0.28 pA. The fraction of opening to the 75% state was 0.29 and the ratio of current means was 0.78.

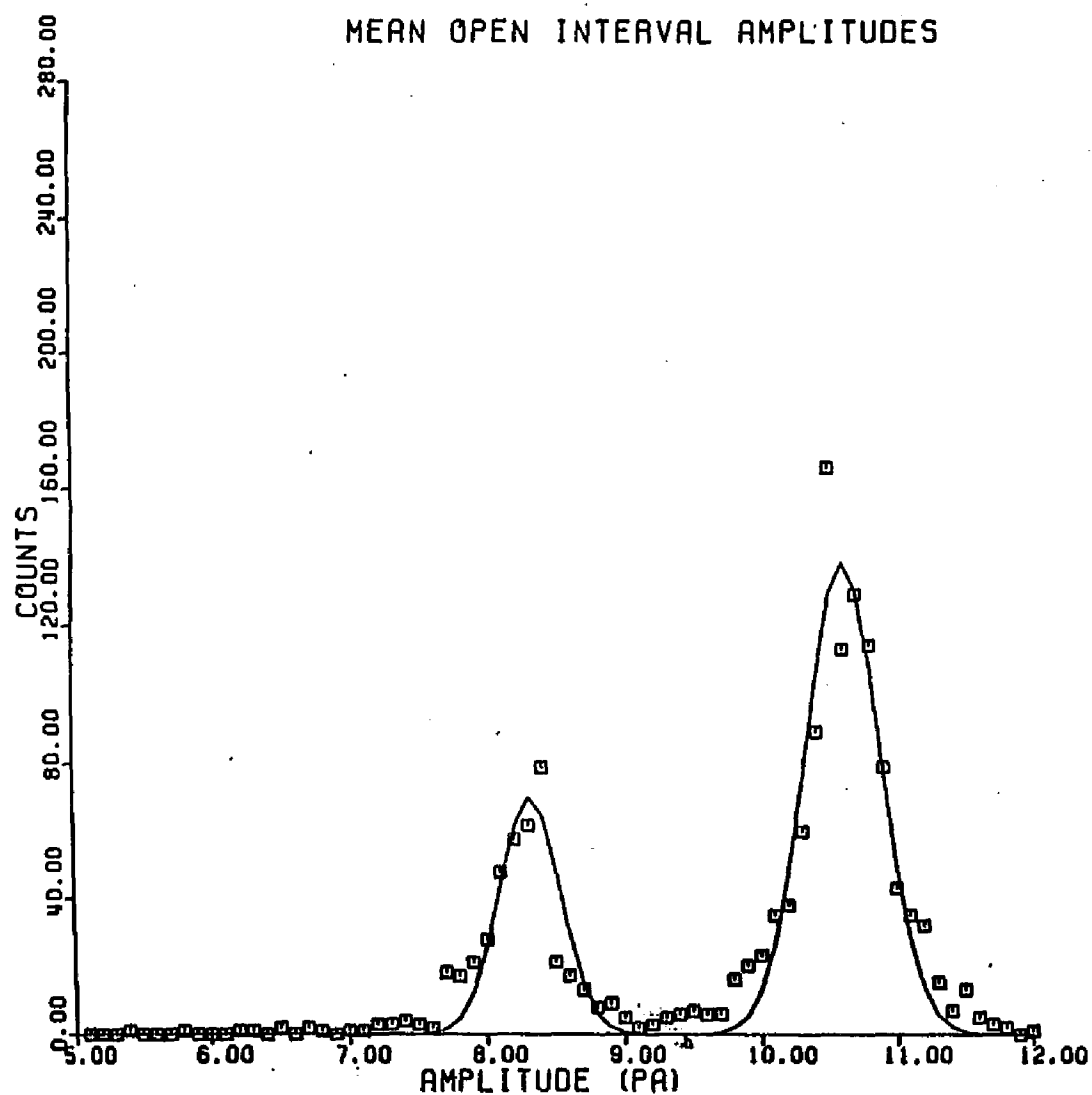


Figure R-12. Amplitude Distribution Functions in a Patch Where the 75% Subconductance State Was Stable

Figure R-13. Amplitude Stationarity of Records in Figure R-12

Measured amplitudes were read from IPROC files and plotted vs. the duration in the entire file. The low conductance state (here 78%) appears intermittently throughout the record until the end where the state is weighted significantly. The means of the two distributions shown in the previous figure were 8.26 ± 0.22 pA and 10.65 ± 0.28 pA. Three standard deviation amplitude windows around these means ($7.7 - 9.0$ pA and $9.8 - 11.5$ pA) allowed segregation of subconductance events for kinetic analysis.

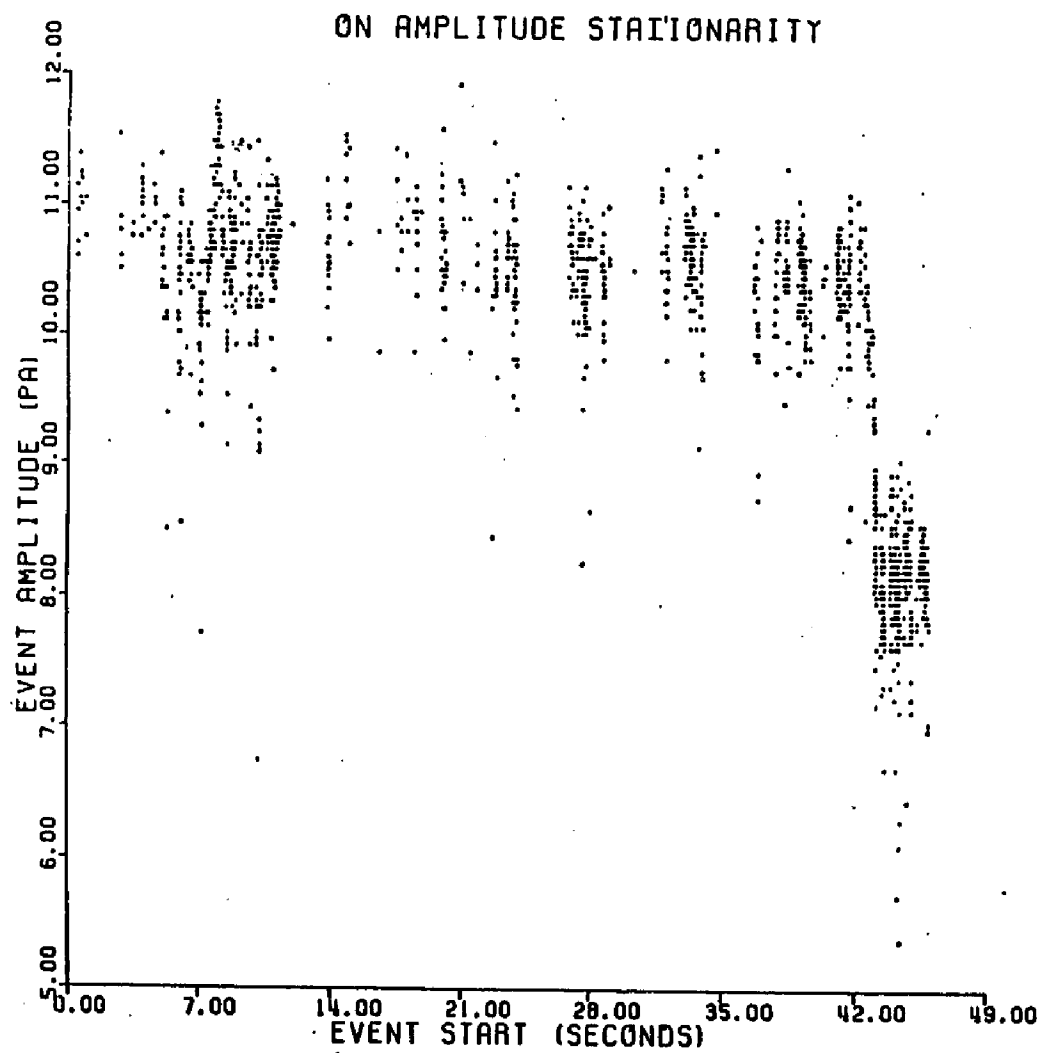


Figure R-13. Amplitude Stationarity of Records in Figure R-12

Figure R-14. Kinetic Analysis of ON Lifetimes Differentiates
Between Subconductance States

Data sets obtained as in Figure R-13 were fit to exponential distributions using FITS. The maximum conductance state has ON times best fit by a single exponential ($\tau = 5.37$ ms) while the lower conductance partner requires two exponential fits (τ s = 0.19 ms and 3.16 ms).

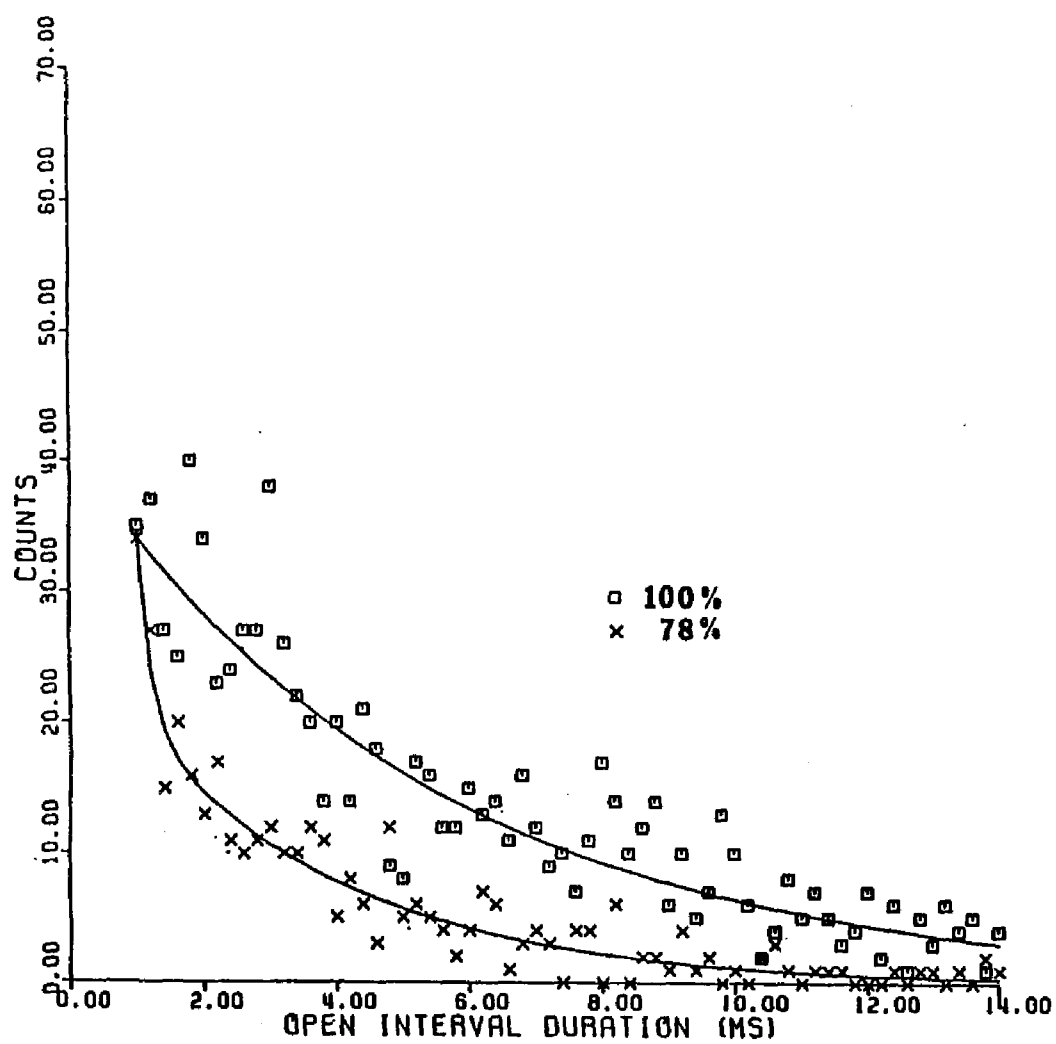


Figure R-14. Kinetic Analysis of ON Lifetimes Differentiates
Between Subconductance States

indicate that the three-quarter level subconductance state is kinetically dissimilar to that of its larger partner. No data was analyzed for the other apparent subconductance states.

B. Kinetic Properties of IR

1. Probability of Opening Versus Voltage

The individual members of an ion channel population are not expected to follow the state function properties of populations of their elements. Instead, the temporal passage from one steady-state to another for a SC must be viewed from the probability of different kinetic states, the relative proportions of each state present at any one time, and the rates of interconversions between states. A first step in understanding how the input parameter (in this case, voltage) governs the gating between any open and closed state is to determine the average amount of time the channel is open or the probability of opening ($P_V(o)$). As for any macroscopic voltage-sensitive K^+ conductance:

$$I(t) = \gamma * N * P_V(t) * (V - V_K)$$

where γ is the SC conductance, N , the number of channels, and $(V - V_K)$, the driving force. The temporal expression of current ($I(t)$) is directly related to the voltage sensitivity of $P_V(o)$. Here $P_V(o)$ is written as $P_V(t)$ to indicate that the probability of being open at any voltage may change with time or be nonstationary (e.g., inactivation). $P_V(o)$ is itself related directly to kinetic mechanism as it gives the proportion of time any one channel is in the open state(s) (or closed states) at any voltage. $P_V(o)$ was determined for kinetic analysis in two ways: (1) by manually sampling a 1 Hz analog filtered average of long stretches (5-20 seconds) of SC currents and

then taking the ratio of the average current to the absolute SC current, or (2) by direct calculation using the open to total time ratio as determined by IPROC (Sachs et al., 1982).

In Figure R-15a are records of current fluctuations in a SC CAP at different steady-state holding voltages with K_0^+ at 140 mM. The I-V curve for this channel is illustrated in Figure R-15b, and shows the steady-state IR property. As appreciated from the data records, depolarization increases while hyperpolarization decreases $P_V(o)$. This is quantified by measurement of $P_V(o)$ vs. ΔV and displayed in Figure R-15c, which shows a Boltzmann function fitted to data from five patches. The $P_V(o)$ vs. voltage relation is very steep around RMP with an e-fold (2.78) change in $P_V(o)$ for about a 34.2 mV change in voltage as measured at the steepest region of the curve. The first-order Boltzmann curve suggests a simple two-state process ($C_{\infty} \rightarrow 0$) but may also represent the average result of the n Boltzmann functions expected for an n -state voltage-dependent mechanism at equilibrium. Such a Boltzmann function for $P_V(o)$ of a SC is similar to the g/g_{max} versus voltage curves used in macroscopic conductance analysis (e.g., Hille, 1984). The steepness cofactor fitted to the $P_V(o)$ vs. ΔV curve is 13.5 mV. Since $k = RT/nF$, where R , T , and F have their usual meaning and n is equal to the number of charges that move across the membrane field during a single gating transition, we calculate that $n = 1.88$ charges. As there is a conformational energy change during channel gating even in the absence of a membrane potential the difference of 1.88 from 2 total charges could represent such energetic loss (Hille, 1984). Therefore, we expect that IR moves a total of 2 elementary charges across the total membrane field during a single

Figure R-15. Voltage Dependence of IR Channel Gating

(a) Voltage sensitivity of channel gating. Representative traces from a CAP with 140 mM K_0^+ show the changes in kinetics upon changes in the holding potential. Changes in the magnitude of the events at -50 mV are due to briefer openings unresolved by the chart recorder.

(b) IR I-V Characteristics. I-V plot from the CAP data represented in (a) with 140 mM K_0^+ .

(c) $P_V(o)$ vs. Voltage. $P_V(o)$ was determined from analog filtering or using IPROC and plotted vs. holding potential for five patches. $P_V(o)$ changes e-fold for 34.2 mV change in holding potential for the steepest region of the curve between RMP and -30 mV. All were CAPs that had 140 mM K_0^+ . For some patches computer-generated $P_V(o)$ estimates showed precipitous changes relative to the boundary points at other voltages. In these few cases it appeared that IPROC had lost the baseline estimation. These points were not included in the Figure.

Figure R-15. - Continued

The $P_V(o)$ vs. V points were fitted with a Boltzmann function:

$$P_V(o) = \frac{1}{1 + \exp(-(\Delta V - \Delta V_0)/k)}$$

using a Marquardt, nonlinear least-squares method where V_0 is a parameter defined as that V at which $P_V(o) = 0.5$. k reflects the steepness of the curve and is equal to RT/nF , where R , T , and F have their usual meaning and n reflects the number of charges moving across the membrane in the gating transition. From the fit $V_0 = -21.3$ mV and $k = 13.512$ mV and, therefore, $n = 1.88$. Therefore approximately two gating charges move across the entire transmembrane field drop or, for example, four charges move across half of the potential drop.

(d) Time-Averaged SC Currents. The I-V plot from (b) was multiplied by $P_V(o)$ (in (c)) at the corresponding voltages to obtain the time-averaged SC current (●). Open channel current (○) is shown for comparison over hyperpolarized ranges of potential.

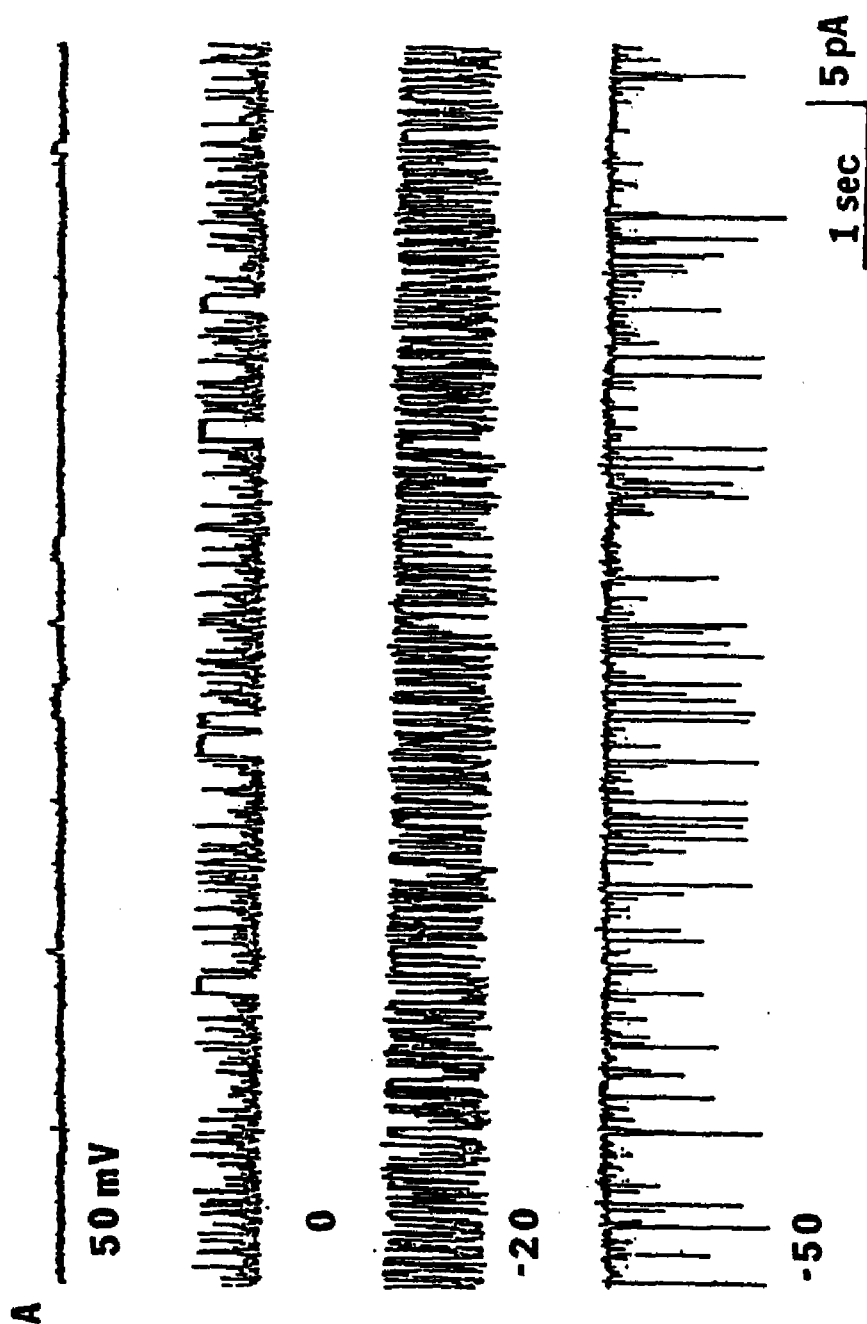


Figure R-15a. Voltage Dependence of IR Channel Gating-- Voltage Sensitivity of Channel Gating

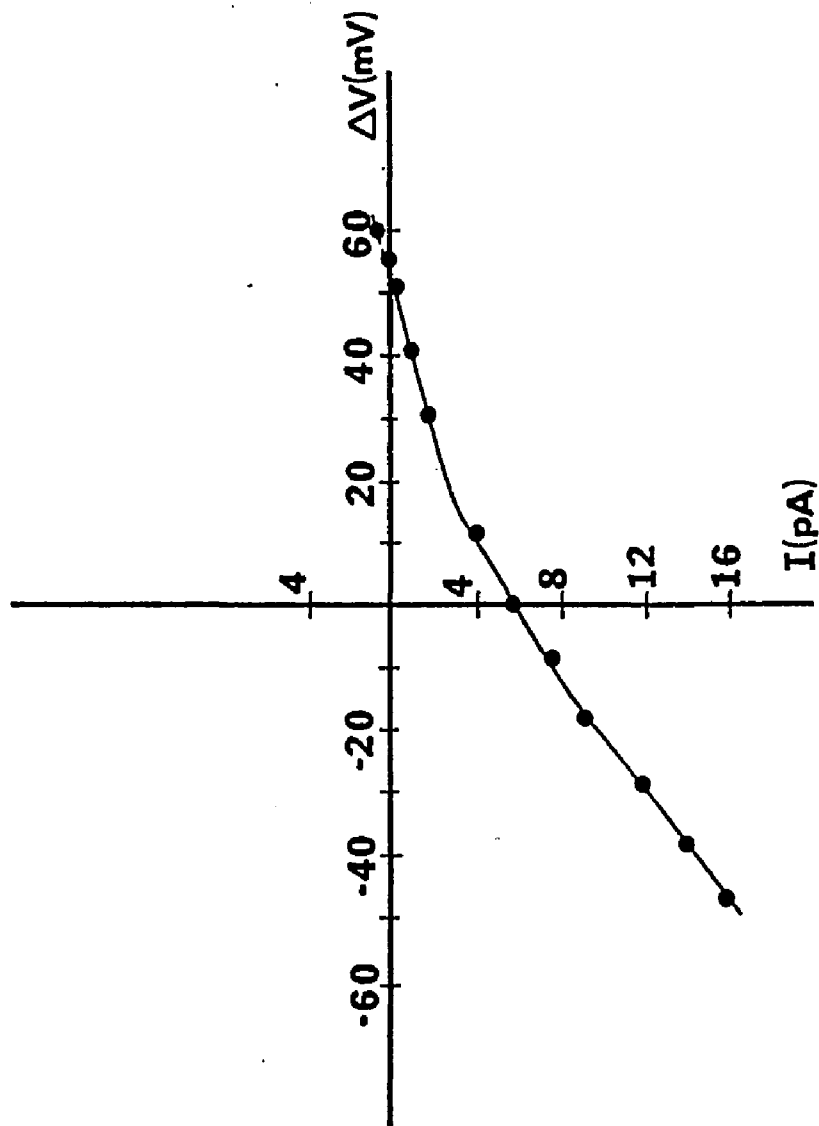


Figure R-15b. Voltage Dependence of IR Channel Gating-- IR I-V Characteristics

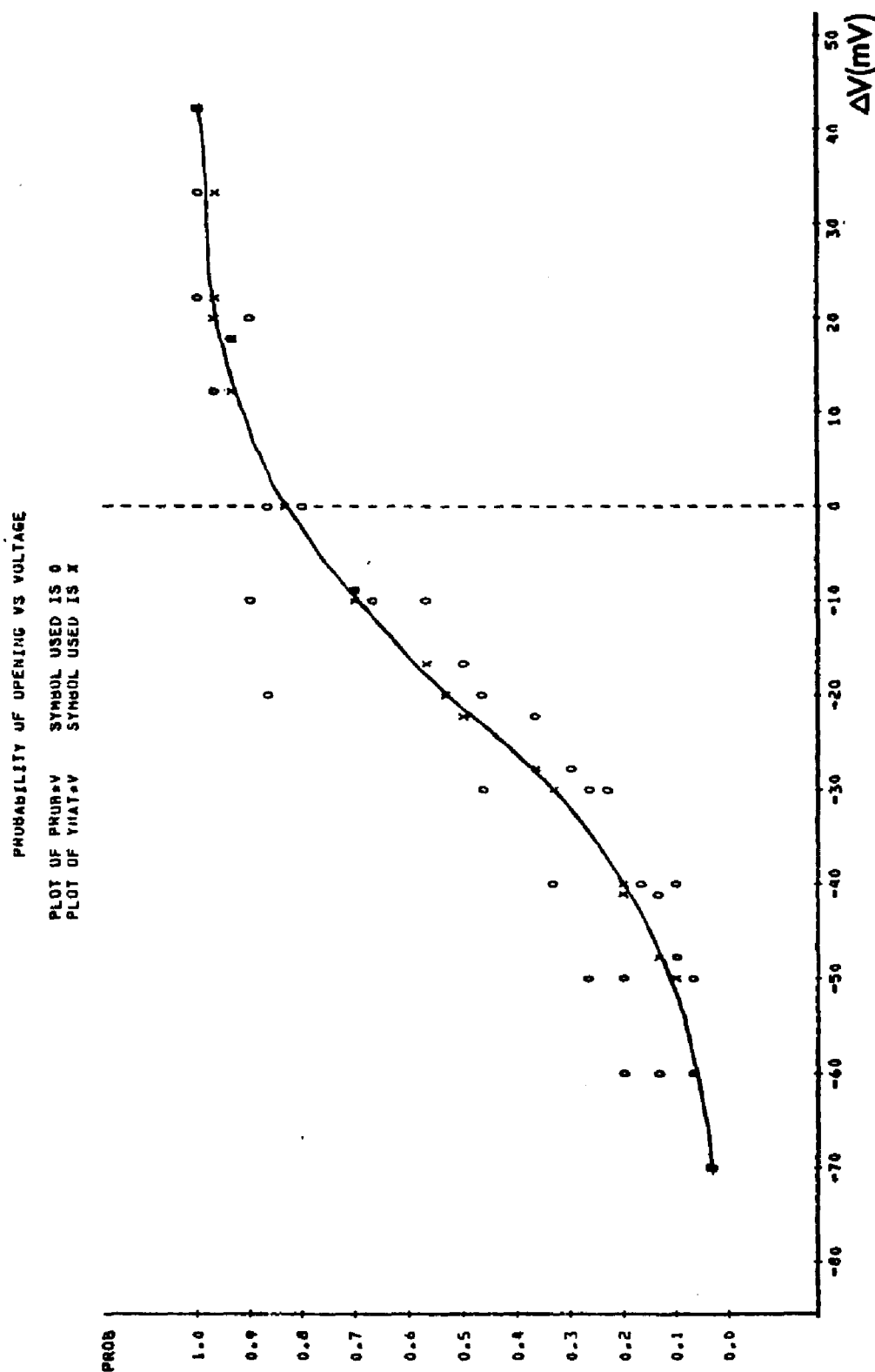


Figure R-15c. Voltage Dependence of IR Channel Gating--
 $P_{V(0)}$ vs. Voltage

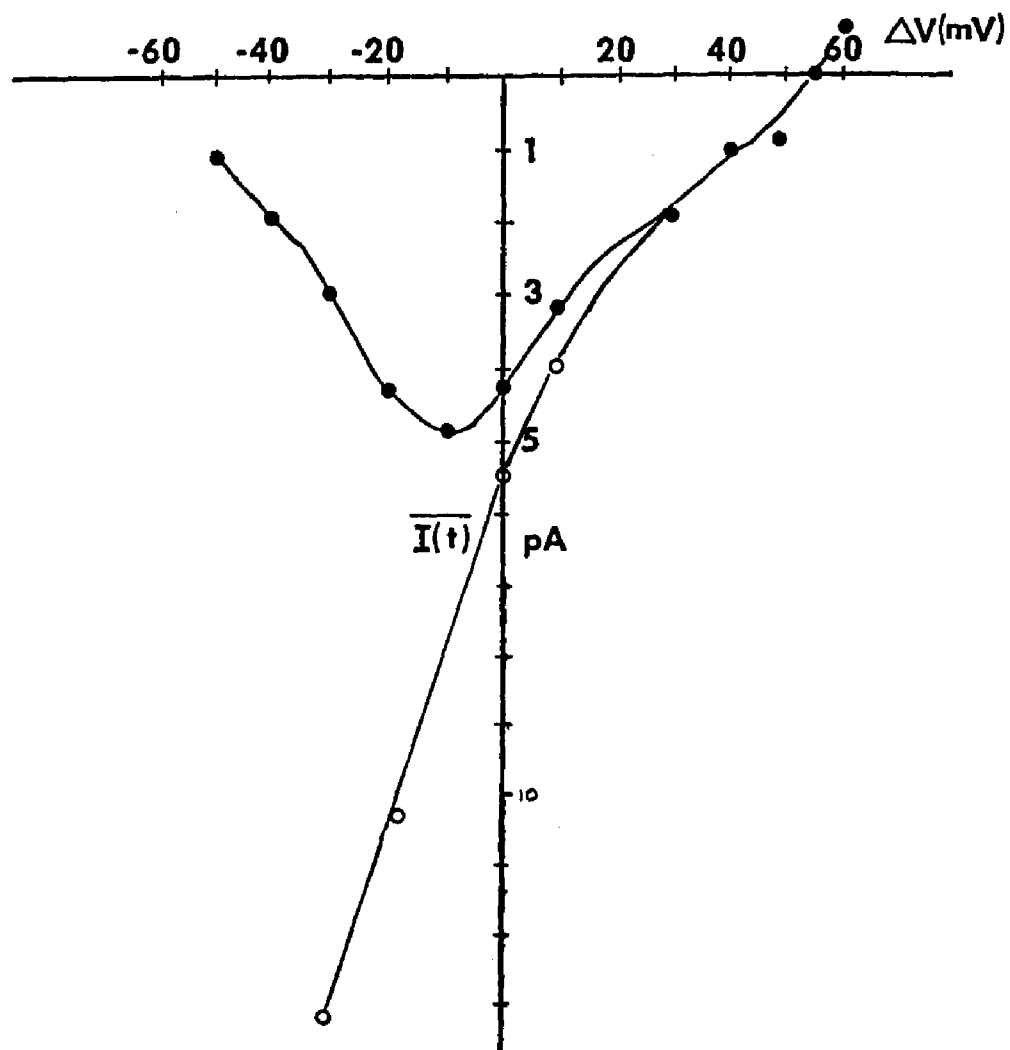


Figure R-15d. Voltage Dependence of IR Channel Gating--
Time-Averaged SC Currents

gating transition or a larger number of charges over a portion of the total membrane potential drop (e.g., 3 charges moving over 66.6% of the membrane field drop). As channel opening often occurs by passage through a series of states smaller than the maximum number available to the channel (because one closed state may be favored by channel closure yet is closer kinetically to the open state than other more distant closed states), the concept of limiting logarithmic potential sensitivity arises (Hille, 1984). This property purports an equivalent gating charge which is always smaller than the actual number of charges that move. As we shall see later the number of (Markovian) closed states appears to equal three for IR, implying three separate transitions to open a SC from the longest lived closed states.

We take particular interest in the fact that the steepest region of $P_V(o)$ is situated slightly hyperpolarized from the RMP. The channel is mostly active at RMP (where $P_V(o)$ is approximately equal to 0.8), half active ($P_V(o)$ is approximately equal to 0.5) at -21 mV, nearly inactive by -60 to -70 mV and fully active ($P_V(o)$ approximately equal to 1.0) by +20 mV. SC IRs of other preparations have a high $P_V(o)$ around RMP but typically require blocking ions to induce kinetic fluctuations (Fukushima, 1981; Omori, 1978, 1980, 1981a,b). It is likely that IR in CHNs has a gating parameter directly responsive to voltage, rather than blocking ion-induced gating transitions, however.

Observation of channel records shows that activity around RMP is patterned into long openings interrupted by very short gaps. These long openings are observable because they are bounded by closures much longer than the brief gaps. Such SC activity has been called bursting. A burst, operationally defined, is a group of openings

bounded serially by brief closures, with groups of such openings separated by longer closures of sufficient duration that the exponential distribution of closed times will represent passage through at least two kinetically distinct closed states (Colquhoun and Hawkes, 1981, 1982).

We next consider the nature of the $P_V(o)$ versus ΔV relationship. One can observe that the burst length (defining burst as groups of openings separated by closures no greater than 2 ms) appears sensitive to the holding potential. As the patch is depolarized from 0 mV, the amount of time spent in long openings (apparent bursts) increases (with the number of brief gaps apparently proportional to the burst length) as does $P_V(o)$. When the patch is hyperpolarized by 10 mV, the burst length has collapsed significantly, corresponding to the steeply decrementing region of the $P_V(o)$ versus voltage curve. Incremental hyperpolarization progressively decreases the burst duration until at ΔV equal to -50 through -60 mV, burst openings are recorded only as ms-order events, as in the limit there appears to be only a single opening per burst. Correspondingly, as the interburst interval increases with hyperpolarization, $P_V(o)$ approaches zero.

Thus, we have achieved a relatively detailed overview of the kinetic operation of IR. It would appear that the voltage sensitivity of $P_V(o)$ is largely a function of the voltage-sensitivity of the burst length (defined as above) and the related interburst interval. The length of the burst and the $P_V(o)$ will be due to the voltage sensitivity of rate constants that provide for the burst duration in any putative mechanism.

The $P_V(o)$ versus voltage function contains a great deal of

information, since the averaging effect is taken over all closed and open states. We can use this function to predict how the average WC currents would appear under identical conditions by point-by-point multiplication of the SC I-V curve by the $P_V(o)$ vs. ΔV curve, which is done in Figure R-15d. This function, scaled by the number of channels of this type in the WC membrane should be similar to WC steady-state I-V plot generated by voltage clamp under identical boundary conditions. In this patch $+50 \text{ mV} \leq \Delta V_{\text{rev}} \leq +60 \text{ mV}$ and the mean (temporally averaged) inward current increases approximately linearly from ΔV_{rev} until around -10 mV where it reaches a maximum and then decreases steeply at more hyperpolarized potentials. The mean plot illustrates a NRR around RMP. A possible function of this negative resistance region in excitability phenomenon will be discussed later. The steeply decreasing region of the curve at large hyperpolarizations is associated with a low $P_V(o)$ and an attenuated burst length. Since these $P_V(o)$ determinations are steady-state estimates (holding potential constant) the channel mechanism should have achieved an equilibrium proportion of states; in all cases observed, the data appeared stationary (in the four patches on which this was tested using IPROC). Transient step-voltage perturbations from potentials where $P_V(o)$ is high to potentials where the steady-state $P_V(o)$ is low would be expected to show transient inward macroscopic currents that are larger than those at steady-state.

Under such circumstances the channel population shifts in time such that closed states gradually predominate the state distribution. This is due to the Markov property of memoryless transitions between states in a Poisson process, coupled with rate constants that instantaneously

respond to the perturbation, and a subsequent temporal redistribution of the probabilities of different states after step impulses (Aldrich and Yellen, 1983; Colquhoun and Hawkes, 1981; Horne and Lange, 1983; Horne and Standen, 1983).

Observation of a number of patches where K_o^+ was varied illustrates that the burst length of $P_v(o)$ may be somewhat sensitive to K_o^+ . This is shown in Figure 16a-c, where traces of data at 30 mM, 140 mM, and 300 mM K_o^+ are shown. One can observe that at "low" K_o^+ (30 mM) the hyperpolarization from RMP required to decrease $P_v(o)$ to about 0.5 is about -20 mV (similar to V at $P_v(o) = 0.5$ at 140 mM K_o^+ in Figure R-15c). However, $P_v(o)$ is near zero at about -50 mV with 30 mM K_o^+ (0.1 - 0.25 at 140 mM K_o^+) so the relationship of $P_v(o)$ to voltage may be somewhat steeper at 30 mM K_o^+ . At 300 mM K_o^+ (here two channels active and assumed identical) the $P_v(o)$ versus voltage data appears shifted by about -20 mV relative to that at 140 mM; it is possible that this is a unique effect of K_o^+ on $P_v(o)$, but one should scale $P_v(o)$ to take into account the presence of two channels. The possibility of K_o^+ participation in gating is compelling, but inasmuch as all potentially controlling variables that affect kinetics are unclear, consideration of this question is delayed for future studies. At present, a possible role for K_o^+ in gating cannot be excluded.

On two patches it was possible to establish the IOP mode and study the sensitivity of channel gating to Ca_i^{2+} . Shown in Figure R-16d is a segment of data from a SC patch with symmetrical 140 mM KCl with 1 mM Ca_i^{2+} and where the channel is clearly closed. Upon introducing the cytoplasmic surface of the channel into a

Figure R-16. Possible Effects of K_o^+ and Ca_i^{2+} on $P_v(o)$.

(a) Representative traces of a CAP at 30 mM K_o^+ .

(b) Representative traces of a CAP at 140 mM K_o^+ .

(c) Representative traces of a CAP at 300 mM K_o^+ .

Displacement potentials from RMP are indicated.

(d) Possible effect of Ca_i^{2+} on IR channel activity

A SC IR patch with 140 mM K_o^+ was isolated into an IOP with 140 mM KCl and 1 nM Ca_i^{2+} in the bath (see Table I). Upon switch to a 140 mM KCl and 10 nM Ca_i^{2+} solution (by perfusion pipette) the channel activity promptly increased. This process was reversed by switching to a 1 nM Ca_i^{2+} and 140 mM KCl solution. The transpatch potential was held at -20 mV throughout.

g_{IR} 30 K_o⁺

a

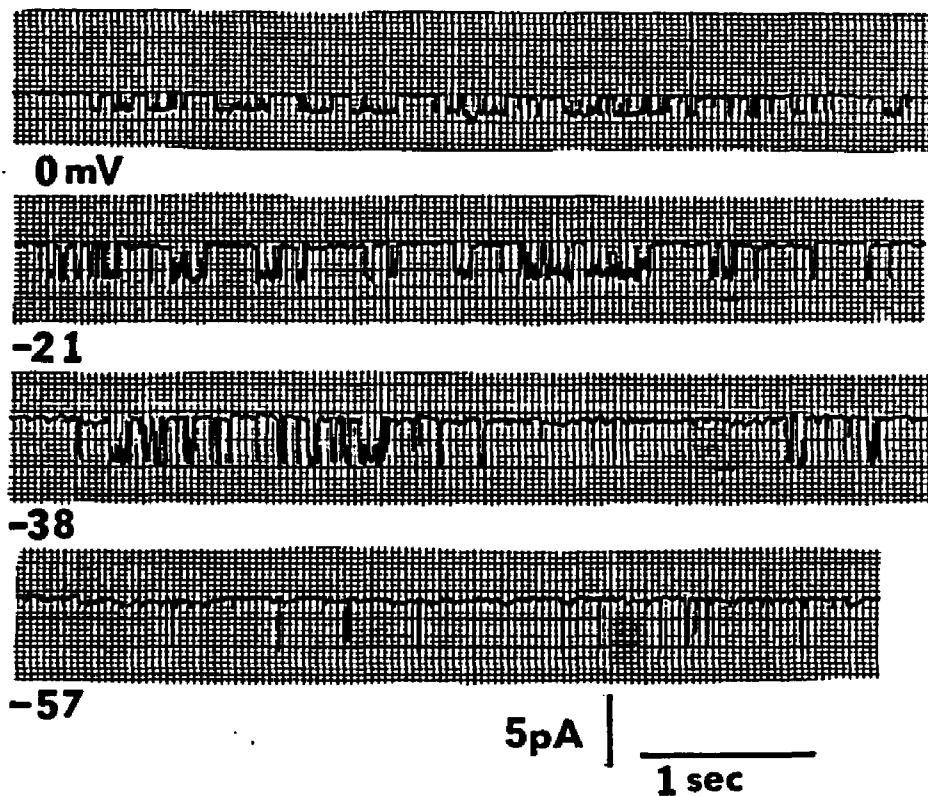


Figure R-16a. Possible Effects of K_o⁺ (30 mM) on P_o(o)

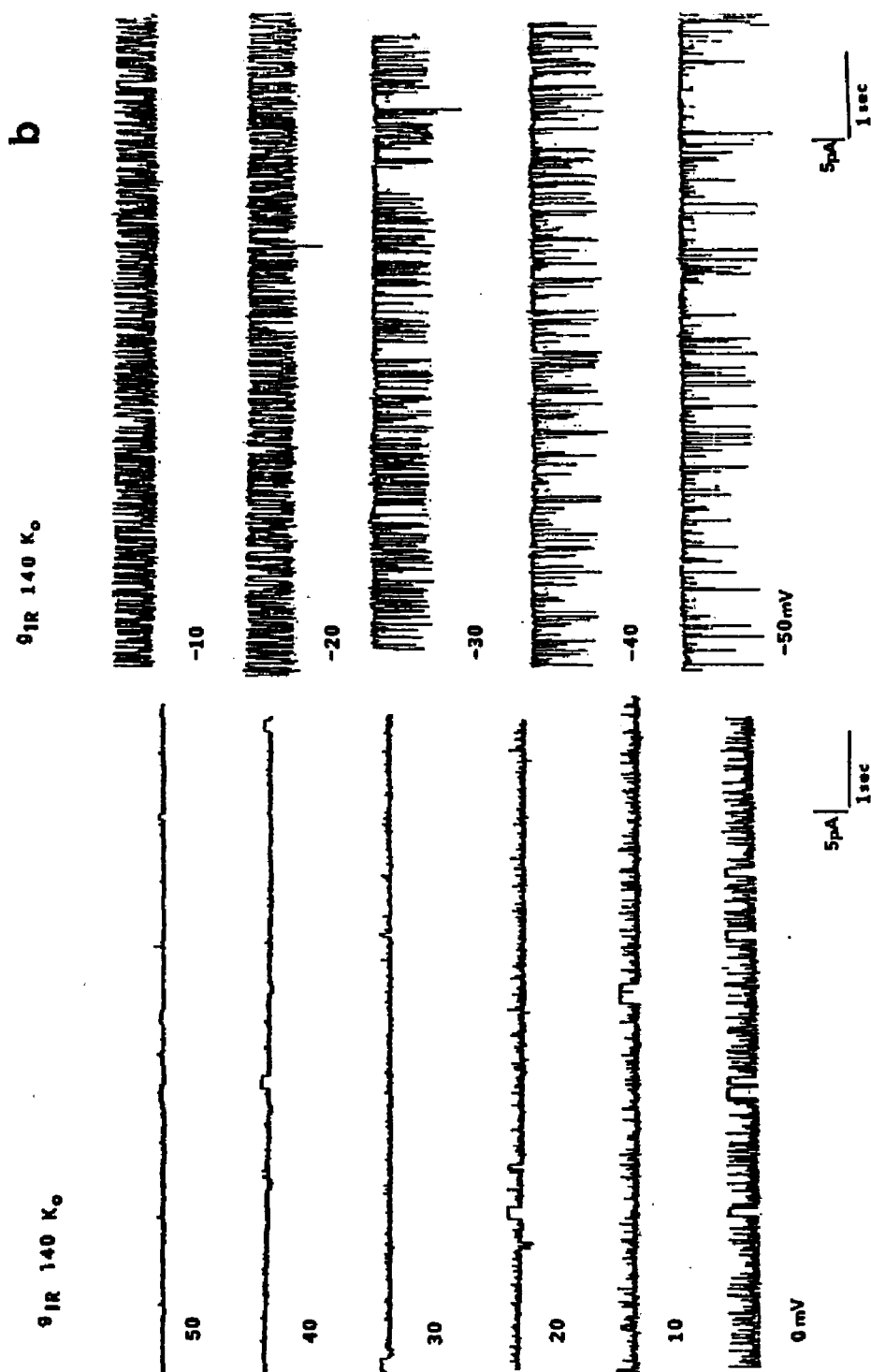


Figure R-16b. Possible Effect of K_o⁺ (140 mM) on P_v(o)

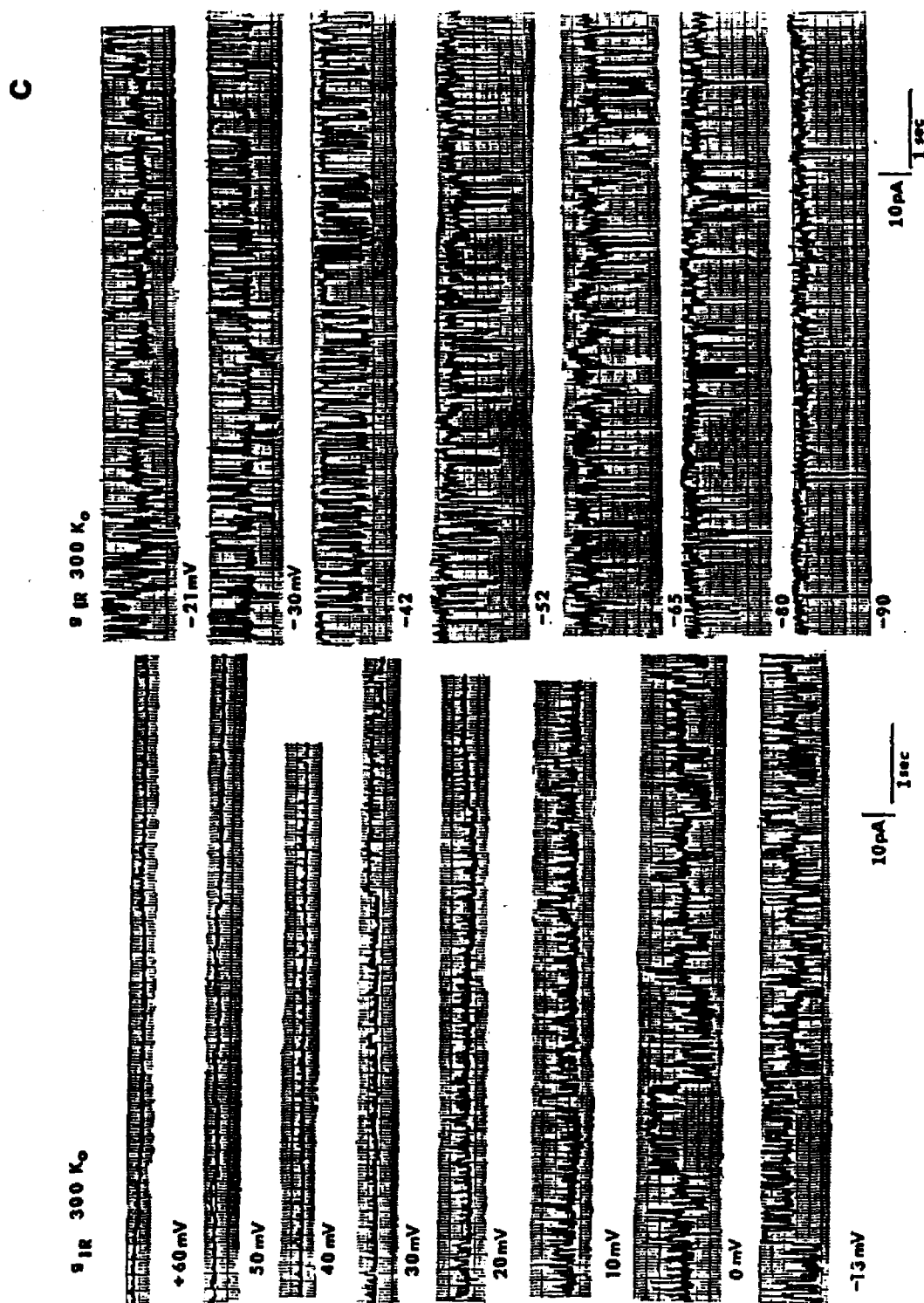
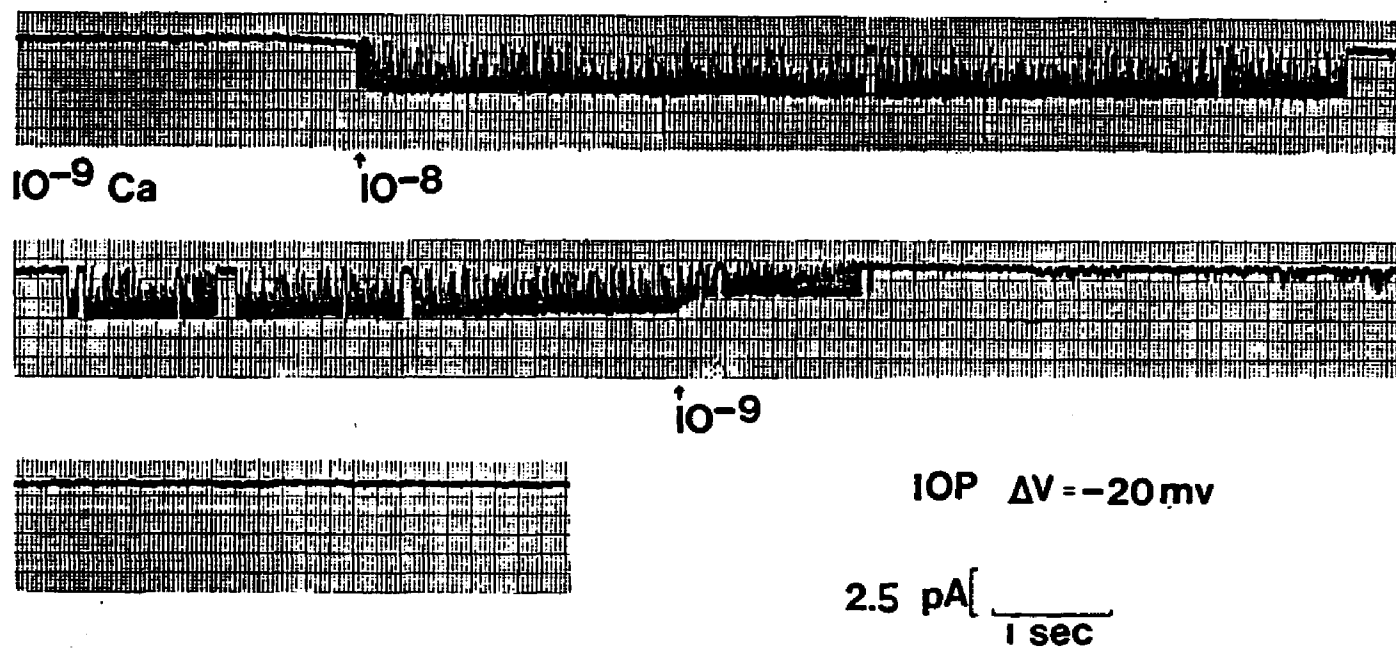


Figure R-16c. Possible Effect of K_o⁺ (300 mM) on P_{V(o)}

Figure R-16d. Possible Effect of Ca_i^{2+} on $P_v(\omega)$



stream of 140 mM KCl plus 10 nM Ca_i^{2+} prompt activation of channel activity occurs at -20 mV (absolute ΔV , would correspond to approximately $\Delta V = +30$ mV, where $P_V(o)$ is approximately 1.0 in CAPs). At this level of Ca_i^{2+} and absolute membrane voltage, $P_V(o)$ is very high (approximating: 0.85 - 0.95). When 140 mM KCl and 1 nM Ca_i^{2+} is again presented to the IR channel activity promptly stops after about 2 seconds. This data as well as data from another experiment indicates that IR is both voltage and Ca_i^{2+} sensitive and that the Ca_i^{2+} sensitivity is about two orders of magnitude greater than other reported CaK channels. In another patch where the IR was found and isolated into an IOP, channel activity was fully established ($P_V(o)$, very high) at 10 nM Ca_i^{2+} . In that same patch a channel with a conductance of over 300 pS became active at depolarized potentials when Ca_i^{2+} levels reached about 1 μM . The behavior of this larger conductance channel was typical of the well studied CaK channels in other systems. Thus, although IR does have a Ca_i^{2+} sensitivity, it is distinguished from larger CaK channels by its on-cell rectification, its smaller conductance, and its high sensitivity to Ca_i^{2+} . Moreover, this data suggests that the intracellular level of Ca_i^{2+} in intact cells is about 10 nM at RMP or slightly less based on comparison of voltages and $P_V(o)$. As the probability of opening increases so dramatically (essentially from 0 to 1) over a ten-fold change of Ca_i^{2+} , its dependence on Ca_i^{2+} must be a very steep Boltzmann function (half probability estimated at 5 nM). This steep relationship could explain the variability of $P_V(o)$ that is seen from patch to patch (e.g., see Figure R-15c) as being related to steady-state intracellular levels of Ca_i^{2+} . The

levels of intracellular Ca_i^{2+} for resting neurons are on the order of 1 to 10 nM, which is consistent with these results. Much further work must be done to more quantitatively establish the apparent relationship of Ca_i^{2+} to the voltage sensitivity of IR.

2. Kinetic Analysis of Open and Closed Times

In this section we will analyze channel kinetics of selected elements of the data set with hopes of supporting or ruling out certain mechanistic models as well as providing a more quantitative interpretation of ensemble and macroscopic currents. Figure R-17 shows an IR channel with 140 mM K_o^+ at -20 mV. Around RMP the IR channel exhibits complicated kinetics with series of brief events (gaps) that clearly group into bursts separated by tens to hundreds of milliseconds. These bursts appear to group into sets or clusters separated by very long closures on the order of seconds. A channel process with gaps, bursts, and clusters requires a minimum of three closed states and one open state for an adequate kinetic description (Colquhoun and Hawkes, 1982; Horne and Standen, 1983). In this analysis, for convenience, the open state will represent the maximum conductance state (100%) for the sake of simplicity - i.e., incorporation of the three other apparent substates would make detailed analysis untractable. Failure to consider substates, however, is justified because they are relatively rare events (except possibly for the three-quarter state) and their probabilities of occurrence are assumed very low ($\ll 1\%$). A potential hazard in this assumption is that the many brief gaps inside bursts are unresolved due to bandlimited frequency response of the recording instrument and may represent closures not to any closed state but rather transitions to an open subconductance state. This could

Figure R-17. Complex Kinetic Patterns Around RMP Show Bursting and Clustering in IR

A continuous stretch of data from a 140 mM K_0^+ CAP at -20 mV is shown to illustrate the complicated kinetics in this voltage sensitive channel. Apparent bursts (b), apparent clusters (c) of bursts, apparent interbursts intervals (ibi), and apparent intercluster intervals (ici) are labeled. The cell was bathed in Solution B.

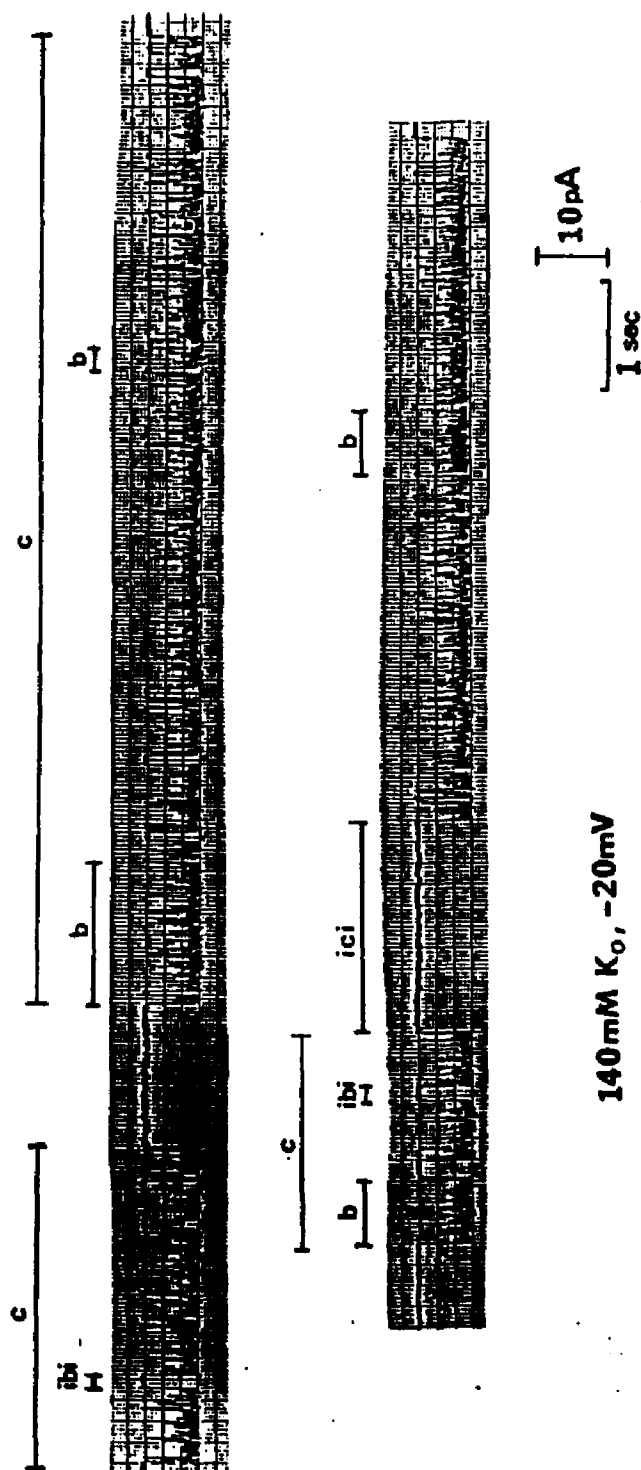


Figure R-17. Complex Kinetic Patterns Around RMP Show Bursting and Clustering in IR

result in a serious overestimation in the measurement of brief closure events (gaps/burst).

The first step in the process of SC kinetic analysis is to carefully measure the open and closed times at as wide a bandwidth as possible. Most of the results to be discussed here were acquired with a Bessel filter set at 2.25 kHz and a tape recorder bandwidth of 2.25 kHz. Figure R-18 shows a segment of data played onto a chart recorder (frequency response about 100 Hz) at one-eighth of original tape recording speed (transfer bandwidth about 275 Hz). Open and closed times were marked off, measured manually and stored in an array. We begin here with data at 140 mM K_0^+ and at $\Delta V = 0$ mV holding potential (RMP) where over 1400 open and closed times were measured.

Initial attempts to fit the closed times to a single exponential distribution function were unsuccessful as events separated into bins of millisecond order, tens of milliseconds, and seconds. Figure R-19 shows the original dispersion of the closed time data plotted as a cumulative distribution function (Siegelbaum et al., 1984). Semi-logarithmic transform of the data illustrates the three exponential decomposition with characteristic τ 's of 9.4 ms, 173.4 ms, and 793.5 ms. From the Markov process assumption, each unique state should fit an exponential distribution function (Horne and Lange, 1983). Using an alternative method in Figures R-20a-c the closed time data were arbitrarily separated into bins corresponding to short duration gaps, interburst intervals, and intercluster intervals and plotted in histograms. As shown in Figures R-20a-c, the natural logarithmic transforms in each case are fit rather well by linear functions displaying the apparent isolation of each distribution. Moreover, the

Figure R-18. Manual Measurement of Open and Closed Times.

Data was from a CAP at 140 mM K_o^+ and $\Delta V = 0$ mV was output from tape (stored at 2.2 kHz) at 275 Hz onto a Gould chart recorder (125 mm/sec, bandwidth of about 100 Hz as measured by this investigator). Open and closed times were manually marked off as illustrated, measured with a centimeter ruler, and stored in tables. Data were then plotted into discontinuous or continuous histograms and their natural logarithmic transforms were taken and plotted to determine fits to single or multiple exponentials.

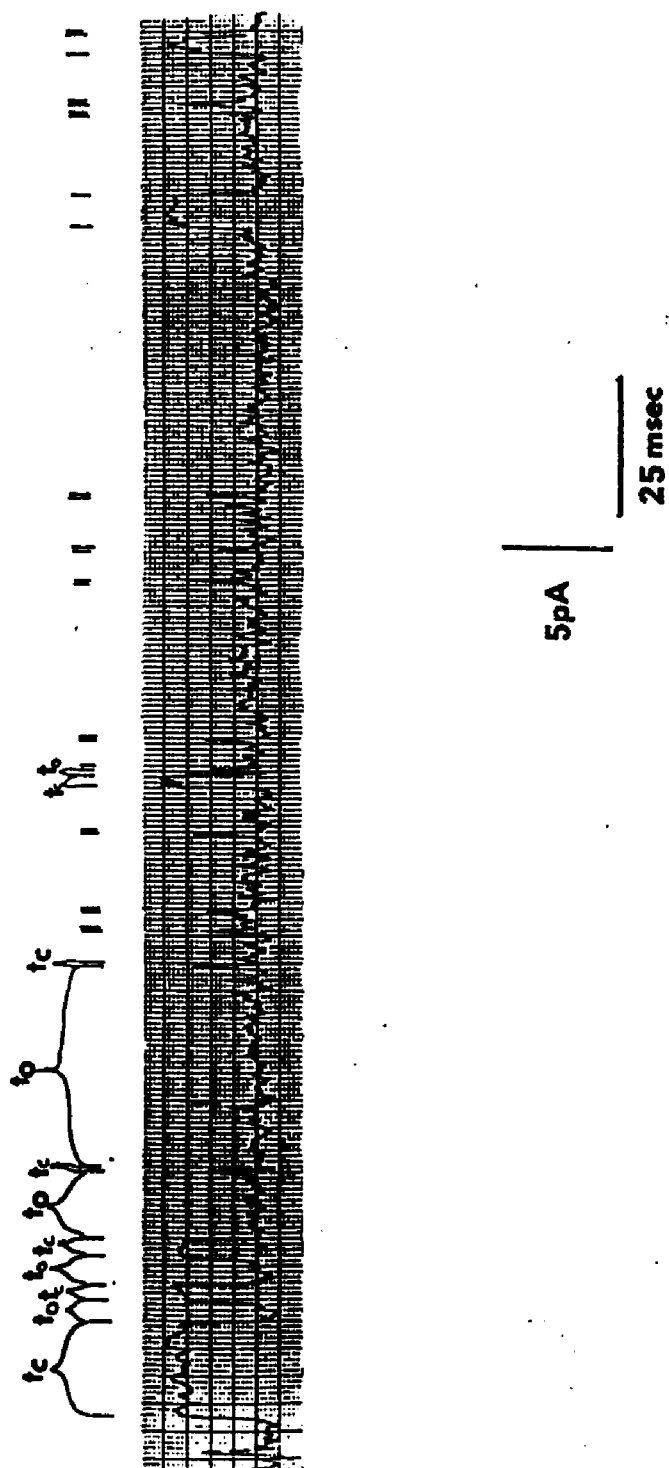


Figure R-18. Manual Measurement of Open and Closed Times

Figure R-19. Cumulative Distribution of Closed Times

Closed time durations were collected manually from a 140 mM K_0^+ CAP held at 0 mV. Cumulative distributions were plotted for all closed times according to the method of Siegelbaum et al. (1984). The total closed time distribution requires three distinct exponentials for fitting of short, medium-long, and long closed times. The natural semilogarithmic transform (inset) of the data illustrates the decomposition to three exponentials (taus of 9.4 ms, 173.4 ms, and 793.7 ms) which were obtained by regression lines fit to the data by eye.

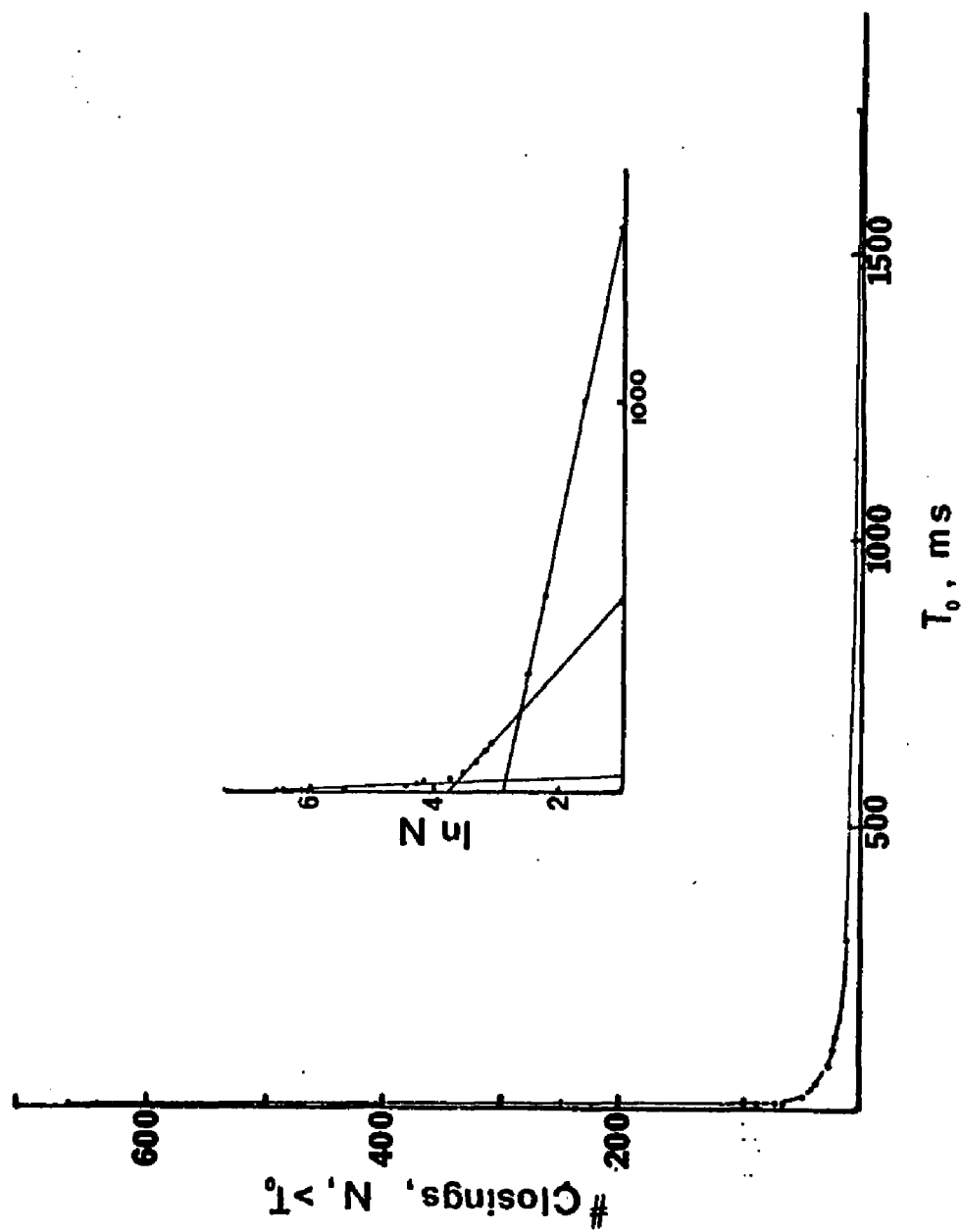


Figure R-19. Cumulative Distribution of Closed Times

Figures R-20a-c. Closed Time Distribution Function at RMP
Decomposes into Three Exponentials

Closed time data from Figure R-19 was decomposed into three temporal categories and plotted vs. bin widths of (upper panels):

- (a) 2 ms (for fast closures),
- (b) 20 ms (for medium-long closures) and
- (c) 200 ms (for long closures).

Data were from a 140 mM K_0^+ patch with a SC present and held at RMP. Taus were calculated from the slopes of the natural logarithmic transform plots (lower panels). All curves and lines were fit visually.

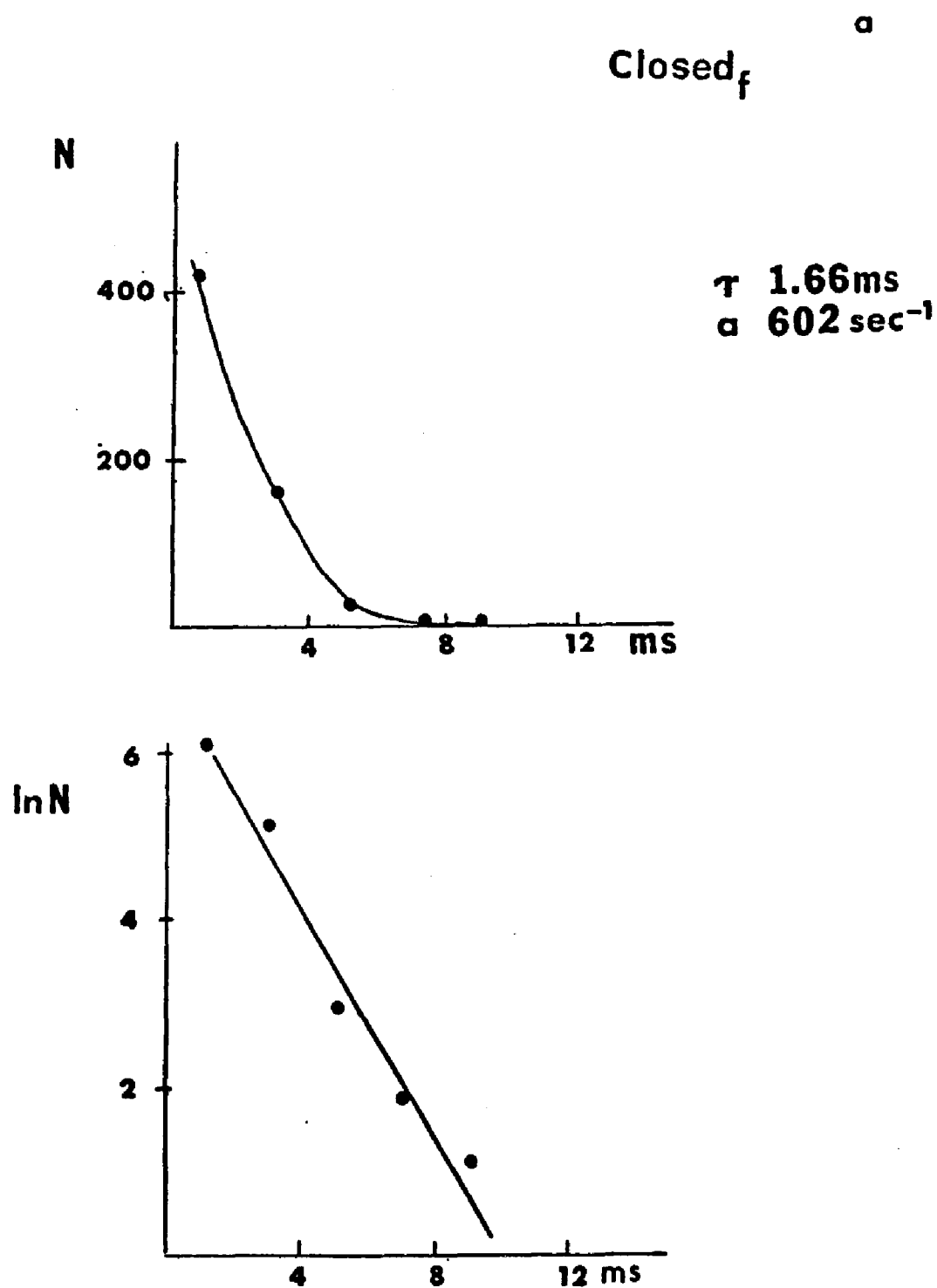


Figure R-20a. Closed Time Distribution Function Decomposes into Three Exponentials (Short Closed Times)

b

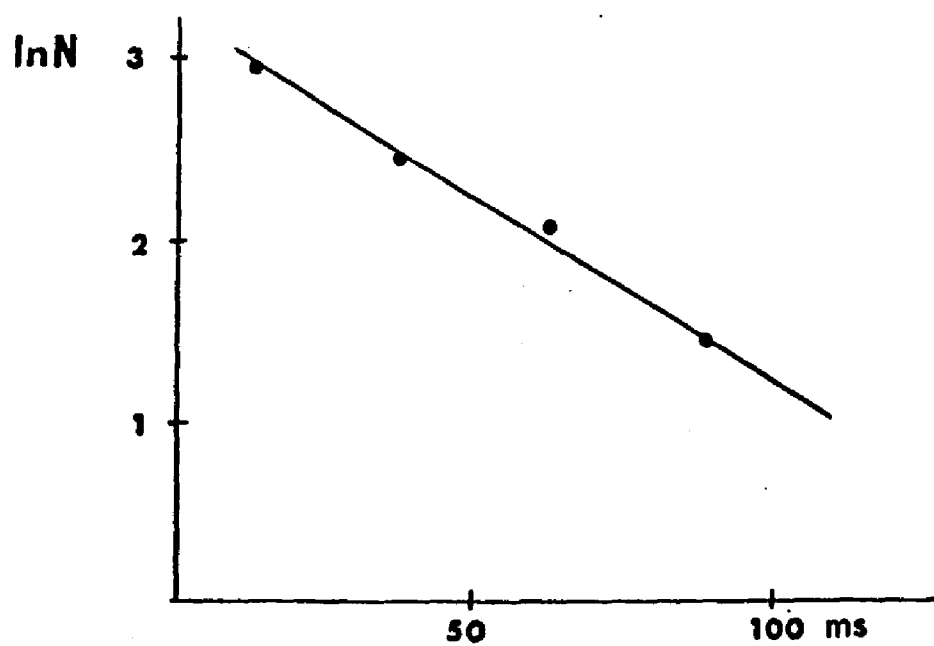
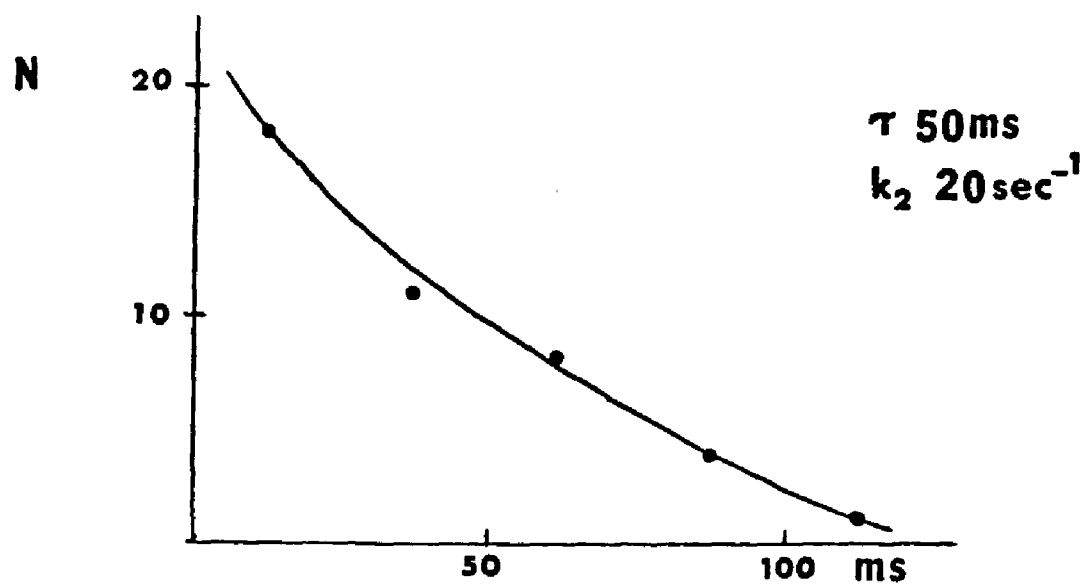
Closed_m

Figure R-20b. Closed Time Distribution Function Decomposes into Three Exponentials (Medium-Long Times)

c

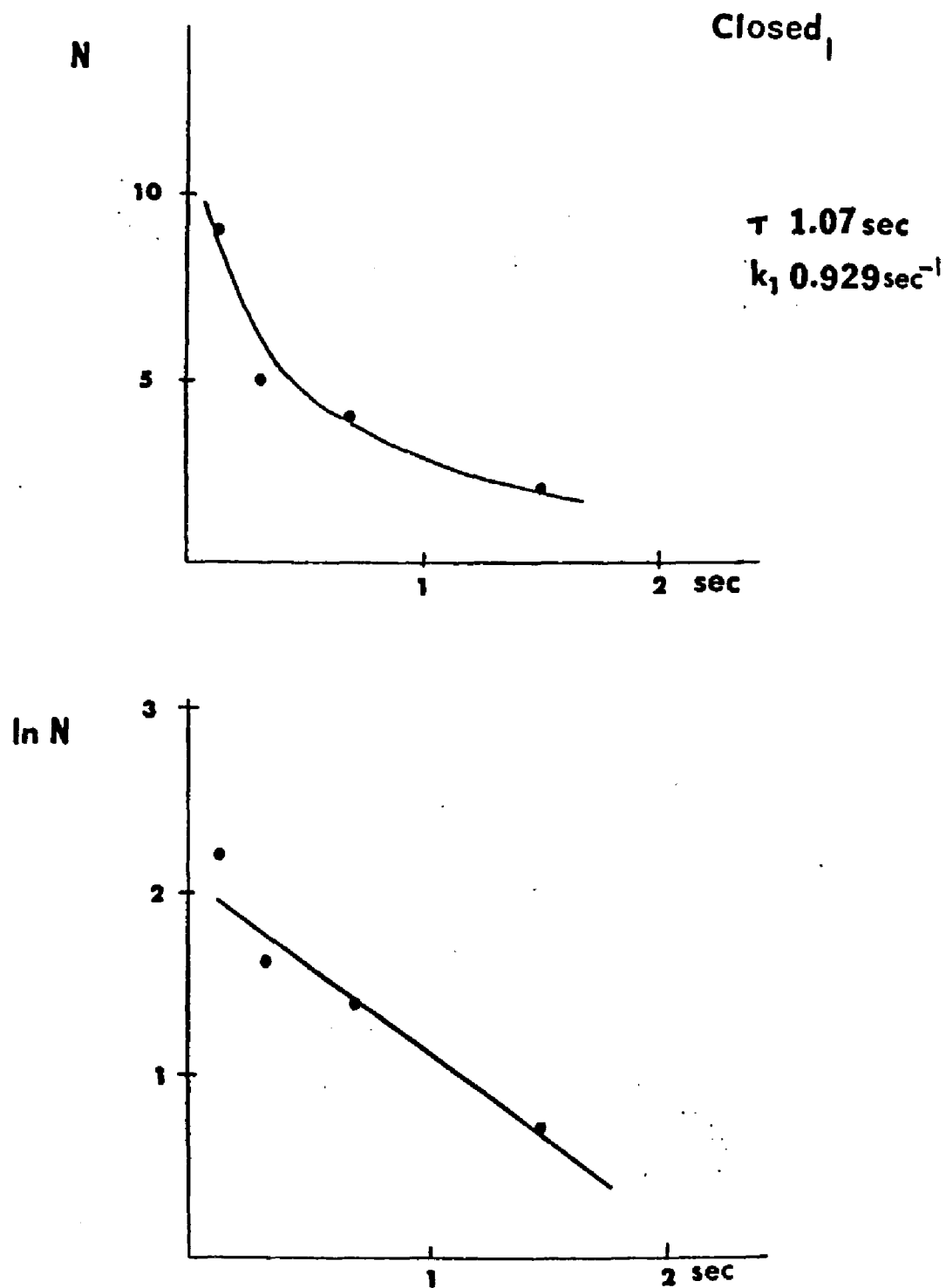


Figure R-20c. Closed Time Distribution Function Decomposes into Three Exponentials (Long Closed Times)

probability that a 50 ms long event originates from an exponential distribution with a 1.6 ms mean or the probability that a 1.0 second closure originates from distribution with a 50 ms mean is very small. From each regression the Tau of each closed state (reflects the inverse of all rates out of that state) is given by the slope of the function; the transition rate is the inverse of the time constant. The fast closed time, which represents the gaps inside of bursts, has a Tau of 1.66 msec or a rate of 602/sec. The medium-closed time, which represents the apparent interburst interval, has a Tau of 50 ms or a rate of 20/sec. The slowest closed time, which represents the apparent intercluster intervals, has a Tau of 1.07 sec or a rate of 0.929/sec. Similar results were obtained by the cumulative distribution of all closed times, which is expected to be the more accurate method because it avoids the bias created by binning the data into apparently separate ranges (Siegelbaum et al., 1984).

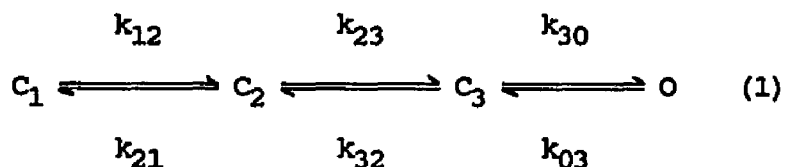
In contrast to the closed times, the open times at $\Delta V = 0$ mV holding potential fit a single exponential distribution as shown in Figures R-21 with a Tau of 18 ms or a rate of 55/sec. A summary of open and closed time distributions at 0 mV is plotted in Figure R-22.

The burst length, defined here as a group of openings separated by no greater than some maximal closed time (2 ms to exclude brief closures), can also be evaluated for its distribution function. We have discussed above the manner in which burst length is an important voltage-sensitive parameter that has an apparent strong influence on $P_V(0)$. We will now quantitate this observation. Shown in Figure R-23 is the distribution function of the burst length at $\Delta V=0$ mV, which fits a single exponential distribution with a Tau of

317 ms or a rate of 3.15/sec. The voltage sensitivity of the burst length is shown by plotting burst length distributions and their natural logarithmic transforms at different voltages, as in Figure R-24 for a SC patch. It can be seen that the burst lengths fit single exponential distribution functions at each voltage. The rate constant (inverse of time constant) at each voltage is plotted versus voltage in Figure R-25. It can be seen that the burst τ 's decrease at hyperpolarized potentials or the average of all possible rates away from the open state increases. At increasingly hyperpolarized potentials, the rates away from the open state would be larger, making the probability of being in the open state progressively smaller. Out-of-burst rates increase e-fold/44 mV while apparent interburst and intercluster rates increase e-fold/93 mV and e-fold/ 538 mV, respectively, with hyperpolarization.

In the last section it was proposed that the closed-state brief gaps were not as voltage sensitive as the burst length. It was also apparent that the number of gaps/burst is directly proportional to the burst length. For the same channel used above the mean number of gaps/burst was determined and plotted vs. burst length in Figure R-26. It can be seen that at all voltages the mean number of gaps/burst is directly proportional to the burst length, suggesting that the longer a channel stays in the bursting state, the more gaps (short closures) one will observe; this result was not unexpected. What was unexpected was that the slope of the line was dependent on voltage. This is illustrated in Figure R-27 where the mean number of gaps/burst*ms increases with hyperpolarization, inasmuch as gaps most likely

represent brief excursions from the open state to the adjacent closed state, which is short lived. For the sequential mechanism:



to be proposed in the Discussion we can more clearly define the meaning of the terms gaps/burst and gaps/burst*msec for mechanistic considerations. Gaps/burst is given by: [(openings/burst) - 1] which in the mechanism here is:

$$k_{30}/(k_{03} + k_{32}).$$

Gaps/burst*msec is given by:

$$(k_{30}*k_{03})/(k_{30} + k_{03}).$$

Such measurements then reflect the equilibrium between the open state and short closed state as well as the burst terminating rate k_{32} .

These considerations can be readily generalized to other mechanisms. That the gaps/burst*ms increases with hyperpolarization could indicate that the rate away from the open state (fast closing rate, k_{03}) increases with hyperpolarization, or that the mean open time decreases with hyperpolarization. We will consider this further in the Discussion.

To summarize, the data analyzed manually appears to fit a three exponential closed-time distribution and a single open-time distribution with a voltage-sensitive burst length and a voltage-sensitive fast closing rate. Data analyzed in IPROC will now be presented to better substantiate these claims.

Figure R-21. Open Times at Resting Potential Fit a Single Exponential Distribution.

The open times from the same patch in Figure R-20 at resting potential were plotted manually vs. a 10 ms binwidth (upper panel). The mean open time is 18 msec, as determined from the semilogarithmic transform (lower panel). The curve and line were fit by eye.

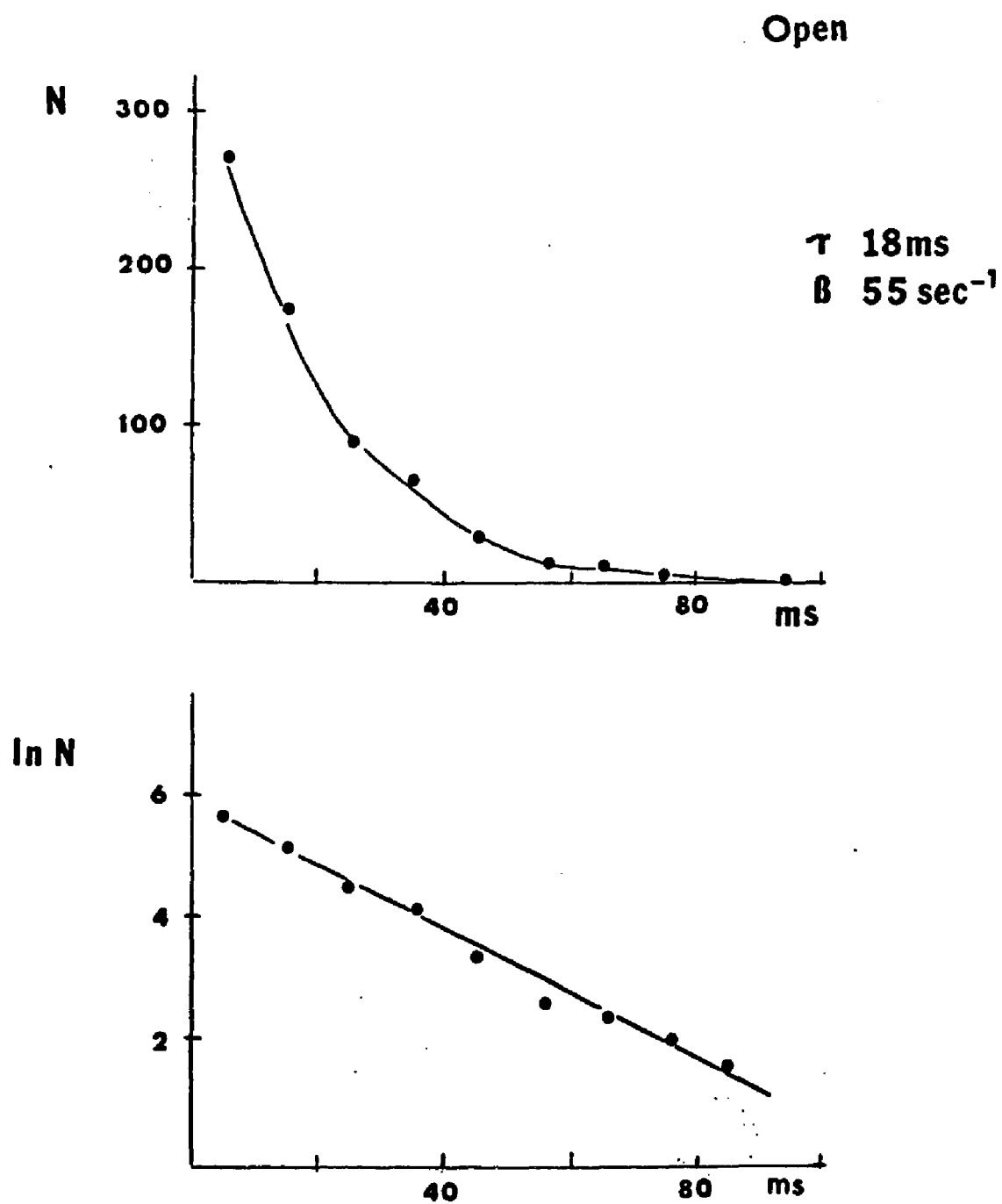


Figure R-21. Open Times at Resting Potential Fit a Single Exponential Distribution

Figure R-22. Summary of Open and Closed Distributions at Resting Potential

Log transforms of data from Figures R-20 and R-21 and the characteristic τ 's are illustrated with a segment of relevant data.

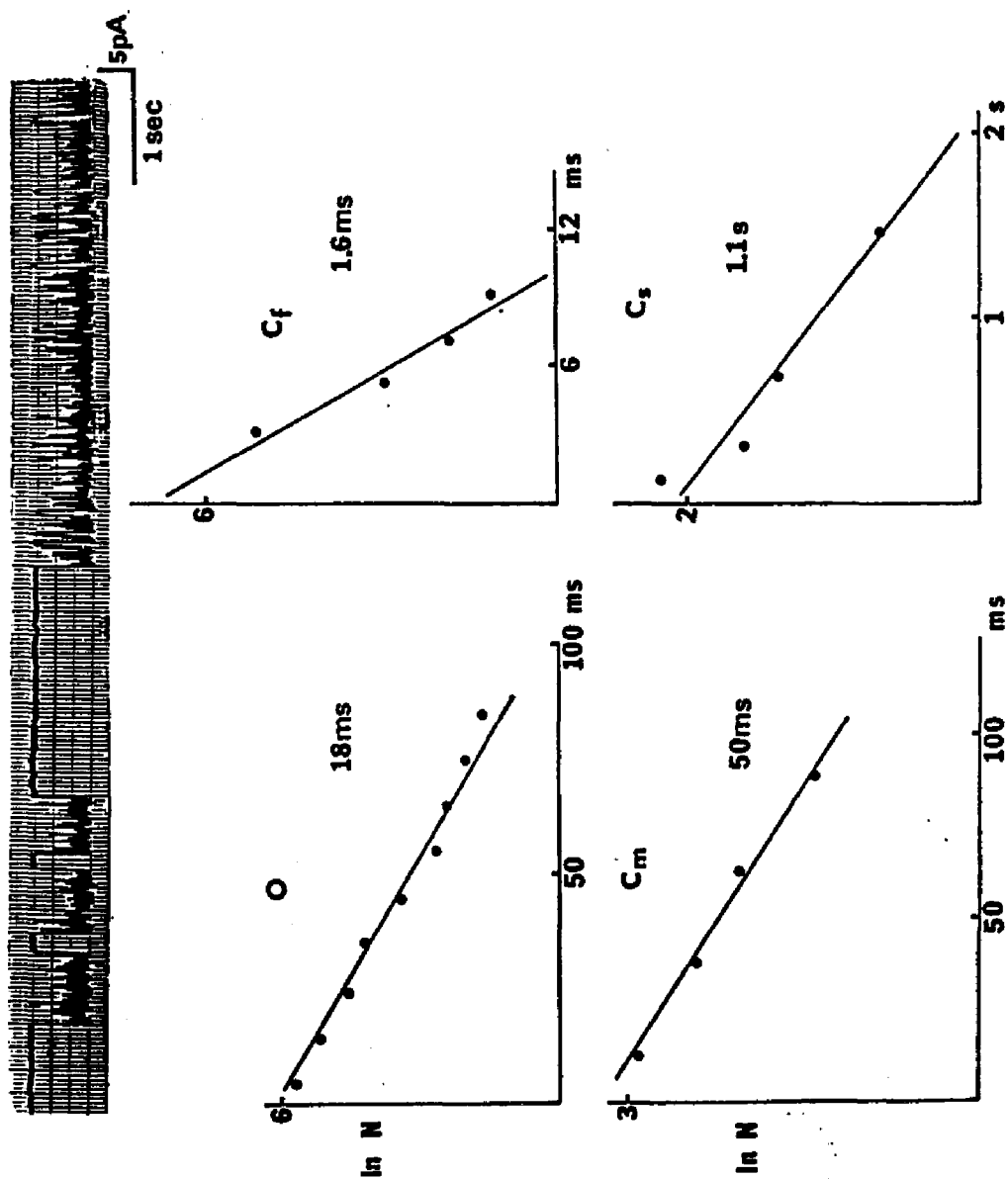


Figure R-22. Summary of Open and Closed Distributions at Resting Potential

Figure R-23. Burst Duration at Resting Potential

Closures greater than 2 ms in duration were used to define a minimum interburst interval (excludes brief closures). Bursts fit a single exponential (upper panel) when the natural logarithmic transform of the distribution is fit linearly by eye (lower panel). Data was from a CAP with 140 mM K_0^+ at 0 mV.

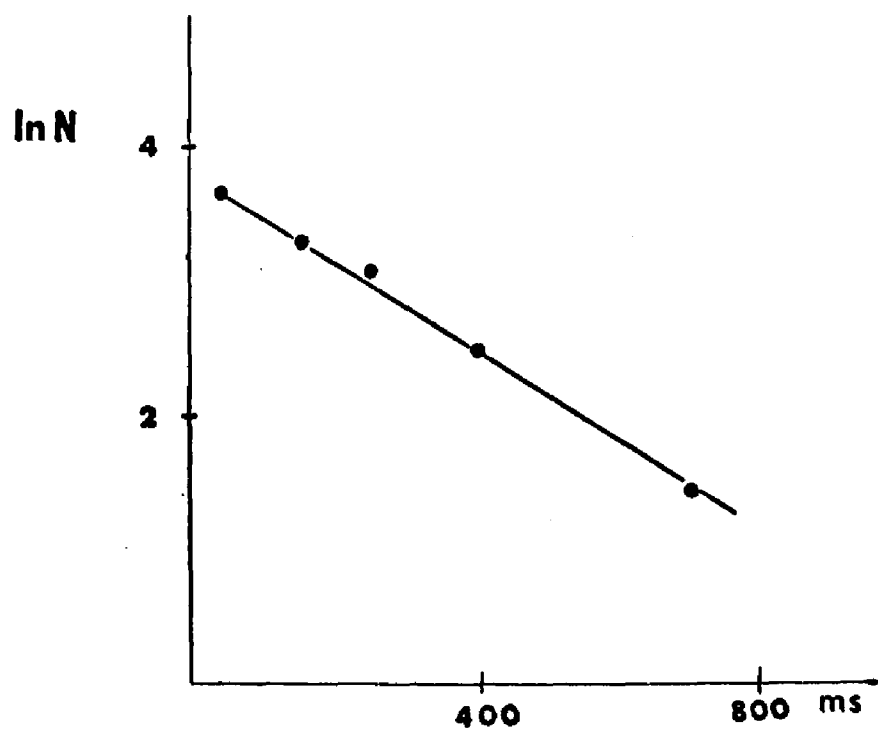
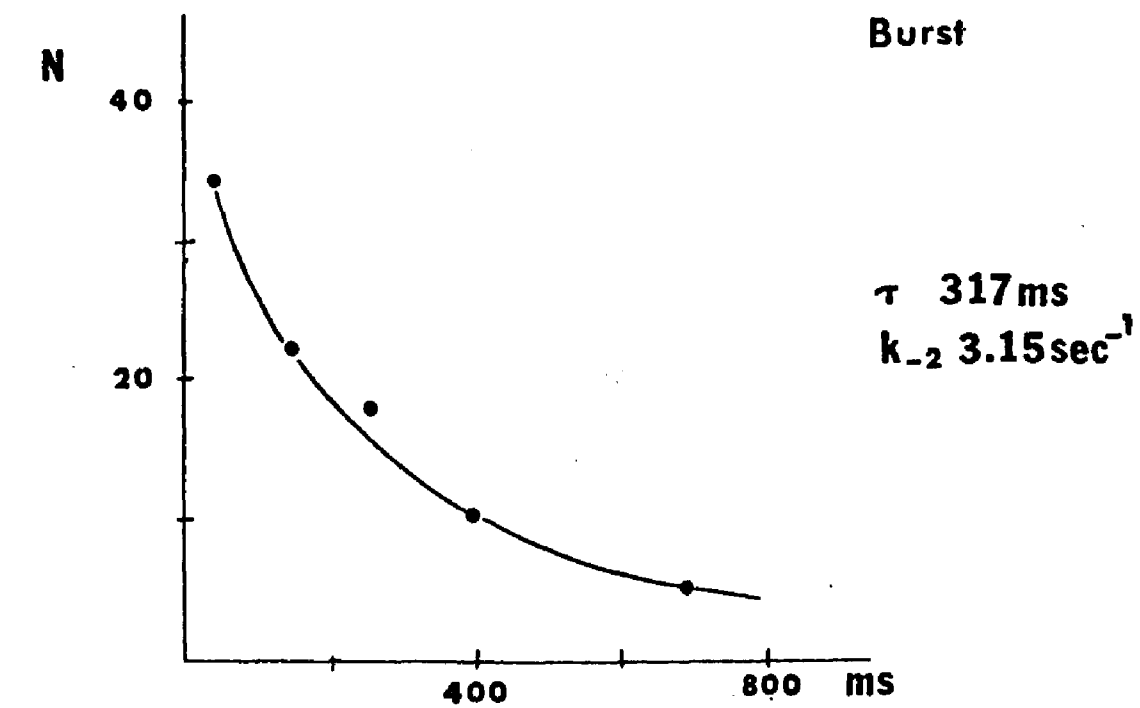


Figure R-23. Burst Duration at Resting Potential

Figure R-24. Burst and Interburst Duration Analysis

(a) Burst Durations are Sensitive to Voltage. Bursts were defined to be any series of openings separated by greater than 2 ms closures. Burst durations were measured manually from the same patch used to construct open and closed times above. An element of the data set is given at each voltage, and the data points are the midpoints of each bin. Semilogarithmic transforms are plotted adjacent to each distribution. All curves and lines were fit by eye.

(b) Interburst Durations are Also Voltage Sensitive. Interburst intervals (includes intercluster intervals) were defined to be closures greater than 2 ms in duration. Events for one CAP at 140 mM K_o^+ were manually tabulated and plotted as continuous distribution functions (bottom panels) for each voltage. Natural logarithmic transforms were used to extract τ aus (lines fit by eye). At each voltage a sample of the data trace is provided, as are the results (top panels) of burst durations analysis (discrete distributions).

CAP 140 K Ω BURST DURATIONS

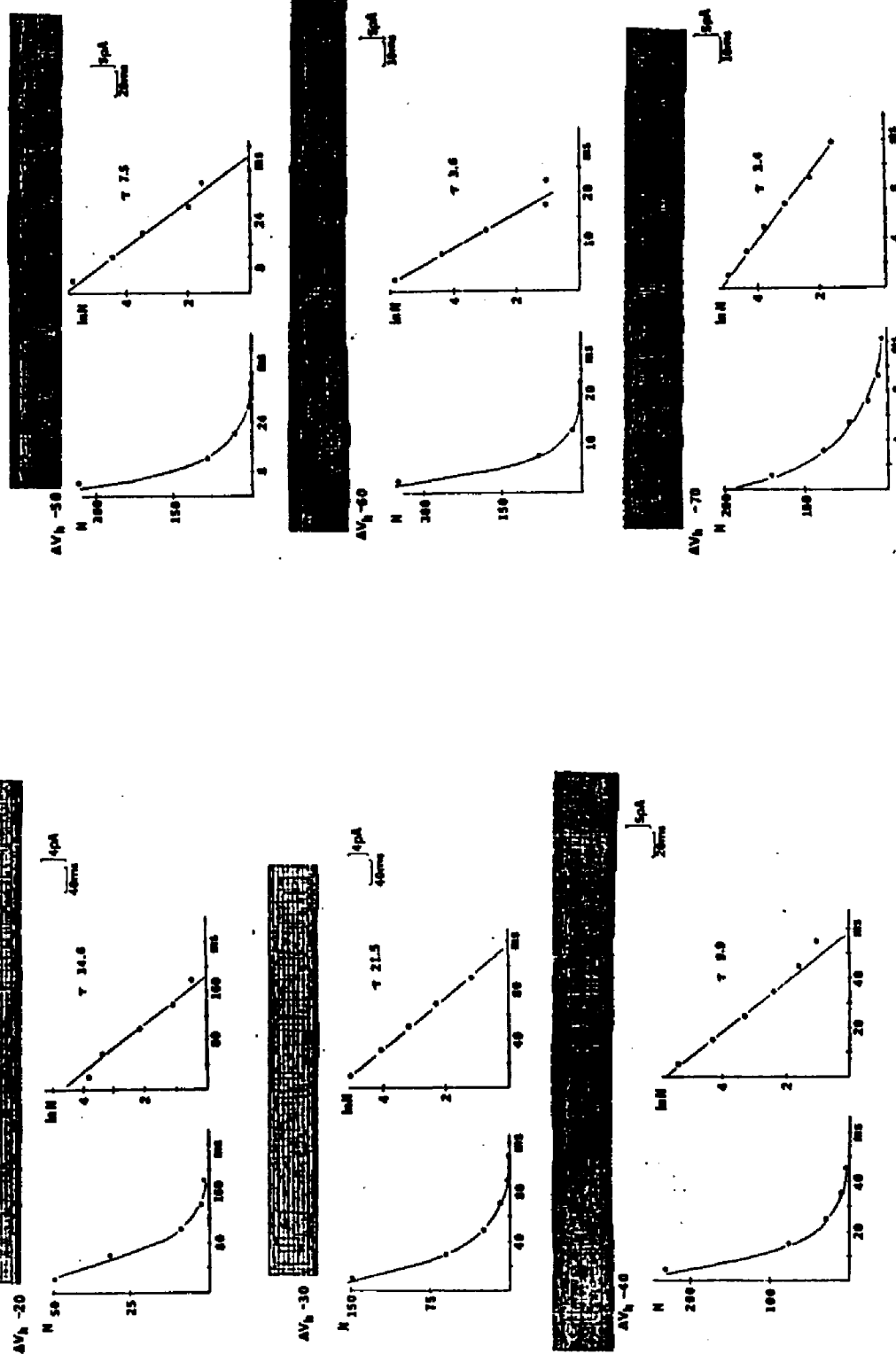


Figure R-24a. Burst and Interburst Duration Analysis-
Burst Durations are Sensitive to Voltage

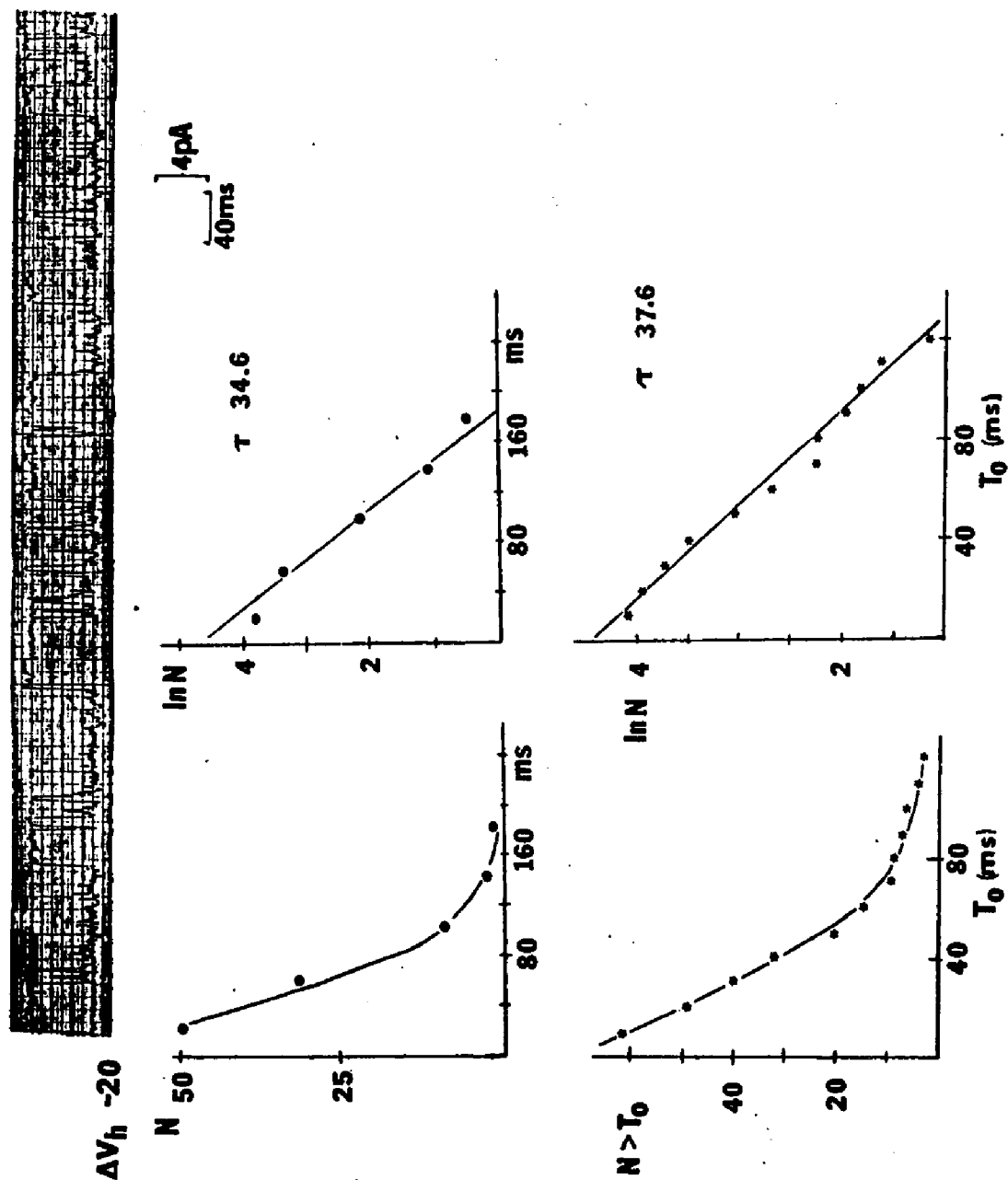


Figure R-24b. Burst and Interburst Duration Analysis (-20 mV)
Interburst Intervals are Also Voltage Sensitive

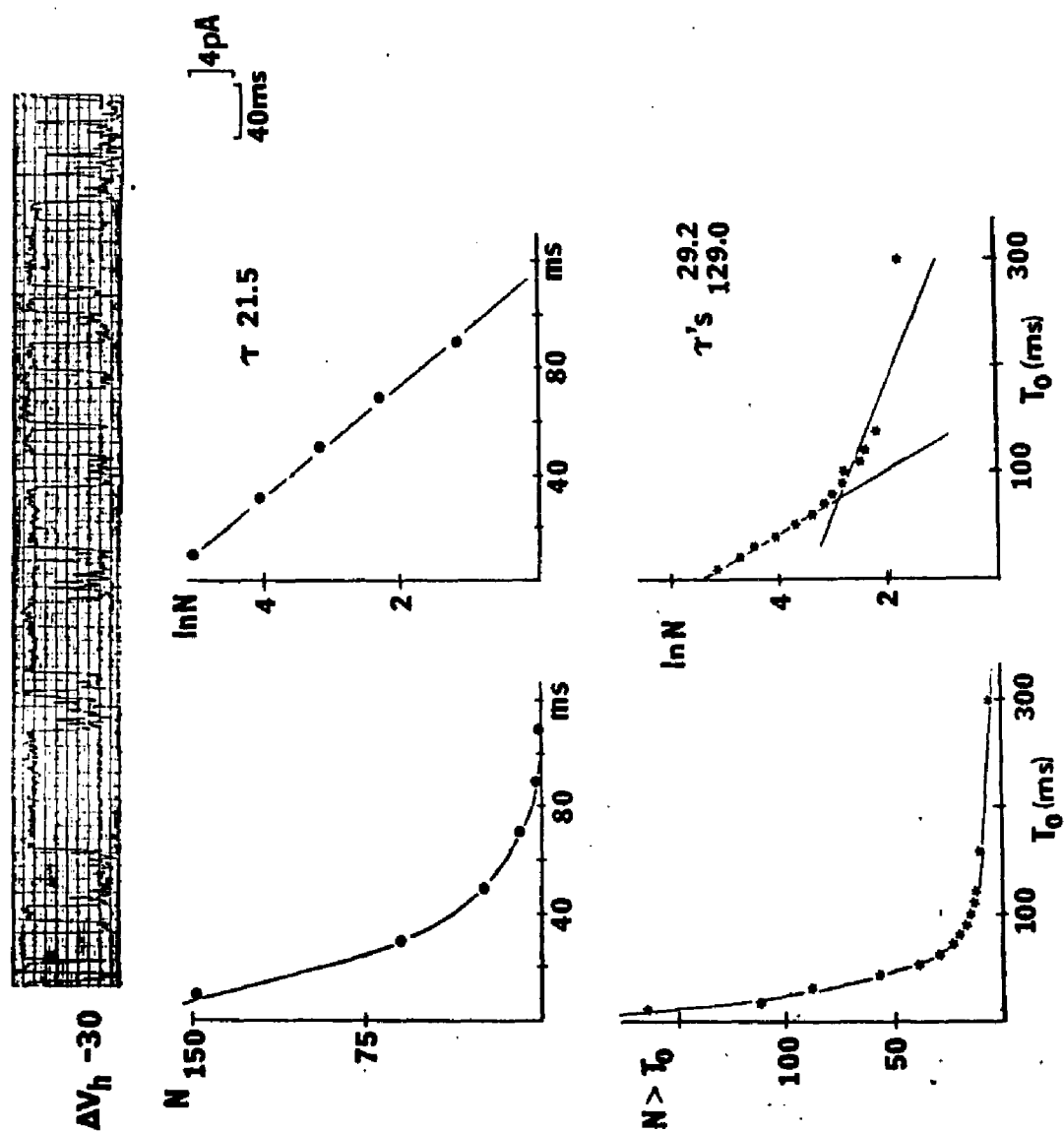


Figure R-24b. Burst and Interburst Duration Analysis (-30 mV)
Interburst Durations are Also Voltage Sensitive

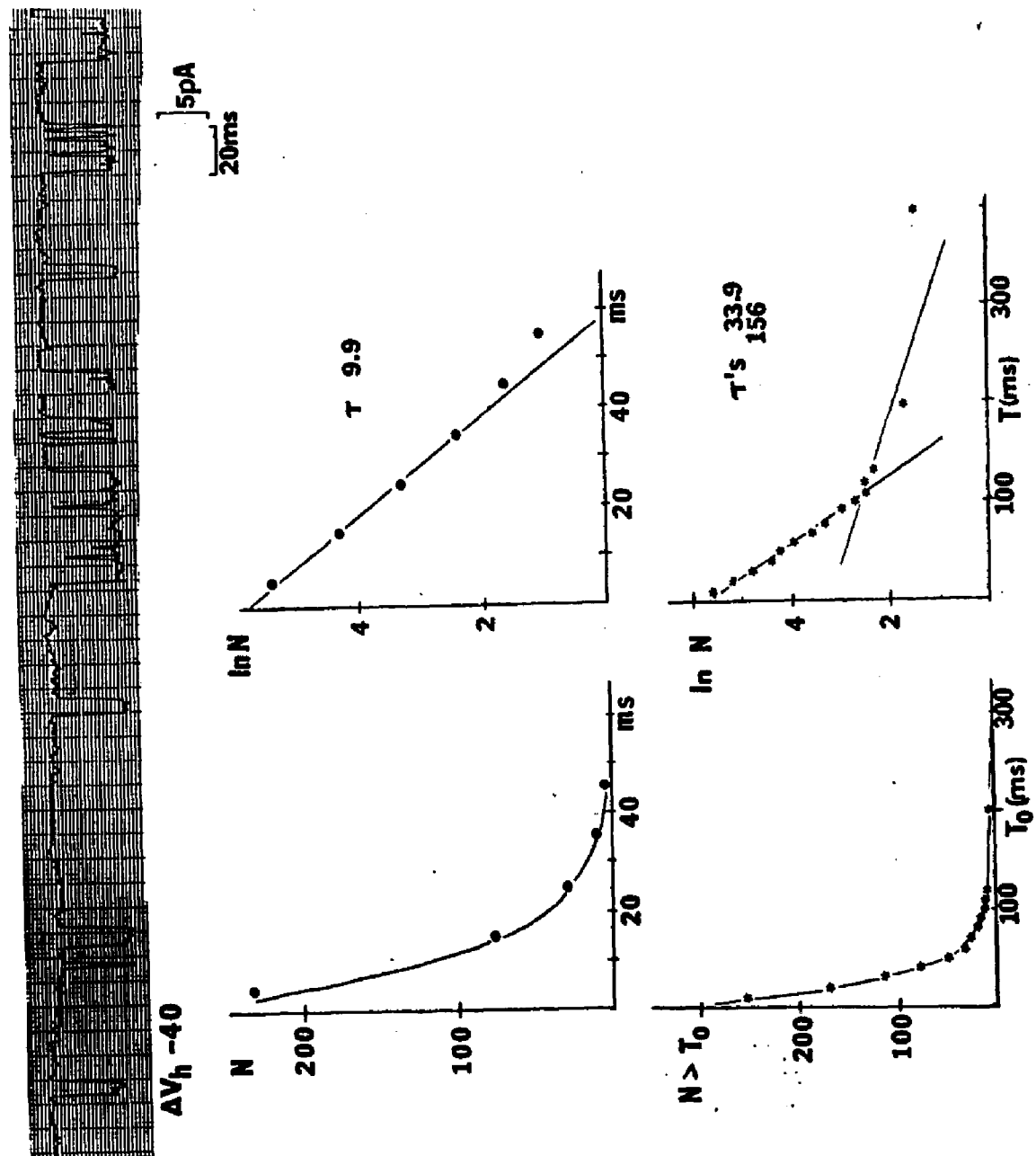


Figure R-24b. Burst and Interburst Duration Analysis (-40 mV)
Interburst Durations are Also Voltage Sensitive

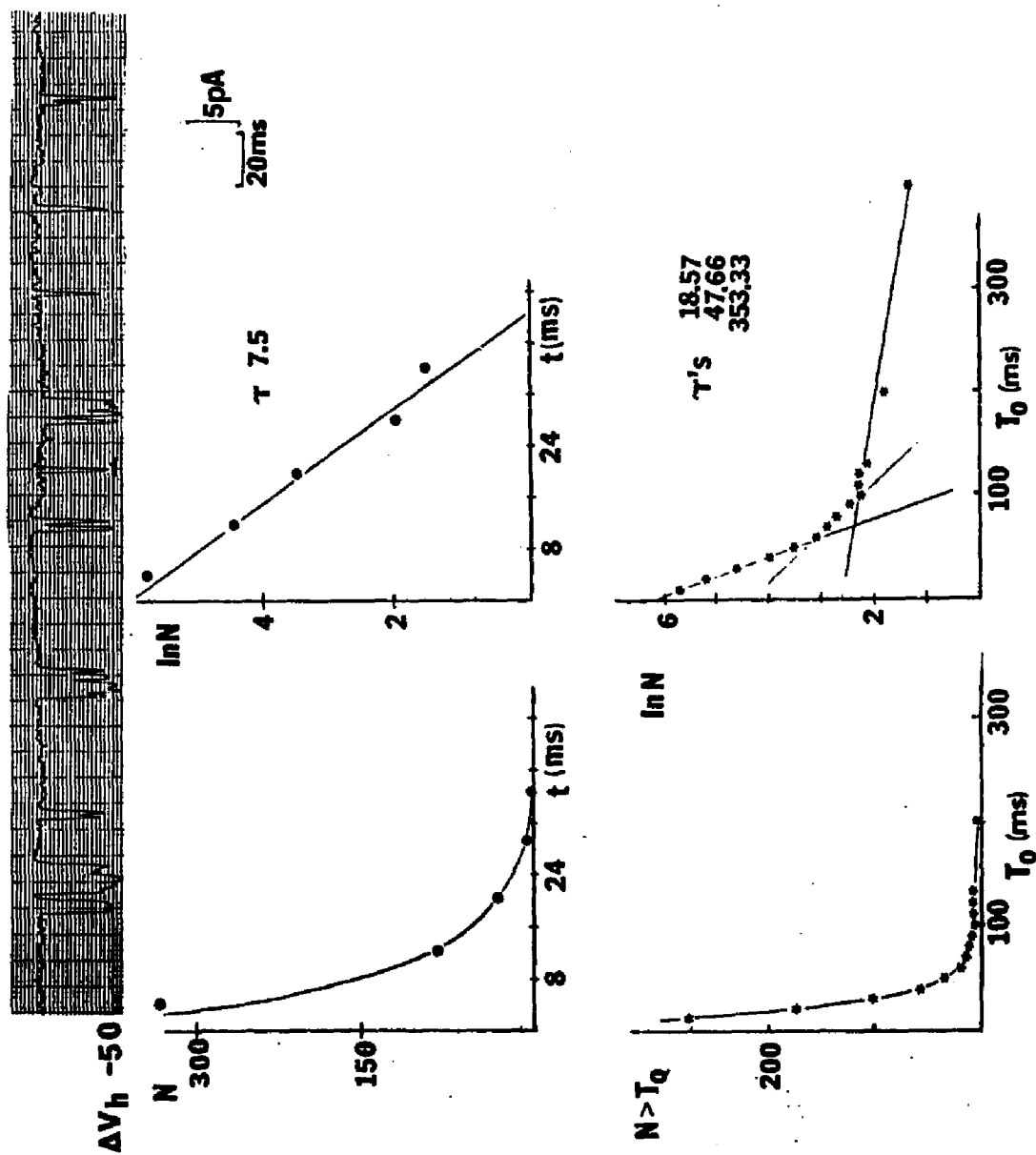


Figure R-24b. Burst and Interburst Duration Analysis (-50 mV)
Interburst Durations are Also Voltage Sensitive

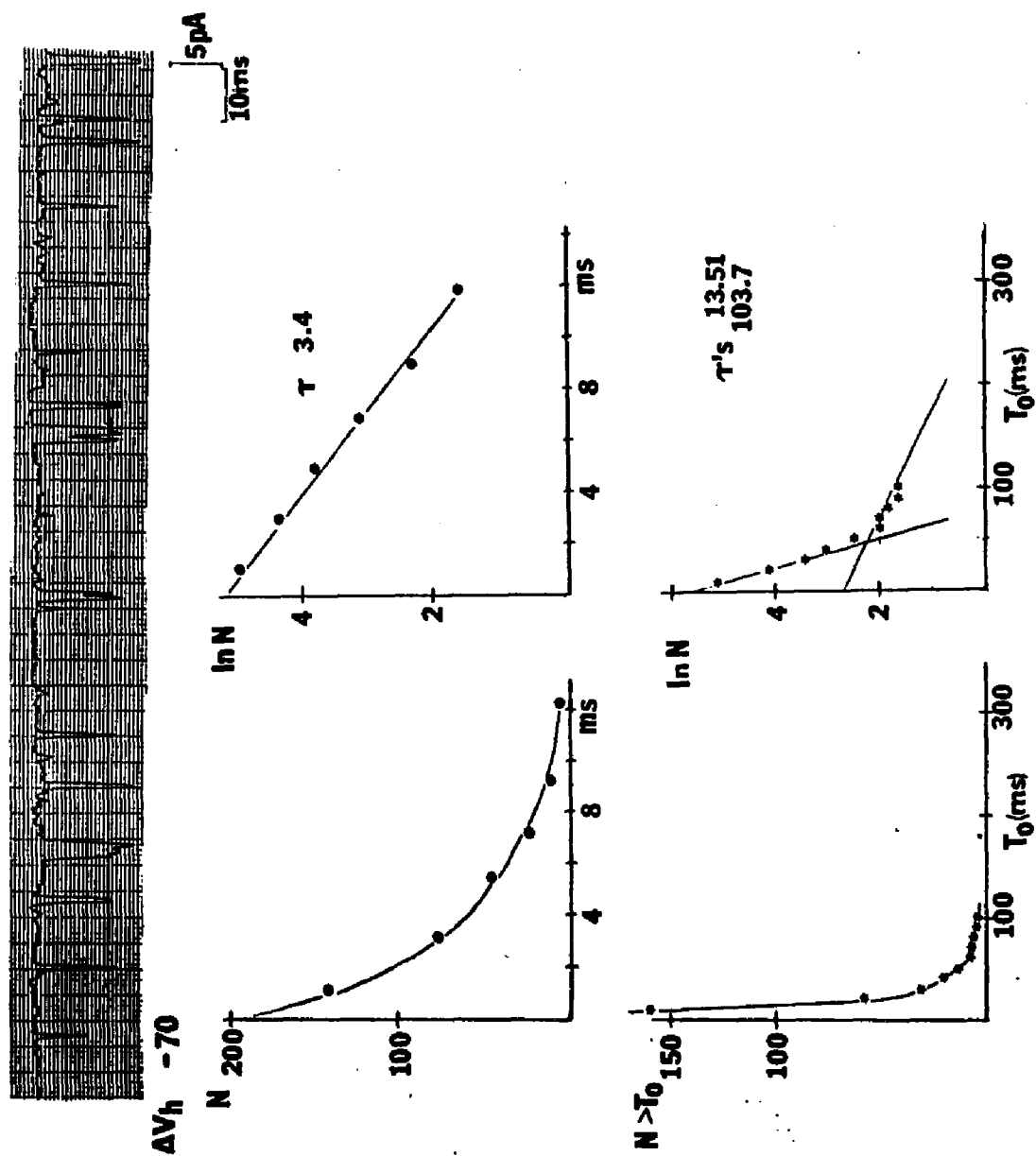


Figure R-24b. Burst and Interburst Duration Analysis (-70 mV)
Interburst Durations are Also Voltage Sensitive

Figure R-25. Strong Voltage Dependence of the Burst and Interburst Durations and Rates

Taus for burst and interburst intervals were determined following manual measurement of burst duration (> 2 ms) and interburst durations for a SC patch with $140 \text{ mM } K_o^+$ held at different potentials, as in Figure R-24. For the burst durations the three points at depolarized potentials were determined from Gaussian means of smaller numbers of events (because burst duration is prolonged at progressively depolarized potentials).

Interburst time constants in some cases ($\Delta V \leq -30 \text{ mV}$) required two exponential fits, reflecting apparent short (interburst) and long (intercluster) closed times; only the shortest and longest Taus were plotted for $\Delta V = -50 \text{ mV}$ even though an intermediate distribution was apparent (Figure R-24b).

Rate constants (inverse of the time constants) for the burst and interburst durations were plotted vs. holding potential (ΔV). The burst rate constant represents a mechanism-dependent weighted average of all rates (paths) of passage out of the open state(s) while the interburst rates reflect an average of relevant rates toward the burst state. Note natural logarithmic scale on the ordinate. Linear

Figure R-25. - Continued

regression equations were found to fit the natural logarithm-transformed data where K here represents the rate determined by inversion of the respective time constant:

$$\ln K = 0.92 - 0.0231 \cdot \Delta V \quad (\text{burst})^*$$

$$\ln K = 0.107 - 0.0114 \cdot \Delta V \quad (\text{short interburst})^*$$

$$\ln K = 0.693 - 0.0019 \cdot \Delta V \quad (\text{long interburst-intercluster})$$

The fitted burst rates increase e-fold/44 mV hyperpolarization while the fitted short and long interburst rates increase and decrease e-fold/93 mV and e-fold/ 538 mV respectively with hyperpolarization.

The fitted rate regressions correspond to the following untransformed distribution functions:

$$\text{Tau} = 0.3985 \cdot e^{0.0231 \cdot \Delta V} \quad (\text{burst})^*$$

$$\text{Tau} = 0.3310 \cdot e^{0.0114 \cdot \Delta V} \quad (\text{short interburst})^*$$

$$\text{Tau} = 0.5000 \cdot e^{0.0019 \cdot \Delta V} \quad (\text{long interburst-intercluster})$$

*indicates significance at the $p < 0.05$ level.

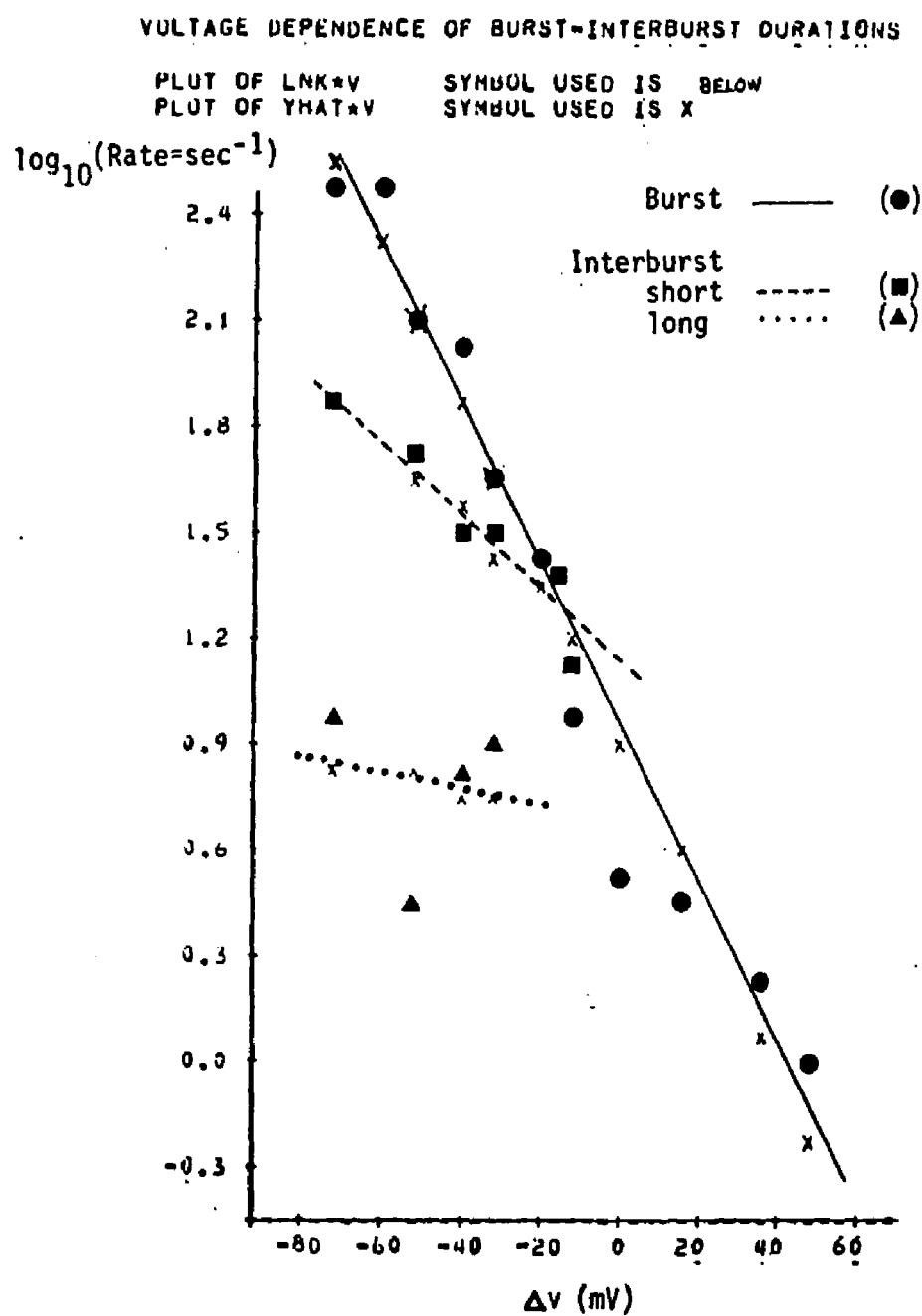


Figure R-25. Strong Voltage Dependence of the Burst and Interburst Durations and Rates

Figure R-26. Mean Number of Short-Gaps/Burst vs. the Duration of Bursts

The number of gaps or closures exceeding the 0.5 of maximal open channel current (threshold) were counted in each identified burst and stored in tables. Over the burst duration binwidths used in Figure R-24 the mean number of gaps/burst was determined and plotted versus the binwidth at the voltage displayed. Note that the three scales on the ordinate are specific to certain voltages. Lines were fit by eye.

Figure R-26. Mean Number of Short-Gaps/Burst vs. the Duration of Bursts

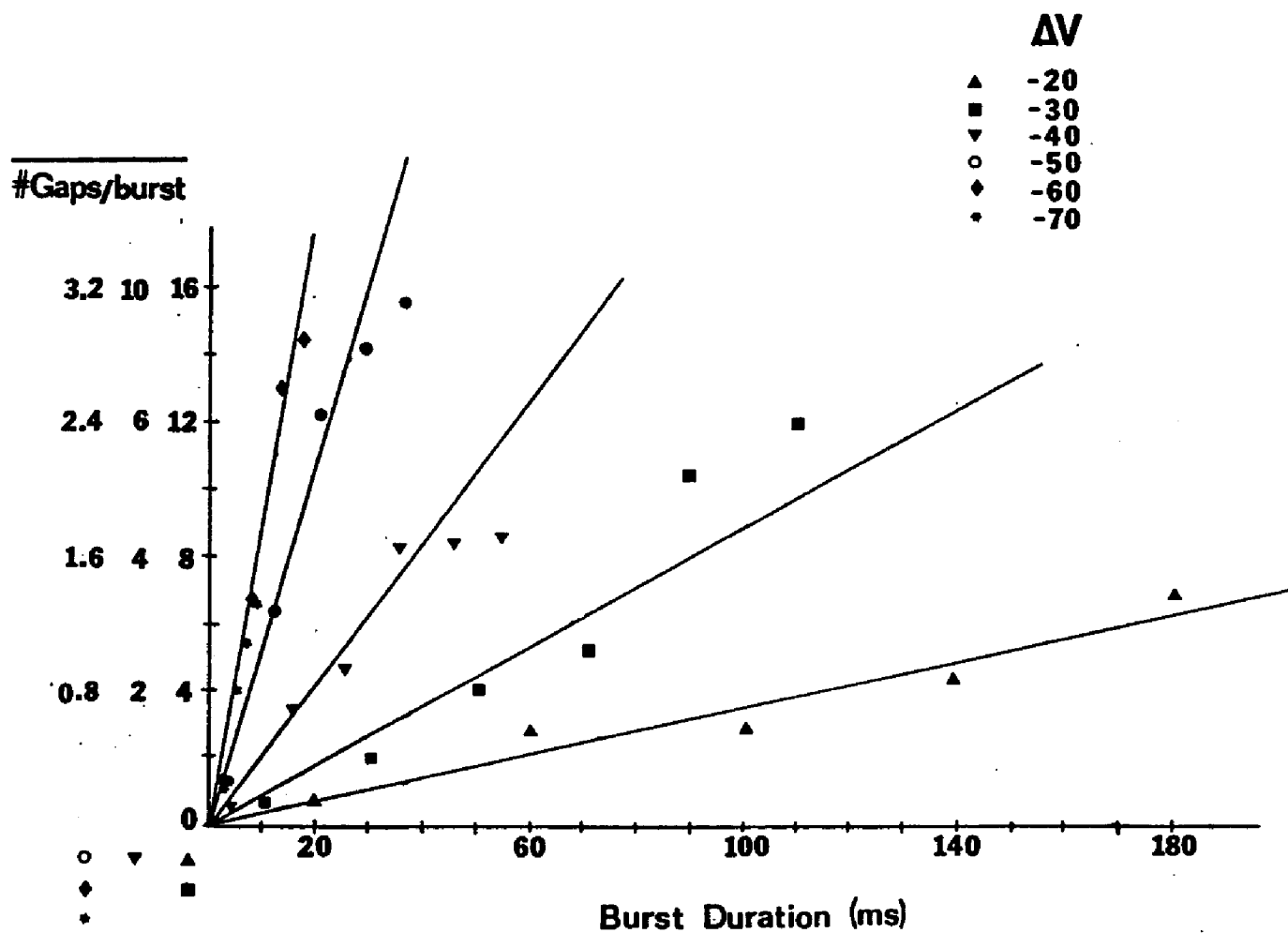


Figure R-27. Rate of Passage from Open State to Short Closed
State Is Voltage Sensitive

Slopes of the lines in Figure R-26 are plotted versus holding potential. The rate of passage to the short lived closed state appears to be voltage sensitive. Data from same patch were used throughout.

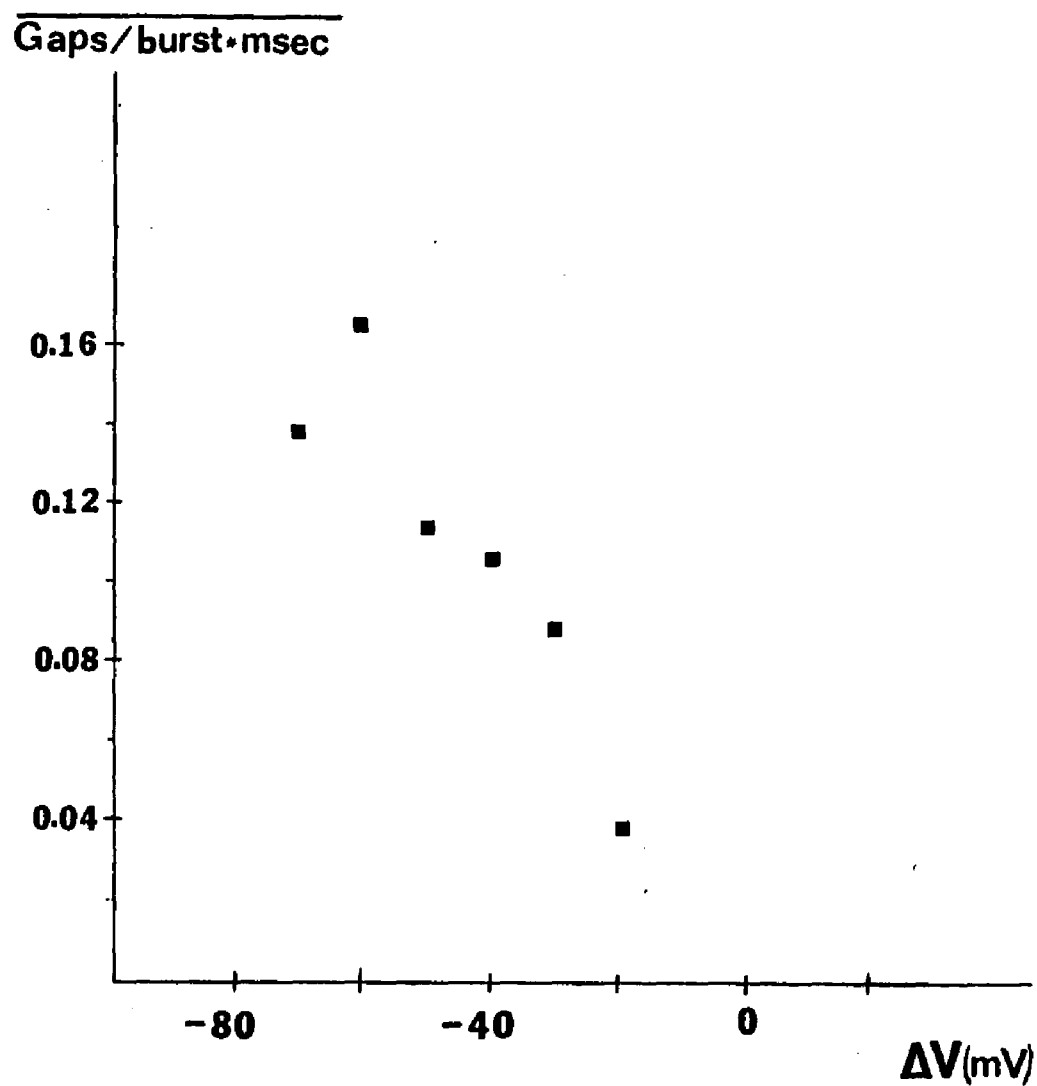


Figure R-27. Rate of Passage from Open State to Short Closed State Is Voltage Sensitive

3. IPROC Analysis of Channel Kinetics

Two representative IRs in CAPs at $140 \text{ mM } K_o^+$ were analyzed thoroughly by IPROC and are presented to illustrate that: (1) the ON and OFF times are slightly voltage sensitive, (2) in patches held at potentials slightly hyperpolarized from RMP, the closed times fit a three exponential distribution function, and (3) voltage sensitivity appears to reside in the medium-long and long closed times and the burst duration. These results imply that voltage sensitivity is largely due to the distribution of closed states and that there are three distinct Markovian closed states in the channel kinetic mechanism. Moreover, manual measurements of burst durations was rational because this serves as an indirect measurement of the medium-long and long closed times.

Figure R-28a displays ON time histograms fitted to data obtained at different holding potentials in a patch. In each case histograms were fit to single exponentials with the Taus listed in the Figures. Although there are slight changes in the Taus across different voltages, there is no apparent significant effect of voltage on duration of the open state. ON time Taus from two patches were plotted and fitted in Figure R-28b where both the variability at some voltages is evident as well as the small decrease in fitted mean open time constant at hyperpolarized potentials. There is a proportional increase (e-fold/68 mV) in the fast closing rate (k_{03}) with hyperpolarization.

Short OFF times from FTTS are displayed for one patch at different voltages in Figure R-29a. Although there is some variability, the steepest exponentials are all very similar (noting that two exponential

fits were used at -41 mV and at -60 mV (the longer exponential is apparently the medium-long closed time discussed above). The fitted OFF Taus results are plotted for two SC patches in Figure R-29b. The Tau data from the two patches and a linear regression fit was found for the OFF Taus. OFF Tau rate constants generated by inversion show that the fast opening rate (k_{30}) increases e-fold/48 mV of depolarization. The combination of an increase in the fast closing rate (k_{03}) combined with a decrease in the fast opening rate (k_{30}) with hyperpolarization would tend to drive the mechanism out of the bursting state.

The set of all OFF times measured was fitted simultaneously using ALLOFF to three exponential distribution functions as displayed in Figures R-30. The insets are a clearer representation of the long OFF distributions. The necessity of three exponential fits to the histograms indicates that a minimum of three distinct Markovian closed states are necessary in any putative mechanism. For two patches the medium-long and long OFF times are plotted in Figure R-31 and fitted to exponential functions of voltage. The time constants for both of these distributions are voltage sensitive with the Tau for medium-long OFF times decreasing and that for long OFF times increasing with hyperpolarization. Inverting, the rates responsible for leaving the medium-long OFF states would increase while the rate for leaving long OFF states would decrease with hyperpolarization. The potential relevance of this data and other SC kinetic data to a putative mechanism will be presented in the Discussion.

Figure R-28. Voltage Sensitivity of ON Times

(a) ON times histograms were determined by IPROC at different holding potentials. Single exponential distribution functions were fitted using the FITS program. Taus are illustrated in each panel. The data was from a CAP with 140 mM K_0^+ .

(b) ON time Taus (τ) are plotted vs. voltage from two different patches where single exponential fits were found (using FITS).

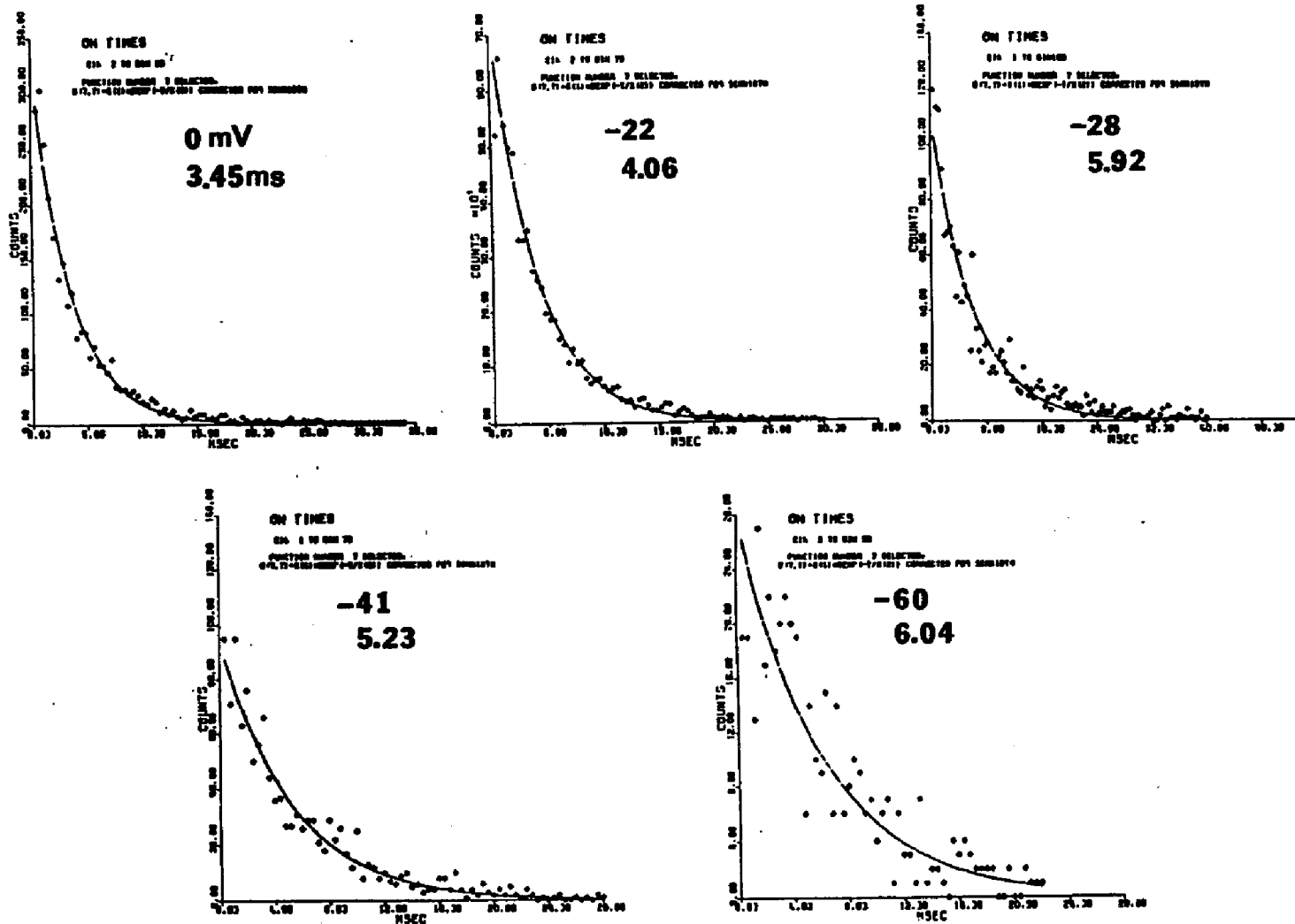
A linear regression fit to the data points was found:

$$\tau = 0.05276 \Delta V + 6.6025$$

which was significant at the $p < 0.05$ level.

The inverse of the fitted ON Tau line ($\odot$) was taken directly from the expected value at voltages over the steepest change of the $P_V(o)$ graph (Figure R-15c) and a line was fitted (by eye) through these points. The resultant rate (k_{03}), increases e-fold/68 mV with hyperpolarization.

Figure R-28a. Voltage Sensitivity of ON Times



ONTIMES VS VOLTAGE

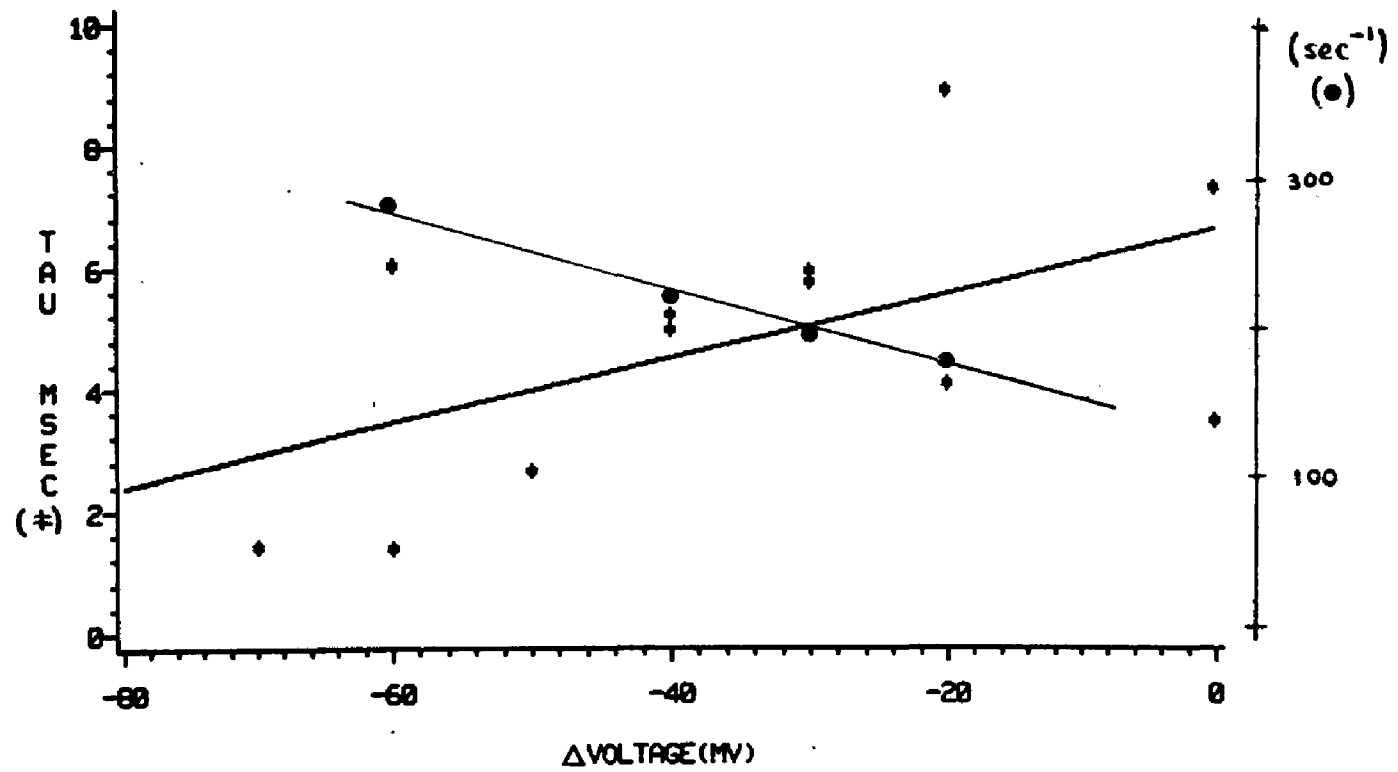


Figure R-28b. Voltage Sensitivity of ON Times

Figure R-29. Voltage Sensitivity of Short OFF Times

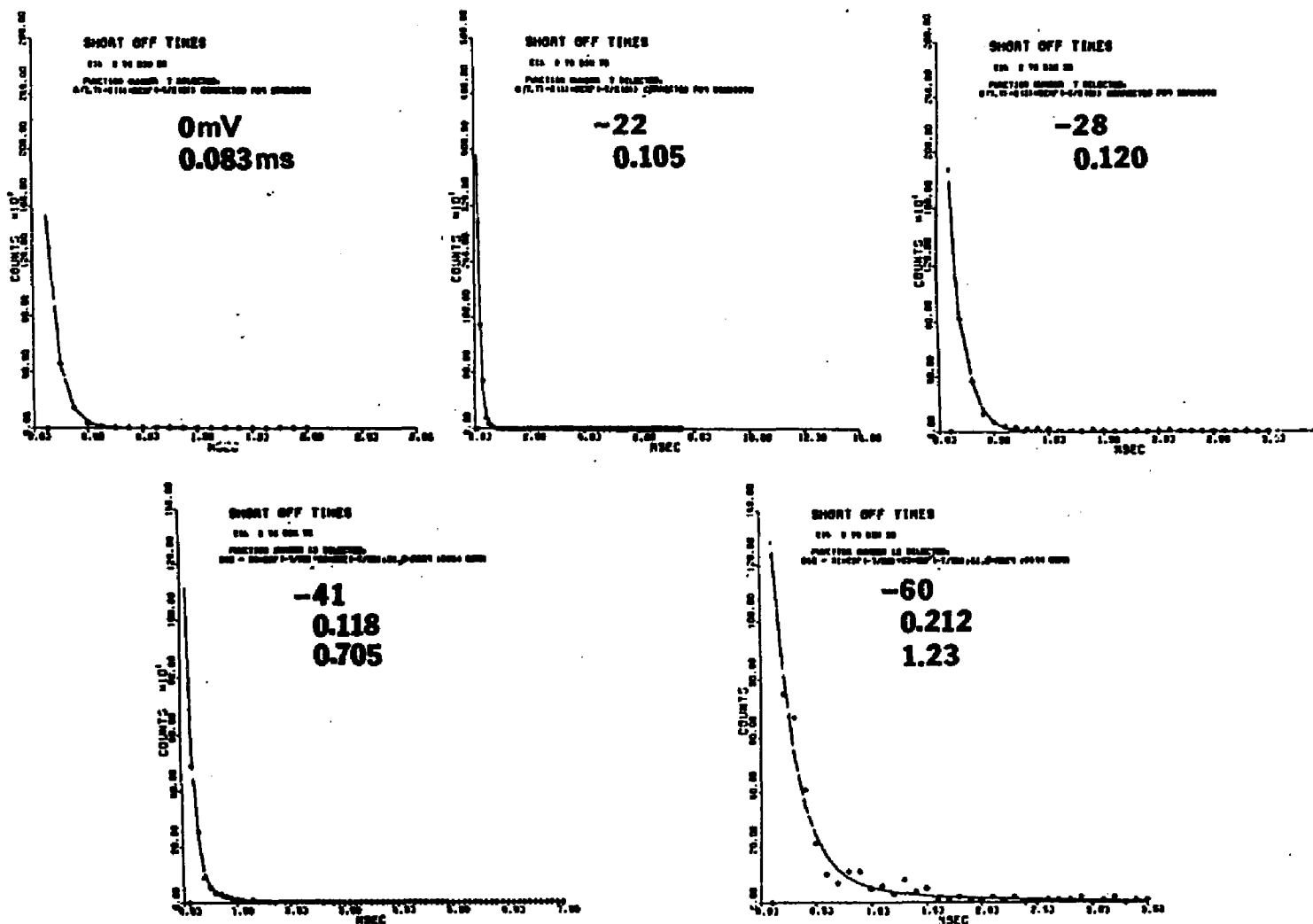
(a) Short OFF time histograms generated by IPROC were fit by single (0, -22, -28 mV) and double (-41, -60 mV) exponentials using FITS. The time constants of the fits are illustrated in each panel.

(b) Short OFF time constants (+) from (a) above and another patch(■) were plotted vs. voltage. Wherever possible, when multiple attempts at fitting (e.g., one or two or three function distributions) were done on a single data set the appropriate means of the data from the multiple sources were taken to attempt to ensure more accuracy. A linear regression fit to the data points was found:

$$\text{Tau} = -0.002064 \Delta V + 0.23789$$

which was significant at the $p < 0.05$ level. The inverse of the fitted short OFF Tau line (●) was taken from the expected value at voltages over the steepest change of the $P_v(o)$ graph (Figure R-15c) and a line was fitted through these points by eye. The resultant rate (k_{30}) increases e-fold/48.2 mV with depolarization.

Figure R-29a. Voltage Sensitivity of Short OFF Times



SHORT CLOSED TIMES VS VOLTAGE

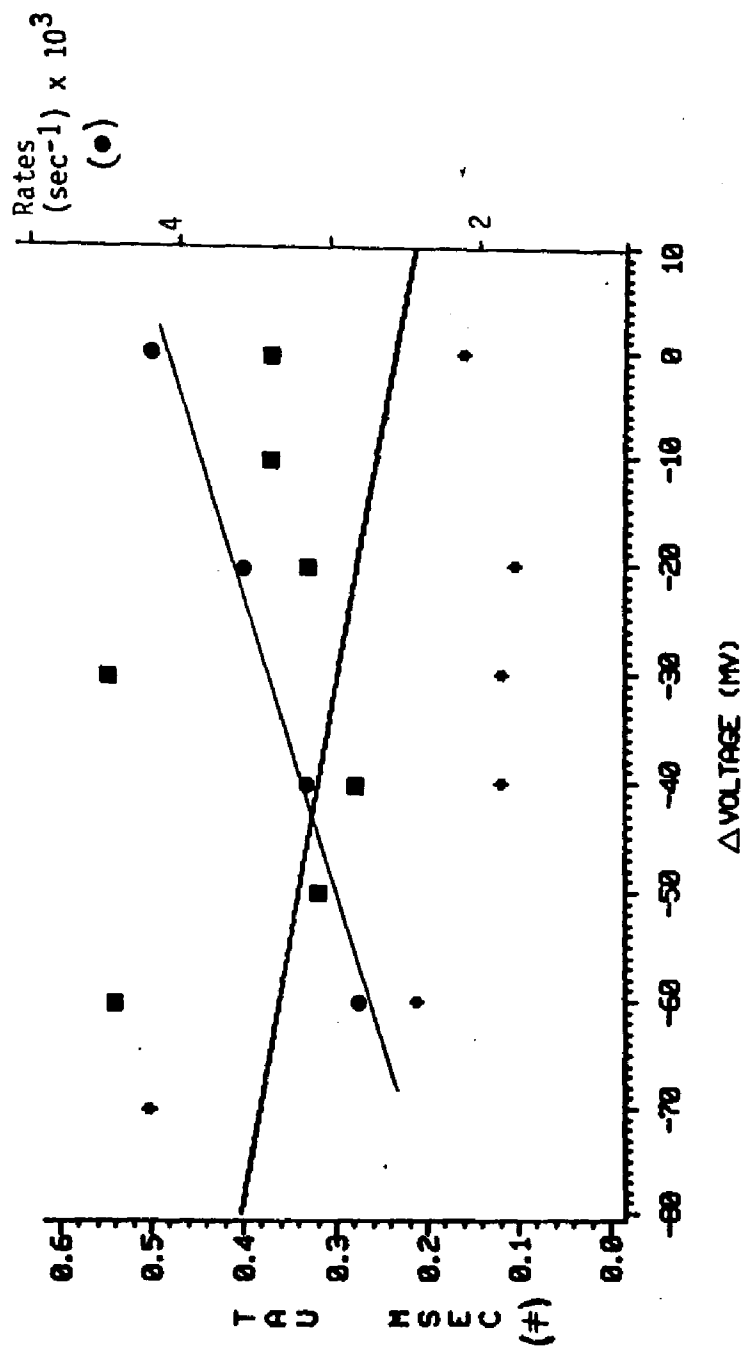


Figure R-29b. Voltage Sensitivity of Short OFF Times

Figure R-30. Total OFF Times Fit Three Exponential Distribution Functions--Further Evidence for a Three Closed State Model

OFF times were fit to three exponential distribution functions using ALLOFF. Because it was difficult to observe the long OFF component in the ALLOFF output, the fitted IPROC version is provided as an inset; in all cases the IPROC inset was similar to the long Tau fitted in ALLOFF. All Taus are stipulated in each figure for each component of the distribution. Voltages were (a) -22, (b) -28, (c) -41, and (d) -60 mV. Data was from a CAP with 140 mM K_o^+ .

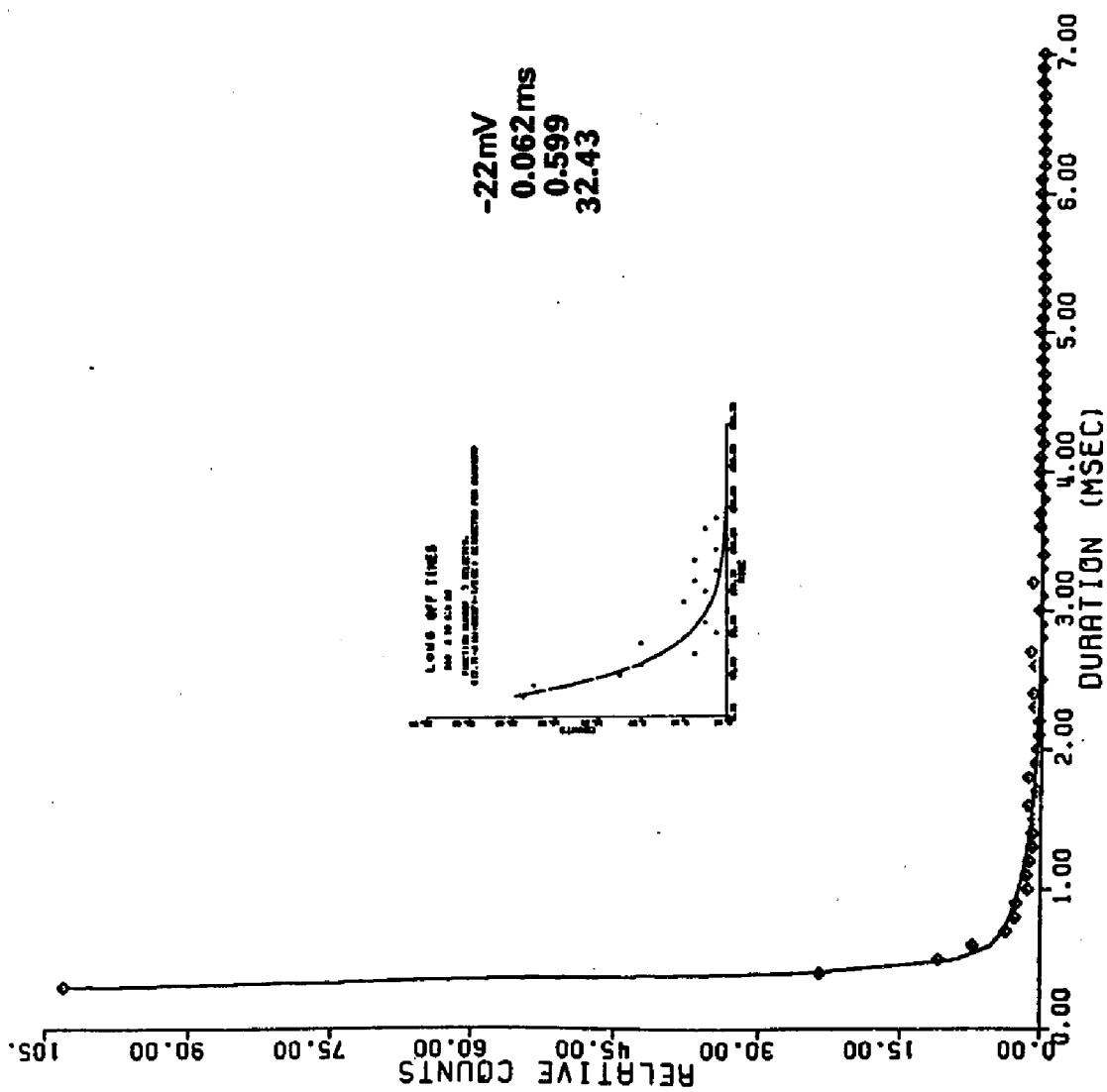


Figure R-30a. Total OFF Times Fit Three Exponential Distribution Functions--Further Evidence for a Three Closed State Model: (-22 mV)

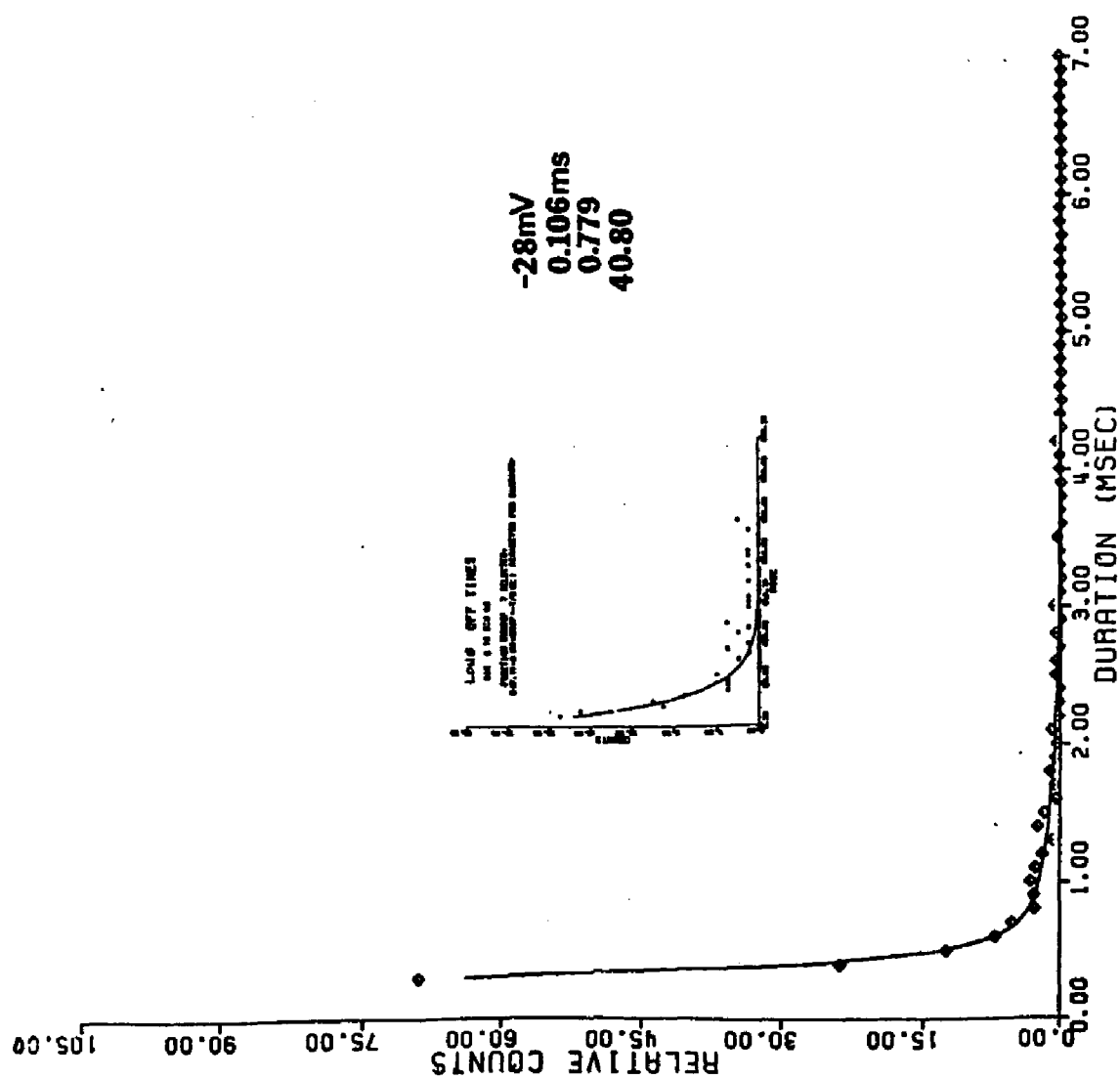


Figure R-30b. Total OFF Times Fit Three Exponential Distribution Functions--Further Evidence for a Three Closed State Model: (-28 mV)

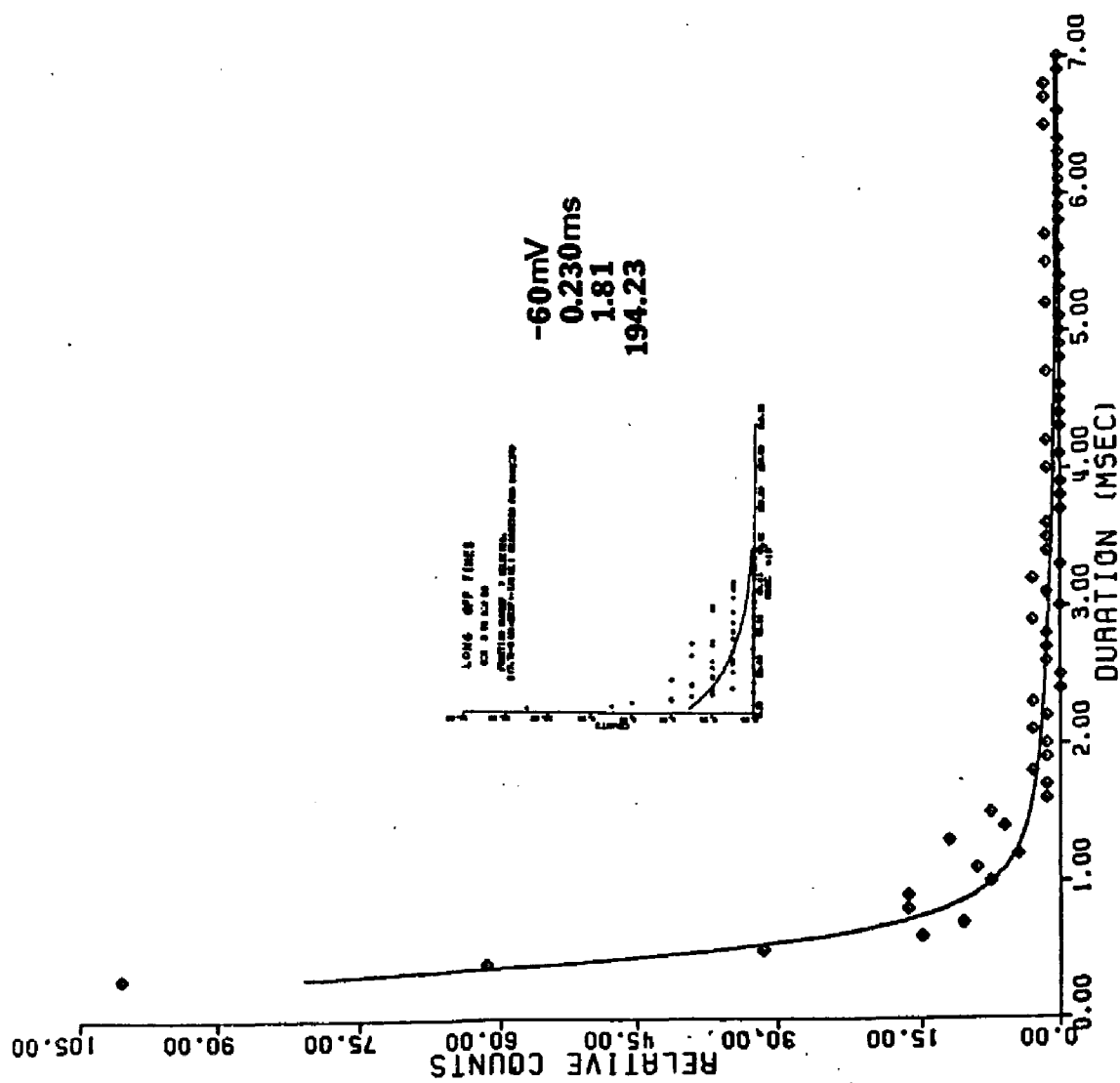


Figure R-30d. Total OFF Times Fit Three Exponential Distribution Functions—Further Evidence for a Three Closed State Model: (-60 mV)

Figure R-31. Medium-Long and Long OFF Taus Exhibit Voltage Sensitivity

Time constants for medium-long and long OFF times from two patches (inclusive of the one above) with distributions fit by FITS and ALLOFF are plotted vs. voltage. Where possible, when multiple fittings were done, means were taken for the data point. Nonlinear least-squares regression (Marquardt) was used to fit each data set to exponential functions:

$$\text{Tau} = 6.6509 * \exp(0.0857 * \Delta V) \quad (\text{medium-long OFFs}) \quad (\bullet)$$

$$\text{Tau} = 23.166 * \exp(-0.0399 * \Delta V) \quad (\text{long OFFs}) \quad (\circ)$$

which were both significant at the $p < 0.05$ level.

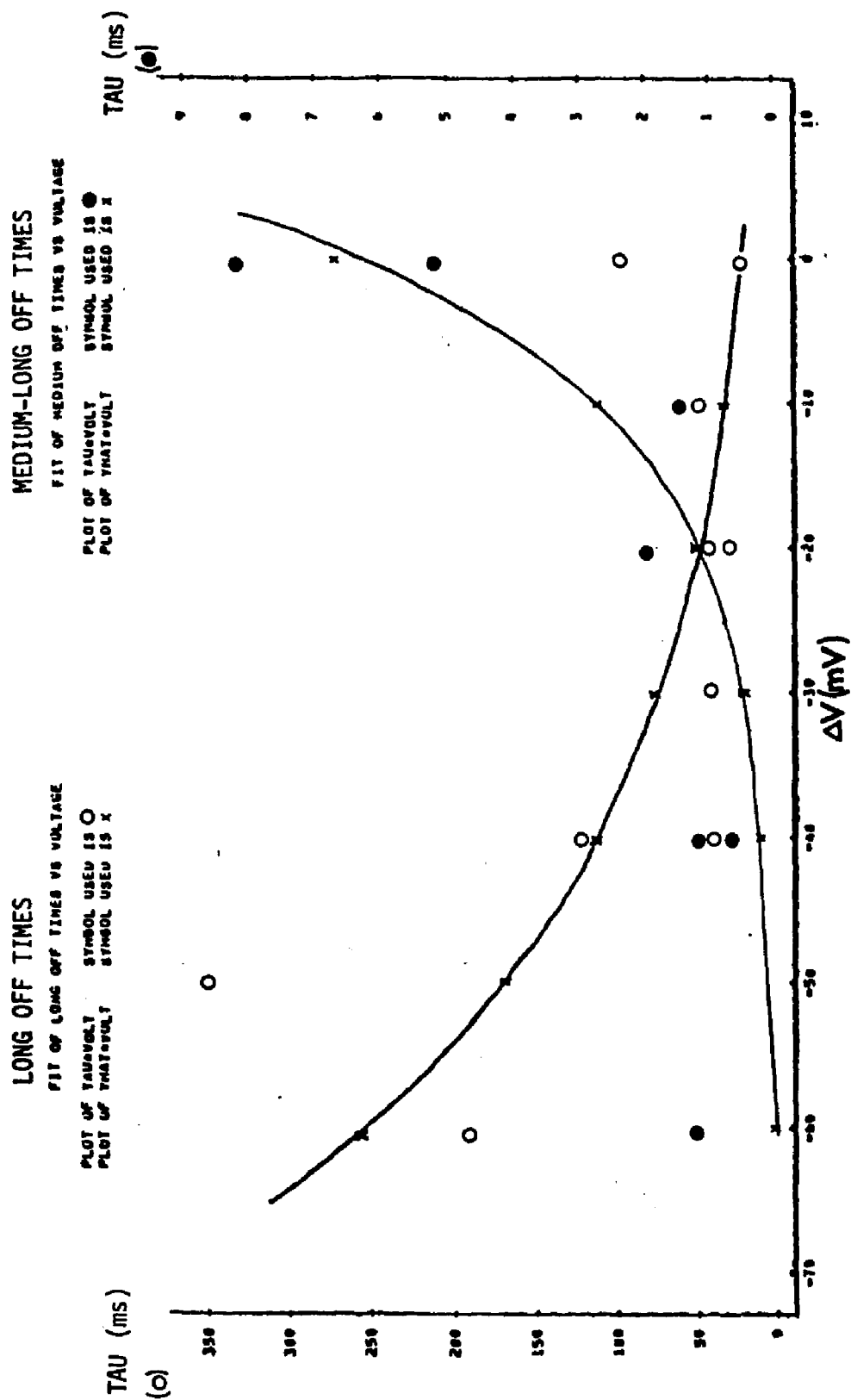


Figure R-31. Medium-Long and Long OFF Taus Exhibit Voltage Sensitivity

4. Single Channel Ensemble Behavior

Assuming independent operation of ion channels, the averaged behavior of a single element exposed repetitively to a large number of voltage impulses should mimic the behavior of a population of such elements exposed to a unit pulse (Aldrich and Yellen, 1983). In this manner, patches exposed to ensembles of voltage pulses provide a means for estimating the kinetic nature of WC currents. The SC behavior can thus be used as a building block to understand WC currents.

Shown in Figure R-32c are averaged ensembles of pulses in a two-channel patch with 140 mM K_0^+ and a holding potential of +20 mV ($P_V(o)$ approximately equal to 1). Thirty-one to thirty-four command voltage pulses were provided to the patch at each command voltage. Currents were stored without signal conditioning and were then signal averaged with identical bin widths at each voltage. One can see that the average currents are larger immediately after the voltage impulse than at steady-state (Figures R-32a,c). This apparently reflects a de-activation process occurring because of temporal redistribution in the probabilities of states in the channel mechanism away from the open state after the voltage pulse. We have already seen how both $P_V(o)$ and the burst duration decrease with hyperpolarization at constant voltages. After the voltage pulse from +20 mV (where $P_V(o)$ is about 1) to more negative voltages, the proportion of time the channel spends in closed states temporally increases or the probability of opening is decreasing over time (nonstationary). With progressively deeper hyperpolarizations the driving force becomes larger across any open channel, but the kinetic trajectory to steady-state is more rapid. One might infer that the rate constants leading from the open to the closed

Figure R-34. Ensemble Voltage Pulse Analysis on IR Predicts Partial Deactivation of Whole-Cell Currents on Hyperpolarization

A CAP with two channels and 140 mM K_0^+ was repetitively exposed to 2-second pulses separated by 4-second resting intervals. The holding potential was +20 mV ($P_V(o)$ about equal to 1). The raw currents were output from tape to a signal averager (9 bit resolution, 2 ms/bin) and 31-34 pulses were averaged at each voltage. The averaged signal was then played onto a chart recorder and is displayed in (c). Transient (●) and steady-state (○) levels of averaged current were plotted in (a). The rates of passage to steady-state were determined by plotting $I(t)$ vs. t on semilogarithmic paper and taking the best exponential fit by eye as the Tau which was plotted in (b). The cell was bathed in Solution A.

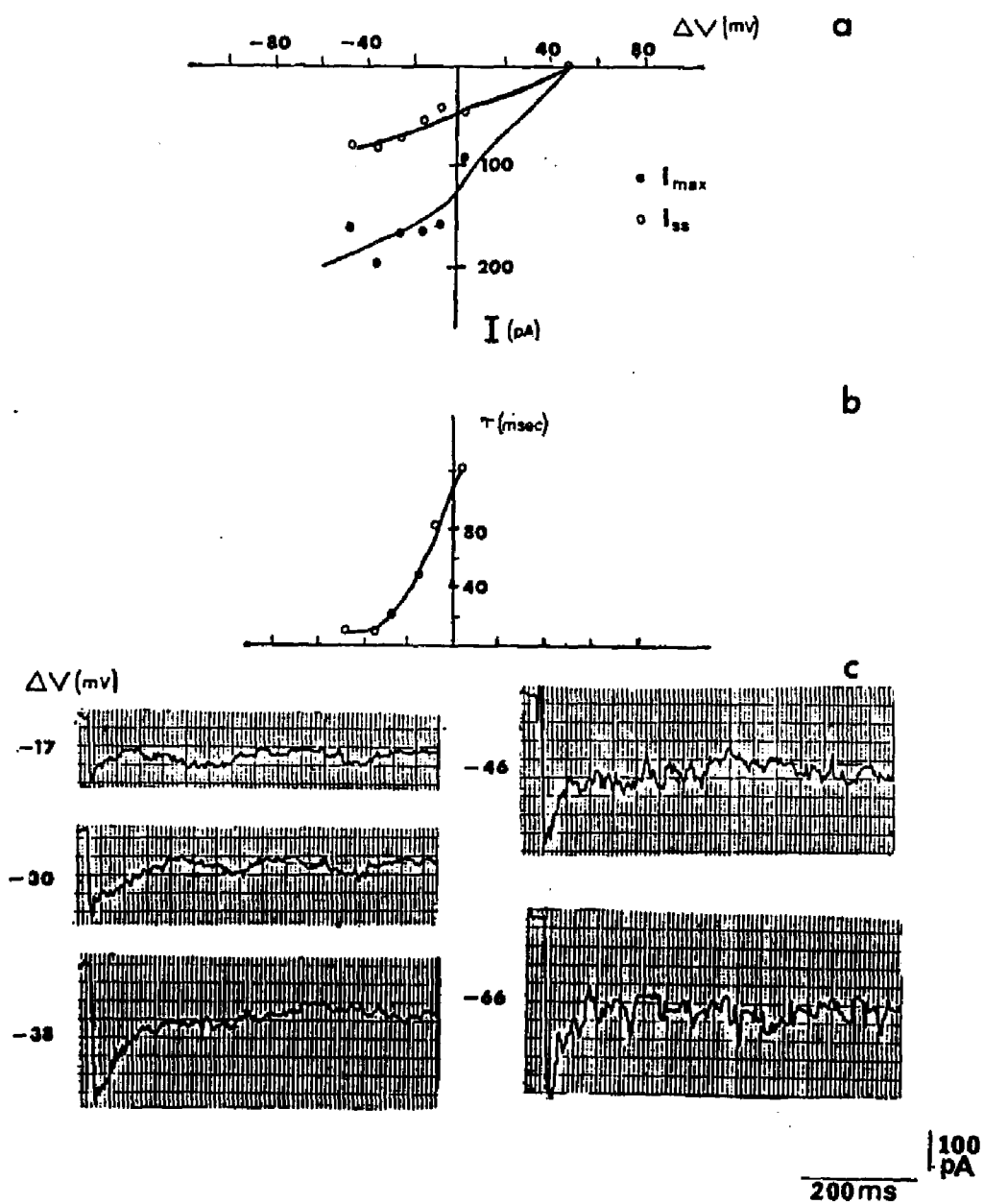


Figure R-32. Ensemble Voltage Pulse Analysis on IR Predicts Partial Deactivation of Whole-Cell Currents on Hyperpolarization

states become faster at more hyperpolarized voltages. This change in the transition rate matrix (Colquhoun and Hawkes, 1981) will occur instantaneously on the pulse, but the rate constants impose that time will pass as the probabilities of states redistribute to a new steady-state. We have already shown that the burst length decreases with hyperpolarization. Proportionally the interburst interval increases with voltage. If each of these rates changed in the transition rate matrix upon the voltage pulse in the manner expected from steady-state analysis, then we would expect a relaxation of the average current as shown in Figure R-32c; the number of ensemble relaxation τ 's in the exponential fits would be an indication of the number of rates affected by hyperpolarization. Figure R-32b shows the fastest rate constants at different voltages determined by logarithmic transform fits to the data; normally there is a slower time constant that is not included here. These changes in time constants may well be represented by voltage dependence of the burst length and interburst intervals; any kinetic model should bear this supposition out.

The temporal changes in probabilities of states impose a time inhomogeneous non-stationary process (Aldrich and Yellen, 1983). Rather than an absorbing barrier for the inactivated state, the rates here represent a process that is more appropriately called a deactivation. The probability of opening is highest when the membrane is held slightly depolarized from the RMP. When hyperpolarization commands are provided the channel still "knows" that it had a high probability of being open (a Markov process senses only the adjacent state of origin). The averaged current at time equal to zero is then

large depending on the driving force. Time-dependent current relaxation is related to the time dependent (nonstationary) change in the distribution of open states, rather than an absorbing inactive state as the channel still opens at deep hyperpolarized potentials but much less frequently--an absorbing state would remove all activity whatsoever.

The relaxation in current here does not represent the I_Q current found in hippocampal neurons in the slice preparation. Those currents activate with hyperpolarization rather than deactivate (see Discussion).

5. Channel Blockade Mechanisms

a) Open-Channel Block by Cesium. Inward rectifying conductances have classically shown a sensitivity to block by the presence of Cs_o^+ (Hagiwara et al., 1976). SC experiments in other preparations have illustrated blocking effects at as low as micromolar concentrations (Fukushima, 1982). Control experiments at 140 mM Cs_o^+ (isosmotic) illustrated no IR "fingerprint" activity, which is not surprising due to a lack of permeant K^+ charge carriers.

With 140 mM K_o^+ , addition of Cs_o^+ in mM or sub-mM concentrations causes very noisy background current levels as well as noisy discrete larger currents (about 5 pA) typically associated with IR in the resting membrane. This effect is seen in Figure R-33, which might be compared with Figure R-18 (open and closed times) also at $\Delta V = 0$ mV. The average current is significantly attenuated at this voltage and Cs_o^+ concentration (5 μM). If one visually estimates the average current through the channel in the presence of Cs_o^+ and compares it to control current levels, it can be appreciated that the

percent decrease in average current through the channel is larger with progressive hyperpolarization. This could indicate that the degree of block is voltage-dependent. Hyperpolarization across the channel might pull the positively charged Cs^+ through the channel mouth where it blocks at some point in the open channel. Each temporal point in a burst is then the average current (on the order of 10^8 charges/sec) through the channel as Cs_o^+ moves into and out of the binding site(s). The rate of Cs_o^+ access to the blocking site must be quite fast since we do not see discrete ON/OFFs inside the burst but rather "noisy" oscillations which are the averages due to the limited bandwidth of the instrumentation; Cs_o^+ block would then be considered a fast flickery block (Yellen, 1984a). It would appear from the records that the rate and degree of block is voltage dependent, as more discrete ON/OFFs are seen at more depolarized voltages. In this patch at 5 μM Cs_o^+ and $\Delta V = 0$ mV a discrete maximum level of current is discernible and fluctuations appear to occur from around this open level; hence, an estimate of the maximum current would be about 3 pA, which is somewhat reduced from patches at 140 mM K_o^+ without Cs_o^+ (4-5 pA). So even at $\Delta V = 0$ mV and 5 μM Cs_o^+ , the block may be sufficiently fast to partially attenuate the burst maximum current. Hyperpolarization of the patch to -38, -56, and -66 mV makes for rapid blocking events so brief and frequent that maximum open-channel current is never approached. The average current through the channel appears to vary during the bursts as well. Perhaps this may be due to the putative multiplicity of K^+ (and Cs^+) binding sites in the channel and that at higher Cs_o^+ concentrations, more negative voltages, or with longer burst times, the sites load up more fully on

Figure R-33. Open Channel Block by Cs_o^+

Data are from a CAP experiment with $140 \text{ mM } \text{K}_o^+$ and $5 \text{ uM } \text{Cs}_o^+$. Holding potential was varied to observe effects of voltage on Cs_o^+ block. Data acquisition occurred at 2.25 kHz . Note flickery block occurs only when channel is open. Note also the different amplitude cursor in the upper left trace vs. the other three. The cell was bathed in Solution A.

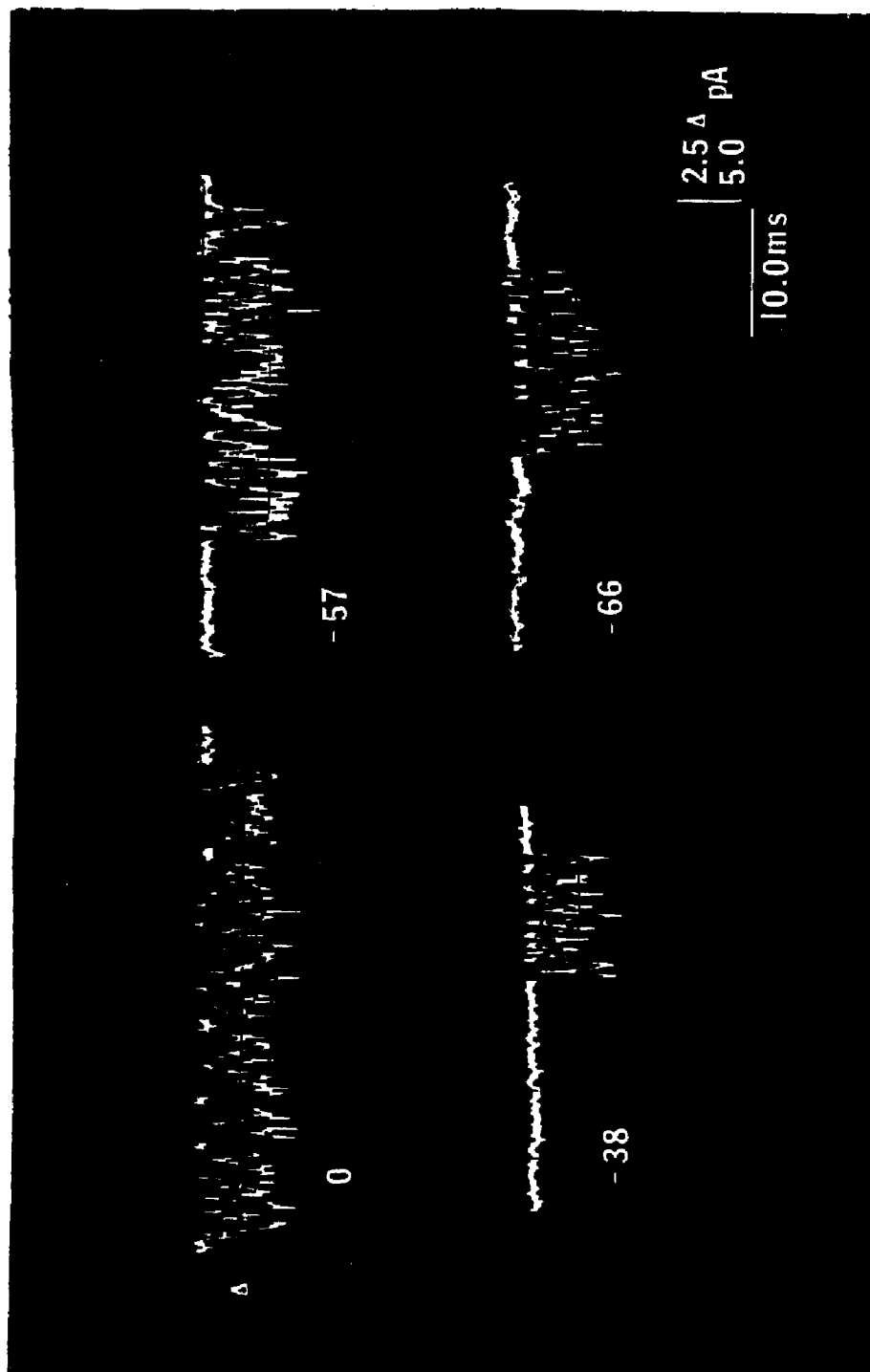


Figure R-33. Open Channel Block by Cs_O^+

average and the current can oscillate between a number of average levels.

The classical estimate of positioning the Cs_O^+ blocking sites in the open channel could be obtained through a detailed study of the concentration- and voltage-dependence of the block. To date, data with less than 5 μM Cs_O^+ are not available. Further studies should clearly explore the Cs_O^+ block as a reporter of the physical sites of blockade within the open channel permeation path.

An interesting question is whether Cs_O^+ integrates into the substate structure of the kinetic mechanism. For example, does Cs_O^+ affect the $P_v(o)$ of substates (if one could resolve them clearly at very low Cs_O^+ concentrations). In support of this premise, substates in other channels may be related to block mechanisms. Curare, a classical nicotinic antagonist, has been shown to exert partial agonist effects on nicotinic channels and can induce the appearance of a substate structure (Trautmann, 1982; Takeda and Trautmann, 1984). Perhaps substates are related to a slow-channel block mechanism (for example, originating from the inner surface of the channel) and that if Cs_O^+ were a slow blocker, the appearance of burst currents might be similar to subcurrent levels seen above.

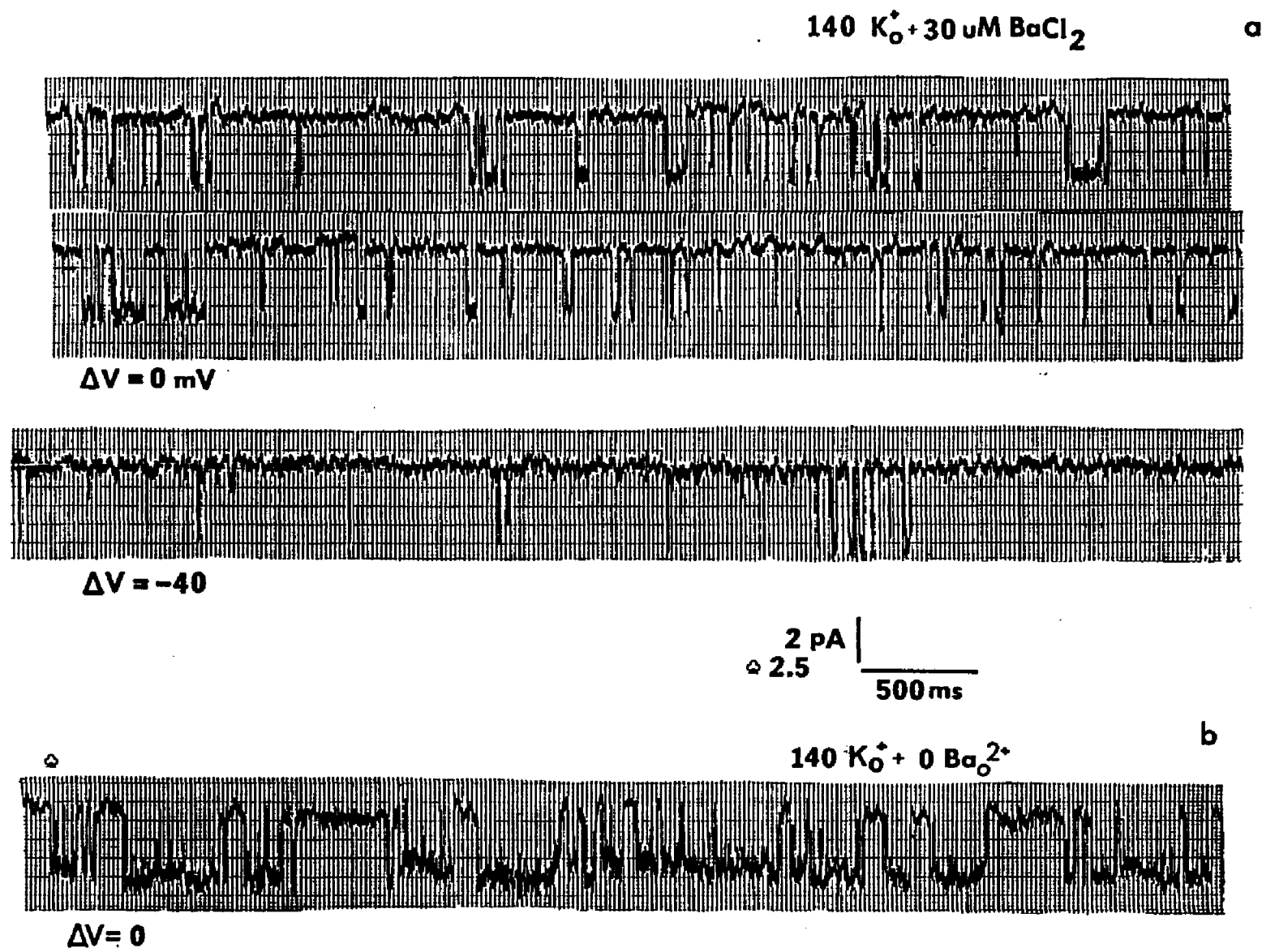
b) Barium May Induce Kinetic Blockade in IR. In addition to blocking the current permeation through the open channel, it is also possible to alter the kinetic rates interconnecting different states with blockers that may bind at sites in the channel conformation that are responsible for the molecular phenomenon of gating. Alterations in the energy levels between different conformers would be expected to alter the rates between and hence change the probabilities of states.

The divalent cation barium (Ba^{2+}) may have such an effect on IR. Figure R-34 shows a single IR channel in the presence of 30 μM Ba_O^{2+} and 140 mM K_O^+ . Shown for comparison is an IR channel exposed only to 140 mM K_O^+ at $\Delta V = 0$ mV where $P_V(o)$ is high (approximately 0.8). In the presence of Ba_O^{2+} at the same voltage, however, the burst length and apparent interburst interval appear radically altered relative to control at $\Delta V = 0$ mV. With Ba_O^{2+} the burst length is much reduced while the interburst interval is longer. The I-V curve and conductance is not significantly altered by Ba_O^{2+} around RMP but at deep hyperpolarized levels the inward current is attenuated as shown in Figure R-35a. However, the $P_V(o)$ vs. voltage relationship is radically affected, as shown in Figure R-35b. This curve was constructed by assuming that short gaps do little to affect the mean ON time during a burst. We can then estimate $P_V(o)$ in the presence of Ba_O^{2+} by the ratio of the sum of burst durations (< 2 msec) to the total of burst durations and interburst intervals. This results in 0.44 and 0.8 for $P_V(o)$ at 0 mV with or without 30 μM Ba_O^{2+} , respectively. Ba_O^{2+} appears to change the voltage-dependence of channel opening probability. At -20 mV the estimated $P_V(o)$ is 0.02, and at -43 mV, 0.05 in this patch. At +10 mV $P_V(o)$ is about 0.52, and at +30 mV, 0.82. In the Figure, a typical $P_V(o)$ vs. voltage curve without Ba_O^{2+} is shown for direct comparison. The slope of the function is slightly decreased with Ba_O^{2+} (37 mV/e-fold change in $P_V(o)$ with Ba_O^{2+} , and 25 mV/e-fold change without Ba_O^{2+}). Also the half probability voltage (K_m) is -21 mV without and +8 mV with Ba_O^{2+} . Thus, Ba_O^{2+} in this channel acted by shifting the $P_V(o)$ vs. voltage

Figure R-34. Possible Kinetic Block of IR by Ba_o^{2+}

Data are from a CAP with 140 mM K_o^+ with (a) or without (b) 30 μM Ba_o^{2+} ($n = 1$). Note by comparison that the burst duration with Ba_o^{2+} is significantly shorter at $\Delta V = 0$ mV than control. Open-channel current shows discrete openings without obvious evidence of fast open-channel block. The same patch with 30 μM Ba_o^{2+} held at -40 mV is also shown to illustrate further the attenuation of kinetic activity. The cells were bathed in Solution A.

Figure R-34. Possible Kinetic Block of IR by Ba_0^{2+}



curve 20-30 mV to more depolarized levels. Since the open-channel currents are not strongly affected around RMP, the block might be largely kinetic and Ba_o^{2+} would affect the rates of transition between states related to the burst length and interburst intervals. It is not possible to rule out a more direct open-channel, voltage-dependent block effect of Ba_o^{2+} at this time, however. More data is needed to appreciate Ba_o^{2+} concentration effects on channel conductance and kinetics.

Point-by-point multiplication of the I-V and $P_V(o)$ curves yielded the time-averaged SC currents as shown in Figure R-35c. Relative to control, the presence of Ba_o^{2+} promotes a profound decrease in the time-averaged current that flows through the IR channel. The NRR remains, however, due to decreases in $P_V(o)$ and conductance at hyperpolarized potentials.

Burst lengths and interburst intervals for this patch at $\Delta V = 0$ mV are plotted in histograms with their controls in Figure R-35d. In the presence of Ba_o^{2+} (30 μM) both the burst and interburst intervals fit single exponential distributions of 53 ms (19/sec) and 135 ms (7.4/sec). These values can be directly compared to the distributions determined without Ba_o^{2+} (140 mM K_o^+) where the burst length and interburst interval time constants were 317 ms (3.15/sec) and 50 ms (20/sec). This represents an 83.4% decrease in the mean burst length and a 270% increase in the mean interburst interval. Thus, Ba_o^{2+} exerts a profound effect on the kinetics of IR. Considerations of the effects of Ba_o^{2+} on a SC mechanism will be found in the Discussion.

Figure R-35. Kinetic Analysis of Ba_o^{2+} Effect on IR

(a) SC currents are decreased in the presence of Ba_o^{2+} at hyperpolarized potentials ($n = 1$).

(b) $P_v(o)$ is decreased by Ba_o^{2+} such that the midpoint of the $P_v(o)$ curve (same slope as control) is displaced about 30 mV to depolarized potentials by Ba_o^{2+} .

(c) Time-averaged SC currents show that Ba_o^{2+} decreases the magnitude of the expected steady-state currents at all voltages. Note the NRR still remains and is due to the maintained decrease in $P_v(o)$ with hyperpolarization.

(d) Histograms of burst duration and (e) interburst intervals at 0 mV show that 30 μM Ba_o^{2+} decreased mean burst duration 83% and increased mean interburst interval 270% relative to a control case. Distributions in all cases fit linear semilogarithmic plots.

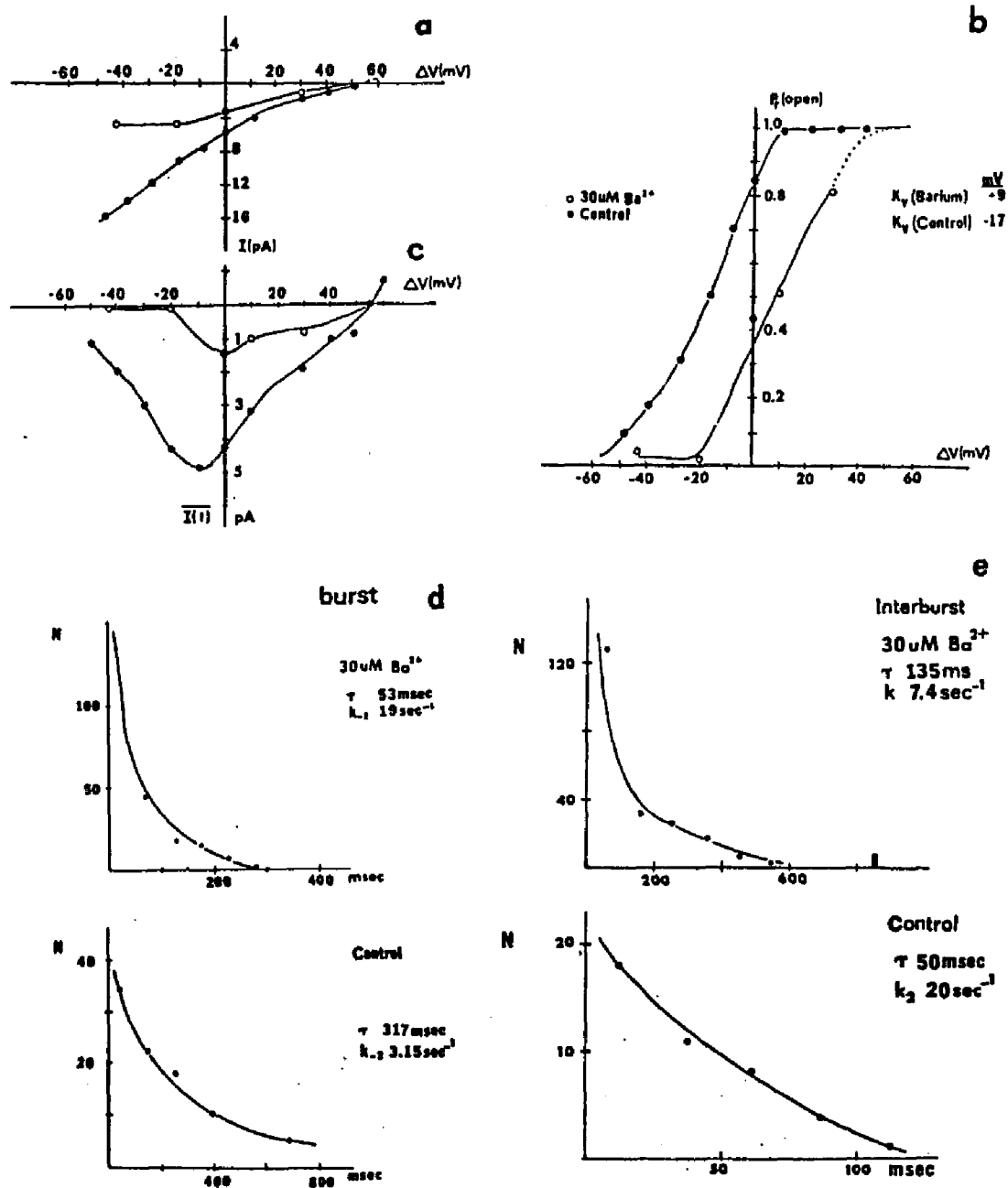


Figure R-35. Kinetic Analysis of Ba^{2+} Effect on IR

6. Whole-Cell Currents

The patch clamp experiments described above were done with on-soma patches. Our objective has been to explain WC excitability or, more explicitly, whole soma-excitability. Since the RMP of the soma affects the information transfer function of the whole neuron, macroscopic excitability in this particular spatial element is of considerable interest.

Two problems are immediately encountered by entering the WC voltage clamp mode: the spatial distribution of capacitative and resistive elements and the complex of unwanted conductance elements. The latter problem can be largely eliminated on the basis of previously established pharmacologic sensitivities of ion conductances in general and specifically in hippocampal pyramidal cells from the slice or in culture. The cells used for these experiments were routinely bathed in physiological saline solutions (Solution A, Table I) that contained 4-AP (to block I_A), TTX (to block fast I_{Na}), manganese or cobalt (to block I_{Ca} and indirectly I_{CaK}). Furthermore, the pipette perfusion solution was chosen judiciously to avoid unwanted conductances; potassium fluoride was the major ion pair where the fluoride ion buffers (in cooperation with EGTA) internal calcium to about 2×10^{-9} M to block I_{CaK} directly. Under these conditions and under hyperpolarizing voltage pulses, the only currents that could flow are slow sodium currents (Dichter, 1983), K^+ currents, or H^+ currents (Barish and Baud, 1984). Chloride currents are separated out on the basis of electrochemical diffusion (the chloride equilibrium potential under these experiments is about -110 mV). Under resting membrane conditions, outward K^+ conductances (e.g., the delayed rectifier)

are not active (Segal and Barker, 1984b); in addition, delayed rectifier channels in this preparation have a much lower conductance than the channels studied here (15-20 pS versus 124 pS with symmetrical 140 K_o⁺). Proton currents, however, would not be segregated out under these conditions and have complicated voltage clamp analysis in other preparations (Barish and Baud, 1984). Under the voltage ranges used in these experiments the currents flowing should be largely K⁺ currents (e.g., IR conductance, delayed rectifier). The remaining problem is whether the membrane is adequately clamped spatially. That is, with all the surface area of membrane (capacitance) to be charged by the voltage steps through the internal resistivity of the cell and the access resistance of the electrode, one must be able to control the voltage of the entire surface membrane or at least the membrane elements of interest. The combination of spatially distributed resistance and capacitance slows down the rate and electrotonic distance at which this control occurs. When voltage and current are controlled by the same electrode as in the WC clamp, the point potential at the electrode tip must spread in time and space sufficiently fast and far to control the conductances of interest. Evidence from hippocampal pyramidal cells in the slice and for the cells in culture suggest that single electrode voltage clamp is quite capable in this regard (Johnston, 1981; Brown et al., 1981; Johnston and Brown, 1983; Segal, 1983).

Hippocampal neurons in the slice have extensive dendritic arborizations (hundreds of micrometers long) yet are electrotonically compact ($1 \leq \rho \leq 2$). Thus, regions far into the dendritic tree would see the potential at the soma. Embryonic neurons used in this study

are not as fully developed as their adult companions since the dendritic tree is only locally distributed (about 50 μm) around the cell, as seen in Figure MM-2. Space clamp for assumed similar electrotonic properties would thus be even better justified in such cultured cells. Segal (1983) has shown that hippocampal neurons in culture are electrotonically compact. Since we were only interested in a somal IR conductance, a somal-proximal dendrite space clamp appeared well supported for low-resistance electrodes (about 5 M Ω) and a small cell (10–15 μm) with membrane resistance of about 300 M Ω ($n = 14$). Moreover, the capacitative currents in these cells relax apparently monoexponentially with a time constant of about 3–4 ms. Since we are only interested in rather slow IR kinetics, the voltage clamp is fast enough to clamp the channel population. As will be seen, the current temporal relaxations are one to two orders of magnitude slower than capacitatively generated currents.

Inasmuch as the resting potential of hippocampal neurons and neurons in general is predominantly a K^+ electrode (Dichter, 1983), the holding potential was set at the calculated V_K knowing now that K_o^+ and K_i were experimentally controlled. Hyperpolarizing and depolarizing pulses were provided to the cell and the currents recorded under conditions of exposure to varying concentrations of K_o^+ ($\text{Na}_o^+ + \text{K}_o^+ = 145 \text{ mM}$). Figure R-36 shows a panel of typical currents elicited under different K_o^+ concentrations (10, 30, 75, 140 mM K_o^+) in different neurons. In all cases the currents show a voltage-dependence where larger hyperpolarizations elicit larger inward currents. Moreover, the macroscopic currents appear sensitive to K_o^+ with currents becoming larger for similar driving forces at

larger K_o^+ ; this occurs for both transient and steady-state currents. Reversal potentials were determined by reversal of current in single or dual pulse paradigms.

I-V relations for these cells are plotted in Figure R-37. The transient currents show a somewhat linear trajectory at low K_o^+ (10, 30 mM K_o^+) with a sublinear bend at deep hyperpolarized levels. This effect is more clearly noted at high K_o^+ concentrations where steady-state currents show a characteristic bend under with hyperpolarization about 30 to 40 mV below V_{rev} . This effect becomes so pronounced by 140 mM K_o^+ that a clear NRR appears centered around a maximum current at -40 mV absolute potential. Negative resistance is typical of bistable conductances (e.g., g_{Na} , g_{Ca}) that can elicit action potentials. It is highly doubtful that IR participates directly in action potential firing but the bistability of potential that could result from current flow through the macroscopic IR conductance may aid in setting unique, stable operating potentials.

The conductance can be estimated from these I-V curves by taking the slope of the steepest curves immediately hyperpolarized below V_{rev} . The conductance increases with K_o^+ in a proportional manner. No attempt is made here to draw functional conclusions based on the conductance vs. K_o^+ relationship, as it has not yet been possible to compare K_o^+ effects on the same cell successfully; nor has WCR been used on enough cells to justify any statistical comparisons as in the SC recording. The important feature of these results is that the WC currents show a conductance dependent both on voltage and the distribution of K_o^+ similar to that found in IRs of other systems.

Figure R-36. Whole-Cell Currents in Different K_o^+ s.

Illustrated is a collage of currents generated from cells at fixed K_o^+ (10, 30, 75, 140 mM K_o^+) under WCR. Cells were typically less than one week old in culture and perisomal dendritic distribution was local and within about 50 μ m (see Figure MM-2). Upon entering the WCR mode (pipette contained WCR intracellular solution--Table I) the membrane was held at the calculated V_K , and hyperpolarizing and depolarizing voltage pulses were used to clamp membrane potential. Potentials illustrated here were total absolute potentials. The bathing solutions were extracellular WCR solutions listed in Table I. Na_o^+ was removed to maintain osmolarity when K_o^+ was increased.

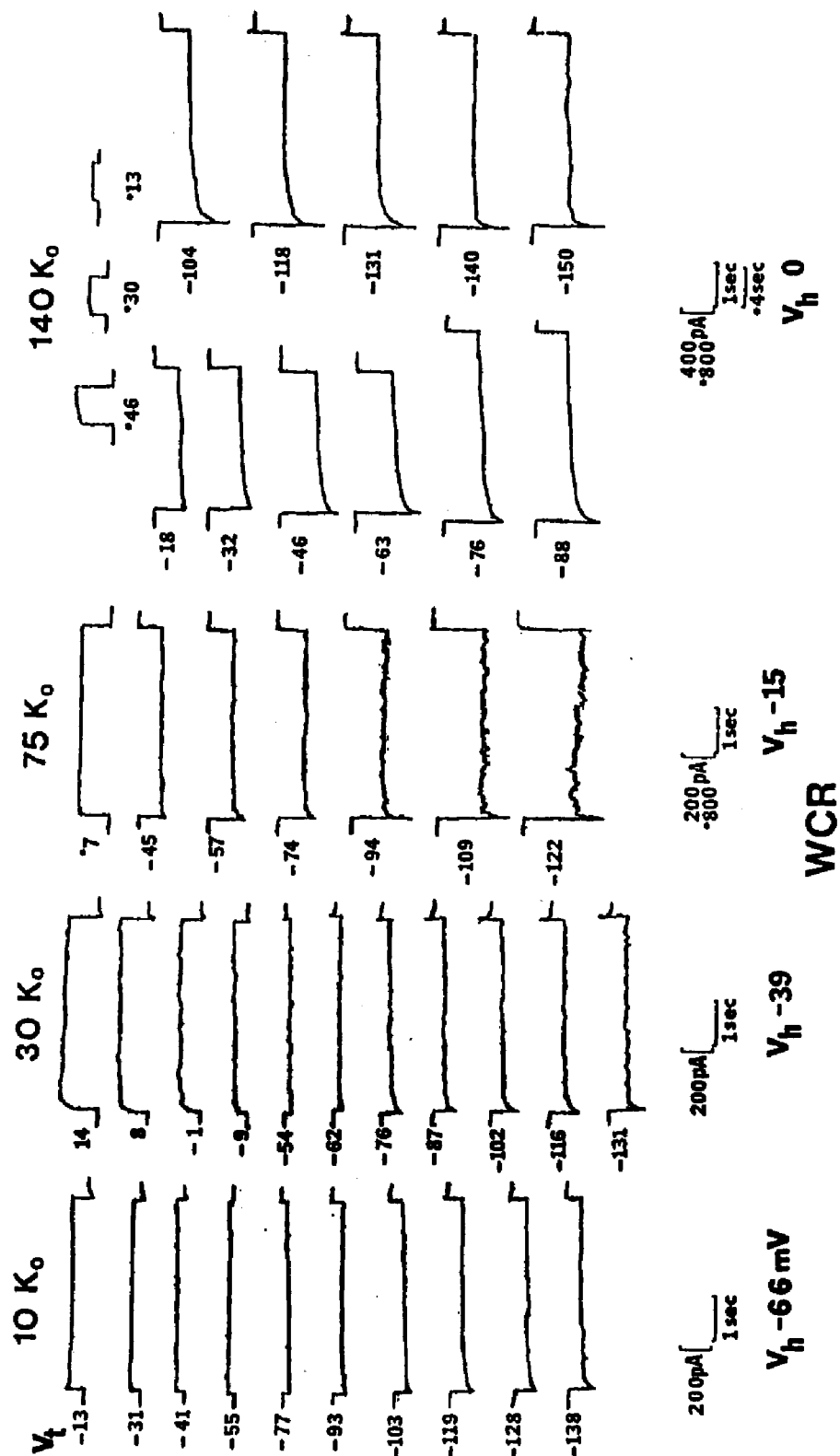


Figure R-36. Whole-Cell Currents in Different K_o^+

Figure R-37. Whole-Cell I-V Relationships.

(a) WCR results from cells that were voltage clamped under different K_o^+ s. I-V curves illustrate the transient ($t = 0$) and steady-state currents from cells as in Figure R-36. Voltages illustrated are absolute membrane potentials. Cells were held at the calculated V_K value from which hyperpolarizing and depolarizing clamp pulses were provided (4 seconds). Symbols for each condition are clear from the inset to the figure. Also inset are three representative clamp pulse transient results from a cell held at 30 mM K_o^+ . Notice that transient currents are always larger than steady-state currents for hyperpolarized pulses while the reverse is true for depolarized clamps. Also note for these cells that increasing K_o^+ increases outward currents (apparent crossover effect) on depolarization as well as increasing inward conductance.

(b) Effects of 5 mM Cs_o^+ on WCR currents of the cells used in (a) at 10 and 140 mM K_o^+ . Note that Cs_o^+ decreases the magnitude of currents relative to control but transient currents still remain larger than steady-state currents. The inset illustrates the response of a cell with 140 mM K_o^+ to a -72 mV clamp pulse immediately after Cs_o^+ was added to the bath (top) and about 20 seconds later (bottom). Cs_o^+ promotes development of an inward current which appears to correlate with the development of block.

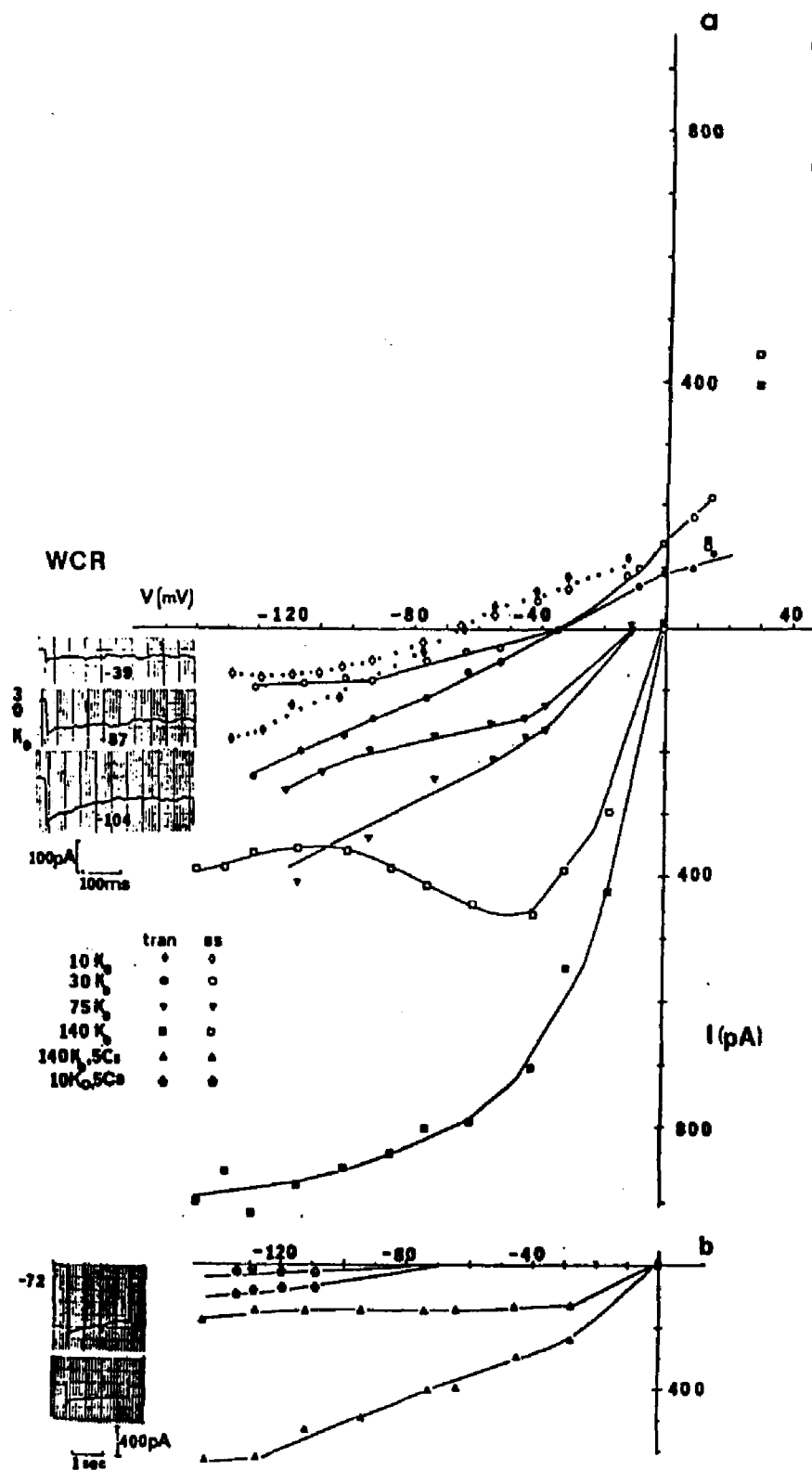


Figure R-37. Whole-Cell I-V Relationships

The macroscopic currents can be blocked by Cs_O^+ . In 140 mM K_O^+ the I-V relationship in Figure R-37a can be compared to results in the same cell after exposure to 5 mM Cs_O^+ (b). The I-V relationship for transient and steady-state currents in the presence of Cs_O^+ are profoundly attenuated. The transient conductance is attenuated about 48%, and the steady-state conductance, 60%, showing the effectiveness of open-channel block by Cs_O^+ on IR as seen in SC experiments. Similar effects for the 10 mM K_O^+ cell are shown in the same panel where the conductance transient is reduced about 45% and the steady-state by 70%. Initial block of WC IR currents occurs coincident with a slowly increasing inward current that could represent block of the "background" conductance. Effects of Ba_O^{2+} on the macroscopic level have yet to be determined.

These results emphasize that SC experiments make accurate predictions about the nature of the WC currents. We can compare the results from WCR to the results obtained from ensemble experiments on SCs. In both cases the transient inward currents decreased temporally to lower steady-state currents at rates determined by the magnitude of the hyperpolarizing command voltages. Our model predicts that these relaxations are due to voltage-induced changes in two or more rate constants. Time constants for current relaxations estimated from the slopes of semilogarithmic plots of currents vs. time are displayed in Figure R-38. It can be seen that the τ values show a steep voltage-dependence and decrease to small values with progressive hyperpolarization. These plots can be directly compared to data in Figure R-32b where the voltage-dependence of the τ values from 140 mM K_O^+ ensemble experiments are plotted, as well as Figure R-25 where

the voltage-dependence of the burst length rate constant is plotted. If the passage toward equilibrium in the distribution of closed states is responsible for voltage dependence, then relaxation constants should show a voltage dependence similar to that of the burst length. These results can be compared by simultaneously plotting current relaxation constants from WCR and ensemble experiments with the burst duration τ_{aus} of SC experiments at each respective voltage. This data is plotted in Figure R-39. It can be seen that the τ_{aus} determined for burst durations, ensemble relaxations, and WCR relaxations all show a similar voltage dependence with τ_{aus} all decreasing with voltage. The correlation of τ_{aus} appears greatest over the range of -100 to -60 mV (absolute potentials) which corresponds to the voltage range $(-50 \text{ mV} \leq \Delta V \leq -10 \text{ mV})$ where the $P_V(0)$ vs. ΔV curve shows the steepest change. Over this range of potentials the distribution of burst lengths adequately explains the ensemble or WCR currents. At more hyperpolarized or depolarized levels perhaps other aspects of the mechanism could be involved (e.g., closed times).

With the above information and some assumptions about surface area we can estimate the number of IR channels in a typical cell. For the 140 mM K_0^+ cell in Figure R-37 the most steep instantaneous conductance is 16.6 nanosiemens (nS). We know from patch experiments that at 140 mM K_0^+ the mean conductance is 124 pS/channel. Direct division gives 133 channels/cell soma. Typically, only one to two channels are active in a single patch and many patches contained no IR channel. Assuming the surface distribution of IR to be homogeneous, we can calculate the expected surface area for a hemisphere (assuming only one-half of the soma is exposed with the other half attached firmly to

the dish) for a cell that is 10 μm in diameter to be about 175 μm^2 . With 133 channels/cell and 1 cell/175 μm^2 , we arrive at about 0.7 channels/ μm^2 . Considering that patches vary from 2-5 μm^2 , the empirically observed channel occurrence appears consistent with the expected channel density. For a channel at low density and high conductance it is interesting to speculate about the role of such elements in the cell (see Discussion).

At low K_O^+ (10 mM) in some cells ($n = 2$) a current was seen that is similar to the I_Q current observed by Halliwell and Adams (1982) and Segal and Barker (1984). This type of current was not seen in cells exposed to higher K_O^+ . The currents observed are shown in Figure R-40. In the Halliwell and Adams (1982) and Segal and Barker (1984b) experiments, the K_O^+ concentration was never manipulated to above the 30 mM level used in this study and so I_Q may represent an effect seen at more typical K_O^+ concentrations (1.4 to 10 mM). On the other hand, it is an empirical observation that the I_Q -like currents were seen in larger, more spherical cells. Whether these cells are more mature pyramidal cells, swollen cells, or an entirely different population of cells is not clear at this time, but might be answered with the recent development of surface-specific monoclonal antibody markers for certain neuronal types in hippocampus (Schaffner and Moskal, 1984).

Figure R-38. Relaxation Kinetics for Whole-Cell Currents

Current magnitudes ($I(t)$) were plotted vs. time on semilogarithmic paper, and τ s were estimated from the trajectories. There was always a fast declining component originating from $t = 0$ and sometimes a slower relaxation. Both of these τ s are plotted for each membrane potential at different K_0^+ s. Both fast and slow distributions became more rapid relaxations with hyperpolarization. Rates of deactivation do not appear to be affected by K_0^+ in the cells examined.

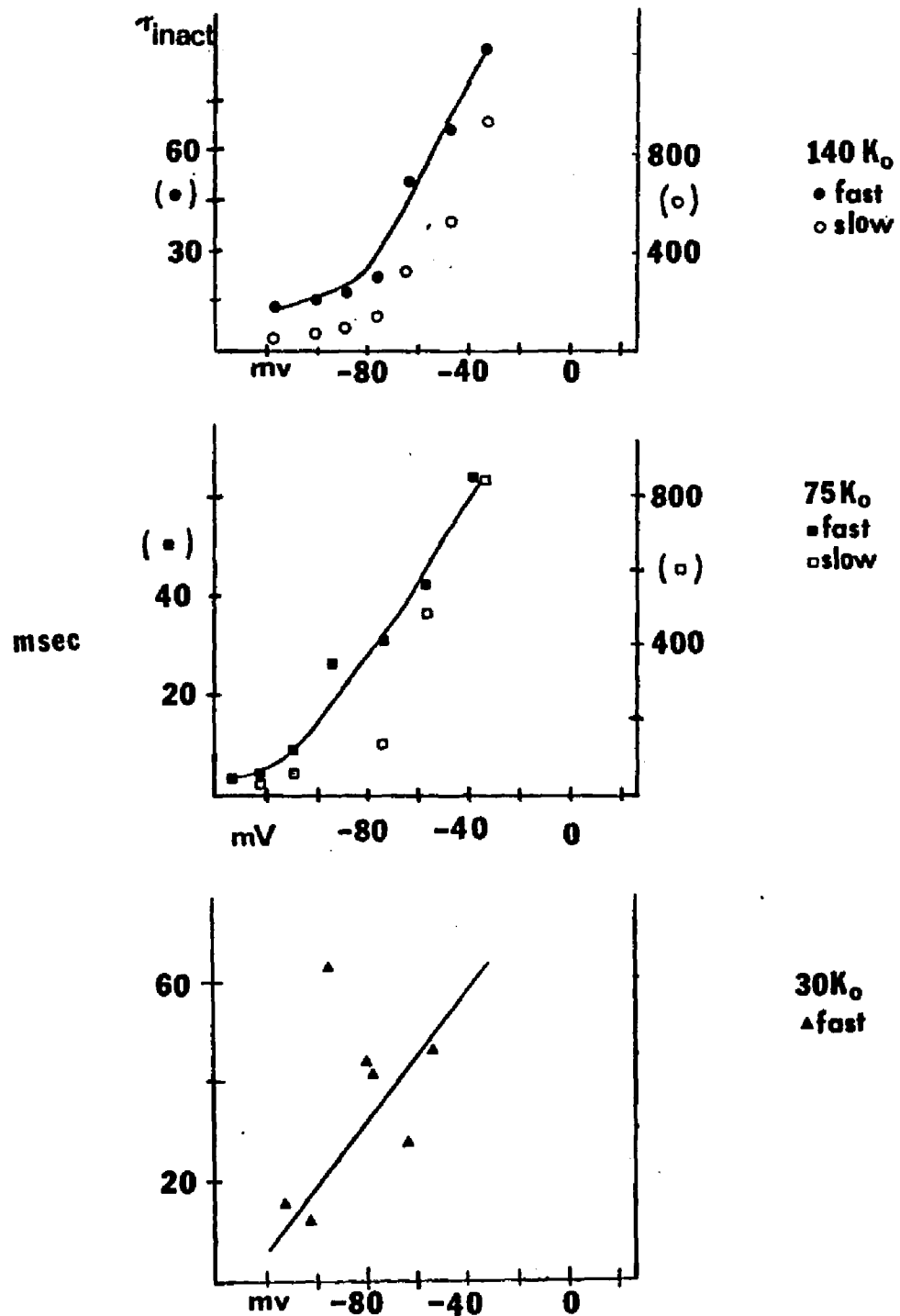


Figure R-38. Relaxation Kinetics for Whole-Cell Currents

Figure R-39. Comparison of Burst Duration Time Constants, Ensemble Time Constants and Whole-Cell Time Constants at Different Voltages

τ determined for the burst durations (B), ensemble average currents (E), and WC currents (W) are plotted on the same axis vs. absolute membrane voltage ($\Delta V - 52$ mV) over the complete range of the $P_V(o)$ vs. ΔV curve. Note the clustering of the ensemble and WC currents around the burst duration times especially over the voltage range (-100 to -60 mV) which corresponds to the steepest change in the $P_V(o)$ vs. ΔV curve (see Figure R-15c). Note also how τ s increase for all measurements with depolarization, although the steepness of the increase is not apparently as large for WC currents.

COMPARISON OF TAUS FROM WCR, ENSEMBLES, AND BURST ANALYSIS

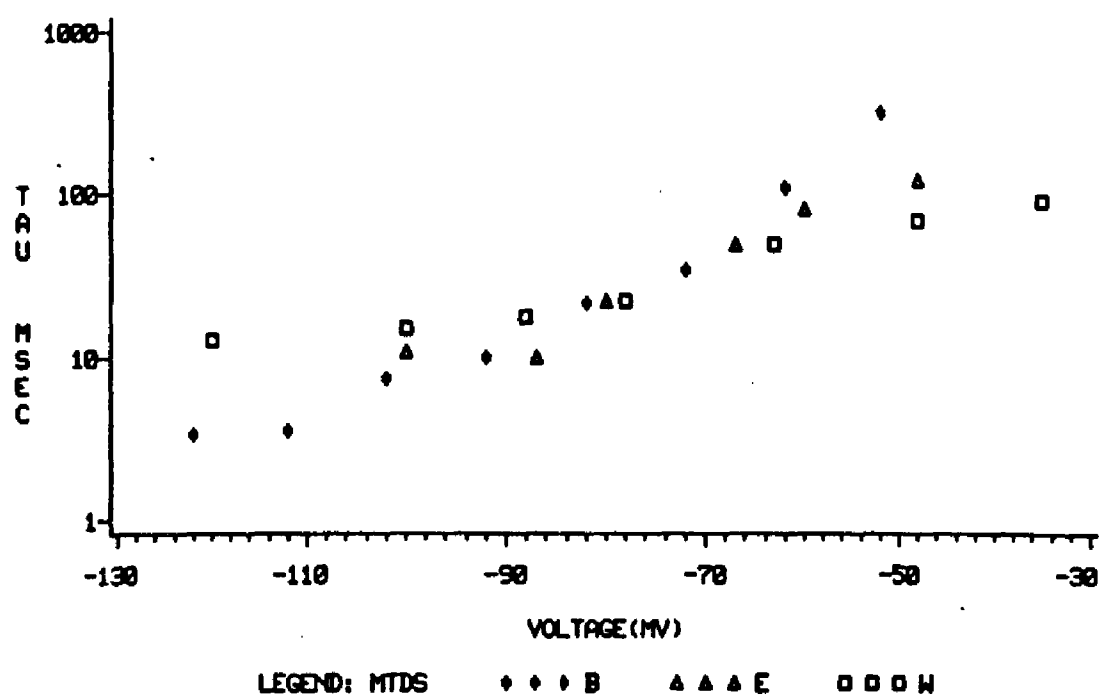


Figure R-39. Comparison of Burst Duration Time Constants, Ensemble Time Constants, and Whole-Cell Time Constants at Different Voltages

Figure R-40. I_Q -Like Currents in Large Cells

(a) WCR of a larger, more spherical neuron with 10 mM K_o^+ .

Cell was held at -66 mV (V_K) and depolarizing and hyperpolarizing pulses were provided to clamp the membrane potential. Shown are traces which result from the respective clamp pulses. Note that command pulses are potential displacements from the holding potential (or V_K). Also note that currents are larger at steady state than at $t = 0$ for pulses greater than 50 mV hyperpolarized from V_K .

(b) I-V plot of the data in (a) above. Instantaneous and steady-state symbols are illustrated. Membrane potential is absolute voltage. The sigmoid region drawn into the I-V plot is meant to indicate the probable participation of inward and outward rectifying conductances.

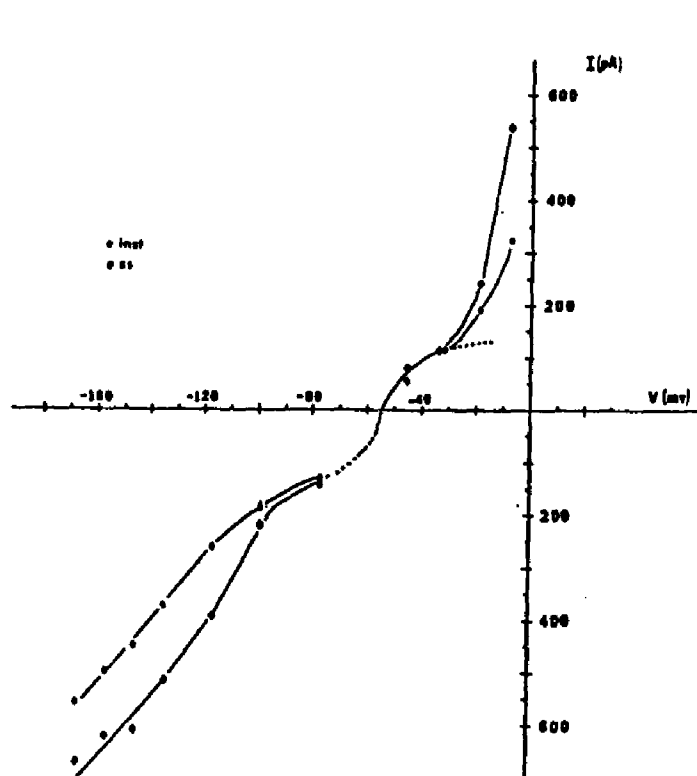
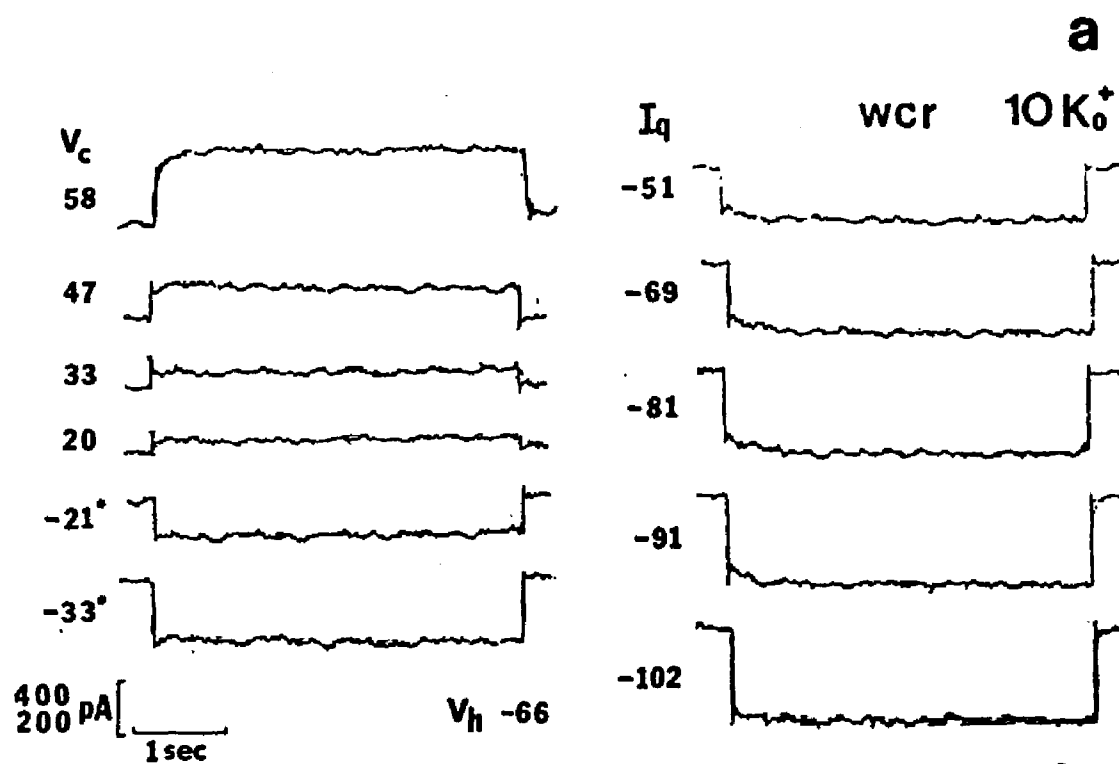


Figure R-40. I_Q -Like Currents in Larger Cells

Chapter IV

DISCUSSION

A. Summary of Results

A large conductance, inward rectifying, K^+ -selective channel has been discovered in CHNs that is active at RMP. This IR has a $g(V-V_K)$ dependence as found in other preparations and also an apparently intricate subconductance state structure. The dependence of conductance on $(K_O^+)^{1/2}$ and the saturation of SC currents with increasing K_O^+ possibly imply a multi-ion pore. Channel kinetics are complex with increases in probability of opening occurring with an apparently opposing decrease in conductance. Three closed and at least one open state will apparently be necessary to explain the kinetics. IR is blocked by Cs^+ and Ba^{2+} acting from the outside. Averages of hyperpolarizing ensemble pulses from resting potential show voltage- and time-dependent decreases in current that can be explained by changes in probabilities of states. WC currents kinetically similar to that expected from SC ensemble experiments provided corroborative evidence.

B. Critique of the Dissociated Hippocampal Neuron in Culture

We know that neuronal information processing is spatially organized into operations performed on topographical surfaces with discrete capabilities. The genome of neuronal cells is well endowed in its ability to support the design of cellular topology or morphology. Typically the "spherical" soma is an integration or summation point to input information coming from dendritic fields and converts information

into a temporally shaped voltage that initiates and maintains impulses of APs which are bussed (transmitted) on cylindrical axons. The complexity of neuronal form has been related to the complexity of function as put forth by Ramon y Cajal (1901, 1911), who initially described the anatomy of the hippocampus: "the form of the cell, insofar as it is the expression of its relations (with other cells), is consequently one of its most important attributes."

The hippocampus being allocortex, a geometrically simplified extension of the contiguous neocortex, has attracted the attention of many neurobiologists and electrophysiologists. One focus of study has been the hippocampal slice preparation, which has provided a great deal of information on the electrophysiological properties of pyramidal neurons. There do remain problems with study of the pyramidal neuron in the slice, however: (1) the small size of the pyramidal neurons makes them difficult to voltage clamp with two microelectrodes, and single electrode clamps are typically much slower in responding, (2) slices are typically hundreds of micrometers thick and diffusion of nutrients into and by-products out of the slice could create spatial gradients of cells in different metabolic states---this potential problem has not been suitably addressed, (3) the complex nature of the extracellular spaces and its inherent matrix creates tortuous paths of currents flowing into or out of cells; coincident complex voltage gradients and ion accumulations in the extracellular spaces could cause offsets in voltage or current clamp data such as to obscure the actual membrane electrophysiology. A detailed analysis of the impedance properties of the hippocampal slice is in order here such as has been done in heart muscle (Levis et al., 1983) and in the lens (Mathias et

al., 1979, 1981; Rae et al., 1982), (4) it is not possible to observe the electrophysiologically monitored cell in the slice continually, so changes in the state of the cell (e.g., swelling) cannot be visually correlated with electrophysiological changes. These potential problems are largely minimized by recent advances in the maintenance of dissociated hippocampal neurons in primary cell cultures which offer a potentially more informative preparation on a macromolecular level than the slice.

The development of methodology to maintain primary dissociated hippocampus in culture has a short history. Banker and Cowan (1977, 1979) were the first to succeed in culturing primary dissociated embryonic rat hippocampal neurons. Peacock et al. (1979) similarly succeeded in the culture of primary dissociated fetal mouse hippocampus. Separation of the cellular elements of the hippocampus allows direct observation of morphologic differentiation for correlation with electrophysiological properties.

Banker and Cowan (1977, 1979) have shown that maintenance of rat hippocampal neurons in culture is sensitive to the state of differentiation of the cells at the time of dispersal. Cells that are postmitotic 12-48 hours prior to plating do best in their culture system. Dissociation of rat hippocampi at 15-18 gestational days results in a neuronal preparation that is highly enriched for pyramidal over granule and basket cells, which in situ develop predominantly postnatally (Angevine, 1965, 1975). Thus, appropriate choice of time of dissociation leads to a highly selective enrichment of pyramidal neurons. Depending on the culturing density, media, types of hormonal supplementation, and substrate (e.g., collagen or polyionic charged

surfaces), the cultures can be maintained typically for two to four weeks or even longer. Certain conditions such as low cell density, defined media, and polyionic substrates can help to maintain predominantly glia-free cultures.

Two criteria can be used to show similarity of pyramidal neurons in culture to intact hippocampus: morphology and electrophysiology. Banker's group (1979) has compared the morphology of rat embryonic hippocampus maintained in culture with that of similar post-gestational aged neonates. Their conclusion is that cellular morphologies distribute into three categorical types in culture: stellate, bipolar, and major-process-dominated (M type). The M type is the most prevalent one in their system and shows remarkable similarity to pyramidal neurons in the neonate or adult hippocampus. In fact, based on dendritic morphology and distribution, they claim that M types can be subdivided into those resembling pyramidal neurons from CA1 (basilar dendrites and an apical dendrite which tapers but does not bifurcate) and CA3 (similar to the above but with the apical dendrite bifurcating at around 100 μ m from the soma) of the neonate or adult rat (see Figures 6 and 7 of Banker and Cowan, 1979). These M type neurons were those selected visually under Nomarski optics in this study. In contrast to apparent isomorphism in the dendritic arborization, it appears that axon-like processes typically originate from dendrites rather than from the basilar soma and so CHNs do not resemble the in vivo case on this point. These results apply to cells maintained in culture from one to three weeks.

Recent studies have dealt with morphologic maturation of hippocampal neurons in culture. As CHNs develop in culture, their

somas increase in size and become more round (Rothman and Cowan, 1981). CHNs form synaptic connections in culture with their axons and dendrites assuming distinct polarities at synapses. In the cultured neuronal system as in vivo, axons are typically presynaptic and dendrites are postsynaptic (i.e., the correct axodendritic or axosomatic synapse polarities); moreover, dendrites in culture contain rRNA and stain for microtubule-associated protein-2, which is not present in axons. In addition, hippocampal neurons can mature morphologically in culture without synaptic contacts which suggests an inborn program of development. Embryonic hippocampal cultures have recently been maintained in serum-free, chemically-defined media as in this study, typically with coculture of spatially segregated glial elements which presumably provide a neurotrophic factor; under these conditions morphologic differentiation parallels development in serum.

It would appear from judicious consideration of the available data that most of the morphological identifying criteria of hippocampal pyramidal neurons are maintained in culture. This is also true in other neuronal culture systems (Ransom et al., 1977a-c). For this study a large body of data supports the use of morphologic criteria to select pyramidally shaped CHNs for electrophysiological analysis.

Recent studies on biochemical (Seifert et al., 1983; Walker and Peacock, 1982) and immunological (Schaffner and Moskal, 1984) properties of CHNs also support the similarity of the pyramidal neurons in culture to those in the intact hippocampus but will not be detailed here.

The next criterion to examine is whether electrophysiological properties extend from the intact animal to dissociated cell culture.

Extracellular and intracellular recordings from the intact hippocampus (Kandel and Spencer, 1961a), the hippocampal slice (Schwartzkroin, 1975, 1977), organotypic explants (Gähwiler, 1981a,b; Gähwiler and Dreifuss, 1982; Zipser et al., 1973), and cells in culture yield similar resting potentials, resting impedances, synaptic (EPSP and IPSP) and action potential firing, time constants, as well as characteristics more typical of epileptogenic processes--the PDS. Peacock et al. (1975) were the first to show morphologic and electrophysiologic differentiation of fetal mouse CHNs, demonstrated resting potentials of -38.2 ± 0.79 mV, time constants of 1-2 msec, input resistances of 30-50 MOhms, spontaneous and driven action and synaptic potentials, fast pre-potentials (Spencer and Kandel, 1961), as well as depolarizing synaptic and hyperpolarizing afterpotentials. Higher order electrophysiologic properties exhibited between cells were reciprocal innervation, multiple innervation, and synchronous bursting in cell populations (Peacock, 1979). Peacock noted that electrophysiologic characteristics of hippocampal neurons in culture were very similar to the intact preparation or to the slice. Since then, intracellular electrophysiological characterization of fetal rat CHNs has provided similar results (Segal, 1983). Acute enzymatic and mechanical dissociation of adult mammalian hippocampus also provides pyramidal shaped neurons that have similar resting potentials, time constants, firing behavior, and sensitivities to neurotransmitters (Gray et al., 1982; Numann et al., 1982). It would appear, therefore, that morphological and electrophysiological differentiation are maintained as stable properties in transfer from intact hippocampus to dissociated cell culture.

Neurons in culture do show a strong tendency to maintain their developmental programming of cytoarchitecture and function: primary dissociated mouse hippocampus can reaggregate if plated at high density to form structures which mimic dentate gyrus and cortical laminations in the intact animal (DeLong, 1970). It would appear that processes intrinsic to the neurons themselves (e.g., surface recognition) allow normal paths of differentiation to occur on dissociation provided that the culture environment permits cell survival. We have routinely observed reaggregation of dissociated neurons in culture but never to the extent of in vivo cytoarchitectural mimicry. Aggregates of cells are typically connected by fiber bundles after about one week in culture. At the same time the neuritic plexus becomes quite dense. The generation of such structures appears dependent on initial cell plating density and the type of substrate provided to the cells for adhesion.

Use of tissue culture systems does raise controversies which need to be mentioned. For example, plating typically starts with embryonic mouse or rat neurons that (in different laboratories and different animals) may have already achieved different states of developmental programming. Culturing clearly disturbs the architecture of the developing hippocampus. Furthermore, to provide environments for cell growth, either serum or chemically-defined media are provided. Without yet knowing what hormonal signals might be lost by architectural dissociation and put back or remain missing in culture, it is impossible to speculate on any alterations in the developmental fate of the neurons. An empirical observation of the relative difficulties of finding the IR in neurons grown in chemically-defined media versus

serum-maintained cells may shed some light on the possibilities of hormonal effects in the state of electrophysiological differentiation of the pyramidal neurons in culture. The expression of ion channel genes may be under such hormonal control. The presence and spatial distribution of ion channels may be related to signals (hormones, trophic factors) arising spatiotemporally from the external milieu and could be causally related to any anisotropic distribution of channels. The "informational content" of the growth medium may also have effects on the differentiated state of cellular metabolism that could affect the appearance or activity of certain channels or their spatial distribution. In fact, steroid hormones (corticoids, gonadal) added to chemically-defined growth media have recently been shown to acutely affect electrophysiological properties of pyramidal neurons in the hippocampal slice (Michal, 1974). On the other hand, the neurotrophic factor produced by glial coculture appears to affect cell viability and neuronal maturation directly (Mueller and Seifert, 1982).

Although other laboratories generate low-density cultures where neurons survive in isolation, we have found that sparse but relatively higher density plating aids in the long term survival of the neurons, perhaps by the formation of some concentration-dependent trophic factor. With high density plating one hopes to generate as near a natural condition to the development of the hippocampal neurons as possible. Indeed, the pyramidal neurons examined in our laboratory show healthy electrophysiologic characteristics. All of the neurons used in this study were from cultures grown 4-30 days in vitro, which represents approximately the first postnatal month for the mouse. It should be mentioned that neonatal pyramidal neurons in the hippocampus

are very labile to epileptogenic phenomenon (Purpura, 1964). Whether this is of any relevance to the results, conclusions, and speculation derived from this study is not clear (see Discussion- Section G).

To summarize, the cultured hippocampal neuronal system is an analytical system with many advantages and similarities to the in situ hippocampus. It is beginning to provide an adequate and useful tool to study electrophysiological properties on a macromolecular and biophysical level which has not previously been possible in hippocampus. One expects with the continual integration of information learned from cultured neurons and that obtained in other systems, that a significant impact on our understanding of the electrophysiological responsiveness of hippocampal neurons will result.

C. A Survey of Inward Rectification in other Systems- Comparison with the Conductance and Kinetic Properties of IR in CHNs

1. Introduction

The SC conductance and kinetic properties of the IR in CHNs will be discussed in terms of their relationships to WC currents found in CHNs from this and another lab as well as to pyramidal cells in the hippocampal slice over similar voltage ranges. Similarities and differences to IR currents found in other preparations will be discussed with special emphasis on the conductance and kinetic properties derived from SC or noise analysis. A progressive development of the IR conductances in other preparations will serve to illustrate, by example, the diversity of macroscopic IR conductances, the respective SC conductance behavior and gating responsiveness. This analysis will allow us to be able to place IR in CHNs into a context of membrane excitability. Within the context of the conductive and

kinetic details of the channel participating in whole membrane excitability, one can review how such a process could function in a pyramidal neuron in a stable state of signal processing and one in an unstable (epileptiform) state.

2. Whole-Cell IR Currents

Preliminary experiments on WC currents activated by hyperpolarization in mouse CHNs showed similar temporal and voltage dependences to SC ensemble currents. Currents were large on increasing hyperpolarization below V_K , the reversal potentials were consistent with V_K values, and currents showed voltage- and time-dependent partial deactivation. Unfortunately, changes in the I-V curve upon changes in bath K_o^+ for a single cell were not technically feasible at the time of this writing, so the functional dependence of macroscopic g_{IR} on K_o^+ are based on extensive SC data but WCR data from only a limited number of cells. In most cells the same type of WC currents were seen on hyperpolarization, that being a rapid unresolved "activation" of currents which partially inactivate over time in a voltage- and time-dependent manner. There is an apparent increase in conductance with voltage and with increasing K_o^+ such that the process appears to exhibit the $g(V-V_K)$ dependence which is consistent with the SC experiments and with macroscopic and SC IR conductances in other preparations (see below). Further studies on mouse CHNs should extend these experiments by comparing WC and SC currents property-by-property. Similar types of WC IR currents (see below) have been found in egg cells, skeletal muscle, heart muscle, macrophages, vestibular hair cells, as well as magnocellular cholinergic neurons and olfactory cortex neurons.

3. Specific Detail of Reports of IR in Preparations of Hippocampal Pyramidal Neurons

There are only four laboratories that have reported results on macroscopic inward-going rectification in hippocampus: the Halliwell and Adams (1982) and Segal and Barker (1984b) voltage clamp studies, as well as the earlier Purpura et al. (1968) and the Hotson et al. (1979) current clamp studies. Segal and Barker (1984b) used CHNs from rat while the others reported experiments performed in the hippocampal slice. The Halliwell and Adams (1982) study was done on CA1 pyramidal neurons in the slice using a single electrode voltage clamp. The Segal and Barker (1984b) study employed, quite remarkably, a two-electrode voltage clamp on small (20 μ M in diameter) rat CHNs (4-6 weeks) that should be similar to CHNs in mouse.

Purpura et al. (1968) found two types of IR conductances in healthy pyramidal neurons, one with and one without a voltage sag during hyperpolarizing current pulses from resting potential. It is interesting that IR is not always correlated with the "sag" in the voltage response under current clamp but, nevertheless, this is usually represented as an identifying property for IR (e.g., Segal and Barker, 1984). The conductance increase occurred at least 10-15 mV more hyperpolarized than the RMP (-50 to -60 mV). No attempts were made to ascertain the ionic nature of the current. In the current clamp study on CA1 and CA3 pyramidal neurons by Hotson et al. (1979), an IR was found over a 15 mV range subthreshold to neuronal firing (slightly depolarized above resting potential). This increase in resistance on depolarization was removed by manganese, a Ca^{2+} channel antagonist,

as well as with the classical Na^+ channel antagonist, TTX, but was enhanced by Ba^{2+} , which increases Ca^{2+} channel currents (Hille, 1984) but blocks K^+ conductances (Armstrong, 1980). The enhancement of this IR by Ba^{2+} occurred concurrently with prolonged depolarizing afterpotentials which were associated with bursts of cell firing (that showed no accommodation) as well as oscillations in membrane potential, typical properties of epileptiform neurons. Hotson et al. (1979) associated these responses with the well studied repetitive firing behavior in molluscan systems (Faber and Klee, 1972) and hypothesized that the subthreshold IR was due to persistent slow inward (mixed) $\text{Na}^+/\text{Ca}^{2+}$ currents combined with slow outward K^+ currents. They did not, however, find the IR of the Purpura et al. (1968) study which activated at -70 to -80 mV. Unable to offer explanations for this discrepancy, they suggested that multiple forms of IR could exist in hippocampal neurons. The same suggestion has been applied to clamped currents below resting and near threshold potential regions in cat motor neurons (Barrett and Crill, 1974).

Halliwell and Adams (1982) used the single-electrode voltage clamp to study the muscarinic-inactivated current (I_M) and I_Q in the hippocampal slice where activation of these voltage-sensitive conductances are separated by a region of potential. Q currents were activated by hyperpolarizations below -70 mV and showed "instantaneous" activations followed by a slowly increasing inward current. In tail current analysis the Q currents reversed at potentials sensitive to the level of K_o^+ but less than one would expect for a purely K^+ -selective process. Replacement of Na_o^+ with choline shifts V_{rev} for I_Q to hyperpolarized potentials, suggesting that Na^+

also permeates through the Q channel. The conclusion of Halliwell and Adams (1982) was that g_Q is a relatively nonselective cation channel favoring K^+ over Na^+ . The Segal and Barker (1984b) study permitted similar conclusions on a current they called I_{AR} in rat CHNs. On the contrary, the IR process described here appears ideally K^+ -selective. Q is profoundly affected by Cs_O^+ , which blocks both the steady-state and, interestingly, the instantaneous "leakage" conductance in the cells temporally observed just prior to turn-on of I_Q . Outward current tails were not significantly affected by Cs_O^+ , which could indicate that other conductances were present or that a voltage-dependent block by Cs_O^+ is removed on depolarization. Voltage-dependent block of IR K^+ channels and other K^+ channels by Cs_O^+ has been well noted (Hagiwara et al., 1976; Gay and Stanfield, 1977). The distinct block of leakage currents by Cs_O^+ may imply that another conductance (e.g., g_{IR}) is active at RMP, and supports a major conclusion of this study on mouse CHNs; a lack of Cs_O^+ effect on outward currents is similar to the results here, where both an outward conductance or/and a release of voltage-dependent block by depolarization could be responsible. Similar effects of Cs_O^+ on g_{AR} occur in the Segal and Barker (1984b) study, except that Cs_O^+ does appear to block outward tail currents significantly. Application of Cs_O^+ in both the Segal and Barker (1984b) and this study results in a slowly increasing inward current which may be due to a block of outward K^+ currents through IR at resting potentials around V_K . This effect of Cs_O^+ also occurs in heart muscle resting potential background currents (i.e. I_{K1}) (Isenberg, 1976; Vereecke et al., 1980) and in skeletal muscle (Beauge

et al., 1973); the effect of Cs_o^+ was not apparent in the Halliwell and Adams (1982) study but several minutes were required for equilibration of bath solution changes into the slice. Only in this study is the conductance sensitive to the magnitude of K_o^+ .

Ba_o^{2+} apparently attenuates IR on a SC kinetic level (not clear on WCR yet) but does not affect macroscopic g_Q or g_{AR} , suggesting that the conductances arise from different channel populations.

The results and conclusions of the Halliwell and Adams (1982) and Segal and Barker (1984b) studies are quite similar and I_Q and I_{AR} probably represent the same conductance. There remain properties of g_{IR} that are similar to g_{AR} and g_Q , however. In all cases with command voltages slightly below RMP, the instantaneous and steady-state currents are nearly identical and currents at these voltages may represent leakage. It is only with further hyperpolarizations that the instantaneous and steady-state currents become disparate and in opposite ways in this study compared with the Halliwell and Adams (1982) and Segal and Barker (1984b) results. IR is a rather voltage-dependent conductance that partially deactivates on prolonged hyperpolarizations below V_K , whereas g_Q and g_{AR} are apparent activators on hyperpolarization. Table II summarizes and compares relevant properties obtained for WCR on hyperpolarized "activating" currents in the three studies. It is clear that there are similarities and differences between this study and the others, and one can provide two alternative explanations: (1) g_{IR} and g_{Q-AR} most likely represent two uniquely different conductances that have IR properties and are both involved in membrane potential control over similar functional ranges, or (2) the conductances are similar, if not

Table II

COMPARISON OF I_{IR} , I_Q , I_{AR} IN HIPPOCAMPAL PYRAMIDAL NEURONS

	I_{IR}	I_Q (HA)	I_{AR} (SB)
Preparation	Cultured mouse hippocampal neuron	Hippocampal slice	Cultured rat hippocampal neurons
Method	Single channel, whole-cell recording	Single-electrode voltage clamp	Two-electrode voltage clamp
Selectivity	K ⁺ -selective ($V_{rev} = V_K$)	Mixed K ⁺ /Na ⁺ currents ($V_{rev} > V_K$)	Mixed K ⁺ /Na ⁺ currents ($V_{rev} > V_K$); chloride sensitive*
Conductance and K_o^+	g dependent on $(K_o)^{1/2}$	g not apparently K_o^+ -sensitive	g is K_o^+ sensitive but not to $(K_o)^{1/2}$
Rectification (inward)	Open channel property	Rectification implied but not clear from data	Rectifies but not clear if conductive or kinetic
Cs_o^+ block	+, apparently voltage and concentration dependence on single channel and whole-cell levels. Open channel block.	+	+
Ba_o^{2+} block	+, on single channels. possible kinetic block.	-	-

Table II—Continued

	I_{IR}	I_Q (HA)	I_{AR} (SB)
Active at Resting Potential	Yes. Cs^+ causes inward current at V_K in cells where Q -like currents are not present.	Opens 10-20 mV below RMP. Cs has no effect on background currents at RMP.	+/-, Cs causes inward current at RMP—probably blocks leakage channels.
Voltage Sensitivity	Very voltage sensitive—partial inactivation with hyperpolarization: $I_{inst} > I_{ss}$ always.	$I_{inst} = I_{ss}$ until -80 mV then $I_{ss} > I_{inst}$. Currents turn on with hyperpolarization. Rates increase with hyperpolarization.	$I_{inst} = I_{ss}$ to -80 or -90 mV; then $I_{ss} > I_{inst}$. Currents turn on with hyperpolarization. Zero to little voltage sensitivity in turn on rates.
Outward Currents	Small if any above V_{rev} for single channel. Outward currents present in WCR could be contaminating conductance (I_K , I_H) or removal of internal block by cell perfusion. Cs fails to block outward currents on depolarization.	Outward current tails on depolarization above V_{rev} probably reflects deactivation of I_Q . Cs fails to suppress these tails completely.	Outward current tails on depolarization above V_{rev} . Cs profoundly affects these tail currents.

* It has been shown that an I_Q -like current in hippocampal pyramidal neurons results from a hyperpolarization-activated chloride conductance that exhibits slow activation kinetics and an inward rectifying conductance (Madison et al., 1986).

Note Abbreviations: HA= Halliwell and Adams (1982); SA= Segal and Barker (1984b); inst= instantaneous ;ss= steady-state.

identical, but the differences in their representation result from the properties of the system that effect measurement and quantification. We will discuss these considerations separately before comparing the properties of g_{IR} here to a similar set of conductances found in other preparations.

The fact that I_{Q-AR} appears to be relatively nonselective puts it in direct contrast to IR. Ba^{2+} sensitivity is also a distinguishing property as is the temporal nature of the currents and the lack of deactivation on hyperpolarization. These properties make it unlikely that these IR and Q-AR currents arise from the same conductance process.

One way in which these currents could be from similar processes is due to voltage clamp of a cell with a REDS. I_Q -like currents occur in heart (I_{K_2}) and have an IR property with kinetics that are partly due to the extracellular depletion of K^+ from a restricted diffusional space (Kline and Cohen, 1984; Noble, 1984; DiFrancesco and Noble, 1985). This may be especially significant as low concentrations of K_0^+ are used in most studies (e.g., 3 mM in the Halliwell and Adams (1982) study, 5 mM in the Segal and Barker (1984b) study). IR should also be studied under WCR conditions at lower K_0^+ than was used in this study (10 mM to 140 mM K_0^+).

It is of course significant that I_Q -like currents have been seen under similar voltage-clamp paradigms using WCR in a few cells in mouse CHNs as well. Two potentially related observations are that these cells were slightly larger with more spherical somas and were found in older cultures that had developed significant neuritic plexi. In the Segal and Barker (1984b) study, where these I_Q -like currents were

common, the age of the rat hippocampal neuron cultures was four to six weeks old, possibly more typical of the adult neurons studied by Halliwell and Adams (1982) in the guinea pig hippocampal slice. The neurons used in the Segal and Barker (1984b) study were large, phase bright, spherical and with significant neuritic plexi and thus similar to the mouse neurons in which I_Q -like currents were found. The observed similarities in the appearances of cells when these currents are found suggests that neuronal development may have an effect on expression of this particular conductance. It is also possible that the architectural development of the extracellular space in such "dense" cultures (after several weeks) has effects on voltage clamp currents possibly by way of REDS (see Discussion, Section G). On the other hand, I_R -like currents have also been observed by Segal and Barker (1984b) in some rat CHNs but the conductance was not characterized. It could be possible that these cells were less developed (younger) or that the extracellular space was not as restricted. It is not clear if the I_R and I_Q -like currents were seen in the same cells.

4. Comparison to Other K^+ Currents in the Slice or in Rat CHNs

This brief discussion will refer specifically to K^+ currents in the different hippocampal preparations, as TTX block of fast Na^+ currents (Segal and Barker, 1984a) and cobalt or manganese block of Ca^{2+} currents (see Hille, 1984 for references) was used in all WCR experiments here.

Thus far six separate K^+ conductances have been found in either cultured pyramidal neurons or pyramidal cells (CA1 or CA3) in the hippocampal slice: I_M , I_A , I_{CaK} , I_K , I_{AR} , and I_{IR} . I_M ,

the muscarinic-blocked K^+ current, may have a significant dendritic origin (Brown et al., 1980, 1982; Brown and Adams, 1980; Brown and Constanti, 1980; Adams et al., 1982), and was found only rarely by Segal and Barker (1984b) in rat CHNs; the WC currents seen in the present study have been observed in the presence of 30 μ M carbachol (unpublished observation) so this consideration is apparently irrelevant here. I_A , the fast depolarized-activating, fast inactivating K^+ current originally found first in molluscan neurons by Connor and Stevens (1971 a,b) and Neher (1971) has been demonstrated in the slice (Gustaffson et al., 1982) and in culture (Segal and Barker, 1984b; Rogawski, 1984) to be sensitive to μ M and mM concentrations of 4-AP. 4-AP was routinely added in mM concentrations to block I_A contributions to WC currents in this study. I_{CaK} , the Ca^{2+} -activated K^+ conductance found in hippocampus (Brown et al., 1982; Brown and Griffith, 1983) was blocked indirectly with Ca^{2+} channel blockers and directly with WC cell dialysis using Ca^{2+} -buffered solutions (2×10^{-9} M free Ca^{2+}) (DeCoursey et al., 1984); single IR channels in IOPs remain fully active at Ca^{2+} levels of 10^{-8} M and classical CaK channels have been found with a conductance of about 400 pS and require μ M concentrations of Ca_i for activation. IR channels isolated into IOPs have shown a Ca^{2+} sensitivity within the range of 10^{-9} and 10^{-8} M, being silent or fully expressed, respectively. Once in the same patch the large conductance classical CaK channel was expressed with a channel with apparent IR characteristics but only at greater than 10^{-7} M Ca^{2+} , being fully activated by 10^{-4} M. So, under the WCR conditions used this would leave the following K^+ currents for

activation in these cells: I_K , I_{AR-Q} , and I_{IR} . As the I_{AR-Q} was not typically seen in these neurons, the only known currents remaining are I_K and I_{IR} where I_K refers to the delayed rectifier and I_{IR} to the conductance found here and similarly in CHNs from rat by Segal and Barker (1984b). In their study they found a conductance that carries current briefly on hyperpolarization, partially inactivates inside of 100 ms, but they did not characterize it. This fast inactivating inward current is remarkably similar to the currents observed in this study which apparently originate from IR SC conductances that show voltage-dependent kinetics. The resulting WC I-V plots in the respective studies can be compared (e.g., Figure 2 in Segal and Barker, 1984b and Figure R-37 here). It is noted that outside of the inversion of transient and steady-state currents below V_K (I_{AR} versus I_{IR}) that the I-V relationships are very similar and both show a large outward conductance above about -40 mV ($K_O^+ \leq 10$ mM).

In the Segal and Barker (1984b) study I_K is a large (0.35 uS) fast activating (< 100 ms), very slowly inactivating (seconds) current that is TEA⁺-sensitive (25 mM), Cs_O⁺ insensitive, and distinct from the smaller g_{CaK} . In this study, a current which was large and outward was routinely observed upon sufficient depolarization and shows remarkable similarity to the Segal and Barker (1984b) I_K current. Its conductance is much larger than the IR currents seen in these cells (a few nS)). The outward current appears Cs_O⁺ insensitive under conditions where IR currents are blocked and therefore could result from a conductance distinct from that generating I_{IR} . Moreover, it inactivates slowly with similar voltage-dependent kinetics to the Segal

and Barker (1984b) I_K which shows a voltage-dependent inactivation that was removable with hyperpolarization in a time-dependent manner. These effects for I_K were observed here also and SCs that appeared to be responsible for this outward macroscopic current have been isolated (J.M. Sullivan, unpublished observation). They are small 15 pS, non-rectifying channels that show slow depolarized inactivation which is removable by hyperpolarizing prepulses. TEA^+ was not used during WCR to attempt to block this I_K current because the concentrations used to block it by Segal and Barker (1984b) are above the concentration used to block IR currents in skeletal muscle, which show similarity to the conductance under consideration here (Stanfield, 1970).

With the above in mind it would appear that the resultant macroscopic I-V plots here are the result of two active conductances, I_K and an IR current that is dissimilar in kinetics and selectivity to the I_{Q-AR} found similarly by Halliwell and Adams (1982) and Segal and Barker (1984b). Since the voltage ranges over which these currents show time dependence are separated by a 20-30 mV range of apparent linearity ("leakage"), the respective conductances appear well separated by voltage alone. Nevertheless, it is not clear what to subtract as leakage currents so the I-V plots from WCR have been kept in raw form. We will now compare IR currents found in voltage clamp experiments below V_K with similar currents in other preparations.

5. Putting IR into Context—Two Classes of Conductances

We will compare the IR K^+ currents in CHNs to those in other systems including egg cells, skeletal muscle, olfactory cortical neurons, spinal sensory ganglion neurons, spinal motoneurons,

magnocellular neurons of globus pallidus, molluscan abdominal neurons, rod photoreceptor cells and horizontal cells of the vertebrate retina, vestibular hair cells, basophilic leukemia cells and macrophages, oligodendrocytes, heart muscle, nodal tissue, red blood cells, and another IR in hippocampal cells. We will begin to appreciate that the behavior of the IR currents in this study appear most like that in egg cells, skeletal muscle, background currents in heart muscle (I_{K_1}), IR currents in oligodendrocytes, macrophages, leukemic basophilic leukocytes, vestibular hair cells, and olfactory cortex neurons, although there are unique differential conductance and kinetic properties. We will also see that IR channels in these preparations serve a variety of purposes. While many, as IR here, are believed to be background voltage-sensitive conductances, there are others that are controlled by neurotransmitters such as acetylcholine, glutamate, serotonin, or substance P, others that are ion modulated in their conductance or kinetics (Na_o^+ , Ca_i^{2+} , H_i^+) and still others that reflect the energy charge of the cell (e.g., ATP sensitive IRs). The diversity of appearances of IR and its modulatory influences suggest that it serves important and possibly diverse roles in the ability of biological membranes to process signals.

There appear to be two general types of IR conductances in most of the above preparations. It is therefore useful to establish these classes for the purpose of better detailing the similarities of the IR in mouse CHNs to that in certain preparations rather than belaboring discussion in preparations where significant differences occur.

Also, since some preparations have only been subject to current clamp investigations of excitability, it appears important to discuss

the usual defining criterion of IR using such a method. In many preparations subjected to current clamp techniques, reports have appeared correlating the appearance of a "sag" in the electrotonic potential on hyperpolarizing current injection with the opening of channels responsible for the IR. However, the IR process identified in voltage clamp may not be associated with such a sag in electrotonic potential (Ito and Oshima, 1965; Ochs, 1967; Nelson and Lux, 1970). In some preparations "IR" sags in the electrotonic potential under current clamp occur over voltage ranges outside the activation ranges for IR under voltage clamp (Purpura et al., 1967, 1968). There is typically a region of linear resistance between RMP (zero current potential) and current intensities where "IR sags" activate. Moreover, these ranges of electrotonic potential show by no means an identical similarity from preparation to preparation. What remains clear, however, is that many preparations show time- and voltage-dependent conductance properties around RMP that appear K^+ -selective and are affected by membrane hyperpolarization. Many factors may contribute to the sag phenomenon under voltage clamp (Ochs, 1967): (1) holding potential current, (2) time-dependent changes (opening or closing of channels) in conductance, (3) spatial inhomogeneities in electrical responsiveness, (4) cell size and morphology, (5) extracellular current flow and depletion-accumulation phenomenon. Hotson et al. (1979) have suggested that such phenomenon indicate that there may be several types of IR conductances as discussed above.

The first class of IR conductances includes the Q current in hippocampus and the I_{h-f-K_2} pacemaker in heart, an IR current in spinal sensory ganglion, spinal motor neurons, and rod photoreceptor

cells. This conductance is not purely K^+ -selective, does not display the $g(V-V_K)$ dependence, and can show effects of anion replacement. We will discuss this class of conductances together in bulk.

The second class of IR conductances is typified by that found in egg cells, skeletal muscle and others that we will discuss separately. These conductances have a $g(V-V_K)$ dependence and are blocked by Ba_O^{2+} as well as Cs_O^+ . This class of channels also includes IR in mouse CHNs.

5. a). Class 1-- I_Q and the I_{h-f-K_2} Pacemaker System

As first noted by Halliwell and Adams (1982) the I_Q current shows remarkable similarity to the I_{h-f-K_2} pacemaker system in heart muscle (McAllister and Noble, 1967; Noble and Tsien, 1968; DiFrancesco et al., 1979; DiFrancesco and Ojeda, 1980; DiFrancesco 1981a,b, 1982; Noble, 1984). This is based on the slow kinetics of activation, similar effects of ion replacement, insensitivity to Ba_O^{2+} , sensitivity to Cs_O^+ and the apparent IR property. The authors noted one dissimilarity to the IR in egg cells and skeletal muscle in that the conductance in the latter preparations is sensitive to block by Ba_O^{2+} . The Q currents seen by Halliwell and Adams (1982) in hippocampus are similar to those activated on hyperpolarization in many preparations including motoneurons (Barrett et al., 1980), sensory ganglion neurons (Mayer and Westbrook, 1982, 1983a,b), rod photoreceptors (Bader et al., 1982), heart muscle (see above) and nodal tissue (Yanagihara and Irisawa, 1980).

In motoneurons a nonlinear response of the membrane to hyperpolarizing current injection has been established (Ito and Ishima,

1965; Ochs, 1967). The sag could be accounted for by an increase in conductance, a slow development of potential over the cell surface, or an accumulation of K_o^+ in association with other phenomenon; the nonlinearity was variable and was not present in each cell. Nelson and Frank (1967) studied this response under hyperpolarizing current clamp and noted the inward (anomalous) rectification of the process. The membrane conductance was increased with hyperpolarization and decreased with depolarization but was not always correlated with the "sag." IR occurred over a voltage region slightly depolarized from RMP and again over a range of hyperpolarized potentials and may involve two processes including a slow inward current (Liebl and Lux, 1975) and a K^+ current. As synaptic potentials (EPSPs) do not always increase with hyperpolarization it was suggested that IR in this preparation decreases the resistance seen to inward synaptic currents and effectively short circuits the process (Kandel and Tauc, 1966; Nelson and Frank, 1967). Recent voltage clamp experiments on motoneurons in vivo by Barrett et al., (1980) have identified a slowly increasing inward current activated at hyperpolarized potentials below RMP, which reversed around RMP, and thus probably has a significant K^+ component. It is not clear whether this current has an IR conductance at its origin. Depolarizing voltage impulses showed no time dependence in the outward currents that were activated. This motoneuron conductance has yet to be characterized but appears similar to the Q-AR conductance found in hippocampal pyramidal neurons. Another anomalous rectification (Liebl and Lux, 1975) found at subthreshold potentials in cat spinal motoneurons appears to have a Ca^{2+} and K^+ component and is similar to the IR found by Hotson et al. (1979).

In vertebrate rod photoreceptors light inactivates a resting inward Na^+ conductance that causes a transient hyperpolarizing sag of the membrane potential. In this case the sag is due to the activation of the Cs_o^+ -sensitive conductance that resides in the rod inner segment (Fain et al., 1978). This inward conductance shows a steady-state IR I-V plot, but its instantaneous I-V plot is linear with a reversal potential (-32 mV) consistent with a conductance that is permeable to K^+ and Na^+ . This conductance shows remarkable similarity to the Q and AR currents found in the Halliwell and Adams (1982) and Segal and Barker (1984b) studies on hippocampal pyramidal neurons. Fain and Lisman (1981) have hypothesized that this Q-like conductance activated by hyperpolarization carries inward current (Na^+ , K^+) after a light flash to help repolarize the cell to resting potential (-30 to -40 mV). They speculate that without the influence of this conductance the hyperpolarization to light could shut down transmitter release and profoundly affect the ability to respond proportionally to small and large input signals. When these currents are blocked by Cs_o^+ (and TEA^+ and cobalt to block delayed rectifier, Ca^{2+} , and Ca^{2+} -activated currents) the resultant currents activated by hyperpolarization are very similar to that observed in this study (see Figures 2 and 4 in Bader et al., (1982) and Figure 10 in Attwell and Wilson (1980)). In the rod cell these remaining currents are believed to be due to a nonspecific background leakage conductance of about 0.5 nS. The IR channels in CHNs coupled with the high probability of opening produce an outward rectifying I-V plot (Figures R-15d and R-37) that is similar to that in rods (Figure 2 in Bader et al., 1982).

Recent studies by Mayer and Westbrook (1982; 1983 a,b) have identified and characterized a hyperpolarization-activated current (I_h) similar to I_Q/I_{f-h-K_2} in mouse embryonic dissociated dorsal root ganglion neurons. "Inward rectification" sags had been previously identified in A and C neurons of dorsal root ganglion (Gallego and Eyzaguirre, 1978; Gorke and Pierau, 1980) and were used as criteria for selection of cells for study. The steady-state I-V curve showed pronounced outward rectification with an IR bend around -70 mV; outward rectifying currents predominate above -50 mV. The instantaneous I-V curves for this I_h are linear, and thus the IR property is apparently due totally to the effect of voltage on $P_V(o)$ such that $P_V(o)$ increases with hyperpolarization (g_{max} at -100 mV, $g_{1/2}$ at about -80 mV) and decreases with depolarization. This puts I_h , I_Q , I_{h-f-K_2} in direct contrast to that found in CHNs where $P_V(o)$ increases with depolarization from RMP. The conductance of I_h does decrease at extreme hyperpolarized potentials ($V < -120$ mV), however. These I_h currents turn on faster at more hyperpolarized potentials but are not suitably fitted with single exponentials, which could reflect cooperative gating (Hodgkin and Huxley, 1952), an additional time- and voltage-dependent conductance or K^+ depletion from the extracellular region about the membrane. A second IR current appears to occur at potentials more hyperpolarized than -90 mV but was not characterized. The reversal potential for I_h in dorsal root ganglion neurons is -30 mV and is similar to that in rod cells (Bader et al., 1981), Purkinje fibers (DeFrancesco and Ojeda, 1980; DeFrancesco, 1981a,b), and sinoatrial node (Yanagahara and Irisawa, 1980), where the conductances are mixed Na^+/K^+ nonselective

processes. K_o^+ may have a modulatory slowing influence on the kinetics of activation in dorsal root ganglia as has been found by Halliwell and Adams (1982) in hippocampal pyramidal neurons and in the I_{K_2} IR of Purkinje fibers (DeFrancesco, 1982). The conductance is not selective to K_o^+ , however, as is found in CHNs and, for example, egg cells. It is not blocked by Ba_o^{2+} but is blocked by Cs_o^+ . IR in CHNs, however, shows inactivation, and is K^+ -selective.

The conductance of the similar I_f current in sinoatrial node has a significant anion component (Seyayma, 1976, 1979; Yanagahara and Irisawa, 1980). Anion replacement causes rapid block of the steady-state I_h current but has no significant effect on the instantaneous I-V curve. Effects of anions on the similar Q current in hippocampus were found by Segal and Barker (1984b) and a recent study (Madison et al., 1986) suggests that g_Q may have a significant anion selectivity. Chloride replacement by isethionate does not appear to affect the behavior of IR in CHNs, however (data not shown).

Although the cellular location and distribution of the similar h, Q, and h-f- K_2 conductances are not clear, one study in dorsal root ganglia has examined the effects of peripheral and central axotomy on the nature of current clamp electroresponsiveness. Czeh et al. (1977) have found that peripheral but not central axotomy profoundly decreases IR responses and peripheral axon conduction velocity. Coincident with peripheral nerve injury is a decrease in axon diameter and somal chromatolytic changes (see references in Czeh et al., 1977). Such evidence may shed some light on the potential location of the channels responsible for this behavior; Marsh (1982) has recently shown that rat

cervical sympathetic trunks containing predominantly C fiber axons have an "anomalous rectification sag" under current clamp. In the Mayer and Westbrook (1983) study on dorsal root ganglion the only significant cellular processes were two axons, while hippocampal neurons in the slice or *in vitro* contained both axons and dendrites. If Q channels were located on processes, this may explain why they have yet to be found using the SC recording technique in neuronal preparations or in mouse CHNs in this study. The Q-like conductance found recently in hippocampal cells has an IR property that is activated by hyperpolarization, exhibits significant Cl^- modulation (permeability), and, moreover, is believed to be distributed on cellular processes distant from the cell soma (Madison et al., 1986).

The I_{h-f-K_2} system found in sinoatrial node and Purkinje fibers has been shown to be similar to Q currents in hippocampus and h currents in sensory ganglia and photoreceptors. Originally discovered in Purkinje fibers as a hyperpolarization-induced deactivating K^+ current (Noble and Tsien, 1968) under conditions of ion depletion and contaminating currents (Noble, 1984), a new interpretation (DiFrancesco, 1980, 1981a,b) shows that the conductance is a hyperpolarized activation of a noninactivating inward current carried by Na^+ and K^+ ($V_{\text{rev}} = -10 \text{ mV}$). The conductance was originally called g_{K_2} and was found to be activated over the pacemaker potential region (-90 to -60 mV, diastolic depolarization) in Purkinje fibers. It is steeply voltage dependent and shows instantaneous and steady-state IR properties; the steady-state I-V curve also shows a NRR in the pacemaker range of potential. The I_{K_2} current has since been found to be similar to the I_{f-h} current first discovered in

sinoatrial node (Senayama, 1976; Yanagihara and Irisawa, 1980; DiFrancesco and Ojeda, 1980; Brown and DeFrancesco, 1980), and it appears that the same conductance is present for pacemaking activity in nodal tissue. This conductance is sensitive to block by Cs_O^+ but not Ba_O^{2+} , thereby allowing its separation from other K^+ currents (see DiFrancesco, 1982; Noble, 1984). No SCs have yet to be found in heart which might explain the voltage-dependent, non-selective behavior of this conductance system (Coronado and Latorre, 1982).

Heart muscle is a system similar to hippocampal pyramidal neurons in one unique respect, the presence of REDS. This REDS was a primary cause of initial confusion of the I_{K_2} pacemaker current since in heart muscle hyperpolarizations below V_K cause ion depletion as K^+ current passes through the inward rectifier, I_{K_1} , which (as we will discuss later) is known as a background current in heart and can be blocked by Ba_O^{2+} . DiFrancesco (1981a), initially realizing that the original estimates of V_rev could be in error due to depletion-accumulation problems (DiFrancesco et al., 1979), showed that when I_{K_1} is blocked with Ba_O^{2+} the hyperpolarization activated increase in a then isolated, characterizable, non-selective conductance, $g_{f-h-\text{K}_2}$ becomes evident. Thus it appears unlikely that the Q currents seen in hippocampus are significantly obscured by other conductances or that I_Q and I_IR are the same conductance.

5. b). Class 2-- $g(V-V_\text{K})$ Dependence in a Broad Class of (Egg Cell-like) IRs

We will compare conductance and kinetic properties of IR in CHNs to IRs in the following preparations: egg cells, skeletal muscle, macrophages, basophilic leukemic leukocytes, HeLa cells, fibroblasts,

horizontal cells in retina, olfactory cortex neurons, cholinergic magnocellular neurons of the globus pallidus, abdominal ganglia neurons of Aplysia, islet cells in pancreas, vestibular hair cells, oligodendroglia, heart muscle and nodal tissue, red blood cells, and another similar IR in acutely dissociated hippocampal neurons. Fortunately, in these cases a number of SC studies are available for comparison to the channel in this thesis. It will become apparent that IR in CHNs is distinct but has properties quite similar to many of the conductances to be described.

The resting potential in egg cells (Miyazaki et al., 1974), skeletal muscle (Hironaka and Morimoto, 1979), hair cells (Ohmori, 1984), Purkinje fibers, heart muscle or nodal tissue (e.g., Reuter, 1984), and cortical neurons (Dichter, 1983; Kandel and Spencer, 1961a) is largely determined by the K^+ equilibrium potential. In all of the preparations just described, hyperpolarizing current clamp elicits electrotonic potentials that may show transient sags while progressively increasing hyperpolarizing voltage clamp steps below V_K elicit inward currents that partially decay to smaller steady-state magnitudes in a time- and voltage-dependent manner. The conductances generally are sensitive to $g(V-V_K)$ and are blocked by Ba^{2+} and Cs^+ . They appear to be controlled by a significant number of modulatory influences including ion modulation, second messengers, and more typically, voltage and neurotransmitters. There appear to be significant diversities in the biophysical properties of this class for reasons which may be related to the diversity of function.

6. Specific Comparisons to IR in CHNs

a). Egg Cells

In immature starfish and sea urchin eggs Hagiwara and Takahashi (1974) originally found with current clamp methods an inward (anomalous) rectification in the potential that was sensitive to K_o^+ and showed the selectivity sequence: $Tl^+ > K^+ > Rb^+ > NH_4^+ > Na^+, Cs^+, Tris^+$. The increase in voltage under current clamp allowed calculation of chord conductances, which increased with hyperpolarization according to a Boltzmann function and in a manner sensitive to $(K_o^+)^{1/2}$ ($g(V-V_K)$ dependence). They also found that the IR conductance and its reversal potential showed concentration-dependent changes which exhibited a minimum in solutions containing varying ratios of Tl_o^+ and K_o^+ or an AMFD (Hagiwara et al., 1977). The $g(V-V_K)$ - and $g([K_o]^{1/2})$ -dependence and AMFD provided evidence for a multi-ion pore with nonindependent ion fluxes. They later hypothesized that an IR channel contains a number of sites for K^+ in the channel (multi-ion pore) and that opening of the channel is due to the movement of charged groups in the membrane when membrane voltage is hyperpolarized from V_K but that the sites fail to gate open at $V = V_K$ and more depolarized potentials such that the conductance is inactivated (Hagiwara and Takahashi, 1974) and an IR I-V property results.

Subsequent kinetic studies on IR in eggs using voltage clamp and noise analysis techniques have shown that the conductance is expressed temporally as two components: activating (too fast to record) and inactivating, such that the instantaneous currents upon hyperpolarization are larger than at steady-state thereby indicating a

time-dependent inactivation (Hagiwara, 1979). In eggs the rectification in current clamp derived from this behavior creates a NRR in the total I-V plot that provides for metastable potential states. The conductance is sensitive to V and K_o^+ as discussed above and requires internal Na^+ and Ca_i^{2+} for activation (Ohmori, 1978; Hagiwara and Yoshii, 1979). The inactivation seen at large hyperpolarizations is dependent on the presence of Na_o^+ , which has both a facilitatory (increase) effect on the macroscopic and SC conductances (Hagiwara and Yoshii, 1979) as well as an inactivating effect, yet Na^+ does not permeate the channel proper (Ohmori, 1978). Upon removal of Na_o^+ the NRR of the I-V plot disappears and the currents upon hyperpolarization appear similar to the I_f -h- K_2 system in heart. The block by Na_o^+ appears then to be the cause of voltage-dependent inactivation. The inactivation process at deep hyperpolarizations in the presence of Na_o^+ is due to the effect of Na_o^+ on inducing a decrease in $P(o)$ through a blocking effect. Inactivation arises because Na_o^+ , entering from the external mouth of the pore, blocks the channel in a voltage-dependent manner when the membrane is hyperpolarized; this could be a kinetic or a conductive or open-channel block (Ohmori, 1978, 1980). The increase in g_K with K_o^+ is due to the effect of K_o^+ on the SC conductance whereas the effect of Ca_i^{2+} on increasing G_K is due to an internal action affecting the number of channels active but not the SC conductance, (about 6 pS from noise analysis). Inactivation induced by Na_o^+ can also be obtained with Cs_o^+ , Ba_o^{2+} , Sr_o^{2+} , hydrazine or Li_o^+ ; the inactivation time course is faster in Cs_o^+ , hydrazine, or Sr_o^{2+} , but is slower with Ba_o^{2+} or Li_o^+ . The

unitary conductance is independent of each inactivating species, which appear to block the channel in an all-or-none concentration- and voltage-dependent manner. The facilitatory effect of Na^+ on the IR conductance is due to a modulatory binding site, largely outside of the potential drop, that affects access of blocker into the channel proper; only one blocker ion is necessary for inactivation.

The voltage dependence of inactivation is not only dependent on the transmembrane potential drop and the concentration of blocking particles, but is also affected by the level of K_O^+ . K_O^+ increases inactivation by blocker at low K_O^+ but suppresses it at high K_O^+ . A number of K^+ binding sites may exist in the channel and a model by Ohmori (1980) proposes a three-site four-barrier model where one of the sites, the K^+ modulatory site, is external and outside the membrane field, whereas the other two sites can be occupied by permeant ion or blocker; the cooperative participation of K_O^+ (low) in voltage-dependent block may be due to the stabilization of the blocker-channel interaction or the prevention of impermeant blocker from leaving by way of the entry route; inactivation is suppressed at high K_O^+ because on average both internal sites tend to be occupied by K^+ . Divalent ion block is not affected by K_O^+ suggesting that the two charges on the blocker are sufficient to block conduction.

The WC currents found in mouse CHNs are qualitatively similar to those found in egg cells in the presence of Na_O^+ in both cases. A voltage-dependent inactivation of inward currents at deep hyperpolarized potentials is found that is similar to inactivation in eggs. Inactivation of egg cell SCs in the presence of external blockers is physically due to the alteration in $P(o)$ due to

voltage-dependent block of the channel and not to significant innate voltage sensitivity to the gating as is found for CHN IR. It remains possible in CHN IR that the voltage sensitivity is due to block independent of Na_O^+ , as Na_O^+ was absent from the patch pipettes. Perhaps block could originate from the inside of the channel as suggested by Armstrong (1965, 1966, 1975) and Ohmori (1984), giving an apparent voltage sensitivity to the kinetics. Preliminary results suggest that K_O^+ may have a facilitatory effect on $P_\text{V}(0)$ in CHNs as in eggs, where high K_O^+ blocks inactivation. Moreover, the sensitivity to block by Cs_O^+ has a similar voltage- and concentration-dependence to Cs_O^+ block in egg cells (Hagiwara et al., 1976; Fukushima, 1982). As in eggs, the IR in CHNs participates in setting a NRR, which is seen in steady state I-V plots. However, comparison of SC IR currents in CHNs to the egg cell IR (with Na_O^+ block) (Fukushima, 1982) shows significant disparate results: (1) the decrease in probability with hyperpolarization is apparently due to block, whereas in IR it is apparently due to voltage-dependent gating, (2) the conductance is very low (5-6 pS) in eggs, (3) the channel kinetics in eggs can be explained by a two-state model with single exponentials for the open and closed times with each closed state representing a blocked state (but instrumentation rolloff of 200-500 Hz could have excluded fast events), whereas a four-state model appears essential to explain IR kinetics. Similarities include: (1) $P_\text{V}(0)$ decreases with hyperpolarization due to a decrease in burst duration in CHN IR and in egg cell IR due to a decrease in mean open time in the presence of blocking ion, as well as changes in the mean closed time that depend on blocking ion, (2) apparent low channel

density, (3) block by μM concentrations of Cs_O^+ and Ba_O^{2+} , (4) SC "instantaneous" IR property, (5) a SC conductance dependent on $(\text{K}_\text{O}^+)^{1/2}$. It would thus appear that the IR in CHN is similar in functional properties but distinct in molecular conductance and mechanism of voltage sensitivity. Unless the IR under consideration here is, in considering the substate complexity, a result of highly cooperative interaction of a large number of smaller egg-like IRs, then these channels are most likely distinct at the molecular level.

6. b). Skeletal Muscle IR

An IR conductance similar to that found in eggs is also found in skeletal muscle. This K^+ -selective conductance is voltage- and $(\text{K}_\text{O}^+)^{1/2}$ -dependent ($g(V-V_K)$ -dependent) and is inactivated by deep hyperpolarization (Almers, 1972; Standen and Stanfield, 1978). It is localized primarily in the T-tubule system (Williams, 1976) and is blocked in a similar manner as in eggs by Na_O^+ , Cs_O^+ , Rb_O^+ , Li_O^+ , Ba_O^{2+} , Sr_O^{2+} , and Tl_O^+ (in the presence of K_O^+) as is the IR in eggs; muscle IR is also blocked by TEA⁺ (Stanfield, 1970) and formaldehyde. Its apparent action in physiological solutions is to set a NRR in the steady-state I-V plot of as yet unknown function for cell excitability. The transient and steady-state I-V plots are remarkably similar to that seen in CHNs (Almers, 1972a, Figure 2; Almers, 1972b, Figure 6), where beyond -120 mV (absolute potential) instantaneous currents are larger than steady-state currents and the rate of relaxation is voltage dependent. In physiological solutions containing Na_O^+ , the I-V plots as well as the off-Taus versus voltage (Standen and Stanfield, 1979, Figures 1-4) are remarkably similar to those found here in WCR; in muscle this

effect on NRR is mostly due to Na_o^+ -induced block of IR at large hyperpolarizations. It is possible that Na_o^+ contributes a blocking effect to K^+ inward WC currents in CHNs as well. Tl^+ is more permeant than K^+ in the IR of skeletal muscle, as in eggs, but induces an inactivation in the current which is not present when K^+ is the major external cation (without Na_o^+). Thus, an AMFD in $\text{Tl}_o^+/\text{K}_o^+$ mixtures is found in muscle, as in eggs, and it is well established that the gating and permeation properties of muscle and egg cell IRs are dependent on the nature of the permeant ion, implying an intrachannel ionic interaction (nonindependence) during charge flow. Permeant ion effects on gating are also known for the delayed rectifier K^+ channel (Swenson and Armstrong, 1981), Ca^{2+} channels (Almers et al., 1984; Almers and McClesky, 1984) and for nicotinic acetylcholine channels (Marchais and Marty, 1979). The suspected multi-ion nature of the IR channel in CHNs may imply that its voltage-dependent gating is also sensitive to the nature of the permeant ion. In eggs, muscle, and CHNs, inactivation (deactivation) of inward K^+ currents is dependent on voltage-dependent processes attenuating conduction that may or may not involve a blocking ion. It has been suggested that ions such as Na_o^+ or Tl_o^+ could cause inactivation of inward IR K^+ currents due to the stabilization of a conformational state, induced by hyperpolarization, that maintains channel closure. Perhaps such ions are unnecessary in IR of CHNs because the rate constants out of the closed states are relatively slow at hyperpolarized potentials (mean open times very brief). Such blocking particles would, of necessity to the experimental setup, have originated from the inner surface of the membrane in the CHNs.

Skeletal muscle IR, unlike egg cell IR, does not appear to be sensitive to the level of Na_i^+ (Hestrin, 1981) and no studies of Ca_i^{2+} effects have appeared.

Schwarz et al. (1981) have studied the IR channel in skeletal muscle using noise analysis when voltage clamped muscle is exposed to Cs_o^+ . The SC conductance was estimated to be about 10 pS with a channel density of $4/\mu\text{m}^2$. Cs_o^+ was used to induce a block effect on IR, and a Lorentzian ($1/f^2$) component appeared.

DeCoursey et al. (1984a,b) have used similar noise analysis techniques to study the IR channels in K_o^+ depolarized frog skeletal muscle under conditions either when no blocker was present or when the blocker Ba_o^{2+} was present and then compared the conductance and kinetic results to those obtained by macroscopic voltage clamp using the vaseline-gap method. With slight hyperpolarization from V_K in K^+ -depolarized muscle (IR is $V-V_K$ dependent) a Lorentzian-type noise was generated over a narrow potential range representing the innate (without blocker) open-close fluctuations ($3-4 \times 10^{-3}/\text{sec}$). This IR has a conductance of about 8 pS, similar to that found by Schwarz et al. (1981) as well as that in eggs (where Na_o^+ induced fluctuations are on the order of $6 \times 10^{-2}/\text{sec}$, about an order of magnitude slower). Moreover, the estimated channel density was very low as in egg cells and in CHNs. DeCoursey et al. (1984a,b) found that the Lorentzian fluctuations disappeared upon depolarization, as if the channel open probability had increased without blocker present, and Leech and Stanfield (1981) as well found that the time-dependent component of muscle IR does not inactivate completely at depolarized potentials. Thus, the low

currents seen on depolarization could be due to a partial kinetic inactivation of the gate or a partial suppression of the conductance (perhaps through a voltage-sensitive substate); the latter suggestion may be relevant to the channel under consideration here. Analysis of current fluctuations due to Ba_o^{2+} block provided similar Lorentzian spectra and gave similar estimates for the conductance.

SC ion currents of skeletal muscle IR have been recorded in cultured clonal rat myotubes by Ohmori et al. (1981). The fluctuations were generated by μM concentrations of Ba_o^{2+} in the pipette. The currents progressively inactivate under hyperpolarization due to apparent voltage-dependent block by Ba_o^{2+} . The conductance (about 10 pS) was sensitive to K_o^+ as expected, and the channel density estimates were low ($2\text{-}3/\mu\text{m}^2$). The channel, then, is very similar if not identical to that found in egg cells and has similarities and differences to IR in CHNs as discussed under the egg cell section above.

Perhaps more interesting is the finding of DeCoursey et al. (1984) that some skeletal muscle fibers undergo a profound increase in conductance during an experiment. This high-conductance state shows IR, is blocked by Ba_o^{2+} , is partially inactivated by hyperpolarization and, from noise analysis, has a similar low channel density to the smaller IR, perhaps suggesting that they arise from the same conductance. However, the conductance estimated from noise was at least 80 pS, which is of the same order as the IR channel in CHNs. DeCoursey et al. (1984), uncertain about the origin of this conductance change, but supposed that metabolic changes (e.g., ATP, calcium) may have occurred in "exhausted" fibers which then affected a change in the

IR conductance; they were unable to rule out the activation of an independence conductance, however. An alternative view on this effect is that the conductance activated is functionally related to the smaller IR conductance (or channels) but that some metabolic event occurs that locks the channel in a high-conductance state (e.g., channel modification, ATP changes, Ca^{2+}) but which allows maintainance of the IR property. One possible way to imagine such a response is an intricate substate structure that is sensitive to some cellular intermediate--perhaps some channels have (as yet unresolvable) a continuum of conductance states (Begenisich and Stevens, 1975).

One is able to conclude, then, that IR in skeletal muscle is functionally similar to IR in egg cells, but is distinct in its conductance and kinetic properties from IR in CHNs. A possibly unique similarity is the high-conductance IR state that is seen in both CHNs and skeletal muscle preparations. Further work could clarify the situation.

6. c). Macrophages

Gallin has shown that macrophages maintain an N-shaped I-V curve with a NRR around resting potential that can cause bistability of the voltage under current clamp (Gallin, 1981) and which can be removed by Ba_o^{2+} or Rb_o^{+} . The N-shaped I-V curve and a transitional region is offset by regions of outward and inward rectification (Gallin, and Livengood, 1981). The channels responsible for IR have a conductance of 16-30 pS (with $\text{K}_o^{+} = 150 \text{ mM}$) and an IR open channel property that appears to correspond to the IR in the macroscopic I-V plots (Gallin, 1984b). The channel kinetics do not appear to be voltage sensitive once activated below V_{rev} and thus could give rise to the

similar slow inward noninactivating relaxations found for I_h and I_Q . However, the Ba_O^{2+} and Rb_O^+ sensitivity make macrophage IR distinct from these Q-like currents but more like IR in egg cells. Sheehy and Gallin (1984) have subsequently studied this IR conductance using WCR and found a Ba_O^{2+} - and Cs_O^+ -sensitive K^+ -selective current arising from a conductance dependent upon $(K_O^+)^{1/2}$. Time- and voltage-dependent inactivation at depolarized potentials may also contribute to the steady-state IR or K^+ inactivation; SC activity ceases above about -30 mV. Some voltage-dependent inactivation occurs below -120 mV. The voltage-sensitive IR coupled with a CaK conductance found recently in macrophages (Gallin, 1984a) could be the basis of a plasmamembrane oscillator. Macrophages can show spontaneous hyperpolarizing oscillations in membrane potential (Gallin et al., 1975) that may have functional significance to the physiology of these cells (e.g., secretion, chemotaxis (see Gallin and Sheehy, 1985)). The class of IR in macrophages thus appears intermediate between that in eggs and that in hippocampus.

Gallin and Sheehy (1985) have studied the conductance properties of the J774.1 macrophage line originally derived from a mouse reticulum cell sarcoma. In suspension cultures, this cell line has a RMP of about -14 mV (Young et al., 1983) but undergoes a large increase in membrane potential (to -77 mV) following cell adherence (Gallin and Sheehy, 1985). The developmental sequence of the expression of K^+ conductances starts with a delayed rectification present transiently from one to twelve hours following adherence and a coincident expression of the IR conductance at two to four hours which persists in all cells and governs the membrane potential. The temporal sequence of

membrane signal transducing elements may have functional significance to cellular development and differentiation (Ypey and Chapman, 1984) as well as cellular growth regulation in response to mitogenic (DeCoursey et al., 1984; Fukushima et al., 1984a,b; Chandry et al., 1984; Cahalan et al., 1985) or perhaps oncogenic stimuli (J.M. Sullivan, work in progress).

The conductances studied by Gallin and Sheehy (1985) employed the WCR method with 150 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1.1 mM EGTA, 0.1 mM $CaCl_2$ ($pCa = 7.7$) and 10 mM Hepes ($pH = 7.3$) as the cellular dialysis solution. Under such ionic conditions K^+ conductances of IR and delayed rectification should be expressible while the CaK conductance (Gallin, 1984a) should be relatively suppressed. Under such conditions hyperpolarized ($V < V_K$) currents were identified that show time- and voltage-dependent inactivation below -110 mV and closely resemble the similarly deactivating IR currents in CHNs (see Gallin and Sheehy, 1985, Figures 2, 3, and 6). After twelve hours in culture when only the IR conductance is expressed, depolarization fails to activate significant outward currents. This IR property has a chord conductance sensitive to voltage with the largest steady-state conductance having a plateau at -110 mV (at potentials depolarized from the inactivation range). The relationship of conductance to voltage in egg cell IR as established by Hagiwara and Takahashi (1974) fits well to the macrophage IR data. In addition a $(K_o^+)^{1/2}$ -dependence of conductance confirmed previous findings (Sheehy and Gallin, 1984). Macrophage IR was blocked by both Cs_o^+ and Ba_o^{2+} which also accentuate the NRR below -100 mV.

In embryonic CHNs both the inward and outward K^+ currents activated on hyperpolarization or depolarization from V_K show remarkable similarity to the J774.1 macrophage currents. However, both conductances are found coincident at the same time in all CHN cells studied so that a clear segregation of conductive properties was not as easily obtained as in macrophages. However, SC currents responsible for both inward (123 pS) and delayed (15 pS) rectifying conductances were found in CAPs and IOPs that have kinetic behavior consistent with and further delineating the WC conductances. This may indicate that the electrical properties of differentiating macrophages may be similar to embryonic CHNs. The electrical properties of CHNs immediately following cell dissociation or cellular attachment have not yet been studied. A similar property of hyperpolarization of the membrane upon cell adherence is also found for fibroblasts (see Section D-6f below). It is interesting that the differentiation of function in macrophages (or leukocytes in general) and fibroblasts is related to membrane IR conductance changes. Perhaps the dedifferentiation of cell function following oncogenic stimuli is also related to prolonged changes in membrane excitability (J.M. Sullivan, work in progress).

It should be mentioned that the IR conductance studied by Sheehy and Gallin (1984) and Gallin and Sheehy (1985) employed 10 mM Na_i^+ as an internal ion. Internal Na^+ ions have been shown to block outward currents through single CaK channels (Marty, 1983) and ATP-regulated IR channels in heart (Takei et al., 1985) at low mM concentrations. Whether this effect is relevant to the lack of significant outward currents in late J774.1 cultures and the

justification of leakage subtraction is not clear at this time. No Na_i^+ was used in the WCR experiments reported here for mouse CHNs.

6. d). IR in Rat Basophilic Leukemia Cells

Ikeda and Weight (1984) have identified WC IR currents in malignant leukocytes that are similar to those found in CHNs in that a voltage-dependent inactivation is present. In this study a voltage-dependent hyperpolarizing activation was identified that occurs within 5 ms; this may be missed in larger, more complicated cell morphologies (e.g., in CHNs and see Kurachi, 1985) as it would not be recognized as distinct from capacitative currents. Activation is steeply voltage dependent being half activated at 16 mV hyperpolarized from V_K . A voltage- and time-dependent inactivation occurs at deep hyperpolarized potentials (< -120 mV) that can be partially relieved by replacement of Na_o^+ with choline. This inactivation induces a negative slope resistance in the I-V plot. The conductance is selective for K^+ , is dependent on $(\text{K}_o^+)^{1/2}$ and is blocked by Cs_o^+ and Ba_o^{2+} in submillimolar concentrations. No SC studies are yet available for a better comparison of leukocyte unitary IR currents with those in CHNs.

6. e). Single Channel IRs in Cultured HeLa Cells

Sauve et al. (1983) have identified a resting membrane IR K^+ channel in cultured HeLa cells, a continuous cell line originally derived from human cervical epidermoid carcinoma. These experiments were performed with only high K_o^+ concentrations in the pipette and with a voltage clamp setup that polarized bath potential (therefore WC membrane potential) rather than the pipette potential. The channel has a maximum inward conductance of about 40 pS and an apparent substate

conductance of 28 pS, or about 70% of maximum. Only at very large depolarizations are small outward current steps noted with a conductance of about 10 pS. The channel is K^+ -selective but its conductance does not appear to follow a dependence on $(K_o^+)^{1/2}$. Around RMP the channel displays complicated kinetics with rapid flickers (ms order) within single bursts of activity and longer (hundreds of ms to secs) closed periods separating the bursts. The burst length appears voltage-dependent (see their Figure 5) and decreases with hyperpolarization. Moreover, analysis of "selected records" shows that as K_o^+ increases, the probability of opening at resting potential increases, possibly indicative of an influence of permeant ions on channel gating kinetics. Most of the above results are very similar to the IR channel found in CHNs except that the conductance in the neurons is about four times larger and a $(K_o^+)^{1/2}$ dependence on conductance is established only for the neurons. The function of this channel in HeLa cells is not clear, although its activity in the resting membrane could contribute to the establishment of this voltage. HeLa cells are not excitable. IR in the I-V plots was explained by an open-channel property or a result of internal blockade.

HeLa cells are known to possess a CaK conductance that is stimulated by electrical or mechanical perturbations that result in increased levels of Ca_i^{2+} (Roy and Sauve, 1983). Sauve et al. (1984) have also studied a K^+ channel in HeLa cells under conditions of normal physiological saline in the patch pipettes. The channel had a conductance less than 10 pS under depolarized conditions which is similar to that found for the IR under high K_o^+ and large

depolarizations. Moreover, the channel was found to be sensitive to 10^{-4} to 10^{-3} M Ca_i^{2+} as studied under outside-out patch conditions. No I-V curve for this Ca_i^{2+} -sensitive K^+ channel was reported. The authors raised the possibility that the IR and the Ca_i^{2+} -sensitive activities could arise from the same molecular structure.

6. f). IR Single Channel Behavior in Fibroblasts

Hosoi and Slayman (1985a) have recently reported evidence of an IR in cultured mouse fibroblasts. The channels are active at RMP and have a conductance of 20 pS. They apparently contribute to the K^+ -selective RMP of these cells.

Hosoi and Slayman (1985b) have also shown that mouse L fibroblasts have a RMP-active IR conductance under WCR conditions. The magnitude of the macroscopic conductance increases dramatically with the presence of Ca_i^{2+} in the pipette, but the rectification is maintained. The channels responsible for this increase in conductance have about a ten-fold larger conductance than that of the lower conductance channel mentioned above and in that respect are similar to CHN IR.

Fibroblasts are known to possess a hyperpolarizing response in membrane voltage that can be activated by electrical, mechanical, or chemical perturbations (Nelson et al., 1972; see also Hosoi and Slayman, 1985b). The conductance responsible for this behavior is K^+ -selective, Ca_i^{2+} -dependent and inward rectifying and, with the resting membrane conductance, contributes to spontaneous or induced oscillations in membrane potential (Okada et al., 1977a,b, 1979, 1982; Roy and Okada, 1979). The oscillation in membrane potential is a stable signal phenomenon and has been suggested to participate in

endocytic activity and cell membrane motility (see Okada et al., 1982). Ongoing work hopes to identify whether membrane ion channels in fibroblasts are modulated by oncogenic proteins (i.e., sarc-tyrosine protein kinase), and whether such modulation promotes the malignant process through a defect in plasmamembrane ionic signalling (J.M. Sullivan, work in progress).

6. g). Two IR Processes in Retinal Horizontal Neurons

Tachibana (1983) has shown that a macroscopic IR conductance is present in dissociated horizontal cells from vertebrate retina. Shingai et al. (1984) were the first to apply the patch clamp recording technique to this preparation. Under CAP configuration with 140 mM K_o^+ and 30 μM Ba_o^{2+} in the pipette, an IR channel is observed with a conductance of 108 pS (plotting the abstract data), similar in conductance to IR in CHNs. The remainder of this study focused on IOPs where the conductance appears as 10-20 pS K-selective channels. In all cases Na_o^+ or Ba_o^{2+} blockers were used in the pipette to induce gating and varying concentrations of Na^+ and K^+ were present in the bath. The IR property is apparently maintained under these conditions. V_{rev} is consistent with a K^+ -selective process with no significant Na^+ permeability. Kinetically, $P_v(o)$ increases with hyperpolarization, the open times being voltage insensitive but the closed times becoming more brief at hyperpolarized potentials or, equivalently, that the opening rate increases (for a two-state channel). Replacement of Na_i^+ with tetramethylammonium decreases the SC conductance as in egg cells where Na_i^+ has a positive modulation on conductance. As gating is apparently due to Na_o^+ or Ba_o^{2+} block, the increase in opening rate with hyperpolarization

may act to increase the dissociation rate of blocker from the channel. This channel is again quite similar to that in eggs and skeletal muscle, except that $P_V(o)$ decreases with hyperpolarization in those channels; the discrepancy is not clear, nor is the similarity of conductance in CAPs to that in mouse CHNs. It may be that a variety of uncontrolled factors act to influence the SC conductance (e.g., see Sakmann and Trube, 1984b, p669).

Recently the glutamate responsiveness in retinal horizontal neurons has been shown to be mediated by a biphasic conductance change. A conductance increase is due to the opening of glutamate channels while the decrease in conductance over certain potentials is due to a block of an IR K^+ -selective conductance (Tachibana, 1985) that has an NRR positive to V_K (Tachibana, 1983). Kaneko and Tachibana (1985) have recently studied the effect of glutamate on IR channels in horizontal cells in CAPs (and WCR). With 124 mM K^+ in the pipette the channel is active at RMP and has a conductance of 30 pS; there may also be a lower subconductance state. The channel is K^+ -selective and has a conductance sensitive to K_o^+ . Replotting the data, the conductance is proportional to $(K_o^+)^{1/2}$ with a power relationship of 0.74 which is larger than that of 0.65 found by Fukushima for egg cells, 0.62 found by Sakmann and Trube (1984a) for IR in heart and 0.51 to 0.66 (depending on assumptions of the conductance behavior at low K_o^+) for IR in CHNs. The approximate SC g_{IR} vs. $(K_o^+)^{1/2}$ relationship then appears to hold for a number of IR channels. The glutamate-modulated channel in horizontal neurons displays no apparent voltage sensitivity even at deep hyperpolarized potentials (see their Figure 5a) and the WC IR does not inactivate (Tachibana, 1983).

Glutamate in the pipette affects the conductance not by changing $P(o)$ or the SC conductance but by reversibly decreasing the number of active channels. The channel is blocked by both Cs_o^+ and Ba_o^{2+} .

The differences between the Shingai et al. (1984) study and the Kaneko and Tachibana study (1985) are not clear. It could be that there are two IRs in horizontal cells, one voltage sensitive and one glutamate sensitive. IR in CHNs again shows similarity to the egg-like IR found by Shingai et al. (1984) but not the glutamate-sensitive rectifier of Kaneko and Tachibana (1985).

6. h). Fast Voltage-Sensitive IR in Olfactory Cortex Neurons

Constanti and Galvan (1983) have recently identified an IR mechanism in a cortical neuron that is similar to that in eggs and skeletal muscle. This was the first identification of such a process in a central neuron. The rectification process is fast, occurring within 50 ms as determined originally using the "sag" criterion (Scholfield, 1978) under single electrode somatic voltage clamp in cortex slices. This kinetic characteristic distinguishes this IR from the slower Q sag in hippocampal neurons, as does the lack of a depolarizing overshoot. As in mouse CHN IR under voltage clamp, there is a region of I-V linearity around the RMP with time dependence occurring in the region hyperpolarized to -100 mV; currents become larger with time, monoexponentially, in olfactory cortex neurons. The conductance becomes progressively activated with further hyperpolarization, and the time constant for activation becomes faster. With hyperpolarization beyond -140 mV, a voltage-dependent partial inactivation occurs (see their Figure 8A) to create a negative slope conductance region in the steady-state I-V plots. The authors

viewed the inactivation as a true process rather than an ion-depletion phenomenon inasmuch as inactivation is accentuated at high K_o^+ (e.g., 15 mM K_o^+). These experimental conditions are similar to the results in CHNs here and provide corroborative support for the inactivation phenomenon being a voltage- and K_o^+ -dependent process, possibly related to the properties of a multi-ion pore.

The conductance is K^+ -selective and shows no effects of anions on the conductances, as had been found in dorsal root ganglion (Mayer and Westbrook, 1983), nodal tissue, Purkinje fibers, and ventricular muscle in heart (Seyama, 1979; Yanagihara and Irisawa, 1980; Carmeliet and Verdonck, 1977; Siegelbaum and Tsien, 1980), and possibly the Q conductance in hippocampus (Segal and Barker, 1984b; Madison et al., 1986) where anion replacement affects conductance. After replacement of chloride with isethionate in CHNs, IR WC currents persisted (unpublished observation, J.M. Sullivan) thereby suggesting that it is unlikely that anions affect IR in CHNs as well; SC IR conductance was similar in KCl, KNO_3 and K-Citrate pipette solutions at the same concentrations. The IR conductance in olfactory cortical neurons shows the $g(V-V_K)$ dependence as found in eggs, muscle, and CHNs. The conductance is sensitive to block by Cs_o^+ and Ba_o^{2+} , which result in a NRR in the I-V plot at potentials more depolarized than -140 mV. Currents elicited by membrane hyperpolarization in the presence of blockers show remarkable similarity (see their Figures 9a, 11b) to that in CHNs, suggesting that part of the inactivation process may be due to an external blocking ion. In CHNs the blocking ion under WCR could have been Na_o^+ , which has blocking effects in eggs, muscle, and basophilic leukemia cells, where it induces NRRs at

hyperpolarized potentials. We recall that $P_V(o)$ for SCs decreases upon greater hyperpolarization, however, even in the absence of Na_o^+ in CHNs. The function of fast IR in olfactory neurons is not yet clear. Constanti and Galvan (1983) expect that this current lies in parallel with a background Cs_o^+ -sensitive leakage conductance that determines the RMP, which is a K^+ electrode in these neurons (Scholfield, 1978). No SC recordings from olfactory cortex neuronal somas are yet available but the IR currents in CHNs could be partially responsible for this behavior. The kinetics are appropriately fast, the $g(V-V_K)$ dependence is present, the voltage-dependent inactivation (K^+ -dependent or Na^+ -dependent) is present, as is the Ba_o^{2+} and Cs_o^+ block. It would be interesting to compare WCR results in CHNs under similar experimental conditions (e.g., low K_o^+) to the conductance in olfactory cortex. Further SC studies on the effects of K_o^+ on $P_V(o)$ are also needed.

6. i). Substance P Blocks Inward Rectification in Cultured Magnocellular Cholinergic Neurons of Globus Pallidus

Substance P, an excitatory central nervous system neurotransmitter, depolarizes cholinergic magnocellular neurons by blocking a RMP-active, IR K^+ conductance. Using the WCR technique Stanfield et al. (1985) have studied this $g(V-V_K)$ -dependent IR conductance that arises from currents showing remarkable similarity to those generated under similar conditions in CHNs in both depolarizing and hyperpolarizing directions. An IR I-V plot is generated by subtraction of currents not sensitive to Substance P or currents remaining after block of IR with Rb_o^+ (or Cs_o^+); such a leakage subtraction process has not yet been done for the CHN IR where significant apparent outward

rectification remains. Due to the similar nature of the WC currents in pallidal neurons and CHNs, it is feasible that the IR in CHNs contributes to the same resting membrane process; no SC experiments were reported by Stanfield et al., (1985), however. The conductance is distinct from g_O in hippocampus since it persists in the absence of Na_O^+ . The blockade of IR by neurotransmitters, a potential teleological reason for the existence of IRs in addition to their RMP contribution, causes prolonged depolarizations and enhanced neuronal excitability or neuromodulation. Other than the similarity of the inward K^+ currents in cholinergic magnocellular neurons to those in CHNs, the effect of Substance P provides an example of the effects of IR on neuronal excitability, a topic we will discuss further below. Moreover, this example provides further support for our lack of understanding of CHN IR, as we do not yet know enough about its controlling variables to predict how neurotransmitters might modulate a voltage-dependent conductance (see Huguenard and Alger, 1985). Perhaps the agonist acts directly or locally on the IR channel (as glutamate apparently does on horizontal cells in retina) or proceeds through some modulating second messenger system that has links with other signal generating mechanisms or cellular metabolism. We will now examine a neurotransmitter effect on IR which proceeds through such intermediates.

6. j). Serotonin (5-HT) Modulates an IR in Molluscan Abdominal Pacemaking Neurons, R15.

An IR, K^+ -selective, $g(V-V_K)$ -dependent conductance is present in molluscan pacemaker neurons that is blocked by Cs_O^+ , Ba_O^{2+} , Rb_O^+ , and enhanced by pure Tl_O^+ solutions (AMFD).

The conductance is progressively activated by membrane hyperpolarizations and is at least partially active at RMP. 5-HT has been shown by Benson and Levitan (1983) to increase the steady-state magnitude of this IR conductance. It does so through a receptor coupled to cAMP-dependent protein kinase (Drummond et al., 1980), which presumably has a modulatory increase on conductance, $P_V(o)$, or the number of active IR channels. This effect could be a direct result of phosphorylation on the inner face of the channel proper or a protein physically coupled to it (Levitan, 1985) and illustrates that second messenger systems can at least affect IR in excitable preparations. Although the raw voltage clamp currents were not reported in the study for comparison to CHN IR and no SC studies are yet available for 5HT modulation of IRs, the macroscopic conductance properties suggest that it belongs in the egg class of IRs. 5HT modulation of SC activity in other systems can occur through direct affects on channel gating structures (Guhary, 1985), illustrating that there are a multiplicity of response capabilities available to a single modality of information transduction.

6. k). Metabolic Control—Glucose Metabolism Blocks IR in Beta Cells of Pancreas.

Glucose stimulation of beta cells in pancreatic insulin-secreting islets of Langerhans causes dose-dependent membrane electrical phenomenon. Membrane electrical behavior ranging from slow prolonged depolarizations to oscillating membrane potential activity promoting bursts of APs results from variation in glucose dose. The depolarizing shift is due to a decrease in K^+ conductance that depends on the

metabolism of glucose in the secreting beta cell. Ashcroft et al. (1984) have recently used the patch clamp technique to examine the effects of glucose on beta cells. They find a K^+ -selective channel active in the resting membrane under CAP conditions ($140 \text{ mM } K_0^+$) in the absence of glucose in the bath. This channel has an V_K reversal potential and shows a prominent decrease in conductance at depolarized potentials; the channel is then another IR with a conductance at negative potentials of about 50 pS. The channel density is low ($1/3.5 \text{ } \mu\text{m}^2$) and total channel number is estimated at 150, similar in conductance, channel densities and absolute channel numbers for IR in CHNs; in both preparations input cell resistance is high. This IR channel has been called the G channel because when glucose is added to the bath, channel activity decreases in a dose-dependent manner. The effect appears to be largely a kinetic one inasmuch as the presence of glucose significantly decreases $P(o)$. In the absence of glucose, channel activity at RMP is complex with burst-type kinetics. Although the authors state that there is no significant effect of voltage on $P(o)$, examination of Figure 1 in their paper suggests a kinetic behavior somewhat similar to IR in CHN where the burst length decreases with hyperpolarization. Ashcroft et al. (1984) found that mannoheptulose, which blocks glucose catabolism (Dean and Matthews, 1970; Dean et al., 1975) can partially restore channel activity in the presence of glucose, but the burst length is still very small relative to control. No reports of blocking ions are available. The SC conductance is sensitive to K_0^+ , and thus this channel may participate in the increased excitability of islet cells in the

presence of glucose and known coincident accumulations of K_o^+ (Perez-Armendariz et al., 1985).

Although it is possible to find IR in CHNs in either the presence (5.6 mM) or absence of glucose, the conductance and rectification properties, as well as the channel density and number are similar to the properties of the G conductance in beta cells. This evidence reflects the second reported case of the participation of a second-messenger system on the activity of an IR channel. When IR is blocked, prolonged depolarizations are induced and oscillatory bursts of action potentials occur; the nature of the second messenger is not yet clear. The involvement of an IR process in hippocampal pyramidal neurons in the state of oscillatory epileptogenesis will be proposed below.

6. 1). IR in Vestibular Hair Cells

Ohmori (1985) has partially characterized an IR conductance in chick vestibular hair cells. The conductance is egg cell-like, is K^+ -selective, apparently $g(V-V_K)$ -dependent and is partially inactivated by hyperpolarization in the presence of Na_o^+ . Absence of Na_o^+ removes inactivation and decreases conductance, but does not effect activation if the internal perfusate is K^+ -EGTA. Inactivation is restored in Na_o^+ -free media if Cs^+ -EGTA is used as the perfusate, thereby suggesting that Cs_i^+ can exert an internal blocking effect at hyperpolarized potentials. SC IRs in CAPs have a conductance of 50 pS at 160 mM K_o^+ and 22.7 pS at 40 mM K_o^+ , and thus show a K_o^+ dependence but the functional relationship is not yet clear. Kinetically, the mean open time decreases with hyperpolarized potentials (as the closing rate constants

increase). The voltage dependence of this SC IR is probably not dependent on the presence of blocking particles, inasmuch as 160 mM K_o^+ -EGTA was used in the cell attached pipettes. Thus, we now have an example of an egg-like IR that has a kinetic mechanism where rates of closure are an inherent part of the molecular gating of the channel rather than a blocking effect due to an external particle. However, in hair cells and in the IR in CHNs where kinetic fluctuations appear to arise from voltage-dependent gating, it is not yet possible to rule out a kinetic block occurring from the inside of the channel (in CAPs). The internal Cs^+ block found in hair cells sets a precedence for this possibility in other preparations. The SC conductances found by Ohmori et al. (1981b) in myotubes and Fukushima (1982) in tunicate eggs have a much lower conductance (about 10 pS) but similar kinetic behavior. It would appear then that the diversity of IR types in different preparations grows larger or that we are not sufficiently knowledgeable about the nature of all the controlling variables. The function of IR in hair cells is not clear, but the previous discussion of IR in oscillatory behavior may have significance to the ability of hair cell membrane potentials to follow the frequency of sensory input (Corey and Hudspeth, 1979; Crawford and Fettiplace, 1980; Ashmore, 1983; Lewis and Hudspeth, 1983).

6. m). IR Conductance in Oligodendroglia

Corey et al. (1985) have recently identified an IR K^+ conductance in cultured oligodendroglia using WCR and patch clamp methods. The conductance in WCR was inward rectifying with 20 mM K_o^+ and 120 mM K_i and was blocked by Ba_o^{2+} but not by external TEA^+ or 4-AP. SC experiments found a channel that is depolarization-activated, shows

a progressive decrease in $P_V(o)$ below RMP, has a 140 pS conductance, and permits little outward current at depolarized potentials. This IR is extremely similar to that in CHNs (Corey, D.P., personal communication). Its function in glia may be the spatial buffering of K_O^+ .

6. n). IRs in Heart Muscle and Nodal Tissue

Most of the outward K^+ currents in Purkinje fibers, heart muscle, or nodal tissue have been shown to be IR conductances (McAllister et al., 1975). These conductances include I_{K_1} , I_{X_1} , the muscarinic-modulated current that we designate I_M , as well as two new channels identified by patch clamping—an ATP-sensitive channel and an Na_1^+ -dependent K^+ channel. We will discuss these five channels because they have IR properties and compare them to IR in CHN.

6. n-1). I_{X_1}

I_{X_1} is the current primarily responsible for repolarization during cardiac APs in Purkinje fibers (Noble and Tsien, 1969; McAllister et al., 1975), myocardium (McDonald and Tautmann, 1978a,b), and sinoatrial node (Noma and Irisawa, 1976; DiFrancesco et al., 1979). G_{X_1} has a threshold of activation at -50 mV and is fully activated by +20 mV, and thus is operative at plateau potentials. It reverses near the K^+ equilibrium potential and has instantaneous and steady-state IR I-V plots without a NRR (Beeler and Reuter, 1977; McAllister et al., 1975). It is blocked by Ba_O^{2+} but not apparently by Cs_O^+ . The I_{X_1} current with I_{K_2} (see Discussion, Section 5b) may participate together in providing for spontaneous pacemaker activity in heart (Hauswirth et al., 1969;

McAlister et al., 1975). The I_{X_1} conductance is not as sensitive to K_O^+ as is g_{K_1} (see below) and does not show the crossover phenomenon with increasing K_O^+ at depolarized potentials.

SC currents apparently responsible for I_{X_1} in embryonic chick heart have been identified by Clapham and DeFelice (1984). The channel has an IR property that appears very similar to that found in mouse CHNs and has a conductance at negative potentials of 62 pS, which is at least four times larger than the delayed rectifier found in squid axons (Conti and Neher, 1980). The channel shows significant voltage sensitivity with $P_v(o)$ increasing with depolarization above about -40 mV but decreasing progressively from about +20 mV, corresponding to the plateau potential range in heart muscle. The decrease in $P_v(o)$ with depolarization into the plateau range promotes rectification by decreasing the mean open time of the I_{X_1} channel (DeFelice and Clapham, 1982). Compared to IR in CHNs this $P_v(o)$ is depolarized shifted over most of its range. Moreover, the kinetics require at least two exponentials to fit the closed times and a single exponential distribution for the open times. As this study largely ignored brief flickers, a major portion of the voltage dependence appears to reside in the burst duration and interburst intervals and thus is similar to IR in CHNs; depolarized burst durations similarly last hundreds of milliseconds. The channel appears mostly selective for K_O^+ , and the channel density is very low. K_O^+ dependence has not been examined (a low K_O^+ of 4 mM was used in the study); on a SC level I_{X_1} does not have a strong dependence on K_O^+ .

A recent study of heart muscle sarcolemmal ion channels in artificial membranes by Coronado and Latorre (1982) has also identified

a channel that could provide for macroscopic I_{X_1} . The SC conductance with 100 mM K_O^+ was 28 pS, was voltage-dependent in $P_V(o)$, and had a P_K/P_{Na} of 5. The discrepancy of conductance of this channel to the Clapham and DeFelice (1984) study is not clear but IR channels with similar kinetic structure can have a range of SC conductances. No studies are yet available to identify block mechanisms. Nevertheless, the channel shows remarkable similarity to that found in CHNs, especially the voltage dependence. Similar macroscopic I_{X_1} -like currents have been found in CHNs, myocardium (McDonald and Trautwein, 1978, Figure 9) and sinoatrial node (Noma and Irisawa, 1976) under hyperpolarizing voltage clamp in the presence of high K_O^+ (> 10 mM). Partial voltage-dependent inactivation of I_{X_1} currents occurs at deep levels of hyperpolarization. Other reports of partial inactivation of hyperpolarizing K^+ inward currents in voltage clamped heart have appeared (Ehara, 1971; Maughan, 1976; Baumgarten and Isenberg, 1977) for Purkinje fibers and myocardium. As in heart, hyperpolarizing inactivation in CHNs reflects a partial voltage-dependent deactivation of the conductance.

Rousseau et al. (1984) have found an IR channel in cultured myocardial cells that has a 25 to 30 pS conductance across a range of K_O^+ concentrations (10.8 mM to 300 mM). The conductance is therefore not K_O^+ -dependent, and is thus consistent with I_{X_1} . No outward currents were recorded at 150 mM or 300 mM K_O^+ as in the IR in CHNs, suggesting that K_O^+ affects permeation through the I_{X_1} channel. The channel in heart has a voltage-sensitive $P_V(o)$ which decreases with hyperpolarization and increases with depolarization from RMP. The relationship of $P_V(o)$ to voltage

becomes less steep as K_o^+ is decreased, thereby suggesting that K_o^+ has a modulatory effect on the kinetics. The channel has ensemble kinetics that reflect nonstationarity in the redistribution of states on sudden perturbations. Other than the lack of K_o^+ dependence on conductance, this channel is also similar to IR in CHNs.

6. n-2). I_{K_1}

The I_{K_1} conductance in heart is another IR that is represented as a background time-independent conductance in Purkinje fibers (McAllister and Noble, 1966), and ventricular (Beeler and Reuter, 1977; McDonald and Trautwein, 1978) and atrial (Momose et al., 1983) myocardium. The conductance is K^+ -selective, and plays the primary role in the generation of the resting potential in cardiac membranes. Both the instantaneous and steady-state conductances show IR. Both Ba_o^{2+} and Cs_o^+ block this current (Vereecke et al., 1980; Isenberg, 1976; Hiraoka et al., 1980). I-V curves show crossover with increasing K_o^+ at depolarized potentials above V_K since outward currents increase with K_o^+ in contrast to expectations from the GHK equation (Hall et al., 1963; Noble, 1965). The steady-state I-V curves show a depolarized region of negative resistance (Beeler and Reuter, 1977). Intracellular injection of Ca^{2+} into Purkinje fibers has been shown to modulate the conductance of I_{K_1} (Isenberg, 1977). Ba_o^{2+} , by blocking I_{K_1} , can induce an automaticity in firing potential of ventricular muscle (Carmeliet et al., 1983; Hiraoka et al., 1980). In Purkinje fibers I_{K_2} induces pacemaker depolarization. However, the actual pacemaker rate may be more closely related to the activities of

the IRs I_{K_1} and I_{K_1} , as there exists a K_0^+ dependence on Purkinje fiber pacemaker activity (Vassalle, 1966), and changes in K_0^+ (decreases or increases from 3 mM) can abolish pacemaking and cause prolonged depolarizations. It is noteworthy that the sinoatrial node has very little I_{K_1} conductance such that even with a REDS, accumulations or depletions of K_0^+ would have little effect on sinoatrial nodal frequency (Noble, 1984). The IR region of the conductance above V_K promotes higher membrane resistance and helps to develop the long depolarized plateau phases of the cardiac action potential.

Carmeliet (1982) has recently provided evidence that the I_{K_1} IR does show time dependence and is responsible for the two stable potential states in Purkinje fibers (-100 mV and -50 mV). Different conditions can cause the membrane to shift between these states in a time-dependent manner such that there is a memory or hysteresis in I_{K_1} macroscopic IR conductance that is different than simple voltage-dependent kinetics. One way to explain such a shift is through consideration of the K_0^+ accumulation-depletion phenomenon, and another is by means of the induction or removal of IR properties, possibly in a manner consistent with the model of Ciani et al. (1978) for an electrochemical gating mechanism for IRs. These phenomena are quite similar to the PDSs observed in hippocampus that appear associated both with K_0^+ accumulation and an induction of some IR property (Konnerth, 1984). The potential involvement of K_0^+ in these induced changes in permeability is supported by the observation that it was difficult to detect activation of resting membrane CHN IR channels when K_0^+ was low (5 mM), possibly suggesting that some

threshold level may be required to activate the channel as in RBCs (Heinz and Passow, 1980; Grygorczyk et al., 1984). Strickholm (1981) has shown that K_O^+ depolarization of the membrane of crayfish axons induces a net membrane conformation change that alters the activities of its ion channels. With these considerations in mind, we examine the unitary behavior of channels that appear responsible for I_{K_1} in heart. There are five reported studies which have examined these channels in heart. The I_{K_1} channel shows remarkable similarity to IR in CHNs, except for its approximately four-fold lower conductance. We will thus come to understand further the potential relevance of IR to hippocampal neurons from the previous discussion.

Sakmann et al., (1981) were the first to report IR SCs in dissociated ventricular myocytes having a K^+ -selectivity and a K_O^+ -dependent conductance (6-25 pS for 11-150 mM K_O^+). The channel exhibited voltage-dependent kinetics and open times which decreased with hyperpolarization. A similar channel (34 pS with 70 mM K_O^+) was found active at the resting potential of atrial myocytes (Sakmann et al., 1983). A recent report by Uehara and Hume (1985) shows that although the conductance of resting channels (30-32 pS with 145 mM K_O^+) in ventricular and atrial myocytes were similar, the kinetics were much slower in ventricular muscle (τ_{open} 223 ms for ventricular versus 1 ms for atrial myocytes, both at 140 mM K_O^+ and $V_{mem} = RMP - 20$ mV). Only the ventricular channel had a decreasing $P_v(o)$ at hyperpolarized potentials such that in WCR the inward K^+ currents decayed with time upon hyperpolarizations below V_K ; no such decay was noted for atrial myocytes. Ventricular myocytes studied with WCR display an IR property with a prominent NRR (which is missing in

atrial cells), thereby suggesting that, as in the CHN IR, the time-averaged mean open channel current would have a NRR. AP duration increases with K_o^+ in ventricle but not in atria. The resting membrane conductance of atrial but not ventricular myocytes is sensitive to acetylcholine, thereby suggesting further that the resting channels in the two cell types are different.

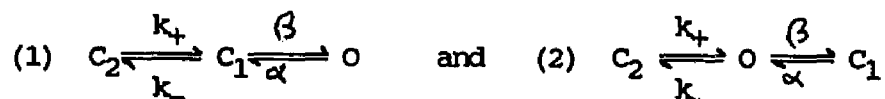
Rousseau et al., (1985) also reported on the K_o^+ -dependence of an IR channel in cultured rat myocardium with a conductance that ranged from 10 to 35 pS with 5.4 to 150 mM K_o^+ and, therefore, an approximate $(K_o^+)^{1/2}$ dependence for the SC conductance. The ratio of permeability of K^+ to Na^+ was 17.8 to 1, Na_o^+ had a facilitatory effect on the unit conductance as in egg cells (Fukushima, 1982), and there was no permeability to Cl^- . The $P_v(o)$ was voltage dependent and decreased with hyperpolarization, as in CHN IR. Here too the kinetics appear voltage sensitive and do not require a blocking ion. Cs_o^+ ions induce an open channel block at submillimolar concentrations that is modulated by K_o^+ levels; Cs_o^+ does not effect SC conductance. 4-AP shifts the $P_v(o)$ relationship to lower probabilities and thus exerts a kinetic block on the channel as Ba_o^{2+} apparently does on IR in CHNs and I_{K_1} channels in heart (Sakmann and Trube, 1984b). It appears that this channel becomes more linear under IOP conditions with pure KCl at the inner surface (Rousseau et al., 1985). The IR property may then originate from some type of internal block (Hille and Schwarz, 1978). Although initial studies were unclear as to whether this channel corresponds to I_{K_1} or I_{X_1} , recent studies of neonatal rat hearts suggests an I_{K_1} -like conductance (Payet et al., 1985).

A thorough study of the SC conductance and kinetic properties of I_{K_1} in ventricular myocytes has appeared recently from two laboratories (Kameyama et al., 1983; Sakmann and Trube, 1984a,b). The conductance and kinetic behavior of the channels appears very similar except that the conductance is about twice as large in the Kameyama et al. (1983) study; that the mean RMP was -81.7 mV in that study but was -50.7 mV in the Sakmann and Trube (1984a) study may partially explain the discrepancy. In both cases channels were active in resting membrane with high K_o^+ in CAPs and showed increasing current with hyperpolarization but with the $P_V(o)$ decreasing progressively. Depolarization increases $P_V(o)$, but the currents become smaller as the trajectory of the I-V relationship approaches V_K . Above V_K little, if any, outward current was observed. $P_V(o)$ approaches 1 at 40-50 mV depolarization levels above RMP in the Kameyama et al. (1983) study and in CHNs but only 0.3 in the Sakmann and Trube (1984b) study. The strong IR property coupled with the decrease in $P_V(o)$ creates a NRR around RMP as was found for CHNs. The I_{K_1} channel was ideally selective for K^+ (49 mV/log K_o^+) (Sakmann and Trube, 1984a) as was the CHN IR channel (58 mV/log K_o^+). In both studies of I_{K_1} IR and IR in CHNs the SC conductance was approximately proportional to $(K_o^+)^{1/2}$ (power was 0.41 in the Kameyama et al. (1983) study, 0.62 in the Sakmann and Trube (1984a) study and 0.51 to 0.65 in the CHN IR study here depending on assumptions at low K_o^+). In both the Sakmann and Trube (1984a) and this study the currents in the IR channels saturated as K_o^+ was increased at constant voltages hyperpolarized from RMP; this behavior is in strict violation of GHK expectations. As the conductance is also proportional to

$(K_o^+)^{1/2}$, this could indicate a multi-ion, single-file channel (Sakmann and Trube, 1984a) where negative cooperativity exists between the K^+ binding sites during ion permeation. In the three studies, discrete substates of conductance were found: (1) in the Kameyama et al. (1983) study, a 65-85% of maximum conductance state appeared as discrete events from the maximum state on return to the closed-state conductance, (2) four and occasionally five states of open-channel conductance were found in the Sakmann and Trube (1984a) study with approximately 25%, 50%, 75% and 100% of maximum levels; the larger than maximum level (125%) and the 25% and 50% levels were of very low probability, and only the 75% level was common, and (3) similar results to Sakmann and Trube (1984a) were found in CHNs with four proportional (see IR conductance models below) conductance substates which all appeared to interconnect kinetically. In the Kameyama et al. (1983) study, depolarization of WC membrane potential by isotonic K_o^+ provided significant outward currents with 50 mM K_o^+ outside the CAP. It would appear that the state of the WC membrane potential affects the nature of IR such that the process that restricts outward current flow (e.g., energy barriers, blocking particles, or rectification gates) is partially inactivated. It is not clear why the IR property is seen with little outward current in some channels (including the CHN IR), while in others there is no measurable outward current, although this could relate to the WC membrane potential, which typically is not measured from cell to cell.

The voltage-sensitive SC kinetics of I_{K_1} and CHN IR also appear very similar with bursting-type activity around RMP and a decrease in $P_v(o)$ with hyperpolarization. The decrease in $P_v(o)$ with

hyperpolarization can be largely explained on the basis of a decreased burst duration when bursts are defined by neglecting fast closures (< 0.5 ms for the Sakmann-Trube (1984b) study and < 2 ms for the Kameyama et al. (1983) study - includes true and band-limited apparent closures and closures to substates). In both studies on I_{K_1} such a simplification removes the ambiguity in the kinetic nature of fast closures and also the fast exponential in the total closed-time distribution. The closed-time distribution then is adequately fit with two exponentials and the open times (in either case) with one exponential. With this simplification there remain two possible three-state sequential mechanisms to explain the voltage-dependent inactivation of the I_{K_1} kinetics:



Kameyama et al. (1983) only considered the first sequential model while Sakmann-Trube (1984b) considered both. Rate constants were determined from fitted time constants using equations specific to each model. In the Sakmann and Trube (1984b) work it was not possible to distinguish between these models on the basis of distributions of open and closed times because the models have related parameters and because of the low voltage sensitivity of the rates. For Model (1) and in Sakmann and Trube (1984b) (with hyperpolarizations from RMP): α increases e-fold/85 mV, k_+ increases e-fold/75 mV, β decreases e-fold/50 mV, and k_- does not change significantly. In the Kameyama et al. (1983) study (with hyperpolarization): α increases e-fold/63 mV, k_+

decreases e-fold/112 mV, β does not change significantly, and k_{-} increases e-fold/61 mV such that the probability of being in the open state decreases, the probability of being in C_1 decreases and the probability in C_2 increases with hyperpolarization; since burst length is partially determined by the value of k_{-} , it is not surprising that this parameter is voltage sensitive. In Model (2) Sakmann and Trube find that only α , which decreases e-fold/120 mV and k_{-} , which increases e-fold/35 mV, are significantly voltage sensitive. Since, in this model, burst length is also partially determined by the value of k_{-} , this model also fits the sensitivity of the burst duration to hyperpolarization.

These models would both explain the voltage-dependent partial inactivation of I_{K_1} upon hyperpolarization. A number of studies have reported on partial inactivation of macroscopic hyperpolarized inactivating I_{K_1} currents in heart. Ehara (1971, Figure 5) showed slow decreases in conductance upon hyperpolarization during current clamp while Maughan (1976, Figure 1), Baumgarten and Isenberg (1977, Figure 4), and DeHemptinne (1978, Figure 2) showed partial inactivation of inward currents upon hyperpolarization by voltage clamp. Although such inactivation was initially thought to represent ion-depletion currents (Baumgarten and Isenberg, 1977; Kline and Cohen, 1984), the current patch-clamp results, where extracellular depletion cannot be a problem, clearly showed that the kinetics are due to voltage-dependence of gating rate constants. In addition, an open channel and gating (kinetic) block of I_{K_1} SCs by Cs_o^{+} and Ba_o^{2+} , respectively, is consistent with the WC voltage clamp data.

Recent work by Trube and Hescheler (1984) has shown that the I_{K_1} IR requires ATP (or ADP) to maintain its activity in the cell or in isolated patches. They also note that diminution of channel activity, even in the presence of ATP, may have other causes, for example, a depletion of internal Ca^{2+} .

Sakmann and Trube (1984a) had originally proposed that the SC rectification at large depolarizations above V_K must be due to a fast process: rectified permeation due to open-channel energy barriers and wells, an internal block mechanism, or a fast rectifying gate. Their data showed (1984b) that the probability of opening was larger at depolarized potentials, reaching a maximum of about 0.3 at a RMP of +60 mV, whereas Kameyama et al. (1983) found a maximum $P_V(o)$ of about 1.0 at depolarized potentials. On the other hand, Kurachi (1985) has shown that $P_V(o)$ decreases from potentials 20-30 mV below V_K (above resting potential). In addition to the inactivation gate in I_{K_1} previously recognized by Kamiyama et al. (1983) and Sakmann and Trube (1984b), Kurachi (1985) has shown that I_{K_1} SCs exhibit activation behavior upon depolarizations into regions depolarized from RMP from voltages slightly above V_K . In this experiment the kinetics of I_{K_1} exemplify the behavior of egg-like IR. This activation was measured using ensemble analysis and indicates that the behavior of $P_V(o)$ above RMP can be explained by an independent activation gate that is closing. Moreover, the kinetic behavior of the I_{K_1} channel can be largely explained on the basis of a two-state ($C \rightleftharpoons O$) activation-gate model at potentials 20-30 mV depolarized from RMP and in the approach to V_K when the channel conductance exhibits IR. The kinetic analysis of open times shows that the initial increase in

τ_{open} over the region above (+40 mV) RMP is consistent with the increase in $P_v(o)$ over the same voltage ranges. At potentials more depolarized than a RMP of +40 mV, mean open time progressively decreases such that at a V_K of +40 mV (the region of strongest rectification) or zero outward current, the mean open times is estimated to be about 1 ms. Since no net average outward current is observable in depolarizing ensembles greater than V_K (Sakmann and Trube, 1984a), the apparent rectification of the conductance could be due to the very fast (unresolved) closure of the activation gate. In fact, Kurachi (1985) was able to analytically obtain a macroscopic I_{K_1} -like IR with a NRR and cross-over at high K_o^+ using a linear (non-rectifying) channel, voltage-dependence of the opening and closing rates for the activation gate, and a conductance of the open pore proportional to $(K_o^+)^{1/2}$ as channel properties for the calculation. Thus, the model for IR suggested by Sakmann and Trube (1984a) with the linear pore and fast rectification (activation) gate appears to be viable. This would be a similar conclusion to the recent study by DeFelice and Clapham (1982) where I_{X_2} rectification is due to a decrease in $P_v(o)$ at potentials above V_K into the plateau potential region. However, this model does not explain the rectification of SC currents at potentials more negative than V_K and cannot rule out a rectification of the open pore, internal blockade, or other models of IR at this time.

In CHNs a fast flickery behavior of the currents inside bursts occurs in depolarizations toward V_K (data not shown). Whether it will be necessary to include the consideration of an activation gate in IR is not currently clear without further detailed kinetic analysis.

In summary, the SC I_{K1} data in ventricular muscle and in atrial muscle (Momose et al., 1983) is remarkably similar to the IR channel in CHNs in $(K_o^+)^{1/2}$ -dependence of conductance, the substate behavior, ion selectivity, pharmacologic block, and kinetics. The essential dissimilarity is the difference in the magnitude of the conductance (about four times larger in CHNs).

We have now examined a number of different egg-like IRs which have similar kinetic behavior but differ in the absolute magnitude of the conductances. It could be that many different types of these IR channels exist, but this is not economical to the genetic design of cellular excitability. On the other hand, the nature of ion channel interactions in membranes or patches is not yet clear. Membrane biophysical studies have illustrated that membrane proteins form domains in the lipid bilayer (Axelrod et al., 1978; Karplus and McCammon, 1983). Perhaps ion channels communicate in such domains under control of unknown cooperative processes (Liebovitch and Fischbarg, 1986). If such domains contain a number of small channels that can couple their gating, then the SC conductance could show the variability that exists. The substate structure could have functional significance to the elementary nature of the cooperating elements, as could the lipid boundary domain and intracellular metabolites. For example, in outside-out patches from CHNs containing multiple GABA-activated chloride channels, it was found that these channels tend not to act independently but rather appear to enter conductance states in correlative patterns (J.M. Sullivan and S.A. Cohen, unpublished observations). Thus, some types of channels may function cooperatively in membrane domains, and this could also be true of IRs.

6. n-3). Na_i^+ -activated IR K^+ channel

Another IR channel was recently found in ventricular muscle (Kaneyama et al., 1984) that has about a 200 pS conductance, is K^+ -selective, shows bursting kinetics with apparent burst lengths decreasing with hyperpolarization, but where $P(o)$ is not very voltage sensitive. Rather, the kinetics appear largely determined by the concentration of Na_i^+ on the inner surface of the channel. The channel activates with Na_i^+ above 20 mM and is fully activated by about 100 mM. Na_i^+ appears to control the length of the interburst intervals (it decreases the intervals) and thus serves the same effect as voltage in IR of CHNs (where interburst intervals are voltage sensitive). The voltage sensitivity to $P(o)$ however, was only determined over the absolute voltage range of -40 to +20 mV which would correspond to depolarized potentials for IR in CHNs where $P_v(o)$ is already very high. Thus, this channel should be reexamined at more hyperpolarized potentials for potential voltage-dependent inactivation which does appear to occur in the WCR records. In WCR changing pipette Na_i^+ (intracellular perfusion) affects the magnitude of outward current and removes the NRR in the steady-state I-V plot. The channel was not Ca_i^{2+} sensitive. Outside of its larger conductance, the rectification behavior of the channel and its kinetics appear similar to I_{K1} and IR in CHNs. It is interesting that Na_i^+ has a modulatory influence on noise analysis estimates of SC conductance and kinetics in egg cell IRs (Hagiwara and Yoshii, 1979). We are not aware if Na_i^+ has any effect on IR in CHNs, although Dichter (1983) has shown that cortical neurons have a significant Na^+ leakage conductance at resting potentials, and epileptiform neurons have some

type of persistent mixed $\text{Na}^+/\text{Ca}^{2+}$ current. In heart, this channel could act to prevent depolarizations of the membrane when $\text{Na}^+/\text{Ca}^{2+}$ exchange or the Na^+-K^+ ATPase function is disturbed. Its function in hippocampus could be to prevent depolarizing shifts in response to the persistent Na^+ inward currents below threshold. However, this channel appears distinct from CHN IR.

6. n-4). ATP_i -Regulated IR Channels

A number of studies have recently reported the presence of an IR channel in atrial and ventricular muscle and atrioventricular nodal cells (Noma, 1983; Soejima and Noma, 1984; Trube and Hescheler, 1984; Kakei and Noma, 1984; Kakei et al., 1985; Noma and Shibasaki, 1985). The channel is not typically active in the resting membrane unless efforts are taken to deplete cellular ATP energy stores. The channel is K^+ -selective, has a conductance sensitive to K_o^+ , and exhibits bursting-type, voltage-sensitive kinetics with $P_v(o)$ and burst length decreasing upon hyperpolarization. In IOPs, removal of ATP_i activates the channel, thereby corroborating the role of a reduced level of ATP_i in the K^+ conductance increase and shortening of AP duration that occurs in dinitrophenol-poisoned or anoxic ventricular myocytes (see Noma and Shibasaki, 1985 for references).

Recent work on the ATP_i -regulated IR channel in heart (Kakei et al., 1985; Noma and Shibasaki, 1985) have further detailed the SC and macroscopic conductive and kinetic properties. The channels are K^+ -selective, show a marked IR on strong depolarizations, and show a chord conductance dependence on $[\text{K}_o^+]^{0.24}$, which is a lower dependence than that for IR in eggs (Fukushima, 1982), heart muscle (Sakmann and Trube, 1984a), and CHNs (Sullivan and Cohen, 1985). The

ATP_i-regulated IR has a conductance of 80 pS at hyperpolarized potentials with 150 mM K_o⁺. This conductance is of the same order of magnitude of the SC conductance in CHNs, except that the rectification occurred at more depolarized levels. The degree of rectification increases with the presence of 45 mM Na_i⁺ on the inside of the saponin-opened cells, thereby indicating an internal voltage-sensitive Na_i⁺ block mechanism. A slightly smaller increase in rectification was effected by 90 mM sucrose, while 45 mM Tris⁺ totally prevented outward current and promoted the most extreme IR in the I-V plots. This result must be taken into account by any proposal of internal block mechanisms for IR, as it shows that the degree of rectification can be modulated by the nature of the internal blocking particle, which could vary with different intracellular conditions. In addition to the maximum conductance state, other subconductance states, which varied over twenty to ninety percent of the maximal state, were found very infrequently. This is very similar to the subconductance behavior of IR in CHNs and the I_{K1} channel in heart (Sakmann and Trube, 1984a). In fact, the ATP_i-regulated and I_{K1} channels were both found in the same heart muscle cells, which complicated the analysis of the ATP_i-regulated channel over hyperpolarized voltage ranges. Similarity of the channel density for the ATP_i-regulated and I_{K1} channels and the presence of similar subconductance levels may indicate that they originate from the same molecular structure. The I_{K1} channel also appears to be sensitive to ATP_i, inasmuch as Trube and Herscheler (1984) have shown that ATP_i is required for maintenance of channel activity in isolated patches (8 mM Na_i⁺), and Sakmann and Trube (1984a) suspected that

cell metabolism exerted some effect on I_{K_1} expression in CAPs. However, no easily distinguishable outward currents were identified in the I_{K_1} channel (Sakmann and Trube, 1984a) but are found in ATP_i -regulated channel where rectification occurs at high depolarized potentials, especially when K_o^+ is high (Takei et al., 1985). Could the variable magnitude of outward current be related to the concentration and type of some internal blocking particle? Sakmann and Trube (1984a) and Kurachi (1985) have suggested the additional presence of a fast voltage-sensitive rectification (inactivation) gate. The conductance of the ATP_i -regulated channel (80 pS at 150 mM K_o^+) is precisely two times that of I_{K_1} (40 pS at 140 mM K_o^+). In their consideration of the complex subconductance structure, Takei et al. (1985) suggested that a number of smaller subunits may contribute to the global channel function, as has been suggested for the I_{K_1} channel (Sakmann and Trube, 1984a) and the CHN IR channel (see Discussion - Section D-4).

TEA_i^+ and 4- AP_i both block the outward flow of current in the ATP_i -regulated channel of heart muscle by an apparent flickery block mechanism (Yellen, 1984a). Ca_i^{2+} depresses channel activity at 100 μM (Noma and Shibakashi, 1985).

The kinetics of the ATP_i -regulated channel appear relatively simply, as compared to I_{K_1} or neuronal IR. The channel kinetics appear independent of voltage, at least at depolarized potentials above V_K , where the ATP_i sensitivity was studied independent of the I_{K_1} . The maximum channel opening probability (0.8) was found at 0.5 mM ATP_i or below and was approximately zero by 2 mM ATP_i . The SC conductance and IR were independent of the ATP_i concentration, and

only the probability of opening was modulated by the kinetic blocking effect of ATP_i . The dose response curve of P_o vs. ATP_i in many channel patches requires a Hill coefficient of greater than 3, which could indicate multiple ATP_i binding sites and positive co-operativity of ATP_i binding. At +20 mV fast ($\tau = 1.1$ ms) and slow ($\tau = 134.9$ ms) time constants were required to fit the SC open times, and fast ($\tau = 1.2$ ms) and slow ($\tau = 69.4$ ms) time constants were also required to fit the closed times. With an increase in ATP_i concentration (0.1 mM), the closed-time components both increased while the open-time components progressively decreased. This data explains the P_o vs ATP_i relationship, since, as the closed times become more prolonged, the P_o drops as ATP_i increases. No kinetic models were proposed, but two distinct closed and open states would be required. Both GTP and UTP blocked the ATP_i -modulated IR channel over similar concentration ranges, thereby indicating a poor base selectivity for the ATP_i binding sites; ADP_i was effective in blocking at an order of magnitude higher in concentration, and AMP_i was ineffective. The ATP analogue, AMPPNP, which has a stable terminal γ - PO_4 bond, fails to block the channel, thereby indicating that ATP_i hydrolysis and phosphorylation are not essential for the kinetic changes, which, thus, are apparently mediated allosterically.

Although voltage effects on SC kinetics have yet to be explored at hyperpolarized potentials, the ATP_i -regulated channel exhibits similar conductance, subconductance, and selectivity as the I_{K1} channel and the IR channel in this study. ATP_i affects the activity of I_{K1} in heart (Trube and Herscheler, 1984), and although we have yet to examine the ATP_i sensitivity of IR in CHNs, Huguenard and

Alger (1985) have found that 0.5 mM ATP_i blocks IR activity in acutely dissociated adult hippocampal pyramidal neurons. It is tempting to speculate that $I_{\text{IR-ATP}_i}$, I_{K_1} , and I_{IR} in CHNs could all originate from the same general molecular mechanism.

Two ATP_i -blocked IR channels have recently been identified in cultured pancreatic islet cells with maximum inward conductances of 75 and 30 pS and maximal outward conductances of 30 and 10 pS, respectively under symmetrical 140 mM KCl (Findlay et al., 1985). Both channels are predominantly K^+ -selective, and the activity of the lower conductance channel (or substate) occurs parallel with the larger conductance process. Both types of channel activity are blocked by ATP_i progressively over the range of 0 to 1 mM. This activity typifies the ATP_i -dependent channels in heart and hippocampal neurons. The ATP_i -sensitive channels dominate the RMP in islet cells. Depolarization of pancreatic, insulin-secreting cells by glucose could be due to membrane-bound phosphoglycerate kinase (Dean et al., 1975) locally generating ATP_i to block outward K^+ conductances. Then, the glucose-blocked IR channel in pancreatic cells (see below) (Ashcroft et al., 1984) could be related to other ATP_i -dependent channels.

6. n-5). Acetylcholine-Sensitive Channel

It has previously been demonstrated that the positive chronotropic effect of acetylcholine or carbachol on nodal tissue or atrial muscle is due to an increase in K^+ permeability that resembles the IR of I_{K_1} in conductance, selectivity, K_o^+ -dependence and block by Cs_o^+ (Arbigo et al., 1983; Momose et al., 1984; Sakmann et al., 1983). Recent patch-clamp studies of the muscarinic-activated

conductance have identified a K^+ -selective channel with an IR characteristic that has a low probability of being open in the resting membrane (Pott and Bechem, 1984; Soejima and Noma, 1984). Basal levels of activity can be attenuated by muscarinic antagonists (atropine or scopolamine). The presence of acetylcholine or carbachol in the pipette (CAP) greatly increases the activity of the muscarinic channel, whereas bath application does not promote as significant effects. A local second-messenger effect is probably involved in the activation of the ion channel after ligand binding.

Although the channel has an IR property, it does not appear to be voltage sensitive, and the conductance is smaller (25–50 pS) than IR in CHNs. A muscarinic conductance is present in hippocampal neurons, where activation of receptors promotes apparent channel closure (Halliwell and Adams, 1982), but these effects appear dissimilar to CHNs IR. In preliminary experiments it was possible to identify IR in CHNs in the presence of muscarinic agonists (carbachol, 30 μ M). It should be noted that Segal and Barker (1984b) have had difficulty identifying the muscarinic conductance in cultured hippocampal neurons of rat.

6. o). IR in Red Blood Cells (RBC)

Blocking Ca^{2+} efflux (Gardos, 1985) or stimulating Ca^{2+} influx (Lew and Ferreira, 1978) leads to a large increase in K^+ conductance for the RBC. This effect is mediated by a CaK channel (Hamill, 1983) that has been shown to exhibit IR (Grygorczyk and Schwartz, 1983, 1985; Grygorczyk et al., 1984). The channel is predominantly selective for K^+ ($P_K:P_{Na}$ of 15:1) and requires K_o^+ and Ca_i^{2+} (in the μ M range) for gating. In CAPs and IOPs with symmetrical high K_o^+

concentrations, an IR I-V plot results with a conductance of 40 pS at hyperpolarized potentials and 10 pS at depolarized potentials above reversal. This data suggests that IR in the RBC is a property of the open pore. Although the most frequently observed conductance level (40 pS) was studied, another subconductance state with about thirty percent higher conductance (57 pS) was infrequently observed occurring with the 40 pS state. Another subconductance state of 17 pS was much more infrequently observed. This subconductance behavior is reminiscent of current-carrying behavior of the IR in CHNs and shows the same general pattern of conductance increase but with the probabilities of the maximal and submaximal states switched in the RBC channel. The RBC channel also shows a logarithm of conductance behavior which can be linearly related to an integer series, as in CHN IR (see Section D-4 below). This behavior can be understood as an extension of the Ciani et al. (1978) model for IR in egg cells, as has been done in this study for IR. Also consistent with this explanation is the requirement of K_o^+ for gating. RBCs incubated in K_o^+ -free solutions for 7-10 minutes become flux-unresponsive to Ca_i^{2+} increases, as if the IR channel has become desensitized (Knauf et al., 1975; Heinz and Passow, 1980). Greater than 500 μM of K_o^+ are necessary to induce the permeability change in response to Ca_i^{2+} and to maintain the channel and net K^+ efflux in an active state (Heinz and Passow, 1980). Increasing K_o^+ increases the rate of K^+ efflux from cells, suggesting that K_o^+ binding to external sites affects the time-average behavior of the channel gating mechanism (Knauf et al., 1975; Heinz and Passow, 1980). SC recordings of RBCs and isolated patches from cells incubated in K_o^+ -free solutions

failed to identify active IR channels in support of the flux data above (Grygorczyk et al., 1984). In the same study comparing the flux data and SC data the authors estimated the total channel number in the cell at about 50 implying a low channel density.

One open state and at least two closed states are required to explain the distributions of open and closed times in IR of RBCs. Ca_i^{2+} increases $P_v(o)$, but at any Ca_i^{2+} level hyperpolarization increases, while depolarization decreases $P_v(o)$, a property consistent with a kinetic description of IR. The Ca_i^{2+} and voltage sensitivity reflects the effects of these variables on the distribution of open times. The open-channel conductance at hyperpolarized potentials decreases in the presence of Cs_o^+ , thereby indicating an open-channel block. Ba_o^{2+} , on the other hand, decreases conductance at hyperpolarized potentials similar to Cs_o^+ , but also decreases SC activity relative to control over the same range of voltages. Ba_o^{2+} acts by increasing the duration of closed times and decreasing the duration of open times at hyperpolarized potentials. Thus, the effect of Ba_o^{2+} is expressed over the conductive and kinetic aspects of the SC function of IR in RBCs. Quinine and quinidine, blockers of K^+ channels in many preparations (see Grygorczyk and Schwarz, 1985), decreases SC IR activity in RBCs by way of changes that they induce in the open and closed times. TEA_o^+ and Na_o^+ both decrease SC conductance of the RBC channel. The effect of Na_o^+ is interesting because the block can be relieved by increasing K_i^+ . Rb_o^+ , known to be a K^+ channel permeant in many systems, decreases SC conductance of IR in RBCs without changing the reversal potential. This effect of Rb_o^+ indicates that

selectivities calculated on the basis of reversal potentials conflict with those determined on the basis of changes in current under different ionic conditions at the same voltage. The block by Rb_o^+ indicates a voltage-dependent selectivity and can be explained by nonindependent permeation in a single-file multi-ion pore (Hille and Schwarz, 1978). The effect of K_o^+ on increasing conductance in RBCs and the relief of Na_o^+ block by K_i^+ are also consistent with a multi-ion single-filing pore (Yellen, 1984b). In parallel with this hypothesis were the voltage-dependent blocking effects of Cs_o^+ , which can be explained by a fast two-ion blockade in the multiion pore, and the Ba_o^{2+} effect, which may decrease SC conductance by both a fast blocking reaction at a site near the external mouth of the pore and a slower binding reaction, more internal in the channel, which alters SC kinetic fluctuations.

The RBC IR channel has some properties similar to those of CHNs IR. First is the IR, K^+ -selectivity, voltage dependence, Ca_i^{2+} sensitivity, multiple conductance states with the logarithmic dependence of conductance on an integer series potentially modeling hypothetical charges near the gating site, the similar properties of Cs_o^+ and Ba_o^{2+} , and the requirement of one open state and at least two closed states to explain channel kinetics. What is distinct, again, is the magnitude of the SC conductance, the activation of the RBC channel by hyperpolarization, and the two- to three-fold lower Ca_i^{2+} sensitivity in RBCs. We have also suggested that the IR channel in CHNs is a multi-ion single-file pore based partly on the conductance vs. $(\text{K}_o^+)^{1/2}$ dependence. Further measurements on the voltage-dependence of Cs_o^+ and Ba_o^{2+} block, on the relief of

block by internal or external ions, as well as a SC AMFD assay using Ti^+/K^+ mixtures could further delineate the nature of permeation in the IR of CHNs. However, the behavior of the channels is comparatively quite similar, with the major discrepancy occurring because of an inverse of the kinetic dependence on voltage. Perhaps such dissimilarities could be understood on the basis of rate constant differences in a similar mechanism. The subconductance behavior of the two channels could suggest that both proteins are designed in similar fashions. Why the probabilities are reversed at the higher conductance levels is not clear but could depend on both the putative voltage or Ca_i^{2+} dependence of the states. As for the functional similarities, RBC IR has been posited as a modulator of serum K_o^+ levels and similar roles may exist for CHN IR by modulating K_o^+ and participating to set the RMP.

6. p). Other Studies on IRs in Hippocampal Neurons

In acutely dissociated guinea pig hippocampal neurons, Wong and Clark (1983) have identified a K^+ channel that exhibits IR with 140 mM K_o^+ in CAPs. The channel carries little if any outward current, and its kinetics do not appear to be voltage sensitive. The conductance was 250 pS, which is larger than IR in mouse CHNs. Ca_i^{2+} apparently increases the flickering of the channel (R.K.S. Wong, personal communication).

After this work was completed, Huguenard and Alger (1985) confirmed these studies with their report of preliminary studies on an IR channel in acutely dissociated hippocampal neurons. With 150 mM K_o^+ and 0 mM Ca_o^{2+} they find a slope conductance of 200 pS at -100 to -120 mV (hyperpolarized from RMP, assumed to be -50 mV), whereas near

the RMP, the conductance was 100 pS. Reversal of current flow at voltages greater than RMP was not possible with CAPs. Isolation of IR into IOPs results in "linearization" of the SC I-V plot and an increase in conductance to 250 pS. In CAPs with 150 mM K_o^+ and 2 mM Ca_o^{2+} , depolarizations to near +100 mV (150 mV depolarizations) activate outward currents through a channel with a conductance of 100 pS.

This IR channel activity is voltage-dependent, with $P_v(o)$ decreasing upon hyperpolarization. Isolated into IOPs, the IR-like SC activity can be reversibly blocked by decreasing Ca_i^{2+} in the bath into the range below 100 nM. ATP_i at 0.5 mM (in 10^{-7} M Ca_i^{2+}) also blocks the channel. Carbachol (10-50 μ M) reversibly blocks the IR channel and the outward conductive channel (with 2 mM Ca_o^{2+}) if, presumably, it is added to the bath during CAP channel activity. The authors incorrectly quoted the results of Sullivan and Cohen (1985) that the IR channel in CHNs is responsible for the Q conductance (Halliwell and Adams, 1982). They correctly stipulate that the channel activity does not, in their hands, fit the behavior of the Q conductance because of the inactivation at hyperpolarized potentials, the sensitivity to very low Ca_i^{2+} concentrations, and the sensitivity to block by carbachol. They are unable to explain the changes in conductance which result from different ionic recording conditions, but suggest the the IR channel could function in neuronal modulation (by cholinergic transmission). If this is true, then the IR channel may be related to the macroscopic M conductance found in hippocampus (Halliwell and Adams, 1982). In this study WCR I_{IR} currents were found with 30 μ M carbachol in the bath during preliminary experiments.

The channels of Wong and Clark (1983) and Huguenard and Alger (1985) in acutely dissociated hippocampal pyramidal neurons have similar conductive but dissimilar voltage sensitivities. The channel studied by Huguenard and Alger (1985) is most similar to that in mouse CHNs except for the somewhat lower SC conductance in CHNs. They confirmed the results of this study with a decrease in $P_V(0)$ upon hyperpolarization, an apparent linearization upon isolation to IOPs, and a Ca_i^{2+} sensitivity above the nM range and apparently much more sensitive to Ca_i^{2+} than typical CaK channels (activity predominantly above 10^{-6} M), which show ATP dependence in some preparations (Findlay et al., 1985) and are carbachol insensitive in adult hippocampal pyramidal neurons (Brown and Griffith, 1982). The 100 pS channels that they find at large depolarizations may be similar to channels seen occasionally in high- K_o^+ CAPs in mouse CHNs depolarized above V_K , but which have a conductance and kinetic behavior distinctly different from that expected of IR by extrapolating its known functional properties (at hyperpolarized potentials) to depolarized potentials. The differences in some basic properties of the experimental systems that could be responsible for such discrepancies include the use of adult vs. embryonic neurons, the much lower membrane resistance in neurons acutely dissociated by treatment with proteolytic enzymes, and the state of cell metabolism in cultured vs. dissociated neurons. Their finding of ATP-induced blockade of IR (at low concentrations), and the carbachol block of both inward and outward conductances is intriguing. The ATP-blocking effect of IR in the Huguenard and Alger (1985) study is then similar to the ATP-dependent channel in heart muscle (Kakei and Noma, 1984; Kakei et

al., 1985), which is blocked over the same concentration ranges. Their study better illustrates IR in CHNs as a neurotransmitter-sensitive channel and suggests by carbachol and Ca_i^{2+} sensitivity that IR in CHNs could contribute to the slow afterhyperpolarization current that regulates spike accommodation in hippocampal pyramidal neurons (Lancaster and Adams, 1986). In addition to the blocking effect of carbachol, both histamine (Haas and Konnerth, 1983; Pellmar, 1986) and noradrenaline (Haas and Konnerth, 1983; Madison and Nicoll, 1986a,b; Lancaster and Adams, 1986) appear to act antagonistically on the afterhyperpolarization conductance (current = I_{AHP}) through histamine receptors and β_1 -noradrenergic receptors coupled to adenylate cyclase.

Although I_{AHP} is difficult to isolate in single-electrode voltage clamp (Lancaster and Adams, 1986), it would be interesting to see if the SC properties of IR in CHNs might explain the afterhyperpolarization conductance studied under WCR where intracellular dialysis (e.g., with cAMP, GTP analogues) is possible. It is discouraging that carbachol did not affect WC currents in this study but under WC dialysis conditions essential components of the receptor-channel signalling cascade could have been missing. It is potentially encouraging that norepinephrine transiently blocked a resting membrane active IR-appearing channel (one experiment, data not shown).

Currently it cannot be clearly stipulated as to the direct participation IR plays in hippocampal neuronal processing outside of its clear role in RMP electrogenesis.

We have now reviewed a large number of "egg-like" IRs in many different preparations. One unifying feature is the apparent diversity

of channel types. IR in CHNs is very similar to many of the channels which show voltage-dependent inactivation and shows closest resemblance, property by property, to the I_{K_1} channel in heart muscle, which serves as the predominant resting membrane conductance. Perhaps IR serves a similar purpose in CHN. We will now discuss models which could explain the conductance and kinetic properties of IR in CHNs specifically.

D. Single Channel Conductance Properties and a Discussion of Models

The WC currents induced by hyperpolarization from RMP arise from the activity of open SCs that display a characteristic IR property in their conductance. In addition, the substate conductance structure, which could have relevance to the IR property, appears quite complex. As the literature already displays a number of unique quantitative models for the nature of an IR conductance, this section will attempt to summarize conductance properties which have been analyzed that may support or refute different models. Each model will be briefly presented and then discussed relative to the data. Some potentially new ways to view an IR conductance property will be discussed.

The first four models to be discussed are (1) the ion carrier model of Adrian (1969), (2) multiple-site models of single-file diffusion with a large asymmetric energy barrier at the inner mouth of the pore (Woodbury, 1971; Jack et al., 1975), (3) the electrochemical gating model of Ciani et al., (1978), (4) an alternative to the Ciani et al. model--IR resulting from voltage and K_o^+ sensitivities in the substate conductance, and (5) the internal-block models of Hille and Schwarz (1978), Armstrong (1975), Standen and Stanfield (1978), and Cleemann and Morad (1979).

1. In the Ion Charged-Carrier Model of Adrian (1969), originally formulated to explain quantitatively the IR property of K_0^+ -depolarized skeletal muscle (Katz, 1949) and later used extensively for IRs in heart (Noble, 1972), postulates a charged carrier mechanism which shuttles K^+ ions back and forth across the membrane with voltage-sensitive rates and with asymmetric binding affinities on either side of the membrane. While this mechanism satisfactorily explains macroscopic IR in skeletal and heart muscles, it was developed before noise analysis studies had predicted the pS range of the channel conductances. Discrete pS-conductance IR channels have since been found in skeletal (Ohmori et al., 1981) muscle and heart (e.g., Sakmann and Trube, 1984a), and it is highly unlikely, as in the case with the neuronal IR here, that carrier mechanisms, having unitary conductances too small to be measured with "gigaseal" technology, could participate in such a process. However, for some channels with substates a carrier mechanism could be conceived to participate in conduction (Lauger, 1985).

2. Single-File Diffusion Models (Heckmann, 1972) for multiple-site or single-site channels can account for nonlinear conductances if it is assumed that the largest, rate-limiting Eyring energy barrier is placed asymmetrically in reference to the assumed linearly decreasing field (GHK theory) (Hodgkin and Katz, 1949, Jack et al., 1975). IR can be modeled with a channel design having a large voltage-sensitive barrier close to the inner membrane surface of the channel (Woodbury, 1971; Noble, 1965; Jack et al., 1975). Jack et al. (1975) have shown that such a model predicts the IR behavior of I_{X_1} , the delayed outward current in Purkinje fibers, nodal tissue, and heart muscle. I_{X_1}

does not show the NRR at depolarized potentials common to I_{K_2} and I_{K_1} (Noble and Tsien, 1968). When IR of CHNs is fully conductive (some outward current) its I-V curve shows distinct similarity to that of I_{K_1} , so this type of model may be appropriate here. With this model and using equation (8.24) of Jack et al. (1975), it is possible to calculate that the maximum change of conductance is 58 mV per tenfold change of current and that, over the region of rectification, conductance should decrease by a factor of about 1.7 times (0.588 ratio). In the patches where outward current was seen, SC conductance decreased by at least sixfold over the rectifying region. Such a conductance decrease would require high cooperativity between different ion-binding sites in a multi-ion pore for such a GHK model to fit the IR curves in this study. That the SC conductance vs. K_o^+ function follows the nonlinear (half-power) relationship might suggest a multi-ion pore with negative cooperativity between the binding sites in the permeation path. Also, the finding that channel current saturated with increasing K_o^+ at constant voltage (Figure R-7) provides evidence against a GHK model as the explanation of rectification (see Section 4 Results: SC conductance vs. K_o^+). Although current evidence would suggest a channel with multiple binding sites, more detailed analytical approaches for ascertaining such properties will be necessary. Further studies to illustrate a multi-ion channel pathway would focus on isolated channels, where the concentration of symmetrical K^+ can be manipulated. In addition, one would also examine IR for AMFD, a property typifying multisite channels. This could be realized with isolated patches exposed to Tl^+/K^+ mixtures, as has been done with the gramicidin A channel (Neher, 1975; Neher et

al., 1978; Sandblom et al., 1977; Eisenman et al., 1977; Eisenman et al., 1983) but not for a biological channel.

3. In the Electrochemical-Gating Model of Ciani et al. (1978), a current-carrying oligomeric pore in the membrane consists of coupled subunits, each of which has its net dipole moments oriented by the transmembrane voltage. Upon voltage-induced gating, the channel exposes external anionic sites (not a part of the pore proper) that specifically bind K_O^+ and stabilize the channel in the open conformation. This model directly incorporates the effects of K_O^+ on conductance by having K_O^+ participating directly in the gating phenomenon (i.e., K_O^+ stabilizes the voltage-oriented subunits comprising the oligomeric structure). This process has been termed electrochemical gating. Once opened by voltage and stabilized by K_O^+ , the conductance dependence on $(K_O^+)^{1/2}$ is assumed to be a property of the open channel, and the $(V-V_K)$ dependence determines the numbers of stable pores that can participate in conductance. Rectification is a consequence of the disappearance of open pores at depolarized potentials ($V-V_K > 0$). Depolarized potentials attenuate the permeability to K^+ by preventing the subunits from orienting perpendicular to the plane of the membrane. A decrease in K_O^+ has a similar effect because the stabilization of oriented conductive pores by K_O^+ is decreased. In contrast, deep hyperpolarized potentials or high K_O^+ have a saturating effect on permeability because all available pores end up being opened and stabilized in the conductive configuration.

Although the open-channel conductance is dependent on $(K_O^+)^{1/2}$, as found for the IR in CHNs, the nature of the

rectification is not yet clear. Inasmuch as IR channels in CHNs have low $P_V(o)$ at hyperpolarized potentials and high $P_V(o)$ at depolarized potentials, the direct application of the electrochemical gating model is inappropriate unless modifications are made (see below); as Ciani et al. (1978) note, a number of similar models could be used to predict such IR behavior.

D. 4a. Alternatives to the Ciani et al. Model--IR as a Property Resulting from Voltage-Sensitive Subconductance States

As an alternative to the Ciani et al. (1978) model, one can postulate an oligomeric channel in which the orientation of each subunit has a probability that depends on voltage. This is in contrast to the Ciani et al. (1978) model, in which a cooperative reorientation of subunits in response to voltage is assumed. Here we will assume that gating is strictly voltage-dependent and not dependent upon K_O^+ , and that the subconductance states either have different voltage sensitivities of occurrence or voltage-dependent lifetimes.

The subconductance states in hippocampal IR most likely result from long-lived conformational states in IR. Recent studies in the field of protein chemistry have shown that conformational substates shift rapidly at physiological temperatures (Karplus and McCammon, 1983; Lauger, 1985; Frauenfelder et al., 1979; Frehland and Faulhaber, 1980). If the probabilities of different substates shift with voltage and the substates have different conductances then it is possible to generate rectifying behavior for the channel (Lauger, 1985) by changes in transpatch potential. As the shifts in conformation could be sufficiently rapid to exceed the frequency response of the patch-clamp amplifier, one might observe that a continuous change in (average)

conductance has occurred rather than a progression through discrete substates. The rectification behavior of IR at depolarized potentials could be related to the substates that have been noted at hyperpolarized potentials where only the "three-quarter" state was seen regularly. It is possible that at depolarized potentials the lower subconductance states become more probable during the course of conformational fluctuations of the channel and it is these states that dominate the current-voltage behavior. In IR channels which carried some outward current, the rectification would require that conductance states lower than the 25% state would also be part of the substate structure, assuming that the same proportion of levels exists at potentials above V_K . Such a substate has not been observed at hyperpolarized potentials, although the signal-to-noise ratio would be very low. It is also possible that a continuum of substates of conductance exist in this channel, but we only observe long-lived transitions or averages between different substates (e.g., Begenisich and Stevens, 1975; Sigworth, 1985; Lauger, 1985).

In channels where no outward current was noted or when the conductance promptly dropped at voltages above V_K during ramp voltage clamps, it is possible that a very low-conductance substate has been activated or that a sudden rectification of the channel has occurred. In fast ramp voltage clamps to depolarized potentials the probability of opening can remain high during the rectification because the channel usually returns to the open state immediately following the end of the ramp. It is possible that a very fast rectification gate can modulate the IR channel conductance in CHNs, on a time frame shorter than a millisecond (normal recording bandwidth 1-2.5 kHz) (Leech and

Stanfield, 1981; Sakmann and Trube, 1984a; Kurachi, 1985). If such a rectification gate existed, then one might recognize its effects on ramp voltage clamps. The abrupt changes in conductance on some ramps around or slightly above V_K may represent such a phenomenon. The process of rectification in the channel appears independent of the gating phenomenon which provides for open-closed transitions--i.e., a rectification gate appears to be distinguished from a kinetic gate. On the other hand, a fast inactivation of the kinetic gate at depolarized potentials may also provide for IR.

Still, it is not clear why some IR channels carry some outward currents while others carry none at all. This phenomenon has been observed in other IR channels studied with the patch-clamp method (e.g., Sauve et al., 1983; Kameyama et al., 1984; Sakmann and Trube, 1984a; Corey et al., 1985). The study of Kameyama et al. (1983) has shown that when WCs are partially depolarized by high K_O^+ , the rectification decreases and SCs carry significant outward current in CAPs (38% lower slope conductance at $V > V_K$ than at $V < V_K$) compared to IR currents in the control state. It is not yet clear why such changes in rectification behavior occur by such a procedure or that this property is easily generalized to other preparations. Rousseau et al. (1985) have found that an IR in cardiac cells has more linear behavior when isolated into an IOP in pure KCl solutions, while Hosoi and Slayman (1985) find the same effect by isolation of IR in fibroblasts (Hosoi, personal communication). Isolation of the I_{K1} channel into IOPs requires ATP to prolong channel activity and may require other as yet unknown diffusable substances to maintain its function (Trube and Hescheler, 1984). Isolation of a few CHN IR

channels into pure KCl solutions also appears to partially linearize the channel conductance. It appears that the IR property of some channels requires an intact cell with an intact RMP to maintain the rectification.

4 b. Alternatives to the Ciani et al. Model--
Subconductance State K_O^+ -Modulation Model for IR

Ciani et al. (1978) proposed a model for the IR of egg cells that could account for the empirically derived expression for this conductance determined originally by Hagiwara and Takahashi (1974), that being:

$$G_K = \frac{B(K_O^+)^{1/2}}{1 + \exp\left(\frac{(\Delta V - \Delta V_h)}{v}\right)} \quad (\text{Eq. D-1})$$

where G_K is the rectifier macroscopic K^+ conductance, ΔV is the displacement of membrane voltage from V_K and RMP, ΔV_h is the displacement voltage (a constant) at which one half the maximal conductance is activated, B is a constant with units $\mu S * M^{1/2}$, and v is a constant representing the steepness of the G_K vs. ΔV curve.

The anomalously rectifying K^+ conductance was experimentally shown to depend upon both the membrane voltage and K_O^+ ($V-V_K$ dependence), and the dependence of conductance on the $\text{SQRT}(K_O^+)$ was assumed to represent a property of the open channel, while the dependence of G_K on ΔV was assumed to represent an effect of K_O^+ on the number of patent channels. The Ciani et al. (1978) proposal

then is an electrochemical gating model in which the external permeant K_0^+ ions participate in the gating or open-close phenomenon. They postulate, in similarity to mechanisms for other model pore formers like alamethicin (see Ciani et al. (1978) for references), that voltage induces the (simultaneous) orientation of three negatively charged subunits in the membrane which aggregate to form a channel. Upon gating, each subunit exposes negative charges on the external mouth of the pore which are complexed by K_0^+ to stabilize the conformation of the channel in the open state. Rectification per se in the model, results because at depolarized potentials the subunits never orient in the membrane (therefore, minimal outward conductance is manifest), and at hyperpolarized potentials the channels are opened and stabilized to varying net average degrees dependent on the concentration of K_0^+ , which complexes unimolecularly with the exposed anionic charge site(s). Under such conditions, the net membrane-specific K^+ conductance is given by:

$$G = \sum_{s=0}^n g_s [As^*] \quad (\text{Eq D-2})$$

where $[As^*]$ is the concentration of oriented channels complexed with ($s = 0, 1, 2$ or 3) K_0^+ ions, and g_s is the conductance of an individual pore with s complexed cations. Although never explicitly stated in the Ciani et al. (1978) model, the parameter g_s may have

different magnitudes at different values of s , and for an individual pore the number of complexed K_O^+ ions could determine the fraction of maximum available conductance from the pore, that is, determine a substate of SC conductance. When averaging the fractional contribution of each SC conductance over the total population one arrives by this summation at the specific conductance of the membrane. By hyperpolarizing the membrane voltage at a particular K_O^+ level the conductance will reach a finite limit as the maximum equilibrium fraction of opened pores achieves its maximum conductance stabilized at that respective K_O^+ . Similarly, at a given hyperpolarized voltage, addition of K_O^+ would stabilize more of the pores at larger conductances, and the average membrane conductance would approach another finite limit as all exposed anionic sites are titrated. Here we will posit an alternative hypothesis, namely, that as the number of SC subunits complexed by K_O^+ increases, the magnitude of the SC conductance also increases. This is not an irrational assumption considering that each subunit or aggregate of subunits (subconductance structure) could add a fractional contribution to the total SC conductance. These considerations result in a model in which the $(K_O^+)^{1/2}$ dependence of conductance must be explained by an open-channel property reflecting the rate of flux through the pore proper (at whatever level of instantaneous conductance). In addition, the direct participation of K_O^+ in the energetics of stabilizing conformational states with different conductances therefore influences the net time-averaged conductance property of the channel over a single gating transition. Although the original Ciani et al. (1978) model explains the properties of the IR conductance in eggs, the

rectification must depend on the disappearance of patent pores at depolarized potentials. This is not necessary with the modified model. Channels can remain open while their conductance is modulated by voltage and/or K_0^+ operating over the range of conformational structures permitting charge flow.

In considering such models, we began to wonder whether K_0^+ might take a more direct molecular role in the process of rectification on a SC level (rather than rectification resulting from channel closure). In doing so, a new model was generated which is functionally similar to the Ciani et al. (1978) model but which explains the data observed in IR of CHNs and is more appropriate in a contemporary way with what has been learned recently about biological ion channels with subconductance states by use of patch-clamp methodology. Such a model might be called an electrochemical rectification mechanism.

The impetus to consider this model grew immediately out of the observation by Dr. K.W. Foster that the magnitude of the subconductance levels of a single mouse CHN IR channel at fixed K_0^+ incremented with a logarithmic dependence on a sequence of consecutive integers. This observation is illustrated in Figure D-1, where the logarithm of the subconductance states for one experiment is plotted vs. a series of four consecutive integers: $n = 0, 1, 2, 3$. That the logarithm of the subconductance states varies with an integer series suggested that the untransformed subconductance magnitudes would have a functional dependence on the exponential of the corresponding integer n . It was immediately apparent that the Boltzmann-like function derived empirically by Ciani et al. (1978) for conductance of egg IR could be operationally related to this observation if we could understand the

Figure D-1 Subconductance State Magnitudes Fit a Logarithmic
Dependence on an Integer Series

Conductance of substates determined in a SC patch (see Figure R-8b) were logarithmically transformed and plotted vs. a series of consecutive integers (N). The line drawn is a least squares fit ($p < 0.05$) to the points and represents the function:

$$\log g_{\text{sub}} = 0.446148*N + 3.154456$$

SUBCONDUCTANCE STATE INTEGER SERIES

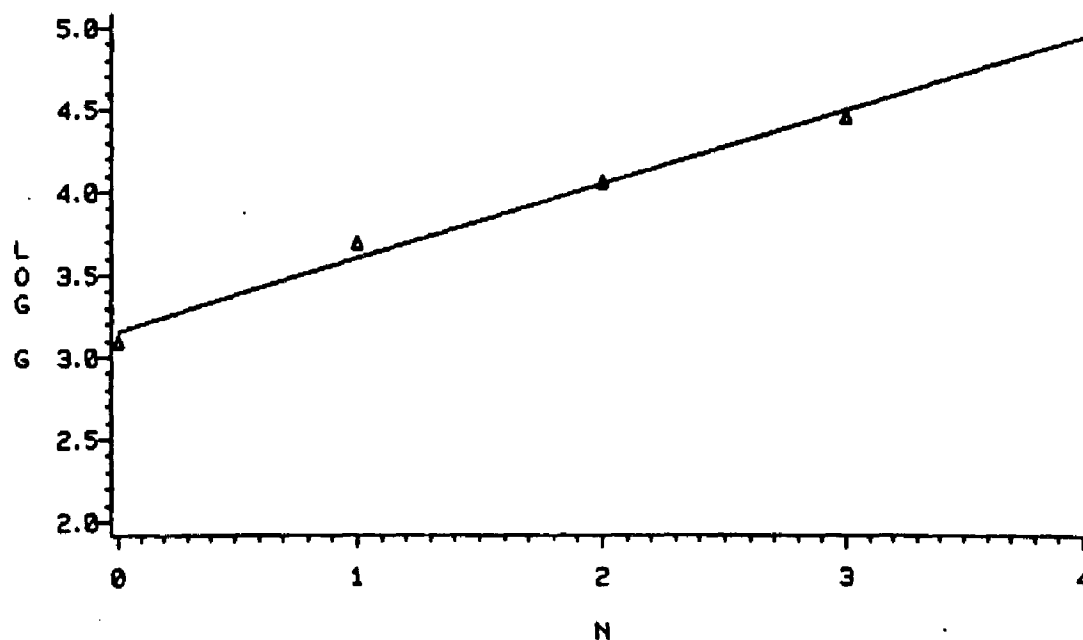


Figure D-1. Subconductance State Magnitudes Fit a Logarithmic Dependence on an Integer Series

equivalence of the parameter n to the current model. In the Ciani et al. (1978) model, n was defined to be equal to 3 and v , the Boltzmann steepness factor equal to RT/nF (where R , T and F have their usual meaning), was fit to 8.4 mV (about 9 mV), which suggested that each oligomer (comprised of three subunits) carried three negative charges and could, therefore, complex three K_0^+ ions. In our electrochemical rectification model we will also view n as an integer representing the number of subunit contributions to a single IR channel. Since a total of four conductance states was observed in the IR-channel fine structure, we imagine an IR-channel architecture with three "staves" surrounding the central pore, as shown in Figure D-2. Each of these staves is assumed to participate in a cooperative gating transition in response to membrane voltage changes such that each subunit exposes a single anionic charged site on the outside surface of the pore where it is not affected by the intramembrane drop in potential. Similarly to Ciani et al. (1978), we will allow K_0^+ to complex with these sites according to Langmuir isotherms or for a SC according to a first-order (diffusion-limited) probabilistic process. If we define n to be the number of anionic sites that are complexed ($n = 0, 1, 2, 3$) by K_0^+ using the Ciani et al. (1978) model one is able to make interesting predictions about the nature of a $g(V-V_K)$ process. We will show that rectification for a SC can occur as a result of the rectification of individual subconductance states and that the presence or absence of rectification is directly related to the number of anionic sites on the external surface of the channel that have complexed K_0^+ . We will show that as we sequentially titrate each anionic site on a modified Ciani et al. channel the magnitude of

Figure D-2. Pictorial Representation of an IR Channel Modeled as an Electrochemical Rectifier

IR channel is drawn with pore opened (by voltage) and with three anionic fixed charge sites exposed, one on each channel subunit. Note that the sites do not occupy the permeation path. K_0^+ ion is shown to interact unimolecularly with each of these sites according to first-order rate theory (K_m , the equilibrium constant, is assumed to be of millimolar order). Permeation path is arbitrarily drawn with a narrowing taper.

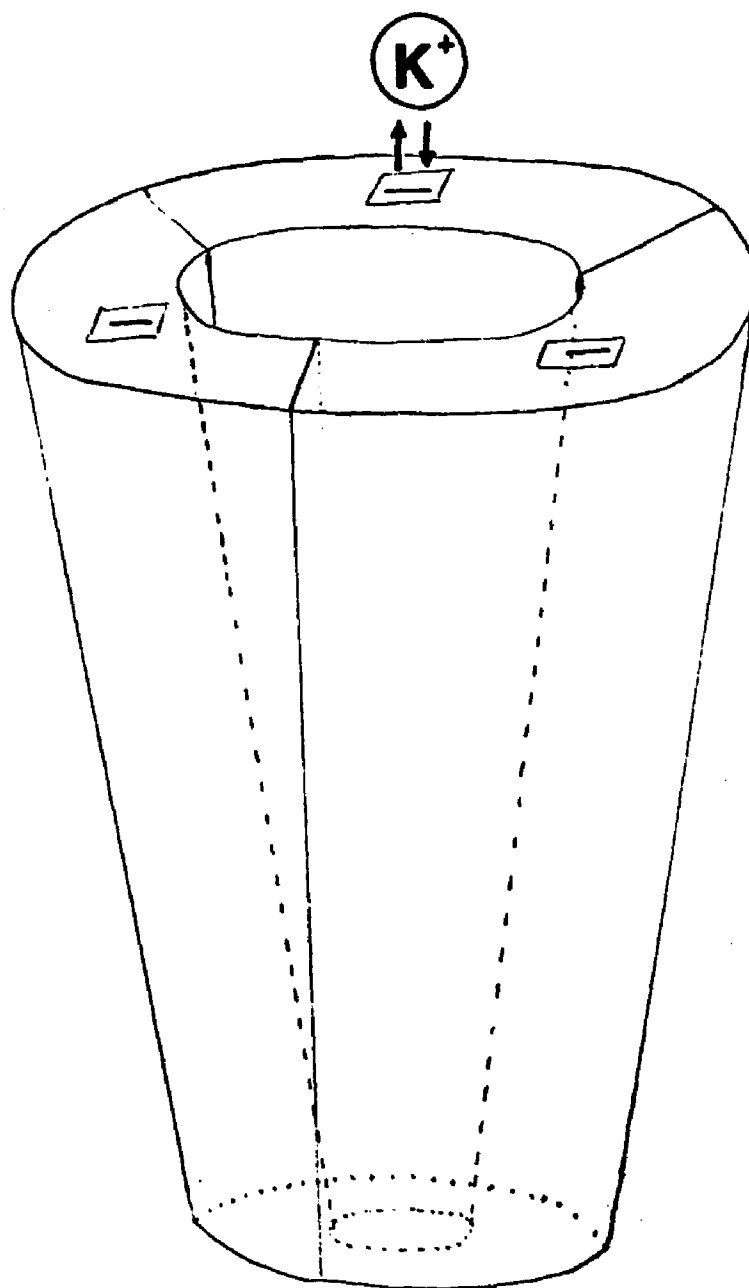


Figure D-2. Pictorial Representation of an IR Channel Modeled as an Electrochemical Rectifier

the inward-going rectification at depolarized potentials will increase exponentially. Also, unexpected from initial hypothetical considerations, as the anionic site titration number increases and IR appears in the predicted I-V plot, NRRs above V_K and the crossover phenomenon occur progressively as K_O^+ binding sites are filled.

The new model may represent a significant extension of the Ciani et al. (1978) model inasmuch as it represents a new way to view IR on the basis of properties of the molecular subconductance structures of a channel and inasmuch as it allows a more cohesive interpretation of K_O^+ effects on IRs.

Rather than work forward from physical principles we will make some specific assumptions that appear well justified and then show that such a modified Ciani et al. (1978) IR model (Equation D-1) will work by applying its principles to the development of the g vs. ΔV curve and subsequently the I-V curve. We will then discuss how the assumptions of the model could be relevant to rectification.

The model assumes:

(1) three subunits which gate together (cooperative) in response to membrane voltage to form an open channel,

(2) each subunit upon undergoing the gating transition exposes one anionic (negative) charge outside the potential drop which binds one K_O^+ in a unimolecular reaction (the K_m for this process is assumed to be on the order of 1 - 100 mM),

(3) the number of K_O^+ -complexed subunits is given by n where $n = 0$ when no K_O^+ ions are bound, $n = 1$ when one subunit is complexed, $n = 2$ when two subunits are complexed, and $n = 3$ when all sites are complexed. This creates a four-state conductive mechanism

dependent on the amount of anionic sites titrated specifically by K_O^+ . We note that the external charge screen around the channel mouth is the only modulatable parameter in the model once the channel has gated to the open conformation as a result of transmembrane potential. Thus, rectification in this model results from open-channel processes, as opposed to kinetic or rectifying gates.

(4) We use a modified form of the Ciani et al. model (Eq. D-1) to characterize the conductance vs. ΔV curve:

$$g = \frac{B(K_O^+)^{1/2}}{1 + \exp\left(\frac{-nF(\Delta V - \Delta V_h)}{RT}\right)} \quad (\text{Eq. D-3})$$

where B is a multiplicative constant (unspecified) assumed equal to one and V_h is assumed to be equal to zero for simplicity, as these constants were not easily obtainable by attempts to form SC chord-conductance vs. voltage curves. Then, ΔV is the difference of the RMP in a cell (assumed equal to -52 mV), and V_K across the patch ($\text{RMP} - V_K$) added to the displacement (disp) potential (pipette potential = $-\Delta V_{\text{disp}}$). V_K is calculated assuming $K_1 = 140 \text{ mM}$, and the fitted reversal potentials for SCs approximated the difference of $(\text{RMP} - V_K)$. Thus, $\Delta V = (\text{RMP} - V_K) + V_{\text{disp}}$ and F, R, T have their usual significance. We stipulate here that n is the average number of titrated anionic sites remaining after the K_O^+ binding reaction, recognizing that at any instantaneous point in time the

probability of site occupation will be given by the level of K_O^+ , and perhaps the voltage and the number of sites already occupied. Applying the equation directly, g vs. V_{disp} curves were constructed at each K_O^+ used experimentally and are illustrated in Figure D-3a-d. Three points are immediately appreciated: (1) the conductance is a constant (linear resistive channel) when $n = 0$ (i.e., when there are no K_O^+ s complexed), (2) as n increases at a fixed K_O^+ , the steepness of the g vs. V_{disp} curve increases (i.e., the ability to carry inward current is enhanced by increasing K_O^+ near the mouth of the pore), and (3) at fixed n , as K_O^+ increases, the steepness of the g vs. V_{disp} curve and its maximum increase, which can be better appreciated in Figure D-4a-c, where g vs. V_{disp} curves at each n are plotted on the same scale for each K_O^+ .

Next, to determine if the process of K_O^+ binding to anionic charged sites at the external pore mouth contributed to SC IR, SC I-V plots were constructed using the g vs. V (disp) data and shown for each n at a respective K_O^+ value in Figures D-5a-d. A number of points are important here: (1) When $n = 0$, the channel is a pure ohmic resistor without rectification, (2) the conductance at hyperpolarized potentials is relatively constant regardless of n (when $\Delta V_h = 0$), (3) rectification appears above V_{rev} when $n = 1$ and becomes steeper and with smaller outward currents as n increases, and (4) a NRR appears above V_{rev} when $n \geq 1$ at all K_O^+ values. Also, as seen in Figures D-6a-c, as K_O^+ increases for fixed n , a crossover phenomenon in the I-V curves becomes apparent above V_{rev} , and the steepness of the NRR is enhanced.

Figure D-3.a-d. Conductance vs. Voltage (Displaced) Curves for the Modified Ciani et al. (1978) Model at Constant K_O^+ and with Variable Binding site Titration (n) for K_O^+

Curves were generated by direct use of Equation D-3, with the assumption that 0, 1, 2, or 3 (the variable n) of the anionic charged sites were complexed with K_O^+ . Note that the scales are different in each graph for each respective K_O^+ : (a), 30 mM K_O^+ ; (b), 75 mM K_O^+ ; (c), 140 mM K_O^+ , and (d), 300 mM K_O^+ .

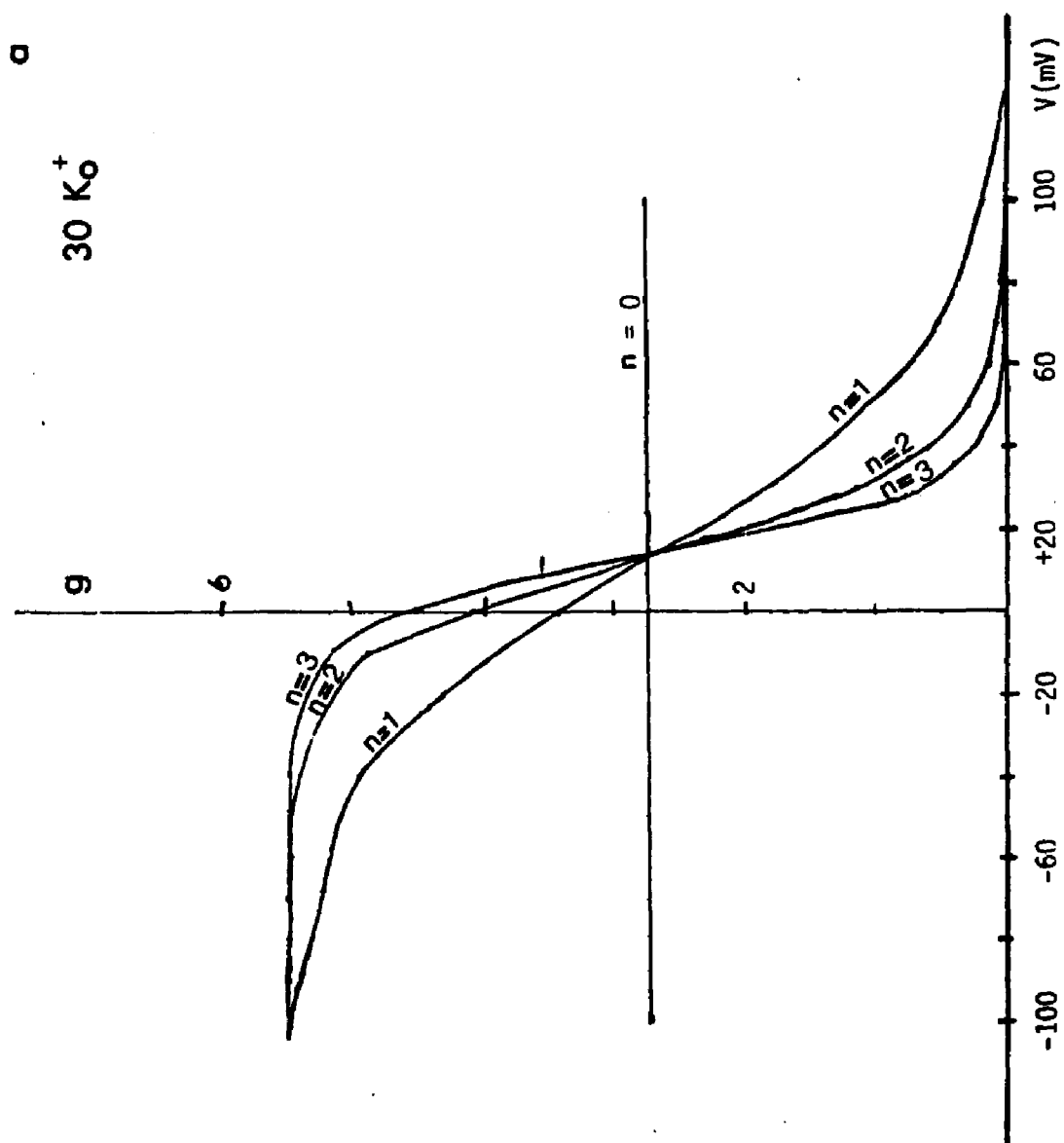


Figure D-3 a.

Conductance vs. Voltage (disp) Curves for the Modified Ciani et al. Model at Constant K_0^+ and with Variable Binding Site Titration (n) for K_0^+ (30 mM)

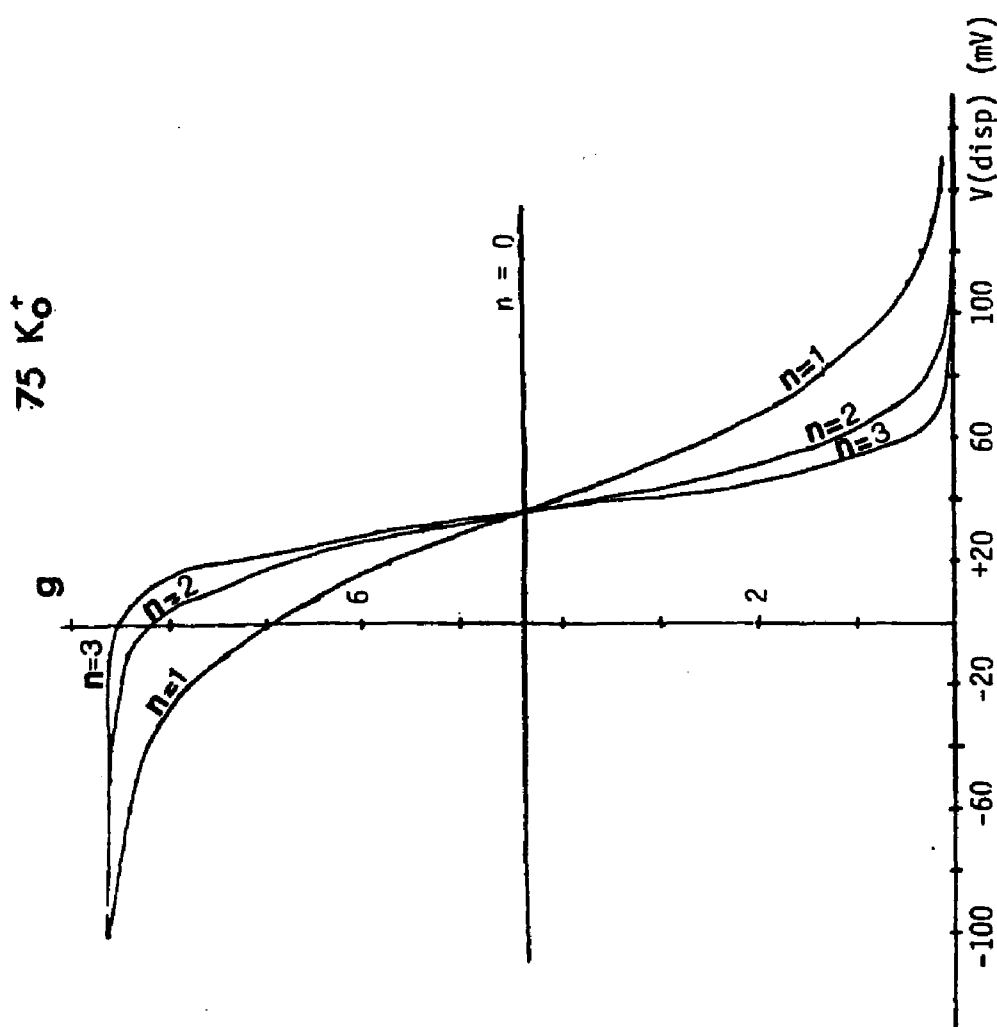


Figure D-3 b.

Conductance vs. Voltage (disp) Curves for the Modified Ciani et al. Model at Constant K_o^+ and with Variable Binding Site Titration (n) for K_o^+ (75 mM)

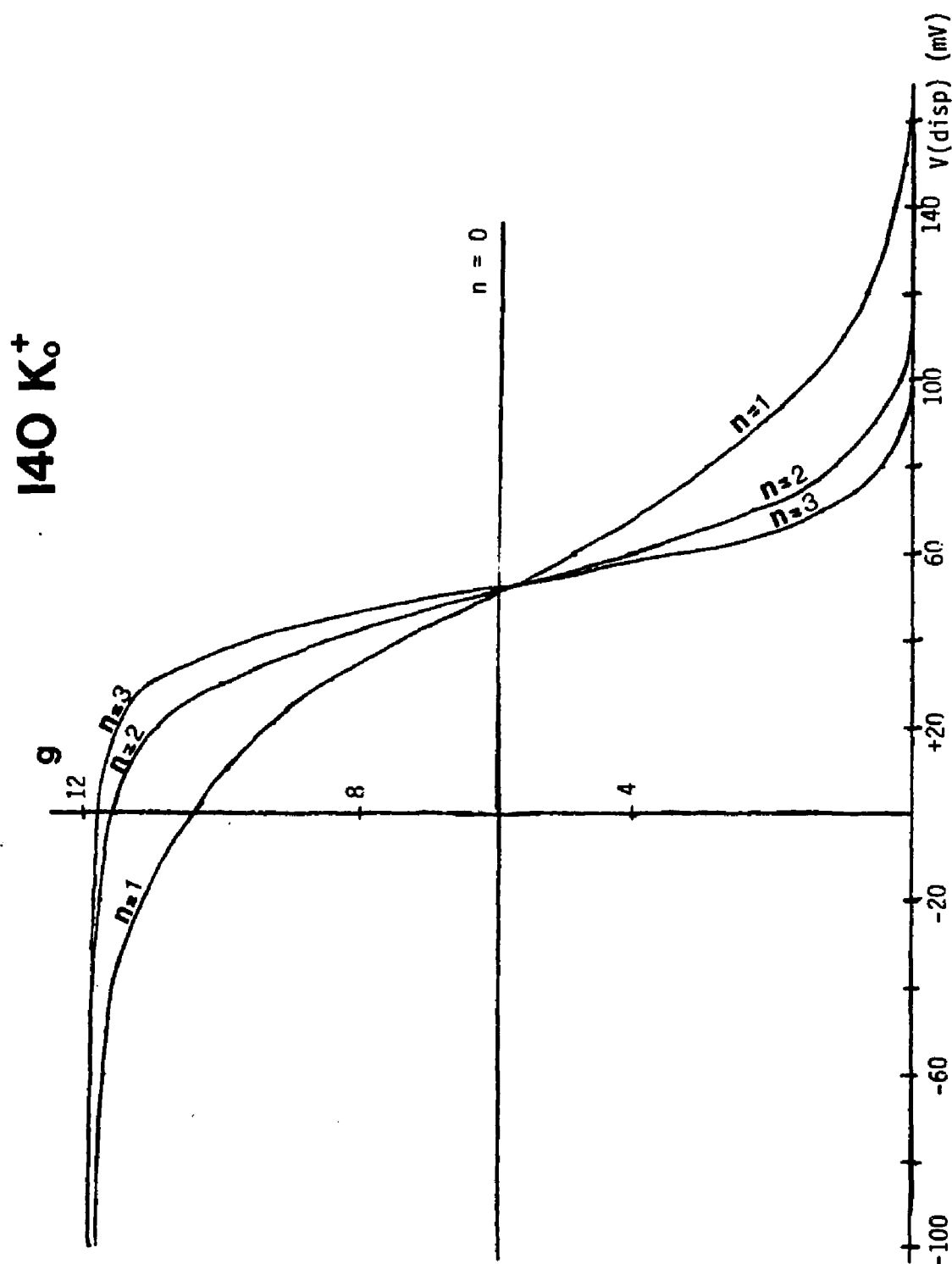


Figure D-3 c.

Conductance vs. Voltage (disp) Curves for the Modified Ciani et al. Model at Constant K_o^+ and with Variable Binding Site Titration (n) for K_o^+ (140 mM)

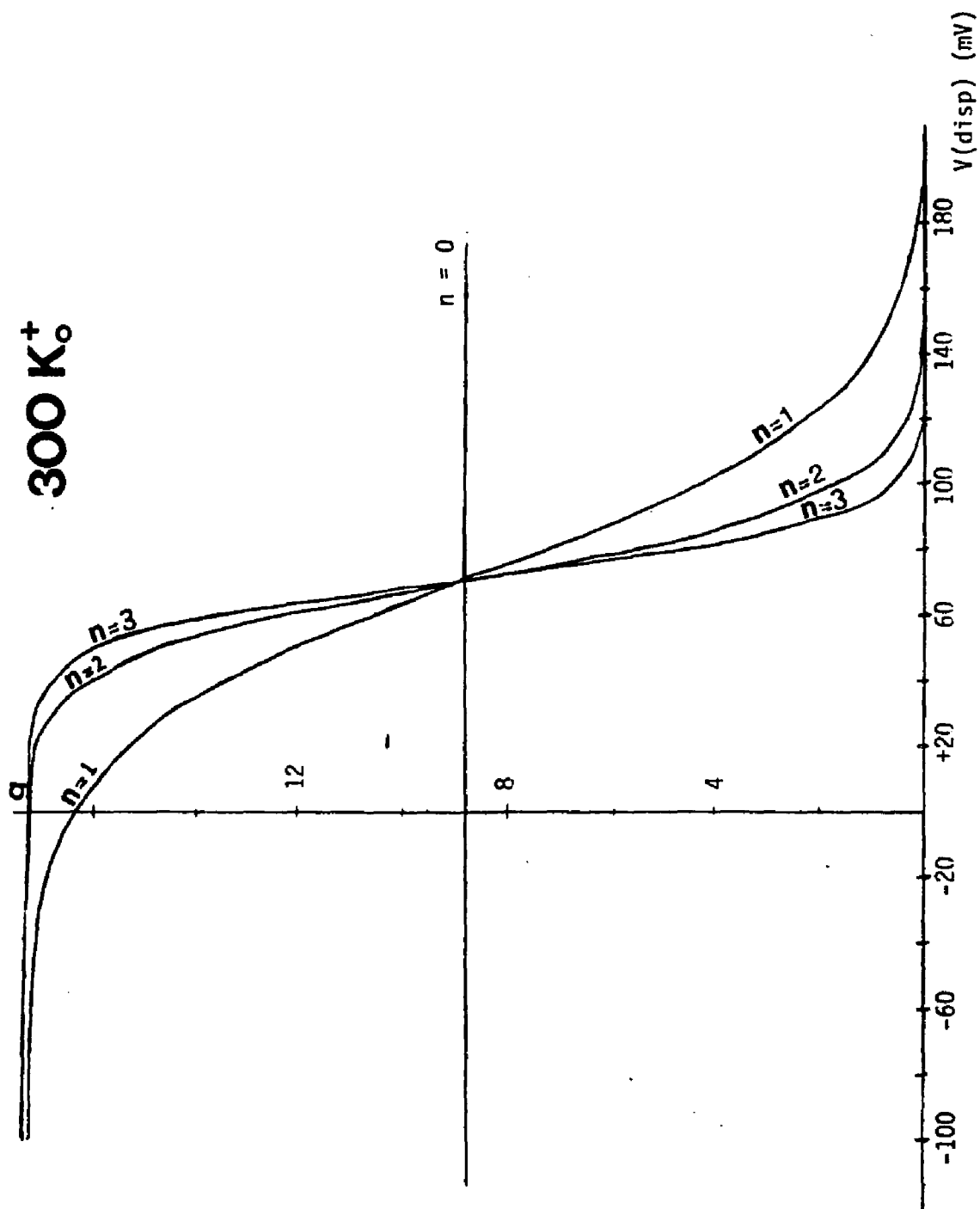


Figure D-3 d.

Conductance vs. Voltage (disp) Curves for the Modified Ciani et al. Model at Constant K_o^+ and with Variable Binding Site Titration (n) for K_o^+ (300 mM)

Figure D-4 a-c. Conductance vs. Voltage (Displaced) Curves for the Modified Ciani et al. (1978) Model at Fixed Binding Site Titration (n) for K_o^+ and with Variable K_o^+

Conductance vs. Voltage (disp) Curves were plotted for a fixed n allowing K_o^+ to vary in the modified Ciani et al. (1978) model. The same data as in Figure D-3 were used except that data for n = 0 is not represented. Data for n = 1, 2, and 3 are plotted in (a), (b), and (c) respectively.

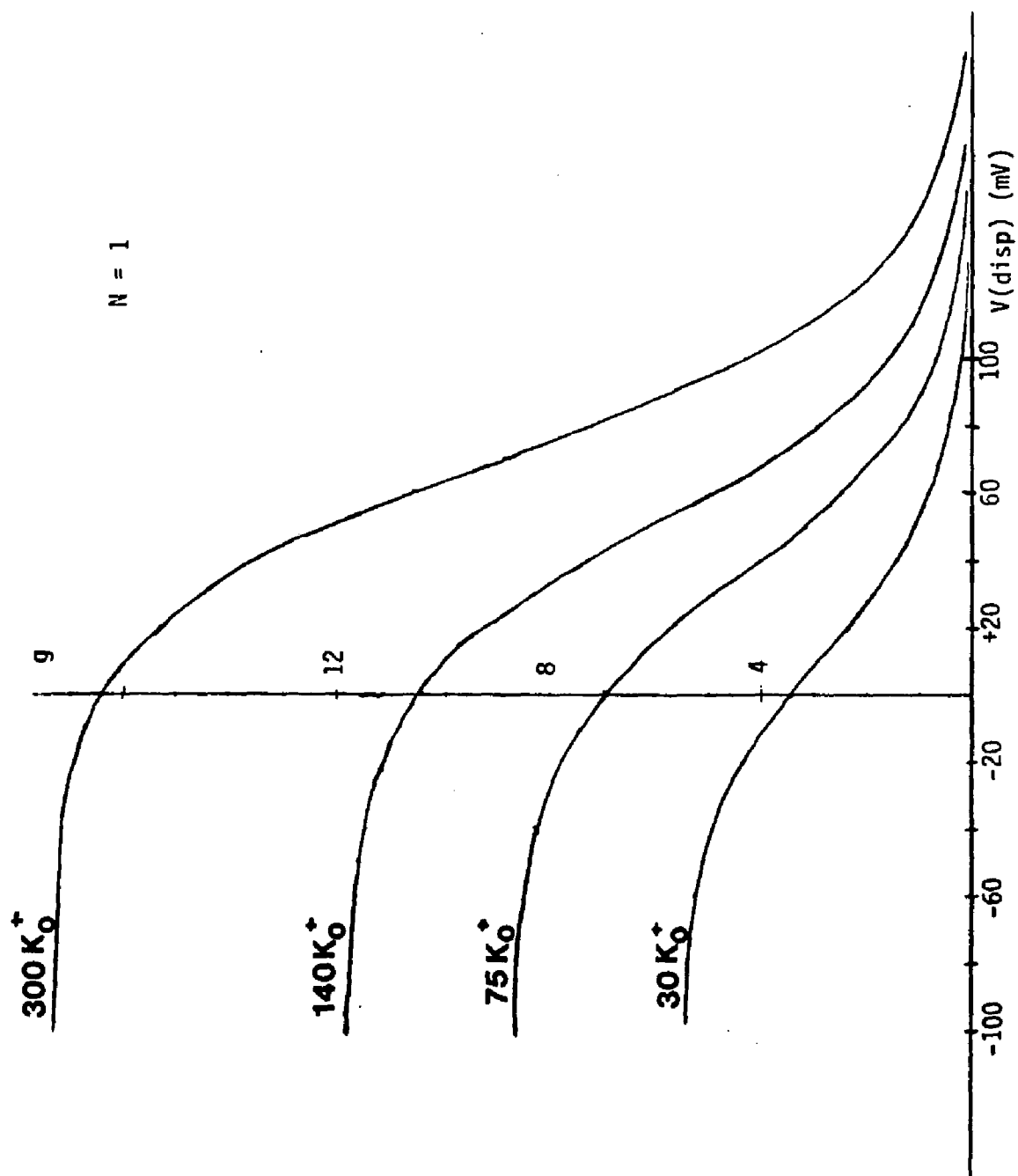


Figure D-4 a.

Conductance vs. Voltage (disp) Curves for the Modified Ciani et al. Model at Fixed Binding Site Titration (n) for K_O^+ and with Variable K_O^+ ($n = 1$)

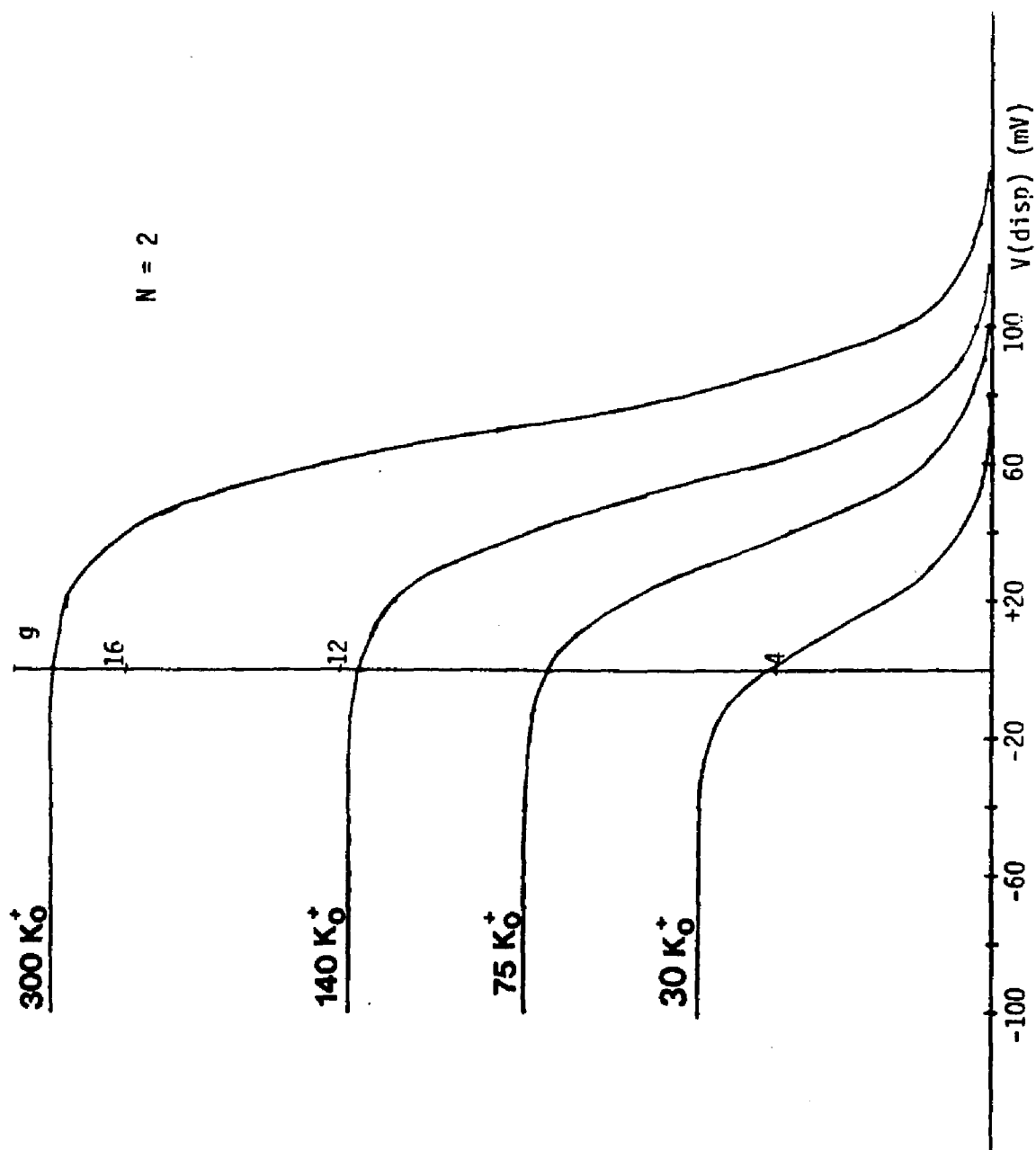


Figure D-4 b.

Conductance vs. Voltage (disp) Curves for the Modified Ciani et al. Model at Fixed Binding Site Titration (n) for K_o^+ and with Variable K_o^+ ($n = 2$)

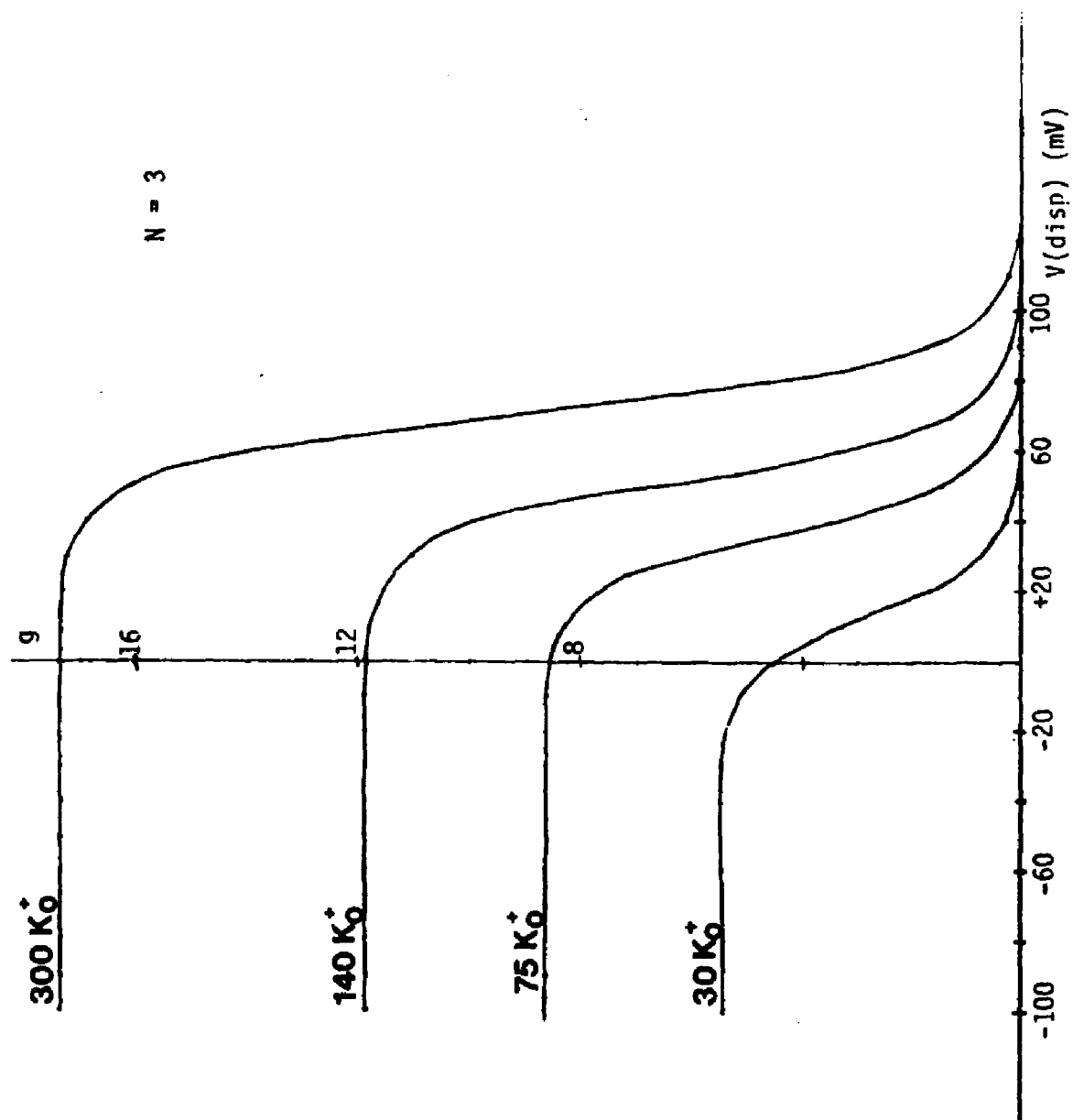


Figure D-4 c.

Conductance vs. Voltage (disp) Curves for the
Modified Ciani et al. Model at Fixed Binding Site
Titration (n) for K_O^+ and with Variable
 K_O^+ (n = 3)

Figure D-5 a-d. Effect of n on I-V Curves from the Modified Ciani et al. (1978) Model for IR at Constant K_o^+

SC subconductance I-V curves were calculated from g_n vs. V (disp) data from the modified Ciani et al. (1978) model. Note that V_{disp} is pipette potential or V as in the Results section Figures (e.g., Figure R-2 for comparison). Note different scales for current amplitudes at each K_o^+ : (a), 30 mM K_o^+ ; (b), 75 mM K_o^+ ; (c), 140 mM K_o^+ ; (d), 300 mM K_o^+ . The value of n used for each curve is stipulated. The equation used to compute I-V data was:

$$I = g_n((RMP - V_K) + V_{disp}) \quad (\text{Equation D-4}).$$

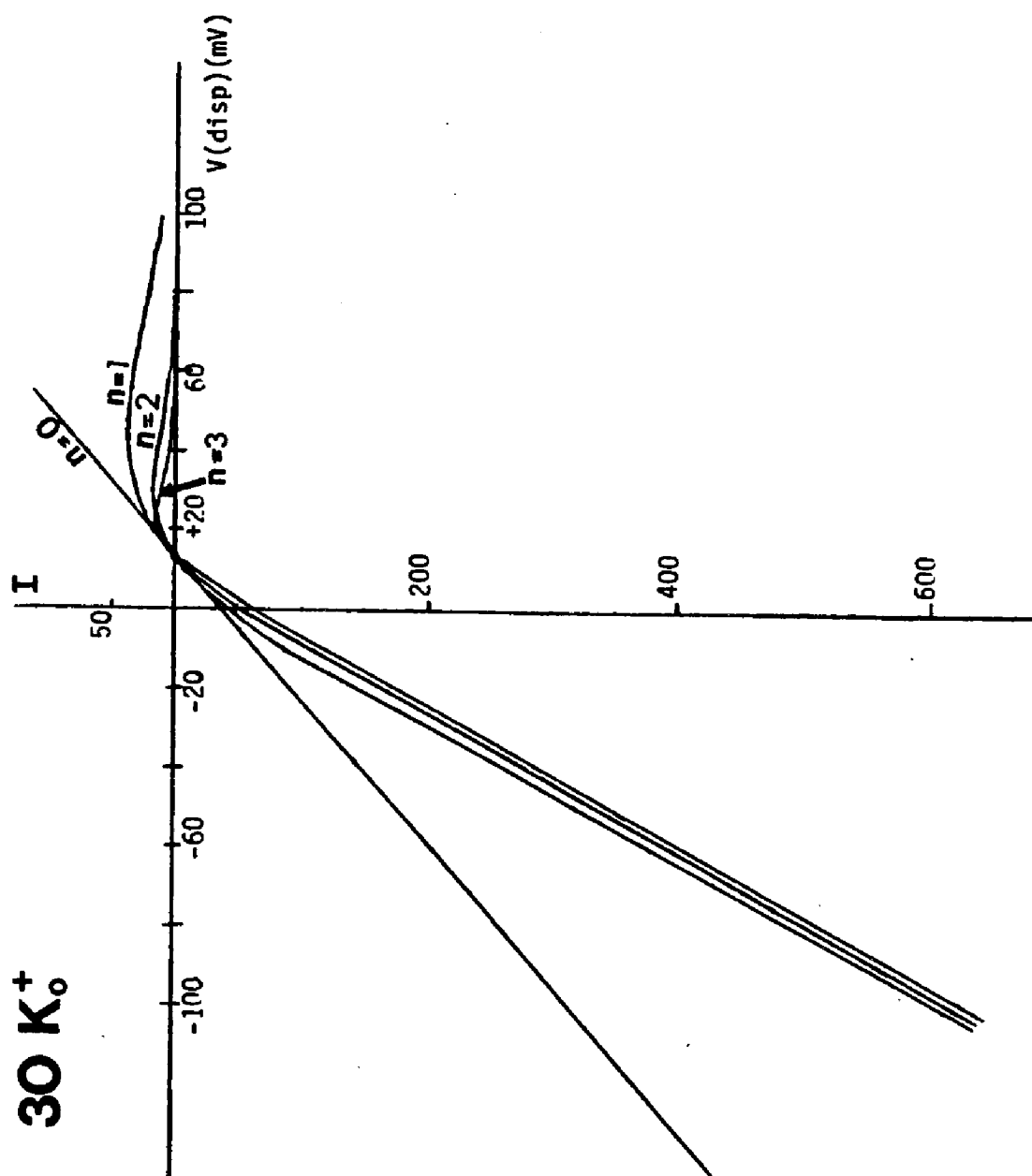


Figure D-5 a. Effect of n on I-V Curves from the Modified Ciani et al. Model for IR at Constant K_o^+ (30 mM K_o^+)

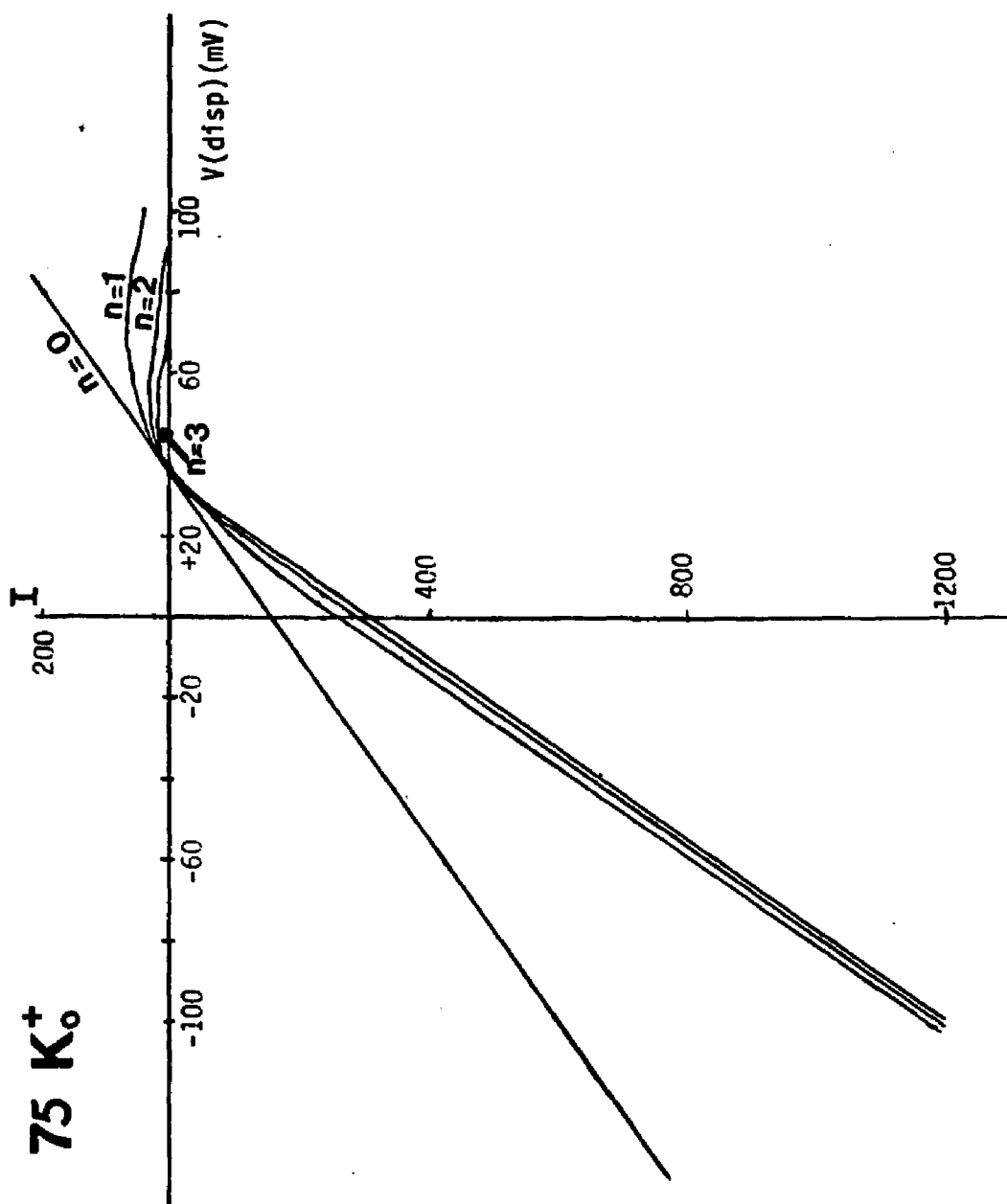


Figure D-5 b. Effect of n on I-V Curves from the Modified Ciani et al. Model for IR at Constant K_o^+ (75 mM K_o^+)

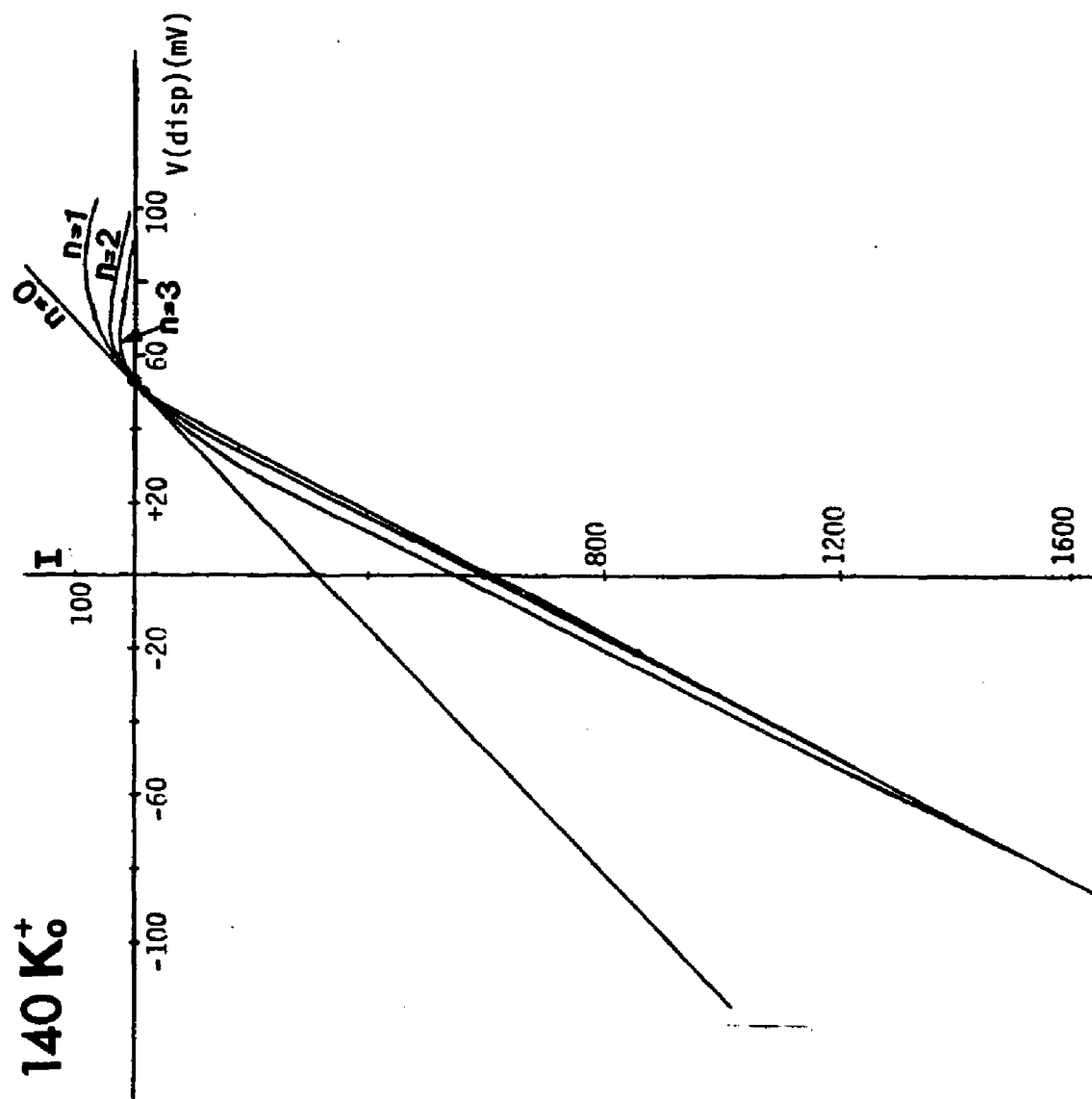


Figure D-5 c. Effect of n on I-V Curves from the Modified Ciani et al. Model for IR at Constant K_o^+ (140 mM K_o^+)

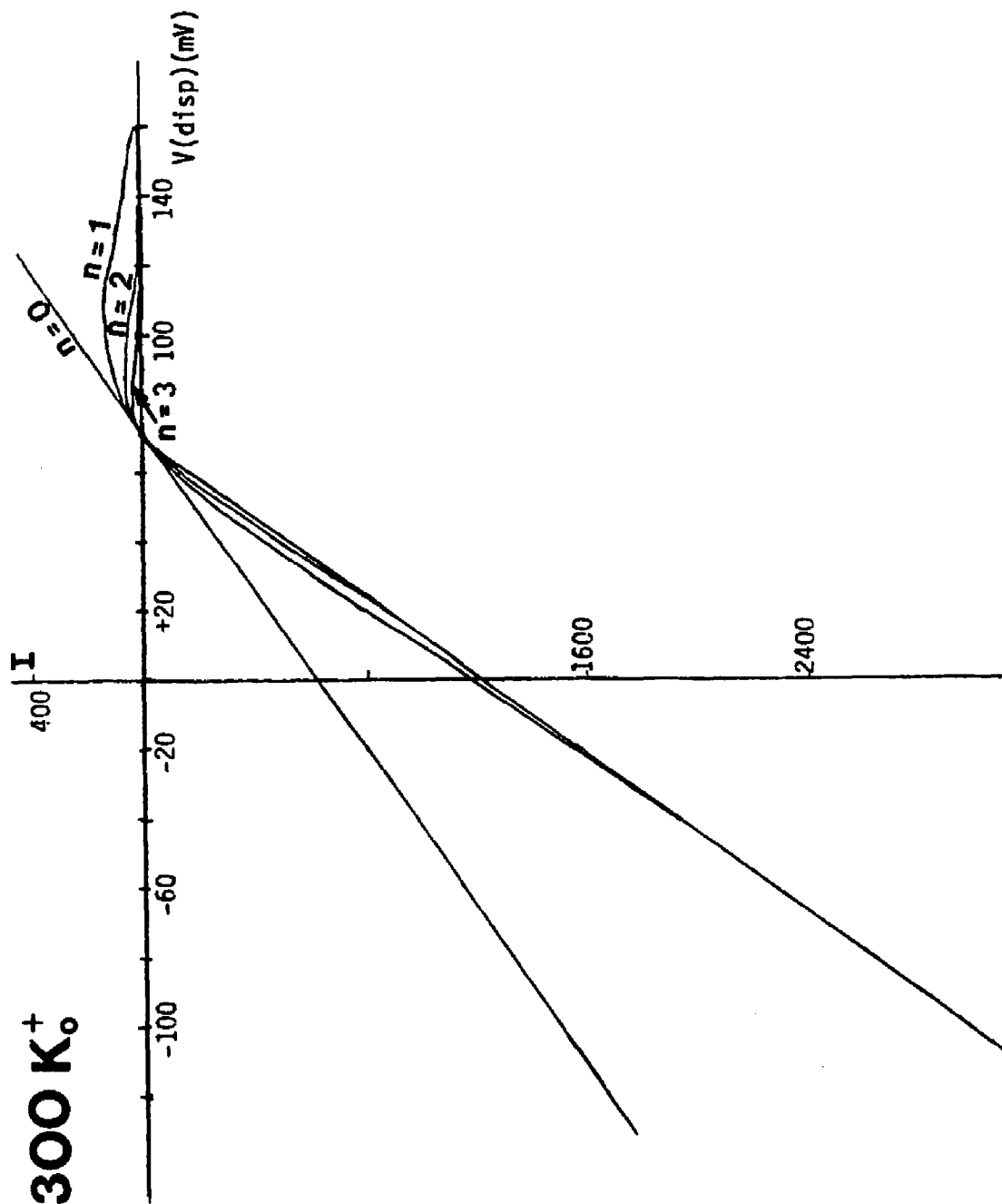


Figure D-5 d. Effect of n on I-V Curves from the Modified Ciani et al. Model for IR at Constant K_o^+ (300 mM K_o^+)

Figure D-6 a-c. Effect of K_o^+ on I-V Curves from the Modified Ciani et al. (1978) Model for IR at Constant n

DC I-V curves were calculated according to the equation in the preceeding Figure legend at constant n ((a), $n = 1$; (b), $n = 2$; (c), $n = 3$) to illustrate the effect of K_o^+ . Note that the scales for outward current are magnified (x4) to better illustrate the crossover phenomenon and NRRs.

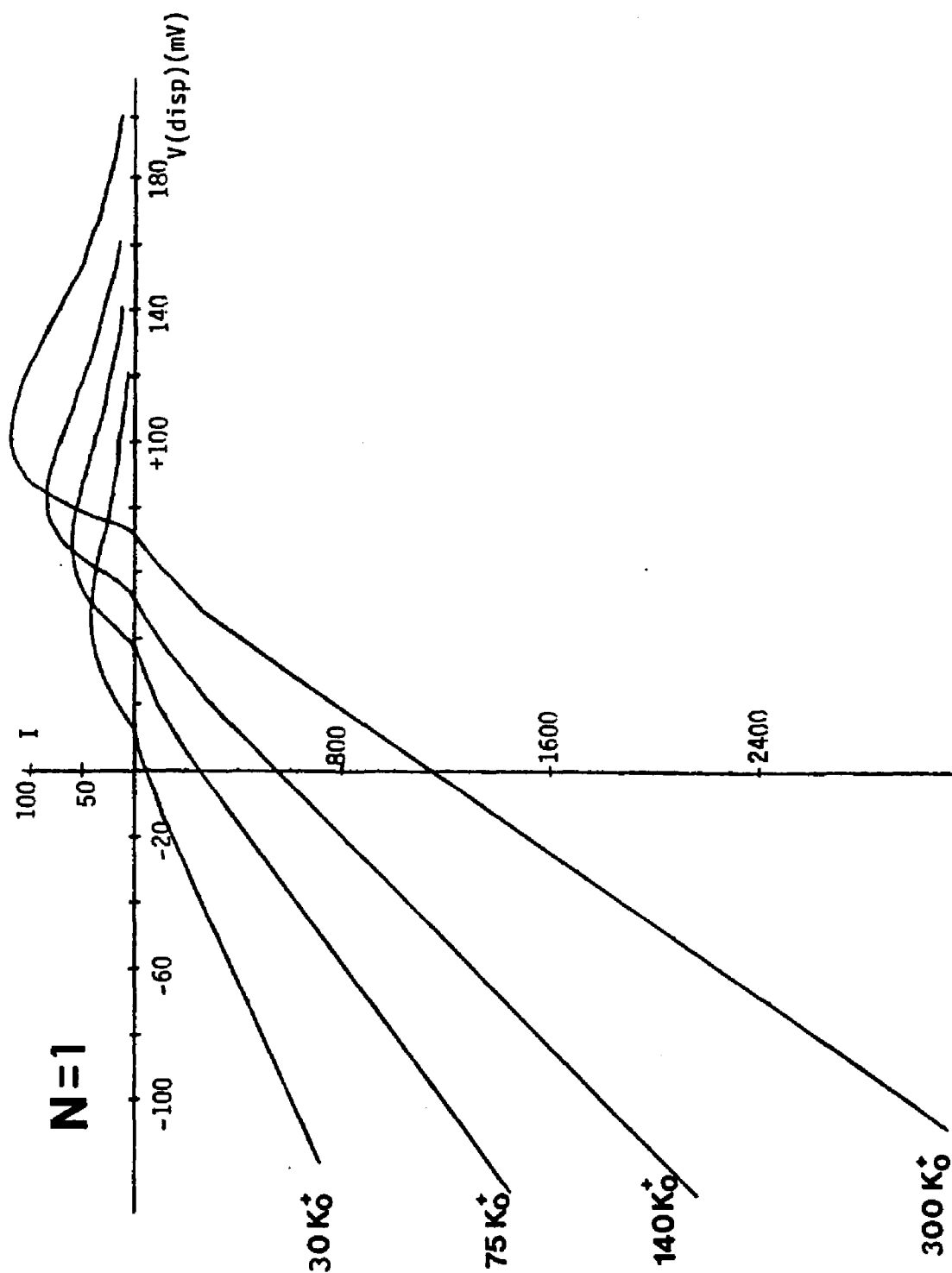


Figure D-6 a.

Effect of K_o^+ on I-V Curves from the Modified Ciani et al. Model for IR at Constant n ($n = 1$)

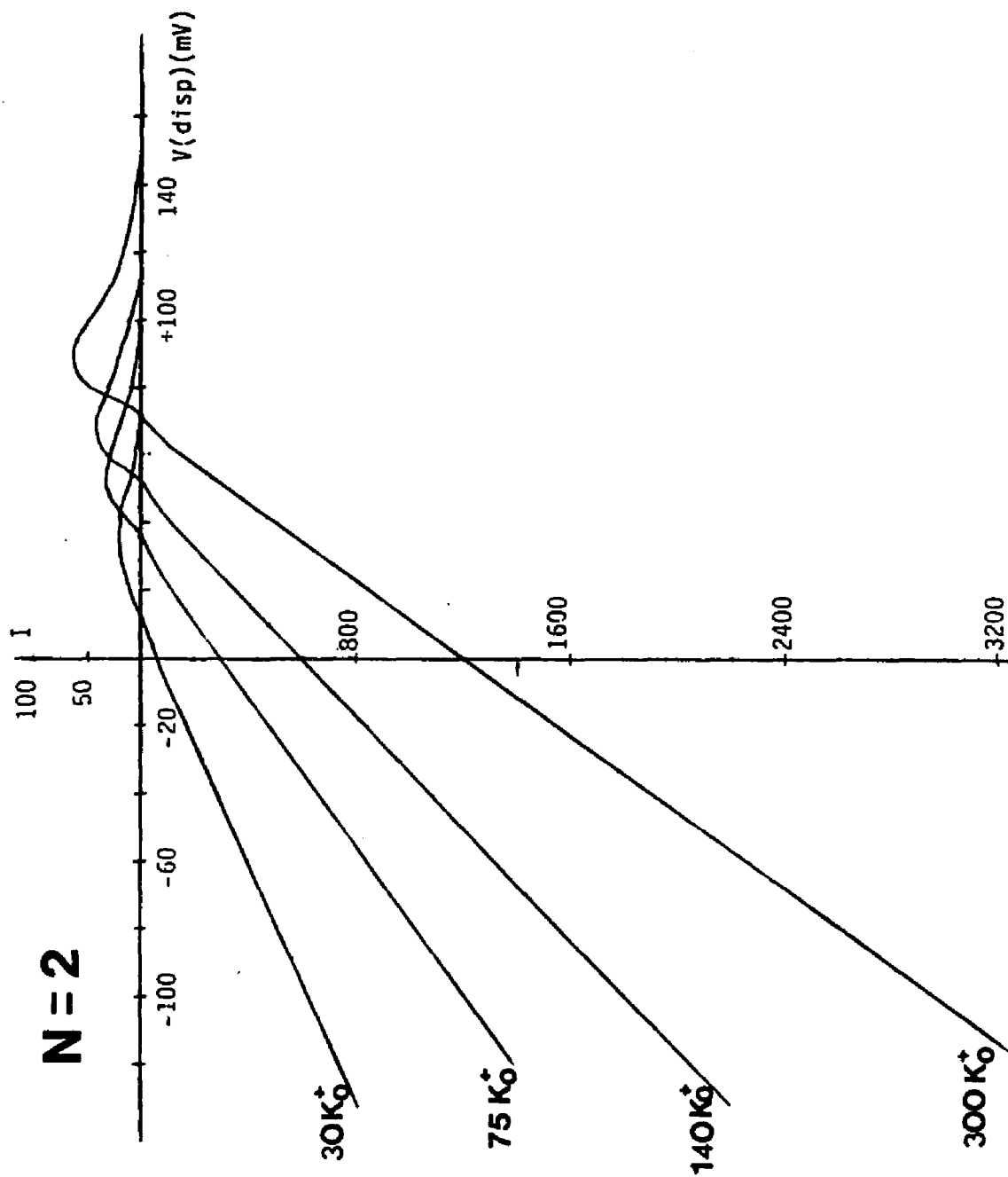


Figure D-6 b.

Effect of K_O^+ on I-V Curves from the Modified
Ciani et al. Model for IR at Constant n ($n = 2$)

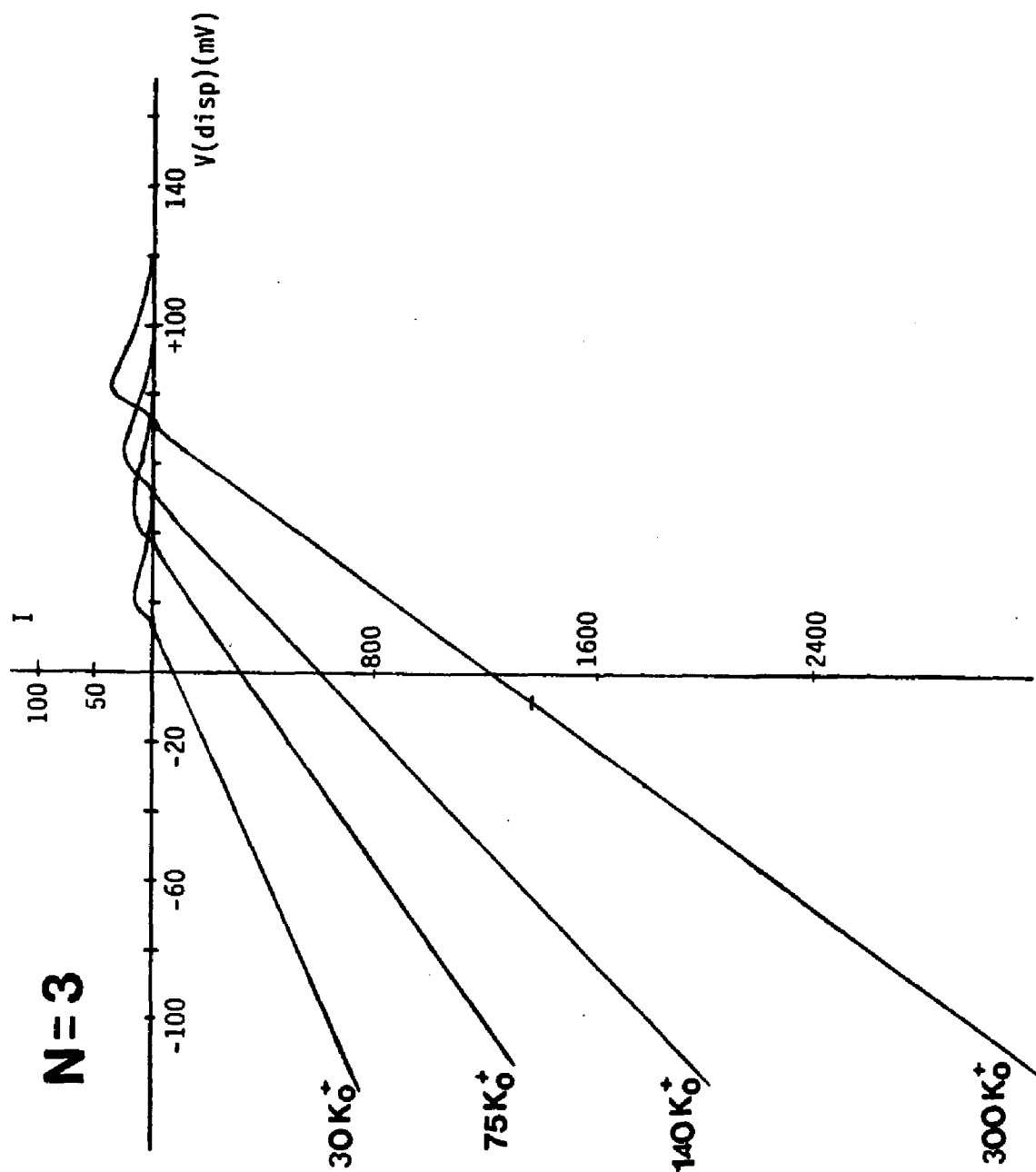


Figure D-6 c.

Effect of K_o^+ on I-V Curves from the Modified Ciani et al. Model for IR at Constant n ($n = 3$)

We have found then that treating IR as a subconductance property, rather than stipulating that channels close at depolarized potentials (Ciani et al., 1978), provides for many features of IR in other systems: (1) a steep change in conductance above V_{rev} with rectification increasing with K_O^+ , (2) crossover phenomenon above V_{rev} , which can be argued to be a property of a multi-ion pore since outward current passes more readily as the outward side is permeant-ion loaded, in direct violation of GHK theory (Jack et al., 1975), and (3) a NRR above V_{rev} similar to that found in many K^+ conductances, especially in heart (e.g., Noble and Tsien, 1968, 1969; Noble, 1984; Carmeliet, 1982)—such NRRs above V_{rev} have been implicated in membrane bistability resulting from K^+ conductances (Carmeliet, 1982; Byerly and Masuda, 1979), and, at least in heart muscle, the I_{K_1} conductance is quite similar to IR in CHNs and can, in isolation, elicit AP-like responses as a consequence of its NRR property when all other conductances are blocked (Tourneau, 1986). The similarity of IR in CHNs to I_{K_1} in heart raises further speculation on the participation of IR in the PDSs correlated with epileptiform activity in hippocampus (see below, Discussion- Section G).

The utility of the current model to the topic of consideration of an IR in CHN is particularly relevant. In most SC experiments it was difficult to distinguish outward current flow through IR by slow changes in potential. Fast ramps often elicited outward current flow, but yet in many ramp clamps, abrupt changes in conductance were noted. In the few circumstances where such ramps were analyzed in detail, a NRR, seen in plotting the I-V curve, has similar features to that of the subconductance model channel for $n \geq 1$ (data not shown). Perhaps

the sudden change in conductance reflects occupation of a subunit by K_0^+ passing out through the channel. This consideration illustrates the dependence of the I-V curves on the rate of change in potential (driving force) across the open channel. As we have modeled the K_0^+ binding sites away from direct participation in permeation through the pore as did Ciani et al. (1978), it is possible that the charge screen which develops on the exterior mouth of the channel has an effect not only on electrostatic repulsion of intrachannel K^+ attempting to exit the channel (hence, rectification) but also on the rate of K_0^+ clearance from the channel mouth by restriction of electrodiffusion (Lauger, 1976). Thus, local time- and diffusion-dependent accumulation of K_0^+ at the external channel mouth during ramp voltage clamp (of the order of 10^{-3} sec for a changing driving force) could lead to expression of a NRR for a channel with high conductance when the rate of ion flux through the open channel is expected to be comparable to the limiting diffusion access rate (Latorre and Miller, 1983; Yellen, 1984a).

Consistent with the model is the finding that rectification becomes steeper with increasing K_0^+ (see Figure R-2). In this case we are considering the fully occupied channel with $n = 3$ in the model (and in the highest conductance state). As can be seen in Figures D-5 and D-6, increasing K_0^+ affects the degree of IR above V_{rev} in the model channel. It may be worthwhile, then to construct experiments where K_0^+ in the pipette is manipulated during a SC experiment to see potential effects of K_0^+ on SC rectification or expression of subconductance states (see below), but this was not attempted during the period of this project due to technical limitations.

We consider that stability of subconductance states is regulated by K_O^+ . As only the "three-quarter" state was seen most frequently (e.g., estimated at < 1% of time at 140 mM K_O^+) we can estimate the K_m for K_O^+ binding assuming 99% occupancy of sites, using Michaelis-Menton kinetics:

$$N_{occ} = \frac{N_{max} * K_O^+}{K_O^+ + K_m} \quad \text{or}$$

$$P(occ) = \frac{N_{occ}}{N_{max}} = \frac{K_O^+}{K_O^+ + K_m}$$

Here N_{occ} is the number of sites occupied by K_O^+ , N_{max} is the maximum number of sites available (3 in our model), and $P(occ)$ is the probability of occupancy estimated for a single site in a SC extrapolating the population Michaelis-Menton model into a probabilistic equation. For $P(occ) = 0.99$ and $K_O^+ = 140$ mM, K_m is 1.6 mM for K_O^+ (assuming non-cooperative binding sites) which is approximately the level of K_O^+ in the normal physiological extracellular solution. If estimates are accurate, then according to this model, under all K_O^+ conditions used (30 mM to 300 mM) in these experiments saturation ($n = 3$) of binding sites and a low probability of occurrence of subconductance states would have resulted. This is consistent with a low probability of observing substates in these experiments. In addition, one would predict from the model that, at low K_O^+ and low occupancy ($n = 0$ or 1), channel conductance would be more linear, which is consistent with the decrease in rectification at lower K_O^+ values. At lower K_O^+ levels near 1.6 mM, one could expect that $0 \leq n \leq 1$ and that significant current

flows out through the channel so as to clamp the resting membrane near calculated V_K . This provides alternative evidence for a channel active in the resting membrane and for significant outward conductance at RMP without arbitrary selection of representative I-V plots for certain degrees of SC rectification (see Discussion Section F and Figure D-8). Also implied in this result is an increase in SC rectification and WC conductance as well as the establishment and enhancement of a NRR above V_{rev} for steady-state g_K as K_O^+ increases even to levels of 20-30 mM, levels consistent with those observed during epileptic seizures or in spreading cortical depression. As a NRR above V_{rev} could provide the membrane operational capacity to develop bistability and a PDS, our subconductance model could have relevance to epilepsy in hippocampus.

Our new model predicts a SC rectification without the necessity of inactivation gates (Clapham and DeFelice, 1984; Gunning, 1983; Kurachi, 1985), rectification gates (Sakmann and Trube, 1984a; Kurachi, 1985), or internal block mechanisms (see below). In this sense, rectification can be understood in terms of the stability of binding of K_O^+ to a subconductance-state-associated SC subunit in the channel proper. We note, however, that SC conductance in this model is controlled by $\text{SQRT}(K_O^+)$, as the model does not predict the subconductance state magnitudes at hyperpolarized potentials. This is because K_O^+ binding does not occur inside the permeation path and, therefore, does not affect open channel conductance directly. We assumed that the substates gate together and that K_O^+ stabilized the open-channel conductance properties depending on the number of sites titrated. Thus, permeation is distinct from rectification in this modification of

the Ciani et al. (1978) model, as it was in the original model. In this sense, the appearance of subconductance states in channel records reflects a conformational transition in the protein that leads to a long-lived change in open-channel resistance. The appearance of such a conformational change could be related to the loss of stabilizing K^+ ion(s) from an external binding site (Strickholm, 1981). In this sense, this model predicts the effects of K_o^+ accumulating at the channel mouth as a result of subunits (substate) of the channel contributing binding sites to effect the outward conductance of IR.

The change in substate conductance at hyperpolarized potentials needs to be explored further in this model. We had initially expected that the model would predict the spread of substate conductance levels at hyperpolarized potentials based upon the logarithmic series in the subconductance state magnitudes (Figure D-1). Reviewing the form of Equation D-3 suggested that the constant ΔV_h would contribute to the form of the results for SC substate I-V plots at hyperpolarized potentials. When ΔV_h was set to the level of the half-maximal voltage (-21.3 mV) of the $P_v(o)$ vs. ΔV curve (Figure R-15c) we found that different subconductance levels were predicted at hyperpolarized potentials. The result is shown in Figure D-7. Although it is not clear how the value of ΔV_h might shift the effect of rectification from depolarized to hyperpolarized potentials, Ciani et al. (1978) and Cleemann (1981) both posited a role for ΔV_h in intracellular effects of K_i^+ on the IR conductance. For a multi-ion pore one might expect that K_i^+ as well as K_o^+ might modulate the character of the SC conductance.

Figure D-7. Changes in V_h of the Modified Ciani et al. Model
Predict the Expected Differences in Subconductance
Magnitudes with Changes in the Titration Number (n).

Using Equations D-3 and D-4, g vs. ΔV curves and I-V curves were calculated. Assuming that at 140 mM K_o^+ RMP and V_K are approximately equal, the voltage terms in the above equations reduce to: ΔV (disp) - ΔV_h , now including V_h as a parameter which we have set equal to the potential (-21.3 mV) at which the $P_v(o)$ vs. ΔV curve is at half maximal (see Figure R-15c). The resultant g vs. ΔV curves (upper panel) and I-V curves (lower panel) are plotted for each titration number, n .

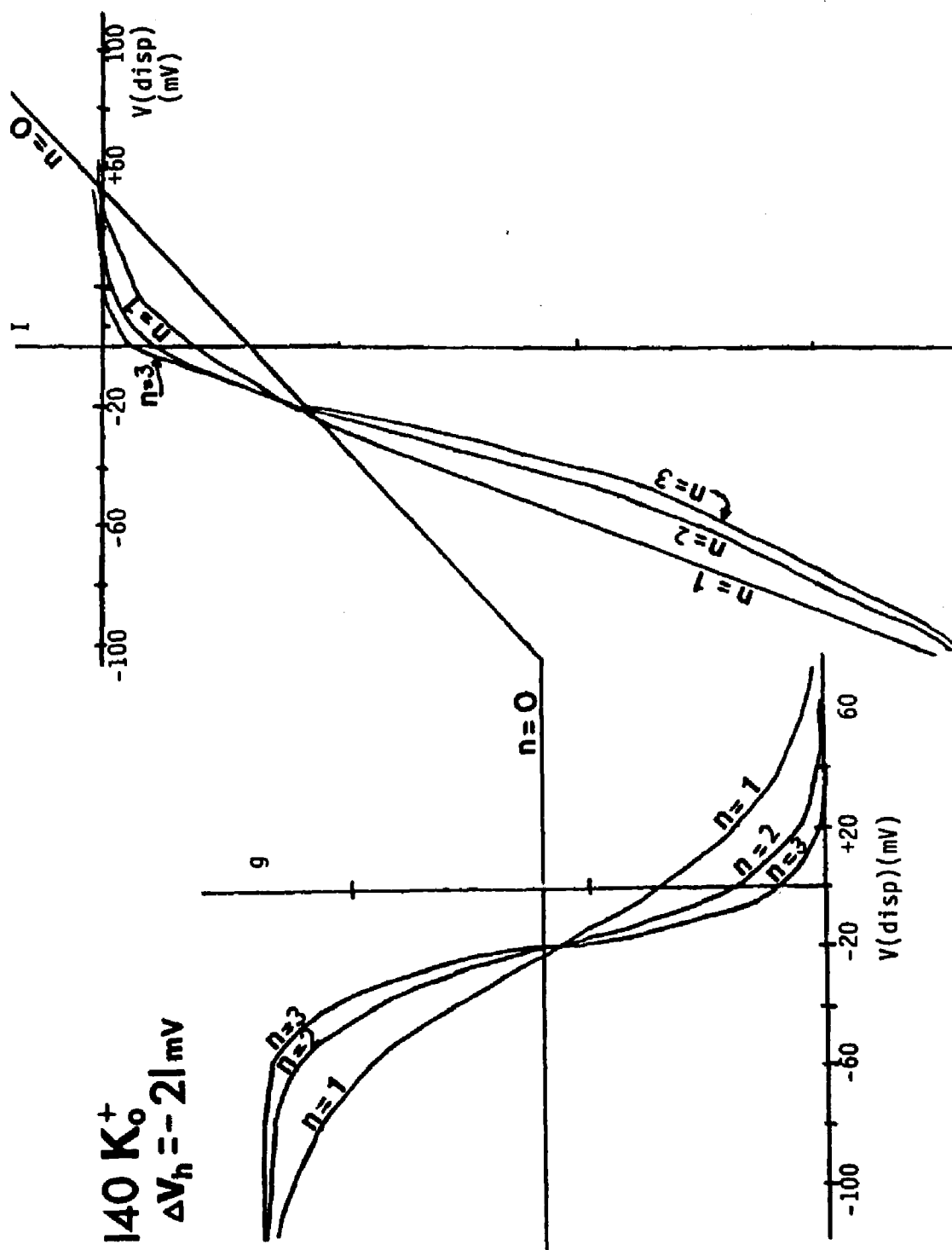


Figure D-7. Changes in V_h of the Modified Ciani et al. Model Predict the Expected Differences in Subconductance Magnitudes with Changes in the Titration Number (n).

5. Internal Block Mechanisms

Armstrong and Binstock (1965) and Armstrong (1966) were the first to show that giant axons which do not normally display an IR K^+ conductance process can be induced to behave in such a manner by internal perfusion with 40 mM TEA^+ . With TEA_i^+ pronounced IR appears in the instantaneous I-V curves (200 microseconds) that has a $g(V-V_K)$ dependence. Armstrong (1966) showed that the presence of the induced IR could give rise to prolonged plateaus in the nerve AP that increased in duration in a TEA_i^+ concentration-dependent manner. Concentration dependence of TEA_i^+ -induced rectification is due to the fact that upon depolarization the linear HH (delayed rectifier) K^+ channels open and are subsequently blocked from the inside by TEA^+ driven into the inner mouth of the pore by depolarization. The probability of block of any one channel and, therefore, block of the outward macroscopic currents upon depolarization is related to the concentration of internal blocking ion relative to that of K_i^+ . When TEA_i^+ is driven into the open channel by depolarization, outward current is blocked, leading to IR. The point of block was later modeled to occur at an inner site in the channel where internal TEA^+ competes with K^+ originating from either inside or outside the neuron. The block site (S), placed outside the membrane field, is filled by blocker at a voltage-independent rate. Rectification results because an adjacent site (more external), when occupied by K^+ , displaces blocker from S and clears the pore for conduction (knock ON mechanism). This occurs readily under hyperpolarized but not depolarized potentials because clearance of blocker then fails to occur (blocker cannot exit the pore), and, on the average, outward current is

attenuated; the rectification that results has an exponential dependence on membrane potential.

In a similar blocking-particle model, proposed by Cleemann and Morad (1979), the blocking site was placed at some fraction of the internal half of the channel so that the rates of blocker access would be voltage- and K_o^+ -dependent. The blocker in this model is postulated to be a particle which is tethered to the inner surface of the membrane and which swings into and out of the pore. Addition of this assumption allows for increasing maximum outward current as K_o^+ increases, thereby leading to crossover in the I-V relationships. Such a model predicts the behavior of I_{K_1} in heart muscle, allows for stabilization of resting potential, the rapid phase of repolarization, and a decrease in AP duration with increasing K_o^+ .

A different sort of blocking particle model for IR in skeletal muscle, proposed by Standen and Stanfield (1978), also places the binding site (R) inside the channel. The K^+ ions entering from either side of the channel then compete for binding at this site with internal blocker (B), which accumulates at R upon depolarization (in a Boltzmann voltage-dependent manner) or whose exit rate of leaving the site is voltage dependent. Since the blocker is not able to exit through the external side of the pore, current flow is thereby prevented. Because the concentration of blocker at R increases on depolarization, the outward current flow decreases through all channels, and IR results. Hyperpolarization, on the other hand, pulls K_o^+ into the channel and clears blocker from the site in a voltage-dependent manner, thereby allowing inward K^+ currents. The

site (R), which binds either K^+ or blocker, accommodates two K^+ charges, two B charges or a single divalent blocker, and, with certain reasonable assumptions about equilibrium constants for K^+ or B at R, IR results with an internal blocker concentration of 10 mM ($K_B = 1$ mM, $K_K = 950$ mM). The predicted conductance is approximately sensitive to the SQRT of K_O^+ , but does not show crossover phenomenon with increasing K_O^+ .

Hille and Schwarz (1978) have modeled the IR channel as a multi-ion single-file pore with a minimum of four Eyring barriers and three sites where the relative energy (e.g., kcal/mole) heights of internal barriers are lower than for the external barriers (out 12/4/4/8 in) and with a blocking particle (B) present on the inner surface. Both B and K_O^+ can occupy all sites, but B cannot permeate past the most external barrier. With a valence of about two for B, IR is generated because depolarization drives B into the channel and blocks outward flow of K^+ . The model, uniquely, can account for the SQRT dependence of K_O^+ on conductance and for crossover phenomenon, but the rectification is not as steep as in eggs, skeletal muscle, and heart.

We have reviewed how IR can result from internal block by impermeant ions. We have also discussed preliminary results that illustrate how isolation of IR channels from CHN or other sources into solutions of pure permeant ions results in a more linear SC I-V plot. Such data suggest the feasibility of further studies on IR resulting from internal block. Although rectification appears to be very fast, it is possible that the rate of access of blocking particles into the internal mouth of the pore from the cytoplasm is very fast (however, see Latorre and Miller, 1983; Yellen, 1984a,b on diffusion

limitations). It is, however, not currently possible to rule out internal block models, Eyring models, electrochemical gating, fast rectification through independent rectifier gates or subconductance-state participation in the process of rectification in the CHN channel.

If blocking particles are relevant in the rectification process, then it would be of interest to determine their physical nature. Different groups have postulated the blocker to be soluble (Hille and Schwarz, 1978; Standen and Stanfield, 1978) or tethered (Cleemann and Morad, 1979; Armstrong, 1975) forms that have direct access to the pore from the inside. Patch clamp studies can be designed to test these assumptions through the use of ionic, chemical, or enzymatic methods for identifying substances that induce or remove IR properties in cell-free SCs. One must also be aware that the biophysical nature of the cytoplasmic bleb in isolated patches is largely unknown (Tank et al., 1982), and substances necessary for IR activity could be lost on prolonged internal dialysis (Trube and Hescheler, 1984). Soluble cationic impermeant blockers (Cs_O^+ , Na_O^+ , Li_O^+ , Rb_O^+ , H_O^+) have been shown to effect outward ionic flow through K^+ -selective channels in excitable membranes (Bezanilla and Armstrong, 1972; Coronado and Miller, 1979; Methfessel and Boheim, 1982; Marty, 1983; Yellen, 1984a,b). Organic cations such as TEA⁺ (Armstrong and Binstock, 1965), N-methylstrychnine, (Cahalan and Almers, 1979), and pancuronium (Yeh and Armstrong, 1978) also have blocking effects on outward currents. The work of Ohmori (1984) on the IR in vestibular hair cells suggests that internal blocking particles (e.g., Cs_I^+) may have effects on the kinetics of IR. It is not

clear yet whether the voltage-dependent kinetics of IR in CHNs arises because of internal blocking particles in the same manner that external blockade occurs in egg cells (Fukushima, 1982, 1982) or skeletal muscle (Ohmori, 1981). The possible involvement of tethered forms of internal blocking molecules may find precedence in the removal of Na^+ channel inactivation by internal perfusion with pronase to break proteolytic linkages (Armstrong et al., 1973). One must also not neglect the potential involvement of other ions in the rates of gating and possibly rectification in IR channels (Ascher et al., 1978; Swenson and Armstrong, 1981; Fohlmeister and Adelman, 1982). In addition, some studies have shown excess current noises in open channels by means of applying power-spectra analysis to SC experiments (Sigworth, 1985); these excess noises could reflect a fast blocking effect occurring from the inside of the channel. The IR channel here shows significant open-channel noise, the nature of which is not entirely clear but appears to arise from a short-lived closed state or fluctuation between unresolved subconductance states.

The WCR experiments on IR conductance were performed under conditions of internal dialysis with an essentially pure KF solution. It can be noted in Figure R-37 that significant currents occur at depolarized potentials, especially as K_o^+ is increased. In this extremely limited data set, the IR is only apparent at lower K_o^+ . The apparent lack of rectification at high K_o^+ could be an effect of masking by other conductances (e.g., I_K), could be an artefact that arises because of uncertainty as to what should be subtracted as leakage, or could result from some diffusible substance (e.g., blocking particles, protein kinase, ATP) being removed by cellular dialysis.

Further studies of IR under WCR could elucidate the nature of the lack of rectification at high K_0^+ .

E. Single Channel Kinetics—Voltage-Dependent Deactivation and Putative Models

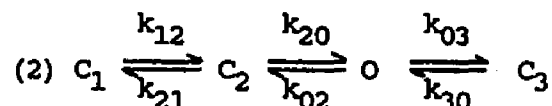
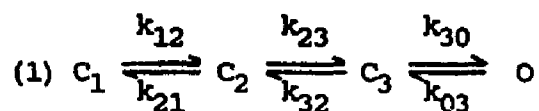
1. $P_V(o)$ versus Voltage Determines Steady-State I-V Plots.

The $P_V(o)$ vs. voltage curve shows a steep trajectory around RMP (e-fold change/34 mV). $P_V(o)$ increases slightly with depolarization and decreases greatly with hyperpolarization. Multiplication of the $P_V(o)$ vs. ΔV relation with the SC I-V curve provides an inverted Gaussian-appearing function centered around RMP. The curve is approximately linear from ΔV_{rev} to the RMP, achieves a maximum inward current and progressively decreases to smaller average currents with further hyperpolarization. The progressive decrease in average current at hyperpolarized potentials is due to the steeply decreasing region of $P_V(o)$ over those voltage ranges. The net result is a NRR centered around or slightly hyperpolarized from RMP. NRRs in steady-state I-V plots imply a region of potential bistability in a free-running membrane. Bistable states in membrane potential that result from K^+ -selective conductances are known for the squid axon (Tasaki and Hagiwara, 1975) and for the I_{K_1} conductance in heart which at least partially determines RMP (Dudel et al., 1967; Carmeliet, 1981, 1982). We will discuss this point further in the last section of the Discussion. Although the NRR would appear in plots of time-averaged SC current at other K_0^+ s, increasing K_0^+ may have an effect of shifting the minimum of the relationship to depolarized potentials, while decreasing K_0^+ would shift it to hyperpolarized potentials. Such displacements of a steady-state NRR with a coincident

changing $V_K(t)$ during K_0^+ accumulation could drive membranes into epileptogenesis (see below). The $P_v(o)$ relationship also accurately predicts that partial inactivation of inward current will occur hyperpolarizing voltage clamp.

2. Kinetic Analysis of Open and Closed Times

Observation of channel records illustrated the complex nature of the kinetics. Around RMP, channel activity grouped into bursts of rapid fluctuations between open and closed states. Bursts were separated by much longer closures which appeared to arise from two distinct lifetime distributions, such that bursts would occur in groups (interburst interval rates: e-fold change per -93 mV) or clusters separated by long intercluster intervals (rates: e-fold change per -538 mV). The total closed-time histogram at RMP was adequately fit manually or by IPROC with three exponentials, thereby indicating that at least three kinetically distinct closed states participated in the voltage-dependent mechanism: one is characterized by a Tau of about a millisecond and is responsible for brief closures during bursts (flicker), a second by a time constant of about a hundred milliseconds and is apparently responsible for the interburst intervals, while the third, by a time constant of hundreds of milliseconds to seconds and appears to represent intervals between clusters of bursts. The open times at resting potential were adequately fit by a single exponential, thereby indicating a single open state. Such a four-state channel could take a number of mechanistic forms—e.g.:



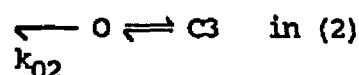
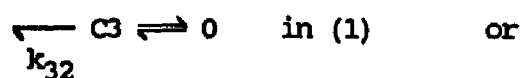
Only the two sequential schemes shown above are considered solely for simplicity.

To attempt to distinguish between such models (if that is even possible, see Sakmann and Trube, 1984b) to explain the $P_V(o)$ vs. ΔV curve and partial inactivation at hyperpolarized potentials, SC patches were analyzed at different holding potentials. Total ON and OFF times were measured in a number of patches and fitted manually or using IPROC, FTIS and ALLOFF. Short OFF times (rates) averaged across two patches were found to be somewhat sensitive to voltage (e-fold per 48 mV) (Figure R-29) with rates of about 6200/sec; these times decreased with progressive depolarization. ON times were always adequately fit by a single Tau, with mean ON times decreasing with hyperpolarization—i.e., the rates away from the ON state (k_{03}) increase with hyperpolarization (e-fold per -68.8 mV). This estimate of k_{03} voltage-dependence is confirmed by the measure of mean short gaps/burst*msec, which increases steeply with hyperpolarization (Figure R-27) (see Section III. B-2 in Results). Use of FTIS and ALLOFF to analyze medium-long, long, or all closed times provided the statistical estimates of closed times that separated apparent bursts and clusters. Shown in Figure R-31 the medium-long closed times decrease

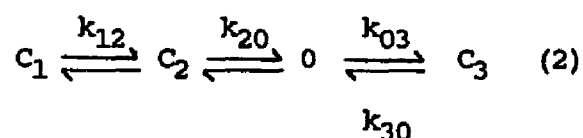
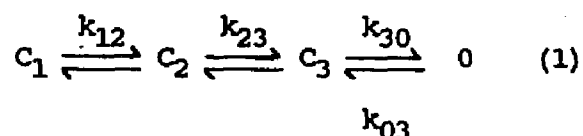
(rates: e-fold increase per -11 mV) while the long closed times apparently increase (rates: e-fold increase per $+25.6$ mV) with hyperpolarization. These distributions of τ s change in an opposite and proportional manner and result in prolonged closed times at hyperpolarized potentials since increasingly long closed times distribute into longer interburst or intercluster intervals, thereby indicating an increased probability of closure ($P_V(c)$) or a decreased $P_V(o)$. At the same hyperpolarized voltages, the burst length is dramatically decreased.

Burst length and interburst intervals were measured manually for a SC patch at 140 mM K_0 . These measurements could be made manually because short closures were typically less than a millisecond and apparently contributed little to $P_V(o)$ as a result of their short average duration.

In all cases, burst lengths (closures > 2 ms) were characterized by a single τ , and the mean τ decreased steeply with hyperpolarization. The rate(s) reflecting passage toward medium-long and long closed states are very voltage sensitive with an e-fold increase in rate per 44 mV hyperpolarization (Figure R-25). Changes in burst length were the most apparent change in channel records with changes in voltage. Interburst intervals were also analyzed and, in most cases, required two time constants for fitting. Rates for the shorter and longer interburst τ s were found to increase e-fold/ -93.06 mV or e-fold/ -538 mV, respectively, with hyperpolarization. Qualitatively, with progressive hyperpolarization the rates out of the bursting states increase dramatically (refer to Four State Models above on page 322):



Rates into the burst states (reflecting the interburst, intercluster or medium and long closed times), e.g.,



increase with hyperpolarization from C_2 (k_{23} or k_{20}) (approximately as steeply as the opposing k_{32} or k_{02} rates increase) but decrease from C_1 (k_{12} in both (1) and (2)). Thus, with hyperpolarization, burst length and interburst (medium-long closures) intervals decrease but intercluster intervals increase. The steep voltage dependence of the burst length (and k_{32} or k_{02}) and interburst interval, as well as the ON times, is probably predominantly responsible for $P_V(0)$ decreasing with hyperpolarization. In fact, both $P_V(0)$ and burst rate change steeply with a similar voltage dependence.

The number of gaps/burst*msec increases steeply with hyperpolarization, thereby suggesting that the rate k_{03} increases with hyperpolarization, which is similar to the result found using IPROC, where it was found that open times decreased with hyperpolarization; IPROC results on medium-long closed times are similar to results using the manual measurement of the faster

distribution of the interburst intervals. Exponential fits to the slow interburst interval were not significant and cannot be compared to the long closed times measured by IPROC.

Nevertheless, with the kinetic information we have it is not possible to test the validity of either model by manual calculation. The 4STATE program was used to attempt to fit the results for τ_{aus} obtained from IPROC, FITS, and ALLOFF to the sequential model (1) for two SC patches with excellent steady-baseline, low-noise data. 4STATE converged to solutions for rates in all cases (as shown in Table III). However, none of the rates show continuous changes with voltage in either patch. Thus, we can conclude from this result either that sufficiently accurate estimates of the time constants were not obtained due to the relatively small numbers of events used in this particular analysis and that these errors were propagated in the model analysis to wide dispersions in rate determinations at the different voltages, or that model (1) is incorrect and possibly model (2) would be more appropriate. A computer program to determine the rates for model (2) was not available, so the question as to which model might be correct cannot be pursued yet. It may be more effective to take the fitted functions of the τ_{aus} versus ΔV and use these expressions to derive the rate constants for either model. This is planned for future work on kinetic modeling of the IR channel. Another way of discriminating between models may be to hold patches at very hyperpolarized potentials so that state C_1 is highly probable (P_{C_1} near one) and then to depolarize quickly to +20 mV and observe the first latency distribution to opening. Model (1) would show a three-exponential fit to first latencies, but model (2) would have a two-exponential fit,

Table III
RESULTS OF A FOUR-STATE SEQUENTIAL MODEL^{a,b}

$ \begin{array}{cccccc} & k_{12} & & k_{23} & & k_{34} \\ c_1 & \rightleftharpoons & c_2 & \rightleftharpoons & c_3 & \rightleftharpoons & 0 \quad (1) \\ & k_{21} & & k_{32} & & k_{03} \end{array} $					
Holding Potential (mV)	k_{34}	k_{32}	k_{23}	k_{21}	k_{12}
<u>Experiment 1</u>					
0	0.80	2.73	1.77	4.44	1.73
-22	0.21	11.21	1.59	2.32	2.48
-28	0.36	5.63	1.56	1.55	0.49
-41	0.53	6.04	0.10	1.89	2.17
-60	0.74	3.17	0.05	0.54	0.45
<u>Experiment 2</u>					
0	0.10	1.78	0.27	0.40	0.27
-10	0.33	1.56	0.28	0.88	0.128
-20	0.43	1.89	0.59	0.57	0.15
-40	0.41	2.22	0.13	0.70	0.18
-50	0.20	2.21	0.26	0.03	0.00005
<u>Average 1 and 2</u>					
0 (n = 2)	0.45	2.26	1.02	2.42	1.00
-10 (n = 1)	0.33	1.56	0.28	0.88	0.128
-20 (n = 2)	0.32	6.55	1.09	1.44	1.31
-28 (n = 1)	0.36	5.63	1.56	1.55	0.49
-40 (n = 2)	0.97	4.13	0.11	1.29	1.15
-50 (n = 1)	0.20	2.21	0.26	0.03	0.00005

^a. The three closed-time distribution (short, medium-long, long) Taus and their relative weights were determined using ALLOFF.

^b. All rates are in units of (sec)⁻¹. k_{03} was not estimated by the algorithm.

inasmuch as only two rates (k_{12} and k_{20}) would have to be involved to open the channel. Data are not currently available to do this kind of analysis.

To summarize, three kinetically distinct closed states and one open state are apparently necessary to explain the temporal behavior of IR in CHNs. Two four-state sequential models were presented, but it is not yet possible to discriminate between them. Voltage sensitivity on hyperpolarization apparently involves four rate constants with the steepest and probably the most relevant changes occurring in the rates leaving the bursting states. The burst length behavior of IR in CHNs is very similar to the I_{K_1} channel in heart (Kameyama et al., 1983; Sakmann and Trube, 1984b), where similar rectification, $P_v(o)$ vs. V curves and mean SC currents with a NRR are found. Partial inactivation upon hyperpolarization is also a similar feature. The channels probably perform similar functions in the two preparations.

3. Ensemble Kinetics Reflect Partial Deactivation of Whole-Cell Currents

Average responses to ensembles of hyperpolarizing pulses illustrate voltage- and time-dependent deactivation (Figure R-32). The rates of relaxation are fitted by at least one steep exponential, while some ensemble averages also have a slow component. The fast rate of relaxation decreases e-fold for 15-20 mV over the steepest region of the curve (around RMP) which again likely represents most strongly the voltage sensitivity of $P_v(o)$ contributed by the burst duration. The averaged currents decay because of temporal nonstationarity in the probabilities of open and closed states after a hyperpolarizing voltage step. What we witness is a voltage-dependent "drift" of the channel

mechanism away from the open state. The time dependence reflects the time (inverse of voltage-dependent rates) it takes to get away from the bursting (open) states, assuming that the rate constants are first order and change instantaneously on changes in potential. Our kinetic analysis adequately predicted the temporal nature of the ensemble currents.

If it is assumed that all IR channels in a space-clamped membrane act with statistical independence, then WC currents should temporally mimic the ensemble currents times the scale factor, N , or the number of channel participants. The WC currents (Figures R-36 and R-37) show partial deactivation with a steep voltage dependence reminiscent of the ensemble average results (Figure R-32). Moreover, the short time constants of partial deactivation for WC currents, ensemble averages, and burst lengths all show a similar dependence on voltage (Figure R-39). This suggests that the underlying voltage sensitivities of the burst length are largely responsible for the partial deactivation of WC currents. The slower relaxation rates in the ensemble averages and WCR currents may relate to the prolonged closed states found in the interburst interval distributions. We conclude that partial deactivation of ensemble-averaged and WC currents by hyperpolarizing voltage clamp is the result of the kinetic sensitivity of IR SCs to voltage.

4. Subconductance States Are Distinguished Kinetically

The unique open-channel subconductance state behavior of the three-quarter substate appears kinetically distinguishable from its maximum conductance relative. In patches where substates were active for prolonged periods, the mean burst duration of the three-quarter

state was about half of that of the maximal conductance state. The differences are expected to be real and not an artifact of unknown membrane potential changes (depolarizations) because the burst duration increases with depolarization but the three-quarter substate has a shorter burst duration (i.e., from -60 mV), than its larger conductance counterpart. Brief fluctuations (ms) between maximal and subconductive states also occur during stable presentation of one state or the other and membrane potential changes would not be expected to occur as quickly or reversibly. Inasmuch as the conditions on the external side of the membrane are fixed and constant, the stable changes in kinetics must be due to some longer-term changes in the structure of the channel or in some modulating influence that alters the energetic barriers between the two open-channel states. Stable changes occurring through enzymatic modification (e.g., phosphorylation, methylation, adenosylation) or a temporal fluctuation in some intracellular messenger (e.g., ATP, Ca^{2+}) could be responsible for kinetically stable subconductance state modalities. We do not know whether the same kinetics exist for brief fluctuations to the three-quarter state or whether the same process might control the appearance, or kinetics of the substate fluctuations in both the transient and stable states. It does appear, however, that the channel has switched into an altered dynamic mode. We did not explore the I-V conductance properties when the three-quarter substate was stably present, so it is not possible to comment on any change in the ability of the channel to carry current. One way to do this would be to apply fast ramp voltage clamps during substate presentation.

As mentioned above, the possibility exists with an intricate substate structure that the conditional transition probabilities between the substates are not symmetrical and the channel is not a passive processor of an energetic gradient at equilibrium. Finkelstein and Peskin (1985) have recently discussed the nature of a voltage-gated channel that is not at equilibrium. If such a SC property does exist for IR in CHNs, then there must be some coupling of energy into the channel kinetics. This may be manifest as a dynamic switching of IR from one mode where the substate appears randomly with low probability (Poisson process) to one where the substate occurs randomly but in a non-Poisson manner. If the probability of getting into or out of the three-quarter substate is not symmetrical, then IR may not be at equilibrium with its energetic environment. Lauger (1985) has recently derived some interesting parallel properties of channels with substates and subpicoampere transport devices (e.g., carriers). If permeation through the channel occurs by way of an energetic coupling (non-equilibrium process) in such a way to promote shifts in the barriers and energy wells of permeation, then such channels could have carrier-like functions.

Obviously, knowledge of the nature of the coupling of energy for such a non-equilibrium process would help us to understand how the molecular structure of the channel relates to its conductive and kinetic performance. The dynamic switching of IR has only been observed at deep hyperpolarized potentials (in both cases, -60 mV). Perhaps this is only because it would have been easier to observe this behavior at potentials where the signal-to-noise ratio is high. On the other hand, the energy of the field across the channel may be used to

couple the subconductance state with the maximum state. Finkelstein and Peskin have suggested that voltage itself may be the source of energy that keeps the channel out of equilibrium. This problem is an extremely interesting one that undoubtedly will be further pursued. Perhaps hippocampal neuron IR will serve as a model system for potential similarities between pores and carriers.

5. Cs_O^+ and Ba_O^{2+} Block IR by Two Apparently Different Mechanisms

We will only briefly review the findings here. Cs_O^+ blocks IR by what appears to be a fast open-channel block (Yellen, 1984a). When open, IR accepts the entry of Cs_O^+ into the channel but is unable to allow total permeation. Cs_O^+ passes across some fraction of the potential drop across the channel and then stabilizes in some energetic well where it binds and blocks K^+ passage. Inasmuch as the degree of block appears voltage dependent and other data suggest a Cs_O^+ concentration-dependence, the rate of access to the binding site in the channel may be enhanced by hyperpolarization and higher concentrations in bulk solution, both of which increase the probability that a Cs_O^+ will encounter the pore mouth. Further studies should examine the voltage and concentration dependence of block to obtain an impression of the fraction of potential drop across the channel where the Cs_O^+ blocks. Cs_O^+ is known to block IR channels in other preparations (Fukushima, 1982; Sakmann and Trube, 1984) and K^+ channels in general (Benzanilla and Armstrong, 1972; Adelman and French, 1978; Coronado and Miller, 1979; Clay and Schlesinger, 1983).

Ba_O^{2+} does not appear to exert an open-channel block except possibly at deep hyperpolarized potentials and discrete or flickery

blocking effects are not seen as with Cs_O^+ . An important feature of the action of Ba_O^{2+} is the displacement of the $P_\text{V}(\text{o})$ vs. voltage curve to depolarized potentials. The blocking effect appears largely kinetic in nature inasmuch as the burst length is profoundly decreased in Ba_O^{2+} relative to that observed in Ba_O^{2+} -free controls. It may be that once open, the pore is blocked by Ba_O^{2+} , but the block is stronger than with Cs_O^+ (possibly due to the divalent nature of Ba_O^{2+} and its similar size to K^+ (Armstrong, 1980)), and the burst duration is shorter because the ON/OFF equilibrium with Ba_O^{2+} is slow. A kinetic modulation by Ba_O^{2+} could be less direct through binding to some site other than the permeation path and altering the free energy differences between the conformations in channel structure that give rise to voltage-dependent gating. Ba_O^{2+} is a classical blocker of K^+ -selective IRs as we have discussed, and it also has blocking effects on other K^+ channels (Armstrong, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982; Vergara and Latorre, 1983).

F. Function of IR in CHNs at Physiological K_O^+

Using the dependence of SC conductance on the $\text{SQRT}(\text{K}_\text{O}^+)$, the $P_\text{V}(\text{o})$ vs. voltage function (assuming isomorphism at 140 mM and 5.6 mM K_O^+) and the rectification of the SC I-V plots around ΔV_rev , one can calculate the expected I-V behavior for IR at normal K_O^+ (e.g., 5.6 mM). The SC conductance for IR was calculated to be approximately 30 pS. For the purposes of this calculation it is assumed that the $P_\text{V}(\text{o})$ vs. voltage curve is similar in shape at 5.6 mM K_O^+ to that in Figure R-15c at 140 mM K_O^+ , except possibly for the displacement along the voltage axis with changes in K_O^+ . This is

done because even though there may be an effect of K_o^+ on the $P_v(o)$ vs. voltage curve (Figure R-16), it is unclear at this time what the functional relationship may be. The rectification around ΔV_{rev} is taken from data where the widest voltage range was obtained and when the greatest outward current was observed. This was done to perform the calculation under the worst rectification conditions because we are currently unable to explain why some SC IRs have no apparent outward currents while others have little outward current at potentials depolarized from ΔV_{rev} . Therefore, the SC "instantaneous" I-V curve was calculated (Figure D-8a) on the basis of the $(K_o^+)^{1/2}$ -dependence of conductance and the assumption that the conductance decreases above ΔV_{rev} to sixteen percent of what it is when hyperpolarized from ΔV_{rev} (e.g., like the channel in Figure R-3). The RMP of the cell was taken as -52 mV and V_K (with 5.6 mM K_o^+ and 140 mM K_i^+ (assumed)) as -81 mV, and, therefore, the reversal potential for the IR K^+ -selective channel under these conditions is the difference between V_K and RMP or -29 mV below RMP ($\Delta V = -29$ mV). The resultant steady-state I-V curve is generated by multiplication of the instantaneous SC I-V curve (Figure D-8a) by the $P_v(o)$ vs. ΔV curve (as was done for Figure R-15d) and is illustrated in Figure D-8b.

It can be easily appreciated (Figure D-8b) that under the worst-case rectification conditions the channel exhibits an outward rectification in the steady-state I-V plot with a NRR 45-50 mV below V_{rev} and a significant outward conductance at RMP ($\Delta V = 0$ mV). In the resting membrane with normal physiological K_o^+ levels, IR would contribute to net K^+ efflux from the cell and, therefore, contribute

to the RMP, or, if it is the only channel active over the hyperpolarized potential ranges, it may solely act to set RMP. In this respect, agents or conditions which act to enhance or block IR as a background resting conductance could have powerful effects on RMP and, therefore, neuronal excitability. K_o^+ loading into the REDS would be expected to increase the conductance but also possibly to promote greater inward rectification through IR (see Figure R-2 and Discussion of substate contribution to inward rectification) and thus would be expected to have a progressively depolarizing effect on the neuron with K_o^+ accumulation. This could lead to prolonged depolarized potentials and the associated epileptogenic transformation (see next section).

Perhaps more interesting to the normal state of excitability and function of the hippocampal pyramidal neuron are the recent findings of Huguenard and Alger (1985) that a similar (if not identical) IR studied in acutely dissociated pyramidal neurons is blocked by low ATP_i and Ca_i^{2+} levels in isolated patches as well as by carbachol (10-50 μM) in CAPs. The level of Ca_i^{2+} and ATP_i can be regulated by the energy charge of the cell (mitochondrial), the activity of voltage- or receptor-dependent Ca^{2+} channels, and any coincidentally operative second messenger systems. IR could thus act in concert with receptor-mediated processes and be tuned up or tuned down by respective excitatory or inhibitory inputs acting on IR directly or indirectly through second messengers. Carbachol, which has both cholinergic and muscarinic activities (Goodman and Gilman, 1980), could, by acting on its respective receptor to block IR, depolarize the pyramidal neuron and, hence, increase firing activity. IR may thus be poised as a

Figure D-8. Calculation of Instantaneous and Steady-State I-V Curves for IR at Physiological K_o^+ (5.6 mM)

(a). The instantaneous SC I-V curve was calculated on the basis of the $(K_o^+)^{1/2}$ -dependence of conductance and the assumption that the conductance decreases above ΔV_{rev} to sixteen percent of what it is when hyperpolarized from ΔV_{rev} (e.g., like the channel in Figure R-3). RMP was taken as -52 mV and V_K (with $K_o^+ = 5.6$ mM and $K_i^+ = 140$ mM (assumed)) as -81 mV. The reversal potential for the K^+ -selective is then -29 mV displaced from RMP.

(b). Assuming that K_o^+ does not markedly affect the $P_V(o)$ vs. ΔV curve, the steady-state I-V curve for IR was calculated using the $P_V(o)$ vs. ΔV curve at 140 mM K_o^+ (Figure R-15c) and multiplying it point-by-point with the instantaneous I-V curve in (a) above.

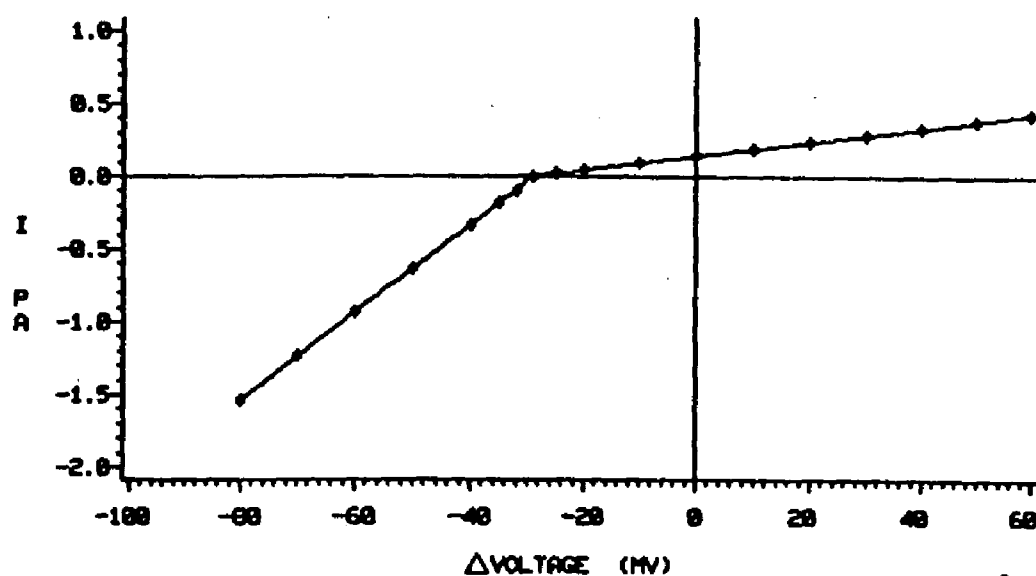
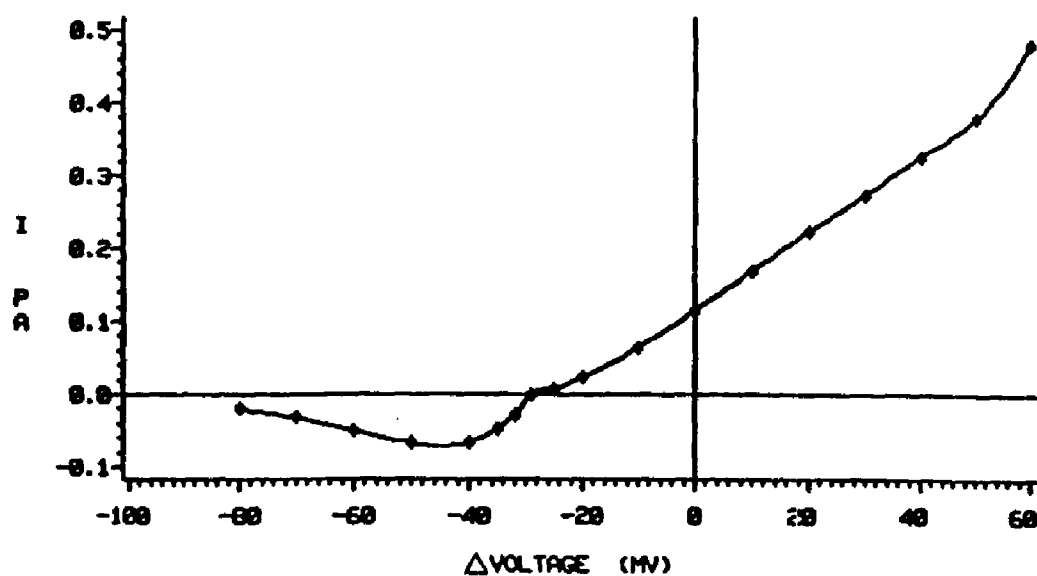
a**SINGLE CHANNEL I-V PLOT FOR IR AT LOW K****b****CALCULATED STEADY STATE I-V FOR IR AT LOW K**

Figure D-8. Calculation of Instantaneous and Steady-State I-V Curves for IR at Physiological K_o^+ (5.6 mM)

critical RMP channel, and agents which act to alter its activity could mediate K^+ -dependent neuronal modulation. If the effect of carbachol is mediated by muscarinic receptors, then the IR channel under investigation here could have a potential relationship to the M conductance of Halliwell and Adams (1982). On the other hand IR is active at RMP in cultured neurons, is turned on by depolarized voltages or small changes in internal Ca_i^{2+} . These properties suggest a potential relationship of IR in CHNs to the unique Ca_i^{2+} -dependent K^+ conductance giving rise to AHPs in adult hippocampal neurons (Lancaster and Adams, 1986).

G. Pacemakers and NRRs, Inward Rectification, K_o^+ , and Paroxysmal Depolarizing Shifts

The intrinsic bursting pacemaker activity of some molluscan neurons (see Faber and Klee, 1972) has been a subject of interest to neurophysiologists concerned with the membrane biophysics of oscillatory potentials and the potential relevance of pacemaking to higher neuronal activity (Prince, 1979). Hippocampal CA3 pyramidal neurons have an intrinsic pacemaker capability, and neurons in CA1 can be driven to operate similarly (Kandel and Spencer, 1961; Wong and Prince, 1978; Johnston et al., 1980; Schwartzkroin, 1975, 1977, 1978; Prince, 1978a,b). The characteristics of an epileptogenic neuron are similar to bursting pacemakers in that rapid oscillatory bursts of APs arise from a PDS in the membrane potential. The membrane of hippocampal neurons has an intrinsic damped oscillatory behavior to depolarizing current injection (Kandel and Spencer, 1961a,c) which may be related to the bursting behavior during PDSs. To generate an oscillator, the membrane must maintain two stable states ($I = 0$) of

potential or be able to time properly the development of inward and outward currents.

One apparently universal attribute of an oscillogenic neuron is the presence of anomalous (or inward) rectification (Kandel and Tauc, 1967) and a NRR over a range of potentials subthreshold to AP firing (Smith et al., 1975; Wilson and Wachtel, 1974; David et al., 1974; Oakley et al., 1972a,b; Schwindt and Crill, 1977; Stafstrom et al., 1982; Faber and Klee, 1972). This type of anomalous rectification and NRR is found in hippocampal pyramidal neurons in CA3 (Hotson, Prince, Schwartzkroin, 1979; Johnson et al., 1980; Prince, 1982; Hotson, 1982). In molluscan bursting pacemakers, the NRR is always present when cells are in a bursting mode. However, the NRR can be experimentally controlled by a decrease in temperature, which results in a linear I-V relationship over the NRR range of potentials (Wilson and Wachtel, 1974, 1978). Under this condition bursting oscillogenesis no longer occurs; raising the temperature restores bursting. Thus, a NRR seems essential to this type of oscillatory behavior. In cortical and motor as well as in molluscan neurons, the convulsant agent pentylenetetrazole (PTZ), which induces seizures, PDS, and bursting activity in silent neurons does so by the induction of a NRR in the I-V relationship at subthreshold potentials (Frazier et al., 1967; David et al., 1974; Ducreux and Gola, 1975). Anticonvulsant drugs change the position of the NRR and can control oscillogenesis (Johnson and Ayala, 1975).

The conductances that are responsible for this NRR and inward rectification in molluscan or higher neuronal systems are, therefore, of special interest to neurophysiologists concerned with oscillogenesis and epileptogenesis. Evidence suggests that the inward rectification

that occurs at potentials slightly hyperpolarized to the zero slope point of the NRR is due to a K^+ inactivation (Nakamura et al., 1965; Nakajima, 1966; Grundfest, 1966) that results from instantaneous inward rectification of the conductance or a steady-state inactivation of the channel at depolarized potentials (Kandel and Tauc, 1967). Agents such as Ba^{2+} , Cs^+ , and Rb^+ which block K^+ conductances enhance IR in many systems. Ba^{2+} enhances the IR studied by Hotson et al. (1979) in hippocampal pyramidal neurons of CA1 and can induce bursting epileptogenesis in these neurons (Hotson and Prince, 1981). The kinetic block of Ba^{2+} in the IR of CHNs would be expected to decrease outward background membrane currents at resting potential and thus increase membrane resistance to persistent inward (depolarizing) currents (see below). The PDSs that occur in the presence of Ba^{2+} may be partially due to its effects on background IR K^+ currents. Ventricular muscle, which is normally silent, can be induced to pacemaker activity by Ba^{2+} (Hiraoka et al., 1980), which partially acts to block the Cs^+ -sensitive IR background I_{K_1} current. In addition Ba^{2+} induces epileptiform burst firing in other cortical neurons (Kmjevic et al., 1971) at least partially by its effects on K^+ conductances (Hotson, 1982). The ability of changes in K_o^+ to induce PDS and epileptiform activity (see below) through changes in K^+ conductances and V_K provides support for the participation of K^+ channels in bursting pacemaking and epileptogenesis (Fertziger and Ranck, 1970; Moody et al., 1974; Ogata et al., 1976; Prince and Schwartzkroin, 1978; Kmjevic et al., 1980).

In addition to the K^+ conductances, a persistent slow inward current is largely responsible for the NRR in bursting systems, which include hippocampal CA1 and CA3 neurons (Eckert and Lux, 1975, 1976; Wong and Prince, 1978; Johnston et al., 1980; Schwindt and Crill, 1977, 1980a-c; Stafstrom et al., 1982; Brown and Griffith, 1983). These inward currents are believed to be carried by Na^+ and Ca^{2+} as partially supported by the results of Dichter (1983) that many cortical neurons have a persistent inward Na^+ conductance that may be active even at RMP. However, even though the NRR is a characteristic of bursting pacemaker or oscillogenic systems, it is also found in nonbursting systems (Partidge et al., 1979), thereby suggesting that other events participate in driving the system into a bursting mode (e.g., some type of depolarizing influence on the cell).

One potential candidate for the depolarizing influence on the cell is the PDS mentioned above. PDSs can arise from synaptic drive, and it has been suggested that they are giant synaptic potentials (Prince, 1968; Johnson and Brown, 1981) or summated dendritic Ca^{2+} spikes (Wong and Prince, 1978; Wong et al., 1979). In the presence of penicillin, GABA activated Cl^- currents are blocked, and a depolarizing influence is induced by disinhibition (e.g., Wilson and Wachtel, 1974; Wong and Traub, 1983). PDSs can also originate from increases in K_o^+ (Zuckermann and Glaser, 1968), and accumulations of K_o^+ occur during PDSs and during seizures up to a ceiling level of about 12 mM (Somjen, 1979). Undoubtedly a number of intrinsic and extrinsic factors can give rise to PDS, to bursting epileptogenesis, and to propagation and synchronization of seizures (e.g., see Chalzonitis and Boisson, 1978). We will focus here on how the

K^+ -selective IR found in CHNs could participate in any intrinsic membrane mechanism that could result from K_o^+ accumulations in REDSs.

Whisler and Johnston (1978) have recently proposed a model for intrinsic bursting oscillogenesis in molluscan and CA3 hippocampal neurons which may help to explain epileptogenesis. They assumed that a neuron is disinhibited (e.g., with penicillin), that the PDS is due to synaptic activity, that K_o^+ accumulation and depletion do occur in the REDS between adjacent pyramidal cell somas, and that such accumulation-depletion phenomenon affects neuronal excitability by way of temporal changes in V_K . Their model includes functional representations for three conductances: (1) the persistent, slow, time-dependent inward Na^+/Ca^{2+} current that has a NRR in its steady-state I-V plot, (2) a time-dependent, depolarized-activating, outward rectifying K^+ conductance responsible for repolarization, and (3) an essentially instantaneous, time-independent anomalous (inward) rectifying K^+ conductance. Time-dependent fluctuations of K_o^+ in REDS are also written in functional form to include typical clearance times (3-5 seconds, Lux and Neher, 1973) and tissue tortuosity. A key feature of the model is that the somal membranes of pyramidal neurons in the hippocampus are naturally separated by about 100 Angstroms with the cells stacked one on top of another (Green, 1961a, 1964). In the model the IR K^+ conductance plays a major role in shaping the region of the I-V plot above RMP and the NRR below and extending above AP threshold. Under normal conditions, the sum of the I-V curves for the three conductances yields an N-shaped I-V function with two stable potential states ($I = 0$) at the voltage axis intercepts. The membrane

exists stably at the resting (more hyperpolarized intercept) potential because the slope resistance is positive at that point. However, the second zero current voltage is located on the negative slope of the I-V plot and would not support stability; such a cell would fire one or more APs if driven by a current source (e.g., an excitatory postsynaptic potential, EPSP); repolarization toward RMP would provide a positive resistance stable point and terminate firing. If K_o^+ is raised twofold, however, and the V_K changed by only 15 mV (-70 to -55 mV), the position of the reversal potential for the IR K^+ conductance shifts. Because of the pronounced rectification above V_{rev} , the maximum positive current point in the total I-V curve (which was above voltage axis) shifts below the voltage axis because IR is shifted out of the range where its outward current stabilizes the K^+ -independent NRR. Under such a condition, no zero-current potential exists (at positive slope conductance), and the model membrane potential oscillates in time, as in a bursting pacemaker, because of the bistability that arises from the NRR conductances. The same effect can be induced by a pulse of depolarizing current (PDS) which causes outward voltage-dependent K^+ current to be activated, supporting local time-dependent K_o^+ accumulation, coincident temporal changes in V_K , and an induction of bursting oscillogenesis. The rate of diffusion out of the REDS, as well as neuronal and glial uptake mechanisms for spatial buffering of K_o^+ , could have profound effects on the stability of such a neuron. The most relevant features of the model then are a REDS, and a time-dependent $V_K(t)$, coupled to an IR K^+ conductance and a persistent Na^+/Ca^{2+} mixed slow inward current. All of these features of the model are consistent

with the properties of hippocampal pyramidal neurons in situ or in the slice.

The results of my study would add to the model that the IR conductance be of the form $g(V-V_K)$. This would make the position of the rectification, and, therefore, the shape of the total I-V plot, sensitive to a conductance increase with K_O^+ . As mentioned above, the REDS is a noted property of the hippocampal cortex and, as in heart muscle, it could profoundly influence the nature of excitability (Attwell et al., 1979a,b; Coulombe and Corabreauf, 1983; Kline and Cohen, 1984) by means of an IR background current similar to I_{K_1} .

This brings up again the question of the role of IR in CHNs. We have shown that IR is active in the resting membrane and, at normal K_O^+ concentrations, would provide for an outward current contribution to V_K and K^+ channel contribution to RMP. Perhaps the unique voltage dependence and conductance of IR functions to aid in repolarization of the neuron following an impulse. Its relaxation kinetics in the population conductance appears somewhat slow for such a contribution, however. On the other hand, we have learned by comparison to other preparations (e.g., I_{K_1} in heart (Carmeliet, 1982), and inward rectification induced by TEA^+ in squid axons (Tashaki and Hagiwara, 1957)) that IR can provide for prolonged depolarized plateau states in the membrane potential. Is it possible that IR in CHNs could take a more direct role in the generation of PDS? Recent studies reviewed here showing that glutamate (Kaneko and Tachibana, 1985; Tachibana, 1983, 1985), acetylcholine (Sakmann et al., 1983; Pott and Bechem, 1984; Soejima and Noma, 1984), serotonin (Drummond et al., 1980; Adams and Levitan, 1982; Benson and Levitan,

1983), substance P (Stanfield et al., 1985) can all have excitatory or inhibitory effects on IR processes. Inasmuch as it has been hypothesized that PDS has a synaptic origin (Prince, 1968; Johnston and Brown, 1981), there may be some credence to the role of neurotransmitters in inducing such a phenomenon through changes in IR. Recent observations on the effect of metabolism (Ashcroft et al., 1984) and changes in cellular ATP (Noma, 1983; Trube and Hescheler, 1984; Kakei and Noma, 1984) on IRs in other preparations and in CHNs (Huguenard and Alger, 1985) may provide a link between cellular metabolism and membrane excitability. It is known that metabolic changes occur in hippocampal pyramidal neurons during epileptogenesis (Lewis and Scheutte, 1975).

As mentioned above, the nature of a REDS in hippocampus, as originally proposed by Green (1961a, 1964) and Kandel and Spencer (1961a,c), would suggest that there would be a problem with a time-dependent accumulation-depletion of K_o^+ and; therefore, a time-dependent $V_K(t)$. Since Zuckermann and Glaser (1968) showed that epileptiform activity could be induced in hippocampus in situ by localized perfusion with high K_o^+ , many studies have been aimed at determining the role of K_o^+ in the initiation, propagation, and termination of seizures (see Somjen, 1979). K_o^+ accumulations occur in spontaneous penicillin- and PTZ-induced seizures (Dichter et al., 1972; Moody et al., 1974; Heinemann and Louvel, 1983). High K_o^+ perfusion also induces epileptogenesis in the hippocampal slice (Ogata 1975, 1976a,b). Moreover, during spontaneous and induced seizures in hippocampus, the highest K_o^+ accumulations occurred in the stratum pyramidale, where the somas of the pyramidal neurons are

located (Fisher et al., 1976). These K_o^+ accumulations typically reach steady-state ceiling levels of 8-12 mM (as measured with K^+ -sensitive electrodes)— levels which reflect a balanced process of K_o^+ accumulation and clearance mechanisms; however, in cortical depression-like swings in potential in stratum pyramidale, levels of accumulation to 30-40 mM can be registered. Functional measurements of K_o^+ accumulation in the REDS of other systems show that accumulations immediately adjacent to the surfaces of cells could be much higher than the typical ceiling levels seen with ion-sensitive microelectrodes (Neher and Lux, 1973). In some tightly packed preparations, one neuron or axon can communicate sufficiently strong so as to induce APs in the adjacent cell through a K_o^+ accumulation (ephaptic) mechanism that does not require synapses (Arvanitaki, 1984; Alkon and Grossman, 1982; Yarom and Spira, 1982). In addition, local K_o^+ microperfusion onto a few hippocampal CA2 pyramidal neurons can activate them to become pacemakers for larger systems of synaptically coupled cells (Wong and Traub, 1983; Traub and Wong, 1982, 1983).

In synaptically uncoupled hippocampus, the synchronization of neuronal firing that occurs in seizures can be duplicated, thereby suggesting that active synaptic mechanisms are not required. Extracellular field bursts that occur in synaptically uncoupled slices are correlated with a transmembrane PDS and bursts of synchronized APs (Jefferys and Haas, 1982; Taylor and Dudek, 1982). Ephaptic synchronization may be an important mediator of intercellular communication in an epileptogenic neuron (Schwartzkroin, 1983). Konnerth et al. (1984) have recently shown that the extracellular field bursts in synaptically uncoupled CA1 spread slowly along the surface

defined by the CA1 pyramidal cell somal layer in a fixed trajectory from the point of origin. Accompanying the negative field potentials, which result from somal depolarizations (Jefferys and Haas, 1982), are bursts of APs, which are temporally correlated with local perisomal accumulations of K_o^+ (up to 7 mM); indeed the form of these K_o^+ accumulations mimics the shape of the field potentials. The CA3 region, where cells are less tightly packed, does not support such a phenomenon. In addition, the same propagation phenomenon can be produced by local pressure perfusion of K_o^+ in a concentration-dependent manner in CA1. The induced K_o^+ accumulation causes depolarization, burst firing, and negative field potentials. Moreover, as in the electrically stimulated case, this activity spreads transversely through the CA1 stratum pyramidale layer. Effectively, the coupling phenomenon is a spatial wave of K_o^+ which has a focal origin in local collections of pyramidal cell somas and spreads unattenuated through the REDS to couple cells into PDSs and bursting oscillogenesis and field potentials, while leaving a wake of neuronal silence (refractory state) behind in its path. Such a K_o^+ wave has properties similar to solitons, which are unattenuated propagating waves in many natural systems (Tuckwell, 1979). Such scroll waves, named because of their spatial appearance, have been hypothesized by Winfree and Strogatz (1984) to cause severe arrhythmias and sudden cardiac death through usurpation of the time-dependent pacemaker currents, which in heart could involve a K_o^+ reactive-diffusive phenomenon. It is possible that IR in CHN could be the functional reporter of these extracellular signals to the transfer function of a neuron.

Konnerth and Lux (1985), moreover, have recently shown that the depolarizing afterpotentials in CA3 has both Na^+ and K^+ components with the apparent slope conductance increasing with K_o^+ . The depolarizing afterpotentials were associated with a decrease in conductance that may involve an inward rectification phenomenon.

Obviously, further work is needed on IR in CHNs. This includes studies of the effects of neurotransmitters and seizure-inducing drugs, as well as the nature of IR modulation (metabolism, Ca_i^{2+} , Na_i^+). Such work has important implications not only for a biophysical understanding of a novel type of IR, resting membrane K^+ channel but also to higher-order properties of the hippocampal system such as neuronal modulation, epileptogenesis, and memory. It is my hope that this work has laid a solid foundation for such future studies.

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