The Role of Kinases and Phosphatases in the Signal Transduction of Hippocampal Pyramidal Cells

by

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Abstract

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The role of the cAMP pathway in long-term potentiation (LTP) was studied in the CA1 region of hippocampus. Widely-spaced trains of high-frequency stimulation (HFS) generated cAMP postsynaptically via NMDA receptors and calmodulin, consistent with the Ca^{2+}/calmodulin-mediated stimulation of postsynaptic adenylyl cyclase. The early phase of LTP produced by the same pattern of HFS was dependent on postsynaptic cAMP. However, synaptic transmission was not increased by postsynaptic application of cAMP. Early LTP became cAMP-independent when protein phosphatase inhibitors were injected postsynaptically. These observations indicate that in early LTP the cAMP signaling pathway, instead of transmitting signals for the generation of LTP, gates LTP through postsynaptic protein phosphatases.

Intracellular and whole-cell recording techniques were used to investigate the interaction between the metabotropic glutamate and β-adrenergic signaling pathways in pyramidal cells from the CAl region of adult rat hippocampal slices. cis-aminocyclopentane-1,3-dicarboxylate (known as *trans*-ACPD), Norepinephrine (NE) and isoproterenol (ISO) blocked the slow afterhyperpolarization (sAHP) evoked by membrane depolarization when applied individually. However, the effects of the combined NE/ACPD or ISO/ACPD application did not exceed that of either drug alone. A larger effect of the combined agonists was obtained when protein kinase C (PKC) was inhibited. The ISO-ACPD interaction could be mimicked by the combination of 8-bromo-cAMP and phorbol dibutyrate. The results indicate that the metabotropic glutamate pathway coupled to PKC limits the b-adrenergic inhibition of the sAHP. The demonstration that PKC can prevent an effect of the cAMP pathway reveals a potentially important motif of interaction between these two pathways, expressed as an assignment of ranks to different neurotransmitters acting on a common effector. Thus, a neurotransmitter of a higher rank (glutamate, coupled to PKC) can override the effect of a lower-ranking transmitter (norepinephrine, coupled to cAMP), enabling the neuron to discriminate among neurotransmitters released in temporal proximity.

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INTRODUCTION

Many aspects of our behavior result from the ability to learn from our encounters and to pattern our behavior around what is experienced. Through learning we acquire languages that enable us to record experience and thereby build complex tools and artifacts, transmit cultures, and create civilizations that are maintained over generations. The complex nature of the judicial system and societal laws and traditions indeed underscores our ability to learn from historical events and past occurrences. We are who we are largely because of what we have learned and what we remember. Without new learning, our attitudes would remain frozen, and there would be no additions to the skills, prejudices and fears that we already possess. Insofar as psychotherapy is successful in treating behavioral disorders, it presumably does so because treatment provides a learning experience that allows the patient to acquire new patterns of behavior. Learning may also produce dysfunctional behavior leading to the genesis of certain mental and somatic diseases and, in the extreme, constituting psychological disorders. Therefore, the study of learning is central to understanding both normal and abnormal behavior, and it is no wonder that learning and memory have long been an area of great importance in psychology. In recent years, research on animal learning and human memory that have emerged from laboratory and psychological studies have come to address many of the same issues. As a result, neurobiology and cognitive psychology have begun to find a common ground, and we are beginning to benefit from the increase in explanatory power that occurs when two initially disparate disciplines converge. Particularly evident in animal studies

of memory and learning, this synthesis will yield revenues as major breakthroughs and insights into mental processes - from the behavioral to the molecular level - providing the infrastructure for a science that will deepen our understanding of behavior and its abnormalities.

Memory founds the pillars of our knowledge, and a better understanding of its nature, how its retained, and how it may be disrupted or improved will broaden our window of knowledge in pursuit of applications we have yet to perceive. Nevertheless, the interface between biology and the study of learning and memory presents one of the most challenging problems in neural science. Studies of memory over the past century have culminated in a commonly used model of the memory storage system. Initially, brain inputs are processed into a short-term memory store of limited capacity which retains less than a dozen items, and, in the absence of rehearsal, persists only for a few minutes. The information is later refurbished into a more permanent long-term form. This long term memory is in turn divided into an intermediate type which is relatively susceptible to disruption, and a truly long-term form, that is stored and maintained by plastic rather than dynamic changes and is very insensitive to disruption. This model necessitates the existence of an additional system which searches and retrieves information from memory stores as demanded by specific tasks.

Concerning the boundaries of this paradigm, a significant clue that its the brain's temporal lobes which are important for memory, came from the observations made by a number of investigating neurosurgeons and psychologists. Before carrying out temporal lobe

surgery for the control of epilepsy, Wilder Penfield (1958) electrically stimulated the exposed temporal lobes in fully conscious patients and observed them reporting vivid experiences of past events upon such stimulation. Additionally, it was discovered that stimulation of the mid-temporal gyrus could result in a brief anterograde or retrograde amnesia. Moreover, it was found that, depending on the duration and intensity of stimulation, the patients exhibited a retrograde amnesia which extended back from several hours to several days, and recovered within 5 minutes to several hours (Bickford et al. 1958). Further evidence for the role of the temporal lobes in memory came from the study of epileptic patients who underwent bilateral removal of the hippocampus and the associated structures in the temporal lobes. It was realized that, although previously acquired long-term memories remained relatively intact, these patients exhibited an irreversible deficit of recent memories and a profound impairment of new, long-term memory formation (Scoville and Milner 1957). These instances argue that, although lesions of the hippocampal region dramatically affect and interfere with the retention of new memories, these structures themselves are not registers or banks for memory storage, but are primarily involved in the process by which memories are placed into or are retrieved from storage. Memory traces are believed to be consolidated elsewhere, presumably in the cerebral cortex, even though the initial storing of long-term memories may occur in hippocampus.

The hippocampus (Ammon's horn) appears as a curved cortical structure, located in close proximity to the amygdaloid body, forming the floor and medial wall of the lateral ventricle. Hippocampus has three major excitatory pathways running from the subiculum to

the CA1 region. The perforant path fibers originating in the subiculum project to the granule cells in the hilus of the dentate gyrus. The axons of the granule cells, in turn, form a bundle, the mossy fiber pathway, that projects to the pyramidal cells of the CA3 region. Subsequently, the pyramidal cells in the CA3 region send excitatory collaterals, called the Schaffer collaterals, to the pyramidal neurons in CA1. The axons of the CA1 pyramids proceed in the alveus to the fornix or to the subiculum, which projects back to the entorhinal cortex (Amoral and Witter 1989).

Stimulating a single pyramidal neuron directly, by passing sufficient depolarizing current through a microelectrode inserted into the cell body of the neuron, past a threshold value initiates a stereotyped series of changes. The cell membrane rapidly depolarizes to become briefly inside-positive and then rapidly repolarizes and returns to a potential near rest. This phenomenon is called an action potential (AP). The repolarization normally continues beyond the original resting potential, so that the membrane becomes transiently hyperpolarized, and then quickly returns to the original resting potential. The last phase is called the fast after-hyperpolarization (fAHP). The rapid depolarization of the membrane is due to inward sodium current, caused by a sudden, large increase in sodium conductance. During the rising phase of the action potential, the sodium conductance exceeds the potassium conductance, and the net inward current drives the membrane potential toward the Na^+ potential (~ +100 mV). As the action potential reaches its peak, the sodium conductance declines, and the potassium conductance increases. The outward potassium current soon exceeds the inward sodium current, and the membrane potential begins to repolarize and

move toward the K^+ potential (~ -90 mV). In hippocampal pyramidal cells, a burst of action potentials prompts the opening of additional channels which induce a slow hyperpolarizing potential lasting several seconds. In these neurons, the Ca^{2+} which enters the cells through voltage-dependent Ca^{2+} channels during the action potential, activates Ca^{2+}-dependent K^+ channels, causing the slow after-hyperpolarizing potential (sAHP) (Storm 1990, 1993). Many kinases have been shown to play a role in modulation of the sAHP phenomenon. Classical studies by Baraban (1985), Muller (1992), Pedarzani (1993) and their colleagues show that PKA, PKC, and CaMKII can block the sAHP channel, and thus reduce the amplitude of this current. Normally, the sAHP substantially diminishes the rate of action potential discharge - a phenomenon referred to as accommodation - and serves to determine the general excitability of cells.

Induction of an AP in a neuron will in turn produce a small excitation (depolarization) in the postsynaptic neuron. This excitatory postsynaptic potential (EPSP) produced by one presynaptic neuron depolarizes the postsynaptic neuron by only 0.2-0.4 mV, which is far below the threshold required for generating an AP. Often a depolarization of 10 mV or more is required to reach threshold. Interneurons also can be recorded from and stimulated intracellularly. Stimulating an interneuron that innervates a pyramidal cell, produces a small inhibitory (hyperpolarizing) postsynaptic potential (IPSP) in the pyramidal neuron. Although a single EPSP is not nearly large enough to elicit an AP, the convergence of many excitatory synaptic potentials from many afferent fibers can be integrated by the neuron to initiate one. On the other hand, IPSP's can counteract the excitatory actions and prevent the membrane

potential from reaching the threshold necessary for the generation of an AP. Moreover, since many neurons in the brain are spontaneously active, IPSP's can hinder the spontaneous generation of action potentials in these cells, and thus determine their firing pattern.

About one century ago, Cajal demonstrated that networks of neurons are not cytoplasmic continuities but communicate with each other at specialized junctions which Sherrington called synapses. This observation prompted the conjecture that information storage in the brain occurs via changes in synaptic efficiency. In 1973 Timothy Bliss and Terje Lomo discovered that a brief high-frequency train of stimuli to perforant path fibers of the rabbit hippocampus produces a long-lasting increase in the EPSP amplitude. It was later discovered that all three afferent pathways of the hippocampus share this property. This rapidly-induced facilitation, named long-term potentiation (LTP), can last for hours in tissue slices, and in the intact animal for days and even weeks. The manifestation of LTP, which ultimately involves a persistent increase in the synaptic efficacy, is not accompanied by generalized changes in the excitability or the passive membrane properties of the postsynaptic neurons (Barrionuevo and Brown 1983). Three plausible explanations for LTP are: an increase in transmitter release, an increase in the sensitivity of the postsynaptic membrane to released transmitter, or a decrease in the series resistance of dendritic spines (Baxter et al. 1985, Briggs et al. 1985). Although there is compelling evidence that some forms of LTP involve a presynaptic modification that increases transmitter release, as will be discussed later, in most synapses there is no basis for ruling out other possibilities. This initial description of LTP opened an area of research that, more than two decades later, continues to generate

considerable excitement, partly because LTP exhibits many properties that make it an ideal synaptic mechanism for memory storage.

LTP can be induced in a number of ways, most conveniently by delivering a tetanus, typically a train of 50-100 stimuli at 100 Hz or more, to the pathway of interest. It can also be evoked by providing the patterns of stimulation within certain critical ranges. Two particularly efficient recipes are termed "theta-burst stimulation" (several bursts of 4 shocks at 100 Hz delivered at an interburst interval of 200 ms) and "primed-burst stimulation" (a single priming stimulus followed at 200 ms by a single burst of 4 shocks at 100 Hz) (Larson et al. 1986, Rose and Dunwiddie 1986). Although the LTP obtained by different schemes of tetanization may not be identical in nature, the significance of these protocols is that synchronized firing patterns at similar frequencies occur in the hippocampus during learning (Otto et al. 1991).

In the CA1 regions of the hippocampus, LTP has three interesting properties:

- **Associativity:** the postsynaptic cell and the participating fibers must be simultaneously active (McNaughton et al. 1978, Levy and Steward 1979).
- **Cooperativity:** a minimum of two fibers need to be activated together to generate LTP (Lovinger and Routtenberg 1988, Malenka 1991).
- **Input-Specificity:** LTP is specific to the stimulated synapses (Anderson et al. 1977, Lynch et al. 1977).

Accounting for these three features, other properties involved in the induction of LTP in the CA1 region of the hippocampus were later discovered: 1) LTP is reduced and even prevented by properly timed inhibitory inputs to or hyperpolarization of the postsynaptic cell during the tetanus (Kelso et al. 1986, Malinow and Miller 1986). Conversely, 2) LTP is greatly facilitated when IPSP's are suppressed (Gustafsson and Wigstrom 1988). Most fascinating, 3) robust LTP can be induced when a weak-stimulus train, not sufficient to produce LTP, is paired repeatedly with a strong stimulus or with a depolarizing current pulse injected into the postsynaptic cell (Kelso et al. 1986, Wigstrom et al. 1986, Sastry et al. 1986). This latter property along with the associativity phenomenon provides a cellular analogue of the Pavlovian classical conditioning (Carew and Sahley 1986). Furthermore, LTP requiring excitation of the postsynaptic cells coincident with activity in the presynaptic neuron provides the first direct evidence for Hebb's rule, proposed in 1949 by Donald Hebb. He proposed a coincident-detection rule in which the synapse linking two neurons is strengthened if the cells are active at the same time.

In hippocampus, the axons of the CA3 neurons that terminate on the pyramidal cells of the CA1 region use glutamate as their major excitatory transmitter. The functional diversity of glutamate is reflected by the presence of disparate glutamate receptors, classified into two broad families: metabotropic and ionotropic. Members of the ionotropic glutamate receptor (iGluR) family are coupled directly to cation channels, whereas the metabotropic glutamate receptors (mGluR) are coupled to effector systems through GTP-binding proteins. iGluRs can

be further divided into three distinct subtypes named for their preferred agonists: NMDA (N-methyl-D-aspartate), AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole propionate) and KA (kainate) receptors; the last two subtypes are often referred to collectively as non-NMDA receptors. NMDA receptors display high Ca^{2+} permeability and voltage-dependent Mg^{2+} block, whereas the non-NMDA receptors pass mainly monovalent cations and mediate fast excitatory synaptic transmission in most regions of the CNS. Recent molecular cloning studies have identified homologous subunits for the AMPA (GluR1-GluR4), kainate (GluR5-GluR7, KA1, KA2) and NMDA (NMDAR1, NMDAR2A - NMDAR2D) receptor classes (Wisden and Seeburg 1993, Bochet and Rossier 1993). Regarding the metabotropics, eight mGluR genes have been cloned and tentatively categorized in three different groups (mGluR1, 5; mGluR2, 3; & mGluR4, 6, 7, 8) on the basis of their sequence homology, pharmacological properties and signal transduction mechanisms (Pin and Duvoisin 1995). Physiological studies have revealed that the non-NMDA receptors dominate in normal synaptic transmission, and modulation of function of these receptors through phosphorylation (Blackstone et al. 1994, Wyllie and Nicoll 1994, Tan et al. 1994, Wang et al. 1994) is the primary mechanism underlying plasticity at excitatory synapses. In other terms, during LTP, only the flow of current through the non-NMDA receptor-channels is enhanced (Kauer et al. 1988, Muller et al. 1988, but also see Bashir et al. 1991, Tsien et al. 1991).

The NMDA receptor-channel, which seems to cluster on the heads of the dendritic spines, is unique in being doubly gated by both transmitter and voltage. This channel is blocked at resting membrane potentials by extracellular Mg^{2+} ions (Ascher and Nowak 1990).

Strong, cooperative input from many presynaptic fibers activates a large number of the non-NMDA receptors which, in turn, lead to postsynaptic depolarization. When the postsynaptic cell is adequately depolarized, the Mg^{2+} ion is expelled from the NMDA channel, and Na^{+} and Ca^{2+} are allowed into the cell. Thus, Ca^{2+} entry through the channel requires the coincidence of postsynaptic depolarization and ligand binding to the NMDA receptor. The Ca^{2+} influx through the activated NMDA receptor-channel, lasting only a few seconds, is critical for LTP. Blocking this receptor-channel with a selective antagonist such as aminophosphonovalerate (APV) or chelating the Ca^{2+} influx by injecting EGTA or BAPTA prevents induction of LTP in CA1 area (Collingridge et al. 1983, Lynch et al. 1983). Conversely, injecting Ca^{2+} into the postsynaptic cell initiates the early phase of LTP (Malenka et al. 1989). There are, however, indications from Ca^{2+} imaging experiments that the Ca^{2+} which permeates NMDA channels is augmented by Ca^{2+} release from intracellular stores. The Ca^{2+} transient associated with the synaptic activation of NMDA receptors is substantially reduced in the presence of ryanodine/dantrolene, or thapsigargin, drugs which inhibit Ca^{2+}-induced Ca^{2+} release and deplete intracellular Ca^{2+} stores, respectively (Obenause et al. 1989, Alford et al. 1992, Harvey and Collingridge 1992, Bortolotto and Collingridge 1993). Moreover, it has been shown that activation of mGluRs leading to generation of inositol 1,4,5-triphosphate (IP_3) can induce LTP by a thapsigargin-sensitive mechanism even when the NMDA receptors are blocked (Bortolloto and Collingridge 1993). There is also evidence that sufficient activation of voltage-dependent Ca^{2+} channels can trigger LTP even when the NMDA receptors are blocked (Grover and Teyler 1990). Whichever route the Ca^{2+} entry may take two Ca^{2+} -dependant protein kinases, namely Ca^{2+}/calmodulin kinase II and protein kinase C, are the

most likely candidates to mediate the persistent enhancement of synaptic transmission in LTP (Nicoll et al. 1988, Tsien and Malinow 1990).

The finding that LTP is involved in many different stages of development and in many areas of the brain besides the hippocampus (Briggs et al. 1985, Cooper et al. 1993, Bliss and Collingridge 1993) raises the question: Is LTP involved in memory storage? Richard Morris and his colleagues (1986) developed a spatial memory task in which a rat has to swim in a water maze, filled with an opaque liquid, to find a platform hidden just underneath the fluid. The animal is released around the pool at random locations and is required to navigate to the escape platform using spatial cues. To reach the platform with minimal distress, the animal must implement the spatial cues it can infer from the walls of the room in which the pool is located. In a nonspatial version of this task the platform is raised above the water surface so that it is visible. Here the rat can swim to the location where it sees the platform. When NMDA receptors in the hippocampus are blocked by injection of APV into the brain's ventricles, animals can navigate the nonspatial version of the task using visual cues but they fail in the spatial version of the task. This experiment is suggestive of the hippocampal NMDAR, and perhaps LTP, to be involved in spatial learning.

Whereas initially the induction of LTP in the CA1 region appeared to depend solely on postsynaptic depolarization, Ca^{2+} influx, and Ca^{2+} second-messenger activation, shortly thereafter evidence accrued in favor of LTP requiring an increase in presynaptic transmitter release. This finding of a presynaptic locus for LTP seems attractive because of its unifying

view of various learning-related forms of plasticity across many species (Kandel 1989). Many lines of evidence support this hypothesis. First, analysis of slice perfusate reveal that LTP is accompanied by an enhancement of glutamate release (Bliss et al. 1985). Second, quantal analyses of transmitter release in both cell cultures and hippocampal tissue slices indicate that LTP involves an increased probability of transmitter release without a change in the sensitivity of the postsynaptic receptor (Bekkers and Stevens 1990, Tsien and Malinow 1990; for contrary evidence see Foster and McNaughton 1991, also see Davies et al. 1989, Kullmann and Nicoll 1992, Larkman et al. 1992). Third, imaging studies with voltage-sensitive dyes in hippocampal monolayer cultures suggest that the induction of LTP by postsynaptic depolarization of a single cell produces LTP in a whole population of neurons (Bonhoeffer et al. 1989). Hence LTP may not be restricted to the cell that was depolarized as might be expected from a strictly postsynaptic mechanism. If the induction of LTP requires a postsynaptic event (activation of NMDA receptors) and maintenance of LTP involves a presynaptic event (increase in transmitter release), then some retrograde plasticity message must be sent from the dendritic spines of the excited postsynaptic cell to the presynaptic neurons. As to the nature of this factor, some neuroscientists denote that arachidonic acid (AA) might be the mysterious retrograde factor, yet other lines of research indicate that gaseous substances such as nitric oxide (NO) or carbon monoxide (CO) may relay the message (Williams et al. 1989, Schuman and Madison 1991, Zhuo et al. 1993). To account for the pathway specificity of LTP, the actions of any membrane permeable retrograde message must be restricted to the recently active presynaptic cells.

The paradigm of LTP can be divided into three parts:

- The initial sequence of events that triggers or sets into motion the modification process, termed "induction."
- The set of mechanisms that constitute the cause of the synaptic enhancement, called "expression."
- The factors that govern the duration of the enhancement, referred to as "maintenance."

A complete understanding of LTP requires investigation of each of these aspects and the way in which they interact.

1. Induction of LTP: As described above, the involvement of several amino-acid receptor subtypes in the induction of LTP has been documented. The NMDAR is an essential trigger for this process, even though the application of NMDA alone only induces a short lasting potentiation (<10 min), called STP (Collingridge et al. 1983, Kauer et al. 1988). AMPA/Kinate receptors, and recently mGluRs (Reymann and Matthies 1989, Izumi et al. 1992, Bashir et al. 1993; for contrary evidence see Manzoni et al. 1995), have also been shown to play a key role. There are also indications that activation of several other receptors can play a facilitory role (Frey et al. 1993; Cahil et al. 1994).

2. Expression of LTP: If LTP is in fact maintained by the continued activity of a protein kinase (as opposed to the presence of a long-lived phosphorylated substrate), then

blocking the catalytic activities of the kinase should suppress the manifestation, as opposed to the underlying cause, of LTP (Schwartz and Greenberg 1987, Lynch et al. 1988). The catalytic activity of two enzymes that have been suggested to participate in LTP-CaMKII and PKC-can be reversibly blocked by H-7. When added after a tetanic stimulation, H-7 reversibly suppresses the expression of LTP (Madison et al. 1988), demonstrating that expression and maintenance are two distinct phases of LTP.

3. Maintenance of LTP: One notion is that the maintenance of LTP depends on conversion of a protein kinase to an activator-independent form. Supporting evidence comes from studies of the effects on LTP of sphingosine (SPH), which blocks PKC and CaMKII activation by preventing ligand binding. When present prior to tetanic stimulation, SPH prevents LTP; however, SPH does not suppress LTP when added after tetanic stimulation. One interpretation is that LTP is maintained by an activator-independent protein kinase (Hannun et al. 1986, Madison et al. 1988a,b).

Several lines of evidence point to a critical role for protein kinases in LTP. One report has demonstrated an NMDA-dependent increase in PKC activity through an LTP-inducing stimulus, and another study has shown that direct injection of PKC into postsynaptic CA1 neurons can produce LTP (Hu et al. 1987, Klann et al. 1991). A recent study has shown that the direct injection of a combination of PKC inhibitors into the postsynaptic neuron blocks the maintenance phase of LTP (Wang and Feng 1992). Conversely, synaptic transmission in the CA1 region is enhanced by application of phorbol esters, although, other studies suggest

that the potentiation induced by phorbol esters occurs via mechanisms distinct from those underlying LTP (Muller et al. 1988, Gustafsson and Wigstorm 1988, Madison et al. 1991). Consistent with this idea, activation of other neurotransmitter receptors that can activate PKC, such as metabotropic glutamate or muscarinic acetylcholine receptors, has been reported to lower the threshold for induction of LTP, possibly by relieving the Mg^{2+} block of NMDA receptors (Ben-Ari et al. 1992). More recently, a mutant mouse strain that does not express α-CaMKII was developed, and these animals were shown to be deficient in both LTP and spatial learning (Silva et al. 1992 a, b). Conversely, hippocampal neurons expressing constitutively active forms of CaMKII show occlusion of LTP (Pettit et al. 1994) Additionally, one group has reported that synaptic strength in CA1 area can be potentiated by activation of PKA (Chavez-Noriega and Stevens 1992). In this regard, a recent study has demonstrated increased levels of cAMP in the CA1 region after tetanic stimulation of the Schaffer collateral pathway; this increase in cAMP may occur through activation of calmodulin-sensitive adenylyl cyclase, since it is dependent on both NMDA receptor activation and increases in intracellular Ca^{2+} (Chetkovich et al. 1991). Finally, there is evidence to suggest that induction of LTP can be blocked by inhibitors of both tyrosine kinase and cGMP-dependent protein kinase (PKG) (O'Dell et al. 1991, Zhuo et al. 1994). Together with the work showing modulation of neuronal and recombinant glutamate receptors by phosphorylation in culture, these studies strongly suggest that phosphorylation of ionotropic glutamate receptor subunits plays a role in the changes that occur at synapses exhibiting LTP (Blackstone et al 1994, Wyllie and Nicoll 1994, Tan et al. 1994, Wang et al. 1994).

In summary, the importance of protein kinases (PK) in signal transduction has long been recognized. A great deal about kinases is now understood, and there is a substantial literature documenting numerous interactions among protein kinases and other members of the signal transduction pathway. Since many PK's seem to converge on sAHP and LTP, interactions between these kinases could be studied via monitoring of these physiological phenomenon. The purpose of this research is to investigate mechanistically certain issues regarding the activation and regulation of PK's. In the first two chapters, assaying sAHP and LTP, we examined modes and effects of PKA activation and in the third chapter we explored the interaction of PKA with PKC pathway[1]:

- Chapter One: PKA and mechanisms of cAMP generation.
- Chapter Two: The gating role of PKA in Long-Term Potentiation (LTP).
- Chapter Three: Inhibition of the cAMP-dependent pathway by mGluR's via PKC.

[1]Many experiments of Chapter One, the majority of Chapter Two and all the experiments of Chapter Three were done by Rabin Nouranifar. The remaining experiments were performed by our technician Mr. Tony Wong in our laboratory.

Chapter 1: **Mechanisms of Adenylate Cyclase activation in CA1 hippocampal cells.**

INTRODUCTION

Activation of PKA is triggered by intracellular cyclic adenosine monophosphate (cAMP), whose formation is catalyzed by integral membrane glycoproteins: adenylyl cyclases (AC). cAMP regulates a variety of biochemical and physiological systems in animal cells. The processes regulated include carbohydrate, lipid, protein and nucleic acid metabolism, as well as synaptic transmission, ion channel function and transcription in neurons. Adenylyl cyclases themselves are regulated by stimulatory and inhibitory receptors which couple to the guanine nucleotide regulatory proteins, G_s, G_o/G_i, and Protein Kinase C (PKC). In addition, modulation of adenylyl cyclase activity by Ca^{2+} and $\beta\gamma$ subunits of G-proteins has been demonstrated in several tissues and it has been proposed that calcium-sensitive adenylyl cyclases may provide coupling between the Ca^{2+} and cAMP signal transduction systems.

Background

Characterization of the adenylyl cyclases was significantly advanced by the isolation of a cDNA clone for Type I adenylyl cyclase from a bovine brain cDNA library (Krupinsky et al. 1989) and cDNA clones for seven additional adenylyl cyclases designated types II-VIII (Yoshimura and Cooper 1989, Bakalyar and Reed 1990, Gao and Gilman 1991, Ishikawa et

al. 1992, Katsushika et al. 1992, Premont et al. 1992). All ACs show considerable sequence homology, and, on the basis of hydropathy plots, can be fitted to a common membrane topology similar to that of transporters and ion channels. All eight adenylyl cyclases contain hyper-variable regions that distinguish each enzyme. The catalytic subunits of these enzymes have a predicted molecular mass of approximately 110-180 kDa (Pfeuffer et al. 1991) and are indicated to contain 12 transmembrane spans in two domains of six spans each and two large cytoplasmic domains. These transmembrane domains possess sequence motifs encoding putative N-linked glycosylation sites. Both the NH_2 and COOH termini are theorized to be cytoplasmic. Two large cytoplasmic domains, a 350-amino acid loop between the first and second set of transmembrane domains and a 250- to 300-amino acid tail after the second membrane domain, are likely to contain the catalytic core of the enzyme based on sequence similarity with cloned guanylyl cyclases. These two large intracellular domains share similarity with each other, and both are highly conserved among the known mammalian adenylyl cyclase sequences. The predicted topography of the mammalian adenylyl cyclases is shown in **Figure 1**. All eight enzymes are stimulated by forskolin and 5-guanylylimido-diphosphate (GppNHp) but differ in their sensitivity to calmodulin and $\beta\gamma$ complex of G proteins. The hatched regions have 60-80% similarity, whereas the solid areas have more than 80% homology over eight amino acid stretches. Although the topographical homology between the mammalian adenylyl cyclases and various ion channels lead to the proposal that the enzyme may have channel activity, there is no evidence for this so far.

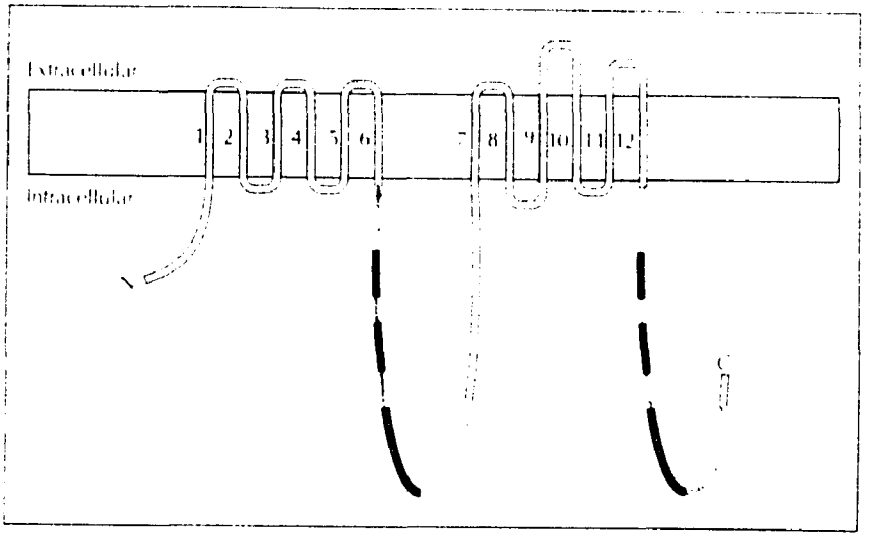

Figure 1 Topographical representation of the mammalian adenylyl cyclases. Modified from Iyengar 1993.

At least five distinct families of mammalian adenylyl cyclases can be classified on the basis of sequence homology. Two of these families have multiple members: the Type 2 family has three members, whereas the Type 5 family has two members (**Figure 2**). Within the brain, *in situ* hybridization analysis for Types 1, 2, 3, 5 and 8 mRNA enzymes has shown distinct regional patterns suggesting that these enzymes do not play a general regulatory role (e.g. regulation of cell metabolism); rather, they may be important for specific neuronal functions. Table 1 shows the distribution pattern of the enzyme in hippocampal region.

Regulation of Adenylate Cyclases

Figure 3 summarizes the knowledge to date of adenylate cyclase regulation. Until recently, the only documented calmodulin-sensitive mammalian adenylyl cyclase was the Type 1 enzyme. However, it is now known that the Type 3 and 8 adenylyl cyclases are also stimulated by Ca^{2+}-calmodulin (Choi et al. 1992, Cooper et al. 1995). In contrast to the Type 1 enzyme, type 3 adenylyl cyclase is not stimulated by calmodulin and Ca^{2+} in the absence of other effectors. However, calmodulin stimulates the type 3 enzyme activity in the presence of forskolin or GPPNHP. Furthermore, at least four β and six γ subunits have so far been identified (Helper and Gilman 1992, Birnbaumer 1992). This molecular multiplicity of the $\beta\gamma$ subunits suggests that additional levels of specificity in regulating the different types of adenylyl cyclases may be achievable. The different combinations may differentially regulate the various adenylyl cyclases, although experiments with recombinant $\beta\gamma$ subunits suggest that this may be unlikely (Iniguez-Liuji et al. 1992).

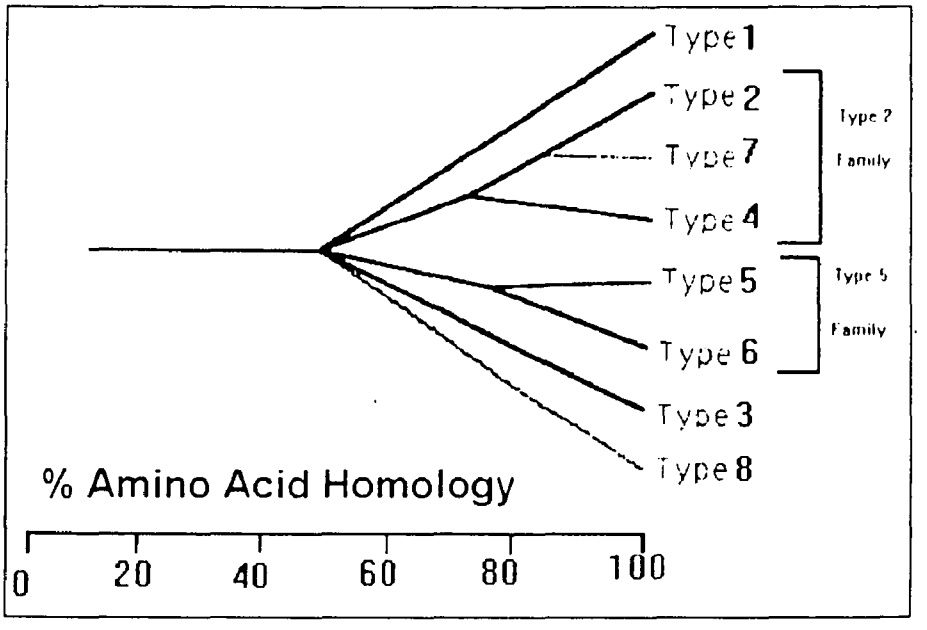

Figure 2 Classification of mammalian adenylyl cyclases on the basis of sequence homology. Modified from Iyengar 1993.

Adenylate Cyclase Type	mRNA Distribution Pattern
Type 1	Dentate gyrus, CA2, anterior extensions of hippocampus. (Xia et al. 1991, Glatt and Snyder 1993)
Type 2	In CA1-CA4 regions, and the dentate gyrus. (Mons 1993)
Type 3	Cerebellum, hippocampus and olfactory bulb. (Glatt and Snyder 1993)
Type 5	In Striatum only. (Glatt and Snyder 1993)
Type 4, 6 & 7	Widely distributed but very scarce in brain. (Pieroni et al. 1993)
Type 8	Dentate gyrus and the pyramidal cells of the hippocampus. (Matsuoka 1992)

Table 1. Localization of Adenylyl Cyclase isoforms in hippocampal and other regions of the brain by *in situ* hybridization studies.

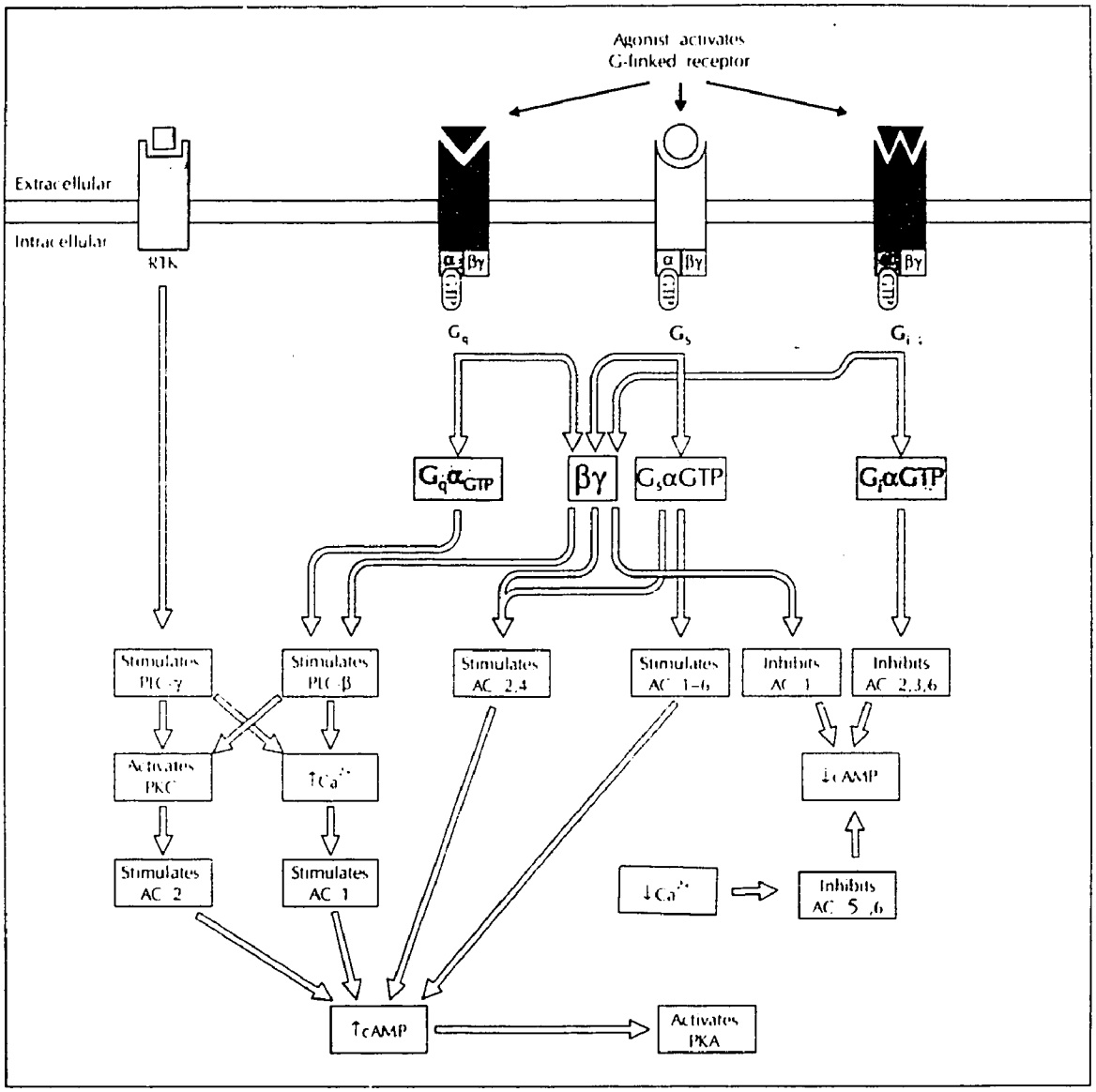

Figure 3 Summarizes the knowledge-to-date of Adenylate Cyclases' regulation by other constituents of signal transduction pathway. Modified from Pieroni et al. 1993.

Here we investigated two questions regarding modes and mechanisms of AC activation in CA1 hippocampal cells:

- Whether AC mediates cholinergic inhibition of I_{AHP}.
- Whether AC is activated by LTP-producing tetani.

METHODS:

Sprague-Dawley rats (125-200 g) were deeply anesthetized with halothane and decapitated. The brain was rapidly removed and placed in ice-cold ACSF containing (in mM) NaCl (118), KCl (3.5), $MgSO_4$ (1.3), $CaCl_2$ (3.5), NaH_2PO_4 (1.25), $NaHCO_3$ (24), and glucose (15), bubbled with 95% O_2/5% CO_2. The hippocampus was then rapidly dissected out and transverse slices of 400 µm thickness were made on a tissue chopper. The slices were maintained in an interface chamber (ACSF and humidified 95% O_2/5% CO_2 atmosphere) at room temperature for at least 1 h before removal for recording. Recording was done in submersion chambers at 29 - 31° C, with the slices immobilized between nylon meshes. In most experiments, the slices were constantly superfused with ACSF gravity fed from reservoirs. For synaptic experiments, a cut was made in each slice between areas CA3 and CA1, and picrotoxin (50 µM; Sigma) was added to the ACSF. A bipolar concentric stainless steel electrode was placed in stratum radiatum of the CA1 region, and the Schaffer collaterals

were stimulated with monophasic pulses 100 μs in width. A series of test pulses (4 pulses at 0.2 Hz) was given every 5 or 10 min, and the excitatory postsynaptic potentials (epsp's) within each series were averaged. Epsp amplitude and maximum initial slope (defined as the greatest slope within any 1 ms interval between the stimulus artifact and the epsp peak) were measured offline for the averaged waveforms. LTP was induced by 3 trains of 100 pulses delivered at 100 Hz, separated by 10 min, a protocol that has been shown to induce cAMP-dependent LTP in the CA1 region (Frey et al., 1993; Matthies and Reymann, 1993). The stimulus intensity during HFS was adjusted to produce a 1 mV field epsp. Whole-cell voltage-clamp recordings were made with the "blind" method (Blanton et al., 1989) with patch pipets having resistances of 2.5 - 4 MΩ. The pipets were filled with solution containing (in mM) K-gluconate (128), HEPES (40), $MgCl_2$ (2), K_2ATP (4), Na_3GTP (0.3), EGTA (0.6), creatine phosphate (20), and creatine phosphokinase (50 U/ml); adjusted to pH = 7.4 with KOH. In these situations, the AHP was evoked, in presence of tetrodotoxin (1uM; Sigma), by clamping the cells at -40 mV and applying a brief depolarizing pulse (100 mV / 200 ms). For intracellular recording with sharp electrodes, cells in CA1 stratum pyramidale were impaled with electrodes containing 3 M KCl (R_e = 60-90 MΩ). The AHP was evoked by a 100 ms, 1 nA depolarizing pulse from a membrane potential of -60 mV. In IBMX, which was generally present for sharp electrode experiments on HFS-induced AHP suppression, the afterhyperpolarization often decreased and synaptic noise increased. Two methods were used to measure the AHP: the amplitude 1 s after the depolarizing pulse was measured directly from the traces, or a single exponential decay was fit to the AHP beginning at 300 ms after the pulse and the extrapolated amplitude of the AHP at t=0 was determined.

In a subset of 14 cells, the effect of HFS was determined using both methods, and the percentage of AHP suppression agreed within 1.4 ± 2.2%. Intracellular epsp's were obtained from a membrane potential of -80 mV. The LTP producing protocol used in HFS, generated an intracellular epsp of 20 - 30 mV. For intracellular injections, cAMP (Sigma), Rp-cAMPS, Rp-8-Br-cGMPS, and $CBP_{281-309}$ (Bachem) were dissolved in the normal electrode solutions. All substances were allowed to diffuse from the recording electrode. Norepinephrine, carbachol, serotonin, IBMX, and APV (all from Sigma) were dissolved in ACSF and applied in the superfusate. RO 20-1724 (Research Biochemicals) was prepared as a stock solution in dimethyl sulfoxide, yielding a final bath concentration of 0.1% dimethyl sulfoxide. Whole-cell recordings were made with the "blind" method (Blanton et al., 1989) with patch pipets having resistances of 2.5 - 4 MΩ. The pipets were filled with solution containing (in mM) K-gluconate (128), HEPES (40), $MgCl_2$ (2), K_2ATP (4), Na_3GTP (0.3), EGTA (0.6), creatine phosphate (20), and creatine phosphokinase (50 U/ml); adjusted to pH = 7.4 with KOH. The afterhyperpolarization was evoked and measured as described for intracellular recording. During whole cell voltage clamp recordings, cells were clamped at -40mV and briefly depolarized by a pulse (100 mV, 200 mS), to generate a slow outward current with a time course typical of the calcium-activated afterhyperpolarization current, I_{AHP} observed in impaled hippocampal pyramidal cells (Lancaster and Adams 1986, Storm 1989). In these experiments, pipets were backfilled with the standard solution containing Rp-cAMPS or the regulatory subunit of PKA (Promega). All data were analyzed using two-tailed t-tests. Membrane potential was amplified using the Axoclamp 2A amplifier (Axon instruments), which was also used to voltage-clamp the cells when necessary. When patch electrodes were

used, the clamping was done in the continuous mode whereas with sharp electrodes the switch mode was applied. Amplitudes of synaptic potentials and AHP as well as the maximum slope of the epsp were measured using Axon's software (PCLAMP). Date were digitized and stored on floppy disks, using an IBM AT PC and Axoclamp interface and software (PCLAMP). Data were displayed on the screen of the computer display, a Tektronix oscilloscope and a Nicolet storage scope. In addition, chart recordings of the resting potential were made using a Gould brush recorder. Summary data are presented as group means with standard error bars. In all traces, the response to the depolarizing current pulse is truncated.

RESULTS

The Cholinergic Inhibition of Afterhyperpolarization in Rat Hippocampus is PKA Independent

Many neurotransmitters have a pronounced excitatory effect due to the inhibition of several K^+ currents. One of these currents, I_{AHP}, underlies the Ca^{2+} -dependent slow afterhyperpolarization which normally limits the response of a pyramidal cell to prolonged or repetitive depolarizing stimuli. Multiple second messenger pathways converge on I_{AHP}. The best documented is cyclic AMP (cAMP), which blocks I_{AHP} by activating cAMP-dependent protein kinase (PKA). This is the pathway through which norepinephrine, acting on β

receptors, inhibits I_{AHP}. Some transmitters, such as acetylcholine acting on muscarinic receptors and glutamate acting on metabotropic receptors, which are not known to directly activate adenylyl cyclase, also block I_{AHP}.

In many systems, muscarinic agonists stimulate AC indirectly, either by increasing intracellular Ca^{2+} concentration and acting on calcium-calmodulin dependent adenylyl cyclase (e.g. AC type I) or by activating protein kinase C to phosphorylate and activate other types of adenylyl cyclase (e.g. AC type II). It is thus possible that muscarinic activation in hippocampus may lead to cyclase activation via one of these two mechanisms. One strategy for blocking the AC pathway is to interfere with the function of the regulatory subunit of PKA by blocking the cAMP binding sites with Rp-cAMPS, thereby preventing the dissociation of the active catalytic subunit.

To test the hypothesis that muscarinic inhibition of I_{AHP} is mediated through indirect activation of AC, we have injected Rp-cAMPS into hippocampal pyramidal neurons, and observed whether this treatment inhibit the effect of muscarinic activation on the I_{AHP}. As a control, we examined the response to norepinephrine in the same cells. These results were published in a paper by our group (Blitzer et al. 1994). Similar experiments have been described in a paper by Pedarzani and Storm (1993), which appeared while our paper was in review.

In cells recorded with whole cell patch mode, addition of norepinephrine to the preparation reduced the amplitude of the I_{AHP}. When pipets contained Rp-cAMPS, the NE effect was significantly diminished at both the 20 min and 40 min after breakthrough (**Figure 4**, left panels). Although carbachol similarly applied inhibited I_{AHP}, intracellular application of Rp-cAMPS had no effect on the response to CCh at either the earliest or the latest times sampled (**Figure 4**, right panels).

The data represented in **Figure 4** clearly show that indirect activation of adenylyl cyclase does not contribute to the muscarinic inhibition of I_{AHP} in the hippocampal pyramidal neuron. These findings agree with those of Winder and Conn (1992), who found no generation of cAMP in hippocampus exposed to CCh. Recently it has been shown that inhibitors of CaMKII interfere with the muscarinic effect (Muller et al. 1992). That finding, together with those reported here and by Pedarzani and Storm (1993), indicate that noradrenergic and muscarinic inhibition of I_{AHP} proceed via clearly distinct transduction pathways.

High-Frequency Synaptic Stimulation Activates the Postsynaptic cAMP Pathway

The cAMP signal transduction pathway is important for learning and memory in a wide variety of species (Bourtchuladze et al., 1994; Frank and Greenberg, 1994; Huang et al., 1994). Mutational inactivation of calmodulin-stimulated adenylyl cyclase interferes with early memory in Drosophila (Levin et al., 1992), and genetic knockout of CREB in

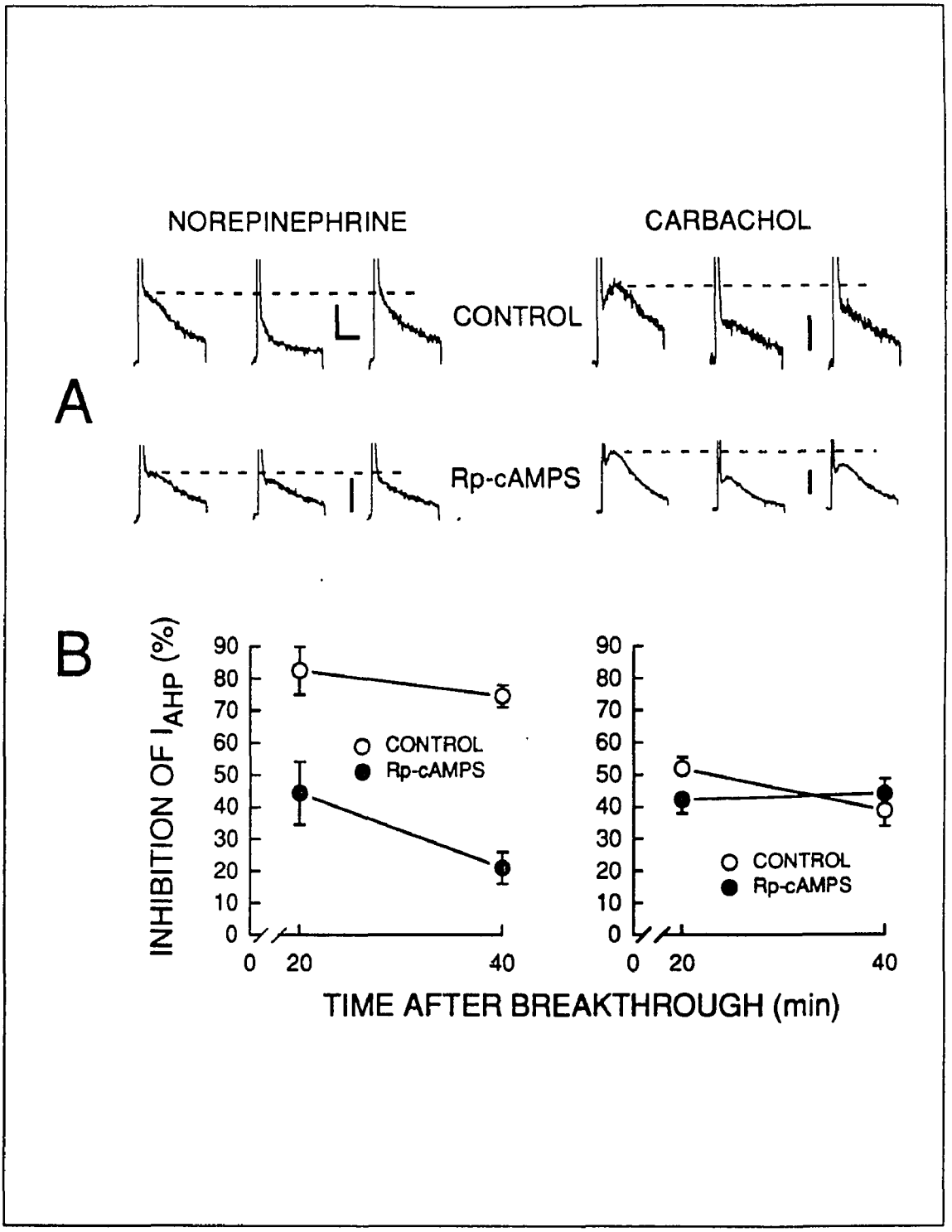

Figure 4 Muscarinic inhibition of I_{AHP} is not cAMP mediated. Norepinephrine (NE; 200nM, 2min) reduced the amplitude of the I_{AHP} by 82 ± 8% (n=5). When pipets contained Rp-cAMPS (1mM, n=6), the NE effect was diminished to 44 ± 10% inhibition of I_{AHP} at the earliest time sampled (20min after breakthrough), and to 21 ± 5 % inhibition after 40min. Carbachol (CCh; 200nM, 2min) inhibited I_{AHP} by 52 ± 4% (n=6). In contrast to the NE results, intracellular application of Rp-cAMPS (n=5) had no effect on the response to CCh at either 20 or 40min after breakthrough. Calibration: 50 pA/2 Sec for upper traces, 100 pA/2 Sec for lower traces.

Drosophila has been shown to affect late memory (Yin et al., 1994). These observations suggest that the cAMP pathway may play a role in multiple stages of learning and memory. In mammalian hippocampus, a role for the cAMP pathway in LTP at the Schaffer collateral - CA1 synapse has been demonstrated. When widely-spaced trains of high-frequency stimulation (HFS) were used, LTP was found to be sensitive to blockers of PKA (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994). These investigators focused on a late phase of LTP, although a PKA-dependent early phase of LTP ($\leq$ 1 h after HFS) has also been observed (Frey et al., 1993; Huang and Kandel, 1994).

The mechanism of the cAMP dependence in early LTP is not known, but it is likely that different mechanisms underlie the PKA dependence of the early and late phases of LTP. The late phase is thought to rely upon protein synthesis, and it has been suggested that PKA contributes to late LTP by regulating protein expression (Nguyen et al., 1994). However, early LTP does not require protein synthesis (Bliss and Collingridge, 1993). To further elucidate the role of the cAMP pathway in LTP, we have investigated whether this pathway is activated shortly after the induction of LTP.

In whole cell recordings, a brief depolarizing current pulse produced an AHP which decayed with a time constant in the range of 1.6 to 3.1 s (**Figure 5**A, left panel). Since our previous work showed that the slow AHP recorded in the whole cell configuration is blocked by activation of the cAMP pathway, changes in AHP should be valuable as an assay for

elevations in postsynaptic cAMP. We therefore attempted to detect HFS-induced increases in postsynaptic cAMP by monitoring the AHP.

In the whole-cell configuration, the delivery of a single HFS train to the Schaffer collaterals resulted in AHP suppression, which was maximal 1-2 min after HFS (**Figure 5**A, middle panel; **Figure 5**D) and recovered within 15 min (**Figure 5**A, right panel). AHP suppression could be repeatedly evoked by subsequent HFS for at least 1.5 h after impalement, and was not associated with any consistent changes in resting membrane resistance, resting potential, or the depolarization produced by the current pulse.

Activation of PKA involves dissociation of the catalytic and regulatory subunits (Krebs and Beavo, 1979). Hence, excess regulatory subunits should inhibit the enzyme. To test whether the HFS-induced suppression of AHP is mediated by postsynaptic PKA, cells were recorded with pipets containing regulatory subunit of PKA. In these cells, HFS-induced suppression of the AHP was blocked (**Figure 5**B,D). The involvement of the cAMP pathway was confirmed with Rp-cAMPS, an inhibitor of PKA (Van Haastert et al., 1984), which also blocked HFS-induced suppression of the AHP (**Figure 5**C,D). The ability of the regulatory subunit of PKA and Rp-cAMPS to prevent AHP suppression indicates that HFS activates the postsynaptic cAMP pathway. A restriction of whole cell recording is the limited recording time during which LTP can be induced (Malinow and Tsien, 1990), which prohibits experiments involving pharmacological manipulations prior to LTP induction. This problem can be avoided by the use of sharp electrodes for intracellular recording. Since the delivery

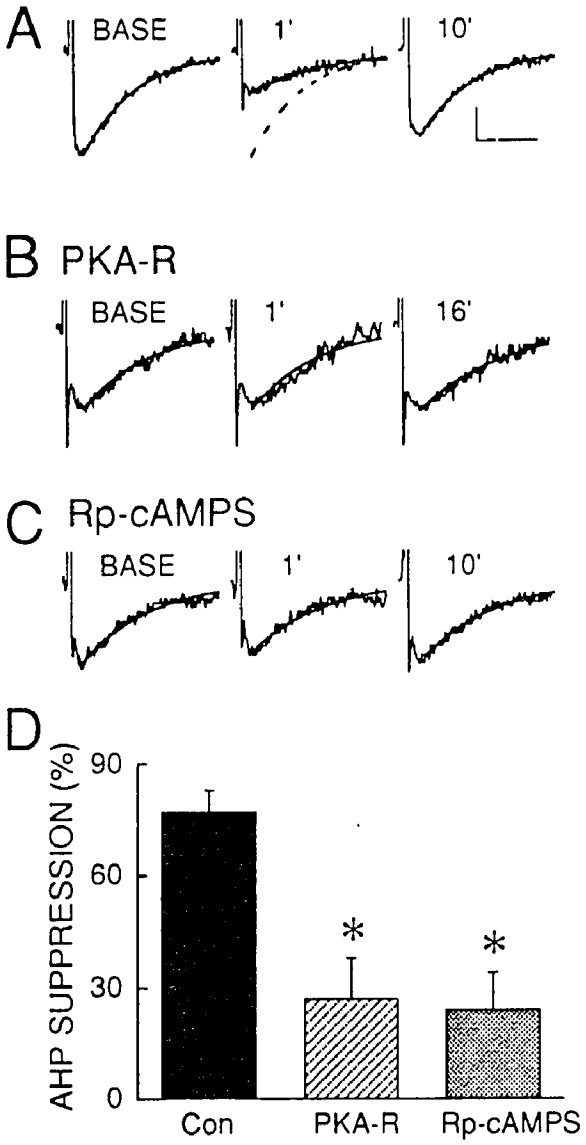

Figure 5 HFS suppresses the AHP via the postsynaptic cAMP pathway. Data were obtained using whole-cell recording method. Time elapsed since HFS is shown, and the smooth lines superimposed on the traces indicate the best single exponential fit to the decay of the AHP. (A) The AHP was transiently suppressed 1 min after a single train of HFS, and recovered by 10 min after HFS. The dashed line represents the fit to the baseline trace. (B) The regulatory subunit of PKA ("PKA-R"; 3 μM in the recording pipet for 65 min), prevented HFS-induced suppression of the AHP. © Postsynaptic application of Rp-cAMPS (200 μM in the pipet for 40 min) also blocked AHP suppression. (D) Summary of whole-cell experiments on HFS-induced AHP suppression. The mean inhibition of the AHP for each treatment is shown. An asterisk indicates a significant difference ($p < .05$) from the control group (n=6). AHP suppression was reduced by 3 μM PKA regulatory subunit (n=5, HFS delivered for 45-77 min after breakthrough) or by 200 μM Rp-cAMPS (n=4, HFS delivered for 40 min after breakthrough). Calibration: 2 mV/2 s.

of substances from sharp electrodes is reduced relative to pipets used in whole-cell recordings, higher concentrations of pharmacological agents are routinely included in sharp electrodes. We determined the electrode concentration of Rp-cAMPS which selectively inhibits the cAMP pathway by testing the effect of Rp-cAMPS on well-characterized cAMP-dependent and cAMP-independent neurotransmitter responses. In control cells, the cAMP-dependent inhibition of AHP by norepinephrine (Madison and Nicoll, 1986) could be reliably repeated for over 2 h. The effect of norepinephrine (NE) was significantly reduced 40 min after impalement with electrodes containing 10 mM Rp-cAMPS, but not 1 mM Rp-cAMPS (**Figure 6**A_1,A_2, D, Table 2). Thus, 10 mM of Rp-cAMPS in the electrode applied for 40 min was adequate but not excessive to inhibit the effect of NE on the AHP. Muscarinic agonists also inhibit the AHP, but in a cAMP-independent manner (Pedarzani and Storm, 1993; Blitzer et al., 1994). Rp-cAMPS did not affect the response to the muscarinic agonist carbachol (**Figure 6**B, D, Table 2). We studied an additional cAMP-independent response, the membrane hyperpolarization produced by serotonin, which is mediated by pertussis toxin-sensitive G-proteins (Andrade et al., 1986). The response to serotonin was also intact in cells impaled with electrodes containing Rp-cAMPS (**Figure 6**D, Table 2). Thus, 10 mM Rp-cAMPS in sharp electrodes was used to specifically probe the role of the cAMP pathway in AHP suppression following synaptic stimulation.

To obtain AHP suppression with sharp electrodes, a more robust stimulation was needed. In the absence of a phosphodiesterase inhibitor, a single train of HFS or three widely spaced trains (10 min apart) did not suppress the AHP (**Figure 7**A, middle panel; **Figure 11**).

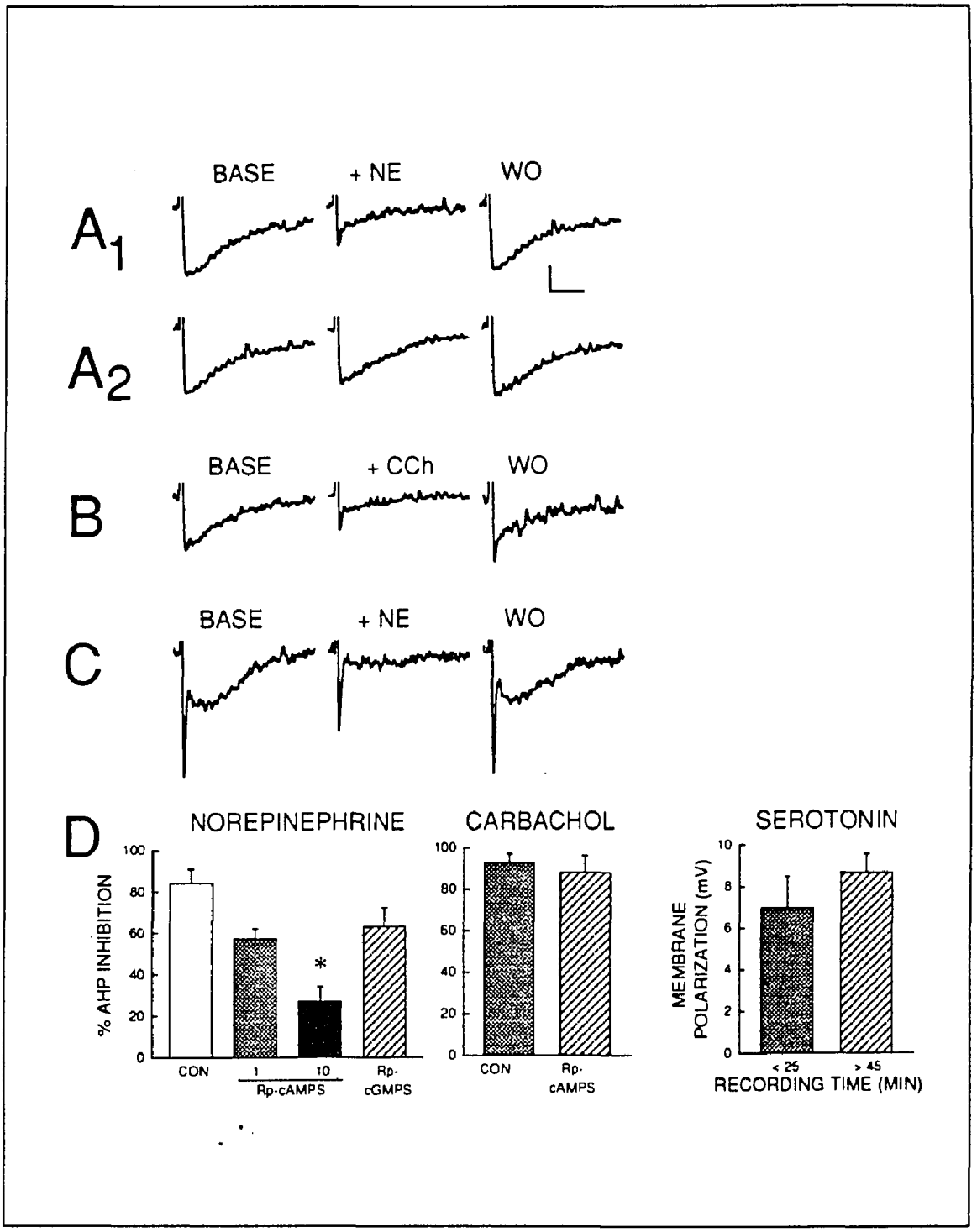

Figure 6 Specificity of Rp-cAMPS Applied in Sharp Electrodes. The times denote duration of recording at the time of agonist application. All agonists were applied in the bath for 2 min. (A_1) Norepinephrine control data were obtained <10 min after impalement with electrodes containing 10 mM Rp-cAMPS. (A_2) In the same cell 40 min after impalement, NE is no longer effective. (B) Carbachol's effect is intact 40 min past impalement with electrodes containing 10mM Rp-cAMPS. Similarly, NE's effect is not diminished in the presence of 10 mM Rp-8-Br-cGMPS (C). (D) Experiments summarizing the specificity of agonists and antagonists with regard to AHP suppression is shown. Calibration: 2 mV/2 s.

Drug	Concentration (mM)	Time (Min)	% Response to Agonist	Number of Observations
Inhibition of AHP by 1 μM **Norepinephrine**				
Control [§]	-	< 10	84 ± 7%	5
Rp-cAMPS	1	40	57 ± 5%	3
Rp-cAMPS	10	40	* 27 ± 7%	5
Rp-8-Br-cGMPS	10	40	63 ± 9%	7
$CBP_{281-309}$	0.200	40	83 ± 14%	3
Inhibition of AHP by 10 μM **Carbachol**				
Control	-	40	92 ± 8%	3
Rp-cAMPS	10	> 42	88 ± 8%	3
Membrane Hyperpolarization by 10 μM **Serotonin**				
Rp-cAMPS	10	< 25	7.0 ± 1.5 mV	3
Rp-cAMPS	10	> 45	8.7 ± 0.2 mV	3

Table 2. Specificity of Rp-cAMPS Applied in Sharp Electrodes. The times denote duration of recording at the time of agonist application. All agonists were applied in the bath for 2 min. (§) Norepinephrine control data were obtained <10 min after impalement with electrodes containing 10 mM Rp-cAMPS. (*) indicates significant difference ($p<.05$) from baseline, based on Newman-Keuls analysis.

However, AHP suppression was reliably produced by the series of three widely spaced trains when phosphodiesterase was inhibited by 3-isobuty1-1-methylxanthine (IBMX) (**Figure 7**B_2, middle panels; **Figure 11**). To rule out the possibility that the effect of IBMX could be due to its inhibition of adenosine receptors (Jacobson et al. 1992), we also used another inhibitor of PDE, 4-(3-butoxy-4-methylbenzyl)-2-imidazolidine (RO 20-1724) (Schultz and Daly 1973, Dismukes and Daly 1974) which also allowed us to observe a reversible post-tetanic suppression of the AHP (**Figure** 7C, middle panel; **Figure 11**). We concluded that preserving the newly formed cAMP, by inhibition of PDE, allowed us to observe the stimulation of AC and the subsequent activation of PKA by the tetanic stimulus. Therefore the requirement of a phosphodiesterase inhibitor for AHP suppression is consistent with the effect being mediated by the cAMP pathway.

The reduced sensitivity of the AHP to HFS when recorded with sharp electrodes agrees with the observation that the AHP recorded in this mode is generally less sensitive to activation of the cAMP pathway than the AHP recorded with the whole-cell technique. Thus, in sharp electrode recordings, bath application of 200 μM 8-Br-cAMP inhibited AHP by 51 ± 5% (n=7), whereas 10 μM 8-Br-cAMP exerted a similar effect in whole-cell recordings (37 ± 8% inhibition of AHP, n=5). It is probable that the washout of some diffusible cytoplasmic factor which normally opposes the effect of PKA, such as a soluble phosphatase, underlies the heightened responsivity of the AHP in whole-cell recording.

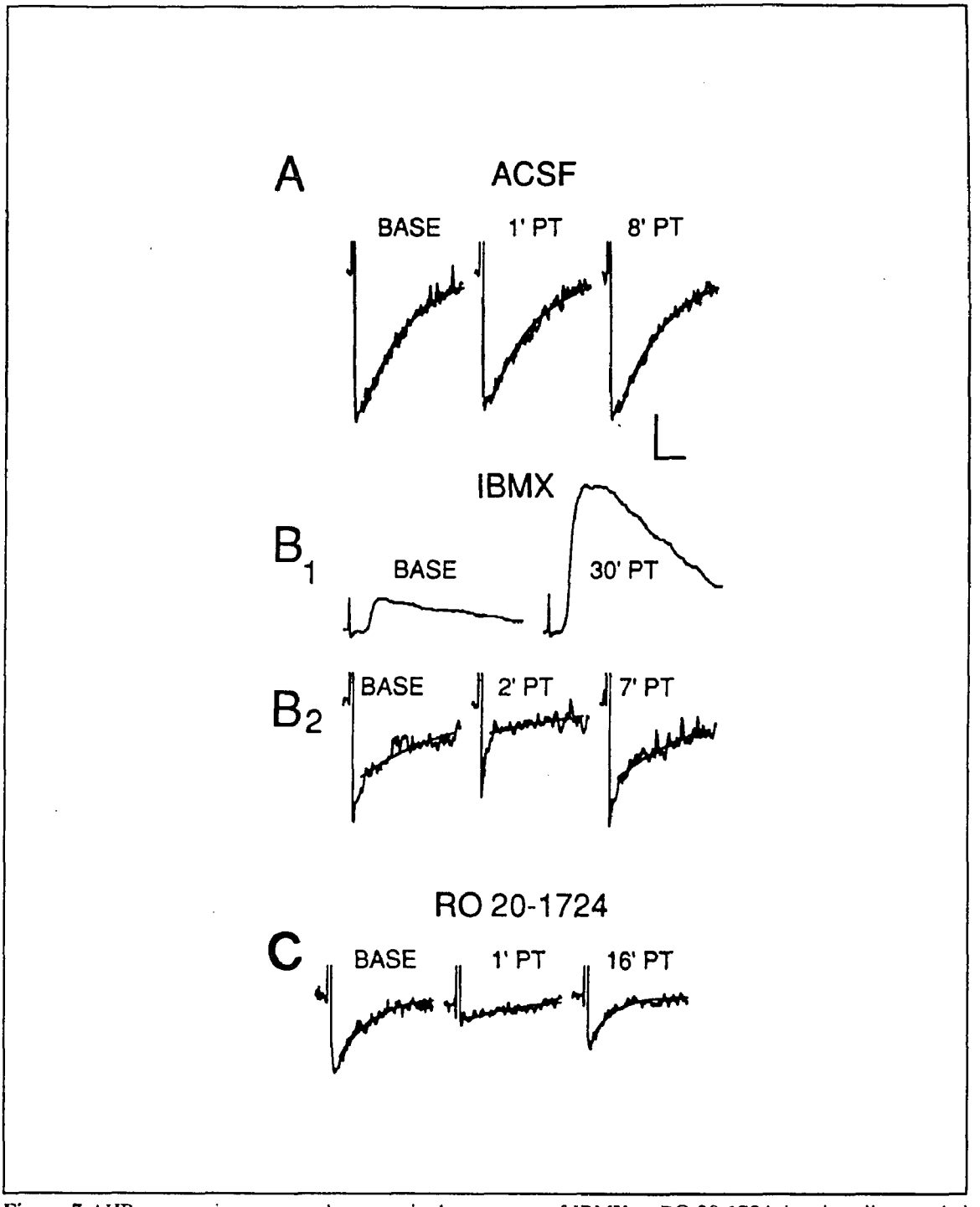

Figure 7 AHP suppression was much greater in the presence of IBMX or RO 20-1724 than in cells recorded without any phosphodiesterase inhibitor. All records were obtained using sharp electrodes. Times since the final HFS train are noted, and fitted lines are as described in methods. (A) In control cells, where a phosphodiesterase inhibitor was not present, the AHP was not inhibited by HFS. (B) AHP suppression and LTP recorded in the same cell, with 1 mM IBMX in the bath. The AHP was transiently suppressed for several minutes after HFS (B_1), and the epsp was potentiated 30 min after HFS (B_2). © AHP suppression in presence of the bath applied 4-(3-butoxy-4-methylbenzyl)-2-imidazolidine (RO 20-1724, 200 μM). Calibration: 2 mV/2 s for AHP traces and 7 mV/20 ms for epsp traces.

Both AHP suppression and LTP could be observed in the same cell (**Figure 7**B_1, B_2). Thus, the same conditions which elevated postsynaptic cAMP also induced LTP.

When 10 mM Rp-cAMPS was included in the recording electrode, HFS-induced suppression of the AHP was blocked (**Figure 8**B; **Figure 11**), in agreement with the effects of Rp-cAMPS and the regulatory subunit of PKA obtained in the whole-cell patch recordings. This indicated that AHP suppression could be used as a functional assay for postsynaptic cAMP elevation using sharp electrodes.

High-Frequency Synaptic Stimulation Activates the Postsynaptic Adenylyl Cyclase in a NMDA-dependent Manner

The mechanism by which HFS elevates postsynaptic cAMP was studied. There are multiple isoforms of adenylyl cyclase, some of which are stimulated by Ca^{2+}/Calmodulin (Iyengar, 1993; Cooper et al., 1995). Since intracellular [Ca^{2+}] increases during HFS due to Ca^{2+} influx through NMDA channels (Perkel et al., 1993), HFS may cause the activation of adenylyl cyclase via Ca^{2+}/Calmodulin. Indeed, biochemical measurements in hippocampal slices have shown that HFS of the Schaffer collaterals results in NMDA- and calmodulin-dependent generation of cAMP in the CA1 region (Chetkovich et al., 1991; Chetkovich and Sweatt, 1993; Frey et al., 1993). However, the cellular source of the cAMP has not been identified. NMDA receptors are located on interneurons in the CA1 region (Manzoni et al., 1994) and on NE-containing terminals (Pittalunga and Raiteri, 1990), and the

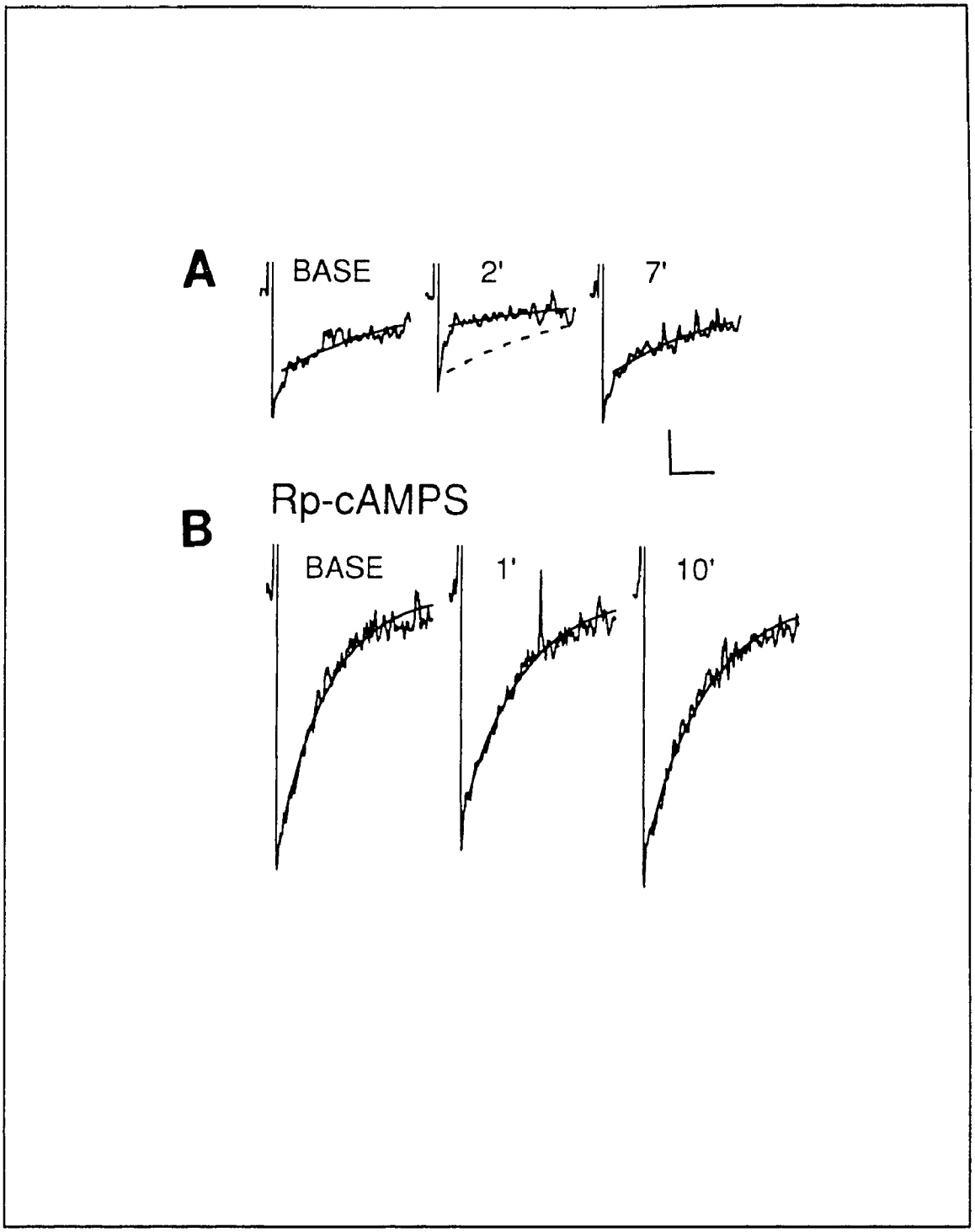

Figure 8 HFS suppresses the AHP via the postsynaptic cAMP pathway. All records were obtained using sharp electrodes. Times since the final HFS train are noted, and fitted lines are as described in Fig. 1. (A) AHP suppression with 1 mM IBMX in the bath. The AHP was transiently suppressed for several minutes after HFS. (B) AHP suppression was blocked by postsynaptic application of Rp-cAMPS (10 mM in the electrode). Rp-cAMPS increased the amplitude of the AHP compared with IBMX controls (5.5 ± 1.4 mV, n=4 vs 2.8 ± 0.3 mV, n=14; $p < .01$), indicating that IBMX inhibited the AHP by elevating postsynaptic cAMP. Calibration: 2 mV/2 s.

cAMP pathway is ubiquitous. Thus, a variety of different cell types may contribute to the cAMP generation measured biochemically in hippocampal slices.

We used HFS-induced suppression of the AHP to investigate the pathway through which postsynaptic cAMP was elevated. The NMDA receptor antagonist aminophosphonovaleric acid (APV) blocked AHP suppression (**Figure 9**, **Figure 11**), indicating that NMDA receptors are involved in the HFS-induced generation of cAMP in the postsynaptic neuron.

High-Frequency Tetanus Stimulates Adenylyl Cyclase in a Ca^{2+}/Calmodulin-Dependent Manner

To test whether the influx of Ca^{2+} through the NMDA receptor channel activated postsynaptic adenylyl cyclase through calmodulin, cells were impaled with electrodes containing a calmodulin-binding peptide ($CBP_{281-309}$), which binds to calmodulin and prevents it from interacting with its effectors (Kelly et al., 1988). In these cells, HFS failed to suppress the AHP (**Figure 10**A; **Figure 11**). The effect of $CBP_{281-309}$ in relieving AHP suppression was specific, since other experiments showed that the peptide did not block the NE-induced inhibition of the AHP (**Figure 10**B; Table 2). These experiments indicate that HFS capable of inducing LTP raises cAMP levels in postsynaptic neurons by NMDA receptor-mediated activation of calmodulin-sensitive adenylyl cyclases (i.e., AC 1 or AC 8).

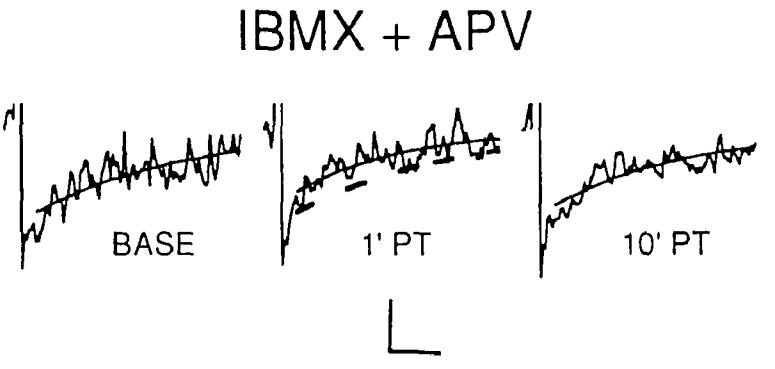

Figure 9 HFS-induced AHP suppression is mediated by NMDA receptors. The effect of HFS in the presence of IBMX was significantly reduced by bath-applied APV (100 μM; n=4). Calibration: 2 mV/2 s.

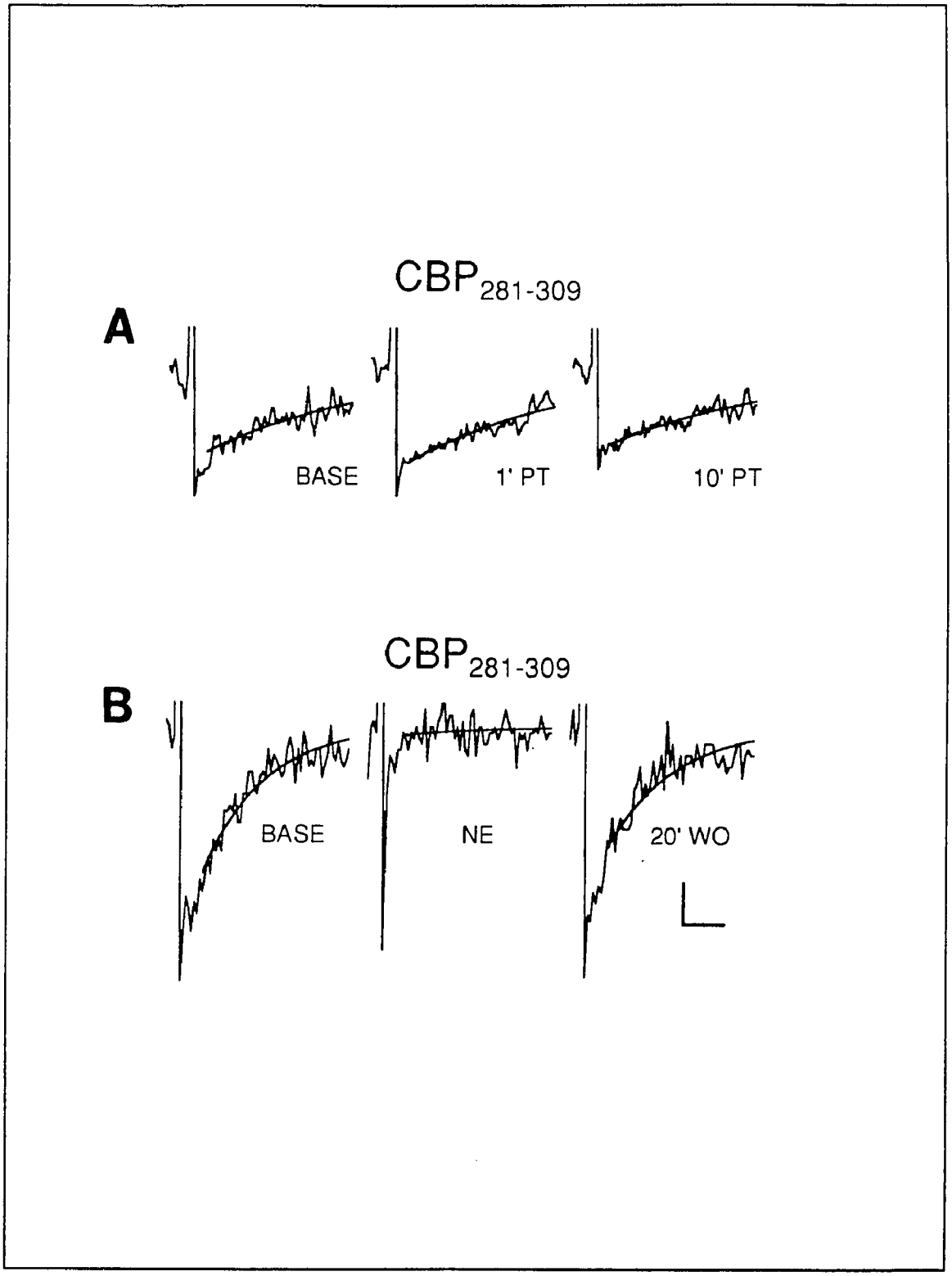

Figure 10 HFS-induced AHP suppression is mediated by calmodulin. (A) The effect of HFS in the presence of IBMX was significantly reduced by intracellular $CBP_{281-309}$ (200 μM for 40 min; n=4). (B) Specificity of $CBP_{281-309}$ applied in sharp electrodes. Norepinephrine (1 μM; n=3) was applied in the bath for 2 min. Calibration: 2 mV/2 sec.

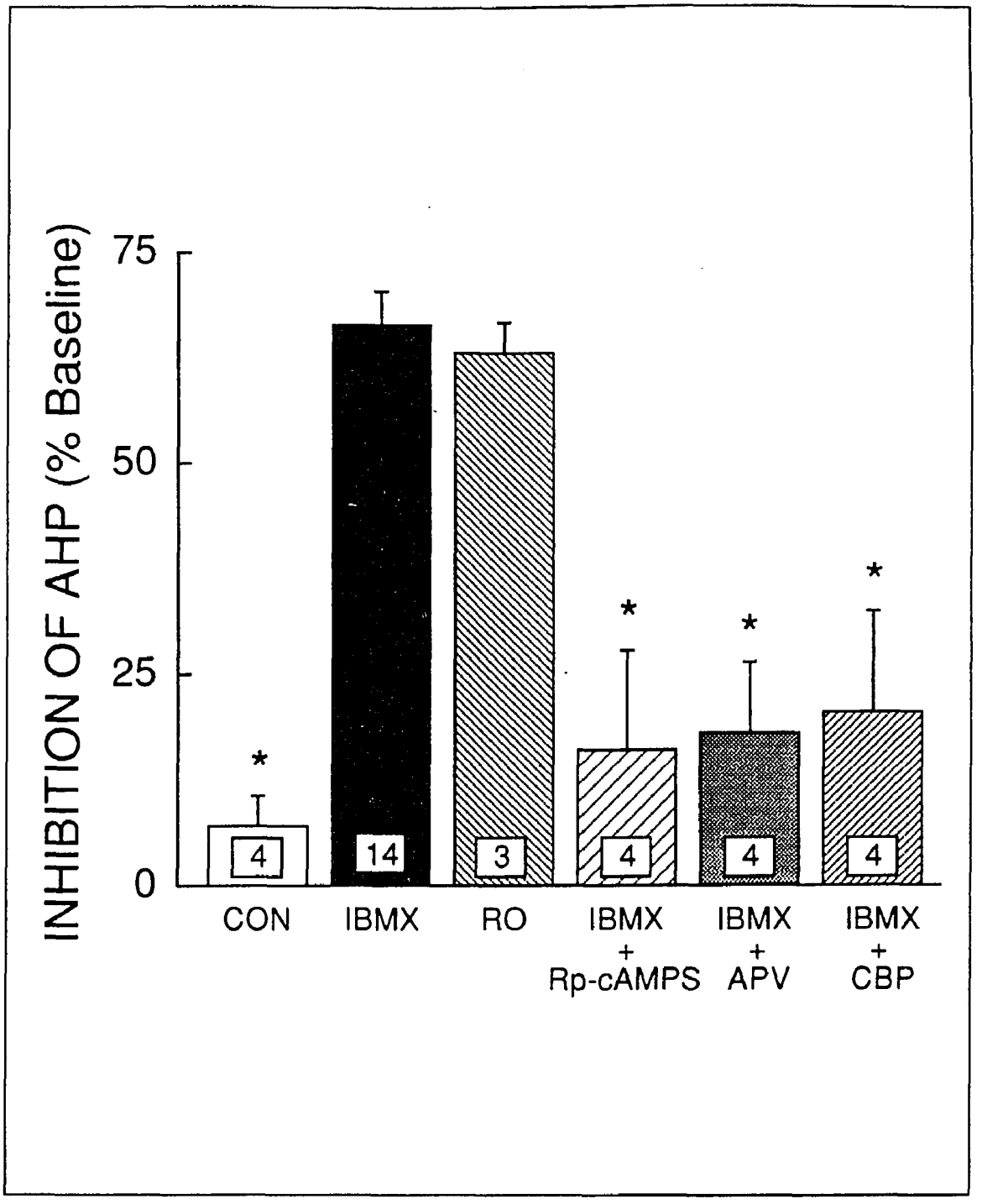

Figure 11 Summary of sharp electrode experiments on HFS-induced suppression of the AHP. The mean inhibition of the AHP for each treatment is shown. The numbers inside each bar indicate the number of observations. Note that AHP suppression was much greater in the presence of IBMX (1 mM) or 4-(3-butoxy-4-methylbenzyl)-2-imidazolidine (RO 20-1724, "RO"; 200 μM) than in cells recorded without any phosphodiesterase inhibitor ("Con"); only these two groups differed significantly from CON. The effect of HFS in the presence of IBMX was significantly reduced by intracellular Rp-cAMPS ("Rp"; 10 mM for 40 min) or $CBP_{281-309}$ ("CBP"; 200 μM for 40 min), and by bath-applied APV (100 μM). Asterisks indicate significant differences from the IBMX group, and bars indicate SEM.

DISCUSSION

The Use of AHP as a Monitoring System

A number of postsynaptic K^+ currents can contribute to membrane afterhyperpolarization following a depolarizing pulse, including I_A, I_C, I_M, I_K, I_D, and I_{AHP} (Storm 1990). Of these currents, only the AHP is sensitive to cAMP-dependent Protein kinase and decays with a tau of > 1 s. We monitored the afterhyperpolarization 1sec after the end of the depolarizing pulse, and single exponential fits of the traces beginning at 1sec time point yielded time constants in the range of 1.1 to 2.9 sec. In addition, the afterhyperpolarization was sensitive to NE. Thus, the kinetic and pharmacological characteristics of the afterhyperpolarization which we studied identify it as mediated by AHP. Although the slow afterhyperpolarization is sensitive to PKA, it can also be blocked by protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II (Baraban 1985; Muller 1992). This sensitivity of the AHP to multiple signal transduction pathways, may complicate the use of this current as an assay for cAMP generation. Nevertheless, the specificity and the inhibitory mechanisms of the agents we used to block AHP identifies the cAMP pathway as the mediator of the AHP suppression. Rp-cAMPS, a derivative of cAMP, selectively blocks the nucleotide binding site on the regulatory subunit of PKA, thus competing with endogenous cAMP for this site and preventing cAMP-induced liberation of the active catalytic subunit from the holoenzyme. The regulatory subunit of PKA acts by binding to the catalytic subunit, so that the resulting holoenzyme is inactive. Thus our work indicates that multiple pathways within the

postsynaptic neurons, including the cAMP signaling pathway may participate in production of LTP.

Activation of Adenylyl Cyclase Following a LTP-Inducing Tetanus

It has been known for some time now that CaMKII and PKC (Malenka 1989, Malinow 1989) are important for the induction of LTP. The role of cAMP pathway has been unclear. Cyclic AMP contributes to a late phase LTP (Frey 1993), an enhancement that persists many hours (3 to 10h) after tetanization and may require the synthesis of new proteins (Frey 1991). However, these experiments did not provide any indication as to whether the cAMP pathway was involved in pre or post synaptic events. Furthermore, it was not known if the cAMP pathway contributed to the earlier phase of LTP, when CaMKII and PKC are operative. Recently, it has been shown that increased transmitter release can be obtained by activation of adenylyl cyclase (Chavez-Noriega and Stevens 1993), suggesting a possible presynaptic contribution of the cAMP pathway in LTP.

In collaboration with Nicole Mons and Dermont Cooper, ultrastructural analysis of synapses in the CA1 region of hippocampus has revealed a striking concentration of adenylyl cyclase immunoreactivity in postsynaptic structures (density), with little expression in presynaptic elements (data not shown). This enrichment of adenylyl cyclase has been manifested in its role in synaptic plasticity. However, the role of the cAMP pathway in the establishment of LTP would be a minor one if the stimulus that produces LTP did not elevate

postsynaptic cAMP levels. To address this issue, we have employed an electrophysiological assay (depression of the AHP) that allows us to monitor elevation of cAMP levels in the same postsynaptic neuron in which LTP is measured. Previously it was shown by biochemical measurements that the cAMP concentration in hippocampal tissue is elevated by tetanic stimulation, an effect which is sensitive to NMDA receptor blockers and calmodulin inhibitors (Chetkovich et al. 1991, Chetkovich and Sweatt 1993, Frey et al. 1993). However, since adenylyl cyclases are found in all cell types in the hippocampus and since NMDA receptors may occur on presynaptic terminals and interneurons (Pittalunga et al. 1990, Huntley et al.), the increase in cAMP levels could be contributed by elements in the tissue other than the pyramidal neuron. The method used here can detect the stimulation of the cAMP pathway in an individual neuron that is the target of synaptic stimulation, and thus provides a clear evidence that stimuli capable of inducing LTP also elevate postsynaptic cAMP levels.

Since the glutamate released during tetanus can activate both the NMDA and metabotropic glutamate receptors, we determined if the tetanus-induced cAMP production in postsynaptic neuron was mediated by the NMDA receptor. Previous work (Chetkovich et al. 1991) suggested that this was likely, but given the plethora of metabotropic glutamate receptors that couple to different G proteins (Nakanishi 1992) it might be expected that some of the effects on adenylyl cyclases in the postsynaptic neuron would be mediated by G protein pathways. By use of the electrophysiological assay for postsynaptic cAMP levels, we find that the NMDA receptor mediates the tetanus-stimulated cAMP production by CaM-sensitive adenylyl cyclases in the postsynaptic neuron. Over the past few years, eight distinct forms of

mammalian adenylyl cyclases have been cloned (Iyengar 1993). It has been proposed that some forms of adenylyl cyclases may serve as signal recognition elements for G_s-independent pathways that raise intracellular cAMP levels (Iyengar 1993). The present observations indicate that postsynaptic NMDA receptors, by raising cellular Ca^{2+} levels, can generate cAMP in a G_s-independent fashion. This indicates that adenylyl cyclases 1, 3 or 8, which are stimulated by Ca^{2+}/CaM (Iyengar 1993, Cali et al. 1994), may be involved. Since tetanus activates the cAMP pathway through the NMDA receptor, and the activity of this pathway is essential for LTP (Frey et al. 1993), it appears that a G protein-independent cAMP pathway plays a crucial role in hippocampal function. To our knowledge this is the first demonstration that the unique signal recognition characteristics of a subset of G protein effector isoforms can play an essential role in determining the physiological response to an external stimulus.

Thus, our results show a clear correlation between the expression and function of adenylyl cyclase in hippocampus, with both the enzyme and its possible role in LTP localized to the postsynaptic neuron. The presence of high adenylyl cyclase levels in the post synaptic density is of particular interest, as this structure may play a role in synaptic plasticity (Kennedy 1993). The postsynaptic density is also enriched in both the cAMP substrate PKA and a PKA anchoring protein, and therefore possesses the enzymatic machinery to both synthesize cAMP and employ it as a second messenger (Siekevitz, 1985; Poeggel et al. 1989; Carr et al., 1992). This concentration of the cAMP signaling apparatus in the postsynaptic density is consistent with the observed involvement of this pathway in long-term potentiation. Therefore, our studies in conjunction with previous work indicate that multiple signaling

pathways within the postsynaptic neuron might be required for long term potentiation of synaptic responses in hippocampus.

Chapter 2: **The Role Of Adenylate Cyclase In Long-Term Potentiation (LTP)**

INTRODUCTION

There is general agreement that LTP in area CA1 of hippocampus depends on the activation of the NMDA type of glutamate receptor on the postsynaptic pyramidal neurons (Bliss and Collingridge 1993). NMDA receptor (NMDAR) activation requires a coincidence of glutamate release from the presynaptic nerve terminals with adequate postsynaptic depolarization. The coincident depolarization is required to remove a plugging Mg^{2+} ion from the NMDA receptor channel (Meyer et al. 1984). The requirement for coincident pre- and postsynaptic changes endows LTP with the properties of associativity (a strong stimulus has a greater chance of producing LTP) and cooperativity (a weak stimulus can produce LTP if coupled to a strong stimulus) (Bourne and Nicoll 1993).

When NMDAR becomes activated, it allows the entry of Ca^{2+} ions into the pyramidal neurons, which in turn activates a number of biochemical processes which lead to enhanced, excitatory transmission lasting many hours and even days. There is a growing consensus that during LTP, both presynaptic glutamate release and postsynaptic glutamate receptor sensitivity are enhanced, although this issue is not yet settled (Bliss and Collingridge 1993).

Other Receptors May Also Be Necessary

Activation of NMDAR is necessary but not sufficient for generation of LTP. Although controversial, there is some evidence that in addition to NMDAR, it is necessary to activate the metabotropic glutamate receptors (mGluR's) on the pyramidal cell (Bashir et al. 1993). The interaction between the mGluR and the β-adrenergic pathway will be discussed in the third chapter of this dissertation. Whether additional receptors, such as the dopamine D1 receptor (Frey et al. 1993) or β-adrenergics (Cahil et al. 1994) play a role is an open question.

Participating Enzymes

A number of enzymes have been shown to participate in LTP. The enzyme most studied is calcium/calmodulin-dependent protein kinase II (CaMKII). It has been shown that its blocking (Malenka et al. 1984) or genetic knock-out (Silva et al. 1992) prevents the establishment of LTP. It is located on the postsynaptic dendritic spines and is capable of phosphorylating and enhancing the sensitivity of the AMPA-type glutamate receptor (Malinow 1989). Another kinase which is involved in LTP is protein kinase C (PKC), and its participation in LTP has also been demonstrated with both chemical blockade and genetic knock-out (Malinow, 1989; Goda, 1993). Its role in generating synaptic enhancement is not clear, however. Another enzyme implicated in LTP is nitric oxide synthase (NOS) (Schuman and Madison, 1991; Zhuo et al., 1993) which is activated by Ca^{2+}/calmodulin and generates nitric oxide (NO) which is believed to be a retrograde messenger, causing the presynaptic changes found in LTP. NO may act by activating presynaptic ADP ribosyl transferase or guanylyl cyclase (GC). Another agent activating presynaptic GC may be carbon monoxide

(CO) (Zhuo et al. 1993) which is generated in the post synaptic cell by cytochrome reductase, although it is not clear how this enzyme is activated following synaptic stimulation.

The Involvement of Adenylyl Cyclases in LTP

As reviewed in Chapter One, recently, eight types of AC's with different modes of regulation have been cloned (Pieroni et al. 1993). Although the most abundant mRNA isoforms in the pyramidal layer of CA1 are of Types 2 and 8, some Type 1 message is also found. AC 1 and 8 are activated by Ca^{2+} and calmodulin and probably will be stimulated by the entry of Ca^{2+} though the NMDA channels. AC 2 may also be activated by the tetanic stimulation, perhaps via the metabotropic glutamate receptors and PKC. We demonstrated that AC is activated during LTP induction; however, at the time, the contribution of these cyclases to the maintenance of LTP was not known.

Phases of LTP

It is customary to distinguish between the induction and maintenance of LTP. A number of procedures may enhance synaptic transmission on a temporary basis (LTP induction), but they may not be sufficient for the maintenance of LTP over many hours or days. There is considerable uncertainty how fast the potentiation decays if LTP is not maintained and whether there are multiple stages in LTP maintenance. The fastest decay of LTP occurs when the synapse is stimulated with NMDA alone or with postsynaptic depolarization which

introduces Ca^{2+} ions into the cells (Kullmann et al. 1992). This short-lived potentiation decays in less than 10 minutes and is called "short term potentiation" (STP) (Malenka 1991). Other modes of LTP inhibition cause a decay over a matter of many minutes to several hours. For instance, inhibition of mGluR causes potentiation to decay over 30 minutes (Bashir et al. 1993). Injecting peptides into the pyramidal neuron which block CaMKII or PKC cause a decay which lasts about an hour. However, upon genetic knock-out of CaMKII, potentiation lasts less than 10 minutes rather than an hour (Silva et al. 1992). Also potentiation induced by the application of NMDA can be made longer if NMDA is applied in the presence of higher bathing Ca^{2+} ion concentrations. Thus STP appears to be a separate phase, since it can be produced even when LTP is minimal. However, any potentiation that lasts longer than STP is probably due to a "failed" LTP. This is based on the view of Lisman (1989) that LTP (or at least its postsynaptic component) is the result of a proper balance between the activity of kinases (predominantly CaMKII) and phosphatases. Thus a "Failed" LTP reflects a situation where the proper balance is not achieved and the potentiation subsequently decays in a rate reflecting the overall dephosphorylation rate at the synapse.

The Role of PKA

The cAMP signal transduction pathway is important for learning and memory in a wide variety of species (Bourtchuladze et al., 1994; Frank and Greenberg, 1994; Huang et al., 1994). Mutational inactivation of calmodulin-stimulated adenylyl cyclase interferes with early memory in Drosophila (Levin et al., 1992), and genetic knockout of CREB in

Drosophila has been shown to affect late memory (Yin et al., 1994). These observations suggest that the cAMP pathway may play a role in multiple stages of learning and memory. In mammalian hippocampus, a role for the cAMP pathway in LTP at the Schaffer collateral - CA1 synapse has been demonstrated. When widely-spaced trains of high-frequency stimulation (HFS) were used, LTP was found to be sensitive to blockers of PKA (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994). These investigators focused on a late phase of LTP, which is thought to rely upon protein synthesis. Thus it has been suggested that PKA contributes to late LTP by regulating protein expression (Nguyen et al., 1994). However, early LTP does not require protein synthesis (Bliss and Collingridge, 1993).

Inhibition of PKA by bath application of Rp-cAMPS causes a slow decay of LTP (Frey et al. 1993, Matties and Reymann 1993). The authors of these papers posit that PKA is involved in the protein synthesis-dependent phase of LTP for two reasons. First, when PKA is inhibited, the decay of LTP is slower than that usually seen with other inhibiting manipulations. Second, the bath application of cAMP analogue, Sp-cAMPS, causes an increase in the size of the excitatory postsynaptic potential (EPSP) which is blocked in the presence of the protein synthesis inhibitor anisomycin (Frey et al. 1993). If this hypothesis is correct, then PKA occupies a similar position in the process of synaptic plasticity in mammals as it does in invertebrates.

There are some problems, however, with these arguments. First, there is some ambiguity in the time course of the decay of potentiation in these studies. Although EPSP

amplitude in the presence of Rp-cAMPS decays over a number of hours, the potentiated EPSP converges with the Rp-cAMPS control at a much earlier time point. It is thus not clear that the rate of decay in the presence of Rp-cAMPS differs from the rate of decay in the presence of other kinase inhibitors. In any case, this rate is much faster than the decay seen in the slice treated with anisomycin (Frey et al. 1988). Also, the effect of Sp-cAMPS is probably mostly presynaptic (Chavez-Noriega and Stevens 1992) but probably too rapid to allow protein transport from CA3 cell bodies. In view of these difficulties it is possible that PKA participates in the generation of regular LTP (LTP1) in addition to its participation in late LTP (LTP2).

Recently, a PKA-dependent early phase of LTP ($\leq$ 1 h after HFS) has been observed (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994). The mechanism of the cAMP dependence in early LTP is not known, but it is likely that different mechanisms underlie the PKA dependence of the early and late phases of LTP. To further elucidate the role of the cAMP pathway in LTP, we have investigated the mechanism by which this pathway contributes to early LTP. In order to simplify the situation we decided to study the effects of Rp-cAMPS and cAMP applied directly into the postsynaptic cell. Our data indicate that PKA is not the kinase responsible for LTP, rather PKA is involved in producing the appropriate balance between kinases and phosphatases participating in this process.

METHODS

Sprague-Dawley rats (125-200 g) were deeply anesthetized with halothane and decapitated. The brain was rapidly removed and placed in ice-cold ACSF containing (in mM) NaCl (118), KCl (3.5), $MgSO_4$ (1.3), $CaCl_2$ (3.5), NaH_2PO_4 (1.25), $NaHCO_3$ (24), and glucose (15), bubbled with 95% O_2/5% CO_2. The hippocampus was then rapidly dissected out and transverse slices of 400 µm thickness were made on a tissue chopper. The slices were maintained in an interface chamber (ACSF and humidified 95% O_2/5% CO_2 atmosphere) at room temperature for at least 1 h before removal for recording. Recording was done in submersion chambers at 29 - 31° C, with the slices immobilized between nylon meshes. In most experiments, the slices were constantly superfused with ACSF gravity fed from reservoirs. In those experiments where Rp-cAMPS was applied in the superfusate, a recirculating chamber similar to one previously described (Thiemann et al., 1986) was used. For synaptic experiments, a cut was made in each slice between areas CA3 and CA1, and picrotoxin (50 µM; Sigma) was added to the ACSF. A bipolar concentric stainless steel electrode was placed in stratum radiatum of the CA1 region, and the Schaffer collaterals were stimulated with monophasic pulses 100 µs in width. Field recordings were obtained with electrodes filled with 2 M NaCl (R_e = 3-5 MΩ) and placed in the stratum radiatum. A series of test pulses (4 pulses at 0.2 Hz) was given every 5 or 10 min, and the excitatory postsynaptic potentials (epsp's) within each series were averaged. Epsp amplitude and maximum initial slope (defined as the greatest slope within any 1 ms interval between the stimulus artifact and the epsp peak) were measured offline for the averaged waveforms. LTP

was induced by 3 trains of 100 pulses delivered at 100 Hz, separated by 10 min, a protocol that has been shown to induce cAMP-dependent LTP in the CA1 region (Frey et al., 1993; Matthies and Reymann, 1993). The stimulus intensity during HFS was adjusted to produce a 1 mV field epsp. Rp-cAMPS and Rp-8-Br-cGMPS (both from Biolog) were dissolved in ACSF and applied in the superfusate. For intracellular recording with sharp electrodes, cells in CA1 stratum pyramidale were impaled with electrodes containing 3 M KCl (R_e = 60-90 MΩ). Intracellular epsp's were obtained from a membrane potential of -80 mV. The protocol for producing and monitoring LTP were as described for field recording, except the criterion for stimulus intensity used in HFS was an intracellular epsp of 20 - 30 mV. In all experiments HFS was delivered at least 40 min after impalement, when the basal epsp had been stable for at least 10 min. For intracellular injections, cAMP, (Sigma), Rp-cAMPS, Rp-8-Br-cGMPS, and Sp-cAMPS (Biolog) were dissolved in the normal electrode solutions. Microcystin-LR (Calbiochem) was prepared as a stock solution in ethanol, diluted to 5% in the electrode. Okadaic acid and nor-okadaone (LC Laboratories) were prepared as stocks in DMSO, diluted to 0.1% in the electrode. All substances were allowed to diffuse from the recording electrode. Membrane potential was amplified using the Axoclamp 2A amplifier (Axon instruments). Data were digitized and stored on floppy disks, using an IBM AT PC and Axoclamp interface and software (PCLAMP). Data were displayed on the screen of the computer display, a Tektronix oscilloscope and a Nicolet storage scope. Amplitudes of synaptic potentials and AHP as well as the maximum slope of the epsp were measured using Axon's software (Axoclamp). Summary data are presented as group means with standard error bars. Data were analyzed using two-tailed t-tests or, where appropriate, ANOVAs

followed by Newman-Keuls tests. Summary data are presented as group means with standard error bars.

RESULTS

The Postsynaptic cAMP Pathway is Necessary for Early LTP

We asked whether LTP requires activity in the postsynaptic cAMP pathway. LTP was induced by 3 widely-spaced HFS trains. To confirm that this protocol induces cAMP-dependent LTP, slices were tested in the presence and absence of Rp-cAMPS (100uM, starting 15min before tetanus) in the bath (**Figure 12**). When HFS was delivered in the presence of Rp-cAMPS, LTP decayed more rapidly than in control slices. By 40 min after the last HFS train, LTP recorded in the Rp-cAMPS-treated slices was significantly smaller than control LTP. One hour after the last train of HFS, the two Rp-cAMPS groups did not differ, indicating that LTP had completely decayed by this time in the presence of Rp-cAMPS.

To determine whether it is the postsynaptic cAMP pathway which is required for LTP, cells in CA1 were impaled with sharp electrodes containing 10 mM Rp-cAMPS 40 to 65 min before tetanization. (**Figure 13**A). Following HFS, the potentiation of the intracellular epsp decayed even more rapidly than that obtained with bath-applied Rp-cAMPS (**Figure 13**; compare to **Figure 12**), perhaps reflecting a higher intracellular concentration of Rp-cAMPS.

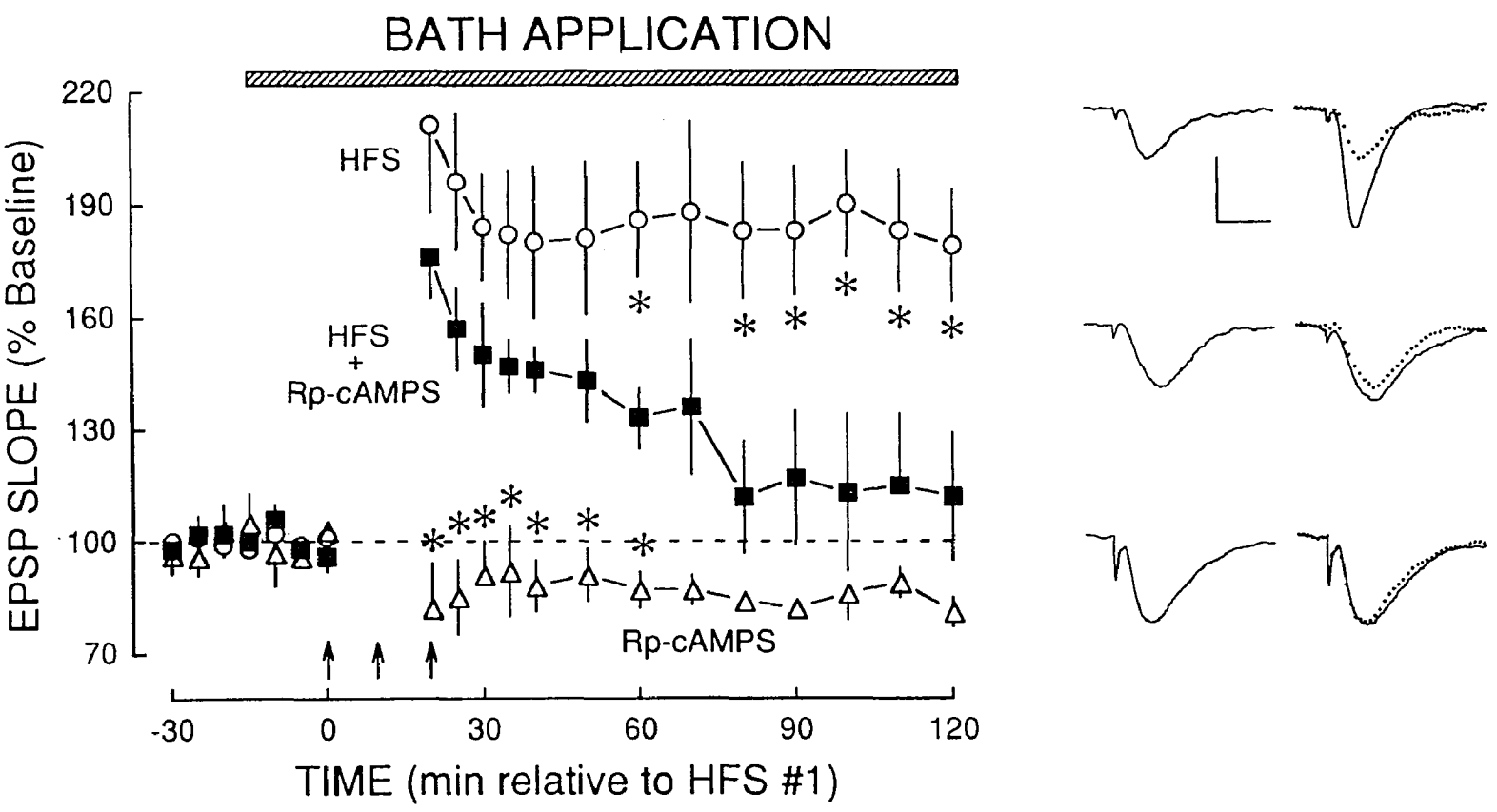

Figure 12 Rp-cAMPS, applied in the bath blocked early LTP in rat hippocampus. Three HFS trains (each 100 Hz for 1 s, separated by 10 min) were delivered as indicated by the gap in the abscissa, with the final train delivered at t=0. (A) Effect of bath-applied Rp-cAMPS. In control slices ("HFS", n=6), LTP was induced with HFS and followed for 2 h after the first train. In the other groups, Rp-cAMPS (100 μM) was introduced to the superfusate 15 min prior to the first tetanus, and was not removed (indicated by hatched bar). One of these groups ("HFS + Rp-cAMPS", n=4) was stimulated in the same manner as the HFS control; the other ("Rp-cAMPS", n=4) served as a control for the effect of Rp-cAMPS, and did not receive HFS. Note that the potentiation obtained in the presence of Rp-cAMPS decayed more rapidly than that of HFS controls. Rp-cAMPS by itself produced a small but significant ($p < .001$) suppression of the epsp. Asterisks indicate significant differences from the HFS + Rp-cAMPS group, and the dashed line represents the baseline slope. On the right, representative traces obtained during the baseline period and 60 min after HFS (arrow) are superimposed. Asterisks indicate significant differences from the HFS + Rp-cAMPS group. Calibrations: 0.5 mV/5 ms.

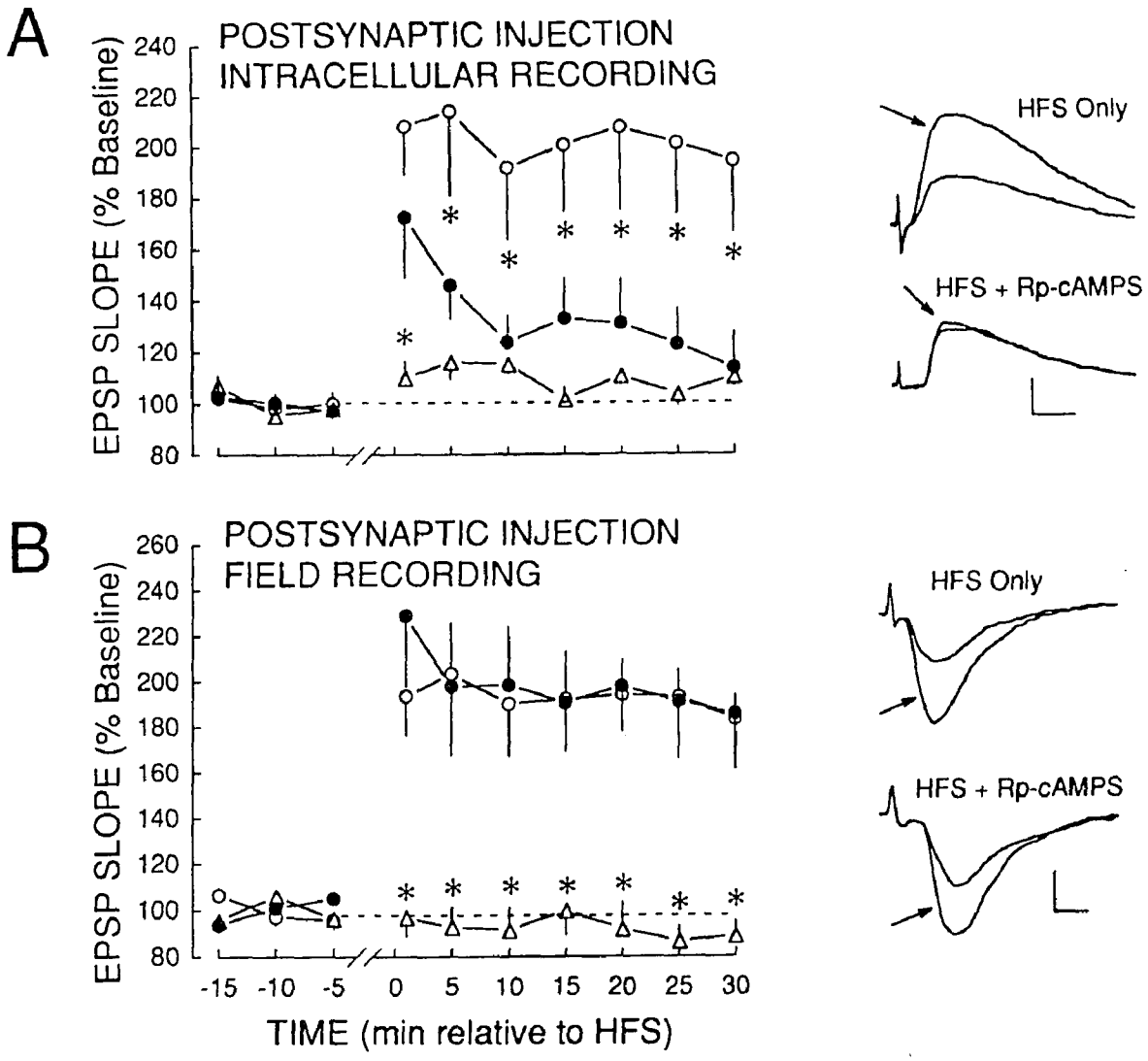

Figure 13 Rp-cAMPS, applied postsynaptically, blocked early LTP in rat hippocampus. Three HFS trains (each 100 Hz for 1 s, separated by 10 min) were delivered as indicated by the gap in the abscissa, with the final train delivered at t=0. (A-B) Intracellular application of Rp-cAMPS also inhibited LTP. Simultaneously recorded intracellular (A) and field (B) epsp summary data and sample traces are shown. Sample traces were obtained during the baseline period and 30 min after HFS (arrow). Intracellular and field traces are from the same slices. (A) Cells impaled with electrodes containing 10 mM Rp-cAMPS (filled circles; n=8) showed a potentiation following tetanus which decayed more rapidly than the potentiation obtained in cells impaled with control electrodes (open circles; n=7). The potentiation in the Rp-cAMPS-treated cells converged with the no-HFS Rp-cAMPS controls (triangles; n=7) over the course of 30 min after HFS. (B) LTP recorded in the field was not different in the slices from which the control and Rp-cAMPS-treated cells were obtained (symbols as in A). Asterisks in A and B indicate significant differences from the HFS + Rp-cAMPS group. Calibrations: A, 5 mV/5 ms; B, 0.5 mV/5 ms.

Five min after the final train, synaptic potentiation in the Rp-cAMPS-treated cells was no longer statistically significant. Simultaneously recorded field epsp's (**Figure 13**B) did not decline over the same period of observation, confirming that the slices remained healthy through the recording period. Thus, the cAMP pathway is required for early LTP, in addition to its previously reported role in late LTP.

In addition to being an inhibitor of PKA, Rp-cAMPS is a relatively weak inhibitor of cyclic GMP-dependent protein kinase (PKG) (Hofmann et al., 1985). To determine if the inhibition of long-term potentiation by Rp-cAMPS could be attributed to effects on postsynaptic PKG, we performed LTP experiments with both bath-applied and postsynaptically-applied Rp-8-Br-cGMPS, a potent and selective inhibitor of PKG (Zhuo et al., 1994). A preliminary experiment determined that Rp-8-Br-cGMPS, injected into the postsynaptic cell, does not inhibit postsynaptic PKA since the inhibition of the AHP by NE was intact in these cells (Table 2, page 36).

Bath application of 0.1 μM Rp-8-Br-cGMPS inhibited LTP (**Figure 14**), in agreement with previously reported results (Zhuo et al., 1994). However, in cells recorded with electrodes containing 10 mM Rp-8-Br-cGMPS, normal LTP was observed (**Figure 15**A-B). Since the concentration of Rp-8-Br-cGMPS in the electrode was 100,000-fold higher than its effective bath concentration, postsynaptic PKG does not appear to play a role in LTP, and the inhibition of LTP by postsynaptic application of Rp-cAMPS cannot be attributed to an effect on PKG. Furthermore, the inability of postsynaptic Rp-8-Br-cGMPS to inhibit LTP

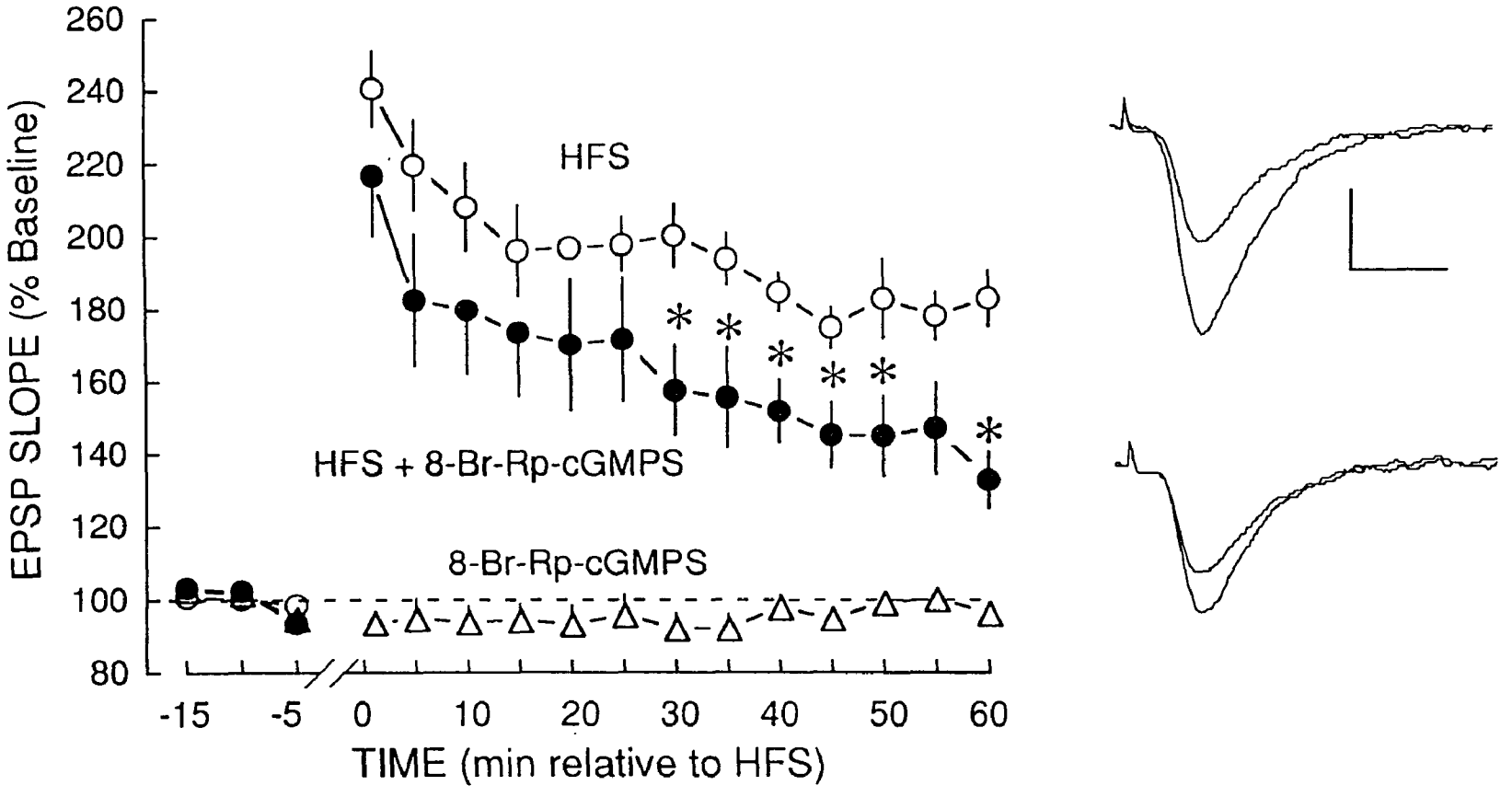

Figure 14 Rp-8-Br-cGMPS inhibited LTP when applied in the bath. HFS was delivered beginning 40 min after introduction of 100 nM Rp-8-Br-cGMPS, which was present through the remainder of the recording period. Asterisks indicate significant differences between the group given HFS in the presence of Rp-8-Br-cGMPS (n=5) and controls given HFS without Rp-8-Br-cGMPS (n=5). Slices treated with Rp-8-Br-cGMPS but not HFS (n=5) showed no change in epsp slope over the recording period. The representative traces were obtained during the baseline period and 60 min after HFS (arrows). Calibrations: 0.5 mV/10 ms.

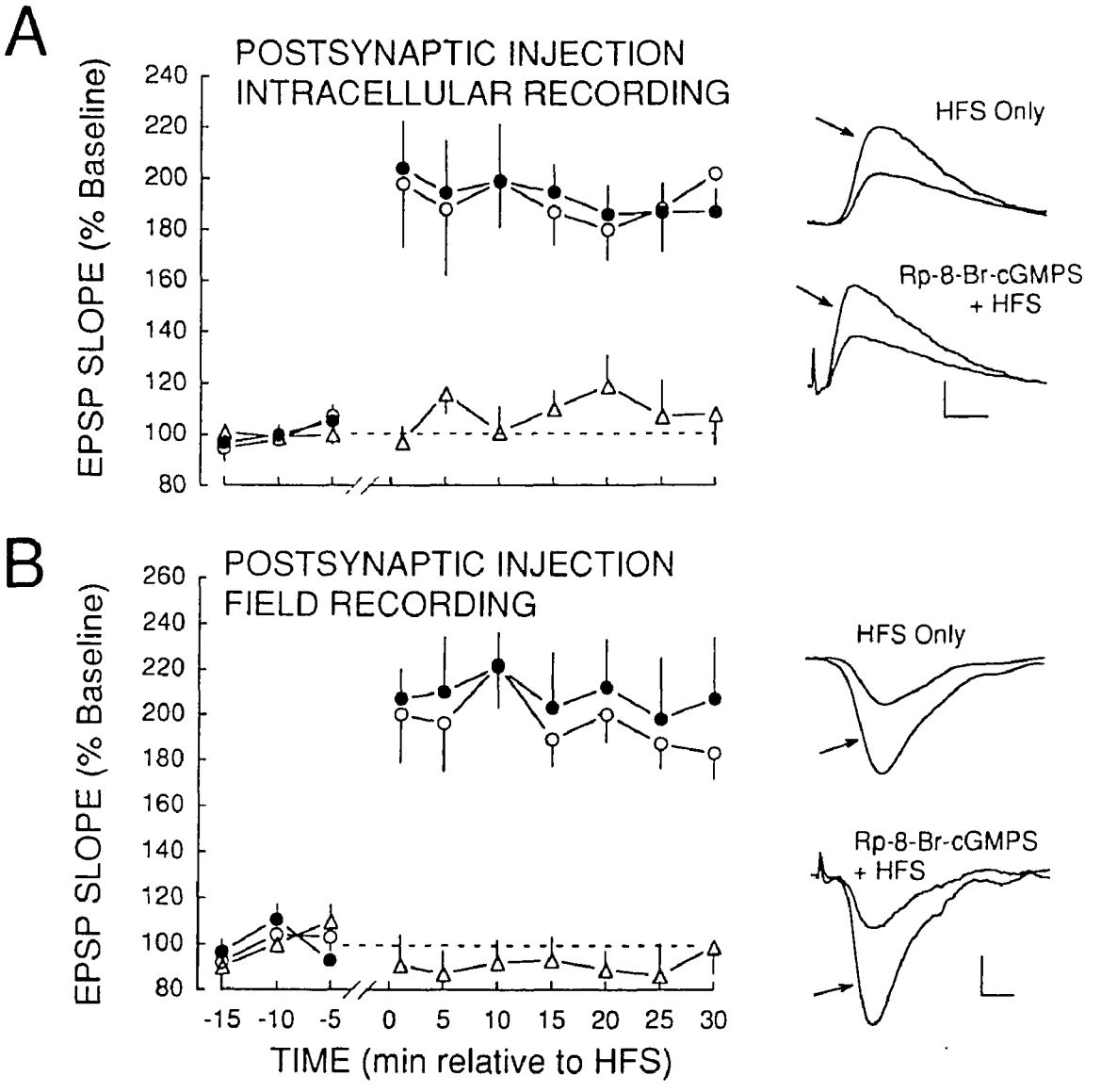

Figure 15 LTP is not inhibited by intracellular application of Rp-8-Br-cGMPS. Simultaneously recorded intracellular (A) and field (B) epsp summary data and sample traces are shown. Traces were obtained during the baseline period and 30 min after HFS (arrows). The intracellular and field traces are from the same slices. (A) Cells impaled with electrodes containing 10 mM Rp-8-Br-cGMPS (40 min) and stimulated with HFS (filled circles; n=5) showed LTP which was indistinguishable from that obtained in cells impaled with control electrodes (open circles; n=5). Cells treated with Rp-8-Br-cGMPS alone (triangles; n=5) did not show any significant changes in synaptic efficiency. (B) LTP measured in the field was also unaffected by intracellular application of Rp-8-Br-cGMPS. Symbols are as described for (A). The two groups which received HFS did not differ statistically at any time point, either in the intracellular or field measures. Calibrations: A, 5 mV/5 ms; B, 0.5 mV/5 ms.

indicates that the effect of Rp-cAMPS cannot be ascribed to any nonspecific effect of cyclic nucleotides. Finally, the high potency of bath-applied Rp-8-Br-cGMPS, together with the relatively high concentration injected into the postsynaptic cell, indicates that the effect of Rp-8-Br-cGMPS was restricted to the postsynaptic cell, and it is likely that postsynaptically injected Rp-cAMPS was similarly confined.

To determine whether PKA-dependent LTP was limited to widely-spaced trains of HFS, we tested a series of 4 trains (each 1 s at 100 Hz) separated by 20 s (**Figure 16**A). Aside from the pattern of HFS, the procedure was identical to that used previously for widely-spaced HFS. Early LTP recorded with Rp-cAMPS-containing electrodes was significantly less than in closely-spaced controls indicating that LTP was dependent on postsynaptic cAMP. However, the effect of Rp-cAMPS was significantly less than that observed following widely-spaced HFS (**Figure 16**B). The reduced PKA dependence of LTP following closely-spaced HFS may help to explain the ineffectiveness of a bath-applied Rp-cAMPS analog in blocking LTP reported by Weisskopf et al. (1994).

Activation of the Postsynaptic cAMP Pathway is not Sufficient to Increase Synaptic Efficiency

Activation of the postsynaptic cAMP pathway is known to enhance responses mediated by AMPA receptors in cultured hippocampal cells (Greengard et al., 1991; Wang et al., 1991). If this mechanism underlies cAMP-dependent LTP, then postsynaptic

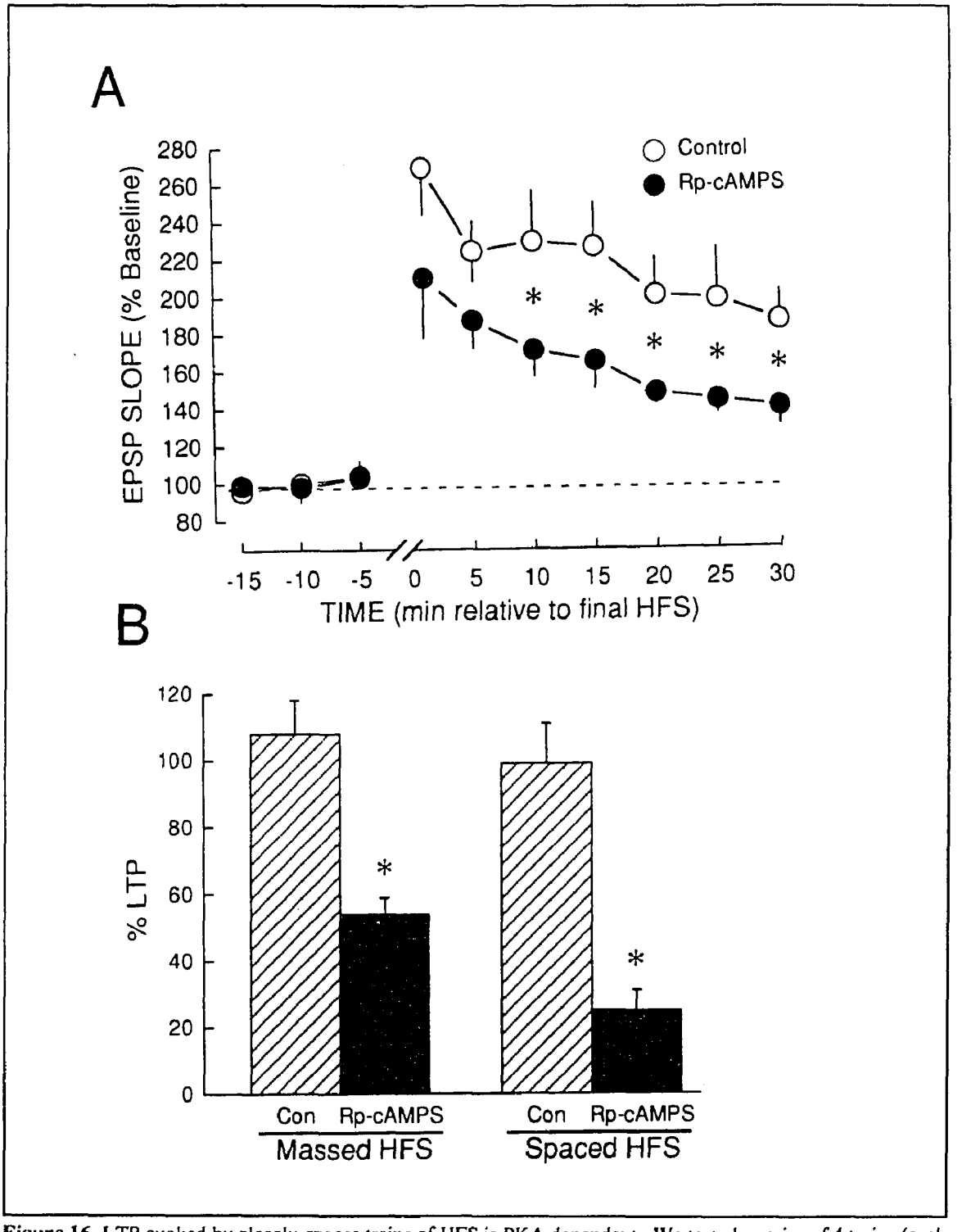

Figure 16 LTP evoked by closely-spaces trains of HFS is PKA dependent. We tested a series of 4 trains (each 1 sec at 100 Hz) separated by 20 s. Aside from the pattern of HFS, the procedure was identical to that used previously for widely-spaced HFS. (A) Early LTP recorded with Rp-cAMPS-containing electrodes was significantly less than in closely-spaced controls (140 ± 10% of baseline at 30 min after HFS, n=5, vs the control value of 186 ± 16%, n=7; $p<.05$), indicating that LTP was dependent on postsynaptic cAMP. (B) the effect of Rp-cAMPS was significantly less than that observed following widely-spaced HFS.

application of cAMP should increase the amplitude of the intracellular epsp. Cells were impaled with electrodes containing the non-hydrolyzable cAMP analogue Sp-cAMPS (10mM), and both the epsp and the AHP were monitored. Sp-cAMPS caused a rapid inhibition of the AHP, which was usually fully blocked by the earliest measurement ($\leq$ 5 min after impalement). However, over a 60 min period following impalement with electrodes containing Sp-cAMPS, the epsp increased only slightly (**Figure 17**B), an effect which was also seen in control cells and which may reflect a gradual recovery of the cell following impalement. Thus, at a concentration and exposure time adequate for Rp-cAMPS to block LTP, Sp-cAMPS did not increase the epsp above control. The absence of an intrinsic effect of postsynaptic Sp-cAMPS within an hour of recording is clearly different from the slow enhancement of the epsp described following bath application of Sp-cAMPS, which has been related to the late phase of LTP (Frey et al., 1993; Nguyen et al., 1994).

Since we found that the AHP was completely blocked very soon after impalement with electrodes containing Sp-cAMPS, it could be argued that the effect of the nucleotide on the epsp was complete before the first measurements were obtained. To rule out this possibility, we performed a series of experiments with electrodes containing cAMP, which caused a more gradual blockade of the AHP (**Figure 17**A_2). However, even under these conditions, there was no significant increase in epsp amplitude (**Figure 17**A_1), indicating that the failure of the epsp to increase in the presence of Sp-cAMPS was not due to a failure to detect an early effect.

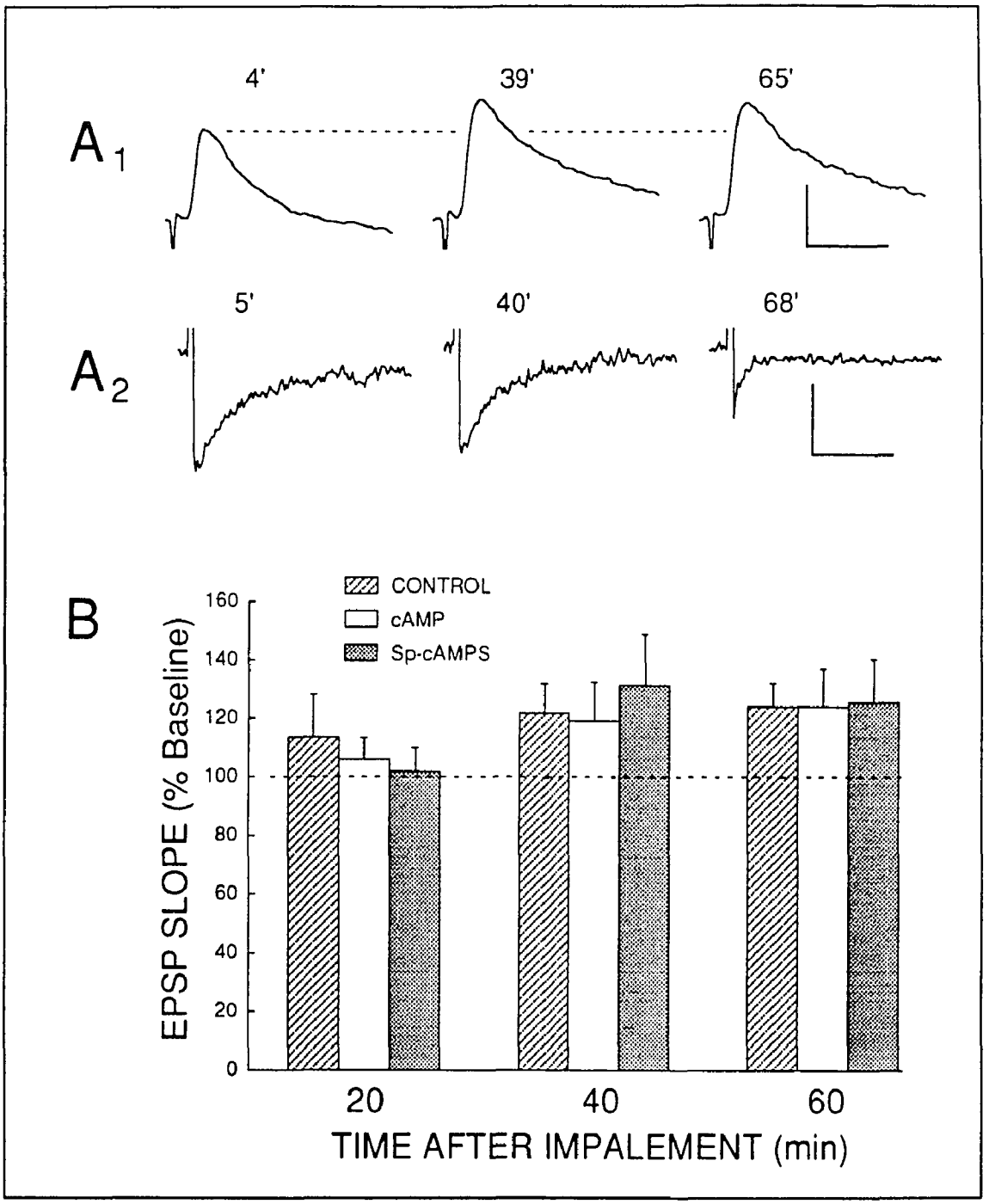

Figure 17 The epsp was not increased by intracellular cAMP or Sp-cAMPS. (A) Traces of the epsp (A_1) and the AHP (A_2) in a cell impaled with an electrode containing 50 mM of cAMP. The times elapsed since impalement are noted. In this cell, epsp slope increased by 20% from 4 min to 65 min after impalement. The AHP was gradually blocked by cAMP over this period. The dashed line indicates the baseline (4 min) epsp amplitude. Calibrations: upper traces, 5 mV/20 ms; lower traces, 5 mV/2 s. (B) Summary of epsp data in cells impaled with control electrodes (n=5) or with cAMP (50 mM; n=3) or Sp-cAMPS (10 mM; n=5). Data are presented relative to the first measured epsp (2-4 min after impalement). Note that there was a small but reliable increase in epsp slope in all groups, including controls. The groups did not differ significantly at any time point. The dashed line indicates the baseline reference.

Inhibition of postsynaptic protein-phosphatase reverses the effect of Rp-cAMPS on LTP

The complete inhibition of LTP by Rp-cAMPS showed that the cAMP pathway did not simply add a component to LTP, but rather that early LTP was entirely dependent on postsynaptic PKA. Since increasing postsynaptic cAMP did not enhance synaptic efficiency, the cAMP pathway does not transmit the signal for early LTP. The behavior of the cAMP pathway is more consistent with that of a gate. A gate would permit signal flow through other pathways, but would not itself be responsible for producing the observed physiological response.

How would a pathway that normally functions to transmit signals act instead as a gate? The answer may lie in the interaction between phosphoprotein phosphatases and protein kinases in regulating signal flow. In the present case, PKA can inhibit protein phosphatases via inhibitor-1 (Huang and Glinsmann, 1976), and may thereby permit signal flow through other pathways involving protein kinases such as CaMKII and PKC, which are required for LTP (Malinow et al., 1989; Silva et al., 1992; Pettit et al., 1994). In this line of reasoning, the protein phosphatases are downstream of PKA, and inhibitors of protein phosphatases should protect LTP from blockade by Rp-cAMPS.

Microcystin-LR is a cyclic hexapeptide which is a specific protein phosphatase blocker (Honkanen et al., 1990; MacKintosh et al., 1990). When microcystin-LR was included in the

recording electrode, it prevented Rp-cAMPS (10mM, 40min) from inhibiting LTP without showing any intrinsic effect on LTP (**Figure 18**; **Figure 20**A). Since microcystin-LR is membrane-impermeant, its ability to protect LTP from Rp-cAMPS indicates that the cAMP pathway is inhibiting postsynaptic protein phosphatases. We also used another protein phosphatase inhibitor, okadaic acid (Nishiwaki et al., 1990), which was applied in the recording electrode. As shown in **Figure 19**A, **Figure 20**B, LTP was intact in the presence of okadaic acid, even with Rp-cAMPS in the electrode. In contrast, the inactive analog nor-okadaone (Nishiwaki et al., 1990) did not protect LTP from Rp-cAMPS inhibition (**Figure 19**B, **Figure 20**B). These data support the hypothesis that the cAMP pathway functions through phosphoprotein phosphatases to gate LTP.

DISCUSSION

Multiple Phases of LTP and the Role of the cAMP Pathway

Distinct phases of LTP have been described, distinguished by their duration and sensitivity to inhibitors of protein kinases or protein synthesis (Bliss and Collingridge, 1993). Thus, a relatively early phase (< 3 h in duration) requires protein kinase activity, but only a later phase depends upon protein synthesis.

When we applied Rp-cAMPS in the bath, LTP decayed within the first hour. An even faster decay was obtained when the inhibitor was directly injected into the cell. The relatively

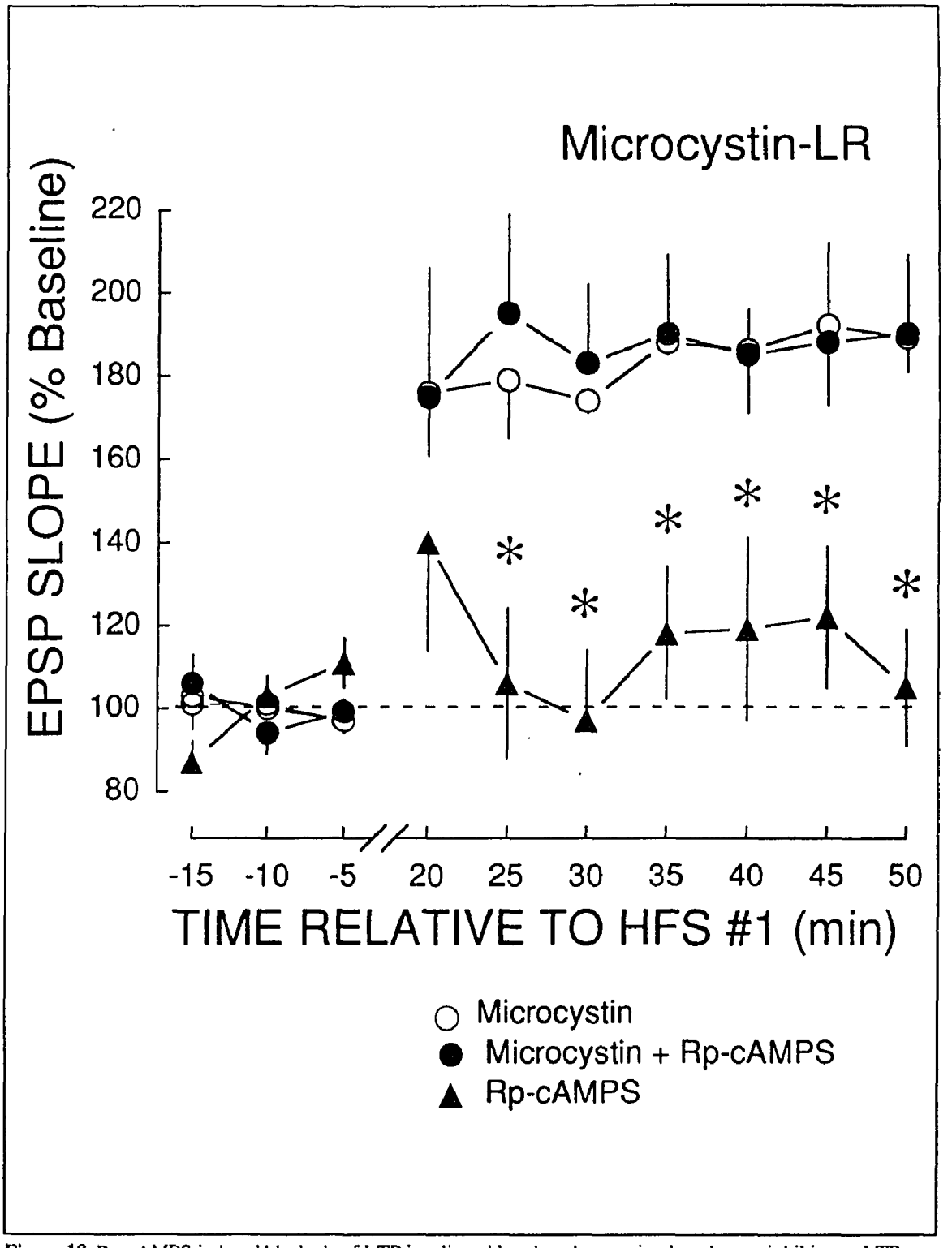

Figure 18 Rp-cAMPS-induced blockade of LTP is relieved by phosphoprotein phosphatase inhibitors. LTP was intact in cells impaled with electrodes containing both 10 mM Rp-cAMPS and 100 μM microcystin-LR (solid circles, n=6) or microcystin-LR alone (open circles; n=3). In cells recorded with electrodes containing only Rp-cAMPS, LTP was blocked (filled triangles, n=4). Asterisks denote differences ($p < .05$) from HFS + microcystin-LR cells. All electrodes contained 5% ethanol.

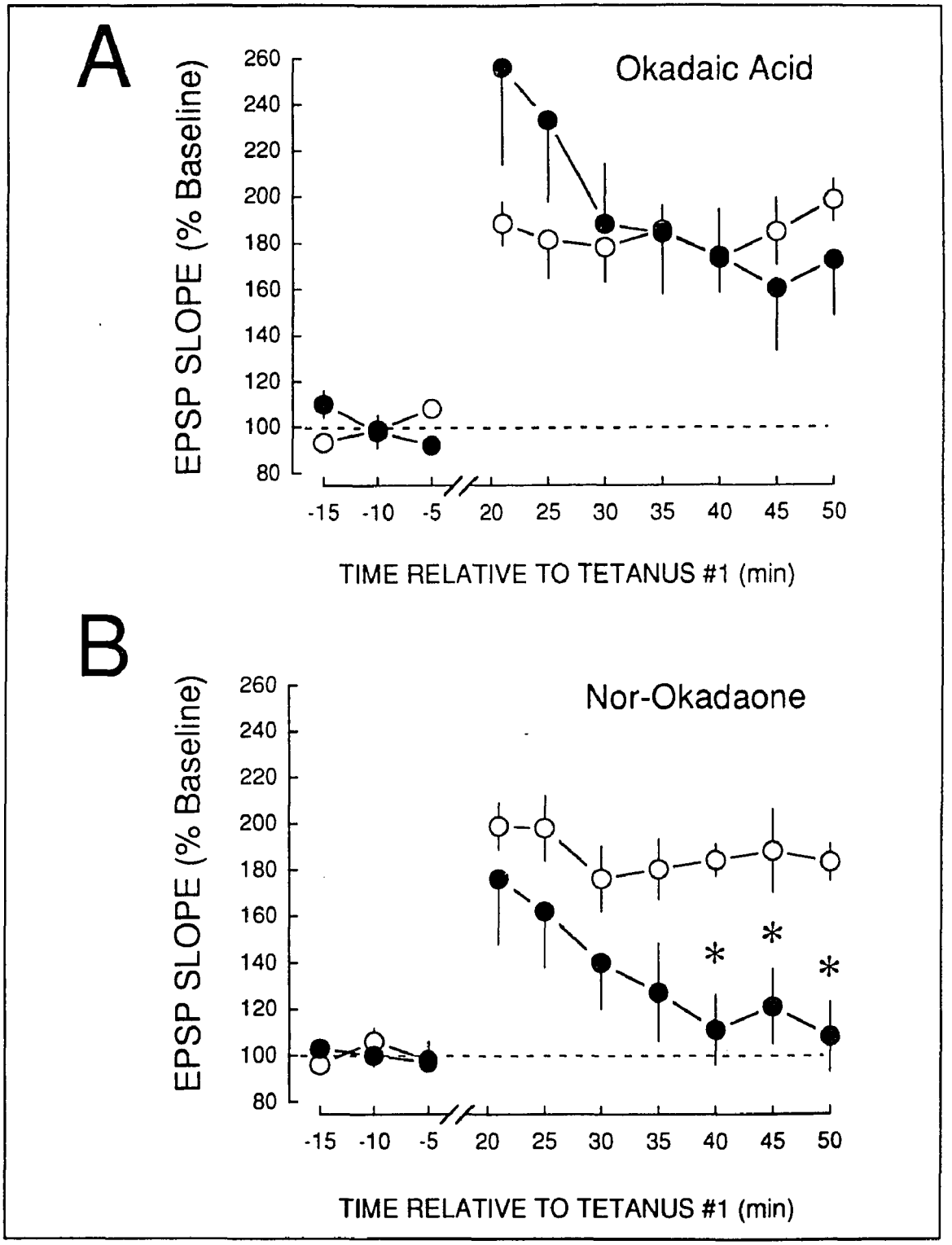

Figure 19 Rp-cAMPS-induced blockade of LTP is relieved by phosphoprotein phosphatase inhibitors. (A) When electrodes contained 10 μM okadaic acid, Rp-cAMPS (10 mM in the electrode; filled symbols, n=7) did not reduce LTP below okadaic acid controls (open symbols, n=4). (B) Nor-okadaone (10 μM in the electrode) did not prevent the blockade of LTP by Rp-cAMPS (10 mM in the electrode; filled symbols, n=5) relative to nor-okadaone controls (open symbols; n=4). Asterisks indicate group differences ($p < .05$).

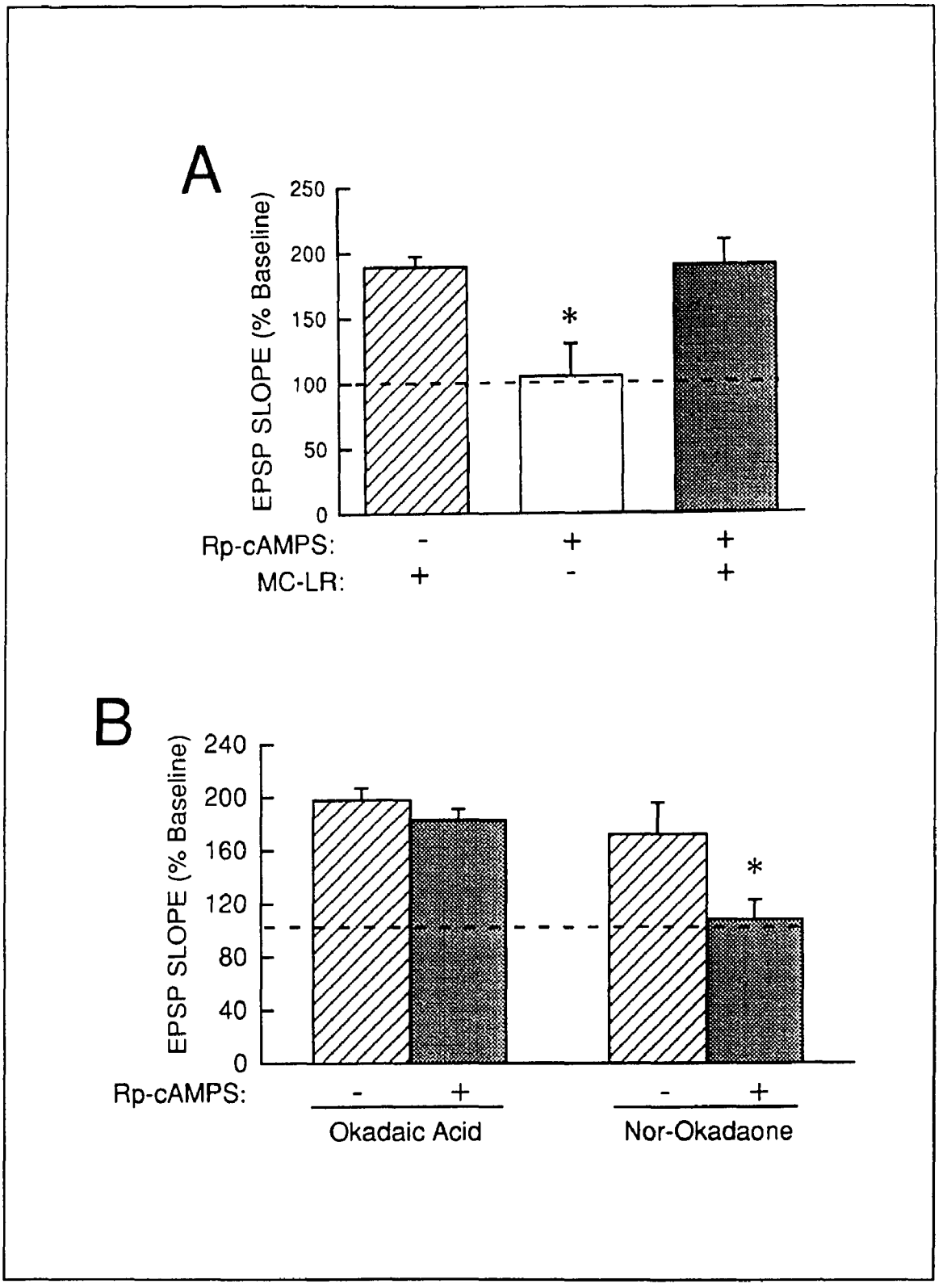

Figure 20 Rp-cAMPS-induced blockade of LTP is relieved by phosphoprotein phosphatase inhibitors. Summary of results obtained with (A) microcystein-LR and (B) okadaic acid/nor-okadaone, at 30 min after HFS. Asterisk indicates a difference ($p < .05$) from the microcystein-LR and the nor-okadaone control groups respectively.

slow decay associated with the extracellular application of PKA inhibitors has led some authors to conclude that the cAMP signaling pathway is involved only in a late phase of LTP (Matthies and Reymann, 1993), whereas others have concluded that cAMP may be responsible for an early LTP component which is superimposed on a cAMP-independent LTP (Huang and Kandel, 1994). Extracellular application of Rp-cAMPS may yield lower intracellular concentrations and weaker effects than those obtained upon intracellular injection of the inhibitor. In the present experiments, the suppression of LTP by Rp-cAMPS was complete within an hour following HFS. We therefore conclude that PKA activity is required for expression of early LTP at the Schaffer collateral-CA1 synapse.

Studies using the PKA activator Sp-cAMPS indicate that cAMP pathway is responsible for the late, protein synthesis-dependent phase of LTP (Frey et al., 1993; Nguyen et al., 1994). Our observations did not extend beyond 60 min following HFS stimulation, and so do not address the role of the cAMP pathway in a later phase of LTP. However, the literature indicates that the cAMP pathway plays a signaling role in late LTP, rather than the gating function shown for early LTP, since the late phase is occluded by the gradual increase in synaptic efficiency produced by bath application of Sp-cAMPS (Frey et al., 1993).

The Gating of the LTP Cascade

The present results suggest an important distinction between signaling pathways which communicate the signals required to evoke LTP, and those which function as gates to regulate

the establishment of LTP. An LTP signaling pathway would be characterized by mimicry and occlusion of LTP, typified by CaMKII. The expression of a constitutively active fragment of CaMKII in the postsynaptic neuron results in enhanced synaptic transmission and occlusion of further potentiation by an LTP-inducing protocol (Pettit et al., 1994). On the other hand, postsynaptic PKA acts to gate LTP, since inhibition of this enzyme prevents LTP but PKA activators do not enhance synaptic transmission.

Our data indicate that the postsynaptic cAMP pathway functions as a gate by regulating the activity of phosphoprotein phosphatases. Signal flow through the transmittal and gating pathways is summarized in **Figure 21**A. A possible mechanism by which the cAMP pathway can function as a gate in LTP is presented in **Figure 21**B. In response to HFS, Ca^{2+} ions enter the cell via the NMDA receptor channels and activate Ca^{2+}/calmodulin (Ca^{2+}/CaM). In turn, Ca^{2+}/CaM activates separate signaling and gating pathways. The signal is transmitted by CaMKII, which can autophosphorylate and thereby maintain itself in an active state (Colbran et al., 1988). The subsequent phosphorylation of target proteins by activated CaMKII results in a persistent enhancement of synaptic transmission (McGlade-McCulloh et al., 1993; Pettit et al., 1994).

Ca^{2+}/CaM can also activate calcineurin and adenylyl cyclase (AC1 or AC 8). Together, these enzymes can determine the phosphorylation state of inhibitor-1 (I-1), which regulates protein phosphatase 1 (Shinolikar and Nairn, 1991). Calcineurin would dephosphorylate and inactivate I-1, allowing PP1 to disrupt the CaMKII signaling pathway

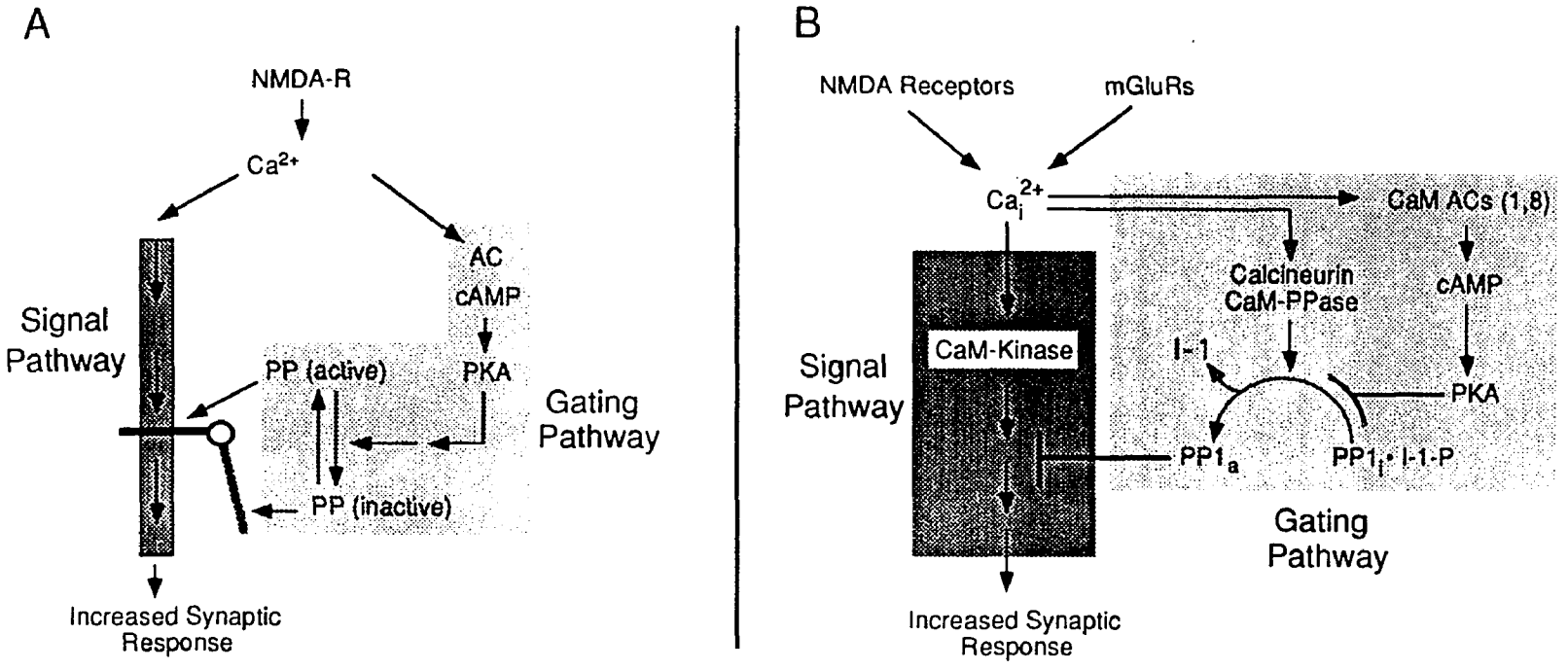

Figure 21 Models of the interaction between gating and signaling pathways in LTP. (A) Summary of the components of the LTP gating pathway identified in this Chapter. (B) An extended model of the signaling and gating pathways for LTP, based on Ingebritsen and Cohen (1983) and Lisman (1989; 1994). The signaling role of CaMKII has been recently described (Pettit et al., 1994). The regulation of phosphoprotein phosphatase activity by calcineurin and inhibitor-1 is speculative for LTP, but has been shown in other systems (Shinolikar and Nairn, 1991; Mulkey et al., 1994). Synaptically released glutamate activates both NMDA and metabotropic glutamate receptors, both of which may contribute to the rise in intracellular Ca^{2+}. Abbreviations: $PP1_a$=activated protein phosphatase 1; $PP1_i$=inactive PP1; I-1-P=phosphorylated (activated) inhibitor-1; mGluR=metabotropic glutamate receptor. Other abbreviations are described in the text.

(Lisman, 1989; Lisman, 1994; Mulkey et al., 1994). If left unopposed, active PP1 may prevent LTP by returning CaMKII and its targets to their unphosphorylated states. However, adenylyl cyclase antagonizes the effects of calcineurin by producing cAMP. The resulting activation of PKA would inhibit PP1 by phosphorylating I-1. Thus, calcineurin should act to close the gate which regulates LTP, while PKA maintains the gate in the open state. When PKA is inhibited, PP1 activity is unopposed and LTP fails.

The Postsynaptic Density Contains the Enzymatic Machinery for the Signaling and Gating of LTP

The postsynaptic density is a submembrane specialization thought to be involved in synaptic plasticity (Kennedy, 1993). With localization of adenylyl cyclase to the postsynaptic density, (in collaboration with N. Mons and D. Cooper, data not shown) almost all the enzymes of the model in **Figure 21B** are known to be present in this structure. CaMKII, the major enzyme of the proposed signaling pathway, is highly concentrated in the postsynaptic density (Kennedy et al., 1983). In the gating pathway, constituents of the postsynaptic density include adenylyl cyclase (Mons et al., in preparation), PKA (Carr et al., 1992), calcineurin (Coghlan et al., 1995), and PP1 (Dosemeci and Reese, 1993). In addition, the postsynaptic density contains AKAP, an anchoring protein which binds PKA and calcineurin (Coghlan et al., 1995). Thus, the two opposing components of the putative LTP gating pathway may be positioned in close proximity and anchored to the postsynaptic density by

AKAP, favoring the regulation of I-1 in or near the postsynaptic density and in the vicinity of its target, PP1. I-1 has yet to be detected in the postsynaptic density, but the ability of postsynaptically-applied I-1-P to regulate phosphatase-dependent changes in synaptic efficiency (Mulkey et al., 1994) suggests that I-1 normally acts on PP1 within the dendritic spine.

Gating by the cAMP pathway Maybe a General Regulatory Mechanism

Cyclic-AMP is one of the most comprehensively studied second messengers which evokes a broad spectrum of well-characterized responses, ranging from modulation of ion channels (Sculptoreanu et al., 1993) to activation of sugar metabolism enzymes such as glycogen phosphorylase (Krebs and Beavo, 1979), and stimulation of transcription factors such as CREB (Lee and Masson, 1993). In each of these instances, cAMP alone is sufficient to mimic the response of receptor-stimulated PKA activation.

A totally different function has also been ascribed to cAMP: that of a gate. As manifested in many biological processes, the cAMP pathway can function as a gate to block signal transmission through other pathways. For instance, elevation of cAMP levels can inhibit transformation of NIH-3T3 cells by H-Ras; yet, alteration of cAMP levels does not by itself block the proliferation of NIH-3T3 cells (Chen and Iyengar, 1994). It has also been shown that increased cAMP levels can hinder the Sonic Hedgehog-induced differentiation in early mouse development. However, cAMP elevation alone is not adequate to halt differentiation

(Fan et al., 1995). Similarly in Drosophila, the cAMP pathway seems to gate the morphogenic Hedgehog signal, rather than functioning as a component of the Hedgehog signaling pathway (Blair, 1995; Jiang and Struhl, 1995; Li et al., 1995; Perrimon, 1995). Moreover, elevation of cAMP levels has been demonstrated to suppress signal transmittal through the MAP kinase pathway (Cook and McCormick, 1993; Graves et al., 1993; Wu et al., 1993; Chen and Iyengar, 1994). Consistent with these results, our data reveal a gating function for the cAMP pathway in early LTP. In contrast with the above cases, the cAMP pathway, in LTP, serves to maintain the gate open, allowing the signal to produce a physiological response. Furthermore, at least in Drosophila development, cAMP seems to play a more passive role, so that endogenous cAMP activity is sufficient to inhibit hedgehog signals (Jiang and Struhl, 1995). However, in LTP, the cAMP pathway is activated by external stimuli, and may function as a stimulus-triggered gate.

Thus, the cAMP pathway acts as a gate in phenomena as diverse as long-term synaptic potentiation, early development and cell proliferation, despite some differences in the detailed operation of the gate in these processes. Such gating may provide a general mechanism by which signals are integrated at the level of a single cell to evoke a complex biological response. Although it is apparent that the cAMP pathway serves to gate signals in numerous responses, it remains to be determined whether gating is a widespread property of other signal transduction pathways as well.

Chapter 3: The Hierarchy of Metabotropic Glutamate Receptor Function in CA1 Hippocampal Neurons

INTRODUCTION

The concept of rank order and its importance

Neuroscientists have for many years experimentally approached the study of nervous system in a linear fashion, that is, event A leads to response B which in turn leads to event C and so on. A familiar example of such an experimental design is one which first characterizes a receptor population such as the noradrenergic receptor and its subtypes (Minneman et al. 1981), then proceeds to investigate the effect or mechanism for that receptor, in this case the generation of the second messenger cAMP (Lefkowitz et al. 1983, Krupinski et al. 1989). Subsequently, the induced response of the second messenger is investigated, such as activation of cAMP-dependent kinase (Browning et al. 1985). The penultimate event in this linear cascade, phosphorylation of substrates including ion channels as well as regulation of eukaryotic gene expression, is then investigated (Browning et al. 1995). The ultimate relevance of this linear sequence of cAMP-induced events has been linked to highly complex and nonlinear responses such as learning and memory (Cahil et al. 1994). One of the strongly appealing features of such an experimental approach is the predictive capability of a linear sequence. Thus, the dose response and time course of cAMP formation in response to norepinephrine or other neurotransmitters is

extremely well characterized and reproducible in a vast number of cellular and cell-free systems. While these predictable systems have led to significant insights into neuronal response potentials, they do not completely reflect the magnitude of complex interactions that occur within the nervous system.

It is estimated that within the CNS one neuron may receive as many as 10,000 synaptic inputs. Upon recognition of this comparatively small degree of complexity relative to the actual complexity of the nervous system in its numbers of neurotransmitters and types of receptors, one must begin to ask what happens when the system is not a simple linear system but actually a scheme of multiple simultaneously occurring interactions. By virtue of asking the question, the experimental approach that must be adopted is a strategy which can consider multiple simultaneously occurring variables.

Although one can mathematically model a complex nonlinear system by producing elaborate equations describing multiple potential outcomes, the combination of nonlinearity coupled with the complexity of the potential outcomes renders the system virtually unpredictable. Because of the unpredictable nature of nonlinear systems, these phenomena remain largely within the purview of computational modeling. Thus the question emerges as to whether one can attempt to study complex nonlinear neuronal systems within the confines of experimental science with sufficient predictability. To resolve this question, we attempted to experimentally study such systems by beginning at the simplest possible level of complexity. One such case would be the interaction between two chemical messengers acting

on the same cell at the same time. In such instances, the simplest paradigm to facilitate the predictability of the outcome of interactions would be a hierarchical classification of the neurotransmitters based on the dominance of their actions.

Convergence of Action Among Various Neurotransmitters

There is general agreement that multiple signal transduction pathways converge on a relatively small number of effectors. This convergence is the basis for crosstalk among the signaling pathways, and could permit other interactive phenomena such as multisite and hierarchical protein phosphorylation. Although well established in the fields of biochemistry and molecular biology (for review see Roach 1991), only a few examples of convergence are known in physiology. In rat superior cervical ganglion cells, for instance, multiple G-protein coupled receptor pathways depress the N-type Ca^{2+} current (See Hille 1992 for review). In CA1 hippocampal neurons, numerous receptors have been shown to inhibit adenylyl cyclase (AC) and to activate an inward rectifying K^+ conductance (Andrade et al. 1986, Zgombick et al. 1989). Under conditions where multiple receptors converge on a common effector by disparate mechanisms, it may be possible to observe a hierarchical relationship among these receptors.

In hippocampal pyramidal cells, a burst of action potentials is followed by a slow afterhyperpolarization (AHP), which reflects the activation of a Ca^{2+}-dependent potassium current. It determines the general excitability of neurons by mediating accommodation of

spike frequency. The AHP provides an ideal physiological assay for the study of neurotransmitter interactions in the hippocampus, since multiple receptors and second messenger pathways converge on it (see Storm 1993 for review). In area CA1 of hippocampus, these receptors include serotonin ($5HT_4$; Andrade and Chaput 1991), norepinephrine (β_1; Madison and Nicoll 1986), histamine (H2; Haas and Greene 1986), acetylcholine (m1; Dutar and Nicoll 1989), dopamine (D_2; Berretta et al. 1990), glutamate (mGluR; Stratton et al. 1990, Charpak et al. 1990), thyrotropin releasing hormone (TRH; Ballerini et al. 1994), lutenizing hormone releasing hormone (LHRH; Chen et al. 1993), and corticotropin releasing factor (CRF; Aldenhoff et al. 1983). The present research addressed the inherent rank order prevalent between two unrelated neurotransmitter receptors (β-adrenergic and mGluR), both of which couple to AHP inhibition. Although the signal transduction pathway of β-adrenergic receptors leading to the PKA-dependent phosphorylation and subsequent inhibition of the AHP has been characterized (Pedarzani and Storm 1993; Blitzer et al. 1994), the mechanism of AHP inhibition by mGluR agonists remains unknown.

Metabotropic Glutamate Receptors

Glutamate not only acts as a major excitatory neurotransmitter, but also plays an important role in neuronal plasticity (Collingridge and Singer 1990), neurotoxicity (Meldrum et al. 1989), memory acquisition and learning (Conn and Desai 1991, Tanabe et al. 1992, Bashir et al. 1993). This functional diversity of glutamate is reflected by the presence of

disparate glutamate receptors which have been classified into two broad families: ionotropic and metabotropic. Members of the ionotropic glutamate receptor family are coupled directly to cation channels, whereas the metabotropic glutamate receptors (mGluRs) are coupled to effector systems through GTP-binding proteins (Schoepp 1990, Desai and Conn 1991). Molecular cloning by cross-hybridization and PCR techniques has revealed the existence of at least eight subtypes of metabotropic glutamate receptors (mGluR1 through mGluR8). Several lines of evidence suggest that mGluRs are involved in synaptic plasticity, both during development and in adult life (Kano 1987; Ito 1989; Linden 1991; Calabresi 1992; Aronica 1993; Bashir 1993).

The eight mGluRs are considerably larger than the other members of the G protein-coupled receptor family but show a common structural architecture with a large extracellular NH_2-terminal domain that precedes the seven transmembrane segments (**Figure 22**) (Tanabe et al. 1992). However, no sequence homology is observed with any other members of the G protein-coupled receptor family (Abe et al. 1992). The amino acid sequences of the mGluRs are highly conserved not only in the seven transmembrane segments but also in their preceding extracellular domains. Among these, cysteine residues, important in structural formation of mGluRs, are remarkably conserved, making the mGluRs represent a novel subfamily of the G protein-coupled receptor family. The cloned mGluRs in the rat have been tentatively assigned to three different groups on the basis of their sequence homology, pharmacological properties and signal transduction mechanisms (review Scheopp

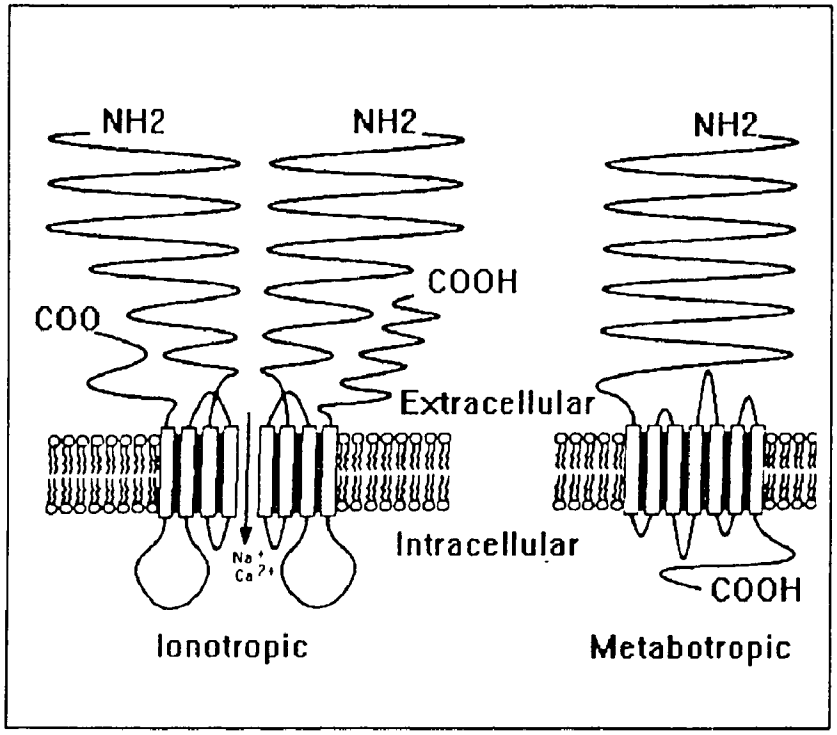

Figure 22 Topological representation of Ionotropic and Metabotropic glutamate receptors. (Modified from Nakanishi 1992).

1994; Pin 1995). These receptor subtypes can be subdivided into three subgroups according to their sequence similarities: mGluR1, 5; mGluR2, 3; mGluR4, 6, 7 and 8 (see Table 3).

Since mGluR5 is highly expressed in pyramidal CA1 neurons of neonates and remains so during both development and adulthood (Nakanishi 1993; Catania 1994), it is suggestive of this receptor to play a role in both synaptogenesis and synaptic transmission of CA1 neurons.

Physiology of Metabotropic Glutamate Receptors

In several brain structures, activation of mGluRs inhibit L and/or N type voltage-dependent Ca^{2+} channels, an effect mediated by a direct coupling between the G protein and the Ca^{2+} channels (Lester and Jahr 1990, Swartz and Bean 1992, Sayer et al. 1992). mGluRs also inhibit several K^+ channels:

- The Ca^{2+}-dependent voltage-independent K^+ channels responsible for the slow afterhyperpolarization (I_{AHP}) which participates in spike accommodation (Charpak et al. 1990; Stratton et al. 1990; Desai and Conn 1991).
- The voltage-dependent K^+ current (I_M) which underlies the medium afterhyperpolarization (Charpak et al. 1990).
- The voltage independent Ba^{2+}-sensitive leak K^+ current (I_{kleak}) (McCormick and Krosigk 1992; Crepel et al. 1994; Guerineau et al. 1994).

The mGluR family			
Receptor	Effector Hippocampal expression sites	Pharmacology	PTX Sensitivity
mGluR1α mGluR1β mGluR1c mGluR1e	IP_3/Ca^{2+}, Arachidonic acid release, cAMP ↑ Dentate gyrus, CA2-4, non-pyramidal neurons in oriens and radiatum of CA1 (Conn and Desai 1991)	Quisqualate ACPD Insensitive to L-AP4 DCG-IV	Yes [2] No
mGluR5a mGluR5b	IP_3/Ca^{2+}, Arachidonic acid release ↑ Hippocampus, CA1-CA4 (Abe et al. 1992)		No
mGluR2	cAMP ↓ Granule cells of the dentate gyrus (Tanabe et al. 1992)	DCG-IV L-CCG-1 ACPD	Yes
mGluR3	cAMP ↓ Granule cells of the dentate gyrus (Tanabe et al. 1993)	Insensitive to L-AP4	Yes
mGluR4a mGluR4b	cAMP ↓ Dentate gyrus and CA3 (Tanabe et al. 1993)	L-AP4 L-SOP	Yes
mGluR6	cAMP ↓ Restricted to retina (Nakajima et al. 1993)		No
mGluR7	cAMP ↓ Dentate gyrus; Some expression in CA1-CA4 (Saugstadt et al. 1993, Okamoto et al. 1994)	Insensitive to ACPD & Quisqualate	Yes
mGluR8	cAMP ↓ Restricted to Olfactory bulb, and Mammillary bodies (Duvoisin et al. 1994)		Unknown

Table 3. A review of the classification, localization and pharmacology of the metabotropic glutamate receptors.

[2] In Oocyte (see Conn and Desai 91).

Additionally, mGluRs participate in slowing of the spike repolarization (Hu and Storm 1991) and could bring about a decrease in both excitatory (Baskys and Malenka 1991) and inhibitory (Desai and Conn 1991) synaptic transmission.

The recent discovery of Cis-aminocyclopentane-1,3-dicarboxylic acid (known as *trans*-ACPD) as selective mGluR agonist has greatly facilitated the study of the effects of mGluR activation in brain slices and primary cell cultures (Pallmer et al. 1989, Desai and Conn 1990). Stimulation of mGluRs by ACPD activates K^+ channels, including the hippocampal Ca^{2+}-dependent K^+ current of intermediate conductance (10-50pS) through the release of Ca^{2+} from intracellular stores (Shirasaki et al. 1994). Once all K^+ currents have been blocked, ACPD still generates an inward current associated with increase in input conductance. In the hippocampus, this current has now been identified and shown to be mediated by the Ca^{2+}-activated nonspecific cationic current (CAN current) (Crepel et al. 1994). CAN currents which had not been described previously in the mammalian CNS, are present in invertebrate neurons and cardiac cells (Swandulla and Partridge 1990). Additionally, selective activation of mGluRs, in hippocampus, also exerts a powerful modulation of GABA and glutamate ionotropic channels. ACPD reduces GABA receptor-mediated currents through a G-protein (Liu et al. 1993), and, via the activation of PKC, ACPD selectively increases NMDA but not AMPA receptor-mediated potentials (Aniksztejn et al. 1992; for contrary evidence see Berretta and Collingridge 1994).

The consequence of these plethora of effects on ionic channels in many brain structures is that mGluRs operate as a booster to enhance excitability. The associated decrease in cell polarity will in turn activate voltage dependent Ca^{2+} channels, which will amplify the excitatory drive even more. However, with further stimulation, mGluRs will halt synaptic transmission by inhibiting voltage-dependent Ca^{2+} channels. This paradigm providing a protective negative feedback prevents potentially excitotoxic stimulations (Baskys and Malenka 1991, Aniksztejn et al. 1992, Glaum and Miller 1992, Glaum et al. 1992). Therefore, mGluRs' activation will enhance the excitatory drive but will also act to prevent sustained excitatory stimulation. This property makes mGluRs highly suitable to provide a self-limiting increase in synaptic drive.

It has recently been demonstrated that receptors which couple to the G-proteins G_i/G_o can enhance the ability of G_s-coupled receptors to stimulate adenylyl cyclase in rat hippocampal cells. It is speculated that this effect is due to the liberation of $\beta\gamma$ subunits from G-proteins synergizing with activated G_s to stimulate certain isoforms of adenylyl cyclase (Gilman 1992; Andrade 1993). Since group 1 mGluRs are also G-protein-linked and expressed abundantly in the CA1 neurons, we expected the inhibition of the AHP by a β-receptor agonist to be enhanced when mGluRs are simultaneously activated. Thus using the AHP as a monitor, we investigated the interaction of these two receptors in adult rat hippocampal pyramidal cells.

METHODS

Sprague-Dawley rats (125-200g) were deeply anesthetized with halothane and decapitated. The brain was rapidly removed and placed in ice-cold ACSF containing (in mM) NaCl (118), KCl (2.5), $MgSO_4$ (1.5), $CaCl_2$ (2.5), NaH_2PO_4 (1.25), $NaHCO_3$ (24), and glucose (15), bubbled with 95% O_2/5% CO_2. The hippocampus was then rapidly dissected out and transverse slices of 400 μm thickness were made on a tissue chopper. The slices were maintained in an interface chamber (ACSF and humidified 95% O_2/5% CO_2 atmosphere) at room temperature for at least 1 h before removal for recording. Recording was done in submersion chambers at room temperature (~25° C), with the slices immobilized between nylon meshes. In all experiments, the slices were constantly superfused with ACSF and tetrodotoxin (1uM; Sigma) gravity fed from reservoirs. Whole-cell recordings were made with the "blind" method (Blanton et al., 1989) with patch pipets having resistances of 2.5 - 4 MΩ. The pipets were filled with solution containing (in mM) K-gluconate (128), HEPES (40), $MgCl_2$ (2), K_2ATP (4), Na_3 GTP (0.3), EGTA (0.6), creatine phosphate (20), and creatine phosphokinase (50 U/ml); adjusted to pH = 7.4 with KOH. Ionophoretic pipets filled with NE (100mM), and NaCl (200mM), with pH adjusted to 4.0. The AHP was evoked by clamping the cells at -40 mV and applying a brief depolarizing pulse (100 mV / 200 ms). For impalement studies, sharp electrodes containing 3 M KCl (R_e = 60-90 MΩ) were used. The AHP was evoked by a 100 ms, 1 nA depolarizing pulse from a membrane potential of -50 to -65 mV (constant throughout each experiment). Two methods were used to measure the AHP: the amplitude 1 s after the depolarizing pulse was measured directly from the traces,

or a single exponential decay was fit to the AHP beginning at 1000 ms after the pulse and the extrapolated amplitude of the AHP at t=0 determined. In a subset of 17 cells, the effect of agonists were determined using both methods, and the percentage of AHP inhibition agreed within -0.42 ± 1.98% (r=0.92). In all experiments the first agonist was delivered at least 20 min after impalement, when the basal AHP had been stable for at least 10 min. For intracellular injections, $PKCI_{19-31}$ (Sigma) was dissolved in 3M KCl and allowed to diffuse from the recording electrode. Isoproterenol, 8-Br-cAMP, Tetrodotoxin (all from Sigma) were dissolved in ACSF and applied in the superfusate. Phorbol-12,13-dibutyrate (Sigma) was prepared as a stock solution in dimethylsulfoxide, yielding a final bath concentration of 0.1% dimethylsulfoxide. Cis-1-aminocyclopentane-1,3-dicarboxylic acid, commonly known as *trans*-ACPD was purchased either from Sigma or RBI, Chelerythrine from Bachem, both of which were dissolved in the superfusate. Summary data are presented as group means with standard error bars. All drugs and agonists, but $PKCI_{19-31}$, were bath applied. All records were digitized and stored in a 80386SX Personal Computer. Data were analyzed using two-tailed t-tests and ANOVA followed by Newman-Keules when applicable. Membrane potential was amplified using the Axoclamp 2A amplifier (Axon instruments), which was also used to voltage-clamp the cells when necessary. When patch electrodes were used, the clamping was done in the continuous mode whereas with sharp electrodes the switch mode was applied. For experiments using Baclofen recording pipets were filled with QX-314 (1mM) to prevent the activation of the inwardly rectifying K^+ current (Andrade 1991). Amplitudes of AHP were measured using Axon's software (PClamp). Date were digitized and stored on floppy disks, using an IBM AT PC and Axoclamp interface and software (PCLAMP). Data were displayed

on the screen of the computer display, a Tektronix oscilloscope and a Nicolet storage scope. In addition, chart recordings of the resting potential were made using a Gould brush recorder. Summary data are presented as group means with standard error bars. In all traces, the response to the depolarizing current pulse is truncated.

RESULTS

Whole-Cell Patch-Clamp Studies of NE-ACPD Interaction

Winder et al. (1993), has biochemically shown that ACPD, a specific activator of mGluR receptors, can enhance the effect of isoproterenol on cAMP accumulation in adult rat hippocampus.[3] Using the I_{AHP} as a monitor of cAMP generation, we investigated whether the activation of mGluRs can intensify the effectiveness of β-adrenergics in the hippocampal pyramidal cells. In order to detect a possible interaction between these receptors, we utilized agonists concentrations near or below their EC_{50}'s for AHP inhibition.

Surprisingly, however, we observed an antagonism between the effects of NE and ACPD on I_{AHP}, using patch-clamp recordings. As shown in **Figure 23**, application of ACPD (10 μM for 5min) suppressed the I_{AHP} by 47% ± 6.5% (n=6), and NE (50nM for 2min) inhibited I_{AHP} by 51 ± 9% (n=7). However, when ACPD and NE were applied together, the

[3]After my work was completed, this effect was found to be mediated by mGluRs' of hippocampal glial cells, since the enhancement of cAMP production was abolished in the presence of glial cell poisons (Personal Communication).

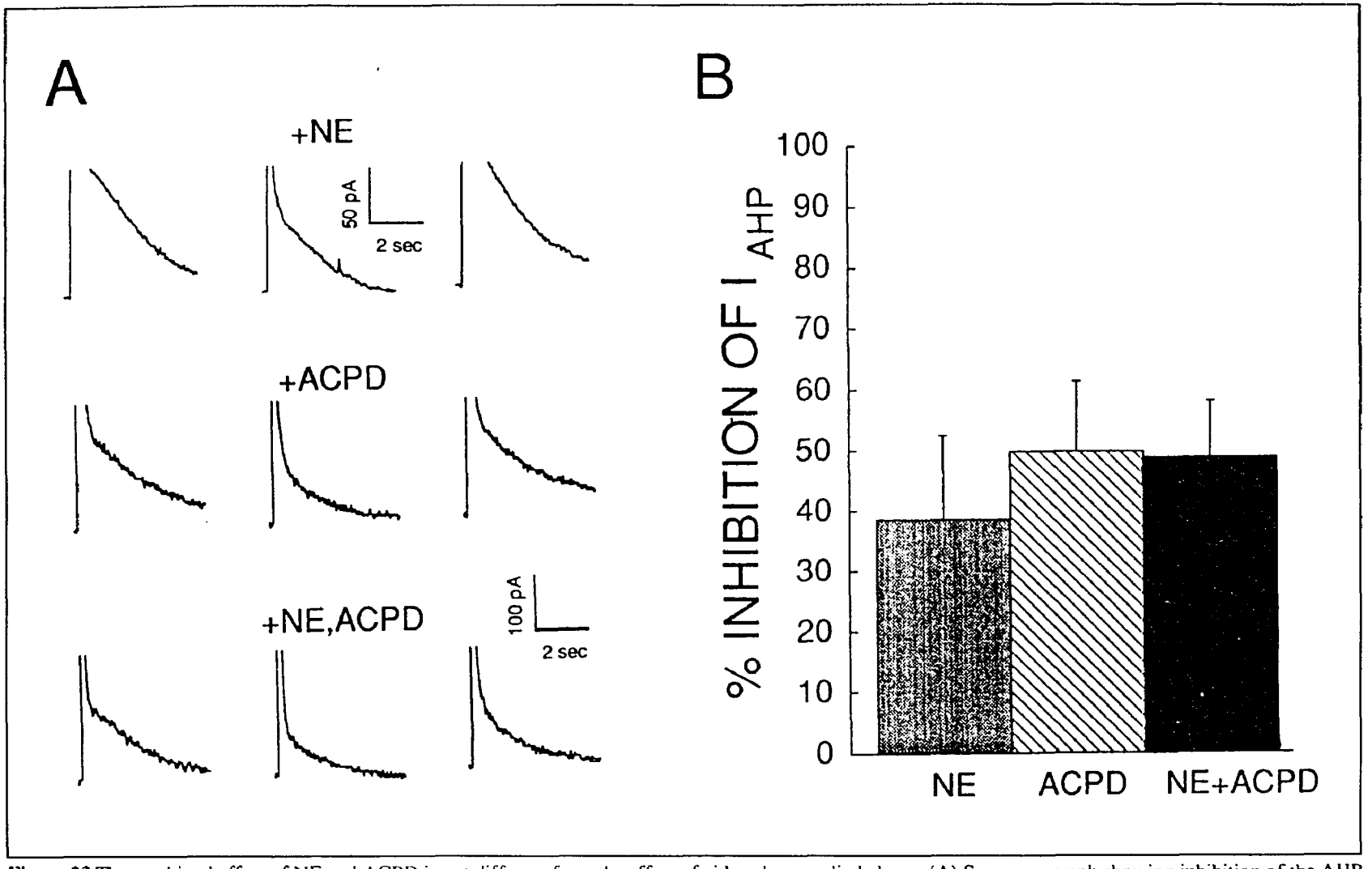

Figure 23 The combined effect of NE and ACPD is not different from the effect of either drug applied alone. (A) Summary graph showing inhibition of the AHP by co-application of NE and ACPD (n=5) not significantly differing from the individual application of NE (50nM for 2min, n=8) or ACPD (10uM for 5min, n=6). (B) In each set of three traces, the left trace illustrates AHP pre-agonist application, the middle trace the agonists peak effect, and the right trace the agonist's washout.

combined effect (48 ± 7%, n=7) was not statistically different from the effect of either drug alone ($p > 0.1$). Since the effects of the individual drugs were submaximal and 100% blockade of the I_{AHP} is demonstrable by higher concentrations of either agonist alone, the absence of any additional influence of the combined treatment does not reflect an occlusion. Rather, the data indicate that the effect of one or both drugs is blocked by the presence of the other. Thus, it appears that ACPD and NE interact at some stage of their signal transduction pathway. The locus of interaction could be at the level of the receptor, the transduction pathway, or further downstream at the AHP channel.

In order to ascertain that an additive or a synergistic effect is detectable in our system, as a control, we replicated the Andrade's (1993) observation using patch recording electrodes. In brief, sharp-electrode studies by Andrade point to the well-known phenomenon that applied alone at low concentrations, neither the GABA nor the 5HT, two G_i-coupled receptors agonists, substantially reduce the AHP. If the G_i-mediated effects of these agonists include inhibition of AC they would be expected to counteract the ability of isoproterenol to block the AHP (Madison and Nicoll 1986). Instead, both agonists do exactly the opposite--they potentiate the β-adrenergic effect, making isoproterenol a more effective antagonist on the AHP. I successfully replicated the Andrade experiments using a whole-cell configuration, and demonstrated the enhancement of both ionophoretic (**Figure 24**) and bath application (**Figure 25**) of NE response in the presence of Baclofen.

The Ionophoretic application of NE to the preparation reduced the I_{AHP} (**Figure 24**). Baclofen alone did not inhibit the I_{AHP}; however, when NE was reapplied in the presence of Baclofen, the I_{AHP} blockade was significantly enhanced (paired t-test, $p < 0.05$). At least 5 min after Baclofen washout, a third application of NE demonstrated that the inhibitory effect of NE on the I_{AHP} had recovered.

Unlike this group of experiments, all the following whole-cell experiments were done between cells and all drugs were applied in the bathing medium. Furthermore, the experiments were designed to avoid receptor-desensitization, to minimize excessive variation of drug effects, and to allow maximum washout possible.

Bath application of NE and Baclofen individually inhibited the I_{AHP} (**Figure 25**). The combined NE-Baclofen effect In the presence of Baclofen, was significantly greater than NE ($p < 0.025$) or Baclofen ($p < 0.025$) alone.

Since the interaction we observed on the I_{AHP} could have occurred at the level of the receptor, AC, PKA or the I_{AHP} channel, we utilized Forskolin to determine whether the inhibition of NE effect by ACPD is up or downstream of AC. ACPD and Forskolin by themselves inhibited the I_{AHP} (**Figure 26**). However, coapplication of ACPD with Forskolin, was not statistically different from the treatment with either drug alone. This experiment is suggestive of the NE-ACPD interaction to occur past the β-adrenergic receptor level.

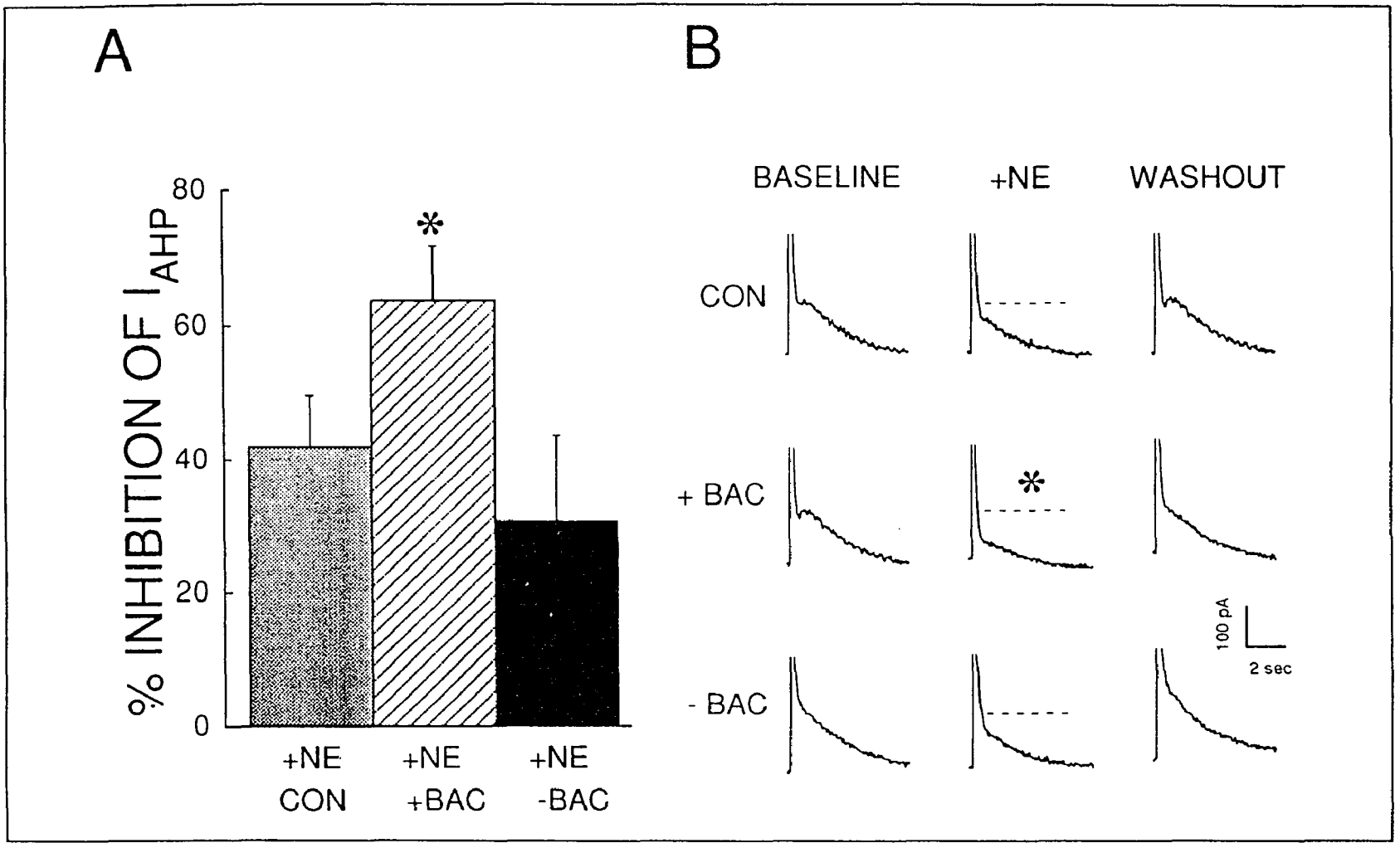

Figure 24 Ionophoretic application of NE in the presence of baclofen significantly reduces the size of the I_{AHP}. (A) Summary graph of paired experiments (n=6) showing the ionophoretic application of NE (10nA, 0.5min) before, during and after baclofen treatment (10uM). Baclofen was applied at least 15 min post NE application when I_{AHP} had recovered. (B) In each set of three traces, the left trace illustrates the AHP pre-agonist application, the middle trace the agonists peak effect, and the right trace the agonist's washout.

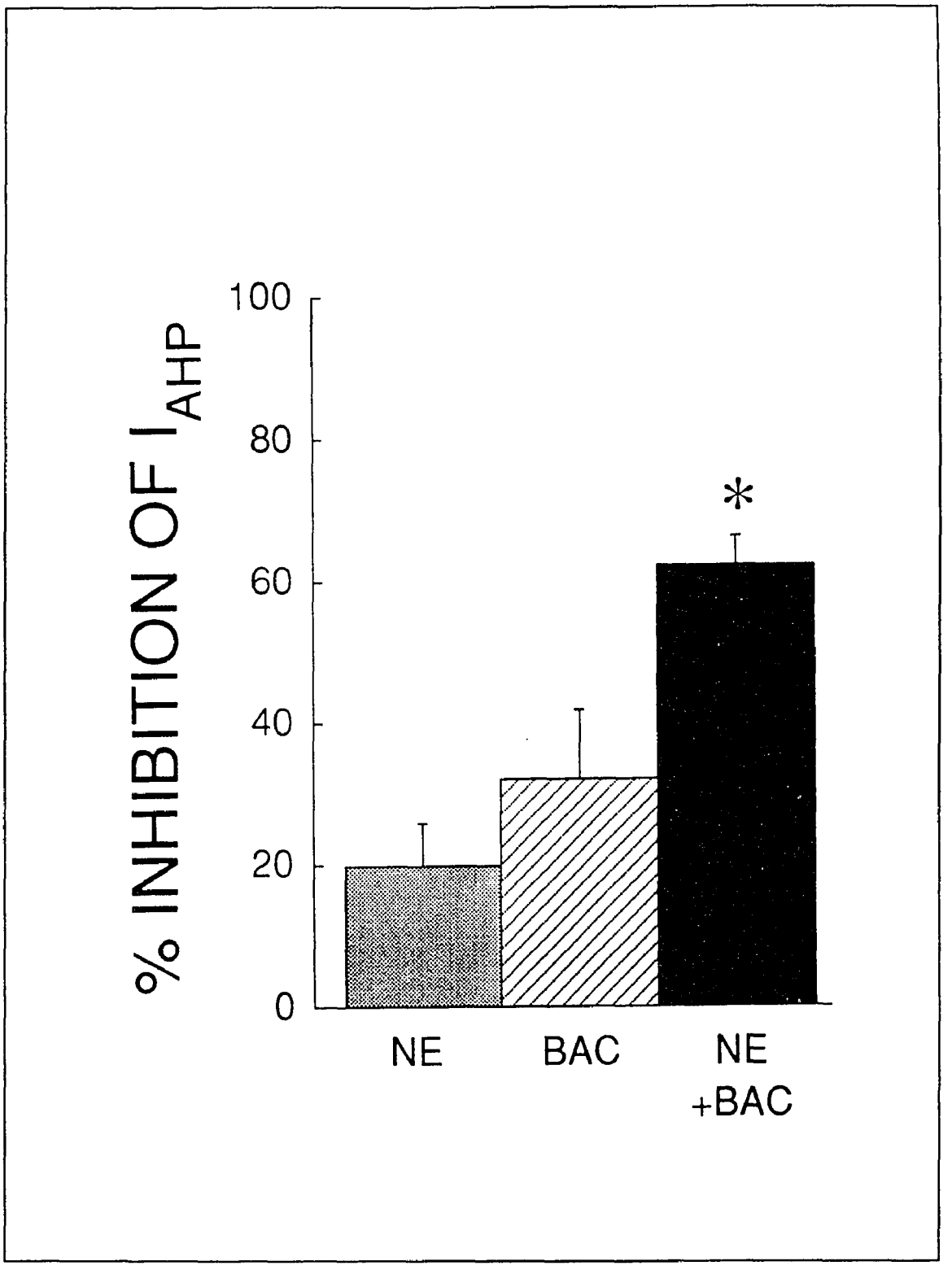

Figure 25 Bath application of NE in the presence of baclofen significantly reduces the size of the I_{AHP}. Summary graph showing inhibition of the I_{AHP} by co-application of NE and baclofen (n=5) significantly differing from the individual application of NE (50nM for 2min, n=8) or baclofen (10uM for 7-15min until a stable baseline was obtained, n=6).

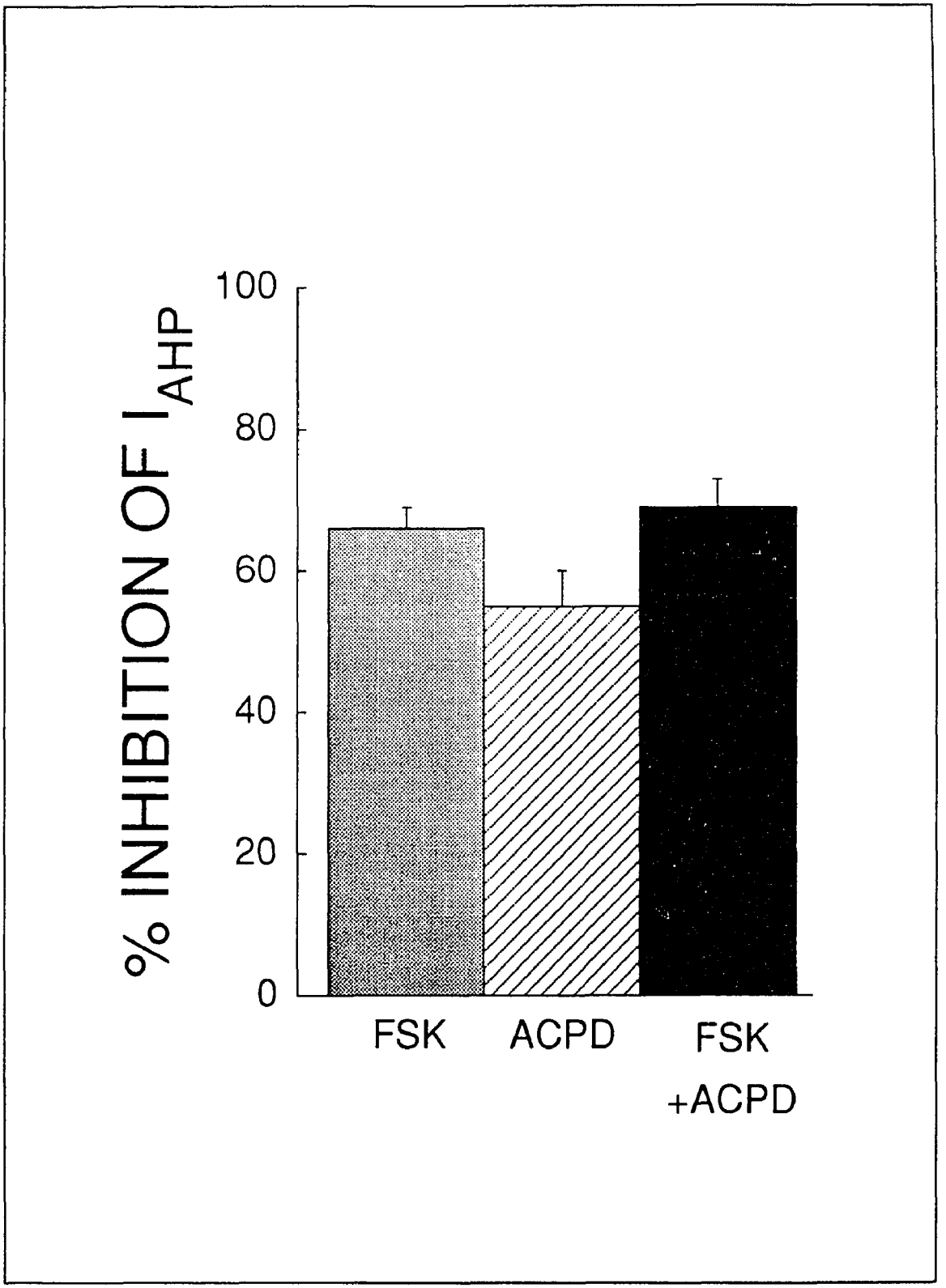

Figure 26 The combined effect of forskolin and ACPD is not different from the effect of either drug applied alone. Summary graph showing inhibition of the AHP by co-application of FSK and ACPD (n=5) did not significantly differing from the individual application of NE (10uM for 2min, n=5) or ACPD (10uM for 2min, n=12).

Sharp-Electrode Studies of ISO-ACPD Interaction

We subsequently confirmed the NE-ACPD interaction phenomenon with impalement studies, verifying that our observation is not an artifact of voltage-clamped patch recording. Here we substituted Isoproterenol (ISO), a more specific β-adrenergic agonist, for NE. In agreement with our whole-cell patch studies, the effect of combined ISO & ACPD, remained statistically indifferent with regard to its corresponding controls.

We utilized concentrations of ACPD and ISO near or below the EC_{50}'s of these drugs for AHP inhibition, in order to detect a possible interaction between the mGluR and β-adrenergic receptors. As shown in **Figure 27**, the combined effect of ACPD and ISO was not statistically different from the effect of either drug alone ($p > 0.5$). Since the effects of the individual drugs were submaximal, the absence of any additional influence of the combined treatment does not reflect an occlusion.

Gereau & Conn (1994) report a long lasting inhibition of AHP when ISO and ACPD (100 μM, 15 min) are co-applied. We saw this late washout effect only when we used a higher concentration of ACPD (100 μM, n=2, data not shown) in combination with ISO.

Since mGluR5 couples to the phosphatidyl inositol pathway, we attempted to mimic the effect of ACPD with an activator of PKC, PDBu. As expected (Baraban et al. 1985), PDBu alone inhibited the AHP (**Figure 28**). The PDBu effect was maximal at 10-20 min

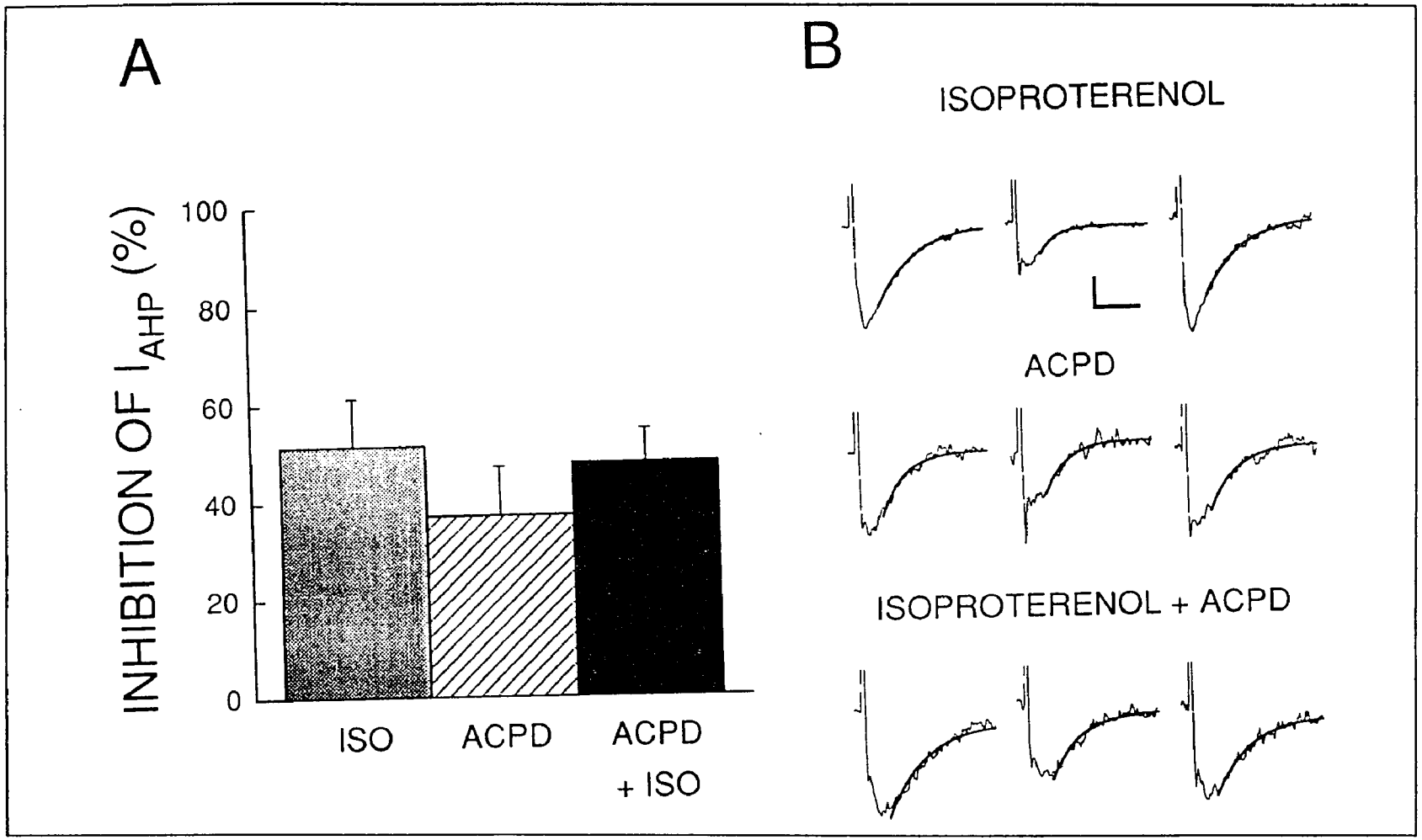

Figure 27 The combined effect of isoproterenol (ISO) and ACPD on the inhibition of AHP does not exceed the effect of either agonist alone. A: Summary graph showing the mean inhibition of the AHP by ISO (10 nM for 2 min, n=7), ACPD (20 μM for 2 min, n=6), and by the ISO-ACPD combination (n=7). There were no significant group differences ($F(2,17)=0.647$, $p>0.53$). B: In each set of three traces, the left trace illustrates the AHP before agonist application, the middle trace the peak agonist effect, and the right trace the agonist washout. Calibration: 2 mV / 2 s.

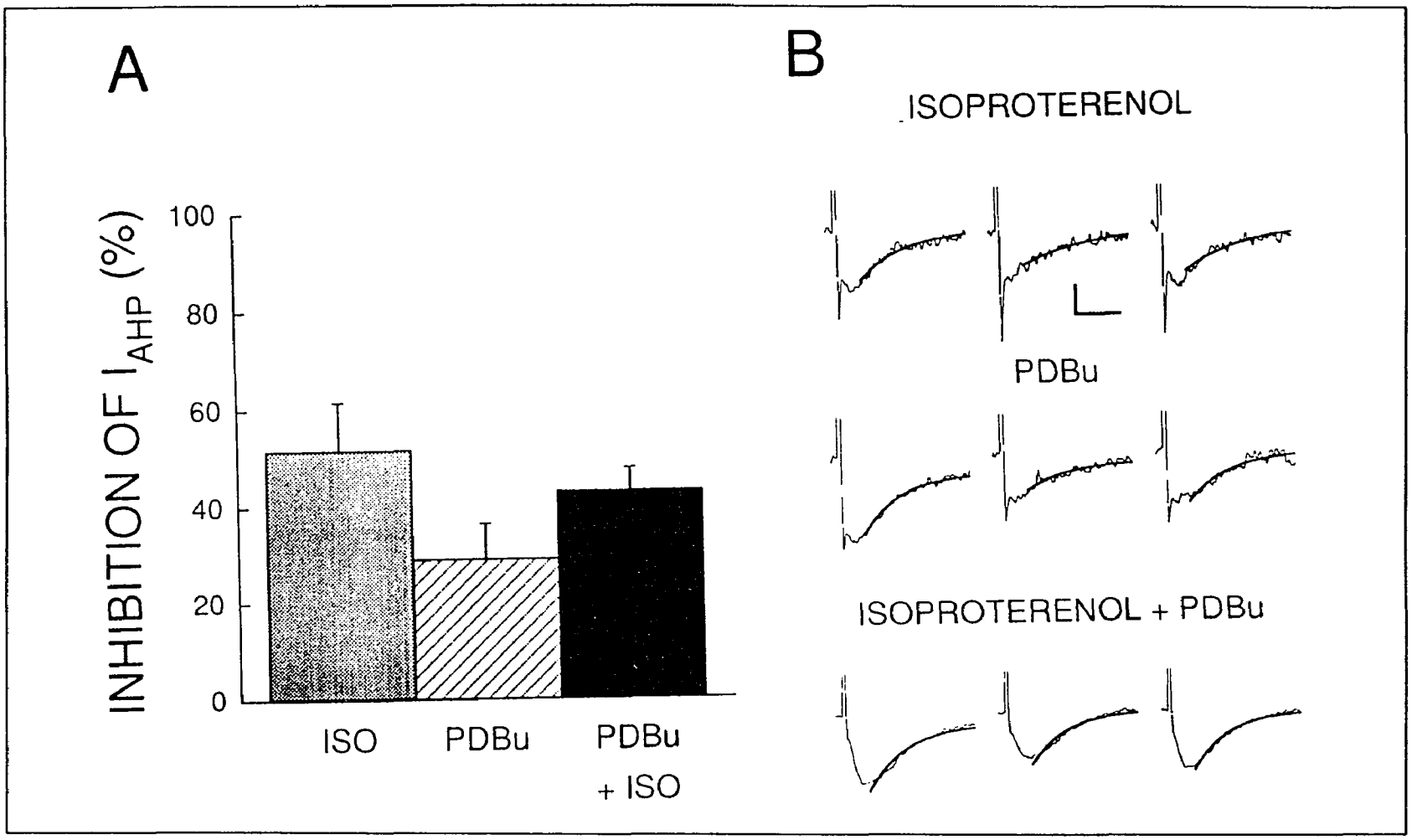

Figure 28 The effect of combined ACPD/ISO is mimicked by PDBu/ISO. A: Summary graph showing the mean inhibition of the AHP by ISO (10 nM for 2 min, n=7), PDBu (50 nM for 10 min, n=7) and by the ISO-PDBu combination (n=3). The effect of the combined treatment did not differ significantly from either agonist alone ($F(2,14)=1.912$, $p>0.184$). The ISO group represents the same data as shown in Fig. 27. B: In each set of three traces, the left trace illustrates the AHP pre-agonist application, the middle trace the peak agonist effect, and the right trace the agonist washout. Calibration: 2 mV / 2 s for the upper two rows of traces, 4 mV / 2 s for the bottom row.

washout, and AHP remained inhibited for an additional 20 min before any substantial washout of the PDBu effect was observed. Since maximal inhibition of the AHP by ISO alone was obtained relatively early (2-4 min washout), ISO was applied 10-20 min after the PDBu washout so that the peak effects of the two drugs coincided. The effect of the PDBu-ISO combination on the AHP was not statistically different from the effect of either drug alone ($p > 0.18$). β-adrenergic receptors do not themselves activate the phosphatidyl inositol pathway (Cotecchia et al. 1990), so the results of this experiment suggest that it is ACPD which reduces the effectiveness of ISO by activating PKC.

We therefore used specific inhibitors of PKC to determine if the ISO-ACPD interaction is PKC mediated. We included PKC_{19-31}, a known inhibitor of the PKC, in the recording electrode and let it diffuse into the cell for 30 min (**Figure 29**). PKC_{19-31} did not alter the effects of ISO or ACPD on the AHP when they were applied individually. However, the combined effect of ACPD and ISO was now significantly greater than the effect of either drug applied alone ($p < 0.002$). These results indicate that it is the mGluR-induced PKC activation which diminishes the effectiveness of the adenylyl cyclase pathway.

The results with PKC_{19-31} were confirmed using chelerythrine, a specific inhibitor of PKC (Young et al. 1994; L'hirondel et al. 1995). Chelerythrine (10 μM) was bath applied at least 30 min preceding addition of any other drug. Under these circumstances, chelerythrine alone slightly inhibited the AHP ($10 \pm 13\%$ n=3). To confirm that chelerythrine inhibits PKC at the planned concentration, we showed that the effect of a high concentration of PDBu was

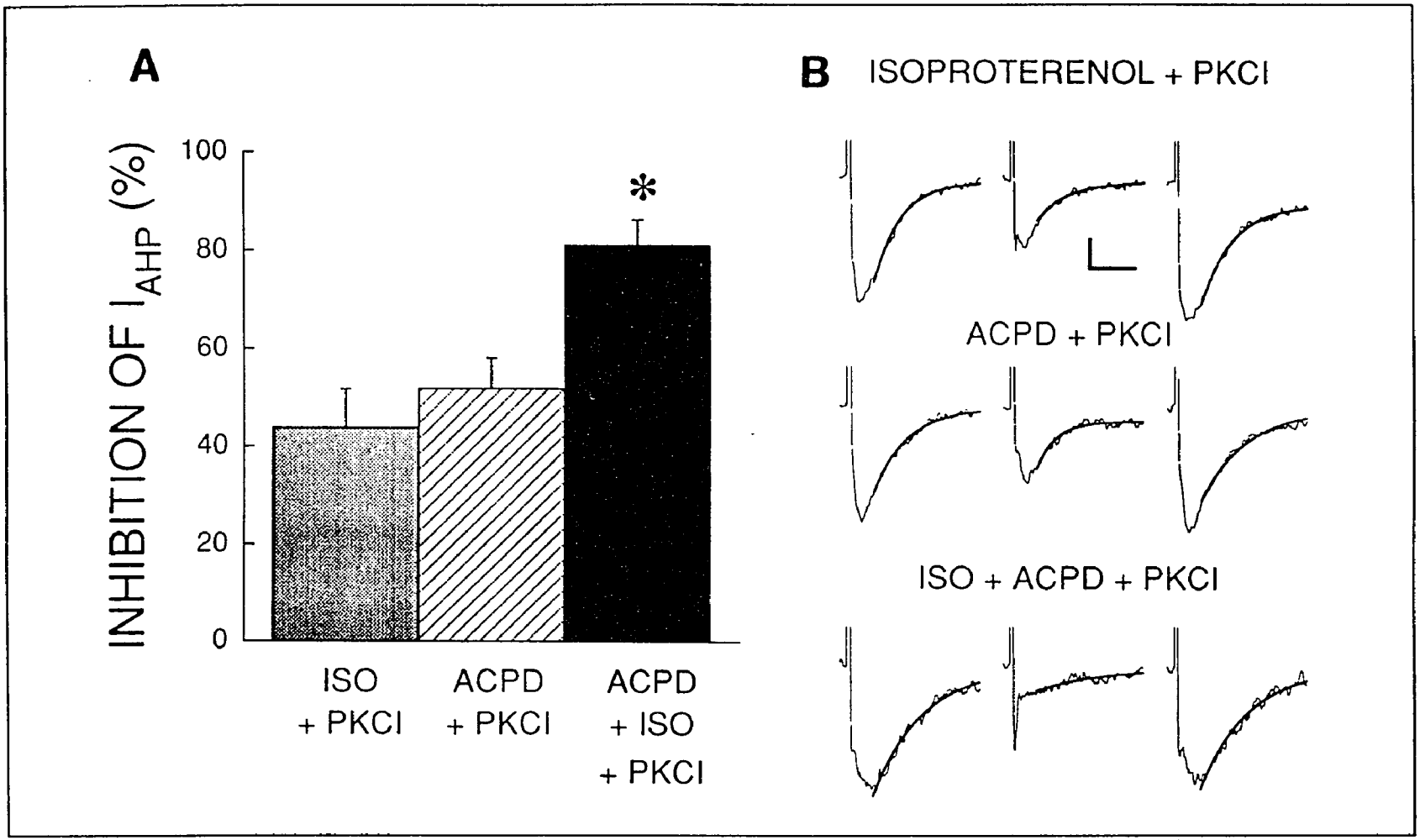

Figure 29 In the presence of $PKC_{19\text{-}31}$, the combined effect of ISO and ACPD is larger than that of either agonist alone. A: Summary graph showing, in the presence of $PKCI_{19\text{-}31}$ (300 μM), the mean inhibition of the AHP by ISO (10 nM for 2 min, n=5), ACPD (20 μM for 2 min, n=4) and by the ISO-ACPD combination (n=7). The asterisk indicates that combined effect was significantly larger than that of either agonist alone ($F(2,13)=11.00$, $p<0.0016$, Newman-Keuls). Note that the effects of ISO and ACPD were intact in the presence of $PKC_{19\text{-}31}$. B: In each set of three traces, the AHP pre-agonist application is shown in the left trace, the peak agonist effect in the middle trace, and the washout in the right trace. Calibration: 2 mV / 2 s.

significantly reduced in the presence of chelerythrine (**Figure 30**). Moreover, the inhibition of the AHP by ACPD (applied at both low and high concentrations) and ISO remained intact in the presence of chelerythrine (**Figure 31** Compared to **Figure 27**). Thus it is concluded that the effects of ACPD and ISO on the afterhyperpolarization are not dependent on PKC activity.

Like PKC_{19-31}, chelerythrine resulted in a combined ACPD-ISO effect which exceeded the individual effects of either drug (**Figure 31**, $p < 0.006$). The data indicate that the inhibition of the AHP per se by ACPD is not PKC mediated, yet PKC serves as the arbitrator to limit the ISO effect when mGluRs are activated.

To determine whether the inhibition of ISO effect by ACPD is up- or down-stream of adenylyl cyclase, we attempted to mimic the ISO-ACPD interaction by combination of 8-Br-cAMP and PDBu. This combination resulted in an inhibition which was significantly different from the effect of 8-Br-cAMP alone ($p < 0.02$, **Figure 32**). The mimicry of the ACPD-ISO interaction indicates that the interaction occurs at or downstream from the cAMP-dependent protein kinase. This interaction, in theory, could take place at either PKA, the AHP channel, or some intermediary modulatory protein.

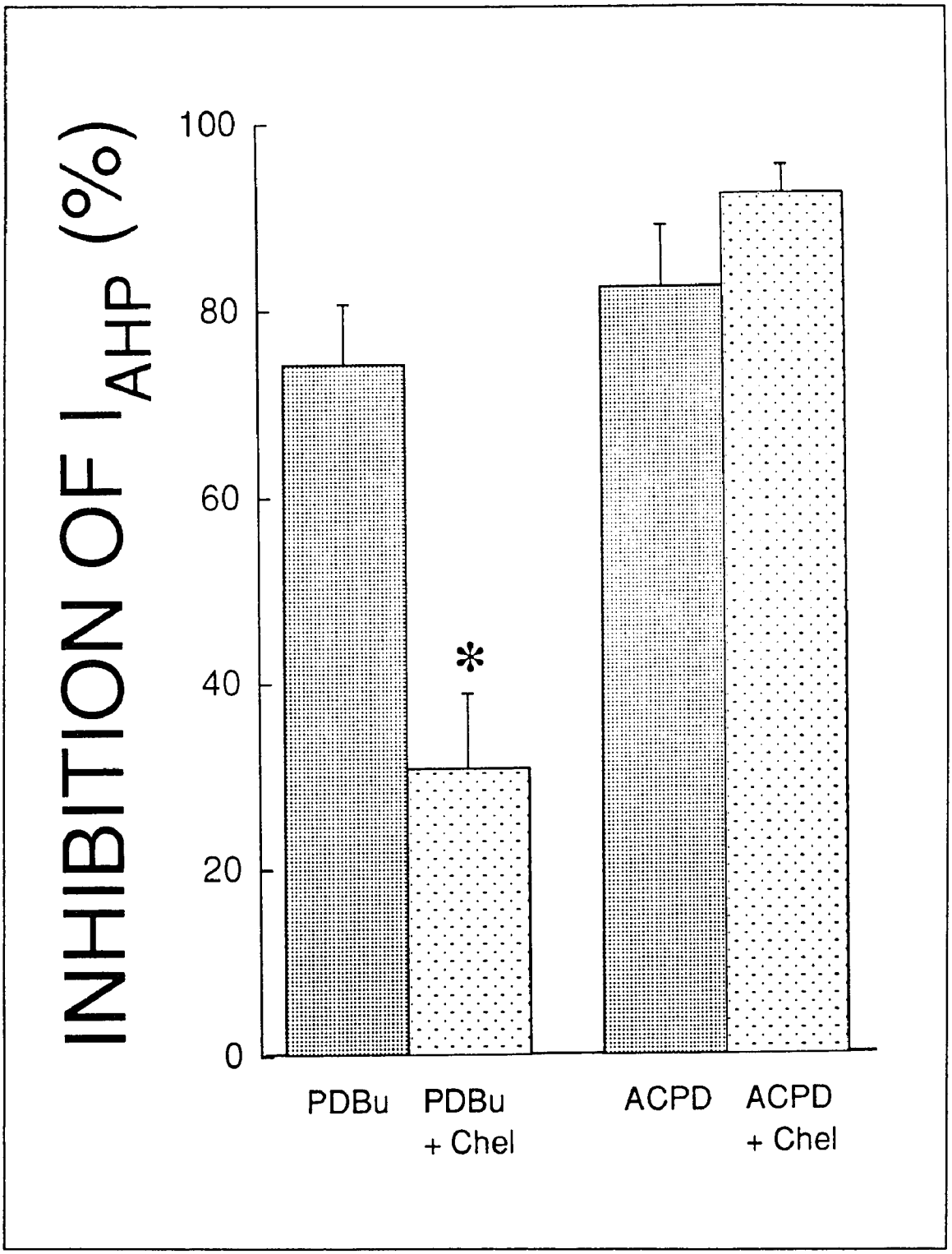

Figure 30 The inhibition of the AHP by ACPD is intact in the presence of the PKC inhibitor chelerythrine. However, chelerythrine does prevent PDBu from blocking the AHP. Left: Summary graph showing the mean inhibition of the AHP by PDBu (10 μM for 2 min) in the absence (n=5) and presence (n=5) of chelerythrine (10 μM). Chelerythrine significantly decreased the effect of PDBu ($p < 0.01$). Right: the effect of ACPD (50 μM for 2 min) in the absence (n=5) and presence (n=5) of chelerythrine. The groups did not differ significantly.

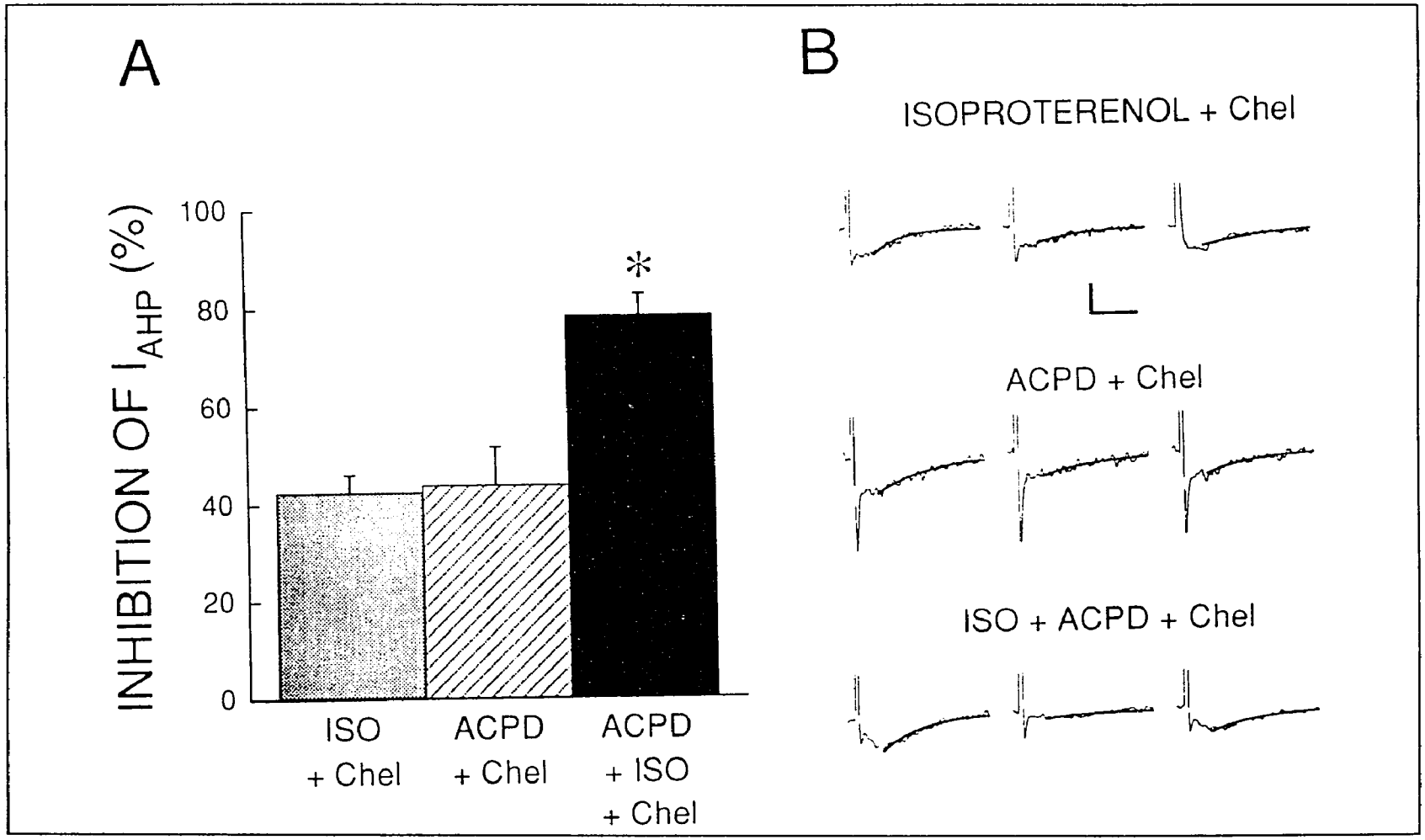

Figure 31 In the presence of chelerythrine the combined effect of ISO and ACPD is larger than that of either agonist alone. A: Summary graph showing, in the presence of bath applied chelerythrine (10 μM), the mean inhibition of the AHP by ISO (n=5), ACPD (n=4) and by the ISO-ACPD combination (n=5). The asterisk indicates that combined effect was significantly larger than that of either agonist alone ($F(2,10)=9.110$, $p<0.0056$, Newman-Keuls). Note that the effects of ISO and ACPD were intact in the presence of chelerythrine. B: In each set of three traces, the AHP pre-agonist application is shown in the left trace, the peak agonist effect in the middle trace, and the washout in the right trace. Calibration: 2 mV / 2 s.

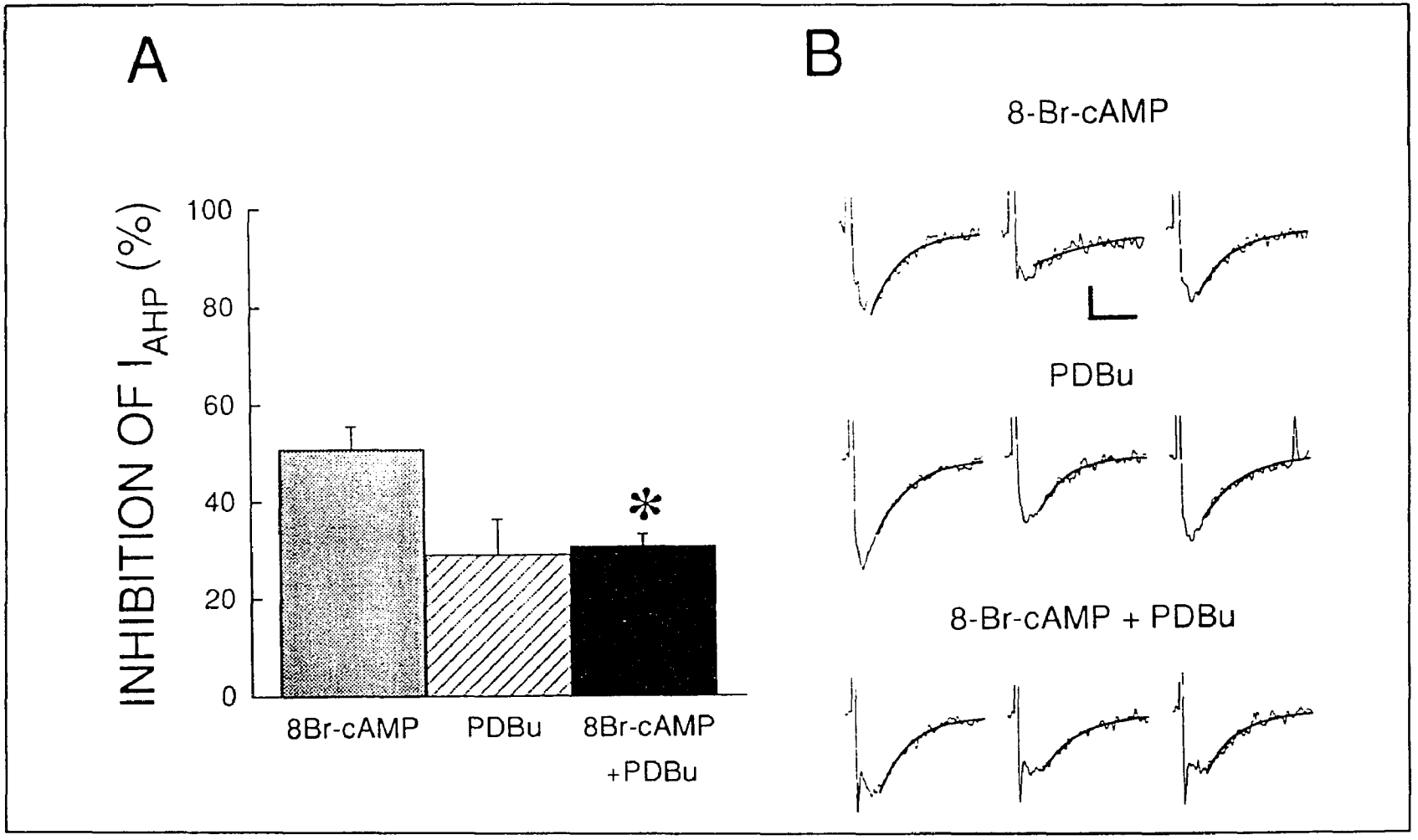

Figure 32 Combined treatment with 8-Br-cAMP and PDBu mimics the effect of ISO/ACPD. A: Summary graph showing the mean inhibition of the AHP by 8-Br-cAMP (bath applied for 10 min, reaching its peak effect at 0-4 min washout, n=7), PDBu (n=7; same data as shown in Fig. 28) and by the 8-Br-cAMP/PDBu combination (n=6). For the combination effect, 8-Br-cAMP was applied at least 10 min after PDBu washout so that the peak effects would coincide. The combined 8-Br-cAMP/PDBu group significantly differs from the 8-Br-cAMP group ($F(2,17)=5.057$, $p<0.02$, Newman-Keuls). B: In each set of three traces, the left trace illustrates the AHP before agonist application, the middle trace the peak agonist effect, and the right trace the washout. Calibration: 2 mV / 2 s.

DISCUSSION

The mechanism by which mGluR5 inhibits the AHP has not been established. However, the involvement of PKA, CaMKII, and PKC has been ruled out (Pedarzani and Storm, 1993; Muller et al., 1992; Gerber et al, 1992). We have confirmed, using PKC_{19-31} and chelerythrine, that the blockade of the AHP by mGluRs is not mediated by PKC. However, mGluR5 does couple to PKC (Sugiyama 1987, Stratton et al. 1990; Hu and Storm 1991; Abe et al. 1992), and our data demonstrate that PKC limits the cAMP-mediated suppression of AHP.

It is intriguing that activation of mGluR both inhibits the AHP and prevents further inhibition of the AHP by the cAMP pathway. This phenomenon defines a hierarchical relationship between two signal transduction pathways which converge on a common effector, with one of the pathways governing the other.

This relationship between mGluR and β-adrenergic receptors can be contrasted with the findings of Andrade (1993). It has been suggested (Andrade 1993; Bourne and Nicoll 1993) that this effect is due to the liberation of βγ subunits from G_i/G_o, which synergize with activated G_s to stimulate certain isoforms of adenylyl cyclase (Tang et al. 1992). The suppressive, rather than facilatory, effect of mGluR activation on the β-adrenergic response may reflect fewer βγ subunits liberated by the activation of G_q, which is not as abundant as G_i/G_o in area CA1 (Pang and Sternweis 1990). Alternatively, the specific limiting effect of

PKC on the adenylyl cyclase pathway may have masked any upstream potentiating effect of βγ subunits on G_s-induced adenylyl cyclase activation.

Our data demonstrate the prioritization of a PKC-linked receptor function. Other instances of PKC suppressing the function of various receptors can also be found. PKC in hippocampus has been shown to inhibit the 5HT effect on $[Ca^{2+}]_i$ accumulation (Takata et al. 1988), to suppress the quisqualate-induced formation of IP_3 (Manzoni et al. 1990), to block the membrane hyperpolarization produced by $GABA_B$ or adenosine A1 receptors (Worley, et al. 1987), to inhibit agonist binding to dopamine D_2 receptors (Rogue, et al. 1990), and to suppress the inhibition of the synaptic transmission by norepinephrine (Scanziani et al. 1993). The novelty of our finding manifests in two parts: 1. PKC acts at the channel, as opposed to the receptor, level to inhibit a response. 2. Unlike the carbachol-GABA interaction, where agonists carry out disparate functions, i.e. carbachol depolarizing and GABA hyperpolarizing the cell (Worely et al. 1987), here both ISO and ACPD serve the same function (to inhibit the AHP).

A model for the interaction of the PKA-PKC pathways on the AHP channel is presented in **Figure 33**. It is well known that either PKA or PKC activation can lead to the inhibition of the AHP. Although PKC phosphorylation sites maybe present on PKA, to the best of our knowledge there is no evidence in any system that such phosphorylations actually occur. Thus, it more likely that the PKA-PKC interaction site is at an intermediary protein such as A-kinase anchoring protein (Rosenmund et al. 1994), or at the AHP channel.

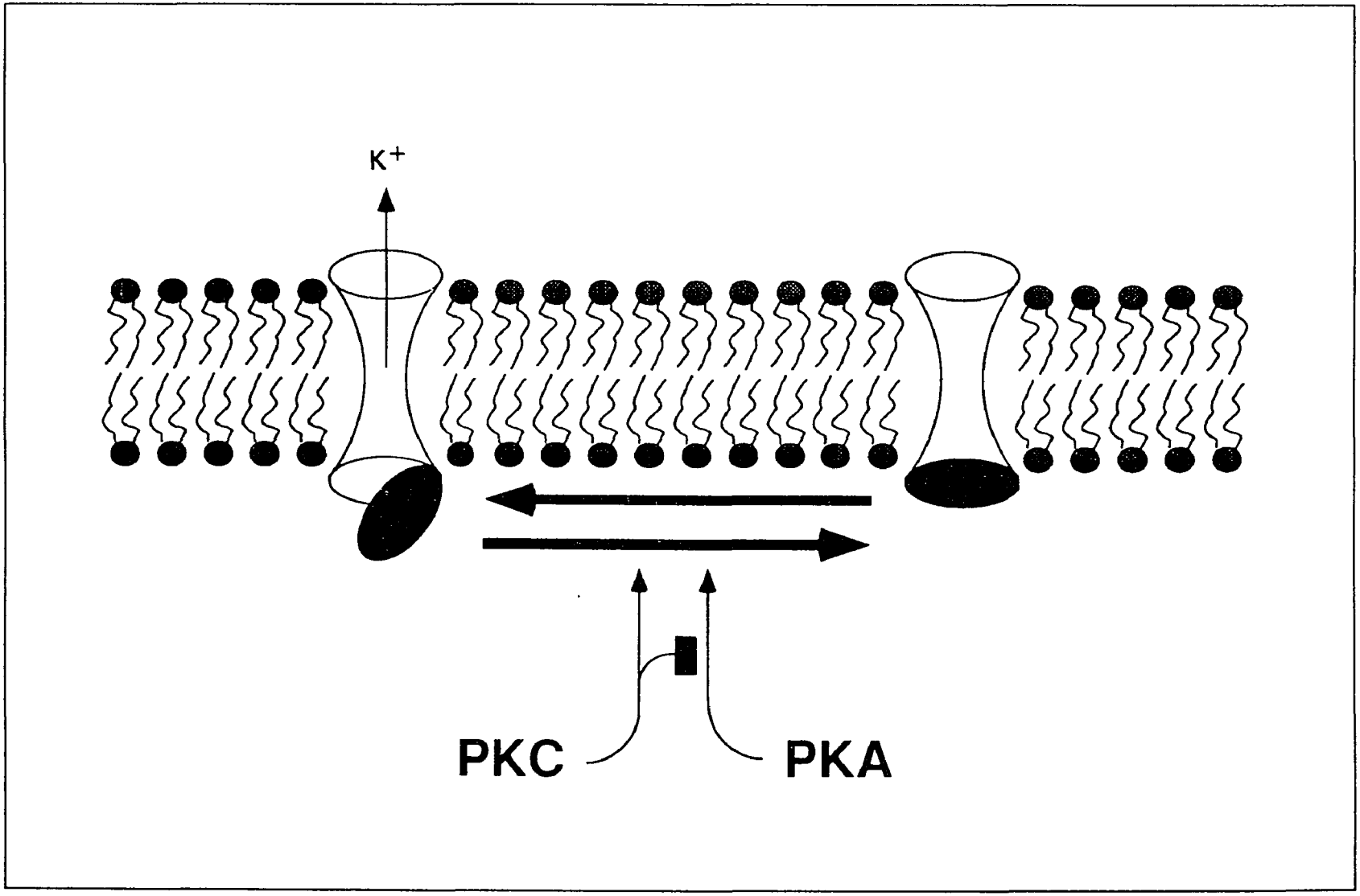

Figure 33 Schematic representation of the AHP channel, and its possible modes of interaction with PKA and PKC.

According to our model, PKC phosphorylates a negative allosteric modulatory site which blocks the effect of PKA-dependent phosphorylation of this channel. This site is not the site through which ACPD inhibits the AHP, since this effect is not blocked by PKC inhibitors. Either PKC precedes PKA in phosphorylating the channel and the induced conformational change hinders PKA's access to its proper site, or PKA still has access but further conformational change is barred. Identification of possible specific sites for this interaction must await cloning of the channel underlying the slow AHP.

Multisite and hierarchical protein phosphorylation resulting in positive or negative allosteric interactions is a well-documented phenomenon (Yeaman 1990; Haystead et al. 1990; Roach 1991). Specifically, proteins and ion channels sequentially phosphorylated by both PKA and PKC have been characterized. For instance, activation of either PKA or PKC dramatically increases the expression of tyrosine hydroxylase. However, simultaneous activation of both pathways has a less than additive effect on enzyme expression (Kedzierski, et al. 1994). In addition, Li et al. (1993) demonstrated positive allosteric interaction between PKA- and PKC-induced phosphorylation, resulting in synergistic closing of rat brain Na^+ channels expressed in CHO cells.

Our demonstration of an interaction between the PKA and the PKC pathways on the AHP, which regulates neuronal responsiveness (Storm 1993), reveals a potentially important motif of interaction between these two pathways. It has been suggested that cellular damage resulting from excessive release of glutamate in the hippocampus may be reduced by K^+

channel openers and conversely increased by K^+ channel blockers (Zini et al. 1993). We have established that activation of mGluRs prevents cAMP pathway-mediated inhibition of the AHP. This phenomenon is consistent with a general neuroprotective role for the mGluRs. mGluRs are known to protect against neurotoxicity (Shinozaki et al. 1994), to prevent apoptosis (Copani et al. 1994), and to accelerate recovery from ischemic injuries (Chiamulera et al. 1992). Under conditions of excessive synaptic stimulation (e.g., during seizure activity), the activation of mGluRs may serve to preserve the AHP and thereby limit the neuronal damage caused by excitatory neurotransmitters (Zhao and Leung 1993; Wasterlain and Shirasaka 1994).

More generally, our data demonstrate a hierarchical relationship between two distinct receptor types converging on a common effector. This arrangement may permit the neuron to discriminate between receptors activated in temporal proximity, so that the effect of a neurotransmitter is modulated (in the present case, limited) by its temporal relationship to the release of a different, dominant neurotransmitter.

General Conclusion:

In the mammalian brain, LTP has been proposed as a synaptic mechanism underlying memory formation (Bliss and Collingridge, 1993). LTP has been most extensively studied in the Schaffer collateral - CA1 pathway of hippocampus, where it is generally accepted that the primary event in the induction of LTP is the elevation of postsynaptic Ca^{2+} (Lynch et al., 1983; Malenka et al., 1988; Malenka et al., 1992). The Ca^{2+} signal may be transmitted through the activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; Pettit et al., 1994; Silva et al., 1992). Other protein kinases implicated in LTP include protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) (Malinow et al., 1989; Frey et al., 1993; Hvalby et al., 1994).

At the time of this undertaking, the mechanism of the cAMP dependence in early LTP was not known, even though it was surmised that different mechanisms underlie the PKA dependence of the early and late phases of LTP. The late phase was known to rely upon protein synthesis, and it had been suggested that PKA contributes to late LTP by regulating protein expression (Nguyen et al., 1994).

To further elucidate the role of the cAMP pathway in LTP, we have investigated the mechanism by which this pathway contributes to early LTP. In summary (Chapter One), high-frequency stimulation (HFS) capable of producing LTP, shortly after its application,

transiently suppressed the AHP, which is known to be blocked by cAMP-dependent protein kinase. Inhibiting PKA by postsynaptic injection of either the regulatory subunit of the enzyme or Rp-cAMPS blocked the AHP suppression, indicating that HFS inhibited the AHP by elevating postsynaptic cAMP levels. The effect of HFS on AHP was also blocked by NMDA receptor inhibition and by postsynaptic injection of a calmodulin antagonist peptide, indicating that both Ca^{2+} entry through NMDA receptor channels and Ca^{2+}/calmodulin-dependent adenylyl cyclases are involved in the postsynaptic generation of cAMP.

Direct assays of LTP (Chapter Two) showed that postsynaptic injection of Rp-cAMPS, but not the cGMP-dependent protein kinase inhibitor, Rp-8-Br-cGMP, blocks intracellular LTP. In the same experiments, LTP measured in the CA1 field was intact. However, postsynaptic injection of cAMP or its analog Sp-cAMPS did not increase the synaptic response of the CA1 neuron, even though the AHP was blocked. Upon the postsynaptic injection of phosphatase inhibitors, the early LTP became cAMP-independent. From these studies it appears that the cAMP and cGMP pathways influence LTP at different loci. Furthermore these observations indicate that in early LTP the cAMP pathway, instead of transmitting signals for the generation of LTP, gates LTP through postsynaptic protein phosphatases. Thus, our data reveal the critical importance of a signaling pathway which plays a modulatory rather than a transmittal role in controlling cellular behavior.

Whether mGluRs play a role in hippocampal LTP is a matter of controversy. mGluRs in CA1 hippocampus are known to activate PKC and generate cAMP, yet mGluR's

suppression of the AHP has been shown to be independent of these elements. The currently available compounds, such as MCPG, are weak mGluR antagonists, and their specificity for the mGluR subtypes is poorly defined. Subsequently, researchers using MCPG as their sole antagonist have obtained ambivalent results (Reymann and Matthies 89, Izumi et al. 92, Bashir et al. 93; for contrary evidence see Manzoni et al. 95). Many speculations and hypotheses regarding MCPG currently exist. One group has suggested that this drug only blocks the mGluRs in neonatal rat hippocampus (Izumi and Zorumski 94); however, there is no evidence that the expression of the mGluR5 subtype is developmentally regulated (Catania et al. 94). Since we investigated the effects of mGluRs on the cAMP pathway, and also studied the role of cAMP in early LTP, our data could shine light on the issue of mGluR activation during the induction of LTP.

mGluR5 is G_q-protein coupled, and liberation of $\beta\gamma$ subunits from G-proteins have been shown to synergize the activity of certain AC isoforms. We therefore hypothesized that direct stimulation of AC concurrent with mGluR activation would potentiate cAMP formation.

Using the AHP as an assay, we investigated the interaction between the metabotropic glutamate and β-adrenergic signaling pathways (Chapter Three). Both ACPD and NE/ISO blocked the AHP; however, their combined effects did not exceed that of either drug alone. A larger effect of the combined agonists was obtained upon the PKC blockade. Interestingly, the inhibitory effect of ACPD on AHP remained intact in the presence of PKC inhibitors. The

interaction of mGlu and β-adrenergic pathway was mimicked by the combination of 8-bromo-cAMP and phorbol esters. The results indicate that the locus of ACPD-NE/ISO interaction is either at the kinase level or further downstream at the AHP channel level. Most importantly, expressed as an assignment of ranks to different neurotransmitters acting on a common effector, the metabotropic glutamate pathway coupled to PKC seems to overshadow and limit the β-adrenergic pathway's inhibition of the AHP.

Taken collectively, however, these data present a problem. On one hand our data (Chapter One) establish the tetanus-induced suppression of the AHP to be exclusively PKA dependent. On the other hand, our results demonstrate (Chapter Three) that activation of mGluRs precludes inhibition of the AHP by the cAMP pathway. If the mGluRs, whose blockade of the AHP is <u>not</u> PKA mediated, are indeed activated during the LTP induction, how could the AHP suppression following a LTP-inducing tetanus be totally PKA dependent?

In our studies (Chapter One), the AHP suppression following tetanus was monitored in 1 min intervals, and the first AHP measurement was obtained 1min after the tetanus delivery. The maximal suppression of the AHP was usually observed at one and sometimes two minutes post tetanus. Studies by Charpak et al. (1990) in hippocampal slice cultures showed that activation of mGluRs via a single electrical pulse to the mossy fibers results in diminution of the AHP. These studies also demonstrated that once NMDA and AMPA/Kinate channels are all blocked, the same stimulation protocol can evoke an inward current which does not desensitize and yet lasts for 30 seconds at most. Based on Charpak's latter

observation, we suggest that the mGluRs effect might have dissipated by the time our first AHP measurement, after the delivery of HFS, was noted. On the other hand, however, this might explain why the peak HFS effect on the AHP was sometimes observed at 2 min post tetanus. Since the mGluRs antagonize the cAMP-dependent inhibition of the AHP, the peak effect of the cAMP was not revealed until the mGluR's influence had ceased.

In brief, learning and memory have risen in all species as "the adaptive strategy" against a rapidly-changing and nonpredictable environment. Yet, in the human culture, learning has become so crucial an activity that the very existence of the culture depends on the ability of its members to learn sets of skills, norms of behavior, facts, beliefs, and so on. In fact, a substantial amount of resources have been invested in creating educational institutions devoted solely to learning so that the majority of people can spend a large proportion of their lives not in doing, but rather in learning to do. Questions about the "best" way to enhance performance are asked by practitioners in a variety of fields and training programs, and large markets exist for techniques to boost performance. It is therefore no wonder that research on memory and learning has been part of psychology since it began as a science in 1800's. At the height of the behaviorist era, around 1950, learning was perceived as the key issue in psychology, and learning was further emphasized from the center stage by the cognitive movement of the 1960's. Since then, cognitive psychology has become increasingly interested in the adaptive function of human memory, which has brought in the biological perspective.

We are exceptional in how adaptable and plastic we are behaviorally; we can learn to live in the world of the Stone Age or in the weightless world of an astronaut orbiting the earth. Yet, the mechanistic knowledge of our own plasticity remains at its infancy. The central nervous system is currently undergoing intense investigation, and this dissertation presents only a glimpse of the basic-science research-efforts invested on some of the issues related to learning and memory. Nevertheless, this work offers a novel perspective on a number of key issues and reveals promising avenues for further exploration of the field of neuroscience.

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