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THE CHARACTERIZATION AND PHYSIOLOGY OF ON- and OFF- BIPOLAR CELLS IN THE SKATE (*RAJA ERINACEA*) BY WHOLE CELL PATCH CLAMP ANALYSIS

by

Frederick J. Rosenstein

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

THE CHARACTERIZATION AND PHYSIOLOGY OF ON- AND OFF-BIPOLAR CELLS IN THE SKATE (*RAJA ERINACEA*) BY WHOLE CELL PATCH CLAMP ANALYSIS

by

Frederick J. Rosenstein

Advisor: Professor Richard L. Chappell

This investigation of the skate (*Raja erinacea*) all-rod retina has identified, through electroretinogram (ERG) and whole-cell voltage clamp recording, independent ON and OFF afferent pathways in outer retina that are attributed to two types of bipolar cells. ON and OFF components of the ERG response could be selectively suppressed with targeted blocking agents. The application of L-2-amino-4-phosphonobutyrate (APB) to block the ON pathway plus picrotoxin (PTX) to inhibit GABAergic input left a functional OFF response that could be suppressed with a solution of APB, PTX plus 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid (KYN)). *N*-methyl-D-aspartate (NMDA), known to be an inner retina blocker, when added to the solutions had no observable effect on the ON and OFF responses. These results suggested the presence of ON and OFF bipolar cells.

Through a comparative study of isolated bipolar cells, using glutamate specific agonists, in conjunction with whole-cell voltage clamp recording, ON and OFF

bipolar cells were identified. Skate ON bipolar cells have metabotropic glutamate receptors that are responsive to the agonists APB and trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD), while the OFF bipolar cells have ionotropic glutamate receptors responsive to the agonists kainate and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). The reversal potential for both cell types are near 0mV and their currents are in opposite directons to each other at positive and negative holding potentials.

In a secondary study, zinc superfused in conjunction with glutamate onto bipolar cells greatly suppressed glutamate-mediated currents in both ON and OFF bipolar cells. Supporting evidence was garnered from ERG experiments. The ON and OFF pathways were isolated with PTX and then using histidine (HIS) as a zinc chelating agent, a solution of PTX plus HIS caused the ON response to become larger. The effect is consistent with findings from isolated bipolar cells. Zinc has an inhibitory effect on the glutamatergic responses in ON and OFF bipolar cells, and chelating zinc would reduce such action. An important conclusion from this ERG experiment is that endogenous zinc in the retina has a neuromodulatory effect in the intact retina.

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Introduction:

Basis for this study:

The all-rod retina of skate (*Raja erinacea and R. ocellata*) may well be unique among vertebrates because of its lack of cones. Nevertheless, the skate retina also has the unusual capability to function, under bright light conditions with an exquisite sensitivity that far exceeds the limits of any other rod system. Other vertebrate retinae, including nocturnal animals, to varying degrees have both rod and cone input, where the rods operate under lighting conditions of low intensity (scotopic vision) and the cones are active at higher intensity (photopic vision). Typically, rods have a greater sensitivity to light than cones (by a factor of 25 times in the mudpuppy (Fain and Dowling, 1973)) and a slower temporal resolution. Cones, however, are suited for bright light conditions, because they do not saturate at such low intensities and have a higher temporal resolution. Moreover, cone systems provide color vision, which results from the presence of two or more types of cone photoreceptors each with its own spectral sensitivity. Most rod systems are achromatic, since they generally possess a single class of rod photoreceptors that are of the same spectral sensitivity.

Rods, in general, saturate several log units before cones, but a particular type of rods in geckos and "red" rods in marine toads have extended light-adapting abilities (Fain, 1974; Kleinschmidt and Dowling, 1975). Skate rods tested with incremental increases in background light were found responsive to an intensity as high as 8 log units above absolute threshold. It is the largest range of any rod

system yet reported, and they adapted in accordance with the Weber-Fechner relationship (Dowling and Ripps, 1972; Dowling, 1987). Furthermore, skate rods recorded using suction electrode techniques, assumed cone-like characteristics when light adapted. They lost their characteristic tail currents and developed fast photocurrents (Cornwall et. al, 1989) as mean intensity increased. Cone-like qualities in light adapted skate photoreceptors measured by the ERG b-wave were also observed. Dark adapted, the skate retina exposed to a flickering light during critical fusion frequency (CFF) tests had a low, sluggish CFF of 10Hz, but given sufficient time to light adapt in the photopic range, the responses became rapid and CFF rose beyond 30Hz (Green and Siegel, 1975). These unusual properties of the skate all-rod retina, in comparison to other vertebrate retinae, raise interesting questions about the neuronal pathways that carry ON and OFF information from the outer to the inner retina.

Rod and cone pathways in the vertebrate outer retina differ, especially with taxonomy, and most broadly between mammals and cold blooded vertebrates, in terms of cell types and the specificity of their synaptic connections. The mammalian outer retina, has ON and OFF cone bipolar cells (Werblin and Dowling, 1969; Kaneko, 1970) as do cold blooded vertebrates, but the cells do not form mixed synaptic connections with rods and cones typical of cold blooded vertebrates. Generally, bipolar cells in the mammalian outer plexiform layer synapse solely with either rods (Kolb, 1970; Boycott and Kolb, 1973; Dacheux and Raviola, 1986) or cones (Boycott and Dowling, 1969; Kolb, 1970; Boycott

and Kolb, 1973; Mariani, 1981; Daw et al., 1990; Wässle and Boycott, 1991), and the greatest specificity in the mammalian cone pathway is with midget bipolar cells. They synapse with only a single cone which enables high visual acuity at the fovea (Kolb, 1970; West and Dowling, 1975).

Bipolar cells in cold blooded vertebrates, unlike those in mammals, for the most part, do not segregate photoreceptor input, rather rod and cone signals are generally merged to some degree through bipolar cell synapses with both types of photoreceptors (Stell, 1967; Scholes, 1975; Kaneko and Tachibana, 1978; Saito et al., 1979; Ishida et al., 1980; Saito and Kujiraoka, 1982; Hensley et al., 1993a; Yang and Wu, 1997). Fish and amphibian bipolar cells form heterogenous and homogenous synapses with rods and cones. In the case of tiger salamander, both depolarizing (ON) and hyperpolarizing (OFF) bipolar cells receive rod or cone dominant input, however one type of bipolar cell synapses solely with cones (Yang and Wu 1997). Fish are generally found to have two types of bipolar cells one of which is a large type of cell that has mixed synapses with both rods and cones (Saito and Kujiraoka, 1982) and the other a small type that only synapses with cones (Stell, 1967).

Significant differences also exist between the rod visual systems of mammals and ectotherms. Cold blooded vertebrates, have ON and OFF bipolar cells that form direct connections with the inner retina, since these are the same cells with heterogenous rod and cone input, but the mammalian rod system has only ON

bipolar cells (Dacheux and Raviola, 1986; Hartveit, 1996). In the outer plexiform layer (OPL), mammalian rod ON bipolar cells synapse exclusively with rods and in the inner plexiform layer (IPL), their axon terminals synapse with A II rod amacrine cells (Kolb and Famiglietti, 1974; Famiglietti and Kolb, 1975; Vaney et al., 1991). These amacrine cells, in turn, form gap junctions with the axon terminals of ON cone bipolar cells and have glycinergic synapses with OFF-center ganglion cells (Daw et al., 1990). It is through the decrease of glycine output by the A II rod amacrines that OFF responses are mediated.

Under light conditions, ON cone bipolar cells are activated at the dendrites directly by cones and indirectly at the axon terminals by ON rod bipolar cells via A II amacrine cell gap junctions. ON Rod bipolar cells depolarize A II amacrine cells and, through gap junctions at the axon terminals, contribute to the depolarization of ON cone bipolar cells. Simultaneously, A II amacrine cells also inhibit OFF-center ganglion cells at their dendrites through the release of glycine. During scotopic vision, depolarizing input by ON cone and rod bipolar cells ceases, as does glycinergic inhibition of the OFF ganglion cells by the A II amacrine cells. This enables the generation of an OFF signal in the proximal retina (Kolb and Famiglietti, 1974; Müller et al., 1988; Daw et al., 1990; Vaney et al., 1991; Wässle et al., 1991).

Although vertebrate retinae have an overall similarity in their organization, there are variations in the outer retina among and between taxonomic groups.

Anatomical differences include squirrel B4 rod bipolar cells which are atypical in having some cone input (West 1978) and cone bipolar cells in fish (Stell, 1967; Scholes, 1975) and salamander that synapse exclusively with cones (Hensley et al., 1993a; Yang and Wu, 1993). In general, nocturnal vertebrates have retinae that are predominantly rod-dominated with little cone input. Similarly, species that inhabit low light environments, such as fish, also have few cone photoreceptors (Romer, 1971). The nocturnal prosimian *Perodicticus potto* has a rod to cone ratio of 300 to 1 (Goffart et al., 1976), and the dogfish, a close evolutionary relative of the skate, has a nearly all-rod retina (Shiells et al., 1981). The skate all-rod retina is exceptional because it is completely devoid of cones, but also has the capability to assume cone-like characteristics once light adapted. The red sensitive cones, however, in *Perodicticus potto* are unusual because they acquire rod-like properties once they dark adapt (Goffart et al., 1976).

The physiology of bipolar cells also differ significantly. L-2-amino-4-phosphonobutyrate (APB) an agonist commonly used to record ON bipolar cells is ineffective at one of two types of glutamate receptors on depolarizing bipolar cells in fish (Nawy and Copenhagen, 1987). Salamanders have a type of bipolar cell that in response to APB, has an unusual potassium mediated outward current with a reversal potential of -70mV (Hirano and MacLeish, 1991). In addition, there are bipolar cells in fish that respond to APB by increasing conductance and have a reversal potential greater than or equal to the chloride

equilibrium potential (Connaughton and Nelson, 2000).

Basis for the following literature discussion:

This research examined skate bipolar cells to determine how ON and OFF information is processed in the outer retina. A secondary examination into the effects of zinc as a neuromodulator was also undertaken. Presented in the next sections is background information on the ERG and the physiology of ON and OFF bipolar cells. Included is a discussion of past and current findings on the metabotropic system of ON bipolar cells and, the effects of zinc as a glutamatergic neuromodulator. Some of the material relates tangentially to the experiments undertaken, for example: calcium and enzyme regulation of the non-specific cation channels, receptor subtypes, and zinc's effects on enzymes in other systems. Derived from these works, however, are conclusions and hypotheses that are applied to this study to explain the physiological mechanisms observed in skate bipolar cells.

Reason and concepts leading to this study:

ERG light adaptation studies were among the first investigations of the skate retina (Dowling and Ripps; 1970, 1972). Subsequent research, primarily using dissociated skate retina cells, has analyzed the GABA receptors and the effects of zinc as a neuromodulator (Lasater et al., 1984; Chappell et al., 1992; Qian et al., 1996, 1997), but few studies of the ON and OFF pathways have been made. Although some glutamatergic responses were recorded from skate OFF bipolar

cells, there was little success recording from ON bipolar cells (Lasater et al., 1984; Chappell et al., 1992). Several factors were the basis for this investigation including observations of skate ERG responses, and immunostaining data of the skate retina (Schlemermeyer and Chappell, 1996).

In his book *The Retina: An Approachable Part of the Brain*, Dowling (1987) describes the ERG of the cone dominated retina as having prominent ON and OFF responses to light that are corneal positive changes in potential. The rod ERG, however, is presented as having only a large ON response with the OFF response, using the cat ERG as an example, as a small corneal negative drop in potential. This statement was of particular interest, since also illustrated was a skate ERG using a brief light stimulus (0.2 sec), which did not have an OFF response. In our laboratory, small but often observable corneal positive OFF responses were present with the skate ERG, when DC recording was used with a flash of longer duration. This left us with the questions, what was the source of this OFF-type response and whether it was derived from the inner retina or outer retina. Preliminary data based on immunostaining of the skate retina provided evidence for the presence of two types of bipolar cells. One kind of cell stained for protein kinase C (PKC), usually associated with ON bipolar cells, and the other cell type stained for serotonin, commonly found with OFF bipolar cells (Schlemermeyer and Chappell, 1996). The immunochemical and ERG observation gave support to the idea that the skate ERG OFF response was independent of ON bipolar cell activity and that, it may result from OFF bipolar

cells.

The ERG was the first method used to answer this question, since limited data has previously been obtained recording glutamate responses using intracellular recording (Lasater et al., 1984) and perforated patch clamp techniques (Chappell et al., 1992). ERG recording with pharmacological agents was used successfully by Stockton and Slaughter (1989) to isolate components of the retinal response in salamander. They used APB to block the ON pathway, KYN to separately block the OFF response and NMDA as an inner retina blocker. A similar approach was taken with this ERG study, using the same drugs plus the addition of picrotoxin, a GABA channel blocker, to inhibit possible interactions from GABAergic pathways and/or between the ON and OFF pathways via the horizontal cells. The results demonstrated the skate has corneal positive ON and OFF components of the ERG that are independent of each other, which suggested the presence of ON and OFF bipolar cells in the skate retina.

The ERG:

The ERG is a complex response recorded transretinally using external electrodes (see Dowling, 1987). In brief, the ERG is comprised of a series of overlapping potentials that can be recorded clinically, or from animal preparations with the eye or retina removed from the animal. Potentials are measured using an active electrode placed on the cornea or vitreal side of the retina, with an indifferent electrode on the forehead, the back of the eye, or distal side of the retina. A light

stimulus of sufficient intensity produces three prominent sequential overlapping responses in the order of a-, b-, and c- waves sometimes followed by a d-wave. In this investigation, each of the components of the ERG were pharmacologically isolated to study the ON and OFF responses, to show they are essentially derived from the activity of different retinal cells in the skate.

The a-wave is a corneal negative response attributed to the photoreceptors. Light hyperpolarizes the photoreceptors causing the corneal negative change in potential and except for the initial downward deflection, it is usually masked by the b- and c-waves that follow. The complete a-wave can be revealed, by recording from a retina isolated from the pigment epithelium (PE), i.e, a retina separated with the PE removed (Dowling and Ripps, 1970), to eliminate the c-wave and, treated with aspartate to block the b-wave (Dowling and Ripps, 1972). The result is an ERG a-wave that is a nearly square corneal negative response to light that lasts the duration of the stimulus.

The b-wave immediately follows the onset of the a-wave, is corneal positive, and it obscures the ongoing a-wave. Although the b-wave can be blocked by interfering with synaptic transmission between the photoreceptors and bipolar cells, the currents generated along the Müller cells were originally believed to be generated by these specialized glial cells (Miller and Dowling, 1970). There is increasing evidence, however, that the bipolar cells themselves may be involved. Müller cells recorded intracellularly have changes in potential that follow a similar

time course to that of the b-wave. Potassium currents that extend from the inner to the outer retina have been recorded which are associated with the b-wave. The currents physically correspond to the length of the Müller cells which span the retina. Müller cells have a current sink at the OPL and two current sources that are distal and proximal to the sink. The distal site is thought to be especially important for the establishment of the current flow around the Müller cell and the b-wave that results. Similarly, bipolar cells are elongated cells oriented from the outer to the inner plexiform layers and can contribute to trans-retinal potential gradients.

The c-wave, which was found to be particularly large in experiments on the skate retina, is produced by the PE and believed to result from changes in potassium concentration around the photoreceptors. Evidence for the c-wave being due to a PE response is its absence from an isolated retina, or when the PE is destroyed with iodate: both situations leave the other ERG components unchanged (Dowling and Ripps, 1970; Noell, 1954). The c-wave is caused apparently by changes in potassium concentration as the photoreceptors hyperpolarize to light. There is a decrease in potassium extruded from the photoreceptors as cation channels close, while the Na^+/K^+ pumps maintain a steady exchange rate. The net effect is a lowering of extracellular potassium concentrations surrounding the inner segments of the photoreceptors. Unlike cone-dominated retinae, the c-wave in rod dominated retinae is large, because of the different response rates by the photoreceptors. Rods have a slower more

prolonged and larger response to light than cones; therefore, they have a greater hyperpolarizing effect on the PE (Oakley and Green, 1976; Matsuura et al., 1978).

Evidence that the origin of the OFF component of the ERG (d-wave) is from OFF bipolar cells is based on studies where an OFF response can be elicited even without an ON response. For example, blocking the ON response with the glutamatergic ON bipolar cell agonist, APB, left an intact OFF response in salamander (Stockton and Slaughter, 1989).

Bipolar cells in the vertebrate retina:

Bipolar cells are second order interneurons that straddle the outer and inner retina, where they synapse with photoreceptors in the outer plexiform layer and amacrine and/or ganglion cells in the inner plexiform layer. Their purpose is to transmit afferent information which they code as either ON or OFF signals, in the form of graded potentials, from the photoreceptors to the inner retina. Under light conditions, ON bipolar cells depolarize and OFF bipolar cells hyperpolarize, but as light diminishes the situation reverses. ON bipolar cells hyperpolarize in the dark, while OFF bipolar cells depolarize (Werblin and Dowling, 1969; Kaneko, 1970; Richter and Simon, 1975; Naka, 1976; Lasansky, 1978; Nelson and Kolb, 1983; Dacheux and Raviola, 1986). These changes in potential are mediated by the glutamatergic system of ON and OFF bipolar cells. Evidence that glutamate is the neurotransmitter released by the photoreceptors comes from

immunocytochemical analyses of glutamate uptake by the photoreceptors (Davenger et al., 1991; Crooks and Kolb, 1992; Peng et al., 1992), *in situ* hybridization of glutamate receptor subunits on dendrites post synaptic to the photoreceptors (Hughes et al., 1992), optical biochemical assay (Ayoub et al., 1989), and responses by cells post synaptic to the photoreceptors to glutamate and its analogues (Daw et al., 1989; Nawy and Jahr, 1990a; Shiells et al., 1981; Yang and Wu, 1991).

Similarities between phototransduction and the metabotropic system:

Much research has been done on photoreceptors, and they are often considered a model toward understanding the physiology of ON bipolar cells. Aspects of the metabotropic system employed by ON bipolar cells are analogous to phototransduction in rods and cones (Nawy and Jahr,1991; Shiells and Falk, 1990). Photoreceptors utilize a membrane bound G-protein, transducin, which is activated by the photolysis of chemically-linked photopigments. ON bipolar cell G-proteins are activated by the glutamate receptor and are not immunoreactive with transducin antibodies (Vardi, et al., 1993). For both photoreceptors and ON bipolar cells, G-proteins mediate the closing of non-specific cGMP-gated cation channels causing the cells to hyperpolarize with reversal potentials near 0mV (Shiells et al., 1981; Atwell et al., 1987; Nawy and Jahr, 1990a,b, 1991; Shiells and Falk, 1990; Hille, 1992; de la Villa et al., 1995; Wu and Maple, 1998). The cGMP gating the photoreceptor channels is hydrolyzed by PDE after it has been activated by transducin. Activated PDE hydrolyzes cGMP to GMP + H^+ causing

the non-specific cation channels in the outer segments to close (McNaughton, 1990; Hille, 1992). It was initially believed ON bipolar cells also used PDE for the hydrolysis of cGMP but blocking agents and immunostaining with rod antibodies to PDE does not support the original suggestion (Nawy and Jahr, 1990a,b, 1991; Nawy, 1999a; Shiells and Falk, 1990; Vardi et al., 1993).

Photoreceptors are often used as a model toward understanding ON bipolar cells. Although analogous in some ways, a direct comparison between the intermediate steps in the metabotropic system and the phototransduction process points as much to their differences as to any similarities. ON bipolar cells have a complexity that may involve several glutamate receptors and more than one glutamatergic pathway with different second messengers.

The metabotropic glutamate receptors:

The metabotropic glutamate receptors (mGluRs) comprise a family of eight G-protein receptors whose amino acid sequences have a high degree of homology among themselves, but their homology is weak with other G-proteins including transducin. The family of mGluRs is divided into three groups with the classification: mGluR1 and mGluR5 forming groupI, mGluR2, mGluR3 and DmGluRA (Drosophila receptor) comprising group II, and those included in group III mGluR4, mGluR6 (present on ON bipolar cells), mGluR7 and mGluR8. Within the groups there is approximately a 70% homology, but among them only 45%, and this is reflected in their sensitivities to the agonists (Pin and Duvoisin, 1995).

The mGluRs in group I, for example, respond to glutamate and its agonists in the order quisqualate>glutamate≥ibotenate>trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD). Those in group II are sensitive to glutamate=tACPD>ibotenate> quisqualate, while group III mGluRs respond to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AP4, also termed APB)>>glutamate>>tACPD>ibotentate and are insensitive to quisqualate (Nakanishi, 1992).

Glutamate is generally considered an excitatory neurotransmitter that depolarizes cells, but working through the mGluRs it also has regulatory effects that can increase or decrease neuronal excitability and suppress synaptic transmission. Suppressive effects associated with neurotransmitter release have been found with mGluR2, mGluR3 and mGluR4. In general, the metabotropic receptors regulate a wide range of ion channels throughout the nervous system. These include different types of potassium, calcium and chloride channels (Anwyl, 1999), as well as the nonspecific cation channels present on depolarizing bipolar cells. They exert their effect, most frequently, on potassium channels in opposite ways by either increasing or decreasing potassium flux. In hippocampal pyramidal cells AP4 lowers synaptic transmission through a presynaptic inhibitory effect, and it has a suppressive effect on cerebellar Golgi cell activity. The same compound, APB, through its binding with mGluR6, causes ON bipolar cells to hyperpolarize. Only ON bipolar cells are known to have mGluR6. The glutamate analog t-ACPD also hyperpolarizes ON bipolar cells (Shiells et al., 1981;

Slaughter and Miller, 1981, 1985; Thoreson and Miller, 1994).

For a class of On bipolar cells, activating mGluR6 mediates the closing of non-specific cation channels (discussed below). In fish, APB also activates a chloride current (Connaughton and Nelson, 2000), and in salamander it induces an outward potassium current (Hirano and MacLeish, 1991). Some fish have glutamate receptors on the same depolarizing bipolar cells that are APB sensitive and APB insensitive coding rod and cone signals, respectively. Glutamate, for these cells, mediates ionic channel closing via the APB sensitive receptors and ionic channel opening through the APB insensitive receptors (Nawy and Copenhagen, 1987).

Identification of G-protein in ON bipolar cells:

ON bipolar cell G-proteins in dogfish have been identified through their reactivity with pertussis toxin (PRTX) and cholera toxin (CTX), which is similar to the dual reactivity of rod transducin (Van Dop et al., 1984; Shiells and Falk, 1992). The toxins, in general, exert their effects by changing the G-protein's capacity to either activate or inactivate by catalyzing ADP ribosylation of the guanyl nucleotide regulatory site. CTX alters the binding of GTP to the G-protein putting it into a period of extended activation (Cassel and Pfeuffer, 1978), while PRTX prolongs the binding of GDP to the G-protein lengthening its period of inactivation (Katada and Ui, 1982). ON bipolar cells recorded from dogfish retinal slices have antagonistic responses to both PRTX and CTX. When the activated PRTX

A-subunit was added to the recording pipette, membrane resistance decreased, conductance increased and the response to flashes of light diminished. These effects were due to an increase in open cation channels, because the G-protein and in turn PDE were inactivated. This caused cGMP concentrations which gate the channels to increase. Adding the activated A-subunit CTX to the pipette had the opposite effects. It kept the G-protein in an active state, and the cells had an increase in membrane resistance. Conductance decreased and there was a loss of light responses due to the overriding closure of channels (Shiells and Falk, 1992).

Although CTX and PRTX sensitivity in dogfish is characteristic of transducin and an indication that ON bipolar cell G-proteins are transducin-like, bipolar cells in the amphibian (salamander) and mammalian retinae are not immunoreactive to transducin antibodies (Vardi et al., 1993). In monkey, cat, rabbit and rat, cone ON bipolar cells, identified by their invaginating synapses with photoreceptors, have been found through immunostaining to have an alpha subunit of $G_o\alpha$ (Vardi, 1998). This G-protein is dissimilar to transducin and is affected by PRTX but not CTX; AP4 in a retinal homogenate, suppresses the effects of PRTX and not CTX (Kikkawa et al., 1993). The highest $G_o\alpha$ concentrations, in monkey and cat, were at the dendritic tips where the mGluR6 receptors are found, but $G_o\alpha$ is also widely distributed in the cell away from the receptor (Vardi, 1998).

mGluR6 activates $G_o\alpha$, and in a mixture of G-proteins, using AP4 as the mGluR6

agonist, $G_o\alpha$ was measured to have an activation 18 times greater than transducin (Weng et al., 1997). Dialyzed $G_o\alpha$ exerts an effect in salamander ON bipolar cells similar to that produced by glutamate (Nawy 1999a). The addition of $G_o\alpha$ to the pipette resulted in a decrease in salamander ON bipolar cell membrane conductance, and the current-voltage relationships were similar to those obtained with glutamate. Furthermore, responses to glutamate were blocked when antibodies to $G_o\alpha$ were dialyzed into the cell, but the subunits $G_i\alpha$ and transducin $G\beta\gamma$ had no effect.

The effects of GTP and cGMP on currents:

Early work identified through pharmacological studies (Slaughter and Miller, 1981; Nawy and Jahr, 1990a; Shiells et al 1981) and molecular cloning (Nakajima et al., 1993) the glutamatergic receptor on depolarizing bipolar cells. Electrophysiological recordings from retinal slices of dogfish, mudpuppy and salamander demonstrated ON bipolar cells to be highly sensitive to APB, but insensitive to kainate and quisqualate. APB caused an increase in membrane resistance, and a reduction of inward conductance. Furthermore, it was observed that with time the magnitude of the responses diminished, and it was postulated to be due to washout of some unknown second messenger components (Nawy and Jahr, 1990b). Recording ON bipolar cells salamander retinal slice whole-cell, the addition of GTP to the electrode caused a decrease in the holding current. Given sufficient time, the nucleotide dialyzed into the cell and, as the holding current decreased and the conductance increased, the

amplitude of the responses to light grew larger. The replacement of GTP with 1mM cGMP also produced a decrease in the holding current, and an increase in conductance.

Retinal slices prepared with Co^{2+} to block input from the photoreceptors and with cGMP in the electrode had currents that were blocked by APB. Similar changes in the holding current occurred with dogfish retinal slices when either GTP or cGMP were added to the electrode. However, the addition of cGMP to the electrode, particularly in higher concentrations, diminished or totally blocked the effects light-stimulated photoreceptors have on ON bipolar cells. 100μM cGMP produced a decrease in ON bipolar cell holding current, an increase in conductance, and it reduced the response to light. These changes occurred more rapidly when the concentration was increased to 250μM cGMP, but now the ON bipolar cell responses to light were eliminated. The high cGMP concentration, presumably, left fewer cGMP gated channels to be opened, so the effect on the ON bipolar cell between dark and a light stimulus was minimized or blocked.

The addition of GTP and/or cGMP causes significant changes in membrane resistance, conductance, and resulting responses when ON bipolar cells are stimulated with glutamatergic agonists. The effects of the nucleotides implicate a second messenger system, and this was examined further using the GTP analogue, GTP-γ-S in the electrode. G-proteins require GTP in their activated

state (as, for example, the binding of GTP in place of GDP to activate transducin). GTP-γ-S is a non-hydrolyzable form of GTP and can substitute for it once the G-protein is activated. The effect observed when salamander and dogfish slice preparations were stimulated with light, was an increase in membrane resistance associated with a block of inward currents (Nawy and Jahr 1990a; Shiells and Falk, 1990). The response was similar to a sustained application of APB, persisting even after the agonist was removed. GTP-γ-S prevented a return to its original pre-APB condition. GDP-β-S has the opposite effect of inactivating the G-protein, and it was demonstrated that the response could be in blocked by this agent as well (Nawy and Jahr 1990a).

It might be thought, that a high concentration of GTP in the previous experiment was working at the G-protein site rather than the cation channels, but this was believed unlikely. A low concentration of GTP (100μM) was erratic in reproducing the effects that occurred at higher concentrations, i.e. decreasing membrane resistance, increasing inward currents, essentially a "cGMP-type" response. For G-protein systems that do not have additional GTP requirements, e.g. nucleotide-gated channels, 100μM GTP is a high enough concentration to keep the system responsive. Nawy and Jahr (1990a), therefore, conclude the GTP effects were at nucleotide gated channels.

Evidence against PDE in ON bipolar cells:

The nonspecific cation channels of photoreceptors and ON bipolar cells are

cGMP-gated, and it is through enzymatic hydrolysis of the nucleotide to GMP+H^+ that the channels close. PDE is the intermediary enzyme in photoreceptors, but is absent or nominally present in ON bipolar cells. Evidence for PDE not being key to closing cGMP-gated channels is based on a lack of immunoreactivity to the enzyme and is also consistent with electrophysiological data. The addition of either 8-bromo-cyclic GMP to the pipette (8-Br-cGMP), a cGMP analogue that is poorly hydrolyzed by PDE, or 8-(4-chlorophenylthio)-cyclic (8-pCPT-cGMP) a very nonhydrolyzable analogue in lieu of cGMP did not decrease the magnitude of ON bipolar cell responses. Furthermore, in a PDE-cGMP system, the compound 3-isobutyl-1-methyl-xanthine (IBMX) a PDE inhibitor should, presumably, decrease membrane resistance. Tested against 1mM glutamate, it was ineffective at reducing responses (Nawy, 1999a). These results contradict previous studies which did show IBMX to have a large effect on responses, by blocking the hydrolyses of cGMP (Nawy and Jahr 1990a; Shiells and Falk, 1990).

It was postulated that IBMX was effective in earlier experiments, because low concentrations of the agonist were being used to stimulate the ON bipolar cells. Therefore, IBMX was capable of blocking this low level of activity. 100μM APB was used on dogfish retinal slices (Shiells and Falk, 1990); 0.5 and 1μM APB was used on salamander retinal slices (Nawy and Jahr 1990a). IBMX was only successful at blocking the response to 0.5μM APB and had minimal effect with 1μM APB. Responses recorded using high glutamate concentrations, 1mM, were not suppressed by IBMX, and this may have been due to its ability at a

higher concentration to override a system with low PDE activity. It might also account for the enhancing effect IBMX had on the standing current. The inhibition of basal PDE activity would open more channels, but high agonist concentrations might overcome the effect (Nawy, 1999a).

Effects of calcium on conductance:

The degree to which glutatmate affects membrane resistance in ON bipolar cells is strongly influenced by intracellular calcium concentrations. In the intact retina, light results in the depolarization of ON bipolar cells, and the opening of non-specific cation channels, which are permeable to sodium, potassium and calcium. The increased opening of non-specific cation channels raises intracellular calcium levels which, in turn, causes the non-specific cation channels to close. Light adaptation in ON bipolar cells is associated with an increase in membrane resistance and is directly regulated by increments in calcium levels. These light adaptating factors are correlated in experiments recording voltage clamped ON bipolar cells from dogfish and salamander retinal slices (Nawy 2000; Shiells and Falk, 1999).

Dogfish ON bipolar cells recorded from retinal slice and voltage clamped were stimulated with a bright light, bleaching 200 rhodopsin molecules per rod ($200Rh^*s^{-1}$), for a two minute interval. Under these conditions, the opening of nonspecific cation channels is maximized, and the cells had a steady diminution in their response back towards zero current during the stimulus. The responses

to short test flashes, $2Rh^*s^{-1}$ that followed were much smaller, because of desenstization, than those before the extended period of illumination, but grew progressively larger with time as the bipolar cell adapted. The light adapting responses, however, were blocked by adding 5mM BAPTA, a calcium chelator, into the pipette, and the effect was overridden by adding 50μM calcium into the pipette (Shiells and Falk, 1999).

The dogfish has a nearly all-rod retina (Shiells and Falk, 1981). When the experiment was performed using high illumination to desensitize the rods (bleaching 10 rhodopsin molecules per rod), the ON bipolar cells also desensitized. The ON bipolar cells lost their sensitivity to superimposed flashes of light. Repeating the experiment, however, with 5mM BAPTA added to the pipette, the ON bipolar cells did not desensitize. They had graded responses to increasing intensities of superimposed flashes of light. OFF bipolar cells which have ionotropic receptors and were tested as a control did not have nearly the same adapting response to light. Calcium is instrumental in reducing the synaptic gain between rods and ON bipolar cells, thereby controlling light adaptation (Shiells and Falk, 1999).

Nawy (2000) found diminished responses by ON bipolar cells could be prevented by controlling the duration that the cation channels were open. Salamander ON bipolar cells in retinal slice preparations were voltage clamped at -40mV and repeatedly tested with glutamate once every thirty seconds, for brief 5 second

intervals. The responses decreased as did baseline currents. In treatments where the time parameters were changed so the bipolar cells were superfused with glutamate for extended periods, two minute intervals, followed by five second interruptions, sensitivity was retained. The responses remainded large with little or no decrement and a steady baseline current was maintained. These results correlate the amount of time the non-specific cation channels are in the open state with the magnitude of the response. For the brief glutamate treatments, the cation channels were open the majority of the time and the responses diminished with repetition. When they were kept predominantly closed, the removal of glutamate gave large consistent responses which returned back to baseline. The phenomena of rundown associated with brief glutamate pulses and extended open times was blocked by the addition of BAPTA to the pipette.

ON bipolar cells have voltage gated channels which were blocked with nifedipine (Nawy 2000), a compound that preferentially inhibits L-type calcium channels (Hille, 1992). The difference in ramps, between nifedipine and control responses, revealed a substantial inward calcium current that was blocked; however, rundown was not prevented, implicating calcium entry at another site. By examing the shift in reversal potential, as calcium concentrations were changed, from 0 to 20mM, the non-specific cation channel was found to be substantially permeable to calcium. In addition, cells tested with glutamate superfused in the middle of a short I-V stimulus (10mV steps) had larger

responses compared to glutamate superfused at the end of a long duration I-V stimulus (also 10mV steps). At negative potentials, calcium has a large driving-force and under conditions of sustainded I-Vs a greater opportunity to enter the cell. The effect of diminished responses, however, could be counteracted with the addition of BAPTA to the pipette. I-V currents for long duration responses with BAPTA were large and similar in size to those of the short duration and demonstrates the reducing effect calcium entry has on currents at negative holding potentials (Nawy, 2000).

Effects of calcineurin:

Calcium has a wide-range of cellular functions that can up- or down- regulate channel permeability, influence cell excitability, extend the duration of response, and alter synaptic transmission. It mediates the activation of $K_{(Ca)}$ and $Cl_{(Ca)}$ ion channels, has self-regulating feedback effects on its own entry through voltage-gated calcium channels, and regulates its own release from internal stores. The IP_3 receptor, for example, is a calcium sensitive channel and, as calcium concentrations rise, calcium release is cut off. In this system, calcium acts as a second messenger that activates other pathways. Glutamatergic responses in retinal neurons include phosphoinositide breakdown with increases in intracellular calcium (Milani et al., 1990).

Calcium can exert its effect on cell conductance through proteins such as calcineurin and calcium/calmodulin kinase II (CAMKII) that modulate ion

channels. Calcineurin is a calcium (Ca^{++})/calmodulin (CAM) activated phosphatase that negatively regulates a wide range of neuronal sites by dephosphorylating membrane proteins. In the brain calcineurin is distributed throughout cells with 50% in the cytosol and 50% bound to the cell membrane, causing a downregulation in cell excitability, transmitter release, and the permeability of ligand and voltage-gated channels. At glutamatergic sites, the inhibition of calcineurin through immunosuppressant drugs augments the presynaptic release of glutamate and the post synaptic response (Yakel, 1997). ON bipolar cells dialyzed with cyclosporan A, an inhibitor of calcineurin, had increased glutamate responses (Nawy, 1999b).

Calcineurin is a metalloenzyme that is strongly bound to zinc and iron, but it also requires another divalent metal in conjunction with CAM for activation. The metal is also necessary to give the enzyme structural stability. $MnCl_2$ serves these functions fully activating purified bovine brain calcineurin and upon its removal deactivating it. Zinc is another of many divalent ions that binds to calcineurin; however, it only partially reactivates calcineurin (King and Huang, 1984). Calcineurin from adrenal cell cytoskeleton, on the other hand, is inhibited by zinc as well as vanadate, fluoride and pyrophosphate (Papadopoulos et al., 1989).

Effects of CaMKII on depolarizing bipolar cells:

Ca^{2+}/Calmodulin-dependent protein kinase II (CAM KII), is present in neurons in large quantities, with a widespread cellular distribution in membranes, cytosol,

cytoskeleton and nucleus (Schulman 1988, Colbran et al. 1989). It is one of the primary enzymes in the brain and is thought to be an important component in bringing about synaptic changes. Because CAMKII is an autophosphorylating enzyme, it can enter a self-perpetuating state of activation, through which long-term potentiation results. The enzyme is part of a cascade that first requires a rise in intracellular calcium. As concentrations increase, calmodulin a protein with a high affinity for calcium, binds with the ion, undergoes a conformational change, and becomes activated. This enables calmodulin, as a calcium calmodulin complex, to bind CAMKII and the activated kinase, in turn, phosphorylates other proteins.

Despite decreases in calcium levels, following a depolarizing event, for example, CAM kinase II can remain active through autophosphorylation. This was demonstrated by activating CAM kinase II with a brief exposure to Ca^{2+}/Calmodulin, followed by treatment with EGTA to remove Ca^{2+}/Calmodulin, with the outcome that CAMKII maintained its activity through autophosphorylation, but at a lower rate. The amino acids autophosphorylated prevents subsequent Ca^{2+}/Calmodulin binding, and different amino acids are phosphorylated when Ca^{2+}/Calmodulin was incubated for extended periods with the kinase. Depending on the treatment with Ca^{2+}/Calmodulin, a variety of kinases resulted with different mobilities on a sodium dodecyl sulfate polyacrylamide gel. After a short 15 second incubation with Ca^{2+}/Calmodulin, the CAMKII α subunit migrated to 50 kDa, and treatment of the α subunit with

Ca^{2+}/Calmodulin for 15 seconds, followed by 5mM EGTA turned some of the kinase into a 52-kDa molecule. A five minute treatment of α-CAM kinase II with Ca^{2+}/Calmodulin changed some of the α subunit into a 54-kDa molecule. When α-CAMKII was incubated with 5mM zinc, the enzyme autophosphorylated and was converted into a 49-kDa structure. It was Ca^{2+}/Calmodulin insensitive with a different activation from α-CAMKII (Lengyel et al., 2000).

ON bipolar cells from retinal slices recorded whole-cell, at -40mV holding potential, had diminished outward currents in response to APB, when either CAMKII inhibitors (S)-5-Isoquinolinesulfonic acid, 4-[2-[(5-Isoquinolinyl-sulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)-propyl]phenyl ester (KN-62) or N-[2-[[[3-(4'-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxy-benzenesulfonamide phosphate (KN-93) were added to the pipette. The bipolar cells, under all conditions, were dialyzed with 1mM cGMP to maximize the number of open cGMP-gated channels. The inhibitors caused a reduced inward holding current which resulted in a smaller outward current in response to APB (Walters et al., 1998).

OFF bipolar cells:

The ionotropic glutamate receptors are subdivided into two broad categories, those that are sensitive to the agonist N-methyl-D-aspartate (NMDA), and the non-NMDA agonists AMPA and kainate. The NMDA receptor is considered separate because of its voltage-dependent block by magnesium and

requirements for glycine as a coagonist. When activated the kinetics of the receptor are slow with a large single channel conductance that has a high calcium permeability (Seeburg, 1993). The NMDA receptor is considered an important component to long term potentiation in the Central nervous system (CNS), but it has also been found in the retina of some species on horizontal cells (O'Dell and Christensen, 1986; Perlman et al., 1987; Anderton and Miller, 1989; Tachibana and Okada, 1991). Hyperpolarizing bipolar cells in salamander were found to have only non-NMDA glutamate receptors, based on their insensitivity to concentrations of 50μM NMDA and less (Wu and Yang, 1991). Subsequent studies have found similar results. Neither rod nor cone dominated hyperpolarizing bipolar cells had any sensitivity to NMDA (Hensley et al., 1993b), nor were NMDA receptors found in cat bipolar cells, or rod dominated rat bipolar cells (Hartveit, 1996; Sasaki and Kaneko, 1996).

AMPA and kainate receptors have an electrophysiology that is quite different from the NMDA receptor. Activation of the non-NMDA receptors is voltage-independent, and does not require glycine, nor are the channels blocked by magnesium. In addition, the AMPA and kainate receptors, do not have long sustained responses to glutamate like NMDA receptors, rather they desensitize rapidly. The AMPA receptor is composed of four transmembrane GluR subunits that are assembled from GluRA, GluRB, GluRC or GluRD. Each subunit is approximately 900 amino acids long, and functioning AMPA receptors can be expressed *in vitro* from a homogenous assembly of any subunit, or a

heterogenous combination of any two subunits. Furthermore, splice variants of the subunits produce AMPA receptors that can either be fast desensitizing, flop, or those more slowly desensitizing with a small sustained after component, flip. RNA splicing determines the ratio of flip to flop and the particular kinetics for a given AMPA receptor. In general, the AMPA receptors are prominent in the CNS because of their fast rates of activation, deactivation, rapid desensitization and very low calcium permeability (Sommer et al., 1990; Seeburg, 1993; Mosbacher et al., 1994; Partin et al., 1996).

The kainate receptors are also comprised of four transmembrane subunits selected from GluR5, GluR6, GluR7, KA-1 or KA-2, each about 900 amino acids long. Different GluR subunits have been found in cells from the CNS, and the particular characteristics of a kainate receptor depends upon its subunit composition. For example, the homeric GluR5 receptor desensitizes more slowly than the heteromeric GluR5/KA2 receptor and has a different I-V profile. Kainate receptors consisting of only GluR6 are insensitive to AMPA, but the addition of KA2 to form a GluR6/KA2 unit makes the receptor AMPA sensitive. Conversely, the homeric AMPA receptors of GluRA, GluRB, GluRC and GluRD composition with their characteristic high affinity for AMPA and rapid desensitization by AMPA or glutamate, are also activated by kainate with sustained responses (Hollman and Heinemann 1994; Nakanishi, 1992; Seeburg, 1993). Receptors that have dual sensitivity to AMPA and kainate are often grouped as AMPA/kainate (AMPA/KA) receptors.

Immunostaining along with pharmacological analyses have provided evidence for AMPA/KA receptors on OFF bipolar cells. Carp OFF bipolar cells express the receptor subunits GluR2/3, GluR4, and kainate subunit GluR5-7 (Schultz et al., 2001), while the subunits comprising the OFF bipolar cells in cat are GluR2/3, GluR4, and kainate subunit GluR6-7 (Vardi et al., 1998; Morigiwa and Vardi, 1999). Kainate receptors in rat were identified by their immunoreactivity to KA2 antisera and were present on OFF-type bipolar cells that made flat synaptic contacts with cone pedicles (Brandstatter et al., 1997). Ground squirrel OFF bipolar cells only have kainate receptors without any other type of glutamate receptor. The addition of GYKI53655 a non-competitive blocker selective for AMPA receptors had no effect on synaptic transmission between cones and OFF bipolar cells (DeVries and Schwartz, 1999).

OFF bipolar cells are predominantly responsive to the glutamate analogues AMPA and kainate. In addition, the broad spectrum inhibitor, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) which blocks AMPA and kainate receptors, when applied to retinal slice preparations, effectively reduces membrane conductance of voltage-clamped OFF bipolar cells. Application of CNQX to the salamander retina blocked spontaneous excitatory post synaptic currents (sEPSC) in newt OFF bipolar cells (Kawai, 1999) and suppressed tiger salamander OFF bipolar cell responses to light (Hensley et al., 1993b). In zebrafish, glutamate and kainate-mediated currents could also be suppressed by CNQX (Connaughton and Nelson, 2000).

The more specific KA/AMPA blocker, DNQX, blocked currents in salamander OFF bipolar cells that were sensitive to light at 500μm (Gao et al., 2000). Mudpuppy rod and cone inputs differ in their responses to the glutamate antagonist kynurenic acid, and this is indicative of two different types of glutamate receptors on the OFF bipolar cells (Kim and Miller, 1993). Cone-driven OFF bipolar cells in cat are responsive to AMPA, kainate and glutamate, but not NMDA or the metabotropic agonists APB and ACPD (Sasaki and Kaneko, 1996). Rabbit OFF-center cone bipolar cells were analyzed with an immunoprobe sensitive to the guanidinium analog, 1-amino-4-guanidobutane. The compound is channel permeable at AMPA and kainate receptors once they are activated, and it was determined the cells respond strongly to kainate (Marc, 1999).

OFF bipolar cell reversal potentials:

In general, reversal potentials for OFF bipolar cells are near 0mV, similar to that found with ON bipolar cells. Salamander reversal potentials range from 0 to -12mV, depending on the preparation (Atwell et al., 1987; Gilbertson et al., 1991; Maple et al, 1994; Maple et al., 1999). Hyperpolarizing bipolar cells in fish (Shiells et al., 1981; Connaughton and Nelson, 2000) and mammals (Hartveit 1997; Sasaki and Kaneko, 1996) were also measured with reversal potentials close to 0mV. These values are consistent with non-specific cation channels permeable to Na^+ and K^+. Reversal potentials equal to 0mV were obtained for kainate and quisqualate induced currents in central neurons when there was an

equal concentration of monovalent cations between the intracellular and extracellular solutions (Ascher and Nowak, 1988). Retinal slice recording of salamander hyperpolarizing bipolar cells had an equal permeability of Na^+ and K^+, but a high Ca^{++} to Na^+ permeability ratio of about 3.2, which exceeds values typical of non-NMDA channels (Gilbertson et al., 1991).

The effects of zinc on the CNS and retina:

Zinc is a prominent constituent of the vertebrate brain. In fish, zinc is present in significant quantities in the diencephalon and to a much lesser extent the telencephalon (Piñuels et al., 1992). In birds, the main architectonic subdivisions, in addition to other regions, stain for zinc (Montagnese et al., 1993). Mammalian hippocampal mossy fibers are immunoreactive for glutamate and contain zinc. The medial cortex boutons of lizards are thought to be a structure homologous to the mammalian hippocampal mossy fibre system. They are also immunoreactive for glutamate and zinc filled (Martinez-Guijarro et al., 1991). Evidence of the importance of zinc in the mammalian brain comes from histological studies of zinc staining patterns. Its widespread distribution includes the visual cortex (Garrett et al., 1991; Garrett and Slomianka, 1992; Dyck et al., 1993), hippocampus, striatum, suprachiasmatic nucleus, thalamus, limbic system, and other structures (Frederickson et al., 1992; Huang et al., 1993; Long et al., 1995; Mengual et al. 2001; Zatt et al., 2001).

Zinc concentrations in hippocampal mossy-fiber neuropil are high, estimated to

be 200 to 300μM (Frederickson et al., 1983). A majority of zinc in the brain, approximately 90%, is associated with proteins providing structural stability, for example, the zinc finger, or acting as an enzymatic catalyst. Other possible functions for this high concentration of zinc include its working as a trophic factor to initiate sympathetic growth (Frederickson et al., 1983). The remaining zinc, roughly 8%, is ionic and can be highlighted by Neo-Timms staining. Axonal boutons in the mammalian brain stain for zinc (Frederickson et al., 1992; Garrett and Slomianka, 1992), and zinc has been found predominantly in glutamatergic synaptic vesicles in the neocortex and hippocampal formation. Electrophysiological data clearly demonstrates zinc can modulate the effects of neurotransmitters on brain and retinal cells (discussed below), and support for this role is provided by evidence of its release and uptake.

Atomic absorption spectrophotometric measurements found approximately 18% of hippocampal zinc was released when the hippocampus was stimulated with high potassium concentrations (Assaf and Chung, 1984). In order for zinc to be effective as a neuromodulator, an efficient uptake system is necessary to reduce concentrations. ZnT-3 has been identified as a zinc transporter based on its homology to the mammalian ZnT-1 and ZnT-2 zinc transporters. All glutamatergic mossy fiber vesicles express immunoreactivity to the transporter ZnT-3. Although it has not yet functionally been proven to be a zinc transporter, 60-80% of the vesicles contained zinc (Wenzel et al., 1997). Zinc might also play a role inside the vesicle stablizing glutamate (Frederickson, 1989), or prevent

neurotoxicity by protecting the cells from high glutamate concentrations (Spiridon et al., 1998 - see below).

Zinc decrements in hippocampal boutons, through chelation or diet deficiency, has been shown to interfere with long term potentiation of mossy fibers (Lu et al., 2000). NMDA receptors are associated with long term potentiation, and when recording from dissociated hippocampal neurons, 50μM zinc blocks NMDA currents (Mayer et al., 1989). The blockage is non-competitive, and is probably independent of the glycine and magnesium binding sites. Increase in glycine concentration was shown to have little effect on the block by zinc, and the block by magnesium was dissimilar to the effects of zinc (Smart et al.,1994).

In a glycine-free solution, hippocampal neurons had their responses to kainate, quisqualate, and glutamate potentiated by 50μM zinc, but non-NMDA glutamatergic receptors had their responses reduced by 1mM zinc (Mayer et al., 1989). Recording from the hippocampal slice preparation, zinc inhibited currents produced by NMDA, 1*S*,3*R*-ACPD and quisqualate, while augmenting non-NMDA-mediated ionotropic responses (Xie et al., 1993). Other hippocampal studies have also reported zinc can increase responses to AMPA as well as quisqualate, but these studies have found zinc has varying effects on kainate responses including no effect at all (Smart et al.,1994). In general, throughout the brain, zinc, augments responses to quisqualate, and it can increase, decrease, or have no effect on kainate responses (Smart et al.,1994).

Xie et al. (1993) found the metabotropic agonist 1S,3R-ACPD produced an inward current in hippocampal cells, and 200μM zinc had a blocking effect on the current rendering the cells unresponsive to ACPD. Zinc also suppressed the after hyperpolarization potential (AHP), a component associated with calcium-dependent potassium channels and the ACPD response. These findings indicate zinc can modulate metabotropic glutamate responses, but the effect may be at a non-receptor site(s); it can regulate a range of channels that include calcium-dependent potassium channels, voltage-gated calcium channels (Smart et al., 1994), and chloride channels (Hille, 1992).

The neuromodulatory effect of zinc on the retina compared to the brain is less well studied, but has been of increasing interest particularly in regard to its ability to regulate GABA and glutamate mediated currents (Wu et al., 1993; Qian et al., 1996, 1997; Han and Yang, 1999; Kaneda et al., 1997; Shen and Yang, 1999). Although other divalent ions are potent blocking agents in the retina, zinc's effects at the GABA and glutamate receptors are of note, especially since Neo-Timm staining reveals significant intracellular concentrations in the photoreceptors. It has been postulated, based on staining patterns, electrophysiological data, and transport rates that zinc may be coreleased with glutamate from the synaptic vesicles and function as a neuromodulator in the retina (Wu et al., 1993; Qian et al., 1997; Spiridon et al., 1998), but its extracellular release by the photoreceptors or other retinal neurons has yet to be demonstrated. Evidence for it being a neuromodulator in the retina are currents

mediated by $GABA_A$ receptors in salamander horizontal cells (Wu et al., 1993) and skate Müller cells (Qian et al., 1996) are blocked by zinc. In carp bipolar cells, currents induced by 100μM GABA are suppressed by zinc but to varying degrees by the $GABA_A$ and $GABA_C$ receptors. The $GABA_C$ receptor is more effectively blocked by 10μM zinc than the $GABA_A$ receptor (Han and Yang, 1999). Skate bipolar cells, however, respond differently to zinc where it can also have an augmenting effect. At low concentrations, zinc enhances currents mediated by $GABA_A$ receptors, while at high concentrations it reduces bipolar cell $GABA_A$ currents (Qian, 1997). Irrespective of the species tested, the effect zinc has on $GABA_C$ receptors is inhibitory causing a reduction in its current (Dong and Werblin, 1995; Qian et al., 1997; Han and Yang, 1999).

Zinc also has varying complex effects on retinal glutamate receptors in vertebrates. Depending on the receptor type and concentration, zinc can diminish, enhance or have no effect on glutamate mediated responses. Perch horizontal cells were reported to have reduced glutamate responses to divalent ions that in order of increasing potency were barium, magnesium, colbalt, nickel and calcium, but zinc was ineffective (Schmidt, 1999). Little zinc effect on the glutamate response was observed in carp horizontal cells, but through pharmacological manipulation, the flip component of the AMPA receptor was revealed to be zinc sensitive (Shen and Yang, 1999).

The AMPA receptor type varies according to its flip to flop splice ratio, i.e., the

ratio of the fast desensitizing flip to the more slowly desensitizing flop components determines the characteristics of the response. The compounds 4-[2-(pheylanl-fonylamino)ethylthio]-2,6-difluoro-pheynoxy acetamide (PEPA) and cyclothiozide (CTZ) are agents that inhibit desensitization, but PEPA preferentially potentiates the flop variant, and CTZ the flip variant. When the carp horizontal cells were tested with solutions of 30μM zinc plus 3mM glutamate in conjunction with 10μM PEPA or 10μM CTZ, zinc had a potentiating effect on the glutamate plus CTZ response, but no effect on the response to glutamate plus PEPA. Furthermore, at a high zinc concentration, a solution of 300μM zinc with 3mM glutamate, and 10μM CTZ, the response of the cells was suppressed compared to the control solution of glutamate plus CTZ (Shen et al., 1999). Thus zinc has a dual effect on carp horizontal cells. The CTZ sensitive component at low zinc concentrations is potentiated but at high concentrations is inhibited.

Salamander horizontal cell glutamate receptors are quite different, since they are not modulated by zinc. Slice preparations superfused with 3mM glutamate depolarized the horizontal cells, and with the co-application of 5μM or 50μM zinc there was little change in the magnitude of the response. The extent to which glutamate depolarized the horizontal cells was not changed whether zinc was present or not (Wu et al., 1993).

Other evidence supporting the role of zinc in the intact retina is the blocking effect it has on glutamate uptake by glial cells. Approximately 45% of the

maximum current generated by the glutamate transporter in Müller cells was reduced by zinc. Based on Michaelis-Menten kinetics, it inhibited glutamate uptake with a K_m of 0.66μM. During ischemia or hypoxia, reverse glutamate transport occurs. When reverse transport is evoked by high extracellular potassium concentrations, zinc suppresses it by up to 40%, with a K_m of 1μM. This could serve a protective purpose minimizing toxicity during ischemia when glutamate concentrations are high (Spiridon et al., 1998).

Methods:

ERG:

Eyecups from the skate (*Raja erinacea*) were prepared by dark adapting the fish for one hour. The skate was double pithed and/or anesthetized with 0.1% 3-aminobenzoic acid ethyl ester (MS 222), prior to removing the eyes. One eye was temporarily stored in a moist dark chamber in the refrigerator for later use, while the other was prepared under red light. Remnant tissue from the exterior of the eye was removed, and then, with the downward slice of a razor blade, the cornea and lens were cut away. The vitreous from the resulting eyecup was drained by capillary action onto a piece of filter paper. Remaining vitreous was drained by capillary action using slivers of filter paper placed on the inner ventral surface of the eyecup. To prepare an isolated retina, the eyecup was then sliced in half. One piece was stored for later use in a moist dark chamber in the refrigerator, and the other was prepared for recording. Using a strip of filter paper, the retina was pulled away from the pigment epithelium and removed from the eyecup. The piece of retina was then lifted from the filter paper, by submerging it in a petri dish filled with Ringer solution, where it was transferred to a small flat piece of filter paper.

Preparations were placed in a superfusion chamber (WP Instruments, Inc., New Haven, CT) which had a Ag/AgCl pellet reference electrode, and the recording electrode was a Ringer-filled glass capillary connected to an electrode holder also containing a Ag/AgCl pellet. The recording electrode was lowered into the

eyecup where a constant level of perfused fluid was maintained. Solutions flowed from a manifold system at a rate of about 1ml/min., with a dead time of about 30s between the switching of solutions. Eyecup preparations were oxygenated with moist oxygen passed through a capillary tube or a Pasteur pipette placed over the edge of the eyecup. Prior to experiments, all solutions were oxygenated and adjusted to pH 7.6. The superfusion chamber, holders, and solutions were enclosed in an electrically shielded cage.

Recording procedures:

The reference and recording electrodes were wired to a DC low noise pre-amplifier (model PARC 118; EG&G Instruments, Princeton, NJ), from which a TM 506 amplifier (Tektronics, Inc., Beaverton, OR) was connected and used in the DC mode to provide suitable DC offset. For the ERG experiments that studied the ON and OFF afferent pathways, the signal was sent to a chart recorder (model 2200S; Gould, Inc., Cleveland, OH) and a reel-to-reel tape recorder (model 3964A; Hewlett-Packard Company, San Diego, CA). Data from the tape was later played back and analyzed with pCLAMP software, version 5.0 (Axon Instruments, Inc., Foster City, CA). Latter ERG experiments using histidine and zinc, the data was sent directly from the TM 506 amplifier to the computer where it was monitored and simultaneously recorded with pCLAMP software, version 6.

The light source stimulating the eyecup was a halogen lamp controlled by a voltage-regulated DC power supply (model SP40-10; Deltron, Inc., North Wales,

PA) set at 6 amps with an unattenuated intensity (log I = 0) of 20μW/cm^2 at the level of the eyecup. Combinations of neutral density filters from log I = -1 to log I = -3 were used in combinations to attenuate the beam from log I = -1 to log I = -8. A shutter activated by a pulse generator (A.M.P.I., Jerusalem, Israel) was programmed to activate the light once per minute, for a 10 second duration, except for the first APB experiment where an 80ms flash was used at 7s intervals.

Cell dissociation:

Skates were double pithed and/or anesthetized with 0.1% 3-aminobenzoic acid ethyl ester (MS 222), prior to removing the eyes. Pairs of eyes were excised, but not dark adapted, for each dissociation. External tissue from the outer surface of each eye was removed, and then the lenses and corneas were cut away with a razor blade. Vitreous from the eyecups was drained on filter paper, and remnant vitreous was wicked away from the ventral surface of each preparation with narrow pieces of filter paper. The eyecups were cut into small pieces, about 3mm^2, with a razor blade, and placed in Ringer solution containing the enzymes protease (20mg/42ml) from *Streptomyces griseus* and papain (90mg/42mls) activated with L-cysteine-HCl (42mg/42ml). Half the enzyme solution was used to separate the retina from the pigment epithelium (PE), and the remainder was saved for later. Approximately 10 to 15 minutes was required before the retina began to peel away from the PE. The retinal pieces were then lifted from the PE, using two pairs of forceps, and transferred to a petri dish containing the

remaining enzyme solution.

The petri dish was placed on a shaker and gently agitated at a slow speed for 80 minutes. Afterwards, the enzymes were washed out by placing the retinal pieces in a conical tube, with 2mls of supplemented L-15 medium and gently triturating with a 10ml Falcon pipette several times. A minute was allowed for the retinal pieces to settle to the bottom of the tube, at which point, the supernatant was discarded and replaced with 2mls of fresh L-15 medium. The entire washing procedure was repeated nine more times. Following the last washing, the pieces of retinae were vigorously triturated 20 times (or more if necessary), to loosen cell connections, and the supernatant containing dissociated photoreceptors was discarded. 2mls of supplemented L-15 medium were again added, and after triturating ten more times, the supernatant containing dissociated cells was plated on 35mm by 10mm Falcon petri dishes filled with supplemented L-15. Five drops of the supernatant were placed in each petri dish till none was left. After another 2mls of supplemented L-15 were added to the conical tube, the retina was further triturated and the dissociated cells were plated. The process was repeated until roughly thirty petri dishes were plated with dissociated cells. One drop of penicillin, streptomycin (Sigma Co.), was added to each petri dish to kill bacteria. The petri dishes were stored at 10°C for later use.

Equipment:

Cells were recorded with electrodes pulled from borosilicate glass (Garner Co.,

Claremont, CA) whose tips were drawn to ~3μM diameter on a P-87 puller (Sutter Instrument Co., Novato, CA) and then flame polished with a MF-90 microforge (Narishige Co., LTD., Tokyo, Japan). The tip of the electrode was filled with internal solution, by applying negative pressure to the back of the electrode through a piece of rubber tubing attached to a syringe. The electrode was then filled from the back with additional internal solution using a fine needle or another syringe making a complete column of fluid within the electrode. After touching the tip of the electrode to the cell membrane, a giga ohm seal was made by reducing the pressure within the electrode with gentle suction, and the membrane under the tip of the electrode was then burst by applying a brief pulse of negative pressure. Cells were voltage-clamped with an Axopatch 1B amplifier (Axon Instruments, Union City, CA), and from the front panel the access resistance and capacitance were compensated. The data was recorded with the pClamp 6 program (Axon Instruments) onto a computer hard drive. The cells were superfused with Ringer and drugs delivered from a manifold system with pinch valves that was pressure driven (Adams and List Assoc. Ltd., Westbury, NY) and usually operated at ~25mmHg. All solutions exited a quartz tip that was 200μM in diameter. Generally, the holding voltages used were -25 and -50mV, because the resting potential for bipolars are within this range, and in response to glutamate, it is below the reversal potential. A few cells were measured at +30mV and with a ramp ranging from -100 to +50mV.

Dissociated cells were viewed through an Olympus microscope equipped with a

Hoffman modulation contrast system. Bipolar cells were photographed using the Hoffman system and 35mm film. Prints were scanned electronically for reproduction.

Drugs:

Patch pipettes were filled with an internal solution whose composition was 191mM cesium acetate, 13mM KCl, 11mM EGTA, 1mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES. The constituents of the control Ringer solution were 250mM NaCl, 6mM KCl, 20mM $NaHCO_3$, 1mM $MgCl_2$, 4mM $CaCl_2$, 0.23mM NaH_2PO_3, 360mM urea, 10mM glucose, and 5mM HEPES buffer, adjusted to pH 7.6 with NaOH. Drugs and chemicals superfused onto the cells were dissolved in Ringer solution. These were (table 1) trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD) (Thoreson and Miller, 1994), (±)-1-aminocyclopentane-cis-1,3-dicarboxylic acid (cis-ACPD), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) (Sasaki and Kaneko, 1996), L-2-amino-4-phosphonobutyrate (APB) (Nawy and Copenhagen, 1987; Shiells et al., 1981; Slaughter and Miller, 1981), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Monaghan et al., 1989; Sasaki and Kaneko, 1996), glutamate, histidine (HIS) (Albert, 1961), 2-carboxy-4-(1-methylethenyl)-3-pyrrolidinacetic acid (kainic acid) (Hartveit, 1996, 1997; Sasaki and Kaneko, 1996), 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid (KYN)) (Stockton and Slaughter, 1989), *N*-methyl-D-aspartate (NMDA), (Stockton and Slaughter, 1989) picrotoxin (PTX) (Chappell et al., 1992), TEA (Malchow et al., 1991; Shiells and Falk, 1999) N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine

(TPEN) (Baba et al., 1991) and zinc (Qian et al, 1997; Wu et al., 1993).

Isolated skate cells were cultured in L-15 medium supplemented with 103mM sodium, 350mM urea, 5.5mM glucose, 21mM HEPES, 5μg/L insulin, 12.5μg/ml taurine, 4.41μg/ml trimethylamine N-oxide (TMAO), 0.5μg/ml linoleic acid-oleic acid-albumin (albumin from bovine serum), 5.9μg/L sodium selenite and 9.8μg/ml holo-transferrin. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results:

ERG analysis of ON and OFF bipolar cells:

An initial electroretinogram (ERG) study of the skate all-rod retina provided evidence for functionally separate ON and OFF pathways, which suggested the presence of at least two types of bipolar cells. These findings laid the foundation for subsequent experiments on isolated bipolar cells including an investigation into the effects of zinc, followed by further ERG experiments using zinc chelators in the skate eyecup preparation. The ERG is a combined response representing the sum of longitudinal voltage contributions recorded across the retina. The approach used in the first set of experiments pharmacologically isolated and selectively suppressed the components responsible for the ON and OFF responses. L-2-amino-4-phosphonobutyrate (APB, L-AP4, or AP4) was used to block the ON response and kynurenic acid (KYN) was used to block the OFF response. The blockage of GABAergic input onto the bipolar cells was achieved with the GABA antagonist picrotoxin (PTX), while some possible inner retinal contributions were suppressed with *N*-methyl-D-aspartate (NMDA).

The ERG has a prominent ON response (b-wave) and, to try and block it, the initial experiments used 100μM APB, an agonist that binds at the AP4 receptor, a short light stimulus (80 msec.), and either a 3 or 4 ND filter. It was found that APB can effectively suppress the ON response (Fig. 1), and recovery was possible when the drug was washed away with Ringer. However, it was quickly apparent that in order see whether or not the elimination of the ON response

produced changes in the OFF component (d-wave), the length of the light stimulus needed to be increased to provide an observable response at light offset. Increasing the flash duration to 10 seconds allowed the ON response to approach a steady state before the OFF response was initiated. Under these conditions, an OFF response was generally visible as a small bump on top of the c-wave. Although the application of APB had no apparent effect on the OFF component (not shown), the OFF response could be blocked with 5mM KYN, an OFF bipolar glutamate receptor antagonist, without affecting the b-wave (Fig. 2). This suggested that the OFF pathway was independent of the ON pathway, but did not exclude the possible input of horizontal cells via a GABAergic mechanism onto the bipolar cells. To examine this possibility, PTX, known to block chloride channels including both $GABA_A$ and $GABA_C$ receptors, was used in combination with the other agents. A concentration of 200-500μM PTX was used in order to rapidly penetrate remaining vitreous, intercellular spaces, and reach the synaptic cleft between cells.

When 500μM PTX was added following the application of Ringer, several effects were observed, these included a suppressed c-wave, and a prolonged ON component and OFF component of the ERG response (Fig. 3). The use of PTX, however, did have a complication. By blocking the GABA receptors, the absence of a tonic GABA input resulted in spontaneous fluctuations in the ERG, and these effects needed to be taken into account by avoiding the use of data where they interfered. The PTX results suggest that $GABA_A$ and/or $GABA_C$ receptors are

present in the skate retina, and that inhibition through GABA input is crucial in both maintaining the overall stability of the trans-retinal potential and in shaping the magnitude of the ON and OFF responses.

In another experiment, after the eyecup was superfused with Ringer containing 200μM PTX and the changes in the ERG reached a steady state, the solution was switched to one containing 200μM PTX and 100μM APB. The ON response was again suppressed, while the OFF response was not (Fig. 4), and recovery of the ON response by washing with Ringer containing PTX alone could be achieved.

Similarly, the enlarged OFF response observed in 200μM PTX plus 100μM APB could be blocked with Ringer containing 5mM KYN in the presence of the PTX and APB. When a Ringer solution containing 100μM APB, 5mM KYN and 200μM PTX was applied, both the ON and OFF responses were suppressed, and a wash with Ringer containing 100μM APB plus 200μM PTX could recover the OFF response (Fig. 5). This suggests that at the level of the bipolar cells the ON and OFF pathways in the outer retina operate independently of each other. It raises the questions, however, do the pigment epithelial cells, or the cells in the inner retina have any effect on the OFF response?

Although PTX was generally effective in reducing the c-wave and could sometimes completely eliminate it, the total blocking effects of the drugs, it was

felt, could be observed by recording from an isolated retina. Separating the retina from the pigment epithelium would also demonstrate the OFF response is completely independent of the c-wave. When the retina was removed from the eyecup and lifted away from the pigment epithelium, the c-wave, which results from decreasing potassium levels surrounding pigment epithelial cells as photoreceptors hyperpolarize, was eliminated. The ERG had only an a-wave, ON and OFF responses; as before, the retina was tested with the same drugs and methods (Fig. 6). 200μM PTX increased the size of the OFF response, while the peak of the ON response stayed relatively unchanged and the decay time increased. 100μM APB quickly blocked the ON response, and its effect became more pronounced with time as can be observed in the response recorded during the treatment with the solution 200μM PTX, 100μM APB and 5mM KYN which followed. This combination of drugs blocked the ON and OFF responses leaving just the a-wave with a small rebound beyond the original dark potential at light off. Recovery of the ON and OFF responses was possible with a 200μM PTX wash and, to a limited extent, further recovery of the control response was possible by washing with Ringer solution (not shown). The same sequence of drugs was again repeated on the preparation, and this time 100μM APB, in combination with 200μM PTX, immediately produced a total block of the ON response. This experiment demonstrates the complete blocking effect of the drugs on an isolated retina, and that the OFF response, which is often obscured by the c-wave, is independent of the pigment epithelium contribution to the response.

To examine the possibility that cells in the inner retina may be contributing to the OFF response, the approach taken by Stockton and Slaughter (1989), using 500μM NMDA as a means towards blocking inner retina contributions, was applied. Although NMDA has been shown to be effective in blocking contributions of third-order neurons in the amphibian retina (Slaughter and Miller, 1983), its efficacy as a blocking agent in the skate inner retina has not been determined. The application of Ringer containing 500μM NMDA plus 200μM PTX, following treatment with 200μM PTX in Ringer did not eliminate the ON and OFF responses. Consistent with previous results, the addition of 5mM KYN with PTX plus NMDA reversibly suppressed the OFF response (Fig. 7). A summary of the ERG experiments is presented in table 2.

Morphology of bipolar cells:

Skate bipolar cells are polymorphic, and when dissociated they can readily be distinguished from the other cells by their unique dendrites. Characteristically, the dendrites arise from a stalk-like structure that is widely variable both in length and degree of arborization. Some cells have dendrites that branch extensively, while for others the branching is limited, merely bifurcated, or simple without any observable branching under 500 power magnification. Figures 8-11 illustrate the typical range of morphologies and sizes, but for some cells the branching patterns are mixed and fall in between categories (Fig. 11). Single non-branching dendrites vary greatly in thickness, and with freshly isolated cells, spines could frequently be seen (400x magnification) at the tips of the dendrites,

but they are not apparent in these photographs. The size of the bipolar cells also varies considerably with regard to the lengths of their axons and dendrites, and the size of the cell bodies. For example, the branching bipolar cells (figs. 8a and b) have a difference in length, measured from the center of the cell body to the axon terminal, of about 100μm, making the axon in figure 8a 2.14 times longer than that of 8b. This study did not investigate the correlation between size, morphology and a cell's position in the retina.

Cell cultures and the dissociation process:

Using the initial protocol described in methods, finding a sufficient number of isolated ON bipolars in good condition and responsive to glutamate was rare. As a result, changes in the dissociation procedure and culture medium were made. These included, wicking away the vitreous from the ventral side of the eyecup with pieces of filter paper, slicing the retina into smaller pieces approximately 16mm^2, and supplementing the enzyme papain with the addition of protease (15mg/42mls). Furthermore, the L-15 medium, originally supplemented with only sodium, urea, glucose, and HEPES, was further augmented with insulin, taurine, TMAO, albumin, and selenium (see discussion). It was also found that optimum results were obtained when the cells were recorded immediately after dissociating, or not more than 48 hours later. The effects these changes had were judged subjectively (no attempt was made to quantify them), but the cells generally appeared healthier for longer periods of time, and the bipolar cells often had much longer and finer dendritic processes. In addition, cells that were

damaged during the dissociation process, and cell remnants, often sprouted aggressive growth cones, which rarely happened in the original medium, and is indicative of favorable growth conditions.

Despite these improvements, few ON bipolars were capable of giving robust sustained responses without the addition of nucleotides to the electrode, even with the use of amphotericin to form perforated patches. Although the cells, after a good dissociation, often appeared healthy, the complexity of the metabotropic system, and the rapid loss of most glutamate responses, suggested ON bipolar cells were quickly becoming run-down, especially after going "whole-cell." Even though high resistance seals were achieved, when the membrane under the tip of the electrode was burst the responses usually diminished as the cell equilibrated with the internal solution. This seemed to necessitate the addition of ATP, GTP and cGMP (only for ON bipolar cell studies), and there was a significant improvement in the number of ON bipolar cells that were recorded subsequent to their addtion. Thereafter, a greater proportion of the cells, 45% (i.e. 15 out of 33 cells for one dissociation) responded to APB or glutamate as ON bipolar cells, but they were not particularly responsive in a dose-dependent manner. The cells often desensitized, or lacked a gradation in sensitivity to the agonists. For some cells, the problem had to do with the initial glutamate responses being marginal from the start, while other cells gave robust responses that were nearly equal across concentrations (discusssed below).

In contrast, dissociated OFF bipolar cells in the same culture medium were much easier to find and record. These cells were sensitive to glutamate and the glutamatergic agonists kainate and AMPA; using kainate, dose-response data was obtained (discussed below). Most of the cells were not simultaneously tested with ON and OFF bipolar agonists, but as an example, in two successive dissociations, half of the cells, 23 out of 46, were found to be OFF bipolar cells. It is not known how many of the 23 not responding were of the ON type or simply damaged OFF bipolar cells, but the outcome is indicative that at least 50% of the isolated cells were OFF bipolar cells. Together, the two studies of the ON and OFF populations of dissociated bipolar cells suggest there was not a pedominance of either cell type that resulted from the dissociation. Qualitatively, however, OFF bipolar cells faired better at retaining their dose-dependent sensitivity. This difference suggests several possibilities: 1) The glutamate receptors of the ON bipolar cells were damaged by the enzymes, or the dendritic tips where they are predominantly located were lost. 2) The dissociation process has a particularly harsh effect on surviving ON bipolar cells and may reflect fundamental changes in the metabolism of the cell. 3) There were irreversible alterations in one or more components of the second messenger system regulating the cGMP channels, or 4) the culture medium lacked the proper nutrients to maintain the cells.

Responses to glutamate and its agonists:

Attempts were initially made to correlate the morphology of the isolated bipolars

with their physiology, i.e., as either ON or OFF bipolars. The intention was to selectively choose each cell type and study the characteristics of its glutamate receptors. The cells were pharmocologically tested with glutamate, and the glutamatergic agonists: APB, trans-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD), 2-carboxy-4-(1-methylethenyl)-3-pyrrolidinacetic acid (kainic acid) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). ON bipolar cells, typically known to have metabotropic glutamate receptors in other species, were identified by their responses to APB and t-ACPD (Fig. 12). OFF bipolars, which are generally known to have ionotropic glutamate receptors, were identified by the ionotropic agonists kainic acid and AMPA (Figs. 13). Many of the bipolar cells did not respond to glutamate, but could be classified as ON or OFF bipolar cells by their response to the more potent agonists. In general, bipolar cells responding to glutamate could be characterized by the direction of their currents as either ON or OFF and were only sensitive to the corresponding set of agonists. ON bipolar cells responding to APB or t-ACPD, were insensitive to the ionotropic agonists, while OFF bipolar cells were responsive to kainic acid and AMPA, but lacked sensitivity to the metabotropic agonists (Figs. 14 and 15).

The majority of metabotropic and ionotropic bipolar cells initially identified by their selective agonists, also differed consistently in the direction of their currents at both positive and negative holding potentials. Metabotropic cells produced outward currents at negative holding potentials, while those that were ionotropic gave inward currents at negative potentials. The situation was reversed at

positive holding potentials, where metabotropic bipolar cells had inward currents and ionotropic bipolar cells had outward currents. Figure 16 is a typical response of an ON bipolar cell to glutamate, and figure 17 is a representative OFF bipolar response to kainate. Three cells having ON bipolar cell characteristics were recorded with ramps in 0.3μM glutamate and subtracted from ramps recorded in Ringer (control) giving an average reversal potential of 7.1 (Fig. 18). OFF bipolar cells tested at negative and positive holding potentials also had reversal potentials near 0mV (Fig. 19). For both types of bipolar cells the reversal potentials were between -25 and +30mV and usually close to 0mV. The direction of these currents are consistent with the responses of ON (metabotropic) and OFF (ionotropic) bipolar cells in other species.

These initial studies have shown, that primarily based on the direction of the currents, and the agonists used for identification, the cells could be clearly separated into two populations: depolarizing (ON) and hyperpolarizing (OFF) bipolar cells. However, despite the dichotomy in their responses, and given the cells' polymorphism, no particular correlation was found between morphology and physiology. Figure 20 (A) and (B) are examples of similar morphological types of ON and OFF bipolar cell, respectively. Because the ON and OFF bipolar cells were indistinguishable, they were primarily selected, in subsequent experiments, on the basis of condition and not morphology.

Changes in membrane resistance:

By measuring the changes in cell membrane resistance during a response by skate ON and OFF bipolar cells, insights into the physiology of the two contrasting glutamatergic systems were gained. The cells were superfused with glutamate and its agonists, and the effects on membrane resistance were investigated by measuring the resultant currents produced by 5mV pulses held for a 50 msec. duration. While superfusing with Ringer, following the start of a trial, a 5mV prepulse was given at 1.3 seconds; a second 5mV pulse of the same duration was given at the peak of the response to the superfused agonist. By subtracting the difference in pulse currents, the change in membrane resistance was calculated. It was found that for ON bipolar cells the size of the initial pulse in Ringer was larger than the second pulse during the response to glutamate or its agonists, which indicates the agonists caused an increase in membrane resistance (Fig. 21 and Table 3).

Consistent with these measurements are the negative sloping ramps in figure 18, obtained by subtracting the ramps in 0.3μM glutamate from those in Ringer. As the voltage along the ramps recorded in glutamate increased, the outward current induced by glutamate decreased, and this is clearly seen in the difference in ramps, glutamate minus Ringer (control). This could have come about with an associated increase in membrane resistance through the closing of channels. Since inward currents predominate at negative potentials, closing cation ion channels would reduce an inward current producing a net outward current. At

positive potentials the situation is reversed with outward currents predominating and the closing of channels producing a reduction in an outward current or a net inward current. The average reversal potential for the three cells recorded with ramps was +7.1mV and, +2.55mV when plotted with data from a fourth cell tested at select holding potentials rather than a voltage ramp. The close proximity of the reversal potential to 0mV is consistent with bipolar cells in other species and the closing of non-specific cation channels predominantly permeable to sodium and potassium (Ashmore and Falk, 1980; Attwell et al., 1987; Yamashita and Wässle, 1991; Thoreson and Miller, 1993), whose reversal potentials have opposite polarities, respectively, but having some calcium conductance as well (Nawy, 2000).

OFF bipolar cells, in contrast, when superfused with glutamate or kainate had a decrease in membrane resistance when tested with 5mV pulses. During the kainate response, they had a larger pulse compared to the pulse in Ringer indicating the agonists for these cells caused an opening of ion channels (Fig. 22 and Table 4). Since they too have a reversal potential around 0mV, but an inward current at negative potentials and an outward current at positive potentials, which are associated with a decrease in membrane resistance. The effect can be due to an opening of non-specific cation channels (Sasaki and Kaneko, 1996).

Dose-response relationships:

A glutamate dose-response investigation of bipolar cells was undertaken with two objectives: first, to study the binding characteristics of the metabotropic and ligand-gated receptors and, second, as a means towards choosing the optimium concentration of agonists for further studies. The ON bipolar cells were tested with glutamate concentrations ranging from 0.3 to 100μM glutamate, and the cells generally gave the most robust and reproducible responses at micromolar concentrations 0.3 to 10μM. At the higher concentrations (those above 10μM), if the cells initially responded, they would greatly desensitize making it impossible to do further experiments or verify any observed effects. Even in the 0.3 to 10μM range, recording was problematic, most bipolar cells desensitized and/or ran-down giving very erratic responses, especially beyond 3μM glutamate. A general diminution of responses due to cell rundown of second messenger components has generally been reported for ON bipolar cells recorded whole-cell (discussed below). Figure 23 is a dose-response curve fitted to three glutamate concentrations using the Hill equation: $y = V_{max}(x^n / k^n + x^n)$ where y is the normalized whole-cell current, x is concentration, k is the E_{50}, n represents the Hill coefficient, and V_{max} the largest response. The best fit was with cells tested from 0.5 to 3μM glutamate. The ON bipolar cells had a Hill coefficient of 1, an EC_{50} of 0.49 and a V_{max} of 1.13. The reduced responses and large variability beyond 7μM glutamate illustrate the common difficulties of recording ON bipolar cells. Although some of the best data was recorded at 0.3 and 1μM, some of it was not included in the curve since the responses diminished at higher

concentrations.

ON bipolar cells were also tested with APB, a more potent agonist than glutamate, and t-ACPD, a substance less potent than glutamate, in dose-response experiments and the situation was similar. Cells were recorded in t-ACPD solutions ranging from 0.3 to 100μM, and it was found that while 0.3μM was an effective concentration, they responded most often to 30μM. Here too, at concentrations both higher and lower than 30μM, bipolar cells would sometimes give more robust responses, but they were less readily reproducible. APB produced effects similar to glutamate and t-ACPD, with concentrations less than and greater than 10μM giving variable responses or none at all, with high concentrations usually causing desensitization. As a result, 10μM APB was decided upon as the best working concentration, but a meaningful dose-response curve with either of these agonists was not obtained. Recording with glutamate, the first objective of the dose-response experiments was partially achieved, in that a Hill equation could be fitted to the data at low concentrations, though limited by the inherent nature of the cells, but the second-- determining the optimum glutamate and t-ACPD concentrations for subsequent experiments-- was accomplished somewhat empirically. Through the process of obtaining dose-response data for these agents, the best working concentrations were determined.

OFF bipolar cell dose-response relationships were studied using kainate over a

concentration range of 1 to 300μM at -50mV, and a sigmoidal curve (Hill equation) was fit to the data. Taking into considering the erratic responses at high kainate concentrations, several approaches were combined to find the best fit for the data. The data was first normalized to 30μM kainate, and responses that were greatly diminished due to desensitization were excluded. Most cells did not respond to 300μM kainate or higher concentrations and some did not respond to 100μM kainate. The data was fitted by the Hill equation, and two responses at two concentrations, 20 and 70μM kainate, were included in the fitted solid curve (Fig. 24; tables 5 and 6). The curve gave a Hill coefficient of 1.42, an EC_{50} of 26.27 and a V_{max} of 1.80. Since the effects of desensitization were apparent at 300μM, a more precise Hill coefficient was calculated by fitting the curve to the maximum data point of 100μM kainate. This truncated curve, represented by the dashed line (Fig. 24), had a Hill coefficient of 1.07, an EC_{50} of 60.07 and a V_{max} of 2.86.

Some of the collected data included responses at 100 and 300μM kainate, but not the value of 30μM kainate to which the data was normalized. Therefore, another set of data was normalized to 100μM kainate, and two data points were gained at 300μM kainate, but two at both 20 and 70μM kainate were lost (fig. 25; tables 5 and 7). With this group of data, 300μM was the maximum concentration where a peak response was measured, beyond which the cells were greatly desensitized by the application of 400μM kainate. The data at the highest concentrations includes only bipolar cells that responded without significant

desensitization. Again, the values were fitted to the Hill equation giving a Hill coefficient of 1.25, an EC_{50} of 23.76 and a V_{max} of 1.16.

A theoretical dose-response curve was formed from the values obtained from the truncated curve normalized to 30μM kainate (Fig. 24) and the one normalized to 100μM kainate (Fig. 25 to obtain Fig. 26). This curve uses the experimental values of an EC_{50} of 23.76 and a V_{max} of 1.16 derived from the data normalized to 100μM kainate, along with the Hill coefficient of 1.07 derived from the truncated fit normalized to 30μM (Fig. 26, dotted curve). The curve normalized to 100μM kainate (Fig. 26, solid curve) is replotted on a log log scale with the assumed dose-response curve (dashed line). The difference in Hill coefficients between the assumed and actual curve results from desensitization and increasing variability obtained experimentally at high concentrations. As concentrations decrease, the percent disparity between the curves increases, since the same EC_{50} and V_{max} were used in plotting both curves and only the Hill coefficient was changed. Note that the assumed curve (Fig. 26, dotted curve) approximates more closely the mean response at low concentrations supporting the assumption that it provides a more appropriate Hill coefficient.

CNQX studies:

The characteristics of the kainate sensitive receptors in OFF bipolar cells were further identified by CNQX studies. The effects of the competitive blocking agent at 1 and 10μM CNQX concentrations was tested against 100μM kainate at a

holding potential of -50mV, and the responses were blocked 36% and 59%, respectively (Fig. 27, Tables 8 and 9). In addition, the effects of 1μM CNQX which was examined over a range of kainate concentrations from 1 to 100μM, reduced the responses of a typical bipolar by 71% at 30μM kainate and 37% at 100μM kainate (Fig. 28).

Initial experiments with zinc plus glutamate agonists:

Zinc is known to have an effect on the GABA receptor of skate bipolar cells (Qian et al., 1997). In order to see if zinc might have an effect on the glutamatergic response, some ON bipolar cells were tested with APB as well as zinc using different concentrations and holding potentials. Zinc blocked the outward APB induced current for all three cells tested (Fig. 29; Table 10). Bipolar 8, held at -25mV and tested with 10μM APB, gave an outward current of 35pA, which was completely suppressed by 3μM zinc. The other two, cells 44 and 45, held at 0mV responded to 3μM APB by producing outward currents of 46 and 23pA, respectively, but with the addition of 0.7μM zinc, APB currents were blocked or reversed. Two cells were also tested with 7μM glutamate, in conjunction with 3μM zinc as well as 200μM PTX. One of these cells was also tested with 7μM zinc plus 200μM PTX. In all cases, the glutamate response was suppressed by zinc, and PTX did not block the suppression of the glutamate response by zinc. This suggests zinc's action is not via the bipolar GABA receptors which PTX blocks (Fig. 30; Table 11).

The effect of zinc on t-ACPD responses:

This series of experiments examined the effect zinc had on the responses produced by t-ACPD using cis-ACPD as a control. Bipolar cell responses to 30μM t-ACPD and 1μM zinc were measured and then compared to the effect resulting from 1μM zinc plus 30μM t-ACPD in combination. It was initially observed, that zinc had a very potent effect on reducing outward t-ACPD currents, however it quickly became apparent, after recording from a few cells without using cis-ACPD that there was a degree of uncertainty about the observed currents. Frequently, it took several minutes for a cell to come to equilibrium with the internal solution and a t-ACPD response to appear. As the current developed and increased in magnitude, it was not always clear whether the response was physiological, or due at least in part to perfusion artifacts (decreased seal resistance and/or an increasingly leaky cell membrane). This was a significant concern, because perfusion artifacts would have increased the subjectivity of analyzing the data and obscured the effects of zinc. Therefore, subsequent experiments with t-ACPD included its inactive enantiomer, 30μM cis-ACPD, as a baseline control to establish the relative magnitude of the t-ACPD response as well as the efficacy of zinc's blocking action. Figure 31 is an example of the sequential application of these drugs, illustrating the blocking effect of 1μM zinc along with recovery of the t-ACPD response.

Five bipolars were successfully recorded using cis-ACPD as a control and, similar to earlier experiments, the results show 1μM zinc effectively blocked 90%

of the 30μM t-ACPD responses (Figs. 32; Table 12). Interestingly, cell 180 produced a small outward current with cis-ACPD, yet no currents were measured with zinc or zinc plus t-ACPD for this cell. When the data was normalized to t-ACPD, and the superfusion currents were taken into account, 100% of the t-ACPD response was blocked by zinc (Fig. 33; Table 13). Multivariate analysis, on the data shows the t-ACPD induced current was significantly different from the control cis-ACPD current at the 0.001 level. Similarly, t-ACPD currents were significantly different from those induced by zinc alone or t-ACPD plus zinc at the 0.004 and 0.001 levels, respectively.

Two cells were successfully recorded with TEA (20mM), a potassium channel blocker, in the electrode. Although cesium was included in the patch electrode for all ON bipolar cell experiments, from the inside of the cell, it only blocks those potassium channels associated with delayed rectification. In order to examine the possibility that other potassium channels might be the site where zinc is having its effect, TEA was added to the electrode. Intracellularly, TEA blocks potassium channels that are delayed rectifiers, as well as types A, $K_{(Ca)}$, and $K_{(ATP)}$, but not inward rectifiers (K_{ir}) (Hill, 1992). The presence of TEA may have decreased the magnitude of the t-ACPD current, but it did not interfere with the blocking action of zinc (Fig. 34; Tables 12 and 13). Zinc probably does not affect K_{ir}, since its blocking action was observed at +30mV. Even if zinc exerts a blocking effect at the K_{ir} channel, it clearly targets another site at positive holding potentials.

Glutamate responses and the effects of zinc:

ON bipolar cells were also tested over a range of glutamate concentrations, alone and with glutamate plus 1μM zinc, at -25, -50 and +30mV holding potentials. The cells were superfused with 0.3, 1, and 10μM glutamate and here, based on experience with the t-ACPD experiments, cells with large standing currents associated with low seal resistances were rejected. Many of the cells, if they did not first deteriorate or desensitize, were recorded at -25 and -50mV holding potentials, and some were also recorded at +30mV. Provided the glutamate response remained comparable to the current resulting from zinc alone, data from the cells was included even when there was desensitization or run-down at higher concentrations.

Eight ON bipolars were recorded, under at least one condition. Three of these were successfully tested at two concentrations, 0.3 and 10μM glutamate and two holding potentials, -25 and -50mV, before responses were lost (tables 14 and 15). Note that also for bipolar cell 81, glutamate sensitivity was examined at three different concentrations at -50mV holding potential and two concentrations at -25mV, but its responses diminished beyond 0.3μM glutamate.

Among the entire group of cells, the currents resulting from 1μM zinc when normalized to their respective glutamate responses ranged from -0.212 to 1.33%. When 1μM zinc was combined with glutamate at all concentrations, it markedly

reduced ON bipolar cell responses to glutamate, and for a majority of cells, the currents were reversed from outward to inward currents. This is shown in figure 35 for a cell recorded with 0.3μM glutamate. Zinc caused the greatest relative change when applied with 1μM glutamate, which resulted in the largest reversals compared to control (Fig.36). At 0.3 and 10μM glutamate, 1μM zinc did not have as potent an effect on the same bipolar cells as it did at 1μM glutamate. This may have been due to desensitization or run-down, possibly involving another component of the second messenger system, indirectly tied to the glutamate mediated response. The ratios of the zinc to glutamate responses did not seem to be a factor that directly determined whether zinc simply blocked or reversed the response to glutamate.

The effect of 1μM zinc was similar for ON bipolars tested below the glutamate reversal potential at -25 and -50mV holding potentials (Fig. 37). Whether zinc blocked or reversed the glutamate response, the magnitude of the normalized currents were comparable between holding potentials (tables 15). Above the 0mV reversal potential for glutamate-induced currents, i.e., at +30mV, the effects of 1μM zinc were similar to those at negative potentials, blocking the glutamate response, or generally reversing it, but now in the opposite direction to the negative holding potentials (Fig. 38). The comparable size of the currents caused by zinc at -25 and -50mV, and the reversal of the current at +30mV suggest zinc regulates an ion channel, or channels, whose reversal is between -25 and +30mV.

Ramp measurements and the effects of zinc:

Using a ramp as the command potential, cells 79, 80 and 81 were recorded while superfusing with solutions of Ringer, 0.3μM glutamate in Ringer, then 0.3μM glutamate plus 1μM zinc in Ringer. Subtracting the ramps, glutamate minus Ringer resulted in negative sloping I-V curves, while subtracting Ringer from the glutamate plus zinc response produced slightly positive sloping curves, although a zero slope was within the envelope of the standard error of the mean (Fig. 39). Even though the glutamate response for bipolar cell 81 had a large zinc effect in the direction of the glutamate response, a similar reversal of current occurred (not shown). Based on the average slope of the ramps, glutamate caused an increase in membrane resistance relative to Ringer with a reversal potential of +7.4mV, but when combined with zinc, although the resistance decreased the reversal potential barely changed to the value of +5.4mV. Similar reversal potentials were obtained when the slopes were plotted from selected holding potentials with a fourth cell added. The glutamate minus Ringer curve had a reversal potential of +2.55mV and the curve for glutamate plus zinc minus Ringer had a reversal potential of -2.55mV. The opposite sign of the slopes between the glutamate and zinc plus glutamate ramps, but the nearly identical reversal potentials, suggests a two-fold action for zinc: The usual channel closing effects of glutamate on the ON bipolar cell are blocked by zinc, i.e zinc prevents the closing of the non-specific cation channels, causing no change in the reversal potential. Furthermore, the presence of zinc in conjunction with the actions of glutamate reverses the slope of the ramps by increasing conductance, possibly

through upregulation of these same non-specific cation channels.

Sequential perfusion of zinc and glutamate agonist:

A primary question to be addressed is whether zinc exert its effect extracellularly, such as via receptors and ion channels, or intracellularly by blocking one or more components of the second messenger system? The approach used to determine the possible site(s) of action was to sequentially superfuse glutamate and zinc then compare the response to the effects of the inverse sequence of agents. Two ON bipolar cells were tested by first superfusing the cells with zinc then immediately following it with the agonist t-ACPD. Both of these bipolar cells were tested by first superfusing the cell for an extended interval (20 seconds) with 30μM t-ACPD and immediately following it with 1μM zinc for 2.5 seconds. Then the sequence was reversed by applying zinc first for 20 seconds followed by glutamate for 2.5 seconds. It was reasoned that if zinc were having an intracellular effect and, not acting primarily on the glutamate receptor or an external ion channel site, then the sequential application of zinc followed by t-ACPD would not require their simultaneous presence at the cell surface to elicit a zinc effect on the t-ACPD response. In other words, a bipolar cell "preloaded" with zinc should be able to block a glutamate response, if its site of action is a component of the second messenger, or elsewhere within the cell.

When the bipolar cell was first superfused with t-ACPD for 20 seconds then zinc for 2.5 seconds, t-ACPD had no observable effect on the zinc-induced current.

In fact, desensitization usually eliminated the responses and prevented further testing, but when the cell was first superfused with zinc for 25 seconds and then t-ACPD for 2.5 seconds, several effects became apparent (Fig. 40). The frequently seen outward current produced by zinc, given sufficient time, returned towards or below the standing current baseline. Then following the extended application of zinc with the short application of t-ACPD, 2.5 seconds, there was at first no initial change from the previous zinc current. The expected outward t-ACPD current seen under control conditions was blocked but subsequently, after a delay, a large inward current occurred that roughly coincided in time with the peak t-ACPD response under control conditions, i.e., Ringer to t-ACPD. Following the return to baseline from this large inward current, there was a temporary overshoot (Fig. 40, lower trace), before the standing current returned to its original value (not shown).

The results were largely similar when tried on another bipolar cell. Zinc in the first 25 seconds produced a transient outward current that returned a bit below the standing current before the application of t-ACPD, and it also had a blocking effect on the initial t-ACPD response (Fig. 41). The outward t-ACPD mediated current observed under control conditions was blocked, and again, there was a precipitous increase in the inward current, following a delay period which nearly coincided with the control peak t-ACPD response without zinc. The control response for this cell had an overshoot below the standing current. Nawy (2000) attributes this to an upregulation of the channels due to the previous closing of

the non-specific cation channels which causes a reduction in intracellular calcium. The sharp increase in inward current under zinc treated conditions coincides with the peak effects of t-ACPD and not the upregulating after-effects of channel closing. In fact, zinc seems to prevent the closing of these channels, although absence of an outward current could be due to a net inward current from another ion channel. Even with the sharp increase of an inward current, the cells repeatedly returned within one minute to their previous standing current after each test. This sequential approach of applying zinc and then the agonist separates in time the simultaneous events that occur with the coapplication of zinc and the agonist. The results suggest the cells can be preloaded with zinc, and when followed with t-ACPD treatment, effects similar to the co-application of t-ACPD plus zinc can be observed.

Kainate response and the blocking effect of zinc:

Kainate is a potent glutamate agonist to which OFF bipolar cells are sensitive. The intent of this set of experiments was to determine whether zinc also has a modulatory effect on the kainate response comparable to its effects on depolarizing ON bipolar cells. Seven OFF bipolars were recorded at -50mV, and the effect of 1μM zinc on the response of these seven cells to 300μM kainate was examined. One additional cell was also tested at this clamp potential with 1μM zinc and 30μM kainate. All bipolar cells, except for one of the seven tested with 300μM kainate had their kainate responses blocked by 1μM zinc (Figs. 42 and 43; Tables 16 and 17), and that cell which gave the same response to 1μM

zinc as it did to 300μM kainate or the combination of the two was not included in further analyses. Based on responses normalized to kainate, the zinc plus kainate combination blocked 100% of the kainate current for three cells and was 75 and 86% effective in suppressing the kainate current for two others. One OFF bipolar cell had a small outward currents when zinc was combined with kainate, probably resulting from the complete blocking effects of zinc combined with a small superfusion artifact. The bipolar cell tested with 30μM kainate had its response reduced by 1μM zinc to 34.8% of its original magnitude.

Responses to zinc:

A group of OFF bipolar cells, all from the same cell dissociation, were tested with with low concentrations of zinc (0.7μM). Three cells responded to 0.7μM zinc alone with large outward currents, while one cell was only sensitive to 7μM and not 0.7μM zinc. These responses were different in shape and magnitude from those observed with ON bipolar cells. The zinc currents seen with the depolarizing (ON) bipolar cells, often grew along with responses to glutamate and its agonists, as the cyclic neucleotides dialyzed into the cell and, unlike these OFF bipolar cells, the currents were not large with sharp transient changes. Based on kainate sensitivity, the cells were identified as the OFF type, and using 5mV pulses, changes in current caused by kainate compared to Ringer were measured. Increases in input resistance calculated for four cells ranged from 1.71 o 12.15 M ohms (table 18). The response of cell 295 to zinc is shown above below and near its reversal potential in figure 44 and a comparison

between the pulse data (Fig. 45A and a ramp (Fig. 45B) for cell 288 is also consistent. Just as the second pulse with zinc was smaller compared to Ringer (Fig. 45A), the difference between the ramp current obtained in Ringer and the ramp in 0.7μM zinc had a negative slope indicating zinc caused a decrease in current by increasing membrane resistance (Fig. 45B). Among these OFF bipolar cells, outward currents were observed at negative holding potentials (-25mV and -50mV). Cells 295 and 302 were also measured at a +30mV holding potential where they were found to have inward currents. The outward currents of these cells in response to zinc at negative holding potentials, coupled with their increase in membrane resistance result, presumably, from the closing of ion channels, and would therefore be the blocking of an inward current.

It is not clear why a few of the cells, all from the same cell culture responded to zinc. If not a fluke, possibly a component of the non-responding cells, either ion channels or enzymes, may have escaped damage or loss during the dissociation, or there may simply be, based on zinc sensitivity, several types of bipolar cells present in the skate retina. The cells seemed to be in good condition but there is the possibility that their physiology was altered by the dissociation. No correlation was observed between cell morphology and sensitivity to zinc.

Effects of zinc chelators on the skate ERG:

The experiments with isolated bipolar cells suggest that zinc is a potent

modulator of the glutamate response; however, it leaves unanswered the question whether *in vivo* there is in fact any extracellular zinc that affects the response of bipolar cells. As a step towards determining this, the effect of zinc chelators in conjunction with picrotoxin was measured using the skate ERG. Following the initial application of Ringer, the eyecups were superfused with 200μM picrotoxin dissolved in Ringer to block the $GABA_A$ and $GABA_C$ receptors. This was necessary, since both GABA receptors have been found to have zinc sensitivity on skate bipolar cells, as do $GABA_A$ receptors on skate Müller cells (Qian et al., 1996; Qian et al., 1997).

Once the changes in the ON and OFF responses reached a steady state with picrotoxin, Ringer containing 100μM histidine was superfused as a zinc chelator in combination with picrotoxin. In a later experiment histidine was substituted with the zinc chelator N,N,N,N-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a 100μM concentration. Changes in the magnitude of the ON and OFF responses were measured and compared to their amplitude in the picrotoxin controls. The effects of the chelators on the ON response was determined by stimulating the eyecup with 5 seconds of light once per minute at a given intensity, either 3 or 4ND and, letting the preparation recuperate in the dark. Repeated measurements of the ON and OFF responses were taken for averaging.

Six dark adapted eyecups (I-VI) were tested with histidine plus PTX. 200μM PTX

increased the ON response (Fig. 46) and the addition of HIS enhanced it further (Fig. 47). The ON responses for these five eyecup preparations are summarized in table 19. Histidine had no effect on eyecup VI.

One dark adapted eyecup was tested with the zinc chelator TPEN, (100μM), also using a 5 second flash of light as a stimulus and its ON response increased by 1.22 (table 20).

DISCUSSION

Overview of research:

This investigation of the skate all-rod retina has provided evidence for independent ON and OFF afferent pathways in the outer retina, which was obtained through pharmacological manipulation of the electroretinogram (ERG). These findings were supported by voltage clamp data recorded from isolated bipolar cells. Autonomous ON and OFF ERG responses were initially demonstrated by recording eyecup preparations, superfused with glutamatergic and GABAergic blocking agents to selectively isolate the ON and OFF pathways. 2-Amino-4-phosphonobutyric acid (APB) and kynurenic acid (KYN) were used to suppress the ON and OFF responses, respectively, and were applied in conjunction with picrotoxin (PTX), a GABA channel blocker which suppressed lateral inhibition via horizontal cells. Superfusing eyecup preparations with APB plus PTX eliminated the ON response, leaving the OFF response, while the application of kynurenic acid KYN plus PTX had the opposite effect, blocking just the OFF response. The discovery of an operative OFF pathway independent of the ON pathway, in the outer retina, suggested the presence of at least two types of bipolar cells and provided the basis for a study to characterize the cells.

Isolated bipolar cells, recorded whole-cell voltage clamp, were found to have two kinds of glutamatergic responses that did not reside within the same cell. One type of bipolar cell was sensitive to the metabotropic agonists, APB and t-ACPD, with ON-like characteristics and, the other had sensitivity to the ionotropic

agonists, AMPA and kainate, with OFF-like characteristics. Glutamate dose-response studies were also completed, and the overall results from this voltage clamp analysis were in accord with the earlier ERG data. In addition, they were in agreement with previous research, which had shown differential staining of ON-like and OFF-like bipolar cells based on their immunoreactivity with protein kinase C (PKC) and serotonin antibodies, respectively (Schlemermeyer and Chappell, 1996). This analysis of the bipolar cells provided the basis for an investigation into the effects of zinc on the glutamatergic responses in ON and OFF bipolar cells and evidence that endogenous zinc is a retinal neuromodulator.

Brain research has shown zinc to be an important neuromodulator of glutamate responses, and in the retina it has been demonstrated to have potent regulatory effects on glutamate- and GABA- mediated currents. Zinc, it is postulated, is co-released with glutamate and acts at the photoreceptor terminals in a feedback system that downregulates any further release of glutamate (Wu et al., 1993). It is also a neuromodulator of the glutamatergic and GABAergic receptors on horizontal cells (Kaneda et al., 1997; Shen and Yang, 1999), as well as the GABAergic receptors on bipolar (Qian et al. 1997; Han and Yang, 1999) and Müller cells (Qian et al., 1996). These widespread effects were the basis for examining the possible role zinc plays on glutamate mediated responses in isolated skate bipolar cells. It was discovered that ON and OFF bipolar cell responses to glutamate are suppressed by zinc, but it remained unknown whether vesicular zinc was released, or similar to other transition metals, it was

simply acting as a blocking agent. Experiments measuring the effects of zinc chelators on the ERG provided *in situ* evidence for its natural presence as a extracellular neuromodulator. These findings are believed to be the first reported for any species, which show that endogenous zinc is a modulator of the light response and may also be an important glutamatergic neuromodulator of both ON and OFF bipolar cells.

ERG recording from skate eyecup preparations:

The skate ERG, recorded in the DC mode, with Ringer alone (control conditons), provided a direct approach to investigate the source of the ON and OFF pathways, since the ON response to light (b-wave) is quite prominent and, under appropriate recording conditions, the response to light OFF (d-wave) could generally be observed. The first experiments investigating the ON and OFF components of the ERG were intended to suppress the ON response with APB and reveal, perhaps, the smaller OFF response. In other species, APB has been proven to be an effective glutamate agonist that binds to the AP-4 receptor of ON bipolar cells (Shiells et al. 1981; Slaughter and Miller 1981, 1985; Nawy and Jahr, 1990a; Wässle et al. 1991; Yamashita and Wässle, 1991; Tian and Slaughter, 1994). When the skate eyecup was superfused with 100μM APB in Ringer, it completely blocked the ON response without altering the rest of the ERG but did not uncover an OFF response with the brief (80msec) flash used (Fig. 1).

It quickly became clear that in order to observe the OFF component a longer light

interval was necessary to separate the ON and OFF responses in time. This had to do with the much smaller OFF component being masked by the very large ON component, which required considerable time to repolarize and reach a steady-state. The initial APB experiments used light flashes of only 80 msec duration and, under conditons of a short light stimulus, the relatively small depolarizing OFF response occurred while the large opposite direction ON response was repolarizing. Consequently, the net potential of the ON component obscured the OFF component. However, when the duration of the flash was lengthened to 10 seconds there was a sufficient separation of the ON and OFF components, allowing the ON response to achieve a steady-state, neither depolarizing or repolarizing before the end of the flash, and at light off a small OFF response was revealed (Fig. 2). Observable ON and OFF components enabled an investigation into the source of these two responses.

Using a 10 second light stimulus, it was found the OFF response, independent of the ON response, could be suppressed with the antagonist KYN, which is a broad spectrum blocker of ligand-gated glutamate channels. This was used previously by Stockton and Slaughter (1989) with the amphibian ERG as an OFF pathway blocker. Superfusing skate eyecup preparations with 5mM KYN dissolved in Ringer, the OFF response was suppressed leaving other components of the ERG unchanged, and by subtracting ERG data, the control in Ringer alone minus KYN treatment, there was a small but observable difference at the OFF response (Fig. 2). This result demonstrated the OFF pathway could

be directly blocked without needing to suppress the ON pathway and that, possibly, it operates independently of the ON pathway. Further proof of this observation might be provided by the survival of an OFF response while simultaneously blocking the ON response as well as lateral inhibition in the outer retina.

Lateral input to bipolar cells presumably occurs via the horizontal cells which release GABA when depolarized in the dark. In order to block horizontal cell input, picrotoxin, a non-specific antagonist of the $GABA_A$ and $GABA_C$ channels was used. Work by Qian et al. (1997) has shown the bipolar cells, type not specified, are sensitive to THIP, a $GABA_A$ agonist, and that responses to THIP could be completely blocked by the $GABA_A$ antagonist, bicuculline. In addition, the combination of 10μM GABA and 100μM bicuculline left a current that resulted from $GABA_C$ receptors. Because of PTX's proven effectiveness, prior to this study (Chappell et al., 1992), its application was anticipated to isolate the bipolar cells from GABAergic input by the horizontal cells and remove a normally present inhibitory current.

Application of 200μM picrotoxin in Ringer had a dramatic effect on the control ERG response in Ringer. It greatly enlarged the ON and OFF responses and had the benefit of reducing the c-wave making the barely discernable OFF response quite prominent (Fig. 3). The c-wave is believed to represent the repolarization of the pigment epithelium (PE), and the effects of PTX on the

c-wave suggest GABA receptors might play a role there as well. A contributing component to the c-wave could also have been due to inhibition at the Müller cells, which in skate have GABA receptors sensitive to PTX (Qian et al. 1996). The time period necessary for the ON response to repolarize from the light stimulus and the OFF response to repolarize from dark stimulus was greatly increased and indicates GABA abbreviates the duration of the responses as well as their amplitudes. Recovery from PTX was possible with a Ringer wash (not shown).

A negative consequence of the PTX effect was an instability in the ERG that occurred during the dark intervals, between the light flashes, when a maximum amount of glutamate is presumably released by the photoreceptors. Although the oscillations appeared about a general mean, the fluctuations point to the important role GABAergic mechanisms have in providing the retina with a baseline of stability. When the experiments with APB and KYN were repeated with PTX in the solutions, the effects were dramatic. 200μM PTX alone altered the ON and OFF responses, by generally increasing their size. After it was superfused for a period of about 10 to 15 minutes, and the magnitudes of the ON and OFF responses reached a steady state, the solution was switched to 200μM PTX plus 100μM APB. The APB + PTX solution, after a few minutes began to suppress the ON response and completely blocked it within 10 to 15 minutes leaving the OFF response (Fig.4). Then a solution of 5mM KYN, 100μM APB plus 200μM PTX was superfused onto the eyecup, and the remaining OFF

response was also blocked leaving just a suppressed c-wave. The OFF response was recovered with the removal of KYN, by washing with APB + PTX solution, providing evidence that the effects were not due to toxicity (Fig. 5). Similarly, the effects of APB and PTX showed at least partial reversal, although the washout of these drugs was more difficult (table 2).

When this experiment was repeated with the same drugs in another preparation, without the PE, as an isolated retina, similar results were achieved. The absence of the c-wave enabled direct observation of the drug effects on the retina, from which it was concluded that neither the ON or OFF responses are derived from the PE. The a-wave was readily observable without the PE and was the only remaining component of the ERG after the retina was treated with a solution of APB, KYN plus PTX (Fig. 6). Similar to eyecup preparations where PTX effectively eliminated the c-wave, the isolated retina treated with PTX had ON and OFF responses that were of comparable magnitude. Under control conditions, in Ringer, the ON and OFF responses of the isolated retina were still not of equal magnitude but, after superfusing the retina with PTX, the OFF response greatly increased. This demonstrates the OFF pathway contribution to the ERG is significantly enhanced when GABAergic input is blocked.

KYN and PTX may also have effects on the inner retina. KYN is a non-specific blocker, and has been shown not only to decrease synaptic input at the bipolar cells, but to also decrease synaptic transmission to third order neurons, from

bipolar cells to the inner retina (Coleman et al., 1986). PTX in the toad, *Bufo marinus,* has the effect of enhancing the ON and OFF response, by blocking GABA input from the amacrine cells (Katz et al. 1991). It was also found in that study and others that these enhanced inner retina responses could be blocked by NMDA, which has been shown to block the amphibian inner retina through its depolarizing effects (Slaughter and Miller, 1983; Stockton and Slaughter, 1989). Although NMDA studies on the inner retina of the skate are not available, it was thought prudent to test the agonist to see if its effects were different from those in salamander and mudpuppy. The generalization that NMDA is an effective blocker of the inner retina may not hold true for other species. Nevertheless, NMDA was used as a potential inner retina blocker in order to assure that it was not a factor here.

When 200μM PTX plus 500μM NMDA in Ringer was applied to eyecup preparations, following 200μM PTX treatment, no observable change was detected in the ERG. The ON and OFF responses were unaffected, and the previous experiments were repeated with NMDA in the solutions to see again if the OFF response could still operate independently of the ON response. Application of 500μM NMDA, 200μM PTX, plus 5mM KYN in Ringer, within 10 to 15 minutes selectively blocked the OFF response without changing the ON response and the OFF response could be recovered with a wash of PTX plus NMDA (Fig.7). In addition, the absence of an effect by NMDA implies that NMDA receptors are not likely to be found on skate bipolar cells as has been reported

for some other vertebrate retinae (Wu and Yang, 1991; Thoreson and Miller, 1993; Hartveit, 1996; Sasaki and Kaneko, 1996; Connaughton and Nelson, 2000).

The effects of all of the drugs, especially KYN, were reversible in that it was possible to recover blocked responses by returning to the previous solution minus the blocking agent (table 2). This is significant, because it demonstrates blockage of the neurotransmitters at their receptors and not the absence of a response due to toxicity. The effects observed by the drugs, for the most part, were very consistent, but there were some difficulties in washout. This can be attributed to the tenacity of the drugs once they penetrate the intracellular space. Relatively high drug concentrations were used to be consistent with Stockton and Slaughter (1989) and comparable to other ERG experiments. High concentrations were believed necessary so that a reasonable dose could penetrate the film of vitreous and reach the cells in a relatively brief time. The objective was to see the drug effect occur quickly on a healthy retina, rather than perform the experiments for extended periods of time and not know if the diminished responses were due to the drugs or cell death. It always took much longer for the drug to wash out than for its effect to occur, and it was frequently a competition between obtaining washout before the retina deteriorated. This accounts for the difference in table 2 between the number of times a drug effect was observed and the number of times it could be reversed.

Dissociated cells

The internal solution:

ON bipolar cell glutamate responses when recorded whole-cell quickly diminish due to the diffusion of second messenger components out of the cell and/or into the patch recording electrode (Nawy and Jahr 1990b; Wässle et al., 1991). In order to compensate for the loss of some key intracellular substances, particularly those relating to the ON bipolar glutamate responses, nucleotides were added to the patch electrode internal solution. The internal solution was supplemented with cyclic GMP (1mM), GTP (1mM) and ATP (1mM). cGMP is necessary for maintaining the non-specific cation channels of ON bipolar cells in an open configuration and, as others have observed (de la Villa et al., 1995; Shiells and Falk, 1990; Nawy and Jahr, 1990a), once it dialyzed into cells, the standing current increased (became more negative). GTP is a necessary constituent for the successful activation of many G-proteins including those that activate PDE. ATP was also added, since it serves many vital functions, which include the running of ionic pumps and is easily dialyzed out of the cell. The addition of these nucleotides to the electrode resulted in a marked improvement in recording and sustaining responses.

Adjustments in the culture medium and dissociating enzymes:

Changes were made with the dissociation process, which were empirically arrived at through a series of gradual adjustments. The dissociating enzyme papain (90mg/42ml) was supplemented with protease (20mg/42ml), and the

combination of enzymes resulted in dissociated bipolar cells with very fine-branching dendritic processes. This minimized the trituration process and, presumably, damage to the cells, their dentrites and associated glutamate receptors. It was noticed that even when the cells looked very good after a dissociation, they rapidly deteriorated within a few days. For this reason as much recording as possible was done the day of the dissociation, but some data was collected in the following days. Since the bipolar cells maintained in the culture medium responded, for the most part, poorly to glutamate and its agonists, especially the ON type, supplements to the medium were added. A number of substances were tried in varying concentrations over many dissociations. Those that resulted in an improvement, better responses and healthier looking cells were kept and the addititives that did not were eliminated from the next dissociation. The decision was made on a subjective basis. A number of substances commonly used in cell cultures were tried successfully.

Urea, a supplement to the culture medium, is a metabolic waste product that compromises cell function, but the cartilaginous fish (Chondrichthyes = Elasmobranchii), unlike other marine animals, use it to their advantage for osmoregulation. They maintain an intracellular osmolarity nearly equal to seawater by concentrating high levels of urea in the cells. Yancey and Somero (1980) undertook a study to determine whether elasmobranch enzymes have an evolutionary adaptation to protect them from the deleterious effects of urea or if the methylamines and, trimethylamine (TMAO) in particular, are responsible for

lessening the harmful effects of urea. Biochemical assays were made on elasmobranch and mammalian enzymes with urea, without urea, and in the presence of urea plus TMAO. It was found for both groups of enzymes tested with urea, TMAO in a majority of the cases lowered the Michaelis constant (K_m), i.e. it mitigated the harmful effects of urea. In addition, it caused an increased V_{max} for some of the enzymes. The protective effects TMAO has on mammalian enzymes supports the concept that it optimizes enzyme function in a high urea environment rather than that the elasmobranch enzymes are unusually adapted. The maximum effect of the methylamine was found to be a urea to TMAO ratio of 2:1, respectively.

In skate, low TMAO to urea ratios have been found in muscle (Forster and Goldstein, 1976), although other methylamines were present. These methylamine analogues in conjunction with TMAO were also effective counter agents to urea. Kinases tested with 400mM urea plus 65mM TMAO, 55mM sarcosine, 50mM β-alanine and 30mM betaine were protected as effectively from urea as TMAO being present in a 2:1 ratio (Yancey and Somero, 1980). In general, the effects of urea on proteins is to increase the solubility of side-chains (Nozaki and Tanford, 1963). Methylamines are similar in structure to stabilizing macromolecules and may work by decreasing the solubility of side-chain groups enabling proteins in an urea environment to retain an optimum conformation (Yancey and Somero, 1980).

TMAO was added to the cell culture medium (0.5μg/ml) for these experiments, and an improvement in the condition of the cells was observed. Several cell dissociations were also tried with TMAO in combination with sarcosine, β-alanine and betaine, but no significant difference was observed. This may have been a problem with the choice of concentrations used, or even the quality of the dissociation itself. Since the primary objective of this research was to obtain a working cell culture (with possible improvements) and not to devise a new culture medium, for simplicity, TMAO was the only methylamine added. TMAO, in fact, has been determined to be a requirement for the establishement and growth of a variety of elasmobranch cell cultures. Shark brain cells were found to be the only tissue capable of limited growth without the addition of TMAO to the medium (Hartmann et al., 1992).

The amino acid taurine is also present in organs throughout the skate (Boyd et al., 1977) and was added to the culture medium (12.5μg/ml). In general, high concentrations of taurine are found in the retina particularly in the photoreceptors, where in the rat it is 79mM in the photoreceptor layer. A number of possible roles for taurine that have been suggested range from being an inhibitory neurotransmitter to those including metabolic functions. One purpose, as a sulfur containing amino acid, may be to protect photoreceptors from the damaging effects of light caused by the formation of oxygen free radicals (Lombardini, 1991). Measuring changes in the ERG, under conditions of hypoxia, taurine was found to have a restorative effect, as determined by the size

of the b-wave. Similar to hippocampal slices, the improvement that taurine provided was thought to derive from its antihypoxic characteristics (Tseng et al., 1990). Beneficial effects were observed with the addition of 12.5μg/ml taurine to skate cell cultures, which included improved condition and better responses.

Other added supplements important for neuronal cell cultures included linoleic acid-oleic acid-albumin (albumin from bovine serum or BSA) (0.5μg/ml), insulin (5μg/ml), transferrin (9.8μg/ml) and sodium selenite (5.9μg/L). Banker and Goslin (1991) ascribe to these additives the following effects: BSA promotes cell growth and may function as an antioxidant or possibly affect nerve growth factor (NGF), by either forming a complex with it, or minimizing its adsorptive loss onto plastic surfaces.

Insulin is important for cell growth and has a variety of effects on cell metabolism including membrane transport, glucose metabolism, and the biosynthesis of fatty acids as well as nucleic acids.

Transferrin is a protein in blood serum, the β-globulin fraction, whose function is the transport of iron. Its purpose in cell culture media may be, in addition to iron transport, the detoxification of trace metals.

Sodium selenium was also included in the medium and is believed to function as an antioxidant protecting membranes.

Characteristics of isolated ON and OFF bipolar cells:

Voltage-clamped skate ON bipolar cells were identified by their sensitivity to metabotropic glutamate agonists, APB and t-ACPD, and the direction of resulting currents at negative and positive holding potentials. APB, a potent agonist that is selective for the group III metabotropic glutamate receptor, mGluR6 (Nakajima et al., 1993) which is unique to ON depolarizing bipolar cells, provides a direct and reliable method for separating metabotropic bipolar cells from those that have ligand-gated glutamate receptors (Shiells et al., 1981; Slaughter and Miller, 1981, 1985; Bloomfield and Dowling, 1985; Wässle et al., 1991). t-ACPD, a general metabotropic glutamate agonist also activates ON bipolar cells through the APB receptor (Thoreson and Miller, 1994; Tian and Slaughter, 1994, 1995). The responsivity of skate ON bipolar cells to APB and tACPD was consistent with the findings in other species. They responded well to the metabotropic agonists, APB and t-ACPD, but were insensitive to the ligand-gated glutamate agonists, AMPA and kainate (Thoreson and Miller, 1993; Euler et al., 1996; Connaughton and Nelson 2000) (Figs. 12 and 14). Although t-ACPD is the least potent agonist, compared to APB and glutamate (Nakanishi, 1992; Tian and Slaughter, 1994), 30μM t-ACPD worked effectively on skate ON bipolar cells. The possibility that t-ACPD may bind to other ON bipolar cell receptors can not be excluded until it is tested, since it is a broad spectrum agonist that also binds to metabotropic glutamate receptors belonging to groups I and II (Nakanishi, 1992). Catfish bipolar cells are immunoreactive with antibodies to metabotropic glutamate receptors belonging to groups I, II and III (Gafka et al, 1999).

Skate OFF bipolar cells were identified by their sensitivity to the ionotropic agonists AMPA and kainate combined with their insensitivity to the metabotropic agonists APB and t-ACPD (Figs. 13-15), just as cat OFF bipolar cells were found sensitive to kainate and AMPA but not to APB or t-ACPD (Sasaki and Kaneko, 1996). AMPA-induced currents had a rapid onset that quickly desensitized leaving a smaller sustained component. These responses were more difficult to record and are indicative of receptors with a high-AMPA/low-kainate affinity (Seeburg, 1993; Hollmann and Heinemann, 1994). Kainate-derived currents were sustained with comparatively little desensitization and, probably resulted from either the high-kainate/low-AMPA affinity receptor, or the high AMPA low kainate affinity receptor, since they do not desensitize to kainate (Hollmann and Heinemann, 1994; Seeburg, 1993). Similar sustained kainate responses were recorded in rat OFF bipolar cells where kainate was used to avoid the problems of rapid desensitization with AMPA (Hartveit, 1997) The competitive AMPA/kainate receptor antagonist, CNQX, blocked glutamate responses in a dose-dependent manner in cat OFF bipolar cells which were insensitive to NMDA (Sasaki and Kaneko, 1996). Kainate mediated responses in skate OFF bipolar cells were effectively blocked by CNQX in a dose-dependent manner (Figs. 27 and 28), but it remains to be determined if they have NMDA receptors, even though the ERG experiments do not seem to indicate that they do.

Skate bipolar cell currents in response to metabotropic and ionotropic glutamate agonists were in opposite directions, similar to that found in other vertebrates

(Shiells et al., 1981; Slaughter and Miller, 1981; Wässle et al, 1991; Euler et al., 1996; Sasaki and Kaneko, 1996). In this study, APB and t-ACPD mediated outward currents at negative holding potentials and inward currents at postive holding potentials, while the bipolar cells responding to AMPA and kainate had the opposite currents, inward at negative holding potentials and outward at positive holding potentials (Fig. 16 and 17). Along with the glutamate analogues, the direction of agonist-mediated currents were an important characteristic for identifying cells as either ON or OFF-type bipolar cells, particularly when recording with glutamate. A few cells tested with APB or t-ACPD produced inward currents at negative holding potentials, and it is not known if this was due to an imbalance with the internal solution. Karschin and Wässle (1990) found out of 93 rat bipolar cells tested with APB recording whole-cell patch-clamp 93 responded with conductance increases, but when they later used nystatin APB caused only a decrease in conductance (Wässle et al., 1991). For this study the addition of cGMP to the internal solution minimized the occurance of conductance increases with cells recorded using APB and t-ACPD agonists (discussed below). ON-type bipolar cells with conductance increases when occassionally observed were not included in the study. They should not, however, necessarily be considered aberrant without further examination.

ON bipolar cells sensitive to APB have been reported that respond to the agonist with an increase in potassium conductance (Hirano and Macleish, 1991) or chloride conductance (Connaughton and Nelson, 2000). In addition, a particular

type of ON bipolar cell in fish has APB sensitive receptors that mediate a decrease in membrane conductance but also has APB insensitive glutamate receptors on that same cell, which mediate an increase in membrane conductance (Nawy and Copenhagen, 1987). The skate OFF bipolar cells were studied using kainate to assure only the ionotropic type cells were being recorded and not, possibly, ON bipolar cells with ionic imbalances.

ON bipolar cell conductance:

Skate ON bipolar cells were identified by their APB-type responses to glutamate and its analogues, from which a further investigation of the glutamatergic response was undertaken. The source of their outward current at negative holding potentials and, inward current when held at positive potentials was unknown. It might result from either an increase in conductance of an outward current, e.g. potassium, or a decrease in conductance of an inward current, such as sodium. To determine changes in membrane resistance, cells were tested two ways, with ramps and applied 5mV pulses. Ramps were recorded in Ringer alone then Ringer plus glutamate and were subtracted; the difference was a ramp that had a negative slope, which indicates a decrease in membrane conductance (Fig. 18). Consistent with these results was the difference in conductance between 5mV pulses applied to bipolar cells in Ringer compared to 5mV pulses during a response to glutamate or its agonists. The conductance measured during a response to glutamate and its agonists was always smaller than that in Ringer (Fig. 21). Both situations represent a decrease in

conductance, from which it can be concluded at negative holding potentials, an outward current results from a reduction in a net inward current, while at positive holding potentials the inward current is due to a decrease in a net outward current.

These ON bipolar cells were determined to have reversal potentials near 0mV based on their ramps, an I-V plot with an additional cell at select holding potentials (not shown), and the reversal of currents observed between -25mV and +30mV holding potentials (Figs. 16 and 18). A reversal potential near 0mV, between reversal potentials for sodium and potassium, is typical of a non-specific type cation channel. Similar to other preparations, skate ON bipolar cell membrane conductance increased with the addition of cGMP to the internal solution as did the glutamatergic responses, which suggests the cation channels are cGMP regulated. These characteristics identified in skate bipolar cells have an overall similarity to depolarizing ON bipolar cells in other vertebrates. For example, cat ON bipolar cells have non-specific cation channels with reversal potentials that move toward more negative voltages, away from 0mV, as the extracellular sodium concentration decreases (de la Villa et al. 1995). APB an agonist specific for the mGluR6 receptor of ON bipolar cells mediates a decrease in membrane conductance through the closing of these cGMP regulated cation channels. However, cGMP and its analogues greatly affect membrane conductance at the non-specific cation channels, and its addition to the recording pipette typically causes the conductance to markedly increase. The responses

that were recorded from skate bipolar cells are the "classic" depolarizing ON-type described in the literature (Shiells et al., 1981; Slaughter and Miller, 1981, 1985; Bloomfield and Dowling, 1985; Attwell et al., 1987; Shiells and Falk, 1990; Nawy and Jahr, 1990a,b, 1991; Wässle et al., 1991; Yamashita and Wässle, 1991; Thoreson and Miller, 1993, 1994; Tian and Slaughter, 1994, 1995; de la Villa et al., 1995; Euler et al., 1996).

OFF bipolar cell conductance:

Skate OFF bipolar cells have characteristics complementary to ON bipolar cells. Glutamate and its ionotropic analogues caused an increase in membrane conductance by opening ligand gated channels, AMPA/kainate type, which in the intact retina would have a depolarizing effect on OFF bipolar cells. Receptor mediated changes in conductance were measured by applying 5mV pulses during responses to kainate and comparing the current to that produced by 5mV pulses in Ringer. The agonist increased conductance, and because the cells had inward currents at negative holding potentials and, outward currents at positive holding potentials, it can be concluded the effect resulted from an opening of ion channels predominantly permeable to extracellular cations (Fig. 22). Based on reversal potentials for these bipolar cells which were near 0mV (Fig. 19), along with the increased conductance at negative holding potentials, sodium is, presumably, the ion primarily, but not exclusively, responsible for the inward current. These characteristics fit the description of non-specific cation channels since 0mV is between the reversal potentials for sodium and potassium, the two

cations with the largest driving-force. Reversal potentials for OFF bipolar cells in other species were close to 0mV (Attwell et al., 1987; de la Villa, 1995; Euler et al., 1996; Hartveit 1997; Sasaki and Kaneko, 1996). The non-specific cation channels in cat OFF bipolar cells was determined to have a permeability ratio of $P_{Na}:P_K:P_{Cs}:P_{Ca} = 1:0.94:1.32:0.57$ (Sasaki and Kaneko, 1996), and in salamander they had an unusually high calcium to sodium permeability ratio of 3.2 (Gilbertson et al., 1991).

The ionotropic glutamate receptor:

Glutamate and its ionotropic analogues typically depolarize OFF bipolar cells by opening non-specific cation channels predominantly permeable to sodium, potassium, and possibly calcium. Evidence that the dark response in OFF bipolar cells is mediated by ionotropic glutamate receptors has been demonstrated by recording from cells superfused with glutamate and/or its ionotropic agonists and antagonists. At negative holding potentials, glutamate produces inward currents in OFF-type bipolar cells, as do the glutamatergic agonists kainate and AMPA (Slaughter and Miller, 1983; de la Villa, 1995; Maple et al., 1999; Euler et al., 1996; Sasaki and Kaneko, 1996; Hartveit, 1997; DeVries and Schwartz, 1999). Consistent with voltage-clamped inward currents, one type of bipolar cell in mudpuppy depolarized in response to kainic acid (Slaughter and Miller 1983). Blocking agents that target the ionotropic glutamate receptor include the nonspecific inhibitors kynurenic acid (KYN) (Kim and Miller, 1993), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and DNQX effective at AMPA/KA

receptors (Hensley et al., 1993b) and, among other compounds, GYKI 53655, a selective blocker of AMPA receptors (DeVries and Schwartz, 1999). The substances cyclothiozide (CTZ) and 4-[2-(pheylanl-fonylamino)ethylthio]-2,6-difluoro-pheynoxy acetamide (PEPA) are selective for AMPA subtypes and function by preventing or modulating desensitization (Shen et al., 1999). In an early study of isolated skate bipolar cells, using intracellular recording, depolarizing responses to kainate and glutamate were observed (Lasater et al., 1984). This investigation, through whole-cell voltage clamp recording, identified the skate OFF bipolar cells to be sensitive to kainate and AMPA with a characteristic voltage-current relationship. In addition, the kainate response was blocked in a dose-dependent manner by CNQX (Figs. 27 and 28) and the OFF response in the ERG by KYN.

Dose-response curve:

Despite the care taken to minimize rundown of the ON bipolar cells, responses were erratic, particularly at concentrations above 5μM glutamate. Some of the problem may have been related to receptor desensitization, but even so, an approximate dose-response curve was obtained. Cells were tested with glutamate from 0.5 to 7μM, and a non-linear curve gives the best fit. Using the Hill equation, a curve was fitted to the three lower glutamate concentrations (0.5μM to 3μM). Obtained is a curve with a Hill coefficient of 1.0, and an EC_{50} of 0.49μM (Fig. 23). Thoreson and Ulphani (1995) did dose-response analysis of glutamate analogues using the b-wave of the ERG rather than record whole-cell

because of the difficulties associated with rundown. As a control, they did, however, record dose-response perforated patch mudpuppy ON bipolar cell responses to APB at the lower concentrations and the Hill coefficient was 1.31, with an EC_{50} of 0.47μM. Their ERG dose-response values with APB were a Hill coefficient of 1.08 and an EC_{50} of 0.85μM. Dissociated cat ON bipolar cells were recorded whole-cell and glutamate dose-responses were measured (de la Villa et al., 1995). Again the difficulties of recording these cells limited the researchers to developing a curve by making pairs of measurements one at the peak response, 100μM glutamate, with another at a lower concentration. Their cells had a Hill coefficient of 1.31 with an EC_{50} of 1.3μM.

Rundown in ON bipolar cells:

Rundown is a problem common to whole-cell voltage clamp recording, where the cytoplasmic contents dialyze out of the cell causing diminished responses (Hille, 1992). Recording glutamatergic responses in ON bipolar cells is particularly challenging, because components of the second messenger system are susceptible to washout. This is what Nawy and Jahr (1990b) surmised to be the reason for decreasing responses recorded from ON bipolar cells in slice preparations. Karchin and Wässle (1990) found that out of 133 isolated rod bipolar cells recorded whole-cell, 93 responded to APB, 88 of which had an *increase* in conductance and only 5 of them had outward currents due to channel closing. The problem was attributed possibly to a washout of cytoplasmic components, and the usage of low calcium concentrations in the electrode, but

by using perforated patch recording they found exclusively bipolar cells that had APB mediated decreases in conductance (Wässle et al, 1991).

To avoid the problems of directly recording ON bipolar cells, indirect methods were used by Hartveit (1996) to prove rat rod bipolar cells are only ON type. The relative latency of bipolar cell responses to ligand-gated glutamate agonists (AMPA and kainate) were compared to the delay in response to GABA and glycine, since these receptors are known to be present on OFF bipolar cells. Hartveit concluded there are no ionotropic glutamate receptors on rat rod bipolar cells, as thought by Karschin and Wässle (1990). They found a predominance of rod bipolar cells that had an increase in membrane conductance in response to glutamate and APB, which was subsequently identified as a possible problem with intracellular washout (Wässle et al, 1991). The approach used by Harveit for recording ON bipolar cells in the rat retina is indirect, but the difficulties of recording dissociated metabotropic bipolar cells were averted. The very contrasting results with the rat retina point to the problem of eliciting direct ON bipolar cell responses whole-cell, with glutamate or its analogues, especially without compensating for cytoplasmic washout. Furthermore, acutely dissociated cells are, most likely, in worse condition, from the enzyme and trituration process, than those recorded from slice preparations.

A method used to minimize washout of cytoplasmic constituents is to do perforated patch recording by adding the antibiotic nystatin or amphotericin to the

electrode. Rather than rupturing the membrane, the antibiotic creates pores (~8Å) that allow only anions and cations to pass (Hille, 1992). However helpful, isolated ON bipolar cell studies that have used perforated patch recording have shown only slight improvements and report relatively few ON bipolar cells that responded to glutamate or its agonists. In an early report of intracellular recording of skate ON bipolar cells, 28% of 75 cells tested (type of bipolar cell not specified) responded to glutamate and/or the glutamatergic agonists (Lasater, et al., 1984). Using amphotericin to do perforated patch recording from isolated skate bipolar cells, the yield of responding cells was still low. One out of eight bipolar cells tested responded to 100μM APB and two out of fifteen cells responded to glutamate (type not specified) (Chappell et al., 1992). Nystatin did give limited results with mammalian rod bipolar cells, but not enough to enable an analysis of the cells other than the observation of a reduction in noise, associated with the closing of nonselective cation channels (Wässle et al, 1991).

Perforated patch using amphotericin was tried for this investigation with meager results. Similar to other researchers, few ON bipolar cells responded and, then, generally with small responses. Therefore, the approach taken was to add cGMP, GTP and ATP to the patch electrode internal solution doing whole-cell patch recordings. Others have commonly added one or more of these nucleotides (Thoreson and Miller, 1993; Pedro de la Villa, 1995; Shiells and Falk, 1999; Nawy, 1999a). This also gave the advantage of allowing the addition of compounds such as TEA, a potassium channel blocker, to the electrode that

would otherwise be impermeable across a perforated patch.

Kainate dose-response:

The problem of rundown, when recording whole-cell glutamate responses from OFF bipolar cells, is not nearly as predomint as with ON bipolar cells. OFF bipolar cells were sensitive to both AMPA and kainate, and the responses had differing characteristics. AMPA responses had a rapid desensitization component and were generally more elusive to record. A dose-response curve was, however, obtained for OFF bipolar cells using kainate, since it gave the best, most reproducible, responses over glutamate and AMPA. A body of data was collected from 1 to 400μM kainate with a small subset of data points, which included 30μM but not 100μM kainate, and another group, which included 100μM but not 30μM kainate (Figs. 24 and 25). The data normalized to 100μM kainate provided the best fitted curve at high kainate concentrations, because there was little desensitization for that set of bipolar cells. The data normalized to 30μM kainate gave a better fitted curve at lower kainate concentrations but a poorer fit at higher concentrations. Two additional intermediate concentrations, 20 and 70μM, could be included when the data was normalized to 30μM kainate, providing additional points along the lower portion of the curve. When the data normalized to 30μM kainate was fitted as a truncated curve up to 100μM kainate, a more precise Hill coefficient was determined, and when combined with V_{max} and EC_{50} from the data normalized to 100μM kainate, an idealized dose-response curve was derived (Fig. 26), which had a Hill coefficient of 1.07. Cat OFF bipolar

cells tested with glutamate between 1 and 100μM had a Hill coefficient of 1.8 and an EC_{50} of 12μM (Sasaki and Kaneko, 1996).

Effects of zinc on bipolar cells:

Some, but not all, bipolar cells in this study responded to zinc alone, but among those that did there were two qualitatively different types of responses. A group of five OFF bipolar cells from the same dissociation had large outward currents mediated by zinc, 150 to 1750pA, at -50mV that were reproducible. Changes in conductance during a response to zinc were measured using 5mV pulses, from which it was determined, zinc caused an increase in membrane resistance (Fig. 45A). There was also an associated decrease in the noise level, which can be attributed to the closing of ion channels. The increase in input resistance caused by zinc was confirmed with a bipolar cell that was also record using a ramp stimulus. A negative slope resulted when the ramp recorded in 0.7μM zinc was subtracted from a ramp in Ringer alone (Fig. 45B). Reversal potential for the ramp was near 20mV, but the bipolar cell in figure 44, recorded at select holding potentials, had a reversal potential near 0mV. More of these cells need to be recorded in order to determine the currents involved. In general, the responses to zinc had the characteristic of a rapid rise time, unlike those observed with the ON bipolar cells, and were present from the start of the experiment. Zinc has been reported to decrease voltage-gated calcium channels, chloride efflux, potassium channel activation, and sodium currents (Smart et al., 1994). An analysis of the outward current was not made, since these responses were rarely

seen. It is interesting that it was from the same dissociation that a group of OFF bipolar cells were very sensitive to zinc. The possibility exists that a component of these cells, not ordinarily seen, escaped damage (or was damaged) during the dissociation process, or that the bipolar cells are representative of a subgroup that is sensitive to zinc at a site seemingly independent of the receptors. Qian et al. (1997) reported skate bipolar cells were insensitive to zinc alone, but it is important to note cGMP was not added to the pipette, and a different dissociation procedure was used which may have protected these cells from internalization of zinc. An increase in membrane resistance to zinc by itself was measured in hippocampal neurons (Xie et al., 1993).

ON bipolar cells were recorded with cGMP, GTP and ATP added to the internal solution, and as the nucleotides dialyzed into the cell, the standing current increased, as did the responses to glutamate and its analogues. When cGMP is added to the pipette, it increases the opening of non-specific cation channels as it dialyzes into ON bipolar cells (Nawy and Jahr, 1990a; Shiells and Falk, 1990). In response to zinc, outward currents in ON bipolar cells were often seen that grew concomitantly along with glutamatergic responses. Initially, the currents were usually negligible, but as the internal solution equilibrated with the cells, the zinc induced current often developed as an outward current, particularly farther away from the reversal potentials, at the more negative -50mV holding potential. In addition to the currents being in the same direction to the glutamate response, they were sometimes of similar magnitude.

ON bipolar cells tested with an extended superfusion time with zinc for 20 seconds (Figs. 40 and 41) had a slow outward current that eventually returned to baseline, or below, while still being superfused with zinc. With other ON bipolar cells tested this way, sometimes the standing current remainded somewhat above baseline and other times it returned below the original standing current. An explanation for these transitory outward currents in ON bipolar cells using short and long perfusion intervals might be related to the regulation of their non-specific cation channels by calcineurin (Nawy, 1999b) and calmodulin-dependent protein kinase (CaMKII) (Walters et al., 1998). Calcineurin is a phosphatase that dephosphorylates proteins, causing ion channels to close and is activated by calcium with the protein calmodulin as an intermediary. CaMKII phosphorylates proteins that possibly have an antagonistic function to keep the channels open Nawy (1999a).

Zinc inhibits calcineurin at millimolar concentrations (Armstrong, 1989), while at micromolar concentrations it can inhibit CaMKII (Weinberger and Rostas, 1991), or transform it making it unresponsive to calcium (Lengyel et al., 2000). Superfusing ON bipolar cells with 1μM zinc might have no effect on calcineurin, but it could inhibit CaMKII shifting the balance between the enzymes in favor of calcineurin. With a short zinc treatment of only a few seconds, outward currents could result from the suppression of CaMKII and the predominace of calcineurin. The net affect would be the closing of the non-selective cation channels which are calcium permeable. With their closing, intracellular calcium levels decrease

(Shiells and Falk, 1999; Nawy, 2000). When followed by a wash with Ringer, intracellular zinc concentrations start to dissipate and the channels open, enabling calcium concentration to rise and, in turn, activate CaMKII through CaM. In a low zinc environment, or under Ringer control conditions, the enzyme regulated channels can maintain a more open state based a high calcium to low zinc equilibrium.

Effects of zinc on glutamatergic responses:

ON bipolar cell responses to APB, t-ACPD and glutamate were blocked with the co-application of zinc. However, zinc solutions in combination with 1μM glutamate had a different effect to those where zinc was combined with 10μM glutamate or 30μM t-ACPD. Concentrations of zinc as low as 1μM, blocked 100% of the current produced by 30μM t-ACPD, and with glutamate concentrations ranging from 1 to 10μM, 1μM zinc more than blocked the currents. To varying degrees, glutamate-induced currents were reversed. Two apparent factors that correspond with these effects, are the currents zinc alone produced and the potency of the glutamatergic agonists.

The size of the currents that zinc alone mediated varied, and as previously mentioned, often increased with time as the nucleotides dialyzed into the ON bipolar cells. Frequently, the zinc mediated current increased concomitantly with the standing current and glutamatergic response. The correlation between the increases in zinc and glutamate responses with the dialyzed nucleotides is

indicative of a cGMP dependent zinc effect. If zinc were simply an ion channel blocker the magnitude of the zinc mediated currents would be expected to remain unchanged. Under those circumstance, as the nucleotides dialyze into the cells, the relative size of the zinc response compared to the increasing glutamate response should decrease which it did not.

The site(s) zinc targeted were not sensitive to the chloride channel blocker PTX (Fig. 30), or the potassium channel blockers TEA and cesium (constituents of the internal solution) (Fig. 34). Although, $K_{(Ca)}$ was not tested for, potassium channels, based on the direction of their currents, are unlikely to be zinc's site of action. If zinc blocked an outward potassium current, the inward current produced by the glutamatergic agonists alone at positive holding potentials should have been enlarged by zinc and not blocked or reversed as happened.

The group of cells tested with t-ACPD, had a smaller zinc response than those tested with glutamate, when a comparison is made to the normalized glutamatergic responses. The maximum reduction achieved by zinc as an inhibitor of the t-ACPD response was that equal to the current measured using the control, cis-ACPD (Fig. 33). In contrast to these relatively modest zinc currents, the bipolar cells tested with glutamate generally had the largest current reversals, which coincided with the largest normalized responses to zinc relative to glutamate (Fig. 36). This observation is in keeping with the idea that larger normalized zinc mediated currents not only block glutamatergic responses, but

produced current reversals based on the intracellular concentrations of zinc achieved. The conditions under which zinc had different effects relates to the degree that membrane conductance was altered as the cells equilibrated with the nucleotides. As the non-specific ion channels opened, mediated by cGMP, presumably, more zinc entered the cell and exerted its own regulatory effects on the channels. Zinc alone may have inhibited CAMKII causing channel closing, but in combination with an activated second messenger system, a new equilibrium with an overriding influence was established.

The ramps recorded in glutamate and glutamate plus zinc show the reversal potentials remained the same, while the slopes changed from negative to positive values (fig. 39). When bipolar cells were tested at select holding potentials of -25 and +30mV their currents also reversed, but the difference, however, in the magnitude between the currents at -25mV and -50mV were small (Figs. 38 and 37, respectively). In toto these effects indicate the glutamate mediated closing of the cation channels was not only blocked by zinc, but there was an increase in conductance at the non-specific cation channels. Most likely, the blocking effect of zinc did not occur at the glutamate receptor, since a glutamate effect is needed to obtain zinc-meditated currents that are opposite to those of glutamate. In addition, a blocking effect at the receptor would probably leave a remaining outward zinc current as observed with zinc alone. Zinc may directly block the closing of the non-specific cation channels, or act further up the second messenger cascade, but an increase in conductance caused by zinc

depends on the activation of a glutamate-mediated second messenger component(s). Once the metabotropic system is fully activated, a new equilibrium between zinc, regulation of the channels, and the second messenger system, possibly, for example, intracellular calcium, is set.

The experiments that sequentially superfused zinc for 20 seconds then t-ACPD for 2.5 seconds supports the previous conclusions regarding the effects of zinc. Zinc mediated a large inward t-ACPD current after it was no longer being superfused and was being washed away by the agonist (Figs 40 and 41). This effect can most easily be interpretted as zinc's site of interaction being intracellular rather than the glutamate receptors. Furthermore, for both bipolar cells, zinc initially blocked the t-ACPD response and there was a delay before a large inward current was produced. The increase in conductance closely coincided in time with the maximum t-ACPD response that was measured under control conditions. One inherent aspect of the metabotropic system is that it requires a longer time to activate and with the cells already "preloaded" with zinc, t-ACPD could not manifest an inward response until the system was fully activated. Interestingly, the initial moments when t-ACPD was superfused, the responses by the bipolar cells were merely blocked and then precipitous inward currents were produced as the second messenger system reached its peak of activation.

The extent to which zinc may have penetrated the cell possibly determined the

difference as to whether it blocked the t-ACPD mediated current or caused its reversal. The majority of cells tested with the simultaneous superfusion of zinc plus t-ACPD did not have current reversals (tables 12 and 13). The two bipolar cells that were tested with the sequential superfusion method did not have their currents reversed or blocked by zinc when they were superfused with a combined solution of t-ACPD plus zinc. However, given a long superfusion time of twenty seconds with zinc, the effect of t-ACPD alone was to produce large inward currents for these cells. Intracellular zinc concentrations may account for the differences among ON bipolar cells whose glutamatergic responses are not blocked, blocked, or reversed.

Another possibility that needs to be considered in conjunction with intracellular zinc concentrations is that glutamate is a more potent agonist than t-ACPD (Nakanishi, 1992). The cells tested with t-ACPD, for the most part, did not have their responses reversed by zinc. Current reversals, on the other hand, were prevalent with solutions of glutamate plus zinc. The cell's second messenger system may be more fully activated by glutamate producing stronger responses, especially at 1μM glutamate. The bipolar cells at 1μM glutamate had the largest reversals, while cells tested at 0.3μM and 10μM glutamate may have been more weakly activated. At 10μM glutamate, the second messenger system might have been suppressed by desensitization limiting the effect of zinc. What the intermediary steps in the metabotropic glutamate system are and how zinc interacts with them are speculative at this point, considering that the original

model for ON bipolar cells is not entirely substantiated.

Possible zinc sites in the metabotropic system:

ON bipolar cell characteristics of a glutamatergic pathway with a G-protein activating PDE, causing hydrolyzation of cGMP and, the closure of cGMP gated channels has parallels to the light response in photoreceptors. Based on immunostaining and electrophysiological data, however, the homology between ON bipolar cells and photoreceptors is now believed to be less close than initially thought with different intermediate steps. Across species rat, cat, cow, monkey and salamander ON bipolar cells are not immunoreactive for the photoreceptor G-protein transducin, isoforms of PDE, arrestin, or the cGMP channel proteins found in photoreceptors (Vardi et al., 1993). The absence of PDE, or its not having a significant role, is supported by the lack of effect the PDE inhibitor, IBMX, had in blocking glutamate responses, as well as the poorly hydrolyzable cGMP analogues 8-Br-cGMP and 8-pCPT-cGMP (Nawy 1999a). The addition, however, of $G_{o\alpha}$, a subunit that does not directly activate PDE, to the pipette reduced glutamate responses through the closing of ion channels. Dialyzed monoclonal antibodies to $G_{o\alpha}$ also suppressed glutamate responses by blocking the effects of the G-protein. Rod and some cone bipolar cells in mammals and salamander stained for $G_{o\alpha}$ (Vardi et al., 1993), and it was identified in the tips of the dendrites of rat ON bipolar cells (Vardi, 1998).

APB is specific for only group III receptors and in the retina mGluR6. Skate ON

bipolar cells tested in this study with APB had their responses blocked by zinc as did the majority of bipolar cells tested with t-ACPD and glutamate t-ACPD (Figs. 29, 31 and 35). Both glutamate and t-ACPD, a glutamate analogue that is not selective for any particular mGluR can activate receptors from groups I, II and III. Rat rod bipolar cells are immunoreactive to antisera against group I receptors mGluR1α and mGluR5 (Koulen et al., 1997); catfish bipolar cells stain for mGluRs from groups I, II and III (Gafka et al., 1999). It is group I that is involved in intracellular calcium release via IP_3 (Nakanishi, 1992) and the group I receptor 1α that was immunoreactive in catfish bipolar cells.

Whether only mGluR6 receptors are activated in skate ON bipolar cells or others were as well, zinc blocked decreases in conductance mediated by glutamate and its analogues and often, zinc plus glutamate caused an increase in membrane conductance. Modulating intracellular calcium levels significantly influences cell function. In particular, increases in intracellular calcium concentrations at the cGMP-gated channels decreases conductance in ON bipolar cells. Light adaptation in ON bipolar cells is associated with a rise in intracellular calcium and a decrease in membrane conductance that can be blocked by the addition of BAPTA to the pipette (Nawy, 2000; Shiells and Falk, 1999). Zinc may be an important means through which dark adaptation occurs. Similar to calcium, zinc's entry into ON bipolar cells may be through the non-specific cation channels, but its effect is opposite to calcium causing an increase in membrane conductance.

Effects of zinc on OFF bipolar cells:

The effects of zinc on ionotropic glutamate receptors are widely variable depending on the type of cell and concentration of zinc. In hippocampal neurons, for example, 50μM zinc blocked NMDA receptors, potentiated kainate and quisqualate receptors, and at high concentrations 1mM zinc blocked responses by non-NMDA glutamatergic receptors (Mayer et al., 1989). Similarly, carp horizontal cells had their AMPA response (flop variants) potentiated at low zinc concentrations, 30μM, and suppressed at high zinc concentrations, 300μM, (Shen and Yang, 1999). In salamander, horizontal cells, however, the glutamate responses were unaffected by zinc (Wu et al., 1993).

This study of skate OFF bipolar cells found kainate-sensitive receptors could be blocked by zinc (Figs. 42 and 43). Concentrations of 300μM kainate produced substantial inward currents that were completely suppressed by 1μM zinc at -50mV and, as a blocking agent, zinc was much more effective than CNQX. 1μM CNQX blocked less than 40% of the 100μM kainate-mediated currents (Figs. 27 and 28).

In order to fully understand the function zinc has on skate bipolar cells a dose-response study at concentrations higher and lower than 1μM zinc should be made. In the hippocampus and carp horizontal cells, low concentrations of zinc, but quantities greater than the 1μM concentration used in this study, have potentiating effects, while even higher concentrations are inhibitory. If zinc can

potentiate skate bipolar cell glutamate responses at higher concentrations, then these ionotropic receptors would be yet another type from those previously described. At higher zinc concentrations, another regulatory dimension of skate OFF bipolar cells may be present that expands their glutamate sensitivity. Either way, zinc can provide an additional level of control to the system. The blocking effect observed by 1μM zinc limits the amplitude of the glutamate response. This may be an important way of preventing OFF bipolar cells from producing peak responses under scotopic conditions.

Effect of zinc chelators on the skate ERG:

Recently, a number of reports have suggested a role for zinc as a neuromodulator of GABA receptors in the outer retina and on Müller cells. Horizontal cell $GABA_C$ receptors are downregulated by zinc in catfish, as are $GABA_A$ and $GABA_C$ bipolar cell receptors in carp (Dong and Werblin, 1995; Han and Yang, 1999). With skate bipolar cells, zinc at low concentrations enhances $GABA_A$ responses and suppresses them at high zinc concentrations (Qian et al., 1997), and $GABA_A$ response of skate Müller cells are also enhanced by zinc at low concentrations (Qian et al., 1996). Evidence for a high zinc concentration near photoreceptor terminals has lead to the suggestion that zinc may be co-released with glutamate, also having feedback effects, regulating further glutamate release by the photoreceptors (Wu et al., 1993).

In the experiment by Wu et al. (1993), using salamander retinal slice preparation

and patch electrodes, the effects of zinc on horizontal cells were monitored. The retina was stimulated with light and zinc solutions were superfused onto the preparation, while changes in potential were measured. Zinc application resulted in the hyperpolarization of the horizontal cells and the loss of their hyperpolarizing light responses. When the retina was superfused with glutamate still in the presence of zinc, the horizontal cells could again depolarize. Zinc, however, could block the depolarizing effect that superfused GABA had on the horizontal cells. The results suggest that in addition to zinc directly blocking the GABA receptors of the horizontal cells, it also has an upstream regulatory effect at the photoreceptors. Presumably, applying zinc blocked the required entry of calcium into photoreceptor terminals to enable the release of synaptic vesicles. Wu et al., 1993 also found using Neo-Timm staining concentrations of zinc at the synaptic terminals. Based on the staining and electrophyiological data, they suggest zinc may be co-released with glutamate from the vesicles. This would constitute a feedback system that inhibits further vesicular release by the photoreceptors. Thus, zinc could have two effects in this system, in addition to reducing the release of glutamate, on the horizontal cells it would also block the $GABA_A$ receptors, but leave the glutamate receptors unaffected.

Histological and electrophysiological data, in general, supports the idea of zinc being released from the photoreceptors to serve as a neuromodulator. As with the salamander, Neo-Timm staining reveals bands of zinc at the terminals of skate photoreceptors (Qian et al., 1997). These findings in conjunction with the

effects of zinc on skate GABA receptors (Qian et al., 1996 and 1997) and glutamate receptors (this study) imply a neuromodulatory role for zinc. It does not, however, prove for the skate retina or any other that this is its purpose *in vivo*. Zinc is an important trace element involved in many metabolic functions including enzyme regulation which could account for its presence, and as for its neuromodulatory capabilities, other divalent ions are also effective blocking agents. Barium, for example, can compete with calcium-regulated systems, and cobalt, like zinc, inhibits the release of glutamate from photoreceptors. For these reasons, it was thought important to try and ascertain whether or not zinc functions as an endogenously released neuromodulator in the retina. Its role as a neuromodulator of retinal light responses was still unresolved and needed to be addressed.

The ERG, it was thought would be a suitable tool to determine *in vivo* any direct functions endogenous zinc may have on the retinal response. Based on information gained from the initial ERG studies on afferent ON and OFF pathways, it was learned that PTX could effectively block GABAergic receptors in the outer plexiform layer (OPL), while leaving ON and OFF afferent pathways intact (Chappell and Rosenstein 1996). Since it blocked $GABA_A$ and $GABA_C$ receptors, the complicating effect of zinc at the $GABA_A$ and $GABA_C$ receptors (i.e., enhancing and suppressing effects) on bipolar cells and $GABA_A$ receptors on Müller cells were blocked. Under these conditions, recording from a retina treated with PTX and, then PTX solutions containing zinc chelators, the removal

of zinc at the photoreceptor terminals and/or the synaptic cleft should be detectable as a difference in amplitude of the ON response.

The amino acid histidine is a potent chelator of zinc which binds the ion in a 1:1 ratio at neutral pH. Zinc concentrations vary from 1nM in the cytoplasm of some cells to 1mM in certain vesicles (da Silva and Williams, 1991), and measurements of zinc in pigment epithelial cells have been found to be 10μM, while those of the hippocampal mossy-fiber neuropil are in the 200 to 300μM range (Frederickson et al. 1983). Consequently, 100μM histidine was considered to be a good concentration to begin testing, based on the potent effect 1μM zinc had on isolated bipolar cells. N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a membrane permeable substance, was the other chelator tried at 100μM in one preparation. Histidine was used preferentially over TPEN because it is not absorbed into cells. This would enable changes in the ERG to be clearly identified as extracellular effects. In addition, TPEN is reported to be quite toxic, so that no effect, or a diminishing ERG response would require substantial experimentation to determine appropriate dosage and the separation of possible toxic effects from zinc effects.

Five eyecups tested with histidine were dark adapted and stimulated during the course of the experiments once every thirty seconds with a ten second interval of light, using either a 3 or 4ND filter to give an ERG response of intermediate size. Using a dark adapted retina stimulated by light, the ON response in 100μM

histidine, normalized to that in PTX, had an average increment of 67% that was significantly different at p = 0.026 (Figs. 47 and 48). The zinc chelating effects of histidine, are consistent with both the results from this study using isolated bipolar cells and the work of Wu et al. (1993), in that the application of zinc reduces the magnitude of responses.

Using isolated bipolar cells, this study has shown that zinc blocks ON bipolar cell responses to APB and t-ACPD and even reverses glutamate induced currents. Based on histological staining (Wu, 1993; Qian, 1997), the supposition that vesicular zinc is released from the photoreceptor terminals would suggest that, in the dark, high levels of extracellular zinc are present when glutamate concentrations are also high (Wu et al., 1993). Under these conditions zinc would exert its maximum effect in reducing the glutamate response as well as on feedback and/or regulating the GABA receptors as proposed by Wu et al. (1993). Removal of zinc by chelating it would reduce the inhibitory effects of zinc at the glutamate receptor. This would increase glutamate mediated hyperpolarization of ON bipolar cells and depolarization of OFF bipolar cells, causing greater changes in potential from light on to light off and vice versa. Chelating the released zinc probably renders the ion inactive at its binding sites.

TPEN an effective zinc chelator, used clinically, was also tried. TPEN (100μM) worked in a similar fashion to histidine, effectively producing increases in the ON responses. An alternative to histidine was tried since the amino acid is a natural

constituent of the retina and there was some concern that histidine could be working as a neurotransmitter or cause metabolic changes.

The results from this ERG study using zinc chelators are in complete agreement with the hypothesis that superfused zinc decreases the glutamate responses on isolated skate bipolar cells and provides evidence that zinc is present in the extracellular space of the retina. This is consistent with the notion that it may have a widespread neuromodulatory effect regulating the responses of the GABA receptors on horizontal, bipolar and Müller cells, as well as the glutamatergic responses of bipolar cells. It is also consistent with the hypothesis that zinc may have an upstream control of the system through direct feedback onto the photoreceptor terminals regulating the release of glutamate (Wu et al., 1993). To date, relatively few studies on the effects of zinc on the retina have been done, but an understanding of its neuromodulatory role, with species-specific differences, is now beginning to emerge.

Conclusion:

The skate retina is atypical among vertebrates because of its lack of cones and its ability, once light adapted, to assume characteristics of a cone system. These special qualities have raised basic questions about the ON and OFF afferent pathways relative to other vertebrate retinae. Mammalian retinae process scotopic information through ON bipolar cells, and an OFF signal is generated via A II amacrine cells at the level of the OFF ganglion cells. At higher light

intensities, photopic information is carried by ON and OFF cone bipolar cells. Cold blooded vertebrates, in general, have ON and OFF bipolar cells with mixed input from rods and cones. The primary objective of this study was to determine the means through which OFF responses arise in the skate retina. Two different approaches were used, one measuring field potentials doing ERG recording on eyecup preparations and the other by whole-cell voltage clamp recording from isolated bipolar cells. The results from both of these experiments provide evidence for an independent OFF pathway from the outer retina that is mediated by OFF bipolar cells.

The ERG experiments demonstrated that the OFF response could be retained after the ON pathway was blocked with APB and the GABAergic input from the horizontal cells inhibited with PTX. The addition of NMDA (known as an inner retina blocker) to the APB + PTX solution left the OFF response unchanged as did the removal of the pigment epithelium. The broad spectrum glutamate inhibitor, KYN, could, however, block the OFF response when it was added to the APB + PTX + NMA solution. These results suggested the presence of OFF bipolar cells which was, indeed, confirmed through whole-cell recording from isolated bipolar cells.

Two types of bipolar cells were identified by their selective responses to glutamate agonists and the direction of their currents in response to glutamate and its agonists at positive and negative holding potentials. Similar to other

vertebrates, skate ON bipolar cells were responsive to the metabotropic agonists APB and t-ACPD, while OFF bipolar cells were sensitive to the ionotropic agonists AMPA and kainate. ON bipolar cells produced outward currents at negative holding potentials and inward currents at positive holding potentials. OFF bipolar cell currents were in the opposite direction to ON bipolar cells at positive and negative holding potentials, but both cell types had reversal potentials near 0mV. These bipolar cell characteristics indicate that the skate all-rod retina has, in addition to metabotropic ON bipolar cells, ionotropic OFF bipolar cells that provide an independent OFF pathway.

A secondary study on skate bipolar cells investigated the effects of zinc as a glutamatergic neuromodulator. In the brain and retina, zinc has been shown to have potent effects on ionotropic glutamate receptors. Zinc has also been reported to be a GABAergic neuromodulator on skate Müller and ON bipolar cells, in addition to its having been been identified in skate photoreceptor terminals by Neo-Timms staining (Qian et al., 1996, 1997). These findings prompted the present study, where it was found by recording from isolated bipolar cells that zinc can block glutamatergic responses. 1μM zinc superfused with APB, t-ACPD, or glutamate blocked ON bipolar responses, measured by whole-cell voltage clamp recording, and in some cases caused a reversal of currents. 1μM zinc also blocked kainate-mediated responses in OFF bipolar cells.

Another objective was to ascertain the neuromodulatory effects endogenous zinc might have in the intact retina, since this has not been demonstrated in any species, although postulated from studies in salamander (Wu et al., 1993) and skate (Qian et al., 1997). The ERG b-wave was monitored, and comparisons were made between control responses and those recorded in the presence of the zinc chelator, histidine. Under control conditions, dark adapted retinae were superfused with PTX to block GABAergic input. Then they were superfused with picrotoxin plus histidine, and there was a marked increase in the b-wave. These results are consistent with (and opposite to) the inhibition observed with the ionic zinc on isolated bipolar cells. This statement is based on the assumption that the chelation of zinc inactivates the ion's inhibitory effect enabling greater changes in potential by the ON and OFF pathways.

This investigation has demonstrated the skate all-rod retina has an independent afferent OFF pathway that is mediated by OFF bipolar cells. It also reports that endogenous zinc has a neuromodulatory effect on glutamate mediated responses in ON and OFF bipolar cells. The ERG and isolated bipolar cell data, considered together, are consistent with the hypothesis that endogenous zinc blocks glutamate responses in bipolar cells, limiting their change in membrane resistance. Zinc reduces glutamate-mediated responses, decreasing membrane resistance in ON bipolar cells and, increasing membrane resistance in OFF bipolar cells. Research with other retinae has demonstrated calcium regulates light adaptation in depolarizing bipolar cells, by increasing membrane resistance,

causing a decrease in the magnitude of the glutamatergic responses (Shiells and Falk, 1999; Nawy, 2000). Zinc, on the other hand, in the dark adapted retina reduces the size of the glutamatergic responses in skate ON bipolar cells, by decreasing membrane resistance. Endogenous zinc may be an important network component of dark adaptation that extends the bipolar cells' range of sensitivity. Reducing the amplitude of glutamate-mediated responses prevents the bipolar cells from reaching their peak output and, in turn, from saturating at low light intensities. The all-rod retina of the skate, once light adapted, can function with cone-like efficiency, and zinc may be a crucial element that enables the ON and OFF bipolar cells to respond across a wide range of luminance, which ever mechanism or mechanisms may be involved.

Table 1. Drugs superfused in electrophysiology experiments.

Drug	Function
t-ACPD	Non-specific glutamate agonist at the metabotropic receptors.
cis-ACPD	Inactive enantiomer of t-ACPD used as a control.
AMPA	Excitatory amino acid agonist that binds to AMPA/kainate receptors.
APB	Glutamate agonist at $mGluR_4$ and $mGluR_6$ receptors.
Glutamate	Excitatory amino acid.
HIS	Amino acid histidine used for its zinc chelating properties.
Kainic acid	Excitatory amino acid agonist selective for the kainate receptor.
NMDA	NMDA receptor agonist.
CNQX	Competitive kainate/AMPA receptor antagonist.
KYN	Kynurenic acid an antagonist at excitatory amino acid receptors.
PTX	Picrotoxin a $GABA_A$ and $GABA_C$ antagonist.
TEA	Potassium channel blocker.
TPEN	Zinc chelator

Table 2. Action and reversibility of pharmacological agents tested.

		Effect on ERG		Times observed	
Drug applied	Concentration (mM)	ON component	OFF component	Effect	Reversal
PTX	0.2 to 2	increase (small)	increase	27	7
APB	0.1 to 0.5	blocked	not blocked	22	9
KYN	1 to 5	not blocked	blocked	16	13
NMDA	0.5	not blocked	not blocked	5	—

Table 3. Changes in membrane resistance of ON bipolar cells during the application of glutamate and t-ACPD.

Cell	Holding Pot. (mV)	Drug (μM)	Response (pA)	5mV Pulse #1 (pA)	5mV Pulse #2 (pA)	Δ Resistance (M ohms)
57	-25	3μM GLU	237	149	133	4.04
58	-25	3μM GLU	68	180	165	2.53
60	-25	3μM GLU	-48	123	117	2.08
62	-25	3μM GLU	-100	185	165	3.28

Table 4. Changes in membrane resistance of OFF bipolar cells during the application of kainate.

Cell	Holding Pot. (mV)	Drug (μM)	Response (pA)	5mV Pulse #1 (pA)	5mV Pulse #2 (pA)	Δ Resistance (M ohms)
304	-50	300μM	-108	160	183	-3.93
318	-50	100μM	-137	150	163	-2.66
372	-50	300μM	-108	157	173	-2.95

Table 5. Kainate dose-response data in pA.

	Kainate concentrations									
Cell	1μM	3μM	10μM	20μM	30μM	70μM	100μM	200μM	300μM	400μM
265	-15	-15	-24		-75		-85			
274	0	-1	-9		-75		-369			
277	0		-18		-38		-50			
294							-77	-48	-66	
320					-30		-35	-52	-48	
375					-98		-150		-148	-244
376					-90		-120		-143	-143
395			-30		-68		-94			
397			-73		-185		-230			
402										
408			-10		-35		-58			
421			0	-24	-48	-86				
422			-23	-38	-45	-55				

Table 6. Kainate dose-response data normalized to 30uM kainate.

	Kainate concentrations								
Cell	1μM	3μM	10μM	20μM	30μM	70μM	100μM	300μM	400μM
265	-0.2	-0.2	-0.373		-1		-1.133		
274	0	0	-0.12		-1		-4.92		
277	0		-0.474		-1		-1.316		
375					-1		-1.531	-1.51	2.492
376					-1		-1.333	-1.589	1.589
395			-0.441		-1		-1.387		
397			-0.395		-1		-1.243		
408			-0.286		-1		-1.657		
421			0	-0.5	-1	-1.792			
422			-0.511	-0.844	-1	-1.222			

Table 7. Kainate data normalized to 100μM kainate.

	Kainate concentrations						
Cell	1μM	3μM	10μM	30μM	100μM	200μM	300μM
265	-0.176	-0.176	-0.282	-0.882	-1		
274	0	0.003	-0.024	-0.203	-1		
277	0		0.36	-0.76	-1		
294					-1	-0.623	-0.857
320					-1	1.486	-1.371
375				-0.653	-1		-0.987
376				-0.75	-1		-1.192
395			-0.316	-0.716	-1		
397			-0.317	-0.804	-1		
408			-0.172	-0.603	-1		
443		-0.244	-0.207	-0.78	-1		

Table 8. The responses of OFF bipolars to kainate and CNQX in pA.

Cell	100μM Kainate	100uM Kainate 1μM CNQX	100uM Kainate 10μM CNQX
214	-73	0	0
234	-98	-72	-81
238	-445	-235	-170
274	369	-232	-
291	-250	-225	-
320	-28	-31	-

Table 9. Data CNQX data normalized to their respective kainate responses.

Cell	100μM Kainate	100uM Kainate 1μM CNQX	100uM Kainate 10μM CNQX
214	1	0	0
234	1	-0.72	-0.83
238	1	0.528	-0.382
274	1	-0.629	-
291	1	-0.865	-
320	1	-1.107	-

Table 10. The effects of APB, zinc, and APB plus zinc on ON bipolar cells (current in pA).

Cell	Holding Potential	3μM APB	0.7μM Zn^{++}	3μM APB + 0.7μM Zn^{++}	10μM APB	3μM Zn^{++}	10μM APB + 3μM Zn^{++}
8	-25				35	5	0
44	0	46	7	-15			
45	0	23	4	0			

Table 11. The effects of 200μM picrotoxin on zinc block of glutamate response of ON bipolar cells (current in pA).

Cell	Holding Potential	7μM GLU	3μM Zn^{++}	7μM GLU + 3μM Zn^{++}	3μM Zn + 200μM PTX	7μM GLU + 3μM Zn^{++} + 200μM PTX	7μM Zn^{++} + 200μM PTX	7μM GLU + 7μM Zn^{++} + 200μM PTX
11	-25	26		2	2	5	0	-11
12	-25	152	14	31	11			
12	+30	-194	-50	-38	-35			

Table 12. Effects of 1μM zinc on 30μM t-ACPD responses at -50mV holding potential.

Cell	30uM t-ACPD (pA)	30uM Cis-ACPD (pA)	1uM Zn^{++} (pA)	30uM t-ACPD + 1uM Zn^{++} (pA)	t-ACPD+Zn^{++} minus Cis-ACPD
159[1]	155	-	12	20	_
160[1]	20	-	13	-8	_
173	59	0	26	0	0
177	77	19	36	25	6
180	58	15	0	0	-15
185	178	-6	11	-27	-21
187	413	70	165	84	14

[1] 20mM TEA in the electrode.

Table 13. Cells from table 12 normalized to their 30uM t-ACPD responses.

Cell	30uM t-ACPD (pA)	30uM Cis-ACPD (pA)	1uM Zn^{++} (pA)	30uM t-ACPD + 1uM Zn^{++} (pA)	t-ACPD+Zn^{++} minus Cis-ACPD
159[1]	1	-	0.077	0.129	_
160[1]	1	-	0.488	-0.89	_
173	1	0	0.441	0	0
177	1	0.247	0.468	0.325	0.078
180	1	0.259	0	0	-0.259
185	1	-0.034	0.062	0.152	-0.118
187	1	0.169	0.400	0.203	0.034

[1] 20mM TEA in the electrode.

Table 14. The effects of glutamate, zinc and glutamate plus zinc on ON bipolar cells in pA.

	-25mV holding potential			-50mV holding potential		
Cell	0.3μM GLU	1μM Zn^{++}	0.3μMGLU+ 1μM Zn^{++}	0.3μM GLU	1μM Zn^{++}	0.3μM GLU+ 1μM Zn^{++}
79	29	-4	-9	38	3	-18
80	98	-10	-50	230	0	-143
81	34	7	-11	40	4	12
83				155	65	-173
	1uM GLU	1uM Zn^{++}	1uM GLU+ 1uM Zn^{++}	1uM GLU	1uM Zn^{++}	1uM GLU+ 1uM Zn^{++}
66	23	1	-27			
67	68	56	-180			
68				15	20	-52
69	51	45	-88			
80	15	-18	-43	37	0	-95
81				17	17	6
	10μM GLU	1μM Zn^{++}	10μMGLU+ 1μM Zn^{++}	10μM GLU	1μM Zn^{++}	10μM GLU+ 1μM Zn^{++}
79	33	-7	-8	43	3	0
80	53	-10	-22	105	0	-48
81	24	10	1	23	7	0

Table 15. Normalized effects of glutamate, zinc and glutamate plus zinc on ON bipolar cells in pA.

	-25mV holding potential			-50mV holding potential		
Cell	0.3μM GLU	1μM Zn^{++}	0.3μMGLU+ 1μM Zn^{++}	0.3μM GLU	1μM Zn^{++}	0.3μM GLU+ 1μM Zn^{++}
79	1	-0.138	-0.310	1	0.079	-0.474
80	1	-0.102	-0.510	1	0	-0.622
81	1	0.206	-0.324	1	0.100	0.300
83				1	0.419	-1.116
	1uM GLU	1uM Zn^{++}	1uM GLU+ 1uM Zn^{++}	1uM GLU	1uM Zn^{++}	1uM GLU+ 1uM Zn^{++}
66	1	0.043	-1.174			
67	1	0.824	-2.647			
68				1	1.333	-3.467
69	1	0.882	-1.725			
80	1	-1.200	-2.867	1	0	-2.568
81				1	1	-0.353
	10μM GLU	1μM Zn^{++}	10μMGLU+ 1μM Zn^{++}	10μM GLU	1μM Zn^{++}	10μM GLU+ 1μM Zn^{++}
79	1	-0.212	-0.242	1	0.070	0
80	1	-0.189	-0.415	1	0	-0.457
81	1	0.556	0.042	1	0.304	0

Table 16. Kainate (KA) responses in OFF bipolar cells and the blocking effect of 1μM Zn^{++}.

Cell	Holding potential (mV)	1μM Zn^{++} (pA)	300μM KA (pA)	300μM KA 1μM Zn^{++} (pA)
359	-50	-8	-33	0
366	-50	-10	-28	0
371	-50	6	-51	-12
372	-50	0	-101	14
374	-50	8	-28	7
375	-50	-	-118	0

Table 17. Kainate (KA) responses in OFF bipolar cells and the blocking effect of 1μM Zn^{++} normalized.

Cell	Holding potential (mV)	1μM Zn^{++} (pA)	300μM KA (pA)	300μM KA 1μM Zn^{++} (pA)
359	-50	0.242	1	0
366	-50	0.357	1	0
371	-50	-0.118	1	0.235
372	-50	0	1	-0.139
374	-50	-0.286	1	-0.250
375	-50	-	1	0

Table 18. Changes in membrane resistance of OFF bipolar cells during the application of zinc.

Cell	Holding Pot. (mV)	Drug	Response (pA)	5mV Pulse #1 (pA)	5mV Pulse #2 (pA)	Δ Resistance M ohms
288	-50	0.7μM Zn^{++}	1260	190	130	12.15
291	-50	0.7μM Zn^{++}	1750	455	370	2.52
295	-50	0.7μM Zn^{++}	192	200	175	3.57
302	-50	7μM Zn^{++}	340	353	315	1.71

Table 19. Effects of picrotoxin and histidine on the skate ERG.

Eyecup	Treatment	Filter (ND)	Light stimulus (μV)		Ratio HIS/PTX	
			ON	OFF	ON	OFF
I	Ringer	3	860	-		
	200uM PTX	3	850	360		
	200uM PTX + 100uM HIS	3	1920	400		
	(PTX+HIS)/PTX	3			2.26	1.11
II	Ringer	3	705	15		
	200uM PTX	3	1566	320		
	200uM PTX + 100uM HIS	3	3160	1165		
	(PTX+HIS)/PTX	3			2.02	3.64
III	Ringer	3	120	155		
	200μM PTX	3	247	59		
	200μM PTX + 100μM HIS	3	319	77		
	(PTX+HIS)/PTX	3			1.29	1.31
IV	Ringer		828	45		
	200μM PTX	4	2115	253		
	200μM PTX+ 100μM HIS	4	2994	305		
	(PTX + HIS)/PTX				1.42	1.21
V	Ringer		126	-		
	200μM PTX	3	205	11		
	200μM PTX+ 100μM HIS	3	287	18		
	(PTX+HIS)/PTX	3			1.40	1.64

Table 20. Effects of picrotoxin and TPEN on the skate ERG.

Eyecup	Treatment	Filter (ND)	Light stimulus (μV)		Ratio TPEN/PTX	
			ON	OFF	ON	OFF
I	Ringer	4	170	-		
	200uM PTX	4	525	200		
	200uM PTX + 100uM TPEN	4	640	253		
	(PTX+TPEN)/PTX	4			1.22	1.27

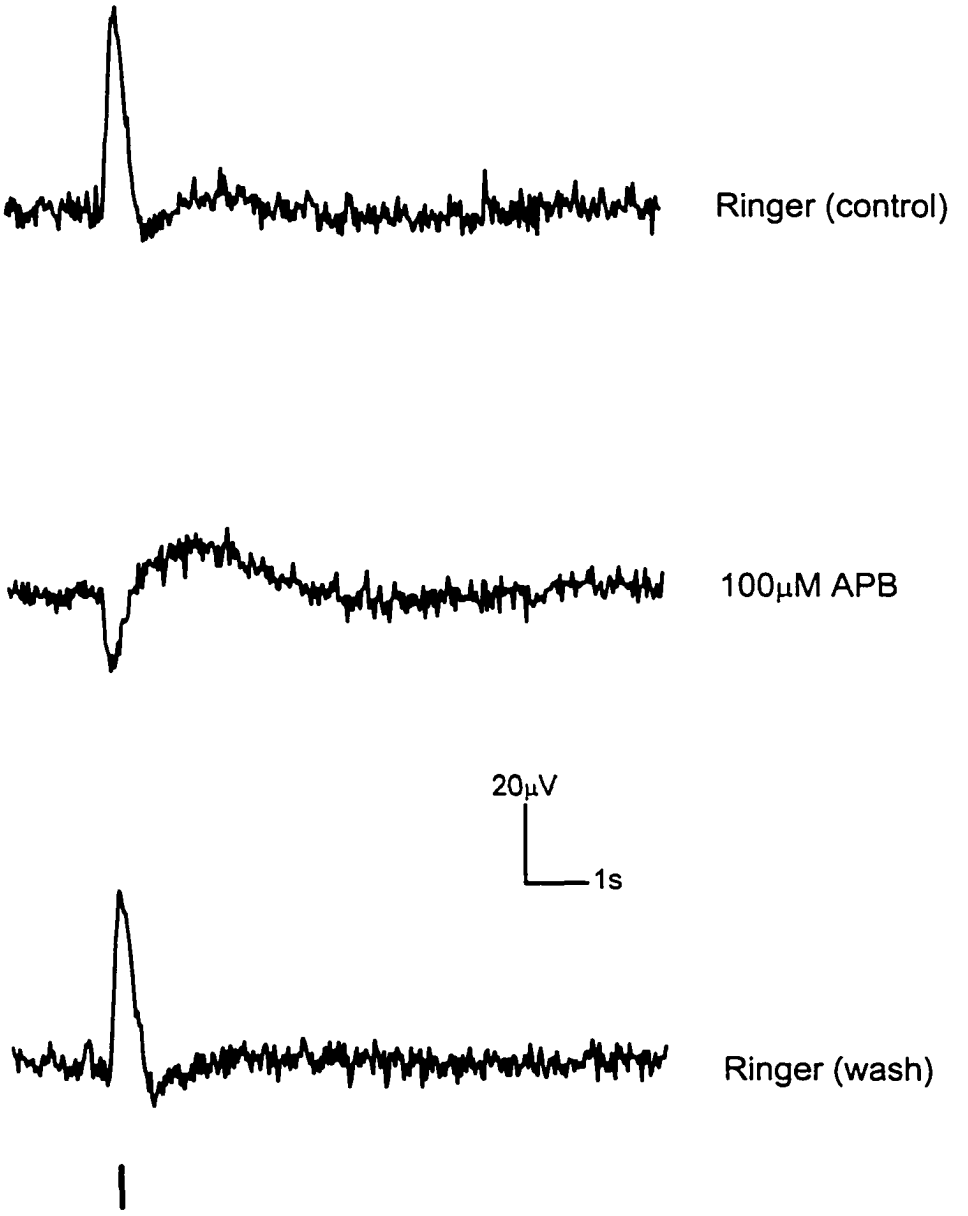

Fig. 1 100μM APB blocks the ON response (b-wave) in the skate ERG. The ON response recorded in normal Ringer solution (upper trace) was blocked by the application of 100μM APB (middle trace). Recovery of the ON response was obtained with a Ringer wash (lower trace). The bar indicates the interval (80 msec) during which light was applied to the eyecup. Flash intensity: Log I = -2

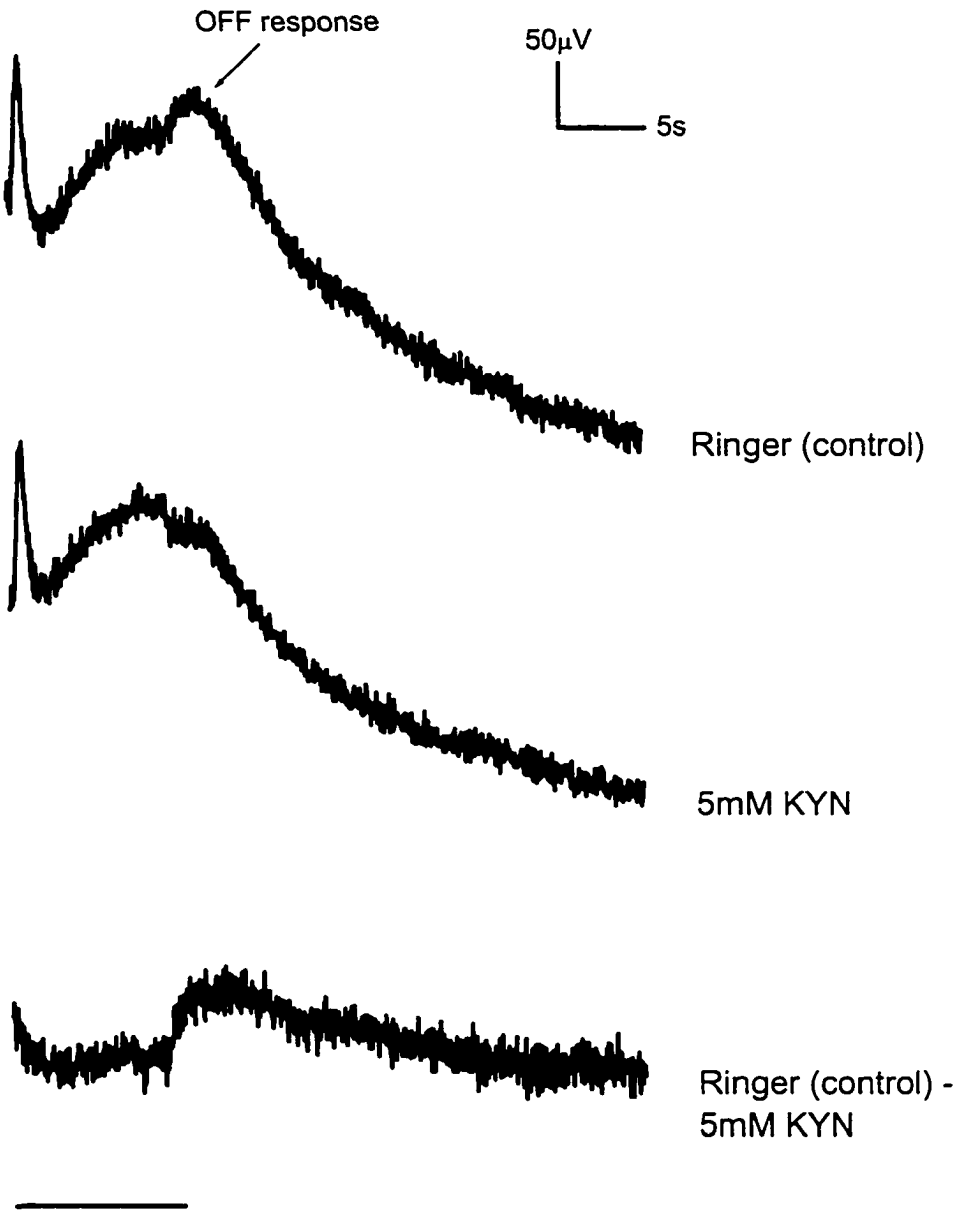

Fig. 2 When the eyecup was also superfused with Ringer and a longer period of light was used (10s), a small OFF response (d-wave) in the ERG was observed (upper trace). The OFF response could be blocked with the application of 5mM KYN (middle trace) and its magnitude measured as a difference between the two ERGs (lower trace). The horizontal bar indicates the interval during which light was applied to the eyecup. Flash intensity: Log I = -4.

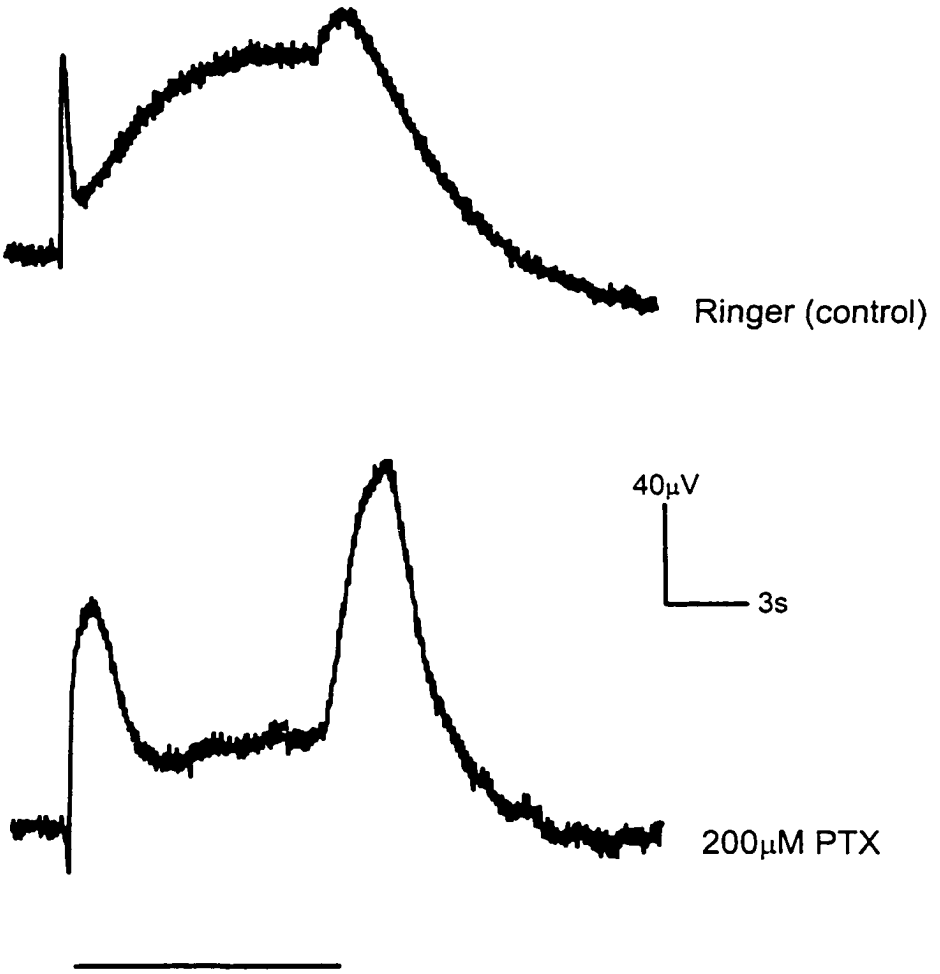

Fig. 3 Application of 500μM PTX altered the control ERG (upper trace) giving enlarged ON and OFF responses (lower trace). PTX also suppressed the c-wave making the OFF response even more prominent. The horizontal bar indicates the interval during which light was applied to the eyecup. Flash intensity: Log I = -3.

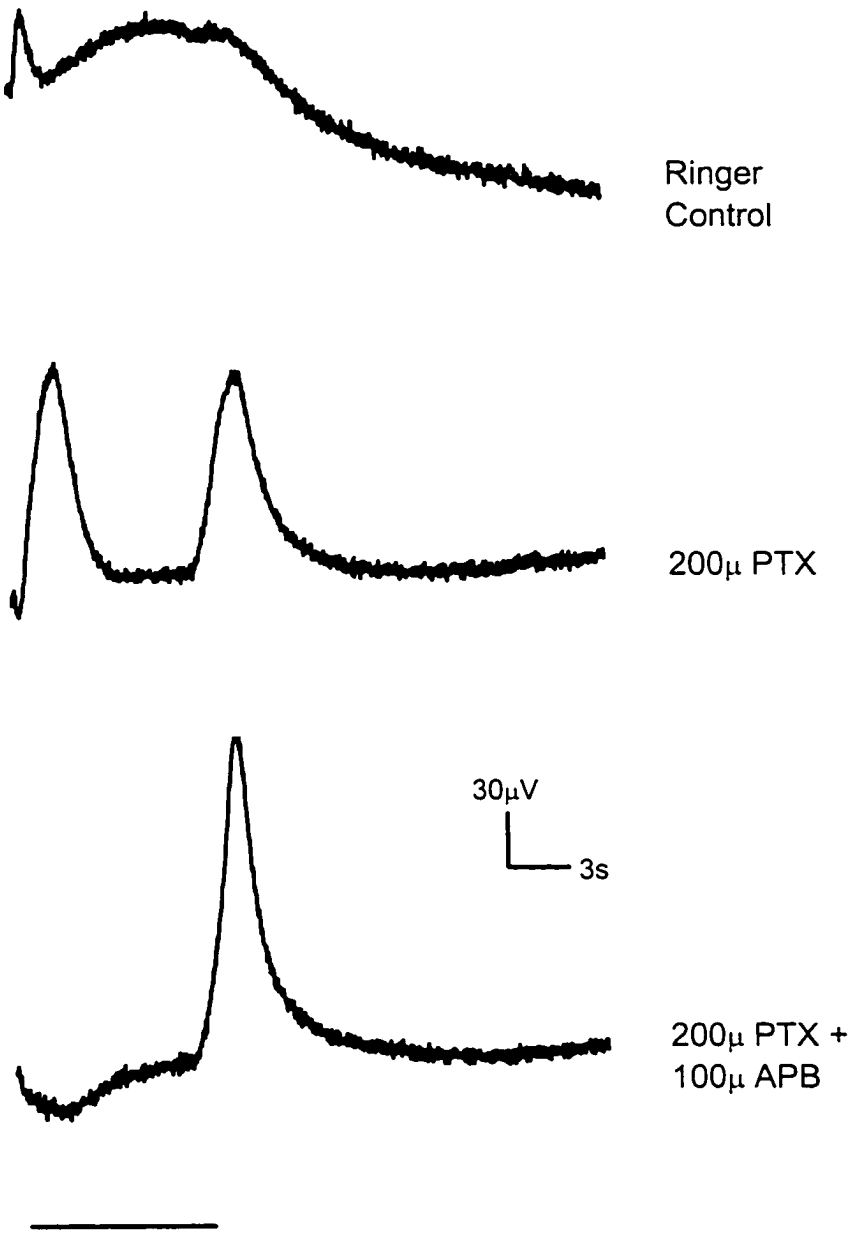

Fig. 4 The ON response in 200μM PTX was blocked by a solution of 200μM PTX plus 100μM APB but left the enhanced OFF response. A wash of 200μM PTX recovered the ON response (not shown). The bar indicates the interval during which light was applied to the eyecup. Flash intensity: Log I = -4

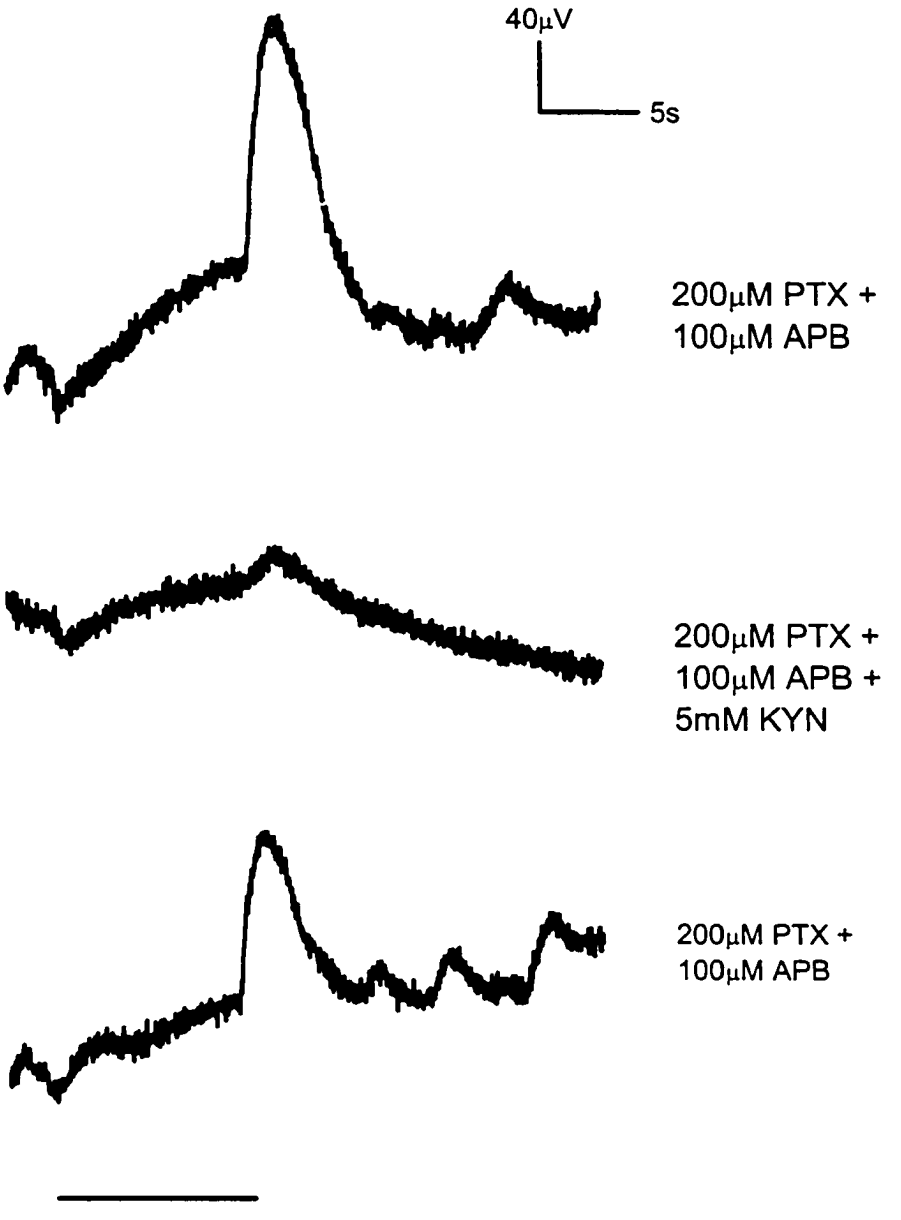

Fig. 5 A solution of 200μM PTX plus 100μM APB blocked the ON response and with the addition of 5mM KYN the OFF response was blocked. Washing with the control solution, PTX plus APB, recovered the OFF response. The horizontal bar indicates the period during which light was applied. Flash intensity: Log I = -3.

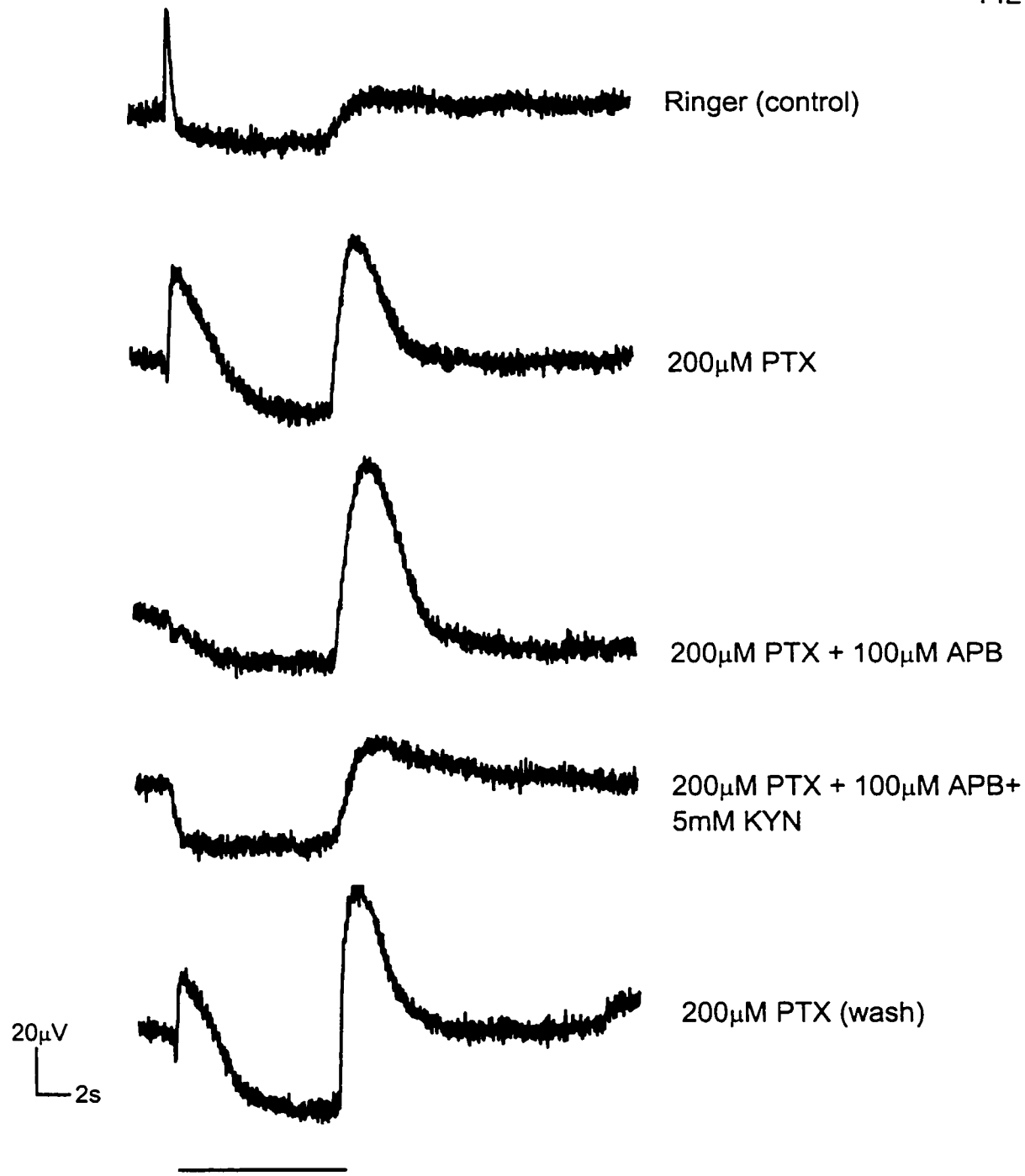

Fig. 6 Isolated retina preparation tested with blocking agents. Separating the retina from the pigment epithelium eliminated the c-wave making the OFF response more distinct in Ringer and more pronounced with 200μM PTX. The application of 200μM PTX plus 100μM APB gave an even larger OFF response, and after the addition of 5mM KYN, only the a-wave from the photoreceptors remained. The ON and OFF responses were recovered with a 200μM PTX wash. The bar indicates the interval during which light was applied to the isolated retina. Flash intensity: Log I = -3.

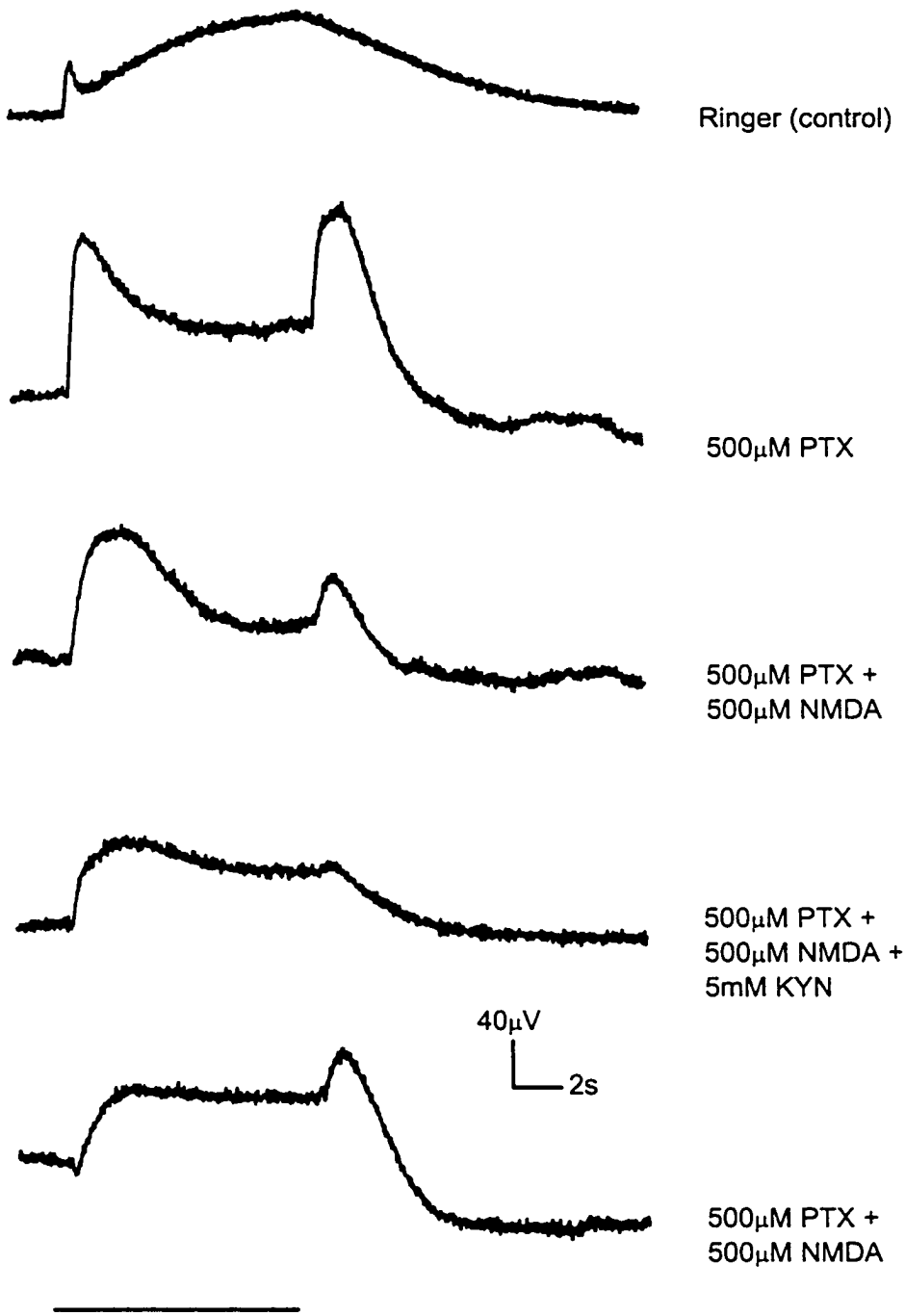

Fig. 7 The ERG OFF response was retained after the PTX treated retina was superfused with 200μM PTX plus 500μM NMDA. The additon of 5mM KYN to the superfusate suppressed the OFF response, but it was recovered with a wash of PTX plus NMDA. Horizontal bar indicates the period during which light was applied. Flash intensity: Log I = -4.

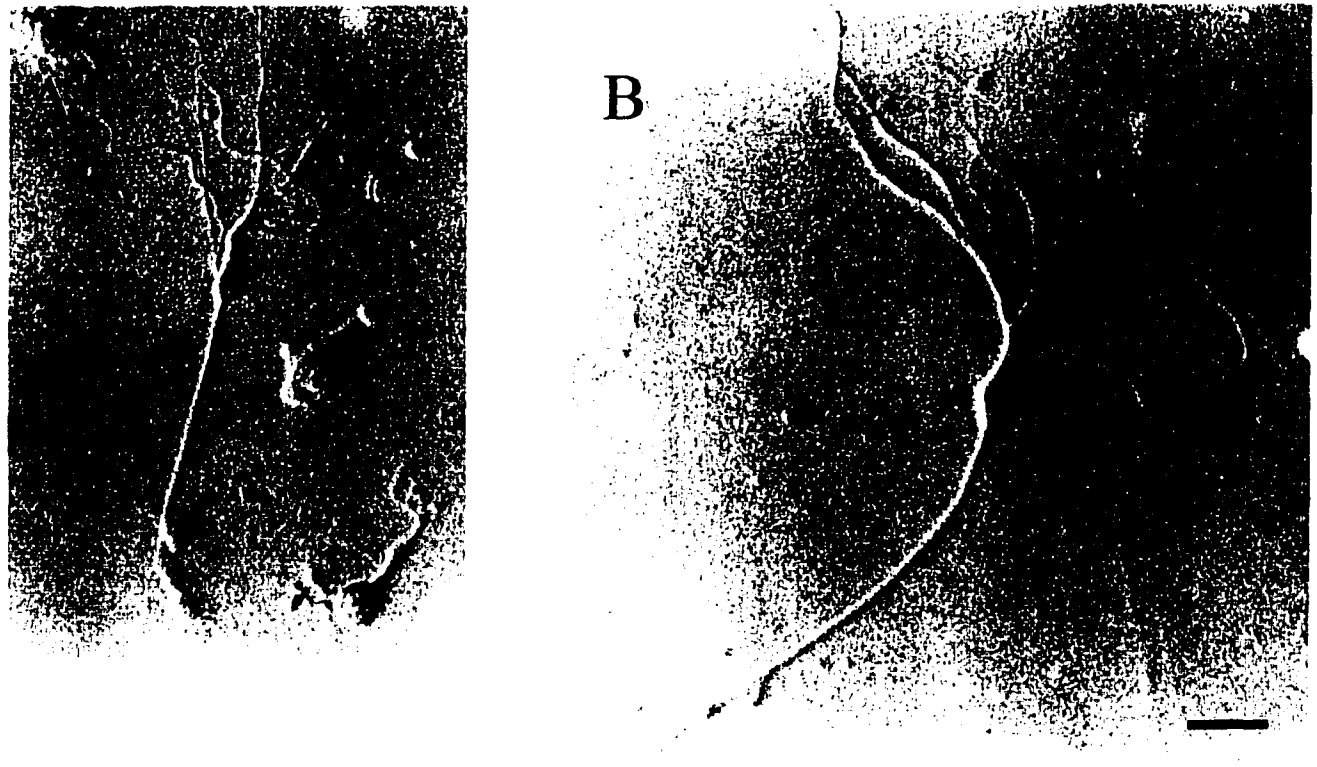

Fig. 8 Branching dendrites. Dendritic branching patterns differ in bipolar cells A and B. The length of the axon measured from the center of the cell body to the axon terminal is 2.14 times longer for bipolar cell B than A. Scale bar = 100μm.

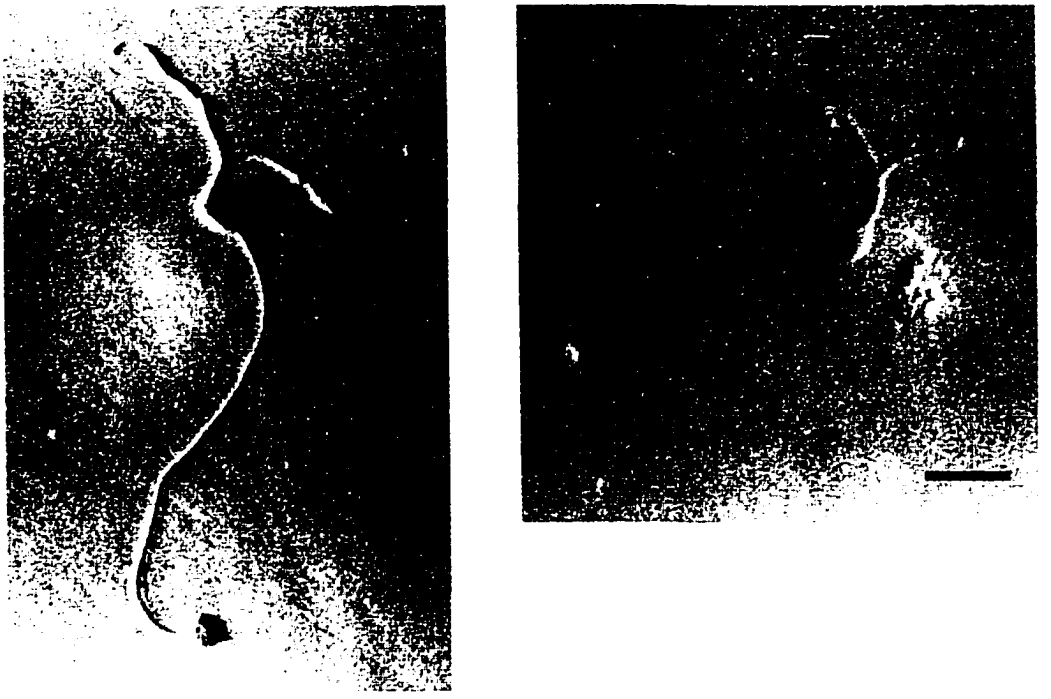

Fig. 9 Bifurcated dendrites. Two examples of bipolar cells with simple bifurcated branching that are frequently observed. Scale bar = 100μm.

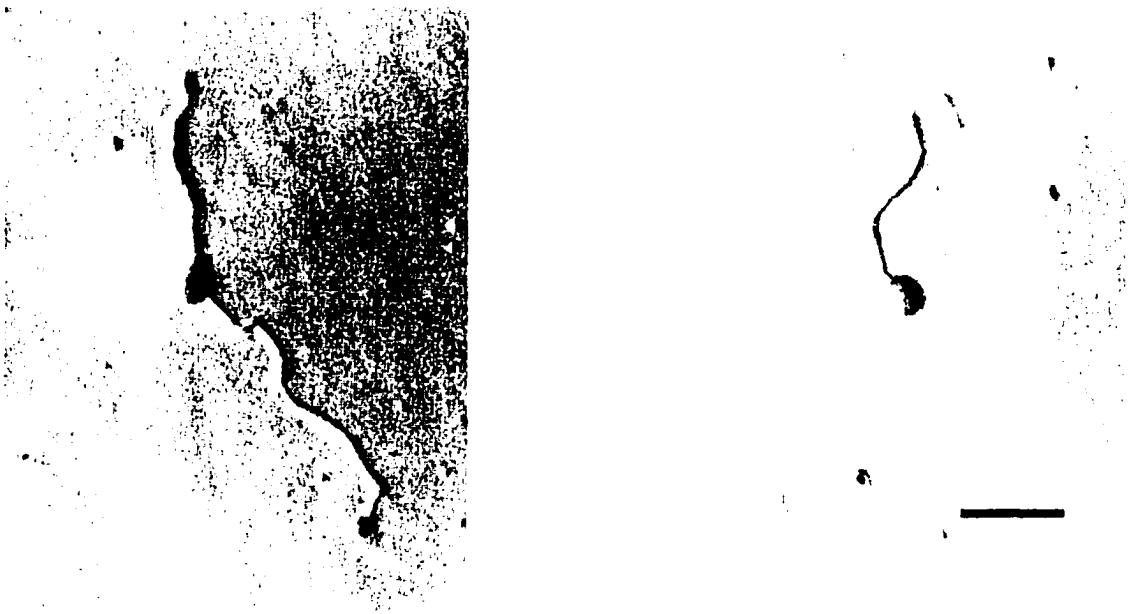

Fig. 10 Non-branching dendrites. These are one of the most commonly observed types of bipolar cells. Although non-branching cells are the least complex, their dendritic patterns do vary. Scale bar = 100μm.

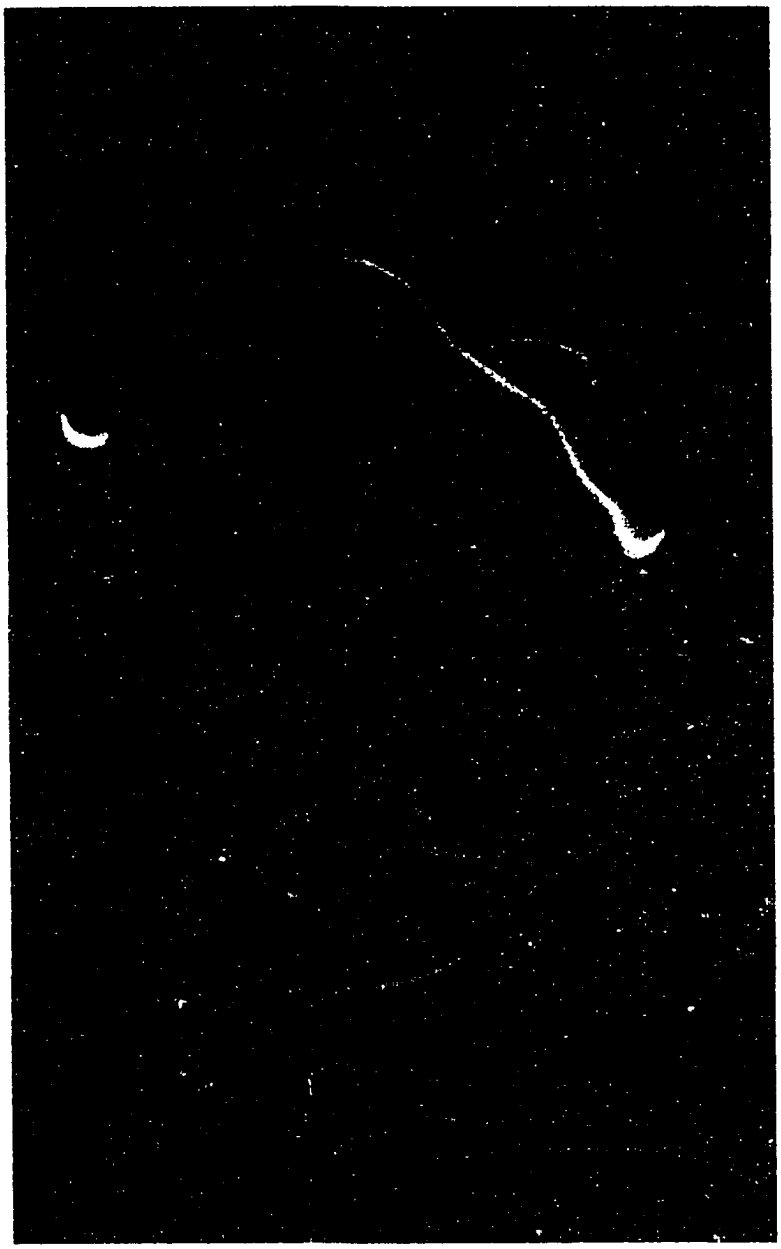

Fig. 11 Bipolar cell with mixed dendrites. Dendritic region has some characteristics of both branching and non-branching bipolar cells. Scale bar = 100μm

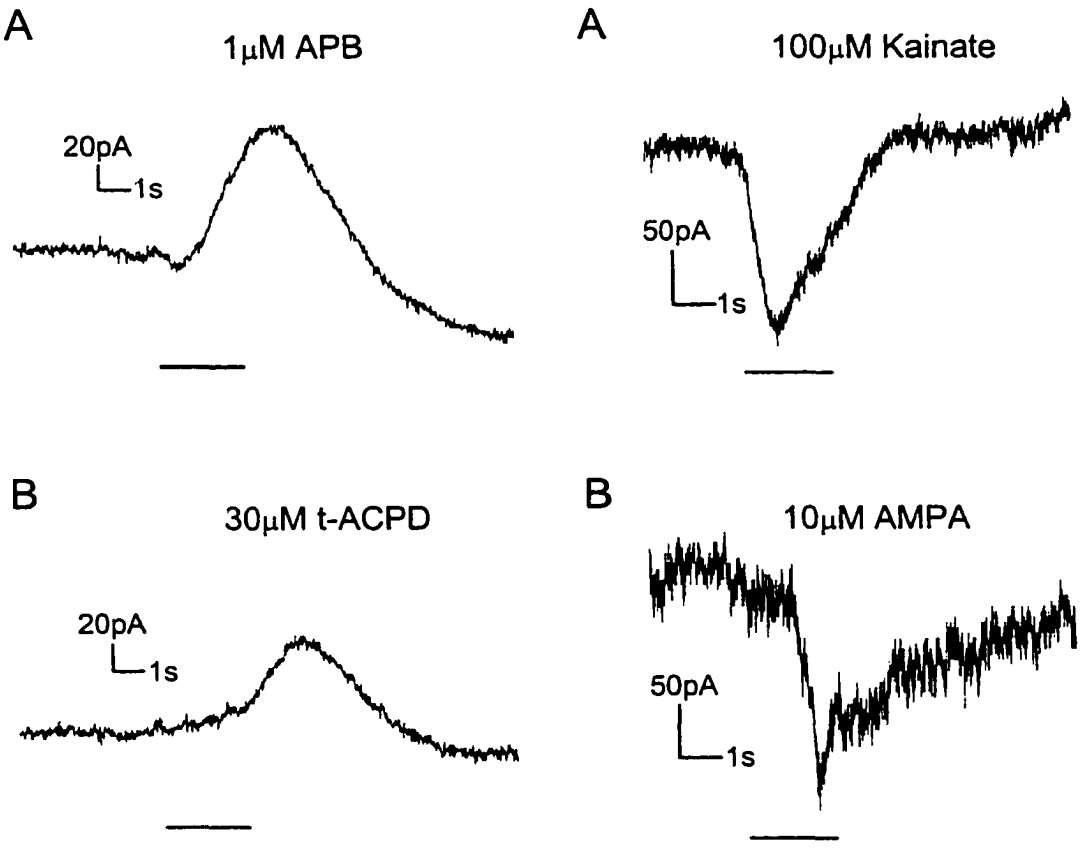

Fig. 12 ON bipolar-like responses. An isolated bipolar cell response to 1μM APB (A) and 30μM t-ACPD (B) V_c = -25mV. Horizontal bar indicates period of drug application.

Fig. 13 OFF bipolar-like responses. An isolated bipolar cell response to 100μM kainate (A) and 10μM AMPA (B) V_c = -60mV. Horizontal bar indicates period of drug application.

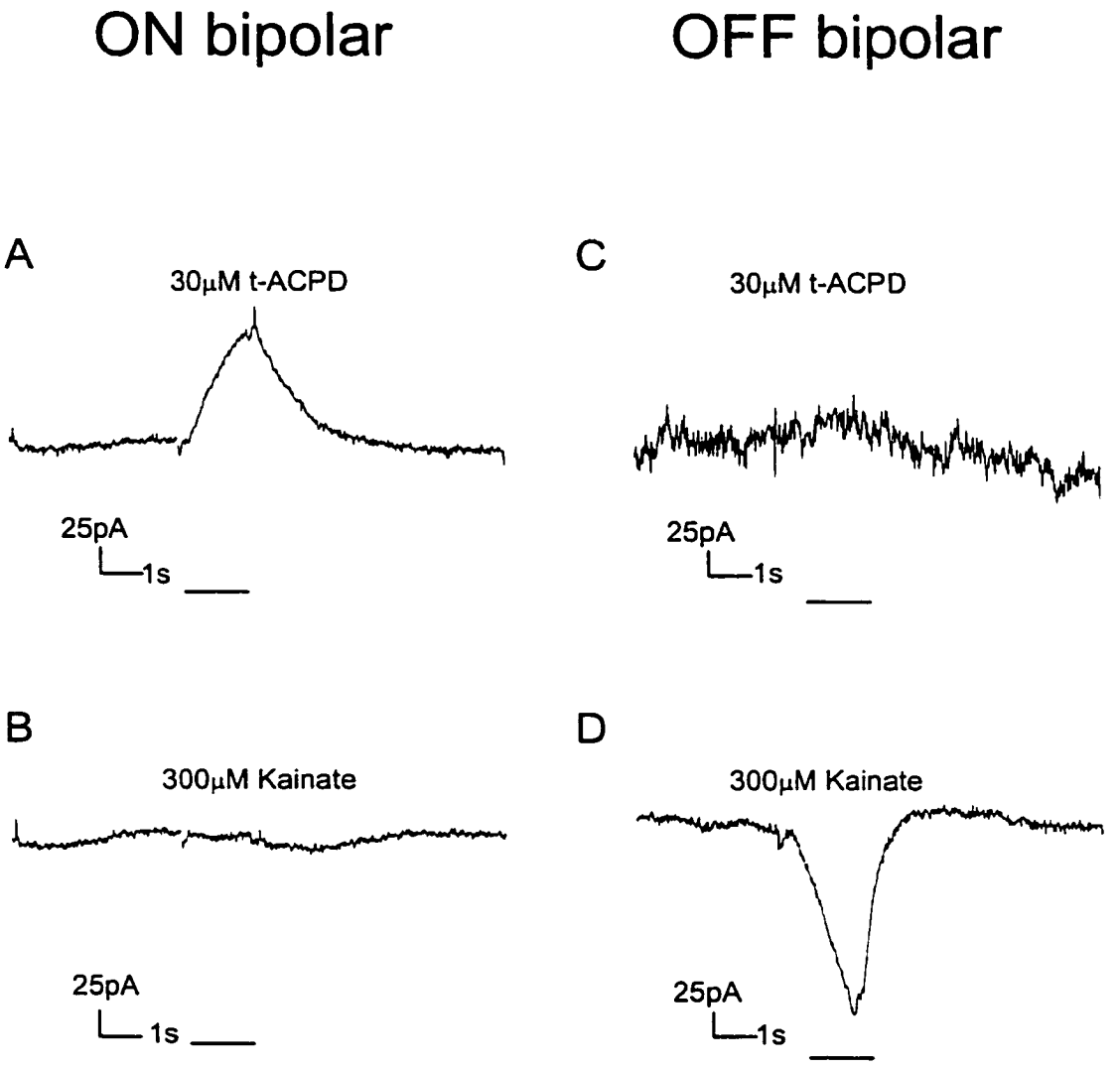

Fig. 14 Comparison of ON- and OFF-bipolar cell responses to t-ACPD and kainate. A typical isolated ON-bipolar cell responded to 30μM t-ACPD with an outward current (A), but not to 300μM kainate (B). An isolated OFF-bipolar cell, on the other hand, showed no response to 30μM t-ACPD (C), but responded to 300μM kainate with an inward current. Both of the isolated bipolar cells were voltage clamped at V_c=-50mV. Horizontal bar indicates the period of drug application.

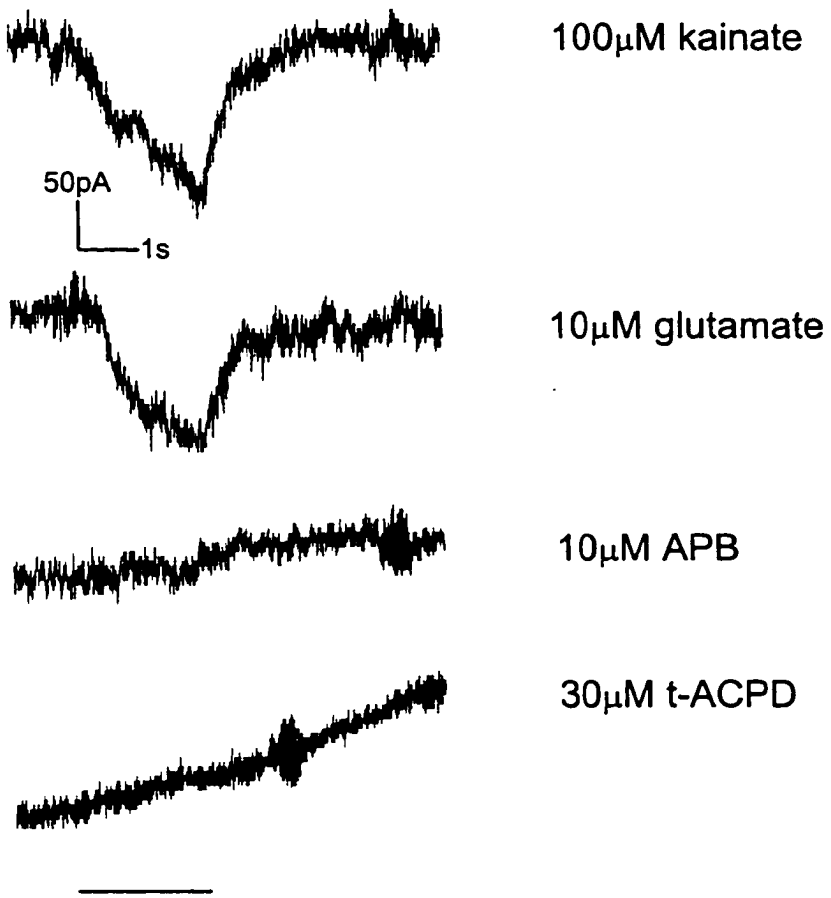

Fig. 15 The effects of glutamate agonists on an OFF bipolar cell. The OFF bipolar cell was sensitive to 100μM kainate and to 10μM glutamate, but insensitive to both 10μM APB and to 30μM t-ACPD. V_c = -60mV. Horizontal bar indicates period of drug application.

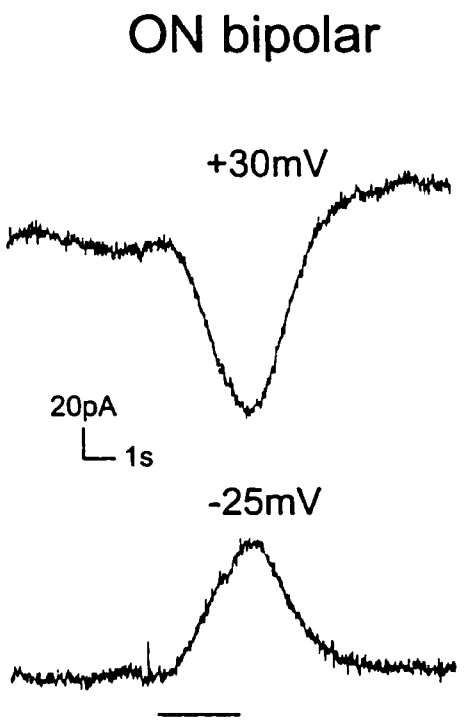

Fig. 16 ON bipolar cell response to 0.3μM glutamate at +30 and -25mV. When voltage clamped at +30mV the ON bipolar cell had an inward current and at -25mV an outward current. Horizontal bar indicates period of drug application.

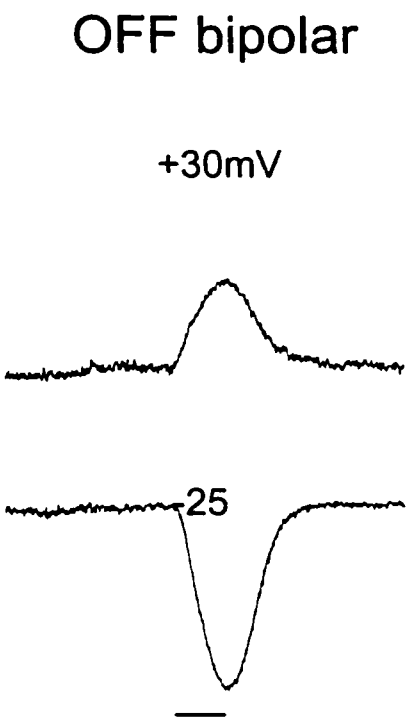

Fig. 17 OFF bipolar cell response to 100μM kainate at +30 and -25mV. When voltage clamped at +30mV the OFF bipolar cell had an outward current and at -25mV an inward current. Horizontal bar indicates period of drug application.

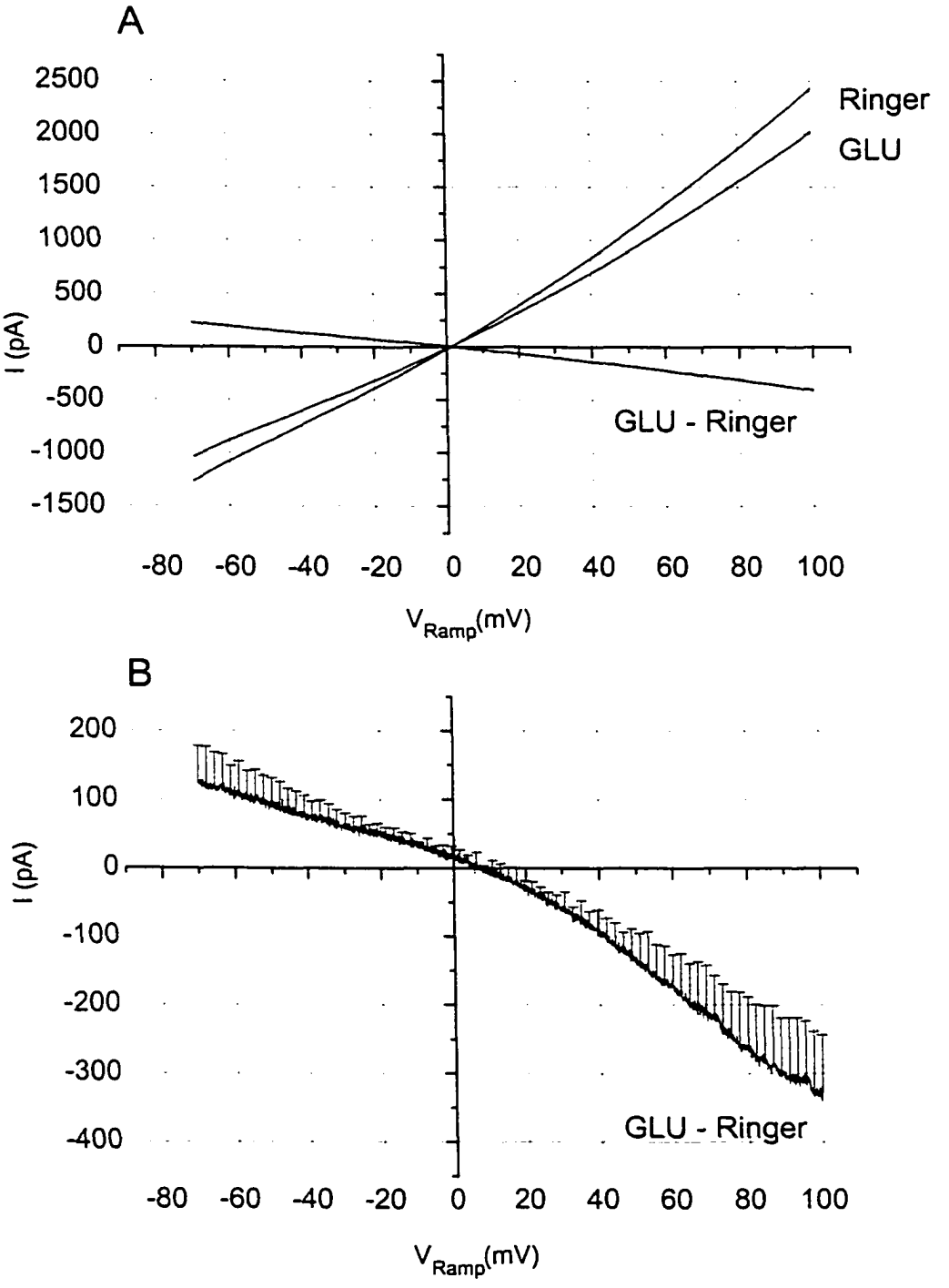

Fig. 18 ON bipolar-like cells recorded in Ringer (control) and 0.3μM glutamate, between -70mV and +100mV using a ramp stimulus. (A) For cell 79, the ramp in Ringer was subtracted from the ramp in 0.3μM glutamate, and the difference (glutamate minus Ringer) is presented. (B) The difference in ramps, glutamate minus Ringer, measured on cells 79, 80 and 81 had negative slopes, which were averaged and presented with +SEM. The negative slope, in general, indicates glutamate caused an increase in membrane resistance for these ON bipolar-like cells.

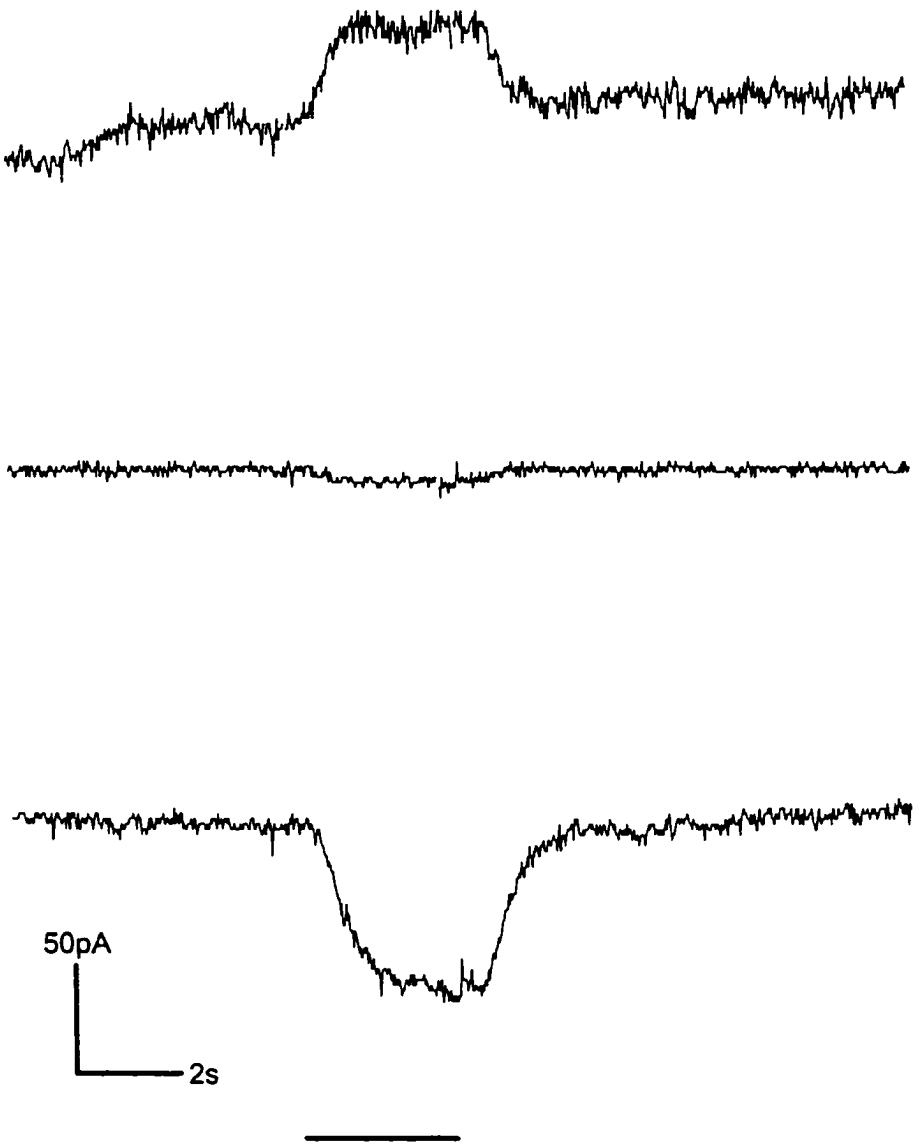

Fig. 19 OFF bipolar cell responses to 100μM kainate at different holding potentials. 100μM kainate produced an inward current at V_c = -25mV (lower trace) and an outward current at V_c = +30mV (upper trace). The current at V_c = 0mV (middle trace) was negligible and indicates the cell was near its reversal potential. Horizontal bar indicates period of drug application.

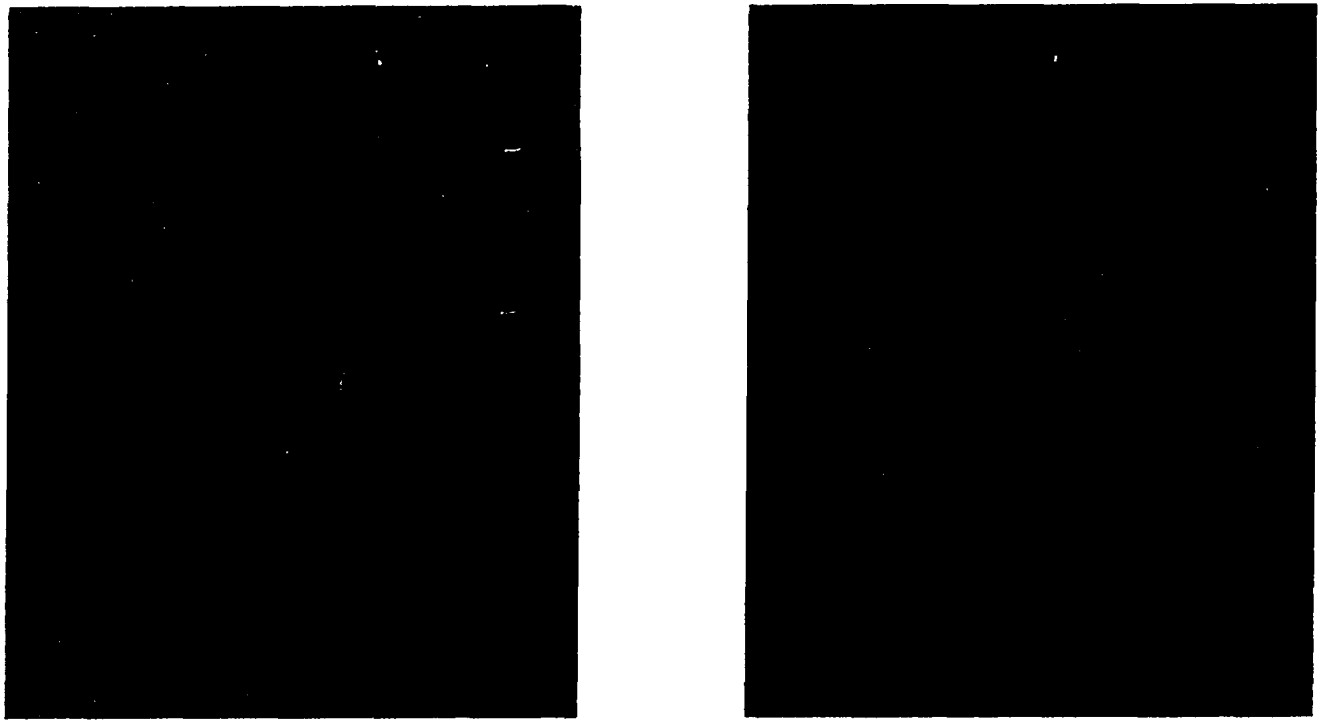

Fig. 20 Morphological similarities between ON and OFF bipolar cells. Examples of bipolar cells with dendritic branching: (A) ON bipolar cell and (B) OFF bipolar cell. Scale bar = 100μm.

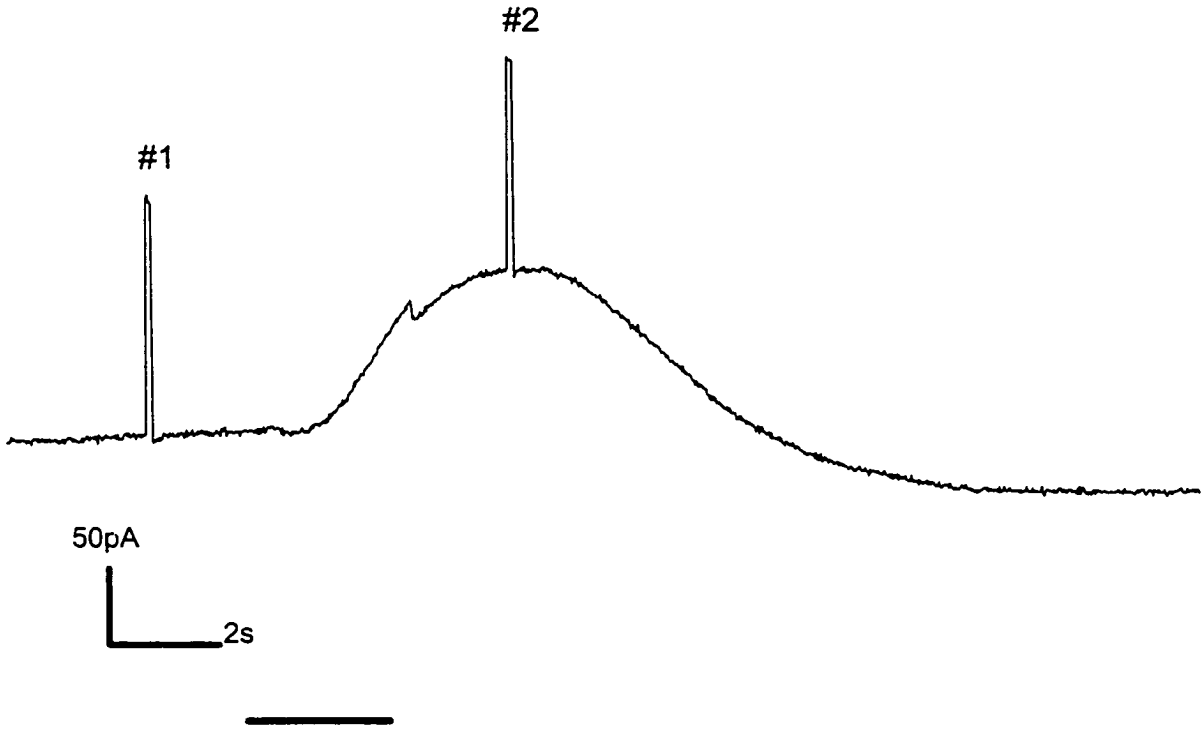

Fig. 21 5mV pulses applied to an ON bipolar-like cell to measure the changes in membrane resistance during a response to 3μM glutamate. Pulse #1 measures 149pA, while pulse #2 is 133pA. Horizontal bar indicates period of drug application (V_c = -25mV).

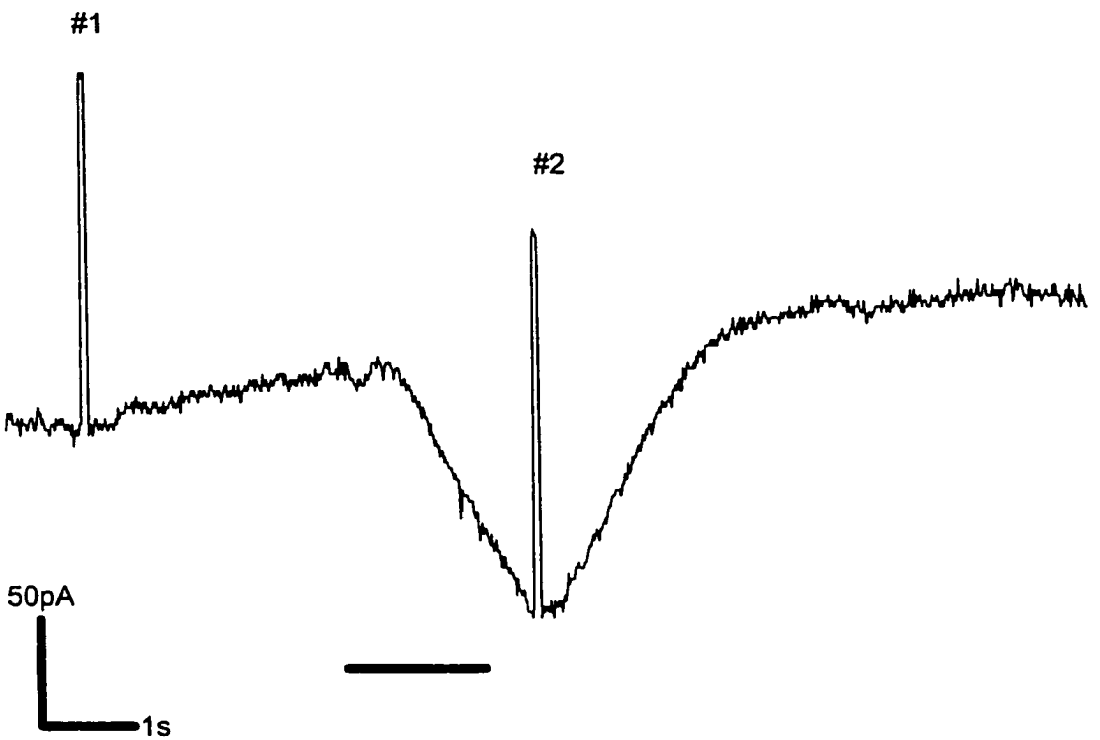

Fig. 22 5mV pulses applied to an OFF bipolar-like cell to measure the changes in membrane resistance during a response to 300μM kainate. Pulse #1 measures 157pA, while pulse #2 is 173pA. Horizontal bar indicates period of drug application (V_c = -50mV).

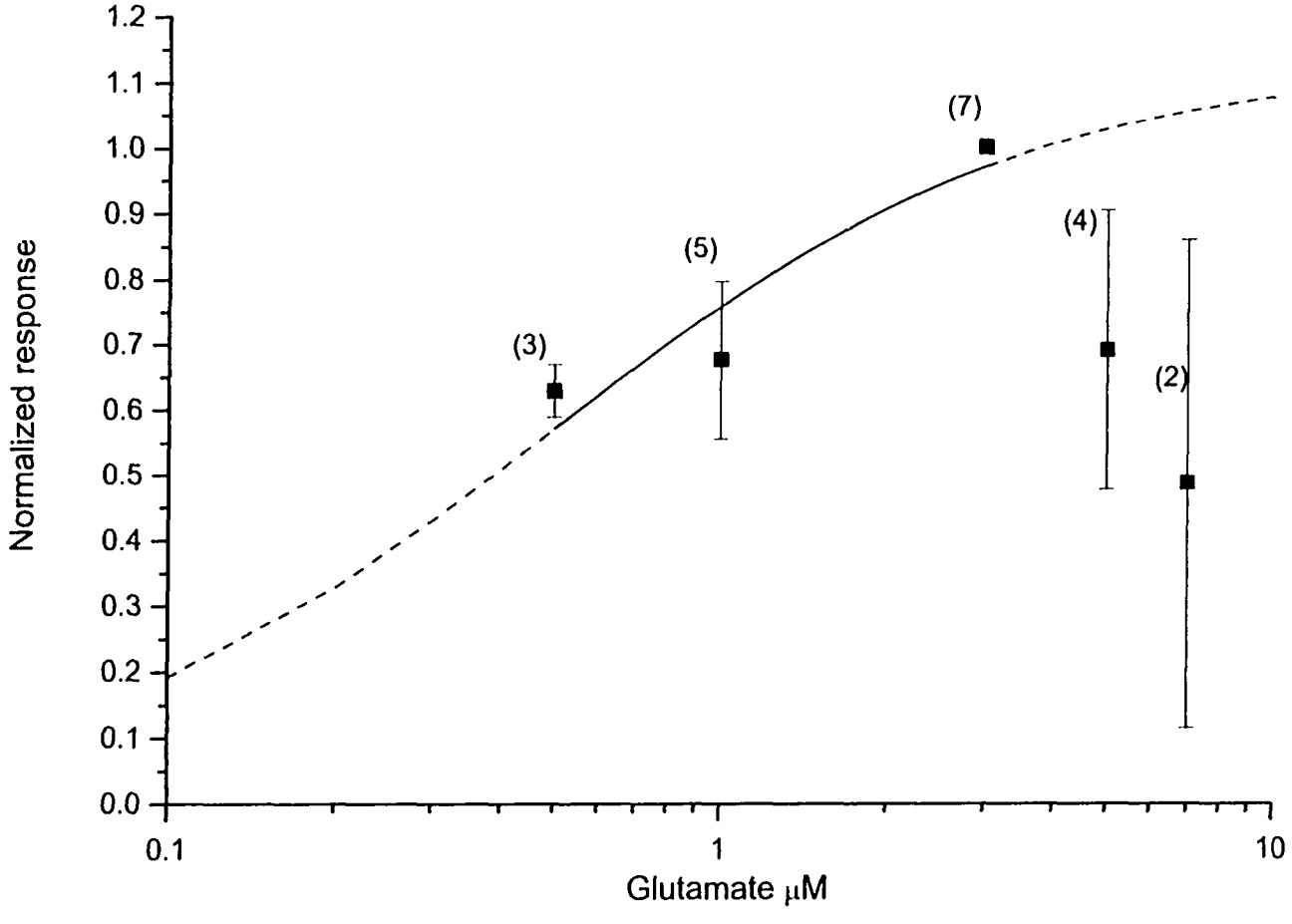

Fig. 23 Dose-response relationship for ON bipolar cells. Data from 0.5 to 3 μM was fitted to the Hill equation and graphed as a solid line. V_{max} was 1.13 ±0.19, EC_{50} 0.49μM ±0.28, and a Hill coefficient of 1.0 gave the best fit. Chi-square (χ^2) and the coefficient of determination (R^2) were, respectively, 0.01 and 0.87. The dashed curve is an extension of the curve plotted from the empirical values and is the assumed dose-response at higher and lower concentrations. The responses were erratic because of cell rundown and desensitization at higher concentrations.

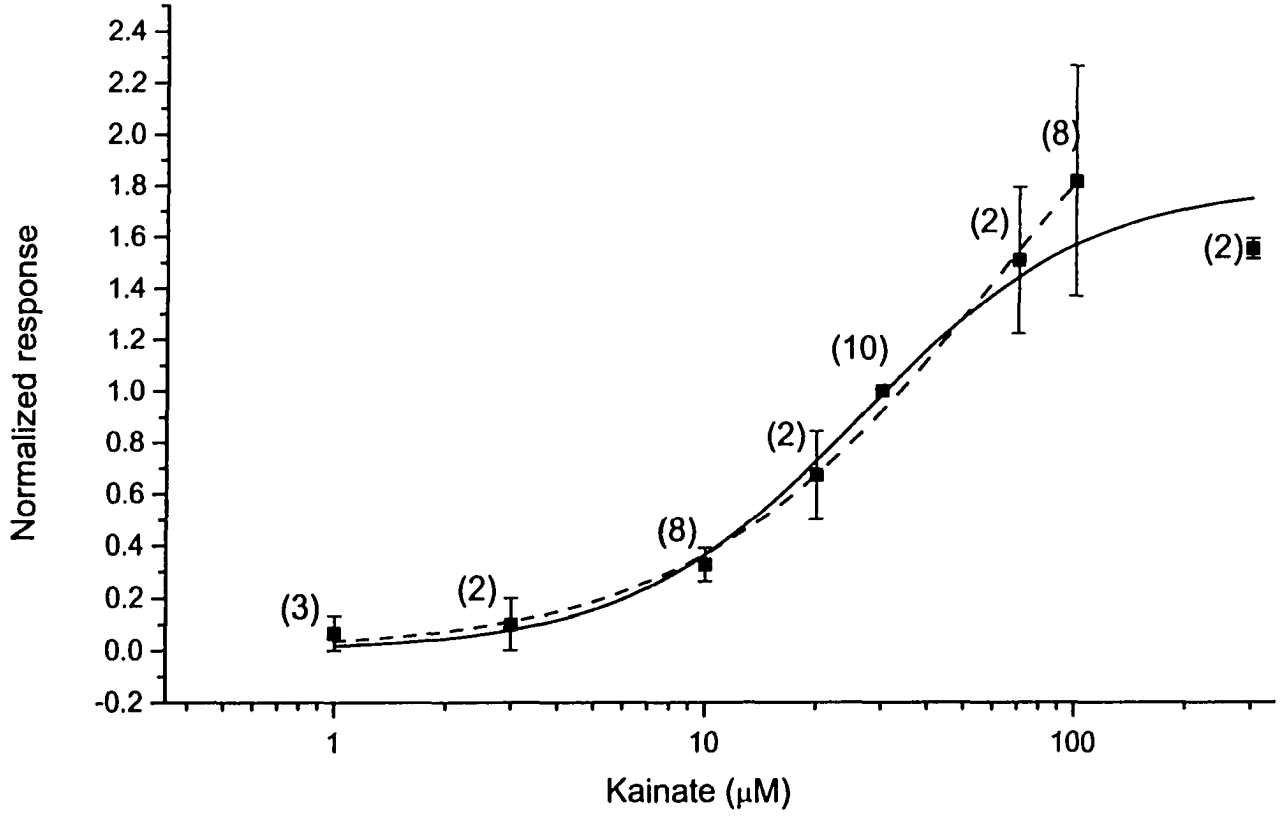

Fig. 24 Kainate dose-response curves normalized to 30μM. Data normalized to 30μM kainate with the solid curve fitted to the complete set of data ranging from 1 to 300μM kainate, and the dashed curve fitted from 1 to 100μM kainate excludes 20 and 70μM kainate. The solid curve had a Hill coefficient of 1.42 ±0.27, EC_{50} = 26.27μM ±6.29 and V_{max} = 1.80 ±0.20 with chi-square (χ^2) and the coefficient of determination (R^2) values equal to 0.02 and 0.98, respectively. The truncated data fitted to kainate concentrations up to 100μM allowed greater precision in evaluating the Hill coefficient by avoiding high kainate concentrations where cells desensitize. The dashed curve had a Hill coefficient of 1.07 ± 0.13, EC_{50} = 60.07μM ±28.01 and V_{max} 2.86 ±0.68 with χ^2 and R^2 values of 0.007 and 0.99, respectively.

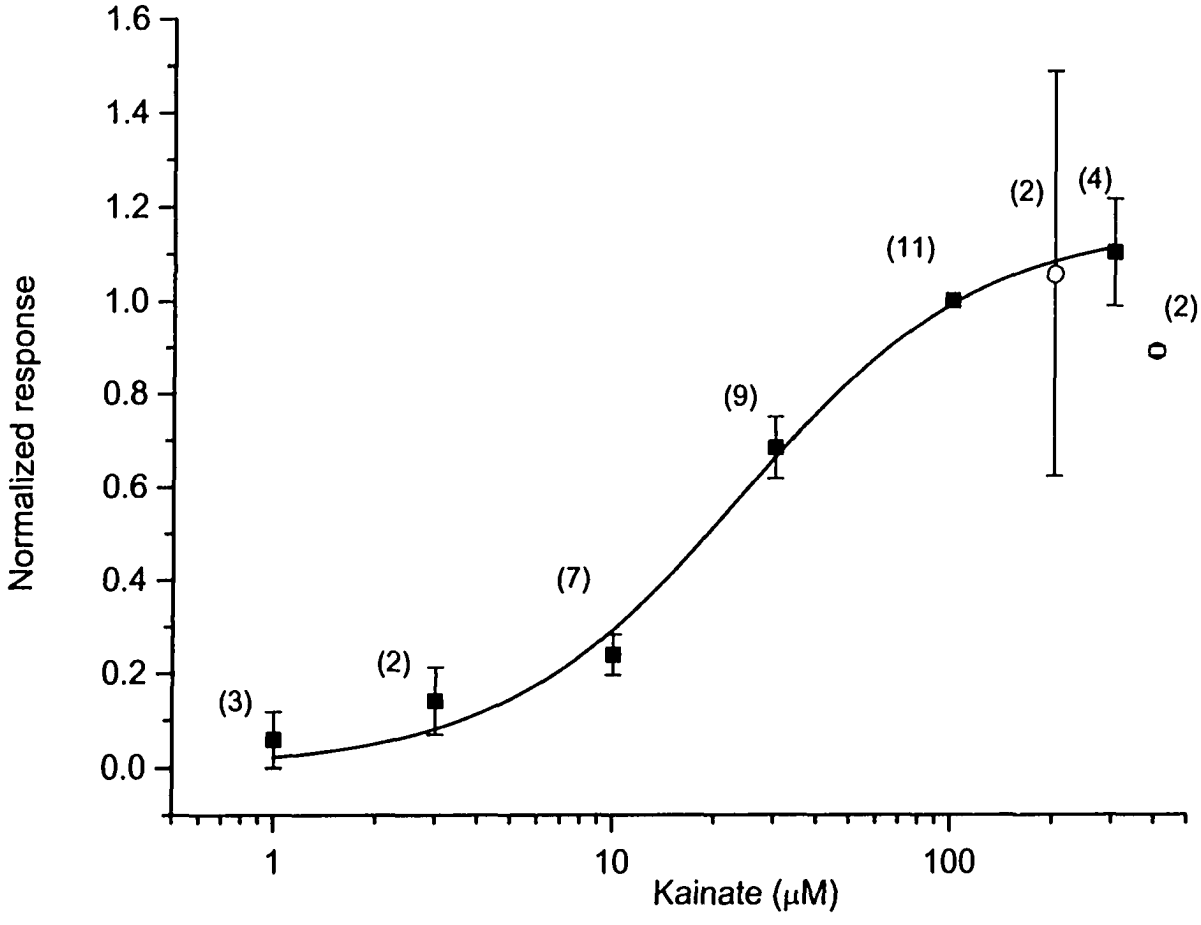

Fig. 25 Kainate dose-response curve normalized to 100μM. Data normalized to 100μM kainate gained two cells at 300μM and lost two cells at 20 and 70μM (see text). The data points at 200 and 400μM were included to illustrate desensitization and variability at high concentrations, but were excluded from the fitted curve. The Hill coefficient was 1.25 ±0.22, EC_{50} = 23.76 μM ±4.33, V_{max} = 1.16 ±0.07 with chi-square (χ^2) and the coefficient of determination (R^2) equal to 0.003 and 0.99, respectively.

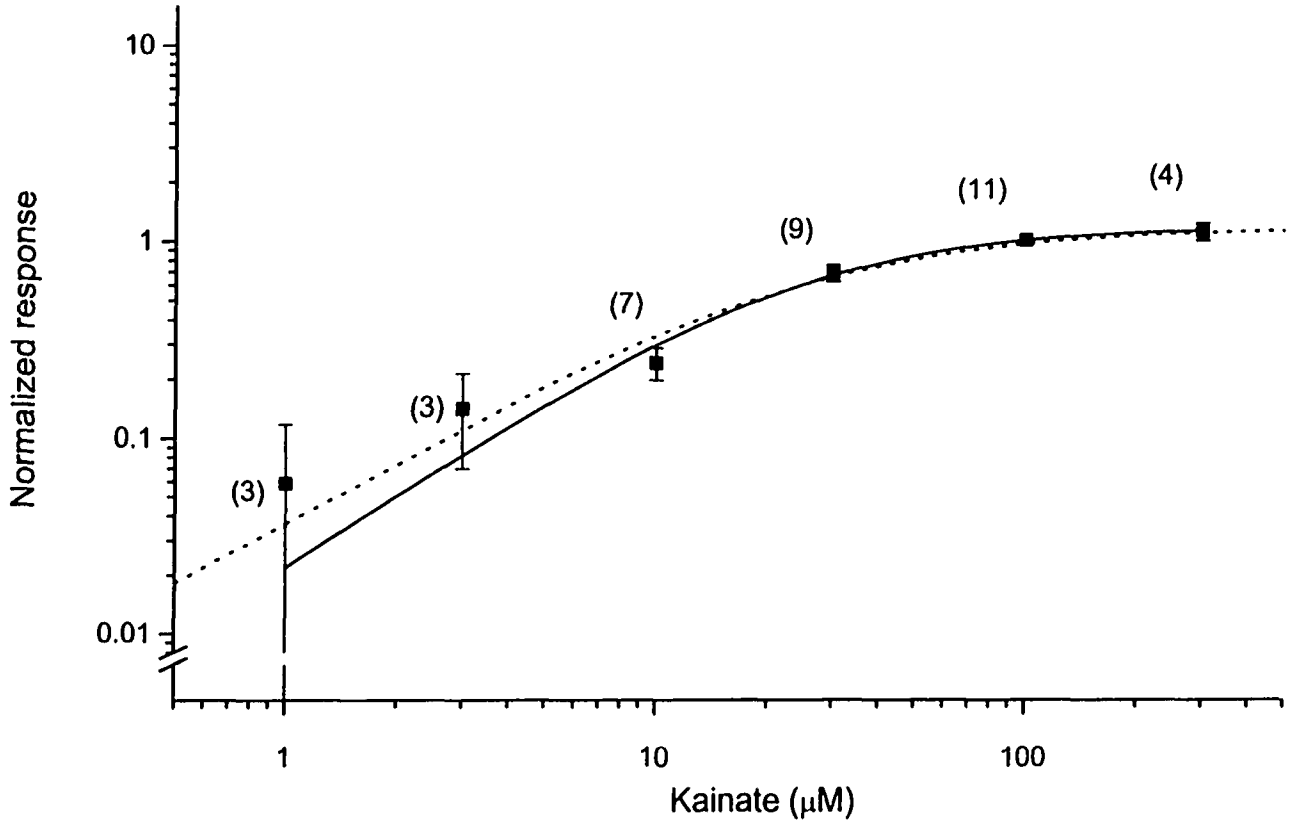

Fig. 26 Assumed and actual dose-response curves for OFF bipolar cells. The solid curve is the actual dose-response data plotted to the Hill equation. The data was normalized to 100μM kainate and fitted from 1 to 300μM kainate. As before, it has the experimental values of Vmax 1.16 ±0.07, EC_{50} 23.76μM ±4.33, and a Hill coefficient of 1.25 ±0.22. The dashed curve is an assumed dose-response relationship. It was replotted using the actual Vmax and EC_{50} values with the more accurate Hill coefficient, 1.07, obtained from the truncated curve normalized to 30μM kainate (Fig. 24, dashed line). The difference between the curves can be attributed to desensitization and greater variability in the responses at saturating kainate concentrations.

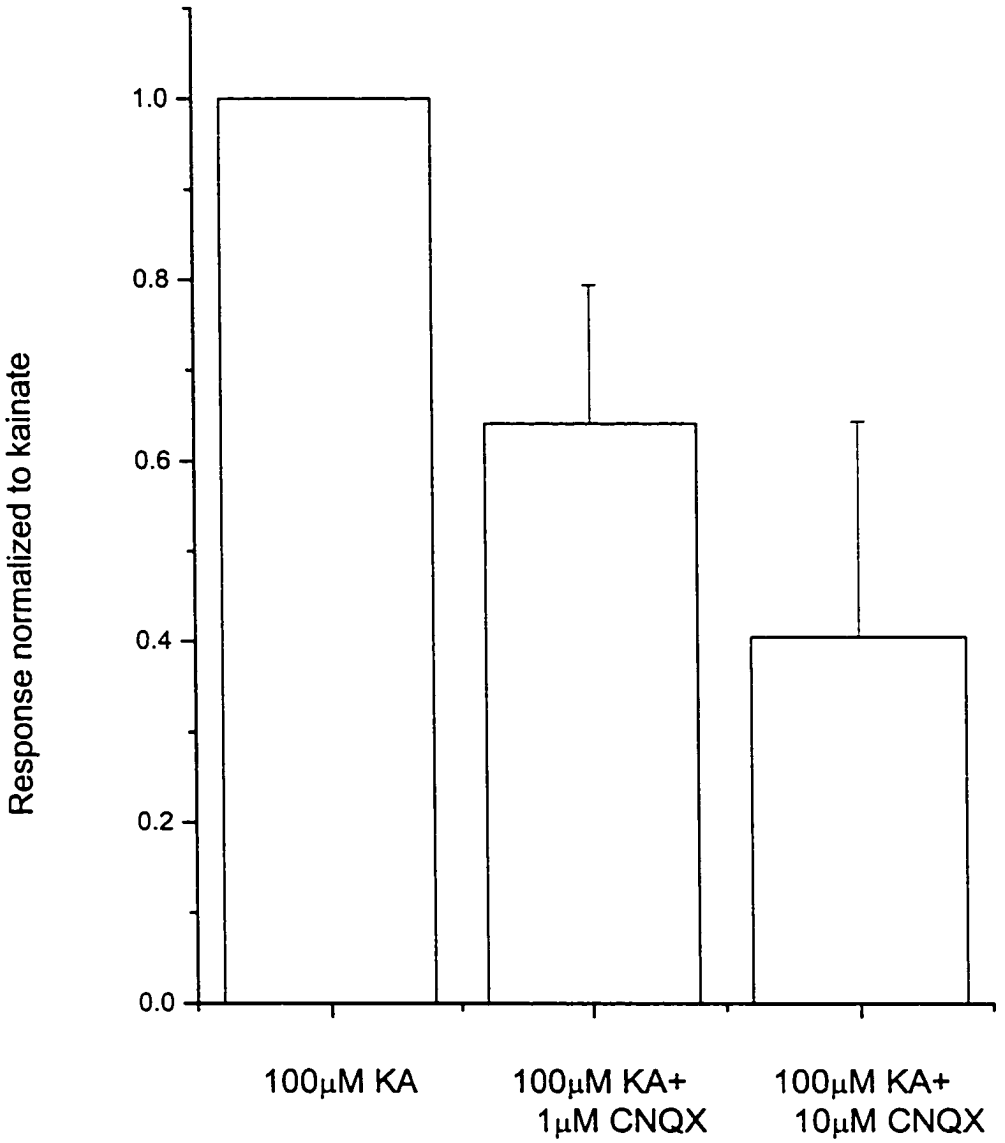

Fig. 27 Suppression of the kainate response in OFF bipolar cells by CNQX. The responses to kainate by the bipolar cells were suppressed by CNQX in a dose-dependent manner. Responses were normalized to each cell's kainate response. N = 6 except for 10μM CNQX where N = 3.

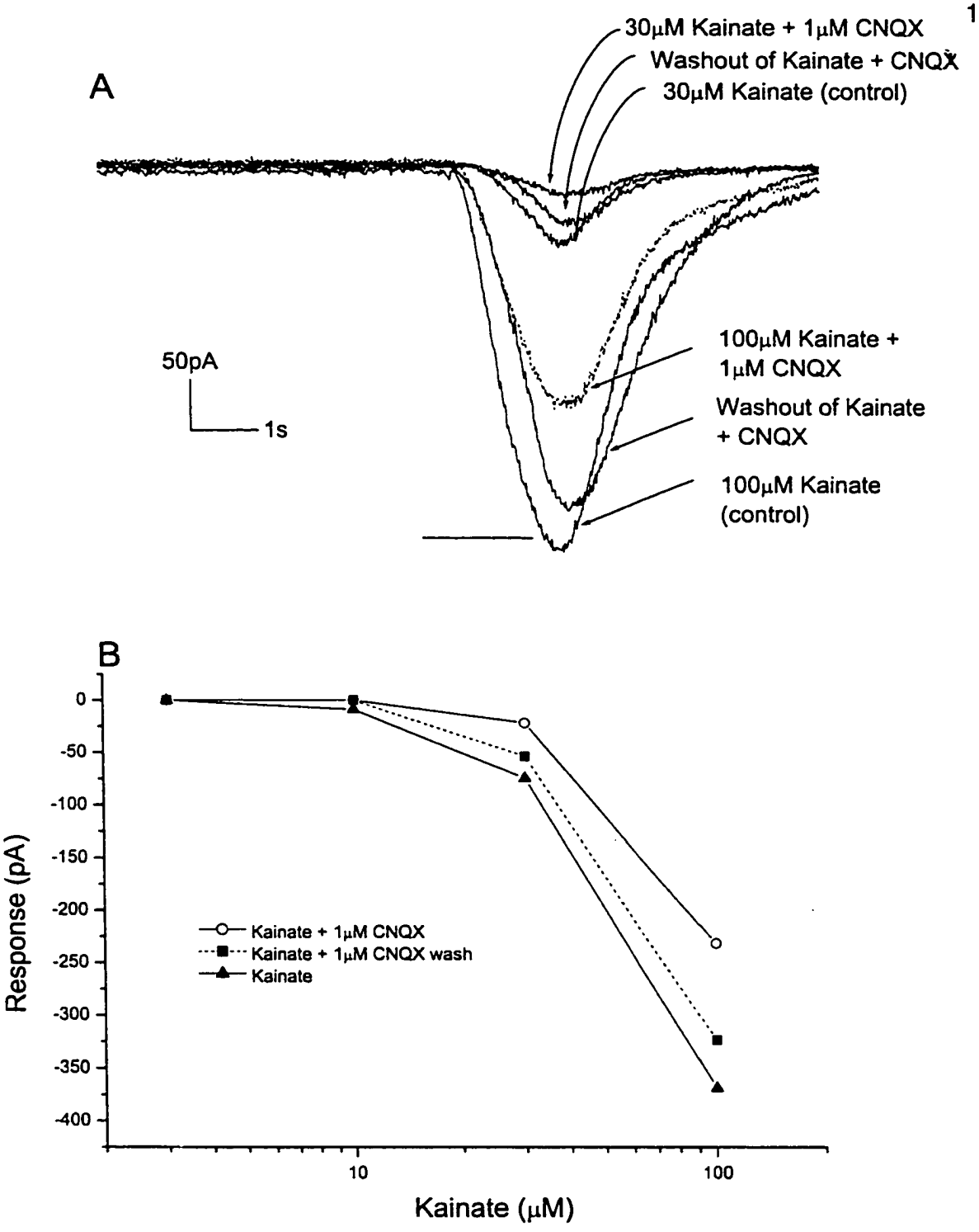

Fig. 28 The effects of 1μM CNQX on kainate responses. (A) OFF bipolar cell responses to 30μM and 100μM kainate, both 30μM and 100μM Kainate in solution with 1μM CNQX, and washout of 1μM CNQX (V_c = -50mV). Horizontal bar indicates period of drug application. (B) Data from the same cell plotted from 1 to 100μM kainate. (V_c = -50mV).

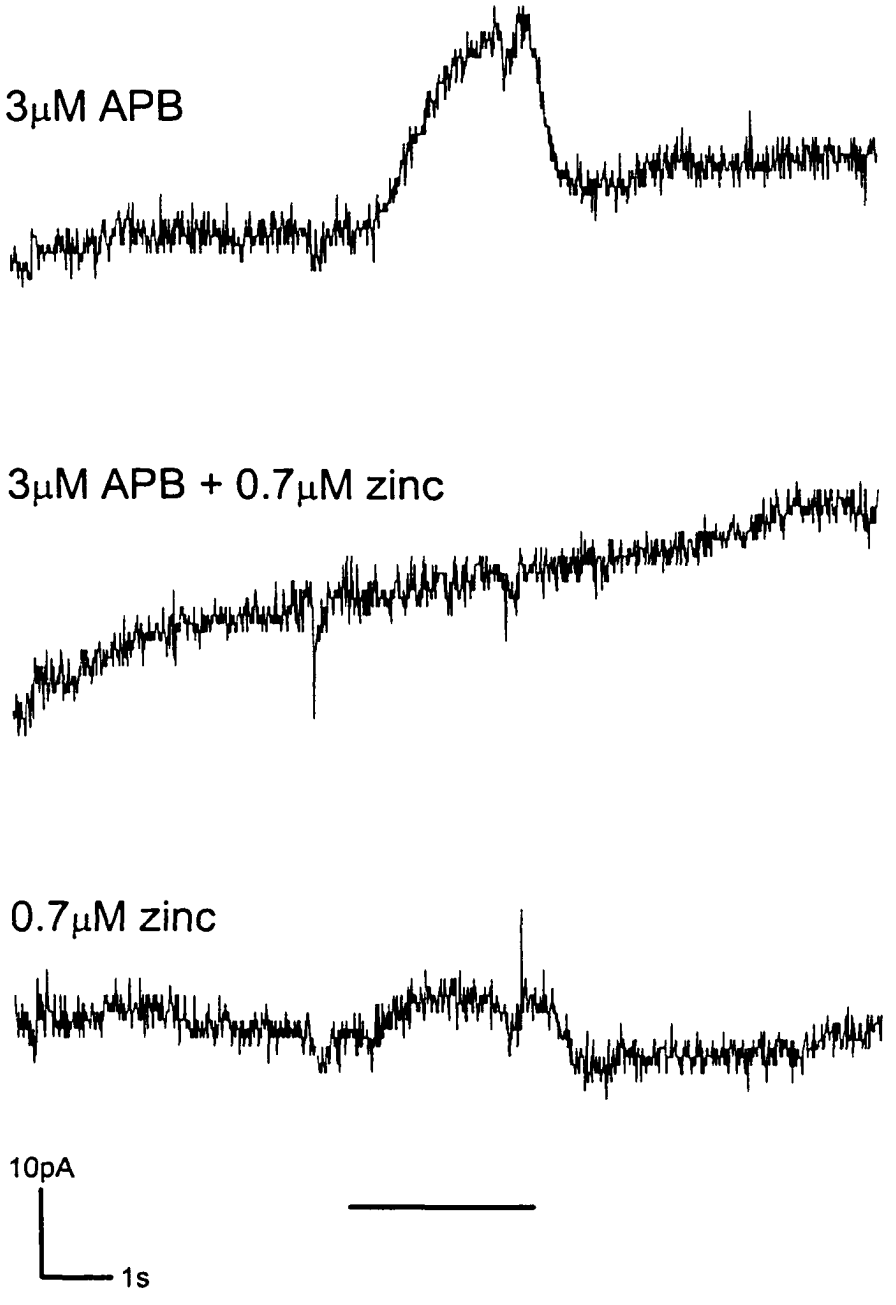

Fig. 29 ON bipolar cell response to 3μM APB blocked by 1μM zinc (V_c = 0mV). Horizontal bar indicates period of drug application.

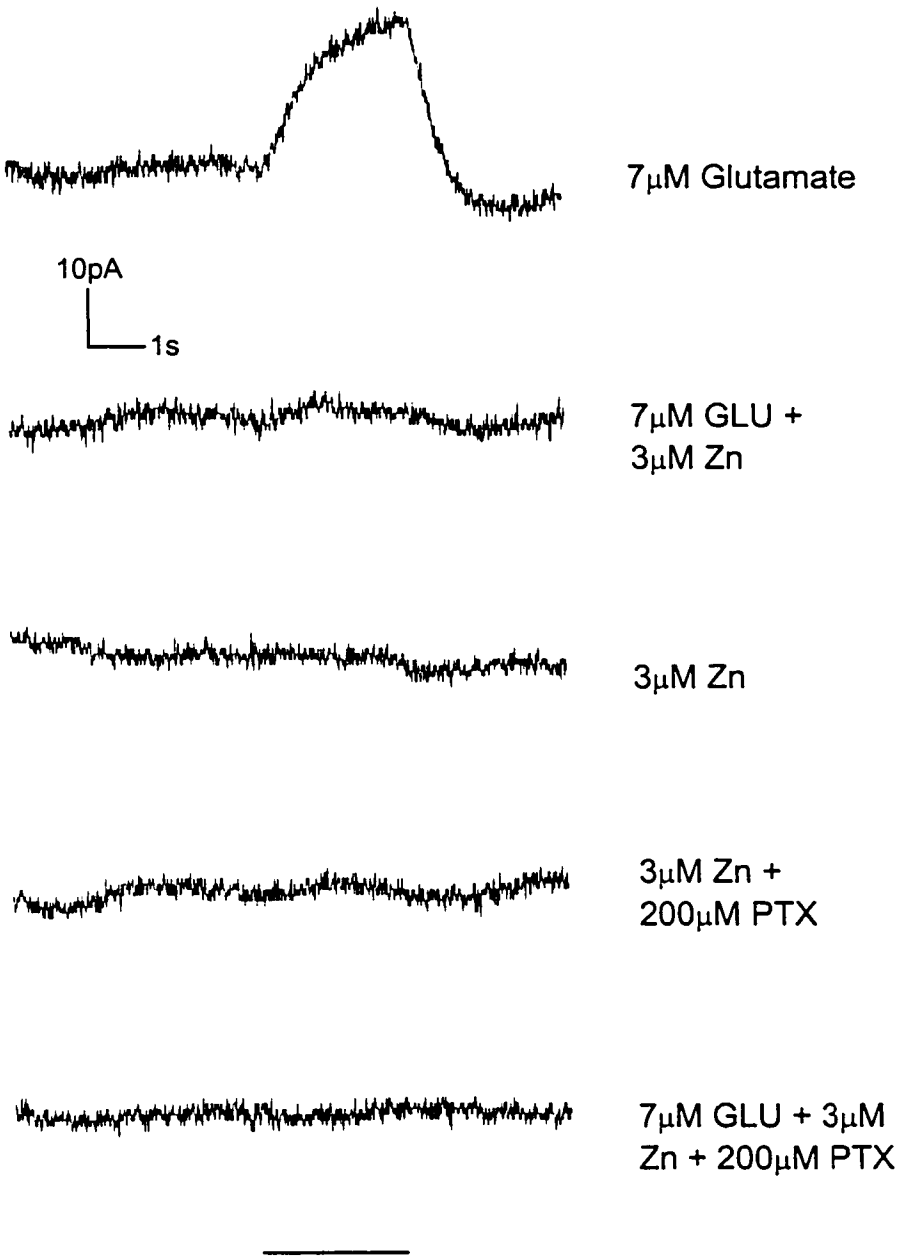

Fig. 30 The effects of zinc and zinc plus picrotoxin on the glutamate response. The response to 7μM glutamate of an ON bipolar cell was blocked by 3μM zinc and 3μM zinc plus 200μ picrotoxin. Horizontal bar indicates period of drug application (V_c = -25mV)

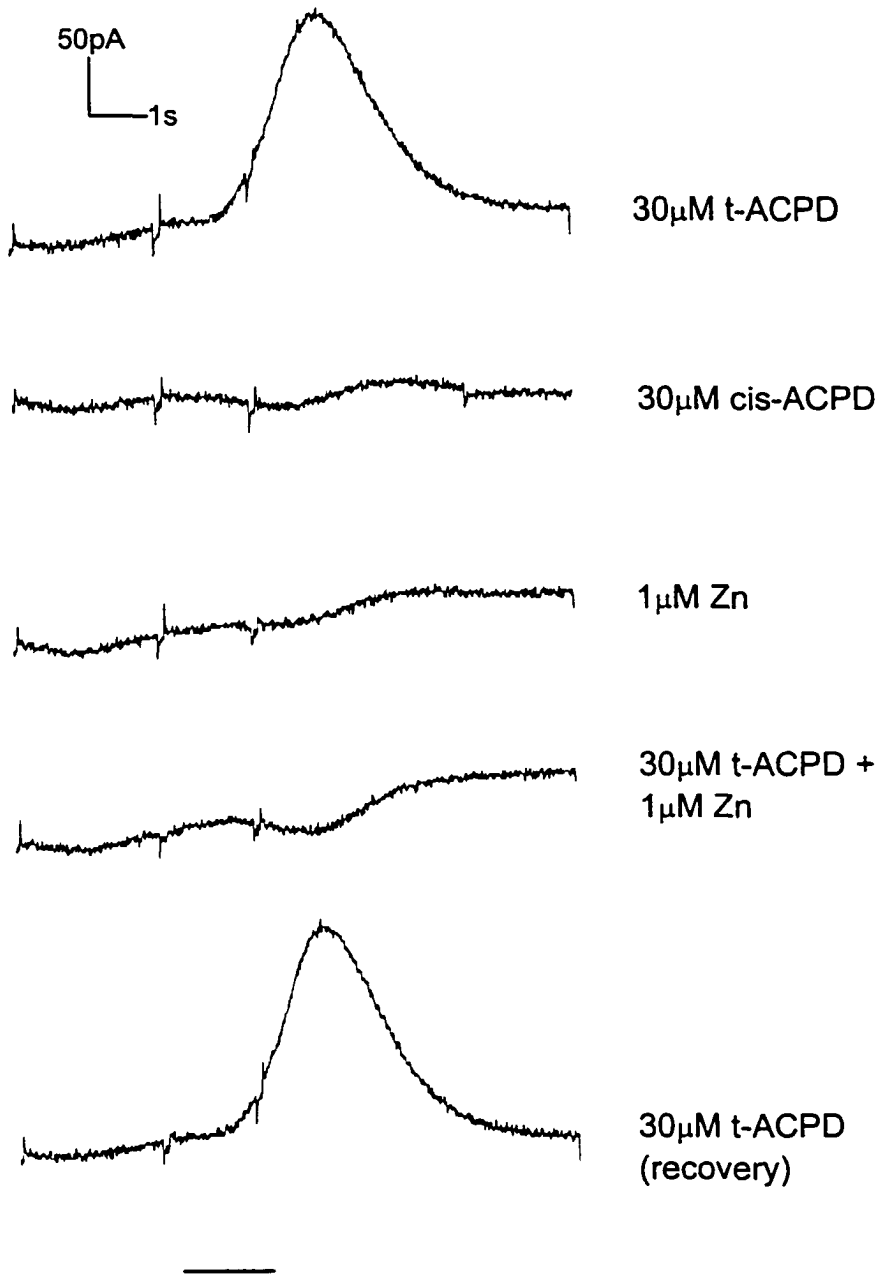

Fig. 31 Effects of zinc on the t-ACPD response at V_c = -50mV. 1μM zinc blocked the response to 30μM t-ACPD reversibly. The cell showed no response to 30μM cis-ACPD or 1μM zinc. The horizontal bar indicates period of drug application (V_c = -50mV).

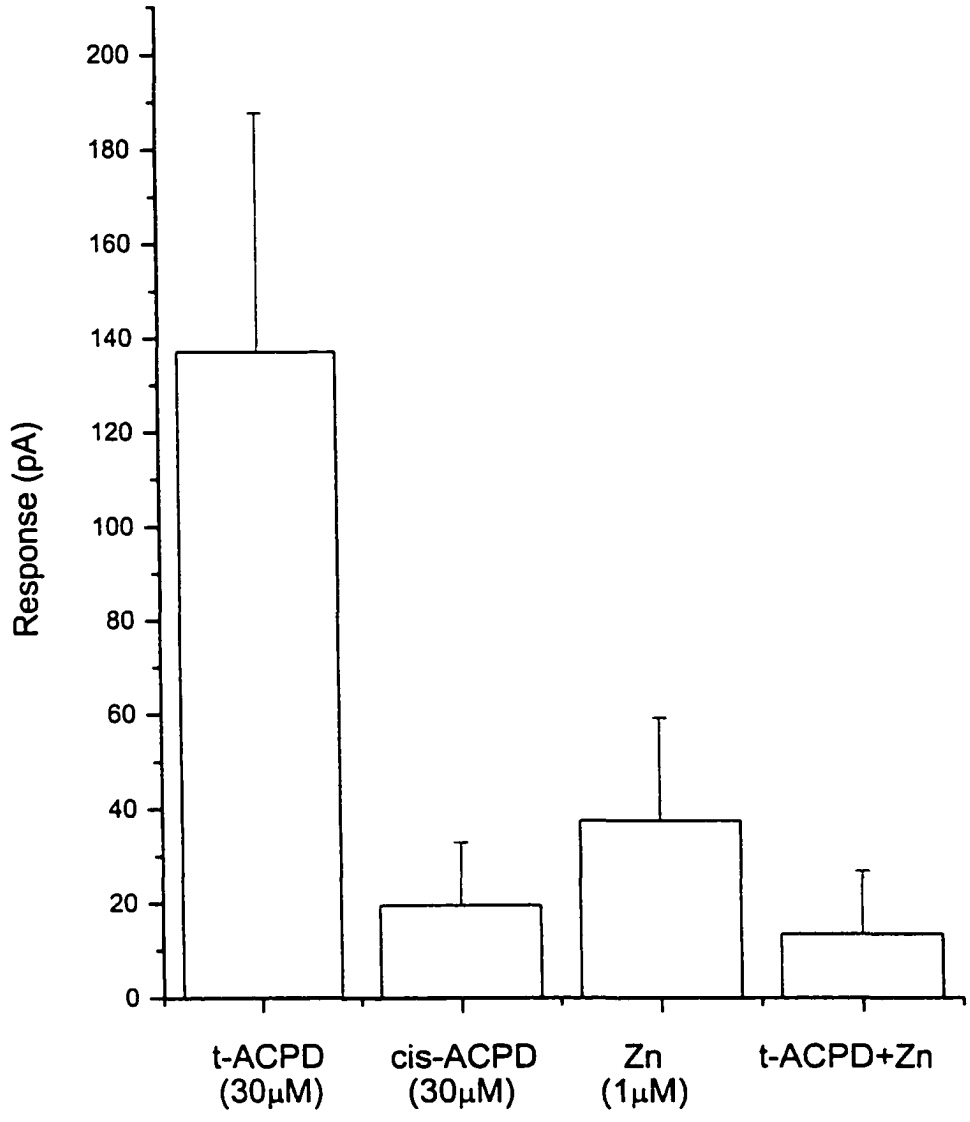

Fig. 32 Suppression of ON bipolar cell responses to t-ACPD by zinc at V_c = -50mV. 1μM zinc blocked the ON bipolar cell response to 30μM t-ACPD. There was no significant response to 30μM cis-ACPD or to 1μM zinc. Error bars indicate ±SEM, n = 5

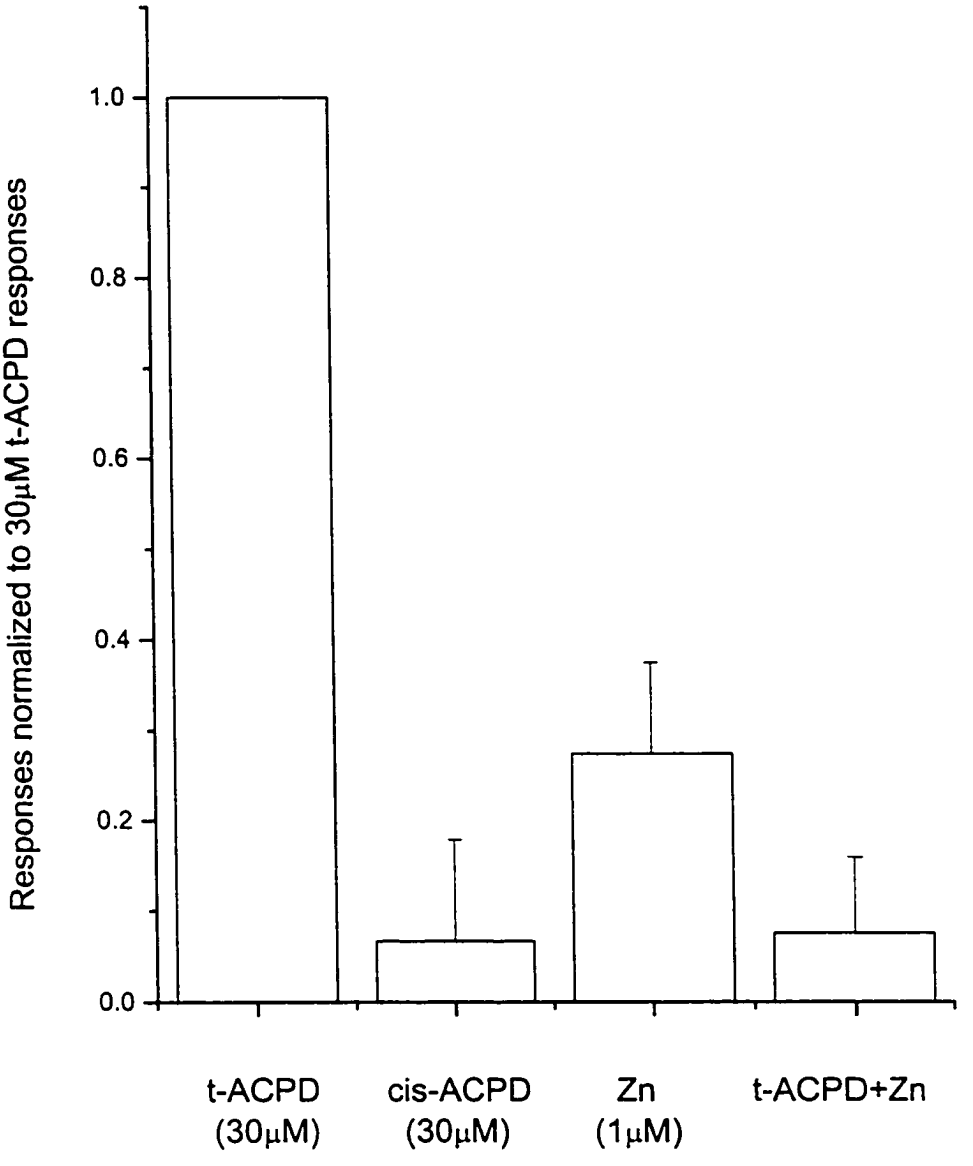

Fig. 33 ON bipolar cell responses to t-ACPD were suppressed by zinc at V_c = -50mV. Data from figure 33 was normalized for each cell to 30μM t-ACPD. Error bars indicate ±SEM, n = 5.

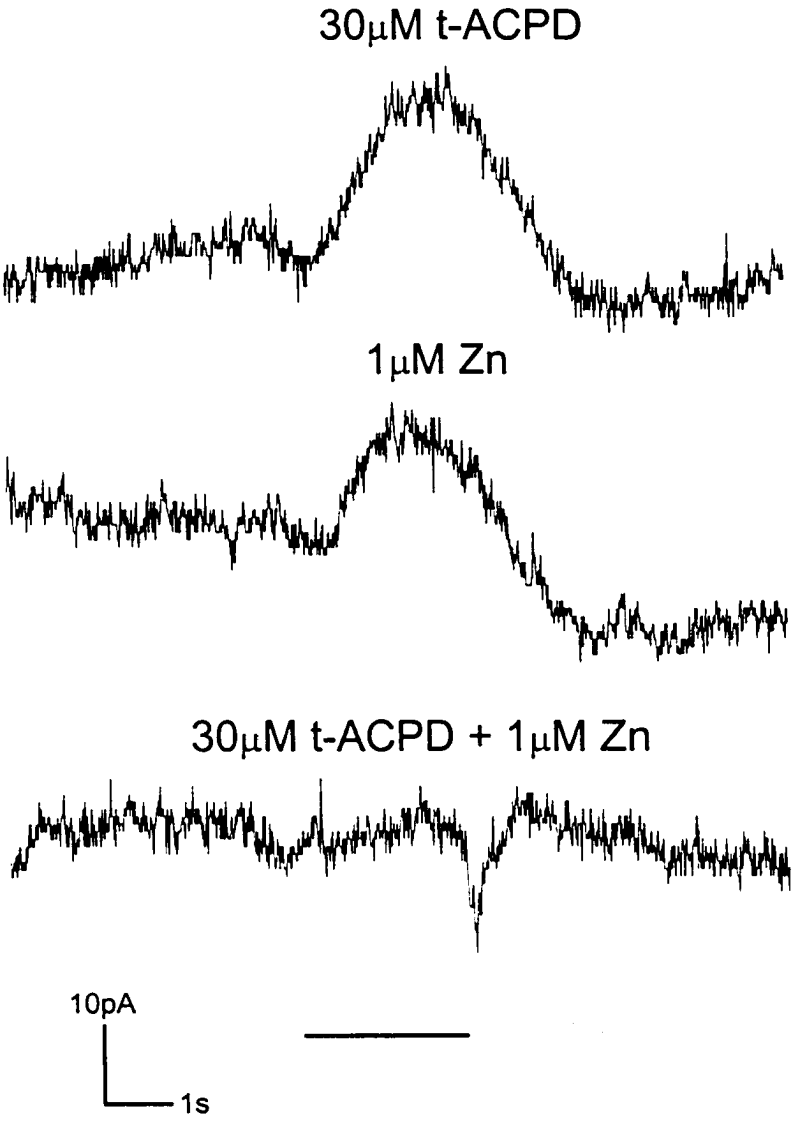

Fig. 34 The effects of zinc plus t-ACPD with 20mM TEA in the patch electrode. This ON bipolar cell gave a response to both 30μM t-ACPD and 1μM zinc. However, the simultaneous application of t-ACPD plus zinc did not produce a response. The horizontal bar indicates the period of drug application.

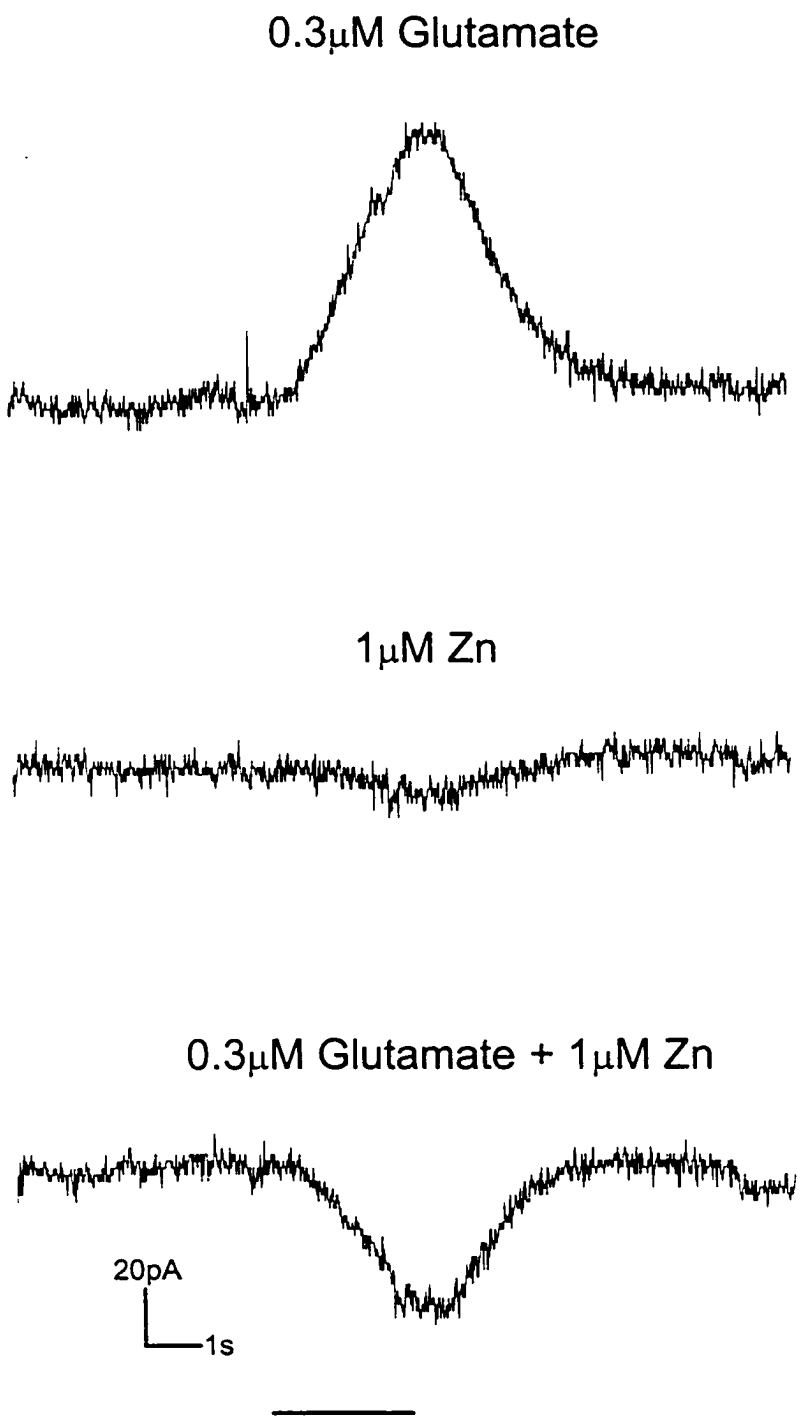

Fig. 35 Reversal of the glutamate response by zinc. Outward currents produced by 0.3μM Glutamate were reversed to inward currents when a solution of 1μM zinc plus 0.3μM Glutamate was superfused (V_c=-25mV). Horizontal bar indicates period of drug application.

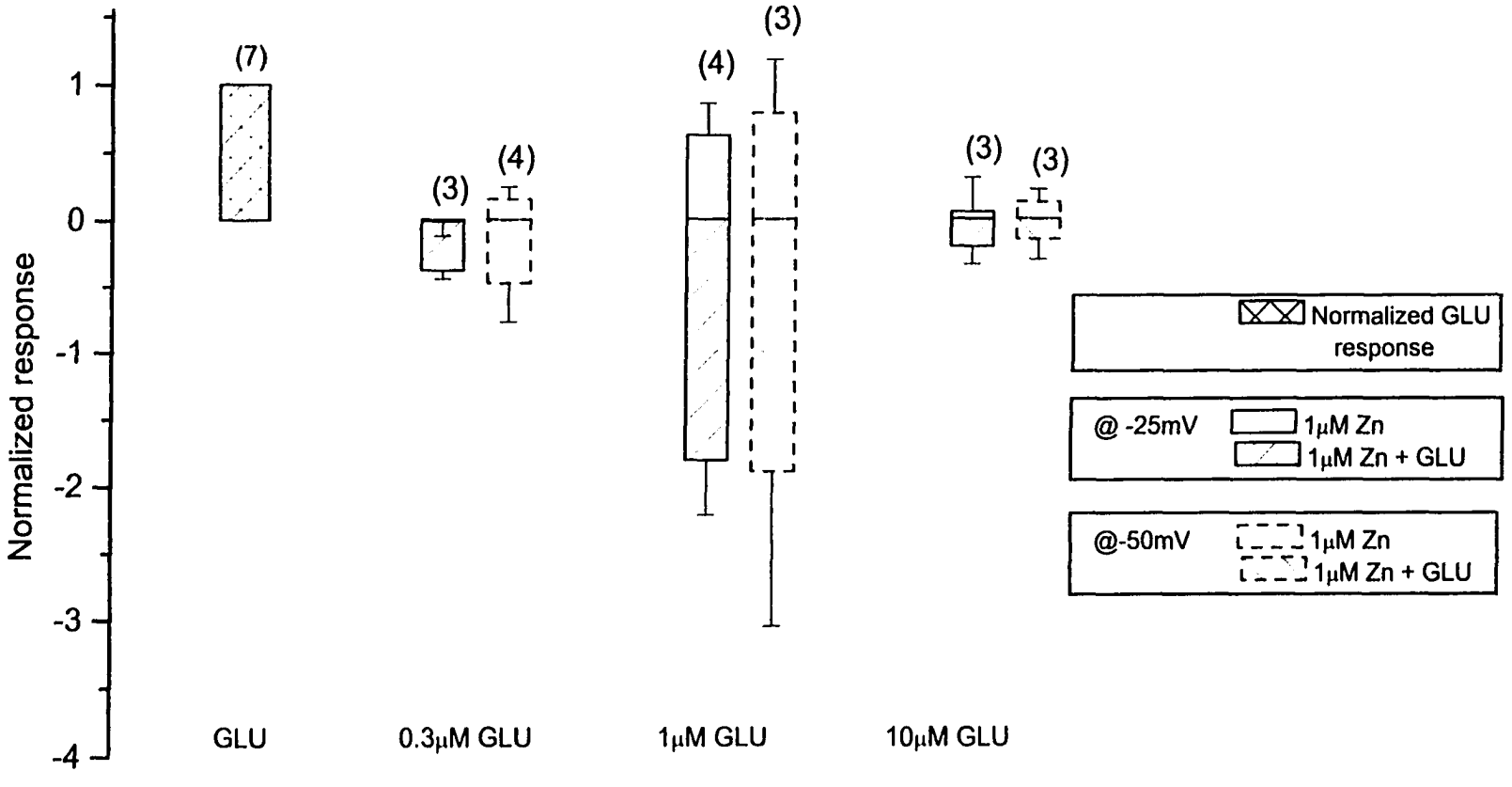

Fig. 36 ON bipolar cells responses to zinc and zinc plus glutamate were measured over a range of glutamate concentrations and normalized to each cell's glutamate response. The responses to 1μM zinc and 0.3, 1, 3 and 10μM glutamate were measured separately and then compared to the combined effect of glutamate plus zinc.

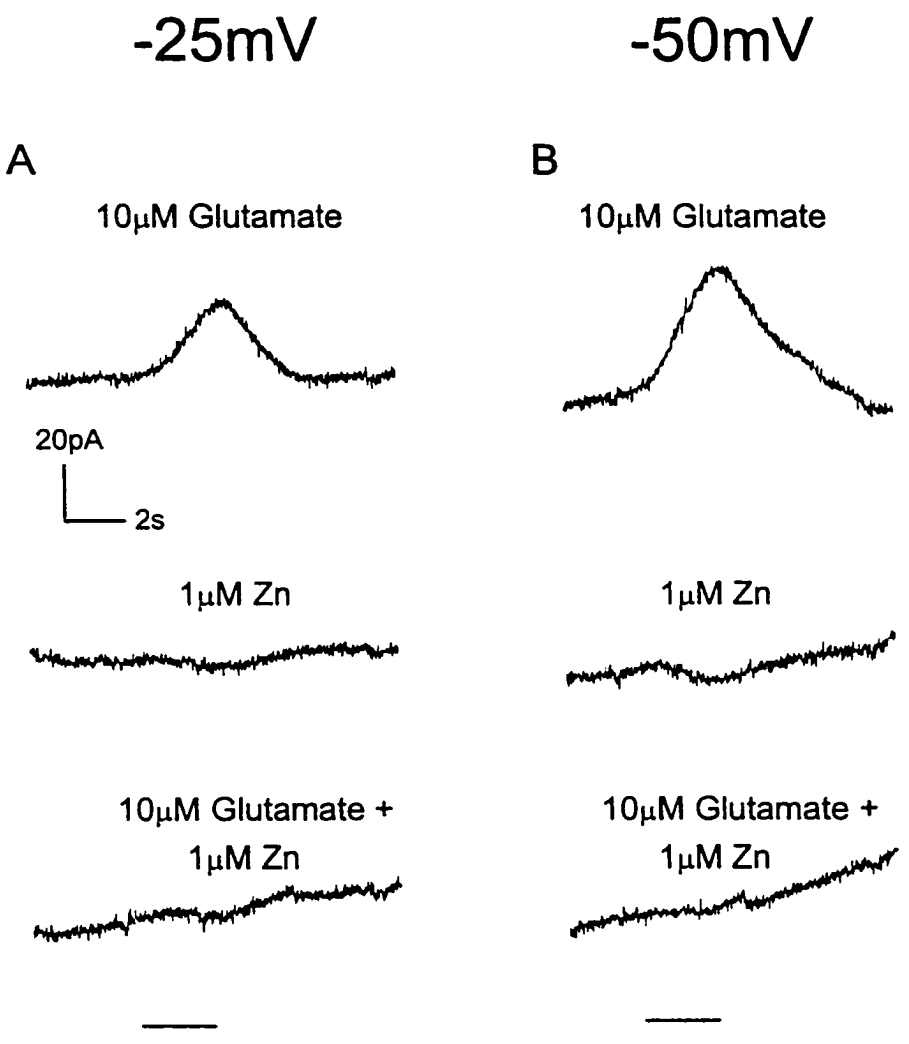

Fig. 37 The blocking effects of zinc at negative holding potentials. When clamped at either -25mV (A) or -50mV (B), the response of an ON bipolar cell to 10μM glutamate was blocked by 1μM zinc. Horizontal bar indicates period of drug application.

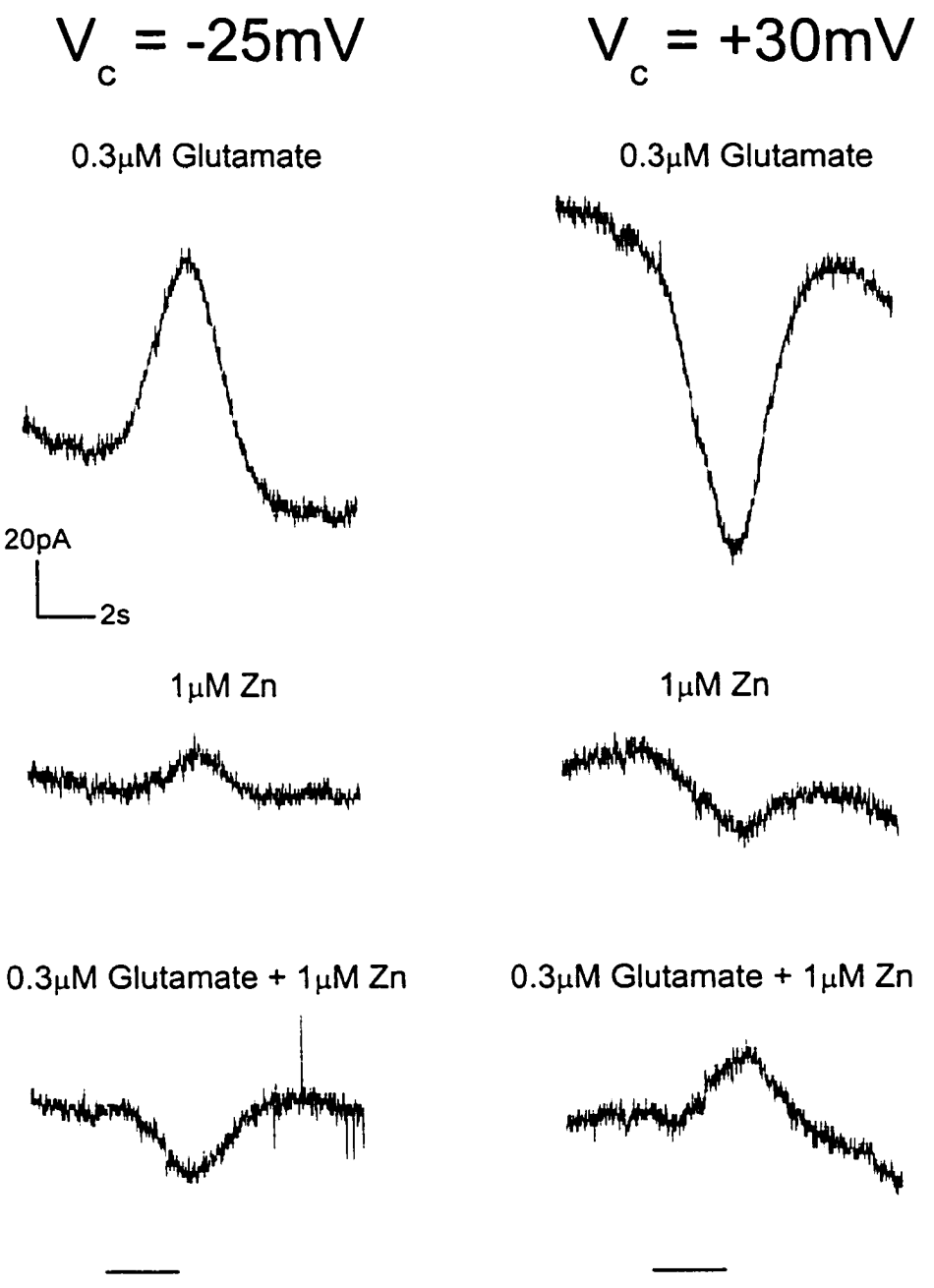

Fig. 38 The effects of zinc on an ON bipolar cell glutamate response at V_c = -25mV and V_c = +30mV. The cell responded to 0.3μM glutamate with an outward current at V_c = -25mV and an inward current at V_c = +30mV. 1μM zinc blocked (or slightly reversed) the glutamate responses. The small response produced by 1μM zinc was in the direction of the response to 0.3μM glutamate alone. Horizontal bar indicates period of drug application.

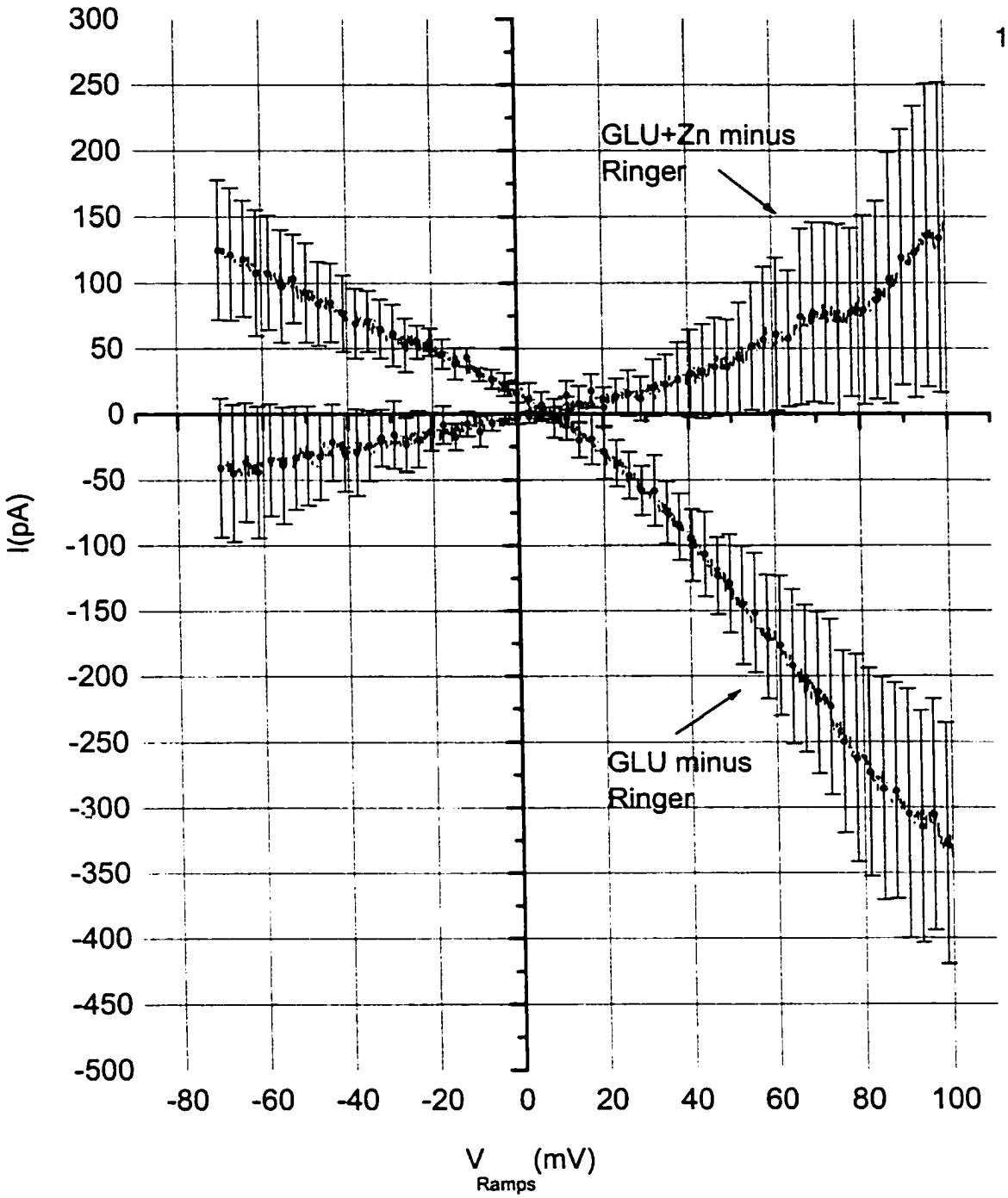

Fig. 39 ON bipolar cells recorded in 0.3μM glutamate and 0.3μM glutamate plus 1μM zinc, between -70mV and +100mV using a ramp stimulus. Cells 79, 80 and 81 were recorded in Ringer (control) and glutamate solutions. The responses were averaged and the ramps recorded in 0.3μM glutamate and 0.3μM glutamate + 1μM zinc were subtracted from those in Ringer and presented with +SEM. E_{rev} for the glutamate ramp is 7.4mV. the addition of zinc reversed the slope of the ramp recorded in 0.3μM glutamate from negative to positive and is indicative of an decrease in membrane resistance and its E_{rev} is 5.4mV.

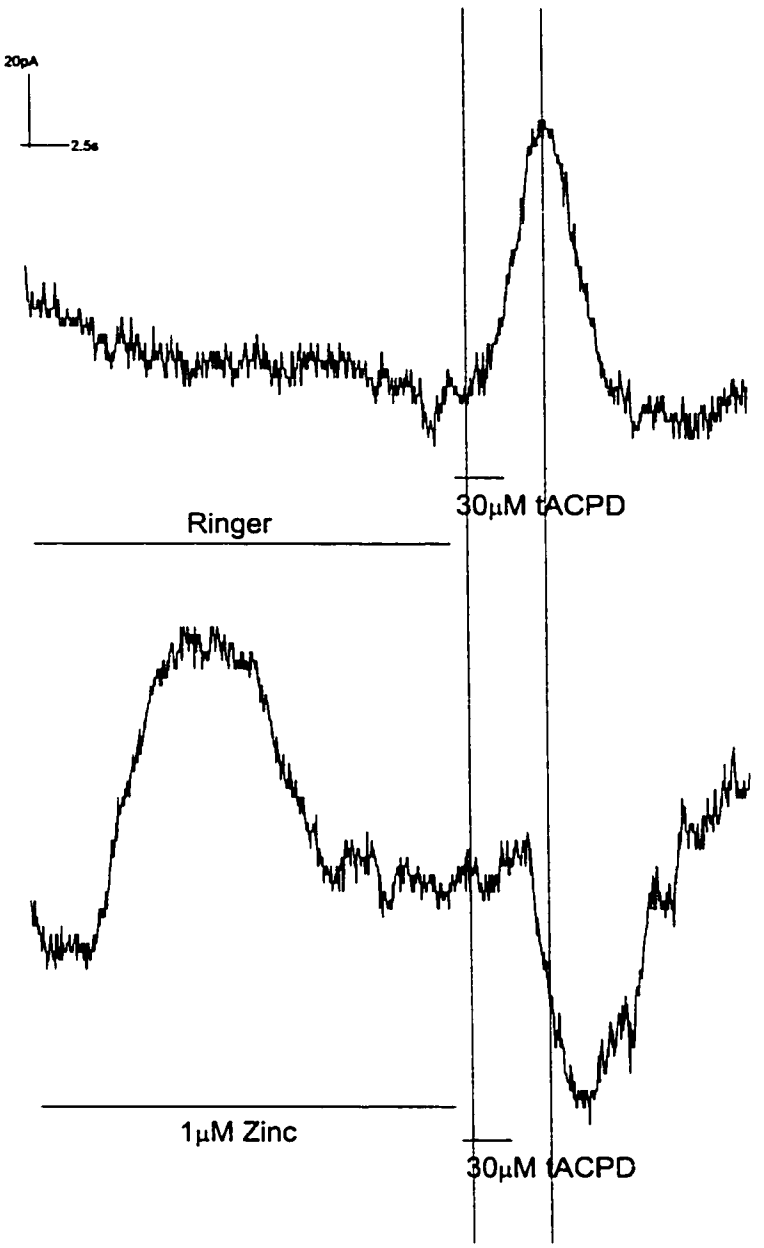

Fig. 40 Effects of sequential superfusion of zinc followed by tACPD on depolarizing bipolar cells. (upper trace) Superfusing an ON bipolar cell in Ringer for 25 seconds followed by 30μM tACPD for 2.5 seconds gave an outward current. (lower trace) Superfusing the cell for 25 seconds with 1μM zinc produced a transient outward current. Following the zinc treatment with 30μM tACPD for 2.5 seconds, the outward current mediated by tACPD under control conditions was blocked for 3 seconds. Then a delayed inward current followed that roughly coincided temporally with the peak tACPD response under zinc free control conditions. tACPD produced a substantial inward current from which the cell rebounded producing an outward current that temporarily surpassed the original baseline standing current. The horizontal bar indicates period of drug application (V_c = -50mV).

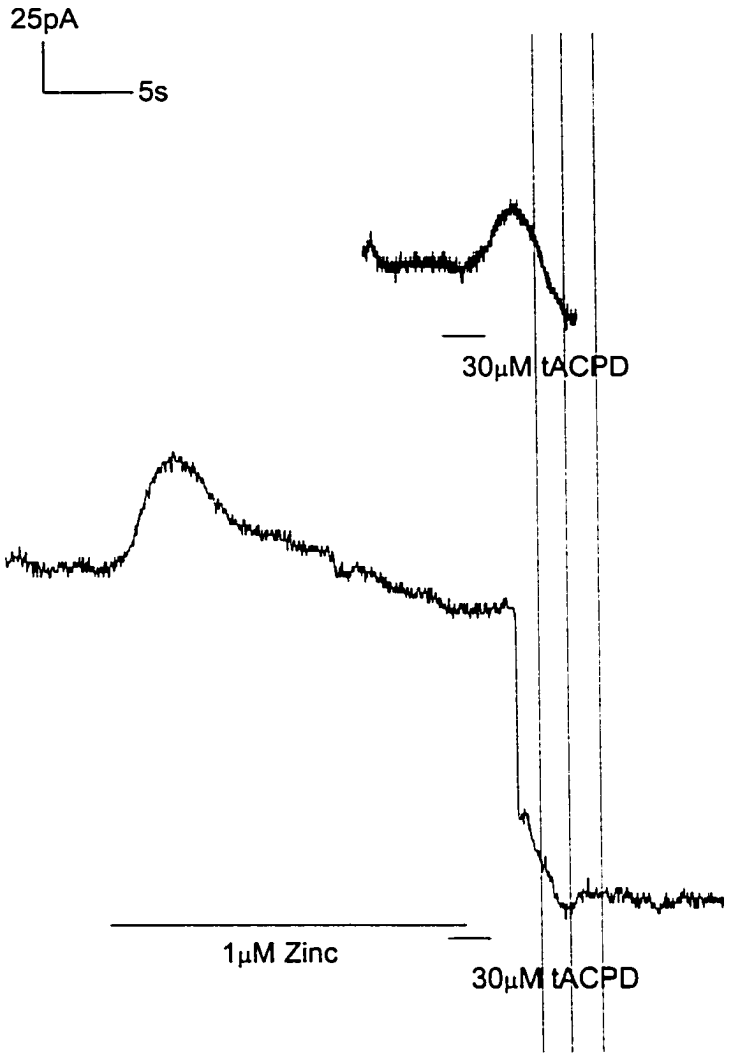

Fig. 41 Response of an ON bipolar cell to tACPD following superfusion of zinc during washout of drugs. Superfusing an ON bipolar cell in Ringer (control) with 30μM tACPD for 2.5 seconds gave an outward current, followed by an inward current after drug washout. The closing of channels during the response had an upregulating effect producing a current temporarily below baseline after the response. Data aquisition terminated before recovery occurred. Pretreatment with 1μM zinc had a similar effect on tACPD mediated currents, as described for the previous ON bipolar cell. It blocked the outward current and with a pecipitous increase produced a large inward current that coincided with the peak tACPD response measured under control conditions. Only a small percent of the total inward current coincided with the inward current measured during washout under zinc free conditions. Within one minute the cell recovered to its original standing current (not shown). The horizontal bar indicates period of drug application (V_c = -50mV).

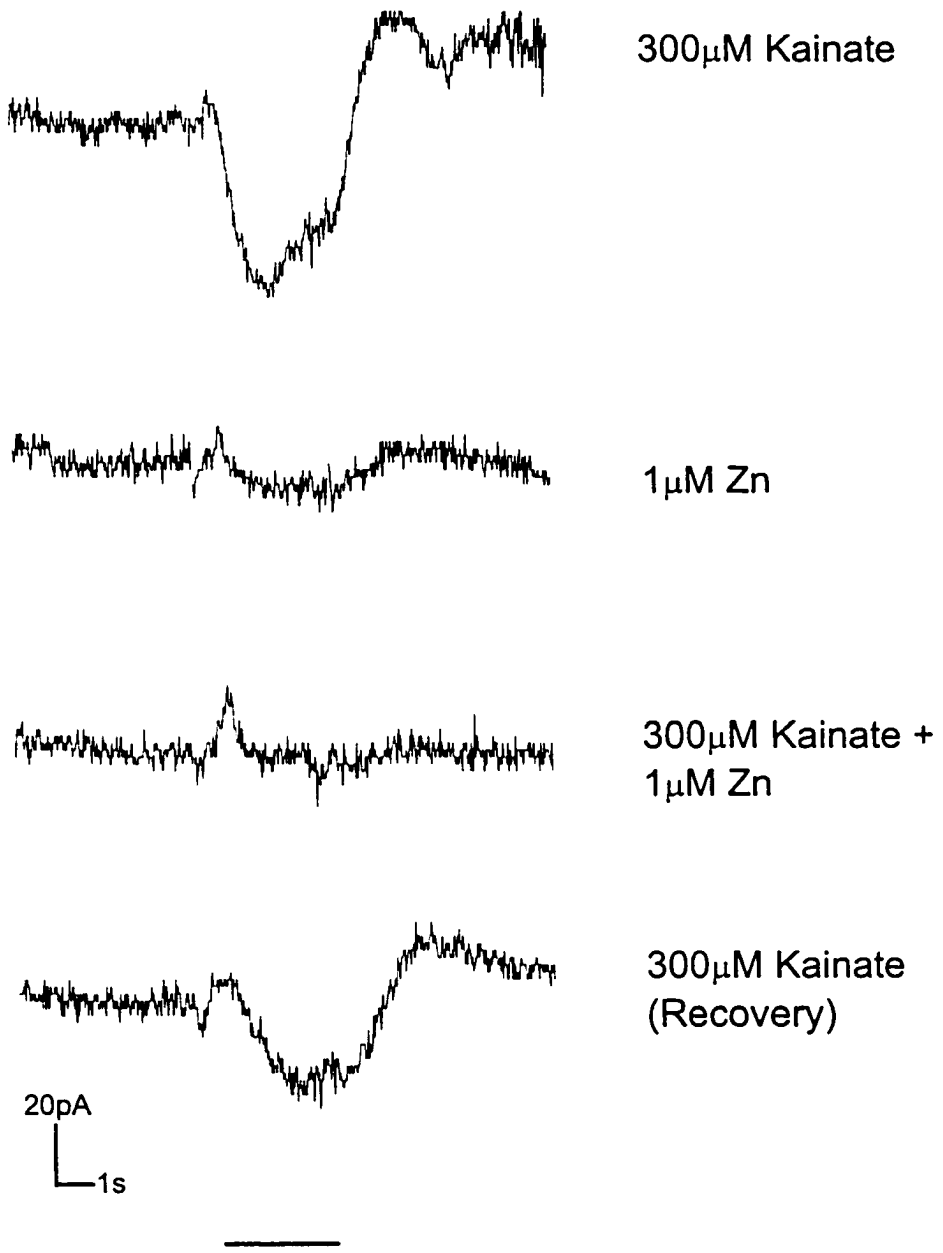

Fig. 42 The OFF bipolar cell response to 300μM Kainate is completely blocked when 1μM zinc is added to the kainate solution. Recovery of the kainate response demonstrates the zinc block is reversible. Horizontal bar indicates period of drug application (V_c = -50mV).

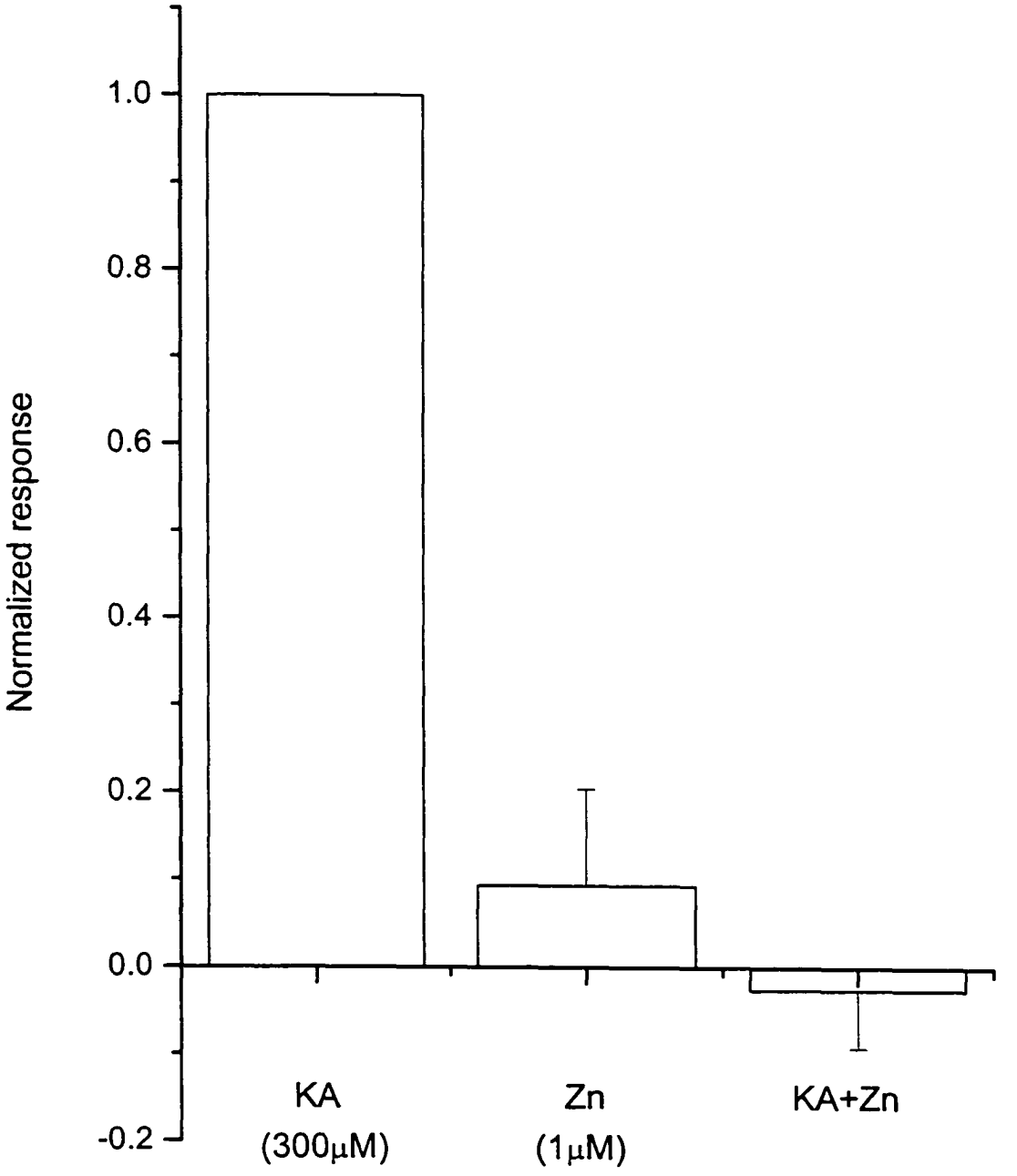

Fig. 43 OFF bipolar cell responses to 300μM kainate were suppressed by 1μM zinc, n = 6 (V_c = -50mV).

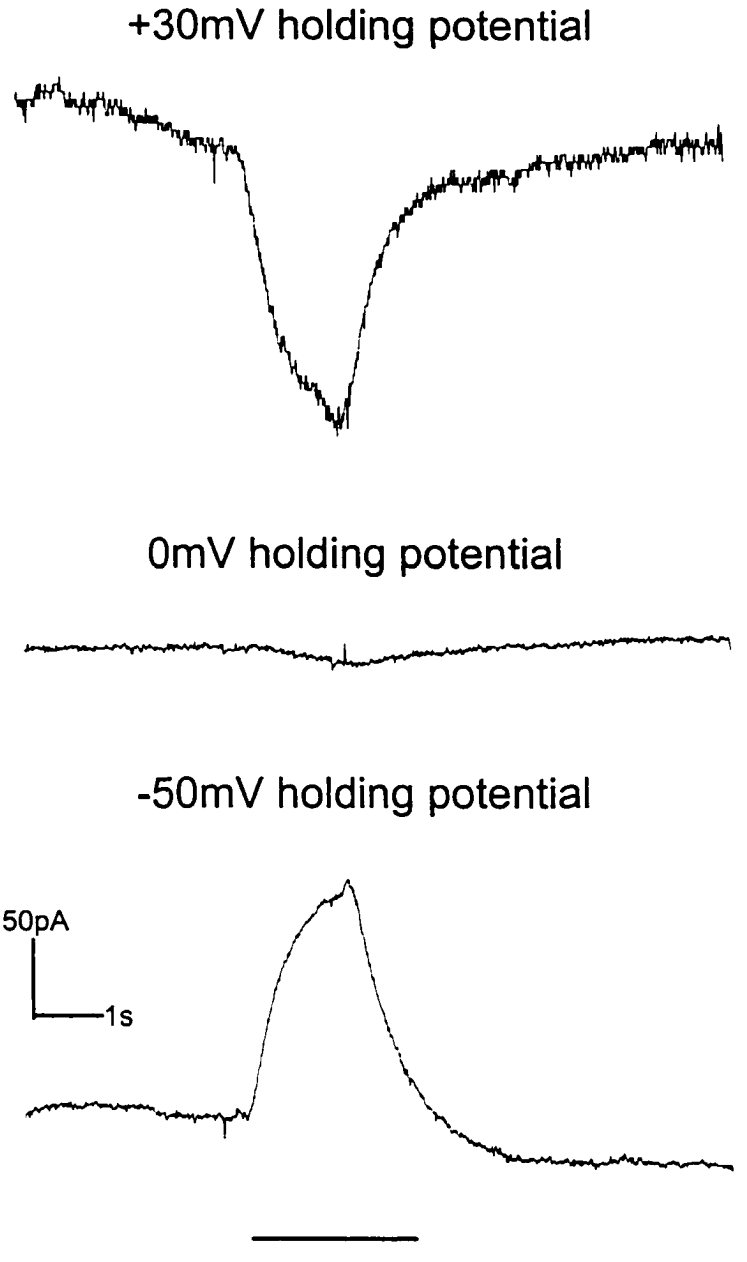

Fig. 44 The currents produced by an OFF bipolar to 0.7μM zinc resulted in an outward current at V_c = -50mV (lower trace) and an inward currents at V_c = +30mV (upper trace). There was only a slight inward current at V_c = 0mV indicating the cell was near its reversal potential. Horizontal bar indicates period of drug application.

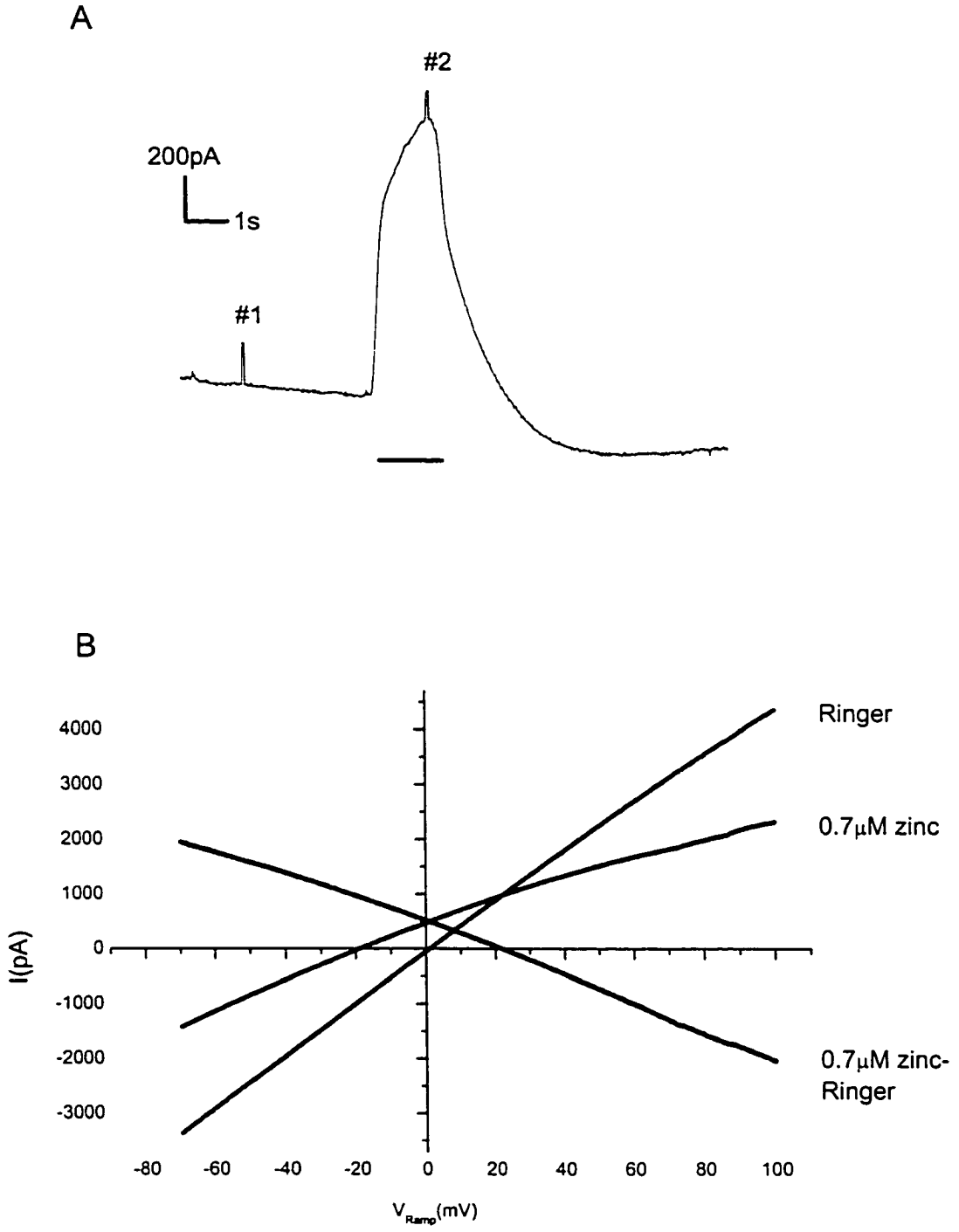

Fig. 45 (A) 5mV pulses were applied to a bipolar cell to measure the changes in membrane resistance during a response to 0.7μM zinc. Pulse #1 measures 190pA and pulse #2 130pA. Horizontal bar indicates period of drug application. (B) On the same cell, a ramp in 0.7μM zinc was subtracted from a ramp in Ringer and presented as the difference. The negative slope indicates zinc caused an increase in membrane resistance.

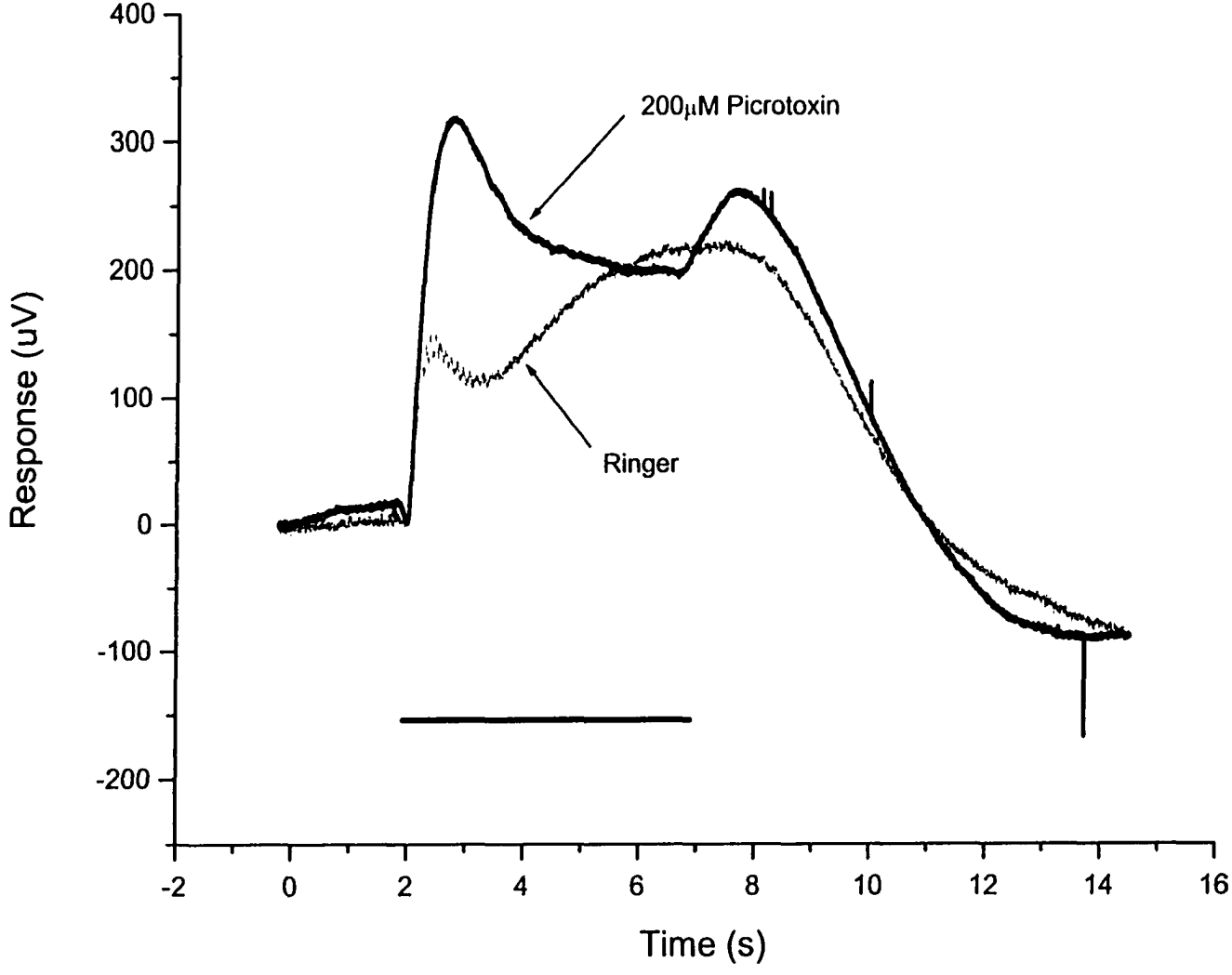

Fig. 46 The effects of picrotoxin on the ERG. After perfusing with Ringer, the eyecup was treated with 200μM picrotoxin, and the effect shown is an average of 5 responses taken after 10 to 12 minutes in the drug. Bar indicates the interval during which light was applied to the eyecup. Preamplifier was AC coupled with a bandpass of 0.01Hz to 1kHz. Log I = -3.

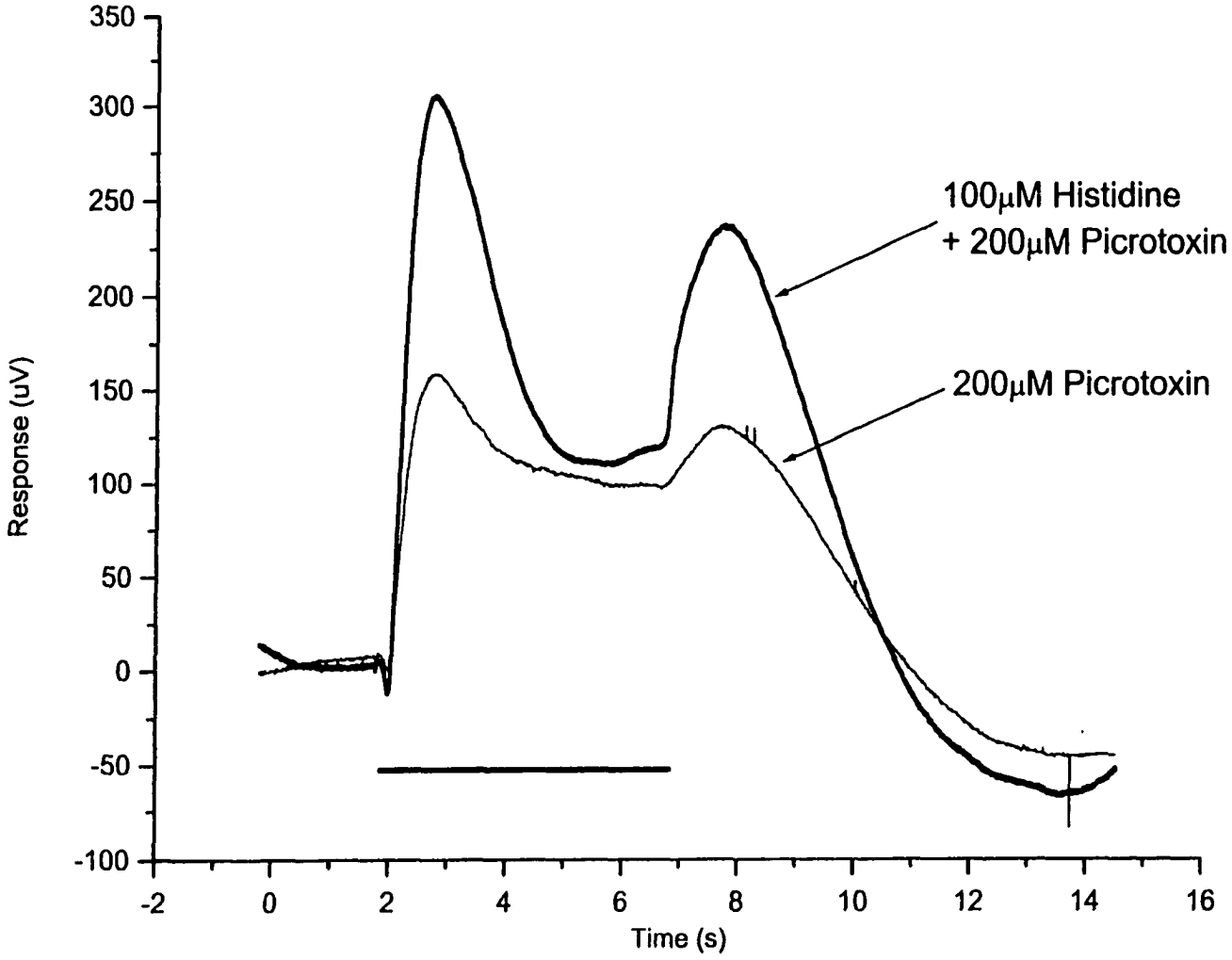

Fig. 47 The effects of histidine on a picrotoxin treated retina. The 200μM picrotoxin ERG is an average of 5 responses taken between 10 and 12 minutes after perfusing the eyecup with picrotoxin. The response was dramatically enhanced with a solution of 100μM histidine plus 200μM picrotoxin, which is shown as an average of 7 responses beginning 11 minutes after perfusing the solution. The bar indicates the interval during which light was applied to the eyecup. Preamplifier was AC coupled with a bandpass of 0.01Hz to 1kHz. Log I = -3.

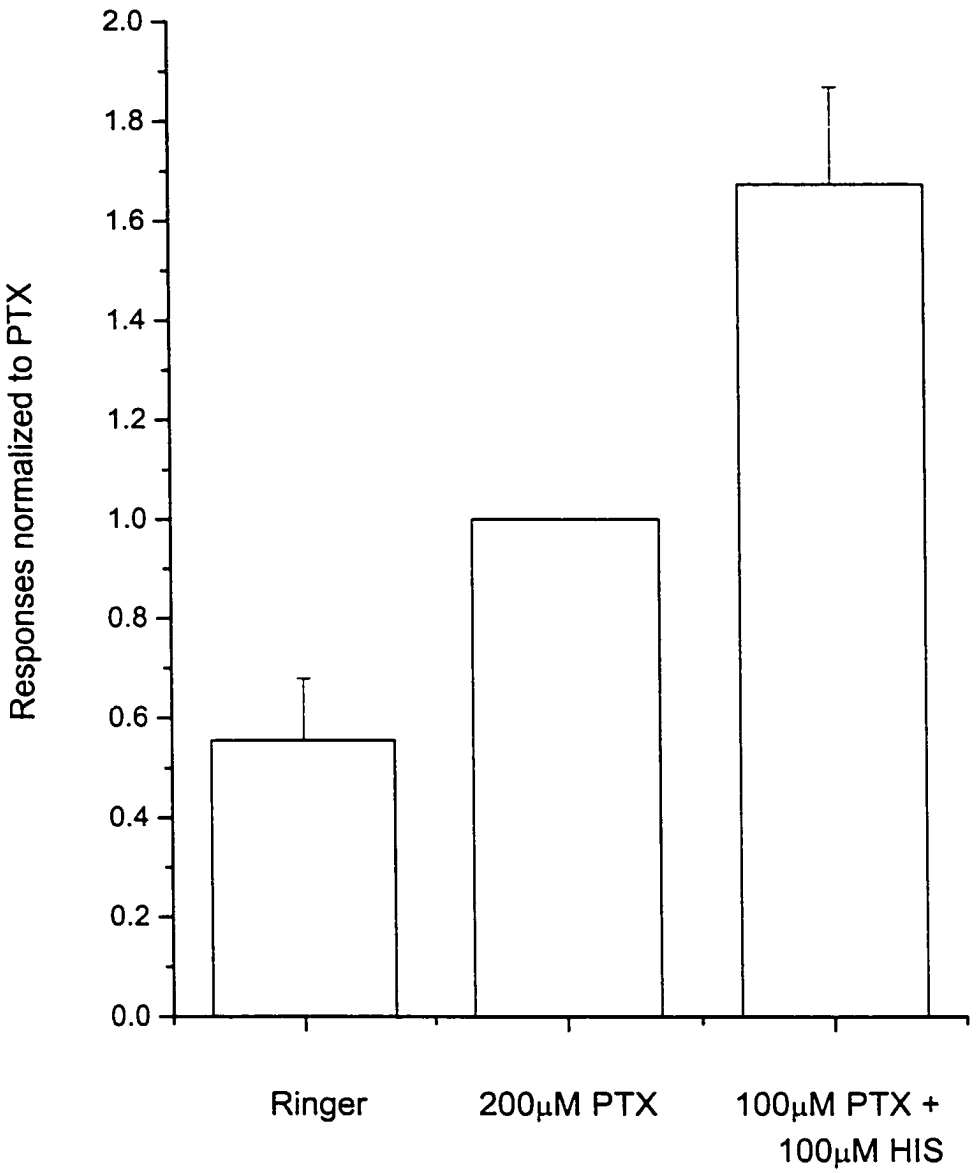

Fig. 48 Effects of histidine on the ERG ON response. Eyecup preparations tested at 3 or 4ND were tested with 200μM picrotoxin, followed by 200μM picrotoxin plus 100μM histidine. The ON responses at both light intensities were pooled and normalized to the average response in picrotoxin. The effect of histidine on the picrotoxin response was compared using the Student t-test, and it was found to be significantly larger at $p < 0.05$, $n = 5$.

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