

A Glutamate-Activated Chloride Current in Cone-Driven ON Bipolar Cells of the White Perch Retina

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Cone-driven ON-type bipolar cells were patch clamped in white perch retinal slices. Application of glutamate activated a current (I_{Glu}) that was mediated by a conductance increase. The reversal potential for I_{Glu} followed E_{Cl} closely when the intracellular chloride concentration was varied. I_{Glu} was not blocked by 100 μM picrotoxin or 1 μM strychnine, indicating that it was not caused by inhibitory input. I_{Glu} is not mediated by a typical ionotropic glutamate receptor since it was not activated by kainate, AMPA, or NMDA, or blocked by kynurenic acid, CNQX, DNQX, or AP-5. Further, I_{Glu} is not mediated by a known metabotropic glutamate receptor since it was not activated by quisqualic acid, AP-4, ACPD, or ibotenate.

I_{Glu} required the presence of extracellular sodium and could be partially inhibited by the glutamate uptake inhibitors THA and tPDC. This is suggestive of sodium-dependent glutamate transport. However, when intracellular sodium was greatly increased, neither the magnitude nor reversal potential of I_{Glu} was substantively affected. Thus, I_{Glu} appears to involve a chloride channel activated by a glutamate receptor with transporter-like pharmacology.

I_{Glu} is localized to the dendrites of the bipolar cell, where bipolar cells receive an endogenous glutamatergic input from photoreceptors. Further, the reversal potential of the light response in these cells is the same as that of I_{Glu} . Thus, it seems likely that I_{Glu} is the current responsible for the cone component of the ON bipolar cell light response in the teleost retina.

[Key words: bipolar cells, ON bipolar cells, glutamate, chloride, on pathway, retina]

Visual information is segregated into ON and OFF pathways at the first synapse in the retina, between photoreceptors and bipolar cells. Since the light response of an ON type bipolar cell is depolarizing and light causes hyperpolarization of photoreceptors, the photoreceptor neurotransmitter glutamate (Lasater and Dowling, 1982; Copenhagen and Jahr, 1989; Ayoub and Copenhagen, 1991) must have a sign-reversing action on ON bipolar cells.

In teleosts the light-elicited depolarizations of ON bipolar cells are mediated by two distinct actions of glutamate (Saito et

al., 1979; Kondo and Toyoda, 1980; Nawy and Copenhagen, 1987; Nawy and Copenhagen, 1990). One effect of glutamate underlies the rod-mediated input to ON bipolar cells. Here glutamate causes a conductance decrease with a reversal potential near 0 mV. This current can be specifically activated by AP-4 (Slaughter and Miller, 1981) and has been particularly well studied. These studies have shown that glutamate acts at a metabotropic glutamate receptor to cause hydrolysis of cGMP. The concomitant reduction in cGMP levels causes cGMP-activated non-specific cation channels to close (Nawy and Jahr, 1990; Shiells and Falk, 1990). Thus, in response to the light-induced decrease in synaptic glutamate concentration, these cation channels open, causing current to flow into the bipolar cells and to depolarize them.

A second action of glutamate underlies the cone-mediated input to teleost ON bipolar cells. Here glutamate opens a conductance with a reversal potential between -40 and -90 mV (Saito et al., 1979; Kondo and Toyoda, 1980). From this finding, it has been inferred that glutamate opens a conductance permeable to either potassium or chloride. Thus, in response to the light induced decrease in synaptic glutamate concentration, this conductance will decrease, causing less current to flow out of the bipolar cells, again resulting in depolarization of the cells.

This second effect of glutamate on ON bipolar cells is not well understood and the literature on it is contradictory (see Discussion). Few investigators have studied this current, although Nawy and Copenhagen (1990) have reported that it could be blocked by intracellular cesium, leading to the conclusion that glutamate was opening a potassium conductance. We have therefore attempted to characterize this current in detail to understand better the synapse from cone photoreceptors onto ON bipolar cells. We have studied the effects of glutamate on ON bipolar cells of the white perch retina and describe here a glutamate-activated current, I_{Glu} , found in these cells. This current has a reversal potential close to E_{Cl} , the Nernst potential for chloride, and it appears to be the current that underlies the light response in cone-driven ON bipolar cells in teleosts.

Materials and Methods

Preparation of retinal slices. White perch (Department of Fish and Game, State of Maine) were stored in a large tank on a 12 hr on/12 hr off light cycle. Prior to each experiment, a fish was dark-adapted for 1 hr and then cooled to 5°C . The animal was pithed and the eyes removed. Retinal slices were prepared using procedures similar to those described by Werblin (1978). Briefly, the lens and iris were removed from the eye, and a small section (5 mm on a side) was cut from the eye and placed into a small chamber filled with Ringer's solution. The retina was separated from the sclera and choroid and placed photoreceptor-side down on a piece of Millipore (Bedford, MA) filter (type HA, 0.45 μm pore size). The filter was removed from the Ringer's solution and inverted onto a second Millipore filter so that the retina stuck to this

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filter with its ganglion cell side down. Vacuum was applied across the filter to improve the bond between the retina and filter. The retina-filter complex was chopped into 200- μm -thick slices using an apparatus consisting of a razor blade mounted on a stage micrometer. Each slice was positioned so that it spanned two Vaseline rails that had been laid down in the recording chamber. The entire procedure was performed under dim red light.

Electrophysiological recordings. Whole-cell patch-clamp techniques (Hamill et al., 1981) were used to record from bipolar cells in the retinal slice. The retinal slice was viewed using a Zeiss 40 \times , long-working-distance, water-immersion lens modified with Hoffman modulation contrast optics (Modulation Optics, Inc., Greenvale, NY). The objective was mounted on a Nikon Labophot microscope modified for a fixed-stage configuration (Micro Video Instruments, Inc., Avon, MA). Patch electrodes of 5–10 M Ω resistance when measured in Ringer's solution were pulled in two stages using a vertical electrode puller (model PP-83, Narishige, Tokyo, Japan). Thin-walled borosilicate glass with an outer diameter of 1.5 mm (TW150F-4, World Precision Instruments, Sarasota, FL) was used. Series resistance during whole-cell recording was typically around 30 M Ω . Consequently, a 100 pA current would produce an error of about 3 mV in membrane potential. Recordings were not compensated for this error.

A patch-clamp amplifier (model 8900, Dagan Corp., Minneapolis, MN) was used to voltage clamp bipolar cells. Currents were low-pass filtered at 1 kHz with an 8-pole Bessel filter and digitized at 10 kHz or 2.5 kHz using a Labmaster DMA (Scientific Solutions, Solon, OH) 12-bit data acquisition card mounted in an IBM PC-AT compatible personal computer (model 4DX-33V, Gateway 2000, North Sioux City, SD). Experimental data were acquired and analyzed using standard electrophysiological software (Grant and Werblin, 1994). Data are presented as mean \pm SD.

Bathing solutions. The Ringer's solution used was composed of (mM) NaCl (110), KCl (2.5), CaCl₂ (2.4), MgCl₂ (1.5), KH₂PO₄ (0.45), glucose (10), and HEPES (10). When a potassium-based intracellular solution was used, the Ringer's solution contained 20 mM TEA-Cl, substituted for NaCl. For recording of light responses and spontaneous synaptic currents, the calcium concentration was reduced to 0.1 mM (Harsanyi and Mangel, 1993). The pH of the Ringer's solution was set to 7.7 by the addition of NaOH.

Picrotoxin at 100 μM and 1 μM strychnine were included in all bathing solutions to block GABA_A and GABA_C (Qian and Dowling, 1993; Lukasiewicz et al., 1994) and glycine (Attwell et al., 1987) inputs to the bipolar cell. Further, all experiments (except those involving the recording of light responses and spontaneous synaptic currents) were performed with 1 mM cobalt chloride in the bath to block calcium-dependent synaptic transmission. In other experiments, synaptic transmission was blocked by using a Ringer's solution in which calcium had been buffered to 10 nM with 6 mM EGTA, or by using cadmium. I_{Glu} was consistently observed under these conditions and was not significantly different from that seen under control (1 mM cobalt) conditions.

(\pm)- α -Amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX), 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid), and (\pm)- α -amino-3-hydroxy-5-isoxazoleacetic acid (ibotenate) were purchased from Research Biochemicals, Inc. (Natick, MA). L-Quisqualic acid (quisqualate), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), and L-trans-pyrrolidine-2,4-dicarboxylic acid (tPDC) were purchased from Tocris Neuramin (Bristol, England). All other chemicals were purchased from Sigma (St. Louis, MO).

Drugs were solubilized without substitution and applied by bath application via a gravity-fed perfusion system or by locally puffing with a Picospritzer (General Valve Corp., Fairfield, NJ) at a pressure of 3 psi. Excess solution was drawn off from the recording chamber by a vacuum-operated siphon.

Electrode solutions. Recording pipettes were filled with either a cesium- or a potassium-based intracellular solution. The formulation of these solutions was (mM) CsCl or KCl (30.68), Cs methanesulfonate or K acetate (93.32), CaCl₂ (1), MgCl₂ (2), EGTA (12.5), HEPES (10), and MgATP (1). The pH of the solution was set to 7.4 by the addition of CsOH or KOH.

The chloride concentration for the intracellular solutions listed above was chosen so that $E_{\text{Cl}} = -30$ mV. Neither GTP nor cGMP was included in the intracellular solutions so that if the bipolar cell under study

had a rod input, this cGMP-dependent component would quickly wash out (Nawy and Jahr, 1990; Shiells and Falk, 1990).

The liquid junction potential was corrected for by using the procedure described by Fenwick et al. (1982). When using the standard Ringer's solution and intracellular solution ($[\text{Cl}]_i = 36.68$ mM, $E_{\text{Cl}} = -30$ mV), the liquid junction potential was -8.7 mV.

Results

Cell identification

An advantage of the retinal slice preparation is that it allows the experimenter to select visually identifiable cell types from which to record. Thus, we were able to identify bipolar cells by their characteristic morphology and location within the inner nuclear layer. However, many bipolar cells in the white perch are small; these cells were difficult to patch clamp and we recorded from few of them. Those that we did record often responded to glutamate with a conductance increase that had a reversal potential near 0 mV. On the basis of these observations, and the morphological similarity of these cells to the type "a" cells described in goldfish (Stell et al., 1977), we classified these cells as OFF bipolar cells. We had more success recording from the larger bipolar cells in the retina that resembled the type "b" cells described in goldfish (Stell et al., 1977), and these cells responded to glutamate in a manner consistent with their being ON bipolar cells.

In some recordings, cells were filled with the fluorescent dye Lucifer yellow (0.5%, included in the electrode solution). The dye-filled cell could be visualized with an epifluorescence attachment after the experiment. By this method we were able to observe the morphology of these cells. All had terminal processes ramifying in the proximal half of the inner plexiform layer, confirming that these were ON bipolar cells (Famiglietti et al., 1977).

Identification of bipolar cells was further facilitated by the presence of several voltage-gated currents that are found typically in bipolar cells. Specifically, when a potassium-based intracellular solution was used, and the cell was held at potentials more negative than -40 mV and stepped to depolarized potentials, a transient outward current resembling an I_A -type potassium current was often seen. This current is characteristic of bipolar cells (Tessier-Lavigne et al., 1988). When this current was blocked with intracellular cesium, we often recorded another current that was small, transient, and voltage gated. This inward current was revealed when the cell was held at potentials more negative than -70 mV and was stepped to depolarized potentials. Based upon the voltage dependence of this current, its kinetics, and sensitivity to cobalt, we conclude that this is a "T" type calcium current, also commonly found in bipolar cells (G. B. Grant and J. E. Dowling, unpublished observations).

Measurement of I_{Glu}

Over the course of this study we recorded from 326 bipolar cells. The effects of glutamate were tested on 235 bipolar cells. Twenty cells did not respond to glutamate. Four bipolar cells responded to glutamate with a conductance increase with a reversal potential near 0 mV (-0.32 ± 5.49). We presume that these are the type "a" or OFF class of bipolar cell because the effect of endogenously released glutamate would be to depolarize them. As discussed above, their small size (mean $C_M = 5.44 \pm 1.1$ pF) made it very difficult to patch clamp these cells successfully, and this accounts for the small number of these cells in our sample. Ten bipolar cells responded to glutamate with a conductance decrease with a reversal potential near 0 mV (-0.82

± 12.99). Since neither cGMP nor GTP was included in the intracellular solution, this current ran down quickly and when it was gone, no other effects of glutamate were noted in these cells. This current was presumably caused by glutamate acting at the AP-4-sensitive glutamate receptor mediating the rod input. These bipolar cells were generally quite large (mean $C_M = 27.44 \pm 14.6$ pF). The remaining 201 bipolar cells, intermediate in size (mean $C_M = 12.12 \pm 4.79$ pF), responded to glutamate with a conductance increase with a reversal potential near E_{Cl} . The input resistance for these cells was approximately $1 \text{ G}\Omega$ ($R_m = 1.09 \pm 1.13 \text{ G}\Omega$, $n = 185$). When using the potassium-based intracellular solution, the mean resting potential was -33 mV ($-32.8 \pm 8.9 \text{ mV}$, $n = 23$). The large number of these cells in our sample is due in part to the fact that they were easier to record than the other bipolar cells in white perch, but is also due to our selecting this class of cell based upon its recognizable morphology. These cells appear to receive mainly cone input, but we cannot rule out that they have some rod input as well. The results presented in the rest of this article deal exclusively with this last category of cells.

Figure 1A illustrates the methodology we used to determine the current–voltage (I – V) relationship of the glutamate-elicited current (I_{Glu}). In this experiment glutamate was puffed onto the bipolar cell during the time indicated by the upper (stimulus) trace. The membrane voltage of the bipolar cell was controlled with a voltage ramp, which was applied both in the presence and absence of glutamate. The whole-cell currents recorded in the bipolar cell are shown in the lower trace (I). On the time scale used during these experiments I_{Glu} was very stable and did not significantly decay over a period as long as a minute. This facilitated recording the I – V relationship of I_{Glu} . The I – V relationship recorded under control conditions is shown in trace 1 of Figure 1B. It is fairly linear since the majority of the significant voltage-dependent potassium and calcium currents in these bipolar cells were blocked. The I – V relationship recorded in the presence of glutamate is shown by trace 2 of Figure 1B. When the control data is subtracted from this, the I – V relationship of I_{Glu} is revealed. This is shown in Figure 1C. I_{Glu} is an inwardly rectifying current with a reversal potential near -30 mV .

I_{Glu} is predominantly carried by chloride ions

In the data shown in Figure 1, the reversal potential for I_{Glu} was -30.8 mV . This was very close to the calculated Nernst potential for chloride in this experiment ($E_{Cl} = -30 \text{ mV}$). To determine the relationship between the reversal potential of I_{Glu} and the intracellular chloride concentration, we recorded from a population of bipolar cells using intracellular solutions with varying chloride concentrations. Figure 2 illustrates data from these experiments. Figure 2A shows the I – V relationships for I_{Glu} recorded from different bipolar cells under conditions of differing intracellular chloride concentration. As the intracellular chloride concentration was reduced, the absolute magnitude of the current at hyperpolarized potentials was reduced, but more significantly, the reversal potential of the current was shifted so that it followed quite closely E_{Cl} , the Nernst potential for chloride.

This is more clearly illustrated in Figure 2B, where we have plotted the intracellular chloride concentration against the average reversal potential of I_{Glu} . The solid line shows the reversal potential predicted by the Nernst equation for an exclusively chloride-permeable channel. The reversal potential for I_{Glu} follows the Nernst equation well at higher intracellular chloride concentrations but deviates somewhat when the intracellular

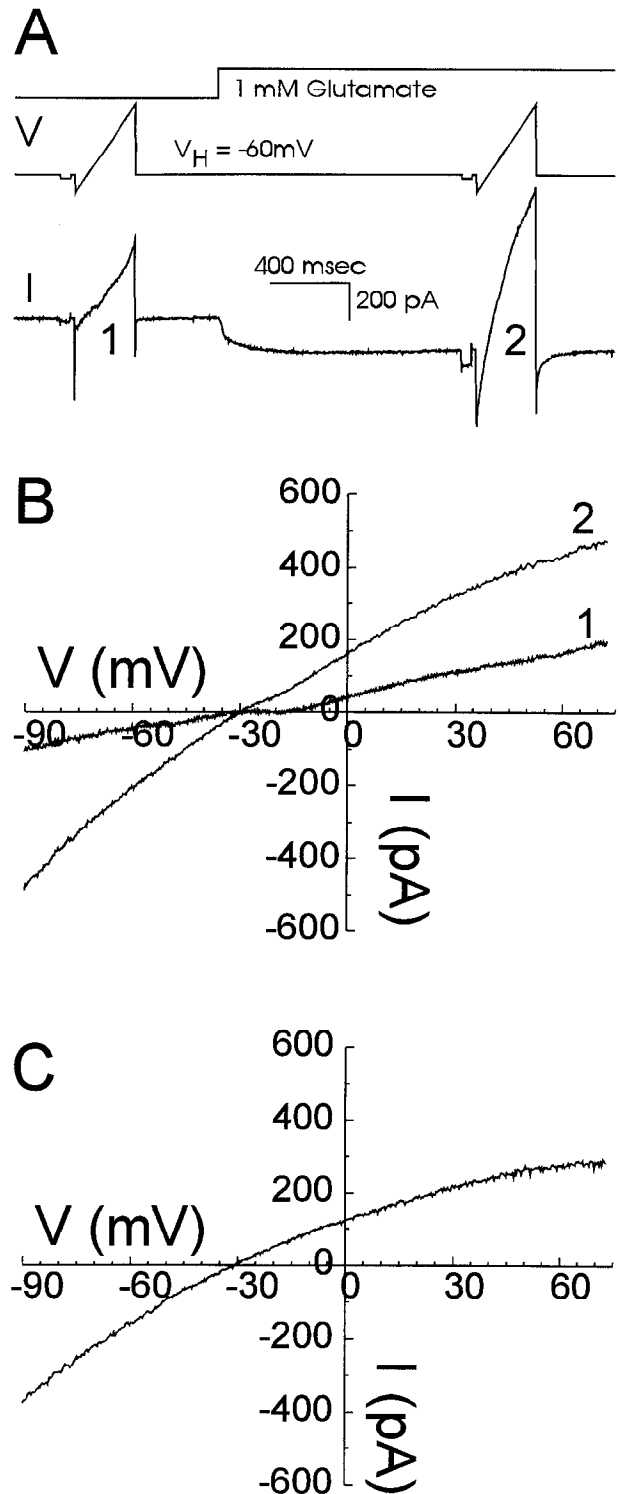


Figure 1. Measurement of the glutamate-elicited current (I_{Glu}) in a bipolar cell. **A**, Glutamate at 1 mM was puffed at a bipolar cell in the retinal slice during the timing indicated by the upper trace. The membrane potential of the bipolar cell was held at -60 mV and two voltage ramps (initial amplitude -100 mV , slope 0.72 mV/msec , duration 250 msec) were applied to the cell, one in the absence and one in the presence of glutamate. The whole-cell current recorded in response to this voltage protocol is illustrated in the lower trace. **B**, The whole-cell currents are replotted on current–voltage (I – V) axes. Trace 1 indicates the current recorded in the absence of glutamate; trace 2 indicates the current recorded in its presence. **C**, The two traces are subtracted (glutamate – control) to generate the I – V relationship of the glutamate-elicited current. A cesium-based intracellular solution was used.

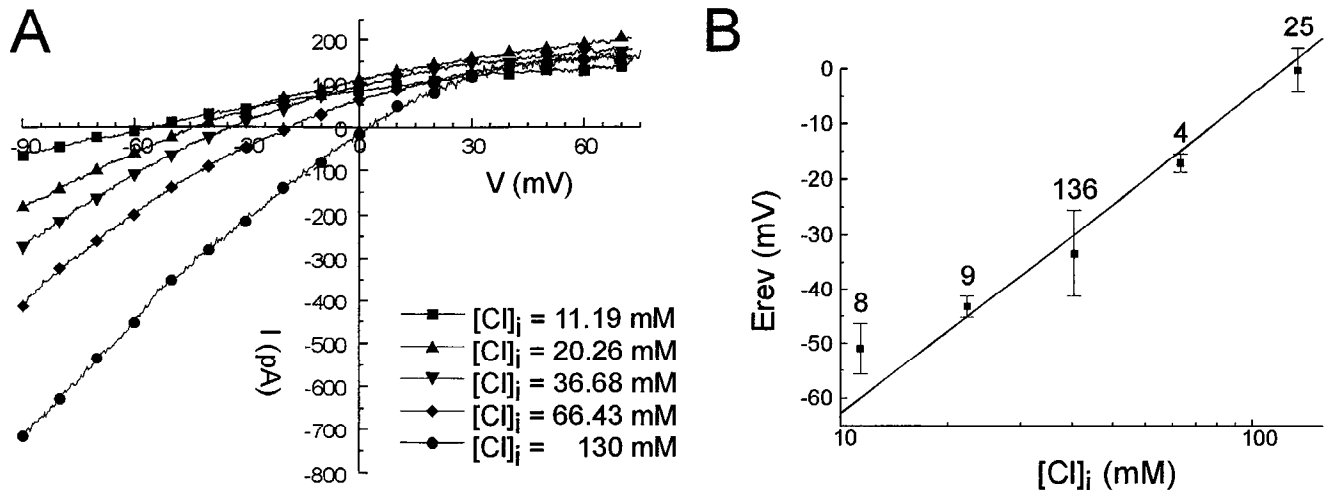


Figure 2. The magnitude and reversal potential of I_{Glu} are affected by changes in intracellular chloride. **A**, Five bipolar cells were recorded using cesium-based intracellular solutions with differing intracellular chloride concentrations (methanesulfonate substitution). The cells were held at -60 mV and I_{Glu} was measured to bath application of 1 mM glutamate. As the intracellular chloride concentration was reduced, the magnitude of I_{Glu} was reduced and its reversal potential shifted in a negative direction along the voltage axis (intracellular chloride concentrations: 11.19 mM, 20.26 mM, 36.68 mM, 66.43 mM, and 130 mM). **B**, The relationship between the reversal potential of I_{Glu} and the intracellular chloride concentration is plotted for all cells. The solid line is the reversal potential predicted by the Nernst equation for a chloride-permeable channel. The data for $[\text{Cl}]_i = 36.68$ mM pools populations of bipolar cells recorded with cesium-based, potassium-based, and sodium-based intracellular solutions. For a typical value of the whole cell current measured near the reversal potential of I_{Glu} , the uncompensated series resistance would distort the reversal potential by -1.5 mV for low $[\text{Cl}]_i$, and 3 mV for high $[\text{Cl}]_i$.

chloride concentration was reduced below 20 mM. This deviation seen at low intracellular chloride concentrations may be due to our inability to fully dialyze the bipolar cell with the low-chloride intracellular solution. Thus, I_{Glu} appears to involve ac-

tivation of a conductance mechanism that is primarily permeable to chloride.

I_{Glu} is not mediated by GABA or glycine receptors

Because I_{Glu} is strongly dependent upon intracellular chloride, one obvious possibility is that the effect of glutamate is an indirect one, caused by exogenously applied glutamate depolarizing neighboring cells, resulting in the release of GABA or glycine onto the bipolar cells. Although we included picrotoxin and strychnine in all bathing solutions, it could be argued that their effects are only partial and that some residual GABAergic or glycinergic input to the bipolar cell remained. We tested for this in the experiment shown in Figure 3. Here I_{Glu} was elicited by bath application of 200 μM glutamate. Initially picrotoxin and strychnine were not contained in the control Ringer's solution. Picrotoxin at 100 μM was then coapplied with the glutamate and there was a slight (10%) but not significant reduction in the magnitude of I_{Glu} . Picrotoxin was then washed out and 1 μM strychnine coapplied with the glutamate. No effect on I_{Glu} was noted. The lack of any significant effect of picrotoxin and strychnine showed that I_{Glu} is not an indirect effect via a GABA- or glycine-activated chloride current.

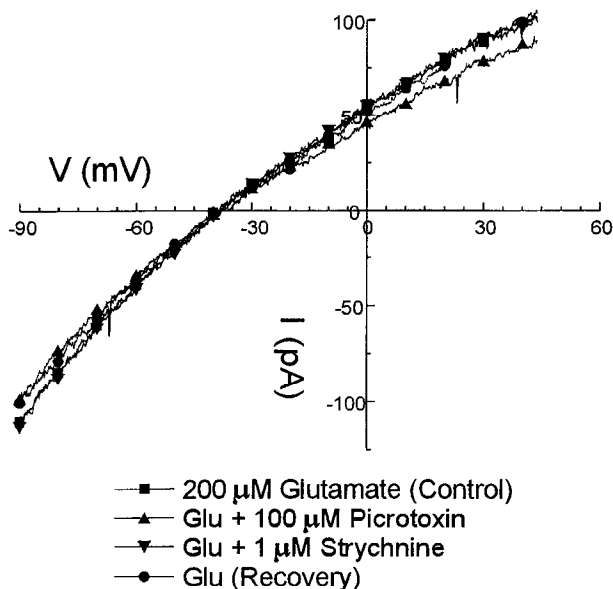


Figure 3. I_{Glu} is not caused by inhibitory GABA_A/GABA_Cergic or glycinergic synaptic inputs. I_{Glu} was elicited by bath application of 200 μM glutamate. Picrotoxin at 100 μM was then coapplied and I_{Glu} was reduced slightly (10%). Picrotoxin was then washed out and 1 μM strychnine was then coapplied with glutamate; I_{Glu} was not substantively affected. Finally, strychnine was washed out and I_{Glu} remained at near control levels. In this experiment cobalt was not included in the bathing solution. A cesium-based intracellular solution was used. $V_H = -60$ mV.

I_{Glu} is not mediated by a known ionotropic glutamate receptor

Figure 4A shows that several agonists that act at ionotropic glutamate receptors are unable to elicit I_{Glu} . In this experiment, glutamate was initially bath applied, and it generated a conductance increase that reversed at -38.0 mV. NMDA, which acts on the NMDA class of glutamate receptor, and kainate and AMPA, which act at the non-NMDA classes of glutamate receptor (Watkins, 1981; Massey, 1990; Nakanishi, 1992), were subsequently applied to the cell. None of these agonists had effects (NMDA, $n = 5$; kainate, $n = 9$; AMPA, $n = 4$). Reapplication of glutamate to the cells following application of the glutamate agonists

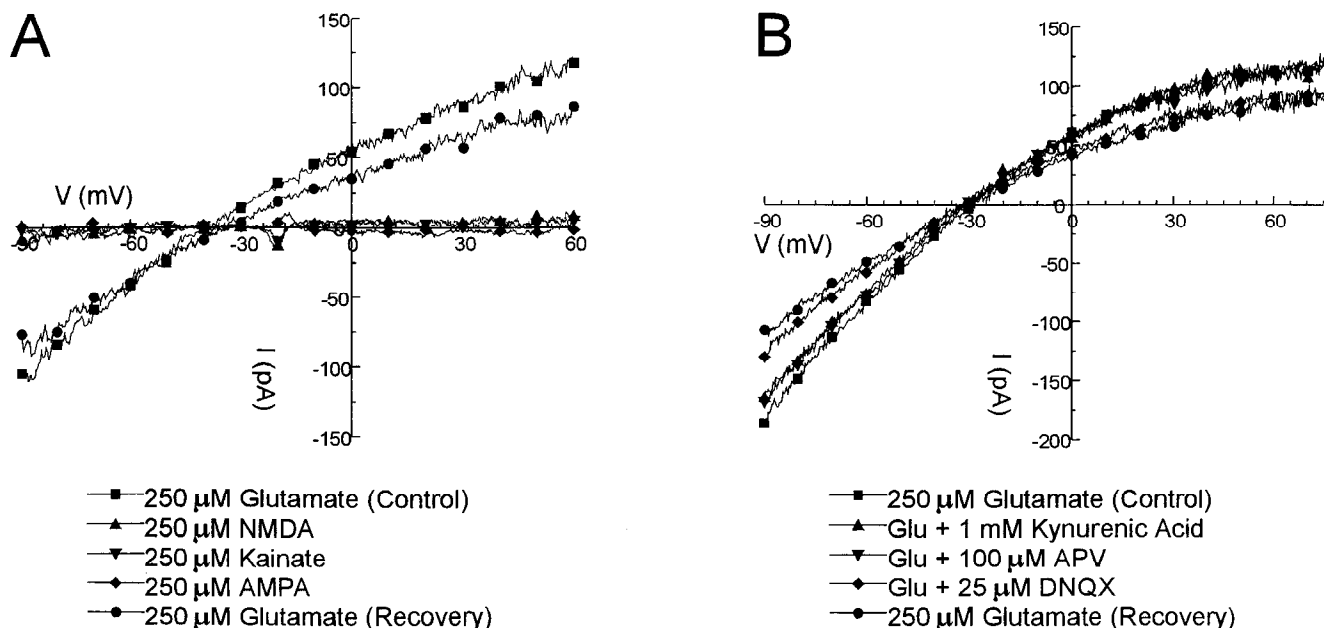


Figure 4. I_{Glu} is not mediated by an ionotropic glutamate receptor. *A*, I_{Glu} was elicited in a bipolar cell to bath application of 250 μM glutamate. Subsequent applications of NMDA, kainate, and AMPA (all at 250 μM) were ineffective at activating I_{Glu} . Finally, reapplication of glutamate elicited a current similar to control. *B*, I_{Glu} was elicited by bath application of 250 μM glutamate. I_{Glu} was not substantially blocked by the coapplication of the ionotropic glutamate receptor antagonists (in order of application) kynurenic acid (1 mM), APV (100 μM), or DNQX (25 μM). A final application of glutamate elicited a current similar to that seen initially. A cesium-based intracellular solution was used. $V_H = -60$ mV.

generated a current similar to the control response, indicating that the cell was still responsive to glutamate. All agonists were applied at a concentration of 250 μM . On several occasions ($n = 4$) we applied kainate to horizontal cells in retinal slices under the same experimental conditions as described above, and the kainate consistently activated a current in these cells with a reversal potential near 0 mV. These experiments showed that the kainate we used in our experiments was fully active.

Figure 4*B* shows that antagonists specific to the ionotropic glutamate receptors are ineffective on I_{Glu} . Here, glutamate was initially bath applied at a concentration of 250 μM , and it generated a conductance increase that reversed at -28.5 mV. This current was not significantly reduced by the general glutamate receptor antagonist kynurenic acid (Tachibana and Okada, 1991) at 1 mM ($n = 2$) or by the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (AP-5) (Ascher and Nowak, 1987) at 100 μM ($n = 2$). I_{Glu} was reduced slightly by the non-NMDA receptor antagonist DNQX (Honore et al., 1988) at 25 μM , but there was no recovery from this treatment. Finally, in other experiments, neither CNQX ($n = 5$) nor DNQX ($n = 2$) had an effect on I_{Glu} .

I_{Glu} is not activated by typical metabotropic glutamate receptor agonists

Having determined that I_{Glu} did not reflect the pharmacology of known ionotropic glutamate receptors, we investigated whether I_{Glu} was generated by a metabotropic glutamate receptor. This class of glutamate receptor is linked to second messenger systems by G-proteins (Nakanishi, 1992). Since in rod-driven ON bipolar cells, a metabotropic glutamate receptor is involved in generating the light-elicited conductance increase to cations (Slaughter and Miller, 1981; Nakanishi, 1992) it seemed possible that I_{Glu} might be acting through another such a receptor on cone

ON bipolar cells. Figure 5 shows, however, that this is not the case: agonists that act at metabotropic glutamate receptors were ineffective at mimicking the effects of glutamate itself.

In the experiment shown in Figure 5*A*, 1 mM glutamate activated a current with a reversal potential of -30 mV. Subsequent applications of ibotenate (Watkins, 1981) at 100 μM ($n = 5$) and 2-amino-4-phosphonobutyric acid (AP-4) (which selectively acts on rod ON bipolar cells; Slaughter and Miller, 1981) at 250 μM ($n = 11$) were without effect on this bipolar cell. Finally, reapplication of glutamate to the cell activated a current similar to control, indicating that the cell remained responsive to glutamate.

In the experiment shown in Figure 5*B*, quisqualate (Tang et al., 1989) at 100 μM ($n = 6$) was without effect on a bipolar cell in which 1 mM glutamate activated a current that reversed at -38.4 mV. Finally, as shown in Figure 5*C*, ACPD (Linn and Christensen, 1992) at 500 μM ($n = 5$) was without effect on a bipolar cell in which 1 mM glutamate activated a current that reversed at -27.5 mV.

Sodium dependence of I_{Glu}

The strong dependence upon intracellular chloride and the lack of effect of ionotropic and metabotropic glutamate receptor agonists caused us to consider the possibility that I_{Glu} might be similar to the glutamate-elicited current described in photoreceptors (Sarantis et al., 1988; Tachibana and Kaneko, 1988; Eliaf and Werblin, 1993). Since that current has a strong dependence upon extracellular sodium, we tested whether the glutamate-elicited current in cone ON bipolar cells had a similar dependence.

Figure 6*A* illustrates that I_{Glu} requires extracellular sodium for its generation. In this experiment I_{Glu} was elicited in a bipolar cell by bath application of 1 mM glutamate. The Ringer's solu-

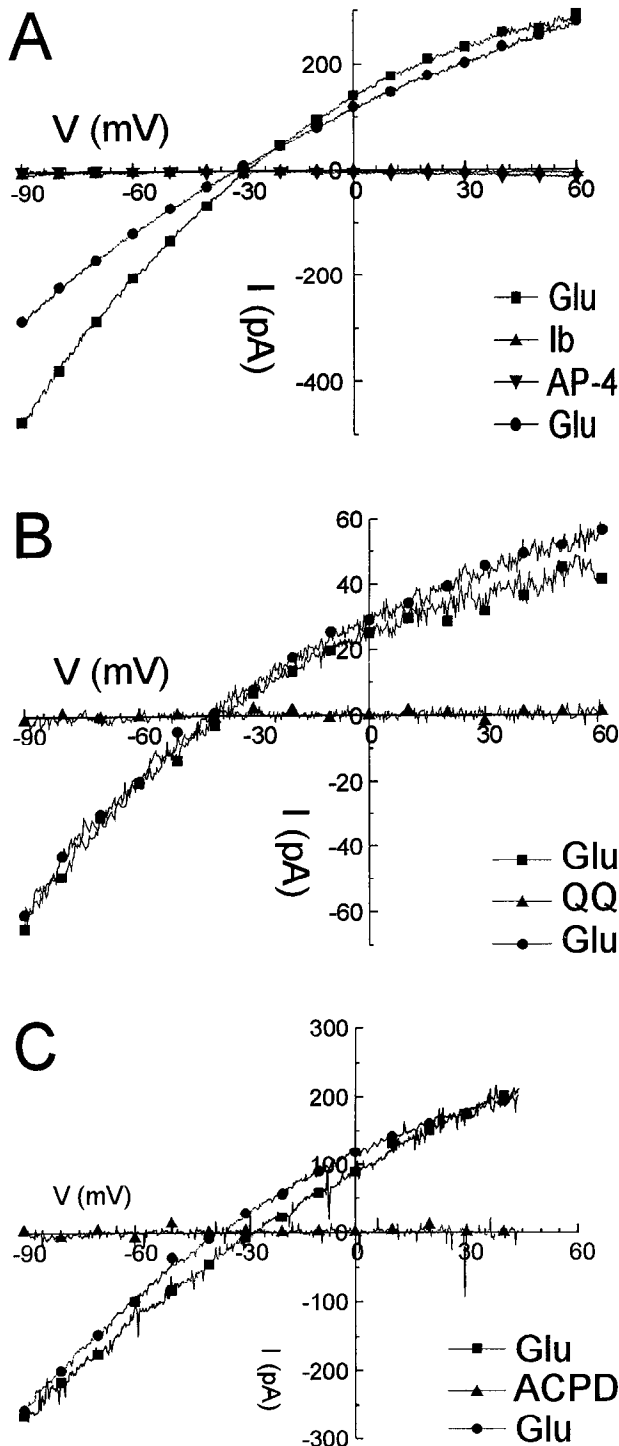


Figure 5. I_{Glu} is not mediated by a metabotropic glutamate receptor. **A**, I_{Glu} was elicited in a bipolar cell by bath application of 1 mM glutamate. Subsequent applications of 100 μM ibotenate (*Ib*) and 250 μM AP-4 were without effect. A second application of glutamate yielded a current similar to control. **B**, I_{Glu} was elicited in a bipolar cell by bath application of 500 μM glutamate. Subsequent application of 100 μM quisqualate (*QQ*) was without effect. A second application of glutamate yielded a current similar to control. **C**, I_{Glu} was elicited in a bipolar cell by bath application of 1 mM glutamate. Subsequent application of 500 μM 1*S*,3*R*-ACPD was without effect. A second application of glutamate yielded a current similar to control. All data were recorded using a cesium-based intracellular solution. $V_H = -60$ mV.

tion was then exchanged for a solution from which all sodium had been removed and replaced with lithium. Under these conditions, application of glutamate generated no current. When the control Ringer's solution was once again introduced to the cell, I_{Glu} recovered to near-control levels. Similar results were observed in six cells.

This dependence upon extracellular sodium raises the possibility that I_{Glu} might be mediated by a sodium-glutamate cotransporter. Many glutamate cotransporters work by coupling the energetically unfavorable movement of glutamate anions into the cell to the energetically favorable movement of sodium ions into the cell. Therefore, if a sodium-dependent glutamate cotransporter was present in cone ON bipolar cells, then I_{Glu} should be dependent upon both the extra- and intracellular sodium concentration.

To study this further, we performed the experiment shown in Figure 6*B*. Here we recorded from two populations of bipolar cells. In one population ($n = 5$) we used a potassium-based intracellular solution, and in the other population ($n = 4$) we used an intracellular solution that contained 124 mM sodium (100% replacement of potassium with sodium). Under both conditions, TEA was included in the extracellular solutions to block potassium currents. From these two populations of bipolar cells we recorded I_{Glu} under similar conditions. The averaged $I-V$ relationships of the respective glutamate-activated currents are plotted in Figure 6*B*.

Under the conditions of high intracellular sodium used here, where $[\text{Na}]_i > [\text{Na}]_o$, I_{Glu} was not significantly different from I_{Glu} recorded under control conditions (low $[\text{Na}]_i$). Indeed, I_{Glu} was slightly enhanced when intracellular sodium was elevated. A similar experiment was also carried out in which the noncontrol population had an elevated intracellular sodium concentration (30.68 mM). Again, under these conditions there was no significant difference in the amplitude of I_{Glu} or its reversal potential between the control (nominally zero $[\text{Na}]_i$) and high $[\text{Na}]_i$ cases.

Thus, I_{Glu} appears have an asymmetric requirement for sodium, it must be present on the extracellular side of the membrane, but dramatic changes in the intracellular sodium concentration have little effect on I_{Glu} .

Glutamate transport blockers reduce I_{Glu}

Because I_{Glu} requires the presence of extracellular sodium it is possible that the pharmacology of the I_{Glu} receptor is similar to that of a glutamate transporter. To test this, we examined the effects of several inhibitors of glutamate transport upon I_{Glu} . Figure 7 illustrates that I_{Glu} can be reduced by several of these inhibitors. In these experiments, I_{Glu} was first recorded by application of glutamate alone (control), then to glutamate and the transporter blocker together, and then to glutamate alone again (recovery). Percentage reductions were calculated relative to the average of the control and recovery data.

Figure 7*A* shows data from an experiment in which DL-threo-3-hydroxyaspartic acid (THA) (Balcar and Johnston, 1972; Balcar et al., 1977) reduced I_{Glu} by 42% when the cell membrane potential was held at -70 mV. On average, 250 μM THA reduced the current elicited by bath application of 250 μM glutamate by 47% at -70 mV (0.472 ± 0.13 , $n = 7$). Figure 7*B* shows data from an experiment in which *L*-trans-pyrrolidine-2,4-dicarboxylic acid (tPDC; Bridges et al., 1991) reduced I_{Glu} by 36.5% at -70 mV. On average, 250 μM tPDC reduced the current elicited by bath application of 250 μM glutamate by 31% at -70 mV (0.313 ± 0.058 , $n = 5$). Finally, Figure 7*C* shows

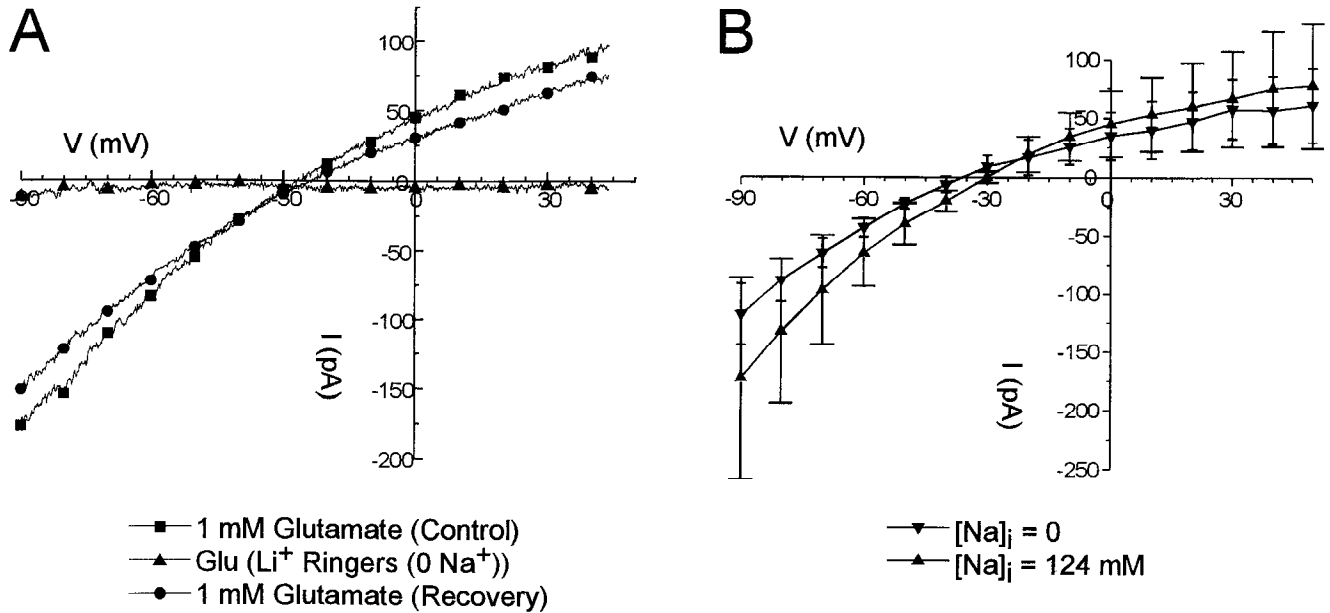


Figure 6. I_{Glu} requires extra- but not intracellular sodium. *A*, I_{Glu} was elicited in a bipolar cell to bath application of 1 mM glutamate. The Ringer's solution bathing the cell was then exchanged for one in which sodium had been replaced by lithium. Under these conditions, glutamate elicited no current. When normal Ringer's solution was restored to the bath, I_{Glu} recovered. A cesium-based intracellular solution was used. $V_H = -30$ mV. *B*, I_{Glu} was measured from two populations of bipolar cells using bath application of 250 μM glutamate. In one population, a potassium-based intracellular solution that contained no added sodium was used. In the other, a sodium-based intracellular solution was used. No substantive difference in absolute current magnitude or reversal potential was seen between the two populations despite the dramatic change in intracellular sodium concentration. $V_H = -40$ mV.

data from an experiment in which dihydrokainic acid DHKA (Johnston et al., 1979) reduced I_{Glu} by 22% at -70 mV. On average, 1 mM DHKA reduced the current elicited by bath application of 250 μM glutamate by 6% at -70 mV (0.055 ± 0.141 , $n = 7$).

When applied in the absence of glutamate, both THA and tPDC generated a small but significant current with a reversal potential close to that of I_{Glu} . DHKA generated no current when applied alone. These observations are consistent with reports that both THA and tPDC act as agonists of moderate potency at glutamate transporters in Müller cells of the retina (Barbour et al., 1991; Sarantis et al., 1993). DHKA is not a substrate for high-affinity glutamate transport and is only a weak agonist there (Johnston et al., 1974). Thus, the ability of THA and tPDC to inhibit I_{Glu} is consistent with their ability to activate I_{Glu} directly.

I_{Glu} is localized to bipolar cell dendrites

Photoreceptors are presynaptic to bipolar cells and release glutamate onto them. Thus, one would expect that a bipolar cell would be maximally sensitive to glutamate in the outer plexiform layer where it makes synaptic contact with photoreceptors. In Figure 8 we localized the sensitivity of ON-bipolar cells to glutamate. E_{Cl} was set at -30 mV and the cells were held at -60 mV; thus, application of 1 mM glutamate by a nearby puff pipette generated an inward current. The glutamate-elicited current was largest and rose to a peak most rapidly when glutamate was pressure ejected in the outer plexiform layer. When the puff was directed at other locations (e.g., into the inner nuclear layer), the magnitude of I_{Glu} was smaller and its kinetics were slower. Thus, the bipolar cell was maximally sensitive to glutamate in the outer plexiform layer where its dendrites reside. Almost no

glutamate sensitivity was found near the bipolar cell terminal in the inner plexiform layer. Similar results were found in five cells.

I_{Glu} is involved in bipolar cell light response

In Figure 8 we illustrated that these bipolar cells are sensitive to glutamate on their dendrites. Thus, we can infer that synaptically released glutamate would act at these receptors. If this were the case, it would be expected that the light response of a bipolar cell that involved these glutamate receptors would reverse at or near E_{Cl} .

Figure 9 illustrates data from an experiment in which this was tested. The timing of the light flash is indicated by the upper trace and the whole-cell currents recorded from the bipolar cell at the indicated holding potentials are below. Shown are the off-components of the light responses, when glutamate concentrations are increasing, that is, when the cones are recovering from the light-induced hyperpolarization. The effect of this dark step was to cause an inward current in the bipolar cell at $V_H = -80$ mV. The size of this current diminished as the cell was held at more depolarized potentials, and it reversed sign between -40 and -20 mV. In Figure 9B, the magnitude of the light-elicited current is plotted against the potential at which the bipolar cell was held. The synaptic current reversed sign at approximately -30 mV, where E_{Cl} was set in this experiment. Thus, the photoreceptor-induced current in this bipolar cell had the same reversal potential as I_{Glu} , suggesting that I_{Glu} is responsible for the light response in this cell. This experiment was carried out in 100 μM picrotoxin and 1 μM strychnine to block inhibitory inputs from amacrine cells (Lukasiewicz et al., 1994).

During the course of these experiments we also recorded from several bipolar cells that lacked light responses yet that dem-

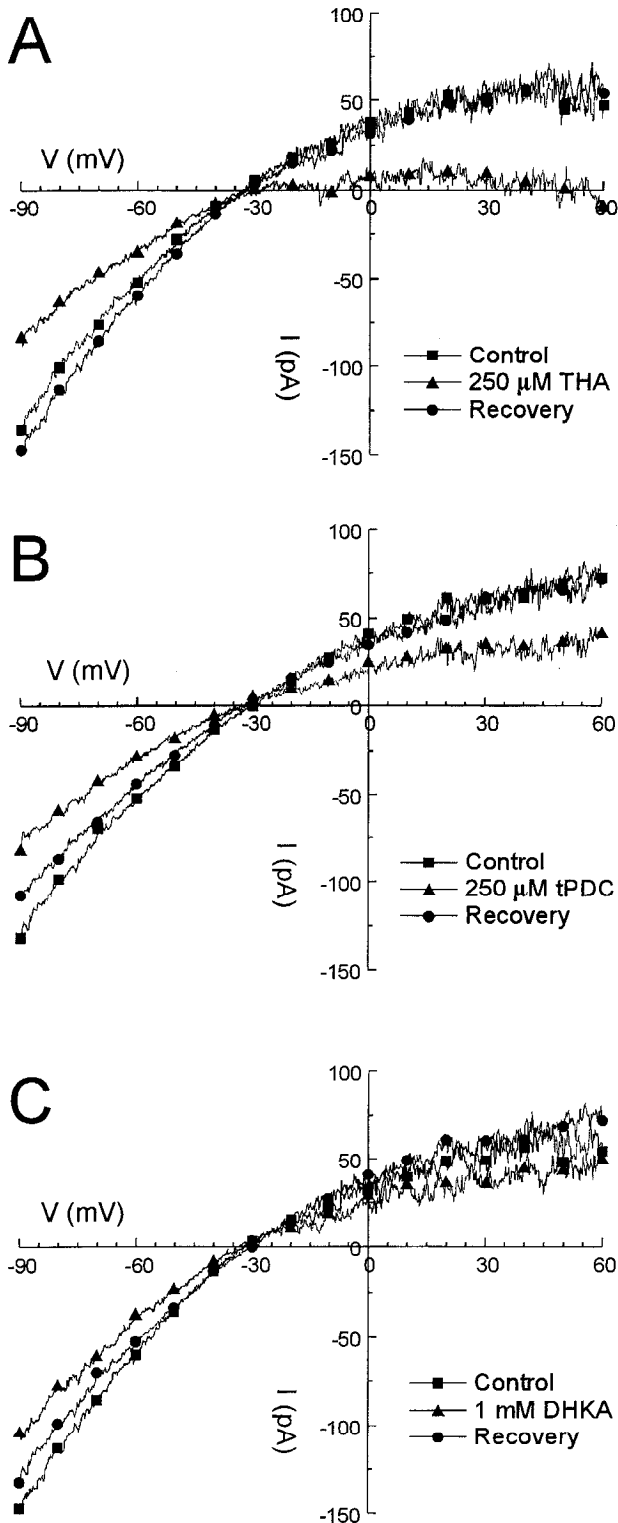


Figure 7. I_{Glu} can be reduced by glutamate transporter blockers. I_{Glu} was elicited in a bipolar cell to bath application of 250 μ M glutamate. The transporter blocker was then coapplied with glutamate. Finally, glutamate was reapplied in order to record a recovery from the effect of the blocker. *A*, I_{Glu} was reduced by 42% at a membrane potential of -70 mV by bath application of 250 μ M DL-threo-3-hydroxyaspartic acid (THA). *B*, I_{Glu} was reduced by 37% at -70 mV by bath application of 250 μ M L-trans-pyrrolidine-2,4-dicarboxylic acid (tPDC). *C*, I_{Glu} was reduced by 22% at -70 mV by bath application of 1 mM dihydrokainic acid (DHKA). Reductions were calculated relative to the average of the

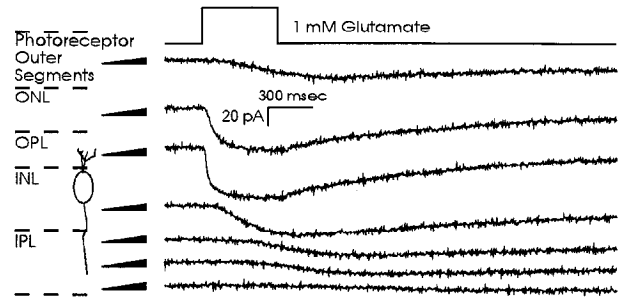


Figure 8. I_{Glu} is localized to the dendrites of the bipolar cell. Glutamate at 1 mM was puffed from a fine-tipped puff pipette at different locations along a bipolar cell in the retinal slice. The puff response was largest and rose to a peak most rapidly when the puff of glutamate was directed into the outer plexiform layer. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. A potassium-based intracellular solution was used. $V_H = -60$ mV.

onstrated many spontaneous blips that resembled currents generated by spontaneously released synaptic quanta. In one case we measured the reversal potential of these blips to be approximately -40 mV. Since this is close to the calculated E_{Cl} of -30 mV, and since these experiments were performed in the presence of picrotoxin and strychnine that should block inhibitory synaptic inputs from amacrine cells (Lukasiewicz et al., 1994), we believe that these synaptic blips arise from glutamate released spontaneously from photoreceptors.

Discussion

Role of I_{Glu}

In carp, Saito et al. (1979) suggested that the center response for a cone bipolar cell is caused by a light-elicited reduction in the membrane conductance to potassium or chloride. Here we provide evidence that in white perch ON bipolar cells, glutamate activates a chloride conductance. We propose that this chloride conductance gives rise to the dark-evoked current in cone-driven ON bipolar cells (I_{Glu}) in white perch. Although we have not investigated whether the same current is present in goldfish or carp, we have recorded it in another teleost, the hybrid bass (a cross between striped bass and white bass). The current recorded in these cells was almost identical to the current that we recorded in white perch. This leads us to believe that this current is found in many if not all teleosts and is not unique to white perch.

For the turning off of this current to be responsible for generating a depolarizing light response in these cells, its physiological reversal potential must be more negative than -30 mV, where we set it in most of our experiments. We were unable to measure accurately the natural reversal potential for this current because under whole-cell patch-clamp conditions, the solutions in the patch pipette quickly dialyze the cell, thus establishing a new value for E_{Cl} . Despite this difficulty, we attempted to get an estimate of the physiological reversal potential for I_{Glu} in several experiments. We did this by measuring the reversal potential of I_{Glu} as quickly as we could after breaking into the cell. These experiments suggest that the normal reversal potential for I_{Glu} is more negative than -45 mV; that is, shortly after cell penetration, we have on occasion measured reversal potentials of up to

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control and recovery data. A potassium-based intracellular solution was used. $V_H = -40$ mV.

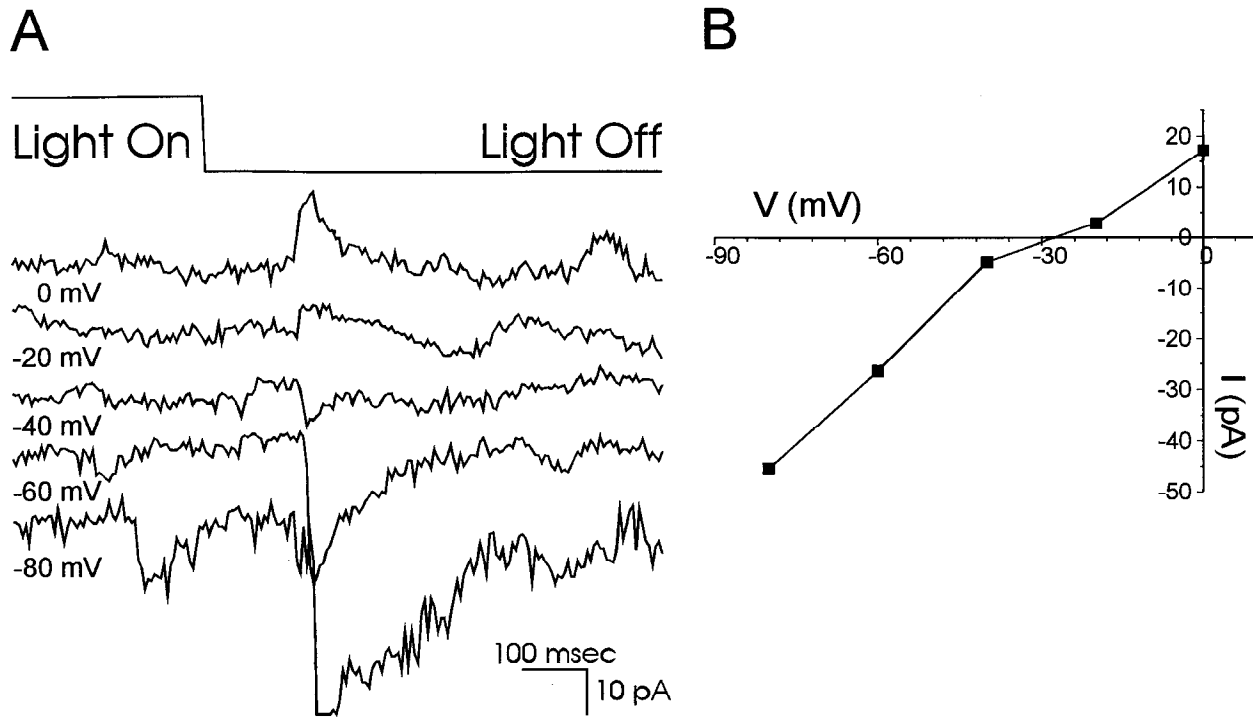


Figure 9. The reversal potential of the bipolar cell light response is near to that of I_{Glu} . A bipolar cell was voltage clamped and a saturating light flash (white light, full field flash) was delivered. The experiment was carried out in the presence of 100 μM picrotoxin and 1 μM strychnine to block inhibitory inputs. **A**, The offset of the light response is shown by the timing trace at the top. The whole-cell currents recorded in the bipolar cell are shown below the flash trace and the holding potential of each is indicated to the left. The dark step generated an inward current at -80 mV that reversed and became an outward current at potentials greater than -20 mV. **B**, The peak amplitude of the light response is plotted on current–voltage axes. The reversal potential of the light-elicited current is approximately -30 mV. This is where E_{Cl} was set in this experiment and is also the reversal potential for I_{Glu} . A cesium-based intracellular solution was used. $V_{\text{H}} = -30$ mV.

-51 mV. This value is more positive than the value of -63 mV for the reversal potential of the center light response but more negative than the value of -28 mV for the average resting potential of cone-driven ON bipolar cells in the dark (Saito et al., 1979). We conclude, therefore, that in the dark, glutamate released from cones would activate I_{Glu} and hyperpolarize the cell. In response to a flash of light, glutamate release decreases, resulting in a decrease of I_{Glu} and depolarization of the cell.

Isolation of I_{Glu}

The retinal slice preparation is synaptically intact. In the absence of blockers it is quite common to record robust excitatory and inhibitory synaptic currents in the cells of a slice. The strength of these inputs leads to the obvious concern as to whether I_{Glu} could be due to the effects of inhibitory neurotransmitters released from cells in the slice.

The dose of picrotoxin (100 μM) we used in our experiments should be more than adequate to block any GABA_A- or GABA_C-mediated chloride conductance increases. Consistent with this, we were unable to record GABA-elicited chloride currents at GABA concentrations as high as 250 μM in the presence of picrotoxin. Furthermore, even if I_{Glu} were due to a GABA-driven chloride conductance increase, the lack of desensitization of this current would suggest that it is a GABA_C receptor-mediated current. In white perch horizontal cells, the IC_{50} for picrotoxin acting on GABA_C receptors is 237 nM (Qian and Dowling, 1994), suggesting that the concentration of picrotoxin that we used is more than adequate to block GABA_C receptors in the fish. Indeed, Qian and Dowling (1993) have shown that 100 μM

picrotoxin blocks almost all GABA_C receptor-mediated currents in bipolar cells in white perch.

Similarly, the dose of strychnine (1 μM) we have used here should be adequate to block any glycinergic inputs to these bipolar cells. We were unable to record glycine-activated currents in our control solutions (i.e., when picrotoxin and strychnine were in the bath). This is consistent with reports of horizontal cell recordings in white perch where the action of 100 μM glycine was blocked with 2 μM strychnine (Zhou et al., 1993). Furthermore, any glycine-mediated input would be expected to desensitize, whereas I_{Glu} does not. This indicates that I_{Glu} is not mediated by glycinergic input to the bipolar cell.

Effective glutamate concentrations

To record I_{Glu} , we found it necessary to use quite high concentrations of glutamate (100 μM to 1 mM). Indeed, we were unable to record I_{Glu} consistently at concentrations lower than 50 μM . If the effective concentrations of glutamate at this receptor are in the range of 0.1–1 mM, it is unlikely that these receptors would be effectively activated *in vivo*. However, all of our recordings were made in the slice preparation where glutamate uptake into neighboring Müller cells decrease substantially the local concentrations of glutamate. In the isolated teleost retina, for example, threshold concentrations for glutamate applied to horizontal cells are 25–200 times higher than those needed when glutamate is applied to isolated horizontal cells (Ariel et al., 1984). If the data are similar for retinal slices and bipolar cells, they suggest that effective glutamate concentrations may be as low as 250 nM on cone-driven ON bipolar cells.

ON bipolar cell currents

Nawy and Copenhagen (1990) reported that when cesium-filled intracellular electrodes are used to record goldfish ON bipolar cell responses, the cone component of the light response is blocked. They inferred from this result that the effect of cesium was to block the cone component of the light response by depressing a potassium conductance. Their result differs from our observations since in most of our experiments we used a cesium-based intracellular solution and recorded a robust I_{Glu} . We did note that I_{Glu} had a tendency to run down slowly over time and eventually it was lost entirely. We did not observe this run-down when we used a potassium-based intracellular solution. However, this effect occurred slowly and did not appear to be due to a direct action of cesium on a potassium channel since such an effect would be expected to occur quite rapidly.

In tiger salamander, Hirano and MacLeish (1991) have reported that glutamate and AP-4 cause an increase in potassium conductance in ON bipolar cells. This current differs from I_{Glu} in that it is carried primarily by potassium and can be activated by AP-4. We have shown here that AP-4 does not activate I_{Glu} in white perch. Similarly, Nawy and Copenhagen (1987) have reported that in goldfish AP-4 acts solely at receptors mediating synaptic transmission from rods and is without effect at the glutamate receptor mediating cone input. Thus, there may be another type of glutamate receptor on ON bipolar cells in tiger salamander, although several other studies have reported that tiger salamander ON bipolar cells possess only AP-4-sensitive glutamate receptors (Attwell et al., 1987; Lasansky, 1992; Thoreson and Miller, 1993).

A consequence of the insensitivity of I_{Glu} to AP-4 would be that in teleosts, photopic ON responses would not be blocked by AP-4. This has been found to be the case in goldfish. DeMarco et al. (1991) showed that intraocular injection of AP-4 severely reduced the amplitude of the b-wave of the electroretinogram, which is generally believed to represent the activity of ON bipolar cell, but did not eliminate it. Furthermore, direct recordings from optic nerves showed that ON responses remained, although reduced in sensitivity. Thus, in teleosts, AP-4 does not block all of the ON pathways, and we surmise that it is the cone ON responses that survive AP-4.

Inhibitory effects of glutamate

We have shown here that glutamate hyperpolarizes cone ON bipolar cells in the teleost retina by opening a chloride conductance; thus, on these cells glutamate acts like a classical inhibitory neurotransmitter. This is an unusual effect for glutamate, which is typically classified as an excitatory amino acid. However there are several other examples where glutamate acts in an inhibitory fashion. As previously mentioned, at the rod-mediated input to ON bipolar cells, glutamate closes channels with a depolarized reversal potential, thus hyperpolarizing the cell (Nawy and Jahr, 1990). This effect of glutamate is indirect, mediated by a second messenger, cGMP. On locust leg muscle, glutamate has a direct inhibitory effect. Here it acts at extrajunctional H-receptors to open chloride channels (Cull-Candy, 1976; Dudel et al., 1989). Similarly, expression of *C. elegans* polyA⁺ RNA in *Xenopus* oocytes yields a glutamate-activated chloride current (Arena et al., 1992). However, both of these currents differ pharmacologically from the one that we measured in that they could be both activated by ibotenate and inhibited by picrotoxin.

Source of I_{Glu}

The current that we have characterized here is quite similar, if not identical, to a glutamate-elicited current reported in cone photoreceptors of tiger salamander (Sarantis et al., 1988; Eliasof and Werblin, 1993) and turtle (Tachibana and Kaneko, 1988). The source of these photoreceptor currents is a matter of controversy. Sarantis et al. (1988) reported that the current was generated by a glutamate-activated chloride channel. Others (Tachibana and Kaneko, 1988; Eliasof and Werblin, 1993) have suggested that the current is mediated by an electrogenic glutamate transporter coupled to a chloride channel. I_{Glu} resembles a glutamate transporter in that it can be reduced by blockers of electrogenic glutamate uptake. The dependence of I_{Glu} upon extracellular sodium can also be used as an argument for its being due to a glutamate transporter. However, when intracellular sodium was increased dramatically, there was no substantive effect upon the magnitude or reversal potential of I_{Glu} . Thus, whereas we cannot completely rule out the source of I_{Glu} as being due to a glutamate transporter, we also cannot rule out that I_{Glu} is mediated by a novel class of glutamate receptor that has pharmacological characteristics like that of glutamate transporters.

Some reports have suggested that transporters and channels are not as distinct as they might seem to be. For example, P-glycoprotein (whose expression confers multidrug resistance on cell lines and human tumors) is a member of the ABC (ATP-binding cassette) superfamily of transporters. However, its expression generates a volume-regulated, ATP-dependent, chloride-selective channel (Valverde et al., 1992). Similarly, treatment of a mitochondrial aspartate/glutamate carrier with mercury reagents transforms it into an unselective pore, reducing its substrate specificity (Dierks et al., 1990). Finally, in retinal Müller cells, Schwartz and Tachibana (1990) have reported that the current generated by activation of the sodium-dependent glutamate transporter reverses sign, a property seemingly more consistent with an ion channel. However, this last point is controversial as other reports (Brew and Attwell, 1987; Barbour et al., 1991) have reported that the glutamate transporter did not generate an outward current.

It is possible that I_{Glu} , the current we have described here, is derived from a hybrid; a glutamate transporter recognition site linked to a chloride channel. The inhibition of I_{Glu} by the glutamate transport blockers would thus be due to direct actions at the recognition site. The dependence upon extracellular sodium could be explained if the opening of this channel required activation of the transporter recognition site by both extracellular sodium and glutamate. An alternative interpretation of the data is that I_{Glu} represents an entirely new class of glutamate receptor linked to a chloride channel. The inhibitory actions of the glutamate transport inhibitors THA and tPDC might then be due to a direct action on this receptor. In hippocampus, for example, tPDC inhibits NMDA receptor responses (Sarantis et al., 1993). The dependence of I_{Glu} upon extracellular sodium is more difficult to explain for this model, but one possibility is that the chloride channel opens when glutamate is bound, but that sodium is needed (perhaps inside the channel) to keep the open channel functioning as an anion-permeable channel (Franciolini and Nonner, 1987).

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