Removal of Extracellular Chloride Suppresses Transmitter Release from Photoreceptor Terminals in the Mudpuppy Retina

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ABSTRACT Removal of extracellular Cl^- has been shown to suppress light-evoked voltage responses of ON bipolar and horizontal cells, but not photoreceptors or OFF bipolar cells, in the amphibian retina. A substantial amount of experimental evidence has demonstrated that the photoreceptor transmitter, L-glutamate, activates cation, not Cl^-, channels in these cells. The mechanism for Cl-free effects was therefore reexamined in a superfused retinal slice preparation from the mudpuppy (Necturus maculosus) using whole-cell voltage and current clamp techniques. In a Cl-free medium, light-evoked currents were maintained in rod and cone photoreceptors but suppressed in horizontal, ON bipolar, and OFF bipolar cells. Changes in input resistance and dark current in bipolar and horizontal cells were consistent with the hypothesis that removal of Cl^- suppresses tonic glutamate release from photoreceptors. The persistence of light-evoked voltage responses in OFF bipolar cells, despite the suppression of light-evoked currents, is due to a compensatory increase in input resistance. Focal application of hyperosmotic sucrose to photoreceptor terminals produced currents in bipolar and horizontal cells arising from two sources: (a) evoked glutamate release and (b) direct actions of the hyperosmotic solution on postsynaptic neurons. The inward currents resulting from osmotically evoked release of glutamate in OFF bipolar and horizontal cells were suppressed in a Cl-free medium. For ON bipolar cells, both the direct and evoked components of the hyperosmotic response resulted in outward currents and were thus difficult to separate. However, in some cells, removal of extracellular Cl^- suppressed the outward current consistent with a suppression of presynaptic glutamate release. The results of this study suggest that removal of extracellular Cl^- suppresses glutamate release from photoreceptor terminals. Thus, it is possible that control of $[Cl^-]$ in and around photoreceptors may regulate glutamate release from these cells. Key words: glutamate • chloride • retina

INTRODUCTION

Chloride ions are important in many different processes in the retina, including inhibition mediated by γ-aminobutyric acid (GABA)[1] and glycine receptors,

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Experiments were begun while Dr. Thoreson was at the University of Minnesota and were completed at the University of Nebraska Medical Center.

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[1]*Abbreviations used in this paper:* ANOVA, analysis of variance; GABA, γ-aminobutyric acid; KA-AMPA, kainic acid/α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; L-AP4, L-2-amino-4-phosphonobutyric acid; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline; OPL, outer plexiform layer.

pH regulation, and regenerative depolarizations in photoreceptors (e.g., Miller et al., 1981; Thoreson and Burkhardt, 1991; Koskelainen et al., 1993). Chloride ions can also regulate neurotransmission from photoreceptors to bipolar and horizontal cells since the removal of extracellular Cl^- selectively suppresses light-evoked voltage responses in depolarizing ON bipolar cells and horizontal cells, but not photoreceptors or hyperpolarizing OFF bipolar cells (Miller and Dacheux, 1973, 1975, 1976). Several different anion substitutes produce the same effect (Miller and Dacheux, 1976).

The finding that Cl^- removal suppressed light responses of ON bipolar and horizontal cells prompted the hypothesis that the photoreceptor transmitter modulates Cl^- conductances in these cell types (Miller and Dacheux, 1976, 1983; Miller and Slaughter, 1985). However, it was subsequently shown that the transmit-

ter released by photoreceptors is L-glutamate (Miller and Slaughter, 1986; Ayoub et al., 1989; Copenhagen and Jahr, 1989) and that glutamate modulates cation conductances in most bipolar and horizontal cells (for review see Wu, 1994). In horizontal and OFF bipolar cells, glutamate released from photoreceptors acts at kainic acid/α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (KA/AMPA) receptors to open nonselective cation channels (Miyachi and Murakami, 1991; Low et al., 1991; Attwell et al., 1987; Gilbertson et al., 1991; O'Dell and Christensen, 1989ab). Although cone-driven ON bipolar cells in the teleost retina possess a glutamate-activated Cl^- conductance (Grant and Dowling, 1995), extensive study of ON bipolar cells in amphibians has failed to reveal a similar conductance (Nawy and Jahr, 1991; Thoreson and Miller, 1993a). Instead, light responses of ON bipolar cells in the amphibian retina arise primarily from the modulation of glutamate receptors sensitive to L-2-amino-4-phosphonobutyric acid (L-AP4) that act to close nonselective cation channels (Slaughter and Miller, 1981; Shiells et al., 1981; Nawy and Jahr, 1990, 1991; Thoreson and Miller, 1993a; Lasansky, 1992). L-AP4-activated K^+ channels also may play a minor role in the generation of ON bipolar cell light responses (Hirano and MacLeish, 1991). Thus, the suppression of light responses in amphibian ON bipolar and horizontal cells after removal of extracellular Cl^- cannot be explained by the presence of Cl^- conductances modulated by the photoreceptor transmitter, L-glutamate, in these cells.

In the present study, we have used whole-cell voltage and current clamp techniques to reexamine the actions of a Cl-free environment on outer retinal neurons. Our results support the hypothesis that removal of extracellular Cl^- suppresses glutamate release from photoreceptor terminals onto all three classes of second-order neurons. The persistence of light responses in OFF bipolar cells, despite the suppression of synaptic currents, results from a compensatory increase in the input resistance of these cells. This finding raises the possibility that changes in Cl^- levels in or around photoreceptors may regulate glutamate release. Some of these results have been presented previously in abstract form (Thoreson and Miller, 1993b).

MATERIALS AND METHODS

Whole-cell patch clamp recordings were obtained from ON bipolar cells in a superfused mudpuppy (*Necturus maculosus*) retinal slice preparation similar to that developed by Werblin (1978) and described in detail by Wu (1987). Surgery was done under dim room lights to preserve light responses. Although this procedure causes some light adaptation, light responses could be recorded from all classes of retinal neurons, including rods (Fig. 1). A single-pass, gravity-feed perfusion system delivered medium to the slice chamber at 0.6–0.8 ml/min. The control medium

contained 111 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES. The Cl-free medium contained 111 mM $NaCH_3SO_4$, 2.5 mM KCH_3SO_4, 1.8 mM $CaSO_4$, 0.5 mM $MgSO_4$, 5 mM glucose, and 10 mM HEPES. Picrotoxin (100 μM) and strychnine (1 μM) were added to both Cl-free and control media to suppress GABA- and glycine-sensitive Cl^- conductances. In some experiments, L-AP4 (5 μM), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX, 10 μM), $CoCl_2$ (2 mM), or $CdCl_2$ (0.5 mM) were added to the medium. The pH was adjusted to 7.8 with NaOH and bubbled continuously with 100% O_2. Experiments were done at room temperature (20–25°C).

Patch pipettes were pulled from borosilicate glass pipettes (omega dot, 1.2 mm OD, 0.95 mm ID) and had tips of ≈2 μM outer diameter (R = 4–10 MΩ). The electrodes contained (in mM): 98 $CsCH_3SO_4$ (or KCH_3SO_4 or CsCl), 3.5 NaCl, 3 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 5 HEPES, 2 D-glucose, 1 glutathione, 1 Mg-ATP, and 0.5 GTP. The pH was adjusted to 7.2 with CsOH. The electrodes also contained 0.025% 5,6-carboxyfluorescein or 0.05% sulfarhodamine 101 to allow fluorescent visualization of the cell after recording.

A 3-M KCl/agar was used as a bridge to the Ag/AgCl reference electrode. The agar bridge was downstream from the retinal slices in the perfusion chamber so Cl^- leached from the agar would not reach the slices. With this arrangement, the junction potential observed after the switch to a Cl-free medium was ≤ 1 mV.

The series resistance was typically ~ 20 MΩ. The input resistance in darkness of ON bipolar cells averaged 987±770 MΩ (n = 26), OFF bipolar cells averaged 901 ± 732 MΩ (n = 17), and horizontal cells averaged 244 ± 201 MΩ (n = 9). Thus, the voltage error introduced by the series resistance was typically ~ 2% for bipolar cells and 10% for horizontal cells. To minimize the voltage error introduced by series resistance, cells were voltage clamped near their resting potentials where little holding current was required. Bipolar cells were therefore voltage clamped at −50 mV and horizontal cells and photoreceptors at −40 mV.

The light stimulus was a diffuse white light generated by a tungsten source and centered on the retina using a fiber optic illuminator (irradiance = 955 μW/cm²). Neutral density filters were used to vary stimulus intensity. Saturating light flashes were used for all experiments reported in this study.

Transmitter release from photoreceptors was directly stimulated by focal application of a hyperosmotic sucrose solution (control medium plus 0.5 M sucrose) applied by pressure ejection (2–20 psi; Picospritzer II; General Valve Corp., Fairfield, NJ) into the outer plexiform layer (OPL) from a large-diameter patch pipette (≈ 4 μM OD). The ejection pipette was placed at the outer edge of the OPL and approximately one cell diameter into the slice.

NBQX was kindly provided by T. Honoré (Novo Nordisk, Denmark), L-AP4 was obtained from Tocris Neuramin (Bristol, UK), and $NaCH_3SO_4$ and KCH_3SO_4 were obtained from Pfaltz and Bauer (Waterbury, CT). Fluorescent dyes were obtained from Molecular Probes, Inc. (Eugene, OR). All other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

ON and OFF bipolar cells and horizontal cells were identified using both physiological and anatomical criteria. The physiological criteria were as follows: (*a*) polarity of the light-evoked current (inward for ON bipolar cells and outward for OFF bipolar and horizontal cells), (*b*) outward current evoked by L-AP4

(5 μM) in ON bipolar cells, (c) current–voltage relationship (e.g., presence of a prominent, sustained, outward-rectifying current in bipolar cells and a prominent inward-rectifying current in horizontal cells), and (d) input resistance (high for bipolar cells and low for horizontal cells). Identification of the cell type was confirmed by examining the fluorescent, stained cell at the end of the experiment. Horizontal cells lie at the outer border of the inner nuclear layer and lack a process extending into the inner retina. OFF and ON bipolar cells have cell bodies in the outer inner nuclear layer and axons that terminate in sublamina A and B, respectively, of the inner plexiform layer. Rods and cones were identified by their characteristic outer segments. Four cells (two putative horizontal cells, one ON bipolar, and one OFF bipolar) were excluded from analysis because of uncertainties regarding their identification.

Although light responses could be recorded at the beginning of the day, bleaching (particularly after fluorescent illumination) typically abolished light responses by the end of the day. However, the finding that cells that were not responsive to light continued to respond to glutamate agonists and exhibited both large input resistances and small holding currents indicates that these were otherwise healthy neurons. These cells were therefore included in the analysis.

Errors are reported as ±SEM. Statistical significance was evaluated with analysis of variance (ANOVA) and paired and unpaired Student's t tests.

RESULTS

Light-evoked Responses and Steady-State Changes in Cl-free Medium

Fig. 1 shows light-evoked currents recorded under voltage clamp from different classes of outer retinal neurons before, during, and after superfusion with Cl-free medium. In control Ringer's, rod and cone light responses averaged 24.7±7.6 pA (n = 3) and 18.3±6.6 pA (n = 4), respectively. In agreement with the intracellular voltage recordings of Miller and Dacheux (1976), the removal of extracellular Cl⁻ did not significantly reduce the amplitude of light-evoked currents in rods and cones (Fig. 1). In a Cl-free medium, rod responses were 83±18% of control whereas cone responses were 120±14% of control.

Unlike the light-evoked currents of photoreceptors, the responses of second-order neurons were suppressed by removal of extracellular Cl⁻ (Fig. 1, see also Figs. 2 and 4). In control medium, ON bipolar cell light responses averaged 16.7±5.3 pA (n = 26) and ranged up to 128 pA. OFF bipolar cell light responses averaged 11.8±3.3 pA (n = 20) and ranged up to 55 pA. Horizontal cell responses averaged 52.3±22.7 pA (n = 12) and ranged up to 250 pA. In Cl-free solution, ON bipolar cell responses were reduced to 15±5% of control (n = 13, P = 0.004, paired t test), OFF bipolar cell responses to 22±6% (n = 11, P = 0.001), and horizontal cell responses to 22±8% (n = 10, P = 0.035).

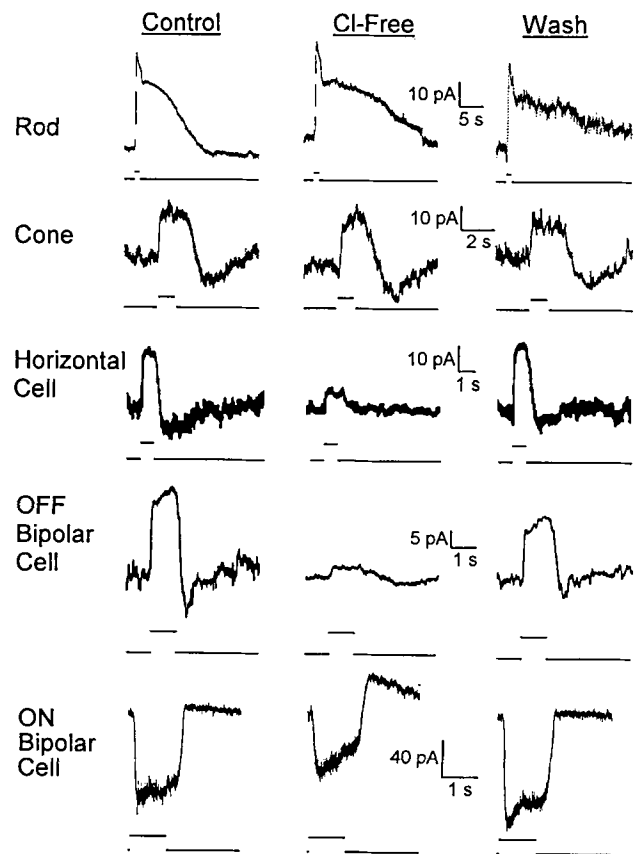The binding of [³H]AP4 and AP4-displaceable [³H]glu-

FIGURE 1. Light-evoked currents recorded under voltage clamp from a rod, cone, horizontal cell, OFF bipolar cell, and ON bipolar cell. At the left are control responses. In the center panel are responses recorded in Cl-free medium. For the ON bipolar cell, Cl-free medium was superfused for 2 min. For all other cells, responses were recorded after 4–5 min superfusion with Cl-free medium. At the right are responses recorded after washout for 5–10 min with the control medium. The photoreceptors and horizontal cells were voltage clamped at −40 mV, and the bipolar cells at −50 mV.

tamate are Cl⁻ dependent (Butcher et al., 1984; Fagg et al., 1983; Mitchell and Redburn, 1988). Thus, the loss of light responses in ON bipolar cells could hypothetically result from the inability of agonists to activate the L-AP4 receptor in the absence of extracellular Cl⁻. However, the ability of bath applied L-AP4 to evoke an outward membrane current was unaffected by removal of extracellular Cl⁻ (data not shown, n = 9), showing that binding to the L-AP4 receptor does not require extracellular Cl⁻.

If removing extracellular Cl⁻ suppresses the release of the photoreceptor neurotransmitter, L-glutamate, the following predictions should be fulfilled:

(a) Since glutamate release from photoreceptors is maximal in the dark, then removal of extracellular Cl⁻ in the dark should suppress an outward current in ON bipolar cells (resulting in a net inward current) and in-

ward currents in OFF bipolar and horizontal cells (resulting in net outward currents). In addition, since the amplitude of the light response reflects a change in glutamate release from photoreceptors, the change in dark current in a Cl-free medium should also be correlated with light response amplitude.

(*b*) The current induced by Cl^- removal should have a reversal potential consistent with cation, not Cl^-, channel activation.

(*c*) The suppression of glutamate release in the dark should decrease the input resistance of ON bipolar cells and increase resistance in OFF bipolar and horizontal cells. The amount of resistance change should also be correlated with light response amplitude.

Besides possible effects on glutamate release, removal of extracellular Cl^- can have direct effects on bipolar and horizontal cells via intrinsic Cl^- conductances in these neurons (Miller and Dacheux, 1983). If these cells possess a significant Cl^- conductance, the removal of extracellular Cl^- will cause an inward current as Cl^- leaves the cell. However, unlike a cation current change induced by suppression of tonic glutamate release, an inward current arising from intrinsic Cl^- conductances should subside as the Cl^- equilibrium is reestablished.

Fig. 2 illustrates the effects of a Cl-free Ringer's solution on the dark current. The traces show that Cl-free Ringer's solution suppressed the light responses of all three cell types and, as predicted, caused a net inward current in the ON bipolar cell and net outward currents in the OFF bipolar and horizontal cells. The initial inward current that is particularly evident for the horizontal cell (Fig. 2 *C*) probably reflects Cl^- efflux from the cell. Consistent with the suggestion that the net outward current in this horizontal cell arises from suppression of glutamate release and the initial inward current from intrinsic Cl^- conductances, the light response persisted during the inward current (*arrow*) and was not suppressed until the subsequent outward current appeared.

There was a significant positive correlation between the change in dark current induced by a Cl-free medium and the amplitude of the light response for all three cell types (data not shown; ON bipolar cells, $n = 24$, ANOVA $P < 0.049$; OFF bipolar cells, $n = 18$, $P < 0.003$; horizontal cells, $n = 9$, $P < 0.002$). The y-intercept was near 0 pA for all three cell types, consistent with the suggestion that the steady-state dark current changes induced by Cl-free Ringer's solution were largely due to changes in tonic glutamate output from photoreceptors.

In cells with light responses >10 pA and in which the major anion in the pipette Ringer's solution was CH_3SO_4 ($E_{Cl} = -59$ mV), the steady-state currents induced by Cl^- removal in ON bipolar, OFF bipolar, and

A ON Bipolar Cell
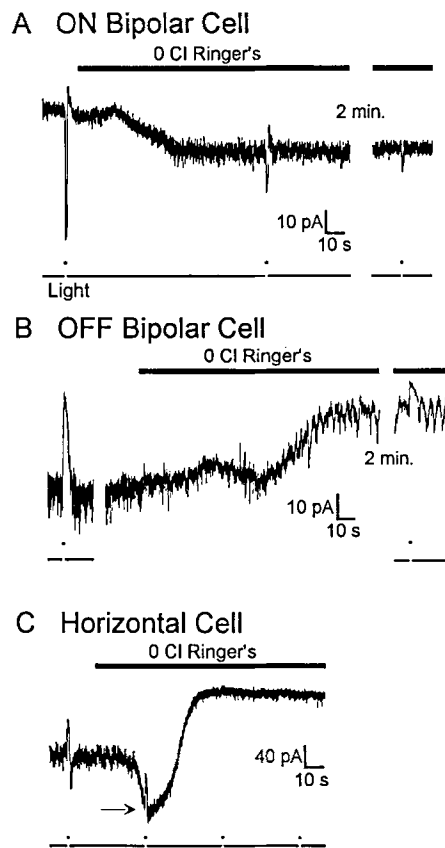
B OFF Bipolar Cell

C Horizontal Cell

Figure 2. Changes in dark- and light-evoked currents in ON bipolar (*A*), OFF bipolar (*B*), and horizontal (*C*) cells induced by Cl-free medium. Stimulus markers for light flashes are shown below each trace. Note that the light response of the horizontal cell was not suppressed during the transient inward current (*arrow*) but only during the subsequent steady-state outward current.

horizontal cells reversed at -11.3 ± 3.1 mV ($n = 10$), -14.3 ± 12.9 mV ($n = 3$), and -2.1 ± 7.8 mV ($n = 5$), respectively. This is similar to reversal potentials for light-evoked and glutamate-activated currents in these cell types and consistent with the hypothesis that these currents arise because of the suppression of glutamate release by Cl^- removal (Gilbertson et al., 1991, OFF bipolar cells, E_{rev} for $I_{Glu} = -1.9 \pm 1.2$ mV; Thoreson and Miller, 1993*a*, ON bipolar cells, E_{rev} for $I_{light} = -13.1 \pm 9.0$ mV; Low et al., 1991, horizontal cells, E_{rev} for $I_{light} = -14.1 \pm 3.4$ mV; Miyachi and Murakami, 1991, horizontal cells, E_{rev} for $I_{light} = +3.8 \pm 2.3$ mV).

Fig. 3 shows the actions of Cl-free Ringer's on the input resistances of bipolar and horizontal cells. The input resistance was calculated from the amplitude of the steady-state current observed in voltage clamp, after a step from -50 to -70 mV for bipolar cells and -40 to -60 mV for horizontal cells. In Fig. 3, *A–C*, the amount of change in input resistance after superfusion with Cl-free Ringer's is plotted against the amplitude of the light response recorded in control Ringer's. For ON bi-
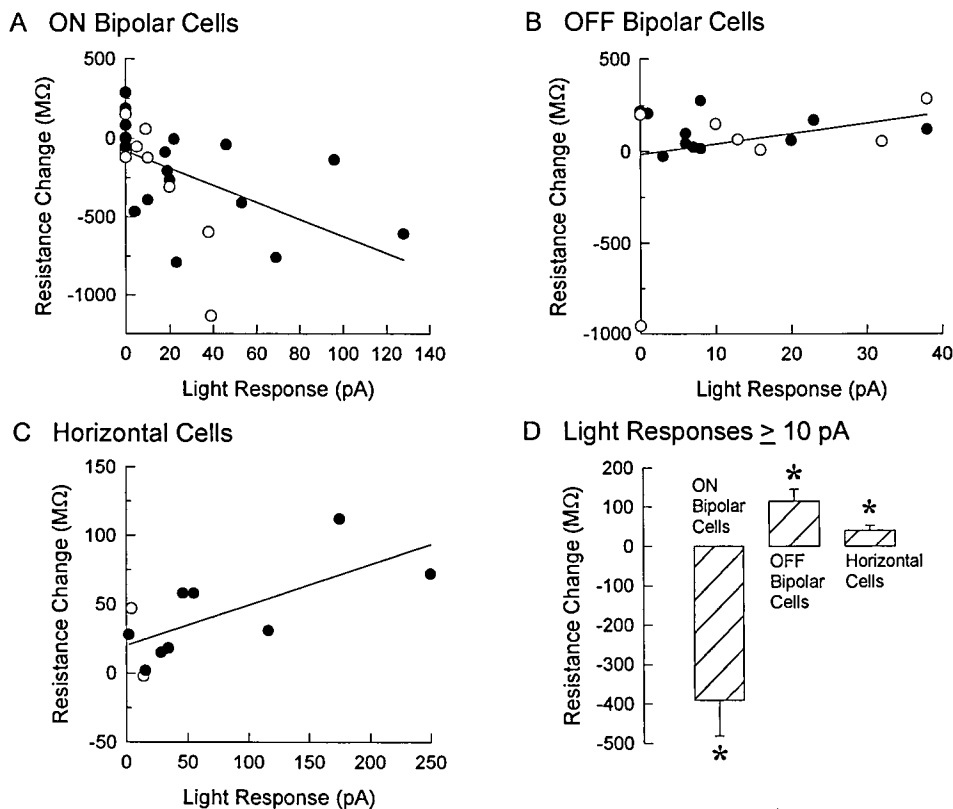
A ON Bipolar Cells

B OFF Bipolar Cells

C Horizontal Cells

D Light Responses ≥ 10 pA

FIGURE 3. Resistance changes in ON bipolar (A), OFF bipolar (B), and horizontal (C) cells induced by a Cl-free medium. Resistance changes are plotted against the amplitude of the control light response. Solid lines represent linear regressions (ON bipolar cells: slope $= -5.324$, y-intercept $= -86.454$ MΩ, ANOVA $P < 0.008$, $n = 26$; OFF bipolar cells: slope $= 5.785$, y-intercept $= -18.626$ MΩ, $P < 0.156$, $n = 18$; horizontal cells: slope $= 0.292$, y-intercept $= 20.281$ MΩ, $P < 0.018$, $n = 11$). Open circles are from experiments with CsCl intracellular Ringer's, and solid circles are from experiments with CsCH$_3$SO$_4$ intracellular Ringer's. The bar graph in D compares the average resistance changes ($\pm$SEM) in those bipolar and horizontal cells that exhibited peak light responses of at least 10 pA. Input resistance was significantly reduced in ON bipolar cells ($P < 0.001$, $n = 14$), OFF bipolar cells ($P < 0.008$, $n = 8$), and horizontal cells ($P < 0.012$, $n = 9$).

polar cells and horizontal cells, there was a significant positive correlation between light response amplitude in control Ringer's and the change in dark current caused by a switch to Cl-free Ringer's. OFF bipolar cells exhibited a similar correlation but it failed to achieve statistical significance for this sample of cells. The y-intercept of the regression line was near 0 for all three cell types, suggesting that the dominant Cl-sensitive conductance in these experiments was correlated with light response amplitude.

Fig. 3 D shows the average resistance change for all cells with light responses ≥ 10 pA. For these cells, there was a significant decrease in the input resistance of ON bipolar cells and a significant increase in the input resistances of OFF bipolar and horizontal cells.

The increase in input resistance in a Cl-free medium found in OFF bipolar cells with at least modest light responses (Fig. 3 D) could account for the persistence of light-evoked voltage responses in these cells, as first reported by Miller and Dacheux (1976), despite the suppression of light-evoked currents in these cells (Fig. 1). Fig. 4 shows an experiment in which input resistance (determined as described above for Fig. 3), voltage responses recorded in current clamp, and light-evoked currents recorded in voltage clamp were measured in both control and Cl-free media. In this experiment, the

67% reduction in light-evoked current in Cl-free medium was accompanied by a 70% increase in input resistance, so that the light-evoked voltage response decreased only 43%. Overall, the light-evoked voltage response was reduced only $32\pm10\%$ ($n = 10$), significantly ($P < 0.001$) less than the $78\pm6\%$ ($n = 11$) reduction in light-evoked current. Thus, like Miller and Dacheux (1976), our results show that light-evoked voltage responses of OFF bipolar cells can persist in Cl-free Ringer's.

Responses to Hyperosmotic Stimulation

To localize the site of the Cl-free induced suppression of ON bipolar cell responses, glutamate release from photoreceptor terminals was focally stimulated with a hyperosmotic sucrose solution (control medium plus 0.5 M sucrose) ejected by pressure (2–20 psi) from a patch pipette positioned in the OPL. When viewed through the microscope, transient movements of the cells in the region near the ejection pipette could be observed during hyperosmotic sucrose ejection. Tissue movement was increased by higher pressures and longer periods of ejection. Recordings from cells were often lost during these abrupt movements, which were probably due to both the pressure pulse and osmoti-
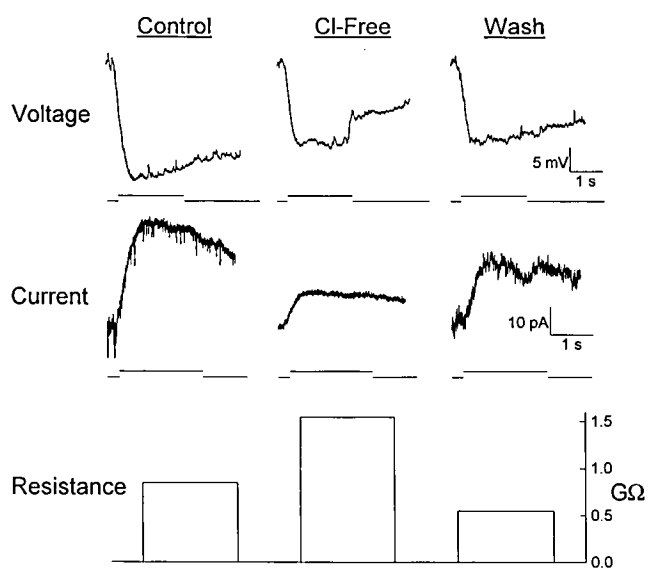
Figure 4. A comparison of light-evoked voltage, light-evoked current, and input resistance in an OFF bipolar cell in control medium, Cl-free medium, and after wash in control medium. The light-evoked current was reduced from 39 to 13 pA in Cl-free medium (33% of control), whereas the light-evoked voltage was reduced from 30 to 17 mV (57% of control). The smaller decrease in the voltage response can be accounted for by the increase in input resistance from 910 to 1,545 MΩ.

cally induced shrinkage. This was particularly a problem when examining sucrose effects in the Cl-free Ringer's solution, perhaps because the absence of extracellular Cl⁻ limited the extent to which Cl⁻ movement could compensate for osmotic transients. The actions of hyperosmotic sucrose on photoreceptors appeared to be limited largely to the osmotically stimulated terminal region of the cell, since sucrose evoked only small currents in photoreceptors (1.6 ± 1.6 pA, $n = 10$).

Fig. 5 A shows responses of a horizontal cell to hyperosmotic sucrose ejected into the OPL. In this cell, hyperosmotic sucrose evoked an inward current of 50 pA. The peak inward current evoked by hyperosmotic sucrose in horizontal cells averaged -26.2 ± 5.2 pA ($n =$ nine cells). An inward current is consistent with stimulation of glutamate release from photoreceptors resulting in the activation of KA/AMPA receptors on horizontal cells. In two cells, hyperosmotic sucrose evoked a small outward current that, as discussed in greater detail for bipolar cells below, may reflect direct actions of sucrose on the horizontal cell. Two other cells failed to respond at all to hyperosmotic sucrose ejected at pressures up to 20 psi.

As shown in the upper right of Fig. 5 A, superfusion with Cl-free medium reduced the amplitude of the osmotically evoked inward current from −50 to −7 pA. The response after washout (Fig. 5 A, control response

for NBQX application) showed partial recovery. The bar graph of Fig. 5 B summarizes results from all cells tested and shows that the inward current was significantly reduced by Cl-free medium to $21.8 \pm 8.5\%$ of control ($n = 7$). This is similar to the degree of suppression of the light response by Cl-free medium.

If hyperosmotic sucrose stimulates glutamate release from photoreceptor terminals and the inward current observed in horizontal cells reflects activation of KA/AMPA receptors, then the KA/AMPA-selective antagonist, NBQX, should suppress the osmotically evoked current. As shown in Fig. 5, A and B, the osmotically evoked inward current was significantly reduced by NBQX ($10 \mu M$). The response obtained after washout of NBQX in Fig. 5 A (the control response for Cd^{2+} application) showed partial recovery.

It was initially hoped that hyperosmotic sucrose would stimulate calcium-independent glutamate release from photoreceptors, as shown by others (Furshpan, 1956; Hubbard et al., 1968; Bekkers and Stevens, 1989; Maple et al., 1994). However, as shown in Fig. 5 (and in Fig. 6 for OFF bipolar cells), superfusion with Cd^{2+} (0.5 mM) or Co^{2+} (2 mM) significantly reduced the inward current evoked by hyperosmotic sucrose. Cd^{2+} and Co^{2+} block Ca^{2+} influx through voltage-sensitive Ca^{2+} channels so the suppression of osmotically evoked inward currents by these agents suggests that a significant fraction of the response to hyperosmotic stimulation is Ca^{2+} dependent.

Fig. 6 shows a series of experiments in OFF bipolar cells similar to those illustrated in Fig. 5 for horizontal cells. Hyperosmotic sucrose evoked inward currents in 13/28 OFF bipolar cells (46%), with the peak current averaging -20.1 ± 6.4 pA. In 11 cells (39%), hyperosmotic sucrose evoked an outward current, and no response was observed to pressures up to 20 psi in four cells (14%). Outward currents in OFF bipolar cells are examined further in Fig. 7.

In a Cl-free solution, the inward current evoked by hyperosmotic sucrose in OFF bipolar cells was significantly reduced to $17.5 \pm 23.9\%$ of control ($n = 5$). In the example shown in Fig. 6 A, a Cl-free Ringer's solution actually caused the response to invert from an inward current to a small outward current. This outward current may be unmasked and perhaps enhanced by the actions of Cl-free Ringer's solution and, as discussed below, may reflect direct effects of hyperosmotic sucrose on the OFF bipolar cell. The response obtained after washout (Fig. 6 A, control response for NBQX application) showed partial recovery.

Similar to the osmotically evoked inward currents in horizontal cells, NBQX ($10 \mu M$), Cd^{2+} (0.5 mM), and Co^{2+} (2 mM) significantly suppressed the inward current in OFF bipolar cells (Fig. 6). The control response for Cd^{2+} application in Fig. 6 A was the response ob-
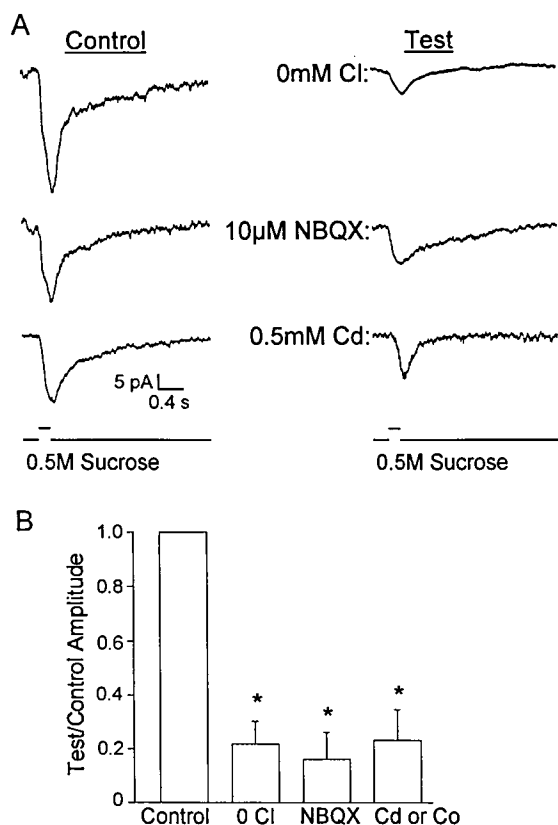
A

Control   **Test**

B

FIGURE 5. Effects on horizontal cells of focal stimulation of photoreceptor terminals with hyperosmotic sucrose solution (control Ringer's solution plus 0.5 M sucrose). (A) Examples of responses of the inward currents evoked by hyperosmotic sucrose in a horizontal cell. The inward current was suppressed by Cl-free medium, NBQX (10 μM), and Cd^{2+} (0.5 mM). The control response for NBQX was the response obtained after washout of Cl-free medium. Similarly, the control response for Cd^{2+} was the response obtained after washout of NBQX. Thus, there was partial recovery in both cases. (B) Bar graph summarizing the results of pharmacological experiments on inward currents evoked by hyperosmotic sucrose in horizontal cells. Responses in the presence of the test solution were divided by the amplitude of the prior response in control solution to yield a fractional response (test/control amplitude). Statistical significance was evaluated with a one-sample t test comparing the test fractional response with the control fractional response (which is by definition equal to 1). The same procedure was used for the bar graph in Fig. 7. The inward currents were significantly reduced by Cl-free medium ($P < 0.001$, $n = 7$), NBQX (10 μM, $P = 0.001$, $n = 5$), and Cd^{2+}(0.5 mM) or Co^{2+} (2 mM) ($P = 0.007$, $n = 4$).

tained after washout of NBQX. The results of Figs. 5 and 6 are therefore consistent with the hypothesis that hyperosmotic sucrose stimulates Ca^{2+}-dependent glutamate release from photoreceptors and that this glutamate release is suppressed by removal of extracellular Cl^-.

As mentioned above, 11/28 OFF bipolar cells evoked outward currents in response to hyperosmotic sucrose.

A

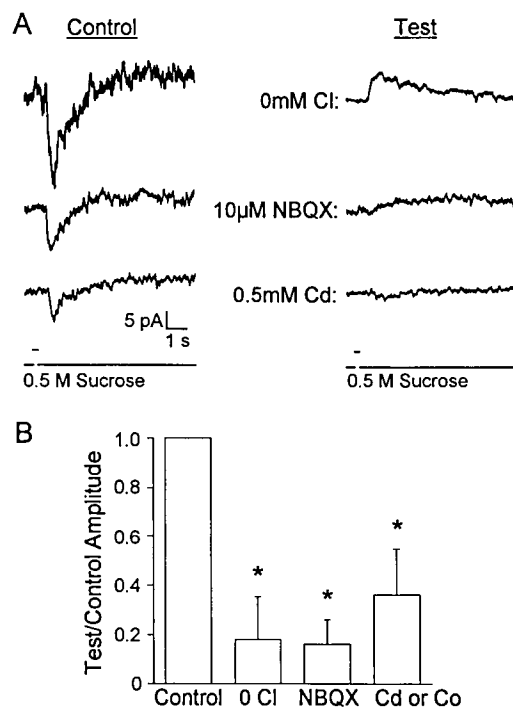**Control**   **Test**

B

FIGURE 6. Effects on OFF bipolar cells of focal stimulation of photoreceptor terminals with hyperosmotic sucrose (0.5 M). (A) Examples of responses of the inward currents evoked by hyperosmotic sucrose in an OFF bipolar cell. The inward current was suppressed by Cl-free medium, NBQX (10 μM), and Cd^{2+} (0.5 mM). The control response for NBQX was the response obtained after washout of Cl-free medium. Similarly, the control response for Cd^{2+} was the response obtained after washout of NBQX. Thus, there was partial recovery in both cases. (B) Bar graph summarizing the results of pharmacological experiments on inward currents evoked by hyperosmotic sucrose in OFF bipolar cells. The inward currents were significantly reduced by Cl-free medium ($P = 0.009$, $n = 5$), NBQX (10 μM, $P = 0.004$, $n = 5$), and Cd^{2+} (0.5 mM) or Co^{2+} (2 mM) ($P = 0.027$, $n = 5$).

Fig. 7 illustrates two experiments designed to determine the origin of these outward currents. Fig. 7 A shows the currents evoked at a series of different holding potentials in an OFF bipolar cell. In this cell, the response consists of a fast current that is temporally correlated with the stimulus and a more slowly decaying component. Both components of the current are outward at negative holding potentials and appear to reverse between -1 and $+20$ mV. In five out of five cells that exhibited outward currents at negative holding potentials, the currents reversed near 0 mV. The intracellular Ringer's solution in the experiment illustrated in Fig. 7 A and in three out of five cells tested contained $CH_3SO_4^-$ as the predominant anion. In the other two experiments, Cl^- was the predominant anion. The finding that the reversal potential for the outward current was near 0 mV regardless of pipette [Cl^-] suggests that the response arises from a nonselective ion current.

A
+40 mV
+20 mV
-1 mV
-22 mV
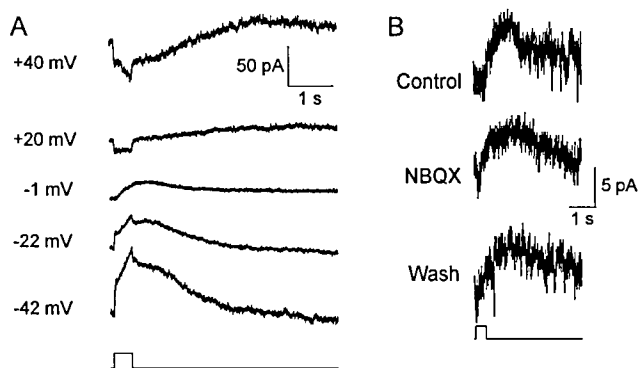-42 mV

50 pA
1 s

B
Control
NBQX
Wash

5 pA
1 s

FIGURE 7. Outward currents evoked in OFF bipolar cells at the holding potential of −50 mV by hyperosmotic sucrose. (A) A series of recordings from an OFF bipolar cell in which hyperosmotic sucrose evoked an outward current that reversed between −1 and +20 mV. The holding potential values in this figure were adjusted to compensate for the series resistance in this experiment. (B) Effects of NBQX (10 μM) on the outward current evoked by hyperosmotic sucrose in an OFF bipolar cell.

Fig. 7 B shows that the outward current associated with hyperosmotic stimulation was not significantly reduced by the application of NBQX, consistent with the hypothesis that the current does not result from the osmotically stimulated release of glutamate from photoreceptors. Similar results were obtained in five additional cells.

Based on the results in OFF bipolar cells, it appears that there are two components to the postsynaptic current evoked by hyperosmotic sucrose, an NBQX-sensitive inward current caused by the osmotically evoked release of glutamate and an NBQX-insensitive outward current due to direct actions of hyperosmotic sucrose on the bipolar cell. In ON bipolar cells, glutamate evokes outward currents (Thoreson and Miller, 1993a), so both the evoked and direct components of the response to hyperosmotic sucrose should produce outward currents.

At the holding potential of −50 mV, hyperosmotic sucrose evoked outward currents in 40/54 ON bipolar cells that averaged +14.2±1.8 pA (e.g., see Fig. 8). Sucrose evoked inward currents in three cells, and no response was observed in 11 cells. The outward currents evoked by hyperosmotic sucrose could be reversed near 0 mV in six cells (data not shown). Five of these six cells were recorded with pipettes containing $CH_3SO_4^-$ as the major cation. In three additional cells, the response was reduced at positive holding potentials but not reversed. These results suggest that the outward current evoked by hyperosmotic sucrose arises from current flow through a nonselective ion channel. If, as suggested above, the actions of hyperosmotic sucrose involve both evoked glutamate release and direct effects on the ON bipolar cell, these two components appear to have similar reversal potentials.

If hyperosmotic sucrose acts directly at ON bipolar cells to stimulate an outward cation current, then making the cation gradient inward during hyperosmotic stimulation should reverse the direct component of the current. In hyperosmotic sucrose, the osmotic depletion of intracellular water transiently raises the intracellular cation concentration, which should generate an outward cation current. If the osmolarity is instead raised with 0.25 M $NaCH_3SO_4$, then despite the osmotic depletion of water from the cell there should still be an inward cation driving force. $CH_3SO_4^-$ is relatively impermeant and thus should not permit an accompanying inward anion current. Consistent with this prediction, in 14 ON bipolar cells, pressure ejection of 0.25 M $NaCH_3SO_4$ produced either a monophasic inward current (6/14) or a biphasic response consisting of a transient inward current followed by a sustained outward current (8/14, data not shown). Whereas the inward current presumably reflects direct actions at ON bipolar cells, the sustained outward current may be due to evoked glutamate release since L-AP4 (5 μM) suppressed the outward current in three out of three cells tested (data not shown).

As shown in Fig. 8, application of Cl-free medium suppressed the outward current evoked by hyperosmotic sucrose in some cells (e.g., cell A in Fig. 8) but enhanced the current in others (e.g., cell B in Fig. 8). Because of this mixture of effects, there was no significant effect of Cl-free Ringer's solution on the mean outward current evoked by hyperosmotic sucrose. The different effects of Cl-free medium in different cells may reflect differences in the relative contributions of the direct and evoked components of osmotically stimulated currents. The observation that osmotically evoked currents in some ON bipolar cells were suppressed in Cl-free medium is consistent with the hypothesis that removal of Cl suppresses photoreceptor transmitter release onto these cells. However, the presence of two components to the outward current greatly complicated interpretation of the results in ON bipolar cells, so responses in these cells were not analyzed further.

DISCUSSION

The results of the present study suggest that synaptic transmission from photoreceptors to second-order neurons is suppressed when external Cl⁻ ions are removed from the bathing medium. There is substantial experimental evidence to support the idea that neurotransmission from photoreceptors is glutamatergic and that the light-evoked currents of second-order neurons (horizontal, ON bipolar, and OFF bipolar cells) in the amphibian retina are dependent on modulation of cation conductances (Miller and Slaughter, 1986; Copenhagen and Jahr, 1989; Ayoub et al., 1989; Lasansky,

1992; Thoreson and Miller, 1993a; Gilbertson et al., 1991; Attwell et al., 1987). Thus, whereas removal of extracellular Cl⁻ suppresses this glutamatergic synapse, it does not appear to do so through Cl⁻ dependence of the charge carrier at glutamatergic synapses. It is possible that a similar phenomenon may occur at cholinergic synapses in the cochlea since Cl-removal suppresses efferent, cholinergic transmission onto hair cells (Desmedt and Robertson, 1975) but acetylcholine activates cation, not chloride, channels in these cells (Fuchs and Murrow, 1992). At most central nervous system synapses, the dominant effect of removing extracellular Cl⁻ is to remove GABA-ergic and glycinergic inhibition, thus enhancing excitability (Barker and Nicoll, 1973; Yamamoto and Kawai, 1967). It appears that an unusual dependence on extracellular Cl⁻ characterizes the glutamatergic neurotransmission from photoreceptors onto second-order neurons. It is therefore important to establish the mechanism by which Cl⁻ ions control this critical site of cell communication for the visual pathway.

Light-evoked Responses and Steady-State Changes in Cl-free Medium

Consistent with the voltage recordings of Miller and Dacheux (1976), removal of extracellular Cl⁻ did not suppress light-evoked currents of rod and cone photoreceptors, showing that phototransduction was not compromised (Fig. 1). In contrast, light-evoked currents were suppressed in horizontal, ON bipolar, and OFF bipolar cells after Cl⁻removal. The suppression of light-evoked currents shows that the suppression of voltage responses in ON bipolar and horizontal cells was not caused solely by shunting of the membrane voltage in Cl-free medium.

Miller and Dacheux (1976) had reported that OFF
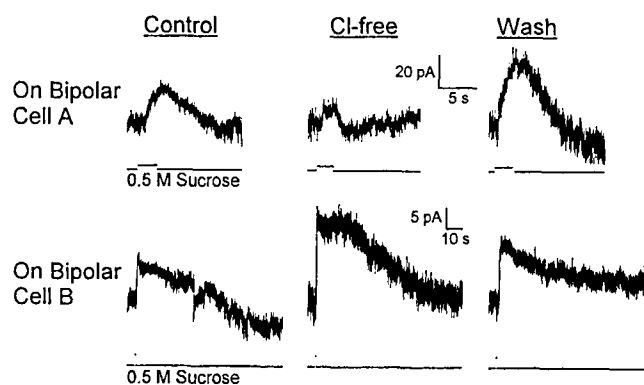

FIGURE 8. Outward currents evoked by hyperosmotic sucrose in two different ON bipolar cells in control medium, Cl-free medium, and after wash in control medium. In cell A, Cl-free medium suppressed the outward current. In cell B, Cl-free medium enhanced the outward current.

bipolar cell light-evoked voltage responses persisted in Cl-free medium, but we found that light-evoked currents of OFF bipolar cells were suppressed. The persistence of light-evoked voltage responses in OFF bipolar cells, despite the suppression of light-evoked currents, appears to be due to a compensatory increase in OFF bipolar cell input resistance (Figs. 3 and 4). Conversely, in ON bipolar cells, the decrease in input resistance should amplify the reduction of light-evoked voltage responses.

The binding of [³H]AP4 and AP4-displaceable [³H]glutamate is Cl⁻ dependent (Butcher et al., 1984; Fagg et al., 1983; Mitchell and Redburn, 1988). However, the outward current evoked by bath application of L-AP4 was unaffected by superfusion with Cl-free medium, which indicates that the binding of L-AP4 receptors in ON bipolar cells is not Cl⁻ dependent. This Cl⁻ independent activation of L-AP4 receptors is consistent with the finding that AP4 binding sites exhibit a different pharmacological sensitivity than electrophysiologically identified L-AP4 receptors and thus probably represent different sites (Fagg and Lanthorn, 1985; Crooks et al., 1986).

The results described above indicate that suppression of light responses in second-order neurons is not caused by suppression of phototransduction, shunting of postsynaptic membrane voltage, interference with receptor binding, or synaptic modulation of Cl⁻ channels in second-order neurons. Instead, we hypothesize that the suppression of light-evoked currents in bipolar and horizontal cells was caused by suppression of glutamate release from photoreceptor terminals.

Light responses of second-order neurons result from the modulation of tonic glutamate release from photoreceptors. If removal of extracellular Cl⁻ suppresses this glutamate release, then, in OFF bipolar and horizontal cells, one should observe a net outward current and increase in input resistance. Conversely, in ON bipolar cells, suppression of glutamate release should produce a net inward current and decrease in input resistance. However, these predictions are only true to the extent that there is tonic glutamatergic input into the second-order neuron. Light responses in neurons in the retinal slice preparation varied considerably in amplitude. Smaller light responses probably resulted from light adaptation, depletion of stores of glutamate in photoreceptors, or loss of postsynaptic glutamate receptors (e.g., severed by the slice technique). The first two would reduce glutamate release from photoreceptors and the last would reduce postsynaptic sensitivity to glutamate. Thus, light response amplitude can be used as a measure of tonic glutamatergic input into a cell. If a Cl-free Ringer's solution reduces glutamate release, then the input resistance and dark current changes should be correlated with light response amplitude. With the exception that the correlation be-

tween resistance change and light response amplitude in OFF bipolar cells did not achieve statistical significance, these predictions are generally fulfilled. Furthermore, the steady-state change in dark current in cells with at least modest light responses (>10 pA) reversed near 0 mV, consistent with suppression of glutamate-sensitive cation currents.

Hyperosmotic Sucrose Experiments

To test our hypothesis further, hyperosmotic sucrose was focally applied to the photoreceptor terminals to stimulate glutamate release directly. The results obtained with this approach suggest that hyperosmotic sucrose evokes a postsynaptic current that consists of two components: one caused by the evoked release of glutamate and the other an outward current caused by the direct actions of hyperosmotic solutions on the postsynaptic neuron. Differences in the proportions of these two components in different experiments may reflect differences in pipette placement and glutamate stores available for release from photoreceptor terminals.

In horizontal and OFF bipolar cells, the inward current evoked by hyperosmotic sucrose was suppressed by the KA/AMPA antagonist, NBQX (Figs. 5 and 6). This suggests that the inward current involves the activation of KA/AMPA receptors in these cells. In support of the hypothesis that removing extracellular Cl$^-$ suppresses the release of glutamate from photoreceptor terminals, Cl$^-$ removal also suppressed the inward current evoked by hyperosmotic sucrose in both horizontal and OFF bipolar cells (Figs. 5 and 6).

An outward current was evoked by hyperosmotic sucrose in 39% of OFF bipolar cells, and this outward current was unaffected by NBQX (Fig. 7), suggesting that this response does not involve glutamate actions at KA/AMPA receptors and is instead a direct effect of hyperosmotic sucrose on the bipolar cell. The reversal potential of the outward current was near 0 mV (Fig. 7 *A*), regardless of pipette [Cl$^-$], suggesting that it may be a nonselective ion current. Increasing the extracellular osmolarity would cause an osmotic efflux of water that would in turn transiently raise the intracellular ion concentration, causing an outward ion flux. The outward current produced by hyperosmotic sucrose could therefore reflect a cation flux out of the bipolar cell. If there is an accompanying Cl$^-$ efflux, this would reduce the amplitude of the outward current. Depletion of intracellular Cl$^-$ after the removal of extracellular Cl$^-$ would reduce this accompanying Cl$^-$ efflux, which would in turn enhance the outward current. This may account for the apparent unmasking of an outward current by a Cl-free medium in the OFF bipolar cell response illustrated in Fig. 6 and contribute to the enhancement of the outward current by Cl-free medium in ON bipolar cell B of Fig. 8.

Direct actions of hyperosmotic sucrose and the actions of evoked glutamate release should both produce outward currents in ON bipolar cells. To test for the presence of a direct cation current, we used a hyperosmotic NaCH$_3$SO$_4$ solution, in which the cation gradient and thus the direct current should be inward. The use of hyperosmotic NaCH$_3$SO$_4$ consistently evoked a biphasic or purely inward current. Thus, like OFF bipolar cells, the response to hyperosmotic sucrose appears to involve both evoked glutamate release and an osmotically stimulated cation efflux from ON bipolar cells.

The complicated interactions between outward currents arising from evoked glutamate release and direct hyperosmotic effects in ON bipolar cells make interpretation of hyperosmotic stimulation in this cell type particularly difficult. Although, when averaging over the sample, there was no significant effect of Cl-removal on the osmotically evoked outward current in ON bipolar cells, removal of extracellular Cl$^-$ suppressed the steady outward current in some cells as predicted (e.g., cell A, Fig. 8). We hypothesize that responses in which the Cl-free medium suppressed the outward current were dominated by evoked glutamate release (e.g., cell A, Fig. 8) and responses in which Cl-free medium enhanced the outward current were dominated by the direct component of the response (e.g., cell B, Fig. 8).

Hyperosmotic sucrose has been shown to enhance the probability of Ca^{2+}-independent vesicular release in the neuromuscular junction, hippocampal neurons, and amphibian retina (Furshpan, 1956; Hubbard et al., 1968; Bekkers and Stevens, 1989; Maple et al., 1994). However, in our experiments with OFF bipolar and horizontal cells, the inward current evoked by hyperosmotic sucrose was suppressed by Co^{2+} or Cd^{2+} (Figs. 5 and 6). Although the results of Maple et al. (1994) show that there is a Ca^{2+}-independent component to the release stimulated by hyperosmotic solutions, our results suggest that most of the glutamate release from photoreceptors observed during hyperosmotic stimulation is Ca^{2+} dependent. A similar Ca^{2+} dependence was observed in the transmitter release evoked by hyperosmotic sucrose in ganglion cells (Yu and Miller, 1995). Thus, although providing support for the localization of the actions of Cl-free medium to the photoreceptor terminals, focal hyperosmotic stimulation does not, unfortunately, allow the separation of Ca^{2+}-dependent and Ca^{2+}-independent steps in the release of glutamate from photoreceptors.

Possible Mechanisms and Functional Implications

The results presented above indicate that removal of extracellular Cl$^-$ suppresses glutamate release from photoreceptor terminals. A number of possible mechanisms could account for this suppression. One possibility is that a depletion of intracellular Cl$^-$ in a Cl-free en-

vironment may disrupt glutamate uptake into the pre-synaptic terminal or synaptic vesicles (Mitchell and Redburn, 1988; Naito and Ueda, 1985). Another possibility is that this suppression is an indirect effect of pH. Acidification can suppress cone to horizontal cell neurotransmission (Harsanyi and Mangel, 1993) by suppressing dihydropyridine-sensitive Ca^{2+} channels in photoreceptors (Barnes and Bui, 1991). However, the most likely way in which Cl^- removal would affect pH is by blocking Cl^-/HCO_3^- exchange, and these experiments were done using HCO_3^--free Ringer's solutions. Furthermore, Cl^- removal does not appear to alter intracellular pH significantly (Nitzan and Miller, 1994). Parallel electrophysiological and imaging experiments show that removal of Cl^- suppresses dihydropyridine-sensitive Ca^{2+} currents in photoreceptors in a pH-independent manner and that this suppression in turn blocks transmitter release (Nitzan and Miller, 1994; Thoreson, 1995).

The present results raise the possibility that regulation of Cl^- levels in and around photoreceptors may in turn regulate glutamate release from these cells. Consistent with this possibility, reducing $[Cl^-]$ by only 10 mM appreciably suppresses light-evoked currents in second-order retinal neurons (Thoreson, 1995). Photoreceptors can regulate $[Cl^-]$ by Cl^-/HCO_3^- exchange, a Cl^- conductance coupled to glutamate up-take, GABA-activated Cl^- channels, and Ca^{2+}-activated Cl^- channels (Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Sarantis et al., 1988; Kaneko and Tachibana, 1986; Koskelainen et al., 1993; Koskelainen et al., 1994). Cl-dependent mechanisms in neighboring cells are also likely to be important in regulating $[Cl^-]_e$ around photoreceptors (Yamashita and Yamamoto, 1991). Given these many Cl^- conductances and transport mechanisms, many possible functions of Cl^- regulation can be imagined. For example, the negative synaptic feedback from horizontal cells to cones involves both GABA-sensitive and Ca^{2+}-activated Cl^- conductances (Kaneko and Tachibana, 1986; Thoreson and Burkhardt, 1991; Barnes and Deschênes, 1992), raising the possibility that cone feedback may not only regulate glutamate release by influencing cone membrane potential, but could also regulate this release via changes in $[Cl^-]$. Activity-dependent changes in $[Cl^-]$ that influence transmitter release might also contribute to postreceptoral processes of light and dark adaptation or alter the balance between the activity of OFF bipolar cells (which are relatively insensitive to Cl^- changes due to the compensatory increase in input resistance) and ON bipolar cells. Further study is needed to evaluate the potential physiological role(s) for Cl^- regulation of glutamate release.

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