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Cone photoreceptors respond to their own glutamate release in the tiger salamander

(presynaptic/transporter/receptor/Cl^- channel/retina)

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ABSTRACT **Pulse-like currents resembling miniature postsynaptic currents were recorded in patch-clamped isolated cones from the tiger salamander retina. The events were absent in isolated cones without synaptic terminals. The frequency of events was increased by either raising the osmotic pressure or depolarizing the cell. It was decreased by the application of either glutamate or the glutamate-transport blockers dihydrokainate and D,L-*threo*-3-hydroxyaspartate. The events required external Na^+ for which Li^+ could not substitute. The reversal potential of these currents followed the equilibrium potential for Cl^- when internal Cl^- concentration was changed. Thus, these miniature currents appear to represent the presynaptic activation of the glutamate receptor with glutamate transporter-like pharmacology, caused by the photoreceptor's own vesicular glutamate release. Using a noninvasive method to preserve the intracellular Cl^- concentration, we showed that glutamate elicits an outward current in isolated cones. Fluorescence of the membrane-permeable form of fura-2 was used to monitor Ca^{2+} entry at the cone terminal as a measure of membrane depolarization. The increase in intracellular Ca^{2+} concentration, elicited by puff application of 30 mM KCl, was completely suppressed in the presence of 100 μM glutamate. Puff application of glutamate alone had no measurable depolarizing effect. These results suggest that the equilibrium potential for Cl^-, E_{Cl}, was more negative than the activation range for Ca^{2+} channels and that glutamate elicited an outward current, hyperpolarizing the cones.**

Vertebrate photoreceptors do not spike; changes in light intensity are coded as continuous changes in membrane potential (1, 2). These potential changes are thought to be communicated to postsynaptic cells as continuous changes in the concentration of the neurotransmitter glutamate at the synaptic terminals of photoreceptors (3–7). Glutamate is thought to be released in the synaptic cleft by conventional Ca^{2+}-mediated exocytosis (3–7) and to be removed by either diffusion or uptake in both retinal glia (8) and photoreceptor terminals (9, 10).

It has recently been shown that exogenous glutamate application elicits a current at the photoreceptor terminal by gating a glutamate receptor with glutamate transporter-like pharmacology coupled to a Cl^- channel (11–15). This Cl^- current was not elicited by agonists of classical glutamate receptors (*N*-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, and kainate) or blocked by glutamate receptor antagonists, but it was elicited by substrates of glutamate transporters and blocked by glutamate-transport blockers (13, 14). The glutamate-gated Cl^- current required the presence of external Na^+ (11, 13), for which Li^+ could not substitute (13), similar to the activation of Na^+-dependent transporters. These glutamate transporter-like properties suggested to us that a glutamate transporter is gating a Cl^- channel in photoreceptors (15). This hypothesis was supported by the expression of several cloned glutamate transporters that coexpressed with a Cl^- conductance in oocytes (16).

In this study, we show that the glutamate-gated Cl^- channel is activated in isolated cones by their own vesicular release of glutamate and that the glutamate-elicited current is outward within the activation range of Ca^{2+} channels in photoreceptors.

EXPERIMENTAL PROCEDURES

Cell Preparation. Cone photoreceptors were mechanically dissociated from the retina of the larval tiger salamander (*Ambystoma tigrinum*). The retina was chopped into small pieces and triturated with a fire-polished Pasteur pipette. Cones were plated on glass and viewed with a phase-contrast objective (×40/0.65). Retinal slices were prepared and viewed as described earlier (17). The Ringer's solution, used for the dissociation and slice preparation, contained 108 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM KCl, 3 mM glucose, and 5 mM Hepes and was titrated to pH 7.75 with NaOH.

Patch-Clamp Recording. Unless specified otherwise, the cones were voltage-clamped at −50 mV with the whole-cell patch-clamp technique (18). Recording pipettes were pulled from a thick-walled glass [SE16, prism, Dagan Instruments (Minneapolis)]. The series resistance during the recording was usually <10 MΩ. Data were filtered through an eight-pole Bessel filter at 500 Hz [Frequency Devices (Haverhill, MA) model LP] and recorded through a T-1 interface [Axon Instruments (Burlingame, CA)] to an IBM-AT computer using the AXOTAPE program (Axon Instruments). Data were digitally filtered at 100 Hz for figure presentation. The recording pipette solution contained 119 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM $BaCl_2$, 0.1 mM Na_3GTP, 5 mM Na_2ATP, 1.27 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA; K^+ salt), and 4 mM Hepes and was titrated to pH 7.4 with KOH; 84 mM potassium gluconate was substituted for an equimolar concentration of KCl in the recording pipette solution to obtain the equilibrium potential for chloride, E_{Cl}, at −30mV. For the voltage-dependence of the events, the solution contained no $CaCl_2$, no $BaCl_2$, and no BAPTA but did contain 0.05 mM EGTA. The bathing solution contained 100.5 mM NaCl, 1 mM $MgCl_2$, 1 mM $BaCl_2$, 0.1 mM $CoCl_2$, 10 mM tetraethylammonium chloride, 3 mM glucose, and 5 mM Hepes and was titrated to pH 7.75 with NaOH. For the voltage-dependence of the events, the solution contained no $BaCl_2$ and no $CoCl_2$ but did contain 0.1 mM $CaCl_2$. The bathing solution was continuously applied via a gravity-driven superfusion system.

Ca^{2+} Optical Recording. The cones were plated on a concanavalin A (0.2 mg/ml type V, Sigma)-coated slide. The dye fura-2 [acetoxymethyl ester (AM); Molecular Probes] was bath-applied at 5 μM with 0.02% pluronic acid for 15 min in

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Abbreviations: mPSC, miniature postsynaptic currents; E_{Cl}, equilibrium potential for Cl^-; GABA, γ-aminobutyric acid.

the Ringer's solution. Optical recordings were acquired with a SIT camera [DAGE–MTI (Michigan City, IN); loaned by R. S. Zucker] and stored with a frame grabber (Imaging Technology, Woburn, MA). Replacing NaCl, 30 mM KCl was pressure ejected onto the photoreceptor terminal for 10–30 ms from a small-tip pipette (<1 μm).

RESULTS

Pulse-Like Currents Require Intact Terminal but No Outer Segment. Fig. 1*A* shows pulse-like currents recorded in isolated cone photoreceptors bathed in a Ca^{2+}-free solution, a condition that normally reduces transmitter release to spontaneous levels (19). These currents were highly reminiscent of miniature end-plate currents (20) normally caused in postsynaptic cells by the presynaptic transmitter release. The pulse-like events were recorded in all cones ($n > 100$) except those ($n = 5$) lacking a tuft of processes at the proximal end of the soma that is thought to represent the photoreceptor synaptic terminal. Most recorded cones had lost their outer segment during the isolation procedure, so the outer segment does not appear to be involved in generating these events. The events were also observed in cones with the outer segment intact ($n > 9$), indicating that they were not caused by the removal of the outer segment. Similar step-like currents were also recorded from cones in retinal slices ($n = 3$), suggesting that this phenomenon was not an artifact resulting from the isolation procedure. They were also observed in isolated rod photoreceptors ($n = 5$) with terminals. The following characterization may therefore apply to events seen in both rod and cone photoreceptors, although it was only carried in cones.

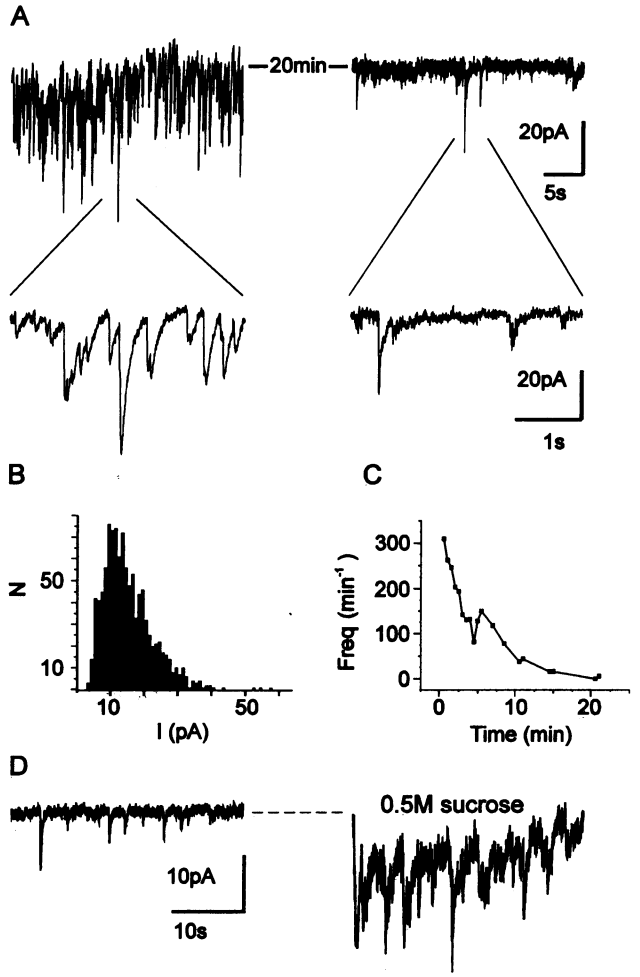

FIG. 1. The exocytotic origin of the pulse-like currents recorded from isolated cone photoreceptors. (*A*) Recording showing the pulse-like currents immediately after and 20 min after obtaining the whole-cell patch in a cone held at −50 mV. A part of each recording section is shown at a larger time scale. (*B*) Amplitude histogram of the pulse-like currents during the recording shown in *A*. N, number of events for the whole 20-min recording in *A*. (*C*) Frequency (Freq) of the pulse-like currents (≥8 pA) during the recording shown in *A*. Frequencies were calculated on 30-sec recording traces. (*D*) Recording showing the increase in frequency of pulse-like currents after application of 0.5 M sucrose.

Similar to the miniature postsynaptic currents recorded in bipolar cells (7), the frequency of events was greatly increased when osmotic pressure was increased by the addition of sucrose 0.5 M to the bathing solution (Fig. 1*D*). The rise time and decay of the currents could be approximated with single exponentials with time constants of 5.2 ± 0.9 ms and 37.0 ± 5.3 ms (SEM, $n = 6$ pulse-like currents), respectively. The currents were of variable magnitude (Fig. 1*A*) and did not follow a binomial distribution (Fig. 1*B*). As discussed for the pulse-like currents recorded in bipolar cells (7), this result does not imply that the events were generated in cones by the release of a single vesicle of transmitter. The frequency of events was observed to decrease in time over a period of about 20 min (Fig. 1 *A* and *C*).

Pulse-Like Currents Are Generated by the Glutamate Receptor with Glutamate Transporter-Like Pharmacology Coupled to the Cl^- Channel. The pulse-like currents appeared to be induced by the activation of a glutamate-gated but not γ-aminobutyric acid (GABA)-gated, Cl^- channel at the photoreceptor terminal. They were unaffected by bath application of 100 μM picrotoxin (PTX in Fig. 2*A*; $n = 5$ cells), a blocker of GABA-elicited currents in cones (21), but they were suppressed by application of exogenous 20–25 μM glutamate ($n = 19$ cells), as shown in Fig. 2*B*, which itself elicited a massive sustained current of 100–200 pA.

The currents had a pharmacology and Na^+ dependence similar to that previously established for the current elicited by application of exogenous glutamate in photoreceptors. Thus, 500 μM D,L-*threo*-3-hydroxyaspartate (THA in Fig. 2), a substrate for glutamate transporters (22), elicited a large sustained current and also suppressed the pulse-like events (Fig. 2*C*, $n = 7$ cells); and 4 mM dihydrokainate (DHKA in Fig. 2), a glutamate-transport blocker (22), greatly decreased the frequency of the pulse-like currents (Fig. 2*D*, $n = 6$ cells). The remaining events can be interpreted as the displacement of the competitive blocker dihydrokainate from the glutamate receptor after massive glutamate release. When Li^+ was substituted for external Na^+, the pulse-like events were also greatly suppressed (Fig. 3*A*, $n = 5$ cells).

The currents, resembling the current elicited by application of exogenous glutamate in photoreceptors (11, 12), appeared to be carried by Cl^-. Fig. 3*B* shows that the reversal potential for the spontaneous pulse-like currents was close to the calculated E_{Cl} at 1 mV ($n = 6$ cells) and −30 mV ($n = 4$ cells) when the concentration of Cl^- in the recording pipette was changed. The pharmacology and the Na^+/Cl^- dependence of these currents are consistent with the notion that the pulse-like currents are due to the activation of the glutamate-gated Cl^- channel.

Transporters were reported to operate asymmetrically and to be externally or internally gated (23–25). Since we have suggested that the Cl^- channel in photoreceptors might in fact be gated by a glutamate transporter, we verified that the synaptic events were not triggered internally. Isolated cones were recorded with a pipette solution containing 20 mM sodium glutamate (replacing 20 mM KCl) to buffer the internal concentrations of Na^+ and glutamate and prevent any large increase in these concentrations. Large events (>10 pA) were observed even 20 min after initiating the whole-cell recording. This observation supports the notion that the events

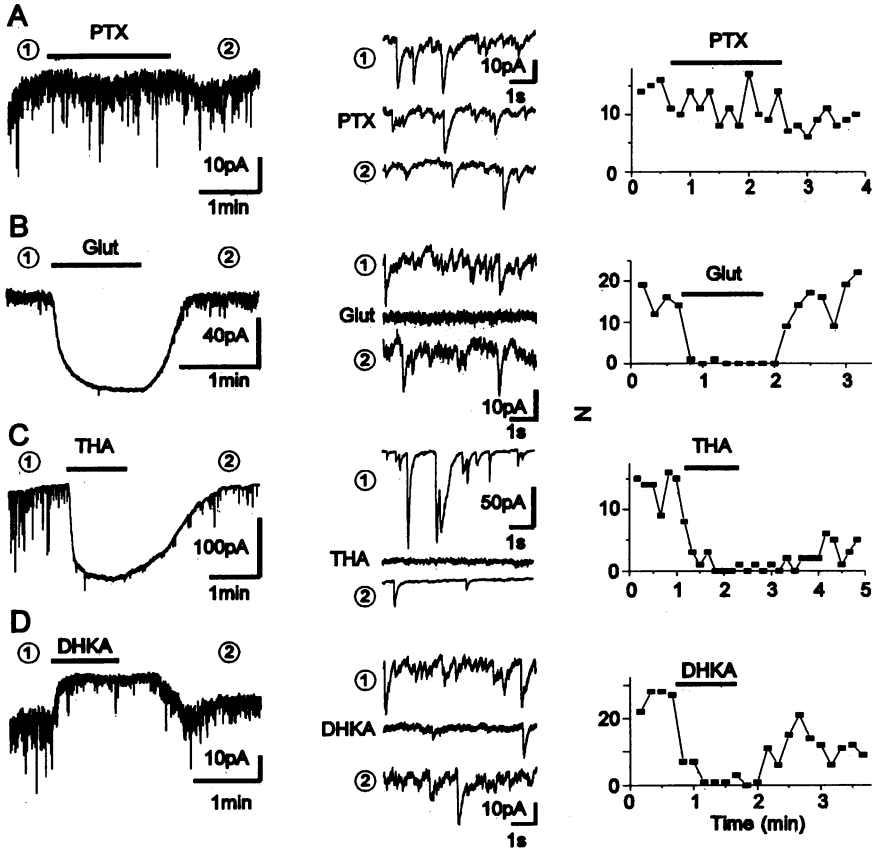

FIG. 2. Pharmacology of the pulse-like currents in isolated cones. The complete recording, before (*Left*), during (*Center*), and after (*Right*) the application of a substance, is always shown with an enlarged sequence in each of the three periods and with a plot representing the numbers of events (N) per 10 s during the recording (events in *A*, *B*, and *D* were > 4 pA and in *C* were > 7 pA). (*A*) Cone recording during application of 100 μM picrotoxin (PTX) showing no effect of this blocker of GABA-elicited Cl^- channel on the pulse-like currents. (*B*) Cone recording during application of exogenous 25 μM glutamate (Glut) showing the suppression of the pulse-like currents by exogenous glutamate. (*C*) Cone recording during application of 500 μM D,L-*threo*-3-hydroxyaspartate (THA), showing the suppression of the pulse-like currents by this substrate for glutamate transporters. (*D*) Cone recording during application of 4 mM dihydrokainate (DHKA), showing the suppression of the pulse-like currents by this glutamate-transport blocker.

were not internally gated but were more likely due to vesicular release of glutamate at the photoreceptor terminal.

Isolated Cones Respond to Their Own Glutamate Release. The glutamate-elicited pulse-like currents suggest that isolated photoreceptors respond to their own vesicular release. To verify that they do not respond to the release from adjacent or attached processes from other cells, we examined whether the frequency of events was dependent upon the voltage membrane of the recorded cone. For this experiments, the bathing solution contained 0.1 mM Ca^{2+}, and the intracellular Ca^{2+} concentration was not buffered as before (see *Experimental Procedures*) because the voltage-dependence of exocytosis is thought to be related to the voltage-dependent Ca^{2+} entry into the cell. When cones were depolarized from −65 mV to −50 mV (n = 5), the frequency of events was observed to increase from 162 to 267 events per min (Fig. 4). Note that the amplitude and the frequency of the events were expected to decrease at −50 mV because the Cl^- driving force decreases from −65 mV at this potential.

Glutamate-Gated Cl^- Current Hyperpolarizes the Cones. Does glutamate gate an inward or outward Cl^- current? To test this, we loaded isolated cones with the membrane-permeable form of the Ca^{2+} indicator dye fura-2 (Fig. 5 *A* and *B*) so that we could indirectly monitor membrane potential at the terminal without affecting the intracellular Cl^- concentration of photoreceptors. As a control, a puff (10–30 ms) of 30 mM KCl elicited a strong fluorescence decrease, indicating a depolarization-elicited Ca^{2+} influx at the cone terminal as shown in Fig. 5*C*. But in the presence of 100 μM glutamate, this Ca^{2+} signal was completely suppressed [99.6 ± 4.2% (SEM), n = 5 cells] (Fig. 5*C*), suggesting that the glutamate-elicited Cl^- current prevented the membrane from depolarizing into the activation range of the Ca^{2+} channels during the KCl puff. We applied a puff of glutamate alone and measured no Ca^{2+} signal, which ensures that the glutamate application did not elicit by itself a depolarization prior to the KCl puff. These results indicate that the glutamate application prevented activation of the Ca^{2+} channels, presumably by generating an outward (hyperpolarizing) Cl^- current maintaining the membrane at a potential more negative than the Ca^{2+} channels' activation range.

DISCUSSION

This study suggests that endogenously released glutamate acts at the glutamate-gated Cl^- channel described previously to generate pulse-like currents in isolated photoreceptors. These events do not appear to result from an artifact of the isolation procedure because they were also recorded from cones in retinal slices. The increase in the frequency of events when the osmotic pressure was increased supports the notion that these events are caused by vesicular release of glutamate. Glutamate appeared to be released by the recorded cone itself and not by processes from other cells impinging on this photoreceptor because the frequency of events was dependent upon the cone membrane potential. Since the glutamate-gated Cl^- channel was related to the glutamate transporter (15), this interpretation is consistent with the notion that transporters are located

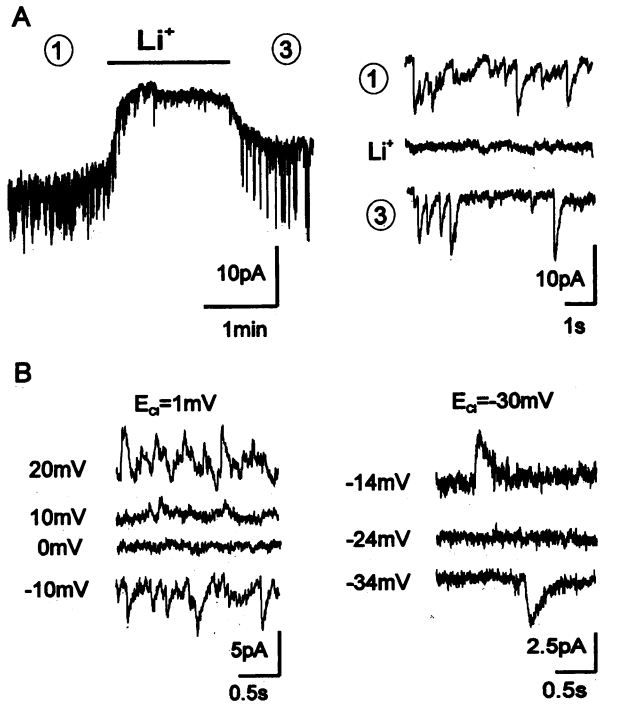

FIG. 3. Ionic dependence of the pulse-like currents. (*A*) Cone recording showing the absolute external Na^+ dependence of the pulse-like currents. Li^+ was substituted for external Na^+ where shown. (*B*) Cone recordings showing the shift in the reversal potential for the pulse-like currents when E_{Cl} was changed. E_{Cl} was set at a different value in each of these two cones by using different Cl^- concentrations in the recording pipette solution. The calculated E_{Cl} are shown above the records, whereas the holding potentials are indicated to the left of the recordings.

close to the site of transmitter release. Photoreceptors appear therefore to respond to their own vesicular release of glutamate.

Sarantis *et al.* proposed that the current generated by the glutamate-gated Cl^- channel was inward (depolarizing) (11). By contrast, our *in vitro* and noninvasive Ca^{2+} measurements suggest that glutamate generates an outward (hyperpolarizing) current. This interpretation is consistent with the notion that E_{Cl} is more negative than the operating range of cone photoreceptors, a notion previously inferred in two earlier studies by

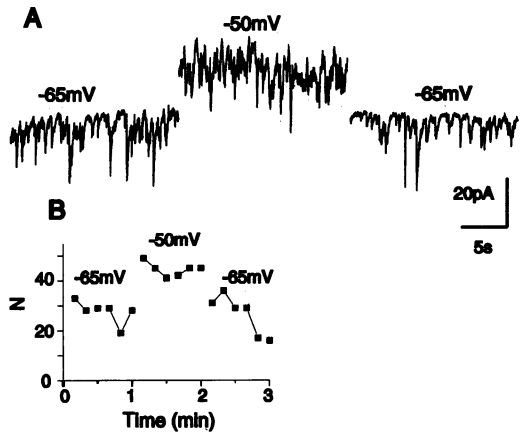

FIG. 4. Voltage dependence of the pulse-like currents. (*A*) Recording showing the increase in frequency of the pulse-like currents when the cone was depolarized from −65 mV to −50 mV. (*B*) Plot showing the numbers of events during three consecutive 1-min recordings at −65 mV, −50 mV, and −65 mV. Each point represents the numbers of events > 4 pA in a 10-sec period.

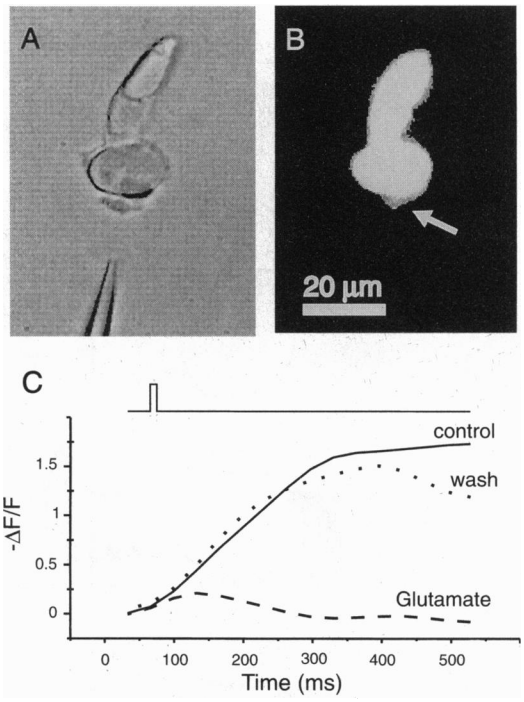

FIG. 5. Effect of the glutamate-elicited current on the Ca^{2+} influx at the photoreceptor terminal. (*A*) Bright-field image showing an isolated cone, typical of those used for experiments, with a puff pipette directed at the terminal. Note that the terminal is indicated by the tuft of processes located at the proximal end of the cell body. (*B*) Fluorescent image (380 nm) of the same isolated cone as in *A* stained with the acetoxymethyl ester form of fura-2. The arrow points to the photoreceptor terminal where the changes in intracellular Ca^{2+} were monitored optically. (*C*) Curves showing the different increases in internal Ca^{2+} concentration (decreased fluorescence) at the photoreceptor terminal after a 10-ms puff of 30 mM KCl in the absence (control, solid line; wash, dotted line) and presence (dashed line) of 100 µM glutamate. The upward deflection of the horizontal line above the curves indicates the timing of the 10-ms KCl puffs.

Attwell *et al.* (26) on the sign-reversing pathway from rods to cones and by Kaneko and Tachibana (27) on the $GABA_a$ receptor in cones. Glutamate may generate a large hyperpolarizing response: a puff of 500 µM glutamate hyperpolarized isolated cones from −30 mV to −43 mV (±1.8 mV SEM, n = 7) with E_{Cl} at −60 mV. However, Cl^- concentrations are dependent upon the homeostasis of all the other ions; therefore, they may differ substantially in cones in the intact retina from those under *in vitro* conditions.

Our interpretation from this study is that photoreceptors respond to the glutamate released at their terminal. The glutamate-elicited Cl^- current could provide a feedback signal about the concentration of glutamate released in the synaptic cleft. Since glutamate release and uptake are voltage-dependent, this feedback signal may enable photoreceptors to adjust their rate of glutamate release and uptake to control glutamate concentration in the synaptic cleft as a continuous function of the photocurrent amplitude (Fig. 5*D*). Such a feedback signal may be necessary because cones are spikeless neurons and light intensities are signaled to postsynaptic cells as graded glutamate concentrations. Therefore, it is necessary to lock the glutamate concentration in the synaptic cleft to the cone membrane potential in spite of variations that may arise in the release and uptake systems. Variations in glutamate content within synaptic vesicles (28), variations in the diffusion rate from the synaptic area, and variations in transporter uptake may occur as a result of dynamic changes in retinal geometry, chemistry, or temperature. These variations, which would otherwise affect the resulting glutamate concentration

in the synaptic cleft, might be offset by the control system we suggest here. The details of this control system, such as its open and closed loop gain as well as its dynamic characteristics, remain to be developed. These factors should determine the degree of control contributed by the transporter–Cl^- channel complex.

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