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Feedback from luminosity horizontal cells mediates depolarizing responses of chromaticity horizontal cells in the *Xenopus* retina

(color vision/glutamate/neurobiotin)

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ABSTRACT **It has been proposed that the depolarizing responses of chromaticity horizontal cells (C-HCs) to red light depend on a feedback signal from luminosity horizontal cells (L-HCs) to short-wavelength-sensitive cones in the retinas of lower vertebrates. In this regard we studied the C-HCs of the *Xenopus* retina. C-HCs and L-HCs were identified by physiological criteria and then injected with neurobiotin. The retina then was incubated with peanut agglutinin, which stains red- but not blue-sensitive cones. Electron microscopic examination revealed that L-HCs contact all cone classes, whereas C-HCs contact only blue-sensitive cones. Simultaneous recordings from C-HC/L-HC pairs established that when the L-HC was saturated by a steady bright red light, C-HCs alone responded to a superimposed blue stimulus. In response to red test flashes, the C-HC response was delayed by ≈30 msec with respect to the L-HC response. Isolated HCs of both subtypes were examined by whole-cell patch clamp. Both responded to kainate with sustained inward currents and to quisqualate or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) with desensitizing currents from a negative holding potential; i.e., both have AMPA-type glutamate receptors. γ-Aminobutyric acid or glycine opened a chloride channel in the L-HC, whereas the C-HC was unresponsive to either inhibitory amino acid. Since glycine has been shown to abolish selectively the depolarizing response of the C-HC, this finding and other pharmacological data strongly implicate the L-HC in the underlying circuit. Moreover, because the C-HC does not respond to γ-aminobutyric acid, the neurotransmitter of the L-HC, by elimination, a feedback synapse from L-HC to blue cone is the most plausible mechanism for the creation of depolarizing responses in C-HCs.**

Horizontal cells (HCs) are second-order neurons of the vertebrate retina. They are categorized into luminosity (L-HC) and chromaticity (C-HC) functional subtypes according to whether they respond only with hyperpolarizations to light stimuli of any color (L-HC) or are hyperpolarized by some colors and depolarized by others (C-HC) (1). L-HCs, in addition to being driven by cones, provided a recurrent input to cones, the so-called feedback synapse (2). Because hyperpolarization of the L-HC results in depolarization of the cone, with a delay, the feedback synapse is thought to be chemical, and in lower vertebrates is presumed to be mediated by γ-aminobutyric acid (GABA), the neurotransmitter of the L-HC (3).

Stell *et al.* (4) proposed that the feedback pathway was crucial to the creation of depolarizing responses in C-HC. They noted that C-HCs in a cyprinid fish contacted blue- or green-sensitive cones but did not make direct synaptic contacts with red-sensitive cones; these same C-HCs were nevertheless depolarized by red stimuli. In their scheme, a four-neuron chain organizes the depolarization: the red cone hyperpolarizes the L-HC, which causes it to relax a tonic GABA-dependent hyperpolarization of green- and blue-sensitive cones. Those cones depolarize, resulting in depolarization of the C-HC. On the other hand, there is evidence that in a turtle retina, C-HCs make direct contacts with red-sensitive cones (5). In addition it was reported (6) that in a holostean fish, the latency of the depolarizing C-HC response was shorter than that of the L-HC, a finding at variance with Stell's model (4). Burkhardt (7) has summarized these and other unresolved problems in relation to a feedback circuit mediating depolarizing responses in C-HCs.

In the present study, we present data on a dichromatic C-HC in the *Xenopus* retina, which is hyperpolarized by blue light and depolarized by red light (8). We show that the C-HC contacts only presumed blue-sensitive cones (9), whereas the L-HC in this retina contacts all cone subtypes. Electrophysiological and pharmacological data obtained from isolated L- and C-HCs and from eyecup preparations indicate that the depolarizing input to the C-HC depends on the responsiveness of the L-HC. Additionally, pharmacological evidence against a direct synaptic input from the L-HC to the C-HC is provided.

MATERIALS AND METHODS

Adult *Xenopus* males were obtained from Nasco (Ft. Atkinson, WI) and maintained in an aerated 300-gallon tank (1 gallon = 3.785 liters) on a 12-h light/12-h dark cycle, lights on at 0600. Prior to decapitation, the animal was anesthetized by a subcutaneous injection of 25 mg of tricaine methanesulfonate (Sigma) in 0.3 ml of Ringer's solution (8). An eyecup preparation superfused with a bicarbonate Ringer's solution (8) at 1.5 ml/min was used for intracellular recordings (cf. ref. 10). A two-channel photostimulator (11) provided light flashes of variable wavelength, duration, and intensity. Data were stored on digital tape for subsequent processing with SPIKE software (Modular Instruments, Taunton, MA). Whole-cell patch-clamp recordings were obtained from HCs isolated by papain treatment and maintained in short-term culture (cf. ref. 12 for patch-clamp methods and properties of isolated HCs in *Xenopus*). Glutamatergic drugs were obtained from Research Biochemicals (Natick, MA); amino acids were purchased from Sigma. For intracellular injections, the electrodes were filled with 4% (wt/vol) neurobiotin (Vector Laboratories) in 0.1 M Tris·HCl (pH 7.6). HCs were identified by physiological criteria and then injected for 10–30 min with a 1-Hz sinusoidal current with a peak-to-peak amplitude of 0.2–0.8 nA. After 30 min in normal Ringer's solution, the retina was isolated from the eyecup and incubated 30 min in 0.25% biotinylated peanut agglutinin (PNA) (Vector). Tissue then was fixed in 4%

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Abbreviations: L-HC, luminosity horizontal cell; C-HC, chromaticity horizontal cell; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA, γ-aminobutyric acid; PNA, peanut agglutinin; DNQX, 6,7-dinitroquinoxaline-2,3-dione.

(wt/vol) paraformaldehyde/0.075% glutaraldehyde in 0.1 M sodium phosphate (pH 7.2) for 4 h, then treated with 1% H_2O_2, and washed for two 30-min periods in ethanol to reduce endogenous peroxidase. Next the retina was incubated in streptavidin-horseradish peroxidase/1% Triton X-100 for 36 h, washed successively in phosphate-buffered saline and Tris buffer, and then treated with 0.05% diaminobenzidine for 10 min followed by 10 min in 0.01% H_2O_2. For light microscopy, the retina was dehydrated, mounted flat, and coverslipped in glycerol. For electron microscopic observations, the tissue was postfixed in 0.5% OsO_4 for 1 h, stained *en bloc* in uranyl acetate in 70% alcohol, and embedded in Durcupan (Buchs, Switzerland).

RESULTS

Connections of L-HCs and C-HCs to Cones. Based upon a prior study using a combination of antibodies and lectins (9), three subtypes of cone were identified in the *Xenopus* retina. Red-sensitive cones were relatively large, with oil droplet diameters of 6–8 μm. They are stained by PNA and constitute ≈89% of the total cone population (Fig. 1*a*). A second population of relatively large cones, the presumed blue-sensitive cones (7% total cones), was not stained by PNA nor were rods (Fig. 1*a*). The *Xenopus* retina also contains a population of miniature cones that reacts with PNA (2). They were easily identified by their small oil droplets (2.0–4.0 μm in diameter) and may correspond to the UV-sensitive cones identified in salamander retina (13). Miniature cones were ignored for the purposes of this study.

L-HCs and C-HCs were identified by their light-evoked responses before being injected with neurobiotin (Fig. 1 *b* and *c*). After the intracellular marking, the retinas were incubated in biotinylated PNA. The dendritic terminals of stained C-HCs were traced to cone pedicles by using electron microscopy (Fig. 1*d*). A total of 860 cones was examined by electron microscopy of which 110 were suitably oriented to trace the connection between outer segment and synaptic base. Sixteen of 17 PNA^- cones received contacts from C-HCs, whereas none of the 93 PNA^+ cones was contacted by C-HC processes. As a further check on data from random sections, we serially sectioned the synaptic bases of 7 PNA^+ red-sensitive cones, none of which contained processes from C-HCs. L-HCs were found to contact both PNA^+ and PNA^- cones. Thus the unstained HC processes invariably observed in PNA^- cones after an injection of neurobiotin into C-HCs (Fig. 1*e*) must come from the L-HC.

Physiology. We recorded simultaneously from 6 L-HC/C-HC pairs under photopic conditions and obtained additional data from >50 L-HCs and >50 C-HCs recorded alone. As shown in Fig. 2*a*, a blue flash or steady background hyperpolarized both L-HCs and C-HCs. On the other hand, when a strong red background was illuminated, the L-HC was hyperpolarized by 21 mV and its responses to blue test flashes were completely suppressed, whereas the C-HC was depolarized by 12 mV and its responses to blue flashes were enhanced (Fig. 2*b*). These data show that blue light drives the C-HC directly

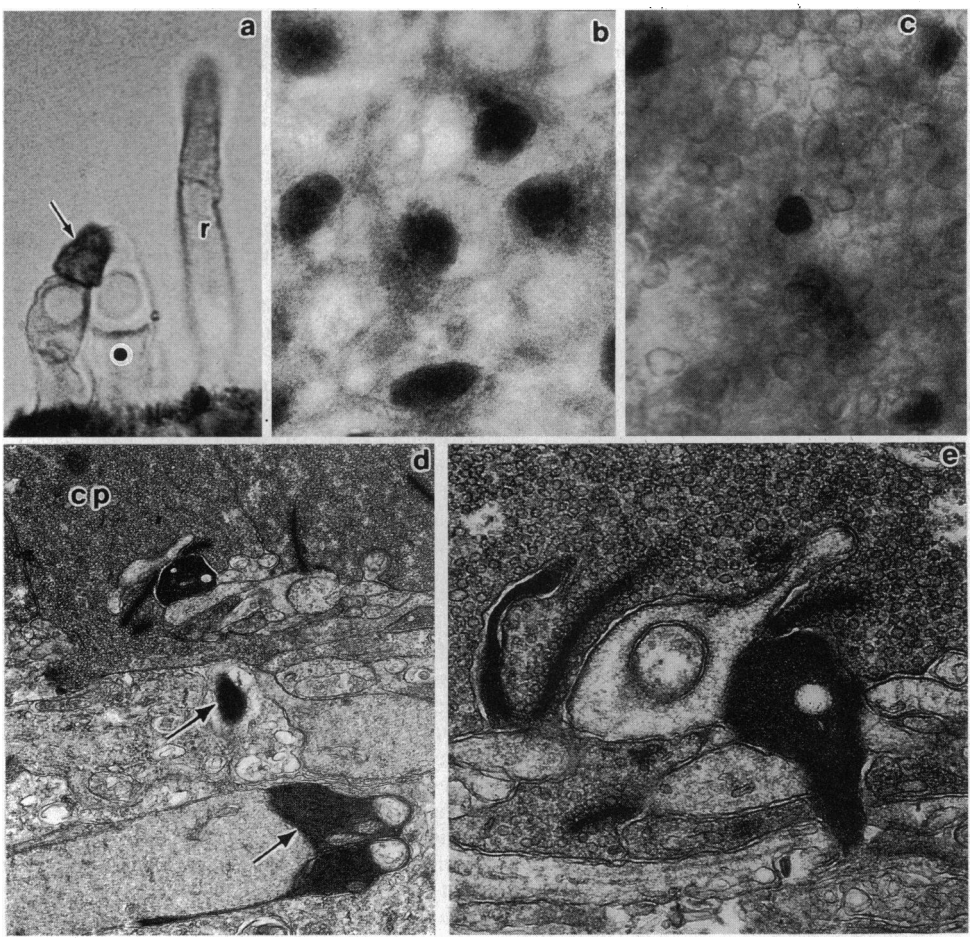

FIG. 1. Connections of the C-HC to cones in the *Xenopus* retina. (*a*) Staining of cones with PNA. The outer segment of a red cone (arrow) is darkly stained, whereas that of a presumed blue cone (dot) is unstained, as are rods (r). (×675.) (*b*) Flat-mount view of whole retina focused on the HC layer. Neurobiotin injected into one L-HC diffused into many perikarya and processes. (×450.) (*c*) An identical view to *b* after injection of a C-HC. (×450.) Unstained profiles are mainly bipolar cells. (*d*) Electron microscopic view showing stained C-HC process (arrows) and a cone pedicle (cp) into which both stained C-HC and unstained L-HC processes have invaginated. (×13,500.) (*e*) Higher power view showing HC processes in relation to the synaptic ribbon (×43,200.)

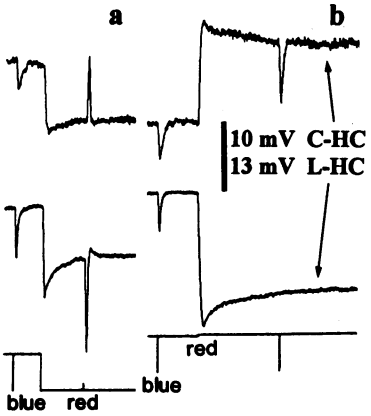

FIG. 2. Simultaneously recorded L-HC and C-HC responses to red and blue light stimuli. (*a*) Both L-HC and C-HC are hyperpolarized by a 20-msec blue (440 nm) light flash. Stimulus duration indicated by downward deflection of lower trace. When steadily illuminated, the same blue light evoked a sustained hyperpolarization in both types of HCs. Superimposed red (650 nm) flashes (upward deflection of lower trace) elicited depolarizations in C-HC but hyperpolarizations in L-HC. (*b*) Responses of a L-HC and a C-HC to a blue light flash before (to the left) and after a strong red field was illuminated. The L-HC hyperpolarized to a saturating level and no longer responded to the blue stimulus. The C-HC was depolarized by 12 mV and its response to the blue test flash increased.

and support the conclusion that the PNA^- cone to which the C-HC connects is blue-sensitive.

Fig. 3 illustrates the simultaneously recorded responses of an L-HC/C-HC pair to a series of red-light stimuli of increasing intensity. The weakest flash did not elicit a measurable C-HC response, but at higher intensities the light-evoked waveforms of the two types of HCs were closely similar. An expectation of the feedback hypothesis is that the C-HC light-evoked response should be delayed with respect to that of the L-HC, and Fig. 3 *Inset* shows that this expectation is met. To make comparison of the time courses easier, the C-HC response was inverted. The initial portion of the C-HC response lags that of

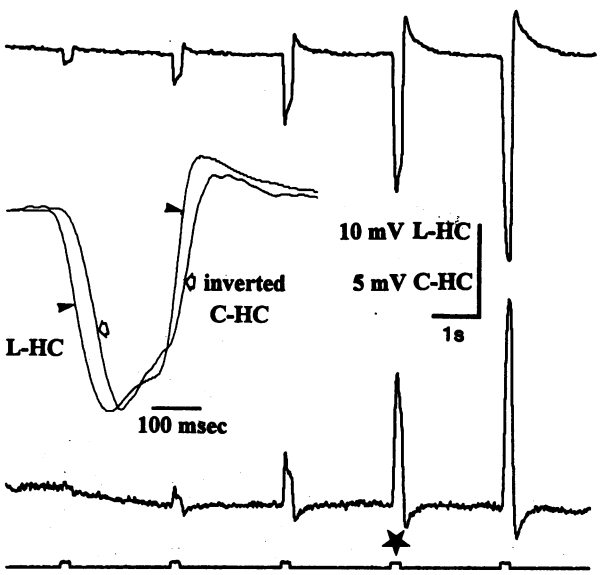

FIG. 3. Kinetics of L-HC and C-HC light responses. The responses of a simultaneously recorded L-HC/C-HC pair to a series of 200-msec 650-nm flashes. Intensity increases by increments of 0.4 order of magnitude from left to right. The pair indicated by a star is expanded and the C-HC response is inverted to compare kinetics. The initial phase of the C-HC response lags that of the L-HC by ≈30 msec.

the L-HC by a delay of ≈30 msec, and this delay was observed whatever the stimulus intensity.

It has been stated (14) that the blue-sensitive cone may not use glutamate as a transmitter. We examined this question by comparing the responses of isolated L-HCs and C-HCs to glutamate and related analogs. We found (Fig. 4*a*) that both HC subtypes responded to puffs of kainate with sustained inward currents from a negative holding potential of −60 mV. From the same holding potential, the responses to quisqualate (Fig. 4*a*) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (data not illustrated) were desensitizing. Thus both L-HCs and C-HCs appear to possess glutamate receptors of the AMPA subtype. Additional supporting evidence came from intracellular recordings in eyecup preparations. In the presence of the quinoxalinedione 6,7-dinitroquinoxaline-2,3-dione (DNQX), a specific antagonist of AMPA receptors, the C-HC hyperpolarized, accompanied by a complete loss of the depolarizing response to red light stimuli and a partial attenuation of the hyperpolarizing response to blue light stimuli (Fig. 4*b*). The same dose of DNQX is sufficient to abolish completely the L-HC response to light in the *Xenopus* retina (10).

It was shown (8) that 5 mM glycine selectively abolished the depolarizing response of the C-HC. We therefore examined isolated L-HCs and C-HCs to see whether one or both cell types had a receptor for glycine. Fig. 5 *Left* illustrates that in symmetrical concentrations of Cl, a puff of 100 μM glycine evoked an inward current in an L-HC. The reversal potential for the glycine-evoked current is near 0 mV. In contrast, the C-HC did not respond to an identical glycine stimulus. As a control against any possible artifact due to malfunctioning of the puffer pipet or miscalculation of the glycine concentration, in each glycine experiment ($n = 7$), an identical strength and duration puff from the pipet used to test the C-HC was found to evoke an inward current in an L-HC or a ganglion cell.

Both the membrane potential of the C-HC and its depolarizing response to light (Fig. 2) appear to be influenced strongly by the L-HC. The question we now consider is whether the L-HC makes a direct synapse onto the C-HC or, instead, acts indirectly through a feedback synapse to the blue-sensitive cone. It is known that the L-HC of the *Xenopus* retina has a high-affinity uptake system for GABA (15) and that GABA is released when the L-HC is depolarized by potassium, glutamate, kainate, or quisqualate (16). We tested for the presence of a GABA receptor on the C-HC by puffing GABA onto isolated C-HCs ($n = 6$). Fig. 5 *Right* illustrates that the C-HC did not respond to puffs of 100 μM GABA, although L-HCs (Fig. 5 *Upper Right*) and ganglion cells (data not illustrated) did. As for glycine, in symmetrical concentrations of Cl, the GABA-evoked current reversed near 0 mV.

Thoreson and Burkhardt (17) reported that 0.5 mM Co blocked a graded depolarization in cones, which was associated with a feedback signal from the L-HC. The same concentration of cobalt did not block the cone to L-HC communication. A prediction of these findings is that submillimolar concentrations of cobalt should attenuate or block the depolarizing component of the C-HC response, if that component in fact depended on a feedback synapse. We tested this prediction on seven C-HCs and five L-HCs recorded in separate experiments. Preliminary experiments showed that 0.4–0.7 mM Co did not block cone-to-L-HC synaptic transfer. The flow rate was fixed at exactly 1.5 ml/min and the exposure time was set at 3 min. Fig. 6 illustrates the results. With respect to the L-HC, exposure to 0.7 mM Co resulted in an average hyperpolarization of the membrane potential level in darkness by 9 mV (range, −4 to −12 mV; $n = 5$). For the same L-HCs, the peak hyperpolarization evoked by a bright red flash of fixed intensity increased by 25% (range, 12–33%). For the C-HC, the results were different in that, although exposure to cobalt also hyperpolarized the cells by an average 12 mV (range, −9 to

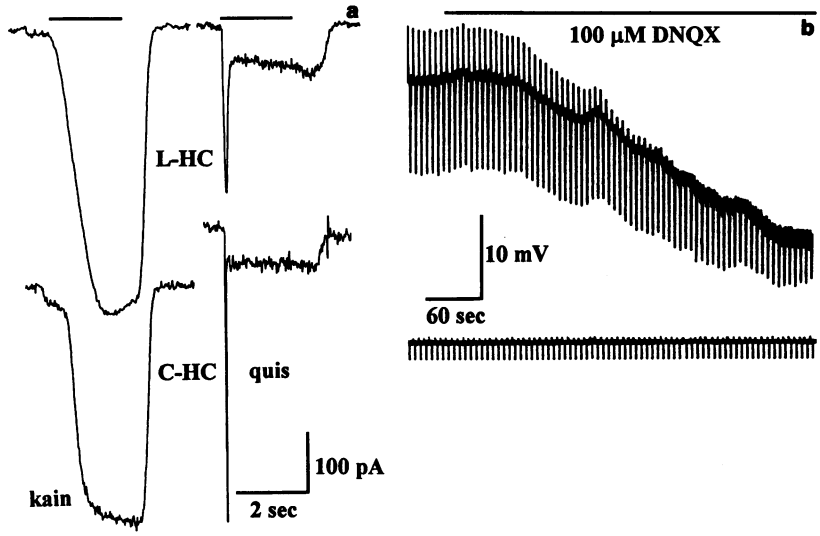

FIG. 4. L-HC and C-HC responses to glutamatergic ligands. (*a*) Currents recorded by whole-cell patch clamp from an isolated L-HC and C-HC in response to 2-sec puffs of 50 μM kainate (kain) or 100 μM quisqualate (quis). Holding potential was −60 mV. Duration of puff is indicated by horizontal bar. In both types of HC, kainate elicited a sustained current and quisqualate elicited a transient current. (*b*) Eyecup preparation. The retina was stimulated alternately by 20-msec flashes of 650-nm (upward deflection of lower trace) and 440-nm (downward deflection) light. Superfusion with 100 μM DNQX in Ringer's solution initially attenuated the depolarizing response. Subsequently, the C-HC hyperpolarized by 18 mV, the depolarizing response to 650-nm flashes was replaced by a small hyperpolarizing response, and the hyperpolarization elicited by 440-nm stimuli was reduced by 50%.

−15 mV; $n = 7$), the amplitude of the depolarizing component was decreased by 31% on average (range, −14 to −55%) and that of the hyperpolarizing component was increased slightly.

DISCUSSION

Stell *et al.* (4) proposed that the depolarizing responses of C-HCs depended on a sign-inverting synapse from L-HCs to short wavelength-sensitive cones. The data of the present report and the findings from an earlier study (8) support the inference that a feedback circuit underlies the origin of depolarizing responses in C-HCs of the *Xenopus* retina. We note, however, that the model of Stell *et al.* (4) may not be applicable to all retinas, for reasons summarized by Burkhardt (7).

As alternatives to feedback, two other possibilities for the creation of depolarizing responses in C-HCs require evaluation. One possibility is that the blue cone-to-C-HC synapse is sign-inverting. Our observation, however, that in the presence of a saturating red field that blocks the L-HC response completely, blue light causes the C-HC to hyperpolarize (Fig. 2*b*) indicates that blue cone-to-C-HC synaptic transfer is sign-conserving, as would be expected at an AMPA receptor. Parenthetically, the results obtained with glutamate analogs on isolated C-HCs and on their light responses in the eyecup (Fig. 4) show that the direct transmission of blue cone information to the C-HCs is mediated by a glutamatergic synapse. To our knowledge, this is the first clear evidence in a vertebrate retina that a blue cone utilizes glutamate as its neurotransmitter. Because the blue cone input is partially blocked by DNQX (Fig. 4*b*) and the isolated C-HC exhibits a sustained response to kainate, but a desensitizing response to quisqualate (Fig. 4*a*), it appears to have an AMPA type receptor, similar to the one found in the *Xenopus* L-HC (10).

A second possible mode for creating depolarizing responses in the C-HC is that red cones directly depolarize blue cones via a sign-inverting synapse. Besides the fact that there is no anatomical basis for chemical transmission between cones, it also is unlikely on functional grounds. The known sign-inverting glutamatergic synapse in the retina is that between photoreceptors and on-bipolar cells. This synapse depends on a metabotropic glutamate receptor and is blocked by 2-amino

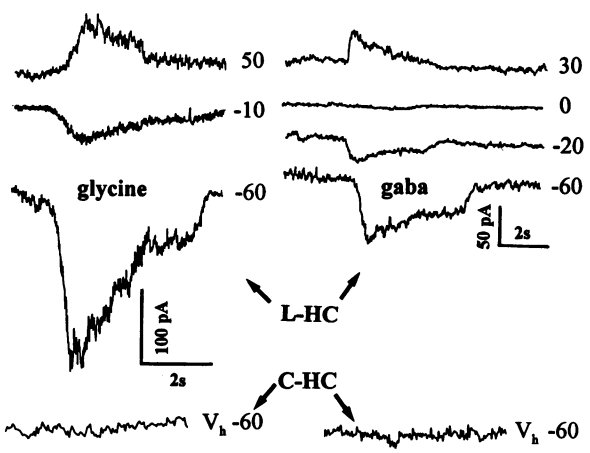

FIG. 5. L-HC and C-HC responses to glycine and GABA. Isolated cells studied by whole-cell patch clamp. In symmetrical 120 mM Cl, glycine- or GABA-induced currents reversed near 0 mV in L-HCs. The same agents elicited no response from C-HCs from a holding potential, V_h, of −60 mV.

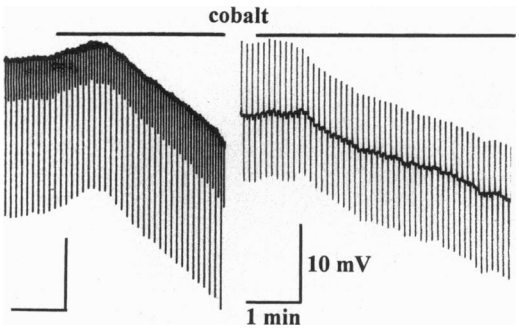

FIG. 6. Effect of submicromolar cobalt on L-HC and C-HC light-evoked responses. (*Left*) Exposure to 0.7 mM Co causes L-HC to hyperpolarize but increases its responses to green or red test flashes. (*Right*) In the presence of 0.7 mM Co, C-HC hyperpolarizes, its hyperpolarizing response to blue light increases, and its depolarizing response to red light decreases.

4-phosphonobutyric acid. The C-HC depolarizing response, however, is not affected by 2-amino-4-phosphonobutyric acid (8). Furthermore, the putative red-to-blue cone synapse would not be expected to be sensitive to glycine or to be blocked by an AMPA antagonist (Fig. 4).

Our data strongly support the inference that the L-HC is implicated in the formation of the depolarizing response of C-HCs. The pharmacological agents that reduce or abolish the light-evoked response of the L-HC selectively reduce the depolarizing responses of C-HCs and the membrane potentials of these two cell types move in opposite directions. For example, glycine selectively abolishes the depolarizing response without affecting the hyperpolarizing response evoked by a blue light stimulus. The isolated L-HC has a glycine receptor that appears to gate a chloride channel, whereas the C-HC lacks a glycine receptor. In the eyecup, glycine depolarizes the L-HC and blocks its light response (18), whereas the C-HC is hyperpolarized by exogenous glycine (8). Secondly, although both L-HCs and C-HCs respond to glutamate and related ligands, the L-HC is more sensitive, i.e., responds at lower concentrations. Thus, when the eyecup is exposed to a relatively weak concentration of *cis*-piperidine dicarboxylic acid, the depolarizing component of the C-HC is completely suppressed before any change in either the membrane potential or the hyperpolarizing response to blue light is observed (8). The same result was achieved with DNQX, the AMPA receptor blocker (Fig. 4).

Two additional experiments favor the feedback hypothesis. The first is that isolated C-HCs do not respond to GABA. We are aware that results with GABA in whole tissue experiments do not always yield readily interpretable data. For example, GABA clearly gates a chloride channel in isolated turtle cones (19), yet superfusion of the turtle retina with GABA does not result in loss or attenuation of the cone depolarization thought to reflect the feedback synapse (20). On the other hand, GABA has clear effects on isolated outer retinal neurons (19, 21), so the apparent absence of a GABA response in the isolated C-HC probably is significant.

The second experiment bearing on feedback is that showing a reduction in the C-HC depolarization induced by submillimolar concentrations of cobalt (Fig. 6). L-HC responses were increased during exposure to 0.7 mM Co, as were the hyperpolarizing responses of C-HCs. In contrast, their depolarizing responses were substantially decreased and this could be explained by a reduction in an intrinsic calcium current of the cone, which is thought to contribute to the graded cone depolarization that is a component of the feedback synapse (17).

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