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Hemichannel-Mediated Inhibition in the Outer Retina

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An essential feature of the first synapse in the retina is a negative feedback pathway from horizontal cells to cones. Here we show that at this synapse, connexin26 forms hemichannels on horizontal cell dendrites near the glutamate release site of the cones. Blocking these hemichannels hyperpolarizes horizontal cells, modulates the Ca^{2+} channels of the cones, and abolishes all feedback-mediated responses. We propose a feedback mechanism in which the activity of the Ca^{2+} channels and the subsequent glutamate release of the cones are modulated by a current through these hemichannels. Because the current through the hemichannels depends on the polarization of the horizontal cells, their activity modulates the output of the cones.

In all vertebrate retinas, photoreceptors project to horizontal cells (HCs) and bipolar cells (BCs). The synaptic complex of this interaction reveals a peculiar and conserved ultrastructure. The cone pedicles are characterized by a presynaptic ribbon (where neurotransmitter release takes place), centrally positioned BC dendrites, and laterally positioned HC dendrites (Fig. 1A). These lateral contacts are thought to be the origin of negative feedback from HCs to cones. In goldfish, this feedback modulates the Ca^{2+} current of the cones. Hyperpolarization of HCs shifts the Ca^{2+} current to more negative potentials, which increases the Ca^{2+} influx and subsequently leads to an increase in glutamate release. Various neurotransmitters have been proposed for this pathway, but this retrograde neurotransmitter has not yet been unequivocally identified (*1*).

In the carp retina, connexin26 (Cx26) immunolabel (*2*, *3*) was restricted to the membrane of the lateral processes of the HCs close to the voltage-dependent Ca^{2+} channels on the opposing cone membrane (Fig. 1, B and C) (*4*). Septilaminar structures indicative of gap junctions between the cones and the HC are not discernible at this site nor have such structures been reported by physiological studies, suggesting that the immunolabel reflects the presence of hemichannels. That functional hemichannels are present on HCs and do not compromise cell viability has been shown in dissociated HCs (*5*, *6*).

The location of Cx26 immunolabel suggested that such hemichannels might be involved in the synaptic interactions between HCs and cones. Thus, we studied the effect of carbenoxolone, a blocker of gap-junctional channels on this feedback pathway (*7–9*). Figure 2A, left, shows the feedback-induced responses of a cone clamped at various membrane potentials (*10*). The feedback-mediated responses in cones can be measured most effectively when the cone response is saturated with a white 20-μm-diameter spot and the retina is stimulated with a full-field white light stimulus (*1*). Such a stimulus induces a shift of the Ca^{2+} current in the cones to more negative potentials, which will be seen as an inward current in a voltage-clamped cone. In the presence of 100 μM carbenoxolone, these feedback-induced responses disappeared (Fig. 2A, right) (n = 13). The effect of carbenoxolone could be washed out within 15 min (Fig. 2B). During the application of carbenoxolone cones hyperpolarized by −4.6 mV ± 1.7 mV (n = 5), whereas their light response amplitude was unaffected (Fig. 2C).

Because blocking the hemichannels led to the disappearance of the feedback-induced responses in cones, the feedback-mediated responses in HCs should also disappear. Carbenoxolone hyperpolarized HCs strongly and reduced their light responses (Fig. 2D). Because of this large hyperpolarization, the effect of carbenoxolone on the feedback-mediated responses was studied before the HCs had hyperpolarized more than about 25% of their maximal hyperpolarization (Fig. 2D, ③). HC light responses show a characteristic transient component (arrow Fig. 2E, left), mainly attributable to negative feedback from HCs to cones (*11*, *12*). In monophasic HCs (MHCs), which hyperpolarize to light of all wavelengths, this pronounced transient component was blocked by carbenoxolone (Fig. 2E, right) (n = 8). Biphasic HCs (BHCs) hyperpolarize to full-field green light stimulation but depolarize upon red light stimulation (Fig. 2F, left). The depolarizing responses are thought to originate from negative feedback from MHCs to middle-wavelength sensitive cones (*1*, *12–15*). Carbenoxolone blocked these depolarizing responses, whereas the hyperpolarizing responses were almost unaffected (Fig. 2F, right) (n = 5).

Is the block of the hemichannels or the hyperpolarization of the HCs responsible for

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the block of feedback? Hyperpolarization of HCs with an intact feedback system shifts the Ca^{2+} current to more negative potentials (*1*). The maximal light-induced shift of the Ca^{2+} current to negative potentials is about −10 mV (*14*). Blocking hemichannels with carbenoxolone also hyperpolarizes HCs, but in this case the Ca^{2+} current was shifted to more positive potentials. The mean carbenoxolone-induced shift of the half-activation potential of the Ca^{2+} current was 4.7 mV (ranging from 0.5 mV to 11.5 mV; n = 8). Although HCs hyperpolarize strongly under carbenoxolone, the Ca^{2+} current is not shifted to negative potentials as would be expected if feedback were still intact. The observed positive shift of the Ca^{2+} current shows that blocking the hemichannels, and not the HC hyperpolarization, is essential for the carbenoxolone-induced block of feedback responses in cones.

Hemichannels have a reversal potential around 0 mV (*5*, *6*). Blocking these channels should hyperpolarize HCs. The size of the carbenoxolone-induced hyperpolarization of the HCs was unexpectedly large (by −44.4 mV ± 3.0 mV; n = 8). This observation suggests that the hemichannel conductance is by far the largest conductance in the HCs. However, carbenoxolone not only blocks the hemichannels but also shifts the Ca^{2+} current of the cone, leading to a reduction of glutamate release, which in turn closes the glutamate-gated channels (*16*). We estimated that the glutamate conductance was eight times larger than that of the hemichannel (*17*).

How do hemichannels mediate negative feedback from HCs to cones? An electrical feedback mechanism has been suggested in which glutamate receptors on the HC dendrites in the cone synaptic terminal form a current sink by which the extrasynaptic potential could be modulated (*18*). Although this original hypothesis was not validated experimentally (*1*), a modified version can account for the observed effects (*19*). Key elements of this mechanism are the relatively high resistance of the extracellular space in the synaptic terminal and the presence of hemichannels at the tips of the HC dendrites. The extracellular potential near these hemichannels will become negative because

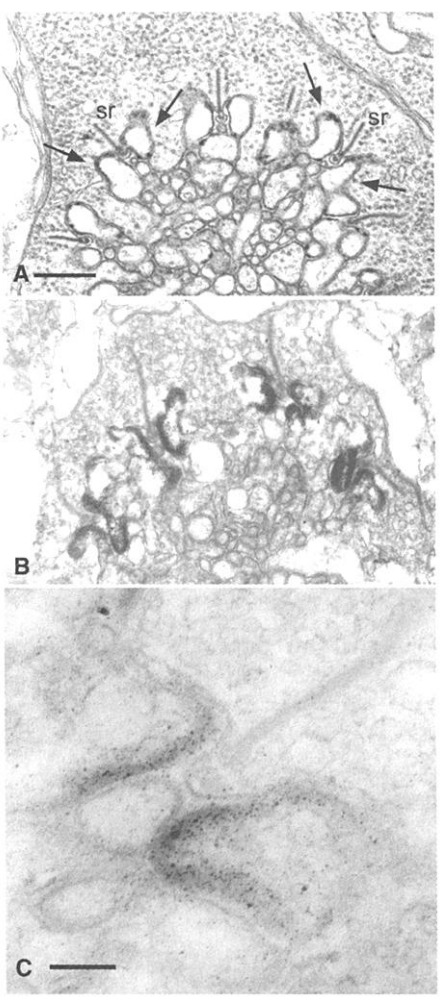

Fig. 1. Localization of Cx26 immunoreactivity. Electronmicrograph of tangential sections of cone pedicles. (**A**) Control section. SR, Synaptic ribbon. Arrows indicate HC dendrites. (**B**) Cx26 immunoreactivity. (**C**) Immunolabel is restricted to the HC dendrites. Bar in (A) indicates 0.5 μm for (A) and (B); bar in (C) indicates 0.1 μm.

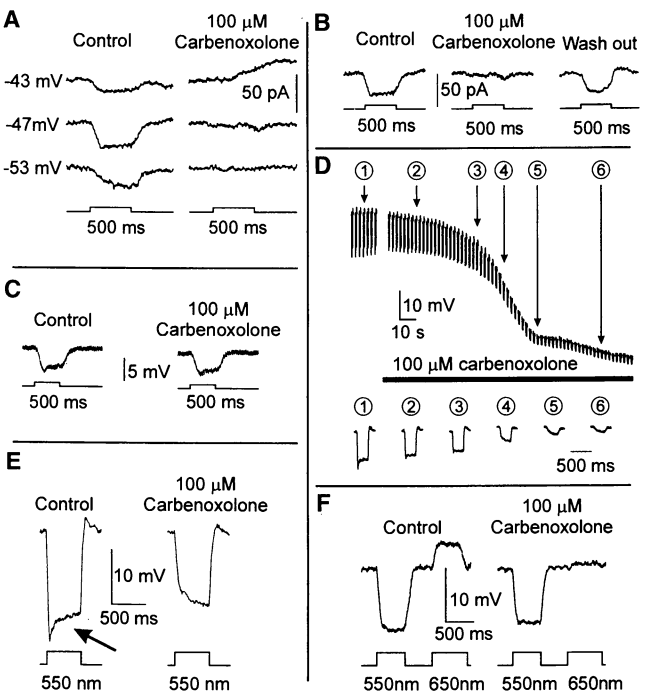

Fig. 2. Carbenoxolone blocks feedback-mediated responses in both cones and HCs. (**A**) Feedback induced response in a cone clamped at various potentials in control solution (left) and in carbenoxolone (right). Feedback-induced responses are maximal around −47 mV and diminished at both hyperpolarized and depolarized potentials. (**B**) Feedback-induced response in a cone clamped at −47 mV in control (left), carbenoxolone (middle), and after 15 min wash (right). (**C**) Carbenoxolone does not affect the response of a cone to light. (**D**) Responses of a monophasic HC (MHC) to 550 nm, full-field stimuli of 500 ms, which are separated by 500 ms during which no stimuli were presented. Expanded light responses at different time points, indicated by the arrows, are given below. (**E**) Carbenoxolone blocks feedback-mediated responses in MHCs. Responses in control (left) and in carbenoxolone (right). (**F**) Responses of a biphasic HC (BHC) in control (left) and carbenoxolone (right). Carbenoxolone blocks the depolarizing response to red light. The responses in (B), (C), (D), and (F) have been shifted such that the baseline is equal.

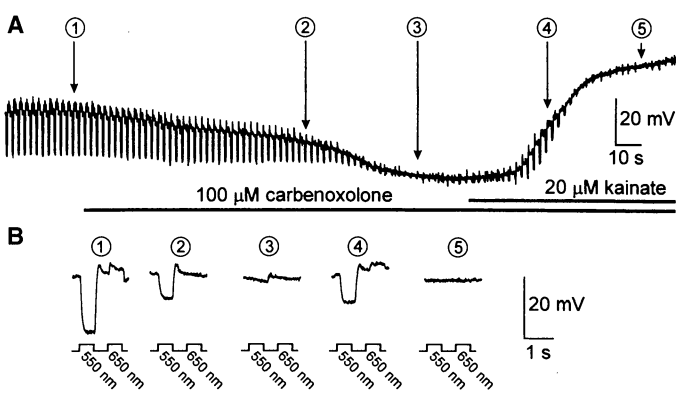

Fig. 3. Glutamate-gated channels are able to mediate feedback. (**A**) Light responses of a BHC during application of carbenoxolone and kainate. (**B**) Expanded light responses of (A) at the time points indicated with arrows. ① Control light responses. ② Light responses just after the application of carbenoxolone. ③ Light responses, taken at the maximal hyperpolarized level. ④ Additional application of 20 μM kainate leads to the reappearance of surround-induced responses. ⑤ Eventually the light responses disappear.

of the current flowing through the high resistance of the extracellular space via the hemichannels into the HCs. Therefore, the voltage-dependent Ca^{2+} channels of the cones will sense a more positive membrane potential, causing an increase in the release of glutamate. Modulation of the HC membrane potential will change the current flowing through the hemichannels, which will subsequently modulate the glutamate release of the cones. In voltage clamp experiments, this modulation of the Ca^{2+} current by the HCs will be observed as a negative shift of the Ca^{2+} current in the cones.

Such an electrical feedback mechanism critically depends on a postsynaptic current sink, near the presynaptic Ca^{2+} channels. This suggests that other channels present in the cone synaptic terminal could also form a current sink and should, therefore, also be able to mediate feedback. Therefore, we studied the contribution of the glutamate-gated channels while hemichannels were blocked with carbenoxolone (Fig. 3). Recordings were made from a BHC during alternating green and red light stimulation. Application of carbenoxolone closed the hemichannels and greatly reduced the glutamate-gated conductance. To study the effect of current flowing through the glutamate-gated channels, we applied kainate. In a restricted time window, only some of the glutamate receptors will be activated by kainate and the others will be modulated by the glutamate released by the cones. In this time window, the light responses and the feedback-mediated depolarizing response to red light stimuli reappeared (Fig. 3, ③) ($n = 3$). This experiment showed that glutamate-gated conductances could, under certain conditions, also act as a current sink and contribute to electrical feedback. Because feedback does not depend on the type of channel on the HC dendrites, these experiments support the hypothesis that it is the current that generates the feedback signal. However, under physiological conditions with full-field white light stimulation, the current through the glutamate-gated channels will reduce with HC hyperpolarization, instead of increase. This happens because the cones reduce their glutamate release. This cascade of events turns the feedback signal via the glutamate-gated channels into a positive one. Therefore, the contribution of the glutamate-gated channels to feedback is negative in cones that are not stimulated by light, whereas it is positive when the cones are directly stimulated by light.

An electrical feedback mechanism strongly depends on the resistance of the extracellular space in the synapse, the conductance of the hemichannels, and their localization. Using morphological data, one can calculate that 13 to 400 hemichannels focally localized at the tips of the HC dendrites yield a large enough current sink (*20*) for the proposed electrical feedback mechanism to function. In contrast to hemichannels that are focally localized, glutamate receptors are expressed more diffusely and also extrasynaptically on the dendrites of HCs (*21*, *22*), reducing the efficiency with which they modulate the calcium channels of the cone.

We propose an electrical or ephaptic feedback mechanism in which hemichannels are key elements that form a current sink near the Ca^{2+} channels of a presynaptic neuron, making the potential sensed by these Ca^{2+} channels dependent on the activity of the postsynaptic neuron.

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16. Blocking hemichannels resulted in a positive shift of 4.7 mV in the Ca^{2+} current. This reduced the Ca^{2+} influx into the cones and hyperpolarized them by −4.6 mV, leading to a relative shift of membrane potential versus Ca^{2+} current of about 9 mV. In conditions where feedback is not active, such a shift reduces synaptic transmission between cones and HCs by about 90% (*15*). This accounts for the large hyperpolarization of the HCs and the reduction of the HC light responses.
17. In the dark, the resting potential of HCs is −34.7 ± 3.6 mV ($n = 12$). Blocking the glutamate-gated conductances with DNQX (an antagonist of ionotropic, non-NMDA glutamate receptors) in the control condition induced a hyperpolarization of −36.7 mV ± 2.8 mV ($n = 7$) in HCs which is significantly less than the carbenoxolone-induced hyperpolarization (−44.4 ± 3.0 mV) (*t* test, $P < 0.05$). Application of DNQX during carbenoxolone further hyperpolarized HCs by −3.6 ± 1.3 mV ($n = 5$), to a final value of −82.7 mV. Taking this as the reversal potential for the potassium conductance and 0 mV as the reversal potential for the cation and hemichannel conductances, one can estimate, using Ohms law, that the hemichannel conductance is about one-eighth of the cation conductance.
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20. The surface of the extracellular space at the base of the cone pedicle in goldfish has been estimated to be between 0.01 to 0.1 μm^2 depending on the fixation procedure used [C. A. V. Vandenbranden *et al.*, *Vision Res.* **36**, 3859 (1996)]. The tips of the dendrites invaginate about 1 μm into the cone pedicle. Assuming that the mobility of the ions in the intersynaptic space is equal to that in the Ringer's solution, one can calculate that the resistance of the intersynaptic space is between 6 and 60 megohms. The shift in the Ca^{2+} current is about −10 mV for a change in HC membrane potential of about −40 mV. Therefore, the total resistance of the hemichannels should be between the 24 and 240 megohms. The single channel conductance of a Cx26 hemichannel is about 270 pS [T. M. Suchyna, *et al.*, *Biophys. J.* **77**, 2968 (1999)], and is almost potential-independent over a voltage range of 60 mV [S. Oh, J. B. Rubin, M. V. Bennett, V. K. Verselis, T. A. Bargiello, *J. Gen. Physiol.* **114**, 339 (1999)]. Various hemichannels are closed by high Ca^{2+} concentrations (*9*). It was estimated that with 1 mM extracellular Ca^{2+} concentration (the concentration used in the present experiments) the conductance of the channels is about 4% of the maximal conductance. Given these numbers, and assuming that the conductance of Cx26 hemichannels is also Ca^{2+}-dependent, one needs between 400 and 4000 hemichannels in the entire terminal to generate enough current to shift the Ca^{2+} current by 10 mV. Because in goldfish cones 5 to 16 synaptic ribbons are present with each at least two HC dendrites per ribbon [W. K. Stell, R. Kretz, D. O. Lightfoot, in *The S-potential*, B. J. Drujan and M. Laufer, Eds. (Liss, New York, 1982), pp. 51–75], one would need about 13 to 400 hemichannels per dendrite.
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