Carbenoxolone Inhibition of Voltage-Gated Ca Channels and Synaptic Transmission in the Retina

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INTRODUCTION

One model of horizontal cell-mediated negative feedback in the retina is the electrical feedback mechanism originally put forth by Byzov and Shura-Bura (1986). They proposed that glutamate-gated ion channels at the tips of horizontal cell dendrites act as current sinks for the changing extracellular currents generated during the light response. Because the dendrites of the horizontal cells are located deep within the invaginating cone synapse, current flow to the sinks can generate a voltage drop as it passes through the bulk extracellular resistance. The voltage drop makes the potential of the extracellular space in the synaptic invagination nonzero, and this can influence the voltage-gated Ca channels present in the cone presynaptic membrane. Normally, hyperpolarization of the photoreceptors in response to light closes Ca channels and reduces the release of glutamate. But, when horizontal cells hyperpolarize in response to annular illumination, the increased current flow to the sinks enhances the negative extracellular potential in the synaptic cleft, reducing the voltage difference across the presynaptic membrane. This, in turn, increases Ca channel open probability and stimulates glutamate release, completing the negative feedback pathway. Thus the ephaptic model predicts that the degree of horizontal cell hyperpolarization dictates the magnitude of the feedback signal by changing the driving force on current flowing through the glutamate-gated ion channels.

However, presynaptic cones hyperpolarize in response to light stimulation and reduce their release of glutamate, shutting off the glutamate-gated postsynaptic ion channels available to act as the necessary current sink. Kamermans et al. (2001) adapted the electrical feedback model by incorporating the discovery that hemichannels composed of connexin26 are located at the tips of horizontal cell dendrites (Janssen-Bienhold et al. 2001). Hemichannels could act as the current sink during horizontal cell hyperpolarization and permit the feedback loop to increase the Ca2+ current into the cone terminal independent of the gating of glutamate-gated channels. To assess the viability of this proposal, the gap junction blocker carbenoxolone was used to eliminate contributions of hemichannels to negative feedback. Application of carbenoxolone strongly hyperpolarized horizontal cells and reduced their response to light. The resulting effects on feedback were interpreted as being the result of carbenoxolone-mediated block of the hemichannels alone (Kamermans et al., 2001).

To draw firm conclusions about the role of hemichannels in feedback, the specificity of carbenoxolone must be considered. Carbenoxolone is used to block gap junctions and assumed to lack side effects common to other gap junction blockers like octanol and halothane, which disrupt excitatory synaptic transmission by interfering with other ion channels (Pocock and Richards 1993; Puil and el-Beheiry 1990; Puil et al. 1990; Rorig et al. 1996). Carbenoxolone was demonstrated to have no effect on synaptic responses, neuronal responses to neurotransmitters, and intrinsic excitability (Kohling et al. 2001; Travagli et al. 1995; Yang and Michelson 2001). Carbenoxolone and its precursor compounds are known to inhibit the Na+-K+-ATPase (Zhou et al. 1996).

Xia and Nawy (2003) investigated the light responses of ganglion cells in the presence and absence of horizontal cell-mediated feedback and concluded that the effects of carbenoxolone must extend beyond those exerted on hemichannels. They found that carbenoxolone reduced light response amplitude in ganglion cells, not surround inhibition, and concluded that the drug affects synaptic transmission in a manner likely involving synaptic Ca channels.

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We investigated carbenoxolone’s inhibition of Ca channels in cells of the retina. Application of the drug to presynaptic photoreceptors reduced voltage-gated calcium channel currents in a dose-dependent manner and reduced intracellular \([\text{Ca}^{2+}]\) concomitantly. The reduction in \(\text{Ca}^{2+}\) influx has a calculable effect on synaptic transmission that explains the hyperpolarization of horizontal cells and the suppression of their light responses. These data suggest that carbenoxolone has broader actions within the retina than previously considered. Because the effect of feedback is to alter Ca channel gating and because carbenoxolone appears to inhibit Ca channels directly, it will be crucial to integrate the data obtained in earlier studies with this additional perspective of carbenoxolone action.

**METHODS**

**Animals and drugs**

Larval tiger salamanders (\(\text{Ambystoma tigrinum}\), obtained from Kons Scientific, Germantown, WI), 10–20 cm in length, were kept at 4°C on a 12-h light/dark cycle. Animals were treated in accordance with guidelines set forth by the Canadian Council of Animal Care.

**Electrophysiology**

Intracellular microelectrode recording in intact retina

Salamander eyecups were placed in a fitted holder, held by light suction, and constantly superfused at a rate of ~1 ml/min with bicarbonate-buffered saline containing (in mM) 95 NaCl, 2.5 KCl, 3 CaCl\(_2\), 8 \(\alpha\)-glucose, and 10 \(N\)-2-hydroxyethylpiperazine-\(N'\)-2-ethanesulfonic acid (HEPES) at pH 7.6. Isolated retinas were kept in RSS on ice. All chemicals were obtained from Sigma Chemical, St. Louis, MO, except where noted. A 10 mM stock solution of carbenoxolone was previously considered. Because the effect of feedback is to alter Ca channel gating and because carbenoxolone appears to inhibit Ca channels directly, it will be crucial to integrate the data obtained in earlier studies with this additional perspective of carbenoxolone action.

**Intracellular microelectrode recording in intact retina**

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**Electrophysiology**

The isolated retina was incubated for 15–20 min at ~28°C in Ca\(^{2+}\)-free RSS and mechanically triturated with a fire-polished Pasteur glass pipette. Aliquots of the resulting cell suspension were plated in dishes precoated with Sal-1 antibody, a mouse monoclonal antibody produced against salamander retinal membranes (a kind gift from Dr. Peter MacLeish) (see MacLeish et al. 1983). Briefly, the dishes were coated with 100 \(\mu\)l 0.2 mg/ml goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by 100 \(\mu\)l 1:100 dilution Sal-1 antibody, both for 1 h at room temperature. Prior to beginning an experiment, isolated cells were kept on ice and allowed to settle to the bottom of dishes for 15–30 min. Ruptured-patch, whole cell recordings were obtained from single and double cones. The standard bath solution to isolate Ca channel current, using Ba\(^{2+}\) as a charge carrier, contained (in mM) 70 NaCl, 2.5 KCl, 5 CsCl, 10 BaCl\(_2\), 15 TEACl, 10 glucose, and 15 HEPES, adjusted to pH 7.6 with NaOH, whereas the pipette solution contained (in mM) 95 CsCl, 0.8 MgCl\(_2\), 0.01 CaCl\(_2\), 0.1: 0.4 BAPTA:CsOH, 5 HEPES, 1 Mg\(^{2+}\)-ATP, and 0.2 Na\(^{2+}\)-GTP, adjusted to pH 7.2 with CsOH. Carbenoxolone was added to the superfusing standard bath solution, which was applied at room temperature (between 21 and 25°C). Patch electrodes were pulled from fire polished micro-hematocrit capillary tubes (WVR Scientific, West Chester, PA) using a two-step vertical pipette puller (Kopf model 730, David Kopf Instruments, Tujunga, CA). Pipette tips were first dipped in filtered (0.22 \(\mu\)m) standard intracellular solutions and then back-filled with the same solution. Filled pipettes had 5–10 M\(\Omega\) tip resistances measured in the standard bath. The bath reference electrode consisted of a bath solution-filled agar bridge with an AgCl wire (World Precision Instruments, Sarasota, FL). Offset currents were nulled before seals were made. Whole cell voltage was clamped with an Axopatch-1D or 200 amplifier (Axon Instruments, Foster City, CA) using whole cell capacitance and series resistance compensation to reduce capacitive artifacts. Signals were filtered at 1–3 kHz (Ithaco 4302 Dual 24dB/octave filter, Ithaca, NY) and digitized at 1 kHz with an Indec Systems interface (Sunnyvale, CA) for storage on the hard disk of a computer running BASIC-FASTLAB acquisition software. BASIC-FASTLAB generated the voltage-clamp commands, and provided some data analysis. Current-voltage (I-V) relations were constructed by calculating the mean current at ~10 ms before the end of each voltage step. I-V relations were fit by dividing the driving force, fitting the resulting activation curve with the Boltzmann function (given by \(I/I_0 = 1 + \exp((V - V_{1/2})/m)\)), where \(V_{1/2}\) is the activation curve midpoint and \(m\) the slope factor), then multiplying back by the driving force. Data were plotted in Sigma Plot 2001 for Windows and were presented in Canvas 9.0 (Deneba Software, Miami, FL). Statistical analysis, performed on raw data, consisted of \(t\)-tests. Data are reported as means ± SE.

**Calcium imaging**

Isolated retinas were sliced with a fine razor blade in a glass petri dish and the resulting mini-slices were transferred to an imaging chamber that had been precoated with 0.01% poly-l-lysine. 1 mM Fluo-4 AM in DMSO (Molecular Probes, Eugene, OR) was added to a final concentration of 10 \(\mu\)M (final DMSO concentration of 0.1%) and left to incubate on ice in the dark for 1 h. All imaging experiments were carried out on a Nikon E800 laser scanning confocal microscope employing an air cooled argon laser producing an excitation wavelength of 488 nm. Fluorescence emission was recorded by a photomultiplier with a cut-off filter of 585 nm. The microscope stage was fitted with a superfusion system and an eight-way valve that provided a flow rate of 1.5 ml/min. Time-lapse movies were captured and analyzed using Nikon EZC1 v. 2.0 with images obtained every 15 s. A region of interest encompassing the synaptic processes of the outer plexiform layer was selected for analysis as shown in Fig. 3. To depolarize the entire slice, 40 mM high-K\(^+\) solution, made equiosmotic by adjusting Na\(^+\) concentration, was applied for one minute. To test the effect of carbenoxolone on depolarization-induced calcium influx, the drug was continuously superfused 60s before and during the high-K\(^+\) stimulus. Statistical analysis, performed on raw data, consisted of \(t\)-tests and were expressed as means ± SD.

**RESULTS**

Carbenoxolone hyperpolarizes and reduces the light response amplitude of HCs

When applied to the dark-adapted, intact retina, 100 \(\mu\)M carbenoxolone caused a reduction in peak amplitude of the HC light response (Fig. 1). Responses to a 500-ms whole field white light stimulus diminished by 92 ± 4\% (\(n = 3\), inset 2) and partially returned to control levels (inset 1), 64 ± 6\% of
Ca channel currents are inhibited by carbenoxolone

The effect of carbenoxolone on voltage-gated Ca channel activity was assessed on both isolated cones and Müller cells. When tested in a 10 mM Ba²⁺ solution, cone Ca channel currents were significantly reduced in the presence of 100 μM carbenoxolone (Fig. 2A). The current traces demonstrate that after 2 min of carbenoxolone superfusion, the depolarizing step to −10 from −60 mV generates less Ca channel current than in control conditions. At the 100 μM dose used by others to assess hemichannel function in the retina (Kamermans et al. 2001, Pottek et al. 2003), carbenoxolone reduced Ca channel current by 37 ± 7% (n = 8; P < 0.05; Fig. 2, A–C). This reduction in current occurs in a dose-dependent manner (Fig. 2C). The dose response curve was constructed with four concentrations of carbenoxolone: 10 μM (n = 4), 30 μM (n = 4), 100 μM (n = 8), and 300 μM (n = 4) and fitted with the Hill equation, giving an EC₅₀ of 48 μM and a Hill coefficient of 1.69. The I-V relations (Fig. 2B) show that carbenoxolone reduces the amplitude of the Ca channel current more at negative voltages. In fact, activation curves constructed from the I-V relations revealed a positive shift of the midpoint of activation of 2.0 mV (from −19.6 ± 1.6 to −17.6 ± 2.1 mV; n = 7). Isolated Müller cell Ca channel currents were inhibited by 52 ± 6% (n = 5; data not shown) in 100 μM carbenoxolone.

Depolarization-induced calcium influx is inhibited in retinal slice preparations

Calcium imaging in retinal slices demonstrated a similar reduction in voltage-gated Ca channel activity in the presence of 100 μM carbenoxolone (Fig. 3). To activate Ca channels, the slices were superfused with 40 mM K⁺ solution, which calculation shows should depolarize the cells to a value between −25 and −20 mV. In control experiments, subsequent depolarizations produced two peaks of similar amplitude (Fig. 3D). The second peak was 15% (±7%; n = 6) smaller than the first, likely the result of photobleaching. The traces in Fig. 3, D and H, are shown as a percentage of the initial peak intensity. When superfused with carbenoxolone for 2 min before the depolarization, the resulting calcium influx was reduced by 72 ± 13% (n = 8; P < 0.001). Corrected for the photobleaching effect, the reduction by carbenoxolone is ~57%. Washout of the drug was not possible during the time scale of the confocal experiments due to the high-intensity laser causing photobleaching.
These data show that carbenoxolone inhibits Ca channels and synaptic transfer directly. With a 37% block of the presynaptic Ca channel current in 100 μM carbenoxolone, an ~90% reduction in postsynaptic response is calculated. Indeed, in this work and others, such a reduction of the horizontal cell postsynaptic signal is observed (Kamermans et al. 2001; Pottek et al. 2003) and is partially attributable to the pronounced inward rectification of the horizontal cell membrane at voltages negative to −60 mV (Tachibana 1983). Horizontal cell hyperpolarization induced by carbenoxolone was interpreted as being due to block of the prominent depolarizing influence of open hemichannels (Kamermans et al. 2001). In fact, hyperpolarizing block has been reproduced by numerous investigators using agents known to inhibit presynaptic Ca channels. Xia and Nawy (2003) found that carbenoxolone inhibited the light response of ganglion cells by ~70%, a value somewhat less than what our model predicts. Considering that carbenoxolone reduces Ca channel currents in cones with an EC50 of ~48 μM, nothing more than suppressed Ca channel activity need be considered to explain the reduction in signaling.

Studies in other neuronal populations have found similar nonspecific effects with carbenoxolone. Rouach et al. (2003) discovered that carbenoxolone inhibits astrocyte-mediated spontaneous activity in cultured hippocampal cell networks in a manner not associated with gap junctions. It has been proposed that gap junctions in astrocytes mediate the network wide activity (Dudek et al. 1999; McCormick and Contreras 2001). The carbenoxolone effects remained in cultures originating from connexin43 knockout mice, and the effects were not mimicked by endothelin-1, a known blocker of astrocytic gap junctions (Giaume et al. 1992). Rouach et al. (2003) suggested that carbenoxolone may be acting at a variety of ion channels, including K and Ca channels. While studying surround antagonism in macaque retinas, Verweij et al. (2003) observed that carbenoxolone reduced the photoreceptor response to light. The present study confirms Ca channels as a conductance affected by carbenoxolone in no way excluding hemichannels as a significant target.

Carbenoxolone, which Kamermans et al. (2001) found hyperpolarized horizontal cells, had additional effects attributed to block of the feedback pathway in the retina. The depolarizing response to red light in biphasic horizontal cells is thought to arise from negative feedback from monophasic horizontal cells to middle-wavelength (green).
sensitive cones (Weiler and Wagner 1984). Carbenoxolone eliminated the depolarizing response to red light in this horizontal cell type (Kamermans et al. 2001). However, the reduced synaptic efficacy of the cone output synapse in the presence of carbenoxolone could be the cause of the lack of depolarizing response. By reducing the hyperpolarizing excursion of the monophasic horizontal cell by 90% as our model and data show, the green-sensitive cone now receives substantially less feedback signal. What little feedback signal remains (~10%) to be passed on as a subsequent depolarization to the biphasic horizontal cell will also be inhibited in the same manner by the Ca channel reduction at the green cone output synapse. By this point, it is hardly surprising that practically no depolarizing signal is detected (10% × 10% = 1%) in the biphasic horizontal cell.

Application of kainate, a glutamate receptor agonist, recovered the horizontal cell light response and surround inhibition while still in the presence of carbenoxolone (Kamermans et al. 2001). This was interpreted as a recovery of the ephaptic feedback mechanism by kainate-induced current sinks, substituting for the carbenoxolone-blocked hemichannel sinks. The result confirms that the feedback mechanism, whatever it is, persists in the presence of carbenoxolone. In terms of the synaptic suppression model described here, rescue by kainate confirms that the photoreceptor synapse is not completely suppressed. By depolarizing the horizontal cell membrane out of the voltage range where strong inward rectification occurs (e.g., bringing the cell positive to −60 mV) (Tachibana 1983), the persistent modulation of glutamate levels by the photoreceptors, albeit reduced in magnitude, reappears.

The electrical feedback model first proposed by Byzov and Shura-Bura (1986) is not mediated by any neurotransmitter but rather by alterations in current flow at the horizontal cell dendrites. Elaborated significantly by the addition of hemichannels which act as an additional, nongated current sink (Kamermans et al. 2001), this ephaptic model remains biophysically unquestionable but yet uncertain in terms of the magnitude of its contribution. Due to the Ca channel effects described here, studies employing carbenoxolone do not permit firm conclusions to be drawn on the nature of the feedback system. These facts necessitate the acquisition of more specific means to knock-down or suppress connexin26-containing hemichannels in the lower vertebrate retinas to reveal their contribution in feedback inhibition.

GRANTS
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