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D₂-like dopamine receptors promote interactions between calcium and chloride channels that diminish rod synaptic transfer in the salamander retina

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Abstract

Activation of D₂-like dopamine receptors in rods with quinpirole stimulates L-type calcium currents (I_{Ca}). This result appears inconsistent with studies showing that D₂-like dopamine receptor activation diminishes rod signals in second-order retinal neurons. Since small reductions in $[Cl^-]_i$ can inhibit photoreceptor I_{Ca} , we tested the hypothesis that enhancement of I_{Ca} with the D₂/D₄ receptor agonist, quinpirole, increases calcium-activated chloride currents ($I_{Cl(Ca)}$) causing an efflux of Cl^- from rods that would provide a negative feedback inhibition of I_{Ca} . In agreement with studies from *Xenopus*, quinpirole reduced rod input to second-order neurons of tiger salamander retina without significantly altering rod voltage responses. Quinpirole also diminished the amplitude of depolarization-evoked increases in $[Ca^{2+}]_i$ measured with Fura-2 in rods, a finding consistent with inhibition of synaptic transmission from rods. Electrophysiological and Cl^- -imaging experiments indicated E_{Cl} in rods is ~ -220 mV. Quinpirole enhanced $I_{Cl(Ca)}$ and elicited an efflux of Cl^- at the resting potential. A similar Cl^- efflux was produced by extracellular replacement of 24 mM Cl^- with $CH_3SO_4^-$ and this low Cl^- solution inhibited Ca^{2+} responses to a similar degree as quinpirole did. When $I_{Cl(Ca)}$ was inhibited with niflumic acid, quinpirole enhanced both I_{Ca} and depolarization-evoked increases in $[Ca^{2+}]_i$. Furthermore, with niflumic acid, quinpirole no longer inhibited rod inputs into horizontal and bipolar cells. These results suggest an initial enhancement of I_{Ca} by quinpirole is followed by a stimulation of Cl^- currents, including $I_{Cl(Ca)}$. The net result is a Cl^- efflux that inhibits depolarization-evoked increases in $[Ca^{2+}]_i$ and synaptic transmission from rods.

Keywords

Outer retina; Calcium-activated chloride current; Calcium current; Electrophysiology; Imaging

Introduction

Dopamine is an important neuromodulator in the retina whose release is regulated by light and by circadian mechanisms. Although circadian mechanisms increase dopamine release during prolonged darkness in the night (Weiler et al., 1997), dopamine release is generally enhanced by increasing illumination (Godley & Wurtman, 1988; Boatright et al., 1989; Witkovsky et al., 1993). It has been proposed that an increase in dopamine levels in daylight illumination reshapes the retinal circuitry, diminishing the strength of rod signals and enhancing cone signals in second-order neurons (Witkovsky et al., 1988, 1989). The effects of dopamine on photoreceptor output are mediated in part by D₂-like (D₂ or D₄) dopamine receptors which are found on amphibian rods and cones (Muresan & Besharse, 1993). One mechanism by which D₂/D₄ receptor activation could reduce rod output would be by inhibiting rod calcium channels. Surprisingly, however, dopamine or the D₂/D₄ receptor agonist, quinpirole, was found to *enhance* rod I_{Ca} in the tiger salamander retina (Stella & Thoreson, 2000) and so would be expected to *increase* rod transmitter release. This result raises the question: how does activation of D₂/D₄ receptors enhance rod I_{Ca} yet suppress rod inputs to its postsynaptic targets?

A complex array of mechanisms regulates synaptic transmission at the photoreceptor synapse, some fraction of which may contribute to reconciling the apparent contradiction in presynaptic and postsynaptic effects of dopamine. For example, dopamine-mediated suppression of rod output might involve other actions of D₂/D₄ dopamine receptor activation in photoreceptors, such as inhibition of Na⁺/K⁺-ATPase activity (Shulman & Fox, 1996), increased rod/cone coupling (Krizaj et al., 1998; Manglapus et al., 1999), and inhibition of I_h in rods (Akopian & Witkovsky, 1996). There may also be contributions from other neuromodulators known to influence rod function, such as nitric oxide, somatostatin, or adenosine (Kurenniy et al., 1994; Akopian et al., 2000; Stella et al., 2002). Furthermore, the net impact of a change in I_{Ca} on synaptic transmission may be influenced by intrinsic feedback interactions involving Ca²⁺-dependent inactivation of I_{Ca} , Ca²⁺-induced Ca²⁺ release, glutamate transporters, and Ca²⁺-activated Cl⁻ channels, among others (reviewed in Thoreson & Witkovsky, 1999).

In the present study, we test the role of feedback between I_{Ca} and $I_{Cl(Ca)}$ in shaping the impact of D₂/D₄ receptor activation on synaptic transmission from rods to second-order retinal neurons. Ca²⁺-activated Cl⁻ channels are localized to photoreceptor terminals (MacLeish & Nurse, 2000). $I_{Cl(Ca)}$ is activated by influx through L-type Ca²⁺ channels on photoreceptors (Corey et al., 1984; Maricq & Korenbrot, 1988; Barnes & Hille, 1989; Yagi & Macleish, 1994; Taylor & Morgans, 1998) and may also be activated by release of Ca²⁺ from intracellular stores (Barnes & Hille, 1989). The Cl⁻ flux through Ca²⁺-activated Cl⁻ channels in photoreceptors can be substantial. For example, during a 1.4-s depolarizing step, the charge movement accompanying activation of $I_{Cl(Ca)}$ in cones has been estimated to be 8.5 times that produced by activation of I_{Ca} alone (Barnes & Hille, 1989). A number of studies suggest that E_{Cl} in rods is positive to the dark resting membrane potential (Bader et al., 1982; Somlyo & Walz, 1985; Burkhardt et al., 1991; Thoreson et al., 2000), so that activation of $I_{Cl(Ca)}$ in rods would result in an efflux of Cl⁻. Reductions in intracellular [Cl⁻]

inhibit calcium channel activity by increasing the time spent in the closed state, thus inhibiting the whole cell I_{Ca} (Thoreson et al., 1997, 2000).

The experiments described herein support the hypothesis that $I_{Cl(Ca)}$ is enhanced by quinpirole, either as a consequence of enhanced I_{Ca} or by direct actions on $I_{Cl(Ca)}$; the accompanying Cl^- efflux provides a negative feedback signal to inhibit I_{Ca} . The regulation of I_{Ca} provided by Cl^- channel activity has a surprisingly strong impact on D₂/D₄ receptor modulation of intracellular Ca^{2+} changes and synaptic output in rods.

Methods

Tissue preparation

Larval tiger salamanders (*Ambystoma tigrinum*, 7–10 inches in length) were cared for according to institutional guidelines. Retinal slices were prepared according to the methods of Werblin (1978) and Wu (1987). Briefly, salamanders were sacrificed by decapitation and pithed, an eye was enucleated, and the anterior portion of the eye including the lens was removed. The resulting eyecup was cut into sections and placed vitreal side down onto a piece of filter paper (Millipore 2×5 mm, Type GS, 0.2- μ m pores). After the retina adhered to the filter paper, the retina was isolated under chilled amphibian superfusate, and the tissue and filter paper were cut into 125- μ m slices using a razor blade tissue chopper (Stoelting, La Jolla, CA). The slices were rotated 90 deg to allow viewing of the retinal layers when placed under a water immersion objective (40 $\times$, 0.7 NA/60 $\times$, 1.0 NA) and viewed on an upright fixed-stage microscope (Olympus BHWI/Nikon EF 600, Tokyo, Japan). Unless otherwise stated, for electrophysiological experiments all procedures were performed under infrared illumination. For imaging experiments, slices were prepared under a dissecting lamp in visible light.

Solutions and perfusion

Solutions were applied by a single-pass, gravity-feed perfusion system that delivered medium to the slice chamber at a rate of 1 ml/min. The normal amphibian superfusate bathing the slices contained (in mM) 111 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 5 glucose. During recordings of light responses, the superfusate was supplemented with the glutamate precursor, L-glutamine (1 mM), to help sustain synaptic transmission in the retinal slice. Light responses recorded in the presence or absence of glutamine did not differ obviously in waveform. For recordings of $I_{Cl(Ca)}$, 10 mM tetraethylammonium (TEA)Cl was added. For recordings of I_{Ca} , the superfusate was switched to a solution containing (in mM) 99 NaCl, 2.5 KCl, 10 $BaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 5 glucose, 0.1 picrotoxin, and 0.1 niflumic acid. The pH of all bathing solutions was adjusted to 7.8 with NaOH and solutions were continuously bubbled with 100% O_2 . For Cl^- replacement experiments, 24 mM Cl^- was substituted for equimolar $CH_3SO_4^-$. $CH_3SO_4^-$ solutions were boiled for 10 min to evaporate residual methanol before glucose was added and the solution brought to its final volume.

Electrophysiology

Patch pipettes were pulled on a Narishige PB-7 vertical puller from borosilicate glass pipettes (1.2 mm O.D., 0.95 mm I.D., omega dot) and had tips of $\sim 1 \mu\text{m}$ O.D. with tip resistances of 10–15 M Ω .

To maintain endogenous second-messenger signaling pathways and minimize response rundown, we used the perforated patch method of whole cell recording with the pore forming antibiotic, nystatin (Rae et al., 1991). Nystatin was mixed in dimethylsulf-oxide (DMSO) at a concentration of 120 mg/ml, vortexed briefly, and then added to the pipette electrolyte solution to achieve a final concentration of 480 $\mu\text{g/ml}$. Fresh nystatin solutions were prepared every 3 h. For measurements of E_{Cl} and recordings of light-evoked voltage responses in rods, we used gramicidin instead of nystatin because of its negligible chloride permeability (Kyrozis & Reichling, 1995). Gramicidin was dissolved in ethanol (5 mg/ml) and then added to the pipette electrolyte solution to a final concentration of 5 $\mu\text{g/ml}$. In successful perforated patch recordings, gigaohm seals were obtained in 30 s or less, and cells were usually well perforated within 5 min of sealing. Following perforation, pipette access resistance typically fell to $< 40 \text{ M}\Omega$. For recordings of I_{Ca} and $I_{\text{Cl(Ca)}}$, the pipette electrolyte solution contained (in mM) 54 CsCl, 61.5 CsCH₃SO₃, 3.5 NaCH₃SO₄, and 10 HEPES. The pH was adjusted to 7.2 with CsOH. For recordings of light-evoked currents, the pipette electrolyte solution contained (in mM) 54 KCl, 61.5 KCH₃SO₄, 3.5 NaCH₃SO₄, and 10 HEPES. The pH was adjusted to 7.2 with KOH. The osmolarity was adjusted, if necessary, to $242 \pm 5 \text{ mOsm}$.

Rods and cones were distinguished by the characteristic shapes of their outer segments. Horizontal cells were distinguished from bipolar cells by the presence of prominent inward-rectifying voltage-dependent currents in the former and prominent outward-rectifying currents in the latter. ON and OFF bipolar cells were readily distinguished from one another by virtue of their inward and outward light-evoked currents, respectively.

Rods were voltage clamped at -60 to -70 mV , horizontal cells were held at -40 mV , and bipolar cells were held at -50 mV using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Currents were acquired and analyzed using Clampex 7.0 software (Axon Instruments, Foster City, CA). Rod and bipolar cell charging curves are typically fit by a single exponential, indicating compact electrotonic structures (Thoreson et al., 1997; Stella & Thoreson, 2000).

Light stimuli were generated using a 50-W halogen lamp focused onto a fiber optic and then reflected through a beam splitter into the condenser path of the microscope. The wavelength of light was selected by use of interference filters (580 and 680 nm) and Wratten gel neutral density filters were used to decrease light intensity. Light stimuli were delivered to slices from complete darkness. The intensity of unattenuated light, measured with a laser power meter (Metrologic, La Jolla, CA), available during recordings from second-order neurons was approximately $1.1 \times 10^6 \text{ photons s}^{-1} \mu\text{m}^{-2}$ at 680 nm and $2.1 \times 10^6 \text{ photons s}^{-1} \mu\text{m}^{-2}$ at 580 nm. During recordings of light-evoked responses from rods, the maximum available intensities were $4.6 \times 10^4 \text{ photons s}^{-1} \mu\text{m}^{-2}$ at 680 nm and $5.6 \times 10^4 \text{ photons s}^{-1} \mu\text{m}^{-2}$ at 580 nm.

Intensity/response data were fit with a form of the Michaelis-Menten equation: $R/R_{\max} = I^a/(I^a + \sigma^a)$, where R = response amplitude, $R_{\max}$ = maximum response amplitude, I = intensity, σ = intensity at which the response is half-maximal, and a is a slope factor (Naka & Rushton, 1966; Baylor et al., 1974).

Statistical significance was chosen as $P < 0.05$ and evaluated with Student's t -test using GraphPad Prism 3.0. Errors are reported as $\pm$ SEM.

Imaging experiments

Digital fluorescent images were recorded with a cooled CCD camera (SensiCam, Cooke Corp., La Jolla, CA) on an upright fixed-stage microscope (Nikon E600FN, Garden City, NY) equipped with a 60 $\times$ (1.0 N.A.) water immersion objective. Axon Imaging Workbench (AIW 2.2) was used to control the camera, filter wheel, and image acquisition. Pixel binning (2 $\times$ 2) of the images was typically used to decrease acquisition time to 1 s. Images were acquired at 5- to 10-s intervals during experimental trials.

For measurements of $[Ca^{2+}]_i$, we used the ratiometric dye, Fura-2 (Molecular Probes, Eugene, OR) (Grynkiewicz et al., 1985). To load retinal slices with Fura-2, slices were incubated at 5 $^{\circ}$ C for 45 min in the dark with 0.5 ml of 10 μ M Fura-20AM + 0.02% pluronic F-127 (Molecular Probes). This was followed by an additional incubation in Fura-2/AM alone for 1.5 h. Isolated cells were loaded by incubation in Fura-2/AM (10 μ M) for 45 min. Intracellular Ca^{2+} increases were stimulated by depolarization with 20 or 50 mM KCl applied for 1 min at 15-min intervals. Measurements were made in the cell soma. For statistical comparisons, the change in the 340/380 nm ratio produced by application of KCl in the presence of a test substance was compared to the average of the 340/380 ratio changes obtained prior to application of the test substance and following washout.

For measurements of $[Cl^{-}]_i$, we used the dye, 6-methoxy-N-ethylquinolinium iodide (MEQ, Molecular Probes) (Biwersi & Verkman, 1991). MEQ was loaded into cells after reducing it to DiH-MEQ by adding 30 μ M sodium borohydride (100 μ l) to MEQ (5 mg) under a continuous stream of nitrogen gas (Wöll et al., 1996). DiH-MEQ that enters cells during the incubation period (15 min) is oxidized and retained in the form of MEQ. Fluorescence emission decreases as Cl^{-} quenches MEQ. The exponential decay in MEQ fluorescence due to dye leakage and bleaching was determined under control conditions and subtracted prior to analysis. For a single-wavelength dye, such as MEQ, changes in fluorescence arising from variations in dye concentration, cell thickness, etc. can be factored out to yield the ion-dependent fluorescence change by determining the change in fluorescence relative to the basal fluorescence ($\Delta F/F = [F - F_0]/F_0$) (Helmchen, 2000).

Results

Effects of quinpirole on I_{Ca}

Accurate measurement of I_{Ca} requires that it be pharmacologically isolated from $I_{Cl(Ca)}$. This was done by using Ba^{2+} carrier and including niflumic acid in the bathing medium. The stimulatory effect of the D_2/D_4 dopamine receptor agonist, quinpirole, on I_{Ca} recorded from a rod photoreceptor under these conditions is illustrated in Fig. 1. When $I_{Cl(Ca)}$ was blocked,

quinpirole (20 μ M) enhanced peak rod I_{Ca} by an average of $71.6 \pm 19.4\%$ ($n = 7$) with little or no effect on the voltage dependence of the current (Stella & Thoreson, 2000).

Effects of quinpirole on rod to second-order neuron synaptic transmission

Witkovsky et al. (1988, 1989) have shown that a D_2/D_4 agonist inhibits rod inputs and enhances cone inputs to horizontal cells in the *Xenopus* retina. Accordingly, we tested whether quinpirole suppressed rod inputs into second-order neurons in the tiger salamander retina by recording the light-evoked currents from voltage-clamped horizontal, ON bipolar, and OFF bipolar cells in the presence and absence of quinpirole. We evaluated the balance of rod and cone inputs by analyzing the relative sensitivity to 580- and 680-nm lights applied to dark-adapted retinas. In the salamander retina, rods are ~ 2.2 log units more sensitive to 580-nm than 680-nm light whereas red-sensitive cones are ~ 0.25 log units more sensitive to 680-nm light (see Fig. 2D; data from Makino & Dodd, 1996). Figure 2A illustrates a family of light-evoked currents recorded from a horizontal cell in control conditions, and Figure 2B shows a family of current records recorded from the same cell in the presence of quinpirole. In quinpirole, the responses at high intensities were shorter and more closely correlated with stimulus duration. This shortened response is consistent with a reduction in inputs from rods (Witkovsky et al., 1988). Figure 2C shows the amplitude/intensity relationships obtained with 580- (circles) and 680- (triangles) nm light in a horizontal cell in the presence (dashed lines) and absence (solid lines) of quinpirole (20 μ M). The intensity necessary to produce a half-maximal current (σ) was used as a measure of the cell's relative sensitivity to 580- and 680-nm light. Quinpirole diminished the sensitivity to both 580- and 680-nm lights, but produced a much greater decrease in the sensitivity to 580-nm light, consistent with a reduction in rod inputs. Quinpirole reduced σ_{580} from -4.171 log attenuation to -2.663 log; following washout σ_{580} recovered to -3.953 log. Quinpirole reduced σ_{680} from -2.565 log attenuation to -1.638 log; following washout σ_{680} recovered to -1.926 log. Thus, the cell was initially 1.61 log units more sensitive to 580- than 680-nm light and in the presence of quinpirole the 580/680 sensitivity difference diminished to 1.03 log units. Following washout of quinpirole, the 580/680 difference increased to 2.03 log units (not shown). Reapplication of quinpirole in this same cell again reduced the 580/680 difference to 1.59 log units and subsequent washout increased it to 2.49 log units. Relative decreases in 580- versus 680-nm sensitivity were observed in all three types of second order neuron. In the control condition, cells were strongly rod dominated, based on their 2.19 ± 0.136 log unit greater sensitivity to 580- than 680-nm light (Fig. 2D; $n = 15$, 6 ON bipolar cells, 4 OFF bipolar cells, and 5 horizontal cells). In the presence of quinpirole, this difference diminished significantly to 1.59 ± 0.254 log units ($P = 0.0016$, Fig. 2D). The absolute sensitivity to 580 nm light was reduced in quinpirole by an average of 0.36 log units ($P = 0.29$). The large reduction in 580-nm sensitivity illustrated in Figure 2 may be due to particularly strong rod inputs into this cell. The decrease in 580-nm sensitivity recovered following washout in this cell; a smaller decrease in 580-nm sensitivity was produced by the second application of quinpirole.

We tested whether quinpirole might have presynaptic effects that alter the voltage responses in rods evoked by light flashes. Figure 3A shows a family of responses evoked in control conditions, and Figure 3B shows responses evoked by the same flash intensities in the

presence of quinpirole. With gramicidin-perforated patch-recording techniques, quinpirole stimulated a small depolarization of the dark membrane potential averaging 1.2 ± 0.46 mV ($P = 0.03$, $n = 11$). Similar to results of Witkovsky et al. (1988), quinpirole caused little change in peak amplitude or waveform. As can be seen from the amplitude/intensity curves for 580- and 680-nm light flashes in Figure 3C and the difference in σ values for 580- and 680-nm lights plotted in Figure 3D, quinpirole did not significantly alter the relative sensitivity of rods to 580- versus 680-nm lights which was in both cases near 2.2 log units, consistent with the light-evoked current measurements of Makino and Dodd (1996). The mean σ value for 580-nm light alone was reduced by 0.22 log units ($P = 0.07$) in quinpirole. These results indicate that the decrease in 580-nm sensitivity relative to 680-nm sensitivity does not arise from changes in the rod voltage response and supports the hypothesis that D₂/D₄ receptor activation in tiger salamander retina decreases the strength of rod input into second-order neurons.

Effects of quinpirole on depolarization-evoked changes in $[Ca^{2+}]_i$

To test whether the D₂/D₄-mediated inhibition of rod output was due to a reduction in Ca^{2+} influx or some other mechanism, such as reduced quantal size (Pothos et al., 1998), we examined the effect of quinpirole on depolarization-evoked increases in $[Ca^{2+}]_i$. Influx through voltage-dependent Ca^{2+} channels in photoreceptors produces an increase in $[Ca^{2+}]_i$ with a component contributed by Ca^{2+} -induced Ca^{2+} release (Thoreson et al., 1997; Krizaj & Copenhagen, 1998). As illustrated in Figure 4, in the absence of niflumic acid or Ba²⁺, application of quinpirole (20 μ M) inhibited the Ca^{2+} increase in rods evoked by depolarization with 50 mM K⁺. Using gramicidin-perforated patch-recording techniques, it was found that 50 mM K⁺ depolarized rods (in retinal slices prepared under visible light as for imaging experiments) from -43.8 ± 7.4 mV ($n = 10$) to -8.9 ± 0.59 mV ($n = 9$). Overall, the 340/380 nm ratio change evoked by 50 mM K⁺ in rods in the retinal slice preparation was inhibited $27 \pm 4.4\%$ by quinpirole compared to ratio changes elicited by 50 mM K⁺ in control medium ($n = 27$, $P < 0.0001$). A similar $33 \pm 12.4\%$ decrease in 340/380 nm ratio change was produced by quinpirole in isolated rods ($n = 7$, $P = 0.037$). Quinpirole also caused a $31 \pm 2.9\%$ ($n = 10$, $P < 0.0001$) inhibition of 340/380 ratio changes evoked by a more modest depolarization to -25.7 ± 1.34 mV ($n = 10$) produced by application of 22 mM K⁺ to rods in the retinal slice preparation. The reduction of depolarization-evoked Ca^{2+} increases is consistent with the decrease in rod synaptic output produced by quinpirole (Fig. 2). Quinpirole did not appreciably alter Ca^{2+} increases evoked by caffeine (10 mM, $n = 10$) suggesting that quinpirole does not affect the Ca^{2+} -induced release of Ca^{2+} from intracellular stores.

For the Ca^{2+} -imaging experiments described above, blockers of $I_{Cl(Ca)}$ were omitted. Niflumic acid is selective for $I_{Cl(Ca)}$ over other currents in photoreceptors (Barnes & Deschenes, 1992) and application of niflumic acid (0.1 mM) reduced the $I_{Cl(Ca)}$ tail current in rods by $46.0 \pm 7.3\%$ ($n = 12$; data not shown). When niflumic acid (0.1 mM) was added to the bath during Ca^{2+} -imaging experiments, the inhibitory effect of quinpirole on depolarization-evoked increases in $[Ca^{2+}]_i$ was converted into a stimulatory one (Fig. 5) resembling the stimulatory effect of quinpirole on I_{Ca} (Fig. 1). In the presence of niflumic acid, quinpirole enhanced the 340/380 ratio increase evoked by 50 mM K⁺ by an average of

$74 \pm 16.5\%$ ($n = 16$, $P = 0.0004$) and enhanced responses to 22 mM K^+ by $76 \pm 11.6\%$ ($n = 17$, $P < 0.0001$).

Immediately upon application of niflumic acid, there was a small decrease in the 340/380 ratio which may result from a small membrane hyperpolarization produced by niflumic acid (-3.9 ± 1.1 mV, $n = 9$). The peak 340/380 ratio evoked by high K^+ in the presence of niflumic acid was typically smaller than that evoked by high K^+ in control solution. The reason for this was not studied further but may also be related to membrane potential effects of niflumic acid.

As a further test that the effects of quinpirole were mediated by Cl^- channel inhibition, we used a structurally different Cl^- channel blocker, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; RBI/Sigma, St. Louis, MO). Application of NPPB (2 μ M) inhibited the $I_{Cl(Ca)}$ tail current by $17 \pm 12\%$ ($n = 7$). Perhaps because of the weak inhibition of $I_{Cl(Ca)}$, there was little or no effect of NPPB on baseline $[Ca^{2+}]_i$ or the amplitude of depolarization-evoked $[Ca^{2+}]_i$ relative to control solution. However, in the presence of NPPB, Ca^{2+} responses evoked by 50 mM K^+ were enhanced by quinpirole an average of $32 \pm 9.8\%$ ($n = 18$, $P = 0.005$; data not shown).

Small reductions in intracellular $[Cl^-]$ can significantly inhibit I_{Ca} (Thoreson et al., 1997, 2000). We suggest that quinpirole enhances Cl^- efflux resulting in feedback inhibition of I_{Ca} . Further tests of this hypothesis, which may explain at least partially the effects of niflumic acid and NPPB on depolarization-evoked Ca^{2+} responses, are described below.

Effects of quinpirole on $I_{Cl(Ca)}$

A series of depolarizing steps (300 ms) from -60 mV to 40 mV was applied to a voltage-clamped rod to activate I_{Ca} and thus stimulate $I_{Cl(Ca)}$. I_{Ca} then was deactivated by repolarizing the membrane to -60 mV. A tail current arising from $I_{Cl(Ca)}$ (Barnes & Deschenes, 1992) persisted long after repolarization (Fig. 6). Quinpirole (20 μ M) enhanced both the outward current evoked by depolarization above E_{Cl} (≈ 20 mV, see Fig. 7) and the inward tail current observed after returning to -60 mV (Fig. 6). After wash-out of quinpirole, both the outward current during the step and the subsequent tail current returned to control levels. The tail current measured 500 ms into the trial was enhanced by an average of $31 \pm 7.8\%$ ($n = 5$, $P = 0.017$). Thus, perhaps as a consequence of the enhancement of I_{Ca} , application of quinpirole increased the amplitude of $I_{Cl(Ca)}$.

Determination of E_{Cl}

For activation of Cl^- channels to produce an efflux of Cl^- , E_{Cl} in rods must be positive to the resting potential. E_{Cl} was determined with both electrophysiological and Cl^- -imaging experiments. Electrophysiological recordings from rods were obtained using gramicidin-perforated-patch whole-cell recordings to avoid artefactual changes in intracellular Cl^- (Kyrozis & Reichling, 1995). After a successful recording was obtained, $I_{Cl(Ca)}$ was activated by applying a 500-ms step from -70 mV to 0 mV. The current/voltage relationship of the cell was measured during the tail current using a ramp voltage change (1 mV/ms from -90 mV to $+60$ mV) applied 50 ms after termination of the step (e.g. Fig. 7A). The same

protocol then was repeated in the presence of niflumic acid (e.g. Fig. 7B). Subtracting the two ramp-evoked currents yields the current/voltage profile for a niflumic acid-sensitive difference current. Figure 7C shows the difference current averaged ($\pm$ SEM) from seven experiments. The niflumic acid-sensitive current reversed about -20 mV.

In the second approach to measuring E_{Cl} , we used a Cl^- -sensitive dye, MEQ, to examine the Cl^- flux that accompanied rod depolarization evoked by bath application of high K^+ solutions (12, 22, 31, 41, 50, and 70 mM K^+). We used gramicidin-perforated patch-recording techniques to measure the membrane potential of rods in the presence of these same high K^+ solutions. For these measurements, slices were prepared under visible light as for imaging experiments but recordings were obtained in darkness. The resting potentials recorded in control superfusate averaged -43.8 ± 1.74 mV ($n = 10$) in rods from slices prepared under visible light and -44.4 ± 1.32 mV ($n = 19$) in slices prepared under infrared illumination. For a single-wavelength dye, such as MEQ, the change in fluorescence relative to basal fluorescence (F/F) can be used as a measure of the change in ion concentration. Figure 7D illustrates the change in $F/F(\times 100)$ plotted as a function of the membrane potential evoked by the different high K^+ solutions. Because MEQ is quenched by Cl^- , an increase in fluorescence reflects an efflux of Cl^- from the cell and a decrease in fluorescence reflects Cl^- influx. Our data indicates that depolarizing the membrane potential to values less than -20 mV stimulated Cl^- efflux, whereas depolarization above -20 mV stimulated Cl^- influx (Fig. 7D). In agreement with the electro-physiological determination of E_{Cl} , Cl^- flux reversed around -19 mV. Thus, the present results support previous studies (Bader et al., 1982; Somlyo & Walz, 1985; Burkhardt et al., 1991; Thoreson et al., 2000) indicating that E_{Cl} is positive to the dark resting membrane potential of rods.

Effects of quinpirole on $[Cl^-]_i$

At the membrane potential of the rod in darkness (-44 mV), enhancement of Cl^- currents by quinpirole will stimulate an efflux of Cl^- . We found that quinpirole evoked an increase in MEQ fluorescence, reflecting a decrease in intracellular Cl^- (Fig. 8). On average, quinpirole produced a $2.0 \pm 0.37\%$ ($n = 11$, $P = 0.0003$) increase in relative MEQ fluorescence ($F/F \times 100\%$). The fluorescence increase was significantly inhibited by 0.1 mM niflumic acid (Fig. 6B; $F/F \times 100\% = 0.44 \pm 0.18\%$, $n = 14$; quinpirole vs. quinpirole + niflumic acid: $P = 0.0005$, unpaired t -test), consistent with the possibility that Cl^- efflux results from an enhanced activation of $I_{Cl(Ca)}$ by quinpirole.

A reduction in intracellular Cl^- similar to that evoked by quinpirole was found when 24 mM Cl^- was replaced by 24 mM $CH_3SO_4^-$; this procedure increased MEQ fluorescence ($F/F \times 100\%$) by an average of $2.6 \pm 0.58\%$ ($n = 15$, $P = 0.0005$, Fig. 9A). Application of this 24 mM $CH_3SO_4^-$ solution inhibited high K^+ -evoked Ca^{2+} responses by an average of $25 \pm 1.9\%$ ($n = 16$, $P < 0.0001$, Fig. 9B), similar to the inhibition produced by quinpirole (Fig. 4).

Effects of quinpirole plus niflumic acid on rod to second-order cell synaptic transmission

If a quinpirole-induced efflux of Cl^- contributes to its ability to inhibit synaptic output from rods, then niflumic acid should inhibit the quinpirole-induced reduction in rod inputs into

second-order neurons. Figure 10A shows a family of light-evoked currents recorded from a horizontal cell in the presence of niflumic acid. Figure 10B shows responses obtained at the same intensities in the same cell recorded after the addition of quinpirole in the presence of niflumic acid. Quinpirole caused an inward shift in the DC holding current and increased the amplitude of the maximal light-evoked current. Both of these actions are consistent with the presynaptic enhancement of I_{Ca} illustrated in Fig. 1. Increases in peak light response amplitude were observed in 7/9 OFF bipolar and horizontal cells and inward DC currents were observed in 8/9 experiments. There was little or no change in the absolute sensitivity to 580- or 680-nm light (Fig. 10C) and, most notably, unlike the effects of quinpirole in control superfusate (Fig. 2), quinpirole added in the presence of niflumic acid produced no significant change in the relative sensitivity to 580- versus 680-nm lights when compared to responses obtained in niflumic acid alone (Fig. 10D, $P = 0.86$, paired t -test; $n = 15$ pairs: 6 ON bipolar cells, 3 OFF bipolar cells, and 6 horizontal cells).

We recorded from rods under the same conditions to test whether the ability of niflumic acid to block the quinpirole-induced 580/680 shift is due to a presynaptic action on the rod light response. Figure 11A illustrates a family of light-evoked voltage responses recorded from a rod in the presence of niflumic acid (0.1 mM), and Figure 11B shows light-evoked voltage responses recorded from the same rod following application of quinpirole (20 μ M) in the presence of niflumic acid. The cell was slightly depolarized by quinpirole and, as seen in most other rods, the initial transient hyperpolarization, as a fraction of the total response, was enhanced. The amplitude/intensity relationships for the tested rods showed little change in absolute sensitivity to 580- or 680-nm light following the application of quinpirole (Fig. 11C). Moreover, the relative sensitivity to 580- and 680-nm light ($\sigma_{580} - \sigma_{680}$) of either the rod peak or plateau voltage was not significantly altered by addition of quinpirole (peak: $P = 0.81$; plateau: $P = 0.53$; $n = 10$ pairs). These data indicate that the ability of niflumic acid to abolish the quinpirole-induced shift in the balance of 580-versus 680-nm sensitivity is not related to changes in the rod light-evoked voltage response.

Discussion

The experimental problem posed by prior findings

The goal of the present study was to reconcile two apparently incompatible findings. The first is that quinpirole, an agonist at dopamine D_2/D_4 receptors (Grandy et al., 1994; Van Tol, 1994), increases cone input to the second-order retinal neurons, horizontal and bipolar cells (Witkovsky et al., 1988; present report). The other is that quinpirole increases I_{Ca} in rods (Stella & Thoreson, 2000), a result that would be predicted to increase transmitter release by rods and enhance rod input to second-order neurons. It is important to note, however, that the quinpirole-induced increase in I_{Ca} was seen when Ba^{2+} was substituted for Ca^{2+} as the current carrier and in the presence of niflumic acid, both which inhibit $I_{Cl(Ca)}$. In the present study, we attempt to rationalize these apparently contradictory data by focusing on chloride in the rod photo-receptor. Our data indicate clearly that when $I_{Cl(Ca)}$ is not blocked, rod input to second-order neurons is reduced in the presence of quinpirole.

Experimental tests of calcium-chloride interactions

$I_{Cl(Ca)}$ is very prominent in both cones (Maricq & Korenbrot, 1988; Barnes & Hille, 1989; Yagi & MacLeish, 1994) and rods (Bader et al., 1982; Chen et al., 2000). The channels carrying this current are confined to the photoreceptor synaptic terminal (MacLeish & Nurse, 2000). The current flux through $I_{Cl(Ca)}$ exceeds that of I_{Ca} by a factor of at least 8 (Barnes & Hille, 1989), and it appears as a prominent tail current which greatly outlasts the stimulus. To evaluate the effects of $I_{Cl(Ca)}$ on I_{Ca} , it is important to establish the value of E_{Cl} . We studied E_{Cl} in two ways: using a Cl-sensitive dye, MEQ, and by determining the reversal potential of $I_{Cl(Ca)}$. Both methods yielded a value near -20 mV, which is substantially positive to the mean membrane potential of rods in darkness, -44 mV. As a consequence, activation of $I_{Cl(Ca)}$ has two effects. It induces an efflux of Cl^- , which depolarizes the rod and indirectly increases I_{Ca} , since the probability of Ca^{2+} channel opening is increased by depolarization (Bader et al., 1982; Corey et al., 1984). The second is that intracellular chloride ions have a direct, if still poorly understood, action on the Ca^{2+} channel that results in increased Ca^{2+} flux (Thoreson et al., 1997, 2000). Thus, Cl^- efflux decreases I_{Ca} through this mechanism. The data of the present report can be interpreted through interactions of I_{Ca} and $I_{Cl(Ca)}$ suggesting that a feedback loop dependent on Cl^- flux and $[Cl^-]_i$ reduces I_{Ca} . Our interpretation is qualitative and does not exclude other mechanisms operating in parallel that also influence I_{Ca} .

We utilized niflumic acid to block $I_{Cl(Ca)}$. Recently, Satoh and Yamada (2001) reported that niflumic acid also inhibits the voltage-dependent Na^+/K^+ current called I_h in rods. This current, however, requires a hyperpolarizing step to be activated and is negligible at potentials equal to, or more positive, than the rod membrane potential in darkness, which is where the Ca^{2+} and Cl^- imaging experiments of the present study were obtained. With regard to light response experiments, the relative sensitivity to 580- versus 680-nm light of either the peak or plateau of the light-evoked voltage response of rods was not significantly altered by quinpirole even in the presence of niflumic acid, so modulation of I_h is unlikely to account for quinpirole-induced changes in the spectral sensitivity of second-order neurons. We found, in agreement with previous investigators (Barnes & Deschenes, 1992; Chen et al., 2000) that niflumic acid reduced $I_{Cl(Ca)}$, hyperpolarized the rod by 3–4 mV and reduced the maximum increase in $[Ca^{2+}]_i$ evoked by exposing the rod to elevated K^+ . The hyperpolarization suggests that there is a baseline activation of $I_{Cl(Ca)}$ in darkness, resulting in a steady Cl^- efflux. This is supported by the finding that rods release transmitter steadily in darkness *via* a Ca^{2+} -dependent process (Schmitz & Witkovsky, 1997).

When quinpirole was applied in the presence of niflumic acid, it was found to *increase* rod input to second-order neurons. Moreover, niflumic acid eliminated the quinpirole-induced shift in the balance of rod/cone inputs to second-order cells. This finding strongly supports the conclusion that Cl^- plays an important role in regulating the actions of quinpirole on rods. Another set of experiments linking Cl^- and I_{Ca} involved measuring changes in $[Ca^{2+}]_i$. The combination of niflumic acid and quinpirole augmented the increases in $[Ca^{2+}]_i$ evoked by high K^+ , whereas quinpirole applied in the absence of niflumic acid, reduced the maximum increase of $[Ca^{2+}]_i$. This particular set of experiments, however, poses a problem of interpretation. According to our hypothesis, Cl^- flux sets in motion two opposing

influences on I_{Ca} : changes in rod membrane voltage and a direct action of Cl^- on the Ca^{2+} channel. In principle, therefore, if $[K^+]_o$ was adjusted to bring the rod membrane voltage to E_{Cl} , then net flux of Cl^- will be zero and so the results in quinpirole and control solution should not differ. Experimentally, what we found was that, in the absence of niflumic acid, quinpirole still reduced the increase in $[Ca^{2+}]_i$ when $[K^+]_o$ was raised to 22 mM which depolarizes the rod membrane to -25.6 mV, close to E_{Cl} . However, in this experiment, quinpirole was applied for 3 min before exposing the retinal slice to high K^+ . Thus, all the quinpirole-dependent changes in I_{Ca} and $I_{Cl(Ca)}$ had been set in motion prior to high K^+ application and we do not know how persistent those changes might be. Furthermore, the inhibition of I_{Ca} by quinpirole existing at the beginning of the high K^+ application will slow the initial rate of activation of Ca^{2+} channels which will in turn slow the rate of Ca^{2+} influx. If the rate of influx is reduced but Ca^{2+} extrusion and buffering rates remain relatively constant, then this slower regenerative activation of Ca^{2+} channels would result in a lower peak Ca^{2+} concentration. Resolution of these issues requires additional experimentation and a more detailed understanding of the Ca^{2+} dynamics in the photo-receptor terminal.

Although many of the results relied on the use of niflumic acid as a tool to block $I_{Cl(Ca)}$, the argument that Cl^- plays a crucial role in regulating I_{Ca} does not depend exclusively on results obtained with niflumic acid. Like niflumic acid, in the presence of the Cl^- channel blocker, NPPB, quinpirole enhanced K -dependent increases in $[Ca^{2+}]_i$. Additionally, quinpirole caused a reduction in $[Cl^-]_i$ and when a comparable reduction of $[Cl^-]_i$ was effected by substituting 24 mM Cl^- by an equimolar amount of methylsulfate, the maximum increase in $[Ca^{2+}]_i$ evoked by elevated K^+ also fell. These data are consistent with previous studies suggesting that Cl^- ions increase Ca^{2+} influx in rods (Thoreson et al., 1997, 2000).

Other possible actions of quinpirole

The observation that the inhibition produced by quinpirole is stronger than expected from simple negative feedback between $I_{Cl(Ca)}$ and I_{Ca} may indicate that quinpirole stimulates a larger Cl^- efflux than would be generated simply from an enhanced activation of $I_{Cl(Ca)}$. One possibility is that, in addition to its effects on I_{Ca} , quinpirole might also enhance $I_{Cl(Ca)}$ directly by altering levels of intracellular second messengers (e.g. cAMP) (Lalevee & Joffre, 1999). Another possibility is that quinpirole might stimulate other Cl^- currents. The major Cl^- current described in photoreceptors is $I_{Cl(Ca)}$, but Cl^- transporter currents also have been described (Eliasof & Werblin, 1993; Picaud et al., 1995; Larsson et al., 1996; Fan & Yazulla, 1997) and other Cl^- channels may be present, since a CIC-2 antibody labels the ONL and OPL and a CIC-3 antibody labels the OPL (Enz et al., 1999; Stobrawa et al., 2001). Cl^- flux through these channels might regulate I_{Ca} activity in a manner analogous to the proposed regulation by $I_{Cl(Ca)}$.

D₂/D₄ dopamine receptors and rod synaptic transfer

In the absence of Cl^- channel blockers, quinpirole increased the relative sensitivity of second-order neurons to 680- versus 580-nm light, consistent with a stronger relative contribution from red-sensitive cones (Fig. 2). The relative sensitivity of rod voltage responses to 680- versus 580-nm light was not significantly altered by quinpirole either in control conditions (Fig. 3) or in the presence of niflumic acid (Fig. 11). However, in the

presence of niflumic acid, quinpirole no longer diminished the expression of rod signals in second-order neurons (Fig. 10). Furthermore, consistent with the increases in rod I_{Ca} , quinpirole applied in the presence of niflumic acid stimulated an inward DC current and increased the amplitude of light-evoked currents in horizontal and OFF bipolar cells, presumably reflecting a postsynaptic response to increased tonic release of glutamate in darkness.

Electrophysiological experiments were generally performed on slices prepared under infrared illumination whereas imaging experiments were performed on more light-adapted slices prepared under room lights and repeatedly stimulated with epifluorescent illumination. Despite this, many of the characteristics of rods that are relevant to this study appeared quite similar. For example, the mean dark resting potential of rods prepared under visible light (-43.8 mV) did not differ significantly from that of rods prepared under infrared light (-44.4 mV). E_{Cl} was found to be near -20 mV in both cases. And to our surprise, light-evoked currents were recorded from rods in slices previously used for Fura-2 experiments and thus liberally exposed to light for at least 2 h (Stella & Thoreson, unpublished observations). Although illumination is reported to stimulate the release of dopamine in the inner retina (Godley & Wurtman, 1988; Boatright et al., 1989; Witkovsky et al., 1993), the D_2/D_4 antagonist spiperone ($10 \mu M$) had no significant effect on the relative sensitivity to 580- versus 680-nm light in horizontal cells from slices prepared under room light, suggesting that little endogenous dopamine reaches the photoreceptor terminals in superfused, thin retinal slices (Thoreson, unpublished observations).

$[Ca^{2+}]_i$ measurements were performed in the cell soma. Although effects of exogenously applied high K^+ and low Cl^- solutions appeared similar in both soma and terminal (Thoreson et al., 1997), Ca^{2+} -activated Cl^- channels appear to be clustered at the rod terminal (MacLeish & Nurse, 2000) and thus Cl^- flux through these channels may be more pronounced in the terminal.

Clearly the rod/cone balance is determined by the strength of cone inputs as well as the strength of rod inputs. D_2/D_4 receptor activation inhibits I_{Ca} in red-sensitive large single cones which are the major cone subtype of the salamander retina, but enhances I_{Ca} in red-sensitive, blue-sensitive, and UV-sensitive small single cones (Stella & Thoreson, 2000). The effects of D_2/D_4 receptor activation of I_{Ca} in red-sensitive double cones have not yet been examined. It is possible, however, that a similar interaction between I_{Ca} and $I_{Cl(Ca)}$ might also operate in cones since both conductances are present (Maricq & Korenbrot, 1988; Barnes & Hille, 1989; Yagi & MacLeish, 1994). In cones, estimates of E_{Cl} range from -65 to at or slightly above the dark resting potential (Kaneko & Tachibana, 1986; Thoreson & Burkhardt, 1991; Picaud et al., 1995; Thoreson et al., 2000). If E_{Cl} is negative to the resting potential, activation of $I_{Cl(Ca)}$ would hyperpolarize the cell and thus limit regenerative activation of Ca^{2+} channels (Maricq & Korenbrot, 1988; Yagi & MacLeish, 1994). On the other hand, activation of $I_{Cl(Ca)}$ in this circumstance would presumably increase $[Cl^-]_i$ which may in turn enhance I_{Ca} by direct actions of Cl^- on Ca^{2+} channels (Thoreson et al., 1997, 2000). Thus, no matter what the value of E_{Cl} , there is likely to be an interplay between these two processes which would help to stabilize the tonic level of I_{Ca} activation, thereby stabilizing synaptic output and preventing potentially damaging increases in $[Ca^{2+}]_i$.

Possible significance of Ca^{2+} – Cl^- interactions for neuromodulation in the retina

In addition to effects on D_2/D_4 receptor modulation, interactions between $I_{\text{Cl}(\text{Ca})}$ and I_{Ca} might also modify actions of other extrinsic modulators (e.g. somatostatin or adenosine). One point to consider when evaluating the conditions under which this feedback mechanism may operate is the sensitivity of $I_{\text{Cl}(\text{Ca})}$ to intracellular Ca^{2+} . The EC_{50} for Ca^{2+} in Ca^{2+} -activated Cl^- channels of amphibian olfactory neurons was found to be $\sim 5 \mu\text{M}$ (Kleene & Gesteland, 1991). Although it has not yet been directly determined for photo-receptors, the finding that quinpirole appeared to enhance $I_{\text{Cl}(\text{Ca})}$ by only 31% whereas it enhanced I_{Ca} by 72% is consistent with a weak Ca^{2+} dependence in rods. If $I_{\text{Cl}(\text{Ca})}$ in photoreceptors exhibits a weak Ca^{2+} dependence then feedback from $I_{\text{Cl}(\text{Ca})}$ may be significant only at relatively high intracellular Ca^{2+} levels (e.g. following enhancement of I_{Ca}).

The working hypothesis presented in this study can account for the paradox that D_2/D_4 receptor activation enhances rod I_{Ca} (when pharmacologically isolated from $I_{\text{Cl}(\text{Ca})}$) but suppresses depolarization-evoked increases in $[\text{Ca}^{2+}]_i$ in rods and the expression of rod signals in second-order retinal neurons. The present results do not rule out contributions from other D_2/D_4 receptor-mediated events in photoreceptors. In addition, other intrinsic photoreceptor mechanisms involving Ca^{2+} -induced Ca^{2+} release from intracellular stores (Krizaj et al., 1999) and Ca^{2+} -dependent inactivation of I_{Ca} (Corey et al., 1984) may contribute to the regulation of I_{Ca} and transmitter release from rods. However, the ability of niflumic acid to disrupt quinpirole's effects on the rod/cone balance and depolarization-evoked Ca^{2+} responses in rods suggests that Cl^- channels play a prominent role in determining the effects of D_2/D_4 receptor modulation on synaptic transmission from rods.

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Fig. 1.
Quinpirole (20 μ M) enhances rod I_{Ca} . Thick dashed line: control. Solid line: quinpirole. Thin dashed line: wash. The voltage was ramped from -90 mV to +60 mV at 1 mV/ms. The leak conductance was assumed to be ohmic and equal to the minimum conductance between -75 and -55 mV and then digitally subtracted.

Fig. 2.

Quinpirole (20 μM) reduces rod signals in second-order neurons of the salamander retina. (A) Currents evoked by 580-nm light (-2.76 , -3.89 , -4.89 , & -5.92 log units) in a horizontal cell in control superfusate. (B) Currents evoked by 580-nm light (-1.13 , -2.13 , -3.26 , & -4.26 log units) in the same cell after >3-min exposure to quinpirole. (C) Sensitivity to both 580- (circles) and 680- (triangles) nm lights was decreased by quinpirole in this same horizontal cell. The intensity required for a half-maximal light-evoked current (σ) was determined from the best fit to the data with a sigmoidal Michaelis-Menten function as described in Methods. In control conditions (solid lines, filled symbols), the intensities required to produce half-maximal light-evoked currents with 580- (σ_{580}) and 680- (σ_{680}) nm lights were -4.17 and -2.57 log units, respectively. Following the application of quinpirole (dashed lines, open symbols), $\sigma_{580} = -2.66$ and $\sigma_{680} = -1.64$ log units. (D) Quinpirole (20 μM) significantly increased the sensitivity of second-order retinal neurons to 680-nm light compared to 580-nm light. Bar graph shows that the difference in σ determined for 580- versus 680-nm lights was reduced significantly from -2.19 log units to only -1.59 log units by application of quinpirole ($P = 0.0016$, paired t -test, $n = 15$ cells: 6 ON bipolar cells, 4 OFF bipolar cells, and 5 horizontal cells). These differences are plotted next to the sensitivity differences expected for pure rod or pure long-wavelength cone input (data from Makino & Dodd, 1996).

Fig. 3.

Quinpirole (20 μM) had little effect on light-evoked voltage responses in rods. (A) Voltage responses evoked by 580-nm light (-1.13 , -2.13 , -3.26 , & -4.26 log units) from a rod in control superfusate. (B) Responses evoked by 580-nm light at the same intensities in the same cell after >3 -min exposure to quinpirole. (C) Amplitude-intensity relationships for 580- (circles) and 680- (triangles) nm lights in the same cell. The intensity required for a half-maximal light-evoked response (σ) was determined from the best fit to the data with a sigmoidal Michaelis-Menten function as described in Methods. In control conditions (solid lines, filled symbols), σ_{580} and σ_{680} were found to be -3.18 and -1.00 log units, respectively. Following the application of quinpirole (dashed lines, open symbols), σ_{580} and σ_{680} were found to be -3.21 and -0.73 log units, respectively. Due to changes in the light path, the maximum available intensity in these experiments was 1.1 log units less than that available for the experiments illustrated in Figs. 2 and 10. (D) Quinpirole (20 μM) did not alter the relative sensitivity to 580- versus 680-nm light (in control, $\sigma_{580 \text{ nm}} - \sigma_{680 \text{ nm}} = 2.20 \pm 0.05$ log units; in quinpirole, $\sigma_{580 \text{ nm}} - \sigma_{680 \text{ nm}} = 2.14 \pm 0.10$ log units; $P = 0.66$, paired t -test, 12 pairs).

Fig. 4. Depolarization-evoked increases in $[Ca^{2+}]_i$ are inhibited by quinpirole ($20 \mu M$). $[Ca^{2+}]_i$ was measured in a rod soma in the retinal slice using Fura-2. The cell was depolarized by 1-min application of 50 mM K^+ . Quinpirole was added 3 min prior to application of high K^+ .

Fig. 5.

In the presence of niflumic acid (0.1 mM), depolarization-evoked increases in $[Ca^{2+}]_i$ are stimulated by quinpirole (20 μ M). Cells were depolarized by 1-min application of 50 mM K⁺. Quinpirole and niflumic acid were added 3 min prior to application of high K⁺.

Fig. 6.

$I_{Cl(Ca)}$ is enhanced by quinpirole ($20 \mu\text{M}$). Rod was depolarized by a 300-ms step from -60 mV to $+40$ mV to activate I_{Ca} . I_{Ca} was then deactivated by repolarization; the tail current reflects activation of $I_{Cl(Ca)}$. K^+ channels were blocked by Cs^+ in the pipette solution and TEA in the bath solution. Control: narrow gray line. Quinpirole: thick black line. Wash: narrow black line. Dotted line indicates baseline current level prior to control voltage step series. Baseline currents (quinpirole: -71 pA; control: -82 pA; washout: -51 pA) were aligned prior to analysis.

Fig. 7.

Rod E_{Cl} estimated with electrophysiological and Cl^- imaging techniques. (A) $I_{Cl(Ca)}$ was activated by applying a 500-ms step from -70 mV to 0 mV during a gramicidin perforated patch whole-cell recording from a rod. A ramp voltage protocol (-90 to 60 mV, 1 mV/ms) was applied during the tail current 50 ms after termination of the step. (B) Same protocol was repeated in the presence of niflumic acid (0.1 mM). The ramp current/voltage relationship obtained in the presence of niflumic acid (B) was subtracted from that obtained in control (A) to yield a difference current. The difference currents obtained in this manner were averaged from seven cells to yield the current/voltage relationship illustrated in (C) (the thick line at the center shows the mean; the thin lines at the boundaries indicate $\pm$ SEM). The niflumic acid-sensitive difference current reversed about -20 mV. The reversal potential determined independently for each of the seven cells averaged -19.8 ± 3.71 mV. (D) The Cl^- sensitive dye MEQ was used to measure Cl^- flux during depolarization evoked by bath application of superfusate containing 12.1 , 21.6 , 31.2 , 40.7 , 50.3 , or 69.9 mM $[K^+]$. These high K^+ solutions depolarized rods to -34.0 ± 1.37 ($n = 10$), -25.7 ± 1.34 ($n = 10$), -19.5 ± 1.26 ($n = 10$), -14.0 ± 0.66 ($n = 10$), -8.91 ± 0.59 ($n = 9$), and -3.0 ± 0.81 mV ($n = 9$), respectively, as determined by gramicidin perforated-patch whole-cell recordings with K^+ -based pipette solutions. MEQ fluorescence increases as intracellular $[Cl^-]$ is reduced. Measurements were made from the somas of rods in the retinal slice preparation. An exponential decay in baseline fluorescence arising from dye bleaching and leakage was subtracted prior to measurement. The change in MEQ fluorescence relative to basal

fluorescence ($F/F * 100$) is plotted against the rod membrane potential determined with each high K^+ solution. Cl^- flux reversed about -19 mV.

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Fig. 8.

(A) Application of quinpirole (20 μM) caused a decrease in $[Cl^-]_i$ measured with the dye, MEQ. (B) In the presence of niflumic acid (0.1 mM), quinpirole did not produce a detectable change in $[Cl^-]_i$. Cl^- quenches MEQ so a decrease in $[Cl^-]_i$ produces an increase in fluorescence.

Fig. 9.

(A) Substitution of 24 mM CH_3SO_4^- for equimolar Cl^- caused a decrease in $[\text{Cl}^-]_i$ (increase in MEQ fluorescence) that is similar to that produced by quinpirole (Fig. 8). (B) 24 mM CH_3SO_4^- inhibited depolarization-evoked increases in $[\text{Ca}^{2+}]_i$ measured with Fura-2 to a similar extent as quinpirole did (Fig. 4).

Fig. 10.

In the presence of niflumic acid, quinpirole does not alter the relative sensitivity to 580- and 680-nm lights. (A) Light-evoked currents evoked by 580-nm light (-4.89 , -6.02 , & -7.02 log units) from a voltage-clamped horizontal cell in control superfusate. (B) Responses evoked by 580-nm light at the same intensities in the same cell after >3 -min exposure to quinpirole. (C) Amplitude-intensity relationships for 580- (circles) and 680- (triangles) nm light flashes applied to the same cell. The intensity required for a half-maximal light-evoked current (σ) was determined from the best fit to the data with a sigmoidal Michaelis-Menten function as described in Methods. In control conditions (solid lines, filled symbols), σ_{580} and σ_{680} were found to be -6.37 and -3.69 log units, respectively. Following the application of quinpirole (dashed lines, open symbols), σ_{580} and σ_{680} were found to be -6.58 and -4.05 log units, respectively. (D) Bar graph shows that, in the presence of niflumic acid, the relative sensitivity to 580- versus 680-nm lights was not significantly changed by quinpirole (niflumic acid: -2.21 log units; niflumic acid plus quinpirole: -2.23 log units; $P = 0.86$, paired t -test, $n = 15$ pairs: 6 ON bipolar cells, 3 OFF bipolar cells, and 6 horizontal cells).

Fig. 11.

Effects of quinpirole (20 μ M) on light-evoked voltage responses of rods recorded in the presence of niflumic acid (0.1 mM). (A) Voltage responses evoked by 580-nm light (-1.13 , -2.13 , -3.26 , & -4.26 log units) from a rod in control superfusate. (B) Responses evoked by 580-nm light at the same intensities in the same cell after >3-min exposure to quinpirole. (C) Amplitude/intensity relationships for 580- (circles) and 680- (triangles) nm lights in the same cell. The intensity required for a half-maximal light-evoked response (σ) was determined from the best fit with a sigmoidal Michaelis-Menten function as described in Methods. In control conditions (solid lines, filled symbols), σ_{580} and σ_{680} were found to be -3.15 and -1.14 log units, respectively. Following the application of quinpirole (dashed lines, open symbols), σ_{580} and σ_{680} were found to be -3.28 and -0.98 log units, respectively. Due to changes in the light path, the maximum available intensity in these experiments was 1.1 log units less than that available for the experiments illustrated in Figs. 2 and 10. (D) Quinpirole (20 μ M) did not significantly alter the relative sensitivity to 580- versus 680-nm light ($\sigma_{580 \text{ nm}} - \sigma_{680 \text{ nm}}$; $p = 0.81$, paired t -test, 10 pairs).