Reciprocal Interactions Between Calcium and Chloride in Rod Photoreceptors

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Thoreson, Wallace B., Eric J. Bryson, and Katalin Rabl. Reciprocal interactions between calcium and chloride in rod photoreceptors. J Neurophysiol 90: 1747–1753, 2003. First published April 30 2003; 10.1152/jn.00932.2002. This study used imaging and electrophysiological techniques in salamander retinal slices to correlate Ca2+ and Cl− levels in rods and thus test the hypothesis of a feedback interaction between Ca2+− and Ca2+-activated Cl− channels whereby Cl− efflux through Cl− channels can inhibit Ca2+ channels. Increasing [K+]o levels produced a concentration-dependent depolarization of rods accompanied by increases in [Ca2+]i measured with Fura-2. The voltage dependence of increases in [Ca2+]i was compared with the voltage dependence of the calcium current (Ica). [Cl−], was measured with the dye, MEQ. Depolarization with high K+ to membrane potentials below −20 mV reduced [Cl−]; larger depolarizations increased [Cl−]o. The Na/K/Cl cotransport inhibitor, bumetanide, shifted the apparent Cl− equilibrium potential (ECl) to more negative potentials, suggesting that this cotransporter helps establish a relatively depolarized ECl. MEQ fluorescence changes evoked by high K+ were inhibited by niflumic acid (0.1 mM), NPPB (2 μM), or replacement of Ca2+ with Ba2+, suggesting that depolarization-evoked Cl− changes result partly from stimulation of Ca2+-activated Cl− channels. Replacing ≥12 mM [Cl−]o with CH3SO4− produced a significant reduction in [Cl−], [Ca2+]o, increases evoked by 20 or 50 mM K+ were also significantly inhibited by replacing ≥12 mM [Cl−]o, with CH3SO4−. Thus modest depolarization can evoke increases in [Ca2+]o that lead to reductions in [Cl−], and conversely, reductions in [Cl−], inhibit depolarization-evoked [Ca2+]o increases. These findings support the hypothesis that feedback interactions between Ca2+− and Ca2+-activated Cl− channels may contribute to the regulation of presynaptic Ca2+ currents involved in synaptic transmission from rod photoreceptors.

INTRODUCTION

Rod photoreceptors possess dihydropyridine-sensitive Ca2+ currents (Ica) (Kourennyi and Barnes 2000; Stella et al. 2002) that mediate Ca2+ influx and thereby regulate glutamate release from photoreceptor terminals. Ca2+ influx through these channels can also activate a very large Ca2+-activated Cl− current (ICl(Ca)) (Bader et al. 1982). In cones, the current flux through ICl(Ca) is at least eightfold greater than the current through Ica (Barnes and Hille 1989). The chloride equilibrium potential (ECl) of salamander rods is about −20 mV (Thoreson et al. 2002). In olfactory receptors, ECl is also positive to the cell’s resting potential and acts to boost the receptor potential (Kleene and Gesteland 1991). In rods, depolarizing responses to darkness would also presumably be boosted by activation of ICl(Ca). In addition, activation of ICl(Ca) at the dark resting potential (around −45 mV) generates a Cl− efflux (Thoreson et al. 2002). The resulting reduction in [Cl−]o has the unusual effect of inhibiting the open channel probability of single Ca2+ channels in photoreceptor terminals (Thoreson et al. 2000). This may promote a negative feedback interaction whereby activation of Ica leads to a Ca2+ influx that activates a Cl− efflux, which in turn feeds back to inhibit Ica (Thoreson et al. 2002). When Ica is enhanced by quinpirole, this negative feedback interaction may help to account for the paradoxical finding that, although activation of D2/D4 dopamine receptors enhances Ica, quinpirole nonetheless inhibits synaptic transmission from rods (Thoreson et al. 2002; Witkovsky et al. 1989).

Is this feedback interaction restricted to conditions where Ica has been enhanced (e.g., with quinpirole) or does it regulate Ica under normal operating conditions at the rod synapse? To address this question, we combined electrophysiology with Ca2+ and Cl− imaging techniques to assess the reciprocal interactions between Ca2+− and Cl− efflux under physiological conditions. The results show an intimate relationship between the two that support the hypothesis of a feedback interaction between Ica and ICl(Ca) operating near the dark potential and defines mechanisms that contribute to maintenance of a positive value for ECl in rod photoreceptors.

METHODS

Tissue preparation

Larval tiger salamanders (Ambystoma tigrinum, 18–25 cm) were cared for according to institutional guidelines. Retinal slices were prepared according to methods pioneered by Werblin (Werblin 1978) and Wu (Wu 1987). Salamanders were pithed and decapitated, an eye was enucleated, and the front of the eye was removed. The resulting eye cup was cut into three or four pieces, and a single piece was placed vitreal surface down onto a piece of filter paper (Millipore 2, 18–25 cm) were

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Solutions and perfusion

Solutions were applied with a single-pass, gravity-feed perfusion system (1 ml/min). The normal amphibian superfusate contained (in mM) 111 NaCl, 2.5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 10 N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 5 glucose (pH 7.8). In some experiments, CaCl₂ was replaced with 2 mM BaCl₂. For Cl⁻ replacement experiments, Cl⁻ was replaced with equimolar CH₃COO⁻: CH₃SO₄⁻ solutions were boiled for 10 min in a fume hood to evaporate any residual methanol before glucose was added and the solution brought to its final volume.

Electrophysiology

Patch pipettes were pulled on a PB-7 vertical puller (Narishige) from borosilicate glass pipettes (1.2 mm OD, 0.95 mm ID, omega dot) and had tips of approximately 1 μm OD with resistances of 10–15 MΩ. For measurements of membrane potentials in rods, we used gramicidin perforated-patch recording techniques (Kyrozis and Reichling 1995). Gramicidin was dissolved in ethanol (5 mg/ml) and then added to the pipette electrolyte solution to achieve a final concentration of 5 μg/ml. For current-clamp measurements of membrane potential, the pipette electrolyte solution contained (in mM) 54 CsCl, 61.5 CsCH₃SO₄, 3.5 NaCl, and 10 HEPES (pH 7.2). Solutions and perfusion

For measurements of [Cl⁻]i, we used the ratiometric dye, Fura-2 (Molecular Probes, Eugene, OR) (Grynkiewicz et al. 1985). Retinal potential, the pipette electrolyte solution contained (in mM) 54 KCl, 61.5 KCH₃SO₄, 111 NaCl, 2.5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, and 10 HEPES (pH 7.2). In some experiments, CaCl₂ was replaced with 2 mM BaCl₂. For Cl⁻ replacement experiments, Cl⁻ was replaced with equimolar CH₃COO⁻: CH₃SO₄⁻ solutions were boiled for 10 min in a fume hood to evaporate any residual methanol before glucose was added and the solution brought to its final volume.

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Imaging experiments

Digital fluorescent images were obtained with a cooled CCD camera (SensiCam, Cooke, Auburn Hills, MI). Axon Imaging Workbench (AIW 2.2, Axon Instruments) was used to control the camera, filter wheel, and image acquisition. Pixel binning (2 × 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 [Ca²⁺]i, we used the dye Fura-2 (Molecular Probes, Eugene, OR) (Grynkiewicz et al. 1985). Retinal slices were loaded with Fura-2 by incubating them at 5°C for 45 min in the dark with 0.5 ml of 10 μM Fura-2/AM + 0.02% pluronic F-127 (Molecular Probes). This was followed by an additional incubation of 1.5 h in Fura-2/AM without pluronic F-127. Intracellular [Ca²⁺]i increases were stimulated by depolarization with elevated 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 with 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-ethylpyrroloquinolinium iodide (MEQ, Molecular Probes) (Biwersi and Verkman 1991). MEQ was loaded into cells after reducing it to DiH-MEQ by adding 30 μM sodium borohydride (100 μL) to MEQ (5 mg) under a continuous stream of nitrogen gas (Woll et al. 1996). DiH-MEQ enters cells during the incubation period (15 min), where it is oxidized and retained in the form of MEQ. Fluorescence emission decreases as Cl⁻ quenches MEQ. As illustrated in Fig. 1, the exponential decay in MEQ fluorescence due to dye leakage and bleaching was determined under control conditions and subtracted prior to analysis. NPPB was used at a concentration of 2 μM in Cl⁻ imaging experiments; concentrations of 10 μM and above generated a detectable intrinsic fluorescence with the MEQ fluorescence cube.

Statistical comparisons were made using Student’s t-test with a significant value of 0.05. Variance is reported as ± SE. Unless otherwise specified, chemicals were obtained from Sigma/Aldrich/ RBI (St. Louis, MO).

RESULTS

To detect changes in intracellular [Cl⁻]i we used the dye MEQ. In its reduced form, MEQ can be readily loaded in cells of the slice where it is oxidized and becomes membrane impermeable. Although the dye MQAE is somewhat simpler to prepare, we prefer MEQ because it is more efficient in retaining in cells than MQAE. A pseudocolor image illustrating photoreceptors in the retinal slice loaded with MEQ is shown in Fig. 1A. Outer segments point toward the bottom of this image as indicated in the figure. As shown in Fig. 1B, MEQ fluorescence decays exponentially during the experiment. This exponential decay in baseline fluorescence was subtracted for analysis (Fig. 1C).

Data from 15 rods.

FIG. 1. Bath application of low Cl⁻ solutions reduces intracellular Cl⁻ levels. A: pseudocolor image of rods in a retinal slice loaded with the Cl⁻ sensitive dye, MEQ. The soma and outer segment (O.S.) of one rod are indicated. B: MEQ fluorescence decreases exponentially during the course of an experiment. Solid line: exponential fit to baseline decay (excluding data during application of low Cl⁻ solutions). C: changes in fluorescence (∆F) after subtracting exponential baseline fluorescence decay. D: mean changes in fluorescence measured after subtracting baseline fluorescence decay divided by the baseline fluorescence level (∆F/F). Data from 15 rods.
Application of a low Cl\textsuperscript− solution caused a reduction in intracellular [Cl\textsuperscript−] that reduces anion quenching of MEQ and thereby increases MEQ fluorescence (Fig. 1, B and C). The experimentally induced change in fluorescence is divided by the baseline fluorescence value (ΔF/Φ). This normalizing procedure helps to factor out changes in fluorescence arising from variations in dye concentration or cell thickness (Helmchen 2000). As shown in Fig. 1D, reducing extracellular Cl\textsuperscript− by 12 mM produced detectable increases in MEQ fluorescence and larger reductions in extracellular Cl\textsuperscript− produced correspondingly larger increases in MEQ fluorescence.

For Fura-2 studies of intracellular [Ca\textsuperscript2+], we stimulated Ca\textsuperscript2+ influx by depolarizing the rods with increases in extracellular [K\textsuperscript+]. We used gramicidin-perforated patch recording techniques to record the membrane potentials produced by the different high K\textsuperscript+ solutions (Fig. 2A). The resulting relationship could be fit with a modified Goldman-Hodgkin-Katz equation with relative permeability of \(\frac{P_{Na}}{P_{K}}\) of 0.13 (line, Fig. 2A). The small deviation from this relationship at more positive potentials may be due to an increasing contribution of Ca\textsuperscript2+ and Ca\textsuperscript2+-activated Cl\textsuperscript− channels to the membrane potential.

After calibrating the solutions in this way, we then used the same high K\textsuperscript+ solutions to stimulate increases in intracellular [Ca\textsuperscript2+]. Fluorescence measurements were made from the somas of rods in the retinal slice. Increasing the concentration of K\textsuperscript+ produced a sigmoidal increase in intracellular [Ca\textsuperscript2+] that was half-maximal at -21.8 mV and reached its peak above -12 mV (Fig. 2B). For comparison, we averaged \(I_{Ca}\) recorded from 12 rods using a ramp voltage protocol (0.5 mV/ms) and gramicidin-perforated-patch recording techniques (Fig. 2C, noisy trace). The half-maximal voltage for the sigmoidal best fit function to the \(I_{Ca}/\text{voltage}\) relationship was -36.2 mV. Thus a small but significant fraction of \(I_{Ca}\) was active at the resting potential of -44 mV. Differences between the voltage dependence of \(I_{Ca}\) and depolarization-evoked increases in [Ca\textsuperscript2+] are considered in the DISCUSSION.

The Cl\textsuperscript− equilibrium potential (\(E_{Cl}\)) of rods was determined by depolarizing cells with identical high K\textsuperscript+ solutions to those used in Fig. 2. As shown in Fig. 1, Cl\textsuperscript− efflux causes an increase in MEQ fluorescence. When cells were depolarized with high K\textsuperscript+ solutions to levels below -20 mV, MEQ fluorescence increased indicating a Cl\textsuperscript− efflux and when cells were depolarized above -20 mV, MEQ fluorescence decreased, indicating a Cl\textsuperscript− influx (Fig. 3A, filled circles, data from Thoreson et al. 2002); these results suggest that \(E_{Cl}\) is around -20 mV. This finding was substantiated by electrophysiological experiments that also indicate that \(E_{Cl}\) in rods is around -20 mV (Thoreson et al. 2002). The small Cl\textsuperscript− efflux evoked by weak depolarization to -35 mV (Fig. 3A) is likely due to additional activation of Ca\textsuperscript2+-activated Cl\textsuperscript− channels by the additional Ca\textsuperscript2+ influx accompanying further stimulation of voltage-gated Ca\textsuperscript2+ channels (Fig. 2).

The fact that \(E_{Cl}\) is positive to the resting membrane potential of around -45 mV in these cells suggests that Cl\textsuperscript− ions are actively accumulated by rods. The most common mechanism by which cells accumulate Cl\textsuperscript− is through the Na/K/Cl co-transporter that mediates the coupled influx of Na\textsuperscript+, K\textsuperscript+, and Ca\textsuperscript2+. Reduced extracellular Cl\textsuperscript− also enhances depolarization-evoked 

\[\text{Ca}^{2+}\] influx (Fig. 2). 

**FIG. 2.** Bath application of high K\textsuperscript+ solutions stimulates a voltage-dependent increase in [Ca\textsuperscript2+]. 

**A:** membrane potential measured with gramicidin-perforated-patch-recording techniques in the presence of different concentrations of extracellular K\textsuperscript+(2.5, 12.1, 21.6, 31.2, 40.7, 50.3, and 69.9 mM). Data from 10 rods. Line shows the modified Goldman-Hodgkin-Katz equation: 

\[E_m = 58 \text{ mV} \log ([K^+]_o + \frac{P_{Na}}{P_{K}}[111 \text{ mM Na}^+])/[98 \text{ mM K}^+]\]  

where the relative permeability of Na\textsuperscript+ to K\textsuperscript+, \(\frac{P_{Na}}{P_{K}}\), is 0.13. [Ca\textsuperscript2+] increases evoked by different concentrations of [K\textsuperscript+] (data points). [K\textsuperscript+] was converted to the corresponding membrane potential using the values shown in A. 

**B:** intracellular [Ca\textsuperscript2+] measured with Fura-2 in the presence of different high K\textsuperscript+ concentrations of [K\textsuperscript+] (10.220.3 mM). 

**C:** normalized current (\(I_{Ca}\)) recorded using a ramp voltage protocol (0.5 mV/ms) and gramicidin-perforated-patch recording techniques (Fig. 2C). 

Data were fit with a Boltzmann sigmoid with \(V_{so} = -21.8 \text{ mV}\) and slope factor of 3.15 (line). Data were from 12 rods in each condition. 

\[\text{Normalized } I_{Ca} = \frac{I_{Ca} - \text{baseline}}{\text{max } I_{Ca} - \text{baseline}}\]  

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Cl\textsuperscript{−} ions (Russell 2000). The Na/K/Cl co-transporter is selectively inhibited by the loop diuretic bumetanide (Russell 2000). We therefore used bumetanide to test for a role for the Na/K/Cl cotransporter. First, the membrane potentials of rods established by solutions containing different concentrations of K\textsuperscript{+} (n = 6) in the presence of bumetanide (0.1 mM) were measured in current-clamp mode, similar to the experiment illustrated in Fig. 2A. Then, changes in intracellular Cl\textsuperscript{−} measured in current-clamp mode, similar to the experiment illustrated in Fig. 2A. Then, changes in intracellular Cl\textsuperscript{−} were evaluated using MEQ. Supporting a role for the Na/K/Cl co-transporter in the accumulation of Cl\textsuperscript{−} by rods, E\textsubscript{Cl} determined from the depolarization-evoked Cl\textsuperscript{−} flux was shifted about 10 mV more negative by application of bumetanide (Fig. 3A, open circles).

The Cl\textsuperscript{−} influx evoked by depolarization to −9 mV with 50 mM K\textsuperscript{+} in control solution (no bumetanide) was strongly inhibited by the Cl\textsuperscript{−} channel blockers 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (2 μM) and niflumic acid (0.1 mM) (Fig. 3B). We also tested the ability of these Cl\textsuperscript{−} channel blockers to inhibit the tail current following a depolarizing step or ramp. This tail current is largely attributable to activation of I\textsubscript{Cl(Ca)} (Barnes and Deschenes 1992). Niflumic acid (0.1 mM) inhibited I\textsubscript{tail} by approximately 50% but NPPB (10 μM) did not significantly inhibit I\textsubscript{tail} (Fig. 3C). Because the inhibition of depolarization-evoked Cl\textsuperscript{−} influx by NPPB cannot be accounted for by block of I\textsubscript{Cl(Ca)}, this suggests it may block another type of Cl\textsuperscript{−} channel in rods. Replacing Ca\textsuperscript{2+} with Ba\textsuperscript{2+} strongly inhibits activation of I\textsubscript{Cl(Ca)} (data not shown) and likewise inhibited the Cl\textsuperscript{−} influx stimulated by high K\textsuperscript{+} (Fig. 3B).

Another Cl\textsuperscript{−} channel blocker, N-phenylanthracilic acid (0.1 mM), had no significant effect on Cl\textsuperscript{−} influx or the tail current (data not shown).

In cones, niflumic acid is a more potent and selective blocker of I\textsubscript{Cl(Ca)} than IAA-94 or fenofamic acid (Barnes and Deschenes 1992) and niflumic acid was the only effective inhibitor of the three Cl\textsuperscript{−} channel blockers tested in this study. We tested the effects of niflumic acid on rod Ca\textsuperscript{2+} currents using Ba\textsuperscript{2+} as the charge carrier to minimize the influence of I\textsubscript{Cl(Ca)} since it is not effectively activated by Ba\textsuperscript{2+}. We found that in rods, niflumic acid inhibited I\textsubscript{Ba} by about 20% (Fig. 3C). Thus at least part of niflumic acid’s ability to block I\textsubscript{Cl(Ca)} resides in its ability to block Ca\textsuperscript{2+} influx. NPPB did not significantly inhibit I\textsubscript{Ba} at 10 μM (Fig. 3C) but substantially inhibited I\textsubscript{Ca} at 0.1 mM (n = 2, data not shown).

Because of its Ca\textsuperscript{2+} channel blocking effects, niflumic acid could not be used as a tool to open the hypothesized feedback loop between I\textsubscript{Cl(Ca)} and I\textsubscript{Ba}. Instead, as a further test for a possible feedback relationship, we determined whether reducing extracellular Cl\textsuperscript{−} by 12 mM also significantly reduced the [Ca\textsuperscript{2+}+] increase evoked by depolarizing rods to −9 mV with 50 mM K\textsuperscript{+} (Fig. 4A). Reducing extracellular Cl\textsuperscript{−} further by replacing it with 24 mM CH\textsubscript{3}SO\textsubscript{4} produced a stronger inhibitory effect (data not shown).

FIG. 3. Mechanisms contributing to regulation of [Cl\textsuperscript{−}] levels in rods. A: rod E\textsubscript{Cl} estimated with Cl\textsuperscript{−} imaging techniques in the presence and absence of an inhibitor of Na/K/Cl cotransport, bumetanide. MEQ was used to measure Cl\textsuperscript{−} flux during depolarization evoked by bath application of the high [K\textsuperscript{+}] solutions used in Fig. 2. MEQ fluorescence is increased by reductions in intracellular [Cl\textsuperscript{−}]. The change in MEQ fluorescence relative to basal fluorescence (∆F/F × 100) is plotted against the rod membrane potential determined with each high K\textsuperscript{+} solution. In control conditions, Cl\textsuperscript{−} flux reversed about −19 mV (filled circles, data from Thoreson et al. 2002). In the presence of bumetanide (0.1 mM, open circles), Cl\textsuperscript{−} flux reversed at more negative potentials (data from 14–17 cells in each condition). Membrane potentials corresponding to the various high K\textsuperscript{+} solutions applied in the presence of bumetanide were calibrated in separate current-clamp experiments (n = 6). B: Cl\textsuperscript{−} influx evoked by application of 50 mM K\textsuperscript{+} that depolarized rods to −9 mV was inhibited by the Cl\textsuperscript{−} channel blockers NPPB (2 μM, n = 10, P = 0.0023) and niflumic acid (0.1 mM, n = 8, P = 0.0062). Replacing Ca\textsuperscript{2+} with Ba\textsuperscript{2+}, which inhibits activation of I\textsubscript{Cl(Ca)}, also significantly reduced depolarization-evoked Cl\textsuperscript{−} influx (n = 14, P = 0.031). C: effects of niflumic acid and NPPB on the peak amplitude of I\textsubscript{Ba} recorded with 2 mM Ba\textsuperscript{2+} as the charge carrier or the amplitude of the tail current (I\textsubscript{tail}) measured 70 ms after a ramp voltage depolarization (−90 to +60 mV, 0.5 mV/ms). Currents recorded in the test solution were normalized to control currents (I\textsubscript{Ba}/I\textsubscript{Ba(control)}, I\textsubscript{tail}/I\textsubscript{Ba(control)}). When possible, control currents were averaged from currents recorded before drug application and currents following washout. Niflumic acid (0.1 mM) significantly inhibited both I\textsubscript{Ba} (P = 0.03, n = 6) and I\textsubscript{tail} (P < 0.0001, n = 22) but NPPB (10 μM) did not significantly inhibit either (I\textsubscript{Ba} (P = 0.39, n = 7) or I\textsubscript{tail} (P = 0.50, n = 9).
or electrochemical activity of Ca^{2+} by 50 mM K^+ solutions inhibited depolarization-evoked [Ca^{2+}]_o increases. Reducing [Cl^-]_o by 12 mM produced detectable decreases in [Cl^-], (Fig. 1) and the reduction in [Cl^-], produced by this low Cl^- solution was in turn sufficient to inhibit depolarization-evoked increases in [Ca^{2+}], (Fig. 4). Reducing [Cl^-]_o by 12 mM also significantly inhibits I_{Ca^2+}, suggesting that the inhibition of depolarization-evoked increases in [Ca^{2+}], by this solution is more likely due to a reduction in Ca^{2+} influx than to changes in other aspects of Ca^{2+} handling (e.g., rates of Ca^{2+} buffering or extrusion) (Thoreson and Stella 2000; Thoreson et al. 1997). Depolarizing rods by only 10 mV from their resting potential of −44 mV evoked detectable Ca^{2+} increases (Fig. 2) that were sufficient to stimulate detectable Cl^- efflux (Fig. 3A; Thoreson et al. 2002). Taken together, the observations that a similar small amplitude Cl^- efflux is stimulated by both weak depolarization and application of low Cl^- solutions and that the latter inhibits depolarization-evoked increases in [Ca^{2+}], support the hypothesis of a negative feedback interaction between influx through I_{Ca^2+} and Ca^{2+}-activated Cl^- efflux.

The finding that weak depolarizations (e.g., to −35 mV) stimulating small increases in spatially averaged [Ca^{2+}] (Fig. 2) evoked detectable Cl^- efflux (Fig. 3A) can be interpreted as suggesting that relatively small increases in [Ca^{2+}], may be sufficient to activate I_{Cl(Ca)}^+. However, another possibility is that Ca^{2+}-activated Cl^- channels are anchored close to Ca^{2+} channels so that influx through these channels produces a high local concentration of Ca^{2+} around the Ca^{2+}-activated Cl^- rods that had been depolarized to −26 mV by applying 22 mM K^+.

Application of 22 mM K^+ evoked an initial transient Ca^{2+} increase followed by a sustained elevation of Ca^{2+}. During the sustained elevation, reducing [Cl^-], by 12 mM or more reduced Ca^{2+} levels close to the baseline concentration existing prior to application of 22 mM K^+. (Fig. 4B).

Ca^{2+}-activated Cl^- channels are concentrated in rod terminals (Macleish and Nurse 2000). However, effects of low Cl^- solutions on the Ca^{2+} increases evoked by 22 mM K^+ were not significantly more pronounced in terminals of enzymatically isolated rods compared with measurements in the soma (12 mM CH_3SO_4, P = 0.87, paired t-test, n = 6; 24 mM CH_3SO_4, P = 0.29, n = 6).

**DISCUSSION**

The results of this study demonstrate a reciprocal relationship between Cl^- and Ca^{2+} over a wide range of membrane potentials and Cl^- levels in the inner segments of rods. Reducing [Cl^-], by 12 mM produced detectable decreases in [Cl^-] (Fig. 1) and the reduction in [Cl^-], produced by this low Cl^- solution was in turn sufficient to inhibit depolarization-evoked increases in [Ca^{2+}] (Fig. 4). Reducing [Cl^-], by 12 mM also significantly inhibits I_{Ca^2+}, suggesting that the inhibition of depolarization-evoked increases in [Ca^{2+}], by this solution is more likely due to a reduction in Ca^{2+} influx than to changes in other aspects of Ca^{2+} handling (e.g., rates of Ca^{2+} buffering or extrusion) (Thoreson and Stella 2000; Thoreson et al. 1997). Depolarizing rods by only 10 mV from their resting potential of −44 mV evoked detectable Ca^{2+} increases (Fig. 2) that were sufficient to stimulate detectable Cl^- efflux (Fig. 3A; Thoreson et al. 2002). Taken together, the observations that a similar small amplitude Cl^- efflux is stimulated by both weak depolarization and application of low Cl^- solutions and that the latter inhibits depolarization-evoked increases in [Ca^{2+}], support the hypothesis of a negative feedback interaction between influx through I_{Ca^2+} and Ca^{2+}-activated Cl^- efflux.

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The finding that weak depolarizations (e.g., to −35 mV) stimulating small increases in spatially averaged [Ca^{2+}] (Fig. 2) evoked detectable Cl^- efflux (Fig. 3A) can be interpreted as suggesting that relatively small increases in [Ca^{2+}], may be sufficient to activate I_{Cl(Ca)}^+. However, another possibility is that Ca^{2+}-activated Cl^- channels are anchored close to Ca^{2+} channels so that influx through these channels produces a high local concentration of Ca^{2+} around the Ca^{2+}-activated Cl^- channels that had been depolarized to −26 mV by applying 22 mM K^+.

Application of 22 mM K^+ evoked an initial transient Ca^{2+} increase followed by a sustained elevation of Ca^{2+}. During the sustained elevation, reducing [Cl^-], by 12 mM or more reduced Ca^{2+} levels close to the baseline concentration existing prior to application of 22 mM K^+ (Fig. 4B).

Ca^{2+}-activated Cl^- channels are concentrated in rod terminals (Macleish and Nurse 2000). However, effects of low Cl^- solutions on the Ca^{2+} increases evoked by 22 mM K^+ were not significantly more pronounced in terminals of enzymatically isolated rods compared with measurements in the soma (12 mM CH_3SO_4, P = 0.87, paired t-test, n = 6; 24 mM CH_3SO_4, P = 0.29, n = 6).
channels despite a relatively small elevation of global Ca\(^{2+}\). Consistent with this possibility, the Ca\(^{2+}\)-activated Cl\(^{-}\) channels in salamander olfactory neurons do not attain half-maximal activation until Ca\(^{2+}\) levels reach 5 \(\mu\)M (Kleene and Gesteland 1991).

The results of this study suggest at least two mechanisms are important in regulating Cl\(^{-}\) levels in rods. First, the effects of bumetanide (Fig. 3A) suggest that the Na/K/Cl co-transporter helps drive the accumulation of Cl\(^{-}\) ions. Second, the finding that experimental manipulations that inhibit I\(_{\text{Cl(Ca)}}\) such as application of niflumic acid or replacing Ca\(^{2+}\) with Ba\(^{2+}\) also inhibited depolarization-evoked Cl\(^{-}\) influx suggests a major role for I\(_{\text{Cl(Ca)}}\) in mediating this flux. However, results of these experiments suggest that other Cl\(^{-}\) channels may also contribute to the influx evoked by strong depolarization. Replacing Ca\(^{2+}\) with Ba\(^{2+}\) more strongly inhibits I\(_{\text{Cl(Ca)}}\) than application of niflumic acid (0.1 mM). However, this manipulation was less effective at inhibiting Cl\(^{-}\) influx than either niflumic acid or NPPB (Fig. 3B). Furthermore, NPPB (2 \(\mu\)M) was equipotent to niflumic acid (0.1 mM) in blocking Cl\(^{-}\) influx but even 10 \(\mu\)M NPPB was ineffective in blocking I\(_{\text{Cl(Ca)}}\) (Fig. 3). These pharmacological differences are probably not due to actions of niflumic acid on I\(_{\text{h}}\) (Satoth and Yamada 2001) since it is minimally active at the dark potential and above. In addition to I\(_{\text{Cl(Ca)}}\), photoreceptors exhibit Cl\(^{-}\) channels coupled to glutamate transporter activity (Eliasof and Werblin 1993; Larsson et al. 1996; Picaud et al. 1995) and may also possess CIC channels 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).

High K\(^{+}\) solutions have been used by a number of investigators for the purpose of depolarizing salamander photoreceptors for Ca\(^{2+}\) imaging studies (e.g., Baldridge et al. 1998; Krizaj and Copenhagen 1998; Thoreson et al. 1997). In this study, we determined the membrane potentials produced by rods from the salamander retinal slice was found to be −36.2 mV. Thus a significant fraction of I\(_{\text{Cl(Ca)}}\) was active at the resting potential of −44 mV in rods used for imaging experiments. Sustained activation of I\(_{\text{Cl(Ca)}}\) at a membrane potential of −40 mV inhibits I\(_{\text{Ca}}\) by more than 50% as the combined result of Ca\(^{2+}\)-dependent inactivation and depletion of synaptic cleft Ca\(^{2+}\) ions (Rabl and Thoreson 2002). The tonic inhibition of I\(_{\text{Ca}}\) that thus accompanies the modest activation of I\(_{\text{Cl(Ca)}}\) at the membrane potential of −44 mV may help explain the relatively small increase in [Ca\(^{2+}\)]\(_{\text{i}}\) evoked by depolarization to −35 mV. Increases in [Ca\(^{2+}\)]\(_{\text{i}}\) also showed a steeper voltage dependence than I\(_{\text{Cl(Ca)}}\). This steeper voltage dependence may arise from the fact that rods used for imaging experiments are not voltage clamped and can therefore produce regenerative Ca\(^{2+}\) action potentials (Burkhardt et al. 1991; Fain et al. 1977). In addition, calcium-induced calcium release (Krizaj et al. 1999, 2003) might further steepen the voltage dependence of [Ca\(^{2+}\)]\(_{\text{i}}\), by boosting the amplitude of depolarization-evoked [Ca\(^{2+}\)]\(_{\text{i}}\) increases.

Corey et al. (1984) reported a \(V_{50}\) for I\(_{\text{Ca}}\) in rods of −22 mV. The more negative activation found in the present study can be accounted for by differences in the superfuse used in the two studies. Corey et al. (1984) used extracellular solutions containing 6 mM Ca\(^{2+}\) and pH 7.2–7.3, whereas we used solutions containing 1.8 mM Ca\(^{2+}\) and pH 7.8. Due to surface potential effects, the higher [Ca\(^{2+}\)]\(_{\text{i}}\) would produce an activation shift of +7 mV (Baldridge et al. 1998) and the lower pH would add another 6- to 7-mV positive shift (Barnes et al. 1993).

The present results suggest that even relatively weak depolarization near the dark potential can produce a sufficiently large Cl\(^{-}\) efflux to cause inhibition of I\(_{\text{Ca}}\)-Ca\(^{2+}\) influx, and synaptic output from rods (Thoreson et al. 1997). This suggests that the feedback relationship between I\(_{\text{Ca}}\) and I\(_{\text{Cl(Ca)}}\) postulated by Thoreson et al. (2002) is not likely to be limited to conditions where I\(_{\text{Ca}}\) has been accentuated (e.g., following suppression of PKA activity). What is the predicted effect of this feedback mechanism on light responses? Hyperpolarization of a rod by bright light would lead to the closure of Ca\(^{2+}\) channels and the resulting reduction in Ca\(^{2+}\) influx would in turn close Ca\(^{2+}\)-activated Cl\(^{-}\) channels. As Cl\(^{-}\) efflux diminished, anion-mediated inhibition of I\(_{\text{Ca}}\) would diminish. Thus more Ca\(^{2+}\) channels would be available to be activated by the depolarization that accompanies light offset thereby accelerating the rate of depolarization as well as boosting synaptic output as a consequence of the increased Ca\(^{2+}\) influx. Unfortunately, none of the Cl\(^{-}\) channel blockers so far investigated are sufficiently selective to allow direct test of this prediction.

Rods produce both spike-like and prolonged regenerative Ca\(^{2+}\) action potentials (Burkhardt et al. 1988; Fain et al. 1977). Generation of a prolonged depolarization produces a substantial shunt reduction of photoreceptor light responses (Burkhardt et al. 1988), and the Ca\(^{2+}\) influx accompanying such a sustained depolarization can be damaging to neurons. A number of mechanisms help to keep I\(_{\text{Ca}}\) activity limited to a tonic low level and prevent more frequent activation of Ca\(^{2+}\) action potentials in photoreceptors. These include J) activation of large outward currents when the cell membrane potential exceeds the dark resting potential (Barnes and Hille 1989; Owen 1987); 2) Ca\(^{2+}\)-dependent inactivation of I\(_{\text{Cl(Ca)}}\); and 3) depletion of Ca\(^{2+}\) from the synaptic cleft during sustained activation of I\(_{\text{Ca}}\) (Rabl and Thoreson 2002). Feedback between I\(_{\text{Ca}}\) and I\(_{\text{Cl(Ca)}}\) may be another mechanism to help assure that I\(_{\text{Ca}}\) activation is maintained at a low level.

DISCLOSURES

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