Reducing Extracellular Cl\(^-\) Suppresses Dihydropyridine-Sensitive Ca\(^{2+}\) Currents and Synaptic Transmission in Amphibian Photoreceptors

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Thoreson, Wallace B., Ron Nitzan, and Robert F. Miller. Reducing extracellular Cl\(^-\) suppresses dihydropyridine-sensitive Ca\(^{2+}\) currents and synaptic transmission in amphibian photoreceptors. J. Neurophysiol. 77: 2175–2190, 1997. A reduction in extracellular chloride suppresses light-evoked currents of second-order retinal neurons (bipolar and horizontal cells) by reducing release of glutamate from photoreceptors. The underlying mechanisms responsible for this action of reduced extracellular Cl\(^-\) were studied with a combination of electrophysiological recordings from single neurons in a retinal slice preparation and image analyses of intracellular Ca\(^{2+}\) (Fura-2) and pH [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester] in dissociated photoreceptors. The results show that reducing extracellular Cl\(^-\) suppresses a dihydropyridine (DHP)-sensitive Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) in photoreceptors. It is proposed that suppression of I\(_{\text{Ca}}\) results in suppression of photoreceptor neurotransmission. The suppressive effect of low Cl\(^-\) on I\(_{\text{Ca}}\) is not due to antagonism by the substituting anion nor is it mediated by changes in extracellular or intracellular pH. We conclude that normal extracellular levels of Cl\(^-\) are important for maintenance of the voltage-gated Ca\(^{2+}\) channels that support neurotransmission from photoreceptors. Several ideas are presented about the mechanisms by which Cl\(^-\) supports photoreceptor neurotransmission and the possibility that modulations of Cl\(^-\) might play a physiological role in the regulation of Ca\(^{2+}\) channels in photoreceptors and, hence, photoreceptor function.

INTRODUCTION

Chloride ions play a number of important, regulatory roles in support of vertebrate photoreceptor function. For example, the ionic gradient of Cl\(^-\) subserves GABAergic feedback inhibition from horizontal cells to cone terminals; this inhibition in turn plays a role in generating center-surround receptive fields in outer retinal neurons (Kaneko and Tachibana 1986). Ca\(^{2+}\)-activated Cl\(^-\) channels help shape prolonged depolarizing responses, which are one consequence of synaptic feedback from horizontal cells to cones (Barnes and Deschenes 1992; Thoreson and Burkhardt 1991). In addition, Cl\(^-\)/HCO\(_3\) exchange mechanisms are important for regulation of photoreceptor pH (Koskelainen et al. 1993, 1994), and Cl\(^-\) may play role in glutamate uptake (Eliasof and Werblin 1993; Picaud et al. 1995; Sarantis et al. 1988).

In addition to these roles, Cl\(^-\) ions modulate neurotransmitter release from photoreceptors (Miller and Dacheux 1973, 1975, 1976; Thoreson and Miller 1996). When extracellular Cl\(^-\) ions are replaced with large, impermeant anions, light-evoked currents are suppressed in all three classes of second-order neurons in the amphibian retina (i.e., ON bipolar, OFF bipolar, and horizontal cells). Transmitter release evoked by the focal stimulation of photoreceptor terminals using a pipette containing hyperosmotic sucrose also is suppressed by reducing extracellular Cl\(^-\) (Thoreson and Miller 1996). A substantial body of evidence indicates that the transmitter released by photoreceptor terminals is L-glutamate, which activates cation channels in amphibian bipolar and horizontal cells (e.g., Ayoub et al. 1989; Copenhagen and Jahr 1989; Gilbertson et al. 1991; Miller and Slaughter 1986; Nawy and Jahr 1991; O’Dell and Christensen 1989; Thoreson and Miller 1993). Although there are glutamate-activated Cl\(^-\) channels in teleost bipolar cells, such channels have not been described in bipolar cells of Urodele amphibians (Grant and Dowling 1995; Nawy and Jahr 1991; Thoreson and Miller 1993). From these and other results, it appears that reducing Cl\(^-\) acts presynaptically to suppress glutamate release from photoreceptor terminals (Thoreson and Miller 1996).

In the present study, we examined the possibility that physiologically relevant changes in extracellular Cl\(^-\) could alter neurotransmission from photoreceptors by examining the effects of comparatively small reductions in extracellular Cl\(^-\) on light responses of second-order neurons. Possible photoreceptor mechanisms for the Cl\(^-\)-induced reduction in neurotransmission were evaluated using imaging methods for Ca\(^{2+}\) and pH as well as whole cell recordings of I\(_{\text{Ca}}\). The results indicate that small changes in extracellular Cl\(^-\) have significant, suppressive effects on neurotransmission from photoreceptors by suppressing DHP-sensitive Ca\(^{2+}\) channels that are located predominantly on rod and cone photoreceptor terminals. The suppressive effect of reducing extracellular Cl\(^-\) does not appear to be due to antagonism by the anion substitute or Cl\(^-\) sensitive pH changes. These results suggest that extracellular Cl\(^-\) may act in concert with presynaptic membrane potential in photoreceptors to regulate transmitter release in the first synapse of the visual pathway.

Some of this work has been published in abstract form (Nitzan and Miller 1994; Thoreson 1995).

METHODS

This study used two complementary approaches: electrophysiological recording and imaging of isolated cells filled with fluorescent indicator dyes. Three types of electrophysiological recordings were used in these experiments: ionic currents recorded with whole cell recording techniques from rod and cone photoreceptors in the superfused retinal slice preparation of the mudpuppy (Necturus maculosus) or larval tiger salamander (Ambystoma tigrinum);
light-evoked currents recorded from bipolar, off bipolar, and horizontal cells in the mudpuppy retinal slice; and intraretinal electoretinogram (ERG) recordings obtained from the superfused mudpuppy eye. Cell imaging experiments were carried out using dissociated photoreceptors from the tiger salamander retina. Two different fluorescent dyes were used to analyze the action of extracellular Cl\textsuperscript{-} ions: a Ca\textsuperscript{2+}-sensitive dye, Fura-2 (Fura-2-AM) and a pH-sensitive dye, 2',7'-bis-(2-carboxyethyl)-5- (and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM).

**Electrophysiology**

The retinal slice preparation used in the present study is similar to that developed by Werblin (1978) and described in detail by Wu (1987). A mudpuppy or tiger salamander was decapitated and pithed rapidly. After enucleation, the front of an eye was removed and the resulting eyeball cut into quarters. A quarter of the eyeball was placed vitreous surface down on a piece of Milipore filter (2 × 5 mm, Type GS, 0.2-μm pores) and the sclera/choroid/retinal pigment epithelium was peeled carefully away under cold Ringer solution to isolate the retina. The isolated retina was then cut into 100-μm slices using a razor blade tissue chopper (Stoelting). The slices were rotated 90° so that retinal layers were visible from above and placed between two beads of vacuum grease in a perfusion chamber. The chamber was then placed on a fixed-stage, upright microscope (Olympus BHWI) and viewed through a water immersion objective (Olympus WPlan40X UV, 0.7 na). All procedures were done under dim room lights to preserve light sensitivity of retinal neurons. A single-pass, gravity-feed perfusion system delivered medium to the slice chamber at 1.2 ml/min and test solutions were bath applied.

In experiments on light-evoked currents in bipolar and horizontal cells, the perfusion medium contained (in mM) 111 NaCl, 2.5 KCl, 1.8 CaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 10 N-2-hydroxyethylpiperazine-N\textsuperscript{-2-ethanesulfonic acid (HEPES)}, and 5 glucose plus 100 μM picrotoxin and 2 μM strychnine. The pH was adjusted to 7.8 with NaOH, and the superfusate was bubbled continuously with 100% O\textsubscript{2}. In Cl\textsuperscript{-} substitution experiments on light-evoked currents, Cl\textsuperscript{-} was replaced with gluconate (C\textsubscript{6}H\textsubscript{11}O\textsubscript{7}N\textsuperscript{7-}), sucrose or glucose was added to maintain osmolarity during Cl\textsuperscript{-} replacement with sulfate.

In photoreceptor recordings, the perfusion medium typically contained (in mM) 101 NaCl, 2.5 KCl, 0.5 MgCl\textsubscript{2}, 20 HEPES, and 5 mM glucose plus (in μM) 100 picrotoxin, 2 strychnine, and 100 nlmic acid (to block Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels (Barnes and Deschênes 1992). In some experiments, a glutamate uptake blocker, dihydrokainic acid (DHKA, 1 mM), also was added. During photoreceptor recordings, the slices were illuminated steadily by a bright white light to suppress the light-sensitive conductance in intact outer segments. In experiments with glutamate, BaCl\textsubscript{2} (1.8 or 10 mM) was used to enhance \(I_{c60}\). When 10 mM BaCl\textsubscript{2} was used, NaGlutamate was reduced to 89 mM. In experiments with methylsulfate, CaCl\textsubscript{2} (1.8 or 3.6 mM) was used because Ba\textsuperscript{2+} forms a precipitate with methylsulfate. Measurements with a Ca\textsuperscript{2+}-sensitive electrode indicated that methylsulfate did not complex with Ca\textsuperscript{2+}. When 3.6 mM CaCl\textsubscript{2} was used, NaMethylsulfate was reduced to 99.2 mM. Whether Ca\textsuperscript{2+} or Ba\textsuperscript{2+} was used as the primary charge carrier, the resulting inward current is referred to as \(I_{c60}\) throughout this manuscript.

Whole cell patch electrodes were pulled on a Narashige PB-7 puller from borosilicate pipettes (1.2 mm OD, 0.95 mm ID, Omega dot) and had tips of ~2 μm OD (R = 4–10 MΩ). The intracellular Ringer solution contained (in mM) 98 CsCl (or CsCH\textsubscript{3}SO\textsubscript{4} or KCH\textsubscript{3}SO\textsubscript{4}), 3.5 NaCl, 3 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 11 ethylene glycol-bis(β-aminoethyl) ether-N,N′,N″-tetraacetic acid, 5 HEPES, 2 mM d-glucose, 1 reduced glutathione, 1 ATP-Mg, and 1 GTP. KCH\textsubscript{3}SO\textsubscript{4} was used for recording light-evoked currents in second-order neurons. CsCl or CsCH\textsubscript{3}SO\textsubscript{4} was used for recording \(I_{c60}\) in photoreceptors. In some experiments on photoreceptors, HEPES was elevated to 20 mM. The fluorescent dye 5,6-carboxyfluorescein (0.025%) was added to permit visualization of cells after recording.

Pipette access resistance was ~20 MΩ. The input resistance (\(R_{in}\)) of rods averaged 404 ± 34 MΩ (n = 24), cone \(R_{in}\) averaged 255 ± 24 MΩ (n = 14), bipolar cell \(R_{in}\) was ~1 GΩ, and horizontal cell \(R_{in}\) = ~250 MΩ (Thoreson and Miller 1996). Therefore, the steady state voltage errors introduced by the access resistance were ~2% for bipolar cells, 5% for rods, and 8% for cones and horizontal cells. Charging curves for bipolar cells, rods, and cones were typically well fit by a single exponential, indicating a compact electrotonic structure (<0.1 λ). Photoreceptors were voltage clamped at ~60 or ~70 mV, bipolar cells at ~50 mV, and horizontal cells at ~40 mV. Rods and cones were identified during electrode placement by their characteristic outer segments. ON bipolar, OFF bipolar, and horizontal cells were identified using both physiological and anatomic criteria as described previously (Thoreson and Miller 1993, 1996).

DC-ERG recordings were obtained using a mudpuppy eyecup preparation (Thoreson and Ulphani 1995). Perfusion entered the eyecup through a broken patch pipette. The DC-ERG was recorded intraretinally using a patch pipette filled with extracellular medium and positioned just proximal to the outer limiting membrane.

KCl (3 M)/agar was used as a bridge to the Ag/AgCl reference electrode. The agar bridge was downstream from the retinal slices in the perfusion chamber so Cl\textsuperscript{-} leached from the agar could not reach the slices. With this arrangement, the junction potential observed following a switch to Cl\textsupersite medium was ~1 mV.

The light stimulus was a diffuse white light generated by a tungsten source and centered on the retina using a fiber optic illuminator (irradiance = 955 μW/cm\textsuperscript{2}). Neutral density filters were used to vary stimulus intensity. Saturating light flashes were used for all experiments.

**Cell dissociation**

Larval tiger salamander (Ambystoma tigrinum) retina was dissociated using enzymatic digestion with papain and trituration as described by MacLeish and Townes-Anderson (1988). Briefly, a salamander was decapitated and pithed. The head was bisected sagittally, and retinas were dissected from the eyecups. The two retinas were incubated at room temperature for 35–40 min in a Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free salamander enzyme solution saturated with 95% O\textsubscript{2}/5% CO\textsubscript{2}. The enzyme solution contained (in mM) 85 NaCl, 25 NaHCO\textsubscript{3}, 1 Na pyruvate, 0.5 NaH\textsubscript{2}PO\textsubscript{4}, 3 KCl, and 16.6 glucose plus 0.1% cysteine HCl, and 14 U/ml papain (Worthington). Tissue then was rinsed three times in a chilled dissociation solution consisting of (in mM) 104 NaCl, 0.5 NaH\textsubscript{2}PO\textsubscript{4}, 3 KCl, 1.8 CaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 0.5 MgSO\textsubscript{4}, 16.6 glucose, 10 HEPES, 1 Na pyruvate, 1 taurine, 0.4 ascorbic acid, 2 glutamine, 0.1% bovine serum albumin, and, in some experiments, 0.1% DNase. The retina was then triturated using a series of fire-polished glass pipettes. The dissociated cell suspension was kept on ice and plated, just before the imaging session, on glass coverslips coated with Sal-1 (kindly provided by Dr. P. MacLeish).

During the imaging session, the glass coverslip containing the attached cells was mounted at the bottom of the perfusion chamber (RC-25, Warner Inst.). Ringer solution entered the perfusion chamber through a Peltier device, which chilled it to 14°C. The perfusion solution was either the dissociation solution described above or a solution consisting of 95% salamander Ringer [which contained (in mM) 110 NaCl, 2.5 KCl, 0.5 MgCl\textsubscript{2}, 10 HEPES, 5 glucose, and 1.8 CaCl\textsubscript{2}] and 5% Leibovitz medium (L-15, Sigma). Ca\textsuperscript{2+} substitutes used were gluconate, methylsulfate, or sulfate. Free [Ca\textsuperscript{2+}] was adjusted to 1.8 mM for each Cl\textsuperscript{-} substitute by using a Ca\textsuperscript{2+}-sensitive electrode (Orion 93-20) and adding the proper CaX (CaCl\textsubscript{2}).


**Ca**²⁺ and pH imaging

**GENERAL.** Cells were loaded with either Fura-2-AM (Molecular Probes) for recording [Ca²⁺], changes or BCECF-AM (Molecular Probes) for intracellular pH changes. Cells were viewed through an inverted microscope (Nikon) with fluorescence quartz objectives (∼20 or ∼40). Images were obtained with a Photometrics cooled CCD camera (Photometrics CH250, Tucson, AZ) driven by an in-house imaging program (Cellview). Excitation light was provided by a Xenon lamp (75 W) and adjusted by neutral density and band-pass filters (Chroma) mounted on a computer-controlled dual-filter wheel and shutter system (LEP Hawthorne, NY).

To maximize contrast while maintaining relatively short exposures (200 ms/frame), images were binned at 5 × 5 or 7 × 7 pixels per unit. This binning still allowed clear identification of the photoreceptor’s terminal. As many as eight regions of interest (ROIs) were chosen for different cells and/or different cell regions; one background ROI was chosen as well. As well as running the camera, shutters, and filter wheels, the program featured a real-time, color-coded graphic display of changes in the emission ratio values for each ROI on the computer screen, which was saved as a spread sheet file for off-line analysis. All images taken during the experiment, as well as background images, were saved first in the Photometrics cache, transferred to the computer hard drive and later onto an optical disk drive (Panasonic 940 WORM drive) for further analysis.

**INTRACELLULAR pH MEASUREMENTS WITH BCECF.** Dissociated photoreceptors were labeled with BCECF using standard procedures (Boyarsky et al. 1988; Newman 1994; Rink et al. 1982). After trituration, retinal cells were incubated in salamander Ringer solution containing the membrane-permeant form of the dye, BCECF-AM (1.3 μg/100 μl), for 10–15 min on ice. Cells then were rinsed and placed on the microscope stage.

Measurements of pH were made, after background subtraction, from ratio images obtained with excitation wavelengths of 490 and 440 nm and an emission wavelength of 515 nm (Filter set 7200, Fura-BCECF, Chroma). The isosbestic point of BCECF excitation spectrum lies near 440 nm; thus BCECF emission intensity due to excitation at 440 nm shows very little pH sensitivity. For excitation at 490 nm, BCECF emission increases as the cell’s pH becomes more alkaline.

Calibration of BCECF was done using the high K⁺-nigerin technique (Chaillet and Boron 1985; Newman 1994). Cells were bathed in high K⁺ solution containing (in mM) 73 KCl, 0.8 MgCl₂, 1.8 CaCl₂, 5 CoCl₂ (to prevent Ca²⁺-induced cell death), 20 HEPES, 10 glucose, and 18.5 N-methyl-D-glucamine. Nigericin (10 μM, Molecular Probes) and valinomycin (2 μM, Aldrich) were added to collapse the pH and K⁺ gradients. When bathed in this solution, the intracellular pH of the photoreceptors equilibrates with the pH of the extracellular bath solution. As the pH increased, the BCECF emission ratio from photoreceptors also increased in a sigmoidal fashion. Calibration curves were obtained regularly, by bathing the cells in a series of high K⁺-nigerin solutions with pH values ranging from 6.0 to 8.0. Calibrations were carried out for different cell regions of the photoreceptor. To compare results from different cells, the ratioed fluorescence values were normalized by the ratio value obtained for a pH 7.0 calibration solution (Newman 1994).

**Ca**²⁺ MEASUREMENTS WITH FURA-2. The results in this paper concerning the free Ca²⁺ concentration are mostly qualitative and are derived from a consideration of the time course, location, and relative fluorescence ratio changes under certain conditions. Measurements were made, after background subtraction, from ratio images obtained with excitation wavelengths of 490 and 440 nm and an emission wavelength of 515 nm (Grynkwicz et al. 1985).

Ca²⁺ influx in dissociated photoreceptors was stimulated by elevating the extracellular K⁺ concentration to 40 mM (which should depolarize the cell by as much as 69 mV) for 10–20 s. Because the flow rate was 1–1.5 ml/min and the chamber volume was ∼200 μl, each stimulus resulted in approximately one change of the chamber’s volume.

**RESULTS**

Light-evoked currents of horizontal, OFF bipolar, and ON bipolar cells exhibit similar sensitivities to small reductions in extracellular Cl⁻. Figure 1A shows examples of light-evoked currents recorded from each cell type as a function of [Cl⁻]. In these examples, gluconate was used as the Cl⁻ substitute during the horizontal and OFF bipolar cell recording whereas methylsulfate was the Cl⁻ substitute for the ON bipolar cell recording. In all three cell types, reducing Cl⁻ by 11.5 mM reduced light-evoked currents. In the horizontal and OFF bipolar cell, there was a monotonic decrease in light-evoked currents with decreased [Cl⁻]. Responses from the ON bipolar cell showed an initial decrease at 97 and 81 mM Cl⁻ followed by a partial recovery at 54 mM Cl⁻. As illustrated in the concentration/response data plotted in Fig. 2, partial recovery at 54 or 81 mM Cl⁻ was typical of the effects observed when using methylsulfate as the Cl⁻ substitute. Recovery from Cl⁻ replacement was slow, requiring ≥30 min. These results and those illustrated in the subsequent figure (Fig. 2) suggest that small, physiologically relevant changes in [Cl⁻] can have functionally significant effects on synaptic transmission at the photoreceptor output synapse.

Figure 2 shows the concentration/response curves for all three second-order neurons (horizontal, ON, and OFF bipolar) using three anion substitutes: methylsulfate (CH₃SO₃⁻, MW = 111, pKₐ = 3.4), sulfate (SO₄²⁻, MW = 96, pKₐ = 2.0), and gluconate (C₆H₁₀O₇⁻, MW = 195, pKₐ = 3.9). Replacing Cl⁻ with methylsulfate, sulfate, or gluconate produced a similar suppression of light-evoked currents in all three classes of cells (Fig. 2). For all three substitutes, reducing [Cl⁻] by 11.5 mM from control (115.1 mM) suppressed light-evoked currents by 22–34%, and complete Cl⁻ removal suppressed the currents by 70–75%. Thus small changes in extracellular Cl⁻ can cause significant changes in light responses of second-order neurons.

Because some anions can complex with Ca²⁺, we measured free [Ca²⁺] using a Ca²⁺ electrode in the different anion solutions. The measured free [Ca²⁺] was 0.5 mM in gluconate Ringer, 1.2 mM in sulfate Ringer, 1.9 mM in methylsulfate Ringer, and 1.8 mM in control Cl⁻ Ringer solution. To assess the influence of Ca²⁺ chelation, we tested the effects on light-evoked currents of reducing bath [Ca²⁺] to 0.5 mM in control Ringer solution. Superfusion for 1–3 min with 0.5 mM Ca²⁺ Ringer solution did not reduce light-evoked currents (I/I_control = 1.14 ± 0.10, n = 8) in ON bipolar (n = 2), OFF bipolar (n = 2), or horizontal cells (n = 4). The mechanism of action of reduced extracellular Cl⁻ cannot therefore be attributed to Ca²⁺ chelation.

The results of Fig. 2 showing that a divalent anion (sulfate) and two different monovalent anions (methylsulfate and gluconate) have roughly similar potencies suggest that the suppression of neurotransmission in low Cl⁻ primarily reflects a reduction in Cl⁻ rather than an action of the substituting anion. These three anion substitutes were also equipotent in suppressing the b-wave of the ERG, although suppression of the b-wave required a greater reduction in bath [Cl⁻].
FIG. 1. Effects of reducing extracellular Cl\(^{-}\) on light responses in a horizontal cell, OFF bipolar cell, and ON bipolar cell. Cl\(^{-}\) was replaced by gluconate with horizontal and OFF bipolar cell and by methylsulfate with ON bipolar cell. Test solutions were bath applied for 3–5 min before recording light-evoked currents evoked by a saturating test flash. Recovery was obtained in the OFF bipolar cell after 24-min washout, horizontal cell after 15 min, and ON bipolar cell after 10 min.

in the eyecup than in the retinal slice, perhaps because of the higher tissue levels of Cl\(^{-}\) in the eyecup preparation. Figure 3 illustrates a second test of the possibility that the substituting anion was responsible for the suppression of synaptic transmission. This experiment compared light-evoked currents recorded in the presence and absence of methylsulfate anion while [Cl\(^{-}\)], was held constant at 108 mM. Three Ringer solutions were used: a normal Ringer solution with 118 mM Cl\(^{-}\) and 10 mM HEPES; a glucose solution in which 30 mM glucose replaced 10 mM NaCl and 5 mM NaHEPES; and a methylsulfate solution in which 15 mM NaMethylsulfate replaced 10 mM NaCl and 5 mM NaHEPES. Figure 3A shows results of one experiment in an ON bipolar cell, and Fig. 3B shows a bar graph that summarizes the results from the same experiment in all three cell types (glucose, \(n = 8\); methylsulfate, \(n = 7\)). Reducing [Cl\(^{-}\)], by 10 mM with the glucose or methylsulfate solutions produced a significant reduction of \(~20\%\) in light-evoked currents, similar to the reduction predicted from the results of Fig. 2. In the cell in Fig. 3A, switching from the high glucose solution to the methylsulfate solution produced a slight increase in the light-evoked current even though [Cl\(^{-}\)], was maintained at 108 mM in both solutions. As shown in the bar graph (Fig. 3B), overall there was no significant difference between the amplitude of light-evoked currents recorded in the high glucose and methylsulfate solutions. If methylsulfate was responsible for the suppression of light-evoked currents when used as the Cl\(^{-}\) substitute, further suppression of the light response would have been expected. Similar experiments using sucrose rather than glucose also showed no difference between response amplitudes in 30 mM sucrose and 15 mM methylsulfate (7 experiments in 3 cells, data not shown). These results clearly point to a reduction in Cl\(^{-}\) and not the substituting anion as being responsible for the suppression.

It has been proposed that DHP-sensitive \(I_{Ca}\) may mediate transmitter release from photoreceptors (Barnes and Hille 1989; Lasater and Witkovsky 1991; Maricq and Kor-
enbrot 1988; Rieke and Schwartz 1994; Wilkinson and Barnes 1996). Figure 4 illustrates examples of an ON bipolar cell recording (Fig. 4A) and the intraretinally recorded b-wave of the ERG (Fig. 4B) before and during the application of the DHP antagonist, nifedipine. The b-wave reflects light responses of ON bipolar cells (Stockton and Slaughter 1989). Both the inward current of the ON bipolar and the b-wave were reduced significantly by nifedipine (40 μM, Research Biochemicals International). Overall, nifedipine (40 μM) reduced light-evoked currents in ON bipolar cells to 55 ± 20% (n = 6) of control and the b-wave to 57 ± 17% (n = 4) of control. Light-evoked currents in two OFF bipolar cells (I/I_{control} = 0.39 ± 0.29) and one horizontal cell (I/I_{control} = 0.13) also were suppressed by nifedipine. These data are consistent with the hypothesis that DHP-sensitive Ca^{2+} channels support neurotransmission from photoreceptors.

If DHP-sensitive I_{Ca} mediates transmitter release from photoreceptors, then DHP-sensitive Ca^{2+} channels should be present in the axon terminals of photoreceptors. This prediction was confirmed by the imaging experiments illustrated in Figs. 5 and 6.

Figure 5 illustrates an imaging experiment to study Ca^{2+} changes in a solitary, tiger salamander rod. The pseudocolored ratiometric images represent higher [Ca^{2+}], as warmer colors (see calibration bar). The outer segment (os), inner segment (is), soma, axon (a), and terminal (t) are labeled in Fig. 5, panel I.

Panels I–A of Fig. 5 show a sequence of [Ca^{2+}] changes before (I), during (2 and 3), and after (4) 60 s application of low extracellular Na^{+} (20 mM, choline substitute). Reducing Na^{+} should suppress Na^{+}/Ca^{2+} exchange in the outer segment and, consistent with this expectation, [Ca^{2+}] was elevated in the outer segment but remained unchanged in other cell regions, indicating tight compartmental restrictions on Ca^{2+} movement. Panels 5–8 of Fig. 5 show the effects of applying an extracellular high K^{+} solution (60 mM) for a period of 15 s. In contrast to the effects of low Na^{+}, high K^{+} increased [Ca^{2+}] in the terminal and soma, while the concentration in the outer segment remained unchanged. In response to high K^{+}, an increase in [Ca^{2+}], was observed first in the terminal (5) and subsequently in the soma (6). The Ca^{2+} increase in the terminal was significantly buffered before that in the soma (7). The finding that the [Ca^{2+}] increase was detected in the terminal before the soma is expected if the terminal has a greater surface to volume ratio than the soma. These results indicate that there is a local source for Ca^{2+} influx in the terminal that is not due to diffusion from the soma.

Figure 6 illustrates ratioed data for Ca^{2+} determined from paired images taken every 4 s. The traces in Fig. 6A show successive applications of elevated extracellular K^{+} (60 mM). Repeated applications of high K^{+} consistently evoked intracellular Ca^{2+} increases. In this experiment, as in most other experiments, the largest Ca^{2+} change occurred in the terminal, with smaller changes in the soma, and yet smaller in the inner and outer segments for which the latency was longer. The large and rapid Ca^{2+} increase in the terminal supports the idea that Ca^{2+} channels are localized to this region.

The experiments illustrated in the remainder of Fig. 6 indicate that the elevation of [Ca^{2+}], in photoreceptors in-
produced by elevated extracellular K⁺ resulted from a Ca²⁺ influx from the extracellular space. Application of the divalent cation, CoCl₂ (2 mM), to the extracellular medium reversibly blocked the high K⁺-evoked Ca²⁺ influx (Fig. 6B, n = 5). Similarly, the DHP antagonists nifedipine (brief application of 80 μM; Fig. 6D) and nimodipine (4 μM; not shown) reversibly suppressed the response to high K⁺ by >60% (n = 7). This suggests that a large fraction of the Ca²⁺ increase is mediated by the activation of DHP-sensitive Ca²⁺ channels.

Figure 7 illustrates the action of reduced extracellular Cl⁻ on the K⁺-evoked changes in intracellular Ca²⁺ in both rod and cone photoreceptors. Replacement of Cl⁻ with gluconate, sulfate, and methylsulfate markedly suppressed the K⁺-evoked Ca²⁺ increases. Similar to the effects on light-evoked currents, the effects of Cl⁻ replace-
LOW Cl\textsuperscript{−} SUPPRESSES PHOTORECEPTOR I\textsubscript{Ca}

I\textsubscript{Ca} suppressed by a DHP antagonist, nifedipine (10–40 \textmu M, \( n = 10 \) cones, 10 rods; Fig. 8, \( \Delta \)). [use of vehicle alone had no effect (dimethyl sulfoxide 1:10,000, \( n = 4 \) cones, \( n = 4 \) rods).] Incomplete block of photoreceptor I\textsubscript{Ca} by nifedipine is largely due to its ineffectiveness in blocking DHP-sensitive Ca\textsuperscript{2+} channels in these cells (Wilkinson and Barnes 1996). The inward current also was suppressed by Cd\textsuperscript{2+} (200 \textmu M, \( n = 2 \) rods).

In the rod recording illustrated in Fig. 8B (right), a transient inward current was revealed by the application of nifedipine. Although it might be hypothesized that this transient current reflects the presence of a transient Ca\textsuperscript{2+} channel subtype, the I-V relation measured at the peak of the transient did not differ from the steady state I-V relation obtained at the end of the 150-ms step. A DHP-resistant, transient component of photoreceptor I\textsubscript{Ca} has been attributed to contamination by slowly developing outward currents or time- and voltage-dependent block of L-type channels (Barnes and Deschenes 1992; Wilkinson and Barnes 1996).

As shown in Fig. 8 (left), a brief (1-min) reduction of [Cl\textsuperscript{−}], suppressed DHP-sensitive I\textsubscript{Ca} in rods and cones. Accompanying this suppression was a left shift in the activation and peak voltages. I\textsubscript{Ca} recovered after washout (control responses, nifedipine experiment). Longer application of low Cl\textsuperscript{−} solutions produced a greater degree of suppression but full recovery was observed rarely. A similar suppression of I\textsubscript{Ca} after a reduction in [Cl\textsuperscript{−}], was seen in rods and cones of mudpuppy (\( n = 13 \) cones, \( n = 21 \) rods) and tiger salamander (\( n = 15 \) rods) whether methylsulfate (with Ca\textsuperscript{2+} as the primary charge carrier) or gluconate (with Ba\textsuperscript{2+} as the charge carrier) was used as the Cl\textsuperscript{−} substitute (methylsulfate, \( n = 13 \); gluconate, \( n = 36 \)). The examples illustrated in Figs. 8 and 10 were obtained in the presence of the glutamate uptake blocker, dihydrokainic acid (1 \textmu M, \( n = 12 \)).

In addition to effects on I\textsubscript{Ca}, reducing [Cl\textsuperscript{−}], also sometimes enhanced an outward current at positive holding potentials. This effect was particularly pronounced when Cl\textsuperscript{−} was replaced with HClO\textsubscript{4}. In experiments on photoreceptors following rundown of I\textsubscript{Ca}, replacing Cl\textsuperscript{−} with HClO\textsubscript{4} enhanced a slowly activating outward current, which was activated at holding potentials more than \(-45 \text{ mV} (n = 6)\). Although detailed studies of this effect have not yet been performed, this observation suggests that anion replacement influences a delayed rectifier K\textsuperscript{+} current as previously suggested for muscle cells (Kao and Stanfield 1968).

Cl\textsuperscript{−} removal significantly increased the input resistance (\( P = 0.001 \), paired \( t \)-test) measured between \(-70 \) and \(-90 \text{ mV} \) from 688.6 \pm 145.2 \text{ M\Omega} to 828.6 \pm 150.0 \text{ M\Omega} (14 trials in 12 photoreceptors in the presence of BaCl\textsubscript{2} (10 \textmu M), strychnine (1 \textmu M), picrotoxin (100 \textmu M), niflumic acid (100 \textmu M), and DHKA (1 \textmu M)).

The sensitivity of I\textsubscript{Ca} to reductions in [Cl\textsuperscript{−}], is illustrated in Fig. 9, which shows a plot of the fractional barium (2 \textmu M) current (\( I_{\text{low Cl}}/I_{\text{control}} \)) as a function of [Cl\textsuperscript{−}], (using gluconate as the primary anion substitute). This illustrates a very tight relationship between [Cl\textsuperscript{−}], and the amplitude of I\textsubscript{Ca} and that reducing Cl\textsuperscript{−} by as little as 11 \textmu M significantly reduced I\textsubscript{Ca}. A longer application time of 3 min contributes to the more pronounced suppressive effect in this figure than the previous one. Rods (\( \triangle \)) and cones (\( \nabla \)) showed a similar sensitivity to reductions in [Cl\textsuperscript{−}].
Similar to the hyperpolarizing shift in the voltage dependence of $I_{Ca}$ illustrated in Fig. 8, Cl$^-$ replacement with frog muscle cells typically caused hyperpolarizing shifts in delayed rectifier $K^+$ currents, voltage-dependent $Na^+$ currents, and DHP-sensitive $I_{Ca}$ (Dani et al. 1983; Delay et al. 1990; Kao and Stanfield 1968). The shifts in voltage dependence
of these currents follow the lyotropic anion series, suggesting that they arise from anion binding to positive charges on the muscle cell surface (Dani et al. 1983). Unlike the present study, the left shifts observed in other studies of anion substitution were not accompanied by reductions in current amplitude (Dani et al. 1983; Delay et al. 1990; Kao and Stanfield 1968). We evaluated one simple explanation for the suppression observed in our study; namely, that a left shift in activation might cause opening and subsequent inactivation of a large fraction of Ca\(^{2+}\) channels. Ca\(^{2+}\) currents in salamander rods exhibit a Ca\(^{2+}\)-dependent inactivation (Corey et al. 1984). Although Ba\(^{2+}\) was substituted for Ca\(^{2+}\) on obtaining a photoreceptor recording, it is possible that sufficient residual Ca\(^{2+}\) remained for inactivation. 

To test a possible contribution of inactivation, we compared I\(_{Ca}\) recorded after holding the photoreceptor for 1 min at either -50 or -70 mV in control Ringer solution. As shown in Fig. 10A, the amplitude of I\(_{Ca}\) recorded when the cell was voltage clamped at -50 mV before the voltage step series was slightly smaller than I\(_{Ca}\) recorded when the cell was voltage clamped at -70 mV before the voltage step series. The peak current was reduced by an average of 17.7 ± 7.8% (n = 10) by this manipulation, suggesting some inactivation occurred. However, this inactivation was significantly less than the 51.7 ± 7.9% (n = 9) suppression induced by Cl\(^-\) replacement with gluconate in the same sample of tiger salamander rods.

If a 20-mV left shift in activation reduced I\(_{Ca}\) by inactivating many of the Ca\(^{2+}\) channels in low Cl\(^-\) medium, then hyperpolarizing the cell an additional 20 mV in low Cl\(^-\) medium should reinstate much of the current. However, as shown in Fig. 10B, the amplitude of I\(_{Ca}\) in Cl\(^-\)-free medium was not greatly enhanced by hyperpolarizing the cell to -90 mV for 1 min before obtaining the voltage clamp series. The peak amplitude of I\(_{Ca}\) in low Cl\(^-\) medium increased only 12.3 ± 7.9% (n = 9) as a result of prior hyperpolarization, so that the current was suppressed by 45.3% when the steady state holding potential was -90 mV and 51.7% when the prior holding potential was -70 mV. The results of the experiments illustrated in Fig. 10 show that inactivation secondary to a left shift in threshold for I\(_{Ca}\) can account for some, but not all of the observed suppression in low Cl\(^-\) medium.

Barnes and colleagues (Barnes and Bui 1991; Barnes et al. 1993) have shown that I\(_{Ca}\) in amphibian photoreceptors can be suppressed by extracellular acidosis. Thus another possible mechanism by which removal of extracellular Cl could decrease I\(_{Ca}\) is extracellular acidosis created perhaps by a change in Cl\(^-\)/HCO\(_3^-\) exchange or altered proton regulation through other anion-dependent mechanisms. Figure 11 shows the effects of extracellular acidification on intracellular Ca\(^{2+}\) using two different protocols for extracellular pH changes: brief extracellular pH changes, in which only the extracellular medium is acidiﬁed, and longer pH changes in which the preparation was acidiﬁed for ~1 min before application of a high K\(^+\) solution set to the same pH. As shown in Fig. 11A, brief application of pH 6.0 caused a significant decrease in the Ca\(^{2+}\) increase induced by high K\(^+\) in the terminal and soma regions. Using the longer application protocol, acidifying the extracellular medium by 0.4 pH units from 7.8 to 7.4 reduced the Ca\(^{2+}\) response (Fig. 11B). Reducing the pH to 7.0 further suppressed the Ca\(^{2+}\) response (Fig. 11, B and C). Similar results were obtained in both rod (Fig. 11B) and cone (Fig. 11C) photoreceptors (n = 8). Under the same conditions as Fig. 11, we did not detect intracellular pH changes using BCECF (n = 8, data not shown). The results of Fig. 11 confirm the results of Barnes's experiments.
FIG. 7. Ca\(^{2+}\) influx monitored with Fura-2 was blocked in the absence of extracellular Cl\(^{-}\). Replacing Cl\(^{-}\) with gluconate in the extracellular solution caused a decrease in the high K\(^{+}\)-evoked increase in [Ca\(^{2+}\)]\(_{i}\) in rod (A) and cone (B) photoreceptors. Partial recovery was achieved after >200 s washout. Replacing Cl\(^{-}\) with sulfate (C) or methylsulfate (D) also reduced the Ca\(^{2+}\) response to high K\(^{+}\) in photoreceptors. In D, return to control solution appeared to produce spontaneous Ca\(^{2+}\) spikes. 1, beginning of 15-s application of high K\(^{+}\) solutions with either Cl\(^{-}\) or the appropriate replacement as primary anion.

FIG. 8. Current-voltage (I-V) relationships in cone (A) and rod (B) photoreceptors. Amplitudes of steady state currents measured at end of 150-ms voltage steps are plotted after adjusting for series resistance and liquid junction potential of pipette. Responses were obtained with CsCl intracellular Ringer solution in presence of 10 mM BaCl\(_2\), 20 mM HEPES, and 1 mM dihydrokainic acid, in addition to usual amphibian extracellular Ringer solution and toxins. In both A and B, graphs at left show I-V plots obtained in control medium (●) and after 1-min perfusion with a solution in which all but 20 mM Cl\(^{-}\) was replaced with gluconate. Control responses on right (●) were obtained after washout of low Cl\(^{-}\) solution and show that the inward current recovered after washout. △, currents in presence of nifedipine (10 μM). Insets: responses evoked by +60-mV steps from holding potential of −67 mV (cone) or −68 mV (rod). Horizontal scale bar in inset: 25 ms. Vertical scale bar (cone): 200 pA. Vertical scale bar (rod): 100 pA. Note that a small, weakly inactivating, inward current remains in rod response after application of nifedipine. I-V profile plotted at peak of this current was indistinguishable from steady state current.
LOW CL\(^-\) SUPPRESSES PHOTORECEPTOR \(I_{Ca}\)

FIG. 9. Concentration/response relationship of effects of reducing Cl\(^-\) on photoreceptor \(I_{Ca}\) recorded with Ba\(^2+\) (2 mM) as predominant charge carrier. Cl-free solution contained 108 mM NaGluconate, 2.5 mM KMethysulfate, 0.5 mM MgSO\(_4\), 10 mM HEPES, 5 mM glucose, 100 \(\mu\)M picrotoxin, 2 \(\mu\)M strychnine, 100 \(\mu\)M niflumic acid. Test solutions were applied for 3 min. Fractional responses were derived from inward currents obtained by stepping cell from a nominal holding potential of \(-60\) to \(-20\) mV and calculated from formula: fractional \(I_{hit} = I_{low \text{Cl}}/I_{control}\). In experiments in which full recovery was not achieved after washout, \(I_{control}\) was adjusted by linear interpolation between initial control responses and responses obtained following washout. Data are plotted for both rods (●) and cones (▲), as well as mean from all cells (○, error bars). Rods: 97 mM Cl\(^-\), \(n = 9\); 54 mM Cl\(^-\), \(n = 6\); 4 mM Cl\(^-\), \(n = 6\). Cones: 97 mM Cl\(^-\), \(n = 5\); 54 mM Cl\(^-\), \(n = 3\); 4 mM Cl\(^-\), \(n = 3\).

et al. (1993) by showing that extracellular acidification suppresses a DHP-sensitive Ca\(^{2+}\) influx in amphibian photoreceptors.

Newman (1996) recently developed a method for measuring extracellular pH changes in the membrane vicinity of dissociated cells using coverslips coated with dextran-conjugated BCECF. Using this method, we examined the effect on extracellular pH of replacing extracellular Cl\(^-\) with glutamate. No extracellular pH change was detected near any photoreceptor tested. Generation of a detectable extracellular pH change would require a substantial transmembrane acid/base flux, which also should alter intracellular pH. Thus these results are in full agreement with the finding described below (Fig. 12) that no intracellular pH change was detected in a Cl-free environment.

As a second test for a possible role of extracellular acidosis in the suppression of Ca\(^{2+}\) influx observed on Cl\(^-\) removal, we tested whether the effect was abolished by buffering the extracellular pH with a high concentration of HEPES (20 mM). However, even in the presence of 20 mM HEPES, Cl\(^-\) removal suppressed Ca\(^{2+}\) influx in dissociated photoreceptors (\(n = 5\)), photoreceptor \(I_{Ca}\) (\(n = 25\); e.g., Fig. 8), and light-evoked currents in second-order neurons (\(n = 12\); e.g., OFF bipolar and horizontal cell in Fig. 1).

Another possible mechanism which might account for the suppression of \(I_{Ca}\) in low Cl\(^-\) is intracellular acidification (Takahashi et al. 1993). We therefore examined the effects of Cl\(^-\) removal on intracellular pH measured with BCECF. Figure 12A illustrates an experiment in which the effects of Cl\(^-\) removal on intracellular pH were compared with pH calibrations in the same cell (performed as described in METHODS). Figure 12B shows the average (mean ± SD)
changes might contribute to the suppression of $I_{\text{Ca}}$, we tested the effects of increasing the concentration of HEPES in the whole cell recording pipette to 20 mM, which should buffer any significant intracellular pH changes. Suppression of $I_{\text{Ca}}$ in Cl-free medium was observed in 4/4 photoreceptors with 20 mM HEPES in the pipette, suggesting that the suppression was not indirectly mediated by intracellular acidification.

**DISCUSSION**

When extracellular Cl$^-$ ions are replaced by large anions, neurotransmission from photoreceptors is significantly reduced (Figs. 1 and 2). The importance of Cl$^-$ for supporting glutamatergic neurotransmission may be unique to photoreceptors. At most CNS synapses, Cl$^-$ replacement removes GABAergic and glycinergic inhibition and thus enhances excitability (Barker and Nicoll 1973; Yamamoto and Kawai 1967). Cl$^-$ replacement has similar actions in the mammalian retina (Miller and Dacheux 1973), suggesting that the results of the present study have broader species implications.

The Cl-free suppression of neurotransmission from photoreceptors is associated with suppression of a voltage-gated, DHP-sensitive, $I_{\text{Ca}}$ in both rods and cones (Figs. 8–10). Electrophysiological demonstration of Cl sensitivity to this current was verified with Ca$^{2+}$/imaging studies in which extracellular acidosis reduces high K$^+$-evoked Ca$^{2+}$ influx monitored with Fura-2. A: brief (15 s) application of high K$^+$ solution at pH 6.0 reduced Ca$^{2+}$ response in a rod photoreceptor. B: perfusion at pH 7.8, 7.4 and 7.0 for 1 min followed by application of high K$^+$ solution in a rod. High K$^+$-evoked Ca$^{2+}$ influx was reduced as pH was reduced. C: Perfusion at pH 7.8 and 7.0 for 1 min followed by application of high K$^+$ solution in a cone. Ca$^{2+}$ influx was strongly suppressed in pH 7.0.

**FIG. 11.** Extracellular acidosis reduces high K$^+$-evoked Ca$^{2+}$ influx monitored with Fura-2. A: brief (15 s) application of high K$^+$ solution at pH 6.0 reduced Ca$^{2+}$ response in a rod photoreceptor. B: perfusion at pH 7.8, 7.4 and 7.0 for 1 min followed by application of high K$^+$ solution in a rod. High K$^+$-evoked Ca$^{2+}$ influx was reduced as pH was reduced. C: Perfusion at pH 7.8 and 7.0 for 1 min followed by application of high K$^+$ solution in a cone. Ca$^{2+}$ influx was strongly suppressed in pH 7.0.

change in BCECF emission ratio measured under the different conditions. In this cell, there was no significant change in the fluorescence ratio when cells were bathed in control or Cl-free (sulfate) Ringer solution. In both control and Cl-free solutions, the intracellular pH was ~7.1. No detectable pH change was observed in three cells using sulfate or methylsulfate as the anion substitute. In two more cells, slight alkalization of ~0.1 pH units were observed. The large emission ratio change observed in Fig. 12 after increasing the pH from 7.0 to 7.5 or reducing it from 7.0 to 6.6 suggests that intracellular pH changes of ~0.2 pH units should be readily discriminable. This resolution was confirmed in similar experiments in which the intracellular pH was calibrated in 0.2 pH unit increments. Thus the measurements of intracellular pH and extracellular pH both suggest that changing [Cl$^-$], in a HEPES-buffered Ringer solution does not generate a substantial transmembrane acid/base flux in dissociated photoreceptors.

As a further test of the possibility that intracellular pH changes in BCECF emission ratio measured under the different conditions. In this cell, there was no significant change in the fluorescence ratio when cells were bathed in control or Cl-free (sulfate) Ringer solution. In both control and Cl-free solutions, the intracellular pH was ~7.1. No detectable pH change was observed in three cells using sulfate or methylsulfate as the anion substitute. In two more cells, slight alkalization of ~0.1 pH units were observed. The large emission ratio change observed in Fig. 12 after increasing the pH from 7.0 to 7.5 or reducing it from 7.0 to 6.6 suggests that intracellular pH changes of ~0.2 pH units should be readily discriminable. This resolution was confirmed in similar experiments in which the intracellular pH was calibrated in 0.2 pH unit increments. Thus the measurements of intracellular pH and extracellular pH both suggest that changing [Cl$^-$], in a HEPES-buffered Ringer solution does not generate a substantial transmembrane acid/base flux in dissociated photoreceptors.

As a further test of the possibility that intracellular pH changes might contribute to the suppression of $I_{\text{Ca}}$, we tested the effects of increasing the concentration of HEPES in the whole cell recording pipette to 20 mM, which should buffer any significant intracellular pH changes. Suppression of $I_{\text{Ca}}$ in Cl-free medium was observed in 4/4 photoreceptors with 20 mM HEPES in the pipette, suggesting that the suppression was not indirectly mediated by intracellular acidification.

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elevation of extracellular K⁺ was used to activate Ca²⁺ channels (Fig. 7). DHP-sensitive Ca²⁺ channels are present in the photoreceptor terminal (Figs. 5 and 6), and blocking these channels with DHP antagonists suppresses light responses in second-order retinal neurons (Fig. 4) (also Rieke and Schwartz 1994), suggesting that transmitter release from photoreceptors can be mediated by these channels. Thus suppression of photoreceptor Iₜₐₜ by Cl⁻ removal could result in the suppression of transmitter release. Consistent with this possibility, photoreceptor Iₜₐₜ and light-evoked currents of second-order neurons show a similar sensitivity to reductions in [Cl⁻]ₒ (Figs. 2 and 9).

Another mechanism that could contribute to the suppression of transmitter release from photoreceptors is a reduction of Cl⁻-dependent glutamate uptake into presynaptic vesicles (Naito and Ueda 1985). However, for this mechanism to play a role in the short-term effects of a Cl⁻-free environment, a reduction in extracellular Cl⁻ would have to empty previously packaged vesicles to cause the rapid changes in postsynaptic responses observed in this study. Although this seems unlikely, vesicular depletion could contribute to the long recovery times typically observed after tissue exposure to a Cl⁻-free environment.

In addition to suppressing Iₜₐₜ, Cl⁻ removal causes a negative shift in the activation threshold. Although the suppression of Iₜₐₜ was greater than that illustrated in Fig. 8, when low Cl⁻ solutions were applied for longer times (e.g., Figs. 9 and 10), this hyperpolarizing shift in activation might compensate partially for the suppression of Iₜₐₜ between −40 and −60 mV, the range over which photoreceptor membrane voltage is modulated by light. Two other factors are likely to influence the effects of Cl⁻ changes on Iₜₐₜ. One is Ca²⁺-dependent inactivation of photoreceptor Ca²⁺ channels (Corey et al. 1984). Although inactivation of photoreceptor Iₜₐₜ is minimal in the present experimental conditions (Fig. 9), inactivation can be significant (ca. 40% in 4 s.) when Ca²⁺ is used as the charge carrier, minimal Ca²⁺ buffering is used in the recording pipette, and Iₜₐₜ is >10 pA (Corey et al. 1984). Thus at a resting membrane potential of −40 mV, a left shift of photoreceptor Iₜₐₜ in vivo is likely to produce significant inactivation. The net effect of inactivation and the shift in activation threshold is dependent on the photoreceptor membrane voltage. Therefore, the other important factor in evaluating effects of Cl⁻ replacement on Iₜₐₜ is the membrane voltage change in photoreceptors in situ. Intracellular recordings indicate that Cl⁻ removal typically causes a small depolarization (Burkhardt et al. 1991; Miller and Dacheux 1976; Thoreson and Burkhardt 1991). Depolarization might accentuate inactivation or, if sufficiently large, depolarize the photoreceptor to a voltage where Iₜₐₜ is activated maximally and thus poorly modulated by light-evoked changes in voltage. Conversely, hyperpolarization could compensate for the negative shift in activation threshold. Thus the effect of Cl⁻ replacement on Iₜₐₜ in situ between −40 and −60 mV depends on the interaction among membrane voltage changes, the shift in activation, the degree of inactivation, and the degree of suppression induced by low Cl⁻ media. However, with this caveat, it nonetheless seems plausible to hypothesize that the suppression of photoreceptor neurotransmission in low Cl⁻ media is a result of suppression (directly caused by low Cl⁻ or as a consequence of inactivation after depolarization) of DHP-sensitive, photoreceptor Iₜₐₜ.

Where does Cl⁻ affect photoreceptor Iₜₐₜ?

The rapid effects of Cl⁻ removal on Iₜₐₜ are consistent with an extracellular site of action. However, Cl⁻ removal causes a significant conductance decrease in photoreceptors, even in the presence of a cocktail of Cl⁻ channel blockers, suggesting that there is transmembrane exchange of Cl⁻. Consistent with this suggestion, during perfusion with Cl⁻-free Ringer solution there is a reduction in [Cl⁻], measured with the Cl-sensitive dye, 6-methoxy-N-ethylquinolinium iodide (MEQ), in dissociated photoreceptors (Nitzan and Miller 1995). These results indicate that Cl⁻ could have an intracellular site of action. One test of whether Cl⁻ acts at an intracellular site is to compare effects on Iₜₐₜ of high and low pipette [Cl⁻]. No difference was observed between these conditions, consistent with an extracellular site of action. However, measurements of membrane capacitance and access resistance suggest that diffusion of Cl⁻ throughout photoreceptors in the slice may take as long as 30 min (Pusch and Neher 1988) by which time our experiments were usually complete. Although the capacitance estimates probably include infoldings of the outer segment membrane in cones and therefore are likely to overestimate the time required for Cl⁻ changes in the soma and terminal, our experiments do not allow exclusion of the possibility of an intracellular site of action.

How does Cl⁻ alter Ca²⁺ currents in photoreceptors?

We evaluated several mechanisms by which reductions in extracellular Cl⁻ might suppress photoreceptor Iₜₐₜ, including: loss of space clamp conditions, actions of the substituting anion, intracellular acidification, extracellular acidification, and inactivation of Iₜₐₜ secondary to a hyperpolarizing shift in the activation threshold.

Space clamp conditions. Although a loss of good space clamp conditions could lead to problems in measuring and activating photoreceptor Iₜₐₜ, studied with electrophysiological techniques, this explanation cannot account for the action of reduced Cl⁻ in suppressing the K⁺-evoked Ca²⁺ increase revealed by imaging experiments. Furthermore, replacing extracellular Cl⁻ with gluconate increases photoreceptor Rₑ and thus should enhance space clamp conditions rather than degrade them. For these reasons, a Cl⁻-free alteration in space clamp conditions is unlikely to explain our findings.

Substituting anion. Two experiments suggest that suppression of Iₜₐₜ and photoreceptor transmission do not result from actions of the substituting anion. The relationship between [Cl⁻]ₒ and amplitude of the light-evoked current in bipolar and horizontal cells is similar for three different anion substitutes (Fig. 2), and all three substitutes also suppressed photoreceptor Ca²⁺ influx. Adding 15 mM methylsulfate to the extracellular medium when [Cl⁻]ₒ was held constant did not reduce the light-evoked current (Fig. 3), although reducing [Cl⁻]ₒ by 10 mM suppressed both light-evoked currents and Iₜₐₜ by ≥20% (Figs. 2 and 9).
EXTRACELLULAR pH. Consistent with the electrophysiological results of Barnes et al. (1993), imaging studies in which the extracellular pH was altered revealed a high pH sensitivity of the K⁺-evoked, DHP-sensitive Ca²⁺ increase in rod and cone terminals (Fig. 11). Thus if a Cl⁻-free environment reduced extracellular pH, then this would suppress $I_{Ca}$. However, results obtained using dextran-BCECF-coated glass coverslips did not reveal any detectable change in extracellular pH. Furthermore, acidosis causes a depolarizing shift in photoreceptor $I_{Ca}$ (Barnes et al. 1993) but Cl⁻ removal causes a hyperpolarizing shift. And finally, the addition of 20 mM HEPES to the extracellular Ringer solution, which should strongly buffer extracellular pH did not eliminate the suppressive effects of a low Cl⁻ environment.

INTRACELLULAR pH. Intracellular acidosis can suppress DHP-sensitive $I_{Ca}$ by proton block of the channel (Takahashi et al. 1993). Experiments on horizontal cells showed that acidification of 0.3 pH units reduced $I_{Ca}$ by $\sim 50\%$ (Dixon et al. 1993). However, our observations with the intracellular pH indicator BCECF did not reveal any pH changes in photoreceptor terminals after a reduction in [Cl⁻]. Calibration experiments indicate that this system is capable of detecting pH changes of $\pm 0.2$ pH units and therefore should detect the degree of pH change necessary to block Ca²⁺ channels.

Two more observations support the pH imaging studies in suggesting that intracellular acidosis did not produce the suppression of $I_{Ca}$. First, the most likely mechanism for pH changes caused by a reduction in extracellular Cl⁻ would be suppression of Cl⁻/HCO₃⁻ exchange. However, all experiments were done in HCO₃⁻-free, HEPES-buffered, Ringer solutions, and if there is any endogenous HCO₃⁻ present, blocking Cl⁻/HCO₃⁻ exchange should produce alkalosis. Second, strongly buffering intracellular pH by including 20 mM HEPES in the recording pipette did not detectably affect Cl⁻ free suppression of photoreceptor $I_{Ca}$. Taken together, these experiments strongly suggest that the suppression of $I_{Ca}$ induced by removal of extracellular Cl⁻ is not mediated by a change in either intra- or extracellular pH.

INACTIVATION SECONDARY TO A LEFTWARD SHIFT IN ACTIVATION. We tested the possibility that the reduction in the peak current is secondary to the opening and subsequent inactivation of a significant fraction of Ca²⁺ channels as the threshold for activation shifts from $-50$ mV to near the typical holding potential of $-70$ mV. However, a 20-mV hyperpolarization in Cl⁻-free medium (which should compensate for the hyperpolarizing shift in threshold) did not counter the suppression of $I_{Ca}$, and a 20-mV depolarization in control Ringer (which simulates the hyperpolarizing shift induced by Cl⁻-free medium) did not induce a similar degree of suppression (Fig. 10). Thus the suppression of $I_{Ca}$ in Cl⁻-free medium is not a secondary consequence of the leftward shift in activation. Although inactivation does not account for the suppression of $I_{Ca}$ observed in the present experiments, inactivation might enhance the suppression of photoreceptor $I_{Ca}$ in situ, as discussed above.

Other possible mechanisms for Cl⁻ effects on photoreceptor $I_{Ca}$

The present experiments eliminate a number of possible explanations for the suppression of $I_{Ca}$ caused by Cl⁻ substitution. The two most plausible remaining possibilities are anion binding to the cell surface and indirect effects of anions mediated by enzymatic mechanisms.

Cl⁻ replacement has been shown to induce a shift in the voltage dependence of $I_{Na}$, $I_{K}$, and muscle $I_{Ca}$, which follows the lyotropic anion series, suggesting that it arises from anion binding to positive charges on the cell surface (Dani et al. 1983; Delay et al. 1990; Kao and Stanfield 1968). The alpha subunit of L-type Ca²⁺ channels cloned from brain possesses a number of positively charged residues that could serve as binding sites for Cl⁻ on both the intra- and extracellular surfaces of the channel (Hofmann et al. 1994). Thus the shift in activation in low Cl⁻ could result from weak anion binding to the cell surface, which influences movement of the voltage sensor, and suppression of $I_{Ca}$ could be due to binding, which influences permeation through the pore.

Because some of the effects of Cl⁻ could be primarily intracellular, the possibility that changes in [Cl⁻], may alter second messenger regulation of $I_{Ca}$ also should be considered. Intracellular Cl⁻ (like F⁻) can stimulate G-protein activity (Higashijima et al. 1987; Nakajima et al. 1992), and G proteins, in turn, can regulate L-type Ca²⁺ channels by actions of the G-protein alpha subunit or phosphorylation by protein kinase C, protein kinase A, or protein phosphatases (Hofmann et al. 1994). A variety of other enzymes, including a family of aminopeptidases, also are stimulated by an increase in [Cl⁻] (e.g., Abramic and Vitale 1989; Horowitz and Meister 1972; Stauch et al. 1989). Under this scenario, reducing Cl⁻ would suppress a tonic, permissive effect of G proteins on Ca²⁺ channels resulting in a suppression of $I_{Ca}$. Consistent with this possibility, a recent report suggests that photoreceptor Ca²⁺ channel activity is enhanced tonically by CaM kinase II activity (Iuvone and Alonso-Gomez 1995).

Functional significance

Changes in [Cl⁻], in response to normal neuronal activity likely are to be quite small. Both increases and decreases of $\pm 5$ mM have been reported (Dietzel and Heinemann 1986; Dmitriev et al. 1995). (Although the effects of increasing [Cl⁻] above 118 mM have not yet been tested, the steep slope of the dose/response function in this range suggests that such an increase should enhance photoreceptor synaptic transmission.) Local changes in the synaptic cleft could be much greater. The present results show that reducing Cl⁻ by 10 mM suppresses photoreceptor $I_{Ca}$ and light-evoked synaptic currents by $\simeq 20\%$ (Figs. 2 and 9). Furthermore, during anoxia, [Cl⁻] in the brain can fall by as much as 40 mM (Jiang et al. 1992). Light-dependent decreases of 20 mM in [Cl⁻], in horizontal cells have been reported (Djamgoz and Laming 1987); if Cl⁻ acts at an intracellular site in photoreceptors and there is a similar reduction in these cells, then sustained illumination might suppress transmitter release by $\simeq 30\%$. Given this range of possible changes in [Cl⁻] and [Cl⁻], it appears that regulation of [Cl⁻] in and around photoreceptors is, at a minimum, likely to have small but significant effects on transmission and, under certain conditions, may have a profound influence on photoreceptor synaptic transmission. Possible functions of Cl⁻ regulation include modulation of horizontal cell to cone feedback, post-receptor light and dark adaptation, and regulation of the
balance between ON and OFF pathways (Thoreson and Miller 1996).

In summary, the present results are consistent with the hypothesis that suppression of transmitter release from photoreceptors in low Cl− media (Thoreson and Miller 1996) is a consequence of the suppression of DHP-sensitive, ICa, localized to the synaptic terminals of rod and cone photoreceptors. Anion effects on ICa likely are mediated by anion binding sites on the cell surface and/or indirect effects on second messenger pathways. The finding that small, physiologically-achievable, reductions in extracellular [Cl−] have significant suppressive effects on photoreceptor ICa and Ca2+-dependent glutamate release at the first visual synapse suggests that regulation of Cl− in the outer retina should have important functional consequences for vision.

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