Research article

Chloride equilibrium potential in salamander cones
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Abstract

Background: GABAergic inhibition and effects of intracellular chloride ions on calcium channel activity have been proposed to regulate neurotransmission from photoreceptors. To assess the impact of these and other chloride-dependent mechanisms on release from cones, the chloride equilibrium potential (ECl) was determined in red-sensitive, large single cones from the tiger salamander retinal slice.

Results: Whole cell recordings were done using gramicidin perforated patch techniques to maintain endogenous Cl- levels. Membrane potentials were corrected for liquid junction potentials. Cone resting potentials were found to average -46 mV. To measure ECl, we applied long depolarizing steps to activate the calcium-activated chloride current (ICl(Ca)) and then determined the reversal potential for the current component that was inhibited by the Cl- channel blocker, niflumic acid. With this method, ECl was found to average -46 mV. In a complementary approach, we used a Cl-sensitive dye, MEQ, to measure the Cl- flux produced by depolarization with elevated concentrations of K+. The membrane potentials produced by the various high K+ solutions were measured in separate current clamp experiments. Consistent with electrophysiological experiments, MEQ fluorescence measurements indicated that ECl was below -36 mV.

Conclusions: The results of this study indicate that ECl is close to the dark resting potential. This will minimize the impact of chloride-dependent presynaptic mechanisms in cone terminals involving GABA_A receptors, glutamate transporters and ICl(Ca).

Background

Regulation of intracellular chloride levels results in a chloride equilibrium potential (ECl) that is hyperpolarized with respect to the resting potential in many nerve cells, but depolarized in others [1-5]. For example, ECl in salamander rod photoreceptors is 25 mV more positive than the dark resting potential [6]. The resting potential of cone photoreceptors in darkness is around -42 to -47 mV and estimates of ECl in cones have ranged from -65 mV to -36 mV [7-11]. Cone photoreceptors possess a number of Cl-conductances that help to shape their responses and synaptic output. As discussed below, the value of ECl in cones is an important parameter for determining the strength and polarity of these effects.

It has been suggested GABA_A receptors in the terminals of cones may mediate inhibitory synaptic feedback from horizontal cells to cones [8]. Under this hypothesis, the light-evoked hyperpolarization of horizontal cells causes a cessation of GABA release and this disinhibition leads to a "feedback depolarization" in cones. There is evidence both for [e.g., [8]] and against [e.g., [12,13]; see review in
ref. [14]) this hypothesis. However, one prediction of the hypothesis is that the Cl equilibrium potential (ECl) must be negative to the resting potential in order for GABA disinhibition to depolarize a cone.

Cone photoreceptors have presynaptic glutamate transporters that are coupled to Cl channels [21-23]. The transporters in cones have been shown to respond to glutamate released from their own terminals [24]. Whether synaptically released glutamate causes cones to hyperpolarize or depolarize depends on ECl. Furthermore, analogous to the negative feedback from ICl(Ca) onto ICa described above, the chloride current produced by activation of glutamate transporters in rods can cause a Cl· efflux that inhibits ICa [25]. As with the feedback between ICl(Ca) and ICa, the strength and polarity of this potential interaction in cones depends on ECl.

Given the importance of ECl in determining the impact of various feedback mechanisms in the photoreceptor terminal, we determined ECl in cone photoreceptors of the salamander retina using a combination of imaging with a chloride-sensitive dye and electrophysiological approaches.

Results

In control superfusate, dark resting potentials of cones from slices prepared under visible light averaged -46.0 ± 2.00 mV (n = 9) after correcting for the liquid junction potential. This is nearly identical to the dark resting potentials of salamander cones prepared under infrared illumination (-46.8 ± 2.03 mV, n = 18).

To measure ECl, ICl(Ca) was recorded using gramicidin perforated patch whole cell recordings and activated by applying a 500 ms step from -78 to -8 mV. This depolarizing step typically evoked a sustained inward tail current arising largely from activation of ICl(Ca) [19]. Only cells that exhibited an inward tail current were used for analysis. As shown in the example of Fig. 1A, the current/voltage relationship of a cone cell was assessed during the tail current by using a ramp voltage protocol (1 mV/ms from -98 to +52 mV) begun 25 ms after the end of the depolarizing step. The same protocol was then repeated after applying niflumic acid (0.1 mM; Fig. 1B). At this concentration, niflumic acid is a selective inhibitor of ICl(Ca) in cones [19]; niflumic acid may not be as selective in rods: [20,26]]. Subtracting the control ramp-evoked current from that obtained in the presence of niflumic acid yields the current/voltage profile for ICl(Ca) (Fig. 1C). In the example shown in Fig. 1C, the difference current reversed around -46 mV. The reversal potential of the niflumic acid-sensitive difference current determined from 8 cones averaged -45.5 ± 2.5 mV. As a control for the possible perturbation of intracellular Cl by possible patch rupture, we repeated the same experiment using a pipette solution with only 3.5 mM Cl-. ECl was not significantly different when measured using the low Cl· pipette solution (-50.4 mV ± 3.4 mV; n = 7; p = 0.49, unpaired t-test). If patch rupture had occurred, ECl would be expected to attain -89 mV with the low Cl· pipette solution and -20 mV with the original pipette solution.

Bath application of GABA evoked small reversible inward currents that averaged -3.4 ± 0.3 pA at the holding potential of -78 mV (not shown). The small size of these currents may be due to receptor desensitization [27]. Consistent with results obtained from measurements of ICl(Ca)-difference currents calculated from ramps applied before and during GABA application indicate that the GABA-evoked current reversed at -46.4 ± 2.7 mV (n = 9).

In a complementary approach for measuring ECl, we used a Cl-sensitive dye, MEQ, to examine the Cl· flux that accompanied cone depolarization evoked by bath application of various high K+ solutions (12, 22, 31, 41, 50 and 70 mM K+). In a separate set of experiments, we used gramicidin-perforated patch recording methods to measure the membrane potentials produced in cones by application of the different high K+ solutions. Slices used for
MEQ experiments and for measurement of membrane potentials in different solutions were prepared using similar techniques under visible illumination; control experiments showed that the fluorescent illumination used during MEQ experiments did not produce any further changes in the cone resting membrane potential \( (n = 3) \). An example of a retinal slice loaded with MEQ is shown in Fig. 2A. Measurements of MEQ fluorescence were made from the cone soma (circle, Fig. 2A). For a single wavelength dye such as MEQ, the change in fluorescence relative to basal fluorescence \( (\Delta F/F) \) can be used as a measure of the change in ion concentration \( [28] \). In the cone in Fig. 2B, bath application of 12 mM K\(^+\), which depolarized cones to -36 mV, produced a 1.2% decrease in MEQ fluorescence. Since MEQ fluorescence is quenched by Cl\(^-\) ions this indicates that depolarization to -36 mV stimulated an influx of Cl\(^-\) ions. Application of a solution with 70 mM K\(^+\), which depolarizes cones to -7 mV, produced a greater influx of Cl\(^-\) as evidenced by the 10% decrease in MEQ fluorescence seen in a different cone (Fig. 2C). Fig. 2D shows the average change in \( \Delta F/F \times 100 \) plotted as a function of the membrane potential evoked by the different high K\(^+\) solutions. The finding that 12 mM K\(^+\) consistently stimulated an influx of Cl\(^-\) indicates that the reversal potential must be below -36 mV.

**Discussion**

The main finding of this study is that \( E_{Cl} \) in salamander cones is close to the dark resting potential (~-46 mV). \( E_{Cl} \) was found to be -46 mV from block of \( I_{Cl(Ca)} \) by niflumic acid; small GABA-evoked currents reversed around the same potential. MEQ fluorescence changes produced by depolarization support these electrophysiological measurements by indicating that \( E_{Cl} \) is below -36 mV.

There can be local variations of \( E_{Cl} \) within cells \([4]\). Large single cones in the salamander retina do not have a distinct axon and terminal; synaptic proteins are instead located at the base of the soma \([29]\). MEQ measurements were made in the cell soma from a region adjacent to the synaptic ending (see Fig. 2). \( I_{Cl(Ca)} \) is localized to the terminal region in rods \([30]\) and these channels are probably also localized to the terminals of cones. Thus, the measurements in the present study are likely to provide estimates of \( E_{Cl} \) in the synaptic terminal and adjacent regions of the cone cell. Measurements of intracellular Cl\(^-\) levels suggest that \( E_{Cl} \) in the inner segment is not significantly different from that measured in the soma \([11]\).

The finding that the Cl\(^-\) equilibrium potential is close to the resting potential does not necessarily mean that Cl\(^-\) is passively distributed. Electrophysiological experiments required that cells be voltage clamped at -70 mV for many minutes. Nonetheless, the value of \( E_{Cl} \) determined from these electrophysiological experiments in which cells were
voltage clamped at -70 mV was similar to the value estimated from MEQ studies in which cells were not voltage clamped and thus at their resting membrane potential. Results from experiments on the prolonged depolarization in cones also suggest that $E_{Cl}$ can be maintained indefinitely at a value above the membrane potential. The plateau phase of the prolonged depolarization, which largely reflects $I_{Cl(Ca)}$ activation [9,19], could remain above the membrane potential established by an adapting background for hours [9]. The ability of cones to maintain $E_{Cl}$ above the membrane potential may arise from activity of the Na/KCl cotransporter as shown in rods (20) as well as from other mechanisms (e.g., CLC-2) [2,31].

**Figure 2**
Intracellular chloride measurements. A. Example of a retinal slice fluorescently stained with the Cl\(^-\) sensitive dye MEQ. The circle indicates the somatic region of a cone in which MEQ measurements were made. B. An example of the reduction in MEQ fluorescence, indicating an increase in intracellular [Cl\(^-\)], produced by application of 12.1 mM K\(^+\) to a cone. C. A greater decrease in MEQ fluorescence was produced by application of 69.9 mM K\(^+\). Different cone from panel B. D. MEQ fluorescence changes produced by bath application of superfusate containing 12.1, 21.6, 31.2, 40.7, 50.3, or 69.9 mM [K\(^+\)]. After correcting for liquid junction potentials, these high K\(^+\) solutions depolarized cones to -35.3 ± 2.90, -26.3 ± 2.82, -20.6 ± 2.82, -15.4 ± 2.84, -11.6 ± 3.05, and -7.4 ± 3.25 mV (n = 9), respectively, determined in current clamp recordings using gramicidin-perforated patch recording techniques. The change in MEQ fluorescence relative to basal fluorescence ($\Delta F/F^*100$) is plotted against the cone membrane potential determined with each high K\(^+\) solution.
**Comparisons with other studies**

$E_{Cl}$ in cones has been estimated in a number of previous studies. The most positive value for $E_{Cl}$ of -36 mV comes from calibration of MEQ fluorescence levels to determine the resting intracellular Cl- concentrations in cones isolated from the salamander retina (11). However, these measurements showed a large variability (range of S.E.M.: -26.5 to -46.6 mV). The most negative estimate of $E_{Cl}$ comes from a study by Attwell et al [7] showing that the sign-reversing pathway from rods to cones reversed around -65 mV. Based on the presumption that this pathway involved disinhibition of GABAergic inputs into cones, this study has been interpreted as suggesting that $E_{Cl}$ is around -65 mV. However, more recent evidence questions whether the horizontal cell to cone feedback pathway thought to underlie this sign-reversing pathway from rods to cones is truly GABAergic [9,13,14]. Other studies have arrived at values for $E_{Cl}$ similar to those found in the present study. 1) By examining the polarity of GABA-evoked currents after patch rupture with either 12 or 24 mM Cl- in the recording pipette, Kaneko and Tachibana [8] estimated $E_{Cl}$ to be around -47 mV in isolated turtle cones. 2) Based on the membrane potential attained by the plateau phase of the prolonged depolarization in turtle cones from the eyecup slice preparation, $E_{Cl}$ was estimated to be at or slightly above the dark resting potential of -42 mV [after correction for a liquid junction potential of -2 mV; ref. [9]]. 3) In a single recording from a salamander cone obtained with a Cl-sensitive electrode, Miller and Dacheux [32] found that $E_{Cl}$ was 2 mV more positive than the dark resting potential. 4) A slightly more negative value for $E_{Cl}$ was found in ruptured patch recordings from goldfish cones by examining the voltage dependence of the $I_{Cl(Ca)}$ tail current [10]. By extrapolating measurements back to the time of patch rupture, Kraaij et al [10] concluded that $E_{Cl}$ was approximately -55 mV.

**Functional implications**

$I_{Ca}$ in cones, like that of rods, can be inhibited by lowering extracellular Cl- [33]. The inhibition of $I_{Ca}$ produced by lowering extracellular Cl- appears to result from a reduction in intracellular Cl- which in turn causes a reduction in the open probability of single Ca$^{2+}$ channels [11]. In rods, where $E_{Cl}$ is positive to the resting potential, activation of Cl channels leads to a Cl efflux thereby producing an inhibition of Ca$^{2+}$ channels [6,11,20]. The present results indicate that activation of Cl channels when the cell is at its resting potential would produce minimal changes in intracellular Cl- in cones. Therefore, the feedback between $I_{Ca}$ and $I_{Cl(Ca)}$ postulated for rod photoreceptors [6,20] would be expected to be minimal in cones in darkness.

Another implication of the finding that $E_{Cl}$ is close to the dark resting potential is that the stimulation of Cl channels associated with glutamate transporters by glutamate released from cone terminals [24] would tend to stabilize the cell membrane potential near the dark potential. In rods, the Cl efflux accompanying activation of glutamate transporters appears to contribute to a glutamate-mediated inhibition of $I_{Ca}$ [25]. As with the feedback between $I_{Ca}$ and $I_{Cl(Ca)}$ considered in the previous paragraph, the finding that $E_{Cl}$ is near the resting potential leads to the prediction that in darkness there would be no Cl efflux accompanying glutamate transporter activation and therefore glutamate would not be expected to inhibit $I_{Ca}$.

Cones hyperpolarize to light, although with prolonged illumination the membrane potential recovers to near the dark resting potential. The impact of chloride-dependent negative feedback between $I_{Cl(Ca)}$ and $I_{Ca}$ or the glutamate transporter chloride current and $I_{Ca}$ would be expected to increase as a cone hyperpolarizes in response to light. By reducing glutamate release, these chloride-dependent negative feedback mechanisms might thus contribute to making post-synaptic responses more transient.

The finding that $E_{Cl}$ is near the resting potential of cones indicates that GABAergic disinhibition near the dark potential should produce little membrane potential change. This result is inconsistent with the postulated role for GABA in generating the feedback depolarization [8] and supports other studies suggesting that GABA is not directly responsible for horizontal to cone feedback [9,13,14].

**Conclusions**

Electrophysiological measurements, supported by experiments using chloride-sensitive dyes, indicate that $E_{Cl}$ in salamander cones is close to the dark resting membrane potential. By minimizing the trans-membrane flux of chloride, this will minimize the presynaptic impact of GABA$_r$ receptors, $I_{Cl(Ca)}$, and glutamate transporter chloride channels.

**Methods**

**Tissue preparation**

$E_{Cl}$ is positive to the resting potential of many neurons in the immature brain [5]. Based on their size, the neotenous tiger salamanders (*Ambystoma tigrinum*, 15–25 cm) used in these experiments are thought to be 2–7 years old out of a life span of ~12 years (34).

Salamanders were handled humanely in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center. Chilled salamanders were rapidly decapitated, an eye was enucleated, and the front of the eye was removed. The resulting eyecup was cut into three or four pieces and a single piece was placed vitreal surface down onto a piece of filter paper (2 × 5 mm, Millipore type AAWP, 0.8 µm
pores). After adhering to the filter paper, the retina was isolated under chilled amphibian superfusate and cut into 125 µm slices using a razor blade tissue chatter (Stoelting Co., Wood Dale, IL). The slices were rotated 90° to view the retinal layers when placed under a water immersion objective (60X, 1.0 NA) on an upright fixed stage microscope (EF 600, Nikon Inc., USA). Slices were prepared under visible light but recordings were performed in darkness. All experiments were done using red-sensitive large single cones selected by anatomical criteria [35].

**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-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and 5 glucose (pH 7.8). The osmolality was measured with a vapor pressure osmometer (Wescor, Logan, UT) and adjusted, if necessary, to 242 ± 5 mOsm. For high K⁺ solutions, various quantities of NaCl were replaced with equimolar KCl. Niflumic acid was diluted (1:10,000) from DMSO stock solutions. Unless otherwise specified, chemicals were obtained from Sigma/Aldrich/RBI (St. Louis, MO).

**Electrophysiology**

Patch pipettes were pulled on a PP-830 vertical puller (Narishige USA, New York) from borosilicate glass pipettes (1.2 mm O.D., 0.95 mm I.D., with internal filament) and had tips of ~1 µm outer diameter with resistances of 10 to 15 MΩ. To maintain endogenous levels of intracellular Cl⁻, we obtained perforated patch whole cell recordings using the cation channel, gramicidin [36]. 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 potentials, the pipette electrolyte solution contained (in mM): 54 KCl, 61.5 KCH₃SO₃, 10 HEPES. The pH was adjusted to 7.2 with KOH. The liquid junction potential (LJP) of this solution was estimated to be -7 mV using the junction potential calculator of PClamp (Axon Instruments). Membrane potential values reported throughout this manuscript were corrected for the LJP. For experiments with niflumic acid or GABA, pipettes were typically filled with a solution containing (in mM): 54 CsCl, 61.5 CsCH₃SO₃, 3.5 NaCH₃SO₄, 10 HEPES (LJP = -8 mV). In some experiments, a low Cl⁻ pipette solution was used containing: 115.5 mM CsCH₃SO₃, 3.5 NaCl, 10 HEPES (LJP = -10 mV). The pH of both solutions was adjusted to 7.2 with CsOH. The osmolality of pipette solutions were also adjusted, if necessary, to 242 ± 5 mOsm. Recordings were made using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA) and PClamp 8 software (Axon Instruments). Cell input resistance calculated using a step from -70 to -90 mV averaged 695 ± 111 MΩ. Access resistance estimated from the peak of the capacitative transient averaged 30.7 ± 4.8 MΩ (n = 24).

**Imaging experiments**

Digital fluorescent images were obtained with a cooled CCD camera (SensiCam, Cooke Corp., Auburn Hills, MI). Axon Imaging Workbench (AIW 2.2, Axon Instruments Inc., Union City, CA) was used to control the camera, filter wheel, and image acquisition. Pixel binning (2 x 2) of the images was used to decrease acquisition time to ≤1 s. Images were acquired at 5 to 10 s intervals during experimental trials.

For measurements of [Cl⁻], we used the dye, 6-methoxy-N-ethylquinolinolium iodide (MEQ, Molecular Probes, Eugene, OR) [37]. 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 [38]. 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. The slow exponential decay in MEQ fluorescence due to dye leakage and bleaching was determined from a 3 min. series of control measurements prior to drug application and subtracted before analysis [11,20]. Variance is reported as ± S.E.M.

**Authors’ contributions**

WT conceived the study and drafted the manuscript. WT and EB participated in all aspects of the experiments but most recordings were performed by EB. Both authors have read and approved the manuscript.

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