Location of release sites and calcium-activated chloride channels relative to calcium channels at the photoreceptor ribbon synapse.

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Abstract

Vesicle release from photoreceptor ribbon synapses is regulated by L-type Ca$^{2+}$ channels which are in turn regulated by Cl$^{-}$ moving through calcium-activated chloride (Cl(Ca)) channels. We assessed the proximity of Ca$^{2+}$ channels to release sites and Cl(Ca) channels in synaptic terminals of salamander photoreceptors by comparing fast (BAPTA) and slow (EGTA) intracellular Ca$^{2+}$ buffers. BAPTA did not fully block synaptic release, indicating some release sites are <100 nm from Ca$^{2+}$ channels. Comparing Cl(Ca) currents with predicted Ca$^{2+}$ diffusion profiles suggested that Cl(Ca) and Ca$^{2+}$ channels average a few hundred nanometers apart, but the inability of BAPTA to block Cl(Ca) currents completely suggested some channels are much closer together. Diffuse immunolabeling of terminals with an antibody to the putative Cl(Ca) channel TMEM16A supports the idea that Cl(Ca) channels are dispersed throughout the presynaptic terminal, in contrast with clustering of Ca$^{2+}$ channels near ribbons. Cl(Ca) currents evoked by [Ca$^{2+}$], elevation through flash photolysis of DM-nitrophen exhibited EC$_{50}$ values of 556 and 377 nM with Hill slopes of 1.8 and 2.4 in rods and cones, respectively. These relationships were used to estimate average submembrane [Ca$^{2+}$], in photoreceptor terminals. Consistent with control of exocytosis by [Ca$^{2+}$] nanodomains near Ca$^{2+}$ channels, average sub-membrane [Ca$^{2+}$] remained below vesicle release threshold (~400 nM) over much of the physiological voltage range for cones. Positioning Ca$^{2+}$ channels near release sites may improve fidelity in converting voltage changes to synaptic release. A diffuse distribution of Cl(Ca) channels may allow Ca$^{2+}$ influx at one site to influence relatively distant Ca$^{2+}$ channels.
Introduction

Visual responses originating in photoreceptor outer segments are transmitted to the rest of the visual system by altering synaptic release from the terminals of rods and cones. Synaptic vesicles are tethered near the active zone at a plate-like structure known as the ribbon (Schmitz 2009). Glutamate release from photoreceptor synapses requires only sub-micromolar levels of Ca\(^{2+}\), much lower than Ca\(^{2+}\) levels typically required for vesicle release at other synapses (Heidelberger et al. 1994; Rieke and Schwartz 1996; Schneggenburger and Neher 2000; Beutner et al. 2001; Bollmann et al. 2000; Thoreson et al. 2004). Therefore, synaptic release from photoreceptors does not necessitate the high levels of Ca\(^{2+}\) that are typically found only in nanodomains immediately adjacent to Ca\(^{2+}\) channels. Nevertheless, L-type Ca\(^{2+}\) channels which mediate vesicle release from photoreceptors are clustered in the terminal (Nachman-Clewner et al. 1999; Morgans 2001; Morgans et al. 2005; Steele et al. 2005; Xu and Slaughter 2005; Specht et al. 2009) beneath synaptic ribbons (tom Dieck et al. 2005) suggesting that release sites are quite close to Ca\(^{2+}\) channels. However, it is also possible that synaptic release from photoreceptors might occur at ectopic sites located some distance from the ribbon, as occurs at bipolar cell ribbon synapses (Zenisek et al. 2003; Zenisek et al. 2008; Midorikawa et al. 2007).

In addition to stimulating vesicle release, Ca\(^{2+}\) influx stimulates Ca\(^{2+}\)-activated chloride (Cl(Ca)) channels localized to photoreceptor terminals (Barnes and Hille 1989; MacLeish and Nurse 2007; Cia et al. 2004). In cones, where the reversal potential of chloride (E\(_{Cl}\)) is -38mV (Thoreson and Bryson 2004), activation of Cl(Ca) channels will tend to stabilize the dark membrane potential. In rods, where E\(_{Cl}\) is -20 mV (Thoreson et
al. 2002), activation of Cl(Ca) channels promotes membrane depolarization. Although depolarization enhances Ca\textsuperscript{2+} channel activity, the dominant effect of this Cl\textsuperscript{-} efflux in rods appears to be an inhibition of Ca\textsuperscript{2+} channel activity by actions mediated at intracellular anion binding sites on Ca\textsuperscript{2+} channels (Thoreson et al. 2000; Babai et al. 2010). In this way, Cl\textsuperscript{-} efflux can act as a negative feedback mechanism to limit excessive Ca\textsuperscript{2+} entry into rods. The strength of feedback interactions between Ca\textsuperscript{2+} and Cl(Ca) channels will be influenced by the distance between the two types of channels.

This study analyzed the spatial relationships between Ca\textsuperscript{2+} channels, vesicle release sites, and Cl(Ca) channels in photoreceptors by comparing electrophysiological measurements to profiles of Ca\textsuperscript{2+}-diffusion predicted from a model developed by Ward and Kenyon (2000). The results indicate an extremely tight coupling between Ca\textsuperscript{2+} channels and ribbon release sites in cones, while Cl(Ca) channels are more dispersed throughout the synaptic terminals of rods and cones. Consistent with physiological data, antibodies to the putative Cl(Ca) channel, TMEM16A, produce more diffuse immunostaining in photoreceptor terminals than antibodies to synaptic Ca\textsuperscript{2+} channels. Similar to the use of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels as submembrane Ca\textsuperscript{2+} sensors (Roberts et al. 1990; Burrone et al. 2002), we also measured the Ca\textsuperscript{2+}-dependence of Cl(Ca) channels and used this relationship to convert Cl(Ca) current measurements into average submembrane Ca\textsuperscript{2+} levels in rod and cone terminals.
Materials and Methods

Retinal Tissue Preparation: Experiments were performed using aquatic-phase tiger salamanders (Ambystoma tigrinum, 18-25 cm, Kons Direct, Germantown, WI or Charles Sullivan Co., Nashville, TN). Care, handling and experimentation procedures were approved by the University Of Nebraska Medical Center Institute for Animal Care and Use Committee or the UCLA Animal Research Committee. Animals were decapitated and pithed, and each eye was enucleated. After surgical removal of the cornea, iris and lens, the resultant eyecup was quartered. Eyecup pieces were placed vitreous side down onto filter paper (2 x 5 mm, AAWP; 0.8 μm pores; Millipore, Bedford, MA) and the retina was isolated in a cold amphibian saline solution containing (in mM): 111 NaCl, 2.5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 10 HEPES, 5 glucose (pH 7.8). Tissue was sliced into 125 μm thick sections using a razor-blade (#121-6, Ted Pella Inc., Redding, CA) tissue slicer (Stoelting, Wood Dale, IL). Slices were placed in a recording chamber for viewing on an upright fixed-stage microscope (E600FN [Nikon, Tokyo, Japan]) fitted with a 60X, 1.0 NA water immersion objective (Nikon).

Calculation of Free Ca^{2+} Profiles: To model profiles of free Ca^{2+}, we used an Excel-based macro program to calculate Ca^{2+} diffusion away from a voltage-gated L-type Ca^{2+} channel in the presence of diffusible buffers (Ward and Kenyon 2000; online at http://www.medicine.nevada.edu/physio/docs/default.htm). Parameters for the simulation are provided in Table 1.
**Electrophysiology:** Patch pipettes were pulled on a PP-830 vertical puller (Narishige USA, East Meadow, NY) from borosilicate glass pipettes (1.2 mm O.D., 0.9 mm I.D., with internal filament, World Precision Instruments, Sarasota, FL) and had tips <2 μm in diameter with resistance values ranging between 12 and 18 MΩ. For Ca²⁺ buffering experiments, we used a pipette solution containing (in mM): 42 CsCl, 48 CsGluconate, 1.9 MgCl₂, 32.9 HEPES, 9.4 TEACl, 9.4 MgATP, 0.5 GTP, plus 0.5 EGTA, 5 EGTA, or 5 BAPTA (pH 7.2). For measurements of depolarization-evoked tail current amplitudes illustrated in Figures 8 and 9, we used a pipette solution with a predicted Eₐ of -39 mV that consisted of (in mM): 11.3 CsCl, 75 Cs Gluconate, 1.9 MgCl₂, 32.9 HEPES, 9.4 TEACl, 9.4 MgATP, 0.5 GTP, 5 EGTA (pH 7.2). We adjusted membrane potentials with this solution for a measured liquid junction potential of -7 mV. Recording electrodes were positioned with Huxley-Wall micromanipulators (Sutter Instruments, Novato, CA) and visualized through the eyepieces or with a video camera (Watec 502H, Rock House Products, New York, NY) mounted on the microscope. Photoreceptors were voltage clamped using a Multiclamp or Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Currents were acquired using a Digidata 1322 interface and pClamp 9.2 software (Molecular Devices). In all experiments, retinal slices were superfused with the amphibian saline solution described above bubbled with 100% O₂.

**Flash Photolysis of DM-Nitrophen:** Flash photolysis of the photolysable Ca²⁺ chelator, DM-nitrophen (Invitrogen, Carlsbad, CA), produces rapid and spatially homogenous increases in [Ca²⁺] throughout the terminal. [Ca²⁺], measured at the center
of the terminal therefore provides a measure of the change in submembrane \([Ca^{2+}]\). The pipette solution for photolysis experiments consisted of (in mM): 10 DM-nitrophen, 5 CaCl\(_2\), 4 DPTA, 4 MgCl\(_2\), 26 Cs Gluconate, 78 HEPES, 6.5 TEACl, 11 Na\(_2\)ATP, 0.5 GTP (pH 7.2). DM-nitrophen was photolyzed by flashes of UV light derived from a Xenon arc flash lamp (Rapp Optoelectronic, Hamburg, Germany). Oregon Green BAPTA 6F (OGB-6F, 0.5 mM, \(K_d = 3 \mu M\), Invitrogen) was also included in the pipette solution to measure \([Ca^{2+}]_i\). Confocal \(Ca^{2+}\) measurements were made using a laser confocal scanhead (UltraVIEW Live Cell Imaging System, Perkin Elmer, Waltham, MA) equipped with a cooled CCD camera (Orca ER, Hamamatsu, Hamamatsu City, Japan) mounted to a fixed stage upright microscope (E600 FN). Images were acquired at 60-150 ms intervals with single frame durations of 48-145 ms and pixel values were binned 2 x 2. To convert OGB-6F fluorescence measurements into \(Ca^{2+}\) levels, we used the following formula (Helmchen 2000):

\[
\Delta [Ca^{2+}]_i = ([Ca^{2+}]_{rest} + K_d (\Delta F/F)/(\Delta F/F_{max})) / (1-(\Delta F/F)/(\Delta F/F)_{max}).
\]

\(\Delta F/F\) represents the fractional change in fluorescence resulting from stimulation. \((\Delta F/F)_{max}\) was determined from the maximal fluorescence change produced by application of 500 ms depolarizing steps to -10 mV. We used the \(K_d\) of 3 \(\mu M\) for OGB-6F provided by Invitrogen. The resting \(Ca^{2+}\) concentration (\([Ca^{2+}]_{rest}\)) for each solution was determined ratiometrically using 0.2 mM Fura-2 as described previously (Thoreson et al. 2004). As a test of \(Ca^{2+}\) measurements obtained with Equation 1, we compared OGB-6F measurements with measurements made using a higher affinity dye, OGB-1 (\(K_d = 0.17\ \mu M\)). We evoked intraterminal calcium changes by applying depolarizing test steps (-70 to -10 mV) of varying duration (50-500 ms). Despite large differences in the \(K_d\), the
same depolarizing stimuli yielded similar intraterminal \([\text{Ca}^{2+}]\) measurements with the two different dyes (Choi et al. 2008).

The increase in Cl(Ca) current as a function of increasing \(\text{Ca}^{2+}\) was fit with a sigmoidal binding curve:

\[
\text{Equation 2: } I = \frac{I_{\text{max}}}{1 + 10^{\left(\log EC_{50} - \log [\text{Ca}^{2+}]\right)h}}
\]

where \(h\) = slope factor and \(I_{\text{max}}\) = the top of the sigmoidal curve. The bottom value was constrained to zero.

**Immunohistochemistry:** Salamanders were placed in an induction tank (~10L) containing MS-222 (2 gm/L). Following induction, the animal was removed, decapitated with large shears and then the brain and spinal cord rapidly pithed. After enucleation, each eye was opened along the ora serrata, and the cornea, lens, and vitreous body were removed and the eyecups were immersion-fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) for 15 min at room temperature. The eyecups were then cryoprotected in 25% sucrose overnight at 4°C. Prior to cutting the tissue with a cryostat the retina was washed with 0.1 M PB and embedded in Tissue-Tek OCT compound (Sakura Finetek Inc., Torrance, CA) and rapidly frozen with dry ice or liquid nitrogen. Cryostat sections of the retina were cut at 12-15 \(\mu\)m, mounted onto gelatin-coated slides, air dried, and stored at -20°C.

All tissue was labeled using the indirect immunofluorescence technique (Stella et al. 2007). Briefly, retinal sections were warmed for 10 min at 37°C. Tissue was preincubated in a 0.1 M PB mixture containing 10% normal goat serum (NGS) or donkey serum (DS) (Invitrogen), 1% bovine serum albumin (BSA) (Sigma-Aldrich, St.
Louis, MO), and 0.5% Triton-X 100 (Sigma-Aldrich) for 1 hour. The sections were then incubated in primary antibodies overnight at 4°C, which were all diluted in 0.1 M PB (pH 7.4) containing 3% NGS or DS, 1% BSA and 0.5% Triton-X 100. The primary antibody/antigen complex was detected using secondary antibodies conjugated to either Alexa 488 or Alexa 568 (Invitrogen, Carlsbad, CA). Following incubation with the antibody, retinal sections were washed three times for 10 min with 0.1 M PB to remove any unbound primary or secondary antibody. For double labeling experiments tissue sections were incubated in a mixture of primary antibodies followed by a mixture of secondary antibodies. All slides were allowed to air dry in the dark at room temperature and cover-slipped with Prolong Gold anti-fade (Invitrogen).

Images of retinal sections were acquired using a Zeiss Laser Scanning Microscope 510 META (Zeiss, Thornwood, NY) with a C-Apochromat 40X 1.2 NA water objective. To identify fluorescent signals, different lasers were used for excitation: for Alexa-488 the 488 nm argon laser line was used and for Alexa-568 the 543 nm HeNe laser line was used. Little or no staining was observed when using the secondary antibodies alone. During acquisition of signals from double-labeled specimens, scans were collected sequentially to prevent spectral bleed-through. Specific band-pass filters were used to achieve proper separation of signals (for double labeling 488/505-530, 543/560LP). In some scans, fluorescent emissions were further separated using a linear unmixing algorithm (Zeiss LSM ver. 4.2, Thornwood, NY). To validate this process and reduce any potential mismatch or bleed-through of fluorescence among different channels, separation of fluorescent emissions was achieved by processing reference samples that were immunostained with a single label to visualize their entire emission.
spectra and localization within the retina. Most images were acquired at a 12 bit resolution of 2048 X 2048, and in some cases 1024 X 1024. To increase signal-to-noise ratio, images were averaged online (e.g. n=4) and the scan speed and photo multiplier detector gain were decreased. The digital fluorescent images were single confocal scans taken in the same planes as corresponding DIC images. Most digital images were acquired at an approximate optical thickness of 0.5 μm or 1.0 Airy units. Digital images were saved as Zeiss .LSM files and final publication quality images were exported in .TIFF format at 300 dpi. Images were processed and adjusted for brightness and contrast using Adobe Photoshop CS4 Extended (Adobe Systems Inc., Mountain View, CA).

**Antibodies:** A rabbit polyclonal antibody to TMEM16A raised against a 620 amino acid peptide was used at a dilution of 1:500 (ab53212; Abcam, Cambridge, MA). This antibody has been shown to react with both human and rodent sequences (manufacturer's data sheet). We also used another rabbit polyclonal antibody raised against a 17 amino acid segment on the N terminus (1:100, SIG5419, Zyagen Inc., San Diego, CA). A mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) was used at a dilution of 1:1000 (Catalog No. CH 22102; Neuromics, Edina, MN) to identify Müller cells in the salamander retina (Sassoè Poggetto et al. 1992). A sheep polyclonal antibody raised against amino acids 712 to 730 of the human α1F calcium channel pore forming subunit (a generous gift from Dr. Catherine Morgans, OHSU, Portland, OR) was used at a dilution of 1:100 to label calcium channels on terminals of photoreceptors (Morgans 2001). Staining in the retina with this antibody was blocked by
the peptide used to develop the antibody (Morgans 2001). A monoclonal antibody raised against the synaptic vesicle protein, SV2 (Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA), was used at a dilution of 1:2000 to label synaptic terminals of photoreceptors in the tiger salamander retina (Mandell et al. 1990; Yang et al. 2002; Zhang and Wu 2009).

Unless otherwise specified, chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The criterion for statistical significance was chosen to be $p < 0.05$ and evaluated using Student’s T-test or one-way ANOVA and GraphPad Prism 4.0. Variability is reported as $\pm$ SEM.
Results

Distance from Ca\textsuperscript{2+} channels to release sites

Ca\textsuperscript{2+} channels are clustered in photoreceptor synaptic terminals (Nachman-Clewner et al. 1999; Morgans 2001; Morgans et al. 2005; Xu and Slaughter 2005; Specht et al. 2009) at the base of the ribbon (tom Dieck et al. 2005), suggesting that release sites are likely to be very close to Ca\textsuperscript{2+} channels. We analyzed the distance between Ca\textsuperscript{2+} channels and vesicle release sites by comparing the effects of different diffusible buffers (EGTA or BAPTA) on synaptic release from cones. BAPTA and EGTA have a similar high affinity for Ca\textsuperscript{2+} but BAPTA has a much faster K\textsubscript{ON} rate, so it chelates Ca\textsuperscript{2+} very rapidly near the mouth of a Ca\textsuperscript{2+} channel. Buffers were introduced into voltage-clamped cones through the patch pipette. To examine effects of the different Ca\textsuperscript{2+} buffers on synaptic release, cones were depolarized from -70 mV to -10 mV for 500 ms, and PSCs were recorded simultaneously from voltage-clamped horizontal or OFF bipolar cells. Recordings were conducted in the presence of cyclothiazide (0.1 mM) to block AMPA receptor desensitization. We waited at least 10 min to allow time for diffusion of the Ca\textsuperscript{2+} buffer to the photoreceptor terminal. Effects of the different buffers on cone-driven PSCs averaged from multiple cell pairs are illustrated in Fig. 1A (0.5 mM EGTA, N=10; 5 mM EGTA, N=12; 5 mM BAPTA, N=7). Cone-driven PSC waveforms were essentially unchanged by elevating the EGTA concentration from 0.5 (dashed line) to 5 mM (black solid line) and diminished, but not fully blocked, by the use of 5 mM BAPTA (gray solid line). EPSCs evoked with shorter 25 ms test pulses were also not blocked by BAPTA (comparison of charge transfer during EPSCs with 0.5 EGTA, 5 EGTA, and 5 BAPTA: P=0.25, one-way ANOVA),
indicating that the persistence of synaptic responses was probably not due to saturation of BAPTA during the 500 ms depolarizing step. Sustained components of the cone-driven PSC were also not blocked by BAPTA, consistent with the hypothesis that both transient and sustained release from cones occurs principally at the ribbon (Jackman et al. 2009).

We also tested effects of these chelators in rods. Like cones, BAPTA did not block PSCs evoked in horizontal cells by test steps applied to voltage-clamped rods (data not shown). However, the persistence of rod-driven PSCs is more difficult to interpret since rod-driven PSCs are at least partly due to synaptic output from neighboring rods coupled to the voltage-clamped cell through gap junctions (Attwell et al. 1984; Zhang and Wu 2004; Cadetti et al. 2005).

We confirmed that Ca^{2+} buffers could diffuse to the synaptic terminal by comparing intraterminal Ca^{2+} levels measured with OGB-6F fluorescence in rods 5-10 min after patch rupture (Supplemental Fig. 1). The rise in intraterminal Ca^{2+} evoked by a 100 ms depolarization from -70 to -10 mV declined after the step much more rapidly when the patch pipette contained 5 mM EGTA (τ=0.22 s, N=6) or BAPTA (τ=0.19 s, N=5) than with 0.5 mM EGTA (τ=51 s, N=6). Although the time course of decline is influenced by extrusion, it is dominated by Ca^{2+} binding to the buffer so these results show that buffers can diffuse through the axon to the rod terminal. Cone pedicles do not possess a thin axon and are thus more accessible to buffers introduced through a patch pipette on the cell body (Sherry et al. 1998; Mariani 1986).

The profiles of [Ca^{2+}] surrounding individual open Ca^{2+} channels predicted for different buffering conditions were simulated using a model developed by Ward and
Kenyon (2000). Parameters for $[\text{Ca}^{2+}]_i$ simulations under each buffering condition are provided in Table 1 and the predicted $\text{Ca}^{2+}$ profiles are illustrated in Fig. 1B. With 5 mM BAPTA, free $\text{Ca}^{2+}$ surrounding a $\text{Ca}^{2+}$ channel should decline below the release threshold of 400 nM (Thoreson et al. 2004; Duncan et al. 2010) within 50 nm. Even if BAPTA levels attained a concentration at the synapse of only 0.5 mM, ten-fold lower than the pipette concentration, then $\text{Ca}^{2+}$ levels should still fall below release threshold within 100 nm of a $\text{Ca}^{2+}$ channel. Thus, conservatively, the persistence of depolarization-evoked PSCs in the presence of BAPTA indicates that $\text{Ca}^{2+}$ channels in cones are <100 nm from synaptic release sites.

**Distance from $\text{Ca}^{2+}$ channels to $\text{Cl(Ca)}$ Channels**

Depolarizing test steps (-70 to -10 mV) applied to rods and cones activate L-type $I_{\text{Ca}}$, voltage-dependent $K^+$, $\text{Ca}^{2+}$-activated $K^+$, and $\text{Cl(Ca)}$ currents (Attwell and Wilson 1980; Bader et al. 1982; Barnes and Hille 1989). Whole cell currents evoked by depolarizing test steps (-70 to -10 mV, 500 ms) were typically dominated by outward currents (Fig. 2). Using a pipette solution in which $E_{\text{Cl}} = -20$ mV, long-lasting inward tail currents were observed after termination of the test step (Fig. 2). The duration of $\text{Cl(Ca)}$ tail currents lasts up to 12 s and exhibits considerable variability, declining in direct proportion to the decline in intracellular $\text{Ca}^{2+}$ levels (MacLeish and Nurse 2007). Tail currents could be inhibited by the $\text{Cl}^-$ channel blocker niflumic acid (0.1 mM) and reversed around $E_{\text{Cl}}$ (N=11 for rods and N=8 for cones, data not shown), consistent with other studies indicating that tail currents reflect $I_{\text{Cl(Ca)}}$ activity (Barnes and Deschenes 1992; Thoreson et al. 2003; MacLeish and Nurse 2007). Tail currents were not
significantly inhibited by bath application of the vesicular glutamate uptake inhibitor DL-threo-β-benzyloxyaspartate (TBOA, 0.1 mM) indicating little contribution from Cl⁻ efflux through EAAT anion channels (Rods: 5 mM BAPTA, P=0.70, paired t-test, N=12; 5 mM EGTA, P=0.70, N=3; 0.5 mM EGTA, P=0.64, N=3. Cones: 5 mM BAPTA, P=0.71, N=7; 5 mM EGTA, P=0.97, N=3; 0.5 mM EGTA, P=0.54, N=3.).

Like Ca²⁺ channels, Cl(Ca) channels are located almost entirely in the synaptic terminals of photoreceptors (MacLeish and Nurse 2007). To analyze the distance between Ca²⁺ and Cl(Ca) channels in rods and cones, Cl(Ca) tail currents in rods and cones were measured after introducing different concentrations of Ca²⁺ buffers (0.5 mM EGTA, 5 mM EGTA, and 5 mM BAPTA) into the photoreceptor through the recording pipette. We waited at least 10 min for buffers to diffuse to the synaptic terminal. Fig. 2 illustrates recordings obtained from rods and cones using the different buffers. The bar graphs in Figs. 2G and H show the average amplitude of tail currents measured 15 ms after termination of the test step with the different buffers. In both rods and cones, tail currents were significantly diminished by increasing the EGTA concentration from 0.5 to 5 mM (t-test, rods, P=0.003; cones P=0.002, Fig. 2G-H). BAPTA caused a further reduction in tail currents but did not block them completely. Although the persistence of Cl(Ca) tail currents in the presence of 5 mM BAPTA may be partly due to partial saturation of BAPTA during the lengthy depolarizing step, it also suggests that a small population of Cl(Ca) channels is located within 100 nm of Ca²⁺ channels.

To estimate the average distance between Ca²⁺ and Cl(Ca) channels, we compared buffer effects on tail currents with profiles of free Ca²⁺ predicted for the three buffering conditions (Fig. 1B; Ward and Kenyon 2000; Naraghi 1997). In rods,
increasing EGTA from 0.5 to 5 mM reduced tail currents by 82% relative to the greater reduction produced by 5 mM BAPTA. By comparison, the predicted Ca$^{2+}$ profiles suggest that increasing EGTA from 0.5 to 5 mM should reduce [Ca$^{2+}$] by 82% relative to the levels predicted for 5 mM BAPTA at a distance of 395 nm from the Ca$^{2+}$ channel (Fig. 1B). In cones, increasing EGTA from 0.5 to 5 mM reduced tail currents by 89% relative to the reduction produced by 5 mM BAPTA. The same buffer-induced change in Ca$^{2+}$ levels should occur at a distance of 490 nm from the Ca$^{2+}$ channel (Fig. 1B).

Measurements of the Ca$^{2+}$ dependence of Cl(Ca) channels described later suggested that their activation is likely to require the binding of two Ca$^{2+}$ ions. If so, the decline in [Ca$^{2+}$] should cause a squared decline in Cl(Ca) activation which would in turn suggest distances of 206 and 263 nm between Ca$^{2+}$ and Cl(Ca) channels in rods and cones, respectively.

In another approach to assess the average distance between Ca$^{2+}$ channels and Cl(Ca) channels, we used the calcium-dependence of $I_{\text{Cl(Ca)}}$ to estimate [Ca$^{2+}$], from $I_{\text{Cl(Ca)}}$ amplitude. Sigmoidal concentration/response curves were generated using the EC$_{50}$ and Hill slope values for Cl(Ca) channels determined from caged Ca$^{2+}$ experiments described later. Peak current amplitudes were assumed to be slightly larger than the average currents recorded with 0.5 mM EGTA (130 and 330 pA for cones and rods, respectively). Using these curves, we converted tail current measurements into [Ca$^{2+}$], and then matched these Ca$^{2+}$ levels to the predicted decline in Ca$^{2+}$. For 5 mM EGTA, this yielded estimated distances of 358 nm and 478 nm in rods and cones, respectively. Estimates with 0.5 mM EGTA are more sensitive to values chosen for the top of the sigmoidal curve but repeating this procedure with data
for 0.5 mM EGTA yielded estimates of 296 and 365 nm for rods and cones, respectively. Distance estimates also depend on parameters used for simulation of the Ca\(^{2+}\) decline. For example, using slightly slower on rates for Ca\(^{2+}\) binding to EGTA from Stern (1992; 1.5·10\(^6\) M\(^{-1}\)S\(^{-1}\)), we obtained distances with 5 mM EGTA of 454 nm and 596 nm in rods and cones. Although the precise values differ somewhat, results of these different analyses all agree that the distance between Ca\(^{2+}\) and Cl(Ca) channels averages a few hundred nanometers. The inability of BAPTA to block tail currents completely in rods and cones indicates that there is also a sub-population of Cl(Ca) channels are very close to Ca\(^{2+}\) channels. Due to the limited spatial spread of Ca\(^{2+}\) when using BAPTA as a buffer, the amplitude of tail currents measured in the presence of 5 mM BAPTA suggested that Ca\(^{2+}\) and Cl(Ca) channels are separated by only ~60 nm. Together, these results suggest that Cl(Ca) channels are dispersed throughout the synaptic terminal, with some channels quite close to Ca\(^{2+}\) channels and others further away.

**Immunohistochemical localization of Cl(Ca) channels**

Based on expression cloning and physiological data, TMEM16A has been implicated as a Cl(Ca) channel (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008; Rock et al. 2009; Hartzell et al. 2009; Romanenko et al. 2010). TMEM16A mRNA is present in the outer retina (Gritli-Linde et al. 2009), and the related protein TMEM16B also appears to form Ca\(^{2+}\)-activated chloride channels localized to mammalian photoreceptor terminals (Stöhr et al. 2009). We tested whether TMEM16A might contribute to Cl(Ca) channel activity in photoreceptors. Consistent with the presence of
TMEM16A protein in photoreceptor terminals, indirect immunofluorescence with an antibody to TMEM16A produced labeling of the outer plexiform layer (OPL) in the tiger salamander retina (Fig. 3A). In addition, TMEM16A labeled cells in the inner nuclear layer (INL) that could also be immunolabeled with antibodies for glial fibrillary acidic protein (GFAP) to label Müller cells (Fig. 3A-D). This is consistent with electrophysiological evidence for Cl(Ca) channels in salamander Müller cells (Welch et al. 2006). Higher magnification of the OPL suggests that TMEM16A immunolabels photoreceptor terminals (arrows in Fig. 3 indicate rod and cone terminals).

To test whether TMEM16A is expressed in photoreceptor terminals, retinal sections and isolated photoreceptors were double-immunolabeled with the TMEM16A antibody and an antibody to the synaptic vesicle protein, SV2 (Zhang and Wu 2009). TMEM16A expression overlapped with and was limited to SV2 expression in both vertical retinal sections (Fig. 4A-C) and within the synaptic terminals of isolated photoreceptors (arrows, Fig. 4D-I), indicating that TMEM16A is localized to the synaptic terminals of photoreceptors. The overlap between TMEM16A and SV2 expression is also consistent with electrophysiological results suggesting that Cl(Ca) channels are dispersed throughout the presynaptic terminal.

The retina-specific protein Ca\(_{\text{V1.4}}\) (\(\alpha_{1F}\)) has been implicated as the pore-forming subunit of the Ca\(^{2+}\) channel underlying transmitter release from photoreceptors (Bech-Hansen et al. 1998; Morgans 2001; Firth et al. 2001). Similar to the pattern observed in other species (Morgans 2001; Firth et al. 2001), antibodies to Ca\(_{\text{V1.4}}\) labeled the OPL, photoreceptor inner segments, and IPL of salamander retina (Fig. 5B). Ca\(_{\text{V1.4}}\) labeling overlapped with TMEM16A in both the OPL and terminals of isolated photoreceptors.
High magnification images showed punctate labeling of CaV1.4 in rod and cone terminals (Fig. 5 G-I, M-O). The TMEM16A antibody produced more diffuse labeling of photoreceptor terminals.

It was not practical to synthesize an antigenic peptide for the Abcam antibody which was raised against a 620 amino acid peptide, so to assess the specificity of labeling in the OPL and photoreceptor terminals, we tested another antibody raised against a different epitope of TMEM16A (Zyagen Inc., San Diego, CA). This antibody showed a similar labeling pattern with immunofluorescence visible in the OPL and IPL, along with diffuse labeling of the terminals of isolated rods and cones (Figs. S2 and S3). Immunolabeling was abolished by pre-incubation with the peptide used to develop the antibody (Supplementary Fig. 2B). We also examined methanol-fixed cells and found that, consistent with results from paraformaldehyde-fixed cells, TMEM16A antibodies produced diffuse staining of the terminal and surrounding regions whereas CaV1.4 antibodies showed a more concentrated distribution in the synaptic terminals of rods and cones (Fig. S3). Together, these anatomical findings are consistent with physiological results suggesting that Cl(Ca) channels are distributed more diffusely than Ca2+ channels.

**Ca2+-Dependence of Cl(Ca) Channels.**

We measured the Ca2+-dependence of Cl(Ca) channels in rods and cones by abruptly elevating submembrane Ca2+ levels in photoreceptor terminals via flash photolysis of the caged Ca2+ compound, DM-nitrophen (10 mM). After introduction of DM-nitrophen into the photoreceptor through the patch pipette, flash photolysis
produces an instantaneous and homogenous rise in intraterminal $[\text{Ca}^{2+}]$ that can be quantified from the change in fluorescent intensity of OGB-6F (Fig. 6). Rods and cones were voltage-clamped at a membrane potential of -77 mV (illustrated in the left panels of Fig. 6A and 6B), below the estimated value of $E_{\text{Cl}} = -39$ mV. The activation of $\text{Cl(Ca)}$ channels by the increase in $[\text{Ca}^{2+}]$ generated inward currents in both rods and cones (Fig. 6). The amplitude of inward currents evoked in rods and cones by flash photolysis of caged $\text{Ca}^{2+}$ did not differ significantly ($P=0.8$, rods; $P=0.54$, cones) between trials in which flash photolysis evoked detectable glutamate release (rods, $N=19$; cones, $N=14$) and trials which failed to evoke a post-synaptic response in simultaneously voltage-clamped horizontal or OFF bipolar cell (rods, $N=69$; cones, $N=20$). This is consistent with effects of TBOA described earlier and suggests a minimal contribution from glutamate transporter currents to the inward currents evoked in photoreceptors by flash photolysis of caged $\text{Ca}^{2+}$.

In the examples shown in Fig. 6, $I_{\text{Cl(Ca)}}$ increased more rapidly in the rod than in the cone. This faster rate is not readily explained by the higher $\text{Ca}^{2+}$ level attained after flash photolysis in the rod since time constants for the rise in $I_{\text{Cl(Ca)}}$ did not show a clear $\text{Ca}^{2+}$ dependence. This may instead reflect an intrinsic difference between rod and cone currents since $I_{\text{Cl(Ca)}}$ activated at a significantly faster rate in rods ($\tau = 3.1 \pm 0.25$ ms) than cones ($\tau = 4.0 \pm 0.30$ ms; $P=0.049$) when compared over a similar range of $\text{Ca}^{2+}$ levels (rods: 2.62 $\pm$ 0.49 $\mu$M; cones: 2.60 $\pm$ 0.50 $\mu$M; $P=0.98$).

Peak current amplitudes from rods and cones were plotted as a function of intraterminal $[\text{Ca}^{2+}]$ (Fig. 7A and 7B). The relationships between amplitude and log $[\text{Ca}^{2+}]$ were assumed to obey standard receptor binding kinetics and therefore fit with
sigmoidal binding curves (see Methods). In most experiments, we obtained only a single
response per cell. Cell-to-cell variability in current amplitude produced scatter in the
data. In a few recordings, we held individual cells long enough for [Ca$^{2+}$]$_i$ to recover to
basal levels between uncaging flashes, allowing us to make more than one
measurement. Fig. 7C and 7D illustrate recordings from a rod and cone, respectively, in
which multiple uncaging flashes were applied. As illustrated by these examples,
repeated measurements in individual cells yielded a Ca$^{2+}$-dependence similar to the
overall sample. The best fit sigmoid to the overall sample from rods exhibited an EC$_{50}$ of
556 nM with slope factor of 1.8 (N=88, Fig. 7A). Data from cones yielded an EC$_{50}$ of 377
nM with slope factor of 2.4 (N=34, Fig. 7B). Comparisons of the 95% confidence
intervals showed no significant differences between the best fit parameters in rods and
cones.

Average submembrane [Ca$^{2+}$]

Similar to the use of Ca$^{2+}$-activated K$^+$ channels as submembrane Ca$^{2+}$ sensors
(Roberts et al. 1990; Burrone et al. 2002), we used the empirically-determined Ca$^{2+}$-
dependence of I$_{Cl(Ca)}$ to assess levels of sub-membrane Ca$^{2+}$ attained at Cl(Ca)
channels in the presynaptic terminal. We chose 5 mM EGTA as the Ca$^{2+}$ buffer for
these experiments because tail currents and post-synaptic responses exhibited smaller
changes over time following patch rupture when using 5 mM EGTA in the pipette
solution compared to experiments with 0.5 mM EGTA or 5 mM BAPTA. This suggests
that 5 mM EGTA is closer to the endogenous buffering capacity. E$_{Cl}$ in the pipette
solution (-39 mV) was matched to E$_{Cl}$ in the DM-nitrophen pipette solution used to
measure Ca\(^{2+}\) dependence. We stimulated rods and cones with a series of voltage steps from -57 mV to -17 mV ranging in duration from 50 ms to 1s. We subtracted capacitative and leak currents using a P/8 protocol. Fig. 8A shows a series of traces illustrating the increase in tail currents evoked in a rod with increasing depolarization from -57 to -17 mV using test steps of 1 s duration. Fig. 8B shows the same measurements performed in a cone. Inward currents due to activation of L-type I\(_{Ca}\) were observed during the test step and inward I\(_{Cl(Ca)}\) tail currents were observed after termination of the test step. Consistent with I\(_{Cl(Ca)}\) being driven by Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (Barnes and Hille 1989; MacLeish and Nurse 2007), both I\(_{Ca}\) and tail currents increased with depolarization above -57 mV. Figs. 8C and D illustrate the effects of increasing test step duration when using a step to -17 mV in a rod and cone, respectively. Tail currents were accentuated by the use of longer test steps, consistent with an increase in [Ca\(^{2+}\)]\(_i\), exceeding the local buffering capacity. The large increase in I\(_{Cl(Ca)}\) observed in the rod with test steps of 500 ms and 1 s may reflect contributions of Ca\(^{2+}\)-induced release of Ca\(^{2+}\) from intracellular stores (Cadetti et al. 2006).

Using the sigmoidal curves fit to rod and cone data in Fig. 6, we converted I\(_{Cl(Ca)}\) amplitude into [Ca\(^{2+}\)]\(_i\), attained at Cl(Ca) channels. Experiments on Cl(Ca) channel localization suggested that Cl(Ca) channels are dispersed throughout the synaptic terminal. This, in turn, suggests that Ca\(^{2+}\) levels attained at Cl(Ca) channels should provide an estimate of the average submembrane [Ca\(^{2+}\)]. Inward Cl(Ca) tail currents exceeding limits of the sigmoidal curve (>153 pA in cones and >125 pA in rods) were assigned a concentration value of 10 \(\mu\)M. Stimuli which failed to evoke inward tail currents were assigned a concentration value of 31 nM. The results of this conversion
are shown in Fig. 9C and D for rods and cones. Consistent with Fura2 measurements (Steele et al. 2005; Szikra and Krizaj 2006; Choi et al. 2008), tail current measurements suggest basal Ca^{2+} levels (assessed from 50-200 ms steps to -57 mV) slightly below 100 nM. Ca^{2+} levels rose steeply as the membrane potential approached the dark resting potential of -40 mV. Application of a 1 s depolarization to -37 mV, slightly above the average dark potential, caused submembrane [Ca^{2+}] to rise to 2 μM in both rods and cones, consistent with estimates obtained using Ca^{2+}-sensitive dyes (Steele et al. 2005; Szikra and Krizaj 2006; Choi et al. 2008). [Ca^{2+}] attained during 1 s steps to -27 and -17 mV may exceed estimated levels since tail currents evoked by these stimuli often exceeded 125 pA in rods or 153 pA in cones.
**Discussion**

Release sites are <100 nm from Ca\(^{2+}\) channels in photoreceptor terminals.

The persistence of cone-driven PSCs in the presence of BAPTA indicates that a number of release sites are <100 nm from L-type Ca\(^{2+}\) channels in cone terminals. These dimensions are consistent with ultrastructural observations that ribbon-associated synaptic vesicles contact the plasma membrane along the flanks of the synaptic ridge at a distance of ~50 nm from edge of the ribbon (Lasansky 1973; Raviola and Gilula 1975). This close proximity between Ca\(^{2+}\) channels and release sites is similar to the squid giant synapse (Adler et al. 1991), GABAergic basket cells in the hippocampus (Bucurenciu et al. 2008), mature calyx of Held neurons (Fedchyshyn and Wang 2005), and retinal bipolar cells (Jarsky et al. 2010). However, it differs from many other CNS synapses where EGTA can significantly depress release indicating that Ca\(^{2+}\) channels are much further away from release sites. These include immature calyx of Held (Borst and Sakmann 1996; Meinrenken et al. 2002), cortical pyramidal cells (Ohana and Sakmann 1998; Rozov et al. 2001), and hair cells (Moser and Beutner 2000). In capacitance recordings from isolated goldfish bipolar cell terminals, 5 mM EGTA diminished vesicle release (Mennerick and Matthews 1996), whereas introduction of 10 mM EGTA into mouse bipolar cell somas did not diminish synaptic release (Singer and Diamond 2003). The more pronounced effects of EGTA observed in the former study may be due to differences in the preparation and cellular access: Mennerick and Matthews (1996) recorded directly from large goldfish bipolar cell terminals whereas Singer and Diamond (2003) recorded from rat bipolar cell somas that are separated from the terminal by a long, thin axon. The finding that release sites are
situated extremely close to Ca\(^{2+}\) channels in photoreceptors is particularly surprising
given the high affinity for Ca\(^{2+}\) exhibited by the release mechanism which requires only
submicromolar levels to stimulate exocytosis (Rieke and Schwartz 1996; Thoreson et al. 2004; Sheng et al. 2007; Duncan et al. 2010). The presence of highly localized Ca\(^{2+}\) nanodomains suggests strong endogenous Ca\(^{2+}\) buffering in photoreceptor terminals.

One consequence of a tight coupling between Ca\(^{2+}\) channels and release sites may be a lower likelihood of ectopic release (i.e., release at sites other than the active zone). In addition to Ca\(^{2+}\) channels in the synaptic terminals of photoreceptors, immunohistochemical studies show faint labeling of the soma and inner segments suggesting some channels may be located at non-synaptic sites (Nachman-Clewner et al. 1999; Morgans 2001; Morgans et al. 2005; tom Dieck et al. 2005). However, electrophysiological measurements suggest that 95% of the Ca\(^{2+}\) channels are localized to the synaptic terminal in rods (Xu and Slaughter 2005) and high-resolution confocal microscopy and immuno-electron micrographs show that Ca\(^{2+}\) channels in the terminal are mainly located beneath the arciform density at the base of the ribbon (tom Dieck et al. 2005; Specht et al. 2009). The finding that both transient and sustained components of synaptic release involve release sites <100 nm from Ca\(^{2+}\) channels is thus consistent with the hypothesis that release occurs predominately at the ribbon in cone photoreceptors (Jackman et al. 2009). However, our data are also consistent with the possibility of non-ribbon release sites located very close to non-ribbon Ca\(^{2+}\) channels. Like cones, rod-driven EPSCs were not blocked by BAPTA in the patch pipette, but interpretation of these responses is complicated by the presence of synaptic output from neighboring, gap-junctionally coupled rods (Cadetti et al. 2005).
Ca\textsuperscript{2+} levels rise to micromolar levels beneath the ribbon when photoreceptors are maintained in darkness (Steele et al. 2005; Szikra and Krizaj 2006; Choi et al. 2009; Jackman et al. 2009; present study, Fig. 9). Micromolar Ca\textsuperscript{2+} levels are sufficient to release many of the vesicles in the readily releasable pool at the base of the synaptic ribbon (Jackman et al. 2009). When maintained in darkness, the sustained release rate is thus determined by the rate at which vesicles replenish the readily releasable pool, not the frequency of individual Ca\textsuperscript{2+} channel openings (Jackman et al. 2009). Linking the rate of release to replenishment rather than individual channel openings may reduce the synaptic noise that can result from stochastic channel openings. By contrast, when photoreceptors are strongly hyperpolarized in bright light, Ca\textsuperscript{2+} channel openings occur principally when the cell depolarizes in response to a light decrement. In this situation, positioning Ca\textsuperscript{2+} channels close to release sites may allow fusion to be closely synchronized with Ca\textsuperscript{2+} channel opening, enhancing fidelity and temporal precision in the conversion of membrane potential changes to synaptic release. The combination of high Ca\textsuperscript{2+} affinity in the release mechanism with a close proximity to Ca\textsuperscript{2+} channels may allow the synapse to switch between different output modes to improve release precision under light-adapted conditions when the occurrence of Ca\textsuperscript{2+} channel openings are closely synchronized to the appearance of a stimulus but reduce synaptic noise in darkness when Ca\textsuperscript{2+} channel openings occur more randomly.

The distance between Ca\textsuperscript{2+} and Cl(Ca) channels averages a few hundred nanometers. In addition to stimulating synaptic release, Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels activates Cl(Ca) channels. The finding that 5 mM BAPTA did not fully eliminate I\textsubscript{Cl(Ca)} in
rods or cones suggests that some Cl(Ca) channels are quite close (<100 nm) to Ca^{2+} channels. However, other Cl(Ca) channels are further away since the distance between Ca^{2+} channels and Cl(Ca) channels averaged 300-600 nm in cones and 200-450 nm in rods. By comparison, the distance between individual ribbons in published electron micrographs of salamander retina is often a micron or more (Lasansky 1978; Townes-Anderson et al. 1985; Pang et al. 2008), consistent with the hypothesis that Cl(Ca) channels are dispersed between ribbons.

Until the identity of the channel(s) contributing to Cl(Ca) currents is fully characterized, measurements of these spatial relationships are necessarily limited to physiological approaches. The putative Cl(Ca) channel, TMEM16B, has been shown to be localized to photoreceptor terminals (Schroeder et al. 2008; Stöhr et al. 2009). The present immunohistochemical results indicate that the related isoform, TMEM16A, is also present at photoreceptor synaptic terminals, consistent with the presence of mRNA for the TMEM16A gene in outer retinal layers (Gritli-Linde et al. 2009; Fig. 3). TMEM16A may therefore participate with TMEM16B in generating I_{Cl(Ca)} in photoreceptors. Staining for TMEM16A antibodies produced labeling of rod and cone terminals that overlapped extensively with labeling for the synaptic vesicle protein SV2 but was more diffuse than the punctate staining observed with antibodies to Ca_{v}1.4 (\alpha_{1F}) Ca^{2+} channels. Punctate labeling of rod terminals by L-type Ca^{2+} channel antibodies was also observed by Nachman-Clewner et al. (1999). Although uncertainties remain about the molecular identity of the Cl(Ca) channel, these results are consistent with electrophysiological results suggesting that Cl(Ca) channels are not clustered as tightly near synaptic
ribbons as Ca\(^{2+}\) channels, but more dispersed throughout the synaptic terminals of rods and cones.

**Ca\(^{2+}\)-dependence of Cl(Ca) channels in photoreceptors.**

Using flash photolysis of DM-nitrophen to elevate submembrane [Ca\(^{2+}\)] instantaneously, we found that I_{Cl(Ca)} could be activated by submicromolar [Ca\(^{2+}\)] levels in photoreceptors. The slopes of sigmoidal fits to Ca\(^{2+}\)-dependence suggest that binding of 2 or more Ca\(^{2+}\) ions is required for activation. This is consistent with TMEM16 expression studies and studies on Cl(Ca) channels in other tissues which show Hill coefficients \(\geq 2\) (Evans and Marty 1986; Arreola et al. 1996; Giovannucci et al. 2002; Reisert et al. 2003; Kuruma and Hartzell 2000; Yang et al. 2008; Pifferi et al. 2009). The EC\(_{50}\) values of 556 nM in rods and 377 nM in cones are similar to the K\(_d\) of I_{Cl(Ca)} in acinar cells (Arreola et al. 1996; Giovannucci et al. 2002) but lower than K\(_d\) values obtained from excised patches containing TMEM16A channels, TMEM16B channels, Cl(Ca) channels from olfactory neurons, or Xenopus oocyte channels (Reisert et al. 2003; Kuruma and Hartzell 2000; Yang et al. 2008; Pifferi et al. 2009). The Ca\(^{2+}\)-dependence of Cl(Ca) channels can be influenced by membrane potential, with lower K\(_d\) values observed at more positive potentials (Hartzell et al. 2005). For example, TMEM16A exhibits a K\(_d\) for Ca\(^{2+}\) of 2.6 \(\mu\)M at -60 mV but only 400 nM at +60 mV (Yang et al. 2008). Thus, differences in the K\(_d\) could mean that a protein other than TMEM16 is responsible for Cl(Ca) currents in photoreceptors, but can also be explained by the presence of protein partners *in situ* that modify the local charge environment to produce a K\(_d\) similar to that observed at positive potentials.
Using Cl(Ca) channels as Ca$^{2+}$ sensors, we found that submembrane Ca$^{2+}$ levels in photoreceptors range from basal levels slightly below 100 nM to 1-2 μM at the dark resting potential, consistent with results obtained using Ca$^{2+}$ sensitive dyes (Steele et al. 2005; Szikra and Krizaj 2006; Choi et al. 2008). Ca$^{2+}$ levels rose steeply as the membrane potential approached the dark potential suggesting that small variations in resting potential can have large effects on resting Ca$^{2+}$ levels. Over much of the normal operating voltage range in cones (-60 to -40 mV), average submembrane [Ca$^{2+}$] remained below the threshold of 400 nM needed to stimulate synaptic vesicle exocytosis (Thoreson et al. 2004; Duncan et al. 2010). This is consistent with Ca$^{2+}$ chelator results indicating that synaptic release is controlled by high Ca$^{2+}$ levels attained in highly localized nanodomains near Ca$^{2+}$ channels.

Significance of Ca$^{2+}$/Cl(Ca) channel relationships.

Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels activates Cl(Ca) channels and activation of Cl(Ca) channels can in turn influence Ca$^{2+}$ channel activity (Thoreson et al. 1997, 2000). In rods, $E_{Cl}$ averages -20 mV, so activation of $I_{Cl(Ca)}$ results in a Cl$^-$ efflux. Although the membrane depolarization that results from Cl$^-$ efflux stimulates $I_{Ca}$, this stimulatory effect is countered by a simultaneous strong inhibitory influence of Cl$^-$ efflux whereby reductions in intracellular Cl$^-$ act at anion binding sites on individual Ca$^{2+}$ channels to directly inhibit channel open probability (Thoreson et al. 2000; Babai et al. 2010). Ca$\text{v}1.4$ Ca$^{2+}$ channels, which are the principal subtype in rods, exhibit slow calcium-dependent inactivation (Koschak et al. 2003; Baumann et al. 2004; McRory et
Inhibition of $I_{\text{Ca}}$ by negative feedback from $I_{\text{Cl(Ca)}}$ activity may provide an alternative mechanism to limit excessive Ca$^{2+}$ entry.

$E_{\text{Cl}}$ in cones averages -38 mV, close to the dark resting potential (Thoreson and Bryson 2004). $I_{\text{Cl(Ca)}}$ evoked by strong depolarizing steps was smaller in cones than rods suggesting a lower Cl(Ca) current density. Thus, Cl(Ca) channel activation is likely to produce only small changes in intracellular Cl$^{-}$ levels that will have only a small effect on Ca$^{2+}$ channel open probability. The activation of Cl(Ca) channels in cones may instead be more important for stabilizing the membrane potential near its dark resting value (Maricq and Korenbrot 1988).

The present results suggest that Ca$^{2+}$ influx through an individual Ca$^{2+}$ channel could potentially influence the activity of Cl(Ca) channels hundreds of nanometers away and thereby indirectly influence the activity of relatively distant Ca$^{2+}$ channels. Such long-range interactions may be boosted by calcium-induced calcium release in rod terminals (Krizaj et al. 1999, 2003; Suryanarayan and Slaughter 2006; Cadetti et al. 2006; Babai et al. 2010). Regulation of relatively distant Ca$^{2+}$ channels by the activation of Cl(Ca) channels may help to ensure similarity in the behavior of Ca$^{2+}$ channels located at different positions along the ~1 µm long synaptic ridge in rods. In amphibian rods with multiple ribbons, long-range interactions might also help to reduce functional heterogeneity between channels at neighboring synaptic ribbons (Frank et al. 2009; Meyer et al. 2009).
Acknowledgments

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### Figures

**Table 1: Parameters to determine free Ca$^{2+}$ around a Ca$^{2+}$ channel in the presence of a diffusible chelator**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{Ca}$</td>
<td>0.2 μm$^2$/msec</td>
<td>Neher 1986</td>
</tr>
<tr>
<td>$I_{Ca}$</td>
<td>0.25 pA</td>
<td>Church and Stanley 1996</td>
</tr>
<tr>
<td>EGTA $K_d$</td>
<td>$1.58\cdot10^{-7}$ M</td>
<td>Naraghi 1997</td>
</tr>
<tr>
<td>EGTA $K_{on}$</td>
<td>$2.7\cdot10^{6}$ M$^{-1}$ S$^{-1}$</td>
<td>Naraghi 1997</td>
</tr>
<tr>
<td>BAPTA $K_d$</td>
<td>$1.8\cdot10^{-7}$ M</td>
<td>Naraghi 1997</td>
</tr>
<tr>
<td>BAPTA $K_{on}$</td>
<td>$4.5\cdot10^{8}$ M$^{-1}$ S$^{-1}$</td>
<td>Naraghi 1997</td>
</tr>
</tbody>
</table>
Figure 1: Effects of the diffusible Ca\(^{2+}\) buffers EGTA and BAPTA on EPSCs evoked in horizontal or OFF bipolar cells by depolarizing steps (-77 mV to -17 mV for 500 ms) applied to voltage-clamped cones. Records in A show the average PSC from multiple cone/horizontal cell and cone/OFF bipolar cell pairs obtained after waiting >10 min for diffusion of 5 mM BAPTA (N=7, solid gray line), 5 mM EGTA (N=12, solid black line), or 0.5 mM EGTA (N=10, dashed line) to the cone terminal. Cyclothiazide (0.1 mM) was present in these experiments to prevent AMPA receptor desensitization. EPSCs were diminished but not fully blocked by the addition of 5 mM BAPTA to the patch pipette solution. B. Decline in [Ca\(^{2+}\)] as a function of distance from an open Ca\(^{2+}\) channel predicted from the “Pore” macro (Ward and Kenyon 2000) with Ca\(^{2+}\) buffering by 5 mM BAPTA (solid gray line), 5 mM EGTA (solid black line), or 0.5 mM EGTA (dashed black line). Model parameters are provided in Table 1.

Figure 2: Effects of the diffusible Ca\(^{2+}\) buffers EGTA and BAPTA on depolarization-evoked Cl(Ca) tail currents in rods and cones. Rod and cone photoreceptors were depolarized by application of a test step from -77 mV to -17 mV for 500 ms. Peak I_{Cl(Ca)} activation was measured 15 ms after the depolarizing step to avoid recording contamination from the capacitative transient. Depolarization-evoked tail currents arising from the activation of I_{Cl(Ca)} persisted in both rods and cones in the presence of 0.5 mM EGTA (A and B), 5 mM EGTA (C and D) and 5 mM BAPTA (E and F). Recordings with 5 mM BAPTA were the largest responses obtained with this buffer. G. Mean tail current amplitude measured in rods with Ca\(^{2+}\) buffering provided by 0.5 mM EGTA (316.0 ± 35.0 pA, N=24), 5 mM EGTA (120.7 ± 21.4 pA, N=14), and 5 mM BAPTA (78.7 ± 14.6 pA, N=23). Tail currents were measured 15 ms after the end of the
Measurements were made at least 10 min. after patch rupture. **H.** Tail current amplitudes in cones with Ca$^{2+}$ buffering provided by 0.5 mM EGTA (123.3 ± 12.4 pA, N=17), 5 mM EGTA (38.1 ± 8.64 pA, N=8), and 5 mM BAPTA (27.0 ± 6.70 pA, N=14).

**Figure 3:** TMEM16A is expressed in both the outer plexiform layer (OPL) and Müller cell bodies which span the entire retina. **A.** Retinal section immunolabeled for TMEM16A (red). **B.** Müller cells immunolabeled in a retinal section with GFAP (green). **C.** Merged fluorescent image of TMEM16A and GFAP. **D.** Merged TMEM16A (red) and GFAP (green) immunofluorescence with the DIC (differential interference contrast) brightfield image. Arrows in panel C indicate labeling of TMEM16A in photoreceptor terminals. **E.** High magnification zoom region in the OPL illustrating TMEM16A immunolabeling in the synaptic terminals of photoreceptors (red). **F.** GFAP labeling of the same high magnification region (green). **G.** Merged fluorescent high magnification image of TMEM16A and GFAP. **H.** Merged TMEM16A (red) and GFAP (green) with the DIC brightfield image. Arrows in G indicate labeling of TMEM16A in individual photoreceptor terminals. OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, and GCL = ganglion cell layer. Scale bars = 10 μm.

**Figure 4:** TMEM16A and SV2 labeling co-localize at photoreceptor terminals. **A.** Immunolabeling of TMEM16A (green) in a vertical retinal section. **B.** Immunolabeling of the same retinal section with a synaptic vesicle marker, SV2 (red). Autofluorescence in the outer segments was observed without secondary antibodies. **C.** Merged fluorescent and DIC image of TMEM16A and SV2. **D.** Brightfield DIC image of a rod photoreceptor
lacking its outer segment immunolabeled with TMEM16 (green). E. Brightfield DIC image of the same rod immunolabeled with SV2 (red). F. Merged brightfield DIC image with both TMEM16A and SV2. G-I: Magnified images of the same rod terminal showing that TMEM16A overlaps completely with SV2. J-L: Brightfield DIC images of a small single cone photoreceptor immunolabeled with TMEM16A (green, J), SV2 (red, K), or both (L). M-O: Magnified images of the same cone terminal showing that TMEM16A overlaps completely with SV2. OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, and GCL = ganglion cell layer. Synaptic terminal regions are indicated by the arrows in panels D and J. Scale bar is 10 μm.

Figure 5: TMEM16A and the Ca\(^{2+}\) channel Ca\(_{v}1.4\) are both expressed at photoreceptor terminals. A. TMEM16A labeling (green) in a vertical retinal section. B. \(\text{Ca}_{v}1.4\) labeling (red) in the same retinal section. C. Merged fluorescent and DIC image of TMEM16A and \(\text{Ca}_{v}1.4\). D-F: Brightfield DIC images of a rod photoreceptor immunolabeled with TMEM16A (green, D), \(\text{Ca}_{v}1.4\) (red, E), or both (F). Autofluorescence in the inner segment region with the green channel was observed without the secondary antibody and is likely due to presence of NADH and NADPH in the mitochondrial rich ellipsoid. G-I: Magnified images of the same rod terminal illustrate the more punctate expression of \(\text{Ca}_{v}1.4\) (H) compared to the diffuse labeling of TMEM16A (G) throughout the terminal region. J-L: Brightfield DIC images of a large single cone photoreceptor immunolabeled with TMEM16A (green, J), \(\text{Ca}_{v}1.4\) (red, H), or both (I). M-O: Magnified images of the same cone terminal illustrates the more punctate expression of \(\text{Ca}_{v}1.4\) (N) compared to the diffuse expression of TMEM16A (M) throughout the terminal region.
region. OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, and GCL = ganglion cell layer. Synaptic terminal regions are indicated by the arrows in panels D and J. Scale bar is 10 μm.

Figure 6: Flash photolysis of caged Ca$^{2+}$ in photoreceptor terminals

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instantaneously elevated intraterminal [Ca$^{2+}$] and stimulated inward Cl(Ca)
currents.

A. Grayscale images of OGB-6F fluorescence in a single confocal section from a rod loaded with DM-nitrophen. Images were obtained prior to flash photolysis (left) and immediately after flash photolysis (right). [Ca$^{2+}$]$_i$ was measured from a region of interest placed in the synaptic terminal (arrows). Fluorescence is brighter in the soma due to the presence of more dye. B. Grayscale images of OGB-6F fluorescence in a single confocal section from a cone before (left) and after (right) flash photolysis of DM-nitrophen. Panels C and D show intraterminal [Ca$^{2+}$] measured at 60 ms intervals in the rod (C) and cone (D). Panels E and F show inward Cl(Ca) currents evoked in the same rod (E) and cone (F). Cells were voltage-clamped at -77 mV. Ca$^{2+}$-activated K$^+$ currents were inhibited by Cs$^+$ and TEA in the pipette solution and $E_{Cl} = -39$ mV. Scale bar = 5 μm.

Figure 7: Ca$^{2+}$-dependence of I_{Cl(Ca)} in rods and cones. The amplitudes of inward Cl(Ca) currents evoked by flash photolysis of caged Ca$^{2+}$ were plotted as a function of intraterminal [Ca$^{2+}$]. Data were fit with sigmoidal concentration/response curves (see Methods). The best fit for data from rods (A) was obtained with an EC$_{50}$ of 556 nM and slope factor of 1.76 (N=88). The best fit for data from cones (B) was obtained with an
EC<sub>50</sub> of 377 nM and slope factor of 2.44 (N=34). Repeated measurements in an individual rod (C) and cone (D) yielded a similar sigmoidal relationship. For these two cells, the best fits were obtained with EC<sub>50</sub> values of 675 and 262 nM and slope factors of 2.56 and 2.44, respectively.

**Figure 8**: Tail currents evoked by depolarizing test steps over a range of voltages (-57 mV to -17 mV) and durations (50 ms to 1s). Panels A and B each show the currents evoked in a rod (A) and cone (B) by changes in the amplitude of a 1 s test step from -57 to -17 mV (10 mV increments). Small inward I<sub>Ca</sub> is detectable during steps to -57 mV and above. Cl(Ca) tail currents become visible with steps to -47 mV and above. Panels C and D each illustrate currents evoked by changing the duration of a strong test step (-77 to -17 mV) from 50 ms to 1 s in a rod (C) and cone (D). Inward I<sub>Ca</sub> were evoked by the step and Cl(Ca) tail currents were observed following termination of the test step with pulses of 200 ms or longer in these examples. Passive R<sub>m</sub> and C<sub>m</sub> were subtracted using a P/8 protocol. E<sub>Cl</sub> = -39 mV.

**Figure 9**: Conversion of the amplitude of depolarization-evoked Cl(Ca) tail currents into submembrane [Ca<sup>2+</sup>] for different test step voltages and durations. I<sub>Cl(Ca)</sub> (A, rods; B, cones) amplitude was converted into [Ca<sup>2+</sup>]<sub>i</sub> (C, rods; D, cones) attained at Cl(Ca) channels using the sigmoidal curves fit to the data in Fig. 7. Currents exceeding 153 pA in cones or 134 pA in rods were assigned a concentration value of 10 μM. Stimuli which failed to evoke inward tail currents were assigned a concentration value of 31 nM.
Table 1: Parameters to determine free Ca$^{2+}$ around a Ca$^{2+}$ channel in the presence of a diffusible chelator

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{Ca}$</td>
<td>0.2 μm$^2$/msec</td>
<td>Neher 1986</td>
</tr>
<tr>
<td>$I_{Ca}$</td>
<td>0.25 pA</td>
<td>Church and Stanley 1996</td>
</tr>
<tr>
<td>EGTA $K_d$</td>
<td>$1.58 \cdot 10^{-7}$ M</td>
<td>Naraghi 1997</td>
</tr>
<tr>
<td>EGTA $K_{on}$</td>
<td>$2.7 \cdot 10^{6}$ M$^{-1}$ S$^{-1}$</td>
<td>Naraghi 1997</td>
</tr>
<tr>
<td>BAPTA $K_d$</td>
<td>$1.8 \cdot 10^{-7}$ M</td>
<td>Naraghi 1997</td>
</tr>
<tr>
<td>BAPTA $K_{on}$</td>
<td>$4.5 \cdot 10^{8}$ M$^{-1}$ S$^{-1}$</td>
<td>Naraghi 1997</td>
</tr>
</tbody>
</table>
A. Average cone-driven EPSCs

B. Diffusible buffer profile
A. 0.5 mM EGTA, rod

B. 0.5 mM EGTA, cone

C. 5 mM EGTA, rod

D. 5 mM EGTA, cone

E. 5 mM BAPTA, rod

F. 5 mM BAPTA, cone

G. Rods

H. Cones

![Bar graphs showing tail current in rods and cones with different concentrations of EGTA and BAPTA.](image)