Regulation of presynaptic strength by controlling Ca\(^{2+}\) channel mobility: effects of cholesterol depletion on release at the cone ribbon synapse

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MATERIALS AND METHODS

Animal care and use. Experimental procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Male and female adult aquatic tiger salamanders (\textit{Ambystoma tigrinum}, 18–25 cm in length; Kons Scientific, Germantown, WI and Charles Sullivan, Nashville, TN) were maintained on a 12:12-h day-night cycle and were killed 1–2 h after the beginning of subjective night. Salamanders were decapitated with heavy shears and immediately pithed.

Retinal tissue preparation. After enucleation, each eye was opened along the ora serrata, and the cornea, lens, and vitreous body were removed. The resultant eyecup was quartered and pressed gently, vitreous side down, onto a 5 × 10-mm piece of filter paper (type AAWP, 0.8-μm pores; Millipore, Billerica, MA). The filter paper and eyecup were then immersed in cold amphibian saline solution, and the retinal pigment epithelium, choroid, and sclera were carefully peeled.
away, leaving the retina adhered to the filter paper. The retina was sliced into 125-μm sections with the use of a razor blade tissue
chopper (Stoelting, Wood Dale, IL) fitted with a no. 121-6 razor blade
(Ted Pella, Redding, CA). Slices were separated from one another and
rotated 90° to view the laminar stratification of the retina under a
water-immersion objective (×60, 1.0 NA) on an upright microscope
(E600FN; Nikon, Tokyo, Japan). Throughout these procedures, the
tissue was submerged in cold, oxygenated amphibian extracellular
saline solution consisting of (in mM) 111 NaCl, 2.5 KCl, 1.8 CaCl₂,
0.5 MgCl₂, 10 HEPES, and 5 glucose (pH 7.8). For experiments on
dissociated photoreceptors, a whole salamander retina was isolated
and incubated in 0.5 mM Ca²⁺ amphibian extracellular saline solution
containing 0.2 mg/ml cysteine and 10 U/ml papain (Sigma-Aldrich,
St. Louis, MO) for 25 min at 20°C. Tissue was washed twice in
low-Ca²⁺ saline solution containing 1% BSA and 1 mg/ml DNase
(Worthington Biochemicals, Lakewood, NJ), followed by two addi-
tional washes in low-Ca²⁺ saline. To isolate individual neurons,
retinal tissue was gently triturated using a fire-polished glass Pasteur
pipette and plated onto 18-mm coverslips (Deckgläser/Thermo,
Braunschweig, Germany) coated with 1 mg/ml concanavalin-A
(Sigma-Aldrich).

Retina tissue fixation and cholesterol imaging. To image membrane
lipids at the terminals of cone photoreceptors, we applied filipin III
(Sigma-Aldrich) to label membrane cholesterol or FITC-CTXB (Sig-
a-ma-Aldrich) to label ganglioside GM1 glycoproteins associated with
cholesterol-rich lipid rafts. Dissociated retinal cells were prepared as
described above and fixed in 0.1 M phosphate-buffered saline (PBS)
containing 4% paraformaldehyde for 30 min. Cells were then washed
in 0.1 M PBS three times (10 min per wash) and bound with either 300
μg/ml filipin III for 90 min or 2 μg/ml FITC-CTXB for 45 min. Cells
were washed an additional three times with 0.1 M PBS for 10 min and
placed at room temperature in darkness until dry. After a brief rinse in
de-ionized H₂O, each slide was covered with ProLong Gold
anti-fade reagent (Invitrogen). Retinal preparations were visualized
under brightfield illumination on an upright microscope (Nikon
E600FN) to identify individual cones. Cones treated with filipin III
were imaged using a DsQi-1 camera (Photometrics, Tucson, AZ),
NIS-Elements software (Nikon), a ×60, 1.2-NA water-immersion
objective (Nikon), a 380-nm excitation/525-nm emission objec-
tive (×60, 1.0 NA; Nikon) using a laser confocal scanhead (Ultra-
View Live Cell Imaging System; Perkin Elmer, Waltham, MA)
equipped with a cooled charge-coupled device camera (Orca ER;
Hamamatsu, Hamamatsu City, Japan). Z-stack image series were
 captures at 1-μm intervals for image analysis by using an exposure of
2 s per image with a 488-nm excitation/525-nm bandpass emission
filter to optimize the FITC signal. Post-image processing was per-
formed in Adobe Photoshop (Adobe Systems, San Jose, CA) to apply
psycolors and optimize the brightness and contrast.

Quantum dot binding and imaging. Quantum dot (QD) attachment
to individual Ca₉ channels at photoreceptor synapses was conducted
as described previously (Mercer et al. 2011b). Briefly, retinal neurons
 were dissociated and incubated with a primary rabbit anti-α₁β₂δ
d subunit antibody (Qin et al. 2002) at a dilution of 1:1,000 in standard
1.8 mM Ca²⁺ saline solution for 3 h at 4°C. Cells were washed three
times in standard saline solution and then incubated with a secondary
biotinylated goat-anti-rabbit IgG (1:2,000; Sigma-Aldrich) for1 h.
Cells were then washed an additional three times with 0.1 M PBS for 10 min and
incubated with 10 mM MitoTracker Red CMFDA (Molecular Probes,
Eugene, OR) at 37°C. Fluorescence was measured using a SpectraMax M5
microplate reader (Molecular Devices), and values were normalized by
retinal tissue protein concentration determined using a BCA protein
assay (Pierce/Thermo Scientific, Rockford, IL). Cholesterol levels
were assayed using the Amplex Red cholesterol assay kit (Invitrogen) and
combined with the

Electrophysiology. To measure the effects of membrane cholesterol
depletion on photoreceptor vesicle release, we recorded simultane-
ously from cone photoreceptors and synaptically connected horizontal
cells using the whole cell patch-clamp configuration. Whole cell
recording electrodes were pulled using a PP-830 vertical puller (Na-

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The location of a QD can be determined with precision exceeding
the diffraction limit by fitting the fluorescence profile with a Gaussian
point spread function (Alcor et al. 2009; Courtly et al. 2006). From
the standard deviation (SD) of displacements exhibited by immobilized
QDs, we estimate the pointing accuracy (full-width half-maximum =
2.35 × SD) to be 35 nm (Mercer et al. 2011b). Videos were captured
at 30 ms per frame for 400 frames using the MetaMorph imaging suite
(Molecular Devices, Sunnyvale, CA). Images were adjusted to opti-
mize QD contrast and smoothed by convolving with a 5 × 5-pixel
Gaussian. HD position was then tracked using NIS-Elements (Nikon)
software. From the x and y coordinates, we calculated the mean
squared displacement (MSD; in μm²/s) (Saxton 1997) using the
following equation:

\[
MSD = \frac{1}{N-n} \sum_{i=1}^{N-n} \left( (X_{i+n} - X_i)^2 + (Y_{i+n} - Y_i)^2 \right)
\]

(1)

where L² provides the surface area that a given channel traverses
within the presynaptic plasma membrane by fitting the data with Eq. 2:

\[
MSD = \frac{L^2}{3} \left( 1 - \exp \left( -\frac{12D^2t}{L^2} \right) \right)
\]

(2)

Pharmacology and cellular cholesterol content. To deplete membrane
cholesterol, sliced or dissociated retinal preparations were incubated with 10 mM MβCD (Sigma-Aldrich) or 2 U/ml COase
(Sigma-Aldrich) in standard amphibian saline solution for 1 h. To
replenish membrane cholesterol, we first treated cells with 10 mM
MβCD for 1 h and then applied a MβCD-cholesterol complex for 1 h,
following established protocols (Albubol et al. 2005; Pucadyil and
Chattopadhyay 2005; Tekpli et al. 2008). To prepare the MβCD-
cholesterol complex, cholesterol (Sigma-Aldrich) was first dissolved in
95% ethanol at 80°C to make a 90 mM stock solution. This stock
solution was diluted to a final concentration of 1 mM cholesterol
by being mixed with an amphibian saline solution containing 10 mM
MβCD for 1 h at 20°C on a rocking platform. MβCD-cholesterol
complex solutions were prepared freshly before each experiment.

We analyzed cholesterol levels in retinal membranes using the
Amplex Red cholesterol assay kit (Invitrogen). Dissected eyecups
were halved, and the retina was carefully isolated from the retinal
glial epithelium and choroid. Retinal tissue was then treated with
MβCD or COase or treated by following the cholesterol repletion
protocol described above. Samples were hydrolyzed with the
cholesterol reaction buffer (0.1 M potassium phosphate, 50 mM NaCl, 5 mM
cholic acid, and 0.1% Triton X-100, pH 7.4) and combined with the
Amplex Red reagent (150 μM), 1 U/ml horseradish peroxidase, 1
U/ml cholesterol oxidase, and 1 U/ml cholesterol esterase for 30 min at
37°C. Fluorescence was measured using a SpectraMax M5 micro-
plate reader ( Molecular Devices), and values were normalized by
retinal tissue protein concentration determined using a BCA protein
assay (Pierce/Thermo Scientific, Rockford, IL). Cholesterol levels
were compared with a standard curve of 0–8 μg/ml cholesterol
activated by the Amplex Red assay kit (R² = 0.992). We performed
the assay for each condition as a triplicate of triplicates: retina halves
were harvested from three different animals, and each sample was
assayed in triplicate.

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rishige USA, East Meadow, NY) from borosilicate glass pipettes (1.2-mm outer diameter, 0.9-mm inner diameter, with an internal filament; World Precision Instruments, Sarasota, FL) with tips 2 μm in diameter and resistance values between 12 and 18 MΩ. Both recording electrodes were positioned using Huxley-Wall micromanipulators (Sutter Instruments, Novato, CA). Cones were voltage-clamped at -70 mV using an Optopatch amplifier (Cairn Research, Faversham, UK), and second-order neurons were voltage-clamped at -60 mV with an AxoPatch 200B amplifier (Molecular Devices). Both amplifiers were controlled using an Axon Instruments Digidata 1322 interface and pClamp 9.2 software (Molecular Devices). We selected cones that required modest holding currents (<200 pA) to voltage clamp the cell at the steady holding potential of ~70 mV. The cone pipette solution contained (in mM) 40 cesium glutamate, 50 cesium gluconate, 9.4 tetraethylammonium chloride (TEACl), 3.5 NaCl, 1 CaCl2, 1 MgCl2, 9.4 MgATP, 0.5 GTP, 5 EGTA, and 10 HEPES (pH 7.2). The presence of glutamate in the patch pipette maintains vesicle release and postsynaptic responses for up to 20 min after the initial whole cell rupture into a photoreceptor (Bartoletti et al. 2010). We measured cone synaptic output by recording glutamatergic EPSCs in horizontal cells with a pipette solution that contained (in mM) 90 cesium gluconate, 10 TEACl, 3.5 NaCl, 1 CaCl2, 1 MgCl2, 10 MgATP, 0.5 GTP, 5.0 EGTA, and 10 HEPES (pH 7.2). Vesicle release was elicited by applying a strong depolarizing voltage step to cone photoreceptors from -70 to -10 mV for 100 ms using a P/8 leak subtraction protocol. The liquid junction potential was estimated from the calculator in pClamp 9.2 to be -13 mV for the cone pipette solution and ~16 mV for the horizontal cell pipette solution. As a vehicle control for cholesterol depletion experiments, we recorded from cones and second-order neurons after treating retina slices with 1:100 dilution of 95% ethanol-amphibian saline solution. These experiments showed no detectable change in cone CaV current (I_Ca, n = 7) or EPSCs in horizontal cells (n = 5) compared with control pairs.

We calculated the number of CaV channel openings contributing to I_Ca and the number of miniature EPSCs (mEPSCs) contributing to EPSCs by deconvolution as described in Bartoletti et al. (2011). Deconvolution of P/8 leak-subtracted I_Ca was performed using OriginPro 8 software (North Hampton, MA). To deconvolve single-channel events during I_Ca, we evoked a step to -10 mV, we used a single CaV channel amplitude of 0.3 pA with decay time constant of 1.1 ms to represent variable channel open times (Bartoletti et al. 2010; Thoreson et al. 2000). The number of channel openings was scaled for an average of 13 ribbons per cone (Bartoletti et al. 2010; Pang et al. 2008). EPSCs were deconvolved using an average mEPSC waveform with amplitude of 5.7 pA and charge transfer of 15.5 pC (Cadetti et al. 2008). Output from a single ribbon contributes ~46 pA to the peak amplitude of the horizontal cell EPSC evoked by a step to -10 mV applied to a cone (Bartoletti et al. 2010). The number of ribbon contacts between a cone and postsynaptic horizontal cell was therefore estimated by dividing the peak amplitude of the EPSC by 46 pA/ribbon. Release events were shifted forward by 300 μs to compensate for the latency to the beginning of the EPSC caused by an instantaneous rise in Ca²⁺ concentration (Duncan et al. 2010). Results of deconvolution were low-pass filtered using a fast-Fourier transform filter with 5–10 points of smoothing. The rate of CaV channel openings was divided by the rate of release events to calculate the number of vesicle fusion events per channel opening at each time point.

Exocytotic capacitance changes were measured in cones using established protocols (Bartoletti et al. 2010; Johnson et al. 2002; Rahl et al. 2005). For these experiments, the shaft near the tip of each recording electrode was lightly coated with dental wax (Integra-Millex, Plainsboro, NJ) to reduce stray capacitance artifacts. Capacitance measurements were made using the “track-in” mode of the Optopatch amplifier by varying membrane potential sinusoidally (500–600 Hz, 30 mV peak to peak) about the holding potential. Series resistance and membrane capacitance were compensated electronically. Track-in feedback gain was increased to its highest stable value. The amplitude of the cone capacitance response was measured 20 ms after the end of the track step to avoid gating charges and to allow time for the phase angle feedback circuitry to settle. We excluded recordings that showed pronounced poststimulus changes in series resistance.

To examine the responses of postsynaptic glutamate receptors independent from presynaptic photoreceptors, we bath-applied 0.5 mM 4-methoxy-7-nitroindoliny1-L-glutamate (MINI-glutamate; Tocris Biosciences, Ellisville, MO) and uncaged glutamate by flash photolysis using UV light derived from a xenon arc flash lamp (JML-C2 Flash Lamp System; Rapp OptoElectronic, Hamburg, Germany). Light flashes (17.5-μm diameter) centered on the horizontal cell were delivered through a quartz fiber optic mounted to the epifluorescence port of the microscope.

mEPSCs were analyzed using MiniAnalysis 6.0.7 (Synaptosoft, Decatur, GA). mEPSCs were detected by the software using an amplitude threshold of 1 pA, and each event was then evaluated individually. If necessary, the length of preceding baseline period was adjusted to improve the amplitude measurement. Double peaks were analyzed using an algorithm within Synaptosoft that extrapolates the exponential decay of the first peak.

Modeling. Vesicle release was modeled using Mathematica (Wolfram Research, Champaign, IL) as described by Bartoletti et al. (2011). When control conditions were simulated, the immediately releasable pool of 20 vesicles was arranged in a square grid with 50-nm center-to-center spacing on the surface of a 250-nm-long ribbon in two rows of 5 vesicles on either side. Fifty-six CaV channels were distributed randomly on the plasma membrane. The plasma membrane, intended to approximate the observed morphology of the cone ribbon synapse (Lasansky 1973; Ravila and Gilula 1975) was constructed as an evaginating arc with 100-nm radius centered at the ribbon apex, giving way to further invaginating arcs on both sides of the ribbon with a 200-nm radius. The range over which channels were distributed was increased from 100 to 150 or 220 nm to simulate effects of COase or MβCD, respectively, on CaV channel domain size. A simulated step to ~10 mV opened CaV channels to a maximal open probability of 0.35, opening in time according to the equation P_o = 0.35 × t/(t + 5 ms). Randomly chosen CaV channels opened for 1.1 ms. Time-dependent Ca²⁺ gradients were modeled in increments of 100 ms, with release binned into 1.1-ms intervals to reflect CaV channel openings (Ait-Haddou et al. 2010). The domains of all open channels were summed linearly. The Ca²⁺ dependence of release was R = 3,634(Ca²⁺)^2/2 μM + [Ca²⁺]^2/2 (Duncan et al. 2010). The K_d for BAPTA and EGTA was 170 nM with K_m for BAPTA of 6 × 10^6 M/s and K_m for EGTA of 9 × 10^6 M/s. The diffusion coefficient for Ca²⁺ was 220 μm²/s, and the diffusion coefficient for Ca²⁺ buffers was 20 μm²/s. We simulated the presence of a diffusion barrier by slowing the rate of Ca²⁺ diffusion and the concentration of diffusible buffer by 1:200. We also examined in the impact of reducing the strength of the diffusion barrier beyond the arciform density to 1:100. Each simulation was repeated for 1,000 trials.

Statistical analysis. Data were compiled in Prism 4 (GraphPad Software, La Jolla, CA) and are presented as means ± SE. Statistical significance between experimental conditions was determined using Student’s t-test. P values <0.05 were considered significant.

RESULTS

Synaptic cholesterol localization and quantification of membrane cholesterol content. Ultrastructural studies by Cooper and McLaughlin (1984) showed evidence of lipid rafts at the margins of the ribbon-style active zone of photoreceptor synapses in the chick retina. To test whether cholesterol levels were also elevated in the presynaptic membranes of salamander cones, we used filipin III (300 μg/ml for 1 h) to stain...
isolated cones for cholesterol. As expected for ubiquitous distribution of cholesterol throughout the membrane, filipin III stained the entire cell (Fig. 1A). However, staining was more intense in the synaptic region at the base of the soma (arrows), consistent with enrichment of cholesterol in the terminal. The presence of cholesterol-rich lipid rafts was further indicated by strong staining of the synaptic terminal region with FITC-CTXB (Fig. 1B), which selectively labels lipid raft ganglioside GM1 subunits (Obrocki and Borroni 1988; Yamamoto et al. 2005). Untreated cones showed only autofluorescence in the inner segment and no fluorescent signal at the synaptic terminal. Dendrites of isolated bipolar cells did not show FITC-CTXB staining (data not shown), consistent with an absence of lipid rafts on postsynaptic membranes in the outer plexiform layer (OPL) (Cooper and McLaughlin 1984). These data show that FITC-CTXB staining of the OPL observed in salamander retina (Mercer et al. 2011b) was primarily due to staining of the synaptic plasma membrane of rods and cones and confirm that cholesterol is enriched in lipid rafts at the cone ribbon synapse (Cooper and McLaughlin 1984).

Cholesterol depletion caused a reduction in staining by FITC-CTXB in the OPL, suggesting a reduction in cholesterol-rich lipid rafts (Mercer et al. 2011b). We quantified changes in whole retina membrane cholesterol content using the Amplex Red cholesterol assay kit. In control retinas, cholesterol averaged 12.6 ± 0.69 ng cholesterol/μg protein. This level dropped to 4.94 ± 0.52 ng cholesterol/μg protein (40.9% of control; P < 0.0001) after cholesterol depletion with MβCD. COase also caused a decrease in total retina cholesterol, diminishing the level to 7.18 ± 0.66 ng cholesterol/μg protein (57.6% of control; P < 0.0001). Repleting MβCD-treated tissue with an MβCD-cholesterol complex restored cholesterol levels to 11.3 ± 0.41 ng cholesterol/μg protein (91.2% of control; P = 0.19). Although we could not measure membrane cholesterol levels specifically in cones, these results show that pharmacological treatment with MβCD or COase produced significant changes in whole retina membrane cholesterol (Fig. 1E).

CaV channel mobility vs. synaptic cholesterol depletion. Experiments using single-particle tracking techniques to visualize individual QD-labeled CaV channels at cone synapses in the retinal slice preparation have shown that channels normally remain close to vesicle release sites, but depleting membrane cholesterol with MβCD enlarges the surface area traversed by an individual channel (Mercer et al. 2011b). To compare release efficiency with changes in CaV channel domain size, we measured changes in the size of the confinement domain in isolated cones using a high-NA (1.45) objective to provide high spatial resolution. Consistent with data obtained from retinal slices using a 1.0-NA objective (Mercer et al. 2011b), single-particle tracking experiments on CaV channels at the synapses of dissociated control cones (n = 15) showed that CaV channels are normally confined to a limited surface area of 0.18 ± 0.000005 μm², but depletion of membrane cholesterol with MβCD (n = 10) expanded the confinement area to 0.88 ± 0.00014 μm² (P = 0.0032 vs. control). Expansion of the confinement area is evident in individual examples of QD trajectory plots and was quantified from the asymptotic level of MSD (Fig. 2). The average confinement areas (L²) determined using Eq. 2 are plotted in the bar graphs in Fig. 2B. To test whether cholesterol depletion also increased the mobility of the presynaptic ribbon complex, we labeled ribbons by patch

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clamping isolated cones and introducing a Hylite 488-labeled peptide that binds to the ribbon protein, ribeye (Mercer et al. 2011b; Zenisek 2008). The small movements of the ribbon did not increase significantly in cholesterol-depleted cells (control: \( L^2 = 0.048 \pm 0.0004 \, \mu m^2, n = 10; \) MβCD: \( L^2 = 0.054 \pm 0.0004 \, \mu m^2, n = 10; \) \( P = 0.67 \)), indicating that increases in synaptic Ca\(_V\) channel mobility are independent of the ribbon complex.

MβCD can have other effects in addition to cholesterol depletion (Zidovetzki and Levitan 2007). Consistent with expansion of the confinement domain being primarily due to cholesterol depletion, repleting membrane cholesterol by applying a complex of MβCD and cholesterol after initially depleting cholesterol with MβCD restored the confinement area to a smaller size of \( 0.26 \pm 0.000015 \, \mu m^2 \) not significantly different from control (\( P = 0.17 \); Fig. 2). We also depleted cholesterol using the enzyme COase. Consistent with a smaller effect on retinal cholesterol levels than MβCD, COase caused a smaller expansion of the confinement area to \( 0.37 \pm 0.000020 \, \mu m^2 \) (\( n = 11, P = 0.014 \); Fig. 2). These results show that depletion of synaptic cholesterol increased the confinement area for Ca\(_V\) channels and that the normal confinement area can be restored by replacing membrane cholesterol.

Effects of membrane cholesterol on neurotransmission. Ultrastructural and electrophysiological data indicate that Ca\(_V\) channels normally remain within \( 50-100 \, \mu m \) of the release machinery at cone synapses (Mercer et al. 2011a; tom Dieck et al. 2005). By allowing Ca\(_V\) channels to move further away from release sites, we predicted that an expanded confinement area would reduce the efficiency with which Ca\(_V\) channel openings stimulate vesicle release. We calculated the change in efficiency between Ca\(_V\) channel opening and fusion by determining the number of individual Ca\(_V\) channel openings and synaptic release events during simultaneous recordings of cone I\(_{Ca}\) and horizontal cell EPSCs (Fig. 3). Cones were voltage-clamped at \(-70 \, mV\) and depolarized to \(-10 \, mV\) for 100 ms to activate I\(_{Ca}\) and stimulate exocytosis of the entire immediately releasable pool of \(~20 \) vesicles per ribbon (Bartoletti et al. 2010). As shown in Fig. 3A, the peak of the horizontal cell EPSC was attained quickly, often before the I\(_{Ca}\) attained its peak amplitude. As described in METHODS, we determined the rate of Ca\(_V\) channel openings by linear deconvolution of cone I\(_{Ca}\), assuming a single-channel current of \( 0.3 \, pA\) with a mean open time of 1.1 ms and 13 ribbons per cone. We determined the number of quantal release events by linear deconvolution of the horizontal cell EPSC, using an average mEPSC waveform and scaling for the number of ribbon contacts estimated from the size of the EPSC (Bartoletti et al. 2010, 2011). As illustrated in Fig. 3B, release efficiency (i.e., the number of vesicle release events associated with a single Ca\(_V\) channel opening) rose and declined quickly as the releasable pool of vesicles was emptied. With depolarizing test steps to \(-10 \, mV\), release from cones attained a maximal efficiency of \( 0.32 \pm 0.029 \) vesicle fusion events per Ca\(_V\) channel opening per ribbon (\( n = 20; \) \( ~3 \) channel openings per fusion event). In retinal slices treated with MβCD, peak fusion efficiency declined significantly to

![Fig. 2. Depletion of synaptic cholesterol increased the confinement domains of individual voltage-gated Ca\(_{2+}\) (Ca\(_V\)) channels. A: mean squared displacement (MSD) calculated using Eq. 1 plotted against time interval. Data were fit with Fig. 2 to determine the size of the confinement area (\( L^2 \)). B: bar graph showing the average confinement areas. Ca\(_V\) channels at the synapses of enzymatically dissociated cones (\( n = 15; \) black squares in A) were confined to a membrane surface area of \( 0.18 \, \mu m^2 \) (control). Depletion of membrane cholesterol with MβCD (\( n = 10; \) white triangles in A) or COase (\( n = 11; \) black diamonds in A) significantly increased the confinement area to \( 0.88 \, \mu m^2 \) (\( **P = 0.003 \) vs. control) and \( 0.37 \, \mu m^2 \) (\( *P = 0.014 \) vs. control), respectively. Repleting membrane cholesterol with the MβCD-cholesterol complex (\( n = 9; \) white circles in A) after an initial MβCD treatment reduced the confinement area to \( 0.26 \, \mu m^2 \) (\( P = 0.17 \) vs. control). C–F: examples of trajectory plots of quantum dot (QD)-labeled Ca\(_V\) channels in isolated cones from different conditions: control (C), MβCD treated (D), COase treated (E), and cholesterol repleted (F).](http://jn.physiology.org/innerimage/2898874.png)
0.22 ± 0.026 fusion events per channel opening (n = 13, P = 0.028; Fig. 3, D and E; ~4.5 channel openings per fusion event). Depletion of cholesterol with COase also caused a significant reduction in fusion efficiency to 0.21 ± 0.033 fusion events per channel opening (n = 9, P = 0.035; Fig. 3E). Repletion of cholesterol restored release efficiency to 0.26 ± 0.030 fusion events per channel opening (n = 12, P = 0.21 compared with control; Fig. 3E). There were no statistically significant differences in cone I_Ca amplitude, EPSC amplitude, EPSC half-width, or EPSC time to peak in any of the treatment conditions (Table 1).

To examine the effects of CaV channel domain size on release further, we simulated release at the cone synapse using a model that incorporates known physiological and anatomic properties of the synapse (Bartoletti et al., 2011). As illustrated in Fig. 3F, the ribbon synapse was modeled as an evaginating arc with 100-nm radius centered at the ribbon apex, giving way above the arcing form density to invaginating arcs of 200-nm radius along the flanks of the ribbon. Fifty-six CaV channels were distributed randomly on the plasma membrane beneath the immediately releasable pool of 20 vesicles positioned in two rows on either side of a 250-nm-long ribbon. To replicate limited effects of diffusible chelators on release efficiency, the model assumed a diffusion barrier that slows the diffusion of Ca^{2+} and Ca^{2+} chelators by a factor of 1/200. In control conditions with 5 mM EGTA as the diffusible buffer, the model predicted a peak efficiency of 0.28 fusion events per CaV channel opening, similar to the measured efficiency of 0.32 fusion events per channel opening. To mimic the expansion of CaV channel domain size caused by COase treatment, we expanded the region within which CaV channel openings and vesicle release events.

Table 1. Properties of I_Ca and PSCs after cholesterol manipulation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>MJCD</th>
<th>COase</th>
<th>Repletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cone I_Ca</td>
<td>141 ± 10.2</td>
<td>145 ± 10.0</td>
<td>134 ± 20.7</td>
<td>145 ± 19.8</td>
</tr>
<tr>
<td></td>
<td>(P = 0.80)</td>
<td>(P = 0.87)</td>
<td>(P = 0.87)</td>
<td>(P = 0.83)</td>
</tr>
<tr>
<td>EPSC amplitude</td>
<td>111 ± 17.2</td>
<td>74.5 ± 10.5</td>
<td>100.0 ± 19.3</td>
<td>94.6 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>(P = 0.11)</td>
<td>(P = 0.72)</td>
<td>(P = 0.72)</td>
<td>(P = 0.51)</td>
</tr>
<tr>
<td>EPSC half-width</td>
<td>12.6 ± 1.8</td>
<td>10.2 ± 1.4</td>
<td>15.2 ± 2.8</td>
<td>14.6 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>(P = 0.33)</td>
<td>(P = 0.09)</td>
<td>(P = 0.09)</td>
<td>(P = 0.20)</td>
</tr>
<tr>
<td>EPSC time to peak</td>
<td>8.8 ± 0.8</td>
<td>8.0 ± 0.6</td>
<td>11.0 ± 2.7</td>
<td>7.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>(P = 0.46)</td>
<td>(P = 0.46)</td>
<td>(P = 0.11)</td>
<td>(P = 0.39)</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>15</td>
<td>9</td>
<td>12</td>
</tr>
</tbody>
</table>

Data are means ± SE, and measurements were compared with control conditions using unpaired Student’s t-test (P values). To test whether cholesterol depletion by methyl-β-cyclodextrin (MJCD) affected other properties of release besides the latency of postsynaptic current (PSC) responses, we measured the peak amplitude of cone voltage-gated Ca^{2+} current (I_Ca) and a number of PSC parameters: peak amplitude, half-width of the PSC waveform, and excitatory PSC (EPSC) time to peak. COase, cholesterol oxidase.
the predicted peak efficiency to 0.12 fusion events per channel opening. However, this version of the model assumed that the diffusion barrier extended unchanged into the cytoplasm beyond the arciform density. Incorporating the plausible assumption that the influence of the diffusion barrier diminished as channels stayed beyond the arciform density (by reducing the diffusion barrier from 1:200 to 1:100 in the light gray region above the dashed line in Fig. 3F) improved model predictions to 0.21 and 0.18 fusion events per channel opening with channel confinement regions of 150 and 220 nm, respectively.

Consistent with earlier results (Bartoletti et al. 2011), we found that replacing 5 mM EGTA with 1 mM BAPTA in the cone pipette solution did not significantly alter peak release efficiency (0.30 ± 0.036 fusion events per channel opening per ribbon, n = 16). With BAPTA as the diffusible chelator, cholesterol depletion with MβCD diminished release efficiency to 0.22 ± 0.033 fusion events per channel opening (n = 10). With 1 mM BAPTA, the model predicted a peak efficiency of 0.31 fusion events per channel opening. Expanding the CaV channel confinement region to 220 nm to simulate MβCD treatment reduced efficiency to 0.18 fusion events per channel opening. Incorporating a reduction in the strength of the diffusion barrier from 1:200 to 1:100 beyond the arciform density increased this predicted efficiency from 0.18 to 0.22 fusion events per channel opening.

In addition to studying release in paired recordings, we also examined exocytosis from cones using capacitance techniques to measure the increase in surface area accompanying vesicle fusion (Bartoletti et al. 2010; Rabl et al. 2005). The time constant for release of the rapidly releasable pool of vesicles is ~3–5 ms (Rabl et al. 2005; Thoreson et al. 2004), so we predicted that MβCD should have a bigger effect on exocytosis evoked by short 5-ms test steps than longer 8-ms test steps. Consistent with this prediction, significantly less vesicle release was evoked by a short 5-ms test pulse after membrane cholesterol depletion (Fig. 4A; control: 143 ± 19.6 fF, n = 6; MβCD: 48.4 ± 18.5 fF, n = 5; P = 0.0072), whereas exocytotic capacitance jumps evoked by an 8-ms test pulse were similar in both conditions (Fig. 4B; control: 130 ± 14.8 fF, n = 10; MβCD: 100 ± 23.5 fF, n = 10; P = 0.27).

It has been reported that MβCD treatment can impair clathrin-mediated exocytosis (Rodal et al. 1999; Subtil et al. 1999). To measure endocytosis, we analyzed the time required for recovery of membrane capacitance following release. In control cones, the time required for the depolarization-evoked capacitance increase to decay by 50% averaged 195 ± 62.1 ms (n = 5) following 5-ms steps and 215 ± 34.5 ms (n = 6) following 8-ms steps. The time required for a 50% decline in the capacitance increase was similar after MβCD treatment, averaging 143 ± 34.6 ms (n = 8; P = 0.46 vs. control) for 5-ms steps and 240 ± 44.8 ms (n = 9; P = 0.70 vs. control) following 8-ms steps. These results suggest that the rapid endocytosis observed in cones was not significantly affected by synaptic cholesterol depletion and is consistent with other evidence that this mechanism is probably not clathrin-dependent at ribbon-style synapses (LoGuidice and Matthews 2007; Wu et al. 2007).

Cholesterol depletion can alter AMPA receptor recycling (Glodowski et al. 2007; Hering et al. 2003), so we tested whether glutamate receptor currents in horizontal cells were altered by cholesterol depletion with MβCD. To elevate glutamate rapidly and stimulate inward AMPA receptor currents, we photolytically liberated glutamate from MNI-glutamate (0.5 mM) by applying a bright UV flash to the OPL while recording from horizontal cells (Rabl et al. 2006). Glutamatergic currents in control neurons reached an average amplitude of 187.1 ± 72.1 pA (n = 5), whereas MβCD-treated horizontal cells exhibited currents with an average amplitude of 216.5 ± 69.3 pA (n = 5; P = 0.999; Table 2). In addition, the time constants for current decay did not differ significantly between control (τdecay = 36.8 ± 11.8, n = 5) and MβCD-treated neurons (τdecay = 21.0 ± 6.02, n = 5; P = 0.27; Table 2). These findings suggest that MβCD treatment did not significantly alter the number or single-channel conductance of glutamate receptors in the postsynaptic membrane, induce glutamate receptor desensitization, or disrupt glutamate reuptake.
Table 2. Effects of cholesterol depletion on glutamatergic currents

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MβCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flash current amplitude, pA</td>
<td>187 ± 72.1 (n = 5)</td>
<td>217 ± 69.3 (n = 5) (P = 0.999)</td>
</tr>
<tr>
<td>Flash decay time constant, ms</td>
<td>36.8 ± 11.8 (n = 5)</td>
<td>21.0 ± 6.02 (n = 5) (P = 0.27)</td>
</tr>
<tr>
<td>mEPSC amplitude, pA</td>
<td>4.40 ± 0.47 (n = 6 cells, 75.2 ± 24.0 mEPSCs/cell)</td>
<td>5.51 ± 1.86 (n = 4 cells, 89.0 ± 18.6 mEPSCs/cell) (P = 0.50)</td>
</tr>
<tr>
<td>mEPSC frequency, Hz</td>
<td>119 ± 11.9</td>
<td>125 ± 18.9 (P = 0.81)</td>
</tr>
<tr>
<td>mEPSC half-width, ms</td>
<td>1.79 ± 0.20</td>
<td>1.47 ± 0.26 (P = 0.35)</td>
</tr>
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</table>

Data are means ± SE. P values for comparisons with control conditions were determined using unpaired Student’s t-test. To examine potential postsynaptic effects of cholesterol depletion, we recorded currents evoked by photolyzed 4-methoxy-7-nitroindolinyl-L-glutamate (MNI-glutamate) and miniature EPSCs (mEPSCs) from horizontal cells voltage-clamped at a holding potential of ~60 mV. We found no significant differences in the amplitude or decay of glutamatergic currents evoked by UV photolysis of caged glutamate. We also found no differences in the amplitude, frequency, or half-width of mEPSCs in control vs. MβCD-treated conditions (P > 0.05 for all parameters).

As a further test of possible effects of MβCD treatment on postsynaptic responses, we analyzed individual mEPSCs and found no significant differences in the amplitude (pA), half-width (ms), or frequency of mEPSCs between control and MβCD-treated retinas (Table 2). Together, these data suggest that cholesterol perturbation alters EPSCs principally by presynaptic actions at cone terminals.

DISCUSSION

The present results show that cholesterol depletion increases the mobility of CaV channels and that this increase in mobility is accompanied by an increase in the number of CaV channel openings associated with release of a single synaptic vesicle at the cone ribbon synapse. Under control conditions, fusion of a single synaptic vesicle at the peak of release is accompanied by the opening of ~3 CaV channels (Bartoletti et al. 2011). After expansion of the CaV channel confinement domain by cholesterol depletion, ~4.5 channel openings were associated with the fusion of each vesicle. These data suggest that when CaV channels spend more time far from ribbon release sites, a greater number of channel openings are needed to guarantee that [Ca2+] rises high enough at release sites to trigger vesicle fusion. In addition to less efficient coupling between channel opening and fusion, we consider below four other possible effects of cholesterol depletion that might cause an apparent reduction in release efficiency.

1) The first effect is changes in quantal amplitude. Treatment with cholesterol-depleting agents could potentially reduce mEPSCs, and thus alter the results of deconvolution, by inhibiting vesicular glutamate transport (Tarasenko et al. 2010) or by allowing postsynaptic receptors to diffuse away from the active zone (Fernandes et al. 2010; Hering et al. 2003; Pinaud et al. 2009; Renner et al. 2009). However, we found no significant change in the amplitude or kinetics of mEPSCs. We also found no changes in the amplitude or kinetics of glutamatergic currents evoked by flash photolysis of caged glutamate. These data suggest that changes in quantal amplitude were not responsible for the apparent change in release efficiency at cone synapses. The finding that exocytotic capacitance increases stimulated by brief 5-ms steps were inhibited by cholesterol depletion also support a presynaptic site of action. The absence of significant postsynaptic effects of cholesterol depletion is consistent with the observation that cholesterol is not a major component of postsynaptic membranes in horizontal cell dendrites (Cooper and McLaughlin 1984).

At nonribbon synapses, depletion of cholesterol has been shown to enhance Ca2+-independent spontaneous release but to diminish evoked release of vesicles (Linetti et al. 2010; Wasser et al. 2007; Zamir and Charlton 2006). At the cone ribbon synapse, we observed no significant change in the amplitude of EPSCs following cholesterol depletion with MβCD or COase. There was also no significant increase in the frequency of mEPSCs in horizontal cells. However, it is worth noting that the vesicle pools that participate in spontaneous and evoked release at cone synapses have not been distinguished experimentally, and many of the spontaneously occurring mEPSCs in horizontal cells are likely to reflect Ca2+-triggered release from neighboring photoreceptor cells with relatively depolarized resting membrane potentials.

2) Changes in single CaV channel properties could potentially produce an apparent change in efficiency by altering the number of channel openings calculated by deconvolution of I_Ca. However, we did not observe a significant change in L-type I_Ca at cone synapses following cholesterol depletion. This contrasts with evidence that cholesterol depletion alters Ca2+ influx through CaV1.3 and CaV2.1 channels (Purcell et al. 2011; Taverna et al. 2004), perhaps because of differences in the type of CaV channels expressed at cone synapses (primarily CaV1.4; Zanazzi and Matthews 2009).

3) A large reduction in the affinity of Ca2+ for the release sensor could potentially diminish release efficiency, but a large reduction in Ca2+ sensitivity would also significantly slow the EPSC time to peak (Duncan et al. 2010), and this was not observed. Small changes in Ca2+ sensitivity would be unlikely to cause significant changes in release efficiency, because Ca2+ levels attained at the release site are thought to exceed 20 μM, nearly two orders of magnitude above release threshold (Thoreson et al. 2004).

4) Cholesterol depletion might influence release efficiency by directly altering SNARE protein function. Although we cannot exclude this possibility, the changes in release efficiency predicted by the model from an expansion of CaV channel domains were sufficient to explain observed reductions in synaptic efficiency. The predictions of the model were especially good if we assumed that the impact of the diffusion barrier declined as channels moved beyond the arciform density. This is consistent with the idea that properties of Ca2+ diffusion within the synaptic evagination differ from those of the bulk cytoplasm (Bartoletti et al. 2011). Taken together, the most parsimonious conclusion of these different experiments is that domain size expansion is the main cause of changes in synaptic efficiency produced by cholesterol depletion.

CaV channel mobility was studied by tracking the movements of individual QDs. Although it is possible for individual
QDs and antibodies to cross-link with multiple CaV channels, we used very low dilutions of all three reagents to produce sparse labeling and minimize this possibility (Bannai et al. 2006). We chose a primary antibody that labels the large extracellular domain of the α2δ2 accessory CaV channel subunit. This subunit is required for proper trafficking of the channel to the membrane and appears to remain associated with the pore-forming subunit (Bauer et al. 2010). Trajectory values showed a unimodal distribution (Mercer et al. 2011b), consistent with the idea that individual QDs are bound to single channels and that α2δ2-subunits rarely, if ever, dissociate from the channel. CaV channels can be endocytosed under certain conditions (Cristofanilli et al. 2007), but the amplitude of ICa did not differ significantly in control vs. treated conditions, suggesting that the number of endocytosed channels was not significantly altered by cholesterol depletion.

To examine the effects of cholesterol depletion on neurotransmission, many studies employ drugs from the cyclodextrin class to chelate and deplete membrane cholesterol. Cyclodextrins are cyclic oligosaccharides composed of six to eight dextrose molecules that complex with cholesterol and remove it from a lipid bilayer (Irie and Uekama 1997; Stella and He 2008) but also can have other effects (Zidovetzki and Levitan 2007). Enzymatically oxidizing membrane cholesterol (Churchward and Coorsen 2009; Puglielli et al. 2005) can target cholesterol in a more specific manner. Cholesterol depletion with both MβCD and cholesterol oxidase reduced retinal cholesterol levels (Fig. 1E), increased CaV channel confinement area (Fig. 2), and reduced synaptic efficiency (Fig. 3). Furthermore, effects of MβCD could be reversed by bathing MβCD-treated tissue with a complex of cholesterol and MβCD to restore cholesterol to the membrane (Abulrob et al. 2005). Together, these results indicate that the observed changes in CaV channel mobility and synaptic efficiency were mainly due to cholesterol depletion and not other effects of MβCD.

Cholesterol-rich lipid rafts are enriched around the margins of the active zone at photoreceptor ribbon synapses (Cooper and McLaughlin 1984). The present results provide further evidence that cholesterol is localized to cone photoreceptor terminals and that depletion of membrane cholesterol enhances the lateral mobility of synaptic L-type CaV channels (Mercer et al. 2011b). P/Q-type (CaV2.1) channels but not L-type CaV1.2 channels appear to accumulate in lipid rafts at central nervous system (CNS) synapses (Davies et al. 2006; Taverna et al. 2004). L-type channels in photoreceptor terminals (Zanazzi and Matthews 2009) may also therefore be unlikely to accumulate in lipid rafts. The presence of membrane cholesterol tends to promote overall membrane fluidity, but removal of cholesterol lipid rafts can nevertheless enhance the mobility of integral membrane proteins by removing barriers to free diffusion (Renner et al. 2009). A removal of lipid rafts surrounding the active zone may explain the expansion of CaV channel confinement areas at photoreceptor synapses caused by cholesterol depletion.

Although the CNS accounts for only 2% of the body’s mass, it contains ~25% of the body’s total unesterified cholesterol (Chattopadhyay and Pailla 2007; Dietschy and Turley 2001). Much of the cholesterol in the CNS is found in glial cells and myelin (Krämer et al. 2001; Saher et al. 2005), but synapses and synaptic vesicles also contain cholesterol (Churchward and Coorsen 2009). The present results provide further evidence that cholesterol-rich lipid rafts are important for clustering CaV channels close to synaptic proteins at the presynaptic active zone (Davies et al. 2006; Gil et al. 2004; Lang et al. 2001; Salaün et al. 2005; Taverna et al. 2004, 2007; Xia et al. 2007). Changes in presynaptic CaV channel mobility might also contribute to cholesterol-dependent modulation of release at other synapses.

Cholesterol biosynthesis and trafficking defects contribute to various forms of neurodegeneration, including retinal degeneration (Liu et al. 2010). Smith-Lemli-Opitz syndrome (SLOS) is the most common pathology of cholesterol biosynthesis (Waterham 2006) and involves a defect in 7-dehydrocholesterol reductase activity (Fliesler 2010; Kretzer et al. 1981). Human patients with SLOS exhibit deficits in rod but not cone pathways (Elia et al. 2003; Garry et al. 2010). A rat model of SLOS created by disrupting cholesterol biosynthesis showed electrophysiological defects in both rod and cone pathways that could be partially ameliorated by a high-cholesterol diet (Fliesler et al. 2004, 2007). Lipid metabolism defects have also been reported to affect electrotetinogram (ERG) b-waves in a rare form of retinitis pigmentosa (Aguirre et al. 1997; Nevet et al. 2010; Zangerl et al. 2006), and impaired cholesterol influx in the lysosomal storage disease Niemann-Pick type C results in retinal degeneration and reduced ERG a- and b-waves (Claudepierre et al. 2010; Phillips et al. 2008). In addition to its location at the ribbon synapse, cholesterol also helps to partition proteins in photoreceptor outer segments (Albert and Boesze-Battaglia 2005; Andrews and Cohen 1983; Niu et al. 2002; Senin et al. 2004; Wang et al. 2008). Changes in the ERG b-wave caused by disorders of cholesterol metabolism appear to be largely due to changes in photoreceptor light responses (Fliesler and Bretteillon 2010), but the present results suggest that changes in synaptic release from cones due to changes in CaV channel mobility might also contribute to visual deficits. Changes in the membrane mobility of CaV channels or other synaptic proteins could also contribute to neurological disorders in other tissues accompanying genetic or metabolic changes that diminish cholesterol levels.

The present results indicate that increases in presynaptic CaV channel mobility caused by cholesterol depletion can alter synaptic strength by increasing the average distance between release sites and CaV channels. Changes in CaV channel mobility caused by other mechanisms, such as disruption of the actin cytoskeleton (Mercer et al. 2011b), might also change synaptic strength. Interactions between transient receptor potential (TRP) channels and the cytoskeleton can be dynamically regulated (Liu et al. 2011), raising the possibility that perhaps cytoskeletal interactions with CaV channels (Cristofanilli et al. 2007; Gregory et al. 2011; Zhang et al. 2005) might likewise be dynamically regulated. The fusion of synaptic vesicles causes a brief expansion of CaV channel confinement area (Mercer et al. 2011b), suggesting that the size of the confinement area, and thus release efficiency, might also be affected by changes in the balance between exocytosis and endocytosis. Changes in presynaptic CaV channel mobility may thus provide a general mechanism for regulating synaptic strength.

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The distribution of filipin-sterol complexes in salamander retinal neurons.


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