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Supporting Material

Roles of Cholesterol in Vesicle Fusion and Motion

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MATERIALS AND METHODS

Amperometry measurement

Exocytosis was induced by superfusing the high K^+ solution or cholesterol containing (2 μM) bath solution through a glass micropipette (tip size of 2~3 μm) positioned ~10 μm away from the cell. Amperometric spikes were recorded by a 5 μm-in-diameter carbon fiber electrode (ALA Scientific Instruments, Westbury, NY) gently positioned onto the cell membrane to avoid diffusion-induced distortion in the signals. The carbon fiber tip was cut to expose a fresh surface before every recording to ensure consistent sensitivity of the electrode. The microelectrode was biased at 700 mV and the amperometry signals were sampled at 4 kHz and filtered at 1 kHz using an EPC-10 double patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany).

The amperometric signals were analyzed using an Igor (WaveMetrics, Lake Oswego, OR) program, Amperometric Spike Analysis 8.15, developed by Dr. Eugene Mosharov (Department of Neurology, Columbia University). Only spikes with amplitudes larger than 2 pA (10 times of the background noise) were considered as true amperometric events and quantified. For foot analysis, only the spikes with foot amplitude > 0.4 pA (2 times of the noise) were considered as true footed-events. Statistics of the spike parameters are calculated as mean ± standard error (SE) and analyzed by Kolmogorov-Smirnov test which is suitable for skewed distributions as assumed by the amperometric parameters (1).

TIRFM imaging

Cells were seeded on high refractive-index coverslip and transfected with 2 μg of NPY-EGFP plasmid (a kindly gift from Dr. Wolf Almers, Vollum Institute, Oregon Health Sciences University) using FuGENE 6 Transfection Reagent (Roche Diagnostics GmbH, Germany), 1-2 days prior to the experiments. Time-lapse digital images were collected at 2 Hz from an inverted total internal reflection fluorescence microscope (Axiovert 200, Carl Zeiss, Germany) equipped with an oil-immersed 100× objective (NA=1.45, Carl Zeiss, Germany) and a CCD camera with pixel size of 0.248 μm. Individual vesicle motion was tracked using Image J (National Institute of Health, Wayne Rasband, USA), and analyzed by Igor routines.

Cell culture and solutions

PC12 cells (ATCC, Manassas, VA) were cultured in advanced RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 5% horse serum, 1% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO_2. PC12 cells were planted on 0.1 mg/ml poly-L-lysine pre-coated coverslips and treated with 5 μM dexamethasone for 3-5 days to enhance neuroendocrine cell phenotype as previously described (2, 3). The bath solution contained (in mM, titrated to pH 7.2 with NaOH): 150 NaCl, 2.4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES. The high K^+ stimulation solution contained (in mM, titrated to pH 7.2 with NaOH): 40 NaCl, 105 KCl, 6 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES. Cholesterol was dissolved in 100% ethanol as stock. In some

experiments, cholesterol was added to the bath solution (final concentration of 2 μM) to stimulate exocytosis. All chemicals were purchased from Sigma (St. Louis, MO).

DEPLETION, REPLETION, AND MEASUREMENT OF MEMBRANE CHOLESTEROL CONTENT

Membrane cholesterol was depleted by pre-incubating cells with 5 mM methyl-β-cyclodextrin (MβCD) for 30 minutes at 37 ºC followed by rinsing. Reduction of cholesterol content was also achieved by culturing cells in medium containing lipoprotein deficient serum (LPDS, Sigma) instead of fetal bovine serum for 4-6 days prior to the experiment (4). In some experiments, membrane cholesterol was sequestered using cholesterol-binding reagent – filipin (5). Specifically, cells were incubated with filipin (4 μg/ml) in dark for 15 minutes at room temperature followed by thorough rinsing before imaging.

Membrane cholesterol can be replenished after MβCD treatment using cholesterol-saturated MβCD (6). To prepare MβCD-cholesterol complex, 5% MβCD aqueous solution was heated to 80ºC, and cholesterol dissolved in chloroform:methanol (1:2) was added drop-wise till the solution was clear to reach MβCD:cholesterol molar ratio of 9.78:1. Water was removed subsequently by evaporation in vacuum for one hour, and the resulting film of MβCD-cholesterol complex was dissolved in water as stock. Cholesterol loading was realized by incubating cells in the bath solution containing 5 mM MβCD-cholesterol complex for one hour at 37 ºC. Analysis of the cholesterol content of the plasma membrane was carried out by centrifuging cells at 4000 rpm for 5 minutes at room temperature, And lysis of cell precipitate with complete RIPA cell lysis buffer (pH 7.4, Santa Cruz, CA) at 4 ºC. The cell lysate was centrifuged for 5 minutes at 500*g* (4 ºC) to remove nuclear fragments, and the supernatant was then centrifuged at 40,000*g* for 30 minutes (4 ºC). The resulting membrane pellet was dissolved in 50 mM Tris buffer (pH 7.4) to final concentration of 0.1 mg/μl. Finally, cholesterol content was determined using the Amplex Red Cholesterol Assay Kit (Invitrogen, CA) and a Flx 800 fluorometer (BioTek, VT).

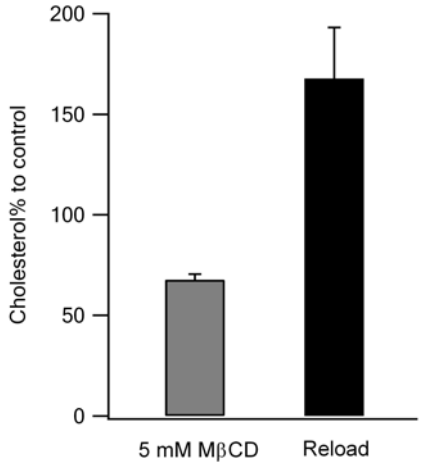

FIGURE S1 Cholesterol level of 5 mM MβCD treated and cholesterol replenished cells (normalized to the control cells from the same passage). Cholesterol was reduced to 67.7 ± 2.8% after 5 mM MβCD application, and it was elevated to 167.6 ± 25.4%) when cholesterol was supplemented by cholesterol loaded MβCD. The statistics was from 3 cell cultures.

CHOLESTEROL DEPLETION DID NOT AFFECT CELL VIABILITY, MORPHOLOGY AND ADHESION

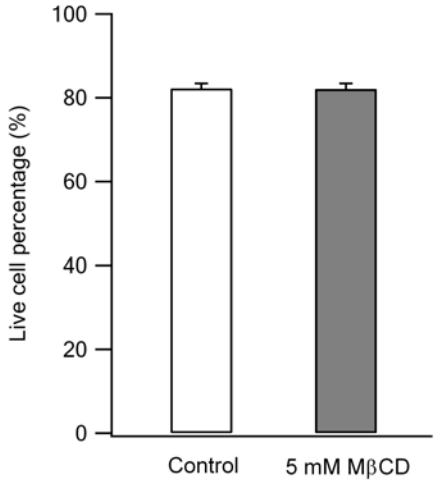

FIGURE S2 Trypan blue assay of viability of the control and 5 mM MβCD-treated cells (cells are from the same passage). MβCD treatment didn't show negative effects comparing to un-treated cells (82.1 ± 1.3% vs. 82.2 ± 1.2%, $p > 0.05$). The statistics was from 3 cell cultures.

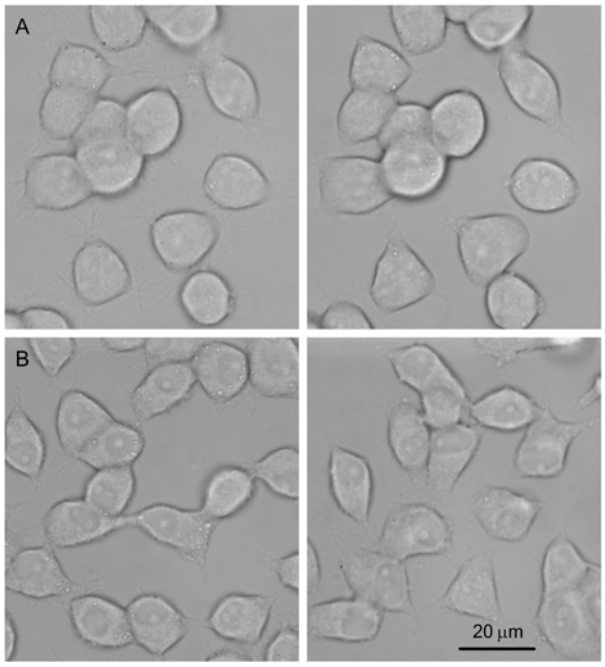

FIGURE S3 Cholesterol depletion does not obviously affect cell morphology and adhesion. (A) Bright-field images of the same PC12 cells before (left) and after 5 mM MβCD treatment (right). (B) Bright-field images of PC12 cells cultured in FBS medium (left) and LPDS medium (right).

LOCAL DELIVERY OF BATH SOLUTION OR ETCHANOL DID NOT EVOKE AMPEROMETRIC RESPONSES

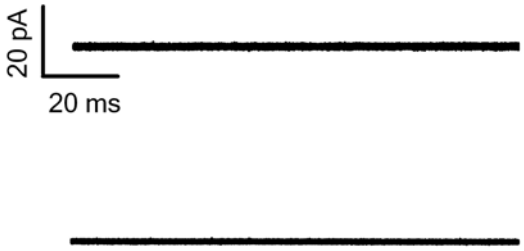

FIGURE S4 Carbon fiber recordings while superfusing bath solution (top) or bath solution containing ethanol as vehicle control (bottom) onto PC12 cells.

PRE-SPIKE FOOT ARE REDUCED BY CHOLESTEROL DEPLETION

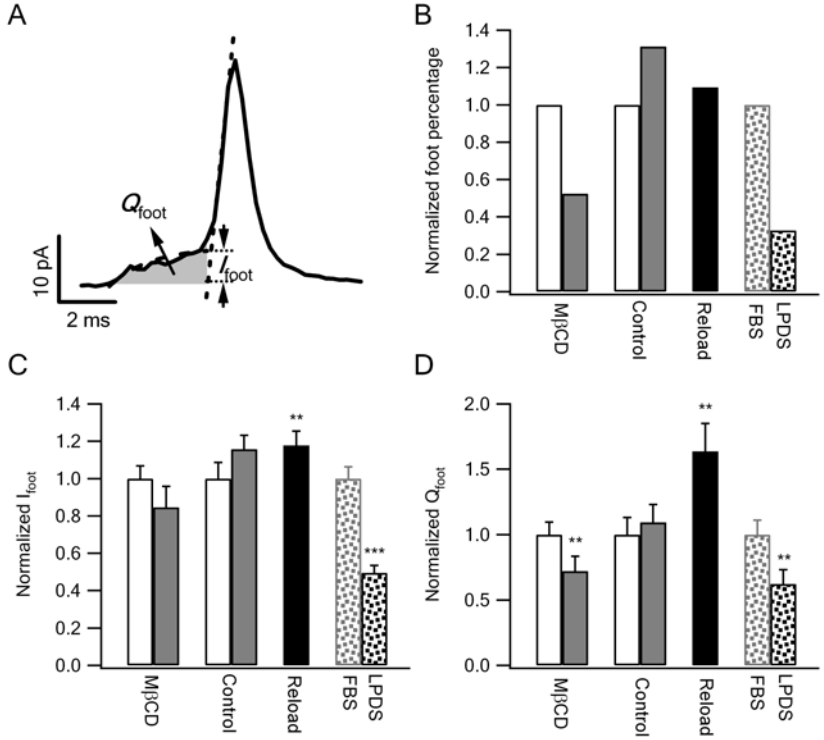

FIGURE S5 Cholesterol depletion reduces the pre-spike foot signal. Analyses are based on the same data set presented in Fig. 1 and 2. (A) An example amperometric signal with pre-spike foot. The duration of the foot segment (t_{foot}) starts from the time point when the current signal exceeds the baseline noise and ends at the time point where the linear extrapolation of the main-spike rising phase intercepts with the baseline (dark dashed line). Q_{foot} is the charge integral of the foot segment. I_{foot} is the approaching plateau value determined by exponential fitting of the foot signal. (B), (C) and (D) Normalized percentage of footed-event, foot amplitude, and foot charge. In 5 mM MβCD protocol, the foot percentage decreased from 30.2% to 15.8%; I_{foot} decreased from 1.56 ± 0.11 pA to 1.32 ± 0.18 pA; Q_{foot} decreased from 6.45 ± 0.62 fC to 4.65 ± 0.74 fC (the first paired columns; normalized to the first exocytotic response; 211 footed-events in the first response and 44 footed-events in the second response from 14 cells). In the control protocol, the foot percentage increased from 24.1% to 36.1%;

I_{foot} increased from 1.43 ± 0.12 pA to 1.65 ± 0.11 pA; Q_{foot} increased from 6.73 ± 0.88 fC to 7.36 ± 0.93 fC (the second paired columns; normalized to the first exocytotic response; 120 footed-events in the first response and 158 footed-events in the second response from 11 cells). When cholesterol was supplemented to the depleted cells foot percentage, I_{foot} and Q_{foot} were recovered to normal (146 events from 17 cells: 33.0%, 1.83 ± 0.12 pA, and 10.56 ± 1.38 fC). In comparison between cells cultured in FBS (232 events from 15 cells) and LPDS (38 events from 15 cells), the reduction in the percentage and the size of foot signal by metabolic depletion of cholesterol was more prominent: (27.6% to 9.0%, 1.42 ± 0.089 pA to 0.70 ± 0.056 pA, 6.66 ± 0.74 fC to 4.14 ± 0.73 fC). $^{**}p < 0.01$, $^{***}p < 0.001$.

LOCAL MICROPIPETTE DELIVERY OF CHOLESTEROL LOADED MβCD ALSO INDUCED AMPEROMETRIC RESPONSE SIMILAR TO PERFUSION OF FREE CHOLESTEROL

In the experiments similar to Fig. 4, cholesterol loaded MβCD (0.5 mM in bath solution) was locally delivered onto individual PC12 cells through an application pipette with a tip size > 5 μm while amperometric responses were monitored. It was found that, similar to adding free cholesterol, cholesterol loaded MβCD was also able to induce amperometric responses, but to a much less extent and with slow release kinetics as compared to high K^+ stimulation. These results further support the notion that addition of negatively curved cholesterol molecules can disturb the delicate energetic balance of the semi-stable hemifusion structure and causes the transition from hemifusion to "spontaneous" full fusion.

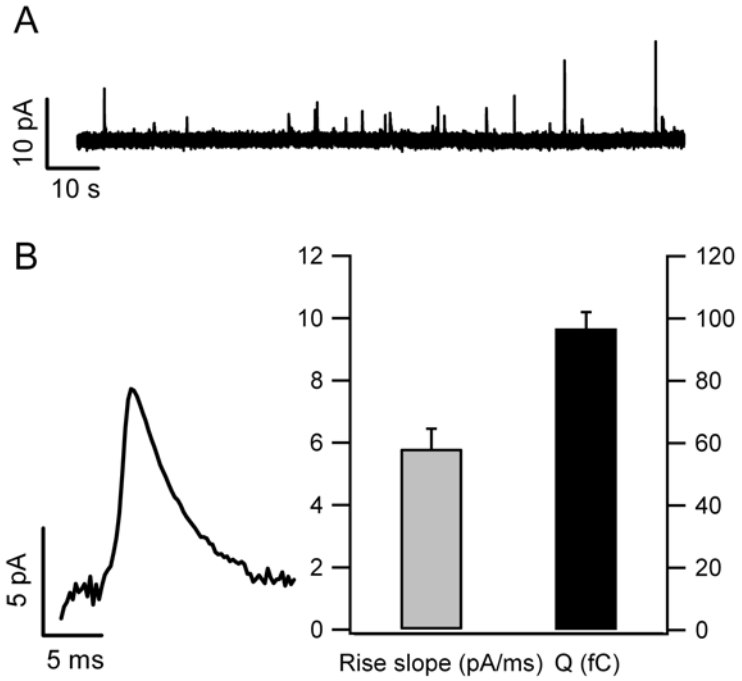

FIGURE S6 Amperometric response induced by cholesterol loaded MβCD. (A) A typical amperometric recording in response to MβCD-cholesterol stimulation. (B) The average amperometric spike averaged from the all the responses from 186 spikes from 17 MβCD-cholesterol stimulated cells (left). The evoked response is much less compared to high K^+

stimulation (10.9 ± 2.7 spikes/cell, $p < 0.001$ *vs.* high K^+ stimulation). The rise slope and quantal size of MβCD-cholesterol stimulated amperometric spikes are 5.81 ± 0.65 pA/ms and 96.87 ± 5.18 fC, respectively (right). As compared to the high K^+ stimulation, MβCD-cholesterol stimulation, similar to free cholesterol stimulation, resulted in slower rise slope ($p < 0.001$) and larger quantal size ($p < 0.001$).

References:

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