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Cholesterol-mediated membrane surface area dynamics in neuroendocrine cells

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ABSTRACT

How cholesterol, a key membrane constituent, affects membrane surface area dynamics in secretory cells is unclear. Using methyl-β-cyclodextrin (MβCD) to deplete cholesterol, we imaged melanotrophs from male Wistar rats in real-time and monitored membrane capacitance (C_m), fluctuations of which reflect exocytosis and endocytosis. Treatment with MβCD reduced cellular cholesterol and caused a dose-dependent attenuation of the Ca^{2+}-evoked increase in C_m (IC_{50} = 5.3 mM) vs. untreated cells. Cytosol dialysis of MβCD enhanced the attenuation of C_m increase (IC_{50} = 3.3 mM), suggesting cholesterol depletion at intracellular membrane sites was involved in attenuating exocytosis. Acute extracellular application of MβCD resulted in an immediate C_m decline, which correlated well with the cellular surface area decrease, indicating the involvement of cholesterol in the regulation of membrane surface area dynamics. This decline in C_m was three-fold slower than MβCD-mediated fluorescent cholesterol decay, implying that exocytosis is the likely physiological means for plasma membrane cholesterol replenishment. MβCD had no effect on the specific C_m and the blockade of endocytosis by Dyngo 4a, confirmed by inhibition of dextran uptake, also had no effect on the time-course of MβCD-induced C_m decline. Thus acute exposure to MβCD evokes a C_m decline linked to the removal of membrane cholesterol, which cannot be compensated for by exocytosis. We propose that the primary contribution of cholesterol to surface area dynamics is via its role in regulated exocytosis.

1. Introduction

Among lipids with roles in regulated exocytosis, cholesterol has often been reviewed [1,2]. A major membrane component, cholesterol and its metabolites are also implicated in other processes, including the modulation of membrane protein function, membrane trafficking, trans-membrane signaling and steroid hormone synthesis [3]. Most (80–90%) cellular cholesterol is located at the plasma membrane [4], and in endocytic and trans-Golgi compartments [5]. It is also enriched in exocytic vesicles, comprising up to 40 mol% of total lipids [6]. Due to its abundance and physical properties, cholesterol is an important factor in the regulation of membrane fluidity/rigidity [7,8] and spontaneous curvature [1]. Changes in these properties can affect membrane protein functions [9] as well as membrane fusion and its sensitivity to local $[Ca^{2+}]_{free}$ [10]. Lipid rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains (reviewed in [2,11–13]), known to concentrate specific exocytosis-related proteins, such as SNAREs [14], G-proteins [15] and voltage-gated Ca^{2+} channels (VGCCs) [16,17]; these may thus serve as sites for vesicle tethering/docking/priming and membrane fusion [14,18]. Moreover, lipid rafts seem to cluster annexin-2, a putative actin network plasma membrane anchor [19,20], implicating actin network in the distribution and formation of the lipid rafts [21]. Microtubules assembly also appears to depend on lipid rafts [22,23]. As a key raft-organizing molecule [24], depletion of cholesterol from membranes or inhibition of its synthesis results in raft disruption [25–27], altering SNARE clustering [26,28], affecting the function of VGCCs [28,29] and disrupting actin membrane anchors [30], thereby possibly interfering with secretory vesicle trafficking; this and the likely disruption of other critical protein functions lead to the attenuation of exocytosis [2,31–34]. Cholesterol also appears essential for clathrin-mediated [35,36] and caveolae-mediated endocytosis [37] as well as massive calcium-activated endocytosis (MEND) [38]. However,

Abbreviations: MβCD, methyl-beta-cyclodextrin; C_m, cell capacitance; G_a, access conductance; G_m, membrane conductance; SNARE, soluble NSF attachment protein receptor; VGCC, voltage-gated Ca^{2+} channels; MEND, massive calcium-activated endocytosis; GM_1, ganglioside GM_1

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it has been reported that cholesterol depletion may also stimulate endocytosis [39]. Thus it is unclear how cholesterol depletion affects total membrane area, which is determined by both exocytosis and endocytosis. An ideal way to study the effect of cholesterol depletion at the cellular level is to monitor membrane area directly. Here we used whole-cell patch-clamp technique to monitor membrane capacitance (C_m), a parameter linearly related to membrane area [40], and monitored cell morphology in real-time by microscopy. Thus, for the first time, in live cells, using a combination of electrophysiology, imaging, and quantitative cholesterol assessments – including real-time measures of changes in plasma membrane cholesterol levels – we have determined that the primary contribution of cholesterol to native surface area dynamics in a secretory cell is via its roles in regulated exocytosis.

2. Materials and methods

2.1. Materials

Methyl-β-cyclodextrin (MβCD) was from Sigma-Aldrich (St. Louis, MO, USA). Membrane raft labeling kit (Vybrant® Alexa Fluor), cholesterol assay kit (Amplex® Red), Vybrant® DiD cell-labeling solution and Alexa Fluor® Dextran 488 were from Invitrogen (San Diego, CA, USA). Fluorescent cholesterol (TopFluor Cholesterol) was from Avanti Polar Lipids (Alabaster, AL, USA) and Dyngo 4a was kindly supplied by Prof. P.J. Robinson (CMRI, Sydney, Australia). All other chemicals used were of highest purity available. Extracellular solution (ECS) contained (in mM): 130 NaCl, 5 KCl, 8 $CaCl_2$, 10 D-glucose and 10 HEPES (N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid) at pH 7.2 (with NaOH). Intracellular solution (ICS) contained (in mM): 150 KCl, 2 $MgCl_2$, 1.74 $CaCl_2$, 2 Na_2ATP, 2 EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid) and 10 HEPES at pH 7.2 (with KOH). MβCD was prepared as a 30 mM stock solution and further diluted to indicated concentrations (Fig. 2C). EGTA and Ca^{2+}-saturated EGTA were prepared as 100 mM stock solutions, as previously described [41]. The intracellular $[Ca^{2+}]_{free}$ was estimated assuming an apparent dissociation constant for the Ca–EGTA complex of 1 μM.

2.2. Animals

The work was performed on primary melanotroph cultures isolated from 30 to 60 day-old Wistar rats. The care of the experimental animals was in accordance with the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and Directive on Conditions for Issue of License for Animal Experiments for Scientific Research Purposes (Official Gazette of the RS, No. 40/85 and 22/87). The research was conducted in conformity with PHS policy and approved by the Veterinary administration of the Republic of Slovenia.

2.3. Cell cultures

Melanotroph primary cultures were prepared by a standard method [42] with modifications. Pituitaries were obtained after decapitation from male Wistar rats and enriched for melanotrophs. Pars intermedia region was physically removed from the pituitary and enzymatically disintegrated by collagenase 3.5 mg/ml (Gibco Life Technologies, Grand Island, New York, USA). Cell covered poly-L-lysine-treated coverslips were kept in the incubator at 37 °C, 95% humidity and in a 5% CO_2 atmosphere for 1–4 days before experimentation. Cell culture purity for melanotrophs was 88 ± 1%, determined by immunocytochemistry (not shown).

2.4. Total cell cholesterol determination

The total cell cholesterol (with cholesterol esters) was determined using the Amplex® Red Cholesterol Assay Kit (Invitrogen, San Diego, CA, USA) according to manufacturer's instructions. Melanotrophs were prepared as described above; however no serum or serum substitutes were used. Prior to the experiment, cell count was determined (~2.5×10^5 per sample). Samples (2 for every group) were treated with various MβCD solutions (Supplemental Fig. S1) for 30 min at 37 °C in 5% CO_2 atmosphere. Subsequently, samples were washed and centrifuged twice at 450 ×*g* for 5 min, and the supernatant was discarded. Then the samples were resuspended in 150 μl of Celytic™ M (Sigma-Aldrich, St. Louis, MO, USA) and shaken at 37 °C for 15 min, which resulted in cell lysis. Samples and standard solutions (with known cholesterol concentrations) were mixed with 300 μM Amplex® Red reagent (containing Amplex® Red reagent stock, horseradish peroxidase, cholesterol oxidase, cholesterol esterase and reaction buffer — PBS) and incubated for 60 min at 37 °C in 5% CO_2 atmosphere. The resulting fluorescence was measured with Anthos Zenyth 3100 fluorescence microplate reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) using excitation at 530 nm and fluorescence detection at 590 nm. Each sample was measured thrice. The results were averaged and background fluorescence was subtracted. Measurements from standard solutions were plotted and second-order polynomial curve was fitted ($y = -0.14x^2 + 11.93x$), from which the total cell cholesterol concentration of the samples was calculated (Supplemental Fig. S1).

2.5. Lipid raft labeling and confocal microscopy

Lipid rafts were labeled according to manufacturer's instructions (Vybrant® Alexa Fluor® 488 Lipid Raft labeling kit, Invitrogen, San Diego, CA, USA). Cell loaded coverslips were treated with various MβCD solutions (Fig. 1, Supplemental Fig. S2) for 30 min at 37 °C in 5% CO_2 atmosphere. Then they were washed with PBS and labeled with fluorescent CT-B conjugate for 10 min at 4 °C and washed again. Subsequently CT-B-labeled membrane rafts were cross-linked with the anti-CT-B antibodies, fixed with 4% paraformaldehyde, mounted using Slow Fade Gold protocol (Invitrogen, San Diego, CA, USA) and placed into recording chamber on an inverted confocal microscope (LSM 510 Meta, Zeiss, Jena, Germany). Fluorescence images were acquired with a plan-apochromatic oil-immersion objective (×63, 1.4 NA) using 488-nm Ar-Ion laser excitation line and filtered with 505-nm LP emission filter (Fig. 1).

2.6. Plasma membrane lipid raft abundance determination

GM_1-positive lipid raft abundance was calculated by drawing 20-px wide band connecting fluorescence labels in a circular shaped ROI (ImageJ, NIH, USA) on the cell periphery (Fig. 1C, Supplemental Fig. S2). This was subsequently used to determine fluorescence signal profile (Fig. 1D). Fluorescence signal threshold was determined by using an iterative selection method built into ImageJ (IsoData protocol) [43] and was used to calculate the % of the length of ROI, occupied by lipid rafts (signal above the threshold). A 20-px wide ROI was used to overlay the plasma membrane, thus it was considered that the % of length of the ROI, occupied by lipid raft corresponded with the percent of the plasma membrane, occupied by lipids rafts in a given optical slice, disregarding the membrane foldings.

2.7. Membrane capacitance measurements

Cell loaded coverslips were transferred into the recording chamber mounted onto an inverted microscope (IM 35, Opton, West Germany) in the recording medium (ECS). The cells were then voltage clamped at −70 mV [40] and a sine wave voltage (1591 Hz, 1.11 mV RMS) was