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FLIM studies of 22- and 25-NBD-cholesterol in living HEK293 cells: Plasma membrane change induced by cholesterol depletion

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ABSTRACT

HEK293 cells stably expressing δ-opioid receptor were labeled first with fluorescent analog of cholesterol, 22-NBD-cholesterol, exposed to cholesterol-depleting agent β-cyclodextrin (β-CDX) and analyzed by fluorescence lifetime imaging microscopy (FLIM). In accordance with chemical analysis of cholesterol level, the total cellular signal of this probe was decreased to half. Distribution of lifetime (τ_{tot}) values of 22-NBD-cholesterol, however, when screened over the whole cell area indicated no significant difference between control ($\tau_{tot} = 4.9 \pm 0.1$ ns) and β-CDX-treated ($\tau_{tot} = 4.8 \pm 0.1$ ns) cells. On the contrary, comparison of control ($\tau_{tot} = 5.1 \pm 0.1$ ns) and β-CDX-treated ($\tau_{tot} = 4.4 \pm 0.1$ ns) cells by analysis of 25-NBD-cholesterol fluorescence implied highly significant decrease of lifetime values of this probe. The observation that 22-NBD-cholesterol appears to be indifferent to the changes in the membrane packing in living cells is in agreement with previous studies in model membranes. However, our data indicate that the alternation of plasma membrane structure induced by decrease of cholesterol level by β-CDX makes the membrane environment of NBD moiety of 25-NBD-cholesterol probe a significantly more hydrated. This finding not only encourages using 25-NBD-cholesterol in living cells, but also demonstrates that previously drawn discouraging conclusions on the use of 25-NBD-cholesterol in model membranes are not valid for living cells.

1. Introduction

Cholesterol constitutes a major component of mammalian plasma (cell) membrane. Its correct distribution among plasma membrane and intracellular membrane compartments is essential for the homeostasis of mammalian cells (Maxfield and Wustner, 2002). Additionally, its lateral and trans-bilayer organization in the plasma membrane determines membrane structure and dynamics (Yeagle, 1985). It is also a crucial constituent of membrane domains characterized by high content of saturated phospholipids, glycolipids and sphingomyelin which are capable to harbor and confine numerous signaling molecules (Babiychuk and Draeger, 2006; Brown and London, 2000; Oh and Schnitzer, 2001) containing high amounts of trimeric G proteins and playing an important role in both positive and negative regulation of trans-membrane signaling through G protein-coupled receptors (GPCR) (Huang et al., 2007; Rahangdale et al., 2006; Xu et al., 2006). We have recently proved that the removal of cholesterol by the depletion with β-cyclodextrin markedly impaired the ability of ≃-opioid receptor (DOR) agonist DADLE to activate G proteins in isolated plasma membranes, while it did not alter the agonist binding site of DOR (Brejchova et al., 2011). The cholesterol depletion in parallel strongly influenced the membrane organization as documented by the Laurdan generalized polarization and DPH anisotropy. As a further step, we intended to characterize the direct changes in the distribution and organization of cholesterol molecules in living cells upon cholesterol depletion employing fluorescence microscopy.

For this purpose, a range of the fluorescent cholesterol analogs were considered (Gimpl and Gehrig-Burger, 2011; Wustner, 2007).

Abbreviations: β-CDX, β-cyclodextrin; CTL, cholestatrienol; DADLE, D-Ala2-D-Leu5 enkephalin; DHE, dehydroergosterol; DMEM, Dulbecco's modified Eagle's medium; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOR, ≃-opioid receptor; DOR-$G_i1\alpha$ cells, HEK293 cells stably expressing DOR-$G_i1\alpha$ (Cys^{351}-Ile^{351}) fusion protein; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; FLIM, Fluorescence lifetime imaging; GPCR, G-protein-coupled receptor; HEK293, human embryonic kidney cell line; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; 22-NBD-cholesterol, 22-{*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]amino}-23,24-bisnor-5-cholen-3-ol; 25-NBD-cholesterol, 25-{*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl] amino}-27-norcholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PM, plasma (cell) membrane; PMSF, phenylmethylsulfonyl fluoride; PNS, post-nuclear supernatant; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PTX, pertussis toxin; T_m, melting temperature; TRH-R, thyrotropin-releasing hormone receptor; TCSPC, Time Correlated Single Photon Counting.

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These molecules bear the fluorophore either at 3β-OH of cholesterol or attached to the side alkyl chain of the sterol. Since the hydroxyl group is essential for the cholesterol functioning we decided to utilize the analogs with the latter approach having the NBD chromophore attached at positions 22 (22-NBD-cholesterol) and 25 (25-NBD-cholesterol). These analogs were thoroughly investigated in the model membranes yielding rather ambiguous conclusions (Chattopadhyay and London, 1987, 1988; Loura and Ramalho, 2011; Ramirez et al., 2010; Scheidt et al., 2003). It was shown that the fluorophore affected dramatically positioning of the NBD-cholesterols in phospholipid bilayers having the sterol moiety oriented up-side-down compared to cholesterol as suggested by NMR (Scheidt et al., 2003). In contrast, earlier study utilizing the parallax quenching proved the 25-NBD cholesterol is deeply buried in the membrane (Chattopadhyay and London, 1987). Even more confusion has been brought by a recent theoretical study proposing the analogs lie parallel along the membrane with the OH and NO_2 groups oriented toward the interface (Loura and Ramalho, 2011). Obviously, controversial conclusions on the probe orientation were reported. Further findings disqualifying the 22-NBD-cholesterol and 25-NBD-cholesterol from being ideal cholesterol analogs are lower ordering ability compared to cholesterol (Scheidt et al., 2003) and significantly reduced partitioning in the cholesterol rich liquid ordered phases (Loura et al., 2001). These conclusions discourage the use of those membrane labels, but were drawn on the basis of data obtained solely on the model systems and no attempts were made to characterize comprehensively the behavior of these dyes in living cells. As pointed out by Pike (2009), the extrapolation of results from model membrane systems to cell membrane may be misleading since the proteins and peptides as well as the lipid trafficking modulate the separation of the lipid phases in the cell plasma membrane. Indeed, recent works on the giant plasma membrane vesicles (GPMVs) prove that the order differences between two separating phases in the natural membranes are much lower than in the model giant unilamellar vesicles (GUVs) (Kaiser et al., 2009; Sezgin et al., 2012). Therefore, we decided to investigate the effect of cholesterol depletion on plasma membrane structure in intact living HEK293 cells by fluorescence lifetime imaging (FLIM) of 22- and 25-NBD-cholesterol. These probes were incorporated into cells under in vivo conditions. The results reveal the suitability and sensitivity of these cholesterol analogs for the membrane studies altering the cholesterol content directly in cells. The outcome of this study is encouraging to use 25-NBD-cholesterol in living cells and demonstrates that conclusions previously drawn on model membranes might not be valid for living cells.

2. Materials and methods

2.1. Chemicals

Complete protease inhibitor cocktail was purchased from Roche Diagnostic, Mannheim, Germany (1697498). 22-NBD-cholesterol (N-1148) and 25-NBD-cholesterol (810250P) were from Invitrogen (Molecular Probes) and from Avanti Polar Lipids, respectively. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and cholesterol were also purchased from Avanti Polar Lipids.

2.2. Cell culture

HEK293 cells stably expressing DOR-$G_i1\alpha$ fusion protein were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% newborn calf serum and 2 mM (0.292 g/l) L-glutamine in humidified 5% CO_2–95% air atmosphere at 37 °C as described by Moon et al. (2001) and Bourova et al. (2003). Geneticin (800 μg/ml) was included in the course of cell cultivation. The cells were grown to 60–80% confluency before harvesting.

2.3. Treatment of HEK293 cells with β-cyclodextrin

Cells were either treated (+β-CDX) or untreated (−β-CDX) with 10 mM β-cyclodextrin in serum-free DMEM for 60 min at 37 °C. Harvesting of cells was performed by centrifugation for 10 min at 300 × *g*. The cell sediment was snap frozen in liquid nitrogen and stored at −80 °C until use.

2.4. Determination of cholesterol level

The cell homogenate or isolated plasma membranes were prepared from the sub-confluent cultures (70–80%) of cells as described by Brejchova et al. (2011) and cholesterol content was determined by an enzymatic method using an Amplex Red cholesterol kit (Molecular Probes, Eugene, OR).

2.5. Labeling of HEK293 cells with 22- and 25-NBD-cholesterol

Cells were grown on LabTek chambered cover-glasses (Nunc 155411) coated with 0.1% poly-L-lysine at 37 °C and 5% CO_2. 22- and 25-NBD-cholesterol were dissolved in pure ethanol at 10 mg/ml, filtered to remove remaining precipitate and actual concentration was determined by spectroscopy at 465 nm (extinction coefficient 22 000 l mol^{-1} cm^{-1} (Haugland, 2002)). This solution was added to HEK293 cell cultures in serum-free DMEM (10^{-4} M final concentration) and incubated for 30 min at 37 °C in 5% CO_2. Medium was then removed, the cells rinsed three times with serum-free DMEM and then treated with β-CDX as described above or left in serum-free DMEM. After the treatment, medium was replaced with colorless DMEM.

2.6. Confocal laser scanning microscopy and FLIM studies of HEK293 cells labeled with 22-NBD-cholesterol in 2-photon microscope

After labeling, the cells were put on microscope stage and fluorescence images were taken using TCS SP2 AOBS Leica confocal microscope. For excitation of 22-NBD-cholesterol, the 488 nm Ar–Kr laser was used and emission was detected in 500–600 nm range. When performing FLIM studies, the cells were grown, attached to the glass support and labeled with 22-NBD-cholesterol in the same way. After insertion on microscope stage, the distribution of lifetime values was determined. Becker & Hickl Simple-Tau 150 TCSPC system connected to TCS SP2 AOBS Leica confocal microscope was used and 22-NBD-cholesterol was excited by 2-photon excitation of IR laser Chameleon Ultra at wavelength of 770 nm. Detection period lasted 5 min. The data were evaluated by SPCImage software as a single exponential decay.

2.7. Fluorescence lifetime and anisotropy imaging of living HEK293 cells labeled with 22- and 25-NBD-cholesterol

Confocal laser scanning microscopy (CLSM) was also carried out in confocal microscope MicroTime 200 (PicoQuant, Germany). The laser light emitted by pulsed diode lasers (LDH-P-C-470, 470 nm) with a 20 MHz repetition rate was focused into the sample with a water immersion objective (1.2 NA, 60×). Laser power lower than 5 μW, was chosen to minimize the photobleaching and saturation. The excitation and emission light was separated by dichroic mirror 505DRLP (Omega Optical, USA) and the fluorescence was guided into detection channel equipped with band-pass filter HQ 515/50 (Omega Optical, USA) and polarization beamsplitter (Ealing, UK), which divides the photons on two SPCM-AQR-14 detectors (Perkin Elmer, Canada) connected to single photon counting system Picoharp 300 (PicoQuant, Germany). The decay curves were then reconstructed for each pixel enabling the determination of