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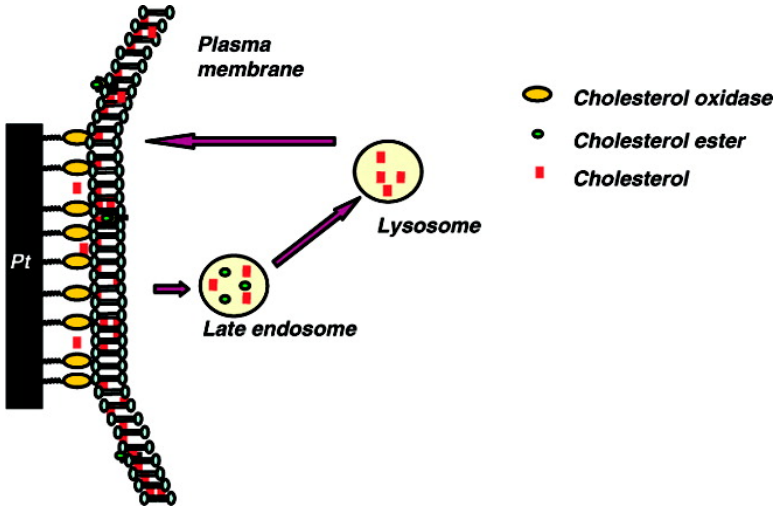

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Direct Electrochemical Evaluation of Plasma Membrane Cholesterol in Live Mammalian Cells

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The plasma membrane is the largest subcellular cholesterol pool and complex trafficking pathways exist within cells to maintain this distribution.[1] Common methodologies for cellular cholesterol analysis such as fluorescent labeling do not yield precise determination of the physiological cholesterol content of the plasma membrane (ca. 800 mM) relative to that of internal compartments. Furthermore, the isolation of subcellular compartments is prone to contamination, and cholesterol may get transferred between compartments during separation.[2] This lack of spatial resolution in traditional cholesterol analysis of subcellular compartments has led to questions regarding how plasma membrane cholesterol is affected under conditions of altered intracellular cholesterol transport. This Communication reports microelectrodes that allow direct real time measurements of plasma membrane cholesterol content in live mammalian cells at physiological temperature. The measurements are rapid and localized and thus do not significantly perturb the total amount of cholesterol in the plasma membrane.

A state-or-the-art assay to estimate plasma membrane cholesterol is the method developed by Rothblat and co-workers where cellular radio-labeled cholesterol is removed by a cyclodextrin solution.[3] Although there is a question as to what cellular cholesterol pools are removed, Rothblat has reported that the initial rate of cellular cholesterol efflux (within ca. 15 s) can be assigned to that from the plasma membrane. Variations of this method have been implemented without regard to this stipulation. Estimates of plasma membrane cholesterol have also been obtained by exposure of cells to cholesterol oxidase solution. A concern with this method is that the enzyme may gain access to internal cholesterol through endocytosis, membrane breakage, and movement of cholesterol during the assay.[2] Our direct electrochemical measurements of plasma membrane cholesterol are localized to the cell surface and require only about 5−10 s thus addressing these caveats.

We recently reported electrochemical detection of cholesterol in the lipid bilayer membrane of a giant vesicle[4] and at the surface of a single oocyte[5] at room temperature using a Pt microelectrode (10 μm diameter) modified with a lipid bilayer membrane containing cholesterol oxidase. This surface architecture is not stable at 37 °C. Here we report direct covalent attachment of cholesterol oxidase to Pt microelectrodes (4 μm diameter) to achieve a surface structure that is stable at physiological temperatures (Supporting Information).[6] Placing the electrode surface in contact with the plasma membrane (Figure 1a) results in enzymatic oxidation of cholesterol at the cell surface. The plateau current increase observed in the first several seconds upon positioning the electrode in contact

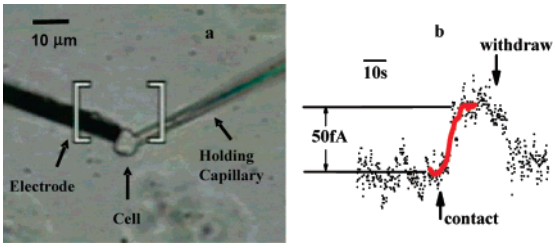

Figure 1. Electrochemical analysis of cell plasma membrane cholesterol: (a) optical photograph showing contact of a captured macrophage with a microelectrode; (b) representative (as collected) amperometric data (dotted line) for contacting (↑) a single macrophage cell with an enzyme modified Pt microelectrode for 20 s and withdrawing (↓) the microelectrode from the contact position. The solid line is smoothed data indicating electrode response on contact.

with the cell surface (for example, Figure 1b) is believed to be limited by the rate of enzymatic catalysis and is proposed to be a measure of plasma membrane cholesterol content. Control experiments using bare Pt electrodes containing no immobilized enzyme do not show oxidative responses (Supporting Information).

Our model for electrochemical detection of plasma membrane cholesterol invokes the aqueous diffusion mechanism proposed by Rothblat for cellular cholesterol efflux to solution phase acceptors such as lipoproteins, vesicles, and cyclodextrin.[7] The aqueous diffusion mechanism is based on studies that show a maximum plateau in the rate of cellular cholesterol efflux with increasing acceptor concentration.[7] The zero-order dependence of efflux on acceptor concentration (at high acceptor concentration) indicates that the rate of cholesterol removal from the plasma membrane is independent of collision frequency between acceptor and the plasma membrane. Thus, movement of cholesterol out of the plasma membrane and solvation in the aqueous phase is proposed as the initial step in efflux of cellular cholesterol to solution phase acceptors. Our idealized physical model for microelectrode detection of cholesterol at the cell surface has a thin aqueous hydration layer between the electrode surface and the plasma membrane.

The cholesterol oxidase modified electrode consumes the aqueous phase cholesterol that exists between the electrode surface and the plasma membrane. This consumption of aqueous phase cholesterol at the cell surface causes further cholesterol efflux from the plasma membrane as cholesterol mass transport occurs from the high concentration in the plasma membrane to the lower concentration at the electrode surface. It is hypothesized that the rate of cholesterol exchange between the plasma membrane and the aqueous layer is fast relative to the rate of cholesterol oxidation at the electrode surface. Mass transport of cholesterol to the electrode contact site likely involves lateral diffusion of cholesterol in the plasma membrane, flip-flop (transbilayer movement) of cholesterol between

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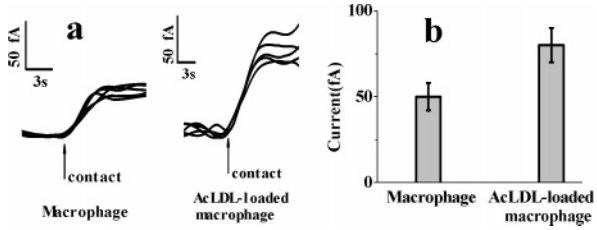

Figure 2. Electrochemical detection of the increased cell plasma membrane cholesterol content of an AcLDL-loaded RAW 264.7 macrophages: (a) representative traces where an AcLDL-loaded macrophage is compared with a macrophage using the same electrode; (b) averaged data from three electrodes; error bars are mean ± SD.

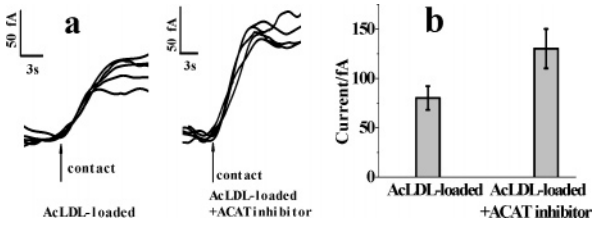

Figure 3. Electrochemical detection of the increased cell plasma membrane cholesterol content of an AcLDL-loaded RAW 264.7 macrophages treated with an ACAT inhibitor: (a) representative traces where an AcLDL-loaded macrophage with an ACAT inhibitor is compared with an AcLDL-loaded macrophage using the same electrode; (b) averaged data from three electrodes; error bars are mean ± SD.

the membrane leaflets, and delivery of cholesterol to the plasma membrane from stores inside the cell. As discussed below, electrochemical data collected at cells with increased cholesterol content indicate a correlation between electrode response and plasma membrane cholesterol content.

Dysfunctions in intracellular cholesterol transport have been linked to several disease states including atherosclerosis where plaque formation in artery walls is triggered by accumulation of low-density lipoprotein cholesterol (LDL-cholesterol, "bad cholesterol") in macrophage cells (i.e., foam cell formation).[2] Figure 2 shows a direct electrochemical comparison of plasma membrane cholesterol content of RAW264.7 macrophages with and without prior incubation with acetylated LDL (AcLDL), a ligand for the scavenger receptor A. Figure 2a shows data collected at a single oxidase-modified electrode for five sequential contacts at a macrophage followed by five sequential contacts at an AcLDL-loaded macrophage. The average electrode response is 50 ± 8 fA for the macrophage and 80 ± 10 fA for the AcLDL-loaded macrophage. The variation in electrode response for sequential contact experiments is due to noise and possibly differences in the nature of the physical contact. The bar graph (Figure 2b) represents averaged data comparing the cells with three different electrodes (five responses each) and three different cell preparations (Supporting Information). Relative to the control cells, the AcLDL-loaded cells have an increased electrochemical response of more than 60%. The faster rate of enzymatic cholesterol oxidation indicates a higher concentration of cholesterol at the electrode surface under steady-state turnover and an increase in plasma membrane cholesterol content. These data provide direct observation of an increase in plasma membrane cholesterol upon accumulation of AcLDL derived cholesterol in macrophages, as suggested by Maxfield.[8]

Accumulation of cholesterol in macrophage cells activates acylcoenzyme A, cholesterol acyltransferase (ACAT), which generates cholesterol esters that can be stored intracellularly in lipid droplets. Treatment of macrophages with an ACAT inhibitor prevents conversion of cholesterol to cholesterol esters and results in an increase of intracellular unesterified cholesterol.[2] Rothblat has reported that the increased accumulation of intracellular unesterified cholesterol, caused by inhibition of ACAT, also results in an increase in plasma membrane cholesterol content.[9] We have conducted electrochemical measurements on AcLDL-loaded macrophage cells with and without treatment with ACAT inhibitor (Figure 3). A comparison of these cells at a single electrode (Figure 3a) shows electrode responses of 80 ± 12 fA for the AcLDL-loaded macrophage and 130 ± 20 fA for the ACAT inhibited AcLDL-loaded macrophage. This trend is independent of the order in which the cells are studied and the bar graph (Figure 3b) represents data from three comparisons using three different electrodes (five responses each) and three different cell preparations. These data independently verify that additional cholesterol is transported to the cell plasma membrane when cholesterol storage through esterification is compromised. This agreement between our electrochemical measurements and those of Rothblat, on the same cellular system, provides validation for both methods in addressing the inadequately understood transport mechanisms involved in movement of cholesterol between internal stores and the plasma membrane.

Microelectrode evaluation of plasma membrane cholesterol content in live macrophages at 37 °C is reported. Electrode responses correlate with biochemically induced increases in plasma membrane cholesterol. The results support the belief that transport of cholesterol to the plasma membrane from stores inside the cell is active in atherosclerotic macrophage foam cells. Work aimed at characterizing the activities of specific cholesterol transport proteins is underway.

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Supporting Information Available: Details for electrochemical measurements for plasma membrane cholesterol detection. This material is available free of charge via the Internet at http://pubs.acs.org.

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