J|A|C|S COMMUNICATIONS

Published on Web 08/11/2009

Observation of Cellular Cholesterol Efflux at Microcavity Electrodes

Danjun Fang,[†] Dechen Jiang,[†] Hui Lu,[‡] Hillel J. Chiel,[‡] Thomas J. Kelley,[§] and James D. Burgess*[,†]

Departments of Chemistry, Biology, and Pediatrics, Case Western Reserve University and Rainbow Babies and Children's Hospital, Cleveland, Ohio 44106

Received May 6, 2009; E-mail: jdb22@case.edu

A fundamental uncertainty in cellular cholesterol homeostasis is the role of cholesterol mass transport by aqueous diffusion between hydrophobic lipid membranes such as the cell plasma membrane and lipoprotein in extracellular space.[1] The solubility of cholesterol in water is low (estimated at 200 nM),[2] and the probable significance of mass transfer by aqueous diffusion to the kinetics of specific cholesterol trafficking pathways is not understood. This communication reports cholesterol oxidase modified platinum microcavity electrodes for detection of plasma membrane cholesterol efflux using a background subtraction charge measurement scheme developed specifically for this application. The data are consistent with the notion that cholesterol in the cell plasma membrane undergoes exchange with cholesterol in solution. Additionally, the microcavity electrode geometry results in slow efflux relative to the expected rate of intracellular cholesterol movement. Thus, depletion of plasma membrane cholesterol at the efflux site is minimized.

Previous work by this research group has demonstrated a disk electrode method for probing plasma membrane cholesterol.[3] The measurements are amperometric and rely on a current increase above baseline upon positioning the electrode in contact with the cell surface. For the charge measurement described here, data acquisition does not require repositioning of the electrode, which is a source of noise in the disk electrode amperometric method. Additionally, the charge measurement scheme should allow planned *in vivo* studies where reference to a baseline current is not possible. Of particular mechanistic concern, the microcavity electrodes more clearly establish the existence of an aqueous layer between the electrode surface and the cell surface, a point of speculation for the disk electrode measurements.

The experimental arrangement employed for the microcavity measurements[4] involves placing the ring end of the glass capillary, extending beyond the enzyme-modified surface of the recessed platinum electrode, in contact with the plasma membrane (Figure 1). This geometry creates a cylindrical compartment ca. 0.4 pL in volume, isolated from the rest of the solution, into which cholesterol efflux from the membrane occurs and is detected through oxidation of accumulated hydrogen peroxide. Efflux of cholesterol from the plasma membrane to solution in the cavity is driven by enzymatic consumption of cholesterol at the electrode surface.

With the microcavity electrode positioned in direct contact with the plasma membrane surface of a single neuron in the buccal ganglion of *Aplysia*,[5] the electrode is poised at an experimentally determined potential that does not oxidize or reduce hydrogen peroxide. This hydrogen peroxide collection potential is held for 5 min to allow cholesterol efflux from the plasma membrane and accumulation of a measurable concentration of hydrogen peroxide in the cavity volume. The hold time can be much shorter, especially

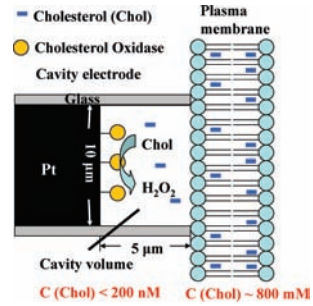

Figure 1. Scheme of microcavity electrode positioned in contact with the cell plasma membrane. The average molecular area of cholesterol in the plasma membrane is estimated at 40 Å^2.[6]

for measurements at 37 °C. Two sequential potential step experiments are conducted for analog chronocoulometric analysis of the accumulated hydrogen peroxide (Figure 2A). The first potential step (Figure 2A, step 1) is from 200 mV (the hydrogen peroxide collection potential) to 600 mV for complete mass transfer controlled oxidation of the hydrogen peroxide confined in the cavity volume (Figure 2B; trace labeled step 1). The potential is stepped back to 200 mV for 30 s (to allow the current to become constant), and a replicate of the first potential step is conducted (Figure 2A, step 2) to gauge the background charge (Figure 2B, trace labeled step 2). Subtraction of the background charge measured in the second potential step from the charge past in the first potential step is an estimate of the accumulated hydrogen peroxide in the cavity volume from steady-state enzymatic oxidation of cholesterol during the hold time (Figure 2C, trace labeled Enzyme modified). The background charge can drift over minutes, and this sequential potential step method allows the background to be continuously tracked and subtracted to give the charge from hydrogen peroxide oxidation (Supporting Information).

The background charge measured in step 2 must contain a contribution from hydrogen peroxide being generated during the 30 s quiet time between the first and second potential step experiments, as enzymatic oxidation of cholesterol is continuous. It is not necessary to wait 30 s for the current to become constant before measuring the background charge. However, in this initial demonstration of the measurement, the contribution to the background charge from hydrogen peroxide is a systematic error where signal is lost. Also, the rate at which hydrogen peroxide escapes from the cavity is uncertain and is also assumed to be a systematic error.

The difference charge at 1 s also reflects faradaic reactions of other species. This faradaic background charge is estimated using the bare platinum microcavity electrode (electrode prior to enzyme modification) positioned in contact with the same cell (Figure 2C, trace labeled Bare). Within 1 s, the difference charge from the enzyme-modified electrode reaches a maximum value relative to the difference charge from the bare cavity electrode (Figure 2C,

[†] Department of Chemistry.
[‡] Department of Biology.
[§] Department of Pediatrics.

10.1021/ja903684f CCC: $40.75

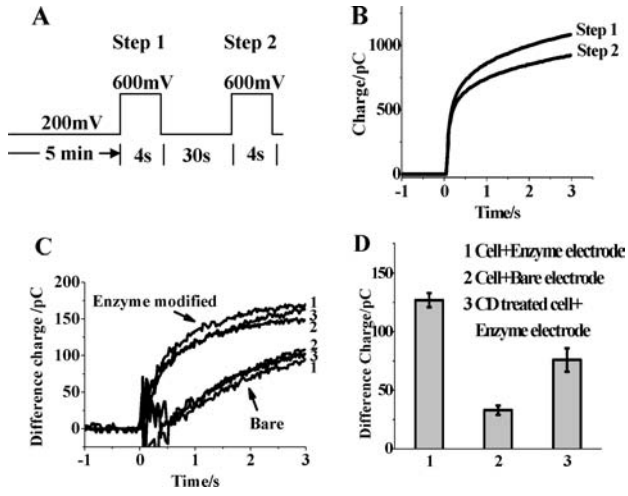

Figure 2. (A) Applied voltage. (B) Overlaid charge for steps 1 and 2. (C) Difference charge for enzyme-modified electrode and bare electrode at the cell (1, 2, 3: chronological order). (D) Three determinations of the difference charge at the cell and cyclodextrin (500 μM; 12 h) treated cell using the enzyme-modified and bare electrodes at 1 s.

1−3 s). The larger difference charge for the enzyme-modified electrode is assigned to hydrogen peroxide oxidation. Hydrogen peroxide oxidation is driven at a mass transfer controlled rate at 600 mV, and the cavity volume is quickly depleted. Enzyme-modified and bare electrode difference charge traces are compared for analysis at 1 s, as the background subtraction can produce noise inside of 50 ms. Comparing averaged data from three sequential and replicate experiments conducted at the bare and enzyme-modified electrode surfaces suggests that ∼75% of the difference charge passed at the enzyme-modified electrode at 1 s (Figure 2D, bars 1 and 2, respectively) is from oxidation of hydrogen peroxide. The average efflux rate (lower limit) is calculated to be 1 pmol/cm^2 s, where the efflux site (contact area of the cavity mouth: ca. 80 μm^2) is 0.5% of the total area of the cell surface. Cells partially depleted of cholesterol using cyclodextrin solution are studied to verify that the measurements are cholesterol dependent. After cholesterol depletion, the electrode showed a smaller difference charge indicating a decreased rate of cholesterol oxidation during the hold time (Figure 2D, bar 3) and, thus, slower efflux of cholesterol from the cell plasma membrane. Exposure of the cells to a cyclodextrin solution with higher concentration results in an additional decrease in cholesterol efflux rate.

It is not yet possible to reproduce the surface area of the etched platinum microcavity electrodes. This causes variation in the amount of enzyme immobilized on different electrodes. Microcavity electrodes with a higher surface area (as gauged by double layer charge) show higher enzymatic activity for oxidation of cholesterol in solution studies and larger responses at cells (Supporting Information).

Characterization of microcavity electrodes for enzymatic activity is conducted in a cholesterol/Triton X-100 solution. While the solution data provide verification that the electrodes exhibit enzymatic activity, it is not possible to directly compare the solution data with data collected at the cell surface. For the cell experiments, the plasma membrane likely acts as a barrier slowing escape of hydrogen peroxide from the cavity volume. Additionally, the plasma membrane gates cholesterol mass transport to the cavity volume, and therefore, it is proposed that the cavity depth largely dictates the thickness of the diffusion gradient.

It is proposed that mass transfer of cholesterol in the plasma membrane (and within the cell) is fast relative to the rate of efflux at the microcavity electrode contact site. A consequence of the cavity electrode geometry and the defined diffusion distance between the electrode surface and the plasma membrane is that cholesterol efflux occurs at a relatively slower rate compared to the disk electrode method[3] developed in this laboratory (ca. 3−4 times slower). Slower efflux at the microcavity electrode, while maintaining the small size of the efflux site, leads to a measurement that is expected to be more reflective of the efflux step and to be less influenced by depletion of cholesterol at the electrode contact site.

The degree to which the microcavity measurement locally depletes plasma membrane cholesterol is of concern regarding the relevance of the measured efflux rate to the unidirectional efflux rate occurring at pseudoequilibrium. The literature regarding cholesterol efflux from cells and model lipid membranes is consistent with the existence of two pools of plasma membrane cholesterol. Most cholesterol molecules in the plasma membrane efflux slowly to the cyclodextrin solution ($t_{1/2}$: ca. 20 min)[7] and are likely associated with phospholipids.[8,9] A smaller amount of the cholesterol, however, effluxes rapidly to the cyclodextrin solution ($t_{1/2}$: ca. 15 s)[7] and likely reflects excess cholesterol above the stoichiometric amount that can be complexed by the cell surface phospholipid.[8,9] This amount of "excess or active" cholesterol probably serves as a switch in cell signaling for cholesterol trafficking.[8,9] The slow efflux at the point of contact for the microcavity electrode is believed to be sustained by the excess cholesterol pool.

The aqueous efflux model for the microcavity electrode measurements assumes no direct contact between the plasma membrane and the electrode surface. It is also assumed that no enzyme is immobilized on the glass walls of the cavity or at the perimeter of the cavity mouth. This issue and the consequences of the physical contact between the electrode tip and the cell surface are uncertain. The product of enzymatic cholesterol oxidation (cholestenone) is believed to influx to the membrane perhaps affecting the measured efflux rate. The charge measurement is, however, a step closer to probing the unperturbed homeostatic plasma membrane cholesterol content of living cells initially in pseudoequilibrium with buffer. The *Aplysia* model will be used in experiments aimed at relating cholesterol cycling to neuronal activity.

Acknowledgment. This work was supported by the Cystic Fibrosis Foundation and the Department of Chemistry, Case Western Reserve University. Helpful discussions with Professors Barry Miller, Dan Scherson, and Ted Steck are acknowledged.

Supporting Information Available: Details for electrode fabrication and method optimization. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Rothblat, G. H.; Phillips, M. C. *J. Biol. Chem.* **1982**, *257*, 4775–2.
(2) Haberland, M. E.; Reynolds, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2313–2316.
(3) Devadoss, A.; Burgess, J. D. *J. Am. Chem. Soc.* **2004**, *126*, 10214–10215.
(4) Zhang, B.; Zhang, Y.; White, H. S. *Anal. Chem.* **2004**, *76*, 6229–6238.
(5) Lu, H.; Chestek, C. A.; Shaw, K. M.; Chiel, H. J. *J. Neural. Eng.* **2008**, *5*, 287–309.
(6) Smaby, J. M.; Momsen, M.; Kulkarmi, V. S.; Brown, R. E. *Biochemistry* **1996**, *35*, 5696–5704.
(7) Haynes, M. P.; Phillips, M. C.; Rothblat, G. H. *Biochemistry* **2000**, *39*, 4508–4517.
(8) Radhakrishnan, A.; McConnell, H. M. *Biochemistry* **2000**, *39*, 8119–8124.
(9) Lange, Y.; Ye, J.; Steck, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11664–11667.

JA903684F