Measurement of Changes in Membrane Surface Morphology Associated with Exocytosis Using Scanning Ion Conductance Microscopy

Wonchul Shin*† and Kevin D. Gillis*†‡
*Department of Biological Engineering, †Dalton Cardiovascular Research Center, and ‡Department of Medical Pharmacology and Physiology, University of Missouri-Columbia, Columbia, Missouri 65211

ABSTRACT The extent that vesicles maintain a distinct identity and morphology after fusing with the plasma membrane is controversial. We used scanning ion conductance microscopy to image changes in the surface membrane of adrenal chromaffin cells after stimulation of exocytosis with a high K^+ solution. Within several minutes after stimulation, punctate depressions, 100–600 nm wide, were noted from 16% of the cells. The depressions were not randomly distributed, but appeared in clusters of two or more within a $\sim$1 μm^2 area and disappeared after several minutes. Increases in membrane surface area, consistent with the fusion and collapse of one or more vesicles into the surface membrane, were observed in 64% of the cells after high K^+ stimulation. Surface area increases did not occur if the high K^+ solution did not contain Ca^{2+}. We conclude that scanning ion conductance microscopy can be used to follow the time course of surface membrane changes resulting from exocytosis and endocytosis.

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Address reprint requests and inquiries to K. D. Gillis, E-mail: gillisk@missouri.edu.

In excitable cells, membrane depolarization leads to Ca^{2+} influx through voltage-gated ion channels and Ca^{2+}-triggered fusion of transmitter-containing vesicles with the plasma membrane in the process of exocytosis. The membrane added by exocytosis is reinternalized by the process of endocytosis. A long-standing controversy is the nature of the exocytosis-endocytosis cycle (reviewed in Burgoyne and Morgan (1)). In particular, does the vesicle retain its shape in a transient ''kiss and run'' fusion event or does the vesicle collapse into the surface with endocytosis occurring at independent sites on the plasma membrane?

We used scanning ion conductance microscopy (SICM) (2) to image changes in the membrane surface morphology of bovine chromaffin cells during stimulation of exocytosis. SICM uses the change in electrical resistance that occurs as a micropipette approaches a nonconducting surface as the feedback signal to maintain the pipette at a fixed distance from an object as the pipette is scanned over the surface in a raster pattern. SICM is particularly useful to image the surface of cells (e.g., Korchev et al. (3)) because, unlike the case of atomic force microscopy, the probe never touches the surface membrane. Using a modulation method of SICM (4), we obtained images of cell surfaces with a resolution comparable to the diameter of the pipette, $\sim$100 nm. Cells were imaged before and after depolarization with a bath solution containing 50–70 mM K^+. Each scan took about 3 min to generate a 512×128 image with scanning areas of either 3×3 μm or 5×5 μm. Fig. 1, *A* and *B*, represent control scans in physiological bath solution demonstrating a typically smooth cell surface with height variations of 140–170 nm. After stimulation with the high K^+ solution, four punctate depressions appeared (Fig. 1 *C*). Fig. 1 *E* presents line profiles before and after stimulation demonstrating two depressions with depths and half-widths of $\sim$100 nm. The depressions were absent in the next scan taken 3 min later (Fig. 1 *D*). Three other sample scans taken $\sim$3 min after high K^+ stimulation that contain punctate depressions are depicted in Fig. 2, *A–C*. The smallest punctate depressions that we could detect had depths of $\sim$30–40 nm and half-widths of $\sim$90–100 nm, whereas feature sizes larger than 600 nm, or features that were not symmetric, were not classified as punctate depressions. Using these criteria, we observed a total of 25 punctate depressions after high K^+ stimulation in nine out of 56 cells tested. The width of the depressions measured at half depth was 196 $\pm$ 25 nm, whereas the measured depth of the depressions was 81 $\pm$ 8 nm (mean $\pm$ SE). In many cases, the $\sim$100 nm outside diameter of the pipette is likely to prevent full probing of the depth of the depressions.

As is evident in Figs. 1 and 2, punctate depressions were generally clustered within an area of $\sim$1–2 μm^2. We performed further analysis of scans obtained from the nine cells that showed punctate depressions to quantify the apparent clustering of these features. The average distances between two depressions was quite similar for the two different scan sizes we used with values of 0.93 $\pm$ 0.13 μm for 3×3 μm images ($N = 16$ depression pairs) and 1.12 $\pm$ 0.16 μm for 5×5 μm images ($N = 11$ pairs). We performed Monte Carlo simulations to determine the expected distances between depressions if they were randomly distributed throughout the image. Random placement resulted in mean distances of 1.56 μm for the 3×3 μm images and 2.60 μm for 5×5 μm images. The mean distances between depressions was

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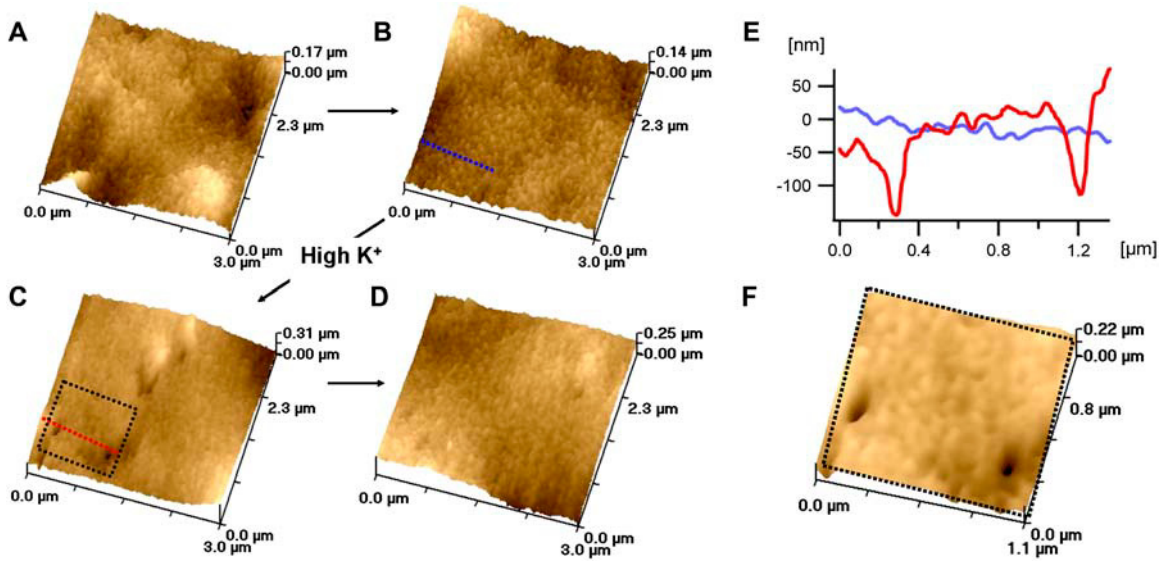

FIGURE 1 Punctate depressions appear after high K^+ stimulation. (*A* and *B*) Cell membrane morphology before stimulation. (*C* and *D*) Cell membrane morphology after stimulation. Punctate depressions appear (*C*), then disappear over several minutes (*D*). (*E*) Line profiles (*blue* from *B* and *red* from *C*). (*F*) Enlarged image from black dashed square from panel *C*.

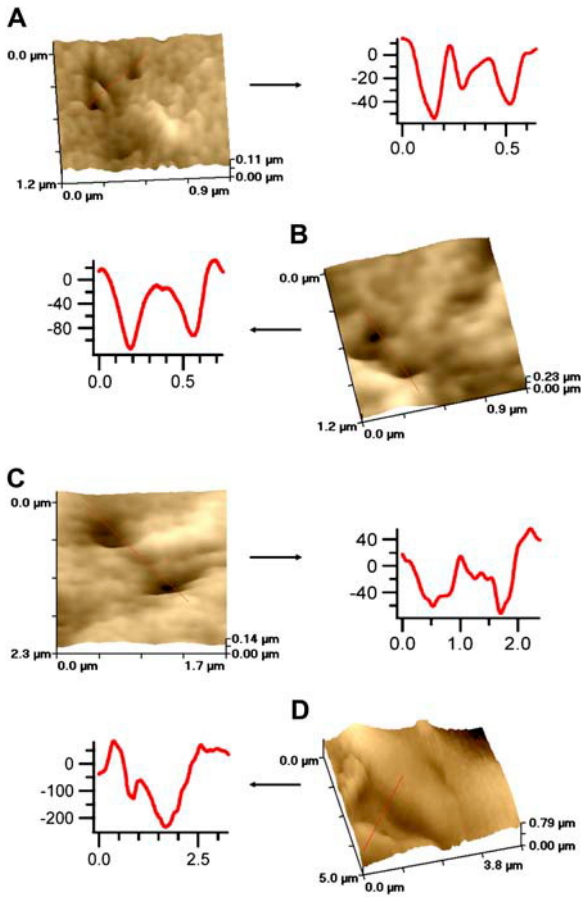

FIGURE 2 Line profiles of punctate depressions (*A–C*) and a valley-type feature (*D*) appearing ~3 min after stimulation; *x* axis units are μm whereas *y* axis units are nm).

significantly smaller than that expected by random placement ($p < .001$ for both image sizes).

Whereas nine of 56 cells exhibited clear punctate depressions, after high K^+ stimulation, most of the remaining cells exhibited ripples or other large morphological changes (e.g., Figs. 2 *D* and 3 *C*) that may have resulted from fusion and collapse of vesicles into the surface membrane. Since increases in membrane surface area are expected to accompany exocytosis, we measured the surface area of scans before and after stimulation. The surface area of scans increased several minutes after stimulation by 0.66 ± 0.24 μm^2 (~7%, $N = 19$) for 3×3 μm scans and 0.76 ± 0.27 μm^2 (~3%, $N = 28$) for 5×5 μm scans. (Fusion of one chromaffin granule should result in an increase in membrane area of ~0.2 μm^2 (5)). In contrast, the difference in area in the two scans before high K^+ stimulation averaged -0.04 ± 0.13 μm^2 for 3×3 μm scans and 0.01 ± 0.12 μm^2 for 5×5 μm scans.

Stimulation of exocytosis requires both membrane depolarization and extracellular Ca^{2+}, so we performed control experiments where cells were exposed to a high K^+ bath solution lacking Ca^{2+}. Fig. 3 presents an experiment, typical of seven cells, where cells were sequentially exposed to a high K^+ solution lacking Ca^{2+} and then a high K^+ solution containing 5 mM Ca^{2+}. In all seven cells, morphological changes (although no obvious punctate depressions) were observed after addition of Ca^{2+} but not upon addition of high K^+ solution lacking Ca^{2+} (Fig. 3 *C*). The change in the area of scans upon addition of high K^+ solution lacking Ca^{2+} was -0.03 ± 0.04 μm^2, whereas the change after subsequent addition of 5 mM Ca^{2+} was 0.41 ± 0.13 μm^2 (3×3 μm scans).

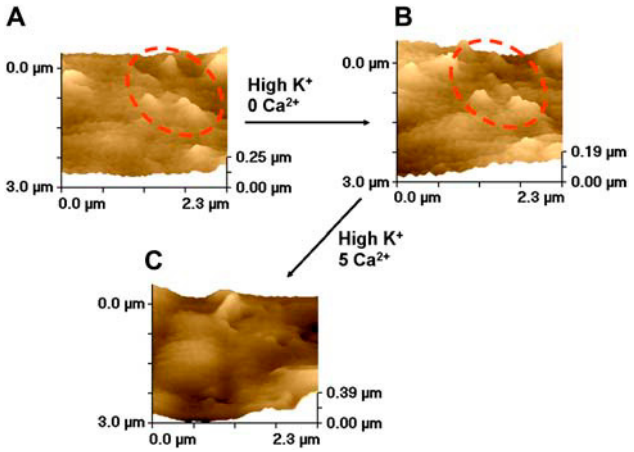

FIGURE 3 Morphological changes require both depolarization and extracellular Ca^{2+}. (*A*) Control image in Ca^{2+} free solution. (*B*) After depolarization with high K^+ solution in the absence of Ca^{2+}. (*C*) Ripples become evident after addition of 5 mM Ca^{2+} to the bath solution. The dashed red ovals show an example of surface features that remain stationary from scan-to-scan and thus demonstrate that the region of the membrane that is scanned remains constant.

In conclusion, we have shown changes of membrane surface morphology associated with exocytosis. The size of punctate depressions, such as those in Figs. 1 and 2, are consistent with that expected for the fusion of individual ~300 nm-diameter chromaffin granules, although a direct size comparison is not possible with the limited resolution of our pipettes, and it is also possible that these depressions represent sites of endocytosis rather than exocytosis. The clustering of punctate depressions we observed is consistent with reports of ''hot spots'' of Ca^{2+} channels and hormone release from endocrine cells (6–8) and with previous observations of clustered depressions in pancreatic acinar cells using atomic force microscopy (9). Punctate depressions were usually only present for a single scan and were therefore dynamic over a timescale of several minutes. On the other hand, punctate depressions, when observed, had a lifetime of at least several tens of seconds—the time required to scan the feature.

We observed net increases in membrane surface area accessible to the scanning pipette upon stimulation that are consistent with previous reports using membrane capacitance to measure net increases in membrane area (e.g., Augustine and Neher (10)). In the majority of cells, increases in surface area resulted from large morphological features such as valleys (Fig. 2 *D*) and ripples (Fig. 3 *C*) that may reflect collapse of vesicle membrane into the plasma membrane. This does not exclude the possibility that some vesicles may instead undergo a transient ''kiss and run'' fusion, because such events would likely escape detection in our experiments. Future experiments combining SICM with fluorescent labeling of vesicle membrane proteins or cargo should help clarify the origin and evolution of surface membrane morphological changes that result from transmitter exocytosis.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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