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Polarized TIRFM Reveals Changes in Plasma Membrane Topology Before and During Granule Fusion

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Abstract

We have recently developed a combination of polarization and total internal reflection fluorescence microscopy (pTIRFM) to monitor changes in plasma membrane topology occurring after fusion of chromaffin granules. In this report, pTIRFM is further exploited to reveal two major findings in regards to the secretory pathway in bovine chromaffin cells. First, we show that changes in membrane topology are sometimes detected even prior to fusion. This occurs with high probability in a small subset of granules that appear in the evanescent field during the experiment. On these occasions, the plasma membrane invaginates with the movement just preceding the appearance of a granule in the evanescent field. Such events may represent a direct interaction of the granule with the plasma membrane. Second, we show that the topological fate of the post-fusion, granule/plasma membrane intermediate is regulated by divalent cation. When Sr^{2+} is used instead of Ca^{2+} to trigger exocytosis, membrane topology in the exocytotic region is stabilized with significant curvature and indentation.

Keywords

TIRFM; Exocytosis; Fusion; Polarization; Granule; Chromaffin cells

Introduction

Chromaffin cells are primary, non-dividing and highly differentiated secretory cells that contain secretory granules (chromaffin granules) which store catecholamine, ATP, and a variety of proteins including chromogranins, opiate peptides, and precursors. Aspects of the secretory pathway elucidated in chromaffin cells have not only been generalized to other endocrine cells, but also to presynaptic pathways (Neher 2006). In fact, much of what is known about the priming and kinetics of secretion were originally elucidated in this system (Holz et al. 1989; Bittner and Holz 1992; Chow et al. 1994; Voets 2000). The stimulus for secretion from bovine adrenal medulla is the activation of nicotinic receptors by acetylcholine released from pre-ganglionic sympathetic nerves. The resulting Ca^{2+} influx

through the nicotinic receptor/channel complex and voltage sensitive Ca^{2+} channels elevates cytosolic Ca^{2+} and is the primary trigger for exocytosis.

We recently described the application of a TIRFM-based polarization technique to reliably—and directly—detect submicron changes in the plasma membrane topology as a result of dense core granule exocytosis (Anantharam et al. 2010). These deformations were detected immediately upon release of a luminal granule marker (within the time resolution of the imaging) and showed a tendency to persist at the fusion site for seconds to tens of seconds after fusion. Furthermore, through computer simulations we were able to interpret the quantitative imaging in terms of likely 3-dimensional membrane topologies of the post-fusion granule/plasma membrane intermediates and their eventual fate (e.g., retained curvature or membrane flattening).

In this report, pTIRFM is further exploited to reveal two major findings in regards to the secretory pathway in bovine chromaffin cells. First, we show that changes in membrane topology are sometimes detected even prior to fusion. On these occasions, the plasma membrane moves toward the interior of the cell, with the movement just preceding the appearance of a granule in the evanescent field. Such events may represent a direct interaction of the granule with the plasma membrane. Second, we show that the topological fate of the post-fusion, granule/plasma membrane intermediate is regulated by divalent cation. When Sr^{2+} is used instead of Ca^{2+} to trigger exocytosis, membrane topology in the exocytotic region is stabilized with significant curvature and indentation.

Materials and Methods

Chromaffin Cell Preparation and Transfection

Chromaffin cell preparation from bovine adrenal medulla and transient transfection was performed as described previously (Wick et al. 1993; Anantharam et al. 2010). Cells were plated onto 25 mm coverslips (refractive index 1.51) that had been coated with poly-D-lysine and calf skin collagen to promote cell adhesion. Cells were transfected with plasmid encoding Neuropeptide-Y-cerulean (NPY-Cer) by Ca^{2+} phosphate precipitation (Wilson et al. 1996). The parent NPY plasmid was a gift from Dr. Wolfhard Almers (Vollum Institute, Oregon Health and Science University, Portland, OR). NPY-Cer is a soluble luminal marker of chromaffin granules that is released upon exocytosis. Experiments were performed 3–7 days after transfection.

Perfusion

Experiments were performed in a physiological salt solution (PSS) containing 141 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl_2 , 0.5 mM MgCl_2 , 5.6 mM glucose, 15 mM HEPES, pH 7.4 at $\sim 28^\circ\text{C}$. Solution was delivered to individual cells through a pipet (100 μm inner diameter) using positive pressure from a computer-controlled perfusion system DAD-6VM (ALA Scientific Instruments, Westbury, NY). Generally, cells were perfused with PSS for 10 s, and then stimulated to secrete with elevated K^+ -containing solution (95 mM NaCl, 56 mM KCl, 5 mM CaCl_2 , 0.5 mM MgCl_2 , 15 HEPES, pH 7.4). Where used, strontium replaced Ca^{2+} at a concentration of 10 mM.

Total Internal Reflection Fluorescence Microscopy

Prismless (through-the-objective) TIRFM was obtained by directing an Argon ion (514 nm) or solid-state (442 nm) laser (Melles Griot, Carlsbad, CA) through a custom side port to a side-facing dichroic mirror z442/514rpc and z442/514m emission filter (Chroma Technology, Brattleboro, VT) on an Olympus IX70 (inverted) microscope (Melville, NY) with the 1.5 $\times$ internal magnifying lens in the emission path. The beam was focused on the

periphery of the back focal plane of a 60×1.49 NA, oil immersion objective (Olympus) so that the laser beam emerging from it was collimated and incident on the coverslip at $\sim 70^\circ$ from the normal giving a decay constant for the evanescent field of approximately 110 nm. The band widths of the emissions were 450–490 nm (Cerulean) and 530–600 nm (Cerulean and DiI). Digital images were captured on a cooled EM CCD camera (Andor iXon, 512×512 pixels, Andor Technology, South Windsor, CT).

Excitation Polarization Optics

The optical setup used to create the p-pol and s-pol 514 nm beams, superimpose their paths, and to further superimpose the 442 nm beam on that path is shown elsewhere (Anantharam et al. 2010). Each of the three beam types is mechanically shuttered independently. The system is programmed to step through a sequence of three openings (one at a time), repeating the cycle without additional delay. The camera takes an exposure synchronous with each shutter opening. Images were acquired at ~ 6.4 Hz with 50 ms exposures and 100 gain (EM setting). At 6.4 Hz, the full cycle of three exposures had a period of 454 ms.

DiI Labeling

1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (diIC18; Sigma, St. Louis, MO) was dissolved in ethanol to make a 1-mM stock solution. DiI was added directly to cells bathed in PSS at a 1:50 dilution. The cells were then quickly washed several times in PSS and used immediately.

Image Analysis

Sequential NPY-Cer, diI s-, and p-polarized emission images were captured using Andor IQ software (Anantharam et al. 2010). Since the footprint of the chromaffin cell is predominantly parallel to the glass coverslip, the diI emission from the s-pol excited sample is brighter than that from the p-pol excited sample over most of the cell surface. However, the p-pol image reveals details not evident in the s-pol image. This is particularly true for cell edges where the plasma membrane orientation is oblique or perpendicular to the coverslip interface (Anantharam et al. 2010). Normalized P/S ratios (emission from the p-polarized excitation divided by the emission from the s-polarized excitation) and $P+2S$ sums (emission from the p-polarized excitation plus two times the emission from the s-polarized excitation) were then calculated pixel-by-pixel for each image, and the transformations were aligned to the NPY-Cer images using custom software written in IDL. The P/S image is sensitive to the orientational distribution of diI, but is insensitive to local fluorophore concentration and illumination intensity. The P/S highlights areas of the membrane with higher p with respect to s. The $P+2S$ image is sensitive to local diI concentration convolved with the TIRF illumination intensity, but is insensitive to the diI orientation. The $P+2S$ images generally show a more uniform fluorescence. Together, the P/S and $P+2S$ images provide complementary pieces of information that can be used to discern topological attributes of the fused granule/plasma membrane complex.

Exocytosis of individual granules was evident from the sudden and complete loss of NPY-Cer fluorescence. Changes in P/S and $P+2S$ were determined in a 292-nm radius ROI centered over localized increases in the P/S ratio at sites of exocytosis. When fusion occurred without an evident increase in P/S , the ROI was centered over the region of the fusing granule.

P/S varies with the relative intensities of the p- and s-pol excitations, biases in the optical system, and interference fringes. To reduce these effects and allow comparisons with theory, P/S ratios from the diI emission were normalized to the ratio obtained with solution containing 10 mM rhodamine 6G (Invitrogen Corp, Carlsbad, CA), which is predicted to be

randomly oriented. The normalization was performed using the mean of the p- and s-polarized rhodamine 6G emissions.

For the purposes of estimating noise in P/S and to set a threshold above which changes in P/S are significant, the P/S of non-fusing granules ($n = 28$) within 1752 nm (3 ROI diameters) of fusing granules was determined at the time of fusion of the neighboring granule. Changes in the P/S for the non-fusing granules had a standard deviation of 2% (50 granules). Changes greater than 6% had a high probability of being significant.

Electron Microscopy

For transmission electron microscopy, cell monolayers were rinsed in serum free medium and then fixed in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4, overnight at 4°C. Following a buffer rinse, they were postfixed for 40 min in 1% osmium tetroxide in the same buffer. After a double distilled water rinse, they were scraped from the culture dishes and pelleted in Eppendorf tubes. For each subsequent step, the cells were resuspended in the next reagent and then pelleted. Next, they were en bloc stained with an aqueous, saturated solution of uranyl acetate for 1 h. They were then dehydrated rapidly in a graded series of ethanol, infiltrated and embedded in Epon, and polymerized. Ultra-thin sections were collected onto copper grids and post stained with uranyl acetate and lead citrate. The sections were viewed on a Philips CM100 at 60 kV. Images were recorded digitally using a Hamamatsu ORCA-HR digital camera system, which was operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

Results

Plasma Membrane Topological Changes Detected Prior to Movement of Granules into the Evanescent Field

It is commonly assumed that a secretory granule must come to the plasma membrane and interact with it prior to fusion. But it is also possible for the plasma membrane to move to the granule to initiate interactions leading to fusion. Indeed, in EM studies performed with stimulated chromaffin cells, granules were sometimes observed to be closely apposed to (Fig. 1A), or appearing to contact (Fig. 1B, C), areas of the plasma membrane that are indented. We were interested in determining whether equivalent observations could be made in real-time with pTIRFM.

For these experiments, successive images of a diI-labeled chromaffin cell were taken with orthogonal excitation polarizations in TIR mode (p-pol—in the plane of incidence, perpendicular to the coverslip; s-pol—perpendicular to the plane of incidence, parallel to the coverslip) followed by a third image of a transfected granule luminal protein, Neuropeptide-Y-Cerulean (NPY-Cer). Regions where the membrane deviates from parallelism with the glass coverslip are highlighted by taking the ratio (p-pol)/(s-pol) (abbreviated P/S) of the membrane-embedded diI fluorescence images excited by the two polarizations (Sund et al. 1999). As previously described (Anantharam et al. 2010), the theoretical analysis indicates P/S is independent of diI concentration and that the sum $P+2S$ of the emissions reports the local fluorophore concentration convoluted with the exponentially decaying evanescent field for a high numerical aperture lens ($NA = 1.49$). Cells were stimulated to secrete by local perfusion of a high K^+ (56 mM) solution.

We first searched for deformations (sites with increased P/S compared to surrounding areas) in the membrane, which were closely apposed to NPY-Cer-labeled granules. In 10 cells examined with pTIRFM, there was no evidence for plasma membrane deformations to be associated with granules in the evanescent field. Secretory events are most commonly observed to occur from these granules (Allersma et al. 2004). There was also no tendency

for plasma membrane deformations to be preferred sites of exocytosis. However, for the small population of granules that appeared in the field during the depolarization (only 13 granules across 10 cells), a preceding topological change was sometimes observed (preceding the appearance of 6 of the 13 granules). Two of the 13 granules appearing in the evanescent field during the depolarization subsequently fused. The appearance of both of these granules was preceded by a plasma membrane deformation.

Figure 2A shows an example of a granule which appeared in the evanescent field during the depolarization and then fused. A change in membrane topology occurred just prior to the appearance of the granule in the field (Fig. 2B, line *a*, time 11.70 s). The change is transient. Over the next several seconds, both the granule and the plasma membrane appear to be moving closer to the coverslip (both NPY-Cer and $P+2S$ increase). By 18.9 s after stimulation, the granule fuses and releases NPY-Cer (not shown).

In 2C, an example is shown of a more stable plasma membrane topological change that precedes the movement of a granule into the evanescent field (without subsequent fusion). NPY-Cer intensity increased over several seconds as the granule approached the glass interface. Line *a* is drawn at 18.9 s which marks the start of an increase in P/S and decrease in $P+2S$. The diI changes that were detected are consistent with a stable invagination occurring in a region that is directly apposed newly arriving granule.

Plasma Membrane Topological Changes upon Fusion Become Long-Lived in the Presence of Strontium

The next set of experiments extends our continuing efforts to understand the regulation of the structural intermediate created by the fusion of a granule with plasma membrane. In a previous study (Anantharam et al. 2010), we showed that although the persistence time of the intermediate is usually quite variable, it is stabilized by addition of dynasore—a suspected inhibitor of the dynamin GTPase (Macia et al. 2006). Strontium has been previously shown to maintain exocytosis but inhibit subsequent rapid endocytosis (Artalejo et al. 2002). It has also recently been suggested to lock the fused granule in an open state (Llobet et al. 2008). In this study, Sr^{2+} was substituted for Ca^{2+} during the high K^+ depolarization. The effect of Sr^{2+} on the localized topological dynamics of the fused granule membrane/plasma membrane intermediate was subsequently investigated (Fig. 3). Stimulated exocytosis of NPY-Cer granules appeared to occur as well in the presence of Sr^{2+} as in the presence of Ca^{2+} (control cells). No qualitative differences in the number of granules or the fraction of granules fusing with a high K^+ stimulus were noted. However, the fraction of exocytotic events with detectable increases in P/S immediately upon fusion increased from 62% (31 out of 50 events, control) to 72% (18 out of 25 events, strontium). Most of the P/S changes were long-lived (Fig. 3A–C). After 22.5 s, curvature persists in 65% of the cells stimulated in the presence of Sr^{2+} , but only in approximately 40% of control (Fig. 3D).

There was also less variability in membrane topology for individual fusion events in the presence of Sr^{2+} than in the presence of Ca^{2+} . The average P/S associated with fusion events demonstrates the strong tendency for curvature to be maintained, which is in marked contrast to control cells (Fig. 3C, left panel). The average $P+2S$, however, between control cells and cells stimulated with Sr^{2+} is not different (Fig. 3C, right panel). Computer simulations used to model this result suggest that granule membrane maintains its curvature (Fig. 3E, b) after fusion in the presence of Sr^{2+} . The initial decrease in $P+2S$ (Fig. 3E, b) is a consequence of the decreased amount of membrane in the vicinity of the fusion pore close enough to the substrate to also be in the exponentially decaying evanescent field. The fusion pore then closes slightly—enough to cause a recovery in $P+2S$, but not enough to significantly change the P/S (c). The data are also qualitatively consistent with another

outcome. It is possible that after the intermediate modeled in Fig. 3E-b, the granule partially flattens (increasing $P+2S$; d) while still maintaining membrane perpendicular to the glass interface at the fusion junction (P/S remains elevated).

Discussion

In previous studies, we and others investigated granule behavior before fusion but without concomitant high spatial and temporal resolution detection of localized plasma membrane topology. With the advent of polarized excitation TIRFM, the ability to monitor granules in the evanescent field and the apposed plasma membrane areas now exists. We initially examined whether chromaffin granules in field were pre-associated with plasma membrane deformations (these types of structures were predicted by EM, Fig. 1) or if these deformations were preferred sites of exocytosis. No tendency emerged to convince us that either of these was the case. But unexpectedly, in the small population of granules that entered the evanescent field during the depolarization ($n = 13$ in 10 cells), six were preceded by localized plasma membrane deformations. The deformations could be transient (Fig. 2A), or long-lived (Fig. 2C), and may represent an interaction of the granule with the plasma membrane.

Why might plasma membrane deformations occur immediately *before* the movement of granules into the evanescent field? The deformations may reflect a process by which the granule is brought to the plasma membrane. It is possible that they occur with even greater frequency but were missed because of the limited time resolution of the experiments (~450 ms). Two of the six granules that entered the field after a plasma membrane topological change subsequently fused within 10 s (none of the seven granules that entered the field without a plasma membrane topological change subsequently fused). This raises the possibility that the granule/plasma membrane associations detected influence the probability of fruitful interactions leading to fusion.

The second part of the study concerns our ongoing efforts to study the regulation of the structural intermediate observed just after fusion of the granule and plasma membrane. In a previous work, we demonstrated that changes in membrane topology with granule fusion could be reliably observed with polarized TIRFM. Although the size and time course of the changes in P/S (i.e., membrane curvature) were variable, they tended to decay over longer times. This suggested to us that retention of curvature after fusion is a common rather than rare occurrence in chromaffin cells. When Sr^{2+} was substituted for Ca^{2+} in the extracellular medium, the topological changes observed at exocytotic sites were altered. The increase in the P/S with fusion was detectable with greater frequency and was longer lived. These effects are consistent with the ability of Sr^{2+} to inhibit endocytosis (Artalejo et al. 2002) and with the suggestion that Sr^{2+} keeps the fused granule in a curved but open state (Lobet et al. 2008). The effects of Sr^{2+} demonstrate that the deformations detected by pTIRFM can be regulated at multiple stages, resulting in distinct topologies. When dynasore, a suspected inhibitor of the dynamin GTPase (Macia et al. 2006), was used to inhibit the post-fusion granule/plasma membrane intermediate, curvature (P/S increase) was also stabilized (Anantharam et al. 2010). However, dynasore application resulted in a stable increase in $P+2S$, consistent with a structure where the granule membrane is connected to the plasma membrane by a neck narrower than the granule diameter. It is likely that dynasore and Sr^{2+} stabilize different post-fusion topologies.

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Abbreviations

diI	1,1'-Diocadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
EM	Electron microscopy
NPY-Cer	Neuropeptide-Y-Cerulean
p-pol	p-polarization
P/S	(p-pol emission)/(s-pol emission)
P+2S	p-pol emission+2(s-pol emission)
PSS	Physiological salt solution
s-pol	s-polarization
TIRFM	Total internal reflection fluorescence microscopy

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Fig. 1. Granules at sites of plasma membrane invaginations viewed by electron microscopy. **A–C** Images taken by EM are shown of granules closely apposed to plasma membrane invaginations (**A**) or appearing to contact the plasma membrane at sites of invagination (**B**, **C**)

Fig. 2.

Plasma membrane topological changes detected prior to movement of granules into the evanescent field. **A** A series of images are shown of the NPY-Cer and apposed *P/S* and *P+2S* intensities for an event where a granule moves into the field and subsequently fuses. Time after application of the high K^+ stimulus is indicated. Interval between frames is 450 ms. **B** A sudden increase in *P/S* intensity (and decrease in *P+2S* intensity) is evident (at 11.70 s, *circled area*) immediately before the gradual increase in NPY-Cer intensity that signals a granule moving into the field (line *a*). Circle diameter is 876 nm. **C** NPY-Cer, *P/S*, and *P+2S* intensities are indicated for a different event. Line “a” indicates the time before the *P/S* increase and *P+2S* decline begin (at 18.90 s). The *P/S* increase is most significant between 21 and 22 s. The *P+2S* decreases gradually between 19 and 21 s. Both measures reach a steady-state after 22 s. The NPY-Cer increase begins around 22 s. In this example, the granule does not eventually fuse

Fig. 3.

Plasma membrane topological changes upon fusion become long-lived in the presence of strontium. Extracellular Ca^{2+} was replaced by 10 mM Sr^{2+} in the high K^{+} depolarizing solution. **A** With fusion of a granule and release of NPY-Cer, a long-lived punctate increase in P/S is observed, along with a transient drop in $P+2S$. Circle radius is 800 nm. **B** Data from **A** is presented in a graph. **C** The P/S and $P+2S$ of diI-membranes of individual fusion events from control ($n = 50$) and Sr^{2+} stimulated cells ($n = 25$) were normalized, aligned to the pre-fusion frame, averaged. The data were also normalized to the average of 10 pre-fusion frames. The average P/S is different between the two groups at every time point after 0.9 s ($P < 0.05$, Student's t test). The $P+2S$ for the two groups are similar. *Error bars* are not shown for clarity. **D** A cumulative frequency histogram was generated to compare the remaining P/S increase from individual fusion events in control (Ca^{2+}) and Sr^{2+} -treated cells at 22.5 s after fusion. With Sr^{2+} replacing external Ca^{2+} , a greater fraction of fusion events were resistant to curvature decay. The distributions are significantly different ($P < 0.05$, Mann-Whitney test). The percent increase in P/S was calculated by taking the difference between the P/S at a particular frame (P/S_f) and the average P/S of 10 pre-fusion frames (Ave_{pre}), divided by the average; $(P/S_f - \text{Ave}_{\text{pre}})/\text{Ave}_{\text{pre}}$. Control data were adapted from (Anantharam et al. 2010). **E** The results from **C** are interpreted in the cartoons