Localized topological changes of the plasma membrane upon exocytosis visualized by polarized TIRFM

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Introduction

The plasma membrane–cytosol interface is a highly specialized domain in which unique and critical cellular functions occur. It is the site of biochemical and ionic signaling through receptors and channels that coordinates cellular processes with the extracellular environment. It is also the site of intercellular interactions, the locus of molecular components important for cell motility, and the site of numerous vesicular trafficking reactions including endocytosis, phagocytosis, viral budding, and exocytosis.

Total internal reflection (TIR) fluorescence microscopy (TIRFM) is superbly suited to visualize the plasma membrane–cytosol interface because the exponentially decaying evanescent field of TIR selectively illuminates the portion of the cell within a distance of 50–100 nm from the glass coverslip upon which the cells reside. TIRFM has been used to characterize the dynamics of fluorescently tagged secretory granules around the time of exocytosis and to visualize individual granule fusion events (Burke et al., 1997; Steyer et al., 1997; Oheim et al., 1998; Han et al., 1999; Tsuboi et al., 2000; Ohara-Imaizumi et al., 2002; Allersma et al., 2004, 2006; Shakiryanova et al., 2005; Silverman et al., 2005; Degtyar et al., 2007). However, the other partner in exocytosis, the plasma membrane, has been much less studied. In this study, we report the implementation of a TIRFM-based polarization technique to detect rapid submicrometer changes in plasma membrane topology as a result of exocytosis. A theoretical analysis of the technique is presented together with image simulations of predicted topologies of the post-fusion granule membrane–plasma membrane complex. Experiments on diI-stained bovine adrenal chromaffin cells using polarized TIRFM demonstrate rapid and varied submicrometer changes in plasma membrane topology at sites of exocytosis that occur immediately upon fusion. We provide direct evidence for a persistent curvature in the exocytotic region that is altered by inhibition of dynamin guanosine triphosphatase activity and is temporally distinct from endocytosis measured by VMAT2-pHluorin.
changes in the plasma membrane itself on living cells labeled with dil (Sund et al., 1999). Recently, polarized TIRF (pTIRF) was used to demonstrate the spread of FM4-64 into the plasma membrane of PC12 cells (Taraska and Almers, 2004) and peptide-induced disorder in supported bilayers (Oreopoulos and Yip, 2009).

The technique involves taking successive images of a sample with orthogonal excitation polarizations in TIR mode: a p-polarization (p-pol; in the plane of incidence and perpendicular to the coverslip) and an s-polarization (s-pol; perpendicular to the plane of incidence and parallel to the coverslip) image. Regions, even submicroscopic ones, in which the membrane deviates from parallelism with the substrate are vividly highlighted by taking the ratio p-pol/s-pol (P/S) of the membrane-embedded dil fluorescence images excited by the two polarizations. This approach is generally applicable for visualizing dynamic topological alterations in the plasma membrane during important cellular processes.

In a theoretical section, we show which combination of p-pol and s-pol excited images reports the local fluorophore concentration independently of the orientation. We also examine the ability of pTIRF to highlight submicrometer morphological distortions in the plasma membrane in the presence of complicating factors including the finite evanescent field depth, near-field emission into the substrate, high aperture observation, membrane foreshortening, optical resolution limitation, and pixelation of the observation region.

In the experimental section, we describe high spatial and temporal resolution images of the granule membrane–plasma membrane complex in living bovine chromaffin cells taken as a repeated triplet sequence: p and s excitation of dil in the plasma membrane and also excitation (at a different wavelength) of the fluorescent protein–marked secretory granules. Guided by the theory section predictions, we demonstrate rapid and varied submicroscopic changes in plasma membrane topology at sites of exocytosis that occur immediately upon fusion (within the time resolution of the imaging) and provide direct evidence for a persistent curvature in the exocytotic region after fusion that is subject to regulation. This occurs immediately upon fusion in at least 60% of events. The curvature decays with varying speeds from as fast as 450 ms to tens of seconds and is strongly affected by the dynamin GTPase inhibitor dynasore. Endocytosis was also imaged by optical techniques. We find that <10% of the fusion events stimulated with elevated K+ result in rapid endocytosis. Thus, most of these topological changes are kinetically distinct from endocytosis and reflect a varying and regulated time course for the flattening of a fused granule membrane into the plasma membrane.

Results

Theory of polarized excitation TIRF

To help interpret our experimental results on the plasma membrane dynamics of secretion from chromaffin cells, we calculate the p-pol and s-pol excited image intensities for a particular geometrical model of secretion: a spherical membrane (the secretory granule) fusing with a planar membrane (the plasma membrane) from within the cell interior, taking into account several possibly countervailing optical effects.

Orientation versus concentration

Idealized geometry. In general, TIRF images show a convolved mix of local fluorophore orientations with concentrations and distances from the substrate. What combination of p-pol and s-pol excited images reports purely orientational distributions, and what combination reports purely concentration and distances?

To calculate the answer, we set up the following polar coordinate system (Fig. 1): the sample plane (at which TIR occurs) is the x-y plane, and the optical axis of the microscope is the z axis. The TIR evanescent field propagates in the x-y plane along x, s-pol excitation is polarized along y, and p-pol excitation is polarized along z (actually, a small amount of p-pol intensity is polarized in the x direction, dependent on the incidence angle of the TIR beam, but we will ignore it for simplicity). In this coordinate system, θ and φ are the polar angle (measured from the z axis) and the azimuthal angle (measured around the z axis), respectively.

For a particular fluorophore whose dipole is oriented in the θ and φ, the probability of excitation caused by p-pol or s-pol light is proportional to the square of the component of the dipole along the z or x axis, respectively. These square components are proportional to cos²θ and sin²θ cos²φ, respectively. Given that the fluorophore becomes excited, the emission can be resolved into two components, one along the z axis (i.e., perpendicular to the substrate) and the other in the x-y plane (i.e., parallel to the substrate). The square of each of these components, proportional to cos²θ and sin²θ, each contribute to the emitted light that is collected by the objective with efficiencies Qz and Qφ, respectively.

An actual sample, of course, contains numerous fluorophores with their dipoles possibly pointing in different directions. Assume that the normalized orientational distribution of dipole is η(θ,φ). The emission light intensities P and S gathered by an objective (and imaged at a single pixel) from p-pol and s-pol excitation, respectively, are

\[ P = \int C \eta(\theta, \phi) \cos^2 \theta (Q_{\perp} \cos^2 \theta + Q_{\parallel} \sin^2 \theta) \sin \theta \sin \phi \, d\phi \]
\[ S = \int C \eta(\theta, \phi) \sin^2 \theta \cos^2 \phi (Q_{\perp} \cos^2 \theta + Q_{\parallel} \sin^2 \theta) \sin \theta \sin \phi \, d\phi, \]

where C is a number proportional to the local effective total amount of fluorophore at the pixel under view. C implicitly includes decaying effect of the evanescent field in the z direction such that fluorophores at larger distances from the surface simply contribute less toward C. C also implicitly includes the foreshortening effect of tangential viewing, all the multiplicative constants and efficiencies of absorption and emission that convert concentration into units of light intensity. The assumptions in Eq. (1) are that no polarizer is used in the emission system, the dichroic mirror introduces no polarization bias, the absorption and emission dipoles are parallel to each other, and...
The plane of incidence (which contains both the incident and reflected beams) is the x-z plane. The incident propagating beam (traveling up along z) is transversely polarized in either the x or y directions, which correspond to p-pol and s-pol, respectively. The evanescent field is polarized either primarily in the z direction for p-pol or entirely in the y direction for s-pol.

The orientational distributions are not a function of z distance to the TIR substrate. Clearly, ratio $P/S$ reports only upon the orientational distribution $\eta(\theta, \phi)$ because the $C$ factors cancel upon taking the ratio. The goal of the following derivation is to find the opposite: a combination of $P$ and $S$ that only reports on $C$ and does not depend on $\eta(\theta, \phi)$.

All of the possible biases of the objective toward gathering light from one excited dipole orientation or another are contained in the factors $Q_1$ and $Q_2$. For a very high aperture (NA $\approx 1.40$), these factors are within 20% of each other (see Materials and methods; Fig. 2 B), so we will set them both equal to unity in Eq. (1). This leads to collapse of the integrand factor involving $Q_1$ and $Q_2$ to unity. Note that this step is valid only for 1.40, 1.45, and 1.49 NA objectives, and even then, it is just an approximation. The normalization requirement is that

$$\int \eta(\theta, \phi) \sin \theta d\theta d\phi = 1.$$  \hspace{1cm} (2)

We now assume azimuthally symmetry in fluorophore orientation so that $\eta(\theta, \phi) = \eta(\theta)$; i.e., there is no particular orientation preference for $\phi$ in the x-y plane. This is expected to be the case in many biological organelles (plasma membrane, secretory granules, etc.) if we gather light from a large enough area. The normalization (Eq. 2) then becomes

$$\int \eta(\theta) \sin \theta d\theta = \frac{1}{2\pi}.$$  \hspace{1cm} (3)

Then, Eq. (3) gives

$$P = \int C \eta(\theta) \cos^2 \theta \sin \theta d\theta d\phi = 2\pi \int C \eta(\theta) \cos^2 \theta \sin \theta d\theta$$

$$S = \int C \eta(\theta) \sin^2 \theta \cos^2 \phi \sin \theta d\theta d\phi = \pi C \eta(\theta) (1 - \cos^2 \theta) \sin \theta d\theta$$

$$= \pi C \eta(\theta) \sin \theta d\theta - \left( \frac{P}{2} - \frac{C + P}{2} \right)$$

$$= \frac{C + P}{2}.$$  \hspace{1cm} (4)

Therefore,

$$P + 2S = C.$$  

This expression is true regardless of the exact form of orientational distribution $\eta(\theta)$ (as long as it is azimuthally symmetric). The sum $P + 2S$ is proportional only to the effective concentration. As long as we view with a very high aperture objective and bin together a large enough image area to ensure that the orientational distribution within the area is azimuthally symmetric, $P + 2S$ is approximately proportional only to the local concentration convolved with the evanescent exponential decay, and invariant with respect to the details of azimuthally symmetric orientation. Because $Q_1$, $Q_2$ undulates as a function of $x$ (Fig. 2), there is no algebraic combination of $P$ and $S$ that is exactly invariant to orientation for all geometrical shapes of the membrane.

We can plug in a particular azimuthally symmetric $\eta(\theta)$ function of interest here: the idealized cases of the fluorophore diI in a planar membrane parallel to the substrate and on a spherical granule much smaller than the size over which $P$ and $S$ are evaluated. We assume for now that diI intercalates into lipid bilayer membranes with its dipole moments parallel to the local membrane surface (Axelrod, 1979). They are (in properly normalized form)

$$\eta(plane) = \left( \frac{1}{2\pi} \right) \delta(\theta - \frac{\pi}{2})$$

$$\eta(sphere) = \left( \frac{1}{4\pi} \right).$$

where $\delta$ is the standard spike $\delta$ function. Using Eqs. 3 and 4 (with $C = 1$ for both plane and sphere, thereby implicitly assuming the same surface area for both) gives

$$P(plane) = 0,$$

$$S(plane) = \frac{1}{2},$$

$$P(sphere) = \frac{1}{3},$$

$$S(sphere) = \frac{1}{3}.$$  \hspace{1cm} (5)

The ratio $P/S$ changes from 0 to 1 in going from a pure plane to a pure sphere. This distinction provides a very sensitive test of local orientation. The sum $P + 2S$ remains constant at 1 and provides an orientationally independent means to measure relative local concentrations.

This idealized calculation must be altered to include numerous real effects: finite evanescent field depth, exocytotic structures containing both truncated spherical and planar regions, granule size on the edge of optical resolvability, and pixelation. These effects, and a general approach to handling them, are considered in detail in Materials and methods. Based
on those considerations, computer-simulated images (Fig. 3) and expected integrated intensities for \( P \) and \( S \) (Fig. 4) can be produced.

The range of \( P/S \) predictions derived from this realistic image simulation (with parameters specified in Fig. 4 A) agree with the range of experimentally observed \( P/S \) values on bovine chromaffin cells (see Test of theory on...). The agreement does not mean that the actual indentations are truncated spheres, but it does suggest that the actual indentations are quite significant. The solid and dashed lines in the \( P/S \) graphs of Fig. 4 A correspond to different granule radii, 150 and 250 nm. For a constant-sized, pixelated region of integration, the \( P/S \) ratio reaches a greater maximum for larger granules in the course of fusion with the plasma membrane.

Fig. 4 B shows that \( P+2S \) is rather insensitive to the depth of indentation until the indentation is beyond a hemisphere. Therefore, any large changes in \( P+2S \) indicate either (a) a deep indentation, (b) an actual increase in fluorophore concentration, or (c) a different geometry than assumed by the sphere/plane model. Large granules (radius \( R \) = 250 nm) do show a decrease in \( P+2S \) in the middle of the indentation depth range, as the simulated image develops a larger dark hole at its center.

**Test of theory on model systems**

According to the idealized theory (Eq. 5), the \( P/S \) ratio should equal 0 if the diI transition dipoles are parallel to the membrane and the membrane is parallel to the coverslip substrate. This prediction was tested with HEK293 cells stained with diI. The plasma membrane was imaged with p- and s-polarized 514-nm excitation. The \( P/S \) ratio of the plasma membrane apposed to the glass coverslip was not uniform. The minimum \( P/S \) ratio was 0.304 ± 0.017 (\( n = 6 \) cells) and was assumed to represent areas parallel to the coverslip. This value could be greater than zero for several reasons. (a) The absorption and/or emission dipoles of diI are not parallel to the bilayer but are at a somewhat oblique angle. A \( P/S \) ratio of 0.3 is predicted for if the transition dipoles are oriented an angle \( \beta \) of 69° rather than 90° from the normal to the bilayer. This value for \( \beta \) was used to generate the curves shown in Fig. 4. (b) The p-pol evanescent field is not purely perpendicular to the glass interface but in reality is elliptically polarized in the x-z plane with a weak x component (Axelrod, 2001) of ~6.8% of the total p-intensity at the incidence angle used in this study. This x component would cause a deviation of \( P/S \) from zero but not nearly accounting for a \( P/S \) of 0.3 and was not considered in the calculations. (c) The evanescent field obtained with through the lens TIRFM is contaminated by impurely polarized propagated light because of internal reflections in the objective (Mattheyes and Axelrod, 2006). The impurity accounts for ~10% of the total intensity at the substrate surface and increasing amounts further into the aqueous phase but was not considered in the calculations. (d) The diI absorption and emission dipole moments are not totally parallel to each other, as discussed in Axelrod (1979). However, because the 1.49 NA objective is relatively insensitive to the orientation of the emission dipole, this nonparallelism probably has little effect.
Eq. 5 also predicts a $P/S$ equal to 1 for an isotropic distribution of fluorophore dipoles. This distribution was approximated by diI-labeled beads. The beads gave a $P/S$ ratio of 0.890 ± 0.042 ($n = 21$), much larger than for the planar membrane on HEK cells and only slightly less than the theoretical value of 1.0. The quantitative discrepancy from theory can be explained. The bead sample does not really qualify as an isotropic distribution of diI; the beads are on the same order of size as the evanescent field depth and are thereby illuminated nonuniformly, with the part closest to the substrate (the part presumably with a small $P/S$ ratio) contributing the most to the fluorescence intensity.

In summary, emissions obtained with p- and s-polarized excitations with diI in defined geometries are similar to the predictions of the theory. Most importantly, the $P/S$ ratio is much greater (threefold) for diI on a spherical bead compared with diI in a bilayer parallel to the coverslip.

**pTIRFM detects topological changes of the plasma membrane at sites of exocytosis**

Chromaffin cells were transfected with neuropeptide Y (NPY)–cerulean (Cer) to label secretory granules and subsequently stained with diI immediately before imaging. As discussed in Materials and methods, a sequence of three images (NPY-Cer, s-polarized diI, and p-polarized diI) was acquired. The footprint of a chromaffin cell is predominantly parallel to the glass coverslip as indicated by the emission from s- being much brighter than from p-pol over most of the cell surface (Fig. S2).

The granule membrane becomes continuous with the plasma membrane upon exocytosis, allowing diffusion of the diI from the plasma into the granule membrane. A high $K^+$ (56 mM) depolarizing solution was perfused locally on the cell of interest to stimulate exocytosis. A short-lived local change in orientation of the diI-labeled membrane occurs around the time of exocytosis. However, extended temporal tracking of the p-pol and s-pol ratios and sums uses the spatially integrated values $P$ and $S$ (without the primes) before forming the $P/S$ and $P + 2S$ combinations. The predictions of the simulations are sensitive to the assumed parameters, which are set close to the actual or expected experimental values: granule radius = 150 nm; Airy disk half-width (out to first minimum) = 211 nm; evanescent field depth = 110 nm; side length of CCD array pixel (as projected onto the image) = 73 nm; angle $β$ between membrane normal and diI dipole = 69°. The $P$ and $S'$ images are shown with the same grayscale; the $P/S'$ and $P + 2S'$ each have their own grayscale.

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the local concentration of diI and/or movement of membrane away into the cell (Fig. 5, A and B). The P+2S emission subsequently returns to baseline.
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The relationship between the fusion-induced topological changes and endocytosis was investigated with vesicular monoamine transporter 2–pHluorin (VMAT2-pHL). VMAT2 is normally present on bovine chromaffin granules and mediates the transport of catecholamine into the granule. Transfected VMAT2 is punctate with 80% colocalization with endogenous dopamine β-hydroxylase, a marker for the granule (Wick et al., 1997). The chromophore in VMAT-pHL is luminal and normally quenched by the low luminal, pH 5.5 (Holz et al., 1983). We found that upon fusion, the VMAT2-pHL gives a bright punctate spot (Fig. 7 A) that often lasts for tens of seconds with some diffusion away from the fusion site. To detect endocytosis, the pH of the extracellular solution was repeatedly changed every 10 s between 7.4 and 5.5 (MES buffer substituted for Hepes; Ferguson et al., 2007). Bafilomycin was present (unless otherwise indicated) to inhibit reacidification of granules that underwent endocytosis (Fernández-Alfonso and Ryan, 2004).

The low extracellular pH quenches surface pHluorin but not pHluorin that had undergone endocytosis as shown in Fig. 7 A. Single endocytotic events were readily detected (two events; Fig. 7 A, circles and arrows). Less than 10% of fusion events were associated with rapid endocytosis in control cells (Fig. 7 B). It is unlikely that there were additional events that the method failed to detect as a result of insufficient VMAT2-pHL because granules with the most VMAT2-pHL were not preferentially detected (Fig. 7 C). Because at least 60% of the fusion events showed distinct topological changes upon fusion, most of them do not correspond to rapid endocytosis. In fact, endocytosis would be predicted to result in an increase in P+2S (as well as in P/S; Fig. 4 B, right), an event that has almost never been observed in control cells (Fig. 6 C and Fig. S4). Thus, the topological changes occurring immediately after exocytosis most likely reflect a varying time course of fusion pore widening and flattening of the granule membrane into the plasma membrane.

Endocytosis could not be detected in the absence of bafilomycin (Fig. 7 B), indicating that acidification normally occurs within seconds. This finding indicates that endocytic vesicles that form at the sites of exocytosis of dense core granules in chromaffin cells are likely to be able to take up catecholamine soon after formation. Acidified vesicles may sequester catecholamine that enters the cell through the plasma membrane catecholamine transporter after release.

**Topological changes upon exocytosis are regulated by dynamin**

Dynamin not only plays an important role in clathrin-dependent and -independent endocytosis but has also been implicated in the regulation of protein release from secretory granules (Tsuboi et al., 2004). Dynamin also tubulates lipid membranes in vitro (Sweitzer and Hinshaw, 1998), raising the possibility that its membrane-shaping function regulates the topology of the granule–plasma membrane complex after fusion.

The role of dynamin in the local topological changes after fusion was investigated by inhibiting the GTPase activities of
logical responses that follow exocytosis. An example of a typical incubation with dynasore caused significant changes in the topology, both of which are expressed in chromaffin cells. A brief preincubation with dynasore (Macia et al., 2006). Dynasore is a membrane-permeant inhibitor of dynamin 1 and dynamin 2, both of which are expressed in chromaffin cells. A brief preincubation with dynasore caused significant changes in the topological responses that follow exocytosis. An example of a typical response is shown in Fig. 8. P/S immediately increased after fusion then decreased somewhat but remained elevated for tens of seconds. Most remarkably, P+2S increased, a response that is rarely observed in control cells (Fig. S4).

The topological changes in the presence of dynasore and controls are summarized and compared in Fig. 6. P/S changes in the presence of dynasore tended to be larger (Fig. 6 A), with a greater fraction of fusion events in the presence of dynasore associated with an immediate increase in P/S (86% dynasore and 60% control; Fig. 6 A). Moreover, P/S increases decayed more slowly to baseline in the presence of dynasore (Fig. 6 B). For example, by 31.5 s, 70% of the P/S changes in the control but only 35% in the presence of dynasore had returned to baseline. Most dramatically, P+2S tended to increase after fusion and stay elevated for tens of seconds (Fig. 6 C). Significant increases in P+2S occurred in 14 of 25 events (56%) in which P/S was elevated in the presence of dynasore but in only 1 of 29 events (3%) in control (Fig. S4).

The increase in P+2S strongly suggests that there is more dil-labeled membrane at the site of fusion, which is consistent with superimposed curvature from the fused granule and flat plasma membrane. A likely structure is a fused granule membrane that retains curvature (R > 1.0; Fig. 4; and Fig. 8 C, b). P+2S tended to undergo photobleaching in control but much less so for events in the presence of dynasore. This is also consistent for the structure proposed in Fig. 8 B (b) because a significant amount of the dil is subject to lower intensity excitation because of the decaying evanescent field in the unfattened granule membrane compared with the plasma membrane. Dil molecules in the fused but recessed granule membrane would be exposed on average to one third the excitation intensity compared with dil in the plasma membrane (Schmoranzer et al., 2000).

Electron microscopy indicates that the inhibition of dynamin GTPase activity by dynasore arrests clathrin-mediated endocytosis with clathrin-coated structures that have connections to the plasma membrane with narrow or broad necks (Macia et al., 2006; Newton et al., 2006). The imaging of the topological changes after fusion suggests that dynasore causes the formation of similar structures within 0.5 s of fusion.

**Figure 6.** The magnitude and duration of P/S and P+2S changes at the sites of fusion differ in control and dynasore-treated cells. (A) A cumulative frequency histogram was generated to compare the frequency and magnitude of P/S changes observed with fusion in control (30 granules) and dynasore (27 granules)-treated cells. A greater fraction of fusion events show an associated increase in P/S with dynasore. The percent increase was calculated by taking the difference between the P/S at a particular frame ([P/S] - [P/S]_p)/[P/S]_p divided by the mean; ([P/S] - [P/S]_p)/[P/S]_p. The two histograms are significantly different (P < 0.05 by Mann-Whitney test). (B) The decay of the initial increase in P/S after granule fusion was followed. Only those events that showed an increase of at least 6% in P/S (compared with the prefusion baseline) in the first 0.45 s after fusion and that could be followed for 31.5 s after fusion were considered (control, n = 29; dynasore, n = 25). The fraction of events with a P/S increase that subsequently declines to the baseline within 4.5, 9, 13.5, 18, 22.5, 27, and 31.5 s are plotted for the two conditions. (C) The P+2S of fusion events with a significant increase in P/S were aligned to their prefusion frames and averaged (control, n = 29; dynasore, n = 25). The data were also normalized to the mean of 10 prefusion frames. The arrow indicates a transient dip in P+2S in control cells. In dynasore-treated cells, an increase in P+2S is usually observed. The two datasets are significantly different at every point after time 0 (P < 0.05 by Student’s unpaired t test). Numbers are presented ± SEM.

Lateral displacement of localized topological change in plasma membrane and the last granule position before fusion

Postfusion increases in P/S occurred close to the last position of the granule before fusion, but they were often not overlapping. A striking example of this lateral displacement is shown in Fig. 9 A and Fig. 5 A. There was a 254-nm displacement from the center of the solid white circle (last position of granule before fusion) to the center of the dashed white circle (position of increased P/S). We determined the lateral displacements for fusion events in which there was a clear increase in P/S using a center of mass calculation as described in Materials and methods (Fig. 9 B). The mean displacement of the P/S change from the last position of the granule was 122 ± 15 nm. Such displacements are consistent with the finding that granules move tens to hundreds of nanometers within 100 ms of fusion (Allersma et al., 2006; Degtyar et al., 2007).
Discussion

In previous studies, we and others have investigated granule motions that immediately precede fusion but without concomitant high spatial and temporal resolution of localized plasma membrane topology. In this study, using a combination of dual labeling and pTIRFM, we have succeeded in visualizing fusion by imaging the release of granule contents and the accompanying changes in the local plasma membrane topology. We have extended the theory of pTIRF for measurement of membrane topology, taking into account the emissions captured by a high NA objective and have predicted the emissions from topological changes anticipated in exocytosis. Experimentally, we have demonstrated with high spatial and temporal resolution imaging of plasma membrane diI with p-TIRFM that the technique is able to capture rapid and varied submicrometer changes in topology at sites of exocytosis in chromaffin cells.

The approach used in the theoretical development of pTIRF and the computer simulations should be generally useful in a wide range of studies of plasma membrane morphological dynamics. We show how, under a set of common assumptions and optical conditions, the ratio $P/S$ reports purely upon local membrane orientation, and the linear combination $P+2S$ reports purely on local concentrations (convolved with distance to the glass coverslip). For a model of the membrane geometry of exocytosis (a spherical shell truncated by and fusing with a plane), we have derived the expected $P/S$ and $P+2S$ measures, taking into account many practical factors: granule radius, evanescent field depth, optical resolution limitation, size of integration area, and detector pixelation. Although it is intuitively expected that $P/S$ should increase whenever the membrane is nonparallel with the substrate, many of the considered factors work in opposite directions so the amplitude of the increase is not easily anticipated. In the case of $P+2S$, the theoretical results are also complicated: some geometries and optical setups can lead to an increase and others to a decrease. Nonetheless, an appropriate model can provide the basis for a reasonable and reliable interpretation of the experimental results.

Direct visualization of fusion pore expansion

Recent work supports the idea that events associated with the fusion pore can take distinct pathways. This has important implications for the extent to which plasma membrane morphology is reordered upon fusion. The classical pathway or model holds that subsequent to fusion pore formation, granules merge completely with and flatten into the plasma membrane. Some granule membrane constituents can diffuse away. The granule lipid and some of its proteins are internalized, and the granule membrane rebuilt through poorly understood intracellular sorting mechanisms (Winkler, 1977). Soluble protein content is likely added during interaction with the Golgi. This pathway may be preferred after intense stimulation in chromaffin cells (Fulop et al., 2005). In the second pathway of fusion/recycling, a fusion pore is also formed through which some or all of the granule cargo is released. However, the pore is not fully expanded as in the classical model but rather is closed after a variable amount of time. Thus, the granule does not collapse fully into the plasma membrane, and the empty or nearly empty granule cavity is recaptured largely intact (Taraska et al., 2003; Tsuboi and Rutter, 2003; Perrais et al., 2004; Tsuboi et al., 2004; Fulop et al., 2005). This model has been termed granule cavity recapture or “cavicapture” (Henkel and Almers, 1996).
within hundreds of milliseconds to seconds after fusion, is regulated by dynamin 1, and is independent of clathrin (Artalejo et al., 2002; Fulop et al., 2008). The electrophysiological approaches that have studied this process were not designed to localize individual exocytotic and endocytotic sites in real time. In this study, individual exocytotic and (rapid) endocytosis events were imaged with VMA T2-pHL. Only $\lesssim 10\%$ of the fusion events were associated with endocytosis within 30 s of fusion. Because at least 60% of the fusion events showed distinct topological changes upon fusion, the changes do not correspond to rapid endocytosis. They likely reflect a varying time course of fusion pore widening and flattening of the granule membrane into the plasma membrane. The fused granule membrane sometimes flattens into the plasma membrane in less than a second but often retains curvature for tens of seconds.

We also routinely detected less than a one–granule diameter shift between the last observed position of the granule and the topological change. This provides further evidence for granule motion in the moments just before the fusion event (Allersma et al., 2006; Degtyar et al., 2007).

Another possibility is that the granule fuses with the plasma membrane and only partially flattens into the plasma membrane, retaining curvature without immediate endocytosis. This type of behavior has been observed after fusion of cortical granules in sea urchin eggs (Terasaki, 1995). The fate of the granule membrane immediately upon exocytosis in mammalian cells has been indirectly surmised but has been difficult to directly image because of the small size of the granules and the rapidity of the events.

In this study, pTIRFM was adapted to monitor the transient changes in the plasma membrane upon exocytosis in chromaffin cells. Upon exocytosis (sudden release of NPY-Cer), there was an immediate and significant localized increase in the $P/S$ ratio in $\gtrsim 60\%$ of the events. Recent, higher frequency imaging indicates that $\sim 80\%$ of the fusion events have significant increases in $P/S$. The increase in $P/S$ likely reflects the union of the granule and plasma membranes with this new entity retaining some of the granule curvature. The topological changes decay with a varying time course from less than a second to many tens of seconds, probably representing different outcomes after the initial fusion event.

One possible outcome for the granule membrane after fusion is recycling by rapid endocytosis. This process occurs within hundreds of milliseconds to seconds after fusion, is regulated by dynamin 1, and is independent of clathrin (Artalejo et al., 2002; Fulop et al., 2008). The electrophysiological approaches that have studied this process were not designed to localize individual exocytotic and endocytotic sites in real time. In this study, individual exocytotic and (rapid) endocytosis events were imaged with VMAT2-pHL. Only $\sim 10\%$ of the fusion events were associated with endocytosis within 30 s of fusion. Because at least 60% of the fusion events showed distinct topological changes upon fusion, the changes do not correspond to rapid endocytosis. They likely reflect a varying time course of fusion pore widening and flattening of the granule membrane into the plasma membrane. The fused granule membrane sometimes flattens into the plasma membrane in less than a second but often retains curvature for tens of seconds.

We also routinely detected less than a one–granule diameter shift between the last observed position of the granule and the topological change. This provides further evidence for granule motion in the moments just before the fusion event (Allersma et al., 2006; Degtyar et al., 2007).
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Topological changes detected by pTIRF are modulated by inhibition of dynamin
GTPase activity

Experiments with dynasore suggest that dynamin plays a role in
determining the topological outcome of exocytosis. This specific
inhibitor of the dynamin GTPase caused qualitative changes in
the topological responses that suggest stabilization of a postfu-
sion intermediate of a granule connected to the plasma mem-
brane. This intermediate was rarely detected in the absence of
dynasore but occurred in >50% of the topological changes in the
presence of dynasore. A function for dynamin immediately upon
fusion had been suspected from a variety of experiments that
measured the release of granule contents (Graham et al., 2002;
Tsuboi et al., 2004; Fulop et al., 2008) and membrane proteins
(Jaiswal et al., 2009). However, it is not possible to say at this
time whether the alterations in membrane topology detected with
dynasore are due solely to dynamin inhibition or to a secondary
effect such as clathrin stabilization at the plasma membrane.

Summary

We have shown that pTIRF can be understood theoretically and
and can be implemented in a manner that should have wide applica-
bility for the study of biological processes that involve plasma
membrane shape changes and deformations. We visualize for
the first time topological features of the expanding fusion pore,
provide evidence that its dynamics are regulated by dynamin,
and distinguish the topological changes from rapid endocytosis.
The experiments support the notion that the granule membrane
and plasma membrane become a unique entity upon fusion
whose dynamics and function are specifically regulated.

Materials and methods

Theoretical details: NA effects

The emission pattern of a dipole near a surface is rather complex, with both
the intensity and polarization as a function of both the observation angle
and the fluorophore orientation. A microscope objective, which gathers
light over a wide range of observation angles, will thereby see an intensity
and polarization that is a strong function of its NA. Aspects of the relevant
classical physical optics theory have been worked out by several groups of
researchers, notably by Burghardt and Thompson (1984), who examined
how much fluorescence is captured by a nearby microscopic objective from
a fluorophore in a dielectric material sandwiched between two other (possi-
bly different) dielectric materials. Using the same general approach but with
some modifications, Hellen and Axelrod (1987) calculated the emission
intensity patterns from a fluorophore located in, e.g., water at or near a
glass substrate interface possibly coated with an intermediate material. The
graphical results and discussion in this study are developed from digital
integration of the relevant equations in Hellen and Axelrod (1987).

The emission intensity patterns can be graphically shown on a polar
plot (Fig. 2 A). The emission intensity in any particular direction is denoted
by the radial distance from the center, at which a fluorophore is located
(Fig. 2 A, small circle). Two patterns are shown: one produced by an excited
fluorophore with its dipole oriented parallel to the surface [Fig. 2 A, thicker
line] averaged over all azimuthal angles; the other with its dipole oriented
perpendicular to the surface. Note that the perpendicular dipole emits very
little light end-on along the z axis toward the objective [out of view below].
Both orientations produce sharp intensity peaks at an angle corresponding
to the critical angle. Both orientations also emit light into the glass at angles
greater than the critical angle, but the intensity of these supercritical angles
drops to zero at an observation angle skimming along the interface. The
super-critical intensity is due entirely to the interaction of the dipole’s near field
(a nonpropagating component of the dipole field, strongest within a wave-
length of the fluorophore) with the surface. For objectives with a small NA,
fluorescence from parallel dipole clearly dominates. For larger NA, fluorosc-
cence from a perpendicular dipole becomes increasingly important.

Fig. 2 A assumes that the dipole resides right upon the surface
(z = 0). For dipoles a little farther out into the solution, the intensity pat-
tern changes as the dipole’s near field interacts less with the surface. The
pattern also changes somewhat because reflection of emitted light off the
surface gives rise to interference effects. The collection efficiency (propor-
tional to the amount of light collected by an objective) for each dipole
thereby depends on z. The collection efficiency is denoted as Q_{||} and Q_{\perp},
respectively.

Fig. 2 B shows the ratio of Q_{||}/Q_{\perp} as a function distance of the dipole
from the surface (in nanometers) for a selection of different NAs. Q_{||}/Q_{\perp}
increases with increasing NA as expected. For 1.49 NA objectives, Q_{||}/
Q_{\perp} is fairly close to 1 (actually 1.0 ± 0.2, depending on the fluorophore’s
distance from the surface). Therefore, 1.49 NA objectives see both parallel
and perpendicular excited fluorophore dipoles (roughly) equally well; they
don’t care what the orientation is of an excited dipole. What matters is only
the total number of excited fluorophores in a region corresponding to a
detector pixel regardless of their orientation. Another consequence of Q_{||}/
Q_{\perp} being close to 1 is that rotational diffusion of the fluorophore after exci-
tation has little effect on the total amount of gathered light.

Theoretical details: specific geometry of exocytotic fusion model and
optical resolution limitations

The goal is to calculate a theoretical P and S fluorescence from a realistic
model of the fusion event as seen through a microscopic and recorded by
a charge-coupled device (CCD) array rather than the idealized plane
and sphere model. The exocytotic fusion structure we use to describe the
stages of secretory granule fusion with the plasma membrane is modeled
in this study as spherical surface of radius R truncated by a flat plane, as
schematically shown near the bottom of Fig. 3 (the theoretical method
to be described is also applicable to other geometries). This fusion structure
is both complicated and on the same order of size as the microscope resolution

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and the evanescent field depth, so the idealized model discussed in the text (submicrometer pure sphere or plane) is not adequate for several reasons. Although the distribution of di l (1,1′-dioctadecyl-3,3′,3′-tetra-methylindocarbocyanine perchlorate) may be azimuthally symmetric as integrated over the whole structure (as required in the previous section), it may not be so symmetric in resolvable subregions. The TIRF illumination intensity, which decreases exponentially with distance from the substrate, may vary significantly over the indentation produced by the fusion. The local azimuthal asymmetry, tangential foreshortening, and uneven illumination may be partially resolvable, but these features are significantly blurred by structure by optical resolution limitations. The di l dipole may not lie exactly in the plane of the membrane. Lastly, the actual image on a digital camera is noticeably pixelated over the size of a fusion site (several hundred nanometers).

Chromaffin cell preparation and transfection

Chromaffin cell preparation from bovine adrenal medulla and transient transfection were performed as described previously (Wick et al., 1993; Holz et al., 1994). Cells were plated onto 25-mm coverslips (refractive index 1.51) that had been coated with poly-D-lysine and collagen to promote cell adhesion. Cells were transfected with plasmid encoding NPY-Cer by Ca2+ phosphate precipitation (Wilson et al., 1995). The super-ecliptic pHluorin was inserted into the luminal-facing domain (between transmembrane domains 1 and 2) of the rat VMAT2 sequence (Liu et al., 1992). The parent NPY plasmid was provided by W. Almers (Yollum 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 d after transfection.

Perfusion

Perfusion experiments were performed in a physiological salt solution (PSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 0.5 mM MgCl2, and 15 mM Hepes, pH 7.4, at ~28°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). Generally, cells were perfused with PSS for 10 s and stimulated to secrete with elevated K+–containing solution (95 mM NaCl, 56 mM KCl, 5 mM CaCl2, 0.5 mM MgCl2, and 15 mM Hepes, pH 7.4). Where used, dynasore was added directly to PSS to a final concentration of 80 mM (Macia et al., 2006; Newton et al., 2006). Bafilomycin A1 (GE Healthcare) was added to PSS to a final concentration of 1 mM. Dil-C18 (from Aldrich) was dissolved in ethanol to make a 1 mM stock solution. Dil was added directly to cells bathed in PSS at a 1:50 dilution. The cells were quickly washed several times in PSS and used immediately.

dil-labeled beads

To label beads with dil, 200-nm diameter nanospheres (Duke Scientific) were incubated in ethanolic dil and washed in PSS.

TIRFM

Prismless (through the objective) TIRFM was obtained by directing an Argon ion (514 nm) or solid-state (442 nm) laser (CVI Melles Griot) through a custom side port to a side-facing dichroic mirror z442/514rpc and z442/514 nm emission filter (Chroma Technology Corp.) on an inverted microscope (IX70, Olympus) with the 1.5x internal magnifying lens in the emission path. The beam was focused on the periphery of the back focal plane of a 60x 1.49 NA oil immersion objective (Olympus) so that the laser beam was incident on the coverslip at ~70° from the normal, giving a decay constant for the evanescent field of ~110 nm. The band widths of the emissions were 450–490 nm (Cer) and 530–600 nm (Cer and Dil). Digital images were captured on a cooled EM CCD camera (Andor Ixon; Andor Technology).

Excitation polarization optics

Fig. 51 shows the optical setup used to create the p-pol and s-pol 514-nm beams, superimpose their paths, and further superimpose the 442-nm beam on that path. 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-exposure exposures and 100 gain (EM setting).

At 6.4 Hz, the full cycle of three exposures had a period of 454 ms.
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