Real-time imaging of plasma membrane deformations reveals pre-fusion membrane curvature changes and a role for dynamin in the regulation of fusion pore expansion

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Abstract
Assays for real-time investigation of exocytosis typically measure what is released from the granule. From this, inferences are made about the dynamics of membrane remodeling as fusion progresses from start to finish. We have recently undertaken a different approach to investigate the fusion process, by focusing not primarily on the granule, but rather its partner in exocytosis – the plasma membrane. We have been guided by the idea that biochemical interactions between the granule and plasma membranes before and during fusion, cause changes in membrane conformation. To enable study of membrane conformation, a novel imaging technique was developed combining polarized excitation of an oriented membrane probe 1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate (diI) with total internal reflection fluorescence microscopy (pTIRFM). Because this technique measures changes in membrane conformation (or deformations) directly, its usefulness persists even after granule cargo reporter (catecholamine, or protein), is no longer present. In this mini-review, we first summarize the workings of pTIRFM. We then discuss the application of the technique to investigate deformations in the membrane preceding fusion, and later, during fusion pore expansion. Finally, we discuss how expansion of the fusion pore may be regulated by the GTPase activity of dynamin.

Keywords: chromaffin cells, dynamin, exocytosis, granule, polarization, TIRFM.

into the plasma membrane. Rather, it may be retrieved largely intact via a rapid form of endocytosis (Henkel and Almers 1996) that does not require the formation of a clathrin coat (Artalejo et al. 1995). The presence of multiple pathways for the recycling of the granule membrane after fusion has important implications for secretion. In the adrenal chromaffin cell, a preparation used in our laboratories, constraining, or conversely, promoting fusion pore expansion will affect the extent to which intercellular signaling molecules (catecholamines and neuropeptides) are released into the circulation (Fulop et al. 2005). These circulating molecules modulate the activity of target organs such as the heart and lungs, affecting how they function during rest, and during stress. They may also have local autocrine and paracrine effects.

Because of the molecular revolution of the past two decades, we now know the identity of many of the important proteins involved in granule fusion and endocytosis. However, there remain significant gaps in our understanding of some of the most basic characteristics of the processes themselves. For instance, is the plasma membrane a passive granule ‘acceptor’ or does it play a more active role in engaging the granule membrane? Once fusion occurs, how wide does the fusion pore expand and what are the kinetics of its expansion? What happens to the granule membrane after fusion – where does it go and how is it recovered? Our understanding is partly limited by the fact that the most widely used assays for exocytosis are not always the most appropriate for answering these questions. Electrochemical detection of catecholamine release from individual granules by amperometry is excellent for investigating the initial fusion pore but is unable to report on later expansion. Conventional fluorescent imaging of granule cargo release provides valuable information about the consummation of fusion, but not about prior or later events. Electron microscopy does the best job of providing detailed information on the various stages of fusion because of its excellent spatial resolution. However, the cells must be fixed and the images capture only single moments in time. The progression of fusion from beginning to end must be pieced together from different events, or even different cells.

Most assays for real-time investigation of exocytosis measure the release of granule contents or increase in plasma membrane area (capacitance). Inferences are often made from these measurements about the dynamics of membrane remodeling as fusion and content release progress. We have recently undertaken a different approach to investigate the fusion process, by focusing not primarily on the granule, but rather its partner in exocytosis – the plasma membrane (Anantharam et al. 2010a,b, 2011). We have been guided by the idea that biochemical interaction between the granule membrane and plasma membranes before and during fusion cause changes to membrane shape. To enable our experiments, we utilized a novel imaging technique that combines polarized excitation of an oriented membrane stain 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (diI) with total internal reflection fluorescence microscopy (pTIRFM). In this mini-review, we will first summarize the workings of the technique and how it may be used to detect submicroscopic changes in plasma membrane conformation in real-time. We then discuss the application of the technique to investigate: (i) localized plasma membrane deformations occurring prior to granule fusion; (ii) the expansion of the fusion pore; (iii) the regulation of fusion pore expansion by the GTPase activity of dynamin.

The theory and practice of pTIRFM

Polarization-based TIRFM detects rapid, submicroscopic changes in plasma membrane topology. The key to the technique is a polarization (p) perpendicular to the glass interface (Fig. 1) that is available in TIRF, but not in epifluorescence (where only polarizations in the plane of the coverslip are possible). Together with polarization in the plane of the coverslip (s), the technique detects the orientation of a plasma membrane fluorophore, diI (Fig. 2). The behavior of diI in lipid membranes is well-documented (Ethier et al. 1983; Wolf 1985; Juhasz et al. 2010). For the polarization studies discussed here, diI is added briefly to the extracellular medium and intercalates in the plasma membrane with its transition dipoles nearly in the plane of the membrane (Axelrod 1979; Sund et al. 1999). A powerful theoretical analysis enables interpretation of the results (Fig. 3; Anantharam et al. 2010b).

Images needed to report membrane deformation are obtained by repeated sequences (as fast as 10 Hz sequence frequency) of three TIR excitations: 442 nm (to obtain the epifluorescence (EPI)), 488 nm (to obtain the total internal reflection fluorescence microscopy (TIRF)), and 514 nm (to obtain the polarization of the evanescent field generated by TIR). The evanescent field generated by TIR permits polarization in the z-direction (p-pol) and in the y-direction s-polarization (s-pol). The polarization in epifluorescence is only x or y (in the plane of the glass coverslip). The white arrow indicates direction of light propagation.
image of a Cerulean labeled cargo protein – usually Neuropeptide-Y); s-polarized 514 nm (parallel to the coverslip and perpendicular to the plane of incidence, to obtain the diI ‘s’ image), and p-polarized 514 nm (perpendicular to the coverslip and in the plane of incidence) to obtain the diI ‘p’ image). Regions - even submicroscopic ones - in which the membrane deviates from parallelism with the glass coverslip, are vividly highlighted by calculating pixel-by-pixel ratios of the emissions from p- and s-polarizations (abbreviated P/S of the membrane-embedded diI fluorescence (Fig. 4). The image ratio P/S reports local membrane deviations from parallelism with the glass coverslip. Any membrane deformation that changes the orientation of the plasma membrane from planar, and parallel to the coverslip, will increase P/S. The amplitude of the increase of P/S can be predicted by computer simulations (Fig. 3; Anantharam et al. 2010b), which show it is quite sensitive even to small deformations. Therefore, P/S is the main parameter used to detect and follow membrane deformations.

The linear combination P+2S approximately reports total diI emission from that location, which, in theory, is proportional to the amount of diI at any x-y-z location convolved with the evanescent field intensity, which decays exponentially with z distance from the coverslip. (The approximation is valid if the collection efficiency of the objective is fairly insensitive to emission polarization over the whole range of z distances, as it is for high-aperture objectives such as the numerical aperture [NA] 1.49). The interpretation of changes in P+2S in terms of geometrical models is more ambiguous than for P/S, with increases, decreases, or no detectable change possible, depending upon various countervailing tendencies arising from geometrical details. Computer simulations (Anantharam et al. 2010b) indicate that P+2S will increase if the geometry results in more diI-labeled membrane close to the glass interface. This could occur if a granule is attached to the plasma membrane through a narrow neck (such as in Fig. 3, structure ‘a’). P+2S decreases if diI diffuses into a post-fusion membrane indentation (Fig. 3, structure ‘c’, 250 nm radius granule) placing diI farther from the substrate, and thereby in a dimmer evanescent field intensity).
Plasma membrane remodeling prior to granule fusion

Electron micrographs sometimes show granules contacting areas of the plasma membrane that are invaginated (Fig. 5a) (Plattner et al. 1997; Anantharam et al. 2010a). We were interested in knowing whether these were snapshots of dynamic changes in membrane shape prior to fusion, or alternatively, long-lived indentations that were preferred sites of exocytosis. Using pTIRFM, 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. There was no evidence for plasma membrane deformations to be associated with granules that were already present for a long period in the evanescent field (>100 granules investigated in 10 cells). Most fusion events occur from such granules (Allersma et al. 2004). There was also no tendency for pre-fusion plasma membrane deformations at sites of exocytosis. However, for the small population of granules (13) that appeared in the field during elevated K+-induced depolarization, six were preceded by a membrane deformation, of which two subsequently fused.

Fig. 5b shows images of one such granule appearing in the evanescent field with elevated K+ depolarization. A transient membrane deformation (P/S increase, line at 11.25 s) occurs just prior to the appearance of the granule in the field. Over the next several seconds, both the granule and plasma membrane appear to be moving closer to the coverslip (both NPY-Cer and P+2S emission intensity increase). By 18.9 s after stimulation (‘time’ in B and C refer to time after the beginning of cell stimulation), the granule fuses and releases NPY-Cer (not shown).
Morphologically, the P/S increase and P+2S decrease in Fig. 5c is expected to result in indentations similar to those shown in the electron micrographs (Fig. 5a). It is unclear how or why such deformations accompany granules arriving at the membrane upon stimulation. They may reflect a process by which the granule is brought to the plasma membrane or a process that primes the interaction for fusion. A more detailed investigation of this phenomenon is required.

Plasma membrane remodeling during the fusion process

The first direct demonstrations of the early fused state were electron micrographs of ‘omega figures’ of the fused granule (Hallade 1975). Subsequently, the initial fusion event was detected using electrophysiological techniques in living cells by the groups of Zimmerberg (Zimmerberg et al. 1987) and Almers (Breckenridge and Almers 1987a,b). The initial fusion pore (<3 nm diameter; Albillos et al. 1997; Breckenridge and Almers 1987b; Zimmerberg et al. 1987) expands to a much wider connection with the extracellular medium after a delay of usually tens to hundreds of milliseconds (Zhou et al. 1996). The expansion is important for controlling the selectivity and kinetics of the release of soluble granule contents (Albillos et al. 1997). The initial fusion pore is well described by amperometry, but the technique is unable to probe its wider expansion. Polarized-TIRFM can follow the wider expansion (Anantharam et al. 2010b, 2011). Because this technique measures deformations in the membrane, its signal persists after granule cargo reporter (catecholamine, or protein), is no longer present.

To perform pTIRFM studies, we depolarized diI -stained chromaffin cells to stimulate exocytosis, while monitoring changes in plasma membrane conformation. A sudden increase in P/S occurred simultaneously with the release of NPY-Cer in ~90% of release events (Anantharam et al. 2011). The increase reflects the union of the granule and plasma membranes, with this new entity retaining some of the granule curvature (Fig. 6a–c). Subsequent changes in P/S followed a variable time course. The deformation could last for less than 1 s, but often decayed more slowly (Fig. 6c), sometimes not returning to baseline for tens of seconds (for examples, see Fig. S3 from Anantharam et al. 2010b).
Regulation of post-fusion deformations associated with pore expansion by dynamin

Dynamin has an established role as a master controller of fission during endocytosis (Hinshaw and Schmid 1995; Takei et al. 1995). It forms rings or collars around the necks of budding endocytic vesicles (Hinshaw and Schmid 1995), triggering cooperative GTP hydrolysis (Warnock et al. 1996) and membrane remodeling, leading to fission (for recent reviews, see Doherty and McMahon 2009; Pucadyil and Schmid 2009; Schmid and Frolov 2011). A number of studies suggest that dynamin can function not only in endocytosis but also immediately upon fusion to regulate the kinetics of granule content release (Graham et al. 2002; Holroyd et al. 2002; Tsuboi et al. 2004; Fulop et al. 2008; Gonzalez-Jamett et al. 2010) and membrane protein dispersion into the plasma membrane (Jaiswal et al. 2009). Indeed, we found that dynasore, an inhibitor of dynamin GTPase activity and clathrin-mediated endocytosis (Macia et al. 2006), slowed the kinetics and altered the characteristics of the membrane changes detected by pTIRFM, suggesting a role for endogenous dynamin in fusion pore expansion (Anantharam et al. 2010a).

The GTPase activity of dynamin plays a critical role in catalyzing membrane fission in endocytosis. Mutants that alter the GTPase activity alter the rates of clathrin-mediated endocytosis. To determine directly whether dynamin GTPase activity regulates fusion pore expansion, adrenal chromaffin cells were transiently transfected with wild-type dynamin-1 (Dyn1WT), Dyn1 mutant T65A (T65A) with reduced GTPase activity, or Dyn1 mutant T141A (T141A) with elevated GTPase activity (Marks et al., 2001; Song et al. 2004). We found that expression of Dyn1 mutants with either decreased or increased GTPase activity substantially altered the characteristics of membrane dynamics detected upon fusion. The effects of the mutants could be distinguished both by the time course of membrane deformations (P/S) and their possible geometries (as suggested by changes in P+2S). Representative examples of P/S and P+2S changes in cells expressing the mutants are shown in Fig. 7 and discussed below.

Cells expressing Dyn1 (T65A) with low GTPase activity display a long-lived deformation upon fusion associated with an increased amount of diI-labeled membrane (i.e. increased P+2S; Fig. 7a–c). This is consistent with a granule membrane connected to a plasma membrane via a short neck. Such an event would occur if expansion of the pore is limited after fusion (Fig. 7c). Long-lived increases in P/S and P+2S might also result from a diI-labeled granule undergoing endocytosis but remaining close to the plasma membrane. However, this outcome is unlikely given the lower rather than higher frequency of endocytosis with Dyn1 (T65A) (Song et al. 2004; Anantharam et al. 2011). Conversely, in cells expressing Dyn1 (T141A) with high GTPase activity, deformations (P/S increases) after fusion are transient and are usually accompanied by decreases, rather than increases, in P+2S. These changes suggest that higher GTPase activity speeds expansion of the pore, resulting in rapid collapse of the granule membrane into the plasma membrane (Fig. 7d–f). Indeed, in the presence of Dyn1 (T141A), 45% of the fusion events were not associated with a change in P/S compared to 13% in control cells, 13% in cells expressing Dyn1WT and 0% in cells expressing Dyn1 (T65A). The increased incidence of fusion events without evident changes in P/S in cells expressing Dyn1 (T141A) likely reflects...
topological changes occurring faster than the temporal resolution of the technique (consistent with the amperometry, below).

The membrane events detected by pTIRFM are associated with intermediate and late fusion pore expansion with deformations detected with a time resolution of approximately 100 ms. To address the possibility that the dynamin GTPase activity regulates fusion pore expansion within milliseconds of exocytosis, carbon-fiber amperometry was performed. Fig. 8a shows an example of an amperometric spike detected after depolarization of a chromaffin cell with a long pre-spike foot (PSF). Expression of Dyn1WT in chromaffin cells had no effect on the percentage of spikes detected with a PSF compared with the control group. Expression of Dyn1 (T141A) with increased GTPase activity significantly reduced the percentage of spikes with a PSF from 46% for Dyn1WT to 34% ($p < 0.05$, Student’s unpaired t-test). The duration of the PSF was also significantly reduced in cells expressing Dyn1 (T141A) compared to cells expressing Dyn1WT (Fig. 8b). Conversely, the duration of the PSF was increased in cells with the slow GTPase mutant, Dyn1 (T65A). Further analysis of these events showed that this mutant significantly increased the fraction of PSFs with relatively long durations (Fig. 8c). These data indicate that the properties of the early fusion pore are affected by dynamin in ways that are self-consistent with dynamin’s effects on later pore expansion (pTIRFM).

The effects of dynamin on the amperometric prespike foot suggested to us that it might already be at or close to fusion sites before exocytosis. Indeed, immunocytochemistry demonstrated that endogenous dynamin (in the absence of transfected dynamin) is highly concentrated on the plasma membrane in adjoining puncta, labeling most of the exposed membrane (Anantharam et al. 2011). Transiently expressed dynamin (detected by immunocytochemistry) also localizes to the plasma membrane (usually as puncta) and is significantly expressed in the adjacent cytoplasm. TIRFM imaging of chromaffin cells revealed GFP-tagged dynamin to be on or

![Fig. 7 Examples of membrane topological changes after fusion in cells expressing dynamin-1 (Dyn1) GTPase mutants. Chromaffin cells were co-transfected with NPY-Cer and either Dyn1 (T65A) or Dyn1 (T141A) mutants. (a) Images are shown of total internal reflection fluorescence microscopy (pTIRFM) responses p-pol emission/s-pol emission (PS) and p-pol emission+2(s-pol emission) (P+2S) to exocytosis in a cell with transfected human Dyn1 (T65A). (b) The PS and P+2S increase is long-lived in a cell transfected with Dyn1 (T65A) with low GTPase activity. (c) One interpretation of the event is considered in the cartoon. The data are also consistent with a 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DI)-labeled granule having undergone endocytosis at the site of fusion and remaining close to the plasma membrane (not shown). Dil transition dipole moment orientation is indicated by direction of arrowheads. (d,e) Conversely, the PS increase after fusion is transient in a cell transfected with Dyn1 (T141A) with elevated GTPase activity. P+2S transiently decreases in region highlighted by white arrow (d). (f) The changes observed are consistent with computer simulations of structures shown in the cartoon. Structures (i) and (ii) represent two possible curvature transitions leading to granule membrane collapse into the plasma membrane. Both structures result in increased PS and decreased P+2S emission. Another possibility not shown is rapid endocytosis with retreat of the dil-labeled granule into the cell.]
near the plasma membrane, sometimes organized into discrete puncta (Anantharam et al. 2011). Live cell imaging did not show localized accumulation of dynamin immediately upon fusion, perhaps because of transient and/or small amounts of accumulation over background. The experiments may also have been limited by the 10 Hz image acquisition and countervailing effects of displacement by the granule membrane or diffusion into the fused and recessed granule membrane.

Possible mechanism of dynamin action to regulate fusion pore expansion

We are struck by the remarkable coherence between current and past studies concerning the effects of varying dynamin’s GTPase activity. Increased GTPase activity increases the rates of fusion pore expansion (Anantharam et al. 2011), rapid endocytosis (Anantharam et al. 2011) and slower, clathrin-mediated endocytosis (Song et al. 2004). One explanation for an effect of dynamin on fusion pore expansion is that it is an indirect consequence of dynamin’s role in endocytosis, with fusion pore expansion being restricted and eventually reversed along the pathway to fission. However, it is difficult to reconcile our data with this model. This scenario would predict that a mutant that increases the frequency of rapid endocytosis would also inhibit fusion pore expansion. Just the opposite occurred. The high GTPase mutant (T141A) increased the incidence of rapid endocytosis and also greatly hastened (rather than slowed) the flattening of granules not undergoing endocytosis (Anantharam et al. 2011).

We favor a mechanism based upon dynamin’s membrane sculpting ability and an additional property that is also essential in endocytosis: its self-limited assembly on lipids in the presence of GTP. GTP hydrolysis is enhanced many-fold by dynamin assembly, which, in turn, reverses assembly and interaction with lipid (Warnock et al. 1996; Ramachandran and Schmid 2008), and only sometimes leads to endocytosis (Bashkirov et al. 2008; Pucadyil and Schmid 2009; Schmid and Frolov 2011). Membrane fission may therefore be considered a stochastic result of GTP hydrolysis. This notion fits well with the results in this study. We speculate that dynamin assembly restricts fusion pore expansion until GTP hydrolysis-stimulated disassembly. Higher GTPase [Dyn1 (T141A)] activity causes the decision between fission (Fig. 9b–c) or fusion pore expansion (Fig. 9b–d) to be made earlier, with a somewhat elevated incidence of rapid endocytosis. Lower GTPase [Dyn1 (T65A)] activity delays the decision, often stabilizing the narrow-neck fusion intermediate (Fig. 9b). Indeed, there is evidence that a high curvature protein/lipid intermediate such as b is rendered unstable when dynamin reduces its lipid interaction upon GTP hydrolysis, leaving a high curvature lipid neck no longer stabilized by interaction with dynamin (Bashkirov et al. 2008; Schmid and Frolov 2011).

Although numerous proteins have been shown to influence fusion pore expansion, dynamin is unique. Its intrinsic membrane-shaping capacity provides a direct biochemical and mechanistic link between fusion pore expansion and membrane fission in endocytosis. Dynamin may not be acting alone. Some of the proteins that have been shown to alter fusion pore expansion (measured by amperometry or protein release) interact with dynamin either directly or indirectly through its proline/arginine-rich domain (PRD). These include actin (Witke et al. 1998; Lee and De Camilli 2002; Berberian et al. 2009; Gu et al. 2010), the SNAREs (Okamoto et al. 1999; Galas et al. 2000; Robinson and Bonifacino 2001; Lam et al. 2008; Ngatchou et al. 2010), and synaptotagmin (Robinson and Bonifacino 2001; Wang et al. 2001; Lynch et al. 2008; Zhang et al. 2011). Thus,
Dynamin may be an integral component of the pathway that controls fusion pore expansion and the release of lumenal contents. A role for dynamin based upon the effects of dynamin inhibitors and dynamin mutants has recently been suggested for another curvature generation process: viral fusion protein-induced cell-to-cell fusion. Thus, dynamin may function in multiple cell biological processes involving high curvature intermediates in addition to membrane fission in endocytosis (Richard et al. 2011).

**Summary**

pTIRFM is a powerful and sensitive technique to investigate biological processes that involve plasma membrane shape changes and deformations. In this review, we discussed the application of the technique to visualize deformations occurring before the fusion event, and later, during fusion pore expansion. Recent work supports the idea that events associated with the fusion pore can take distinct pathways (Taraska et al. 2003; Tsuibo and Rutter 2003; Perrais et al. 2004; Fulop et al. 2005). This has important implications for the extent to which plasma membrane morphology is reordered upon fusion. It is from this perspective that we have approached the study of fusion pore expansion—not by studying what is released from the granule, but rather the dynamics of the membrane itself.

Our studies provide three important insights into the sequence of events accompanying fusion. First, in a small number of cases, plasma membrane invaginations precede the appearance of a granule within the evanescent field (Anantharam et al. 2010a). The deformations indicate an intimate interaction of the granule with the plasma membrane. They may reflect a process by which the granule is brought to the plasma membrane or a process that primes the interaction for fusion.

Second, we find that upon fusion, the granule membrane often does not rapidly collapse into the plasma membrane (Anantharam et al. 2010b). Rather, the deformations detected suggest a varying time course of fusion pore widening, usually leading to substantial collapse of the granule membrane into the plasma membrane. The fused granule sometimes collapses into the plasma membrane in less than a second, but typically, it retains curvature for many seconds. We also found that upon elevated K⁺-induced depolarization, rapid endocytosis with retrieval of the granule membrane occurs infrequently.

Third, we find that the post-fusion fate of the granule membrane is regulated by dynamin GTPase activity (Anantharam et al. 2010b, 2011). When dynamin GTPase activity is reduced (by inhibitor or expression of a dynamin mutant), expansion of the fusion pore is slowed with concomitant long-lasting membrane deformations. When dynamin GTPase activity is elevated by mutation, fusion pore

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**Fig. 9** Possible functions of dynamin upon granule fusion with plasma membrane. Dynamin may have a role in the enlargement of the fusion pore (a–b) and the ‘decision’ (b) to either undergo rapid endocytosis (c) or relatively stable incorporation of the granule membrane into the plasma membrane (d). The decision is stochastic, the timing of which is dependent upon dynamin GTPase activity. Slow, clathrin-dependent endocytosis (also dynamin dependent, not shown) follows the incorporation of the granule membrane into the plasma membrane. There is evidence that dynamin-1 is involved in rapid endocytosis (c) and dynamin-2 in slow, clathrin-mediated endocytosis. A role for PIP₂ is also indicated. Dynamin interaction with PIP₂ through its PH domain is required for its function in membrane fission. PIP₂ is also required for exocytosis.
expansion is accelerated, with more rapid curvature transitions. The ability to identify specific effects of the GTPase activity suggests a mechanism that may explain its function. The findings expand the membrane-sculpting repertoire of dynamin to include the regulation of immediate post-fusion events in exocytosis and provide a direct biochemical and mechanistic link between secretion and endocytosis.

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References


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