REVIEW

Secretory granule behaviour adjacent to the plasma membrane before and during exocytosis: total internal reflection fluorescence microscopy studies

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Abstract

Our current notions of different granule pools, granule interaction with the plasma membrane, and ultimately granule and plasma membrane soluble N-ethylmaleimide-sensitive-factor attachement protein (SNARE) interactions, result largely from inferences based upon biochemical alterations of secretion kinetics. Another view of events comes from studies using total internal reflection fluorescence microscopy (TIRFM) to investigate granule behaviour immediately adjacent to the plasma membrane. The motions of secretory (chromaffin) granules in bovine chromaffin cells visualized by TIRFM are highly restricted, as if granules are caged or tethered. These small motions are regulated by ATP and Ca^{2+}, two factors that increase priming of the secretory response. There is no evidence that granules decrease their motion immediately before secretion. To the contrary, there is a tendency for granules to increase their motions and travel within a few hundred milliseconds of fusion. Hence, the notion of a long-lived docked state as a prelude to fusion does not encompass the physical reality of molecular scale motions, multiple tethering states and significant travel immediately preceding the exocytotic event. Increased travel may increase the probability of granules interacting productively with the plasma membrane constituents, thereby, increasing the probability of fusion.
Keywords chromaffin cells, exocytosis, secretory granule, SNARE proteins, total internal reflection fluorescence microscopy.

Virtually all models of exocytosis envision granule movement to the plasma membrane and a plasma membrane docking step. The precise nature of these events has been surmised but not directly investigated. 'Morphological' docking has been defined by static electron microscopic images (Nakata & Hirokawa 1992, Vitale *et al.* 1995, Steyer *et al.* 1997). Inferences concerning the dynamics of events immediately before fusion are based upon the kinetics of secretion. In these experiments granule pools are defined by the rates at which granules fuse with the plasma membrane after a Ca^{2+} stimulus and the manner in which the kinetics are altered by biochemical manipulations. Another view of

granule behaviour comes from quantitative studies of granule motion using wide field, confocal and especially total internal reflection fluorescence microscopy (TIRFM) of fluorescent-tagged secretory granules. The latter technique selectively illuminates the aqueous phase immediately adjacent to a glass interface with an exponentially decaying excitation (the evanescent field, decay constant 50–100 nm) (Axelrod 1981, 2003). A distance into the cell extending less than a granule diameter is selectively visualized. Motions of granules labelled with green fluorescent protein (GFP)-tagged lumenal cargo or granule membrane protein can be determined with resolution of ~10 nm.

Motions perpendicular to the glass interface (z motions) are calculated from changes in fluorescence intensity, whereas granule motions parallel to the glass interface (R motions) are determined by interpolated centre of intensity measurements (Johns *et al.* 2001, Allersma *et al.* 2004). A common finding in TIRFM studies in chromaffin cells is that most (>98%) of the granules adjacent to the plasma membrane are highly restricted in their motion (Steyer *et al.* 1997, Oheim *et al.* 1998, Han *et al.* 1999, Johns *et al.* 2001, Ohara-Imaizumi *et al.* 2002) as if tethered or caged. The size of the motions, tens of nanometres, is the size of large proteins and of individual steps sizes of molecular motors (5–10 nm for myosin II and 37 nm for myosin Va) (Mehta *et al.* 1999, Murphy *et al.* 2001, Veigel *et al.* 2003, Yildiz *et al.* 2003).

Techniques

In the experiments described below, we used objective-based TIRFM, which was obtained by focusing an Argon ion laser (488 nm) on the periphery of the back focal plane of a 100×1.65NA, oil immersion objective (Olympus, Center Valley, PA, USA). The laser beam was incident on the coverslip at 58–64° to the optical axis, resulting in a ∼55 nm decay constant of the evanescent field. Digital images were captured on a cooled CCD camera. Images were acquired at 10 Hz with exposure times of 20–50 ms.

A major issue for interpretation of the experiments is the need for demanding image analysis. Granules were identified and tracked through a time-sequence stack of images using software written in interactive data language (ITT Visual Information Solutions, Boulder, CO, USA). The procedures permitted the analysis of vast amounts of information. Granules undergoing exocytosis were visually identified. From the stack of images, a data set was generated containing x and y coordinates, granule intensity and local background for each granule in each frame. The x and y coordinates were determined from band-pass-filtered images. Positions were calculated from the peaks of inverted parabolas determined by the intensities of the brightest pixel and each immediately adjacent pixel in the diagonals. Changes in position perpendicular to the glass interface (Z direction) were calculated from changes in granule intensity resulting from the exponential decay of the excitation. Total granule intensity was determined in unfiltered images from the average pixel intensity within a circular region (600 nm diameter, 81 pixel area) around the granule centre after the local background was subtracted. The local background was the average intensity of a ring of 32 pixels just outside the circle. The data set was then analysed to track granules, calculate inter-frame motions and determine measures of granule travel in the xy plane.

Monolayers of bovine chromaffin cells were transfected with a plasmid encoding atrial natriuretic peptide (ANP)-GFP, a marker of the secretory granule lumen or vesicle associated membrane protein (VAMP)-GFP, a marker of the secretory granule membrane. Cells were transfected using calcium phosphate precipitation within 16 h of dissociation. Experiments were performed 4–7 days later, which is sufficient time for labelled granules to mature and attain the cellular distribution and secretory characteristics of endogenous secretory granules. These characteristics are important because newly synthesized granules have different trafficking and secretory characteristics than the majority of endogenous granules that are responsible for secretion in these highly, differentiated, non-dividing cells (Duncan *et al.* 2003).

Granule motions are regulated

Granules undergo priming steps before secretion that are regulated by ATP and calcium (Holz *et al.* 1989, Bittner & Holz 1992, Rüden & Neher 1993). These co-factors also affect granule motion (Allersma *et al.* 2006). Removal of ATP in permeabilized cells caused granule motion to decrease. Motions of individual granules were tracked during incubation with ATP and during a following change to a solution either with or without ATP. Average granule motions in the *XY* and *Z* directions were unchanged in the continuing presence of ATP but decreased by ∼20% within 1 min of ATP removal. A larger effect of ATP removal was observed when the motions of a subpopulation of granules that were especially slow moving in the *XY* plane were analysed. Motions of these granules increased 38% in the continuing presence of ATP whereas upon ATP removal, motions were further decreased by 13%. Hence, a major effect of ATP is to increase the motion of slow moving granules. Removal of ATP is associated with a loss of plasma membrane PtdIns-4,5-P_2 (Eberhard *et al.* 1990, Holz *et al.* 2000) and a loss of cortical actin (Bittner & Holz 2005). The interplay between changes in the environment of granules visualized by TIRFM and granule motions is not understood but could involve molecular motors such as myosin II and myosinVa. Despite numerous investigations over the years, the roles of actin in late stages of the secretory pathway are poorly understood. TIRFM studies indicate that cortical actin does not play a major role in restricting granule motion (Lang *et al.* 2000, Allersma *et al.* 2006).

Nicotinic stimulation causes a small calcium-dependent increase in granule motion (Allersma *et al.* 2006). The analysis of the motion of 330 non-fusing granules revealed a 17% increase in the median motion in the *XY* and *Z* directions ($P < 0.001$). The effect of Ca^{2+} in increasing granule motion has been observed in other tissues. For example, in the *Drosophila* neuro-

muscular junction Ca²⁺, calmodulin and calmodulin kinase II have been implicated in increased motion (Shakiryanova *et al.* 2005, 2007).

Of major interest is the motion of the subpopulation of granules about to undergo exocytosis. Surprisingly, these granules undergo relatively large increases in motion and travel within several hundreds of milliseconds of fusion, as described below. This effect is not observed for neighbouring, non-fusing granules.

Visualization of exocytosis with VAMP-GFP

Secretory granules labelled with the v-SNARE, VAMP-GFP (GFP luminal), showed distinct signatures upon exocytosis when viewed by TIRFM (Allersma *et al.* 2004). In approximately 90% of fusion events, we observed a large increase in fluorescence intensity coupled with a transition from a small punctate appearance to a larger, spreading cloud with free diffusion of the VAMP-GFP into the plasma membrane (Fig. 1). Quantitation indicates that these events reflect two steps in the fusion process: (1) initially, a fused and spherical granule resulting in an approximately twofold increase in fluorescence due to the increase in lumenal pH, and (2) subsequent granule flattening into the plasma membrane and/or free diffusion of VAMP-GFP through the fusion junction into the plasma membrane. Fluorescence spreads and further brightens approximately fivefold as VAMP-GFP is brought closer to the glass interface (Allersma *et al.* 2004). The distinctive fluorescence changes upon of fusion obtained with VAMP-GFP-labelled granules permitted timing of the beginning of fusion events to within 0.1–0.2 s.

Granules can move tens to hundreds of nanometres within 100 ms of fusion

Most of the granules that undergo secretion in chromaffin cells are present in the evanescent field for tens of seconds to many minutes. Motions both in the *XY* plane and *Z* direction increase within the last seconds before fusion (Allersma *et al.* 2006, Degtyar *et al.* 2007) with an average twofold increased in the last 100 ms before fusion. There is considerable variability in the absolute magnitude of the motions. However, almost all of the granules had motions in the *XY* plane within 100 ms of fusion that was well above instrumental noise (Allersma *et al.* 2006). The motions ranged from <10 to 200 nm.

The travel of granules destined to fuse is increased immediately before exocytosis

The above studies focused on the average motions during 100 ms intervals. Important aspects of granule dynamics are suggested by inspection of granule paths parallel to the glass interface over many seconds. In Figure 2, a granule is followed for 5 s before stimulation (black track beginning at 'O') and then for 9 s during nicotinic stimulation (red track) until it fuses with the plasma membrane at 'X'. The granule moves less than its diameter during the entire sequence. The high spatial resolution measurements suggest that the granule increases its *travel* upon stimulation. Just before fusion the granule moves to an area it has not visited previously.

Analysis of 72 tracks of granules that are destined to fuse confirms these impressions (Degtyar *et al.* 2007). Granules extend their travel during stimulation and often move to areas not previously visited in the last few hundred milliseconds before fusion. This was most evident for granules that fused after 8 s of nicotinic stimulation. Fifty-eight per cent of these fusion events occurred in granules that had suddenly moved to an area that they had rarely or never visited before (0–2 visits). In contrast, during the rest of the track (29 positions), only 12% of the time did granules move to a position which had been visited twice or less. When the motions of the same granules were analysed in the
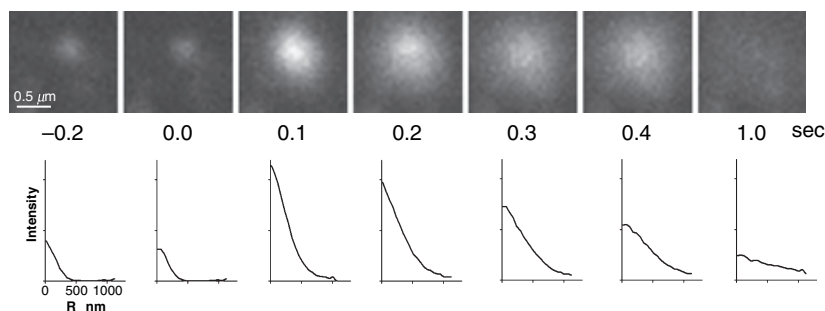
Figure 1 An intensity burst characterizes the fusion of an individual VAMP-GFP-labelled granule with the plasma membrane. Successive frames show the fusion of a VAMP-GFP-containing granule and the subsequent spread of the VAMP-GFP in the plasma membrane in TIRFM. The frame just prior to fusion is *t* = 0.0 s. Below each image is the VAMP-GFP intensity (with background subtracted) averaged as a function of radial distance *r* away from the granule centre. Peak intensity is in the centre of the intensity profile. The fluorescence almost completely disappeared after 1 s (from Allersma *et al.* 2004).
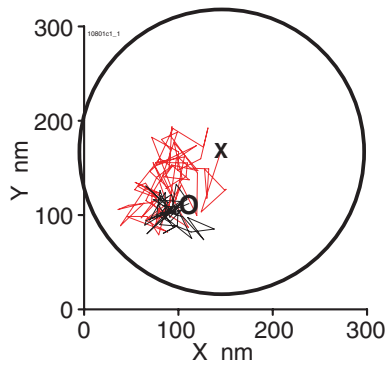
Figure 2 Track of a chromaffin granule before and during stimulation until fusion. The granule was followed for 5 s before stimulation (black path) and for 9 s during stimulation with DMPP (red path) until fusion (marked with 'X'). The large black circle represents a 300-nm granule centred on the last position before fusion. 'O' indicates the beginning of the track.

same way for 30 frames before nicotinic stimulation, there was no difference between the last position and the 29 previous positions in the tendency of granules to visit new regions. The frequency of 0–2 previous visits to the same area for the last position was only 17%. The tendency to move to new areas was acquired within 0.7 s of fusion. It was not observed for non-fusing granules analysed at the same time as their fusing neighbours.

These observations of granule motion before exocytosis contrast with the common assumption that granules that are morphologically close to the plasma membrane (within a granule diameter) are stably interacting with the membrane and that this bound state is required for priming and subsequent exocytosis. Granules with very small motions (<10 nm) may indeed be interacting with proteins involved in the fusion reaction. However, those with large motions (>50 nm) probably engage the fusion machinery only within the last 100 ms.

There are at least two explanations for the increased motion and travel of granules before exocytosis. One is statistical. Some granules are favourably positioned at the time of stimulation to interact with key proteins or lipids, thus allowing fusion to occur relatively quickly with little travel. These granules may be part of the rapidly releasable pool detected after flash photolysis of caged Ca^{2+} (Rettig & Neher 2002). However, many granules are not in a favourable location for fusion. For such granules, sampling the plasma membrane in new territory rather than in domains that the granule had already visited unproductively increases the probability of subsequent fusion. Indeed, the greater tendency of late fusing granules to travel to new areas in the last few hundred

milliseconds before fusion supports this notion. The increased travel could reflect the action of molecular motors or sudden granule untethering.

Alternatively, the increased motion may be part of the fusion mechanism itself. For example, spatially asymmetrical, successive zippering of multiple SNARE complexes (10 nm motions; Liu *et al.* 2006) may increase motion of the granule along the plasma membrane just before fusion.

In summary, it is evident from our work (Johns *et al.* 2001, Allersma *et al.* 2004, 2006) and the work of others (Steyer *et al.* 1997, Oheim *et al.* 1998, Lang *et al.* 2000, Huet *et al.* 2006, Toonen *et al.* 2006, Tsuboi & Fukuda 2006, Nofal *et al.* 2007) that there are different states of granule tethering adjacent to the plasma membrane. High resolution measurements of granule motion indicate that the highly restricted motion is regulated. Quantitative measures of granule motion and travel of fusing granules during nicotinic stimulation indicate that the notion of a long-lived docked state as a prelude to fusion does not encompass the physical reality of molecular scale motions, multiple tethering states and significant travel immediately preceding the exocytotic event. To the contrary, our recent analyses suggest that granules with greater travel and the ability to interact with more regions along the plasma membrane can be favoured to undergo fusion.

Conflict of interest

There is no conflict of interest.

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