Localization of Phosphatidylinositol 4,5-P_2 Important in Exocytosis and a Quantitative Analysis of Chromaffin Granule Motion Adjacent to the Plasma Membrane

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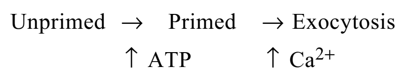
ABSTRACT: A slow ATP-dependent priming step precedes a rapid, Ca^{2+}-dependent triggering step in exocytosis in chromaffin cells and in most, if not all, differentiated secretory cells. A major component of ATP-dependent secretion in permeabilized cells reflects the maintenance of the polyphosphoinositides, especially PtdIns-4,5-P2. Here we summarize recent experiments with PH-GFP (binds to PtdIns-4,5-P2) that indicate that PtdIns-4,5-P2 is localized primarily on the plasma membrane in chromaffin cells, and that it is this pool that plays a role in exocytosis. It is demonstrated that transiently expressed PH-GFP inhibits secretion in subsequently permeabilized cells. Recent studies using total internal reflection fluorescent microscopy (TIRFM) to measure chromaffin granule motion adjacent to the plasma membrane are also summarized. The quantitative analysis indicates that chromaffin granule motion is highly restricted and suggests that chromaffin granules are caged or tethered immediately adjacent to the plasma membrane.

KEYWORDS: phosphatidylinositol 4,5-P_2; exocytosis; chromaffin granule motion; pleckstrin homology domain; green fluorescent protein; pro-atrial natiuretic peptide

THE ATP-DEPENDENCY OF SECRETION AND THE MAINTENANCE OF PHOSPHATIDYLINOSITOL 4,5-P_2

Studies in permeabilized chromaffin cells demonstrated that the secretory pathway could be separated into distinct kinetic steps with different biochemical characteristics.[1–3] A slow ATP-dependent priming step precedes a rapid Ca^{2+}-dependent triggering step.



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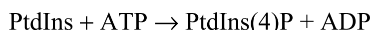
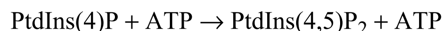
Similar pathways exist in PC12 cells,[4] melanotrophs,[5] and bipolar neurons[6] and are likely to operate in most if not all differentiated secretory cells. It was subsequently discovered that a major component (as much as 70%) of the ATP dependency of secretion in permeabilized chromaffin cells reflects the maintenance of the polyphosphoinositides.[7] (Another component is NSF-mediated, ATP-dependent reactions.) The enzymatic removal of phosphatidylinositol (PtdIns) in permeabilized cells results in the subsequent decline in phosphatidylinositol 4,5-P_2 (PtdIns(4,5)P_2) and PtdIns(5)P and the specific inhibition of ATP-dependent secretion.[7] The study demonstrated that removing either substrate of PtdIns-4 kinase, PtdIns or ATP, inhibited exocytosis. Studies in PC12 cells strongly advanced the concept of the role of the polyphosphoinositides in secretion. Two cytosolic factors that are necessary for ATP-dependent priming of exocytosis were identified as a phosphatidylinositol transfer protein[8] and PtdIns(4)P-5 kinase.[9] These studies implicated the following reactions in ATP-dependent priming of secretion:

PtdIns-4 kinase


$$\text{PtdIns} + \text{ATP} \rightarrow \text{PtdIns(4)P} + \text{ADP}$$

PtdIns(4)P-5 kinase


$$\text{PtdIns(4)P} + \text{ATP} \rightarrow \text{PtdIns(4,5)P}_2 + \text{ATP}$$

The experiments in chromaffin[7] and PC12 cells[8,9] provided the first evidence that the polyphosphoinositides play important roles in vesicular trafficking reactions. Subsequently, polyphosphoinositides were implicated in numerous vesicular trafficking pathways (reviewed in De Camilli *et al.*[10] including synaptic vesicle exocytosis,[11,12] ARF1-dependent trafficking from the endoplasmic reticulum, endocytic recycling of synaptic vesicle membrane, and endocytosis of G-protein coupled receptors.[13] Polyphosphoinositides labeled in the 3 position of the inositol ring are necessary for vesicular trafficking between the Golgi and vacuole/lysosome in yeast and mammalian cells.[14,15]

PtdIns(4,5)P_2 is generally thought to be located on the plasma membrane.[16–18] It has also long been known, however, that PtdIns-4 kinase is an integral membrane protein of the chromaffin granule membrane.[19–22] PtdIns-4 kinase is also associated with synaptic vesicles[11] and mast cell granules.[23] It is, therefore, possible that PtdIns(4,5)P_2 could be synthesized on the secretory granule membrane through the sequential action of granule PtdIns-4 kinase and PtdIns(4)P-5 kinase.

We investigated the localization of PtdIns(4,5)P_2 that is involved in exocytosis by transiently expressing in chromaffin cells a pleckstrin homology (PH) domain that specifically binds PtdIns(4,5)P_2 and is fused to green fluorescent protein (GFP). PH domains are approximately 100-amino-acid motifs with a common tertiary structure with little sequence homology and different binding activities (for reviews, see Refs. 24 and 25). The PH domain of phospholipase C δ_1 is responsible for the binding of the enzyme to its substrate, PtdIns(4,5)P_2, on the plasma membrane.[26] The isolated PH domain binds specifically and with high affinity to PtdIns(4,5)P_2 and to Ins(1,4,5)P_3[27–29] because of strong interactions between PH domain residues and the position 4 and 5 phosphates on the inositol ring.[30] The phospholipase C δ_1 PH domain fused to GFP (PH-GFP) identified the plasma membrane as the main pool of PtdIns(4,5)P_2 in cells types not specialized for Ca^{2+}-dependent secretion.[31,32]

Transiently expressed PH-GFP almost exclusively labeled the plasma membrane of chromaffin cells, with no detectable labeling of chromaffin granules[33] (FIGS. 1 and 2). Localization of PtdIns(4,5)P_2 was also investigated with rhodamine-labeled neomycin. Neomycin is an aminoglycoside antibiotic that strongly binds PtdIns(4,5)P_2.[34–36] Rhodamine-neomycin also labeled the plasma membrane in digitonin-permeabilized cells. The labeling of the plasma membrane with rhodamine-neomycin occurred in the presence of MgATP (which maintains PtdIns(4,5)P_2), but not in its absence. Labeling of chromaffin granules by rhodamine-neomycin was not apparent. The data suggest that the major pool of PtdIns(4,5)P_2 in chromaffin cells is on the plasma membrane, with little PtdIns(4,5)P_2 on chromaffin granules.

The specific labeling of plasma membrane PtdIns(4,5)P_2 by PH-GFP provided the opportunity to determine whether this pool of PtdIns(4,5)P_2 is the pool required for exocytosis. We used four different means of stimulating exocytosis, and, in every case, the expression of PH-GFP significantly inhibited secretion.[33] DMPP- and Ba^{2+}-induced secretion from intact cells expressing PH-GFP was inhibited by greater than 50%. Because the inhibition could have been caused by a PH-GFP-induced change in either Ca^{2+} or Ba^{2+} influx in the transfected cells rather than by a downstream effect on the secretory response, secretion from permeabilized cells in which the Ca^{2+} concentration is directly controlled was investigated. The expression of PH-GFP inhibited secretion directly stimulated by 30 μM Ca^{2+} by 50%. Finally, plasma membrane capacitance increases due to exocytosis (normalized to Ca^{2+} influx) were similarly inhibited by the expression of PH-GFP. Taken together, these experiments indicate an important role for plasma membrane PtdIns(4,5)P_2 in maintaining secretion in response to a Ca^{2+} signal.

If the PH domain inhibits secretion by binding PtdIns(4,5)P_2, then its expression should preferentially inhibit ATP-dependent secretion. Indeed, expression of the PH domain inhibited ATP-dependent secretion by 80% and ATP-independent secretion by 40% (FIG. 3). Secretion that is independent of ATP in permeabilized cells reflects the prior effect of ATP in intact cells immediately before permeabilization.[1] The experiments suggest that, in intact cells, transiently expressed PH-GFP shifts the dynamic equilibrium between primed and unprimed secretion, so that there is reduced primed secretion. This interpretation predicts that neomycin, which binds PtdIns(4,5)P_2, should inhibit only ATP-dependent secretion when added to permeabilized cells and not secretion that was already primed by PtdIns(4,5)P_2 in intact cells. Indeed, neomycin inhibited ATP-dependent secretion with no effect on secretion stimulated in the absence of ATP (manuscript in preparation).

Recently, the regulation by neuronal activity of presynaptic PtdIns(4,5)P_2 was investigated in cultured hippocampal neurons transiently expressing PH-GFP.[37] As in chromaffin cells, PH-GFP selectively labeled the plasma membrane. Synaptic vesicles were not labeled. Upon electrical stimulation, PH-GFP accumulated in the center of boutons where endocytic, clathrin-coated vesicles accumulate. Because PtdIns(4,5)P_2 is metabolized by the lipid phosphatase synaptojanin to permit uncoating of clathrin from endocytic vesicles,[38] the dynamics of PH-GFP labeling may reflect a PtdIns(4,5)P_2 cycle important in exocytosis and endocytosis. Newly formed endocytic vesicles contain PtdIns(4,5)P_2 from the plasma membrane but must lose the lipid in order to mature into synaptic vesicles. The lack of PtdIns(4,5)P_2 in the membranes of both synaptic vesicles and secretory granules from the protein biosyn-

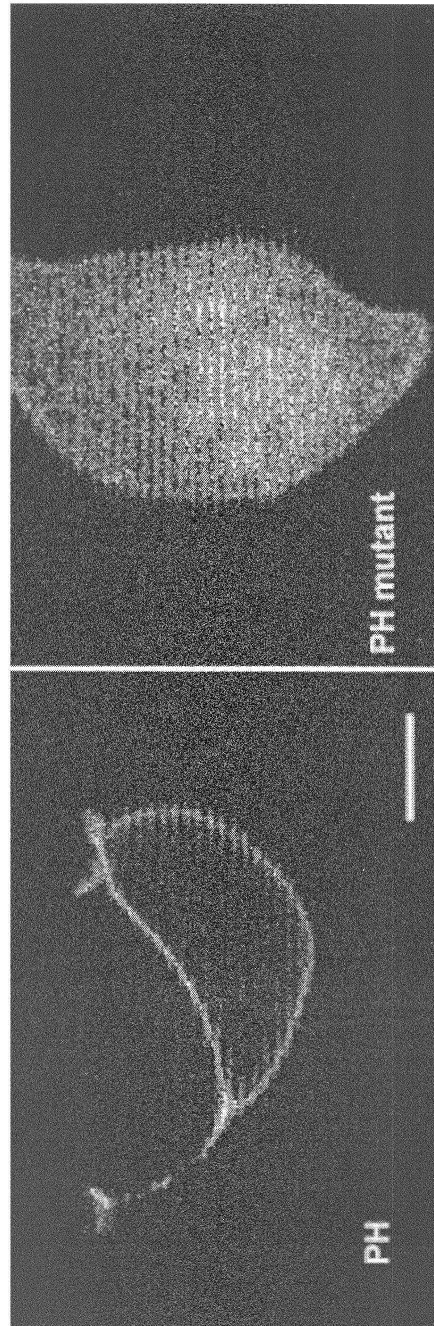


FIGURE 1. PH-GFP specifically localizes to plasma membrane. Chromaffin cells were transfected with a plasmid encoding wild-type PH-GFP or a mutant PH(S34T,R40L)-GFP that does not bind PtdIns(4,5)P_2. Four days later, the cultures were examined by confocal microscopy. Note that PH-GFP, but not the mutant construct, specifically binds the plasma membrane. The calibration bar is 5 μm. (From Holz *et al.*;[33] used with permission.)

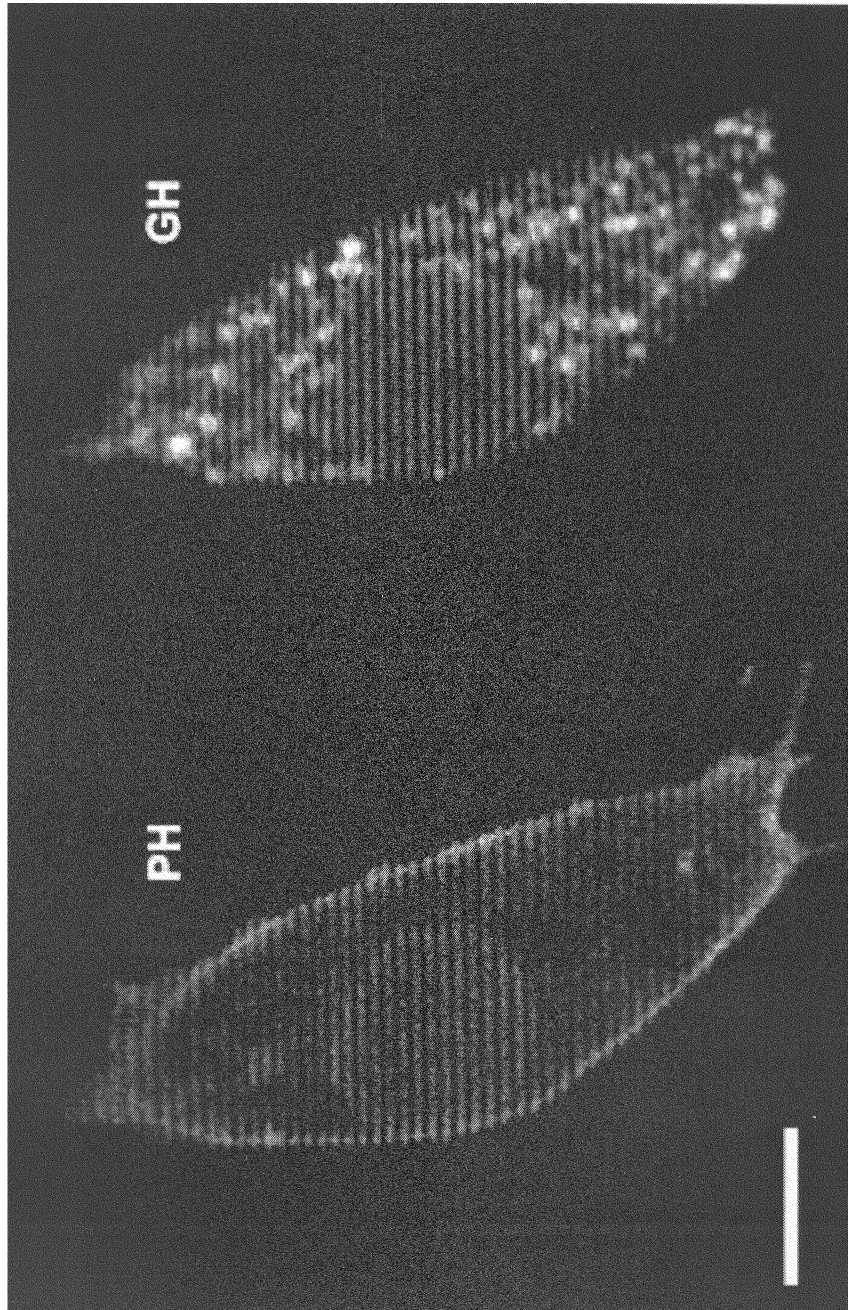


FIGURE 2. *See following page for legend.*

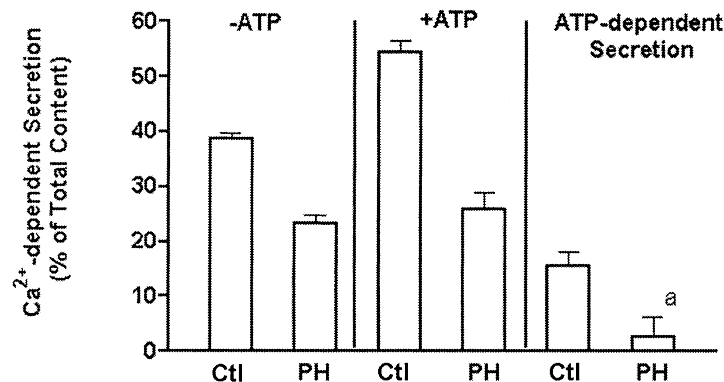


FIGURE 3. The ATP-dependency of the inhibition of secretion by transiently expressed PH-GFP. Chromaffin cells were transfected with plasmids encoding PH-GFP or control plasmid (Ctl) and human growth hormone. Four days later, the cells were permeabilized with 20 µM digitonin ± 30 µM Ca^{2+} in solution containing 139 mM potassium glutamate, 10 mM PIPES, and 2 mM MgATP. Ca^{2+} was buffered with 5 mM EGTA. Ca^{2+}-dependent secretion of hGH was determined as previously described.[55] There were four samples/group. $^{a}P < 0.01$ versus Ctl.

thetic pathway (e.g., chromaffin granules) may have important implications for the process of exocytosis itself.

How might plasma membrane PtdIns(4,5)P_2 be involved in exocytosis? The early experiments ruled out the involvement of PtdIns(4,5)P_2 in secretion as a substrate for phospholipase C.[7] Instead, the lipid is likely to act in the secretory pathway as an allosteric regulator of specific protein function. It is intriguing that there are now three proteins associated with the chromaffin granule membrane that bind in a specific manner: PtdIns(4,5)P_2-synaptotagmin,[39] Rabphilin3,[40] and calcium-dependent activator protein for secretion (CAPS).[41] The Ca^{2+}-regulated interaction of one or more of the proteins with PtdIns(4,5)P_2 in the plasma membrane may modulate protein function and could possibly be directly involved in the fusion reaction.

Additionally, the requirement of plasma membrane PtdIns(4,5)P_2 in secretion may reflect a role for the lipid in regulating cytoskeletal dynamics immediately adjacent to the plasma membrane during exocytosis. Chromaffin cells have a 200-nm-thick actin layer immediately adjacent to the plasma membrane.[42] The polyphosphoinositides, including PtdIns(4,5)P_2 and 3-phosphorylated forms, interact with and

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FIGURE 2. PH-GFP does not label secretory granules. Chromaffin cells were cotransfected with plasmids encoding wild-type PH-GFP and hGH. Five days later, cells were fixed and permeabilized. Immunocytochemistry with anti-hGH identified secretory granules; fluorescence of GFP identified the PH domain. There is virtually no labeling of hGH-containing chromaffin granules by PH-GFP. The calibration bar in the confocal image is 5 µm. (From Holz *et al.*;[33] used with permission.)

regulate numerous cytoskeletal proteins and proteins involved in vesicular trafficking (reviewed in Refs. 15 and 43).

RESTRICTION OF SECRETORY GRANULE MOTION NEAR THE PLASMA MEMBRANE REVEALED BY TOTAL INTERNAL REFLECTION FLUORESCENT MICROSCOPY

The vast majority of studies concerning secretion measure only the final events of fusion and release of granule contents. Although a great deal has been learned about the very late steps in the secretory pathway, these studies have allowed only inferences to be made about the preceding steps, including the freedom or restriction of granule motion near the membrane before secretion. Recent studies on chromaffin cells by two groups[44–47] employed an optical technique, total internal reflection fluorescence microscopy (TIRFM; see Refs. 48 and 49 for descriptions of the theory and practice of TIRFM), to provide for the first time quantitative details about granule motion near the plasma membrane preceding and during exocytosis. TIRFM selectively illuminates subcellular features close to the plasma membrane at cell–substrate contact regions, without interference from optical signals from deeper within the cell. The previous TIRFM studies with chromaffin cells mainly utilized fluorescent weak bases that accumulate in acidic compartments within the cell, including chromaffin granules. In one of the studies, stimulation with a secretagogue resulted in a decrease in the surface density of punctate fluorescent spots. In addition, the movements of several spots were tracked over several minutes with decreased motion observed before the disappearance of granules by exocytosis.[44,46,50] The previous studies quantified some aspects of the basal granule motion, predominately in the lateral direction (the "*x–y*" plane parallel to the membrane).

Weak bases label all acidic compartments in the cell including those that are not chromaffin granules. We have taken a different approach to labeling chromaffin granules.[51] We have expressed by transient transfection in chromaffin cells a GFP-tagged protein that is sorted to secretory granules. The protein, pro-atrial natriuretic peptide–green fluorescent protein (ANP-GFP) has been shown to be selectively stored in secretory granules in PC12 cells and to be secreted into the medium by secretagogues.[52] The construct was successfully used to monitor secretory granule motion using epifluorescence and confocal microscopy.

We investigated granule motion in unstimulated cells with optics that penetrated ~300 nm (one granule diameter) into the cell.[51] Our emphasis was on the quantitative analysis of *z*-motion (toward and away from the plasma membrane), which is evident as a flickering fluorescence in the exponentially decaying evanescent field. Motion in the *z* direction is of great importance for exocytosis, since it brings the secretory granule into contact with the plasma membrane with which it fuses. Because the decay of the evanescent field with distance from the substrate is very steep (e-fold in 80 nm in our setup) and the granules are quite bright, measurements of granule motion in the *z* direction are extremely sensitive, with granule movements of 4 nm readily detectable. Measurements of granule motion in the *z*-axis are approximately an order of magnitude more precise than those in the *x–y* plane.

We observed a wide variety of motions including, but not limited to, granules that approached the membrane and then receded back into the cell. In fact, examples of

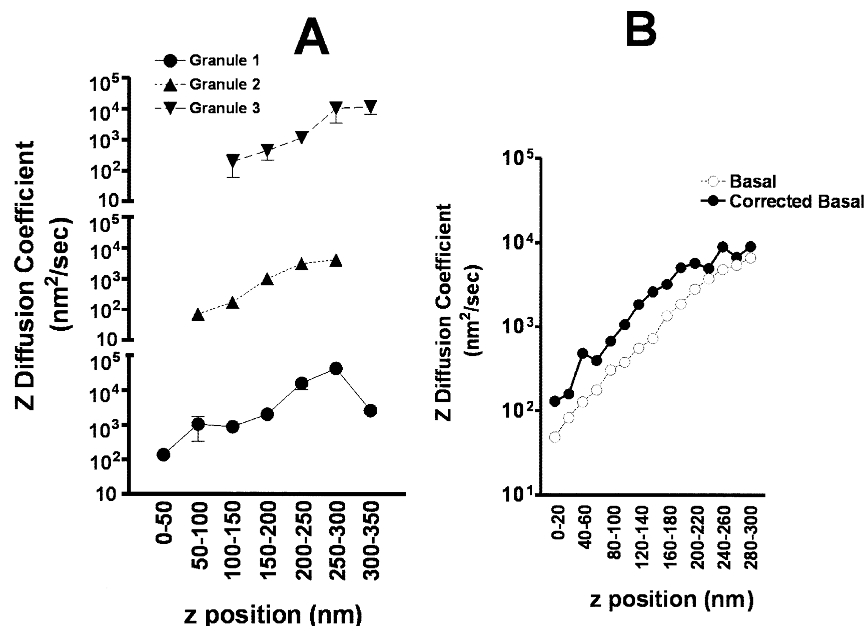

FIGURE 4. Chromaffin granule motion becomes dramatically more restricted upon approach to the plasma membrane. **(A)** Three typical chromaffin granules are tracked by imaging at 0.5-s intervals for 120 seconds. As each granule is located in the indicated *z* range, it undergoes small steps toward and away from the plasma membrane. The size of the steps is determined by changes in the fluorescent intensity of the granule. The local diffusion constant is calculated from these small motions. Free diffusion of a particle the size of a chromaffin granule in a medium the viscosity of cytosol (10 times the viscosity of water) would be ~ 1×10^5 nm^2/s. **(B)** The diffusion coefficients at various distances from the plasma membrane of all granules appearing in at least two successive frames is presented. The plot is based upon 21,090 individual granule motions. The corrected data (*filled circles*) take into account the actual distribution of granule intensities measured by confocal microscopy. (Adapted from Johns *et al.*[51])

almost any type of behavior could be seen. We took a statistical approach that evaluated the movements in a much larger population of tracked granules than previous studies. We found the following:

(a) Large movements in the *x–y* plane or *z* direction are rare. Less than 2% of the granules move more than several granule diameters in the *x–y* plane over a period of several seconds.

(b) Granules rarely underwent unidirectional motion in either the *x–y* plane or the *z*-axis.

(c) Motion in the *z* direction could be quantitated with great accuracy. The mean granule movement of a large number of granules in the *z* direction was 26.6 ± 0.3 nm in 0.5 sec ($n = 21{,}530$ individual granule motions).

(d) Granules became strikingly more restricted in motion as they approached the plasma membrane, with short-term diffusion coefficients decreasing 100-

fold as granules moved just 300 nm (a granule diameter) closer to the membrane (FIG. 4).

Despite the dominant randomness of the motions, two features were inconsistent with a purely Brownian model: (a) the apparent caging of long-distance motion, and (b) a tendency to reverse directions (contrary to unidirectional motion) as shown by the negative autocorrelation of granule *z* velocity.

We investigated the possible roles of the v-SNARE, VAMP, and the t-SNARE, SNAP-25, in granule mobility. A plasmid encoding the light chain of either tetanus toxin (cleaves VAMP) or botulinum neurotoxin type A (cleaves the COOH-terminus of SNAP-25) was cotransfected with the plasmid encoding ANP-GFP, and granule motion investigated. In parallel experiments, the transiently expressed light chain of the toxins strongly inhibited secretion of cotransfected human growth hormone, indicating that the transiently expressed toxins cleaved their respective substrates. Neither TeNT/A nor BoNT/A altered the precipitous decrease in short-term diffusion coefficients of granules upon approaching the plasma membrane. However, a more subtle effect of the toxins was observed. A subpopulation of granules—those granules that were present in at least 40 successive frames and moved through a range of at least 100 nm—was tracked to characterize the motion histories of individual granules. These granules spent a smaller fraction of time near the plasma membrane in toxin-expressing cells than did granules in a similarly defined subpopulation in control cells. The relative reduction in time spent by granules in toxin-expressing cells close to the plasma membrane is consistent with the notion that SNARE proteins are important in mediating granule–plasma membrane interactions. The granules may be spending less time "sampling" the plasma membrane, a possible priming process for exocytosis.

It is likely that the mechanisms involved in slowing granules as they approach the plasma membrane do not involve VAMP2 or SNAP-25. Instead, there is evidence that the cortical actin cytoskeleton that is within ~200 nm of the plasma membrane[42] plays a role in the restricted motion of granules.[53,54] It is possible that plasma membrane PtdIns(4,5)P_2, which is maintained by ATP, plays a role in regulating the cytoskeleton, and thereby, granule motion.

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