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The actin binding protein Scinderin acts in PC12 cells to tether dense-core vesicles prior to secretion

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Abstract

Mechanistic understanding of the control of vesicle motion from within a secretory cell to the site of exocytosis remains incomplete. In this work, we have used total internal reflection (TIRF) microscopy to examine the mobility of secretory vesicles at the plasma membrane. Under resting conditions, we found vesicles showed little lateral mobility. Anchoring of vesicles in this membrane proximal compartment could be disrupted with latrunculin A, indicating an apparent actin dependent process. A candidate intermediary between vesicles and the actin skeleton is the actin binding protein Scinderin. Co-transfection of an shRNA construct against Scinderin blocked secretion, and also increased the mobility of vesicles in the membrane-proximal section of the cell, indicating a dual role for scinderin in secretion; tethering vesicles to the cytoskeleton, as well as liberating them following stimulation through the previously described calcium dependent actin severing activity. Analysis of lipid dependence indicates that scinderin exhibits calcium dependent binding to phosphatidyl-inositol monophosphate, providing a possible mechanism for vesicle binding.

Keywords

vesicle exocytosis; actin cytoskeleton; TIRF microscopy; scinderin

Introduction

Despite the rapid advances in our understanding of the process of vesicular exocytosis, there remain many areas that are poorly understood. One such is – how are vesicles guided to their eventual sites of exocytosis? There is now extensive evidence that the actin cytoskeleton plays a role in exocytosis, both at fast synapses (Cole et al., 2000; Morales et al., 2000; Richards et al., 2004) and in neuroendocrine cells (Nakata and Hirokawa, 1992; Vitale et al.,

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1995; Lang et al., 2000; Johns et al., 2001; Wang and Richards 2011). In electron micrographs of deep-etched freeze-fractured chromaffin cells, a lattice of actin filaments running parallel to the plasma membrane was observed, with clear attachment of the filaments to secretory vesicles (Nakata and Hirokawa, 1992). This and other studies (Vitale et al., 1995; Lang et al., 2000) led to the hypothesis that the actin cytoskeleton formed a barrier to secretion located just within the plasma membrane. Vitale and colleagues (Vitale et al. 1995) showed that vesicles were excluded from a depth of approximately 50nm inside the plasma membrane, and that stimulation allowed vesicles to move closer to the plasma membrane. This provided a simple mechanism for two pools of releasable vesicles in these cells (Gil et al., 2000); a readily releasable pool comprising vesicles already present at the plasma membrane, and a reserve pool of vesicles which are recruited upon depletion of the first pool. This model has required re-evaluation in the light of more recent findings that actin may influence the exocytic event itself (Neco et al., 2004; Doreian et al., 2008). In more recent years this has continued to be an area of extensive research, generally extending the core hypothesis (reviewed in Meunier and Gutiérrez, 2016) and suggesting that the architecture may even influence the spread of the triggering calcium signals and the organization of the secretory machinery (Torregrosa-Hetland et al., 2011).

In chromaffin cells, Scinderin was isolated as a factor that provided a calcium dependent ability to sever actin filaments and thus allow vesicles access to the plasma membrane (Rodriguez-DelCastillo et al. 1990), while more or less concurrently being isolated from adrenal medulla, where it was named adseverin (Maekawa and Sakai, 1990). Scinderin is a member of the gelsolin family of actin-binding proteins (Rodriguez –Del Castillo et al. 1990, Maekawa and Sakai, 1990), and both are found in secretory cells (Rodriguez –Del Castillo et al. 1990). Both scinderin and gelsolin are able to bind actin monomers in addition to severing and capping actin filaments. Recombinant scinderin protein dialyzed into cells, was found to potentiate Ca^{2+} -evoked exocytosis from permeabilized chromaffin cells (Zhang et al. 1996) and antisense scinderin decreased scinderin expression and stimulation-evoked release from the same cells (Lejen et al. 2001).

In this work, we have used TIRF microscopy to examine the motility of vesicles immediately adjacent to the plasma membrane (~100nm). Under both resting conditions, we find these vesicles to be relatively static with respect to lateral motion, and that this vesicle immobility is dependent on the actin cytoskeleton. RNA interference knockdown of the calcium dependent actin severing protein Scinderin greatly increased the motion of membrane proximal vesicles, while also inhibiting secretion. Analysis of recombinant scinderin binding to immobilized lipids indicates that scinderin exhibits calcium dependent binding to phosphatidyl-inositol monophosphate, providing a putative mechanism for vesicle binding.

Materials and Methods

Cell culture

PC12 cells (ATCC) were maintained in flasks in F12 medium (Invitrogen) supplemented with 15% horse serum (ATCC) and 2.5% Fetal bovine Serum (Invitrogen). Prior to experiments they were split, transfected, and seeded into 6 well dishes containing acid-

washed, cover slips previously coated with GelTrex (Invitrogen). Transfections were carried out using electroporation in a Gene Pulser (Bio Rad) using 20 µg DNA.

Molecular Biology

ANFP-EmGFP was a generous gift from Dr Ed Levitan, University of Pittsburgh, and the insert subcloned into pmCherry vector (Clontech). Scinderin was directly cloned from mouse brain cDNA by RT-PCR, and inserted in-frame into the pAcGFP vector from Clontech. Mutations to convert it to a shRNA suppression resistant form, as well as PIP binding domain mutations, were introduced by standard PCR. All constructs were fully sequenced by CCHMC sequence center to ensure accuracy and reading frame. Protein expression and purification used the BioRad Profinity system. Protein concentrations were measured by BCA assay. Gene knockdown was targeted for the sequence identified in Figure 1, and inserted into the bi-cistronic LMP vector (a kind gift from Dr. Roger Briesewitz, Ohio State University), together with either GFP, or a GFP-shRNA resistant scinderin fusion. The cytoplasmic domain of synaptobrevin/VAMP (amino acids 1–91, cdVamp) was created by PCR from VAMP2 purchased from Open Biosystems, and cloned into the AcGFP vector to allow visualization of transfected cells. Transfections were carried out using electroporation in a Gene Pulser (Bio Rad) using 20 µg DNA.

Imaging

Cells were imaged 3 days after transfection, in saline consisting of 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 5.5 mM glucose, 20 mM HEPES buffered to pH 7.3 using NaOH. Stimulation was achieved through depolarization elicited by substitution of an additional 20mM KCl for NaCl in the bathing solution. Cover slips with cells were mounted in an imaging chamber (Warner Instruments) and viewed from beneath with conventional epifluorescence optics and an APO N 65x 1.49 NA objective optimized for fluorescence (Olympus). Image acquisition was controlled by a computer running Slidebook 4.2 (Intelligent Imaging Innovations). Imaging was carried out on an Olympus ix80 inverted microscope with integrated high precision focus drive. Fluorescence excitation was provided by a rapid switching DG4 light source (Sutter Instruments) attached by liquid light guide, and overall image acquisition rates was 4 Hz. The cooled CCD camera was a Hammamatsu ORCA R2, communicating to the host computer via firewire interface. TIRF illumination was achieved through the objective, using 488 and 536 nm lasers (Melles Griot), with fine adjustment by micrometers to optimize TIRF illumination. The depth of evanescent field was assessed using apparent size of ring-stained 1 micron beads, and geometric calculations suggest an estimate of 80–100nm.

Image analysis

TIRFM secretion experiments were analyzed as described previously (Wang and Richards 2011). Briefly, we generated a minimum intensity projection for each time series, and subtracted this image from each frame to remove static artifacts from the image sequence. This subtracted any permanent features of the image from the stack, allowing arriving vesicles to be clearly seen. As this was a flat numerical subtraction, it did not affect vesicle rise and fall times. A maximum intensity projection image of this new stack was then used to identify regions of exocytosis, and each spot was surrounded by a 9 pixel region of

interest (ROI). This ROI was then extracted as an average intensity over time plot, and analyzed for peaks using Microcal Origin. Since fluorescence peaks can be ascribed to both axial movement and expulsion of contents, identified peaks were manually checked for loss of fluorescence complete within 4 seconds, as this is consistent with the rate of fluorescence loss from vesicles where there is a clear 'halo' following secretion. This analysis does not include vesicle which are already present prior to secretion. Number and amplitude of peaks were then compared between conditions. Vesicle tracking was carried out as previously described (Chicka et al., 2016), and based upon centroid tracking of vesicles moving laterally within the TIRF field.

Lipid binding

Lipid binding was assessed using PIP-strips (Echelon; Jin et al., 2006), hydrophobic membranes that have been spotted with 100 pmol of all eight phosphoinositides and seven other biologically important lipids. Membranes were blocked with 5% BSA in PBS-tween, then incubated with 200ng/ml of recombinant HA-tagged scinderin for 1 hour. After washing, the membranes were incubated with anti-HA HRP conjugated antibody (Sigma) in 3% BSA for 1 hour, and then washed again. Detection was by ECL and film.

Results

In a previous series of experiments, we extended work indicating the importance of actin in secretion of dense-cored vesicles, and demonstrated that one role for actin was in regulating membrane accessibility for secretion (Wang and Richards, 2011). This led to the obvious questions (a) how are vesicles guided through the actin cortex found adjacent to the membrane, and (b) how is the actin cortex regulated during secretion? To try to answer these questions, we generated shRNA constructs to knock down the protein Scinderin (also known as adseverin), which has previously been shown to have calcium dependent actin severing ability, and to be required for secretion (Zhang et al., 1996, Lejen et al., 2001). We then used reverse transcription of cDNA extracted from transfected PC12 cells to look for successful knockdown of scinderin mRNA. We chose the most effective construct to continue with the study, and generated an alternative version of scinderin with the targeted sequence modified to generate a synonymous mutated form of scinderin that was resistant to shRNA knockdown. A representative gel, together with targeting and modified sequences, is shown in figure 1A.

As previously described (Lang et al., 1997, Oheim and Stuhmer 2000), TIRF microscopy restricts fluorescent excitation to a small layer, approximately 100 nm deep, immediately above the cover glass. With this technique, individual vesicles labeled with ANF-EmGFP can be resolved (Han et al., 1999) as small spots of fluorescence that appear and then disappear on stimulation. Figure 1B shows a typical TIRF field prior to stimulation. In figure 1C we show example intensity traces over time of regions of interest centered on individual vesicles, illustrating the clear temporal resolution of vesicle arrival and fusion, as well as the stimulation (20mM KCl) dependent increase in rate of fusion. This is a mild stimulus, and consequently not the large increase in secretory rate that can be seen in PC12 cells following more robust stimulation (eg Zhang and Jackson 2008); it was chosen to clearly track

individual vesicle arrival at the membrane. Panel D summarizes the rates of vesicle fusion in control cells, those transfected with anti-scinderin shRNA, and those co-transfected with anti-scinderin shRNA and the shRNA resistant scinderin construct, before and after stimulation. Consistent with previous reports indicating a requirement for scinderin in catecholamine secretion (Zhang et al., 1995, Lejen et al., 2001), scinderin knockdown caused a significant downregulation of secretion (18% of control, after stimulation, $p < 0.01$) which was rescued by co-expression of the synonymous rescue scinderin construct (90% of control, ns). Interestingly, this includes both spontaneous (basal) and stimulated secretion. In order to identify what proportion of vesicles represented “true” membrane fusion (productive exocytosis) as opposed to vesicle arrival and departure, we carried out additional experiments where we blocked SNARE-dependent fusion with transfection of the cytoplasmic domain of the v-SNARE synaptobrevin/VAMP (‘cdVAMP’; Weber et al 1998, Tucker et al., 2004); this indicated that more than half of detected vesicles showed SNARE dependent fusion. The residual portion of vesicles not blocked by cdVAMP probably reflects some combination of incomplete block due to non-saturating levels of cdVAMP, as well as a small number of vesicles demonstrating plasma membrane binding and unbinding with sufficiently rapid kinetics to be included in the analysis.

An unexpected finding in these experiments was an impression on reviewing the timelapse image series, that the vesicles in the scinderin knockdown cells were less rigidly held in place. Previously, we examined secretory vesicle motion in platelets (Chicka et al., 2016), and we used the same approach to quantify the mobility of secretory vesicles in PC12 cells. Through tracking the visual center of mass of the vesicles over time, we can build up a description of the vesicles displacement over time. Figure 2A shows tracks of vesicles in control and scinderin knockdown cells; these tracks were acquired for > 200 vesicles from at least 4 separate cell platings per condition. This allowed us to calculate the mean squared displacement over time for vesicles in wild type cells, as well as those with scinderin knockdown and knockdown plus rescue construct. For comparison, we also examined vesicles in cells where the actin cytoskeleton was disrupted by treatment with $10 \mu\text{M}$ latrunculin A. Disruption of the actin cytoskeleton caused a marked increase in the rate of vesicle motion as has been described previously (Oheim et al., 2000); using the relation that $\text{MSD} = 4Dt$ (Qian et al., 1991), this gives a value for D of $3.73 \times 10^{-3} \mu\text{m}^2\text{s}^{-1}$ for control cells, rising to $19.25 \times 10^{-3} \mu\text{m}^2\text{s}^{-1}$ for latrunculin A treated cells, $28.58 \times 10^{-3} \mu\text{m}^2\text{s}^{-1}$ for scinderin knockdown cells, and a recovery to $5.40 \times 10^{-3} \mu\text{m}^2\text{s}^{-1}$ for the rescue condition.

How might scinderin be responsible for vesicle tethering? Analysis of the scinderin protein structure has revealed multiple gelsolin-like actin binding domains, as well as two regions with homology for PIP2 binding (Zhang et al., 1996). Since it is unlikely that scinderin is restricted to the inner leaflet of the plasma membrane where PIP2 is found (Holz et al., 2000), we speculated that perhaps scinderin might bind other phospholipids. We investigated this using PIP-strips, which contain spots of different phospholipids immobilized on a membrane (see figure 3A, left panel). Incubation with HA-tagged purified recombinant scinderin allowed binding between the protein and different phospholipids, which could then be visualized using an anti-HA antibody. The right panel illustrates an experiment of this type, where multiple strips have been exposed in the presence of different concentrations of calcium, and then were exposed to film simultaneously for 2 minutes. In this way, we could

measure the calcium dependent binding between scinderin and different lipids (figure 3B). This revealed strongest binding for the mono-phosphorylated forms of phosphatidyl inositol. Figure 3C shows data extracted from this, revealing a bell shaped calcium dependence of binding between scinderin and phosphatidyl inositol 4-phosphate.

As a starting point for potential future studies on the structural basis of scinderin-lipid binding, we carried out simple domain deletion experiments, where we removed each of the putative PIP2 binding domains in turn (amino acids 112-120 – ‘1’ and 138-146 – ‘2’), and then together (‘1+2’), within the shRNA resistant form of scinderin. Using the same PIP-strip assay, we found that loss of either domain removed lipid binding from recombinant scinderin (figure 3D; note that these blots are deliberately overexposed in order to show that there really is almost zero protein binding). We also carried out experiments using secretion as a within-cell assay for scinderin function, and found that these null-binding mutants were unable to rescue secretion in scinderin knockdown cells (figure 3D).

Discussion

In this work we have reexamined the role played by scinderin in the exocytosis of dense cored vesicles from PC12 cells, taking advantage of the ability granted by TIRF microscopy to visualize the behavior of individual secretory vesicles as they approach and fuse with the plasma membrane. While our findings support previous models of the role played by scinderin in clearing a path for vesicles through the actin matrix in response to stimulation, we also found evidence for a role for scinderin in the tethering of vesicles prior to secretion.

Over the years a number of techniques have been used to demonstrate a necessary role for scinderin in secretion, ranging from blocking effects of scinderin peptide fragments (Zhang et al., 1996), to inhibitory effects of antisense RNA (Lejen et al., 2001). Interpretation of blocking peptides is difficult, and antisense techniques have been superseded by RNA interference, making it valuable to revisit this question.

In this preparation, we found that shRNA-mediated knockdown of scinderin resulted in a profound depression of secretion (~80%, figure 1D), more so than seen with either antisense or blocking peptides (~50–60%). This confirmation of the older experiments using different techniques for both functional block of scinderin *and* measurement of secretion, provides compelling evidence for scinderin’s necessary role in catecholamine secretion from dense cored vesicles.

The benefit of studying secretion with TIRF microscopy is that it allows us to monitor the behavior of individual vesicles as they approach and fuse with the plasma membrane – the trade-off being that there is lower temporal resolution to study the kinetics of individual secretory events, as one might with electrophysiological techniques such as amperometry or capacitance. In the present study, this spatial tracking proved valuable as it revealed increased vesicle mobility in the scinderin knockdown cells. Two possibilities present themselves for how resting vesicle mobility could increase; loss of tethering, meaning that vesicles are held less tightly, or enlargement of caging volume, if their diffusion is limited by a confining cage (Torregrosa-Hetland et al., 2011; Pasche et al., 2012). While it is difficult to

distinguish between these possibilities, three lines of argument support a loss of tethering interpretation. Firstly, the shape of the MSD curve in the scinderin knockdown cells has a relatively constant slope, indicative of unconstrained motion, similar to that seen in Munc13-4 knockout platelets (Chicka et al., 2016), although this could also reflect a confounding combination of active transport (positive change in slope) and caging (asymptotic slope). Secondly, in these experiments scinderin knockdown and actin depolymerization have similar effects on vesicle mobility, which is consistent with a comparable mechanism of action, specifically a reduction in actin dependent vesicle tethering. The third line of argument is that the canonical function of scinderin is actin severing activity, and so one might predict that scinderin knockdown would result in stabilization of the actin cytoskeleton; a different function of scinderin is thus implicated in this finding.

If scinderin does act as a vesicle tether, how might it do so? Some of the earliest work on scinderin revealed that scinderin could bind to phospholipids, with higher affinity for PIP2 than phosphatidyl serine (Rodriguez-Del Castillo et al., 1992). A pair of sequences with homology for PIP2 binding sites were later identified within the scinderin sequence (Zhang et al., 1996). In conjunction with the highly conserved actin binding sites concomitant with gelsolin homology domains, this in principle provides a pair of binding sites capable of linking the actin cytoskeleton and biological membranes. Preliminary experiments show that loss of either of these lipid binding domains is sufficient to abolish scinderin's binding activity in PIP-strip assays, and also prevents scinderin from functioning in secretion assays. It is to be hoped that future work will be able to elucidate the structural basis for these effects.

These lipid binding findings would be more consistent with plasma membrane binding since PIP2 is localized to the plasma membrane (Holz et al., 2000, Cremona and de Camilli 2001), however no systematic investigation of lipid binding targets was made. In this paper, we have investigated the binding of scinderin to 15 membrane-immobilized phospholipids under varying calcium concentrations. We identify the mono-phosphorylated forms of phosphatidyl inositol as stronger binding partners for scinderin, with a steeper, bell shaped, calcium dependence than exhibited by PIP2 – although the data we see for PIP2 is broadly consistent with that described previously (del Castillo et al. 1992) with the difference that we went to a higher concentration (50 μ M) to see the full effect of high calcium allowing release of scinderin from PIP2 and PIP. While a concentration of 50 μ M will not be seen in the bulk of the cytoplasm of cell even under conditions of stimulation, this is below the peak levels of calcium proposed for microdomains at sites of secretion (reviewed in Neher and Sakaba, 2008)

What might this mean for scinderin's role in secretion? At the low levels of calcium experienced at rest, scinderin will bind both the actin cytoskeleton and lipid membranes of secretory vesicles (figure 4). This appears to be a significant factor in the immobilization of resting vesicles in the region adjacent to the plasma membrane (figure 2). On stimulation, high levels of calcium at release sites will have two actions: activation of the actin cleaving activity of scinderin (Zhang et al., 1995), and release of lipid binding (figure 3). Since scinderin also binds PIP2 in the plasma membrane, which is itself implicated in secretion

(Holz et al., 2000, Kruse et al., 2016), there may be a role for scinderin in directing vesicles to membrane regions where synaptotagmin can then trigger membrane bending and targeted fusion (Bai et al. 2004, Hui et al 2009). These twin actions explain the necessary role for scinderin in secretion from PC12 cells (figure 1).

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Highlights

- Scinderin is required for dense-core vesicle exocytosis in PC12 cells
- In resting cells, Scinderin acts to prevent vesicle motion
- Scinderin exhibits bell-shaped dependence of calcium binding to mono-phosphorylated forms of phosphatidyl inositol.
- Loss of lipid binding domains prevents scinderin's function in secretion

Figure 1.

Scinderin is required for secretion in PC12 cells. A. The targeted portion of scinderin sequence is shown, together with corresponding amino acid position, while beneath is the sequence of the synonymous scinderin mutant used for rescue expression. RT-PCR was used to confirm both knockdown and rescue, as shown. B. A representative field from a TIRF experiment, where the red spots correspond to ANF-mCherry vesicles present within the evanescent field. Scale bar 2 μm . C. Fluorescence over time is plotted for three example regions of interest, before and after stimulation, taken from wild-type PC12 cells, scinderin knockdown cells, and cells where scinderin knockdown has been rescued by co-transfection with the rescue construct. Identified peaks corresponding to secretory events are indicated with an asterisk. D. Quantification of secretion followed for 1 minute under the three conditions from C; control, knockdown ('shRNA') and rescue, plus an additional condition using the cytoplasmic domain of synaptobrevin/VAMP ('cdVAMP') to test for SNARE dependent fusion. n=6–8 experiments from at least 4 separate transfections.

Figure 2.

Scinderin dependent tethering of secretory vesicles in resting cells. A. Example images of cell regions showing tracks of individual vesicles over time. B. Mean squared displacement (MSD) plotted against time, for vesicles in control (square symbols), latrunculin A treated (circles), scinderin knockdown (triangles) and scinderin knockdown plus rescue construct (inverted triangles). Loss of scinderin or actin resulted in a marked increase in diffusional mobility of ANF-mCherry labeled vesicles

Figure 3.

Calcium dependent lipid binding of recombinant scinderin. A. On the left is a diagram indicating the location of different lipid species on the PIP strip. On the right hand side, is an example of 6 PIP strips incubated with recombinant HA-tagged scinderin, in different calcium concentrations, exposed simultaneously to the same film for ease of quantitation. B. Lipid binding was measured for each condition, normalized between zero (background) and 100% (Phosphatidyl inositol 4-phosphate at 100nM calcium). C. Calcium dependence of scinderin binding to Phosphatidyl inositol 4-phosphate. At physiological resting calcium (~100nM) there was significant scinderin binding. This could be reversed by elevating calcium to micromolar levels, or by pulling the calcium to zero through the addition of an effective buffer (2mM EGTA). D. Representative film exposure showing PIP strips exposed to wild type scinderin or one of three mutant forms (deleted amino acids 112-120 – ‘1’, 138-146 – ‘2’, and both ‘1+2’). Very similar results were seen in 4 other experiments, however quantification was difficult due to the need to overexpose the wild type protein as a

result of the extremely low binding seen in the lipid mutant forms. E. Expression of lipid-binding mutants of scinderin was unable to rescue secretion in scinderin knockdown cells. N=6–8.

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Figure 4.

a model for the role of scinderin at rest and in stimulated cells. At rest (upper panel) the actin cytoskeleton is intact, and scinderin binds simultaneously to PIP on vesicle membranes and the actin cytoskeleton; a pairing that is sufficient to account for effective immobilization of secretory vesicles. It is also possible that there is significant binding to PIP2 in the inner leaflet of the plasma membrane. Following stimulation (lower panel) locally elevated calcium results in the severing of actin filaments, and the release of vesicles from scinderin. One or both of these reactions are necessary for secretion to occur in these cells.