Review

Coupling actin and membrane dynamics during calcium-regulated exocytosis: a role for Rho and ARF GTPases

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Abstract

Release of neurotransmitters and hormones occurs by calcium-regulated exocytosis, a process that shares many similarities in neurons and neuroendocrine cells. Exocytosis is confined to specific regions in the plasma membrane, where actin remodelling, lipid modifications and protein–protein interactions take place to mediate vesicle/granule docking, priming and fusion. The spatial and temporal coordination of the various players to form a “fast and furious” machinery for secretion remain poorly understood. ARF and Rho GTPases play a central role in coupling actin dynamics to membrane trafficking events in eukaryotic cells. Here, we review the role of Rho and ARF GTPases in supplying actin and lipid structures required for synaptic vesicle and secretory granule exocytosis. Their possible functional interplay may provide the molecular cues for efficient and localized exocytotic fusion.

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1. Introduction

Exocytosis, i.e., the fusion of membrane-bound secretory vesicles/granules with the cell plasma membrane and consequent expulsion of vesicular contents, is a fundamental cellular process involved in many physiological functions including cell migration, wound repair, neurotransmission, enzyme secretion and hormone release. Exocytosis occurs constitutively in all eukaryotic cells and is up-regulated in some cell types like neurons and endocrine cells in response to extrinsic stimuli leading to elevation in cytosolic calcium. Calcium-regulated exocytosis has been the topic of intense investigation for decades [1–6]. In most cells specialized for calcium-activated secretion, exocytotic vesicles/granules are present in at least two compartments, the release-ready vesicle pool and the reserve pool comprising the vast majority of vesicles. The traffic of vesicles between these two compartments is subject to a fine regulation by the actin cytoskeleton [7–12]. Vesicles at the plasma membrane are docked in apparently two stages: non-primed (fusion incompetent) and primed (fusion competent). ATP is required for priming whereas Ca$^{2+}$ triggers the late ATP-independent fusion reaction [13,14]. Details of the molecular machinery underlying some of these steps have been described. For instance, interaction of vesicles with the plasma membrane is mediated by soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) found on secretory vesicles (v-SNAREs) and on the plasma membrane (t-SNAREs). These proteins form a stable complex with coiled-coil interactions [15], providing thereby sufficient energy to pull membranes into close proximity and dock vesicles to the plasma membrane [16]. ATP-dependent priming reactions are then required to render docked vesicles competent for calcium-triggered fusion [14]. These involve NSF-mediated priming of
SNARE protein complexes, ATP-dependent synthesis of phosphoinositides and protein kinase-mediated protein phosphorylations. Proteins that function specifically in vesicle priming have been discovered, including Munc13, a major priming factor in neurons and neuroendocrine cells [14,17], and CAPS, a phosphoinositide-binding protein required at a pre-fusion step in dense-core granule exocytosis [18]. The mechanism of membrane fusion per se is an aspect that continues to be debated [19,20]. SNAREs are able to drive liposome fusion in vitro [16], but with rather slow kinetics suggesting the requirement of additional factors to achieve physiological membrane fusion. Hence, many observations are in agreement with a lipid nature of the fusion reaction and the addition of exogenous lipids affects various biological fusion reactions at a stage that lies downstream of SNARE complex formation [21,22]. It is therefore likely that lipids are essential partners for proteins in the basic fusion machinery [21–24].

A convergence of biochemical and cellular studies supports the notion that the actin cytoskeleton is coupled to calcium-regulated exocytosis in neuronal cells although the precise roles played by actin filaments are not yet clear [7,12,25]. Early ultrastructural studies of actively secreting cells, such as pancreatic or chromaffin cells, reveal a dense subplasmalemmal actin network [26,27]. Though primarily needed to give cells their shape, one could also predict that this zone poses an obstacle that impedes exocytosis and subsequent endocytosis. Hence, active cytoskeletal rearrangements have been shown to accompany vesicle transport and fusion events in a variety of cell systems [7,28,29]. The data from many of these experiments have been interpreted in support of the actin-physical-barrier model and that transient depolymerization of F-actin is needed so vesicles can gain access to their appropriate docking and fusion sites [7]. Most recent findings confirm that actin filaments play a major role in the function of the exocytotic machinery although they do not support the simplified actin-barrier model but reveal that actin plays probably multiple, both inhibitory and activatory, roles in exocytosis [25,30,31].

Within the past 10 years, two families of small GTPases, namely ADP-ribosylation factor (ARF) and Rho proteins, have emerged as master players in coupling actin dynamics to membrane trafficking events in eukaryotic cells. There are six mammalian ARFs and many more ARF-like proteins. ARF1 and its activities at the Golgi complex have been extensively studied [32]. ARF6 is often located at the cell periphery where it influences membrane trafficking at the plasma membrane [33]. ARFs are thought to act through the recruitment of cytosolic coat proteins onto membranes to facilitate sorting and vesicle formation, activation of lipid-modifying enzymes and modulation of actin structures. Rho proteins are also established regulators of actin cytoskeletal dynamics [34]. Around 20 Rho family proteins have so far been identified and of these, RhoA, Rac1 and Cdc42 have been the most widely studied for their effects on actin organization. Like most small GTPases, ARF and Rho proteins cycle between GDP-bound, inactive and GTP-bound, active states. Thus, they represent acutely regulated signal transducers that respond to upstream signals originating from membrane receptors to further propagate them to downstream effector molecules to carry out their functions.

Here, we will review current evidence concerning the role of ARF and Rho proteins in providing actin structures and lipids required for calcium-regulated exocytosis. Additionally, we shall discuss the possible role of specialized scaffolding proteins in spatially and temporally coordinating ARF and Rho-signalling pathways, thereby imparting to the exocytotic machinery efficiency and site specificity [35–37].

2. ARF-dependent lipids at the exocytotic sites in neuroendocrine cells

ARF GTPases have been linked to regulated secretion in various cell types including melanotrophs [38], adipocytes [39], neutrophils [40], mast cells [41], gastric parietal cells [42] and insulin-secreting pancreatic β cells [43]. In chromaffin and PC12 cells, ARF6 is associated with the dense core secretory granules [44,45]. Evidence that ARF6 plays a role in chromaffin granule exocytosis came first from studies showing that myristoylated peptides corresponding in sequence to the amino-terminal end of ARF6 inhibit noradrenaline secretion when introduced in the cytosol of permeabilized chromaffin cells [44]. These results were then substantiated by the fact that, among various inactive or constitutively active ARF mutants, ARF6 is the sole protein able to modify the secretory response in PC12 cells [45]. It is interesting to note that the activation/inactivation cycle of ARF6 is intimately linked to the exocytotic reaction. Subcellular fractionation experiments revealed that ARF6 bound to secretory granules is in its inactive GDP-bound state whereas active GTP-bound ARF6 was detected only in plasma membrane-containing fractions prepared from secretagogue-stimulated cells [45]. Accordingly, several results indicate the presence of ARNO, a guanine nucleotide exchange factor promoting the activation of ARF6, on the plasma membrane in chromaffin and PC12 cells [45,46]. Moreover, expression of a catalytically inactive ARNO mutant in PC12 cells or introduction of anti-ARNO antibodies in the cytoplasm of permeabilized chromaffin cells inhibits secretion to an extent similar to the inactive GDP-bound ARF6 mutant [46], implicating ARNO in the exocytotic pathway through the regulation of ARF6. Since ARNO is specifically associated with the plasma membrane [45,46], ARF6 can be activated only following the recruitment and docking of granules to the plasma membrane in secretagogue-stimulated cells.
The specific activation of the granule-associated ARF6 at the plasma membrane is an appealing feature as it implies that effector activation occurs near the exocytic sites. Two lipid-modifying enzymes have been proposed as the effectors mediating the function(s) of ARF6 in regulated exocytosis, namely phosphatidylinositol 4-phosphate 5-kinase (PIP5-kinase) leading to the production of phosphatidylinositol 4,5-bisphosphate (PIP2) and phospholipase D (PLD) producing phosphatidic acid (PA). In PC12 cells, expression of the ARF6(N48I) mutant that cannot activate PLD inhibits secretion, demonstrating that activation of PLD is a major downstream effect of ARF6 that leads to exocytosis [45]. The role of PLD in secretion has been investigated by using primary alcohols, which interfere with the PLD-induced production of PA, and by expressing various wild-type and mutated PLD proteins. PLD activation was found to be an important event for exocytosis in many secretory cell types [41, 47–49]. We previously microinjected a catalytically inactive PLD1 mutant into chromaffin cells and monitored secretion by amperometry [48]. Analysis of individual exocytic events in injected cells revealed an apparent reduction in the initial rate of release, possibly reflecting a defect in a late step of exocytosis close to fusion pore formation and/or expansion [48]. The precise role of PLD-produced PA at the site of fusion remains to be elucidated. PA is a multifunctional lipid that has been proposed to serve as protein attachment site, to activate selected enzymes or to represent the starting material for the production of additional signalling lipids. PA is also a cone-shaped lipid and a predicted effect of its generation at the granule docking site would be to promote a negative curvature of the cytoplasmic plasma membrane leaflet (Fig. 1), thereby facilitating the formation of the hemi-fusion intermediates required for the fusion of two membranes [21–23]. Additional experiments are now required to prove or refute any of these possibilities.

ARF6 is also a direct activator of PIP5-kinase responsible for generating PIP2 [50], a major plasma membrane phosphoinositide involved in many membrane trafficking and actin rearrangement events [51–53]. In chromaffin and PC12 cells, PIP5-kinase has been implicated in the synthesis of the plasma membrane PIP2 required for the ATP-dependent priming reactions preceding fusion [54, 55]. Although the general role of PIP2 in exocytosis remains to be defined, it is likely that PIP2 recruits the specific PIP2-binding proteins acting in the exocytotic pathway, such as CAPS involved in the priming of docked secretory granules for fusion [18]. Indeed, ARF6 has been described as playing its role in exocytosis through the regulation of PIP5-kinase for the synthesis of the plasma membrane pool of PIP2 [43, 56]. Surprisingly

Fig. 1. Hypothetical model for the role of PLD1-produced phosphatidic acid (PA) in membrane fusion. ARF6 and/or Rac-dependent PLD1 activation hydrolyzes the membrane phospholipid phosphatidylcholine to generate the cone-shaped PA and choline. In stimulated cells, when vesicle and plasma membranes are brought in close proximity through the formation of SNARE complexes, the local elevation of PA promotes membrane bending and the hemi-fusion intermediates required for the formation of exocytic fusion pores.
for an ARF-effector complex [50,57], the interactions between ARF6 and PIP5-kinase in PC12 cells were reported to be governed by a calcium-dependent mechanism involving the phosphorylation of the PIP5-kinase rather than the guanine nucleotide-bound state of ARF6 [56]. This is an intriguing observation but it may shed some light on the mechanism by which ARF6 could control two distinct lipid-modifying pathways at the exocytotic sites. For instance, ARF6-dependent activation of the plasma membrane-bound PLD might occur through a classical GTP-dependent switch of conformation whereas the activation of the ARF6/PIP5-kinase complex might be regulated by calcium signals. In other words, in the sequence of events leading to exocytosis in stimulated cells, recruitment and docking of secretory granules to the plasma membrane in response to cytosolic calcium elevation may first result in the activation of PIP5-kinase by the granule-bound ARF6. Consequently, PIP2 formed at the plasma membrane may contribute to the recruitment of PIP2-dependent priming factors (CAPS for instance) and ARNO to the granule docking sites. ARNO then stimulates nucleotide exchange on ARF6, which in turn activates PLD1 and the formation of PA required for the final fusion event. Whether this is a universal mechanism applicable to all secretory cell types remains to be established. Nevertheless, these observations place ARF6 at a strategic position to provide adequate lipids for the exocytotic machinery.

3. Rho GTPases and the remodelling of actin in the course of exocytosis

In view of their well-established effects on actin organization, Rho proteins appear as ideal candidates to provide actin structures required for exocytosis. In chromaffin and PC12 cells, RhoA is associated to secretory granules whereas Rac1 and Cdc42 are found in the subplasmalemmal region [58]. Using Clostridium botulinum exo-enzyme C3 (an ADP-ribosyltransferase that specifically inactivates Rho), we found that RhoA is a downstream partner of the granule-associated Go protein, an heterotrimeric GTPase that regulates the ATP-dependent priming of secretory granules in chromaffin cells [59,60]. Activation of RhoA through the stimulation of Go stabilized the cortical actin cytoskeleton visualized with rhodamine-conjugated phalloidin in secretagogue-stimulated cells [60]. Similarly, expression of an active GDP-bound RhoA mutant in PC12 cells enhanced the rhodamine-phalloidin fluorescence in the periphery of stimulated-cells, suggesting the formation of actin filaments (Fig. 2A). GTP-bound Cdc42 had a comparable

![Fig. 2. Effect of GTP-loaded RhoA and Cdc42 on peripheral actin and secretagogue-evoked growth hormone (GH) secretion in PC12 cells. (A) Active Cdc42L61 and RhoAV14 stimulate the formation of actin filaments in the periphery of secretagogue-stimulated cells. PC12 cells expressing GFP-Cdc42L61 or HA-RhoAV14 were stimulated for 10 min with 59 mM K+, fixed and stained with rhodamine-conjugated phalloidin to visualize actin filaments. The asterisks indicate non-transfected cells displaying a classical disruption of cortical actin in response to K+-stimulation. Bar, 5 μm. (B) Effect of dominant active Cdc42L61 and RhoAV14 on GH release from PC12 cells. Cells co-transfected with GFP-Cdc42L61 or HA-RhoAV14 along with a plasmid encoding GH were incubated for 10 min in calcium-free Locke’s solution (basal release) or stimulated for 10 min with 59 mM K+. The net secretory response was obtained by subtracting the basal release from the K+-evoked release. Basal release ranged from 5% to 8%. As control, GH release was measured in cells expressing an empty vector. Data are given as mean values±S.E. (n=3).]
effect on peripheral actin in PC12 cells: it enhanced the rhodamine-phalloidin staining in stimulated cells, suggesting the de novo formation of actin filaments in the subplasmalemmal region (Fig. 2A). Yet, these two GTPases produced opposite effects on PC12 cell exocytosis: expression of the dominant active RhoA mutant inhibited secretion whereas the active Cdc42 mutant significantly stimulated it (Fig. 2B). This observation suggests that these two members of the Rho family form distinct actin structures unresolved at the optical level, and can play negative and positive roles in the exocytotic machinery. In other words, the actin cortex, classically viewed as a barrier that hinders the movements of granules to the plasma membrane, may as well play a secondary active role to drive the late steps of the exocytotic process. In line with this idea, agents that completely depolymerize actin filaments (like latrunculin B or C. botulinum C2 toxin) increase secretion at low concentrations and progressively inhibit it at higher doses as reported in chromaffin and PC12 cells [61,62], or completely inhibit the exocytotic response in pancreatic cells [63,64] and in mast cells [65]. In the latter, the appearance of newly synthesized actin filaments simultaneously with the disassembly of the cortical network has been observed during degranulation [66]. This dual role of actin was also illustrated in PC12 cells using evanescent wave microscopy, in which actin was found to both hinder and mediate movements of GFP-labeled secretory granules in the subplasmalemmal region [30].

Thus, it is tempting to imagine that the actin remodelling needed for exocytosis may include a rearrangement to form structures that provide either tracks permitting docking of secretory granules to appropriate sites or a force which spatially constrains components of the exocytotic machinery.

Although the precise role of actin and the mechanisms underlying its dynamic regulation remain to be dissected, the findings that Rho proteins play a role in secretion provide a potential link to the pathway coupling actin reorganization to exocytosis. In chromaffin cells, experiments designed to identify the effectors by which the secretory granule-associated RhoA protein inhibits exocytosis and affects the cortical actin cytoskeleton led us to propose that RhoA exerts its control on actin dynamics through a phosphatidylinositol 4-kinase (PI 4-kinase) also associated with secretory granules [67]. PI 4-kinase produces phosphatidylinositol 4-phosphate, a phosphoinositide that can be subsequently phosphorylated by the PI(P)5-kinase to generate granule-bound PIP2. PIP2 interacts with and regulates numerous cytoskeletal proteins [68]. Local production of PIP2 on membranes has also been shown to initiate actin nucleation and regulate membrane–cytoskeleton interactions [69,70]. Thus, activation of the Rhoa/PI 4-kinase pathway and formation of PIP2 residing on the granule membrane may promote the formation of granule-associated actin filaments and/or contribute to the stabilization of the peripheral actin barrier by decreasing the severing activity of scinderin known to be involved in the actin reorganization underlying exocytosis [71]. In other words, PIP2 produced at the surface of secretory granules may be involved in maintaining a standby position of the exocytotic machinery whereas the PIP2 generated at the plasma membrane may define the active sites of exocytosis. This rather attractive hypothesis remains, however, to be explored experimentally.

We recently investigated the molecular cascade by which Cdc42 is able to enhance actin polymerization and secretion in PC12 cells [31]. Several functionally distinct Cdc42 effectors have been identified [72]. Among them, the neural Wiskott–Aldrich syndrome protein (N-WASP) links Cdc42 to actin polymerization though the actin-related protein-2/3 (Arp2/3) complex, which promotes actin nucleation and polymerization [73,74]. We found that Cdc42 is activated at the plasma membrane in secretagogue-stimulated PC12 cells [31]. Expression of a constitutively active Cdc42 mutant increased the secretory response, recruited cytosolic N-WASP to the plasma membrane, and enhanced actin polymerization in the subplasmalemmal region [31] (Fig. 2).

Moreover, expression of N-WASP produced a stimulatory effect on secretion that was comparable to the effect obtained with active Cdc42, by a mechanism dependent on its ability to induce actin polymerization at the cell periphery. Co-expression of a N-WASP mutant unable to polymerize actin completely abolished the stimulatory effect of GTP-bound Cdc42 on secretion, demonstrating that N-WASP lies downstream of Cdc42 in the exocytotic pathway [31]. Finally, we observed that Arp2/3 is associated with secretory granules and that it accompanies granules to the docking sites at the plasma membrane upon cell activation [31]. All together, our results provide for the first time a molecular support for the de novo formation of actin filaments in the course of exocytosis. Secretagogue-evoked stimulation induces the sequential ordering of Cdc42, N-WASP and Arp2/3 at the interface between granules and the plasma membrane, thereby providing a way to specifically target local actin filament polymerization at the granule docking sites. What might be the role of these newly formed actin filaments at the sites of exocytosis? In yeast, Cdc42p/WASP triggers the formation of actin filaments that accumulate on docked vacuoles and seem to be involved in the terminal steps leading to homotypic membrane fusion [75]. In Xenopus eggs, Cdc42/N-WASP-induced actin filaments formed at the surface of exocytosing cortical granules are able to drive closure of the exocytotic fusion pore [76]. Recent work has established that for dense core granules in endocrine cells, the fusion pore opening–closing time can be modulated by various signalling pathways, allowing a control of the amount of hormones released per granule [77,78]. Thus, the possibility that actin filaments formed at the granule docking site regulate expansion and/or closure of the fusion pore, thereby providing a molecular
basis for control of quantal release, will be an interesting future issue.

In summary, actin plays a composite function in the exocytotic process in neuroendocrine cells. Both RhoA and Cdc42 emerge as important signalling molecules for regulating granule exocytosis and the accompanying actin cytoskeletal rearrangements. Through their ability to interact with a number of downstream targets, RhoA and Cdc42 can coordinate the actin-based processes to the successive molecular tasks required to drive a secretory granule to membrane fusion.

4. ARF, Rho and their downstream partners in neurotransmitter release

Evoked release of neurotransmitter in neurons has many homologies with hormone secretion performed by neuroendocrine cells; in both cell types, secretion arises from an entry of Ca$^{2+}$ that in turn triggers the fusion of a sub-population of primed vesicles docked at the plasma membrane [79]. In general, the localization and functions of the molecular components participating to the release machinery are conserved between neurons and neuroendocrine cell. However, neurons stand out from other secretory cells by an extremely fast kinetic of fusion and by their ability to sustain secretion over a wide range of frequency. To be able to maintain neurotransmitter release during prolonged synaptic activity, neurons must rapidly refill the releasable pool of vesicles. As a consequence, the recycling machinery and the movement of vesicles inside the nerve terminals need to be tightly coupled to the exocytotic process [80]. Because ARF and Rho-related proteins are well-known regulators of various organelle trafficking processes, they have been involved in neurotransmitter release and pre-synaptic plasticity, both at the stage of exocytosis and in the endocytotic counterpart that underlie the cycling of synaptic vesicles in nerve endings.

Among the ARF family, ARF6 is the sole member that has been found in nerve terminals and implicated in synaptic vesicle trafficking and neurotransmission. In cortical neurons, GTP-bound ARF6 was found concentrated at synapses [81]. Subcellular fractionation of brain synaptosomes indicated that ARF6 is present on the plasma membrane and associated to clathrin-coated vesicles [81], suggesting a role for ARF6 in synaptic vesicle endocytosis. This hypothesis was supported by experiments showing that the constitutively active GTP-bound ARF6 mutant increases the binding of proteins of the endocytotic machinery, like AP-2 and clathrin, on purified synaptosomal membranes [81]. Furthermore, pull-down experiments from rat brain extracts revealed that GTP-bound ARF6 interacts with the PIP5-kinase Iγ, an isoform highly enriched in brain [82]. This interaction leads to the stimulation of PIP5-kinase Iγ, and in turn, to the formation of PIP2 at the presynaptic plasma membrane. Since a large body of data implicates PIP2 in the regulation of clathrin mediated endocytosis [83], it has been suggested that ARF6 regulates synaptic vesicle trafficking and neurotransmission by promoting the budding of nascent endocytotic vesicles via the formation of PIP2 micro-domains at the pre-synaptic endocytotic sites. Several arguments support also the participation of ARF6 in synaptic vesicle exocytosis. For instance, in Xenopus spinal neurons, microinjection of a guanine nucleotide exchange factor for ARF proteins (mSec7-1) increases the frequency of spontaneous release [84]. mSec7-1 was found to enhance the amplitude of evoked responses without affecting the size of the quantum, and it increased the depression rate of synaptic depression induced by repetitive stimulation [84]. From these data, it has been proposed that mSec7-1, most likely by activating ARF6, facilitates the probability of a mature vesicle to fuse in response to Ca$^{2+}$ entry and/or increases the size of the readily releasable pool of synaptic vesicles. In neurons, Munc 13-1, implicated in the priming of synaptic vesicles, and synaptotagmin, supposed to be the Ca$^{2+}$-sensor of exocytosis, are both potentially regulated by phosphoinositides [85–87]. Thus, ARF6 may modulate neurotransmitter release by inducing PIP2 micro-domains near the release sites and thereby regulating synaptotagmin, Munc 13-1 or any other pre-synaptic phosphoinositide-binding protein required for synaptic vesicle exocytosis.

Several members of the Rho family have been found in pre-synaptic nerve terminals. These include RhoA, RhoB and Rac1 present in cytosolic and membrane-bound fractions isolated from rat brain synaptosomes [88]. Using highly purified synaptic vesicles, we demonstrated that only Rac1 is directly associated with these organelles [88]. The pre-synaptic functions of Rho-related proteins have been probed by using various clostridial toxins that specifically and differentially inactivate members of the Rho family by covalently fixing a sugar or a nucleotide near the effector loop [89]. Exoenzyme C3 from C. botulinum, toxin B from C. perfringens and lethal toxin from C. sordellii are all inhibitors of neurotransmitter release when injected in Aplysia cholinergic neurons [88]. The most potent effects were obtained with lethal toxin, which inactivates various Ras-related GTPases and Rac [90]. In Aplysia neurons, lethal toxin acts with an efficiency that is comparable to tetanus toxin which is known to block neurotransmitter release through the proteolytic cleavage of the vesicle-associated SNARE protein VAMP [91], indicating that lethal toxin blocks a crucial step of the synaptic vesicle cycle. The use of several variants of lethal toxin that affect a different spectrum of small GTPases has led to the idea that Rac inactivation accounts for the lethal toxin-induced inhibition of neurotransmitter release [92]. Interestingly, the blockade of acetylcholine release induced by lethal toxin can be
fully reversed in about 1 s by applying sustained stimulations [88]. This time scale is smaller than the replenishment kinetics of the release sites [93] and the residency time of synaptic vesicles on the plasma membrane before fusion estimated around 2–5 s [94,95]. Therefore, recovery from lethal toxin-induced blockade involves most likely synaptic vesicles that are recruited from a population that is already docked at the plasma membrane. By inference, this observation suggests that Rac is involved in a post-docking step in neuronal exocytosis. In line with this idea, analysis of the variance of the post-synaptic response amplitudes, which allows to determine several parameters of quantal release [96–99], indicated that the inhibitory action exerted by lethal toxin on neurotransmitter release was neither due to an alteration of the release probability nor to a reduction in the quantal size [92]. The only consequence of lethal toxin action was the reduction of the apparent number of active release sites [92], in agreement with a role of Rac in a stage close to the final fusion event.

The implication of PLD1 in neuroendocrine cell exocytosis led us to probe the idea that PLD1 fulfills a role in neurotransmitter release. PLD1 is present in membrane fractions prepared from rat brain synaptosomes and it colocalizes with synaptophysin, a marker of synaptic vesicles, in cultured cerebellar granule cells [99]. Thus, PLD1 is present in nerve terminals in areas specialized in neurotransmitter release. To determine the possible involvement of PLD1 in neurotransmitter release, a catalytically inactive PLD1 mutated protein was injected into Aplysia cholinergic neurons. This mutant was found to be a potent inhibitor of acetylcholine release [99] and, as revealed by analyzing the fluctuations in amplitude of post synaptic responses, it affected the number of release sites. Thus, by analogy with the function proposed in hormonal secretion, PLD1 may induce lipid modifications in the pre-synaptic plasma membrane required for fusion of synaptic vesicles and neurotransmitter release (Fig. 1). The mechanisms regulating PLD1 activity in neuronal exocytosis remain to be explored. PLD1 is known being regulated by ARF and Rho proteins [100,101] but the possible functional links between ARF6, Rac and PLD1 in neurons have not yet been investigated. It is, however, interesting to note that lethal toxin, which inactivates Rac, and the inactive PLD1 mutant similarly inhibit acetylcholine release from Aplysia neurons by reducing the number of functional release sites without affecting the probability of release or the size of the quantum [92,99]. Thus, the synaptic vesicle-associated Rac might well be an activator of the plasma membrane-bound PLD1 in the cascade leading to neurotransmitter release.

The role of Rac in neurotransmitter release raises also the question of the actin cytoskeleton as a downstream effector of Rac in the neuronal exocytotic pathway. The implication of actin in trafficking and fusion of synaptic vesicles is still subject to debates [12]. Depending on the type and/or the age of the synapse studied, actin has been alternatively proposed to have no function at all [102], to be positively or negatively involved in synaptic vesicle mobilization before fusion [103–108] or to act after fusion in the recycling of empty vesicles [109]. These contradictory results may reflect the diversity in function, size and morphology of the synapses studied. To date, there is no direct evidence that actin filaments are required for the fusion of synaptic vesicle to occur since drugs that depolymerize (latrunculin, cytochalasin) or stabilize (phalloidin, jasplakinolide) actin have little or no effect on the amount of neurotransmitter released per stimuli [103–108]. Thus, because the inactivation of Rac leads to a complete and fast inhibition of neurotransmitter release [88,92], it is rather improbable that actin filaments serve as downstream effectors of Rac in controlling the number of pre-synaptic release sites. On the other hand, one cannot completely exclude that a remodeling of actin filaments controlled by Rac or any other member of the Rho family participates in a fine regulation of synaptic vesicle movement and trafficking inside the nerve terminal. This sort of control cannot be observed in neurons exposed to lethal toxin as the toxin completely blocks the late fusion stages in neurotransmitter release. In this context, it is interesting to note that N-WASP, the downstream partner of Cdc42 in remodeling actin for large dense-core granule exocytosis [31], has been detected in pre-synaptic nerve terminals after subcellular fractionation of brain synaptosomes [110]. Nerve terminals are also enriched in LIM-kinase [111], a serine/threonine kinase that blocks actin depolymerization upon Rac activation [112,113]. At the postsynaptic site, a long-lasting increase in F-actin content is observed in dendritic spines after induction of Long-Term Potentiation (LTP) [114]. Since LTP is impaired in hippocampal neurons of the LIM-kinase1 knock-out mice [115] and in neurons exposed to lethal toxin [116], LTP is likely to involve actin filaments and the regulation of their dynamics via the Rac/LIM-kinase pathway. The occurrence of a similar molecular cascade at the pre-synaptic site is an interesting line for future investigations.

5. Coupling ARF and Rho signalling pathways at the sites of exocytosis

The functional characteristics of the sites of exocytosis that ensure tethering of vesicles to the appropriate active zones at the plasma membrane, as well as organization of the exocytotic machinery for rapid and efficient release, remain poorly understood. Spatial and temporal coordination of the components involved in exocytosis may be achieved by dynamic macromolecular complexes, which are based on protein–protein and/or protein–lipid interactions. SNARE complexes in imparting site specificity seem incompetent given their intracellular localization that is
not specifically restricted to zones of active exocytosis. In neurons, several large scaffolding proteins specifically localized in the presynaptic active zone have been identified. These proteins are typically composed of multiple protein-binding domains that are able to link networks of synaptic constituents and, in some cases, the underlying cytoskeleton [117,118]. Given the role played by Rho and ARF proteins in different stages of calcium-evoked exocytosis, molecules that are able to couple Rho and ARF signalling pathways at the granule docking and fusion sites are also expected to assemble exocytotic sites into effective functional units.

Scribble is a cytoplasmic protein, first characterized in epithelial cells where it plays an important role in maintaining apical polarity and in tumour suppression [119]. Scribble contains several consensus binding motifs including PDZ domains which have been described as protein-interacting modules that play a central role in organizing diverse signalling assemblies [120,121]. At synaptic junctions, PDZ domain-containing proteins are thought to be responsible for the organized assembly of components involved in neurotransmission and synaptic plasticity [122]. Accordingly, a role for Scribble in sustaining synaptic vesicle concentrations at their sites of release during high-frequency stimulation has been reported [123]. Interestingly, Scribble is able to directly interact with β-PIX, a nucleotide exchange factor for Rac1 and Cdc42, and with GIT1, a GTPase activating protein that inactivates ARF6 [124]. In PC12 cells, Scribble present at the plasma membrane recruits cytosolic β-PIX and GIT1 to the cell periphery upon secretagogue-induced stimulation [124]. Expression of mutated Scribble proteins that remain in the cytosol and thereby sequester β-PIX away from the plasma membrane significantly reduce secretion. Moreover, β-PIX itself increases secretion whereas a catalytically inactive mutant defective in nucleotide exchange activity reduces it, implicating β-PIX in the exocytotic pathway in PC12 cells [124]. Taken together, these results reveal that Scribble may act as a membrane anchor for a β-PIX/GIT1 complex devoted to regulated exocytosis. In other words, Scribble is a prime candidate to define a spatial landmark in the plasma membrane where Rho and ARF signalling pathways converge to the sites of vesicle/granule docking and fusion.

Piccolo is another potential applicant to fulfill the integration of Rho and ARF signals. Piccolo is a large (~530 kDa) protein that is spatially restricted to active zones within nerve terminals, together with RIM1, Munc13-1 and structurally related proteins such as Bassoon [125,126]. RIM1 was originally identified as a target protein of the small G protein Rab3A implicated in the docking of synaptic vesicles [127] whereas Munc13-1 that binds both RIM1 and the SNARE protein syntaxin regulates the priming of synaptic vesicles [128]. These proteins associate into large molecular complexes to form with the actin cytoskeleton a meshwork thought to functionally organize the site of neurotransmitter release [129]. In neuroendocrine cells, the role of Piccolo and associated proteins in determining the sites of exocytosis is less well understood. However, Piccolo is expressed in PC12 cells and pancreatic β cells and recent studies suggest that it might serve as a calcium sensor in insulin secretion [130]. Interestingly, Piccolo directly interacts with GIT1 through a small region that is not conserved in the closely related protein Bassoon [131]. In cultured neurons, Piccolo and GIT1 co-localize at synaptic sites [131]. In brain, Piccolo forms a complex with GIT1 and several GIT-associated proteins including βPIX [131]. From these observations, it is tempting to speculate that the convergence of ARF and Rho signalling pathways to the sites of exocytosis is based on a Piccolo-induced protein network. The fact that Piccolo is a C2 domain-containing protein with putative calcium-binding sites might provide to the scaffold the calcium-sensitivity expected for the spatial and temporal regulation of exocytosis in neurons and neuroendocrine cells.

6. Conclusion

Chromaffin granule exocytosis differs in many physiological aspects from neuronal synaptic vesicle exocytosis. While different mechanisms are certainly operating to suit the physiologically different cellular functions, it is now clear that neurons and neuroendocrine cells use similar proteins to mediate and regulate the docking and fusion of synaptic vesicles and granules with the plasma membrane. ARF and Rho GTPase signalling occurs in neurons and neuroendocrine cells and they are likely to represent additional components of the conserved machinery for hormone and transmitter release (Fig. 3). Intimate links between Rho and ARF GTPases have emerged along with the evidence that many regulators and effectors of ARF and Rho families are often intertwined. These functional relationships between ARF and Rho proteins could provide neurosecretory cells with a way of tightly coordinating vesicular traffic and fusion under the plasma membrane with the accompanying actin dynamics. Spatial control of exocytosis requires also molecular mechanisms that identify localized sites of membrane fusion, and link them to the intracellular machinery of vesicle targeting and fusion. The scaffolding proteins described to interact with both ARF- and Rho-regulating proteins are attractive candidates to act as molecular devices that integrate the many components of the exocytotic pathway into a finely tuned machinery for secretion. We have to keep in mind, however, that most of our models and paradigms derive from studies in tissue culture cells. The challenge in the future will be to determine whether regulated secretion in living organisms obeys similar rules and mechanisms.
Fig. 3. Hypothetical model for the role of ARF6 and Rho GTPases in chromaffin granule (A) and neuronal synaptic vesicle (B) exocytosis. (A) In resting chromaffin cells, secretory granule-associated RhoA contributes to the stabilization of cortical actin. Stimulation with a secretagogue triggers the recruitment and docking of secretory granules to the plasma membrane. Docking allows the formation of Cdc42-dependent actin structures required for the late stages of exocytosis. Granule docking permits also the activation of ARF6 by ARNO, which in turn stimulates PLD1 to produce membrane-localized PA. The local elevation of PA at the granule docking site facilitates the formation of the exocytotic fusion pore. (B) In synaptic terminals, vesicle-associated Rac1 activates membrane-bound PLD1 to render pre-synaptic release sites competent for fusion. ARF6 regulates neurotransmission by stimulating the formation of PIP2 micro-domains at the pre-synaptic membrane, thereby priming vesicles to fuse or promoting the formation of nascent vesicles.
References


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