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REVIEW

Cytoskeletal control of vesicle transport and exocytosis in chromaffin cells

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Abstract

Chromaffin cell exocytosis is a fascinating interplay between secretory vesicles and cellular components. One of these components is the cytoskeleton and its associated regulatory proteins. Transport of chromaffin secretory granules from their site of biosynthesis towards the active site of exocytosis requires both F-actin fine remodelling as well as microtubule trails. At least two molecular motors, myosins II and V, seem to play a crucial role in the control of F-actin dynamics and vectorial vesicle displacement respectively. Vesicle movement experiences spatial restrictions as they approach the cell cortical region, where the F-actin meshwork constitutes a barrier-limiting vesicle access to the plasmalemma. During secretion, cortical F-actin is locally disrupted providing access of vesicles to release sites on the plasmalemma. Removal of the stimulus restores cortical F-actin. Two pathways (Ca^{2+}- scinderin and PKC-MARCKS) control F-actin changes during the secretory cycle . Furthermore, GTPases such as RhoA, that controls F-actin network integrity, and Cdc42 signalling which induces the formation of local actin filaments at active sites, provide additional evidence on the importance of F-actin as a key element in vesicle transport and in the exocytotic machinery of chromaffin cells.

Keywords exocytosis, F-actin, myosin, Rho GTPases, scinderin, vesicle transport.

Mechanisms of chromaffin granule transport in chromaffin cells

Chromaffin granules are biosynthesized using the exocytotic pathway for membrane organelles (Winkler *et al.* 1987), implying an initial formation of an immature vesicle in the endoplasmic reticulum (ER), posterior maturation through the different compartments of the Golgi apparatus and final transport to the active sites for exocytosis. Therefore, the transport of these specialized vesicles is an essential element to understand how this neuroendocrine cell model accomplishes is physiological function.

Transport of vesicles in the interior of chromaffin cell cytoplasm

Initial phases of vesicle transport take place deep in the interior of the cytoplasm where microtubules are dense and provide together with F-actin an intricate network supporting ER and Golgi cisternae architecture and vectorial transport (Caviston & Holzbaur 2006). Therefore vesicle movements visualized by dynamic confocal microscopy appear to be sensitive to microtubule-affecting chemicals in regions adjacent to the chromaffin cell nucleus (Neco *et al.* 2003), whereas F-actin inhibitors affect granule motion in the interior

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(Neco *et al.* 2003, Giner *et al.* 2005), as well as in the cortical region (Lang *et al.* 2000, Oheim & Stuhmer 2000). The distribution and density of these cytoskeletal elements appear as an essential factor contributing to the dominion of actin-based transport in the proximity of the releasing sites for secretion. Microtubules have a radial distribution in chromaffin cells concentrating in internal regions and touching tangentially the cell periphery (Fig. 1), whereas F-actin localizes preferentially in the cortical region forming a peripheral barrier limiting vesicle access to the immediate vicinity of the plasmalemma (Lee & Trifaró 1981, Trifaro *et al.* 1985, Cheek & Burgoyne 1986, Aunis & Bader 1988; Fig. 1). Accordingly, with this gradient of cytoskeletal structures, F-actin-altering chemicals affect fast as well as slow secretory phases whereas microtubule inhibitors have a minor impact on secretion affecting only the slow secretory components recruited by repetitive stimulation (Neco *et al.* 2003).

F-actin dense meshwork influences vesicle motion in the proximity of the plasma membrane

Total internal reflection fluorescence microscopy provides us with a detailed description of vesicle movement within the cortical region, as the evanescent field spans 100–200 nm from the cell limits, demonstrating that vesicles suffer restriction in their motion when approaching the plasma membrane (Steyer & Almers 1999, Lang *et al.* 2000, Oheim & Stuhmer 2000, Johns *et al.* 2001). This restriction may arise from a combination of two factors: the vesicle interaction with docking or tethering elements (Toonen *et al.* 2006) as well as from vesicle imprisoning in cytoskeletal cages (Steyer & Almers 1999, Oheim & Stuhmer 2000). The existence of an intricate network of F-actin was evidenced *in vitro* (Cheek *et al.* 1986), and the formation of empty polygonal cages during secretion was shown by electron microscopy (Tchakarov *et al.* 1998). Recently, the use of transmission light and confocal microscopy has provided a compelling evidence of the importance of the organization of the F-actin network in dynamic cages which control vesicle motion in different areas of the chromaffin cell cytoplasm (Giner *et al.* 2005, 2007). These studies show the cytoskeleton as a dynamic network increasing the density of F-actin in the cortical area, where small polygonal cages slightly larger than the vesicles allow the motion of the organelles in a restricted space (Giner *et al.* 2007), therefore the motion of the cytoskeletal network itself and the space in the interior of such cages are determinant factors explaining the difference in granule motion in different areas of the chromaffin cell cytoplasm.

Molecular motors involved in chromaffin vesicle motion

The dynamic nature of the cytoskeletal network controlling vesicle motion suggests the presence of

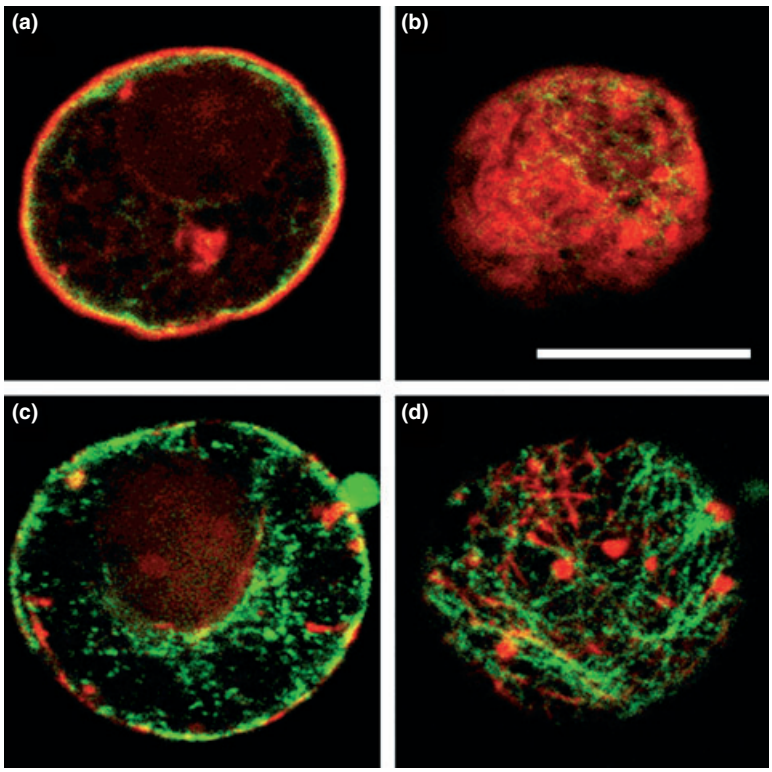

Figure 1 Distribution and density of major cytoskeletal structures in chromaffin cells. Fixed and permeabilized chromaffin cells were stained with rhodamine-phalloidin, a probe for filamentous actin (red fluorescence), and FITC coupled to anti-tubulin (green fluorescence) was employed to visualize microtubules. Confocal images depict equatorial (a, c) and polar confocal sections (b, d). (a, b) Evidence of the high density of F-actin in the cortical region and the presence of cytoskeletal cages of F-actin in the cytoplasm (a). In the lower panel, F-actin was disassembled using latrunculin A for better appreciation of the microtubule density in the cortical region. Radial distribution of microtubules (c), with much lower density in the cortical area (d) is evident. Bar: 10 μm.

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molecular motors at two different levels: the protein/s responsible for causing the changes in the F-actin network itself and the molecular motor/s responsible for the displacement of vesicles along the F-actin trails. Multiple evidence indicates that myosin II plays a role in the access of vesicles to releasing sites, the use of myosin light chain kinase (Lee *et al.* 1987) or inhibitors (Reig *et al.* 1993, Kumakura *et al.* 1994), and that its phosphorylation accompanies secretion (Cote *et al.* 1986, Lee & Holz 1986, Gutierrez *et al.* 1988, 1989). Studies have shown the direct implication of myosin II in the motion of chromaffin granules (Neco *et al.* 2002, 2004) and furthermore in controlling F-actin dynamics in the chromaffin cell cytoplasm (Giner *et al.* 2005). Nevertheless, myosin II is a conventional structural motor causing remodelling of F-actin, but unconventional myosin V is likely to play the pivotal role taking chromaffin granules as cargo providing vectorial displacement along the F-actin rails. Accordingly, myosin V head antibodies inhibit the sustained phase of secretion associated with vesicle transport between the reserve and ready releasable vesicle pools (Rose *et al.* 2003), and the expression of a dominant-negative domain of myosin Va results in the loss of the cortical restriction and motility of the vesicles in the actin cortex of PC12 cells (Rudolf *et al.* 2003). For its role in motility, myosin Va interacts with MyRIP and Rab27A linking vesicles to F-actin (Desnos *et al.* 2005). Furthermore, myosin Va could modulate the activity of syntaxin-1 (Watanabe *et al.* 2005), and consequently, the possibility of interaction with the fusion machinery itself.

Molecular mechanisms controlling F-actin dynamics during secretion

Cell stimulation triggers cortical actin microfilament network reorganization

Chromaffin cells are among neurosecretory cells that have subplasmalemmal areas largely devoid of chromaffin vesicles (Fig. 2c; Cheek & Burgoyne 1991, Vitale *et al.* 1995). As indicated above, the chromaffin cell cortical area is occupied by a network of actin filaments (Fig. 2a,b; Vitale *et al.* 1991). Most vesicles are apparently retained within an actin network. Fluorescence microscopy has demonstrated that exocytosis is accompanied by focal and transient disruption of the cortical

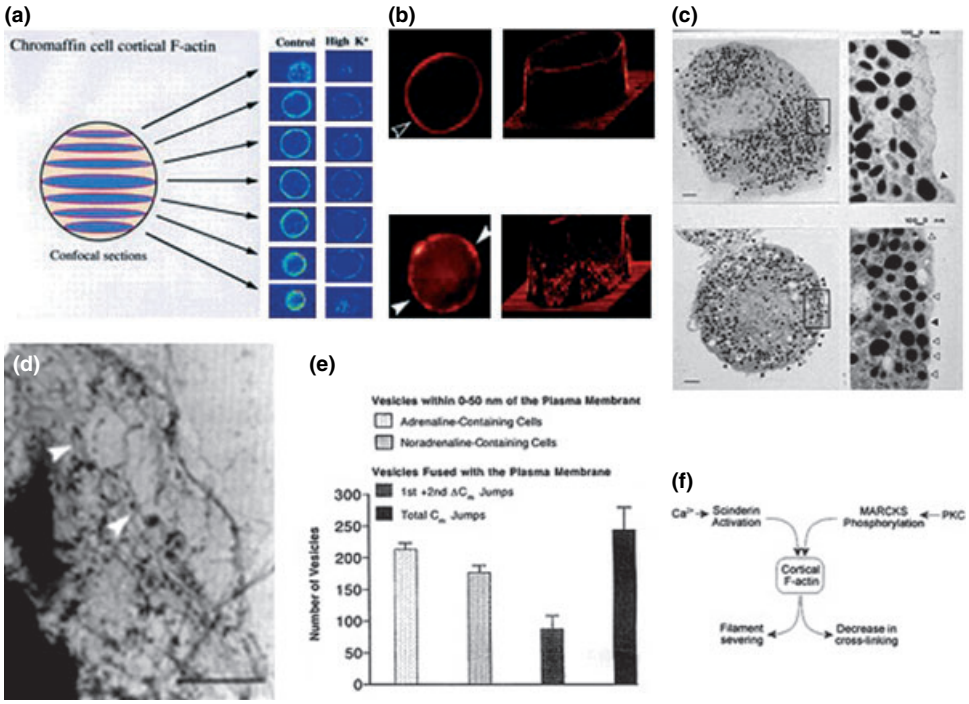

Figure 2 (a) Confocal microscopy of a resting and a high K^+ stimulated cell stained as in (b). Optical sections were 1.5 μm apart. (b) Cortical F-actin (rhodamine-phalloidin staining) in resting (top two panels) and nicotine stimulated for 40 s (bottom two panels) chromaffin cells. The two right panels show the three-dimensional fluorescence image analysis of the cells shown in the two left panels. (c) Electron microscopy of chromaffin cells. Effect of PMA on cortical vesicle distribution: control cell [10^{-7} M 4α-PMA] in (a) and (a′) and PMA treated [10^{-7} m × 6 min] cell in (b) and (b′). Open arrows indicate vesicles within 0–50 nm from plasma membrane. (d) Electron micrograph showing the presence of fine actin filaments in a subplasmalemmal area of cortical F-actin disruption (arrowheads). (e) Correlation between morphology and patch clamp data on the number of vesicles in the release-ready pool. (f) The two pathways which control and modulate the cortical F-actin network.

actin network (Fig. 2e; Vitale *et al.* 1991). F-actin disassembly seems to precede catecholamine release (Vitale *et al.* 1991). A decrease in cell F-actin with a concomitant increase in G-actin also occurs upon stimulation (Cheek & Burgoyne 1986). Moreover, in intact or permeabilized cells, the presence of substances that destabilize actin networks, such as cytochalasin D, DNase1 or latrunculin, enhanced stimulation-induced exocytosis (Cuchillo-Ibanez *et al.* 2004). These observations suggest that the cortical F-actin must be locally removed for secretion to occur. On the other hand, findings reveal that actin may also play both an inhibitory and a facilitator role during exocytosis in chromaffin and PC12 cells (Matter *et al.* 1989, Gasman *et al.* 2004). Moreover, a few and organized actin filaments have been observed in the focal areas of cortical actin disassembly (Fig. 2d; Tchakarov *et al.* 1998). Removal of the stimulus (i.e. nicotine, high K^+) restores the cortical F-actin through an increased actin polymerization (Vitale *et al.* 1991). Cortical F-actin network disruption is also observed upon treatment of cells with phorbol myristate acetate (PMA). In this case, the fluorescence images of cortical F-actin are similar to those observed upon cell depolarization (Vitale *et al.* 1995). Therefore, the cortical F-actin network disruption and reconstitution observed during exocytosis is a cyclic, dynamic event (Giner *et al.* 2005).

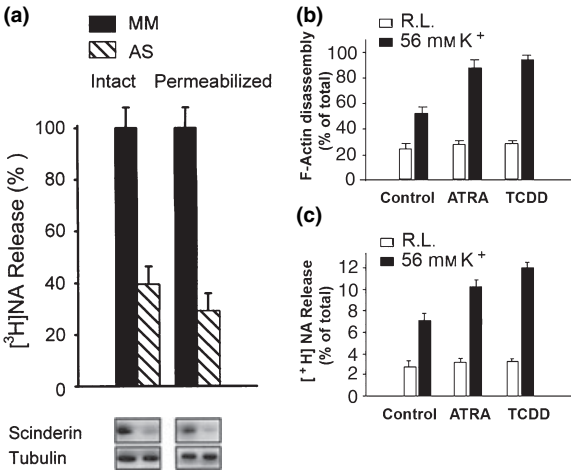

Figure 3 (a) Intact or permeabilized chromaffin cells were stimulated for 60 s with 56 mM K^+ or 20 M Ca^{2+} respectively. Cell cultures were previously incubated for 72 h with either scinderin antisense oligodeoxynucleotide (AS) or its oligodeoxynucleotide mismatch (MM, control). At the bottom, western blots of samples from the cultures showing decreased scinderin expression in the AS-treated cells. (b, c) Scinderin gene promoter stimulation: cell cultures were treated with 10 μM ATRA or 10 nM TCCC for 48 h and then, stimulated by high K^+. (b) F-actin disassembly and (c) [^{3}H]-noradrenaline output. R.L., regular Locke's solution.

Role of scinderin on cortical F-actin disassembly/assembly and chromaffin vesicle availability for exocytosis

Chromaffin cell scinderin is a Ca^{2+}-dependent F-actin severing protein (Rodriguez Del Castillo *et al.* 1990) with two Ca^{2+}- and three actin binding sites. Immunocytochemistry has shown a subplasmalemmal continuous staining for scinderin (Vitale *et al.* 1991). Upon stimulation, F-actin and scinderin cortical fluorescent rings are disrupted, suggesting redistribution of scinderin together with the disassembly of F-actin (Fig. 2d). The N-terminal half of scinderin has two F-actin severing domains and two phosphatidylinositol 4,5-bisphosphate (PIP_2) binding sites.

Four approaches have demonstrated the role of scinderin in secretion:

(1) Ca^{2+}-induced F-actin disassembly and exocytosis from permeabilized cells was increased by recombinant scinderin (Zhang *et al.* 1996). No effects were observed when recombinant scinderin was devoid of actin sites 1 and 2. Recombinant scinderin-evoked increases in exocytosis and F-actin disassembly were inhibited by exogenous G-actin, by two peptides with sequences corresponding to the two scinderin actin binding sites and by PIP_2, an inhibitor of scinderin activity (Zhang *et al.* 1996).

(2) An antisense oligonucleotide targeting the scinderin gene decreased the expression of scinderin and decreased depolarization and/or Ca^{2+}-evoked F-actin disassembly and exocytosis in intact or permeabilized cells (Fig. 3a; Lejen *et al.* 2001).

(3) The promoter region of the scinderin gene has four dioxin responsive element sequences, the binding sites for the transcription factor aryl hydrocarbon receptor (AhR), a receptor present in chromaffin cells. Ten nanomolar TCCD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin) or 10 μM ATRA (all *trans*-retinoic acid); both ligands for the AhR increase scinderin expression and potentiate F-actin disassembly and exocytosis (Fig. 3b,c).

(4) Similar potentiation of F-actin disassembly and exocytosis was observed with the vector-mediated expression of scinderin or its active segments (Dumitrescu Pene *et al.* 2005).

All these effects were probably due to an increase (1, 3 and 4 above) or decrease (2 above) in the number of chromaffin vesicles at release sites. Additional experiments suggest that scinderin acts as a molecular switch during the secretory cycle; thus inducing F-actin disassembly when intracellular Ca^{2+} increases and, later

in the cycle, when Ca^{2+} levels has decreased, promoting actin nucleation (third actin site of scinderin) and polymerization with restoration of cortical actin networks.

Modulation of cortical F-actin networks and chromaffin vesicle pools by protein kinase C

Published evidence suggests the involvement of protein kinase C (PKC) in secretion (Knight & Baker 1983, Pocotte *et al.* 1985, Bittner & Holz 1990). Many cytoskeletal-related proteins are targets of PKC (Hartwig *et al.* 1992, Elzagallaai *et al.* 2000). One of these proteins is MARCKS, a myristoylated alanine-rich C kinase substrate (Aderem 1992, Elzagallaai *et al.* 2000) known to be present in chromaffin cells (Rose *et al.* 2001, Cuchillo-Ibanez *et al.* 2004), to bind actin and cross-links actin filaments (Hartwig *et al.* 1992). The latter is inhibited by PKC-induced MARCKS phosphorylation. PMA treatment potentiates cortical F-actin disruption and exocytosis in response to stimulation, effects blocked by PKC inhibitors (Zhang *et al.* 1995, Cuchillo-Ibanez *et al.* 2004). PKC also controls cortical F-actin when exocytosis is triggered by Ca^{2+} release from intracellular stores (i.e. histamine; Zhang *et al.* 1995). Similarly, inhibitors of mitochondria Ca^{2+} uptake (i.e. CCCP) potentiate secretion through activation of the PKC-MARCKS pathway (Cuchillo-Ibanez *et al.* 2004). PMA also releases catecholamines from permeabilized cells. This effect is blocked by MPSD, a peptide with the sequence of the phosphorylation site and the actin binding domain of MARCKS (Rose *et al.* 2001). On the other hand, peptide Ala-MPSD in which the four serine residues of MPSD are substituted for alanines was ineffective in blocking secretion (Rose *et al.* 2001). MARCKS is phosphorylated by PKC and phosphorylated MARKS is unable to cross-link actin filaments with the consequent F-actin network disassembly.

In resting cells, electron microscopy revealed the presence of a 200 nm wide peripheral area almost devoid of secretory vesicles; actin filaments occupy this area (Vitale *et al.* 1995). Very few secretory vesicles (1.2–2.5% of the total vesicles) are found in the area 50 nm wide between the cortical F-actin and the plasma membrane (Fig. 2c,e; Vitale *et al.* 1995). PMA acute treatment disrupts F-actin networks in some cortical areas and causes a two to threefold increase in the number of vesicles within 0–50 nm of the plasma membrane (Fig. 2c,e). This enhanced the initial rate of exocytosis in response to stimulation (Vitale *et al.* 1995). Using membrane capacitance to measure exocytosis, a threefold increase in membrane capacitance (i.e. a threefold increase in the number of vesicles fusing with the plasma membrane) was detected during the first and second depolarizations (80 ms) of the train (Fig. 1e). PMA-treated cells released vesicle contents at the rate of 1240 vesicles s^{-1} whereas untreated cells released at the rate of 511 vesicles s^{-1}. The total number of vesicles released derived from membrane capacitance correlated well with the number of vesicles occupying the 0–50 nm peripheral zone obtained by electron microscopy (Fig. 2e; Vitale *et al.* 1995).

In summary, regardless of the stimulus, both pathways of modulation of cortical F-actin intervene (Fig. 2e); the Ca^{2+}-scinderin pathway is the most important when responses result from the entry of Ca^{2+} whereas the PKC-MARCKS pathway seems to be fully activated during intracellular Ca^{2+} release from stores or when Ca^{2+} sequestration is blocked.

Dual function of Rho GTPases in actin remodelling at vesicle release sites

Rho proteins are well known for their effects on the actin cytoskeleton in a variety of cellular processes including membrane trafficking events (Ridley 2006). Over the past years, efforts have been concentrated in understanding the functional relationship between Rho GTPases and actin dynamics within the process of neuroendocrine secretion. By investigating the intracellular distribution of Rho proteins in chromaffin cells, RhoA was found associated with the membrane of secretory granules while Rac1 and Cdc42 were preferentially localized in the subplasmalemmal region (Gasman *et al.* 1999).

Surprisingly, inhibition of the granule-bound RhoA by Clostridium botulinum exo-enzyme C3 does not affect exocytosis whereas expression of an active GTP-bound RhoA ($RhoA^{V14}$) enhanced the F-actin content in the periphery of stimulated-PC12 cells and inhibited secretion (Gasman *et al.* 1998, Bader *et al.* 2004). These data suggest that activation of RhoA is required to maintain the actin filament network in the vicinity of secretory granules and/or to prevent the secretagogue-induced reorganization of actin cytoskeleton. RhoA exerts its regulation on actin dynamics through the granule-associated phosphatidylinositol-4 kinase (PI 4-kinase) that produces phosphatidylinositol 4-phosphate (PI4P; Gasman *et al.* 1998). PI4P can be subsequently phosphorylated by phosphatidylinositol-5 kinase to generate PIP2, a phosphoinositide that has been largely implicated in regulated secretion of hormones (Gong *et al.* 2005). As PIP2 regulates numerous cytoskeletal proteins and initiates actin nucleation (Yin & Janmey 2003), it is tempting to imagine that RhoA/ PI 4-kinase-induced formation of PIP2 facilitates the association of granule with the actin network and contributes to the stabilization of

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the peripheral actin barrier. One of the most attractive hypothesis would be that PIP2 inhibits the actin-severing activity of scinderin (see above and Zhang *et al.* 1996).

In contrast to RhoA, expression of the constitutively active GTP-loaded mutant of Rac1 ($Rac1^{L61}$) or Cdc42 ($Cdc42^{L61}$) facilitated secretory response in chromaffin and PC12 cells, respectively, suggesting that these GTPases play an active role in secretion (Li *et al.* 2003, Gasman *et al.* 2004). In line with this idea, reduction in endogenous Cdc42 by short interference RNA experiments drastically blocked the secretory activity of PC12 cells (Malacombe *et al.* 2006). Interestingly, the active $Cdc42^{L61}$ also enhanced actin polymerization in the subplasmalemmal region (Gasman *et al.* 2004). In consequence, Cdc42 could trigger the formation of actin filaments at the exocytotic sites of the plasma membrane, thereby optimizing the efficiency of the exocytotic machinery.

Among distinct Cdc42 effectors, the neural Wiskott-Aldrich syndrome protein (N-WASP) links Cdc42 to actin polymerization through the actin-related protein-2/3 (Arp2/3) complex, which promotes actin nucleation and polymerization (Rohatgi *et al.* 1999). In stimulated PC12 cells, Cdc42 is activated and recruits cytosolic N-WASP to the plasma membrane (Gasman *et al.* 2004). Expression of N-WASP stimulates secretion through a mechanism that is completely dependent on its ability to induce actin polymerization at the cell periphery. Moreover, a portion of the Arp2/3 complex is associated with secretory granules and it accompanies granules to the docking sites at the plasma membrane upon cell activation. These results are the first demonstration that 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 an actin structure that makes the exocytotic machinery more efficient.

To summarize, Rho GTPases constitute key components of the exocytotic machinery by playing a composite function during secretion in neuroendocrine cells. Whether Cdc42-induced local production of actin filaments serves for docking, priming, fusion pore formation/expansion or subsequent granule endocytosis remains to be explored experimentally. Moreover, the link between the reported effects of Rac1 on exocytosis and actin dynamics requires further investigations.

Final remarks

The use of a multidisciplinary (molecular biology, microscopy, electrophysiology, etc.) approach has allowed a better understanding of the role of the cytoskeleton and its related proteins in the secretory cycle (vesicle synthesis, transport, exocytosis and retrieval). Although a functional picture of the cytoskeleton in secretion is beginning to emerge, technical limitations prevent firm conclusions concerning the organization and regulation of the actin network. Further work is now necessary to determine the fine-tuning of the cytoskeleton components in different stages of the exocytotic process. In particular, efforts have to be focused in developing innovative live cells assays to image and analyse cytoskeleton dynamics in real time, with high spatial resolution and under physiological conditions.

Conflict of interest

There is no conflict of interest for this study.

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