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Exocytosis, endocytosis and recycling of secretory vesicles in neuroendocrine cells, and its regulation by cortical actin

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Abstract: The cortical actin network is a mesh of filaments distributed beneath the plasmalemma that dynamically reacts in response to stimuli. This dynamic network of cortical filaments, together with motor myosin partners, adjusts the plasmalemma tension, organizes membrane protein microdomains, remodels the cell surface and drives vesicle motion in order to fine-tune exocytosis, endocytosis and recycling of secretory vesicles. In this review, we discuss how these mechanisms work in secretory cells.

Introduction

The cytoskeletal actin network is a highly dynamic mesh of filaments formed by globular actin monomers (G-actin) that assemble to form actin filaments (F-actin). Six actin isoforms have been described in mammals. Among them, the cytoplasmic isoforms β-actin and γ-actin are components of the cytoskeleton, with β-actin mostly expressed in stress fibers, contractile rings and cell-cell contacts, whereas γ-actin is mainly present in a dense branched meshwork at the cell cortex (cortical actin) and lamellipodia (Dugina *et al.*, 2009). Nucleation, elongation, ramification, and depolymerization of these filaments are tightly orchestrated by a set of enzymes and auxiliary proteins such as the ARP2/3 complex, N-WASP, cortactin, Src kinases, scinderin, and cofilin, among others (Gasman *et al.*, 2004; Olivares *et al.*, 2014; González-Jamett *et al.*, 2017; Carman and Dominguez, 2018). This armamentarium of proteins acts in concert to rearrange cortical actin filaments in response to stimuli (Rottner *et al.*, 2017; Li *et al.*, 2018), remodeling the cell surface, driving vesicles and other organelles to the plasmalemma as occurs during exocytosis (Papadopulos, 2017; Miklavc and Frick, 2020; Venkatesh *et al.*, 2020), and/or removing patches of the plasmalemma and directing the formed vesicles to a given target membrane, as occurs during endocytosis (Houy *et al.*, 2013; Hinze and Boucrot, 2018). Together with motor partners, such as myosin II, the cortical actin network further provides the membrane tension and drives forces for these processes to occur (Chugh and Paluch, 2018; Sonal *et al.*, 2018; Svitkina, 2020). In this review, we will focus on the role of the cortical F-actin network on regulated exocytosis, compensatory endocytosis and vesicle recycling in secretory cells, with an emphasis on adrenal chromaffin cells.

How Does the Cortical Actin Control Exocytosis in Secretory Cells?

A selective set of proteins regulates Ca^{2+} and SNARE-dependent fusion of secretory granules with the plasmalemma to tightly control the release of neurohormones (Cárdenas and Marengo, 2016) and neurotransmitters (Jahn and Fasshauer, 2012). Among these proteins are the actin filaments (Marengo and Cárdenas, 2018). In this regard, increasing evidence during the last 40 years has demonstrated that cortical actin plays a pivotal role in different processes associated with the activity of secretory cells. In the early 1980s, it was noted that the actin filaments are densely localized in the periphery of neurosecretory chromaffin cells (i.e., cortical actin) (Lee and Trifaro, 1981), and interact with secretory granule membranes in a Ca^{2+}-dependent manner (Fowler and Pollard, 1982). Thus, it was initially proposed that this cortical mesh of F-actin acts as a barrier that retains secretory vesicles away from the subplasmalemmal area, and that, upon a rise of cytosolic Ca^{2+} concentrations, this F-actin network disassembles, releasing the secretory vesicles to allow them to reach the cell periphery (Fowler and Pollard, 1982; Burgoyne and Cheek, 1987; Aunis and Bader, 1988). A similar

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mechanism was proposed later in pancreatic beta cells (Trexler and Taraska, 2017) and mast cells (Singh *et al.*, 2013). According to this idea, treatment of chromaffin cells with cytochalasin D, a fungal metabolite that prevents the incorporation of new monomers into actin filaments by binding to the barbed ends (Cooper, 1987), or with latrunculin A, a toxin that sequesters actin monomers and promotes subunit dissociation from the F-actin ends (Fujiwara *et al.*, 2018), increases the number of vesicles fusing with the plasmalemma (Berberian *et al.*, 2009). These treatments facilitate a slow secretory component, as was visualized by electron microscopy and measured by single-cell amperometry (Gil *et al.*, 2000). The disassembly of the F-actin network triggered by Ca^{2+} involves scinderin, an actin-binding protein that promotes disassembly of actin filaments upon cytosolic Ca^{2+} rises (Rodriguez Del Castillo *et al.*, 1990), and MARCKS, a myristoylated alanine-rich C kinase substrate that in its non-phosphorylated state binds and cross-links actin filaments, but upon its PKC-induced phosphorylation such MARCKS´s properties are inhibited, allowing F-actin disassembly (Rosé *et al.*, 2001). More recent studies show that MUNC18-1 also regulates the cortical F-actin mesh, as Munc18-1 knock-out (KO) chromaffin cells show a twice as dense F-actin network (Pons-Vizcarra *et al.*, 2019). Curiously, the expression of a MUNC18-1 mutant (V263T) that recovers recruitment, docking and fusion of secretory vesicles in KO cells, does not rescue the F-actin phenotype (Pons-Vizcarra *et al.*, 2019), suggesting that a dense cortical F-actin meshwork is not a rate-limiting barrier for the motion of the secretory vesicle towards the exocytotic sites.

As demonstrated by us, Ca^{2+} concentrations that trigger exocytosis not only promote disruption of a preexisting cortical actin network but also induce the formation of new actin filaments (Olivares *et al.*, 2014; Figs. 1A and 1B), indicating that the cytoskeletal actin mesh is actively remodeled and adapted to facilitate different steps of the neurosecretory process (Fig. 1C). Among the proteins involved in *de novo* formation of actin filaments are the small GTPase Cdc42 and αII-spectrin, which recruit N-WASP, a promotor of Arp2/3-mediated actin nucleation, to the plasmalemma (Gasman *et al.*, 2004; Houy *et al.*, 2020). Src kinase and its substrate cortactin also contribute to the Ca^{2+}-dependent formation of F-actin, as established by the enhanced incorporation of Alexa Fluor 488-G-actin into filaments (Olivares *et al.*, 2014; González-Jamett *et al.*, 2017). On the other hand, the β2a subunit of voltage-dependent Ca^{2+} channels reduces both the incorporation of G-actin monomers into actin filaments, and Ca^{2+} and Na^{+} currents in bovine chromaffin cells, indicating that the F-actin cortex also influences the traffic of vesicle carrying ion channels (Guerra *et al.*, 2019).

The intensity of the stimulus, and therefore the magnitude of Ca^{2+} entry, determines the degrees of F-actin disassembly and assembly (Olivares *et al.*, 2014) and their impact on the exocytotic release of transmitters (Doreian *et al.*, 2008). The variable effects of F-actin disassembly/assembly on neurosecretion include increase or reduction of the number of exocytosis events, or the amount of transmitter released per event, as determined by single-cell amperometry (Doreian *et al.*, 2008; Berberian *et al.*, 2009; González-Jamett *et al.*, 2013; Olivares *et al.*, 2014; González-Jamett *et al.*, 2017). Probably these effects are determined by a differential activation and/or recruitment of actin partners, such as those that promote actin assembly, like N-WASP (Gasman *et al.*, 2004) and cortactin (González-Jamett *et al.*, 2017), or F-actin disassembly, like scinderin (Rodriguez Del Castillo *et al.*, 1990), among others. However, future investigations should be conducted to determine the submembrane Ca^{2+} levels that selectively recruit or activate proteins involved in F-actin assembly or disassembly, including protein kinase C, Src kinase, scinderin, among others.

The rearrangement of the cortical actin cytoskeleton is required for secretory vesicle mobility (Desnos *et al.*, 2003; Neco *et al.*, 2004; Wen *et al.*, 2011). Biochemical and morphological analyses, together with live-cell imaging experiments, in PC12 cells, a cell line obtained from a pheochromocytoma of rat adrenal medulla, have shown that this mobility depends on myosin Va and VI, which bind to secretory vesicle and promote their motion towards the cell periphery (Rudolf *et al.*, 2003; Tomatis *et al.*, 2013).

The actin cytoskeleton also plays a role in the organization of the exocytotic site (Torregrosa-Hetland *et al.*, 2011). As visualized by total internal reflection fluorescence microscopy (TIRFM) and Förster resonance energy transfer (FRET), SNARE proteins and voltage-dependent Ca^{2+} channels appear to be clustered together with F-actin structures (Torregrosa-Hetland *et al.*, 2013). This actin function seems to involve annexin A2 (Gabel *et al.*, 2015), an actin-binding protein that binds and remodels lipid membranes in a Ca^{2+}-dependent way (Gabel and Chasserot-Golaz, 2016). In this regard, biochemical assays in chromaffin cells, together with immunogold electron microscopy, reveal that annexin A2 is recruited to the plasmalemma upon cell stimulation, where it bundles actin filaments that organize lipid platforms for docking and exocytosis of secretory vesicles (Gabel *et al.*, 2015).

The cortical actin cytoskeleton further provides the membrane tension required for the late stage of the exocytotic process (Bretou *et al.*, 2014; Shin *et al.*, 2018; Wen *et al.*, 2016). In this regard, measurements of exocytosis with single-cell amperometry shows that the disruption of the F-actin cortex, with cytochalasin D or latrunculin A or by interfering with the function of N-WASP or cortactin, delays the enlargement of the fusion pore (González-Jamett *et al.*, 2013; Olivares *et al.*, 2014; González-Jamett *et al.*, 2017), a transient structure formed during exocytosis (Álvarez de Toledo *et al.*, 2018). The membrane tension provided by cortical F-actin importantly depends on myosin II (Bretou *et al.*, 2014). Indeed, inhibition of myosin II function also impairs the fusion pore expansion (Neco *et al.*, 2008; Doreian *et al.*, 2008; Berberian *et al.*, 2009). Regarding the mechanism by which myosin II might control membrane tensions, optical trap experiments suggest that myosin-1a contributes to the adhesion between the plasmalemma and the F-actin cortex (Nambiar *et al.*, 2009). Then, it remains to be proved that this mechanism is also valid for myosin II.

Although some of the findings aforementioned appear to be controversial, it is currently accepted that the cortical actin cytoskeleton is a dynamic structure that is constantly rearranged at specific submembrane regions in response to different stimulus intensity. This tightly controlled assembly and disassembly of actin filaments facilitates different stages

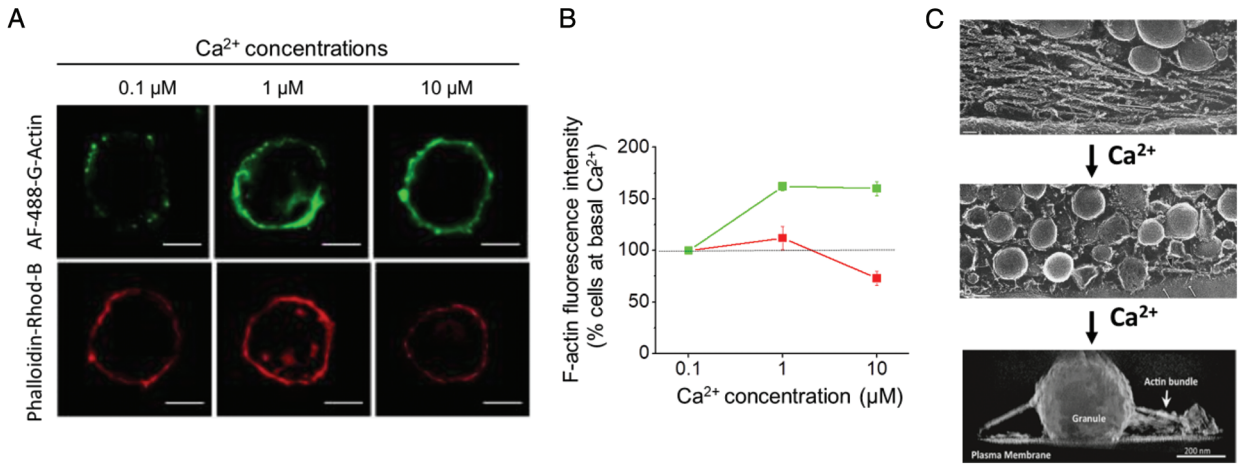


FIGURE 1. Ca^{2+} concentrations that induce exocytosis promote both disruption of the preexisting cortical actin network and formation of new actin filaments. A–B: As measured in permeabilized chromaffin cells, formation of new actin filaments (green) is observed at 1 and 10 μM free Ca^{2+} concentrations, whereas a significant disruption of the actin network (red) is manifested at 10 μM free Ca^{2+} concentrations. Figures reproduced from Olivares *et al.* (2014). C: Electron microscopy analyses show that in unstimulated chromaffin cells a dense cortical actin mesh separates the secretory vesicles from the plasmalemma (upper panel), whereas in stimulated cells, the secretory vesicles that reach the plasmalemma are still surrounded by actin filaments (middle panel), and their exocytosis is favored by actin bundles linking secretory vesicles (granule) to the plasmalemma (bottom panel). Upper and middle images adapted from Nakata and Hirokawa (1992); Copyright 1992, Society for Neuroscience, and bottom image adapted from Gabel *et al.* (2015).

of the secretory process, including secretory vesicle mobility, and its docking and fusion at exocytotic sites, wherein SNARE proteins and Ca^{2+} channels are organized together with actin structures. Despite different actin partners participating in these distinct events have been identified, the underlying mechanisms still remain ambiguous, in particular those that control membrane tension, and the signaling pathways activated by the stimulus that determine local assembly or disassembly of actin filaments. This should be resolved with the use of modern instruments with high temporal and spatial resolution.

How Does the Cortical Actin Drive Endocytosis and Vesicle Replenishment in Secretory Cells?

The cortical actin cytoskeleton plays a critical role in several forms of endocytosis by providing force for the invagination of endocytic pits (Kessels and Qualmann, 2021) and their later fission (Wu *et al.*, 2016; Gormal *et al.*, 2015). For example, using time-lapse imaging of Lifeact–GFP-transfected bovine chromaffin cells in combination with fluorescent 70 kDa dextran, Gormal and collaborators demonstrated that an acto-myosin II ring constricts the neck of nascent big vesicles or cisternae during bulk endocytosis, allowing the retrieval of large amounts of plasmalemma in response to sustained stimulation (Gormal *et al.*, 2015). Actin polymerization can also provide the additional work against the membrane tension needed to complete membrane bending during clathrin-mediated endocytosis in cells (Boulant *et al.*, 2012). In this regard, Huntingtin-interacting protein 1-related (HIP1R), a binding partner for clathrin light chains, is required for the productive interactions of clathrin coated vesicles with the actin cytoskeleton (Poupon *et al.*, 2008). Additionally, using 'flash-and-freeze' electron microscopy, Watanabe and collaborators revealed that polymerized actin, possibly through the modulation of membrane tension, is needed for membrane invagination during the ultrafast endocytosis occurring in less than 0.1 s at sites flanking 100 nm the active zone in hippocampal pre-synapses (Watanabe *et al.*, 2013). Moreover, Wu and collaborators, using measurements of membrane capacitance and fission pore conductance, imaging of vesicular protein endocytosis, and electron microscopy, also reported that cortical actin mediates overshoot (endocytosis that surpasses previous exocytosis), slow (time constant > 10 s) and rapid endocytosis (time constant ~1 s) in the calyx of Held, possibly by exerting mechanical forces required to bend membranes and thus to generate membrane pits (Wu *et al.*, 2016). Finally, actin also has a fundamental participation in clathrin- and dynamin-independent endocytosis, a mechanism that is coordinated by the small GTPases Arf1 and Cdc42 and Bin/Amphiphysin/Rvs (BAR) domain proteins (Sathe *et al.*, 2018). BAR domain proteins further provide a link between membrane remodeling and the actin cytoskeleton, coordinating the modulation of membrane curvature and actin assembly during cellular processes such as endocytosis and organelle trafficking (Carman and Dominguez, 2018).

We have recently found, by using membrane capacitance measurements in mouse chromaffin cells, that the fast dynamin-dependent endocytosis (time constant ~800 ms) which develops after the exocytosis triggered by action potential-type (ETAP) stimulus (Moya-Díaz *et al.*, 2016, 2020) also depends on cortical F-actin (Montenegro *et al.*, 2021). Particularly, depolymerization of actin filaments, induced by an increase of cytosolic Ca^{2+} or by pretreatment with cytochalasin D, significantly decelerates this endocytosis (Montenegro *et al.*, 2021). This endocytic process is tightly associated with the replenishment of the group of vesicles secreted during ETAP. Inhibition of GTPase dynamin by different experimental approaches (cell dialysis with dynamin 829–842 blocking peptide or specific blocking antibodies against dynamin), modifications of cytosolic $[Ca^{2+}]$, as well as depolymerization of F-actin decelerate both processes in parallel (Moya-Díaz *et al.*, 2016; Montenegro *et al.*, 2021).

We propose two alternative hypotheses (Fig. 2) to explain this tight relationship between fast dynamin-dependent endocytosis and rapid dynamin-dependent ETAP replenishment. One hypothesis is based on the idea that ETAP exocytosis is produced by a kiss-and-run type of mechanism. Through this process, the secretory vesicle is retrieved directly after the formation of a fusion pore, without collapse of the vesicle, and after the release of some portion of its content (Saheki and de Camilli, 2012). In such a case, vesicles would be rapidly retrieved and replenished locally, resulting in a rapid recovery of ETAP exocytosis. This hypothesis is supported by previous publications of the Corey Smith group showing that fast endocytosis, measured as membrane capacitance changes, and kiss-and-run, evaluated by small amperometric spikes and internalization of fluid phase fluorescent markers of small sizes, predominate in response to stimulation with action potentials applied alone or at low frequencies (Chan and Smith, 2001; Fulop *et al.*, 2005). Later studies from the same group proposed that cortical F-actin plays a key role in stabilizing the kiss-and-run fusion event, whereas a stronger stimulation, as well cytochalasin D treatment, disrupts the actin cortex, driving full granule collapse (Doreian *et al.*, 2008). The other possible hypothesis is that, after ETAP exocytosis, a fast endocytotic mechanism facilitates rapid vesicle replenishment by clearance of exocytotic materials from active zones, restoring the structure of the exocytotic sites (Hosoi *et al.*, 2009). Following this hypothesis, if endocytosis is impaired by F-actin disruption (or by inhibition of dynamin) vesicle replenishment will be affected as well. In addition, the actomyosin network is also essential in the transport of vesicles to the exocytotic sites at the plasmalemma (Neco *et al.*, 2003; Papadopulos *et al.*, 2015). Therefore, in agreement with our experimental results, both hypotheses consider a pivotal role of cortical actin in the recycling of secretory vesicles occurring after ETAP. Future investigations directed to the fusion pore dynamics during ETAP should be conducted to discriminate between both hypotheses.

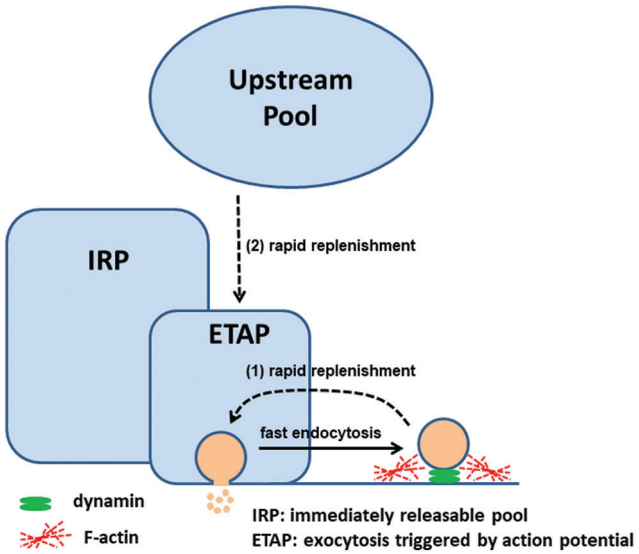


FIGURE 2. We propose two possible mechanisms for ETAP rapid replenishment: (1) local vesicle recovery through kiss and run, and/or (2) vesicle mobilization from upstream vesicular pools after clearance of exocytic materials by fast endocytosis. Both depend on a dynamin-dependent fast endocytosis and on an organized cortical F-actin. Low cytosolic [Ca^{2+}] favors F-actin polymerization, and in consequence, accelerates fast endocytosis and rapid ETAP replenishment. Figure adapted from Moya-Díaz *et al.* (2016).

Conclusions

The localization of cortical filamentous actin in the sub-plasmalemmal region of secretory cells is strategic for the multiple roles that this protein has on diverse processes associated with secretion, as was summarized in this viewpoint. Cortical F-actin co-localizes with the secretory vesicles at different stages of the secretory process, even when secretory vesicles dock and fuse with the plasmalemma. Also, in this cortical area the compensatory membrane retrieval is produced for the reestablishment of plasmalemma surface and composition after vesicle fusion, and the transport of new endosomes to their respective membrane targets is initiated. Similar events have been observed in other systems, like in epithelial cells which undergo regulated secretion (Khandelwal *et al.*, 2013, 2010). Some of the contributions of cortical actin to secretory vesicle exocytosis, endocytosis and recycling were already described in chromaffin cells, in pancreatic beta cells, in mast cells and in some neuronal models, as it was mentioned in this viewpoint. However, the strategic localization and properties of cortical actin open the door to many other regulatory functions associated with secretion. As aforementioned, how these many functions are distinctly and tightly regulated should be further investigated in the future.

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