Phosphatidylinositol-4,5-bisphosphate: Actin Dynamics and the Regulation of ATP-Dependent and -Independent Secretion

Mary A. Bittner and Ronald W. Holz

Department of Pharmacology, University of Michigan Medical School, University of Michigan, Ann Arbor, Michigan

Received October 18, 2004; accepted January 5, 2005

ABSTRACT

It has long been believed that the cortical actin cytoskeleton plays an important role in regulating the secretion of hormones and neurotransmitters. In this study, we investigated the control of actin dynamics in primary neuroendocrine cells and determined the relationship of actin dynamics to various components of the secretory response. The amount of cortical f-actin depends more strongly on PIP2 than on the availability of g-actin. Not only does PIP2 regulate actin, but actin regulates the level of PIP2, as revealed by PIP2-labeling studies. Manipulation of cortical actin had differing effects on the ATP-dependent and -independent components of secretion. ATP-dependent secretion was particularly sensitive to changes in cortical actin stability and was inhibited by expression of a protein (Yersinia pestis protein kinase A) that disassembles cortical f-actin and by pharmacological agents that promote either disassembly or stabilization of actin. The data suggest that an ATP-dependent component of secretion requires rapid changes in actin dynamics. These results point to a complex web of interactions involving PIP2, actin, and the secretory response.

The inositol phospholipid PIP2 has long been known to be important in exocytosis in neuroendocrine cells (Eberhard al., 1990; Hay et al., 1995) and has recently been implicated in exocytosis from nerve terminals (Micheva et al., 2003). This requirement for PIP2 is independent of the lipid being a substrate for phospholipase C (Eberhard et al., 1990). Several studies suggest that PIP2 regulates the function of proteins involved in exocytosis, including synaptotagmin (Schiavo et al., 1996; Bai et al., 2004), CAPS (Grishanin et al., 2002), and rabphilin3 (Chung et al., 1998). Another role, which has received less attention, is the regulation of the cortical actin cytoskeleton in which secretory granules adjacent to the plasma membrane are embedded. PIP2 regulates a number of proteins involved in the generation and maintenance of the actin cytoskeleton (Yin and Janmey, 2003). PIP2 stimulates actin polymerization (Lassing and Lindberg, 1985) and inhibits the actin-severing abilities of both gelsolin and actin depolymerizing factor/cofilin (Janmey and Stossel, 1985; Ojala et al., 2001). Thus, an increase in PIP2 can lead to increased targeting of anchoring proteins to the plasma membrane, an increase in cytoskeleton-plasma membrane linkages, and a decrease in the activity of actin-severing proteins, leading to an overall increase in actin filaments.

There is also evidence for the regulation of PIP2 at sites of actin assembly. Rac and Rho (low-molecular-weight GTPases that stimulate membrane ruffling and stress-fiber formation, respectively) both recruit the enzyme responsible for PIP2 synthesis, PIP 5-kinase, to the plasma membrane (Chatah and Abrams, 2001). Another GTPase, Arf6, also recruits and directly activates PIP 5-kinase (Honda et al., 1999; Skippen et al., 2001). Both Rac (Li et al., 2003) and Arf6 (Galas et al., 1997) have been implicated in the regulation of secretion in chromaffin cells.

Although PIP2 is an important regulator of actin, other factors, such as the availability of actin monomers, may also modify actin dynamics. Cytoskeletal disruptors (latrunculin, mycaloid, and cytochalasin) and stabilizers (phalloidin and

ABBREVIATIONS: PIP2, phosphatidylinositol-4,5-bisphosphate; Alexa-phalloidin, Alexa Fluor 568 phalloidin; DMPP, dimethylphenylpiperazinium; GFP, green fluorescent protein; CAPS, Ca2+-dependent activator protein for secretion; KGEP, potassium glutamate-, EGTA-, and PIPES-containing solution; PIPES, piperoxan-(-N,N-bis(ethanesulfonic acid); PH-GFP, pleckstrin homology domain of phospholipase Ca fused to green fluorescent protein; YpkA, Yersinia pestis protein kinase A; PIP, phosphatidylinositol phosphate.
jasplakinolide) have allowed investigators to perturb actin dynamics in vitro (Sampath and Pollard, 1991) and in situ (Gallo et al., 2002; Peterson and Mitchison, 2002) and examine the role of the cortical actin cytoskeleton in secretion. Early studies were based on the hypothesis that cortical actin acts as a barrier to secretion, preventing access of secretory granules to the plasma membrane. Cortical actin disruption sometimes occurs upon stimulation, and various agents were used to stimulate or mimic this effect (Burgoyne et al., 1988; Sontag et al., 1988). Other studies investigated the effects on secretion of endogenous actin regulatory pathways: scinderin, an actin-severing protein that is stimulated by Ca\(^{2+}\), myristoylated alanine-rich C-kinase substrate phosphorylation by protein kinase C (Trifaro et al., 2002), and Rac (Li et al., 2003).

There are two ways in which the actin cytoskeleton might affect secretory granule motion, and evidence exists that supports both. Granules might be tethered to or caged by actin, restricting their motion. Consistent with this is the observation that granule motion is indeed restricted (Oheim et al., 1998; Han et al., 1999; Steyer and Almers, 1999; Johns et al., 2001). Further support for this notion comes from a study in which mycalolin B (which, like latrunculin, can bring about the depolymerization of f-actin) increased granule mobility in the processes of differentiated PC-12 cells (Ng et al., 2002). On the other hand, actin might be required for some granule motions, either by serving as a track for molecular motors or for the generation of actin comets. This would be consistent with studies that suggest that latrunculin decreases granule mobility in PC-12 cells (Lang et al., 2000) and in chromaffin cells (Oheim and Stuhmer, 2000).

This study focuses on actin dynamics in primary neuroendocrine cells and the relationship of actin dynamics to different components of the secretory response. We demonstrate that cortical f-actin stability is dependent strongly on PIP\(_2\) but not on the availability of g-actin and that actin may in turn regulate the levels of PIP\(_2\). ATP-dependent and -independent secretion were differentially affected by agents that alter actin dynamics. Sequestration of g-actin by latrunculin specifically inhibited ATP-dependent secretion but prolonged ATP-independent secretion.

**Materials and Methods**

**Chromaffin Cell Preparation and Transfection.** Chromaffin cell preparation and transfection were performed as described previously (Holz et al., 1994). For [\(^{3}H\)norepinephrine and human growth hormone secretion experiments, cells were plated in 96- or 12-well plates (22.6 mm well diameter), respectively. For immunocytochemistry, chromaffin cells were plated on glass coverslips (Fisher, 1 thickness) fastened to the bottom of punched-out wells on 12-well plates. Cover slips were sequentially coated with poly-o-lysine and calf-skin collagen to promote cell adhesion. Ca\(^{2+}\) phosphate precipitation was used for transfection.

**Secretion Experiments.** Human growth hormone secretion experiments were generally performed 5 to 6 days after transfection at 27°C. Intact cell experiments were performed in a physiological salt solution containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 5.6 mM glucose, and 15 mM HEPES, pH 7.4. Secretion from permeabilized cells was performed in potassium glutamate solution (KGE) containing 139 mM potassium glutamate, 20 mM PIPES, pH 6.6, 2 mM MgATP, 20 \(\mu\)M dигитонин, and 5 mM EGTA with either no added Ca\(^{2+}\) or sufficient Ca\(^{2+}\) to yield 30 \(\mu\)M buffered Ca\(^{2+}\). There were four wells or dishes per group. Human growth hormone was measured with a highly sensitive chemiluminescence assay from Nichols Institute (San Juan Capistrano, CA). Secretion was expressed as the percentage of the total cellular human growth hormone that was released into the medium. There was usually 0.5 to 2.0 ng of human growth hormone and 60 nmol of catecholamine/22.6 mm diameter well.

Nontransfected cells were labeled with [\(^{3}H\)norepinephrine as described previously (Bittner and Holz, 1992). Release was calculated as the amount of [\(^{3}H\)norepinephrine released into the incubation medium divided by the total [\(^{3}H\)norepinephrine (i.e., [\(^{3}H\)norepinephrine released + [\(^{3}H\)norepinephrine remaining in the cells). Ca\(^{2+}\)-dependent release was calculated as the difference between release in the presence and absence of Ca\(^{2+}\). Data are expressed as mean ± S.E.M. unless otherwise indicated. Significance was determined by Student’s t test or by one-way analysis of variance (for three or more groups). Error bars smaller than symbols were omitted from figures.

Drugs were from the following sources: latrunculin B, mycalolin B, and jasplakinolide were from Calbiochem (San Diego, CA); and cytochalasin B was from Sigma-Aldrich (St. Louis, MO).

**Plasmids.** The plasmids encoding the pleckstrin homology domain of phospholipase C\(_{6}\), fused to GFP (PH-GFP) was constructed as described previously (Varnai and Balla, 1998). The plasmid encoding Verrsinia pestis protein kinase A (YpkA) was a gift from Dr. Jack Dixon (University of California, San Diego, La Jolla, CA). Identification of YpkA-transfected cells for immunocytochemistry was accomplished by cotransfection with a plasmid encoding ANP-emerald GFP (ANP-GFP; generously provided by Dr. Edwin Levitan, University of Pittsburgh, Pittsburgh, PA). ANP-GFP is directed to the regulated exocytotic pathway and packaged into secretory granules.

**Confocal Microscopy and Immunocytochemistry.** After experimental manipulation, cells were fixed in 4% paraformaldehyde, permeabilized with acetone, and incubated with Alexa-phalloidin (Molecular Probes, Eugene, OR) to visualize f-actin. Cells were visualized with an MRC600 Laser Scanning Confocal Microscope with a 100× objective lens (numerical aperture, 1.4) with a pinhole aperture setting of 6 (Bio-Rad, Hercules, CA). Neutral density filters were used to reduce light intensity to a level that was just sufficient to obtain satisfactory images. Within an experiment, all cells were treated with the same concentration of Alexa-phalloidin, and all images were taken at the same microscope settings to allow direct comparison. Average pixel intensities were obtained of outlined membrane segments using Scion Image (Scion Corporation, Frederick, MD). Histograms (containing the number of pixels at each intensity) of the outlined regions of interest were imported into a spreadsheet and graphics program for statistical analysis.

**Results**

**Effect of the Pleckstrin Homology Domain of Phospholipase C\(_{6}\) on the Actin Cytoskeleton.** The pleckstrin homology domain of phospholipase C\(_{6}\) (tagged with GFP) binds specifically PIP\(_2\) and its polar metabolite Ins-1,4,5-P\(_3\) (Rebecchi et al., 1992; Lemmon et al., 1996). We had used previously PH-GFP to demonstrate that the PIP\(_2\) that is important in secretion is localized to the plasma membrane (Holz et al., 2000). We now find that the sequestration of plasma membrane PIP\(_2\) by the pleckstrin homology domain of phospholipase C\(_{6}\) reduces the cortical actin cytoskeleton. PH-GFP was transiently expressed in cultured bovine adrenal chromaffin cells. Cells were fixed, stained for f-actin with Alexa-phalloidin, and imaged by confocal microscopy (Fig. 1). The pleckstrin homology domain substantially decreased the intensity of peripheral Alexa-phalloidin staining (cell indicated by arrow, Fig. 1, A–C). Quantification of the intensity...
of the fluorescence in representative segments of the cell periphery revealed a shift to lower values in cells expressing the pleckstrin homology domain (Fig. 1D). The mean intensity for PH-GFP-expressing cells was 14.56 ± 0.98 versus 22.79 ± 1.46 U/pixel for nontransfected cells (p < 0.0001). Only 19.9 ± 3.8% of pixels in the PH-GFP-expressing cells were above the median pixel intensity (21 units) of those cells not expressing PH-GFP (p < 0.00001; n = 35 cells/group).

**Effect of Removing ATP on the Actin Cytoskeleton.** If plasma membrane PIP$_2$ is required to maintain the cortical cytoskeleton, then reducing the levels of PIP$_2$ by other means should also have the same effect. We (Holz et al., 1989; Eberhard et al., 1990) and others (Hay and Martin, 1992; Hay et al., 1995) have demonstrated previously that ATP is required to maintain a secretory response; the major part of this effect is caused by the generation of PIP$_2$. We asked whether removing ATP alters the actin cytoskeleton in digitonin-permeabilized chromaffin cells. Chromaffin cells were permeabilized for 4 min in potassium glutamate solution containing 20 µM digitonin with or without 2 mM MgATP, fixed, and then incubated with Alexa-phalloidin to visualize f-actin (Fig. 2). Removal of ATP caused a loss of f-actin in the periphery of the cell (Fig. 2A–C). Quantification of the fluorescence confirmed this conclusion. The distribution of pixel intensities adjacent to the plasma membrane was shifted to lower intensities in cells incubated in the absence of ATP (Fig. 2C). In the presence of ATP, the mean intensity in the cell periphery was 27.5 ± 1.76 versus 18.62 ± 0.91 U/pixel in cells without ATP (p = 0.0002). Only 13.4% of pixels in cells incubated in the absence of ATP had an intensity greater than the median intensity in the cells with ATP (p = 0.00002; n = 12 cells/group).

**Expression of YpkA Disassembles Cortical f-actin and Inhibits ATP-Dependent Secretion.** Because ATP is involved in numerous cellular processes, it is difficult to ascribe its effects on secretion to its effects on the cytoskeleton. To investigate the relationship between the cytoskeleton and secretion, we used a completely independent means of manipulating actin. YpkA is activated by and phosphorylates actin, disassembling actin stress fibers (Juris et al., 2000). We asked whether the expression of the YpkA protein in chromaffin cells altered the cortical actin cytoskeleton. Cultured chromaffin cells transiently expressing the YpkA protein were fixed and stained for f-actin. YpkA expression caused a profound loss of cortical f-actin (Fig. 3, A–C). In 14 (61%) of 23 cells, cortical actin was entirely abolished, whereas in the remaining 9 cells, only traces of actin were visible. For the cells in Fig. 3, A to C, this is shown graphically in E. Note that the intensity peak for the YpkA-expressing cell is indistinguishable from the off-cell background.

We asked whether the loss of cortical actin altered the secretory response. Chromaffin cells coexpressing YpkA and human growth hormone were permeabilized for 4 min in the presence or absence of ATP and then stimulated for 2 min with 30 µM Ca$^{2+}$ in the continuing presence or absence of ATP. YpkA inhibited the ATP-dependent but not -independent human growth hormone secretion (Fig. 3D). This experiment suggests that the f-actin is required for continuing ATP-dependent secretion.

**Effects of Latrunculin B on Secretion.** The actin cytoskeleton is also susceptible to pharmacological intervention. Latrunculin binds soluble g-actin and alters the f-actin/g-actin equilibrium, thereby causing f-actin disassembly in vitro. We investigated the effects of latrunculin on both ATP-dependent and -independent catecholamine secretion. The effects were surprisingly complex. Permeabilization for 2 min in the presence of 10 µM latrunculin B completely blocked the ATP-dependent component of secretion (Fig. 4A). In contrast, latrunculin was much less effective at inhibiting ATP-dependent secretion after a longer permeabilization. After 8 min of permeabilization with latrunculin, ATP-dependent secretion was inhibited by only 35% (Fig. 4B). The longer permeabilization decreased subsequent Ca$^{2+}$-dependent secretion. To test whether the degree of inhibition by latrunculin was largest when secretion was most vigorous, secretion was stimulated by optimal (30 µM) or suboptimal (1 µM) free Ca$^{2+}$ concentrations after a 4-min permeabilization (Fig. 4C). Latrunculin inhibited Ca$^{2+}$- and ATP-dependent catecholamine release stimulated by 30 µM Ca$^{2+}$ by 58% (p < 0.0001), and by 1 µM Ca$^{2+}$ by only 19% (not significant).

---

**Fig. 1.** Effect of PH-GFP on the actin cytoskeleton. Chromaffin cells cultured on glass coverslips were transfected with a plasmid containing the GFP-tagged pleckstrin homology domain of phospholipase C6 (PH-GFP). After 5 days, the cells were fixed, permeabilized with acetone, blocked with 0.5% bovine serum albumin, and incubated with Alexa-phalloidin to visualize cortical actin. Confocal sections were taken through the center of the cells of interest (A–C). The arrow indicates a cell expressing PH-GFP. Scale bar, 5 µm. Representative areas of the cell periphery were selected, and the intensity of cortical actin labeling was quantified using Scion Image. D, pixel intensities <13 units (below the solid line in D) were indistinguishable from background values outside of the cell. Untransfected cells in the same field served as controls. n = 35 cells/group.
Thus, latrunculin was most effective in inhibiting secretion in vigorously secreting cells. ATP-independent secretion decays over a time scale of minutes after permeabilization (Holz et al., 1989). Although latrunculin inhibited ATP-dependent secretion, it enhanced ATP-independent secretion (Fig. 4, A and B). In another experiment, cells were again permeabilized for various lengths of time with 10 μM latrunculin in the absence of MgATP (Fig. 4D). As the length of the permeabilization time increased, cells permeabilized without ATP rapidly lost the ability to secrete catecholamine, whereas secretion from cells without ATP but with latrunculin decayed more slowly.

Fig. 2. Effect of removing MgATP on the actin cytoskeleton. Chromaffin cells cultured on glass coverslips were permeabilized with 20 μM digitonin in KGEP with or without 2 mM MgATP for 4 min. The solution was removed, and the cells were incubated for 15 min in KGEP in the continuing presence or absence of MgATP. The cells were then fixed, and actin was visualized as in Fig. 1. A and B, representative confocal sections of cells incubated with or without MgATP. Scale bar, 5 μm. C, quantification of Alexa-phalloidin–stained actin in the periphery of cells with or without ATP. n = 12 cells/group.

Fig. 3. YpkA reduces cortical actin and inhibits ATP-dependent secretion. A to C, cultured chromaffin cells transiently co-expressing YpkA and ANP-GFP (to mark the transfected cells) were fixed, and the cortical actin was visualized as in Fig. 1. A, ANP-GFP; B, f-actin stained with Alexa-phalloidin; C, overlay. The arrow points to a transfected cell. Scale bar, 5 μm. D, cultured chromaffin cells were co-transfected with YpkA and human growth hormone. After 5 to 7 days, cells were permeabilized for 4 min in KGEP with 20 μM digitonin in the presence or absence of 2 mM MgATP and then stimulated for 2 min with or without 30 μM Ca²⁺ in the continuing presence or absence of MgATP. n = 4 wells/group. E, quantification of Alexa-phalloidin–stained actin in the periphery of cells with or without YpkA shown in A to C. The peak labeled "background" represents the intensity of an equivalent area of the off-cell image (i.e., no actin present).
Thus, after 8 min of permeabilization, cells without latrunculin retained only 9% of their original secretory capacity, whereas cells permeabilized with latrunculin retained 23% of their ability to secrete. Rather than inhibiting ATP-independent secretion, latrunculin helped to maintain it.

Other cytoskeleton effectors, including cytochalasin B (10 μM, 15 min) (data not shown) and mycalolide B (0.25–2 μM, 15 min) (Fig. 5A), were similarly able to stabilize ATP-independent secretion while inhibiting ATP-dependent secretion. For example, 1 μM mycalolide B inhibited ATP-dependent secretion by 80% and at the same time enhanced ATP-independent secretion by 50%. Like latrunculin, both cytochalasin B and mycalolide B were much more effective at inhibiting ATP-dependent secretion after a short (2 min) rather than a long (8 min) permeabilization (data not shown).

Jasplakinolide stabilizes actin filaments. Preincubation of intact cells with modest concentrations of jasplakinolide (0.01–0.3 μM) enhanced ATP-independent secretion but had little effect on ATP-dependent secretion (Fig. 5C). Higher concentrations of jasplakinolide strongly inhibited ATP-dependent secretion.

Because these agents are able to penetrate the plasma membrane, we were able to examine their effect on secretion from intact cells. Preincubation with mycalolide (Fig. 5B), jasplakinolide (Fig. 5D), and latrunculin (data not shown) had little or no effect on secretion stimulated by the nicotinic agonist dimethylphenylpiperazinium (DMPP) at concentrations that strongly inhibited ATP-dependent secretion from permeabilized cells. The data suggest that release from intact cells more closely resembles ATP-independent rather than ATP-dependent secretion.

**Effects of Latrunculin B on the Actin Cytoskeleton.** The unexpected ability of latrunculin and mycalolide to prolong ATP-independent secretion was further explored. Given their ability to sequester g-actin, these agents would be predicted to decrease the amount of cortical f-actin. Chromaffin

---

**Fig. 4.** Latrunculin effects on catecholamine secretion. Cultured chromaffin cells were labeled with [3H]norepinephrine. A and B, cells were permeabilized for 2 min (A) or 8 min (B) in KGEP containing 20 μM digitonin with or without 10 μM latrunculin B and with or without MgATP. The permeabilization solution was removed, and secretion was stimulated for 10 min by 30 μM free Ca2+ in KGEP in the continuing presence or absence of latrunculin and MgATP. Ca2+-dependent release was calculated by subtracting the fraction of [3H]norepinephrine released in the absence of Ca2+ (usually 1–2%) from that released in the presence of 30 μM Ca2+. n = 3 wells/group. C, cells were permeabilized for 4 min in KGEP containing 20 μM digitonin with or without 10 μM latrunculin B and with or without MgATP. The permeabilization solution was removed, and secretion was stimulated for 15 min by 1 or 30 μM free Ca2+ in KGEP in the continuing presence or absence of latrunculin and MgATP. Ca2+ - and ATP-dependent release was calculated by subtracting the amount of Ca2+-dependent release in the absence of MgATP from that released in the presence of MgATP for both 1 and 30 μM Ca2+. n = 3 wells/group. D, cells were permeabilized for various times in KGEP containing 20 μM digitonin with or without 10 μM latrunculin B and without MgATP. The permeabilization solution was removed, and secretion was stimulated for 10 min by 30 μM free Ca2+ in KGEP in the continuing presence or absence of latrunculin. Cells having 0-min preincubation had digitonin added to the Ca2+-containing stimulation solution. n = 3 wells/group.
cells were incubated for 15 min in physiological saline with or without 10 μM latrunculin B, fixed, permeabilized, and f-actin–labeled with Alexa-phalloidin. As was reported previously (Lang et al., 2000), the cortical actin network in latrunculin-treated cells (Fig. 6A) was disrupted compared with the smooth, even appearance of the actin cortex in untreated cells (Fig. 1A, nontransfected cells). Similar segmentation of actin was seen in mycalolide-treated cells (Fig. 6B). It is surprising that quantification of the actin staining revealed no significant difference between treated and untreated cells in the average amount of phalloidin-labeled actin per unit length of membrane (Fig. 6C). However, the distribution of stained actin differed, with a larger fraction of the pixels of latrunculin- or mycalolide-treated cells at background levels [32.4 ± 3.6% of pixels (latrunculin) and 27.54 ± 2.3% (mycalolide) vs 15.4 ± 2.7% for untreated cells, p = 0.0008]. The patches of membrane in which actin was present were somewhat brighter in the drug-treated cells. Rather than simply dissociating the cortical f-actin, these drugs elicited its rearrangement. The segmented appearance of actin after treatment of intact cells with latrunculin or mycalolide persisted during subsequent permeabilization with digitonin (data not shown).

We continued to explore these effects in permeabilized cells, this time concentrating on the effects of latrunculin. Again we got an unexpected result. Chromaffin cells were permeabilized in the presence or absence of MgATP with or without latrunculin and then were fixed and incubated with Alexa-phalloidin (Fig. 7, A and B). In the absence of latrunculin, cortical f-actin was decreased in the absence but not in the presence of ATP (see Fig. 2). It is surprising yet consistent with its ability to maintain secretion in the absence of ATP that latrunculin maintained cortical f-actin in the absence of ATP (Fig. 7B). In fact, the average pixel-intensity profile of cells treated with latrunculin in the absence of ATP was indistinguishable from that of cells with ATP alone (Fig. 7C) but differed from the –ATP group (p = 0.0071). In representative segments of the cell cortex, cells incubated without MgATP had only 25.8 ± 4.0% of pixels above the median value for control cells with ATP, compared with 52.2 ± 3.0% for cells without ATP but with latrunculin.

Latrunculin and PIP₂ Levels. In the experiments described above, latrunculin exhibited an unexpected ability to maintain both cortical actin and secretion in the absence of ATP. Because both of these effects could be caused by increases in PIP₂, we determined the effects of latrunculin on

Fig. 5. Mycalolide and jasplakinolide effects on catecholamine secretion. Cultured chromaffin cells were labeled with [3H]norepinephrine. Cells were then incubated with the indicated concentrations of mycalolide B (A and B) or jasplakinolide (C and D) for 15 or 60 min, respectively. Cells in B and D were stimulated for 5 min with 20 μM DMPP in physiological salt solution. Cells in A and C were permeabilized for 4 min in KGEP containing 20 μM digitonin with or without MgATP. The permeabilization solution was removed, and secretion was stimulated by 30 μM free Ca²⁺ in KGEP for 12 min in the continuing presence or absence of MgATP. Stimulated release was calculated by subtracting the amount of release in the absence of secretagogue (Ca²⁺ or DMPP) from that released in the presence of stimulation. n = 3 wells/group.
PIP$_2$ levels. Chromaffin cells labeled to isotopic equilibrium with [myo-$^3$H]inositol were permeabilized for 4 min with or without 10 μM latrunculin in the absence of MgATP, and the amounts of phosphatidylinositol, PIP, and PIP$_2$ were determined. Latrunculin increased the PIP$_2$/phosphatidylinositol ratio from 1.24 ± 0.05 to 1.55 ± 0.10 ($p = 0.032$), an increase of 25%. A second experiment gave a similar result. Because these experiments were performed in the absence of ATP, the increase probably reflects decreased degradation of the phospholipid rather than an increase in its synthesis.

**Discussion**

In this study, we investigated the relationship between PIP$_2$, actin, and secretion. We began by recognizing that there is conflicting evidence for a role for actin in regulated

![Fig. 6. Latrunculin and mycalolide cause rearrangement of cortical actin in intact chromaffin cells. Chromaffin cells cultured on glass coverslips were incubated with 10 μM latrunculin B (A) or 1 μM mycalolide B (B) in physiological saline for 15 min. The cells were then fixed, and actin was visualized with Alexa-phalloidin and quantified as in Fig. 1. $n = 12$ cells/group. Scale bars, 5 μm.](image)

![Fig. 7. Latrunculin preserves cortical actin in the absence of MgATP. A to C, chromaffin cells cultured on glass coverslips were permeabilized with 20 μM digitonin in KGEp with or without 2 mM MgATP and with or without 10 μM latrunculin B for 4 min. The solution was removed, and the cells were incubated for 15 min in KGEp in the continuing presence or absence of MgATP. The cells were then fixed, and actin was visualized and quantified as in Fig. 1. In this experiment, background fluorescence was $<7$ units. $n = 12$ cells/group. Scale bars, 5 μm.](image)
secretion. Some studies suggest that cortical actin forms a barrier to secretion; others suggest a requirement for actin. Here, we define for the first time two different roles for actin in secretion: one to maintain a component of ATP-dependent secretion, and the other to stabilize ATP-independent secretion. We also demonstrate that PIP\(_2\) is a major regulator of cortical actin, suggesting that one of the roles of PIP\(_2\) in exocytosis is to modulate the actin cytoskeleton. Indeed, we found that maintaining normal actin dynamics as well as f-actin levels is critical for the normal secretory response.

**PIP\(_2\) is a Major Regulator of Cortical Actin Dynamics.** The control of actin dynamics by PIP\(_2\) has been studied previously in rapidly turning over actin filaments (e.g., stress fibers, filopodia, and lamellipodia). We found that this regulatory function of PIP\(_2\) also holds true for the less dynamic cortical actin cytoskeleton. Sequestration of plasma membrane PIP\(_2\) by the pleckstrin homology domain of phospholipase C\(\delta\) reduced the intensity of Alexa-phalloidin staining of cortical actin in transfected chromaffin cells (Fig. 1). Removal of ATP, which also decreases the levels of cellular PIP\(_2\), had a similar effect (Fig. 2). Loss of cortical actin occurred rapidly, within a few minutes after permeabilization, without ATP. In contrast, cortical actin was much more stable to actin depolymerizing drugs. In intact chromaffin cells, latrunculin and mycalolide caused a rearrangement rather than a dispersal of cortical actin (Fig. 5), and in permeabilized cells, latrunculin actually stabilized actin against the depolymerizing effects of ATP withdrawal. Thus, in permeabilized cells, the sequestration of g-actin by latrunculin has little impact on the integrity of the actin cortex in the time scale of these experiments, whereas the actin cortex is modified rapidly upon loss of PIP\(_2\).

**Latrunculin Reveals Reciprocal Regulation between PIP\(_2\) and Actin.** On the basis of numerous precedents for the regulation of actin stress fibers by PIP\(_2\), we had reason to expect that cortical actin would be subject to regulation by PIP\(_2\). Our data show that the converse is also true. Altering actin dynamics with latrunculin caused an increase in PIP\(_2\). This is the first evidence for a reciprocal relationship in the regulation of actin and PIP\(_2\). Because the latrunculin experiment was done in the absence of ATP, there was no ATP to support increased PIP\(_2\) synthesis. Thus, the maintenance of PIP\(_2\) levels probably reflected a decrease in degradation, perhaps through inhibition of lipid phosphatases or phospholipase C. The regulation of PIP\(_2\) by actin may represent a feedback mechanism to protect the relatively stable cortical actin from rapid changes.

**Actin Dynamics Play an Important Role in ATP-Dependent Secretion.** YpkA, a protein kinase from *Y. pestis* that is activated by and phosphorylates actin, causes disassembly of actin stress fibers in fibroblasts (Juris et al., 2000) and a profound loss of cortical actin in chromaffin cells (Fig. 3). Latrunculin binds g-actin. Both YpkA expression (Fig. 3) and latrunculin (Fig. 4) preferentially inhibited ATP-dependent secretion.

Inhibition of ATP-dependent secretion was seen both in cells permeabilized with latrunculin, in which the actin cytoskeleton remained intact, and in cells in which cortical actin was rearranged by a pretreatment of the intact cells with latrunculin or mycalolide. Because ATP-dependent secretion was inhibited under both circumstances, neither the stabilization nor rearrangement of f-actin can account fully for latrunculin or mycalolide’s effects. It is likely that these effects on secretion (Gil et al., 2000; Li et al., 2003) owe more to changes in actin dynamics caused by the binding of g-actin than by the changes in f-actin levels or distribution.

Inhibition of ATP-dependent secretion by latrunculin and mycalolide was largest when secretion was most vigorous. How might this occur? One possibility is that when cells are strongly stimulated by Ca\(^{2+}\) before significant rundown of the secretory response (e.g., after 2 min of permeabilization), vigorous ATP-independent secretion depletes the granules near the plasma membrane (Fig. 8, A and B). New granules need to be moved into position before becoming secretion-competent, and this movement requires actin. This notion is consistent with what has been observed in the calyx of Held (Sakaba and Neher, 2003), in which ATP-dependent replenishment of secretion-competent vesicles was inhibited by latrunculin. In contrast, cells permeabilized for a longer time before stimulation (e.g., 8 min) or stimulated with submaxi-

**Fig. 8.** ATP-dependent priming after rundown or secretion. A resting chromaffin cell is illustrated in A. ●, secretion-competent granules adjacent to the plasma membrane (not necessarily docked, but within the cortical actin layer). These granules can be released without the cell undergoing additional priming. ○, secretion-incompetent granules. B, after vigorous secretion, the granule population near the membrane is depleted. Granules in the interior of the cell require actin to move to the periphery. C, granules are still in place after rundown. Actin is not required to replenish the granule population near the membrane. In both cases, the ATP-dependent priming process itself may be identical. The difference lies in the population of granules that are available to become secretion-competent and the requirement for actin-based movement for the granules in B.
Atp reduces levels of Pip2 (Eberhard et al., 1990) and reactivity of the secretory pathway and other cellular processes. Regardless of the explanation, the experiments indicate that the ATP dependencies for secretion under these different conditions reflect contributions of different ATP-dependent processes in the secretory pathway.

Such considerations may also explain the refractoriness of secretion from intact cells to inhibition by latrunculin, myosin, and jasplakinolide. Prior permeabilization and rundown, chromaffin cells contain a substantial number of granules able to be released without an additional requirement for ATP (Fig. 4D, 0 permeabilization time). These granules (approximately 15–20% of the total) probably correspond to the pool of granules that undergo release during stimulation of intact cells. In fact, when Ca2+- and digitonin were added together in the absence of ATP, latrunculin had virtually no effect (Fig. 4D). This result is similar to the lack of effect of these agents in intact cells (Fig. 5, B and D), and suggests that release from intact cells represents the component we are calling “ATP-independent” secretion.

**Regulation of the Secretory Response Is a Complex Interplay between Atp, Pip2, and Actin.** Removal of ATP reduces levels of Pip2 (Eberhard et al., 1990) and reduces cortical actin. It is surprising that treatment with latrunculin, which binds g-actin and alters actin dynamics, reduced or prevented the loss of cortical actin in the absence of ATP. As discussed above, latrunculin added to permeabilized cells in the absence of ATP caused an increase in Pip2. Thus, although latrunculin tends to depolymerize f-actin because of changes in f-actin/g-actin dynamics, it may also stimulate a compensatory effect through increased Pip2, resulting in f-actin stabilization. Coincident with these effects was the stabilization of ATP-independent secretion. Likewise, jasplakinolide, which stabilizes actin filaments, also enhanced ATP-independent secretion and strongly inhibited ATP-dependent secretion.

Thus, the experiments with latrunculin suggest that changes in actin dynamics play a critical role in a distinct component of ATP-dependent secretion and point to a web of interactions involving Pip2 and actin. These interactions ensure the stability of the secretory pathway and other cellular processes.

**Acknowledgments**

The plasmid encoding YpkA was a gift from Dr. Jack Dixon.

**References**


Eberhard DA, Cooper CL, Low MG, and Holz RW (1990) Evidence that the inositol phospholipids are necessary for exocytosis: loss of inositol phospholipids and inhibition of secretion in permeabilized chromaffin cells caused by a bacterial phospholipase C and removal of ATP. Biochim Biophys Acta 1038:25–45.


**Address correspondence to:** Dr. Mary A. Bittner, Department of Pharmacology, University of Michigan Medical School, 1301 MSRB III, Ann Arbor, MI 48109-0632. E-mail: mbittner@umich.edu