Changes in actin network during calcium-induced exocytosis in permeabilized GH3 cells: calcium directly regulates F-actin disassembly

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Abstract

Using digitonin-permeabilized GH3 cells, we investigated both the release of prolactin (PRL) and changes in the cytoskeleton. We determined that permeabilized GH3 cells released PRL in a dose-dependent manner upon addition of micromolar Ca^{2+} . Phalloidin, a filamentous actin (F-actin) stabilizing agent, inhibited both Ca^{2+} -dependent and -independent PRL release, whereas cytochalasin B, a destabilizing agent, had almost no effect on the release. Observation with a confocal laser scanning microscope revealed that F-actin existed mainly in the cortical region in the quiescent state. Increased cytosolic Ca^{2+} induced a change in F-actin distribution: F-actin in the cortical region decreased, whereas F-actin inside the

cells increased. This change in F-actin distribution was not observed when phalloidin was added. Addition of cytochalasin B induced patchy F-actin spots, but the pattern of the changes of F-actin distribution did not change. The time course of change in F-actin distribution showed that the F-actin network in the cortical region was reduced within 1 min, and Ca^{2+} -dependent release of PRL continued for up to 20 min. These results suggest that the F-actin network near the membrane acts as a barrier to exocytosis and that Ca^{2+} directly controls the cytoskeletal changes.

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Introduction

The pathway of signal transduction in exocytosis has been investigated in various cells, and it has been clarified that an increase in the cytoplasmic Ca^{2+} concentration is the key step in triggering exocytosis (Hutton 1986, Augustine *et al.* 1987). However, the precise mechanism of exocytosis resulting from increased Ca^{2+} remains unclear. The molecular mechanisms by which Ca^{2+} regulates secretory granule transport, cytoskeletal rearrangements, secretory granule–plasma membrane interaction and exocytotic fusion are now under investigation. In the 1980s, numerous reports suggested involvement of the cytoskeleton in exocytosis (Cheek *et al.* 1986, Trifaró 1990). In particular, the actin network was considered to be the most important part of the cytoskeleton with regard to the regulation of the traffic of secretory granules (Aunis & Bader 1988, Segawa & Yamashina 1989). Most of the experiments using phalloidin and cytochalasin, which induce actin fiber polymerization and depolymerization respectively, showed that depolymerization of F-actin was required for exocytosis (Lelkes *et al.* 1986). Therefore, it is considered that

actin fibers form a network and act as a barrier to secretory granules moving towards the plasma membrane. Trifaró *et al.* (1992) supported this hypothesis by showing that concomitant and similar redistribution of F-actin and scinderin, an actin filament-severing protein, occurred upon nicotinic stimulation and that the redistribution would produce subplasmalemmal areas of decreased viscosity and increased mobility of secretory granules in chromaffin cells. However, these findings are still circumstantial and it is also unknown whether depolymerization of F-actin itself is sufficient for exocytosis (Muallem *et al.* 1995), because the F-actin depolymerizing agents themselves, such as cytochalasin, do not induce but only potentiate exocytosis (Sontag *et al.* 1988).

Recent progress in the study of neurotransmitters has revealed that the final step in the regulated fusion of vesicles with the plasma membrane requires a complex that includes synaptobrevin, syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) (Rothman 1994). However, the final step is not clear in endocrine cells, and even the participation of these proteins is not yet clear. The precise steps in which Ca^{2+} regulates the traffic of

secretory granules have not been established. Moreover, there has been no report about the relationship between this protein complex and the changes in F-actin network. Adrenal chromaffin cells have often been used in studies of the molecular mechanism of exocytosis. However, these cells have the characteristics of both endocrine cells and neurons, and do not represent endocrine cells. Only a few reports indicated a change in F-actin distribution during stimulation of endocrine cells other than chromaffin cells (van de Moortele *et al.* 1991, Kiley *et al.* 1992, Carbajal *et al.* 1997), and we know of very few studies that determined both secretion of hormone and the changes in F-actin distribution at the same time (Sontag *et al.* 1988, van de Moortele *et al.* 1991, Carbajal *et al.* 1997).

In the present study, we used digitonin-permeabilized GH3 cells, which were derived from the rat pituitary adenoma and release prolactin (PRL), as a model of endocrine cells for examining the precise mechanisms by which Ca^{2+} regulates exocytosis. Permeabilization of the cells allows direct access to the cell interior and makes it easier to analyze each step in exocytosis. Using a confocal laser microscope, we analyzed the changes in the actin network quantitatively. We examined the precise relationship between the release of PRL and the changes in the cytoskeleton under the same experimental conditions.

Materials and Methods

Materials

Ham F-10 medium was purchased from Gibco Laboratories (Grand Island, NY, USA). Digitonin was obtained from Merck Laboratories (Darmstadt, Germany). All other reagents were obtained from Sigma Chemical (St Louis, MO, USA). Plastic cell-culture flasks and multiwell plates were purchased from Falcon, Becton Dickinson Labware (Oxnard, CA, USA). Glass-bottom microwell dishes coated with poly-D-lysine were obtained from MatTek Corp. (Ashland, MA, USA).

Cell culture

GH3 cells were obtained from the Japanese Cancer Research Resources Bank. The cells were fed with Ham F-10 medium supplemented with 1.2 g/l NaHCO_3 , 5% fetal calf serum (FCS) and 12.5% horse serum (HS), without antibiotics. They were maintained in 75 cm^2 cell culture flasks at 37 °C in an atmosphere of 5% CO_2 , 95% humidified air, and subcultured every 5–7 days. Two or three days before the experiments, the cells were seeded onto 24-well plates at a density of about 10^6 /well for studies of PRL release or onto glass bottom microwell dishes at 0.2×10^6 /well for morphological studies.

Release of PRL from permeabilized GH3 cells

The experiment on PRL release was performed according to the following procedure. GH3 cells, which were

cultured on 24-well plates, were washed twice with 1 ml Ham F-10 medium supplemented with 1.2 g/l NaHCO_3 and 0.1% bovine serum albumin (BSA). They were then incubated for another 30 min at 37 °C in fresh medium, and washed again with the same medium. After being washed, the cells were simultaneously permeabilized and stimulated in 0.4 ml Kglu buffer (20 mM potassium acetate, 120 mM potassium glutamate, 20 mM Hepes, 1 mM EGTA, 5 mM D-glucose, 0.1% BSA, pH 7.4) containing 10 μM digitonin, various doses of CaCl_2 and other agents for 20 min. The free Ca^{2+} concentrations were calculated as described previously (Nishizaki *et al.* 1992). After the incubation, buffers were aspirated into tubes on ice and centrifuged at 1000 g for 10 min at 4 °C to remove detached cells. The supernatants were collected and stored at –20 °C until the PRL assay. The concentrations of PRL were determined by radioimmunoassay (RIA), using the agents and procedure supplied under the National Institute of Diabetes, Digestive and Kidney Disease (NIDDK) Rat Hormone Distribution Program. In this assay, the sensitivity and intra-assay coefficient of variation are 100 pg/tube and 10.0% respectively.

F-actin staining by FITC-labeled phalloidin

The GH3 cells in the glass-bottom microwell dishes were washed twice with 1 ml of BSSG buffer (5 mM KCl, 142 mM NaCl, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 14.3 mM NaHCO_3 , 10 mM Hepes, 5 mM D-glucose, 0.1 mM EGTA, pH 7.2). To confirm the distribution of F-actin in quiescent state cells, the cells were fixed with cold Zamboni solution (2% paraformaldehyde, 0.13 M phosphate saline buffer, 0.21% picric acid) without stimulation. After 2 days' fixation at 4 °C, cellular F-actin was stained with 1.6 μM fluorescein isothiocyanate (FITC)-labeled phalloidin in Kglu buffer for 10 min, followed by washing twice with 1 ml Kglu.

In order to determine the effect of Ca^{2+} stimulation, cultured GH3 cells were washed twice with BSSG buffer. They were then simultaneously permeabilized and stimulated in Kglu buffer containing 10 μM digitonin and 1 μM free Ca^{2+} for 10 min. This was followed by a wash with 1 ml Kglu without Ca^{2+} and digitonin. In case of fixed cells, the cells were fixed with cold Zamboni solution for at least 2 days at 4 °C and F-actin was stained by the same procedure as for the quiescent cells. In case of live cells, immediately after washing, the cells were treated with 1.6 μM FITC-labeled phalloidin in Kglu buffer for 10 min, followed by washing twice with 1 ml Kglu.

Confocal laser scanning microscope

Kglu buffer was mounted on FITC-phalloidin stained cells, and fluorescent images were obtained using a confocal laser scanning microscope system (LSM 410, Carl Zeiss Inc. Germany) at an excitation wavelength of

Figure 1 PRL secretion by digitonin-permeabilized GH3 cells. After three days' culture in Ham F-10 medium containing 5% FCS and 12.5% HS in 24-well culture plates, the GH3 cells were washed twice, then permeabilized and stimulated by incubation in Kglu buffer containing 10 μ M digitonin. (A) The time course of PRL secretion was determined for various times of incubation with (●) and without (■) 1 μ M free Ca²⁺. (B) Ca²⁺-dependent PRL release was determined. GH3 cells were stimulated for 20 min, then the buffer was collected and centrifuged at 1000 *g* for 10 min. The amount of PRL in the supernatant was determined by RIA.

488 nm and an emission wavelength of 515 nm. Each parameter of the confocal laser microscope was fixed in every experiment. In this condition, full-width half-maximum (FWHM, half maximum width of optical section) was 1 μ m. The equatorial plane of focus was defined as the midpoint between the top and the bottom of the cell; the nucleus, which does not contain F-actin, usually lies on this plane. Each image of cell was saved immediately after the observation until the analysis. To control for the effect of bleach of the fluorescence, we confirmed that averages of overall intensity of the first and last ten cells that were observed in one experiment were not different.

Analysis of F-actin distribution

Images of equatorial slices were quantified by radial line scan analysis. The fluorescence intensity of FITC-phalloidin along the horizontal line that goes through the center of the cell was measured for each cell using an 'NIH image' program. The edge of the cell was defined as the

beginning of the sharp slope of the intensity profile. Each value for the intensity seen at the same distance from the edge was summed and averaged to form the intensity profile for each set of experimental conditions. One pixel represents a distance of 0.0992 μ m.

Presentation of data

All PRL release experiments and FITC-phalloidin staining experiments were carried out using at least three different cell preparations with good reproducibility. All the figures are representative; in the case of PRL release experiments, all data are given as the mean of duplicate determinations of same cell preparations.

Results

Ca²⁺ dependent PRL secretion from permeabilized GH3 cells

For both PC12 cells and bovine chromaffin cells, it has been established that pores formed by digitonin in plasma

Figure 2 Effects of phalloidin and cytochalasin B on PRL secretion by permeabilized GH3 cells. (A) GH3 cells were incubated for 20 min with Kglu buffer containing 10 μ M digitonin, various concentrations of free Ca²⁺ and 0 (●), 1 (▲), 5 (■) or 10 (○) μ M phalloidin. (B) GH3 cells were incubated for 20 min with Kglu buffer containing 10 μ M digitonin, various concentrations of free Ca²⁺ and 0 (●), 10 (▲) or 50 (■) μ M cytochalasin B. After collection, the buffer was centrifuged at 1000 *g* for 10 min. The amount of PRL in the supernatant was measured by RIA.

membranes do not impair the process of exocytosis and they are large enough to allow macromolecules to pass in both directions (Schäfer *et al.* 1987, Holz 1988). A high-potassium buffer was used in the permeabilization experiments. We studied the effect of various concentrations of digitonin on GH3 cells (data not shown), and confirmed that 10 μ M digitonin was sufficient to permeabilize the cell membrane and adequately preserve Ca²⁺-dependent exocytosis. To examine the time course of PRL release from the permeabilized GH3 cells, GH3 cells seeded on 24-well plates were incubated for 0, 5, 10, 20, 40 or 80 min in Kglu buffer containing 10 μ M digitonin with or without 1 μ M free Ca²⁺ at room temperature. As shown in Fig. 1A, PRL release from the permeabilized cells reached a plateau within 20 min in the presence of Ca²⁺. In contrast, the release increased linearly up to 80 min in the absence of Ca²⁺. This linear increase in PRL release without Ca²⁺ may be due to a direct effect of digitonin on secretory granules (Grant *et al.* 1987). The

difference in PRL released in the presence and in the absence of 1 μ M Ca²⁺ reached a maximum at 20 min. The effect of Ca²⁺ concentration on PRL release after 20 min of incubation was then studied (Fig. 1B). Over the physiological Ca²⁺ concentration range (0.1–10 μ M), PRL release was regulated by the Ca²⁺ concentration. Nearly the same pattern of Ca²⁺-dependent exocytosis was observed as in digitonin-permeabilized bovine chromaffin cells (Pocotte *et al.* 1985) and GH3 cells permeabilized by electric field discharge (Ronning & Martin 1985). ATP was not included in Kglu buffer and the addition of ATP to Kglu buffer had no effect on PRL release (data not shown).

Effects of phalloidin and cytochalasin B on PRL secretion from GH3 cells

We tested the effects of two agents, which affect the equilibrium between filamentous and monomeric actin,

Figure 3 Confocal images of FITC-phalloidin-stained quiescent and stimulated GH3 cells fixed with Zamboni solution. GH3 cells were subcultured on glass-bottom culture dishes in Ham F-10 medium containing 5% FCS and 12.5% HS. After 2 days' culture, the cells were washed twice with BSSG and fixed with cold Zamboni solution (A) or permeabilized with 10 μ M digitonin with (C) or without (B) 1 μ M free Ca^{2+} for 10 min and fixed. After 2 days' fixation in a cold room, GH3 cells were stained with 1.6 μ M FITC-labeled phalloidin and observed under a confocal laser microscope. The bar in C represents 10 μ m.

Figure 4 Confocal images of FITC-phalloidin-stained permeabilized living GH3 cells. GH3 cells were subcultured on glass-bottom culture dishes in Ham F-10 medium containing 5% FCS and 12.5% HS. After 2 days' culture, the cells were washed twice with BSSG and permeabilized with 10 μ M digitonin then left unstimulated (A, C, E) or stimulated (B, D, F) with 1 μ M free Ca^{2+} and other agents for 10 min. Then the GH3 cells were stained with 1.6 μ M FITC-labeled phalloidin and observed under a confocal laser microscope. The permeabilization buffer contained no other agents (A and B), 5 μ M phalloidin (C and D) or 5 μ M cytochalasin B (E and F). The bar in F represents 10 μ m.

on PRL release in permeabilized GH3 cells. One to ten micromolar phalloidin, which stabilizes F-actin, was added to the buffer when GH3 cells were stimulated with various concentrations of free Ca^{2+} . PRL secretion was suppressed by phalloidin at any concentration of Ca^{2+} (Fig. 2A). We also tested the effect of cytochalasin B, a microfilament-destabilizing agent that blocks the filaments' barbed ends. However, no effect of cytochalasin B on PRL release was observed except for mild suppression at high concentrations (Fig. 2B).

F-actin distribution in the quiescent and Ca^{2+} -stimulated GH3 cells

In order to confirm the distribution of actin network in the quiescent GH3 cells, FITC-labeled phalloidin was used to stain F-actin of GH3 cells in microwell dishes that were fixed without any treatment. FITC-phalloidin staining of quiescent GH3 cells revealed F-actin to be present mainly in the cortex (Fig. 3A). Although the intensity of the cortex ring was almost homogeneous, close observation at high magnification showed weak heterogeneity in the intensity of the ring. To determine the effect of permeabilization, GH3 cells were permeabilized without or with $1\ \mu\text{M}$ free Ca^{2+} and fixed with Zamboni solution (Fig. 3B and 3C respectively). There was no influence of permeabilization by digitonin on F-actin distribution in the quiescent GH3 cells (Fig. 3A and 3B). Stimulation of permeabilized GH3 cells with $1\ \mu\text{M}$ free Ca^{2+} induced changes in F-actin distribution: the staining of the cortex region of the cell was disrupted and the overall intensity decreased, whereas the intensity of the interior region increased diffusely (Fig. 3C). To observe the change in actin network in live cells in detail, GH3 cells were stained with FITC-phalloidin without fixation immediately after permeabilization and stimulation. F-actin distributions were same as in fixed GH3 cells, except for very subtle staining in the nucleus of fixed cells (Figs 3B, 3C and 4A, 4B respectively). Up to 20 min, incubation with digitonin had no effect on F-actin distribution in the quiescent cells (data not shown). Because phalloidin is a probe of F-actin, the decrease in FITC-phalloidin fluorescence intensity indicates disassembly of the actin network at the plasma membrane.

Quantitative analysis of changes in F-actin distribution

The changes in the Distribution of F-actin detected after stimulation of permeabilized cells were further analyzed quantitatively. Images such as those shown in Fig. 4 were quantified using radial line scan analysis of equatorial slices, as described in Materials and Methods. The FITC fluorescence intensity profiles for each set of experimental conditions were pooled. Figure 5A confirms and quantifies the effects of $1\ \mu\text{M}$ free Ca^{2+} seen in Fig. 4A and 4B. A selective decrease and increase in the intensity of F-actin in the cortical and cytoplasmic regions respectively were demonstrated.

Effect of phalloidin and cytochalasin B on F-actin distribution

To investigate the effect of phalloidin on F-actin distribution, GH3 cells were incubated with $5\ \mu\text{M}$ unlabeled phalloidin along with Ca^{2+} , and stained with FITC-labeled phalloidin. Because of the competition between unlabeled ($5\ \mu\text{M}$) and labeled ($1.6\ \mu\text{M}$) phalloidin to bind to F-actin, the binding of FITC-labeled phalloidin was markedly inhibited (Fig. 4C). The effect of Ca^{2+} on F-actin distribution was almost free (Fig. 4D). Quantitative analysis showed only very mild increases in intensity in the interior region after Ca^{2+} stimulation (Fig. 5B).

Although cytochalasin B produced a markedly patchy staining intensity of F-actin, it had a small effect on the overall change in F-actin distribution (Fig. 4E and 4F). Quantitative analysis showed that the intensity of the cortex region decreased and that of interior region increased on Ca^{2+} stimulation as it did with the control, but the amounts of decrease and increase in intensity were reduced (Fig. 5C).

Time course of changes in F-actin distribution in Ca^{2+} -stimulated GH3 cells

To examine the time course of changes in F-actin distribution, GH3 cells were permeabilized and stimulated with Ca^{2+} for 0, 1, 2 or 5 min and stained with FITC-phalloidin. Confocal laser microscope images were analyzed by the same procedure as for Fig. 5. Within 1 min, F-actin in the cortex region had already decreased to the same level as after 5 min of stimulation. In contrast,

Figure 5 Quantitative analysis of the change in F-actin distribution induced by Ca^{2+} . Densitometric line scan analyses were performed on confocal images of FITC-phalloidin-stained GH3 cells. A horizontal scan line was obtained per cell, which extended from the exterior to the center of the cell. The intensity profiles of the lines obtained under each experimental condition were pooled to form a mean profile, as described in Materials and Methods. Data shown are the averages of the intensity of pixels. The X-axis represents the distance from the edge of a cell. The experimental conditions are identical to those described in Fig. 4. (A) GH3 cells were permeabilized with $10\ \mu\text{M}$ digitonin and stimulated with (●) and without (■) $1\ \mu\text{M}$ free Ca^{2+} ; $n=55$ cells. (B) GH3 cells were permeabilized and incubated with $5\ \mu\text{M}$ phalloidin, with (●) and without (■) $1\ \mu\text{M}$ free Ca^{2+} ; $n=30$ cells. (C) GH3 cells were permeabilized and incubated with $5\ \mu\text{M}$ cytochalasin B, with (●) and without (■) $1\ \mu\text{M}$ free Ca^{2+} ; $n=30$ cells. Values are means $\pm$ S.E.M.

Figure 6 Time-dependent changes in F-actin distribution in Ca^{2+} -stimulated GH3 cells. GH3 cells, which were cultured in glass-bottom culture dishes, were incubated with 10 μM digitonin and 1 μM Ca^{2+} for 0 (●), 1 (▲), 2 (■) or 5 (○) min, and then incubated with FITC-phalloidin after washing. Confocal laser microscope images were subjected to densitometric line scan analysis. A horizontal scan line was obtained per cell, which extended from the exterior to the center of the cell. The intensity profiles of these lines for each incubation time were pooled to form a mean profile; $n=40$ cells for each incubation time. Data shown are the averages of the intensity of pixels. The X-axis represents the distance from the edge of a cell. Values are means $\pm$ or $-$ S.E.M.

F-actin in the interior region did not change until 5 min, and then increased slowly (Fig. 6).

Discussion

In neuroendocrine cells, it has been established that stimulation induces an increase in the cytosolic Ca^{2+} , which triggers exocytosis. Many agents, such as protein kinase C (Burgoyne *et al.* 1989, Nishizaki *et al.* 1992), calmodulin (Burgoyne 1991, Cheek 1991), fodrin

(Burgoyne & Cheek 1987, Perrin *et al.* 1987), gelsolin (Bader *et al.* 1986, Trifaró & Vitale 1993), scinderin (Rodríguez *et al.* 1990, Vitale *et al.* 1995), calpactin (Burgoyne *et al.* 1991), and 14–3–3 proteins (Roth & Burgoyne 1995) and GTP-binding proteins (Norman *et al.* 1994, Price *et al.* 1995), have been studied as Ca^{2+} target proteins in the regulation of exocytosis, though the exact mechanisms by which Ca^{2+} regulates exocytosis remain unclear. Immunocytochemical studies showed that the cortical actin network was disassembled upon nicotine stimulation of bovine adrenal chromaffin cells (Cheek & Burgoyne 1986, Trifaró & Vitale 1993). However, it remains controversial whether or not the disassembly of actin filaments is essential for exocytosis, and the exact relationship between the increase in cytosolic Ca^{2+} and actin network disassembly has not been well documented. To our knowledge, only one report has shown that increased cytosolic Ca^{2+} directly induced catecholamine release and the morphological change in F-actin distribution; that study used streptolysin-O-permeabilized chromaffin cells and rhodamine-phalloidin (Sontag *et al.* 1988). Permeabilization of the cells made it easier to control the cytosolic Ca^{2+} concentration directly without changing any other components of the cells. In this report, we investigated more specifically the direct effects of an increase in cytosolic Ca^{2+} on both exocytosis and the change in F-actin distribution under the same experimental conditions. We determined the time course minutely and studied the effect of Ca^{2+} on the release of hormone and the change in the actin network. A confocal microscope made it possible to analyze the changes in the actin network quantitatively.

GH3 cells were permeabilized with the detergent digitonin, and we confirmed that free Ca^{2+} in micromolar concentrations directly induced exocytosis of PRL without requiring any co-factor. Immunofluorescence and cytochemical studies demonstrated that F-actin is localized mainly in the cortical region of chromaffin cells (Vitale *et al.* 1991, 1995) or mast cells (Koffer *et al.* 1990) in the resting state. In the case of cultured normal anterior pituitary cells (Carbajal & Vitale 1997) and pituitary-derived GH4C1 cells (Kiley *et al.* 1992), these cells exhibited bright cortical actin staining, but in GH3B6 cells, the staining was diffuse in the cytoplasm and displayed a patchy aspect at the cell periphery (van de Moortele *et al.* 1991). The GH3 cells in our experiments showed a staining pattern similar to those of chromaffin cells and GH4C1 cells.

The changes in the cytoskeleton during cell stimulation have been studied using a variety of cells, such as adrenal chromaffin cells (Zhang *et al.* 1995), mast cells (Norman *et al.* 1994), leukemia cells (Pfeiffer *et al.* 1985) and pancreatic acinar cells (Muallem *et al.* 1995). F-actin network disassembly has been observed in these cells upon stimulation. Classically, pituitary cells have been widely used for studies of the mechanisms of hormone secretion,

and the effect of cytochalasin has been investigated (Liu & Jackson 1986). However, only a few reports have shown changes in the cytoskeletons of pituitary cells during cell excitation (van de Moortele *et al.* 1991, Kiley *et al.* 1992, Carbajal & Vitale 1997). The changes in F-actin distribution during thyrotropin-releasing hormone (TRH)- or K^+ -induced stimulation of GH3-derived cells were not identical in those reports. In GH3B6 cells, thick actin bundles were formed in the cytoplasm and ran parallel to the cell surface after stimulation with TRH. In contrast, in GH4C1 cells, TRH caused a generalized decrease in fluorescence intensity, and K^+ did not alter the F-actin network.

Our present findings of a change in F-actin distribution – a reduction in F-actin in the cortical region and an increase in F-actin in the interior region – have also been observed in mast cells and adrenal chromaffin cells (Norman *et al.* 1994, Vitale *et al.* 1995). This change in F-actin was different from the changes observed in both GH4C1 cells and GH3C6 cells. The reduction in the cortical F-actin network is completed within 1 min, followed by the increase in F-actin in the cytoplasm a few minutes later (Fig. 6). Because of this time lag between the decrease in F-actin in the cortex and the increase in the cell interior, the change in F-actin distribution could not be explained solely by the simple relocation of F-actin. In mast cells, it was demonstrated by two-step staining that both *de novo* polymerization of actin and relocation of F-actin contributed to the increase in the internal staining (Norman *et al.* 1994). In our experiments, cytochalasin B reduced both the decrease in cortical F-actin and the increase in the cell interior (Fig. 5C). This result also suggests that the increase in F-actin in the cell interior is due to *de novo* polymerization, and that cytochalasin B suppresses this polymerization. Compared with the time course of PRL release (Fig. 1A), the reduction in the cortical F-actin network was quicker. These observations support the hypothesis that disassembly of the actin network removes the barrier to secretory granule traffic towards the plasma membrane and that granules freely dock and fuse with the plasma membrane to complete exocytosis. Electron microscopic studies (Senda *et al.* 1989, 1994, Nakata *et al.* 1992, Tchakarov *et al.* 1998) revealed the localization of F-actin and secretory granules in chromaffin and pituitary cells: the subcortical actin filaments are distributed unevenly near the plasma membrane, and secretory granules are preferentially seen in the region where actin filaments are sparse. These findings were consistent with our present observations and provide morphological support for the concept of the role of actin filaments.

Effects of phalloidin, which is known to bind to F-actin more tightly than to globular actin (G-actin) and shifts the equilibrium between filaments and monomers toward the filaments (Cooper 1987), were observed in both secretion and distribution changes in F-actin. Phalloidin completely

inhibited Ca^{2+} -induced release and inhibited Ca^{2+} -independent constitutive secretion of PRL (Fig. 2A). In fluorescence studies, phalloidin itself competed with FITC-labeled phalloidin to bind with F-actin, so the staining with FITC-phalloidin decreased, but we observed no change in F-actin distribution upon Ca^{2+} stimulation without a very mild increase in the interior region (Fig. 5B). Because of the weak staining of the cortex of unstimulated cells, it was hard to show the difference between Ca^{2+} -stimulated and -unstimulated cells. These results are also in agreement with the idea that the actin network acts as a barrier to the traffic of secretory granules – that is, phalloidin blocks the depolymerization of actin filaments so that the actin network is preserved and inhibits the traffic of granules and exocytosis of PRL. In contrast, cytochalasin B, which binds to the barbed end of the actin filaments and inhibits both polymerization and depolymerization of the end of the filaments, has no effect on Ca^{2+} -induced secretion (Fig. 2B) or the overall distribution of F-actin in the quiescent state (Fig. 5C), although the cortical F-actin ring was more heterogeneous (Fig. 4E). On Ca^{2+} stimulation, distribution of F-actin to the interior region and a punctate staining pattern of F-actin were observed (Fig. 4F). The effect of cytochalasin on hormone secretion was not identical with all cell types and experimental conditions. In the experiments on permeabilized adrenal chromaffin cells, cytochalasins usually potentiated calcium-induced secretion (Lelkes *et al.* 1986, Aunis & Bader 1988, Sontag *et al.* 1988). However, Roth & Burgoyne (1995), who also used digitonin-permeabilized chromaffin cells, reported that cytochalasin had no effect on basal or stimulated catecholamine secretion. We have no reasonable explanation for these differences in the effects of cytochalasin on secretion. Sontag *et al.* (1988) reported the effect of cytochalasin on F-actin distribution upon stimulation of chromaffin cells, and they also observed a punctate staining pattern, but what they meant by a 'punctate staining pattern' is unclear.

In the present study, we clarified the direct effect of an increase in cytosolic Ca^{2+} on both exocytosis and the change in the cytoskeletal actin network. Our results support the hypothesis that Ca^{2+} directly regulates the F-actin network, which acts as a physical barrier preventing secretory granule traffic towards the plasma membrane. A variety of proteins have been proposed as target proteins of Ca^{2+} , but the exact relationships among these proteins are unknown. A recent study showed two stages of exocytosis: an ATP-dependent priming reaction and a Ca^{2+} -triggered fusion reaction (Hay & Martin 1992). As in the case of synapse of neurons, it is suggested that neuroendocrine cells also require an *N*-ethylmaleimide sensitive factor (NSF)-soluble NSF attachment protein (SNAP)–SNAP receptor (SNARE) complex and Ca^{2+} to trigger fusion of secretory vesicles and the plasma membrane (Banerjee *et al.* 1996). Our results support the

hypothesis that, in the exocytotic reaction, disassembly of the actin network and docking and fusion of secretory granules are separate steps, and Ca^{2+} is required for both steps. Actin network disassembly may be insufficient for exocytosis. Disassembly of the actin network allows the secretory granules to move freely, but exocytosis does not occur because the docking and fusion steps are dependent on Ca^{2+} . Our findings that phalloidin blocked both exocytosis and the change in the actin network in spite of an increase in Ca^{2+} support the hypothesis that actin network disassembly is essential for exocytosis. Further investigations are necessary to clarify more precisely the mechanism and proteins that are regulated by Ca^{2+} .

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