The Participation of Annexin II (Calpactin I) in Calcium-evoked Exocytosis Requires Protein Kinase C

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Abstract. Permeabilized adrenal chromaffin cells secrete catecholamines by exocytosis in response to micromolar calcium concentrations. Recently, we have demonstrated that chromaffin cells permeabilized with digitonin progressively lose their capacity to secrete due to the release of certain cytosolic proteins essential for exocytosis (Sarafian T., D. Aunis, and M. F. Bader. 1987. J. Biol. Chem. 34:16671–16676). Here we show that one of the released proteins is calpactin I, a calcium-dependent phospholipid-binding protein known to promote in vitro aggregation of chromaffin granules at physiological micromolar calcium levels. The addition of calpactin I into digitonin- or streptolysin-O-permeabilized chromaffin cells with reduced secretory capacity as a result of the leakage of cytosolic proteins partially restores the calcium-dependent secretory activity. This effect is specific of calpactin I since other annexins (p32, p37, p67) do not stimulate secretion at similar or higher concentrations. Calpactin I requires the presence of Mg-ATP, suggesting that a phosphorylating step may regulate the activity of calpactin. Calpactin is unable to restore the secretory activity in cells which have completely lost their cytosolic protein kinase C or in cells having their protein kinase C inhibited by sphingosine or downregulated by long-term incubation with TPA. In contrast, calpactin I prephosphorylated in vitro by purified protein kinase C is able to reconstitute secretion in cells depleted of their protein kinase C activity. This stimulatory effect is also observed with thiophosphorylated calpactin I which is resistant to cellular phosphatases or with phosphorylated calpactin I introduced into cells in the presence of microcystin, a phosphatase inhibitor. These results suggest that calpactin I is involved in the exocytotic machinery by a mechanism which requires phosphorylation by protein kinase C.

A new group of calcium-binding proteins that associate reversibly with biomembranes has recently been identified in a wide range of mammalian cell types and tissues (for review see Geisow et al., 1987; Burgoyne and Geisow, 1989). The common key feature of these proteins, named annexins, is their ability to associate specifically with phospholipid bilayers in a calcium-dependent manner. Sequence data indicate that annexins are a family of structurally similar proteins consisting of two regions: the tail with the phosphorylation sites and the core containing the binding sites for calcium, phospholipids and cytoskeletal elements (Glenney and Tack, 1985; Glenney, 1986; Johnsson et al., 1986; Glenney et al., 1987).

Calpactin I is one of the best characterized components of the annexin family. Calpactin I is a protein that occurs in cells as a 36-kD monomer (the heavy chain) and as a 90-kD complex containing two copies of the 36-kD heavy chain and two copies of an 11-kD light chain (Erikson et al., 1984; Gerke and Weber, 1984; Glenney and Tack, 1985). Calpactin I is a major substrate for protein-kinase C and for the Rous sarcoma virus enzyme pp60-v-src (Gould et al., 1986; Gerke and Weber, 1984, 1985; Powell and Glenney, 1987).

The tyrosine and serine phosphorylation sites for pp60 src and protein kinase C have been identified in the NH₂-terminal region of the p36 heavy chain (Glenney and Tack, 1985; Gould et al., 1986). This region also contains the binding site for the p11 light chain (Glenney et al., 1986; Johnson et al., 1986). Although no definite function has been established for any member of the annexin family, the observation that annexins interact with membranes in a calcium-dependent manner suggests that they may participate in calcium-regulated traffic such as exocytosis. Indeed, calpactin is able to aggregate phospholipid-containing liposomes in a calcium-dependent manner (Glenney, 1986a, b; Glenney et al., 1986, 1987; Blackwood and Ernst, 1990) and a recent study revealed that calpactin I complex can promote the fusion of secretory granules at micromolar calcium concentrations, in the presence of arachidonic acid (Durst and Creutz, 1988). Therefore, calpactin appears to be a possible candidate for functioning as a docking protein between secretory vesicles and the plasma membrane during the exocytotic process.

For several experimental reasons, chromaffin cells from the adrenal medulla provide an excellent system to study the molecular mechanisms underlying exocytosis (Trifàro, 1982;
Livett, 1984; Aunis and Bader, 1988). Postreceptor events controlling exocytosis have been extensively studied in these cells as they have proven amenable to cell permeabilization techniques (Lee and Holz, 1986; Baker and Knight, 1981; Bader et al., 1986; Sontag et al., 1988). Permeabilized chromaffin cells secrete catecholamines by exocytosis in response to micromolar calcium in the incubation medium. We have previously shown that the ability of digitonin-permeabilized chromaffin cells to respond to calcium in the medium declines with time after permeabilization, and that this decline in calcium responsiveness is due to the leakage of cytosolic proteins required for exocytosis (Sarafian et al., 1987). Since secretion can be fully restored in extracted permeabilized cells by reintroducing the leaked cytosolic proteins, such cells can be used to systematically identify the essential components of the exocytotic machinery. We show here that calpastatin I is present among the cytosolic proteins released by digitonin-permeabilized cells and that the reintroduction of calpastatin into the cells partially restores the calcium-dependent exocytotic response. Moreover, our observations indicate that phosphorylation by protein kinase C may be required for the involvement of calpastatin I in the secretory machinery.

Materials and Methods

Culture of Chromaffin Cells

Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase and purified on self-generating Percoll gradients (Bader et al., 1986a,b). They were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and containing cytosine arabinoside (10 μM), fluorodeoxyuridine (10 μM), streptomycin (50 μg/ml), and penicillin (50 U/ml). Cells were usually cultured as monolayers on 24 multiple 16-mm well plates (Costar, Data Packaging Corp., Cambridge, MA) at a density of 2.5 x 10⁶ cells/well and used within 3-7 days after plating. In some experiments where proteins were isolated from permeabilized cells, cells were plated in 50-mm-diam plastic culture dishes (Falcon Labware, Oxnard, CA) at a density of 20 x 10⁶ cells/dish.

Streptolysin-O

Streptolysin-O (SLO) (Institut Pasteur, Paris, France) was activated with 4 mM dithiothreitol, purified by precipitation with 75% saturation ammonium sulphate at 4°C and stored as a suspension in 75% ammonium sulphate at 4°C. Each preparation of toxin was assayed for hemolytic activity against 2.5% rabbit erythrocytes in 25 mM phosphate-buffered saline (145 mM NaCl, 25 mM potassium-phosphate, pH 7.5). The dilution of toxin hemolyzing 50% of red cells (ED50) was estimated and the reciprocal of this value was taken as the number of hemolytic units per milliliter of the nondenatured toxin solution. Cultured chromaffin cells (2.5 x 10⁶ cells) were permeabilized for 5 min at 37°C in 200 μl calcium-free permeabilizing medium (150 mM glutamate, potassium salt, 10 mM Pipes, 5 mM NTA, 0.5 mM EGTA, 0.2% bovine serum albumin, pH 7.0) adjusted with 1 M KOH, 5 mM Mg-ATP, and 4.5 mM magnesium-acetate containing 18 U/ml of SLO.

[3H]Noradrenaline Release from Permeabilized Cells

Catecholamine stores were labeled by incubation of intact cells with [3H]noradrenaline 16 Ci/mmol; (Amersham France, Les Ulis, France) for 30 min. Cells were then washed four times with Locke's solution containing calcium (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 11 mM glucose, 0.56 mM ascorbic acid, and 15 mM Hepes, pH 7.5) and twice with calcium-free Locke's solution (containing 1 mM EGTA); washing times were 10 min. Cells were subsequently permeabilized with SLO (18 U/ml). Secretion was induced with permeabilizing media containing various amounts of CaCl₂ to yield the indicated free calcium concentration. The exact free calcium concentration in permeabilizing media was calculated as described by Flodgaard and Fleron (1974) using the stability constants given by Silfen and Martell (1971). Catecholamine release was monitored by determining the radioactivity present in the incubation media after centrifugation at 12,000 g and in the cells after precipitation with 10% TCA. Release of [3H]noradrenaline is expressed as the percentage of total radioactivity present in the cells before calcium-induced stimulation. The amount of [3H]noradrenaline released during permeabilization remained close to 5% of the total radioactivity present in the cells before permeabilization.

Purification of Annexins

Calpastatin I monomer (p36) and tetramer (p90) were purified independently either from pig brain or from spinal cord according to the procedure of Shadle et al. (1985) and Glenny et al. (1987) with slight modifications as described by Regnouf et al. (1991). The two forms of calpastatin I were tested for their calcium-dependent binding to phosphatidylserine liposomes and to F-actin and for their ability to aggregate chromaffin granules (Drust and Creutz, 1988). Annexin I (calpastatin II, p37) was purified from bovine adrenal medulla as described by Regnouf et al. (1991). Calelecrin (p57) and endonexin (p32) were purified from bovine adrenal medulla and from pig brain respectively according to the procedure of Regnouf and Pradel (1989).

Production and Purification of Anti-Calpastatin I Antibodies

Purified brain calpastatin I monomer was subjected to preparative SDS gel electrophoresis. The band corresponding to the protein was excised, homogenized, and dialyzed against 0.15 M NaCl, 10% methanol, and 0.1% Triton X-100 for 48 h, then against 0.15 M NaCl, 0.01% Triton X-100, 1% methanol for 24 h, and finally against phosphate-buffered saline. The homogenate was emulsified with an equal volume of complete Freund's adjuvant and used directly for the following booster injections. Antibodies were raised in rabbits by subcutaneous injections of 65 μg antigen at 7-d intervals over a period of 1 mo. Production of specific antibodies was detected 5 wk after the initial immunization. Immunoblot experiments with a crude annexin preparation or with purified proteins indicated that the antisera does not cross react with other members of the annexin family such as p37 (calpastatin II), p57, and p32.

Preparation of Chromaffin Cell Membrane Proteins, Cytosolic Proteins, and Leaked Proteins from Permeabilized Cells

Chromaffin cells, plated at a density of 20 x 10⁶ cells in 50 mm-diam culture dishes, were permeabilized for 10 min with 2% of calcium-free permeabilizing medium containing 20 μM digitonin. Cells were then incubated for 30 min in calcium-free permeabilizing medium in the absence of bovine serum albumin and without detergent. The extracellular fluid, containing the released cytosolic proteins, was then collected and centrifuged for 30 min at 100,000 g to remove detached cells and cellular debris. Cells were then scraped in ice-cold permeabilizing medium and sonicated twice for 5 s at setting 9 on Ultrasonic Cell Disruptor. The homogenate was centrifuged at 100,000 g for 30 min to separate soluble proteins (supernatant) from membrane proteins (pellet). Membrane, soluble, and leaked proteins were dialyzed against water containing 0.1 M PMSF and lyophilized.

Measurement of Protein Kinase C Activity

Chromaffin cells in culture (2.5 x 10⁶ cells) were scraped off and homogenized in 0.6 ml of ice-cold buffer composed of 20 mM Tris-HCl, pH 7.5; 2 mM EDTA, 2 mM DTT, 2 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotonin, and 20 μg/ml pepstatin. After sonication, the homogenate was centrifuged at 100,000 g for 20 min. The supernatant was removed and the pellet (membrane fraction) was suspended in 0.3 ml of homogenizing solution containing 0.1% Triton X-100 detergent. A 20-μl sample of the supernatant (soluble protein kinase C) or of the membrane fraction (membrane-bound protein kinase C) was added to the protein kinase C assay medium containing 1.75 mM CaCl₂, 16 μg/ml phosphatidylserine, and 100 μM diacylglycerol to reduce the effect of Triton X-100 on protein kinase activity.

1. Abbreviation used in this paper: SLO, streptolysin-O.
C activity (Masmoudi et al., 1989). Protein kinase C was assayed using the protocol described by Castagna et al. (1982). The activity of protein kinase C was determined by measuring the enzymatic transfer of \(^{32}P\) from \([\gamma-^{32}P]ATP\) to histone H1 as substrate. The protein kinase C activity was determined in the presence of calcium plus phosphatidylserine. Non-specific enzymatic activity was measured in the absence of phosphatidylserine and subtracted from the total activity.

**Purification of Protein Kinase C and In Vitro Phosphorylation of Calpactin I (p90)**

Protein kinase C was purified according to the procedure of Le Pesch et al. (1983) with some modifications as described by Zwiller et al. (1985). The enzyme was stored at 4°C in 20 mM Hepes, pH 7.5, 1 mM DTT, 0.1 mM EDTA, and 50% glycerol and dialyzed against calcium-free permeabilizing buffer for 3 h just before use in order to get rid of glycerol. Calpactin I (2-8 nM) was phosphorylated in 100 µl of phosphorylating medium (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 2 mM PMSF, 5 mM Mg-acetate) containing 1.75 mM CaCl\(_2\), 160 nM TPA, 1.6 µg phosphatidylserine, and 0.2 µg protein kinase C. Calcium was omitted when calpactin I was phosphorylated for reconstitution experiments in permeabilized cells. Some experiments were performed in the presence of 1 µM microcystin, a phosphatase inhibitor (Honkanen et al., 1990).

Phosphorylation reaction was initiated by the addition of 20 µM ATP, ATP-\(\gamma\)-S or \([\gamma-^{32}P]ATP\) (25 µCi/ml) and incubations were carried out at 30°C for 30 min. Proteins were then either directly solubilized in SDS sample buffer for polyacrylamide gel electrophoresis or diluted twice in 300 mM glutamate, potassium salt, 20 mM Pipes, pH 7.0, 10 mM NTA, 1 mM EGTA and 10 mM Mg-ATP and immediately reintroduced into permeabilized cells. Control experiments in permeabilized cells were performed with protein kinase C preincubated for 30 min in phosphorylating medium containing the indicated substrates but in the absence of calpactin I.

**Electrophoresis and Immunoblotting**

Samples of proteins were solubilized in 10 mM Tris (pH 8.0), 1 mM EDTA, 3% SDS, 10% beta-mercaptoethanol, 10% glycerol and electrophoresed on 10% mono-dimensional polyacrylamide gels. Electrophoresis was performed at 8 mA for 1 h and then at 15 mA for 12 h. Gels were stained with 0.05% Coomassie brilliant blue. Electrophoretic transfer was carried out in 0.025 M Tris-HCl, 0.192 M glycine (pH 8.2), 20% methanol, at a constant current of 500 mA for 3 h. The nitrocellulose blots were incubated 2 h with anti-calpactin I antibodies at a final dilution of 1:500. After washing, the blots were either incubated 2 h with \(^{125}I\)-labeled anti-rabbit immunoglobulins (Amersham International, Amersham, England) at a final dilution of 1:1,000 dilution. Immunolabeled bands were then revealed with 0.02% chloro-2-naphtol and 0.01% H\(_2\)O\(_2\). The dried blots were exposed at -70°C to Amersham Hyperfilm MP with an intensifying screen. Autoradiographs were quantified by scanning densitometry with an LKB 2202 Ultrascan (Pharmacia, Saint-Quentin-En-Yvelines, France) laser scan densimeter at 633 nm.

**Other Assays**

Proteins were measured by the method of Bradford (1976) with bovine serum albumin as standard. Radioactivity was determined by liquid-scintillation counting using BIOFLUOR (New England Nuclear, Dreieich, Germany) in a Minaxi Tri-Carb 4000 counter (United Technologies Packard, Rungis, France).

**Presentation of Data**

All experiments described were carried out on at least three different cell preparations. In the figures and tables which are representative of a typical experiment, data are given as the mean of triplicate determinations on the same cell preparation ± SEM. Error bars smaller than the point symbols were not drawn. Unless indicated in the legend, data are given as the net secretory values obtained by subtracting the release obtained in the absence of calcium during the stimulation period (7.8 ± 0.5%; n = 20) from the release measured in the presence of calcium.

**Results**

**Effect of Calpactin I on the Secretory Activity of SLO-permeabilized Chromaffin Cells**

We have observed that in chromaffin cells permeabilized...
We examined the effect of the reintroduction of purified calpactin I on the capacity of permeabilized cells to release catecholamines. Chromaffin cells were permeabilized with SLO, incubated with various concentrations of calpactin I (p90) for 30 min and then stimulated with 20 μM free calcium. As illustrated in Fig. 2A, increasing the incubation time between permeabilization and the calcium-induced stimulation resulted in a marked progressive inhibition of the secretory activity. A 30-min period in calcium-free medium before stimulation decreased secretion to 26% of that obtained from cells stimulated immediately after permeabilization. However, the addition of calpactin I during this intermediate incubation period reduced the dose-dependent manner this secretory run down observed in SLO-permeabilized cells (Fig. 2B). Secretion after 30-min incubation with the concentration of calpactin I producing the maximal effect (8 μg/well) remained close to 75% of that obtained in cells immediately stimulated. The basal release estimated in the absence of calcium was not modified, at any of the calpactin concentrations tested.

In contrast to the stimulation of secretion by calpactin I (p90), endonexin (p32), calpactin II (p37), or calelectrin (p67) had no effect on the secretory activity in SLO-permeabilized cells even at high concentration (20 μg/well), indicating that the stimulating effect of calpactin I is specific to calpactin I and cannot be elicited by other annexins (Fig. 3).

with either digitonin (Sarafian et al., 1987) or SLO (Sarafian, T., J. M. Sontag, D. Aunis, and M.-F. Bader, unpublished observation) secretory responsiveness runs down after permeabilization as the cells leak several soluble proteins essential for calcium-evoked exocytosis. Secretion can be fully restored by reintroducing the leaked cytosolic proteins into permeabilized cells (Sarafian et al., 1987). Fig. 1 shows the electrophoretic profile of cytosolic proteins, membrane proteins, and proteins leaked from digitonin-permeabilized chromaffin cells within 30 min. Calpactin I was identified on nitrocellulose sheets by cross reaction with a specific anti-calpactin heavy chain (p36) antibody. Calpactin I was detected in the cytosolic and membrane fractions and also among the proteins leaked from permeabilized chromaffin cells, suggesting that calpactin I may be one of the essential proteins for exocytosis responsible for the loss of secretory activity in permeabilized cells.
control experiments, neither boiled p36 nor boiled p90 proteins had any effects on catecholamine release.

Times more effective than p36 (Table I), the calpactin tetramer (p90) is probably necessary to maintain the secretory capacity after prolonged permeabilization. Fig. 5 shows that the presence of 20 μM free calcium during the incubation period between permeabilization and stimulation significantly reduced the loss of secretory activity, indicating that some proteins involved in the exocytotic machinery may be retained within the cells in the presence of calcium. To test whether calpactin is one of these proteins, we examined the effect of reintroducing purified calpactin (p90) into SLO-permeabilized cells when calcium was present during the intermediate incubation period. We observed that calcium decreased the stimulatory effect of calpactin I on secretion by 73 ± 9% (± SEM; n = 6), a result which suggests that calcium may be able to reduce the leakage of endogenous calpactin (data not shown).

Effect of Mg-ATP on the Stimulatory Activity of Calpactin I

As secretion in chromaffin cells is triggered by both calcium and Mg-ATP (Bader et al., 1986b), we examined the effect of Mg-ATP on the calpactin-dependent reconstitution of secretion in permeabilized chromaffin cells. Cells were permeabilized with either digitonin or SLO, then incubated with calpactin I (p90) in the presence or absence of Mg-ATP and subsequently stimulated. Table II shows that calpactin I was unable to maintain the secretory activity after permeabilization in the absence of Mg-ATP. In other words, exogenous calpactin I requires the presence of Mg-ATP to restore secretion, an observation that suggests that a phosphorylating step may regulate the activity of this protein.

Effect of Protein Kinase C Modulators on the Stimulatory Activity of Calpactin I

Since calpactin I is known to be a substrate for protein kinase C (Gould et al., 1986) and tyrosine kinase (Glenney et al., 1986), we examined the effect of reintroducing purified calpactin heterotetramer p90 which was previously boiled for 10 min. (Gerke and Weber, 1985; Glenney, 1986). Thus, calcium could reduce the leakage of these proteins from permeabilized cells. Fig. 5 shows that the presence of 20 μM free calcium during the incubation period between permeabilization and stimulation significantly reduced the loss of secretory activity, indicating that some proteins involved in the exocytotic machinery may be retained within the cells in the presence of calcium. To test whether calpactin is one of these proteins, we examined the effect of reintroducing purified calpactin (p90) into SLO-permeabilized cells when calcium was present during the intermediate incubation period. We observed that calcium decreased the stimulatory effect of calpactin I on secretion by 73 ± 9% (± SEM; n = 6), a result which suggests that calcium may be able to reduce the leakage of endogenous calpactin (data not shown).

Calcium Regulation

The presence of calcium in the incubation medium may permit a translocation of several cytosolic proteins from a soluble to a membrane-bound compartment, including calpactin which is a calcium-dependent phospholipid-binding protein (Gerke and Weber, 1985; Glenney, 1986). Thus, calcium could reduce the leakage of these proteins from permeabilized cells. Fig. 5 shows that the presence of 20 μM free calcium during the incubation period between permeabilization and stimulation significantly reduced the loss of secretory activity, indicating that some proteins involved in the exocytotic machinery may be retained within the cells in the presence of calcium. In control experiments, neither boiled p36 nor boiled p90 proteins had any effects on catecholamine release.
**Figure 5.** Effect of calcium on the loss of secretory activity observed in SLO-permeabilized cells. SLO-permeabilized chromaffin cells were incubated for the indicated times in permeabilizing medium depleted in Mg-ATP in the presence (solid squares) or absence (open squares) of 20 μM free calcium. Cells were subsequently stimulated for 15 min with permeabilizing medium containing 5 mM Mg-ATP and 20 μM free calcium. The presence of calcium reduced the loss of secretory activity observed when there is a delay between permeabilization and stimulation.

Terbush and Holz (1986) have reported that permeabilization of chromaffin cells with digitonin causes the leakage of protein kinase C. Accordingly, the protein kinase C activator TPA was unable to potentiate the calcium-evoked secretion when the incubation period before stimulation exceeded 40 min (Fig. 6, open triangles). As shown in Table III, permeabilization produced a progressive leakage of soluble protein kinase C activity as soon as the plasma membrane became permeable; in contrast membrane-bound protein kinase C was more resistant, being retained in the cells during the first 40 min. Preincubation of the cells with TPA before permeabilization decreased the rate of release of cellular protein kinase C by inducing a shift of the enzyme from a soluble to a membrane-bound form (Table III). The remaining soluble form was still rapidly released, but the membrane-bound protein kinase C was retained; by 80 min the amount of particulate enzyme was still 75% of the initial membrane-bound activity (Table III). Under these conditions, the addition of TPA during the stimulation period potentiated the secretory response irrespective of the incubation period introduced between permeabilization and stimulation (Fig. 6, solid triangles), an observation that is consistent with the retention of membrane-bound protein kinase C. Interestingly, the ability of calpactin I to restore secretion was also prolonged (>60 min) when the cells were preincubated with TPA before permeabilization (Fig. 6, solid squares), suggesting that the retention of protein kinase C was linked with the effect of calpactin I on the secretory process.

To strengthen the hypothesis that stimulation of secretion by calpactin I may implicate protein kinase C, we examined the effect of calpactin on SLO-permeabilized chromaffin cells preincubated with sphingosine in order to block protein kinase C activity (Hannun et al., 1987; Merrill et al., 1986; Wilson et al., 1986). As illustrated in Fig. 7A, treatment of permeabilized cells for 10 min with 100 μM sphingosine completely inhibited the reconstitution of secretion induced by calpactin. In many cell types including chromaffin cells, chronic exposure to active phorbolesters results in down-regulation of protein kinase C with a concomitant loss of enzymatic activity (Hii et al., 1987; Hepler et al., 1988; Bader et al., 1989; Simon et al., 1989). We also examined the effect of calpactin I (p90) on calcium-evoked secretion from chromaffin cells treated for 24 h with 1 μM TPA before permeabilization. The presence of TPA and/or calpactin during the

**Table II. Effect of Mg-ATP on the Calpactin I-dependent Reestablishment of Secretion in Digitonin- and SLO-permeabilized Chromaffin Cells**

| Permeabilization | Incubation condition between permeabilization and stimulation | Net [³H]noradrenaline release (±SEM) |
|------------------|-------------------------------------------------------------|-------------------------------------|
|                  | −Calpactin + Mg-ATP | −Calpactin − Mg-ATP | +Calpactin + Mg-ATP | +Calpactin − Mg-ATP | +Calpactin − Mg-ATP | +Calpactin + Mg-ATP | +Calpactin + Mg-ATP |
| Digitonin        | 12.8 ± 1.0          | 6.5 ± 0.5             | 7.1 ± 0.2             | 7.0 ± 0.2             | 11.8 ± 0.6           |
| SLO              | 37.7 ± 0.7          | 14.8 ± 1.1            | 15.6 ± 0.4            | 16.0 ± 0.8            | 26.0 ± 0.4           |

Chromaffin cells were permeabilized with 20 μM digitonin for 10 min or 18 U/ml SLO for 5 min in calcium-free permeabilizing medium in the presence or absence of 5 mM Mg-ATP. Permeabilized cells were then either immediately stimulated with 5 mM Mg-ATP and 20 μM free calcium (Control) or incubated for 15 (Digitonin) or 30 min (SLO) in calcium-free permeabilizing medium in the presence or absence of Mg-ATP and/or calpactin I (p90). Cells were subsequently stimulated for 10 min with permeabilizing medium containing 5 mM Mg-ATP and 20 μM free calcium. Calpactin I requires the presence of Mg-ATP to reconstitute the calcium-dependent secretory process.
30-min incubation period between permeabilization and stimulation increased the amount of secretion in control cells but neither TPA nor calpactin was able to restore the secretory response of cells preincubated for 24 h with TPA (Fig. 7B). In other words, calpactin I has no effect on secretion when protein kinase C is inhibited by sphingosine or down-regulated by long-term incubation with TPA, suggesting that calpactin I may restore the secretory activity in permeabilized cells by a mechanism involving protein kinase C.

**Effect of Phosphorylated Calpactin I on Secretion**

Since calpactin I was unable to exert its restoration effect on secretion in chromaffin cells having their protein kinase C inhibited (Fig. 7), we examined whether prephosphorylation with protein kinase C could restore the effectiveness of exogenous calpactin. Therefore, calpactin I (p90) was prephosphorylated by incubation for 30 min with purified protein kinase C in the presence or absence of phosphatidylserine, TPA and calcium, and then analyzed by gel electrophoresis and autoradiography (Fig. 8). As shown, incorporation of Pi into the 36-kD subunit of calpactin I was totally dependent on the presence of protein kinase C, being maximal when calcium, TPA and phosphatidylserine were present but calcium was not absolutely required (Fig. 8). The effect of protein kinase C-dependent phosphorylation on the ability of calpactin I to maintain the secretory activity of permeabilized chromaffin cells is illustrated in Fig. 9. In control cells, the stimulatory effect of phosphorylated calpactin on calcium-evoked exocytosis was not significantly different from that observed with the nonphosphorylated protein. In agreement with the preceding experiment, calpactin I was unable to enhance the secretory activity in cells having their protein kinase C downregulated by 24-h incubation with TPA. How-

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**Table III. Effect of TPA on the Release of Protein Kinase C from SLO-permeabilized Chromaffin Cells**

| Incubation period (min) | Protein kinase C activity | Soluble | Membrane bound |
|-------------------------|---------------------------|---------|----------------|
|                         | -TPA                      | +TPA    | -TPA           | +TPA           |
|                         | pmol/min/mg protein      |         |                |                |
| 0                       | 280.2                     | 114.6   | 194.7          | 779.1          |
| 30                      | 85.9                      | 51.6    | 169.3          | 894.2          |
| 40                      | 72.9                      | 16.3    | 200.2          | 837.7          |
| 80                      | 36.8                      | 8.1     | 46.4           | 594.1          |

Chromaffin cells were preincubated for 10 min in calcium-free Locke's solution in the presence or absence of 200 nM TPA, permeabilized with SLO for 5 min and incubated for the indicated times in calcium-free permeabilizing medium. Cells were then immediately scrapped in ice-cold homogenizing buffer (see Materials and Methods), and the suspension was sonicated and centrifuged to separate membranes from soluble fractions. The results are from one typical experiment with three wells per group. Similar results were obtained on three different cell preparations.
Figure 7. Effects of sphingosine and long-term treatment with TPA on the calpactin I-dependent restoration of secretion in SLO-permeabilized chromaffin cells. (A) Chromaffin cells were permeabilized for 5 min with SLO. Cells were then either immediately stimulated for 15 min with 20 μM free calcium (Control), or incubated for 10 min with permeabilizing medium in the presence (+Sphingosine) or absence (30 min period) of 100 μM sphingosine and for a further 20 min with permeabilizing medium, or incubated for 10 min with permeabilizing medium in the presence (+p90 + Sphing.) or absence (+p90) or sphingosine and then for 20 min with 20 μg/well calpactin I. Cells were subsequently stimulated for 15 min with permeabilizing medium containing 20 μM free calcium. (B) Cultured chromaffin cells labeled with [3H]noradrenaline were maintained for 24 h in the presence (24h TPA) or absence (−) of 1 μM TPA. Cells were then permeabilized with SLO, immediately stimulated with 20 μM free calcium (Control) or first incubated for 30 min in calcium-free permeabilizing medium (30 min period) or in calcium-free medium containing 20 μg/well calpactin I (+p90) or 200 nM TPA (+TPA) or 20 μg/well calpactin I and 200 nM TPA (+TPA + p90). Cells were subsequently stimulated with permeabilizing medium containing 20 μM free calcium. Sphingosine, a potent inhibitor of protein kinase C, and a long-term incubation with TPA, a treatment known to cause a progressive decline of protein kinase C activity, reverse the stimulating effect that TPA and calpactin I are able to exert on the calcium-dependent secretory response.

Effect of TPA on the Intracellular Distribution of Calpactin I

Although the major serine phosphorylation site for protein kinase C has been identified (Gould et al., 1986), the effect of this phosphorylation on the function of calpactin is as yet undetermined. To determine the possible relationship between calpactin and protein kinase C, we have examined the effect of TPA on the intracellular distribution of calpactin. Cells were pretreated with TPA and then permeabilized for 30 min with digitonin. The presence of calpactin among the membrane and cytosolic proteins, and among the soluble proteins diffusing from the cells, was analyzed and quantified by immunochromatographic detection on nitrocellulose blots, using a specific anti-p36 antiserum. As illustrated in Fig. 10, pretreatment of the cells with TPA reduced the leakage of cytosolic calpactin provoked by the permeabilization of the plasma membrane with digitonin by 52%. Moreover, this TPA-induced retention of calpactin was almost entirely due to an increase (62%) in membrane-bound calpactin that became associated with the cells. The amount of soluble calpactin remaining in the cells after a 30-min digitonin treatment was the same for cells maintained in the presence or absence of TPA. Thus, it is possible that the activation of protein kinase C with TPA results in the phosphorylation of calpactin, thereby promoting the binding of calpactin to intracellular membranes.

Discussion

In this report, we have shown that calpactin I partially reestablishes secretion in digitonin- or SLO-permeabilized
Figure 9. Effect of calpactin I prephosphorylated by protein kinase C on secretion in chromaffin cells having their protein kinase C downregulated by 24-h incubation with TPA. Cultured chromaffin cells labeled with [3H]noradrenaline were maintained for 24 h in the presence (TPA24h) or absence (Control) or 1 μM TPA. Cells were subsequently permeabilized with SLO and incubated for 30 min in calcium-free permeabilizing medium (30 min) or in calcium-free permeabilizing medium containing 200 nM TPA (30 min + TPA) or 8 μg/well calpactin I (30 min + p90) or 8 μg/well calpactin I prephosphorylated with protein kinase C (30 min + p90-P). Cells were subsequently stimulated with 20 μM free calcium. Protein kinase C downregulation abolished the stimulatory effect of TPA and calpactin I on exocytosis but did not affect the reconstitution of secretion obtained by reintroducing prephosphorylated calpactin I.

PKC
- + + 0 + + + + + +
p90
- - + + + + + + + +
PS
- + - + - - + + + +
Calcium
- + - + + - + + + +
TPA
- + - + + + + + + +

Figure 8. Autoradiograph of the one-dimensional SDS-polyacrylamide gel of calpactin I after in vitro phosphorylation by protein kinase C. Calpactin I (2 μg) was incubated for 30 min with purified protein kinase C (0.2 μg) in phosphorylating medium containing [γ-32P]ATP and as indicated 1.75 mM CaCl2, 16 μg/ml phosphatidylserine (PS) and 160 nM TPA. Control experiments were performed with calpactin I alone (C and D) or protein kinase C alone (A and B). The reaction was terminated by solubilizing proteins in electrophoretic sample buffer. The numbers indicate the mobilities of molecular mass standards in kilodaltons (kD). PKC, protein kinase C; p36, phosphorylated 36-kD subunit of calpactin I.

The phosphorylation of calpactin I was totally dependent on the presence of phosphatidylserine and TPA but calcium was not absolutely required.
proteins (Funakoshi et al., 1987; Sudhof et al., 1988) reveals properties of the light-chain known to enhance the binding levels. These observations may be related to the regulatory tetramer (p90) aggregated granules at micromolar calcium and Creutz (1988) reported also that subunit association is in vitro aggregation and fusion of chromaffingranules (Drust et al., 1988) and the calcium-dependent phospholipid-binding protein that promotes in vitro aggregation and fusion of chromaffin granules (Drust and Creutz, 1988; Nakata et al., 1990). Interestingly, Drust and Creutz (1988) reported also that subunit association is critical since p11 or p36 subunits were ineffective in promoting chromaffin granule aggregation whereas the calpain tetramer (p90) aggregated granules at micromolar calcium levels. These observations may be related to the regulatory properties of the light-chain known to enhance the binding of calpain I to phospholipids (Powell and Glenney, 1987).

Calpain I belongs to a family of calcium-binding proteins, the annexins (Geisow et al., 1987). A comparison of the amino acid sequences now known for several of these proteins (Funakoshi et al., 1987; Sudhof et al., 1988) reveals that annexins are similar in their structure in that they possess a conserved core region consisting of four or eight repeats of a 70-amino acid segment sharing 40–60% homology between family members. It has been proposed that the 70–amino acid segment represents the putative calcium/phospholipid binding domain in each protein underlying the ability of these proteins to aggregate phospholipid vesicles and secretary granule membranes (Geisow et al., 1987). This led us to examine whether other members of the annexin family may reconstitute secretion in permeabilized chromaffin cells. We found that the stimulatory effect on calcium-dependent exocytosis was specific to the calpain I tetramer (p90) and cannot be elicited by the p36 subunit alone or by other annexin such as endonexin I (p32), calpain II (p57) or calelectrin (p67). One explanation for this lack of effect may be that structurally the p36 heavy chain of calpain I, although similar to other annexins in its conserved core, differs because it possesses a unique NH\textsubscript{2}-terminal tail which contains the binding site for the p11 light chain. The calpain I tetramer (p90) is also the only member of the annexin family that can aggregate chromaffin granules at physiologically relevant calcium concentrations (Drust and Creutz, 1988). Taken together these results suggest that the association of p11 to the NH\textsubscript{2}-terminal tail of p36 plays a critical role in the modulation of the calcium sensitivity of the lipid-binding sites contained in the core of p36 and also in the ability of calpain I to restore the exocytotic process.

In a recent study, Drust and Creutz (1991) have reported the presence of five members of the annexin family in the adrenal medulla. They determined their subcellular localization by analyzing fractions from sucrose gradients and found that calpain I was predominantly associated with plasma membrane- and chromaffin granule-containing fractions. Synexin fractionated into both cytoplasmic and plasma membrane fractions, calpain II and endonexin I were almost entirely cytosolic, and calelectrin (p67) was present exclusively in the plasma membrane. These differences in subcellular distribution suggest that annexins may play distinct cellular functions. The finding that calpain I is the sole annexin associated with both chromaffin granules and the plasma membrane provides further support for its role in the exocytotic process.

Recent studies using quick-freeze deep-etch electron microscopy revealed the presence of fine strands cross-linking opposing membranes when liposomes or chromaffin vesicles were mixed with calpain in the presence of calcium (Nakata et al., 1990). Similar cross-linking strands were observed between chromaffin granules and the plasma membrane after stimulation of chromaffin cells with acetylcholine. Moreover immunocytochemistry showed that calpain I is located between the plasma membrane and the facing surface of the chromaffin granule in stimulated chromaffin cells (Nakata et al., 1990). Thus, calpain I may be involved in the fusion process between secretory vesicles and the plasma membrane during exocytosis.

Calpain I has been shown to be a substrate for the Rous sarcoma virus enzyme pp60v-src (Gerke and Weber, 1984; Glenney and Tack, 1985) and for protein kinase C (Khanna et al., 1986; Gould et al. 1986) both in vitro and in vivo. The tyrosine and serine phosphorylation sites are both found within the 25 NH\textsubscript{2}-terminal amino acids of the calpain heavy-chain (p36). This NH\textsubscript{2}-terminal tail region of calpain I also contains the binding site for the calpain I light chain (Glenney et al., 1986; Johnsson et al. 1986) but neither protein kinase C nor pp60v-src modify the light-chain binding to the tail (Powell and Glenney, 1987). Thus, the functional significance of the phosphorylation of calpain I still awaits elucidation. Here, we show that the stimulatory effect of calpain I on secretion is ATP dependent. Moreover, calpain I is unable to restore the secretory activity in cells that have completely released their cytosolic protein kinase C during a 60-min permeabilization period or in cells having their protein kinase C inhibited by sphingosine or downregulated by long-term incubations with TPA. These observations suggest that phosphorylation of calpain by protein kinase C may be required for the activity of calpain in the exocytotic machinery. Additional support for this idea comes from the observation that in contrast to nonphosphorylated calpain, calpain prephosphorylated in vitro by purified protein kinase C was able to reconstitute secretion in cells.

| Phosphorylating medium | Net [H]noradrenaline release | Relative release* |
|------------------------|-----------------------------|------------------|
| KG medium              | 14.4 ± 0.6                  | 14.0 ± 0.5       | 97 |
| +PKC                   | 17.5 ± 0.6                  | 23.0 ± 0.7       | 132 |
| +ATP                   | 14.5 ± 0.9                  | 21.1 ± 0.1       | 146 |
| +PKC +ATP-γ-S          | 15.2 ± 0.9                  | 25.7 ± 1.0       | 169 |

Chromaffin cells were pretreated for 24 h with 1 μM TPA, permeabilized with SLO for 5 min, and then incubated for 30 min with calcium-free permeabilizing medium (KG medium) in the presence or absence of 8 μg/well of non-phosphorylated or phosphorylated calpain I (p90). Calpain I was prephosphorylated as described in Materials and Methods by preincubation with protein kinase C (PKC) in phosphorylating media containing either ATP, or ATP-γ-S, or ATP and the phosphatase inhibitor microcystin (1 μM). For control, phosphorylating media were introduced 30 min into cells in the absence of calpain. Cells were subsequently stimulated with 20 μM free calcium in permeabilizing buffer. Calpain I requires a prephosphorylation step with protein kinase C to enhance the calcium-evoked secretion in chromaffin cells with reduced protein kinase C activity. The presence of a phosphatase inhibitor or the use of thiolphosphorylated calpain I which is resistant to cellular phosphatasessignificantly enhanced the effect of phosphorylated calpain. Data are given as means (± SEM) of nine determinations on three different cell preparations.

* Expressed relative to the response obtained in cells incubated in phosphorylating medium in the absence of calpain.

Table IV. Effect of Phosphorylated Calpain I on Secretion in SLO-permeabilized Chromaffin Cells Pretreated for 24 h with TPA
Figure 10. Effect of TPA on the intracellular distribution of calpactin I. Chromaffin cells (20 x 10^6/dish) were preincubated for 15 min in the presence (+) or absence (-) of 200 nM TPA, permeabilized for 10 min with 20 µM digitonin, and incubated for 30 min in calcium-free permeabilizing medium in the presence or absence of 200 nM TPA. The extracellular fluids containing the released cytosolic proteins were collected, cells were scraped in permeabilizing medium, and soluble and membrane-bound proteins were separated as described in Materials and Methods. Released cytosolic proteins (A), soluble proteins (B), and membrane-bound proteins (C) were subjected to one-dimensional gel electrophoresis (200 µg protein in each lane), and the presence of calpactin I was revealed on immunoblots with anti-p36 antibodies and ¹²⁵I-anti-rabbit immunoglobulins, and subsequent autoradiography. The amount of calpactin I associated with each fraction was quantified by scanning densitometry of the autoradiograph (below). TPA reduced the leakage of calpactin from permeabilized cells by enhancing its binding to intracellular membranes.
having their protein kinase C downregulated by phorbol esters. Interestingly, the presence of microcystin, a phosphatase inhibitor, or the use of ATP-γ-S to thio phosphorylate the protein potentiates the reconstituting ability of exogenous calpactin. Thiophosphorylated proteins are known to be relatively resistant to cellular phosphatases (Eckstein, 1985). Our interpretation of these results is that the involvement of calpactin in the exocytotic machinery requires phosphorylation by protein kinase C.

Calpactin I was previously identified by Creutz et al. (1987) among the chromobindins, a group of soluble proteins of the adrenal medulla that bind to the chromaffin granule membrane in a calcium-dependent way (Creutz et al., 1983). Calpactin was found to undergo phosphorylation predominantly on serine residues during stimulation of chromaffin cells with nicotine (Creutz et al., 1987). Since protein kinase C is activated during nicotine stimulation (Tebusch and Holz, 1986), it becomes a good candidate for mediating the phosphorylation of calpactin during exocytosis. In addition Creutz et al. (1987) observed in several experiments traces of alkali-resistant phosphorylation of calpactin after nicotinic stimulation, suggesting that some phosphorylation on tyrosine residues may also occur. Indeed the presence of a tyrosine-specific protein kinase has been described on both secretory granule membrane and plasma membrane fractions of chromaffin cells (Parsons and Creutz, 1986; Grandori and Hanafusa, 1988) and recent studies support the relevance of tyrosine phosphorylation to secretagogue-induced exocytosis in chromaffin cells (Ely et al., 1990).

Thus calpactin is phosphorylated when chromaffin cells are stimulated and our present report indicates that phosphorylation by protein kinase C is required to permit exogenous calpactin to reestablish secretion in permeabilized cells. We also found that stimulation of protein kinase C with phorbol esters modifies the properties of cellular calpactin. TPA reduced the leakage of calpactin from permeabilized cells by enhancing its association with intracellular membranes. In other words, phosphorylation of calpactin may have a stimulatory effect on the binding to phospholipids or to an unknown target site present in membranes. It should also be noted that TPA-induced phosphorylation of tyrosine residues has been described (Gilmore and Martin, 1983). Whether the binding of calpactin to membranes may be catalyzed by phosphorylation of serine or tyrosine residues remains therefore unclear although in vitro experiments have demonstrated that phosphorylation of calpactin by pp60s-src has an inhibitory rather than a stimulatory effect on phospholipid binding (Powell and Glenney, 1987). Nevertheless, clarification of this point requires detailed phosphopeptide and phosphoamino acid analyses of calpactin in TPA-treated chromaffin cells. An attractive speculation is that protein kinase C and tyrosine-specific kinase(s) may modulate the exocytotic events in chromaffin cells by exerting opposite regulatory effects on the binding of calpactin to membranes.

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