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 pp60v-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 NH_2-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 (Trifaro, 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 calpactin I is present among the cytosolic proteins released by digitonin-permeabilized cells and that the reintroduction of calpactin 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 calpactin 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 × 10^5 cells/well and used within 3–7 d 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 × 10^6 cells/dish.

Streptolysin-O

Streptolysin-O (SLO)[1] (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% saturated ammonium sulphate at 4°C. Each preparation of toxin was assayed for hemolytic activity against 2.5% rabbit erythrocytes in 25 mM phosphate buffer saline (145 mM NaCl, 25 mM potassium-phosphate, pH 7.5). The dilution of toxin hemolyzing 50% of red cells (EC 50) was estimated and the reciprocal of this value was taken as the number of hemolytic units per milliliter of the nondiluted toxin solution. Cultured chromaffin cells (2.5 × 10^5 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 CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 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

1. Abbreviation used in this paper: SLO, streptolysin-O.

media containing various amounts of CaCl$_2$ 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 Sillen 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

Calpactin 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 Glenney et al. (1987) with slight modifications as described by Regnouf et al. (1991). The two forms of calpactin 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 (calpactin II, p37) was purified from bovine adrenal medulla as described by Regnouf et al. (1991). Calelectrin (p67) 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-Calpactin I Antibodies

Purified brain calpactin I monomer was subjected to preparative SDS gel electrophoresis. The band corresponding to the protein was excised, homogenized, and dialyzed against 0.15 M Na phosphate, 10% methanol, and 0.1% Triton X-100 for 48 h, then against 0.15 M Na phosphate, 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 (first injection) or incomplete (second injection) 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 antiserum does not cross react with other members of the annexin family such as p37 (calpactin II), p67, and p32.

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

Chromaffin cells, plated at a density of 20 × 10^6 cells in 50 mm-diam culture dishes, were permeabilized for 10 min with 4 ml 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 scrapped in ice-cold permeabilizing medium and sonicated twice for 5 s at setting 9 on Ultrosonic 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 mM PMSF and lyophilized.

Measurement of Protein Kinase C Activity

Chromaffin cells in culture (2.5 × 10^6 cells) were scrapped 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 aprotinin, 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 resuspended in 0.3 ml of homogenizing solution containing 0.1% Triton X-100 detergent. A 40-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 CaCl$_2$, 16 μg/ml phosphatidylserine, and 100 μM diacylglycerol to reduce the effect of Triton X-100 on protein kinase

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 [γ-^{32}P]ATP to histone H1 as substrate. The protein kinase C activity was determined in the presence of calcium plus phosphatidylserine. Nonspecific 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 Peuch 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 μg) 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-γ-S or [γ-^{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 1:500 or with horseradish peroxidase–labeled anti–rabbit immunoglobulins at a 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 Ultroscan (Pharmacia, Saint-Quentin-En-Yuelines, France) laser scan densitometer 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
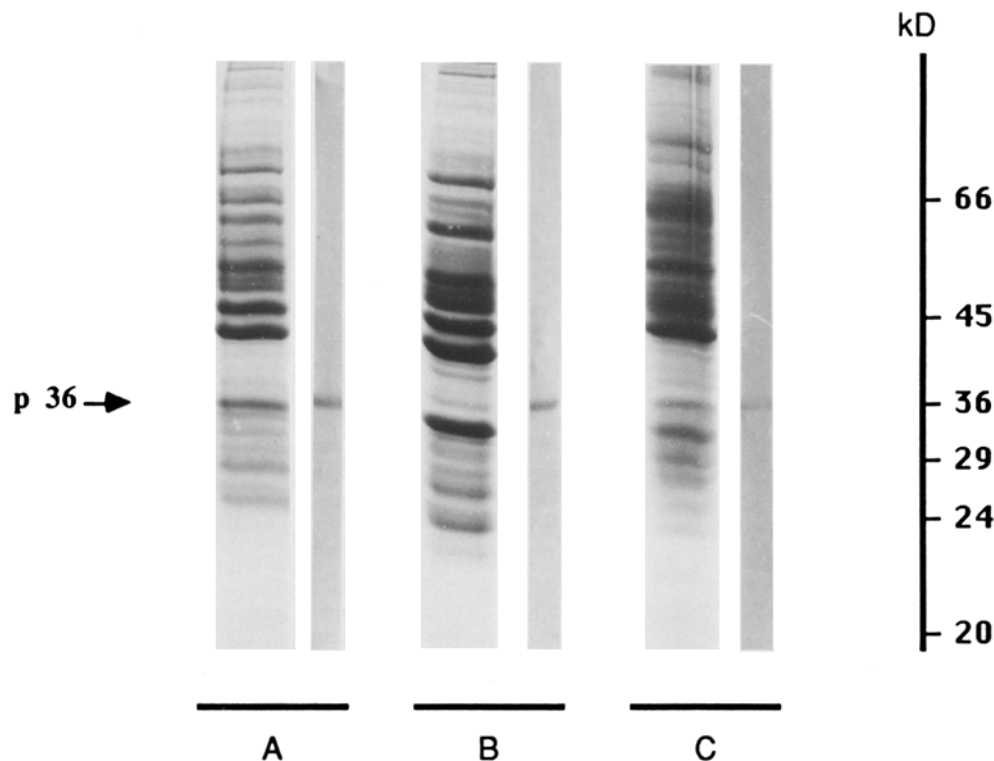
We have observed that in chromaffin cells permeabilized

Figure 1. Immunodetection of calpactin I among the cytosolic proteins released from digitonin-permeabilized cells. Chromaffin cells (20 × 10^6/dish) were permeabilized for 10 min with 20 μM digitonin and then incubated for 30 min in calcium-free permeabilizing medium. The extracellular fluid containing the released cytosolic proteins was collected, cells were scraped in permeabilizing medium, and the 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 separated on monodimensional (10% acrylamide) gel electrophoresis (200 μg protein in each lane). Proteins were transferred to nitrocellulose sheets and the blots were subsequently incubated with anti–p36 antiserum at a 1:500 dilution. Calpactin I (*arrow*) was detected in the soluble and membrane-bound fractions and among the cytosolic proteins released from permeabilized cells.
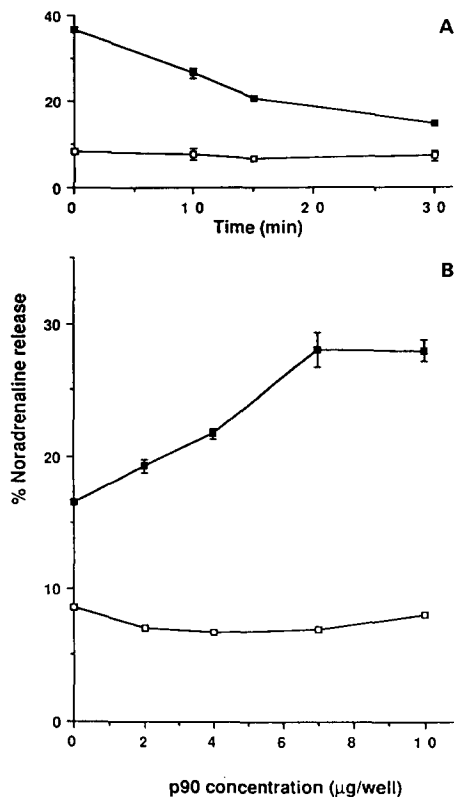
Figure 2. Reconstitution of secretion by calpactin I in SLO-permeabilized chromaffin cells. (*A*) Chromaffin cells (2.5 × 10^5/well) were permeabilized for 5 min with 200 μl of calcium-free permeabilizing medium containing 18 U/ml SLO and then incubated for the indicated times in 200 μl calcium-free permeabilizing medium. Cells were subsequently stimulated for 15 min in the presence (*solid squares*) or absence (*open squares*) of 20 μM free calcium. [^{3}H]Noradrenaline release is expressed as the percentage of the total radioactivity present in the cells before stimulation. 30 min of incubation between permeabilization and calcium-induced stimulation produced a 74% inhibition of the secretory response from SLO-treated cells. (*B*) Chromaffin cells permeabilized with SLO were incubated for 30 min in calcium-free permeabilizing medium containing the indicated concentrations of purified calpactin I (p90). Cells were subsequently stimulated for 15 min in the presence (*solid squares*) or absence (*open squares*) of 20 μM free calcium. The addition of calpactin I promoted a dose-dependent restoration of the calcium-dependent secretory activity.

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.

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 Fig. 2 *A*, 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 in a dose-dependent manner this secretory run down observed in SLO-permeabilized cells (Fig. 2 *B*). 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).
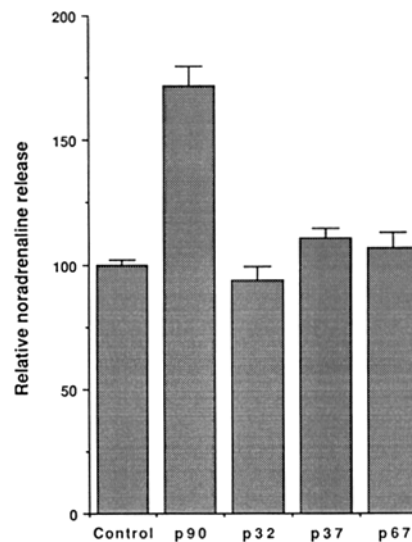

Figure 3. Comparison of the effect on secretion of calpactin I (p90) with that of other proteins from the annexin family. Chromaffin cells were permeabilized with SLO and then incubated for 30 min in calcium-free permeabilizing medium (*Control*) or in calcium-free permeabilizing medium containing 8 μg/well of either calpactin I (p90), calpactin II (*p37*), endonexin I (*p32*), or calelectrin (*p67*). Cells were then stimulated for 15 min with 20 μM free calcium. [^{3}H]Noradrenaline release during the stimulation period was calculated as the percentage of the total radioactivity present in the cells before stimulation. Results are expressed relative to the response obtained in control cells (13.5 ± 0.3% of [^{3}H]noradrenaline cell content). The basal calcium-independent release (7.6 ± 0.4%) was not significantly modified by the different protein tested and was substracted. Calpactin I is the sole annexin able to stimulate calcium-evoked secretion in SLO-permeabilized chromaffin cells.
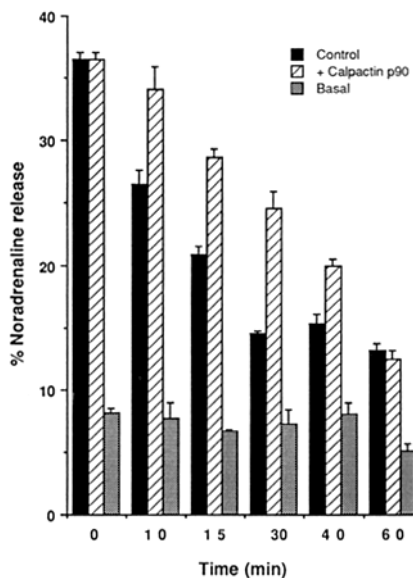
Figure 4. Effect of calpactin I on the capacity of chromaffin cells to release catecholamines after various incubation periods between permeabilization and calcium-induced stimulation. Chromaffin cells were permeabilized for 5 min with 18 U/ml SLO and then incubated for the indicated times in 200 μl calcium-free permeabilizing medium in the presence (*+calpactin p90*) or absence (*control*) of 8 μg/well calpactin I. Cells were subsequently stimulated for 15 min with permeabilizing medium containing 20 μM free calcium. [³H]Noradrenaline release is expressed as the percentage of the total radioactivity present in the cells before stimulation. Basal release, determined by incubating cells for 15 min in calcium-free permeabilizing medium, was not significantly modified by the presence of calpactin I over the range of intermediate incubation periods tested.

Next we examined the ability of calpactin I to restore secretion in SLO-treated cells at various times after permeabilization. Fig. 4 shows an experiment in which cells were permeabilized with SLO for 5 min, incubated for various times in the presence or absence of 8 μg per well of calpactin I (p90), and then stimulated with calcium. Calpactin I enhanced catecholamine release during the first 40 min of incubation but was ineffective when an incubation period >60 min was introduced between permeabilization and stimulation. In addition, it can be seen that calpactin was unable to restore secretion to the level obtained in control cells stimulated immediately after permeabilization when the delay before stimulation exceeded 10 min. These results suggest that calpactin I is probably not the sole protein responsible for the loss of secretory capacity after prolonged permeabilization.

Calpactin I may be found as a monomer (p36) and also as a tetramer (p90) consisting of two heavy chains of relative molecular mass 36K (p36) and two light chains of 11K (p11) (Gerke and Weber, 1984; Glenney et al., 1986). We compared the effect of the various molecular forms of calpactin on secretion from permeabilized cells. Since p90 was four times more effective than p36 (Table I), the calpactin tetramer (p90) is probably necessary to maintain the secretory responsiveness in SLO-permeabilized chromaffin cells. In control experiments, neither boiled p36 nor boiled p90 proteins had any effects on catecholamine release.

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. 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.,

Table I. Effect of Calpactin I Heavy Chain (p36) vs. Calpactin I Tetramer (p90) on the Secretory Activity of SLO-permeabilized Chromaffin Cells

Addition	Net [³H]noradrenaline release	Relative release*
	%	
None	15.3 ± 1.1	100
+ p36	18.1 ± 1.0	118
+ p90	26.7 ± 0.4	174
+ boiled p90	14.3 ± 0.1	93

Chromaffin cells were first permeabilized with SLO in calcium-free permeabilizing medium. Cells were then either incubated for 30 min in 200 μl of calcium-free permeabilizing medium, or in calcium-free permeabilizing medium supplemented with 8 μg/well of the calpactin heavy chain p36 or with 8 μg/well of the calpactin tetramer p90 and then stimulated for 15 min with 20 μM free calcium. For control, cells were incubated for 30 min with 20 μg/well of calpactin heterotetramer p90 which was previously boiled for 10 min.
* Expressed relative to the control response obtained in cells incubated for 30 min in permeabilizing medium in the absence of calpactin.
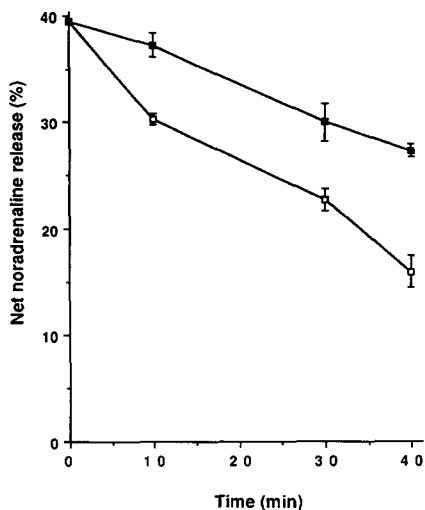
Figure 5. Effect of calcium on the loss of secretory activity observed in SLO-permeabilized cells. SLO-permeabilized chromaffin cells were incubated for the indicated times in permeabilizing medium depleted in Mg-ATP in the presence (solid squares) or absence (open squares) of 20 μM free calcium. Cells 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.

1986), we addressed the question of whether protein kinase C was involved in the calpactin capacity to restore secretion.

As described in Fig. 4, calpactin enhanced the secretory response in permeabilized cells when the incubation period introduced between permeabilization and stimulation did not exceed 40 min. The maximal effect of calpactin, calculated as the percentage of secretion obtained in cells incubated for the indicated period in the presence of calpactin relative to the response obtained in the absence of calpactin, was observed after a 40-min incubation period between permeabilization and stimulation (Fig. 6, open squares). The lower effect of exogenous calpactin at the beginning of the time course may be attributed to endogenous calpactin still present in the cells. The lack of effect of calpactin after 60 min-incubation may be explained by the leakage of either another protein essential for the exocytotic mechanism or a protein required for the activity of calpactin I itself.

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. 7 A, 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 phorbol esters results in downregulation 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

		Net [³H]noradrenaline release			
		Incubation condition between permeabilization and stimulation			
Permeabilization	Control	−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.
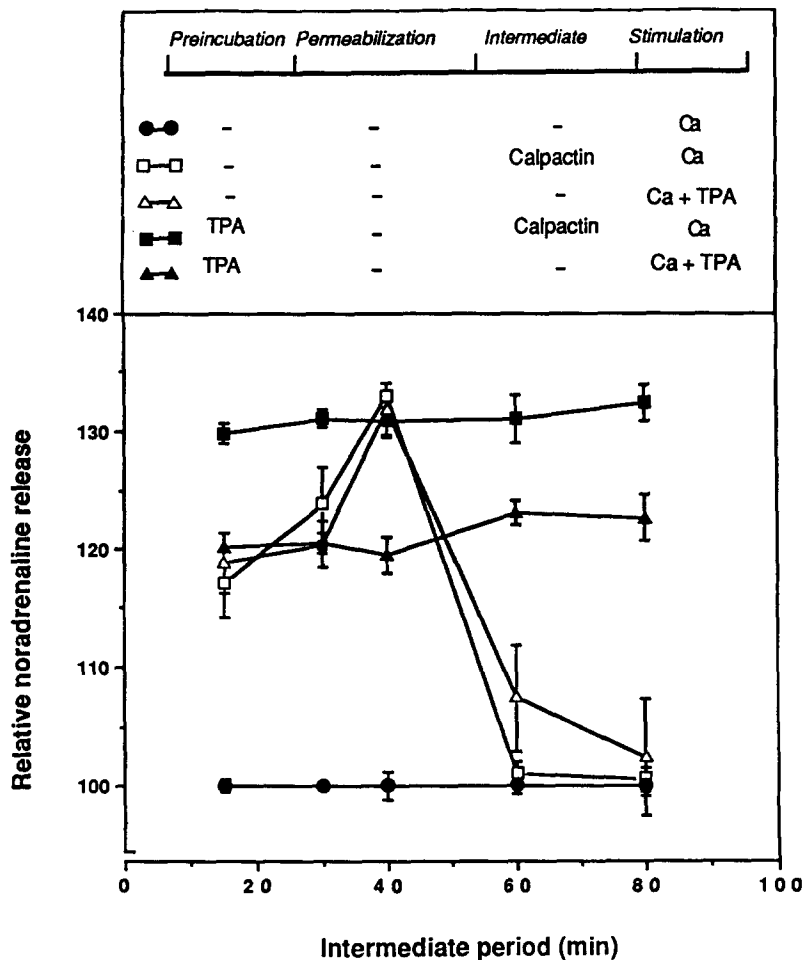
Preincubation	Permeabilization	Intermediate	Stimulation
●—● -	-	-	Ca
□—□ -	-	Calpactin	Ca
△—△ -	-	-	Ca + TPA
■—■ TPA	-	Calpactin	Ca
▲—▲ TPA	-	-	Ca + TPA

Figure 6. Effect of TPA on the calpactin I–dependent reconstitution of secretion in SLO-permeabilized chromaffin cells. Chromaffin cells were preincubated for 15 min in the presence (*solid symbols*) or absence (*open symbols*) of 200 nM TPA. Cells were subsequently permeabilized with SLO and then either incubated for the indicated times in the presence of 20 μg/well calpactin I (p90) and stimulated with 20 μM free calcium (*squares*) or incubated in calcium-free medium and stimulated with 20 μM free calcium and 200 nM TPA (*triangles*). Control cells (*circles*) were permeabilized with SLO, incubated for the indicated times in calcium-free medium, and then stimulated with 20 μM free calcium. For each incubation period, results are expressed relative to the response obtained from control cells. Preincubation of the cells with TPA before permeabilization maintains the stimulating effect of TPA and calpactin I on the calcium-evoked catecholamine release.

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. 7 *B*). In other words, calpactin I has no effect on secretion when protein kinase C is inhibited by sphingosine or down-

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

	Protein kinase C activity			
	Soluble		Membrane bound	
Incubation period	−TPA	+TPA	−TPA	+TPA
min	*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.

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-
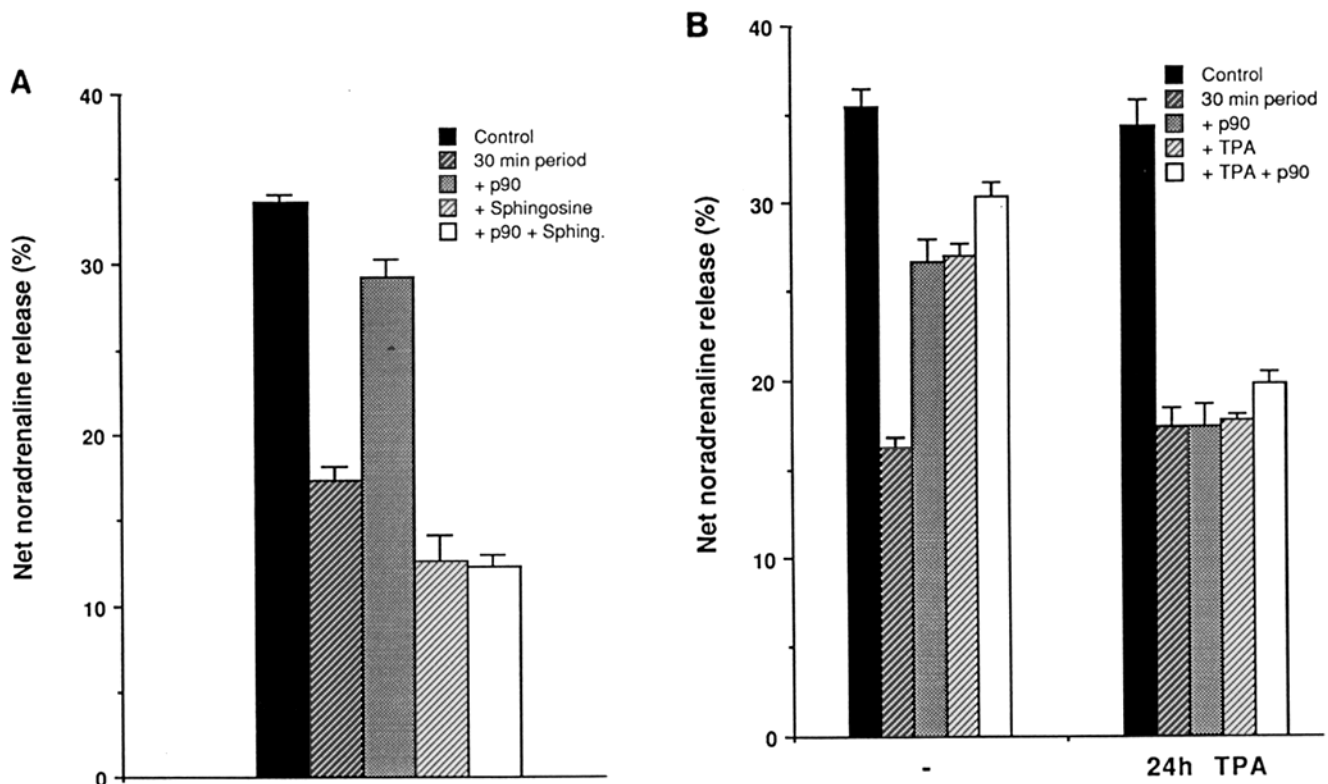
Figure 7. Effects of sphingosine and long-term treatment with TPA on the calpactin I–dependent restoration of secretion in SLO-permeabilized chromaffin cells. (*A*) Chromaffin cells were permeabilized for 5 min with SLO. Cells were then either immediately stimulated for 15 min with 20 μM free calcium (*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.

ever secretion in these cells could be restored with identical efficiency by the introduction of calpactin I pre-phosphorylated by protein kinase C (Fig. 9). These results strongly suggest that the action of calpactin I on exocytosis requires the participation of protein kinase C. Interestingly, the introduction of phosphorylated calpactin I in the presence of a phosphatase inhibitor (microcystin) or the use of calpactin phosphorylated with a non-hydrolyzable ATP analogue (ATP-γ-S), significantly enhanced the effectiveness of phosphorylated calpactin (Table IV), indicating that a dephosphorylation step is probably not involved in the mechanism by which calpactin I stimulates the exocytotic process.

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 immunochemical 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

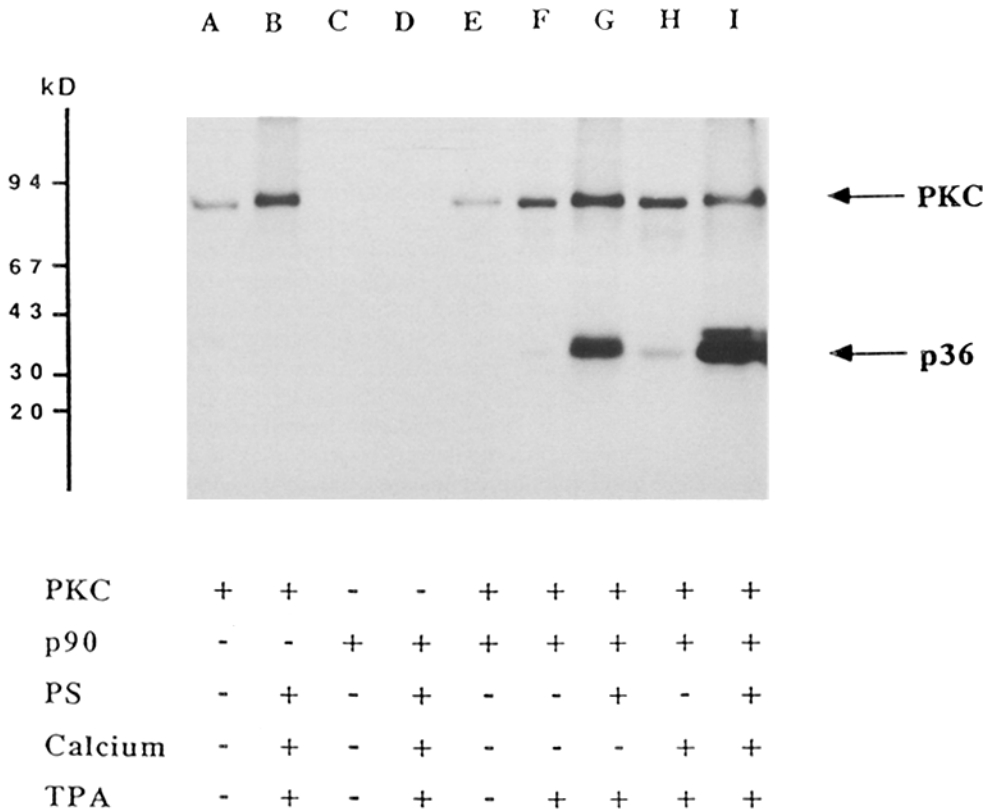|	A	B	C	D	E	F	G	H	I
PKC	+	+	-	-	+	+	+	+	+
p90	-	-	+	+	+	+	+	+	+
PS	-	+	-	+	-	-	+	-	+
Calcium	-	+	-	+	-	-	-	+	+
TPA	-	+	-	+	-	+	+	+	+

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-^{32}P]ATP and as indicated 1.75 mM CaCl$_2$, 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.
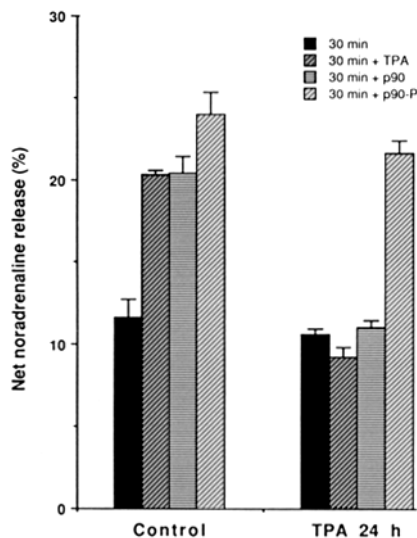

Figure 9. Effect of calpactin I prephosphorylated by protein kinase C on secretion in chromaffin cells having their protein kinase C downregulated by 24-h incubation with TPA. Cultured chromaffin cells labeled with [^{3}H]noradrenaline were maintained for 24 h in the presence (*TPA 24 h*) or absence (*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.

chromaffin cells that have a reduced secretory response as a result of leakage of cytoplasmic proteins. In our hands, digitonin-treated cells release 15% of their cytosolic components within 30 min. Previous work from our laboratory has demonstrated that secretion can be fully restored by reintroducing the leaked proteins at a concentration estimated to be close to that in intact cells, i.e., 500 μg proteins per 2.5 $\times$ 10^5 cells (Sarafian et al., 1987). Calpactin I represented 2% of the cytosolic proteins released by permeabilized cells, as estimated by determining the surface area of the calpactin (p36) peak in a densitometric scan. Based on this calculation, the introduction of 10 μg calpactin per 2.5 $\times$ 10^5 cells should have been sufficient to completely reconstitute secretion if calpactin is the sole essential protein responsible for the loss of secretory activity by permeabilized cells. However, we show here that calpactin even at maximal concentration restores only partially the calcium-evoked catecholamine release. Moreover, calpactin does not reconstitute the secretory activity after prolonged run down, indicating that permeabilized chromaffin cells probably release other cellular proteins that are crucial to the exocytotic mechanism. In contrast, Ali et al. (1989), following our initial experimental procedure (Sarafian et al., 1987), have recently reported that calpactin I is sufficient to completely reconstitute exocytosis in digitonin-permeabilized cells. They indicate also that the association of p36 with p11 is not required for the stimulatory activity of calpactin (Ali and Burgoyne, 1990). Our results differ in that we show here that the dissociation of calpactin I (p90) into its subunits (p36) clearly reduced the effectiveness of exogenous calpactin on calcium-dependent exocytosis in SLO-permeabilized cells. Calpactin I is a cal-

Table IV. Effect of Phosphorylated Calpactin I on Secretion in SLO-permeabilized Chromaffin Cells Pretreated for 24 h with TPA

Phosphorylating medium	Net [³H]noradrenaline release		Relative release* (B/A) × 100
	−calpactin p90 (A)	+calpactin p90 (B)	
	%		
KG medium	14.4 ± 0.6	14.0 ± 0.5	97
+PKC +ATP	17.5 ± 0.6	23.0 ± 0.7	132
+PKC +ATP-γ-S	14.5 ± 0.9	21.1 ± 0.1	146
+PKC +microcystin	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 prephosphorylated calpactin I (p90). Calpactin 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 calpactin. Cells were subsequently stimulated with 20 μM free calcium in permeabilizing buffer. Calpactin I requires a prephosphorylation step with protein kinase C to enchance the calcium-evoked secretion in chromaffin cells with reduced protein kinase C activity. The presence of a phosphatase inhibitor or the use of thiophosphorylated calpactin I which is resistant to cellular phosphatases significantly enhanced the effect of phosphorylated calpactin. 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 calpactin.

cium-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 calpactin 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 calpactin I to phospholipids (Powell and Glenney, 1987).

Calpactin 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 secretory 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 calpactin I tetramer (p90) and cannot be elicited by the p36 subunit alone or by other annexins such as endonexin I (p32), calpactin II (p37) or calelectrin (p67). One explanation for this lack of effect may be that structurally the p36 heavy chain of calpactin I, although similar to other annexins in its conserved core, differs because it possesses a unique NH₂-terminal

tail which contains the binding site for the p11 light chain. The calpactin 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 NH₂-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 calpactin 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 calpactin I was predominantly associated with plasma membrane- and chromaffin granule–containing fractions. Synexin fractionated into both cytoplasmic and plasma membrane fractions, calpactin 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 calpactin 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 calpactin 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 calpactin I is located between the plasma membrane and the facing surface of the chromaffin granule in stimulated chromaffin cells (Nakata et al., 1990). Thus, calpactin I may be involved in the fusion process between secretory vesicles and the plasma membrane during exocytosis.

Calpactin 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 NH₂-terminal amino acids of the calpactin heavy-chain (p36). This NH₂-terminal tail region of calpactin I also contains the binding site for the calpactin 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 calpactin I still awaits elucidation. Here, we show that the stimulatory effect of calpactin I on secretion is ATP dependent. Moreover, calpactin 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 calpactin by protein kinase C may be required for the activity of calpactin in the exocytotic machinery. Additional support for this idea comes from the observation that in contrast to nonphosphorylated calpactin, calpactin prephosphorylated in vitro by purified protein kinase C was able to reconstitute secretion in cells
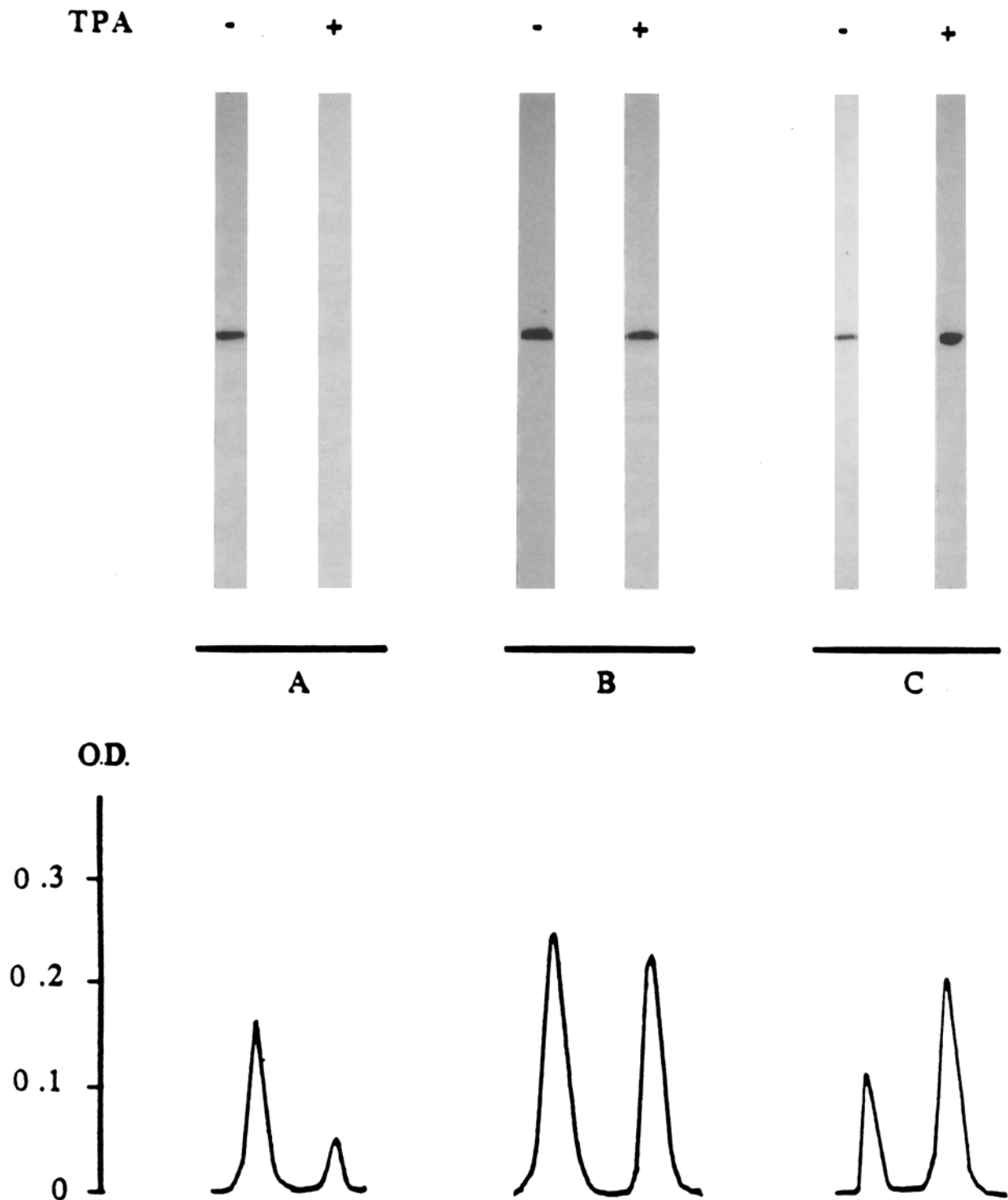
Figure 10. Effect of TPA on the intracellular distribution of calpactin I. Chromaffin cells (20 × 10⁶/dish) were preincubated for 15 min in the presence (+) or absence (−) of 200 nM TPA, permeabilized for 10 min with 20 μM digitonin, and incubated for 30 min in calcium-free permeabilizing medium in the 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 ^{125}I-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 thiophosphorylate the protein potentiate 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 nicotinic stimulation (Terbush 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 pp60v-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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