Ca^{2+} and pH Determine the Interaction of Chromaffin Cell Scinderin with Phosphatidylserine and Phosphatidylinositol 4,5,-Biphosphate and Its Cellular Distribution During Nicotinic-receptor Stimulation and Protein Kinase C Activation

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Abstract. Nicotinic stimulation and high K^+-depolarization of chromaffin cells cause disassembly of cortical filamentous actin networks and redistribution of scinderin, a Ca^{2+}-dependent actin filament-severing protein. These events which are Ca^{2+}-dependent precede exocytosis. Activation of scinderin by Ca^{2+} may cause disassembly of actin filaments leaving cortical areas of low cytoplasmic viscosity which are the sites of exocytosis (Vitale, M. L., A. Rodríguez Del Castillo, L. Tchakarov, and J.-M. Trifaró. 1991. *J. Cell. Biol.* 113:1057–1067). It has been suggested that protein kinase C (PKC) regulates secretion. Therefore, the possibility that PKC activation might modulate scinderin redistribution was investigated. Here we report that PMA, a PKC activator, caused scinderin redistribution, although with a slower onset than that induced by nicotine. PMA effects were independent of either extra or intracellular Ca^{2+} as indicated by measurements of Ca^{2+} transients, and they were likely to be mediated through direct activation of PKC because inhibitors of the enzyme completely blocked the response to PMA. Scinderin was not phosphorylated by the kinase and further experiments using the Na^+/H^+ antiport inhibitors and intracellular pH determinations, demonstrated that PKC-mediated scinderin redistribution was a consequence of an increase in intracellular pH. Moreover, it was shown that scinderin binds to phosphatidylserine and phosphatidylinositol 4,5-biphosphate liposomes in a Ca^{2+}-dependent manner, an effect which was modulated by the pH. The results suggest that under resting conditions, cortical scinderin is bound to plasma membrane phospholipids. The results also show that during nicotinic receptor stimulation both a rise in intracellular Ca^{2+} and pH are observed. The rise in intracellular pH might be the result of the translocation and activation of PKC produced by Ca^{2+} entry. This also would explain why scinderin redistribution induced by nicotine is partially (26–40%) inhibited by inhibitors of either PKC or the Na^+/H^+ antiport. In view of these findings, a model which can explain how scinderin redistribution and activity may be regulated by pH and Ca^{2+} in resting and stimulated conditions is proposed.

It has been proposed that the actin microfilament network localized underneath the plasma membrane of chromaffin cells (Lee and Trifaró, 1981; Trifaró et al., 1984; Cheek and Burgoyne, 1986) acts as a barrier to the movement of secretory granules, blocking their access to exocytosis sites at the plasma membrane (Trifaró et al., 1982, 1984, 1989; Cheek and Burgoyne, 1986, 1987; Burgoyne and Cheek, 1987; Burgoyne et al., 1989; Burgoyne, 1991). Thus, removal of the actin barrier would allow the free movement of granules and their subsequent interaction with the plasma membrane (Lelkes et al., 1986). In this regard, it has been shown that nicotinic receptor stimulation of chromaffin cells induces cortical filamentous actin (F-actin) disassembly (Cheek and Burgoyne, 1986; Trifaró et al., 1989; Vitale et al., 1991; Marxen and Bigalke, 1991). The existence of actin binding proteins, which regulate G-actin/F-actin equilibrium (Yin and Stossel, 1979; Craig and Pollard, 1982; Bader et al., 1986; Maekawa et al., 1989; Rodríguez Del Castillo et al., 1990) suggest a role for these proteins in the reorganization of cortical F-actin networks during secretion. Recently, we have published evidence for the presence of scinderin, a new Ca^{2+}-dependent actin-filament severing protein, in adrenal chromaffin cells (Rodríguez Del Castillo et al., 1990) as well as in other tissues with high secretory activity (Tchakarov et al., 1990; Rodríguez Del Castillo et al., 1992). Immunofluorescence microscopy studies on chromaffin cells revealed that under resting conditions scinderin shows a diffuse cytoplasmic staining and a continuous cortical fluorescent ring, suggesting some interaction of the protein with plasma membrane elements. Nico-

tinic stimulation causes the fragmentation of scinderin cortical fluorescent ring, suggesting the release of the protein from binding sites in some areas of the subplasmalemmal region (Vitale et al., 1991). This redistribution, which occurs along with F-actin disassembly, is a Ca^{2+}-dependent process which precedes exocytosis (Vitale et al., 1991). Moreover, a close relationship between cell stimulation-induced scinderin redistribution and F-actin disassembly is observed because areas devoid of scinderin are also devoid of F-actin. Furthermore, we have also demonstrated that the sites of exocytosis are preferentially localized to these cortical areas (Vitale et al., 1991). These results suggest that stimulation evoked-Ca^{2+} influx induces the association of scinderin with actin filaments and promotes its severing activity. On the other hand, when the stimulus (nicotine) is removed, the recovery of scinderin continuous cortical distribution is faster than cortical F-actin reassembly indicating that when Ca^{2+} concentrations returns to basal levels, cortical scinderin is not associated with cortical F-actin networks. This would suggest that scinderin interacts with other membrane components (Vitale et al., 1991) and that even though Ca^{2+} has a key role in this process, other intracellular messengers may also modulate scinderin distribution and activity.

Several lines of evidence suggest the participation of protein kinase C (PKC[1]; Takai et al., 1979) in catecholamine secretion from bovine chromaffin cells. Activation of PKC increases evoked-catecholamine release in intact (Pocotte et al., 1985; Morita et al., 1985; TerBush et al., 1988; Bittner and Holz, 1990) as well as in permeabilized chromaffin cells (Knight and Baker, 1983; Brocklehurst et al., 1985; Knight et al., 1988; Bittner and Holz, 1990; Tachikawa et al., 1990; TerBush and Holz, 1990; Isosaki et al., 1991). Moreover, nicotinic stimulation induces translocation of PKC from cytoplasm to membranes (TerBush et al., 1988), a process known to be associated with the activation of the enzyme. However, the sites of action of PKC in the secretory process still remain unknown. One possibility is that PKC is involved in the reorganization of the cortical cytoskeleton preceding exocytosis, because PKC interacts with many cytoskeleton and cytoskeleton-associated proteins (Naka et al., 1983; Katoh et al., 1983; Phatak et al., 1988; Georges et al., 1989; Miyamoto and Wu, 1990; Zalewski et al., 1990; Apgar, 1991).

The present paper describes the partial contribution of PKC activation to nicotine-induced scinderin redistribution and the role of intracellular Ca^{2+} and pH in the regulation of scinderin localization during resting and stimulated conditions. The results demonstrated that PMA, a compound known to activate PKC (Castagna et al., 1982), induced cortical scinderin redistribution in a Ca^{2+}-independent manner and with a slower rate of onset than that observed during nicotinic stimulation. PMA-induced scinderin redistribution was completely blocked by three PKC inhibitors. However, the PKC inhibitors only partially (26–40%) inhibited nicotine-induced scinderin redistribution. Scinderin was not phosphorylated by PKC. PMA and nicotine induced a rise in intracellular pH, which was blocked by a Na^+/H^+ antiport inhibitors. Immunofluorescence microscopy studies revealed that amiloride completely blocked PMA-induced but partially (30–40%) blocked nicotine-induced scinderin redistribution. Furthermore, the experiments described here indicate that scinderin might be associated with membrane phospholipids such as phosphatidylserine (PS) and phosphatidylinositol 4,5-biphosphate (PIP_2) in a Ca^{2+}- and pH-dependent manner. Our results indicate that nicotine stimulation and Ca^{2+} entry activates PKC, which in turn activates the Na^+/H^+ antiport with the subsequent rise in intracellular pH. These cellular changes (increases in Ca^{2+} and pH) might modulate scinderin activity and its interaction with its targets (phospholipids and actin).

Materials and Methods

Chromaffin Cell Culture

Bovine adrenal glands were obtained from a local slaughterhouse and chromaffin cells were isolated by collagenase digestion and further purified using a Percoll gradient (Trifaró and Lee, 1980). Cells were used either immediately after isolation for preparation of cytosolic fractions for phospholipid binding studies or plated on collagen-coated glass coverslips contained within plastic Petri dishes (0.3×10^6 cells/35-mm diameter dish for fluorescence microscopy studies or 1.5×10^6 cells/35-mm diameter dish for intracellular Ca^{2+} measurements) or in collagen-coated Petri dishes (3×10^6 cells/35-mm diameter dish) for PKC assay, respectively. Plated chromaffin cells were grown at 37°C in a humidified incubator under CO_2 + air atmosphere for 48 h and cultures were periodically examined under phase contrast optics.

Immunofluorescence Microscopy Studies

Cultured chromaffin cells were rinsed three times with regular Locke's solution (in mM: NaCl, 154; KCl, 2.6; K_2HPO_4, 1.25; KH_2PO_4, 0.50; $MgCl_2$, 1.2; $CaCl_2$, 2.2; and D-glucose, 10.0; pH 7.2), when Ca^{2+}- free Locke's solution was used, Ca^{2+} was removed and 1 mM EGTA was added. Cells were then treated with different drugs as described in the respective experiments, and immediately fixed in 3.7% formaldehyde in Locke's solution for 20 min and processed for immunofluorescence microscopy as previously reported (Lee and Trifaró, 1981; Vitale et al., 1991). Briefly, chromaffin cells were permeabilized by three successive exposures of 5 min each to 50, 100, and 50% acetone. After washing several times with PBS (in mM: NaCl, 130; Na phosphate, 100; pH 7.0) preparations were incubated at 37°C with scinderin antiserum 6 (1:80 dilution) for 60 min. Coverslips were thoroughly rinsed with PBS and were next incubated at 37°C with goat anti-rabbit immunoglobulin FITC-IgG (1:160 dilution) for another 60 min. At the end of this period, preparations were washed with PBS and mounted in PBS: glycerol (1:1, vol/vol). Control experiments were carried out with (*a*) FITC-IgG alone and (*b*) scinderin antiserum previously adsorbed with scinderin. Scinderin antibodies were raised in rabbits against the purified bovine protein (Rodríguez Del Castillo et al., 1990), scinderin antiserum 6 has been previously characterized (Tchakarov et al., 1990; Vitale et al., 1991).

Slides were observed with a Leitz Ortholux fluorescence microscope equipped with a 200-W high pressure lamp (E. Leitz, Inc., Rockleigh, NJ) and an incident light illuminator containing an I-filter block (KP 490 plus 1-mm GG 455 exciting filter, TK dichroic beam splitting mirror, and K 515 suppression filter) (Ploemopack II, Toronto, Ontario). Photographs were taken with Kodak-Tri-X pan films (400 ASA, Eastman Kodak Co., Rochester, NY). One hundred cells per coverslip were examined and classified as having either a "continuous cortical staining" or a "discontinuous cortical staining". To avoid personal bias, code numbers were given to each coverslip to be examined and the procedure was conducted without knowing whether cells were from control or treated preparations; only after the coverslips were examined and the results recorded were the codes revealed to identify the experimental conditions used (single-blind design).

Protein Kinase C Activity

Protein kinase C activity in particulate fractions was assayed as described by TerBush and Holz (1986). Cells were rinsed three times with regular

1. *Abbreviations used in this paper*: BCECF, 2',7'-bis(carboxyethyl)5(6)-carboxyfluorescein; BCECF/AM, 2',7'-bis(carboxyethyl)5(6)-carboxyfluorescein acetoxymethyl ester; PIP_2, phosphatidylinositol 4,5,-biphosphate; PKC, protein kinase C; PS, phosphatidylserine.

Locke's solution and incubated at room temperature with 0.1% DMSO in Locke's solution alone or containing 10^{-7} M 4α-PMA or PMA for 1 or 6 min. After these treatments, cells were immediately scraped off in 900 μl ice-cold buffer A (in mM: potassium glutamate, 139; Pipes, 20 (pH 6.6); EGTA, 5; and leupeptin, 50 μg/ml). Cell suspensions were sonicated for 5 s twice in a sonic dismembrator (model 300; Fisher Scientific, Ottawa, Ontario, Canada). Homogenates were centrifuged at 78,000 *g* for 90 min and sediments were resuspended in 900 μl buffer A, diluted 10-fold with buffer A and enough Triton X-100 was added in order to reach a final concentration of 0.1%. 20 μl of particulate fraction preparations were incubated at 30°C for 10 min with 180 μl of PKC assay buffer (final concentration; potassium glutamate, 42 mM; Pipes, 20 mM (pH 6.6); $MgCl_2$, 10 mM; DTT, 10 mM; BSA, 0.8 mg/ml; leupeptin, 5 μg/ml; mercaptoethanol, 25 μM; Triton X-100, 0.01%; ATP, 30 μM (300,000 cpm of [γ^{32}P]-ATP, sp act, 10 Ci/mmol); histone Type III-S, 50 μg/ml; EGTA 5 mM; Ca^{2+}, 0.8 mM; ± phosphatidylserine, 167 μg/ml; ± 1,2 diolein, 26.7 μg/ml. The reaction was stopped with 1 ml of 5% TCA containing 0.25% Na_2VO_4, 15 mM NaH_2PO_4, and 2 mM ATP and 100 μl of a 6.5 mg BSA/ml solution. Tubes were kept on ice for 20 min and then centrifuged at 10,000 *g* for 10 min. Sediments were dissolved in 0.1 ml 0.5 M NaOH. The precipitation/dissolution step (with 1 ml 5% TCA, 0.25% Na_2WO_4/100 μl 0.5 M NaOH) was repeated three times. Radioactivity was determined in the resuspended sediments by liquid scintillation spectrophotometry (model LS 7800, Beckman Instruments Inc., Fullerton, CA). PKC activity was considered to be the difference between the activity in the presence of Ca^{2+} plus phosphatidylserine-diolein and the activity in the presence of Ca^{2+} and without phosphatidylserine-diolein. PKC activity was expressed as pmol-[^{32}P]/min/mg membrane protein. Under the experimental conditions described, PKC specific activity was linear for samples containing 0.5–2.5 μg protein.

Measurement of Intracellular Ca^{2+}

Intracellular Ca^{2+} concentrations were measured by using the fluorescent Ca^{2+} indicator Fura-2 (Grynkiewicz et al., 1985). It has been described that cell suspensions can be resistant to display changes in intracellular Ca^{2+} concentrations in response to stimulation (Tucker et al., 1990). To avoid this possibility, cells attached to collagen-coated coverslips were used in the present work. Chromaffin cells, grown on glass coverslips at a density of 1.5×10^5 cells/35 mm diameter dish, were rinsed three times with assay buffer (in mM: NaCl, 118; KCl, 4.6; Hepes, 20 [pH 7.20]; $CaCl_2$, 1; D-glucose, 10). Coverslips were next incubated with Fura-2 (10^{-8} M, final dilution) for 40 min at 37°C. After the loading step, cells were rinsed five times with assay buffer and coverslips were inserted into the cuvette loaded with 2 ml assay buffer. The coverslips were immobilized in such a way that the same group of cells were facing the incoming excitation light beam. Fluorescence of the attached cells was monitored using a dual wavelength luminescence spectrophotometer (LS-50; Perkin-Elmer Instrument Division, Norwalk, CT). R_{max} values were obtained from cell preparations lysed with 1% Triton X-100 and R_{min} values by adding enough EGTA to reach 10 mM.

Phosphorylation of Scinderin

PKC-catalyzed phosphorylation of scinderin was performed according to Kawamoto and Hidaka (1984) by evaluating ^{32}P incorporation into scinderin. Bovine adrenal medullary scinderin and PKC were purified as previously described by Rodríguez Del Castillo et al. (1990) and by Uchida and Filburn (1984), respectively. The reaction mixture (final vol: 300 μl) contained 25 mM Pipes (pH 6.6), 10 mM MgCl, 30 μM [^{32}P]ATP (10^6 cpm; sp act: 10 Ci/mmol), 0.8 mM Ca^{2+}, 100 μg scinderin/ml and 1.5 μg PKC/ml in the presence or absence (control) of 60 μg PS/ml and 26.7 μg 1.2 diolein/ml. Histone Type III (50 μg/ml) was used as a control. The reaction mixture was incubated at 35°C for 30 min. At the end of the incubation, the tubes were immersed in ice and 50 μl of a solution containing 8 M urea, 3% SDS, 100 mM DTT, 0.005% bromophenol, 70 mM Tris-HCl (pH 6.7) were added. Samples were run on SDS-PAGE (Doucet and Trifaró, 1988) and proteins were electrotransferred onto nitrocellulose membranes (Towbin et al., 1979). Dried membranes were exposed for autoradiography to Kodak AR(XAR-5) x-ray film for 24 h at −80°C. After development, scinderin was localized in the membranes by immunoblotting using scinderin antiserum 6 (Rodríguez Del Castillo et al., 1990; Tchakarov et al., 1990).

Measurement of Intracellular pH

Intracellular pH (pHi) of cultured chromaffin cells was determined according to Kao et al. (1991) and the calibration curve was obtained using 2′,7′-bis (carboxymethyl)5(6) carboxyfluorescein (BCECF) as previously described by Thomas et al. (1979). Briefly, chromaffin cells, grown on glass coverslips were loaded with 5 μM 2′,7′-bis (carboxymethyl)5(6) carboxyfluorescein acetoxymethyl ester (BCECF/AM) in assay buffer (in mM: NaCl, 137; KCl, 4.4; KH_2PO_4, 1.2; $CaCl_2$, 2.2; $MgCl_2$, 0.7; glucose, 10; Hepes, 5; pH 7.2) at 37°C for 15 min. After this step, cells were washed three times with assay buffer. The coverslips were inserted into the cuvette with cells facing the incoming excitation (500 nm) light beam (spectrofluorometer, Aminco Bowman Inc., Silver Spring, Maryland). At the indicated times nicotine or PMA were added and the fluorescence emission at 530 nm was measured with an aperture slit of 3.0 nm.

Electrophoresis, Immunoblotting and [^{125}I]Protein A Method

Monodimensional SDS-PAGE were performed according to Doucet and Trifaró (1988). The protocol for immunoblotting was as described by Towbin et al. (1979). After electrophoresis, proteins were electrotransferred onto nitrocellulose membranes, previously blocked with 3% BSA in PBS and then incubated for 4 h at room temperature with scinderin antiserum 6 (1:500 dilution), monoclonal (clone GA-2C4) antibody against gelsolin (1:500 dilution) or monoclonal (clone M C 5) antibody against protein kinase C (1:500 dilution). Membranes were next washed and incubated for 1 h at room temperature with goat antirabbit immunoglobulin G-alkaline phosphatase conjugate (1:3,000 dilution) in the case of scinderin antiserum or with goat antimouse immunoglobulin G-alkaline phosphatase conjugate (1:3,000 dilution) in the case of mAbs. Color was developed by treatment with a mixture of *p*-nitroblue tetrazolium chloride and 5-Br-4-Cl-3-indolyl phosphate-toluidine salt. Quantification of scinderin was performed by the [^{125}I]protein A method described by Burnette (1981) as modified by Rodríguez Del Castillo et al. (1990). Nitrocellulose membranes were incubated with scinderin antiserum 6 (1:1,000 dilution) for 60 min followed by an incubation with [^{125}I]protein A (200,000 cpm/ml 1% BSA in PBS; sp act: 30 mCi/mg protein A). A standard curve was obtained with purified scinderin. Dried membranes were exposed to Kodak AR(XAR-5) x-ray film for 18 h at −80°C. Autoradiograms were scanned in a transmission densitometer (model EC910; E-A Apparatus Corp., Philadelphia, PA). The sensitivity of the assay allows the detection of 10 ng of scinderin.

Preparation of Protein Samples and Phospholipid Binding Studies

Cytosol from bovine adrenal chromaffin cells was obtained by homogenizing 150×10^6 freshly isolated chromaffin cells in 2.5 ml 0.3 M sucrose in either buffer B (in mM: Tris, 20; NaCl, 100; $MgCl_2$, 2; dithiothreitol, 1; PMSF, 1; EGTA, 5; pH 7.1 adjusted with HCl) or buffer C (in mM: MES, 10; NaCl, 100; $MgCl_2$, 2; DTT, 1; PMSF, 1; EGTA, 5; pH 6.8 adjusted with NaOH). Homogenates were centrifuged at 100,000 *g* for 1 h, sediments were discarded and supernatants (cytosolic protein fraction) were used. Scinderin was purified from bovine adrenal medullae as previously described (Rodríguez Del Castillo et al., 1990). Cytosolic protein fractions and scinderin preparations were dialyzed overnight at 4°C against buffer B or C. Phosphatidylinositol 4,5-biphosphate (sodium salt, PIP_2) was dissolved in buffer B or C at a concentration of 1 mg/ml. A chloroform/methanol suspension of phosphatidylserine (PS) was evaporated at 0°C under a N_2 atmosphere and resuspended in buffer B or C at a concentration of 1 mg/ml. Phospholipid suspensions were mixed by shaking in a vortex for 15 min and further sonicated for 20 min (Mettler Electronics Corp., Anaheim, CA). Liposomes thus obtained (300 μl) were incubated with 300-μl protein samples (scinderin or cytosolic protein fraction) in a final volume of 700 μl. Free Ca^{2+} concentrations (from 10^{-9} to 10^{-5} M) were adjusted according to Caldwell (1970). The mixtures were incubated at 35°C for 30 min, centrifuged at 78,000 *g* for 30 min and supernatants and sediments were separated. Sediments were dissolved in 700 μl 50 mM Tris-HCl (pH 8.5), 2% SDS, 1 mM EDTA. Experiments on the binding of PKC to phosphatidyl serine liposomes were also performed in order to compare binding characteristic of a known protein to those of scinderin. The presence of scinderin and PKC in supernatants and sediments was analyzed by electrophoresis followed by immunoblotting.

Affinity Chromatography Studies

Actin-DNase I-Sepharose 4B and a polyacrylamide-immobilized phosphatidylserine columns were prepared according to Bader et al. (1986) and Uchida and Filburn (1984), respectively. The phosphatidylserine column

(first column) was connected in series to the actin-DNase I sepharose 4B column (second column) and both were equilibrated with buffer D (in mM: Tris-HCl, 20 (pH 7.1); NaCl, 100; EGTA, 5; DTT, 1; PMSF, 1; the free Ca^{2+} concentration was adjusted to 10^{-5} M). Bovine adrenal medullae were homogenized in 0.3 M sucrose, 20 mM imidazol (pH 6.8); 1 mM EGTA, 1 mM ATP, 1 mM PMSF (1 g of medulla in 4 ml buffer). The homogenate was centrifuged at 1,000 *g* for 10 min and the resulting supernatant was centrifuged at 100,000 *g* for 1 h. To the supernatant thus obtained, enough ammonium sulphate was added to reach 65% saturation. The mixture was stirred for 20 min and then centrifuged at 15,000 *g* for 20 min. The sediment was resuspended in buffer D and dialyzed against the same buffer overnight. The dialyzed material was clarified by centrifugation at 100,000 *g* for 30 min. The supernatant was then loaded onto the system of two consecutive columns. The columns were extensively washed with buffer D. Proteins in each column were separately eluted with buffer D without Ca^{2+}. Eluates were concentrated under pressure and the presence of scinderin, protein kinase C and gelsolin in the samples was investigated by electrophoresis followed by immunoblotting using the corresponding antibodies.

Protein Determination

Protein concentrations were determined by the method of Bradford (1976) using BSA as standard.

Chemicals

PMA, amiloride (hydrochloride), staurosporine, ionomycin, A23187, sphingosine, nicotine (hydrogen tartrate salt), L-α-phosphatidyl-L-serine, L-α-phosphatidylinositol 4,5-biphosphate, 1,2-diolein, FITC-IgG, mAb against gelsolin (clone GS-2C4) and cyanogen bromide–activated Sepharose 4B were purchased from Sigma Chemical Co. (St. Louis, MO). 5-(*N*-methyl-*N*-isobutyl)-amiloride was obtained from Research Biochemicals Inc. (Natick, MA). mAb against PKC (clone M C 5) and [^{125}I]protein A were from Amersham Canada (Oakville, Ontario, Canada). [γ^{32}P]ATP was obtained from New England Nuclear (Boston, MA). Materials for electrophoresis and immunoblotting were purchased from BIO-RAD Laboratories (Mississauga, Ontario, Canada). 4α-PMA and calphostin C were obtained from LC Services Corp. (Woburn, MA). 2',7'-bis(carboxyethyl)-5-(6)-carboxy-fluorescein and 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester were obtained from Calbiochem (La Jolla, CA). Fura-2 acetoxymethyl ester was a gift from Dr. M. Quik (McGill University, Montreal, Canada).

Results

Cortical Scinderin Redistribution Induced by PMA: A Fluorescence Microscopy Study

2-d-old cultured chromaffin cells were incubated with 0.1% DMSO alone for 6 min (control) or in the presence of 10^{-5} M nicotine for 40 s or 10^{-7} M PMA for 1 or 6 min and then processed for scinderin immunofluorescence microscopy (Fig. 1). Control cells showed, as expected, a diffuse cytoplasmic staining and a bright and continuous cortical fluorescent ring (Fig. 1 *a*). As we have previously shown (Vitale et al., 1991), after nicotinic stimulation the cortical fluorescent ring appeared fragmented (Fig. 1, *b* and *c*). Moreover, incubation of chromaffin cells with PMA for 1 or 6 min (Fig. 1, *d* and *e*, respectively) also produced a disruption of the scinderin cortical fluorescent ring.

Time Courses of Nicotine- and PMA-induced Cortical Scinderin Redistribution

Chromaffin cells were incubated for 0, 5, 20, 60, 120, 180, or 360 s with 10^{-5} M nicotine (Fig. 2, □), 10^{-7} M PMA (Fig. 2, ○) or 10^{-7} M 4α-PMA (Fig. 2, ◇). As previously described (Vitale et al., 1991), nicotine induced a sharp increase in the percentage of cells displaying cortical scinderin redistribution (Fig. 2, □). 5 s of exposure to nicotine raised the percentage from 20 ± 3% to 60 ± 3% (total cells examined, n = 600) and maximum scinderin redistribution was observed after 40 s of nicotine treatment. Nicotine exposure for periods longer than 60 s resulted in lower percentages of cells showing a disrupted cortical fluorescent ring; cells incubated with nicotine for 180 s showed a similar percentage of scinderin redistribution than control preparations (0 s incubation) (Fig. 2, □). Furthermore, at least one min incubation with PMA was necessary to induce scinderin redistribution (Fig. 2). The percentage of cells displaying scinderin

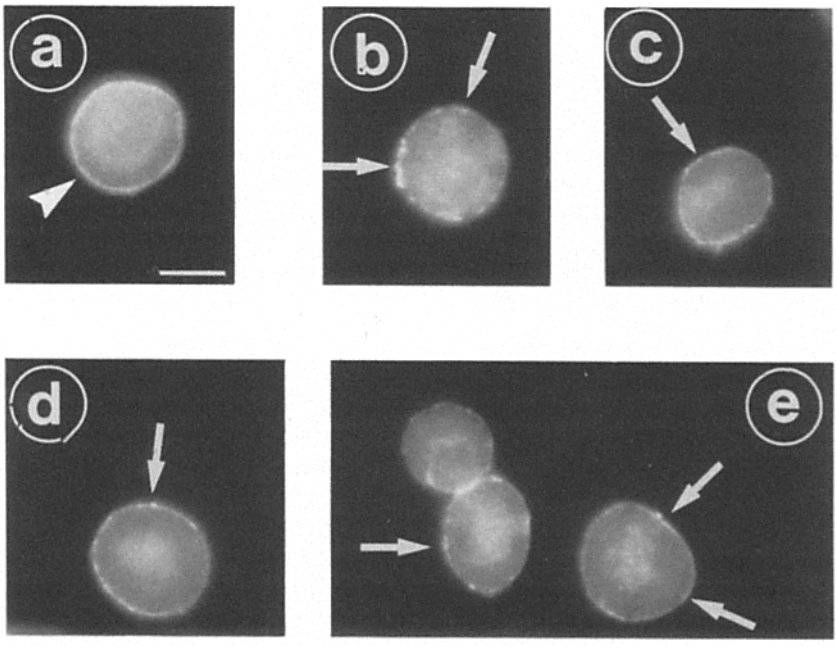

Figure 1. Localization of scinderin by immunofluorescence microscopy. Effects of nicotine and PMA on scinderin cortical distribution. 2-d-old chromaffin cells were incubated with Locke's solution containing 0.1% DMSO for 6 min (control) or with the same buffer containing either 10^{-5} M nicotine (for 40 s) or 10^{-7} M PMA (for 1 or 6 min) and immediately processed for fluorescence microscopy using scinderin antiserum 6. A control cell shows a diffuse cytoplasmic staining and continuous ring of fluorescence (*a*, *arrowhead*). Incubation of chromaffin cells with nicotine for 40 s (*b* and *c*) caused the disruption of the cortical fluorescent ring. PMA treatment for 1 min (*d*) or 6 min (*e*) also caused the disruption of the cortical fluorescent ring. Patches of remaining cortical scinderin immunofluorescence are indicated by arrows. Bar, 10 μm.

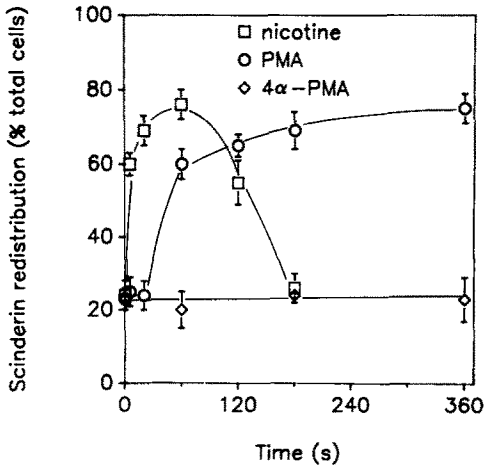

Figure 2. Time course of scinderin redistribution in response to nicotine, PMA and 4α-PMA. Chromaffin cells grown in culture for 48 h were incubated with either 10^{-5} M nicotine (□), 10^{-7} M PMA (○), or 10^{-7} M 4α-PMA (◇) for 0, 5, 20, 60, 120, 180, and 360 s. After these periods of time, cells were stained for immunofluorescence microscopy using scinderin antiserum 6. 100 cultured cells per coverslip were examined and classified as having either a "continuous cortical fluorescent pattern" (see Fig. 1 *a*) or having a "discontinuous cortical fluorescent pattern" (see Fig. 1 *b*). To avoid personal bias, code numbers were given to each coverslip to be examined and the procedure was conducted without knowing whether cells were from control or treated preparations; only after all coverslips were examined and the results recorded were the codes revealed to identify the experimental condition used (single-blind design). The percentage of scinderin redistribution for each condition was then calculated and plotted. Each value shown is the mean ± SEM of the percentage of discontinuous cortical scinderin fluorescence staining of four to six coverslips (400–600 cells for each value), containing cells from two different cultures.

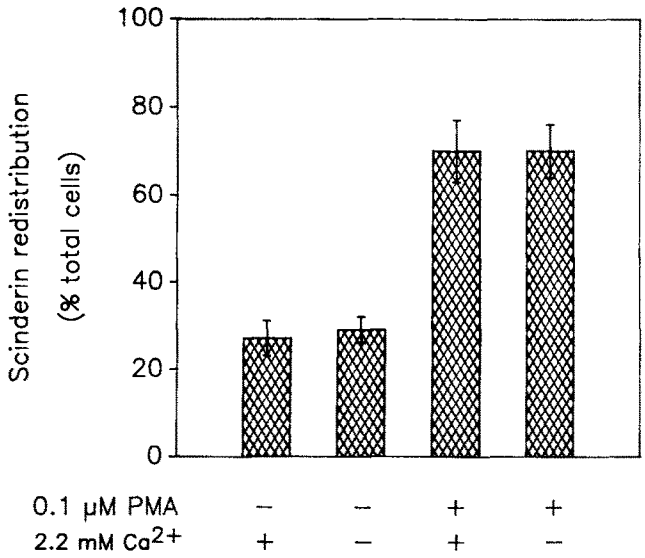

Figure 3. Effect of extracellular Ca^{2+} on PMA-induced subplasmalemmal cortical scinderin redistribution. Two-day old chromaffin cell cultures were incubated for 6 min in Locke's solution containing 0.1% DMSO alone or 0.1% DMSO and 10^{-7} M PMA each case containing either 2.2 mM Ca^{2+} or 1.0 mM EGTA (Ca^{2+}-free medium). Following the incubations, cells were processed for scinderin immunostaining. The peripheral scinderin fluorescence was examined and one hundred cells per coverslip were examined and classified according to the criteria mentioned in legend to Fig. 2. Values shown are the mean ± SEM of eight coverslips (800 cells for each experimental condition) containing cells from three different cultures.

redistribution rose from 1 to 6 min of incubation (Fig. 2, ○). 4α-PMA, an inactive phorbol ester, did not cause subplasmalemmal scinderin redistribution at any time studied (Fig. 2, ◇).

Effect of Ca^{2+} on PMA-induced Cortical Scinderin Redistribution

Nicotine- or high K^+-induced cortical scinderin redistribution are absolutely dependent on the presence of extracellular Ca^{2+} (Vitale et al., 1991). However, the increases in scinderin patched fluorescence induced by 10^{-7} M PMA treatment were not affected by removal of Ca^{2+} from the medium, indicating that the effect of PMA was independent of extracellular Ca^{2+} (Fig. 3). Moreover, the percentages of cells displaying a patched cortical fluorescent ring for scinderin in either Ca^{2+} free- or Ca^{2+} free-0.1% DMSO Locke's solution (controls), were not different from cells incubated in the presence of Ca^{2+} (Fig. 3). Experiments with Fura-2 showed that 10^{-7} M PMA (final concentration) did not cause any alteration in intracellular Ca^{2+} levels either in the presence (Fig. 4 *A*; PMA) or in the absence (1 mM EGTA) of extracellular Ca^{2+} (Fig. 4 *B*, PMA). Furthermore, as expected, 10^{-5} M nicotine (final concentration) produced a sharp increase in intracellular Ca^{2+} in the presence of extracellular Ca^{2+} (Fig. 4 *A*; NiC) and it did not cause a release of Ca^{2+} from intracellular stores (Fig. 4 *B*, NIC).

Protein Kinase C Activity in PMA-treated Cells

It is known that activation of PKC by phorbol esters is associated with translocation of the enzyme from the cytoplasm to the cell membranes (Kraft and Andersson, 1983; Ko et al., 1985; TerBush and Holz, 1986). Incubation of cultured chromaffin cells with 10^{-7} M 4α-PMA (an inactive phorbol ester) for 1 or 6 min did not change the membrane PKC activity with respect to controls (Fig. 5). On the other hand, treatment of chromaffin cells for 1 or 6 min with 10^{-7} M PMA increased threefold the membrane associated PKC activity with respect to control values (Fig. 5).

Effects of Inhibition of PKC Activity on Nicotine and PMA-induced Cortical Scinderin Redistribution

The effects of the PKC activity inhibitors, sphingosine (Hannun et al., 1986), staurosporine (Tamaoki et al., 1986) and calphostin C (Kobayashi et al., 1989), on nicotine- and PMA-induced cortical scinderin redistribution were also studied (Fig. 6 *A–C*). Chromaffin cells were preincubated for 6 min with increasing concentrations of either sphingosine (0–10^{-4} M; Fig. 6 *A*), staurosporine (0 to 10^{-6} M, Fig. 6 *B*) or calphostin C (0–10^{-6} M, Fig. 6 *C*) and they were next challenged for 40 s with 10^{-5} M nicotine or for 6 min with 10^{-7} M PMA. Control preparations (*filled symbols*) were incubated with the highest concentration of each inhibi-

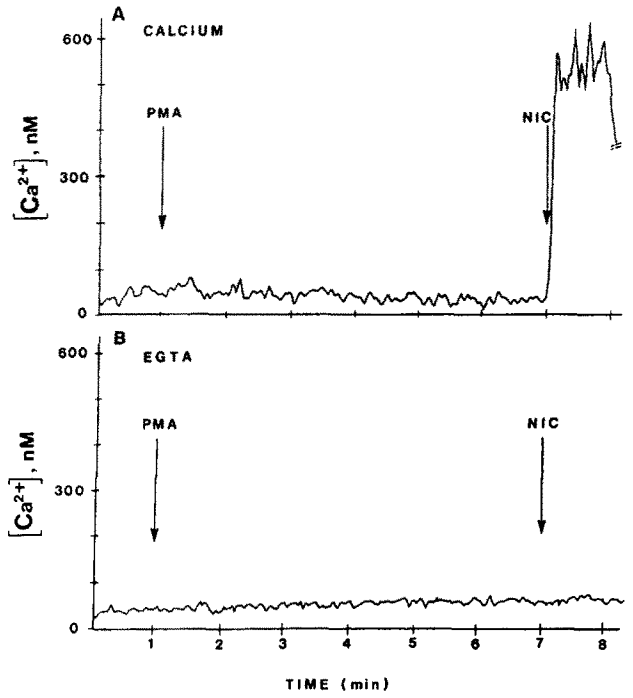

Figure 4. Effects of PMA and nicotine on intracellular Ca^{2+} levels in chromaffin cells in the presence or in the absence of extracellular Ca^{2+}. Chromaffin cells grown on collagen-coated coverslips were loaded with Fura-2 for 40 min and the changes in intracellular Ca^{2+} were monitored using a dual wavelength luminescence spectrophotometer. Coverslips were introduced in a cuvette containing assay buffer (1 mM Ca^{2+}) (*A*) or Ca^{2+}-free assay buffer (2 mM EGTA) (*B*). The coverslips were immobilized in such a way that the same group of cells were always facing the incoming excitation light beam. At the times indicated by the arrows, cells were sequentially challenged with PMA (*PMA*: 10^{-7} M, final concentration) and nicotine (*NIC*: 10^{-5} M, final concentration). The figure shows traces of representative experiments.

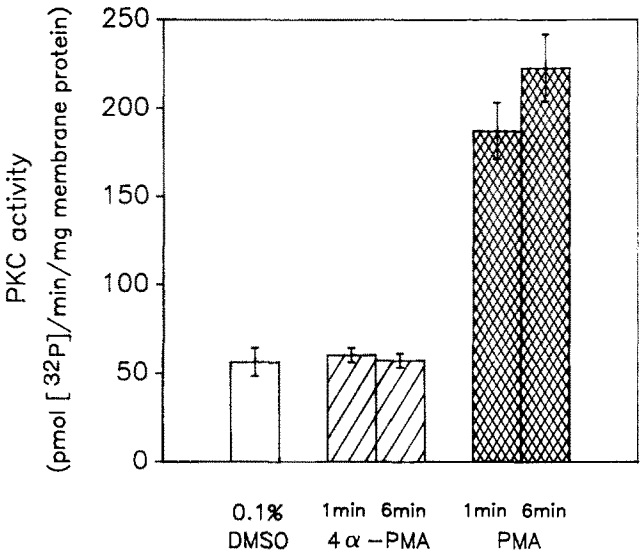

Figure 5. PKC activity in chromaffin cells. Chromaffin cells cultured for 48 h were incubated for 1 or 6 min in regular Locke's solution containing 0.1% DMSO alone or in the presence of 0.1% DMSO plus either 10^{-7} M 4α-PMA or 10^{-7} M PMA. Following these incubations, cells were scraped and resuspended in PKC-assay medium, sonicated and centrifuged at 78,000 *g* for 90 min. Sediments were separated, resuspended and added to PKC-assay buffer containing 0.1% Triton. PKC activity was measured as described in Materials and Methods. Phosphorylated proteins were precipitated with 5% TCA and radioactivity of the samples measured. Membrane PKC activity is expressed as specific activity ([^{32}P] pmol/min/mg protein). Values shown are the mean ± SEM of four culture dishes.

tor. Preincubation of chromaffin cells with any of the PKC inhibitors tested did not affect cortical scinderin distribution (Fig. 6). However, the three PKC blockers inhibited in a dose-dependent manner nicotine (Fig. 6, □) and PMA (Fig. 6, ○) induced scinderin redistribution. The maximal inhibitory effect of sphingosine on nicotine-induced redistribution was 40% and was observed with 50 × 10^{-6} sphingosine, higher concentrations of this PKC inhibitor did not increase the inhibition (Fig. 6 *A*, ○). Incubation of chromaffin cells with 0.5 × 10^{-6} M staurosporine produced an inhibition of 30% on nicotine-induced redistribution (Fig. 6 *B*, □). Higher concentrations of staurosporine did not increase the inhibitory effect (Fig. 6 *B*, □). PMA-induced cortical scinderin redistribution was completely inhibited (100%) by 1.0 × 10^{-6} M staurosporine (Fig. 6 *B*, ○). Furthermore, as can be observed in Fig. 6 *C* (□), 0.5 × 10^{-6} M calphostin C reduced by 26% the number of cells displaying a discontinuous scinderin fluorescence staining as a result of nicotinic receptor stimulation. In contrast, PMA-induced scinderin redistribution was completely abolished (100% inhibition) by micromolar concentrations of this PKC inhibitor (Fig. 6 *C*, ○). As indicated in Fig. 6, staurosporine and calphostin C were 100 times more potent than sphingosine in inhibiting nicotine- and PMA-induced cortical scinderin redistribution.

Ca^{2+} and pH Dependent Interactions of Scinderin with Phospholipids (PS and PIP_2)

Recent published work has demonstrated the interaction of actin-filament severing proteins with phospholipids (Janmey and Matsudaira, 1988; Yin et al., 1988; Maekawa and Sakai, 1990; Sakurai et al., 1991*a*,*b*). This together with the observations on the localization of scinderin in chromaffin cell subplasmalemmal areas and that, under resting conditions (10^{-8} M Ca^{2+}), scinderin does not interact with subplasmalemmal F-actin (Vitale et al., 1991) prompted us to study the interaction of scinderin with phospholipids. Preliminary studies showed that when scinderin was incubated with liposomes (0.4 mg phospholipid/ml) in the presence of 10^{-6} M free-Ca^{2+} concentration, binding of scinderin to liposomes was saturable at concentrations of the protein ranging from 20 to 30 nM. Furthermore, under these experimental conditions, incubations lasting from 30 min to 3 h did not increase the amount of scinderin bound to liposomes. Therefore, the following experiments were performed using a concentration of scinderin six times higher (180 nM) than the concentration known to elicit saturation (30 nM) upon 30 min of incubation. The fact that during cell stimulation intracellular Ca^{2+} concentration varies dramatically within seconds, whereas scinderin remains constant, suggests that under physiologi-

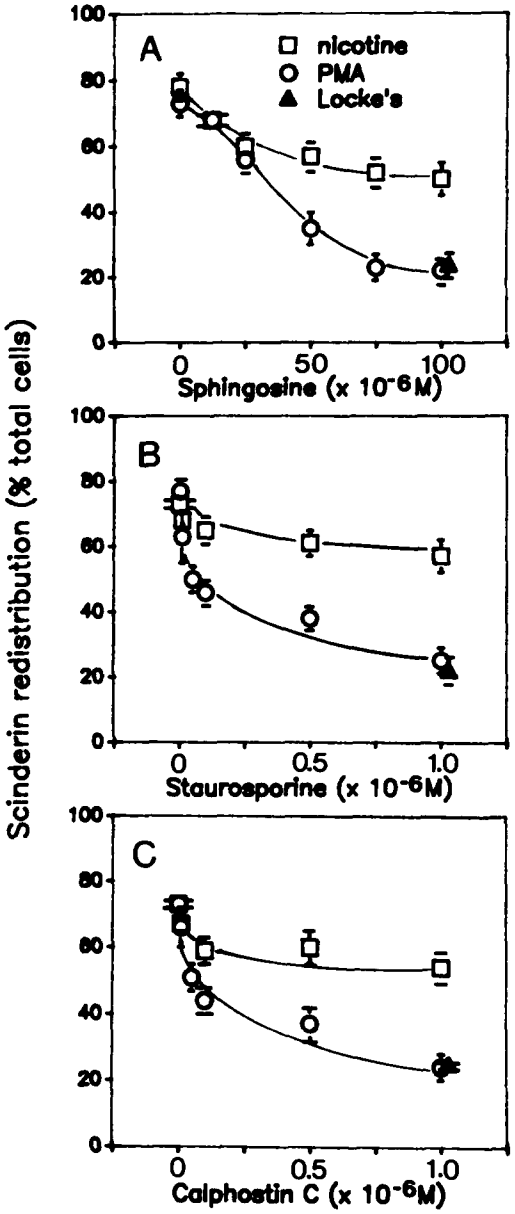

Figure 6. Dose-dependent inhibitory effect of PKC activity inhibition on nicotine and PMA-induced cortical scinderin redistribution. Chromaffin cells cultured for 48 h were preincubated for 6 min in the presence of increasing concentrations of sphingosine (*A*) or staurosporine (*B*) or calphostin C (*C*). After this period, cells were treated for 40 s with 10^{-5} M nicotine (□) or for 6 min with 10^{-7} M PMA (○) in the presence of the corresponding concentration of each inhibitor. Control preparations were incubated for 12 min with the highest concentration of each inhibitor (*filled symbols*). After the incubations, cells were immediately fixed in 3.7% formaldehyde and stained for scinderin immunofluorescence. Preparations were viewed under the fluorescence microscope and single-rounded cells were classified as described in legend to Fig. 2. Each value represents the mean ± SEM of the percentage of cells with disrupted cortical fluorescent rings in four coverslips (400 cells examined for each value).

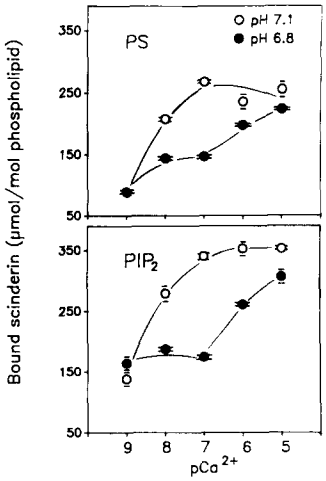

Figure 7. Effects of Ca^{2+} concentrations and pH on scinderin binding to PS and PIP_2 liposomes. Scinderin (180 nM) was incubated with either PS or PIP_2 liposomes (0.4 mg phospholipid/ml) in the presence of increasing free Ca^{2+} concentrations (pCa^{2+}; from 9 to 5) at two different pHs (6.8 and 7.1) as described in Materials and Methods. The mixtures were incubated at 35°C for 30 min and then centrifuged at 78,000 *g* for 30 min. The amount of scinderin in sediments (liposomes) was measured by the [^{125}I]protein A method. The figure shows the saturation plots for PS (*upper panel*) and PIP_2 (*lower panel*). Each point represents the mean ± SEM calculated from results obtained in three separate experiments.

cal conditions Ca^{2+} is one of the limiting factors in the binding of scinderin to either actin or phospholipids. Purified scinderin (180 nM) was incubated in the presence of increasing concentrations of Ca^{2+} at pH 6.8 or 7.1 with either PS or PIP_2 liposomes. The plots shown in Fig. 7 indicate that scinderin was found to bind both types of liposomes in a Ca^{2+}-dependent manner at the two pHs tested. The B_{max} for the binding of scinderin to PS were 256 μmol/mol phospholipid at pH 7.1 and 224 μmol/mol phospholipid at pH 6.8 (Fig. 7, *upper*). The B_{max} for the binding of scinderin to PIP_2 were 354 μmol/mol phospholipid at pH 7.1 and 307 μmol/mol phospholipid at pH 6.8 (Fig. 7, *lower*). At pH 7.1 maximum binding of scinderin to either phospholipid was obtained at 10^{-7} M Ca^{2+} whereas at pH 6.8 maximum binding was observed at 10^{-5} M Ca^{2+} (Fig. 7).

Many cytosolic proteins have been shown to interact with membrane phospholipids (i.e., PKC, gelsolin, calmodulin, et cetera). These proteins may have different binding affinities and consequently they may compete with each other for the available binding sites. Therefore, additional binding studies were performed using total cytosolic protein (competition studies) instead of purified scinderin. Under the same experimental conditions described above, binding of scinderin to PS at pH 6.8 was only observed at 10^{-8} M free-Ca^{2+} (Fig. 8, pH 6.8, scinderin [SC]). On the contrary, at pH 7.1 scinderin was bound to PS liposomes at concentrations of 10^{-7} M free-Ca^{2+} and above. The binding was in these cases inversely proportional to Ca^{2+} concentrations, being the maximal binding observed at 10^{-7} M free-Ca^{2+} (Fig. 8; pH 7.1, SC). In this last group of experiments, PKC binding to PS liposomes was used as control (Fig. 8; PKC). The PKC binding under these experimental conditions was slightly affected by pH changes; at pH 7.1, PKC showed a higher affinity for PS liposomes than at pH 6.8 (Fig. 8, PKC; compare signals at 10^{-8} M Ca^{2+} for pH 6.8 and 7.1). Binding studies carried out with PIP_2 liposomes (Fig. 9) revealed that at pH 6.8, scinderin did not bind to PIP_2 liposomes at any Ca^{2+} concentration. However, at pH 7.1 scinderin was bound to PIP_2 liposomes at free Ca^{2+} concentrations of 10^{-7} M and above (Fig. 9). Higher Ca^{2+} concentrations did not increase the amount of scinderin bound to PIP_2.

The experiments indicate that in presence of other cytosolic proteins and at 10^{-8} M Ca^{2+} and pH 6.8, conditions

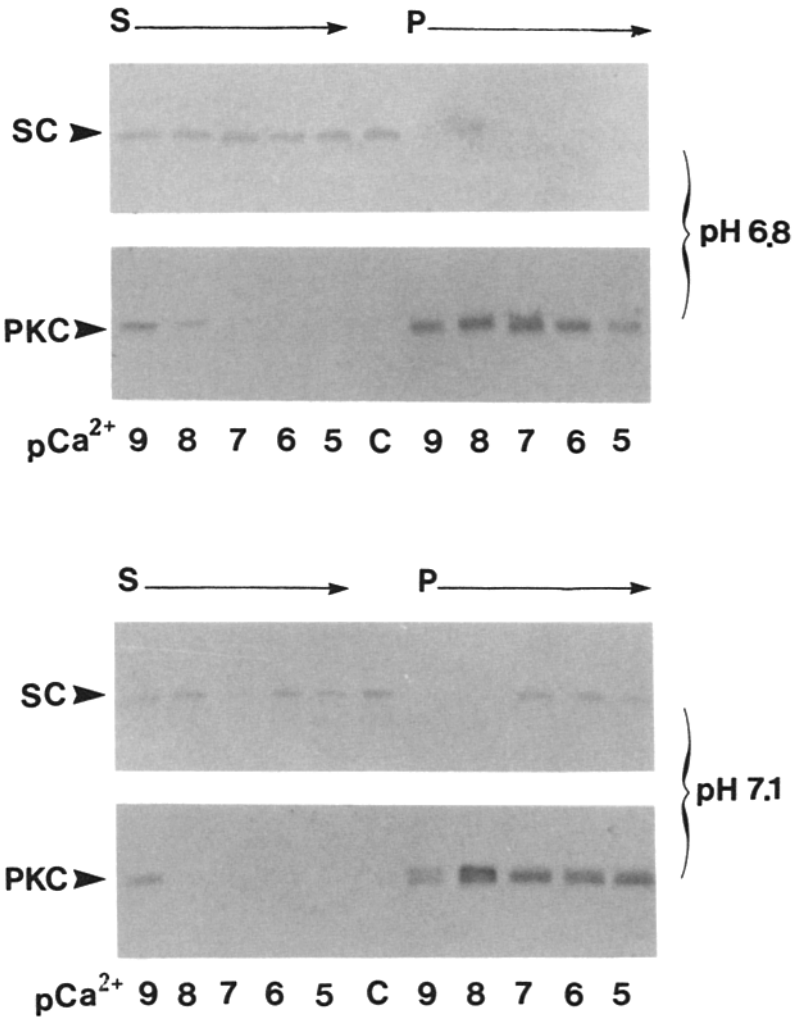

Figure 8. Effects of cytosolic proteins on the Ca^{2+}-dependent binding of scinderin and PKC to PS liposomes (competition studies). Total cytosolic proteins (400 μg) were incubated with PS liposomes (0.4 mg PS/ml) in the presence of increasing free Ca^{2+} concentrations (pCa^{2+}; from 9 to 5) at two different pHs (6.8 and 7.1) under the same experimental conditions described in legend to Fig. 7. The figure shows the immunoblottings for scinderin (*SC*) in supernatants (*S*) and pellets containing liposomes (*P*). Protein kinase C (*PKC*) binding to PS liposomes was used for comparison (control). (*C*) cytosolic protein sample incubated without liposomes in the presence of 10^{-5} M free Ca^{2+}.

similar to those found in resting cells, scinderin binds only to PS (Fig. 8). Moreover, alkalinization of the medium (pH 7.1) without changes in the Ca^{2+} concentration, a condition produced by protein kinase C activation (Na^+/H^+ antiport activation, see below), displaces scinderin from PS (Fig. 8).

Competition studies between scinderin, actin and PS were carried out by a two column-affinity chromatography approach as described in Materials and Methods (Fig. 10). The results obtained showed that at pH 7.1 (when scinderin showed its maximal affinity for PS) and in the presence of 10^{-5} M free Ca^{2+}, the amount of scinderin eluted from the PS column (*first column*) was only 5% of the total amount of scinderin present in the original sample (Fig. 10 *SC*, lane *1*). The remaining 95% of scinderin was eluted from the

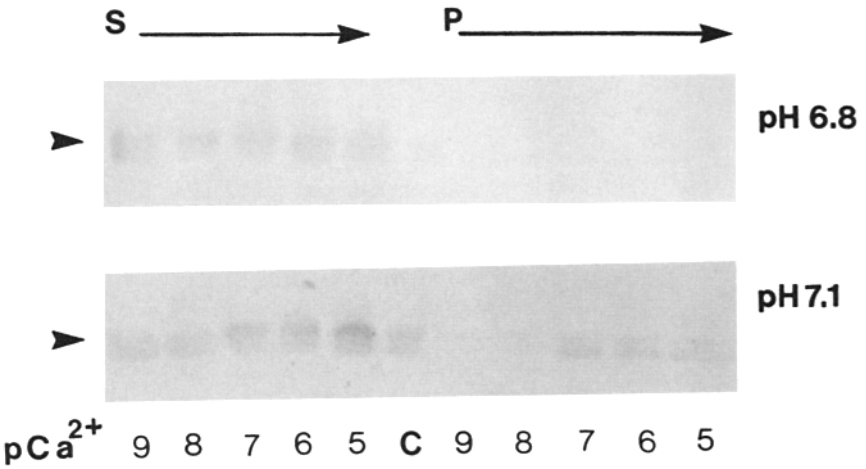

Figure 9. Effects of cytosolic proteins on Ca^{2+}-dependent scinderin binding to PIP_2 liposomes (competition studies). Total cytosolic proteins (400 μg) were incubated with PIP_2 liposomes (0.4 mg PIP_2/ml) in the presence of increasing free Ca^{2+} concentrations (pCa^{2+}; from 9 to 5) at two different pHs (6.8 and 7.1) under the same experimental conditions described in legend to Fig. 7. The figure shows the immunoblotting for scinderin (*arrowheads*) in supernatants (*S*) and pellets containing liposomes (*P*). (*C*) cytosolic protein sample incubated without liposomes in the presence of 10^{-5} M free Ca^{2+}.

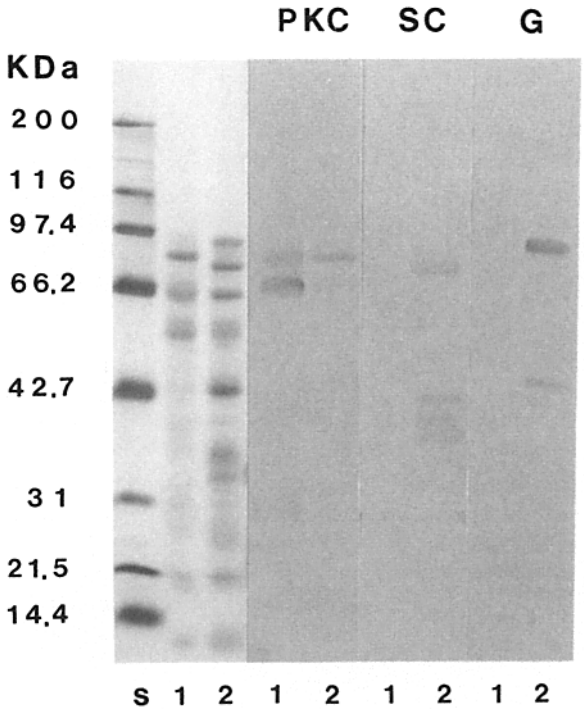

Figure 10. Interactions of scinderin with G-actin and PS, an affinity chromatography study. To study the interactions of scinderin with G-actin and PS an affinity chromatography approach was employed. A chromaffin cell cytosolic protein preparation was treated with ammonium sulphate (65% saturation), the mixture was stirred for 20 min, centrifuged at 100,000 *g* for 60 min and the supernatant was dialyzed overnight against buffer D (10^{-5} M free Ca^{2+}, pH 7.1). The sample was then loaded onto a system consisting in a phosphatidylserine column (first column; 1) in tandem with an actin-DNase I Sepharose 4B column (second column) and recycled overnight. After this step, the system of the two columns in tandem was extensively washed with buffer D (10^{-5} M free Ca^{2+}). Proteins retained by each column were separately eluted with Ca^{2+}-free buffer D (5 mM EGTA). Aliquots of eluates from columns 1 and 2 (25 μg protein) were analyzed for the presence of protein kinase C (*PKC*), scinderin (*SC*), and gelsolin (*G*) by SDS-PAGE followed by immunoblotting with the respective antibodies. S indicates the molecular weight standards. The bands (mol wt <45 kD) which appeared in PKC 1 and 2, Sc 2 and G 2 are storage degradation products of PKC, scinderin and gelsolin, respectively.

actin-DNase I Sepharose 4B column (second column, Fig. 10 *SC*, lane *2*). Under the same experimental conditions, 90% of PKC, also a PS-binding protein, was retained by the PS column (Fig. 10 *PKC*, lane *1*) whereas the remaining 10% was associated with the actin-DNase I Sepharose 4B column (Fig. 10 *PKC*, lane *2*). Gelsolin, another actin-binding protein, interacted only with the actin-DNase Sepharose 4B column (Fig. 10 *G*, lanes *1* and *2*). Moreover, none of the three proteins (PKC, scinderin, and gelsolin) were recovered in the flow-through of the system, suggesting that the proteins interacted either with PS or actin. The experiments show that at conditions (10^{-5} M Ca^{2+}, pH 7.1) which provide optimal binding of scinderin to PS, the protein has greater affinity for actin as indicated by its recovery (95%) in the eluate from the second column. These results do not rule out the possibility that other actin-binding proteins might have stronger affinities for actin. However, the present results as well as our earlier published experiments (Rodríguez Del Castillo et al., 1990) demonstrate that both gelsolin and scinderin are equally retained by actin affinity columns.

Effect of PMA or Nicotine on Intracellular pH

The experiments described above indicated that the binding of scinderin to phospholipids depend not only on Ca^{2+} concentrations but also on the pH. It has been suggested that activation of PKC brings about the phosphorylation of different cellular proteins which are accompanied with changes in intracellular pH (Burns and Rosengurt, 1983). Experiments carried out in our laboratory to determine whether scinderin was a direct substrate for PKC indicated that, under conditions in which histone type III showed increased phosphorylation, PKC did not phosphorylate scinderin (data not shown). Although a direct effect of PKC on scinderin was not observed, it became necessary to determine the effects of PKC activation and nicotine receptor stimulation on chromaffin cell pH. Fig. 11 shows that resting pHi values of BCEF/AM loaded chromaffin cells suspended in incubation buffer averaged 6.98 ± 0.9 (n = 7). The pHi was recorded for 3 min before adding either PMA (*A*) or nicotine (*B*). Addition of both substances caused a rise in pHi, after 6 min incubation in the presence of PMA the chromaffin cell pHi increased by 0.15 pH unit above resting values (Fig. 11,

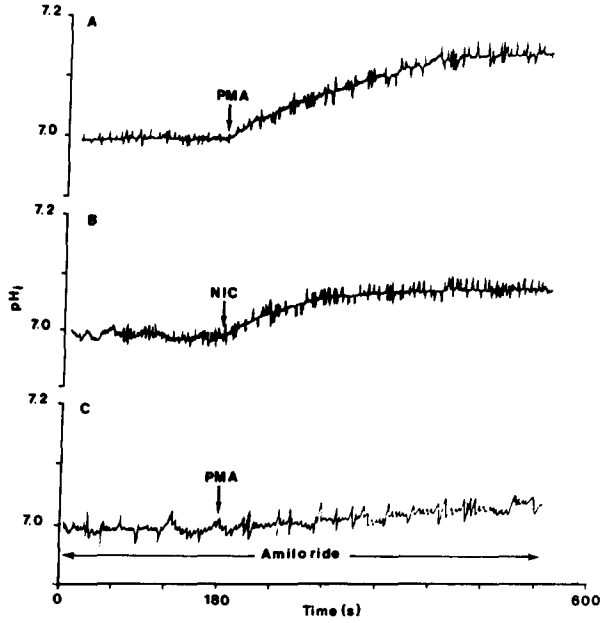

Figure 11. Effects of PMA, nicotine and amiloride on pHi. Chromaffin cells cultured on coverslips were loaded with BCECF/AM as described in Materials and Methods. Coverslips were transferred to a cuvette containing 2 ml of assay buffer (in mM: NaCl, 137; KCl, 4.4; KH_2PO_4, 1.2; $CaCl_2$, 2.2; $MgCl_2$, 0.7; glucose, 10 mM; Hepes, 5; pH 7.2) and excited with a 500-nm light beam; fluorescence emission was recorded at 530 nm. After 3 min of baseline recording 10-μl aliquots of either PMA (*A*) or nicotine (*B*) were added to reach a final concentration of 10^{-7} M and 10^{-5} M respectively. Addition of PMA and nicotine caused increases in pHi. *C* shows the effect of the presence of 10^{-3} M amiloride in the incubation medium on PMA-induced rise in pHi. Calibration of pHi was performed as described in Materials and Methods.

PMA), whereas nicotinic stimulation increased pHi by 0.08 pH unit (Fig. 13, NIC). PMA- (Fig. 11, *lower panel*) or nicotine- (data not shown) induced rises in pHi were not observed when the cells were previously incubated (3 min) with 10^{-3} M amiloride.

Inhibitory Effects of Amiloride and Methyl-isobutyl Amiloride on Nicotine and PMA-induced Cortical Scinderin Redistribution

The changes in chromaffin cell pH described above suggest that cellular scinderin distribution in response to different stimuli (nicotine, PMA) might be due, at least in part, to changes in intracellular pH. Consequently, the effect of amiloride and methyl-isobutyl-amiloride, Na^+/H^+ antiport inhibitors (Moolenaar et al., 1983) on scinderin distribution was studied. Preincubation of chromaffin cells for 3 min with increasing concentrations of amiloride or its analogue, methyl-isobutyl-amiloride (Talor et al., 1989), did not affect cortical scinderin redistribution but inhibited in a dose-dependent manner the subsequent nicotine- or PMA-induced scinderin redistribution (Fig. 12, *A* and *B*, *open symbols*). However, nicotine- and PMA-evoked effects showed different sensitivities to Na^+/H^+ antiport inhibition. A millimolar concentration of amiloride caused only 40% inhibition on nicotine-induced scinderin redistribution (Fig. 12, *A*, (□) whereas the same concentration completely blocked PMA-induced scinderin redistribution (Fig. 12 *A*, ○). Methyl-isobutyl-amiloride, although 10 times more potent than amiloride, also had a higher inhibitory effect on PMA- than on nicotine-induced scinderin redistribution (Fig. 12 *B*). A concentration of 10^{-4} M methyl-isobutyl-amiloride caused a 100% decrease in the percentage of cells displaying cortical scinderin redistribution in response to PMA (Fig. 12 *B*, ○) whereas the inhibitor caused only a 30% decrease in the redistribution of scinderin as a result of nicotinic receptor stimulation (Fig. 12 *B*, □). These experiments suggest again that the redistribution of scinderin induced by nicotine is only partially (30–40%) due to PKC activation.

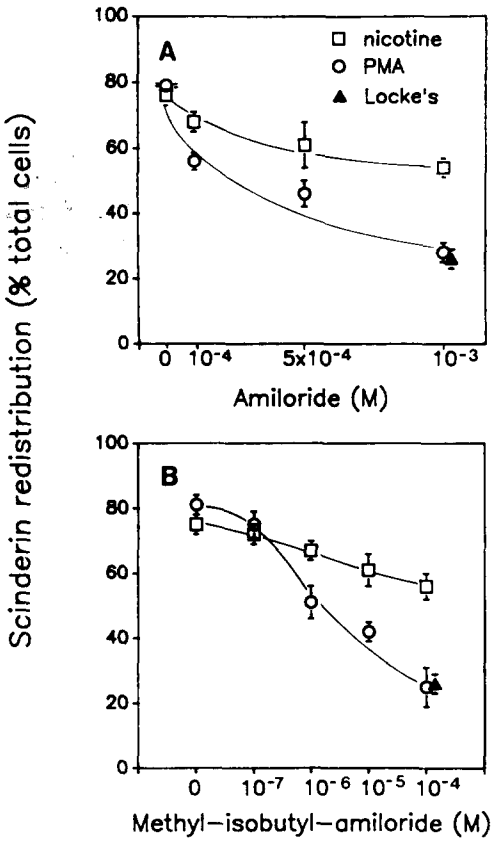

Figure 12. Dose-dependent effect of Na^+/H^+ antiport activity inhibitors on nicotine- and PMA-induced cortical scinderin redistribution. 2-d-old chromaffin cells cultured on collagen coated coverslips were preincubated for 3 min in the presence of increasing concentrations of the Na^+/H^+ antiporter inhibitors, amiloride (*A*) or methyl-isobutyl-amiloride (*B*). Following this period cells were treated for 40 s with 10^{-5} M nicotine (□) or for 6 min with 10^{-7} M PMA (○) in the presence of the corresponding concentration of each inhibitor. Control preparations were incubated for 9 min with the highest concentration of each inhibitor (*filled symbols*). After the incubations, cells were immediately fixed in 3.7% formaldehyde and stained for scinderin immunofluorescence. Preparations were viewed under the fluorescence microscope and single-rounded cells were classified as described in legend to Fig. 2. Each value represents the mean ± SEM of the percentage of cells with disrupted cortical fluorescent rings in 5-4 coverslips (500–400 cells examined for each value).

Effects of Ionophores and of Ammonium Chloride on Scinderin Redistribution

Either direct increases in intracellular pH or increases induced indirectly as a result of Ca^{2+} entry appear to evoke scinderin redistribution (Fig. 13 *A*). NH_4Cl (15 mM), which increases intracellular pH (Kao et al., 1991), induced an immediate increase in scinderin distribution, which was maximal at 5 s and decayed to control values by 90 s. Ionomycin (1 μM) and A23187 (1 μM), two different calcium ionophores (Pressman, 1976; Negishi et al., 1990), also induced similar scinderin redistributions. Nicotine, however, induced a greater and more prolonged scinderin redistribution that was maximal after 40 s of stimulation (Vitale et al., 1991). Thus, a different or additional mechanism of action appears to follow stimulation by nicotine.

To determine whether the effects of NH_4Cl and ionophores were additive, scinderin redistribution was measured after the addition of combinations of these drugs (Fig. 13 *B*). Approximately the same response was observed whether the drugs were used singly or in any of the combinations tested, suggesting that all three substances may increase intracellular pH. In fact, ionomycin has been shown to increase intracellular pH through stimulation of the Na^+/H^+ antiporter via a Ca^{2+}- and calmodulin-dependent mechanism (Negishi et al., 1990). A23187 may act similarly although this has not been shown.

Discussion

Recent work from our laboratory has demonstrated that cortical scinderin redistribution, induced by either nicotine or a depolarizing concentration of K^+ is a Ca^{2+}-dependent event which precedes exocytosis (Vitale et al., 1991). These results have suggested that Ca^{2+} entry after cell depolarization may modulate the activity of Ca^{2+}-dependent actin filament-severing proteins such as scinderin. As a consequence of the activation of these proteins, the peripheral cytoskeleton is reorganized allowing exocytosis to occur.

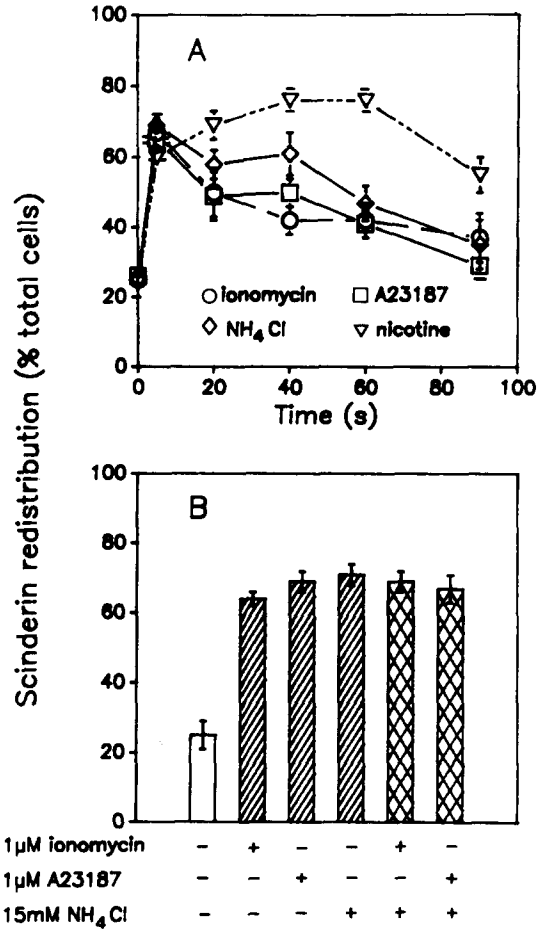

Figure 13. Cortical scinderin redistribution induced by nicotine, Ca^{2+} ionophores and NH_4Cl. (*A*) Time course: two-day old chromaffin cell cultures were incubated either with 10^{-5} M nicotine (▽), 10^{-6} M ionomycin (○), 10^{-6} M A23187 (□) or 15 mM NH_4Cl (◇) for 0, 5, 20, 40, 60, and 90 s. After these periods of time, cells were stained for immunofluorescence microscopy using scinderin antiserum 6. 100 cells per coverslip were examined and classified as having either a "continuous cortical fluorescent ring" (Fig. 1 *a*), or having a "discontinuous cortical fluorescent ring" (Fig. 1 *b*). This procedure was conducted as described in legend to Fig. 2. The percentage of cells showing cortical scinderin redistribution (discontinuous cortical fluorescent pattern) was then calculated and plotted. Each value shown is the mean ± SEM of the percentage of discontinuous cortical fluorescence staining of three coverslips (300 cells for each value). (*B*) Effect of combination of Ca^{2+} ionophores and NH_4Cl (intracellular alkalinization) on cortical scinderin redistribution. Chromaffin cells cultured for 48 h were incubated for 5 s with Locke's solution alone (□) or Locke's solution containing either ionomycin, A23187, or NH_4Cl (▨). The following combinations: ionomycin + NH_4Cl or A23187 + NH_4Cl were also tested (▩). After the incubations, cells were stained for scinderin and examined and classified as explained above (*A*). Each value shown represents the mean ± SEM of the percentage of discontinuous cortical fluorescence staining of cells present in three coverslips (300 cells for each value).

Here we report that PKC modulates cortical scinderin distribution by controlling the interactions between scinderin, membrane phospholipids (PS and PIP_2) and actin through the activity of the Na^+/H^+ antiport. Short-term incubations (1–6 min) of chromaffin cells with the phorbol ester PMA, a PKC activator (Castagna et al., 1982), induced redistribution of cortical scinderin. This PMA-induced effect is likely to be mediated by activation of PKC because under the experimental conditions described here: (*a*) PMA produced translocation of PKC from the cytoplasm to membranes, a process known to activate PKC in chromaffin cells (TerBush and Holz, 1986); (*b*) 4α-PMA, a phorbol ester which does not activate PKC, did not produce any disruption of scinderin fluorescent ring pattern; (*c*) PKC activity inhibitors, such as staurosporine, sphingosine and calphostin C, inhibited PMA-induced effects on cortical scinderin in a dose-dependent manner; and (*d*) PMA did not cause intracellular Ca^{2+} transients. Although it is generally accepted that PKC is the only known target for phorbol esters, the possibility that PMA may act through a still unknown pathway which is independent of activation of PKC can not be completely ruled out.

Studies on the time course of PMA-induced scinderin redistribution demonstrated that chromaffin cells must be incubated with the phorbol ester for at least 1 min in order to detect disruption of the cortical scinderin staining. This effect of PMA was quite slow when compared to nicotine-induced scinderin redistribution. 5 s of nicotinic stimulation was enough to increase the percentage of cells showing scinderin discontinuous fluorescent rings from 20 ± 3% to 60 ± 3%. The fact that PMA must cross the plasma membrane to reach its target (PKC) might explain the slow onset of scinderin redistribution. In this regard, it has been demonstrated that stimulation of nicotinic receptors as well as depolarization of chromaffin cells with 56 mM K^+ induce a shift of PKC from cytoplasm to membrane within 2 s (TerBush et al., 1988), whereas PMA needs a longer time than nicotine to induce the translocation of PKC from the cytoplasm to membranes (TerBush and Holz, 1986; TerBush et al., 1988).

Another important difference between PMA- and nicotine-induced cortical scinderin redistribution is that PMA effects are completely independent of extracellular Ca^{2+} whereas nicotine-induced effects are fully dependent on the presence of this divalent cation (Vitale et al., 1991). Moreover, our results indicate that PMA elicited no effect on basal intracellular Ca^{2+} levels either in the presence or in the absence of extracellular Ca^{2+}. Thus, it seems likely that PMA effects on scinderin redistribution were mediated through an activation of PKC and not by a change in the intracellular Ca^{2+} concentration. It has been demonstrated that phorbol esters increase the sensitivity of PKC for Ca^{2+} (Castagna et al., 1982). Therefore, under resting conditions ($pCa^{2+} \approx 8$), PMA would be able to activate PKC. In contrast, nicotine-induced cortical scinderin redistribution is absolutely dependent on extracellular Ca^{2+}. However, the possibility exists that the Ca^{2+} influx that follows nicotinic receptor stimulation activates PKC and consequently cortical scinderin redistribution. To test whether PKC activation participates in nicotine-induced cortical scinderin redistribution, experiments were performed in which PKC activity was inhibited before incubation of chromaffin cells with nicotine. These experiments showed that blockade of PKC activity with different inhibitors (sphingosine, staurosporine, calphostin C) reduced in 26–40% the percentage of cells displaying cortical scinderin redistribution. Thus, activation of PKC mediates, only partially, nicotine-induced cortical scinderin redistribution.

It has been reported that actin-binding proteins such as vinculin, filamin (Kawamoto and Hidaka, 1984), talin (Litchfield and Ball, 1986), and caldesmon (Umekawa and Hidaka, 1985) are substrates for PKC. However, under our experimental conditions, PKC failed to phosphorylate scinderin. Moreover, because the effect of PKC on scinderin was Ca^{2+}-independent it can be concluded that the effect on scinderin could be the result of other process(es) which is (are) dependent on PKC activation. One such possibility is that PKC affects the interaction of scinderin with the membrane phospholipids because immunocytochemical evidence indicates that scinderin could be associated with some components of the plasma membrane. This is based in the following observations: (*a*) in resting conditions scinderin appears as a cortical fluorescent ring underneath the plasma membrane; (*b*) upon nicotinic stimulation, scinderin and F-actin cortical fluorescent rings appear fragmented with similar patterns of distribution; (*c*) when the stimulus (nicotine) is removed, the recovery of scinderin continuous cortical fluorescent ring is faster than F-actin reassembly, suggesting that when Ca^{2+} concentration returns to basal levels, cortical scinderin is not associated with cortical F-actin networks but with other membrane components (Vitale et al., 1991); and (*d*) the activities of the other actin-filament severing proteins are inhibited by phospholipids such as PIP_2, PIP, and PS (Janmey and Matsudaira, 1988; Yin et al., 1988; Maekawa and Sakai, 1990; Sakurai et al., 1991*a*,*b*). Results described here show that scinderin binds to PS and PIP_2 in a Ca^{2+}-dependent manner. Moreover, in the presence of cytosolic proteins and at low Ca^{2+} concentrations, alkalinization of the medium (pH = 7.1) decreases the binding affinity of scinderin for phospholipid liposomes. Competition studies using liposomes and affinity chromatography column also demonstrated that G-actin and phospholipids (PS and PIP_2) compete for binding to scinderin, suggesting that the phospholipid binding site and one of the two actin binding sites (Trifaró et al., 1992) are localized in the same domain of the scinderin molecule. Moreover, phospholipids could be more easily displaced from scinderin by G-actin at acid than alkaline pHs providing Ca^{2+} is present. These results clearly indicate the important role of the anionic charges in the binding between scinderin and phospholipids.

The fact, that Ca^{2+} concentrations and pH changes determine the binding of scinderin to either phospholipids or actin led us to think that, perhaps, PMA effects on scinderin redistribution could be mediated by changes in intracellular pH, because under our experimental conditions, PMA did not increase intracellular Ca^{2+}, PMA-induced effects were independent of extracellular Ca^{2+} and scinderin was not phosphorylated by PKC. Evidence that PMA increases pHi by activation of the Na^+/H^+ antiport in chromaffin cells has been recently reported (Negishi et al., 1990). The PMA-induced rise in pHi was completely blocked by amiloride, a Na^+/H^+ antiport inhibitor, and by staurosporine, a PKC inhibitor (Negishi et al., 1990). Our results indicate that PMA produced an increase in chromaffin cell pHi which was inhibited by amiloride. Other substances known to increase intracellular pH such as NH_4Cl (Kao et al., 1991) and ionomycin (Negishi et al., 1990) also induced scinderin redistribution. The fact that the ionophores, ionomycin and A23187, induced scinderin redistribution could also be explained by an increase in intracellular pH because it has been demonstrated that in chromaffin cells, ionomycin increases intracellular pH through stimulation of the Na^+/H^+ antiport via a Ca^{2+}-calmodulin-dependent mechanism (Negishi et al., 1990). Nicotine also induced a rise in pHi, which, although it was lower than that produced by PMA, was also blocked by amiloride. These results agree with those reported by Yanagihara et al. (1990) using carbachol. However, Burgoyne et al. (1988), have demonstrated that nicotine failed to modify pHi and Rosario et al. (1991), on the other hand, observed that nicotine induced a decrease in pHi which was not blocked by amiloride. Na^+/H^+ antiport inhibitors (amiloride and *N*-methyl-isobutyl-amiloride) reduced by 100% PMA-induced cortical scinderin redistribution whereas only 30–40% inhibition was observed when nicotine was the stimulus. These results indicate that PKC is, although only partially, involved in the mechanism of nicotine-induced cortical scinderin redistribution. Moreover, the rise in intracellular pH observed with nicotine might be the result of a translocation and activation of PKC produced by Ca^{2+} entry. This also would explain why scinderin redistribution is partially inhibited by inhibitors of PKC.

Taking into consideration all data presented in this paper, the following model for the regulation of scinderin is proposed (Fig. 14). Under resting conditions (pCa = 8; pH = 6.98), scinderin is divided into two pools, a soluble one and a pool of scinderin bound to membrane phospholipids (Fig. 14 *A*). These two pools correspond to the cytoplasmic and cortical stainings observed by immunofluorescence microscopy respectively. Under these conditions, the binding of scinderin to phospholipids is possible because the half-maximal binding to phospholipids is observed at 1×10^{-8} M Ca^{2+}. Moreover, scinderin binding to actin does not occur under these conditions because actin filament severing activity is evident at 1×10^{-7} M Ca^{2+} (Rodríguez Del Castillo et al., 1990). It appears then, that the physiological role of scinderin binding to membrane phospholipids is to keep cortical scinderin in the vicinity of its substrate (F-actin). Nicotinic stimulation causes a fast increase in intracellular Ca^{2+} concentrations (pCa $\cong$ 6-5) and a slow rise in pHi (Fig. 14 *B*). High intracellular Ca^{2+} concentrations induces release of scinderin from the membrane-associated pool (discontinuous scinderin cortical fluorescent ring), and binding of scinderin to F-actin (colocalization of scinderin and F-actin in cortical fluorescent patches) (Vitale et al., 1991) with activation of scinderin severing activity (cortical F-actin disassembly). An increase in pHi only takes place after Ca^{2+} influx activates PKC, and this enzyme activates the Na^+/H^+ antiport. This rise in pHi is also involved in nicotine-induced scinderin redistribution because the blockade of the Na^+/H^+ antiport by amiloride partially inhibits the nicotine-induced effect. Therefore, for a complete displacement of scinderin from membrane phospholipids by actin, an increase in intracellular Ca^{2+} with a subsequent rise in pHi are necessary (Fig. 14 *C*). Under these conditions the low affinity second Ca^{2+} binding site of scinderin might be activated. During recovery (Fig. 14 *D*), intracellular Ca^{2+} decreases due to extrusion or sequestration, and the scinderin-actin complex begins to dissociate (low affinity Ca^{2+} binding site). When Ca^{2+} concentrations are $\sim 10^{-7}$ M and pHi is still high (7.1), conditions in which scinderin expresses its maximal affinity for phospholipids, the remaining scinderin-actin complex is dissociated by competing phospholipids (PS and PIP_2). This would explain why scinderin continuous cortical fluorescent ring is recuperated faster than

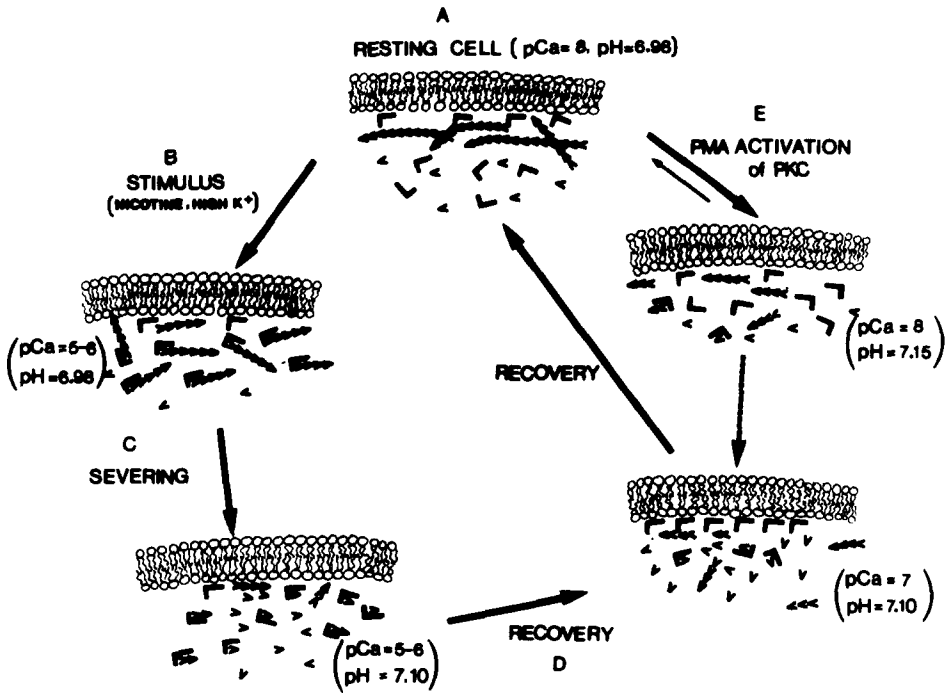

Figure 14. Schematic representation of the interactions of scinderin with actin and membrane phospholipids (PS and PIP_2) and of the effects on intracellular pH and free Ca^{2+} concentrations on these interactions. See text for explanation. ⌐ = scinderin; ⋘ = F-actin; < = G-actin; ∫ = membrane phospholipids.

F-actin reassembly (Vitale et al., 1991). Finally, when Ca^{2+} concentrations return to basal values (pCa = 8), PKC activity decreases and chromaffin cell pHi reaches resting values (pH 6.98), scinderin is in equilibrium between its membrane bound and cytoplasmic pools (Fig. 14 *A*). Therefore, PKC might modulate nicotine-induced scinderin redistribution at two different steps: (*a*) an increase in pHi allows the displacement of scinderin from the phospholipids to actin during the disassembly step and more important, (*b*) during the recovery step, producing an increase in the affinity of scinderin for phospholipids limiting the actin filament severing activity of scinderin. PMA-induced effect on scinderin redistribution (Fig. 14 *E*) is not physiological because even though there is a rise in intracellular pH, the intracellular Ca^{2+} concentrations are not high enough (lack of Ca^{2+} influx and because Ca^{2+} is chelated by other proteins) to activate scinderin (Fig. 14 *E*) and the protein is simply released from the membrane (cortical discontinuous staining). Nevertheless, the use of PMA and ionophores in the experiments described in this paper has been of great importance in the elucidation of the role of Ca^{2+} and intracellular pH in the distribution of scinderin in the chromaffin cell, under resting and stimulated conditions.

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