Cortical Filamentous Actin Disassembly and Scinderin Redistribution during Chromaffin Cell Stimulation Precede Exocytosis, A Phenomenon Not Exhibited by Gelsolin

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Abstract. Immunofluorescence and cytochemical studies have demonstrated that filamentous actin is mainly localized in the cortical surface of the chromaffin cell. It has been suggested that these actin filament networks act as a barrier to the secretory granules, impeding their contact with the plasma membrane. Stimulation of chromaffin cells produces a disassembly of actin filament networks, implying the removal of the barrier. The presence of gelsolin and scinderin, two Ca^{2+}-dependent actin filament severing proteins, in the cortical surface of the chromaffin cells, suggests the possibility that cell stimulation brings about activation of one or more actin filament severing proteins with the consequent disruption of actin networks. Therefore, biochemical studies and fluorescence microscopy experiments with scinderin and gelsolin antibodies and rhodamine-phalloidin, a probe for filamentous actin, were performed in cultured chromaffin cells to study the distribution of scinderin, gelsolin, and filamentous actin during cell stimulation and to correlate the possible changes with catecholamine secretion. Here we report that during nicotinic stimulation or K^+-evoked depolarization, subcortical scinderin but not gelsolin is redistributed and that this redistribution precedes catecholamine secretion. The rearrangement of scinderin in patches is mediated by nicotinic receptors. Cell stimulation produces similar patterns of distribution of scinderin and filamentous actin. However, after the removal of the stimulus, the recovery of scinderin cortical pattern of distribution is faster than F-actin reassembly, suggesting that scinderin is bound in the cortical region of the cell to a component other than F-actin. We also demonstrate that peripheral actin filament disassembly and subplasmalemmal scinderin redistribution are calcium-dependent events. Moreover, experiments with an antibody against dopamine-β-hydroxylase suggest that exocytosis sites are preferentially localized to areas of F-actin disassembly.

C HROMAFFIN cells of the adrenal medulla store their secretory products in specialized organelles, the chromaffin granules (Smith, 1968; Trifaró, 1977). In response to cholinergic stimulation and upon Ca^{2+} entry, the granules fuse with the plasma membrane and release their soluble contents to the cell exterior by exocytosis (Trifaró, 1977; Viveros, 1974). Immunofluorescence and cytochemical studies have described the presence of a mesh of filamentous actin (F-actin) underneath the chromaffin cell plasma membrane (Lee and Trifaró, 1981; Trifaró et al., 1984; Cheek and Burgoyne, 1986). It has been proposed that actin networks act as a barrier to the secretory granules by blocking their movement towards the plasma membrane (Trifaró et al., 1982, 1984, 1989; Cheek and Burgoyne, 1986, 1987; Burgoyne and Cheek, 1987; Burgoyne et al., 1989). Evi-

dence obtained from different experimental approaches has demonstrated that stimulation of chromaffin cells brings about a disassembly of cortical F-actin networks, suggesting the removal of the physical barrier to granule movement (Cheek and Burgoyne, 1986, 1987; Burgoyne and Cheek, 1987; Burgoyne et al., 1989; Trifaró et al., 1989). The existence of actin-binding proteins that regulate the dynamics of actin networks (Yin and Stossel, 1979; Craig and Pollard, 1982; Stossel et al., 1985; Maekawa et al., 1989; Rodriguez Del Castillo et al., 1990) strongly suggests a role for these proteins in the disassembly of actin filaments triggered by cell stimulation. Therefore, it was of interest to investigate the participation in this process of gelsolin (Yin and Stossel, 1979) and scinderin (Rodriguez Del Castillo et al., 1990) two Ca^{2+}-dependent actin-binding proteins that control actin filament length.

Gelsolin is an actin filament capping and severing protein found in many cells, including chromaffin cells, and in extracellular fluids (Yin and Stossel, 1989; Yin et al., 1981; Stossel et al., 1985; Trifaró et al., 1985; Bader et al., 1986).

Previous work from our laboratory has described the presence in chromaffin cells of another actin binding protein that can be eluted by an EGTA containing buffer from actin–DNase I affinity columns along with gelsolin (Bader et al., 1986). Recently we have isolated, characterized, and given the name of "scinderin" to this new protein (Rodriguez Del Castillo et al., 1990). Scinderin is an 80-kD cytosolic protein that shortens actin filament length provided Ca^{2+} is present in the medium (Rodriguez Del Castillo et al., 1990).

The fact that, when chromaffin cells are stimulated there is an entry of Ca^{2+} (Douglas, 1968) with a consequent increase in its intracellular level (Cheek et al., 1989) prompted us to investigate whether this condition would influence gelsolin and/or scinderin distributions in a way that could be correlated to actin filament disassembly. This paper describes biochemical and immunocytochemical experiments performed in cultured chromaffin cells. We have studied the cellular localization of scinderin and gelsolin under different experimental conditions and compared their subcellular redistribution with F-actin disassembly and catecholamine secretion. The present experiments demonstrate that during cell stimulation, subplasmalemmal scinderin, but not gelsolin, is redistributed in chromaffin cells, that this redistribution precedes exocytosis, and that exocytosis sites are preferentially localized to areas of F-actin disassembly. The redistribution of scinderin is mediated by nicotinic receptors. The results also show that the redistribution of scinderin and F-actin disassembly are Ca^{2+}-dependent events and that similar patterns of distribution for scinderin and F-actin are observed during stimulation. A preliminary account of this work has been presented elsewhere (Vitale, M. L., A. Rodríguez del Castillo, L. Tchakarov, M. L. Novas, and J.-M. Trifaró. 1990. *J. Cell Biol.* 111:424a).

Materials and Methods

(a) 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 plated on collagen-coated glass coverslips contained within plastic Petri dishes at a density of 0.25×10^6 cells/35-mm dish for fluorescence microscopy studies or in collagen-coated plastic Petri dishes at a density of 0.5×10^6 cells/35-mm dish for catecholamine release studies. Cells were grown at 37°C in a humidified incubator under a CO_2 + air atmosphere for 48 h as previously described (Trifaró and Lee, 1980).

(b) Source of Antibodies

Polyclonal antibodies were raised in rabbits against purified bovine scinderin, gelsolin, and dopamine-β-hydroxylase (DβH)[1] as previously described (Bader et al., 1986; Rodriguez Del Castillo et al., 1990; Trifaró et al., 1976). Scinderin antiserum 6 thus obtained does not recognize gelsolin and gelsolin antiserum does not cross-react with scinderin (Bader et al., 1986; Rodriguez Del Castillo et al., 1990; Tchakarov et al., 1990). Moreover, scinderin was the only protein immunoprecipitated from an adrenal medullary cytosolic preparation by antiserum 6 (Tchakarov et al., 1990). Anti-DβH IgG has been previously characterized (Trifaró et al., 1976). A mouse mAb against gelsolin (clone GS-2C4) was purchased from Sigma Chemical Co. (St. Louis, MO). This antibody is specific for an epitope localized on a 47-kD peptide derived from a chymotryptic cleavage of human gelsolin (Chaponnier et al., 1986).

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1. *Abbreviations used in this paper*: DβH, dopamine-β-hydroxylase.

(c) Extraction of Chromaffin Cell Cytoskeletal Proteins

Chromaffin cell cytoskeleton was prepared essentially as described by Bader et al. (1984). Briefly, chromaffin cells were cultured in a 100-mm-diam plastic Petri dish at a density of 60×10^6 cells/dish. After 24 h in culture, the cells were resuspended by washing several times with buffer A (PBS: 100 mM sodium phosphate, 130 mM NaCl, pH 7.2 containing 5 mM EGTA, and 2 mM PMSF). Cells were collected by centrifugation at 5,000 g for 10 min. The sediment thus obtained was resuspended in 1 ml buffer A containing 600 mM KCl, 10 mM MgCl₂, 1% Triton X-100, and 0.5 mg DNAse I/ml. The mixture was centrifuged at 27,000 g for 30 min and the sediment containing Triton-insoluble proteins (cytoskeleton) was washed three times with buffer A, resuspended in 1 ml electrophoresis buffer and boiled for 5 min.

(d) Preparation of Total Proteins and Actin-binding Proteins from Bovine Adrenal Medulla

Bovine adrenal medullae (60 g) were washed in ice-cold Locke's solution to remove the blood and then homogenized in 300 mM sucrose, 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM DTT, 1 mM PMSF, 5 mM N-ethylmaleimide, 1 mM EGTA and 1 mM Na-ATP (1 g of medulla in 2 ml of solution), using a Sorvall omnimixer (Sorvall Instruments Div., Newton, CT). The homogenate was centrifuged at 1,000 g for 10 min. The supernatant thus obtained (total protein sample) was centrifuged at 100,000 g for 60 min and CaCl₂ was added to obtain a final concentration of 2 mM. The preparation was then applied to a DNase I-Sepharose 4B column prepared as described by Bader et al. (1986). The column was pre-equilibrated with buffer A (20 mM Tris-HCl pH 7.5, 2 mM CaCl₂, 0.5 mM Na-ATP, 1 mM DTT, and 1 mM PMSF) containing 100 mM KCl. The column was then washed extensively (300 ml) with the same buffer A but this time containing 300 mM KCl. Finally the Ca^{2+}-dependent actin binding-proteins were eluted with buffer B (20 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM DTT, 2 mM EGTA, and 1 mM PMSF). Samples from either total proteins or EGTA eluates were used in the experiments.

(e) Preparation of Scinderin

Scinderin was purified from bovine adrenal medullae following a chromatographic procedure previously described (Rodriguez Del Castillo et al., 1990) except that the last chromatographic step (HPLC) was omitted. Therefore, the preparation obtained in this case also contains small amounts of gelsolin and other Ca^{2+}-dependent actin-binding proteins.

(f) Electrophoresis and Immunoblotting

Monodimensional SDS-PAGE was performed according to Doucet and Trifaró (1988); gels were usually run at 60 V overnight in a Bio-Rad Protean I apparatus (Bio-Rad Laboratories, Inc., Richmond, CA). The protocol for immunoblotting was as described by Towbin et al., (1979). After electrophoresis, proteins were electrotransferred onto nitrocellulose membranes (Hoefer Scientific Instruments, San Francisco, CA). Membranes were first blocked with 3% BSA in PBS and then incubated with scinderin antiserum 6 (1:500 dilution) or gelsolin antiserum (1:500 dilution) for 60 min. Membranes were next incubated with goat antirabbit immunoglobulin G-alkaline phosphatase conjugate (1:3,000 dilution) for another 60 min. Color was developed by treatment with a mixture of p-nitroblue tetrazolium chloride and 5-bromo-4-chloride-3-indolyl phosphate-toluidine salt.

(g) Fluorescence Microscopy

Single Staining. Experiments were started by rinsing the cultured cells three times with regular Locke's solution (in millimolar: NaCl, 154; KCl, 2.6; K₂HPO₄, 2.15; KH₂PO₄, 0.85; MgCl₂, 1.2; CaCl₂, 2.2; and glucose, 10.0; pH 7.2). Cells were then incubated for different periods of time with Locke's solution in the absence (control) or presence (stimulated) of different compounds. Chromaffin cells were fixed in 3.7% formaldehyde in Locke's solution for 20 min at different times after initiated the stimulation and processed for fluorescence microscopy as described previously (Lee and Trifaró, 1981). Briefly, cells were permeabilized by three successive exposures of 5 min each to 50, 100, and 50% acetone. Preparations were washed several times with PBS and then incubated at 37°C with either scinderin antiserum 6 (1:20 dilution), gelsolin antiserum (1:20 dilution) or mouse monoclonal antibody against gelsolin (1:40 dilution) for 60 min.

Coverslips were thoroughly washed with PBS and were next incubated at 37°C with goat antirabbit immunoglobulin G-fluorescein isothiocyanate conjugate (FITC-IgG) (1:80 dilution) for another 60 min. When a mouse mAb against gelsolin was used, goat antimouse immunoglobulin G-tetramethylrhodamine isothiocyanate conjugate (TRITC-IgG; 1:256 dilution) was used as a second antibody. Coverslips were washed several times with PBS and mounted in glycerol-PBS (1:1; vol/vol). Control experiments were performed with (a) second antibody alone and (b) first antiserum after adsorption with the correspondent antigen.

Double Staining. Chromaffin cells were incubated with Locke's solution for different periods of time in the absence (control) or presence (stimulated) of different compounds. For scinderin/F-actin or gelsolin/F-actin staining, fixation and acetone-induced permeabilization were as above. Permeabilized cells were incubated at room temperature for 40 min with 0.3 μM rhodamine-labeled phalloidin, a probe for filamentous actin (Faultish et al., 1988). Coverslips were then washed six times with PBS and incubated with scinderin antiserum 6 or gelsolin antiserum followed by FITC-IgG as described above.

For DβH/F-actin staining, cells incubated for 40 s with 10 μM nicotine were fixed with 3.7% formaldehyde and then incubated with anti-DβH IgG (1:100 dilution) for 60 min, washed as indicated above, and further incubated with FITC-IgG as described previously. This was followed by incubation for 40 min with 0.3 μM rhodamine-labeled phalloidin.

Preparations were observed with a Leitz Ortholux II fluorescent microscope equipped with a 200-W high-pressure lamp and a Ploemopack II incident light illuminator equipped with an I-filter block (KP 490 plus 1 mm GG 455 exciting filter, TK dichroic beam splitting mirror, K 515 suppression filter) for fluorescein and an M-filter block (2 mm BG 36 plus S 546 exciting filter, TK 580 dichroic beam splitting mirror, K 580 suppression filter) for rhodamine. Photographs were taken with Kodak Tri-X pan films (400 ASA). 100 cells per coverslip were examined and classified as having either "continuous staining" as shown in Fig. 3 A, a. or having "discontinuous patched staining pattern" as can be seen in Fig. 3 A, b. This was done without knowing whether cells were from control or stimulated preparations (single blind design).

(h) Catecholamine Release Studies

Catecholamine output was evaluated by measuring [³H]noradrenaline ([³H]NA) output essentially as described previously (Trifaró and Lee, 1980; Kenigsberg and Trifaró, 1980). Previous experiments from our laboratory have demonstrated that when chromaffin cells are loaded with [³H]NA under controlled conditions, there was a concomitant and parallel release of endogenous catecholamines and [³H]NA upon stimulation (Trifaró and Lee, 1980; Trifaró and Bourne, 1981).

Chromaffin cells were plated on collagen-coated plastic dishes (0.5 × 10⁶ cells/35-mm dish) and grown for 48 h. Dishes were washed three times with special medium (in millimolar: NaCl, 110; NaHCO₃, 40; KCl, 5; MgSO₄, 1; NaH₂PO₄, 1; NaPyruvate, 1; CaCl₂, 2; Fe(NO₃)₃, 2.5 × 10⁻⁴; ascorbic acid, 0.1; pH 7.2 adjusted with Hepes). Chromaffin cells were incubated at room temperature for 5 min with 1 ml of special medium containing 0.1 nmol [³H]NA (42.1 Ci/mmol sp act; New England Nuclear, Boston, MA). After this labeling step, each dish was incubated with six changes of 1 ml regular Locke's solution over a 60-min period before the experiments were commenced. Cells were then incubated with 1 ml regular Locke's solution with or without 10 μM nicotine or 56 mM K⁺ for different periods. After these periods of time, the entire (1 ml) incubation medium was removed and radioactivity determined. [³H]NA cell content was determined by treating the dishes with 1 ml of 10% TCA for 10 min followed by two washes of 0.5 ml 6% TCA; the three aliquots were combined and the radioactivity was measured in a liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA). Total [³H]NA cell content was calculated by adding the [³H]NA released into the medium to the [³H]NA extracted with TCA. The assay allowed the determination in 1 ml sample of a level of [³H]NA equivalent to 0.35% of the total cell catecholamine content.

(i) Protein Determination

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

(j) Chemicals

Nicotine (hydrogen tartrate salt), muscarine (chloride), D-tubocurarine (chloride), monoclonal anti-gelsolin clone GS-2C4, TRITC-IgG, and FITC-IgG were purchased from Sigma Chemical Co. Materials for SDS-PAGE and
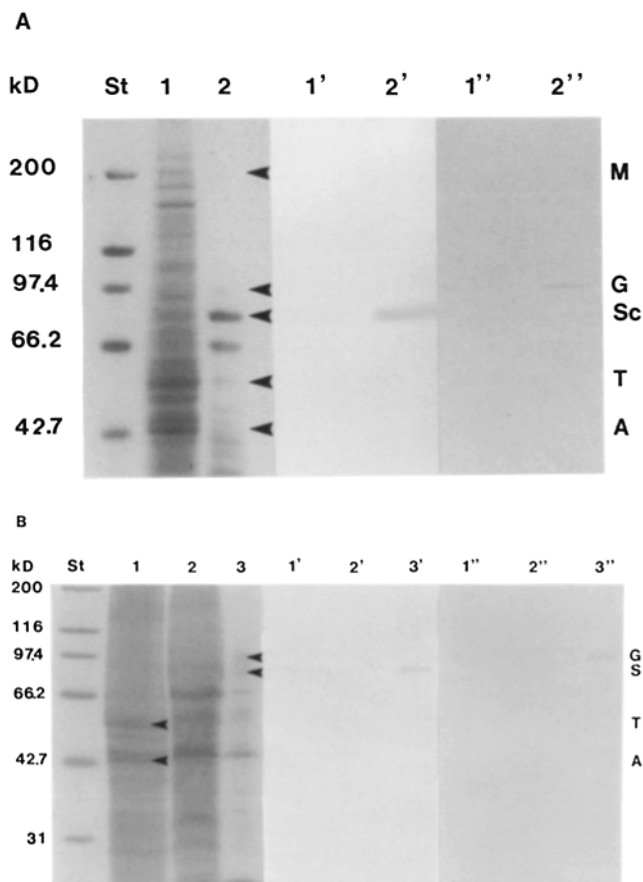

Figure 1. Specificity of scinderin and gelsolin antibodies. (*A*) Chromaffin cell cytoskeletal proteins (50 μg, lane *1*) and a partially purified scinderin preparation (5 μg, lane *2*) were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Lane *St* shows the position of molecular weight standards. Lanes *1'*, *2'*, *1''*, and *2''* are the immunoblots of the same preparations after incubation with scinderin antiserum 6 (lanes *1'* and *2'*) or gelsolin antiserum (lanes *1''* and *2''*). The presence of myosin (*M*), tubulin (*T*), and actin (*A*) in the chromaffin cell cytoskeleton and of scinderin (*Sc*) and gelsolin (*G*) in a partially purified scinderin preparation is indicated by arrowheads. (*B*) Cytoskeletal proteins (200 μg, lane *1*), adrenal medullary total proteins (200 μg, lane *2*) and DNase I-actin affinity-purified Ca²⁺-dependent actin-binding proteins (30 μg, lane *3*) were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Lane *St* shows the position of molecular weight standards. Lanes *1'–3''* are the immunoblots of the same preparations after incubation with scinderin antiserum 6 (lanes *1'*, *2'*, and *3'*) or gelsolin antiserum (lanes *1''*, *2''*, and *3''*). A faint reaction is observed with scinderin antiserum 6 (lanes *1'* and *2'*) and with gelsolin antiserum (lanes *1''* and *2''*). The presence of gelsolin (*G*), scinderin (*Sc*), tubulin (*T*), and actin (*A*) is indicated by arrowheads.

immunoblotting were from Bio-Rad Laboratories, Ltd. (Mississauga, Ontario, Canada). Rhodamine-labeled phalloidin was from Molecular Probes, Inc. (Eugene, OR).

Results

(a) Specificity of Scinderin and Gelsolin Antisera

Scinderin and gelsolin distribution in bovine adrenal chromaffin cells in culture was studied by using polyclonal an-
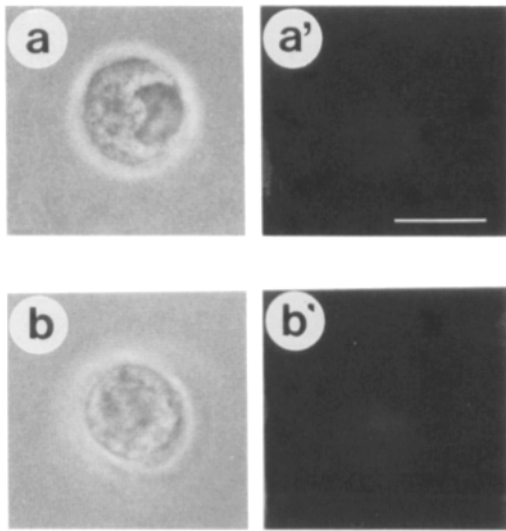
Figure 2. Control experiments for immunocytochemical staining. No staining was observed when chromaffin cells were incubated with antisera preadsorbed with the correspondent antigens (a', scinderin; b', gelsolin). a and b show phase contrast images of cells present in a' and b', respectively. Bars, 10 μM.

tisera against both proteins. To study the distribution of scinderin and gelsolin in bovine adrenal chromaffin cells in culture, it was important to demonstrate the specificity of the polyclonal antisera to be used. Previous work from our laboratory demonstrated that scinderin and gelsolin antisera are specific for these proteins and that do not cross-react with any other chromaffin cell cytosolic protein (Bader et al., 1986; Rodriguez Del Castillo et al., 1990; Tchakarov et al., 1990). Cross-reactivity of both antisera with chromaffin cell cytoskeletal proteins was also investigated. Fig. 1 A shows electrophoretic patterns of chromaffin cell cytoskeletal proteins (lane 1) and partially purified scinderin preparation also containing a small amount of gelsolin (lane 2). Myosin (M), tubulin (T), and actin (A) can be recognized as components of the cytoskeleton. Preparations of chromaffin cell cytoskeleton (Fig. 1 A, lanes 1', 1'') and partially purified scinderin (Fig. 1 A, lanes 2', 2'') were transferred onto nitrocellulose membranes and incubated with either scinderin (Fig. 1 A, lanes 1' and 2') or gelsolin antisera (Fig. 1 A, lanes 1'' and 2''). Scinderin antiserum 6 did not cross-react with either gelsolin or any cytoskeletal protein. Furthermore, gelsolin antiserum did not cross-react with any cytoskeletal protein or scinderin. To determine whether small amounts of scinderin or gelsolin could be associated with the cytoskeleton, gels (Fig. 1 B) were overloaded with four times more cytoskeletal protein (200 μg of 1% Triton-X-100 insoluble proteins). The immunoblots obtained from these gels indicated the presence in the cytoskeletal preparation of small amounts of both scinderin (Fig. 1 B, lane 1') and gelsolin (Fig. 1 B, lane 1''). However, it should be pointed out that the amount of scinderin associated with the cytoskeleton represents <1% of the total cellular scinderin. In addition, to discard a possible cross-reactivity of scinderin or gelsolin antisera with any chromaffin cell protein, gels were also overloaded with 200 μg of total protein. Here again, the immunoblots showed bands corresponding to either scinderin (Fig. 1 B, lane 2') or gelsolin (Fig. 1 B, lane 2''), and indicated no cross-reac-

tivity of antisera with any additional chromaffin cell protein. These results show the specificity of antisera against scinderin and gelsolin. Therefore, changes in the distribution of either of the two antibodies will reflect changes in scinderin or gelsolin subcellular localization.

(b) Distribution of Scinderin, Gelsolin, and of F-Actin in Resting and Nicotine-stimulated Chromaffin Cells: A Fluorescence Microscopy Study

Chromaffin cells, cultured for 48 h, were incubated with regular Locke's solution alone or in the presence of 10 μM nicotine for 5, 20, or 40 s. At the end of these incubation periods, cells were processed for immunofluorescence using scinderin or gelsolin antibodies. Control experiments demonstrated that chromaffin cells were not stained when incubated with antisera adsorbed with the correspondent antigens (Fig. 2).

To investigate within the same cell the subcellular organization of scinderin or gelsolin together with that of F-actin some preparations were also stained with rhodamine-labeled phalloidin, a probe for filamentous actin. Scinderin distribution in control cells showed a bright and continuous cortical fluorescent ring and a less intense and diffuse cytoplasmic fluorescence (Fig. 3, a and e'). Nicotine stimulation caused a fragmentation of the bright fluorescent ring suggesting a redistribution of cortical scinderin. Patches of scinderin appeared clearly as a fragmented fluorescent ring at the equatorial plane of the cells (Fig. 3 A, b, c, and d). The effect of nicotine on scinderin reorganization was seen as early as 5 s of stimulation (Fig. 3 A, b).

Distribution of F-actin in chromaffin cells under resting conditions showed a continuous cortical fluorescent ring (Fig. 3 B, e and Fig. 4 B, a'). Stimulation of cells with 10 μM nicotine produced a disruption in the rhodamine-phalloidin cortical fluorescent pattern (Fig. 3 B, f, g, h; and Fig. 4 B, b', c'), suggesting depolymerization of F-actin. Interestingly, in 88 ± 3% of the nicotine-stimulated cells showing scinderin reorganization (916 of 1,200 total cells examined), there was a concomitant distribution of F-actin and scinderin (compare f and f', g and g', h and h' in Fig. 3 B).

Gelsolin distribution in chromaffin cells was studied using monoclonal and polyclonal antibodies (Fig. 4, A and B, respectively). Cells incubated with regular Locke's solution and treated with gelsolin polyclonal antiserum (1:20 dilution) showed a cortical cytoplasmic fluorescent pattern (Fig. 4 B, a'). The fluorescent ring underneath the plasma membrane was weaker than that observed in anti-scinderin-stained cells. Under resting conditions, only 14 ± 3% of antigelsolin-labeled cells (700 cells examined) showed a discontinuous cortical fluorescent pattern for gelsolin. Although exposure of chromaffin cells to nicotine for 40 s produced the disassembly of cortical F-actin network it did not cause any modification in the gelsolin cortical fluorescence (compare b' and b'', c' and c'' in Fig. 4 B). Additional experiments, using a mouse mAb (1:40 dilution) against the 47-kD chymoptryptic fragment of gelsolin, showed a diffuse cytoplasmic staining (Fig. 4 A, a, b, and c). To be certain that there was not redistribution of gelsolin or that gelsolin redistribution in response to cell stimulation was masked by a strong fluorescence due to a relative high concentration of antibodies used in the staining, different higher dilutions of either polyclonal (1:40, 1:80,

A

Single staining

0 s   5 s   20 s   40 s
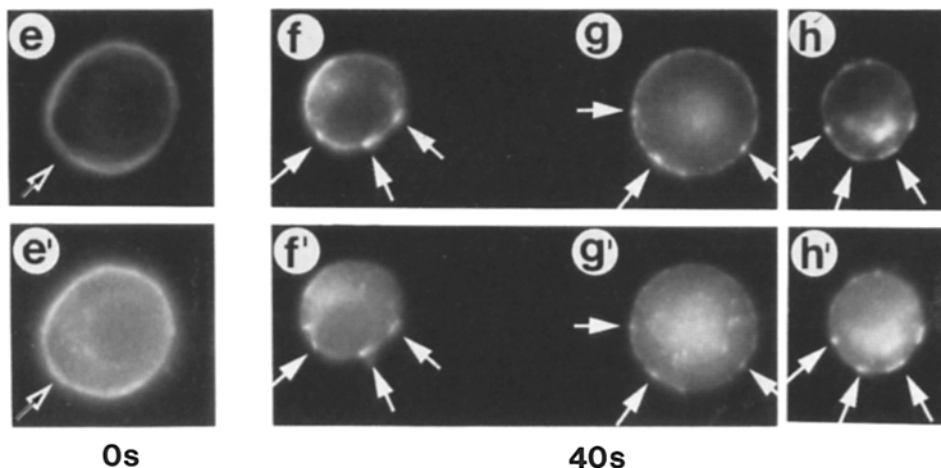
B

Double staining

0s   40s
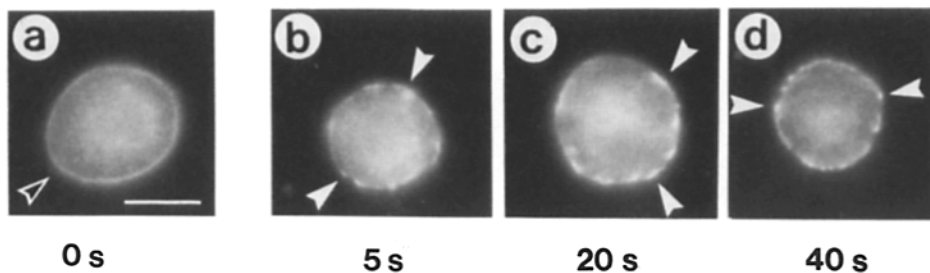
Figure 3. Localization of scinderin and actin by fluorescence microscopy. (*A*) Single staining: chromaffin cells were stained by incubation with scinderin antiserum 6 followed by FITC-anti-rabbit IgG. In resting cells (*a*) scinderin antibody fluorescence consists in a bright and continuous peripheral ring (*open arrowhead*) and a less intense and diffuse cytoplasmic staining. Incubation with 10 μM nicotine for 5 (*b*), 20 (*c*), or 40 s (*d*) produces a disruption of the cortical fluorescent pattern. Some patches are marked by arrowheads. Bar, 10 μM. (*B*) Double staining: chromaffin cells were incubated with Locke's solution for 40 s in the absence (*control*) or presence (*stimulated*) of 10 μM nicotine. Cells were sequentially stained with rhodamine-labeled phalloidin followed by scinderin antibody and FITC-anti-rabbit IgG. A control cell in (*e, e'*) shows continuous and intense rings of fluorescence for F-actin (*e*) and scinderin (*e'*) colocalized at the subplasmalemmal region (*open arrows*). Stimulated cells display a disrupted cortical fluorescent pattern either for F-actin (*f, g, h*) or scinderin (*f', g', h'*). There is a correspondence between the patched distribution of both actin and scinderin in each cell (compare *f* and *f', g* and *g', h* and *h'*). Some patches are indicated by arrows.

and 1:100) or monoclonal (1:80 and 1:160) gelsolin antibodies were tested. The results obtained from these experiments were similar to those described above with higher concentrations of antibodies.

(c) Time Courses of F-Actin Disassembly and Scinderin Redistribution in Chromaffin Cells Stimulated with Nicotine

Cells displaying scinderin redistribution in response to 10 μM nicotine stimulation also showed a similar time course for actin filament disassembly (Fig. 5). However, upon removal of the stimulus, the rate of recovery of cortical scinderin distribution was faster than that of cortical actin filament reassembly (Fig. 5). 80 s after the removal of nicotine from the incubation medium, 24 ± 3% (*n* = 300) of cells showed normal cortical distribution of scinderin with a concomitant fragmented cortical rhodamine-phalloidin fluorescence (Fig. 5).

(d) Time Courses of Scinderin and Gelsolin Redistribution and Catecholamine Release in Chromaffin Cells Stimulated with Either Nicotine or a Depolarizing Concentration of K+

In view of the earlier redistribution (5 s) of scinderin ob-

served upon cell stimulation, time courses of scinderin redistribution and catecholamine output were performed and compared. Chromaffin cells were incubated for 0, 5, 10, 20, or 40 s with either 10 μM nicotine or 56 mM K+ or 40 s with either 10 μM nicotine or 56 mM K+ followed by 50 or 80 s with regular Locke's solution. After these incubation periods, the cells were immediately fixed and processed for scinderin (antiserum 6) and gelsolin (antiserum) immunofluorescence microscopy. Cells were classified as having a continuous or a discontinuous cortical fluorescent pattern and the percentage of cells showing redistribution was plotted. During nicotinic stimulation, there was a sharp increase in the percentage of cells displaying a redistribution of the cortical scinderin fluorescence (Fig. 6 *A*, ▼). The maximum value (78 ± 2%) was reached 40 s after nicotinic stimulation was started. Removal of the stimulus at that point produced a decline in the number of cells showing a discontinuous fluorescent pattern. 80 s after the removal of nicotine from the incubation medium, scinderin redistribution reached control values.

In contrast to these observations the number of cells displaying a disrupted cortical fluorescent ring for gelsolin (9 ± 1%) was not modified by nicotinic stimulation (Fig. 6 *A*, ◆). Moreover, incubation with 10 μM nicotine for longer periods of time (up to 180 s) did not increase this percentage

A Single staining

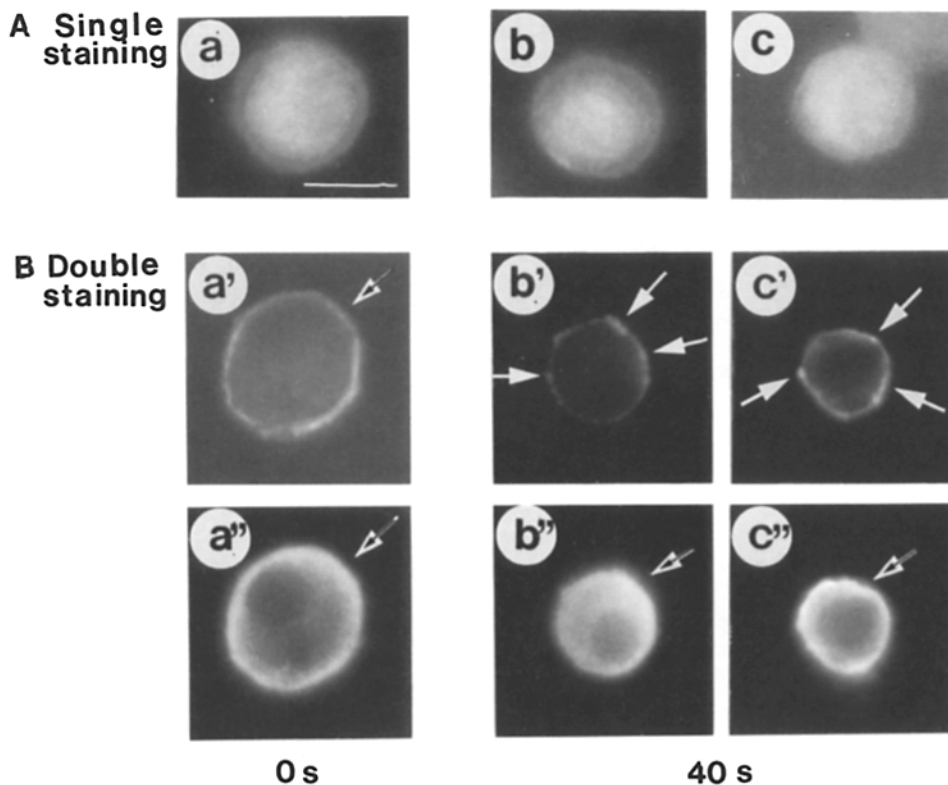## B Double staining

0 s   40 s

Figure 4. Localization of gelsolin and actin by fluorescence microscopy in cultured chromaffin cells. (*A*) Single staining: chromaffin cells were stained after incubation with a monoclonal antibody against gelsolin (1:40 dilution) followed by TRITC-anti–mouse IgG. Control cells (0 s) or cells incubated for 40 s with 10 μM nicotine (*b* and *c*) showed similar staining for gelsolin. Bar, 10 μM. (*B*) Double staining: chromaffin cells grown for 48 h were stained for F-actin with rhodamine-labeled phalloidin and then with gelsolin antiserum followed by FITC-anti-rabbit IgG. Control cells (0 s) display a bright peripheral fluorescent pattern for F-actin (*a'*) and a less intense cortical ring and a diffuse cytoplasmic staining for gelsolin (*a"*). Incubation of cells with 10 μM nicotine for 40 s produces a disruption of the F-actin cortical fluorescent pattern (*b'*, *c'*, *arrows*) without affecting gelsolin staining (*b"*, *c"*). Continuous peripheral fluorescent rings are marked by open arrows.

(data not shown). Control values for gelsolin redistribution were always lower than those for scinderin redistribution (9 ± 1 vs. 22 ± 3%).

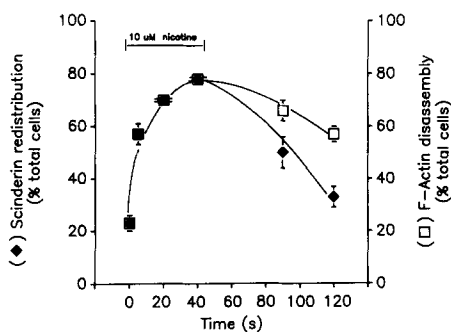Catecholamine output also rose sharply during nicotinic

Figure 5. Time courses of F-actin disassembly and scinderin redistribution in response to nicotine stimulation in cultured chromaffin cells. Chromaffin cells cultured for 48 h were incubated for 0, 5, 20, or 40 s with 10 μM nicotine or for 40 s with nicotine followed by an additional 50 or 80 s with regular Locke's solution. After these periods of incubation cells were immediately fixed, permeabilized, and processed for fluorescence microscopy using rhodamine-phalloidin and scinderin antiserum 6 as described in Materials and Methods. 100 cells per coverslip were examined and classified as having either a "continuous cortical fluorescent pattern" (see Fig. 3, *e–e'*) or a "discontinuous cortical fluorescent pattern" (see Fig. 3, *f–f'*). This was done without knowing whether cells were control or stimulated with nicotine. Each value shown represents the mean ± SEM of the percentage of discontinuous cortical fluorescence patterns of 6–8 coverslips (600–800 cells for each value) containing cells from three different chromaffin cell cultures. Upon removal of the stimulus the rate of recovery was different for scinderin and F-actin (90 and 120 s).

stimulation (Fig. 6 *A*, ○). However, the increase in catecholamine release lagged 15–20 s behind scinderin redistribution. [³H]NA output leveled off when nicotine was removed from the medium (Fig. 6 *A*, ○).

To test whether direct cell depolarization also induces scinderin redistribution, chromaffin cells were exposed to high K⁺ concentrations. Depolarization of chromaffin cells with 56 mM K⁺ also caused a reorganization of subplasmalemmal scinderin (Fig. 6 *B*, ▼). An increase in the number of cells displaying a patched fluorescent pattern could be detected 10 s after K⁺ depolarization was initiated. The maximum percentage of cells displaying a scinderin rearrangement (68 ± 7%) was observed after 20 s of depolarization; this value was lower than that obtained for nicotinic stimulation (78 ± 2%). Replacement of high K⁺ medium for regular Locke's solution produced a decline in the number of cells showing a disrupted scinderin cortical fluorescence. However, 80 s after lowering K⁺ concentration the values for scinderin redistribution were still higher than those of controls. The distribution of gelsolin was not modified by K⁺ depolarization (Fig. 6 *B*, ◆).

K⁺ depolarization stimulated catecholamine output (Fig. 6 *B*, ○); a sharp increase in release was detected 20 s after initiation of the K⁺ challenge. Removal of high K⁺ by replacing the incubation medium for regular Locke's solution leveled off [³H]NA release (Fig. 6 *B*, ○). As in the case of nicotinic stimulation, the rise in the percentage of cells showing discontinuous scinderin cortical fluorescent ring preceded the increase in catecholamine output.

(e) Characterization of Nicotine-induced Scinderin Redistribution: Evidence for a Nicotinic Receptor-mediated Event

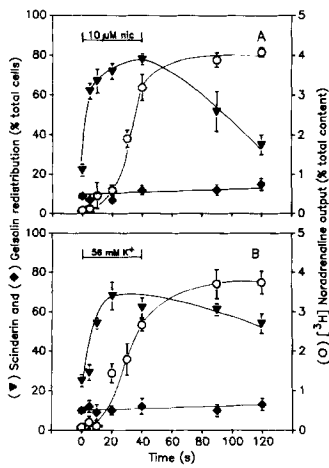We have previously demonstrated that stimulation of chro-

Figure 6. Time courses of scinderin and gelsolin redistribution and [³H]NA output in response to stimulation in cultured chromaffin cells. (A) Nicotinic stimulation: chromaffin cells cultured for 48 h were incubated for 0, 5, 10, 20, or 40 s with 10 μM nicotine or for 40 s with nicotine followed by an additional 50- or 80-s period with regular Locke's solution. After these periods of incubation, cells were immediately fixed, permeabilized, and processed for immunofluorescence microscopy using either scinderin (▼) or gelsolin (♦) antisera. 100 cells per coverslip were examined and classified, as described in the legend to Fig. 5. This was done without knowing whether cells were control or stimulated with nicotine. Each value plotted represents the mean ± SEM of the percentage of discontinuous cortical fluorescent pattern of 6–8 coverslips (600–800 cells for each value) containing cells from three different cell cultures. [³H]NA output (○): cultured chromaffin cells with catecholamine stores labeled with [³H]NA were incubated for 0, 5, 10, 20, 30, or 40 s with 10 μM nicotine or for 40 s with 10 μM nicotine followed by an additional 50- or 80-s period with regular Locke's solution. After each of those periods media were removed and their radioactivity measured. Basal [³H]NA output was determined by incubating the cells with nicotine-free Locke's solution for the same periods of time as above. Basal values (0.7–1.0%) were subtracted from the corresponding data obtained during stimulation. Nicotine-induced [³H]NA secretion is expressed as percentage of total [³H]NA cell content. Each point represents the mean ± SEM of values obtained from three different culture dishes. (B) K⁺ depolarization: Chromaffin cells grown in coverslips for 48 h were incubated at room temperature for 0, 5, 10, 20, or 40 s with a Locke's solution containing 56 mM K⁺ (high K⁺) or 40 s in high K⁺ followed by an additional 50- or 80-s period with regular Locke's solution (regular K⁺). [³H]NA output (○): chromaffin cells with their catecholamine stores labeled with [³H]NA were incubated with a 56 mM K⁺ Locke's solution for 0, 5, 10, 20, 30, or 40 s or for 40 s with high K⁺ Locke's solution followed by 50 or 80 s with regular Locke's solution. The procedure followed in these experiments was as described above for nicotine stimulation (A).

maffin cells by acetylcholine induces disassembly of cortical actin networks (Trifaró et al., 1989). Because acetylcholine stimulates both, muscarinic and nicotinic receptors, experiments were performed to determine whether scinderin redistribution was exclusively a nicotinic mediated response or was also affected by muscarinic receptor stimulation. Stimulation of chromaffin cells with 10 μM nicotine for 40 s produced a fourfold increase in the percentage of cells showing scinderin reorganization when compared with control levels (Fig. 7). The redistribution of scinderin was blocked when 10 μM D-tubocurarine (dtc), a nicotinic antagonist, was present in the incubation medium (Fig. 7). Moreover, incubation of cells with 10 μM muscarine (musc) for 40 s did not cause any rearrangement of subplasmalemmal scinderin (Fig. 7) or F-actin disassembly (control: 20 ± 4%, n = 300 cells and musc: 18 ± 3% disassembly, n = 300 cells). The results clearly indicate that scinderin redistribution is a nicotinic receptor-mediated event.
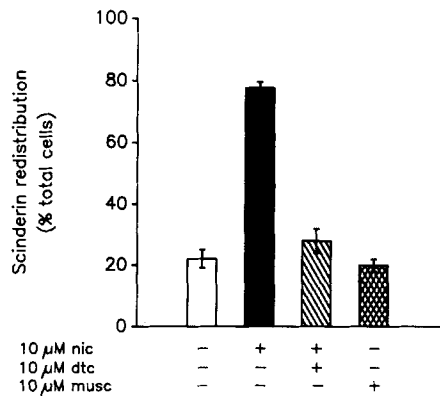

Figure 7. Effects of cholinergic and anticholinergic drugs on scinderin redistribution in chromaffin cells in culture. 48-h-old chromaffin cell cultures grown on collagen coverslips were incubated for 40 s under the following experimental conditions: (a) regular Locke's solution alone (control), Locke's solution containing (b) 10 μM nicotine (nic), (c) 10 μM nicotine plus 10 μM D-tubocurarine (dtc) or (d) 10 μM muscarine (musc). After these treatments, cells were fixed, permeabilized, and processed for immunofluorescence using scinderin antiserum 6. 100 cells per coverslip were examined and scinderin distribution was classified as described in the legend to Fig. 5. Each value represents the mean ± SEM of the percentage of discontinuous scinderin distribution of 6–8 coverslips (600–800 cells for each value) containing cells from three different cell cultures.

(f) Calcium Dependence of Scinderin Redistribution and F-Actin Disassembly by Nicotine-stimulated or K⁺-depolarized Chromaffin Cells

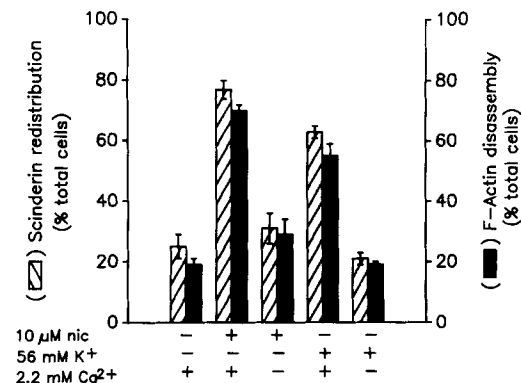Catecholamine release from chromaffin cells in response to

Figure 8. Effect of extracellular Ca²⁺ on nicotine-evoked or high K⁺-induced scinderin redistribution and identical F-actin disassembly in cultured chromaffin cells. Chromaffin cells grown for 48 h on collagen coated coverslips were incubated with Locke's solution (control) or with Locke's solution containing 10 μM nicotine (nic) or 56 mM K⁺ either each case containing either 2.2 mM Ca²⁺ or 0.1 mM EGTA (Ca²⁺ free). Following these incubations, cells were fixed, permeabilized, and processed for double staining fluorescence microscopy using rhodamine-labeled phalloidin and scinderin antiserum 6 as indicated in Materials and Methods. 100 cells per coverslip were examined for scinderin (fluorescein staining) and for F-actin (rhodamine fluorescence) peripheral distribution and were classified as having a continuous or a patched cortical staining. Each value represents the mean ± SEM of the percentage of discontinuous scinderin and F-actin distribution of 4–5 coverslips (400–500 cells for each value) containing cells from two different cell cultures.
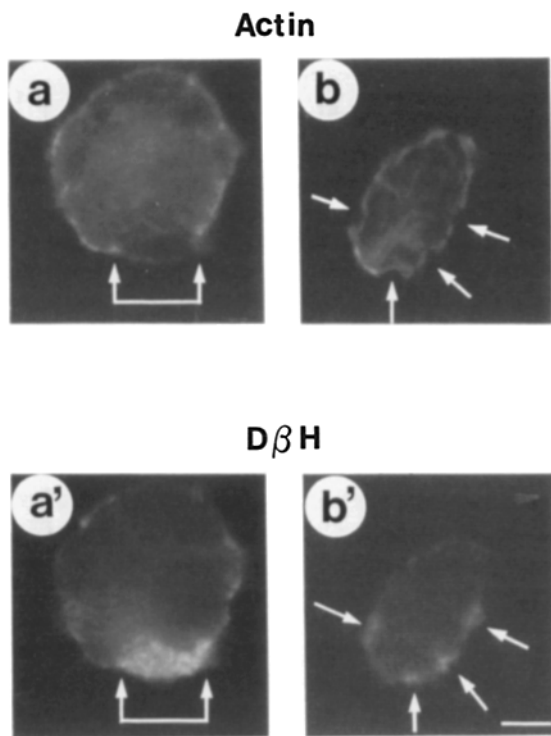
Actin

DβH

Figure 9. Presence of surface DβH in cortical areas devoid of rhodamine–phalloidin staining. Chromaffin cells grown for 48 h were incubated for 40 s with 10 μM nicotine, fixed and stained for DβH and F-actin as described in Materials and Methods. Fluorescent patterns of two stimulated cells (a, a' and b, b') are shown as they appeared after incident light illumination for rhodamine (a and b) and fluorescein (a' and b'). Arrows show the absence of rhodamine–phalloidin staining (F-actin) and the presence of FITC-IgG staining. Bar, 5 μM.

stimulation is triggered by the entry of extracellular Ca^{2+} through so-called slow Ca^{2+} channels. Consequently, it was of interest to determine whether scinderin redistribution also requires the presence of extracellular Ca^{2+}. In resting conditions, 25 ± 4% of chromaffin cells showed discontinuous distribution of subplasmalemmal scinderin (Fig. 8). This percentage rose when the cells were incubated for 40 s with 10 μM nicotine or were exposed for the same period of time to 56 mM K^+ (77 ± 3 and 63 ± 2%, respectively) (Fig. 8). F-actin disassembly either during resting conditions or stimulation also followed a similar pattern of distribution (Fig. 8). Disruption of scinderin and actin cortical fluorescent rings in both experimental conditions was not observed in the absence of extracellular Ca^{2+} (Fig. 8). The results indicate that stimulation evoked Ca^{2+} influx is a necessary requirement for scinderin redistribution and F-actin depolymerization in response to either nicotine stimulation or K^+ depolarization. Moreover, the data also show that Ca^{2+} entry affects F-actin disassembly and scinderin reorganization in the same manner, suggesting again a close functional relationship between the two proteins.

(g) Exocytosis Is Observed in Areas of F-Actin Disassembly

The disassembly of cortical F-actin produced by cell stimulation would suggest that subplasmalemmal areas devoid of

F-actin are formed and that these might be zones of low cytoplasmic viscosity and probably high secretory granule mobility. The absence of a cytoskeletal barrier in these areas, would allow the interaction of secretory granules with plasma membranes with the subsequent release of granule contents to the cell exterior by exocytosis. To test the possibility that exocytotic pits might be present in plasma membrane areas devoid of F-actin, chromaffin cells were stimulated with 10 μM nicotine for 40 s, fixed, and incubated with anti-DβH-IgG to detect the presence of chromaffin granule membranes on the cell surface. Fig. 9 (*arrows*) shows DβH cell surface staining in areas devoid of F-actin as indicated by the absence of rhodamine phalloidin fluorescence.

Discussion

Work from our laboratory, as well as others, has demonstrated that filamentous actin is mainly localized in the cortical surface of the chromaffin cell (Lee and Trifaró, 1981; Trifaró et al., 1984, 1989; Cheek and Burgoyne, 1986). We have also suggested that cortical F-actin acts as a barrier to the secretory granules, impeding their contact with the plasma membrane. Chromaffin granules contain α-actinin (Aunis et al., 1980; Trifaró et al., 1982) and fodrin (Perrin and Aunis, 1985), anchorage proteins which mediate filamentous actin association with these vesicles. Stimulation of chromaffin cell produces disassembly of actin networks and removal of the barrier (Cheek and Burgoyne, 1986, 1987; Burgoyne et al., 1989; Trifaró et al., 1982, 1984, 1989). This interpretation is based on the following evidence. Cytochemical experiments with rhodamine-labeled phalloidin and actin antibodies indicated that in resting chromaffin cells, a filamentous actin network is visualized as a strong cortical fluorescent ring (Lee and Trifaró, 1981; Cheek and Burgoyne, 1986, 1987; Trifaró et al., 1989). Nicotinic receptor stimulation produces a fragmentation of this fluorescent ring leaving cell cortical areas devoid of fluorescence (Cheek and Burgoyne, 1986, 1987; Trifaró et al., 1989). These changes are accompanied by a decrease in F-actin associated with a concomitant increase in G-actin as evaluated by the DNase I inhibition assay (Cheek and Burgoyne, 1986; Trifaró et al., 1989). These changes are also accompanied by a decrease in the amount of F-actin recovered with the Triton-X-100 insoluble (cytoskeleton) protein (Burgoyne et al., 1989; Trifaró, 1990). F-actin network disassembly has also been observed in mast cells upon stimulation (Koefer et al., 1990) and in depolarized (high K^+) synaptosomes (Bernstein and Bamburg, 1985).

The present experiments clearly demonstrate that stimulation of chromaffin cells with either nicotine or a depolarizing concentration of K^+ causes disassembly of cortical F-actin networks and redistribution of subplasmalemmal scinderin. Gelsolin on the other hand does not show such a rearrangement. To be certain that this was the case, polyclonal and monoclonal antibodies against gelsolin were used and these were tested at different dilutions on resting and stimulated cells. Under these conditions, no rearrangement or changes in the fluorescence pattern of gelsolin were observed. These observations ruled out the possibility that gelsolin redistribution in response to cell stimulation was masked by a strong antibody fluorescence. Thus, the effect of cell stimulation seems to be quite specific for scinderin. Previous studies

from our laboratory have demonstrated that scinderin is a structurally different protein from gelsolin (Rodriguez Del Castillo et al., 1990). Scinderin and gelsolin have different molecular weights, isoelectric points, amino acid composition and yield different peptide maps after limited proteolytic digestion (Rodriguez Del Castillo et al., 1990). Both proteins have an actin filament severing activity which is Ca^{2+} dependent; in the case of gelsolin, severing activity is inhibited by phosphatidylinositol 4,5 biphosphate (Yin et al., 1988). Further work from our laboratory has demonstrated different tissue expressions for scinderin and gelsolin. Scinderin seems to be expressed in neuronal, endocrine, and exocrine tissues (Tchakarov et al., 1990) systems in which secretion is a main function. Immunocytochemical studies showed that in chromaffin cells scinderin has a diffuse cytoplasmic and a more dense subplasmalemmal distribution (Rodriguez Del Castillo et al., 1990). Instead, gelsolin only showed a diffuse cytoplasmic distribution (Rodriguez Del Castillo et al., 1990). Therefore, experimental data suggest that gelsolin and scinderin are two distinct Ca^{2+}-dependent F-actin severing proteins that may also differ in their fine regulation by intracellular messengers.

The present studies also show that scinderin redistribution and actin filament disassembly, induced by either nicotine or high K^+, precedes catecholamine release. The lag period observed between scinderin redistribution/F-actin disassembly and catecholamine release was not due to the lack of sensitivity of the catecholamine release assay used. We have previously demonstrated that the [³H]NA taken up by cultured chromaffin cells is stored and released together with endogenous catecholamines and that the measurement of [³H]NA in the incubation medium gives a precise indication of total catecholamine release (Trifaró and Lee, 1980; Kenigsberg and Trifaró, 1980; Trifaró and Bourne, 1981). The catecholamine assay used in the present experiments detect catecholamine concentrations equal to 0.35% of total content. The present results show that after 5 s of initiated cell stimulation, 65% of the cells showed scinderin redistribution and F-actin disassembly. The catecholamine assay could easily have detected 65% (2.5% of total catecholamine content) of the total release (4.0% of total catecholamine content) observed at the end of the stimulation period if catecholamines were released concomitantly with scinderin redistribution and F-actin disassembly. Moreover, similar time courses and amounts of catecholamine release during the first minute of stimulation have also been previously observed by us (Côté et al., 1986) and other laboratories (Baker et al., 1985; TerBush et al., 1988; Bittner and Holz, 1990). It can also be argued that the lag period observed was due to a slow diffusion of catecholamines into the incubation medium. This seems unlikely, since release experiments on cultured cells eliminates physiological barriers such as capillary endothelial walls, et cetera.

The rates of F-actin disassembly and scinderin redistribution during stimulation were found to be similar and subplasmalemmal areas showing filamentous actin also showed cortical scinderin. Immunocytochemical studies have also shown that caldesmon (Burgoyne et al., 1986) and fodrin (Perrin and Aunis, 1985) are preferentially localized in the cortical region of the chromaffin cell. Moreover, in one of these studies (Perrin and Aunis, 1985) a redistribution of cortical fodrin antibody fluorescence was observed upon nicotinic or high K^+ stimulation. However, in this case the time course of fodrin redistribution was much slower than that described for scinderin in the present experiments. In view of the observations described in this paper, it can also be argued that scinderin shows a subplasmalemmal distribution because it is bound to filamentous actin. However, this notion should be discarded since after removal of the stimulus the rate of recovery of scinderin cortical fluorescence was faster than that of rhodamine-phalloidin fluorescence. In other words, during the post stimulation period, a significant number of cells displayed cortical continuance of scinderin fluorescence in the presence of a fragmented ring of rhodamine-phalloidin fluorescence. This would suggest that scinderin is retained in the cortical region of the resting cell through its binding to a site other than filamentous actin. Maekawa and Sakai (1990) have shown the presence in chromaffin cells of a 74-kD actin filament–severing protein which binds to phosphatidylinositol and phosphatidylserine in a Ca^{2+}-dependent manner. Although the possibility exists that scinderin (79.6 kD) and the 74 kD protein described above are the same protein, there is no evidence of these at the present time. Moreover, scinderin seems to be bound or retained in the cortical region of the cell under resting conditions and during recovery from secretion, conditions which are characterized by low intracellular Ca^{2+} levels. Furthermore, muscarinic stimulation does not release catecholamines (Wilson and Kirshner, 1977; Fisher et al., 1981), redistribute scinderin or produce actin filament disassembly. It is known that, in adrenal chromaffin cells, nicotine and high K^+ induce the entry and a rise in cytosolic Ca^{2+}, which is necessary for catecholamine secretion (Douglas and Rubin, 1961; Douglas, 1968; Cheek et al., 1989; Kim and Westhead, 1989; O'Sullivan et al., 1989). Muscarine produces mobilization of Ca^{2+} from intracellular stores (Wilson and Kirshner, 1977; Kim and Westhead, 1989) an effect which is independent of extracellular Ca^{2+} (Kao and Schneider, 1985) and is mediated by inositol 1,4,5 triphosphate (Hughes and Putney, 1990). The reduced and localized release of Ca^{2+} induced by muscarine is not enough to trigger catecholamine release and, as shown in these studies, scinderin redistribution. Therefore, only secretagogues that induce Ca^{2+} entry are able to redistribute subplasmalemmal scinderin and produce the disassembly of F-actin networks leaving cytoplasmic areas devoid of these two proteins. We have previously demonstrated by low shear viscometry that the concentrations of Ca^{2+} required by scinderin to induce a fall in the viscosity of actin gels are in the range of Ca^{2+} concentrations expected to be found in the chromaffin cell cytoplasm as a result of cell stimulation (Rodriguez Del Castillo et al., 1990). These observations suggest that Ca^{2+} entry might regulate the actin filament–severing activity of scinderin. One is tempted to speculate that cell stimulation and Ca^{2+} entry bring about activation of proteins such as scinderin with a consequent severing of cortical actin filament networks. This should produce subplasmalemmal areas of decreased viscosity and high secretory granule mobility, allowing subsequent interaction of granules with the plasma membrane. The experiments with anti-DβH described here seem to indicate that exocytotic pits are preferentially present in plasma membrane areas devoid of F-actin. Bound DβH is a chromaffin granule component with a specific membrane topology (Joh and Hwang, 1982). No granule surface DβH can be found and the en-

zyme has an intragranular domain recognized by the antibody. Therefore, when secretory vesicle membranes are inserted into plasma membranes during exocytosis, antigenic DβH sites are exposed on the cell surface, allowing visualization of plasma membrane exocytosis sites (Phillips et al., 1983).

Activation of scinderin and actin filament disassembly seem to precede exocytosis. However, to consider this as the only important phenomenon in secretion would be an oversimplified notion of what might be the fine regulation of exocytosis in which intervention of other messengers and modulators such as calmodulin (Kenigsberg and Trifaró, 1985), cAMP (Cheek and Burgoyne, 1987), G-proteins (Matter et al., 1989; Bader et al., 1989), polyphosphatidylinositol breakdown (Janney and Stossel, 1987; Forsher, 1989), et cetera, may occur.

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