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 Ca2+-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.

Chromaffin cells of the adrenal medulla store their secretory products in specialized organelles, the chromaffin granules (Smith, 1968; Trifarò, 1977). In response to cholinergic stimulation and upon Ca2+ entry, the granules fuse with the plasma membrane and release their soluble contents to the cell exterior by exocytosis (Trifarò, 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 Trifarò, 1981; Trifarò 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 (Trifarò et al., 1982, 1984, 1989; Cheek and Burgoyne, 1986, 1987; Burgoyne and Cheek, 1987; Burgoyne et al., 1989). Evidence 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; Trifarò 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 Ca2+-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; Trifarò et al., 1985; Bader et al., 1986).
Bovine adrenal glands were obtained from a local slaughterhouse and density of 0.25 x 10^6 cells/35-mm dish for fluorescence microscopy using a Percoll gradient (Trifar6 and Lee, 1980). Cells were plated on collagen-coated glass coverslips contained within plastic Petri dishes at a 37°C in a humidified incubator under a CO2 + air atmosphere for 48 h as cells/35-mm dish for catecholamine release studies. Cells were grown at previously described (Trifar6 and Lee, 1980).

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. 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. Therefore, the preparation obtained in this case also contains small amounts of gelsolin and other Ca^2+ dependent actin-binding proteins.

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 PMSE, 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 PMSE) containing 10 mM KCl. The column was then washed extensively (300 ml) with the same buffer 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 PMSE). Samples from either total proteins or EGTA eluates were used in the experiments.

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.

Monodimensional SDS-PAGE was performed according to Dauvet and Trifar6 (1988); gels were usually run at 60 V overnight in a Bio-Rad Protein 1 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 anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (1:3000 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.
Coverslips were thoroughly washed with PBS and were next incubated at 37°C with goat anti-rabbit 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 (Paulish 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 D2H/F-actin staining, cells incubated for 40 s with 10 μM nicotine were fixed with 3.7% formaldehyde and then incubated with anti-D2H 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 Plomopack 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.

**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 I', 2', 1', and 2' are the immunoblots of the same preparations after incubation with scinderin antiserum 6 (lanes I' and 2') or gelsolin antisem (lanes I' 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 I'-3' are the immunoblots of the same preparations after incubation with scinderin antisem 6 (lanes I', 2', and 3') or gelsolin antisem (lanes I', 2', and 3'). A faint reaction is observed with scinderin antisem 6 (lanes I' and 2') and with gelsolin antisem (lanes I' 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 Antiserum

Scinderin and gelsolin distribution in bovine adrenal chromaffin cells in culture was studied by using polyclonal ant-
of both scinderin (Fig. 1 B, lane 1') and gelsolin (Fig. 1 B, lane 2'). The immunoblots obtained from these gels indicated the presence in the cytoskeletal preparation of small amounts of gelsolin or any cytoskeletal protein. To discard a possibility of scinderin associated with the cytoskeleton represents <1% of the total cellular scinderin. In addition, scinderin antiserum 6 did not cross-react with any cytoskeletal protein (200 µg of 1% Triton-X-100 insoluble protein) or gelsolin (lane 1'). Myosin (M), tubulin (T), and actin (A) can be recognized as components of the cytoskeleton. Preparations 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 cytoskeletal proteins (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-

(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 redistribution of cortical scinderin. Patches of scinderin appeared as small as 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 concurrent 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 antisera (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 cytoplasmic 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, 1:400, 1:800, 1:4,000) were used. 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.
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 observed 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, v). 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.
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 stimulation (Fig. 6 A, o). 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, o).

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, v). 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, o).

K+ depolarization stimulated catecholamine output (Fig. 6 B, o); 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, o). 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.

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

We have previously demonstrated that stimulation of chro-
Catecholamine release from chromaffin cells in response to nicotine stimulation and [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 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 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 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 nicotine-evoked or high Ca²⁺-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.
The relationship between the two proteins.

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 mM 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. Cytotoxic 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 [\(^3\)H]NA taken up by cultured chromaffin cells is stored and released together with endogenous catecholamines and that the measurement of [\(^3\)H]NA in the incubation medium gives a precise indication of total catecholamine release (Trifaro and Lee, 1980; Kenigsberg and Trifaro, 1980; Trifaro 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, etc.

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-D\(^{3}\)H described here seem to indicate that exocytotic pits are preferentially present in plasma membrane areas devoid of F-actin. Bound D\(^{3}\)H is a chromaffin granule component with a specific membrane topology (Joh and Hwang, 1982). No granule surface D\(^{3}\)H can be found and the en...
zyme has an intragranular domain recognized by the anti- 
body. Therefore, when secretory vesicle membranes are in-
serted into plasma membranes during exocytosis, antigenic 
DPIV sites are exposed on the cell surface, allowing visual-
ization 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 
over-simplified notion of what might be the fine regulation of 
exocytosis in which intervention of other messengers and mod-
ulators such as calmodulin (Kenigsberg and Trifaro, 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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