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Dynamic Changes in Chromaffin Cell Cytoskeleton as Prelude to Exocytosis

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Contents

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Abstract

Earlier work by us as well as others has demonstrated that filamentous actin is mainly localized in the cortical surface of chromaffin cell. This F-actin network acts as a barrier to the chromaffin granules, impeding their contact with the plasma membrane. Chromaffin granules contain α-actinin, an anchorage protein that mediates F-actin association with these vesicles. Consequently, chromaffin granules crosslink and stabilize F-actin networks. Stimulation of chromaffin cell produces disassembly of F-actin and removal of the barrier. This interpretation is based on: (1) Cytochemical experiments with rhodamine-labeled phalloidin indicated that in resting chromaffin cells, the F-actin network is visualized as a strong cortical fluorescent ring; (2) Nicotinic receptor stimulation produced fragmentation of this fluorescent ring, leaving chromaffin cell cortical areas devoid of fluorescence; and (3) These changes are accompanied by a decrease in F-actin, a concomitant increase in G-actin, and a decrease in the F-actin associated with the chromaffin cell cytoskeleton (DNAse I assay). We also have demonstrated the presence in chromaffin cells of gelsolin and scinderin, two Ca^{2+}-dependent actin filament-severing proteins, and suggested that chromaffin cell stimulation activates scinderin with the consequent disruption of F-actin networks. Scinderin, a protein recently isolated in our laboratory, is restricted to secretory cells and is present mainly in the cortical chromaffin cell cytoplasm. Scinderin, which is structurally different from gelsolin (different pI_s, amino acid composition, peptide maps, and so on), decreases the viscosity of actin gels as a result of its F-actin–severing properties, as demonstrated by electron microscopy. Stimulation of chromaffin cells either by nicotine (10 μ*M*) or high K+ (56 m*M*) produces a redistribution of subplasmalemmal scinderin and actin disassembly, which preceded exocytosis. The redistribution of scinderin and exocytosis is Ca^{2+}-dependent and is not mediated by muscarinic receptors. Furthermore, our cytochemical experiments demonstrate that chromaffin cell stimulation produces a concomitant and similar redistribution of scinderin (fluorescein-labeled antibody) and F-actin (rhodamine phalloidin fluorescence), suggesting a functional interaction between these two proteins. Stimulation-induced redistribution of scinderin and F-actin disassembly would produce subplasmalemmal areas of decreased cytoplasmic viscosity and increased mobility for chromaffin granules. Exocytosis sites, evaluated by antidopamine-β-hydroxylase (anti-DβH) surface staining, are preferentially localized in plasma membrane areas devoid of F-actin.

Introduction

The discovery of the synaptic vesicle by De Robertis and Bennet (1954,1955) and the subsequent description of exocytosis by De Robertis and Vaz Ferreira (1957) started a new era of research on the mechanism of neurotransmission. The process of exocytosis is a fascinating interplay between cellular components and the secretory vesicle. It has become clear in recent years that secretion by cells can take two forms: constitutive and regulated (Tartakoff et al., 1978; Gumbiner and Kelly, 1982; Green and Shields, 1984; Kelly, 1985). Constitutive secretion is the form of secretion that is unregulated and closely follows the rates of synthesis of secretory products. This form of secretion occurs in almost all cell types including lymphocytes, liver, and yeast cells (Buckley and Kelly, 1985; Kelly, 1985; Rothman et al., 1985). The other form of secretion is highly regulated and characteristic of endocrine and exocrine cells as well as neurons (Smith, 1968; Trifaró, 1977,1990).

Cells displaying regulatory secretory pathways store their secretory products in membrane-bound secretory granules or vesicles (Smith, 1968; Trifaró, 1977; Trifaró and Poisner, 1982). Vesicle storage not only allows secretory tissues to store a large amount of secretory material in a relatively small vol but also protects this material for intracellular degradation and provides a very efficient means of transporting and releasing fixed amounts (quanta) of secretory substances (Trifaró, 1977; Trifaró and Poisner, 1982). Regulated secretion is triggered by an increase in intracellular calcium. However, despite the fact that the role of Ca^{2+} in secretion was observed several years ago (Houssay and Molinelli, 1928;

Harvey and MacIntoch, 1940; Douglas and Rubin, 1961; Douglas, 1968), the exact mechanism by which Ca^{2+} is involved in the secretory process is still poorly understood.

One attractive hypothesis is that the action of Ca^{2+} in secretion is mediated through the control by this ion of cellular cytoskeleton networks (Trifaró, 1990). A group of the specific proteins, some of them previously known from studies on muscle, form a cytoplasmic network in all nonmuscle cells including neurons and neurosecretory cells (Trifaró, 1978; Birchmeier, 1984). Immunocytochemical studies have shown at least three types of filament systems: microfilaments, microtubules, and intermediate filaments (neurofilaments in neuronal tissue). In addition to the three filament systems, a large variety of cytoskeleton-associated proteins has been characterized (Trifaró et al., 1984; Trifaró et al., 1985a,b). These cytoskeleton-associated proteins can be classified according to their functions: e.g., proteins that favor (actinogelin) or disrupt (gelsolin, scinderin) microfilament formation, proteins that serve as anchorage for cytoskeleton elements to membranes (vinculin, spectrin or fodrin, α-actinin, ankyrin), and proteins that regulate the phosphorylation of cytoskeleton elements (calmodulin).

On the basis of their well established role in muscle contraction, it is generally assumed that the prime function of contractile proteins, as part of the cytoskeleton in nonmuscle cells, is to generate the force required in many expressions of cell motility (Trifaró, 1978). Cells move and subcellular organelles (mitochondria, lysosomes, and secretory vesicles) move within cells; some of these movements might require the intervention of contractile proteins. In this regard, it has also been observed that the cytoskeleton system interacts not only with the plasma membrane but also with different organelles (Trifaró et al., 1985a; Trifaró et al., 1985b; Trifaró et al., 1988; Trifaró, 1990). Alternatively, cytoskeleton elements may oppose the movement of organelles by controlling cytoplasm viscosity (Trifaró et al., 1982; Trifaró et al., 1985a,b).

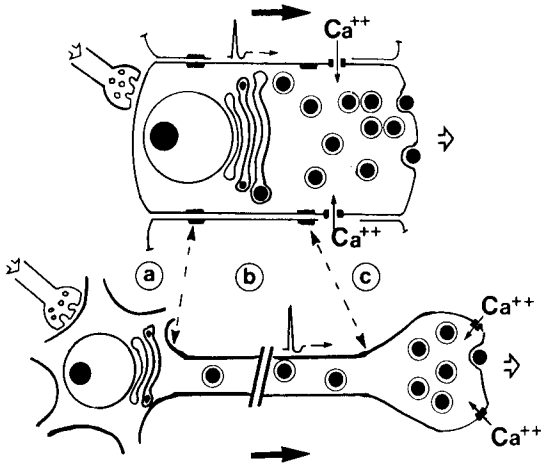

Fig. 1. Schematic representation of a neuron and paraneuron. The functional characteristics in three different regions—a, b, and c—are indicated by broken lines. The input region **(a)** is the reception site (receptors) for the stimulus. The stimulus-induced membrane changes in **(a)** are accompanied by the inward movements of Na^+ and Ca^{2+}, with the generation of "all-or-none" action potentials that travel down the intermediate conductive region (b). The arrival of the action potential to the output region (c) produces an opening of slow Ca^{2+} channels with a concomitant rise in intracellular Ca^{2+}. The increase in intracellular Ca^{2+} triggers the secretory machinery, and the secretory vesicles discharge their content by exocytosis. Paraneurons are then functionally polarized cells receiving information in one end and releasing material for export at the opposite side (Trifaró, 1990).

Chromaffin cells are secretory cells among those nonmuscle cells from which cytoskeleton proteins have been isolated and characterized (Trifaró and Ulpian, 1976; Lee et al., 1979; Aunis et al., 1980; Trifaró et al., 1982,1984,1985b,1985a, 1988,1989). The cells of the adrenal medulla are derived embryologically from the neural crest sharing a common origin with the sympathetic neurons and common subcellular features with many endocrine cells. One such feature is the storage of secretory products and membrane-bound organelles. Secretory cells with this characteristic display regulated secretion and have been named "paraneurons" (Fig. 1), a term that embraces cells that are generally and traditionally not considered neurons and yet should be regarded as "relatives" of neurons on the ba-

sis of their structure and function (Trifaró, 1982, 1984, 1990). The cytoskeleton is a dynamic structure that is changing according to different stages of cell function. In the following pages, the dynamic changes observed in the cytoskeleton during the secretory process of chromaffin cell are discussed.

Actin Microfilaments and Actin-Binding Proteins

Actin

Work on isolated chromaffin cells treated with 25% glycerol and exposed to fluorescein-labeled heavy meromyosin first suggested the presence of actin in chromaffin cells (Trifaró and Lee, 1978). However, the unequivocal demonstration of the presence of actin in chromaffin cells can be found in subsequent studies on the isolation and characterization of this protein. In these studies, actin was purified from chromaffin cell cytosol by DNAse I affinity chromatography (Lee et al., 1979). An SDS-PAGE of the fraction eluted from DNAse I column by 3*M* guanidine hydrochloride revealed two bands: a major one (92%) of mol mass 42 kDa, which comigrated with muscle actin, and a minor one (10%) of mol mass 90 to 91 kDa, which was probably an actin-binding protein such as gelsolin (Lee et al., 1979).

Two-dimensional electrophoresis performed on adrenal actin, muscle actin, and a mixture of both actins revealed the presence in chromaffin cells of two isomeric forms of actin (β and γ) that have the same mol wt of muscle actin (α) but different isolectric points (Lee et al., 1979). Antibodies against actin were also used to investigate localization of actin in cultured bovine chromaffin cells by indirect immunofluorescence (Trifaró and Lee, 1978; Aunis et al., 1980; Lee and Trifaró, 1981). The staining pattern obtained showed actin to be widely distributed in the cells and associated with many cellular structures. A strong membrane and a weak cytosol fluorescence were observed when freshly isolated or 1-d-old cultured cells were used (Fig. 2A).

Membrane patching and capping patterns were also apparent (Lee and Trifaró, 1981). More recent studies using rhodamine-labeled phalloidin, a substance with affinity only for filamentous actin, demonstrated the presence of fluorescence in the subplasmalemma area of chromaffin cells, suggesting again the presence of an actin network in this region (Burgoyne and Cheek, 1988; Trifaró et al., 1989). Seven- to eight-day-old chromaffin cell cultures exhibited, in addition to membrane fluorescence, a fine granular fluorescence in the cytosol of the cell body, neurites, and terminal cones (Fig. 2B) (Lee and Trifaró, 1981). The granular fluorescent pattern obtained with antiactin was similar to that observed when chromaffin cells were stained with an antibody against DβH (Fig. 2C) (Lee and Trifaró, 1981; Trifaró et al., 1984). The similarity in the staining patterns suggested that actin could be in part, associated with chromaffin granules.

Consequently, antiactin was used to examine actin sites at the electron microscopic level. The protein A-gold technique (Bendayan et al., 1982) used in these studies revealed the localization of electron opaque gold particles in the proximity of the granule-dense core, thus suggesting that actin-binding sites were in close association with secretory granules (Trifaró et al., 1985). In addition to the labeling around the granules, a small number of gold particles was also seen in the cell web underneath the plasma membrane. Furthermore, stereo-electron microscopy of polyethylene glycol-embedded rat adrenal medulla has revealed a three-dimensional lattice of microtrabeculae (actin filaments?) that is continuous with the surface of chromaffin granules and also with the inner surface of the plasma membrane (Kondo et al., 1982).

In other studies, the membrane proteins of chromaffin granules isolated by two density gradient procedures, differing in ionic strength, were then compared. Densitometric scanning of the SDS-polyacrylamide gels of granule membrane proteins revealed the presence of up to 46

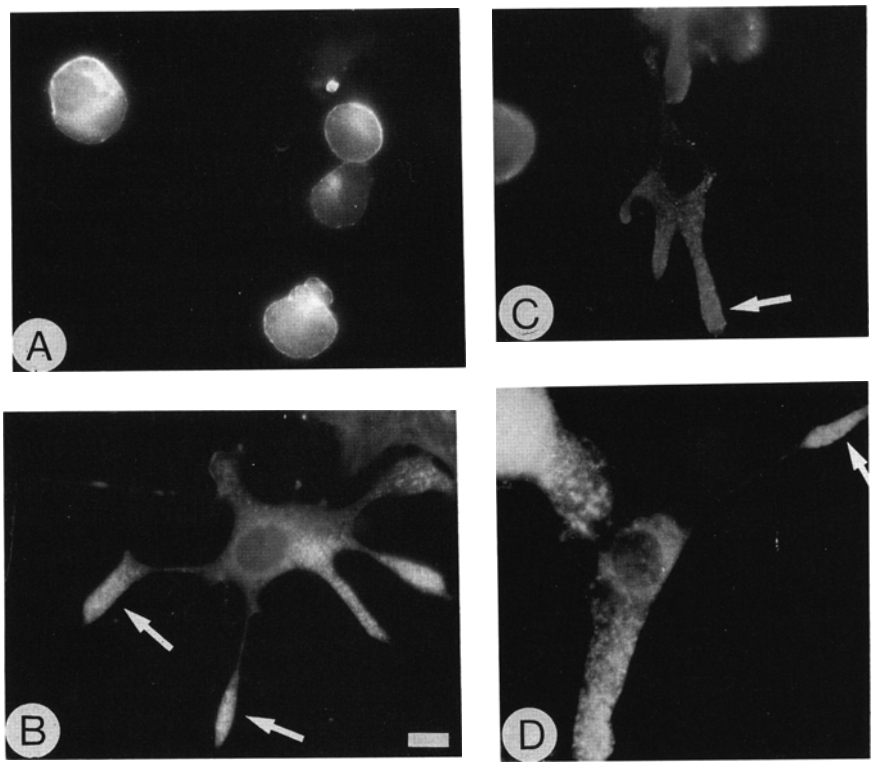

Fig. 2. Cultured chromaffin cells as they appear after staining by antibodies against chicken gizard actin (**A,B**), bovine chromaffin granule dopamine β-hydroxylase (**C**), and chicken skeletal muscle α-actinin (**D**). Chromaffin cells cultured on collagen-coated cover slips for 1 (**A**) and 7 (**B, C, D**) d were fixed and stained with corresponding antibodies as previously described (Lee and Trifaró, 1981). The preparations were examined by incident light fluorescent microscopy (epifluorescence). Strong membrane fluorescence is observed in 1-d-old cultures (**A**). After a few days in culture, the chromaffin cells developed processes (**B,C,D**) with growth cone-like structures at the terminals (arrows). A fine granular fluorescence is observed in the cytosol of the cell bodies, the processes, and especially in the terminal cones where fluorescence has the highest intensity. The bar in **B** indicates 10 μm (Trifaró, 1990).

protein bands (Trifaró et al., 1982). The main component of these bands was DβH. Another protein band was found to comigrate with actin; the presence of this protein was confirmed by immunoblotting using antibodies against actin (Trifaró et al., 1984,1985; Trifaró et al., 1985).

α-Actinin and Fodrin

The interaction of actin with secretory vesicles might require the presence in chromaffin granule membranes of actin anchorage proteins, similar to those present in the Z-line of the skeletal muscle. Alpha-actinin is the richest component of this area of the muscle, and it is known to serve as an anchorage for actin filaments. With antibodies prepared against muscle α-actinin, the localization and presence of α-actinin in cultured chromaffin cells was investigated by indirect immunofluorescence (Trifaró et al., 1984,1985; Trifaró et al., 1985). The staining pattern obtained showed α-actinin to be distributed over a whole chromaffin cell body. Some diffused fluorescence was detectable in the cytoplasm around the nucleus, but the nucleus itself was not stained (Fig. 2D).

In addition, a punctate staining pattern was observed in the cytoplasm and along the neurites.

The staining was very intense in the growth cones, the structures where α-actinin appeared to accumulate (Fig. 2D). A similar staining pattern was observed when chromaffin cells were labeled with DβH (Fig. 2C) or with chromogranin A antibodies to visualize secretory granule distribution (Trifaró et al., 1984,1985a,b). The staining appeared as a granular pattern along the neurites, and a very strong fluorescence was observed in the nerve endings where granules accumulate. The fluorescence distribution observed with α-actinin antibodies suggested an association of α-actinin with chromaffin granule membranes (Fig. 2C,D). Therefore, an actin-binding protein similar to α-actinin might be expected to be present in the secretory organelles, since it has been demonstrated that actin is associated with secretory granule membranes. An SDS-PAGE of chromaffin granule membranes showed the presence of a protein component that comigrated with purified α-actinin (Trifaró et al., 1982,1984,1985a,b).

In addition, an α-actinin–like protein was extracted from purified chromaffin granules using conditions known to extract α-actinin from myofibrilar Z-lines. The extracting protein of mol mass 97 kDa and isolectric point 6.4 was recognized by α-actinin antibodies as seen by immunodiffusion and immunoblotting (Trifaró et al., 1984; Trifaró et al., 1985a,b). The results obtained with pronase digestion of intact and broken granules suggested localization on the cytoplasmic surface of the granule for both actin and α-actinin (Bader and Aunis, 1983). Recently, another actin-binding protein, fodrin, was found to be present in the cytoplasmic surface of chromaffin granules and on the inner surface of the plasma membrane (Aunis and Perrin, 1984). The presence of two different actin-binding proteins (α-actinin and fodrin) in the granule membranes may indicate the existence of two different actin secretory granule associations, with each binding protein involving one specific type of interaction (Trifaró et al., 1984; Trifaró et al., 1985b).

Chromaffin granule membranes contain a significant number of actin nuclei that are able to promote actin polymerization and formation of membrane-bound actin filaments. Granule membranes preincubated with α-actinin antibodies show a reduced number of binding sites, an observation that indicates the absence of actin nuclei in those pretreated membranes (Trifaró et al., 1985b). Therefore, α-actinin molecules are either the nuclei themselves or they stabilize the actin nuclei, probably by anchoring these nuclei to the granule membrane.

In summary, actin microfilaments not only interact with the inner surface of the plasma membrane but also with the cytoplasmic surface of chromaffin granule, probably through their binding to α-actinin and fodrin, two actin anchorage proteins. Moreover, chromaffin granule membranes are able to crosslink actin filaments and increase the viscosity of F-actin gels in a Ca^{2+}-dependent manner. Consequently, chromaffin granules stabilize actin networks; and at the same time, the network of actin microfilaments, because of crosslinking by secretory granules, seems to increase the viscosity of the cytoplasm and therefore opposes the free movement of secretory granules.

Chromaffin Cell Actin Filament-Severing Proteins and Cell Viscosity

Gelsolin

First isolated from rabbit lung macrophages, gelsolin is a globular protein that regulates the network structure of actin filaments (Yin and Stossel, 1979). In the presence of micromolar concentrations of Ca^{2+}, gelsolin binds to Ca^{2+} and actin microfilaments and prevents gelation of these filaments by actin-binding proteins (Yin and Stossel, 1979). Moreover, gelsolin is complexed with Ca^{2+} and binds the end of actin microfilaments, to which monomers add during elongation. The final average length of actin microfilaments formed in the presence of gelsolin–Ca^{2+} is shorter than those formed in their

absence. In this respect, gelsolin behaves as an endogenous cytochalasin-B.

In our early experiments on the isolation of chromaffin cell actin by DNase I affinity chromatography, it was noticed that in addition to actin, another polypeptide (91 kDa; pI = 6.0–6.2) was eluted from the column by guanidine hydrochloride-containing buffer (Lee et al., 1979). In view of this, we decided to repeat our early experiments on the isolation of chromaffin cell actin. However, in this case, the DNase I affinity column was eluted first with EGTA-containing buffers prior to elution with 3*M* guanidine hydrochloride. Under these conditions, the EGTA buffer eluted 91 and 79 kDa polypeptides. The 91 kDa polypeptide was immunologically (immunoblotting) reactive with antibodies against rabbit lung macrophage gelsolin (Trifaró et al., 1985). Chromaffin cell gelsolin binds reversibly to actin and inhibits active polymerization in a Ca^{2+}-dependent fashion, as measured by the low shear falling ball viscometer technique (Bader et al., 1986; Trifaró et al., 1988). We also have demonstrated by electron microscopy that actin filaments obtained in the presence of Ca^{2+}–gelsolin were much shorter as a result of the fragmentation of the actin network than those obtained in the absence of gelsolin (Bader et al., 1986; Trifaró et al., 1988).

Scinderin

The second protein isolated by DNase I affinity chromatography is a 79 kDa protein that was recently isolated and purified in our laboratory (Rodríguez Del Castillo et al., 1990). It is a novel Ca^{2+}-dependent actin-severing protein that binds to actin and severs actin filaments with a consequent decrease in the viscosity of actin gels. We have named this protein "scinderin" (a name derived from the Latin scindere, meaning "to cut") because of its actin filament-severing properties (Rodríguez Del Castillo et al., 1990). The apparent mol wt of scinderin as determined by SDS-PAGE and confirmed by immunoblotting is 79,600 ± 450 Daltons. Equilibrium dialysis experiments indicated that scinderin has two Ca^{2+}-binding sites (K_d $5.85 \times 10^{-7}M$, B_{max} 0.81 mol Ca^{2+}/mol protein and K_d $2.85 \times 10^{-6}M$, B_{max} 1.87 mol Ca^{2+}/mol protein).

The addition of scinderin did not modify the viscosity of actin solutions when Ca^{2+} was absent. However, in the presence of Ca^{2+}, a decrease in the viscosity of actin solutions was observed with molar ratios of scinderin to actin of more than 1:3200 (Fig. 3A). At the scinderin–actin molar ratio of 1:800, a decrease in viscosity from 480 to 10 cP was observed (Rodríguez Del Castillo et al., 1990). The effect of Ca^{2+} concentrations on scinderin-induced decreases in actin viscosity was also tested at a fixed molar ratio (1:1600). Changes in the viscosity were observed between Ca^{2+} concentrations of 10^{-8} and $10^{-6}M$, with the maximum fall in viscosity observed at $10^{-6}M$ Ca^{2+} (Fig. 3B). The interaction of scinderin with actin was also investigated by electron microscopy.

Under conditions suitable for polymerization—actin alone, in the presence of $10^{-5}M$ Ca^{2+}, or in the absence of Ca^{2+} and in the presence of EGTA and scinderin—formed a network of very long filaments (Fig. 4A). Short filaments were never observed under these conditions. In contrast, only short filaments were observed when scinderin was added to actin networks in the presence of Ca^{2+} (Fig. 4B) (Rodríguez Del Castillo et al., 1990). Under these conditions, it was possible to measure the length of the actin filaments (Fig. 4C). The filaments observed were shorter than 0.8 μm with more than 50% of the filaments with lengths of 0.2 μm or shorter. The average filament length observed under these conditions was 0.32 ± 0.04 μm (n = 183). This length corresponds to filaments formed by approx 58 actin monomers (Rodríguez Del Castillo et al., 1990).

Additional experiments indicated that two molecules of actin formed a complex with one molecule of scinderin and that this interaction was Ca^{2+}-dependent (Fig. 5). This would suggest the presence in scinderin of two actin-binding sites. Moreover, two main fragments (40 and 38 kDa) can be obtained from scinderin by limited proteolytic digestion with chymotrypsin (Fig.

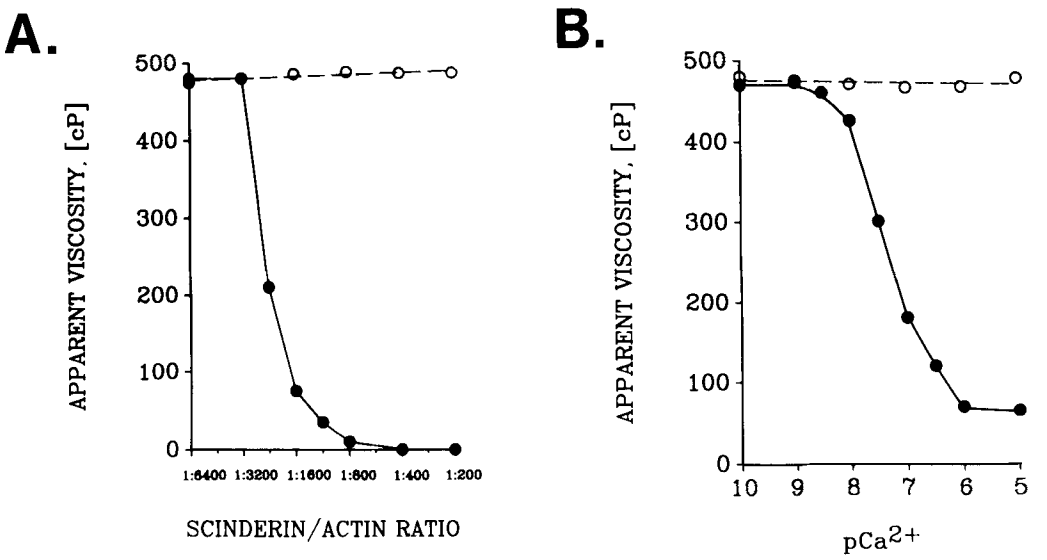

Fig. 3. Effect of scinderin on the apparent viscosity of F-actin. Effect of various concentrations of scinderin **(A)**. G-actin (10 mg/mL) in 2 m*M* Tris-HCl, pH 8, 0.2 m*M* ATP, 0.2 m*M* $CaCl_2$, and 0.005% NaN_3 was polymerized for 2 h at 4°C by addition of 100 m*M* KCl, 1 m*M* $MgCl_2$, and 0.8 m*M* ATP. The F-actin thus obtained was sedimented by centrifugation at 80,000*g* for 3 h and resuspended in incubation buffer (40 m*M* PIPES, pH 6.8, 100 m*M* KCl, 2 m*M* $MgCl_2$, 2 m*M* ATP, 5 m*M* EGTA, and 0.05% NaN_3. Apparent viscosity was determined using a falling ball viscometer; F-actin (final concentration 1.91 mg/mL = 44.37 μ*M*) and scinderin (final concentration 17.6–0.56 μg/mL = 0.22–0.007 μ*M*) were mixed together in the presence of either $CaCl_2$-EGTA buffer (10^{-5}*M* free Ca^{2+}, ●) or 5 m*M* EGTA (○). The sample (200 μL) was shaken at high speed (Vortex) for 10 s and approx 100 μL were drawn into a capillary tube. The tube was sealed with plasticine at one end and incubated in horizontal position for 120 min at 25°C. At the end of the incubation period, the tube was mounted at an angle of 45°, and apparent viscosity was measured and calculated. Effect of free Ca^{2+} concentration on scinderin activity **(B)**. F-actin (1.91 mg/mL = 44.37 μ*M*) was incubated in a capillary tube with 2.21 μg scinderin/mL (molar ratio to actin 1:1600) for 120 min at 25°C. The incubation buffer was the same as above containing 5 m*M* EGTA (○) and different concentrations of $CaCl_2$ (●) to obtain the free Ca^{2+} concentrations indicated in the abscissa. The pCa^{2+} values were calculated using the binding constant for Ca-EGTA at pH 6.8 of 2.14×10^6. Filled circles and solid lines represent incubation of F-actin and scinderin in the presence of 10^{-10}–10^{-5}*M* free Ca^{2+}, and open circles and broken lines represent incubation of F-actin alone in the presence of the same Ca^{2+} concentrations (Rodríguez Del Castillo et al., 1990).

6A). Each fragment interacts with G-actin in a Ca^{2+}-dependent manner, yielding actin-fragment complexes of molar ratios 1:1 (Figs. 6B,C,D). The results suggest that each fragment contains an actin- and a Ca^{2+}-binding site. Furthermore, amino acid sequence experiments have also indicated that both scinderin and the 40 kDa fragment have their *N*-terminals blocked. Consequently, the 40 kDa fragment might be derived from the *N*-terminal region of scinderin. Amino acid sequence data of cyanobromide fragments of scinderin also indicated no sequence homology between the fragments and gelsolin.

Immunofluorescence studies with antiscinderin on cultured chromaffin cells showed a weak and diffused cytoplasmic staining and a strong fluorescence ring pattern at the cortical cytoplasmic region (Fig. 9A), thus suggesting a preferential subplasmalemmal localization for this actin-severing protein (Rodríguez Del Castillo et al., 1990; Vitale et al., 1991). A more diffused cortical cytoplasmic fluorescence pattern was obtained when antigelsolin was used (Vitale et al., 1991). Scinderin antibodies were also used to determine if scinderin was expressed in other tissues. Cytosol fractions from bovine brain, anterior and posterior pituitary, kidney, salivary gland, testis, liver, skeletal muscle, heart muscle, platelets, and plasma were prepared and incubated in the presence of Ca^{2+} with actin DNase I

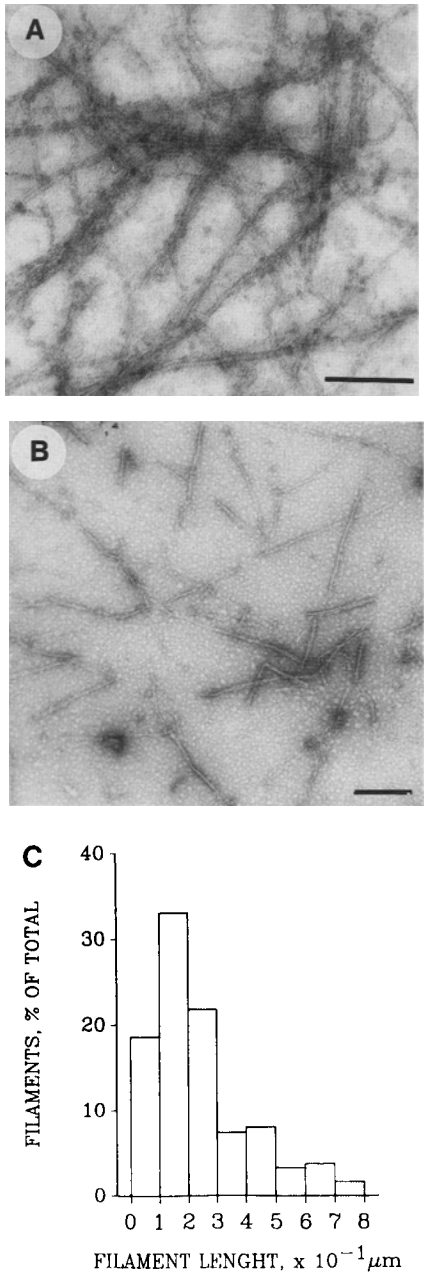

Fig. 4. Electron micrographs of negatively stained mixtures of F-actin filaments and scinderin (scinderin to actin molar ratio of 1:20) in the absence (**A**) or presence (**B**) of $10^{-5}M$ free Ca^{2+}. A mesh of interconnecting filaments can be observed in (A). The bar indicates 0.1 μm. In (B), as a result of the Ca^{2+}-activated effect of scinderin on F-actin, only short filaments are observed. The bar represents 0.1 μm. A histogram with the distribution of the length of 183 short F-actin filaments is shown in (**C**) (Rodríguez Del Castillo et al., 1990).

Sepharose 4B beads (Tchakarov et al., 1991; Rodríguez Del Castillo et al., 1991). The beads were extensively washed with the same buffer and then washed one more time with EGTA-containing buffer to elute Ca^{2+}-dependent actin-binding proteins. Samples of this last wash were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes. Immunoblots with antiscinderin antibody demonstrated the presence of the actin-severing protein in all of the abovementioned tissues (Fig. 7) with the exception of liver, plasma, and skeletal and heart muscle, thus suggesting that scinderin might be expressed in secretory tissues. Gelsolin, on the other hand, was expressed in all of the above tissues (Tchakarov et al., 1990).

Comparison of Scinderin and Gelsolin

In addition to the difference in tissue distribution among these two actin filament-severing proteins (Tchakarov et al., 1990), other differences were found. A partially purified preparation containing scinderin and gelsolin was subjected to SDS-PAGE followed by transfer to nitrocellulose membranes. Polyclonal antibodies raised against chromaffin cell gelsolin did not crossreact with scinderin when tested in immunoblots, and scinderin polyclonal antibodies did not recognize gelsolin (Rodríguez Del Castillo et al., 1990; Vitale et al., 1991). Not only were the mol wt and pI of gelsolin and scinderin different, but also, the one- and two-dimensional peptide maps obtained after limited proteolytic digestion with either Staphylococcus V8 protease or chymotrypsin showed marked differences in peptide composition (Rodríguez Del Castillo et al., 1990).

Two-dimensional electrophoresis showed the presence of three isoforms of pI = 5.8, 5.9, and 6.0 for adrenal medullary gelsolin and three distinct isoforms for scinderin (pI = 6.0, 6.1, and 6.2). The mol wt of adrenal medullary gelsolin fragments obtained by chymotrypsin digestion were similar to those previously described for

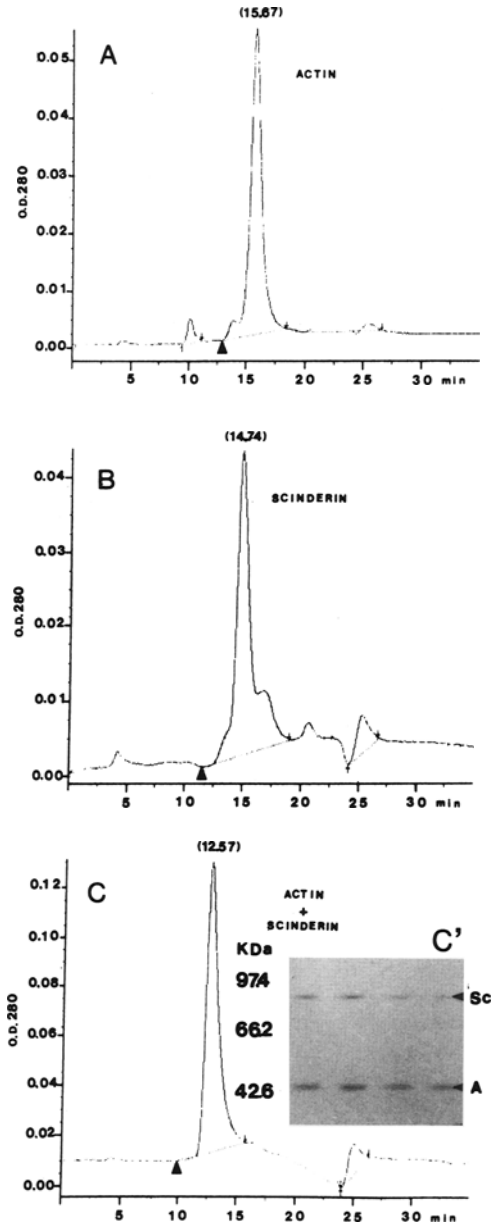

Fig. 5. Formation of actin–scinderin complexes. Interaction of scinderin with G-actin was studied by gel filtration. Scinderin (1 μ*M*) and G-actin (2.5 μ*M*) were incubated either alone or together for 1 h at room temperature in 20 m*M* Tris, (pH 7.5), 150 m*M* NaCl, containing either 1 m*M* Ca^{2+} (buffer A) or 2 m*M* EGTA (buffer B). Proteins either alone or together were then chromatographed on a Waters I 125 column in tandem with a Waters 300 SW column equilibrated with buffer A or B. The flow rate was 1 mL/min. Panels **A** and **B** show the G-actin and scinderin elution profiles. The retention times of the proteins were 15.67 and 14.74 min, respectively. When actin and scinderin were first incubated in the presence of Ca^{2+}, the chromatographic profile of the mixture showed a retention time of 12.57 min (panel **C**) and a mol wt of 160 kDa when compared with standard proteins of known mol wt. Electrophoresis of the proteins in this peak (fractions 11–13 min) after staining with Coomassie blue are shown in (C). From scanning of the gel, a molar ratio of 2 mol of actin per mol of scinderin was calculated. When actin and scinderin were incubated in buffer B (EGTA), no complexes were formed. Sc, scinderin; A, actin; and arrowheads on baselines indicate the beginning of each peak.

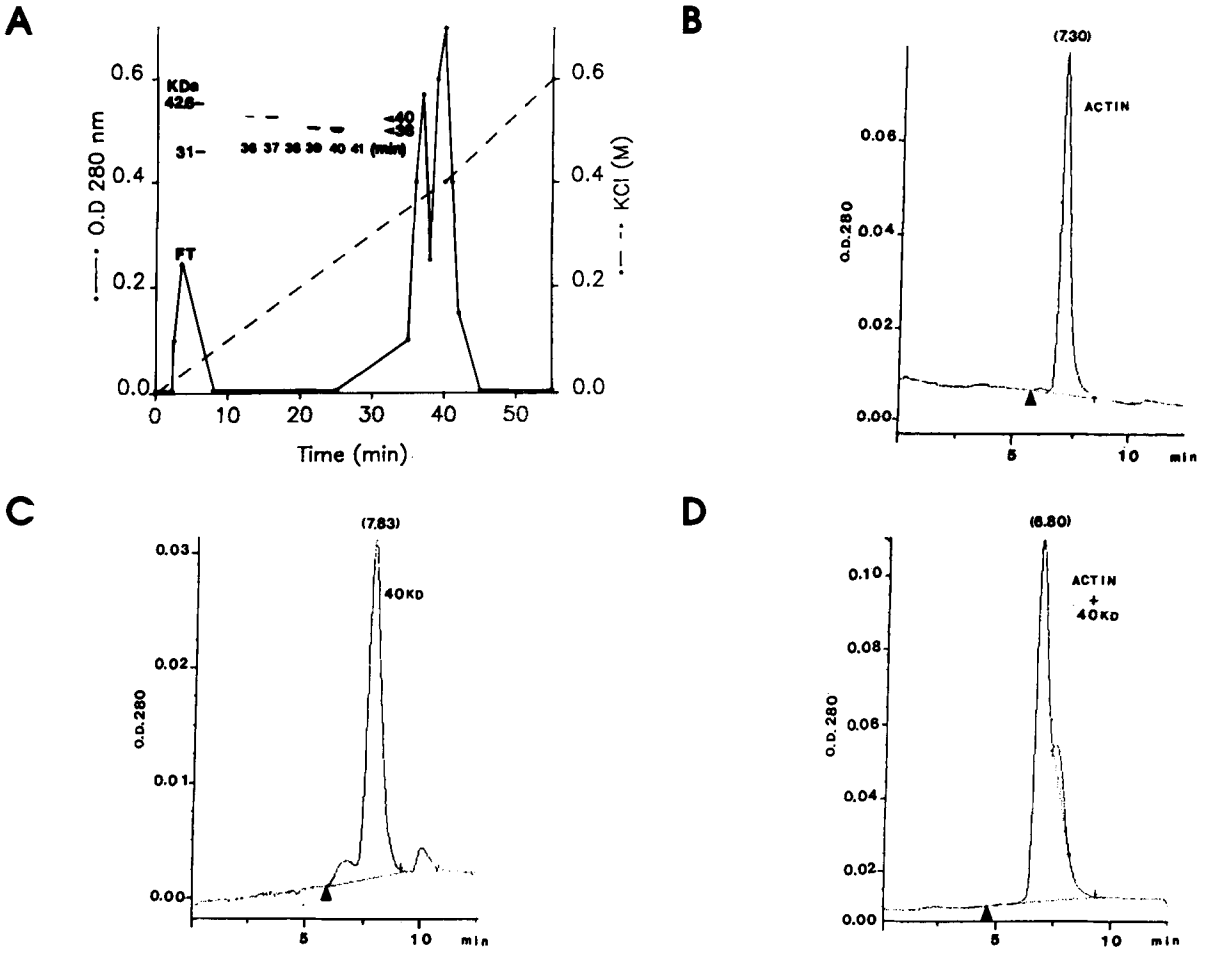

Fig. 6. Purification of scinderin fragments by DEAE-chromatography and localization of actin-binding sites. Chymotryptic digestion of scinderin gives two main proteolytic fragments in equal molar ratio, with mol wt 40 and 38 kDa, respectively, and a small amount of a 32 kDa polypeptide, all of them with different isoelectric points *(see above)*. This made possible their purification by anion-exchange chromatography. Here, we report the purification of the 40 and 38 kDa polypeptides. Three hundred micrograms of scinderin in 20 m*M* Tris, 200 m*M* KCl, 0.1 m*M* EGTA (pH 7.5) were incubated with 1.5 μg of chymotrypsin for 15 min at 25°C; the digestion was stopped by adding enough ice-cold PMSF (100 m*M*) and di-iso propyl fluorophosphate (DIPF, 100 m*M*) to reach a final concentration of 1 and 0.5 m*M*, respectively. The preparation was applied to a Waters DEAE-5 PW column preequilibrated with the buffer as indicated above but in addition, containing 20 m*M* KCl. The column was eluted with a KCl gradient from 20 to 400 m*M*) in 40 min. The flow rate was 0.8 mL/min, and 0.4 fractions were collected and analyzed by SDS-PAGE (15% polyacrylamide gel). Panel A shows the elution profile of this column. Two peaks were eluted at 36 and 41 min. An SDS-PAGE of these fractions followed by staining with Coomassie blue showed the presence of two polypeptides of mol wt of 40 and 38 kDa, respectively (insert). Interactions between the 40 and 38 kDa fragments of scinderin with G-actin were carried out at molar ratios of 2.5 μ*M* actin/1 μ*M* of either 40 or 38 kDa peptide. The incubation medium and method followed were as described for scinderin–actin complexes in Fig. 5. However, in this case, the chromatography procedure was performed with only one column (Waters I 125). Under these condi-tions, retention times for G-actin and the 40 kDa fragment were 7.30 and 7.83, respectively (panels B and C). When the 40 kDa fragment was incubated with G-actin in the presence of Ca^{2+} (buffer A), the chromatographic profile yielded one peak (panel D) close to the void vol with a retention time of 6.80 min (complex). The small shoulder at 7.40 min corresponded to the retention time of free G-actin. Similar results were obtained when a 38 kDa peptide–G-actin mixture was used in presence of Ca^{2+}. No complexes between G-actin and the fragments were formed when buffer B (EGTA) was used. The integration of the peak areas in both cases (polypeptides 40 or 38 kDa) yielded actin–fragment molar ratios of 1:1. Arrowheads on baselines show the beginning of each peak and FT indicates flow through.

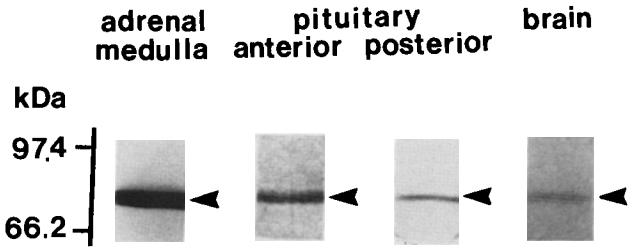

Fig. 7. Tissue expression of scinderin. Samples (150 μg protein) of concentrated EGTA washes from DNase Sepharose 4B beads were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Bovine adrenal medullary extract was used as control. Bovine anterior and posterior pituitary glands and brain were tested and found to displace positive immunoreactivity when tested against scinderin antibodies at antibody dilution of 1:500. The position of scinderin in the immunoblots is indicated by arrowheads.

human gelsolin (Kwiatkowki et al., 1985). Upon incubation using a protein to enzyme ratio of 400:1, five fragments were obtained for gelsolin. Molecular weights and pI of these fragments were 80 (pI = 6.0), 50 (2 isoforms of pI = 5.5 and 5.7), 46.5 (pI = 5.5), 31 (pI = 5.5), and 16 (pI = 6.1).

Chymotrypsin digestion of scinderin under similar experimental conditions gave a quite different peptide pattern. The polypeptide pattern showed two main proteolytic fragments of mol wt 40 (2 isoforms of pI = 6.0 and 6.1) and 38 kDa (2 isoforms of pI = 5.7 and 5.9) and a small 32 kDa (pI = 5.8) fragment. The amino acid composition of purified scinderin was also determined and compared with gelsolin composition. The content of lysine is lower in scinderin than in gelsolin; however, scinderin is a more basic protein than gelsolin, since the content of acidic residues (aspartic and glutamic) is much greater in the latter. Moreover, the content of isoleucine and tyrosine residues is higher in scinderin than gelsolin, thus indicating that scinderin cannot be a breakdown product of gelsolin (Rodríguez Del Castillo et al., 1990). Furthermore, there was no homology between the amino acid sequences of cyanobromide fragments of scinderin and gelsolin *(see above)*.

Dynamic Changes in Cortical Actin Microfilament Networks During Exocytosis

Stimulation-Induced Actin Disassembly

Work from our laboratory as well as others has demonstrated that filamentous actin is mainly localized in the cortical surface of the chromaffin cell *(see Actin above)*. We have also suggested that cortical F-actin acts as a barrier to the secretory vesicles, impeding their contact with the plasma membranes (Trifaró et al., 1982; Trifaró and Fournier, 1987; Trifaró, 1990). As indicated above, chromaffin vesicles contain α-actinin and fodrin, anchorage proteins that mediate filamentous actin association with these organelles. 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 (Figs. 8B, 9B,C,D) (Lee and Trifaró, 1981; Cheek and Burgoyne, 1986,1987; Trifaró et al., 1989). Cholinergic receptor stimulation produces a fragmentation of the fluorescent ring leaving subplasmalemmal areas devoid of fluorescence (Figs. 8B,9B,C,D) (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 DNase I inhibition assay (Fig. 8A) (Cheek and Burgoyne, 1986; Trifaró et al., 1989). These changes are also accompanied by a decrease in the amount of F-actin recovery with the Triton X100 insoluble (cytoskeleton) protein (Burgoyne et al., 1989; Trifaró, 1990).

F-actin network disassembly has also been observed in mast cells upon stimulation (Koffer et al., 1990) and in depolarized (high K^+) synap-

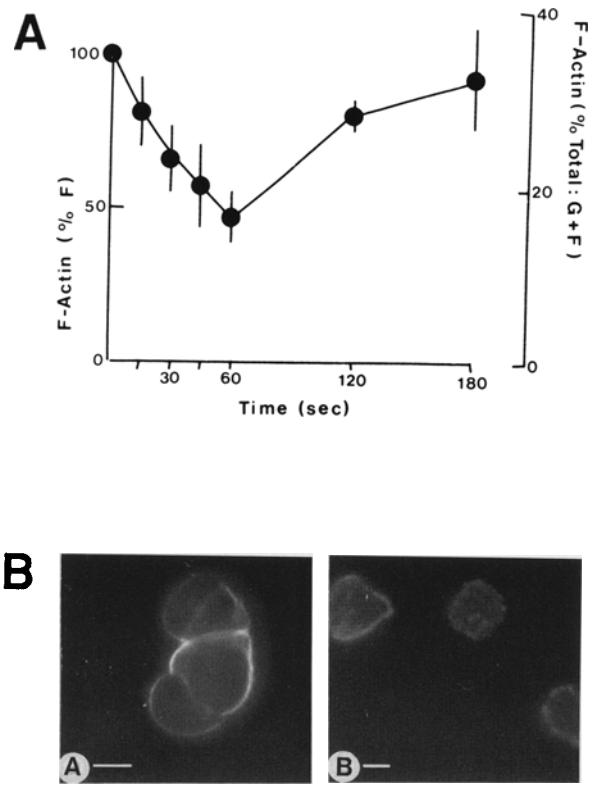

Fig. 8. Cultured chromaffin cell F-actin was measured (DNAse I inhibition assay) at different times after stimulation with $10^{-4}M$ acetylcholine **(A)**. One-day-old cultured chromaffin cells fixed with 3.7% formaldehyde and stained for F-actin with rhodamine phalloidin **(B)**. Control **(A)** and chromaffin **(B)** cells fixed 45 s after stimulation with $10^{-4}M$ acetylcholine. The fluorescent ring observed in the subplasmalemma area in **(A)** appeared fragmented in **(B)** (Trifaró, 1990).

tosomes (Bernstein and Bamburg, 1985). The presence of gelsolin and scinderin, two Ca^{2+}-dependent actin filament-severing proteins, in the chromaffin cell suggests the possibility that stimulation brings about activation of one or more actin filament-severing proteins with the consequent disruption of actin networks. Therefore, experiments with scinderin and gelsolin antibodies were performed in cultured chromaffin cells to study the distribution of scinderin and gelsolin during resting and cell stimulation and to correlate the possible changes with actin disassembly and catecholamine secretion.

Stimulation-Induced Scinderin Redistribution

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 (Vitale et al., 1991). 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. 9A). Nicotine stimulation caused fragmentation of the bright fluorescent ring, suggesting a redistribution of cortical scinderin (Figs. 9B',C',D'). Patches of scinderin appeared clearly as a fragmented fluorescent ring at the equatorial plane of the cells (Vitale et al., 1991). The effect of nicotine on scinderin reorganization was seen as early as 5 s of stimulation (Vitale et al., 1991). Stimulation of cells with nicotine also produced, as indicated above, a disruption in the rhodamine-phalloidin cortical fluorescent pattern, suggesting depolymerization of F-actin (Figs. 9B,C,D). Interestingly, in 88% of the nicotine-stimulated cells showing scinderin reorganization (916 of 1200 total cells examined), there was a concomitant distribution of F-actin and scinderin (Vitale et al., 1991).

Cells stained with antigelsolin showed a cortical cytoplasmic fluorescence pattern. The fluorescence ring underneath the plasma membrane was weaker than that observed in antiscinderin-stained cells. 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 (Vitale et al., 1991). Similar results were obtained using a mouse monoclonal antibody against the 47 kDa chymotryptic fragment of gelsolin (Vitale et al., 1991).

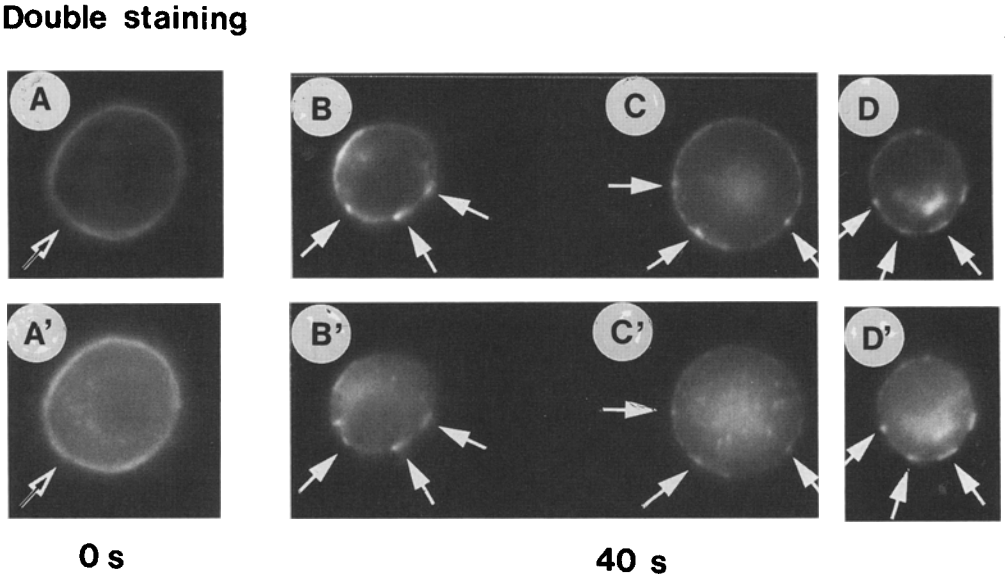

Fig. 9. Localization of scinderin and actin by double-staining fluorescence microscopy. Chromaffin cells were incubated with Locke's solution for 40 s in the absence (control) or presence (stimulated) of 10 μ*M* nicotine. Cells were sequentially stained with rhodamine-labeled phalloidin followed by scinderin antibody and FITC-anti-rabbit IgG. A control cell in **(A, A')** shows continuous and intense rings of fluorescence for F-actin **(A)** and scinderin **(A')** colocalized at the subplasmalemmal region (open arrows). Stimulated cells display a disrupted cortical fluorescent pattern either for F-actin **(B,C,D)** or scinderin **(B',C',D')**. There is a correspondence between the patched distribution of both actin and scinderin in each cell (compare B and B', C and C', D and D'). Some patches are indicated by arrows.

Scinderin Redistribution and Cortical Filamentous Actin Disassembly Precede Exocytosis

In view of the earlier redistribution (5 s) of scinderin observed upon cell stimulation (Vitale et al., 1991), time-courses of scinderin redistribution and catecholamine output were performed and compared. During stimulation with either nicotine or a depolarizing concentration of K^+ (56 m*M*), there was a sharp increase in the percentage of cells displaying redistribution of cortical scinderin fluorescence (Figs. 10A,B). Maximum values were reached 20 s (high K^+) to 40 s (nicotine) after stimulation was started. After removal of the stimulus, the number of cells showing a discontinuous fluorescent pattern returned to 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 cell stimulation (Figs. 10A,B) (Vitale et al., 1991).

Catecholamine output also rose sharply during either nicotine or high K^+ stimulation. However, the increase in catecholamine release lagged 15–20 s behind scinderin redistribution (Figs. 10A,B). Catecholamine output leveled off when nicotine or high K^+ was removed from the medium. Therefore, the studies indicate that scinderin redistribution and also actin filament disassembly induced by either nicotine or potassium preceded catecholamine release. The lag period observed between scinderin redistribution/actin disassembly and catecholamine release was not caused by the lack of sensitivity of the catecholamine release assay used, since the method can detect catecholamine concentrations equal to 0.35% of total content. We have demonstrated previously that radioactive noradrenaline

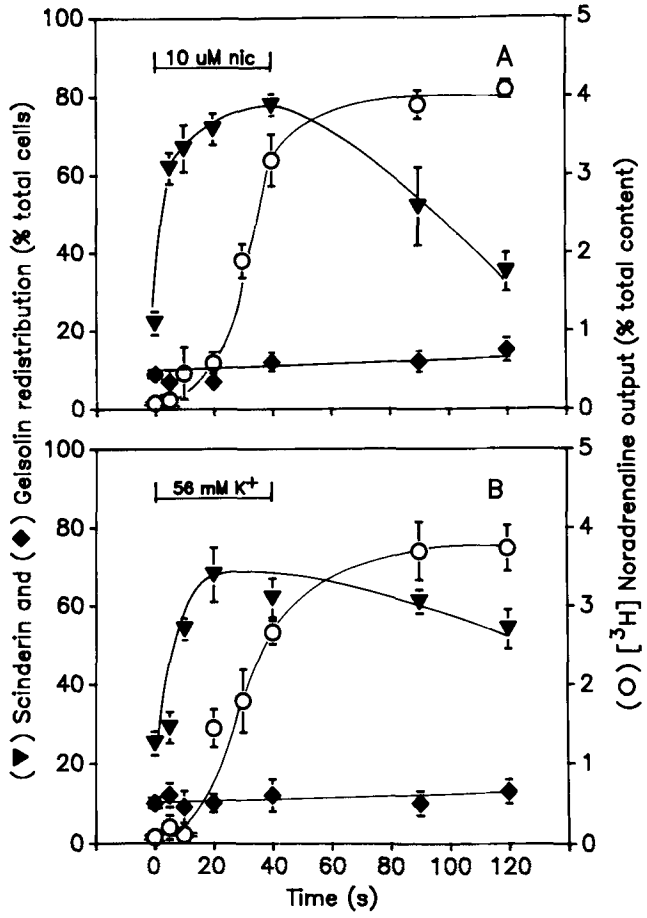

Fig. 10. Time-courses of scinderin and gelsolin redistribution and [^{3}H]NA output in response to stimulation in cultured chromaffin cells. Nicotinic stimulation: chromaffin cells cultured for 48 h were incubated for 0, 5, 10, 20, or 40 s with 10*M* nicotine or for 40 s with nicotine followed by an additional 50- or 80-s period with regular Locke's solution **(A)**. After these periods of incubation, cells were immediately fixed, permeabilized, and processed for immunofluorescence microscopy using either scinderin (▼) or gelsolin (◆) antisera. One hundred cells per coverslip were examined and classified, as described in the legend to Fig. 11. This was done without knowing whether cells were control or stimulated with nicotine (single-blind design). 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. [^{3}H]NA output (O): cultured chromaffin cells with catecholamine stores labeled with [^{3}H]NA were incubated for 0, 5, 10, 20, 30, or 40 s with 10 μ*M* nicotine or for 40 s with 10 μ*M* 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 [^{3}H]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 [^{3}H]NA secretion is expressed as percentage of total [^{3}H]NA cell content. Each point represents the mean ±SEM of values obtained from three different culture dishes. 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 K^+ 56 m*M* 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^+) **(B)**. [^{3}H]NA output (O): chromaffin cells with their catecholamine stores labeled with [^{3}H]NA were incubated with a 56 m*M* 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)** (Vitale et al., 1991a).

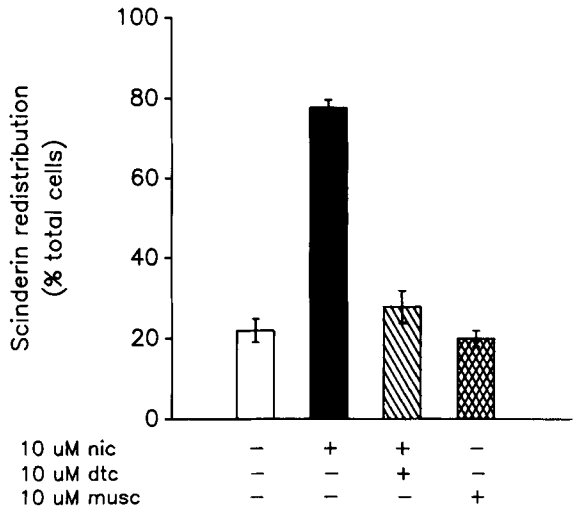

Fig. 11. Effects of cholinergic and anticholinergic drugs on scinderin redistribution in chromaffin cells in culture. Forty-hour-old chromaffin cell cultures grown on collagen-coated coverslips were incubated for 40 s under the following experimental conditions: (1) regular Locke's solution alone (control), (2) Locke's solution containing 10 μM nicotine (nic), (3) 10 μM nicotine plus 10 μM d-tubocurarine (dtc), or (4) 10 μM muscarine (musc). After these treatments, cells were fixed, permeabilized, and processed for immunofluorescence using scinderin antiserum #6. One hundred cells per coverslip were examined, and scinderin distribution was classified as having either a "continuous cortical fluorescent pattern" (*see* Fig. 9A,A') or a "discontinuous cortical fluorescent pattern" (*see* Figs. 9B,B', C,C',D,D'). This was done without knowing whether cells were control or stimulated (single-blind design). 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 cultures (Vitale et al., 1991a).

taken up by cultured chromaffin cells is stored and released together with endogenous catecholamines and that the measurements of radioactive noradrenaline in the incubation medium give a precise indication of total catecholamine release (Trifaró and Lee, 1980; Kenigsberg and Trifaró, 1980; Trifaró and Bourne, 1981).

The rates of actin disassembly and scinderin redistribution during stimulation are similar, and subplasmalemmal areas showing filamentous actin also show cortical scinderin (Vitale et al., 1991). Other immunocytochemical studies also have shown that caldesmon (Burgoyne et al., 1986) and fodrin (Perrin and Aunis, 1985) localized in the cortical region of chromaffin cells. Moreover, in one of these studies, a redistribution of cortical fodrin antibody fluorescence was observed upon nicotinic or high potassium stimulation (Perrin and Aunis, 1985). However, in this case, the time-course of fodrin redistribution was much slower than that described for scinderin in the experiments discussed here. It also can 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 (Vitale et al., 1991). 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.

Furthermore, muscarinic stimulation does not release catecholamines (Wilson and Kirshner, 1977; Fisher et al., 1981), redistribute scinderin (Fig. 11), or produce actin filament disassembly (Vitale et al., 1991). It is known that in adrenal chromaffin cells, nicotine and high K^+ induced the entry and a rise in cytosolic Ca^{2+}, which is necessary for catecholamine secretion (Douglas and Rubin, 1961; Trifaró and Bourne, 1981; Cheek et al., 1989; Kim and Westhead, 1989). Muscarine produces mobilization of Ca^{2+} from intracellular stores (Wilson and Kirshner, 1977; Kim and Westhead, 1989), an effect that 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 intracellular release of Ca^{2+} induced by muscarine is not enough to trigger catecholamine release and scinderin redistribution (Vitale et al., 1991).

Exocytosis in Areas of F-Actin Disassembly

We have discussed above that low shear viscometry studies indicate that the concentration of Ca^{2+} required by scinderin to induce a fall in the viscosity of actin gels is in the range of Ca^{2+}

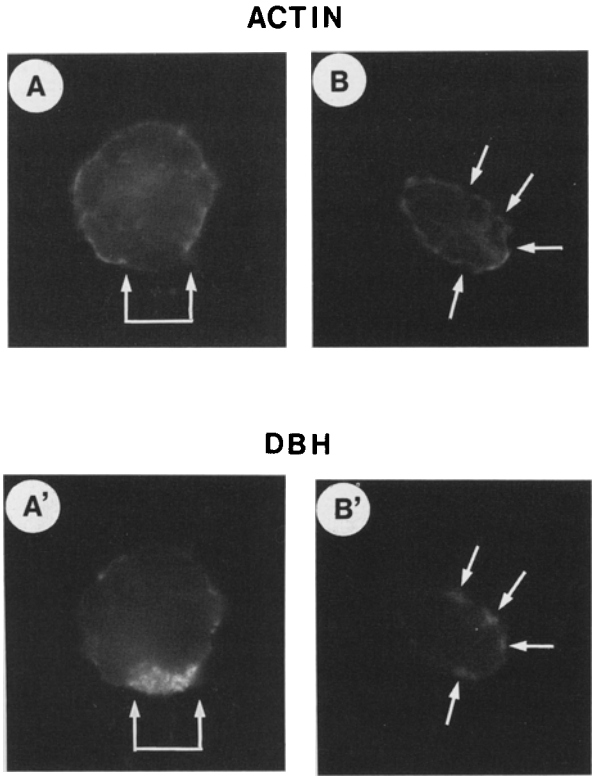

Fig. 12. Presence of surface DβH in cortical areas devoid of rhodamine-phalloidin staining. Chromaffin cells grown for 48 h were incubated for 40 s with 10 μ*M* nicotine, fixed and stained for DβH and F-actin. Fluorescent patterns of two stimulated cells **(A,A',B,B')** are shown as they appeared after incident light illumination for rhodamine **(A,B)** and fluorescein **(A',B')**. Arrows show the absence of rhodamine-phalloidin staining (F-actin) and the presence of FITC-IgG staining (DβH). Bar, 5 μ*M*. (Vitale et al., 1991a).

concentrations expected to be found in the chromaffin cell cytoplasm as a result of cell stimulation. These observations might 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 a protein such as scinderin with a consequent severing of cortical actin filament networks. Consequently, the disassembly of cortical F-actin produced by cell stimulation would suggest the presence of subplasmalemmal areas devoid of F-actin and that these areas might be zones of low cytoplasmic viscosity and probably high secretory vesicle mobility. The absence of a cytoskeleton barrier in these areas will 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 possiblity that exocytosis pits might be present in plasma membrane areas devoid of F-actin, chromaffin cells were stimulated with nicotine for 40 s, fixed, and incubated with anti-DβH IgG to detect, the presence of chromaffin granule membranes on the cell surface. Bound DβH is a chromaffin granule component with a specific membrane topology (Joh and Hwang, 1987). No granule surface DβH can be found, and the enzyme has an intragranular domain recognized by the antibody. Therefore, when secretory vesicle membranes are inserted into plasma membrane during exocytosis, anti-DβH sites are exposed on the cell surface, allowing visualization of plasma membrane exocytosis sites (Phillips et al., 1983). Our experiments indicate that DβH immunofluorescence is present in areas devoid of F-actin, as indicated by the absence of rhodamine-phalloidin fluorescence (Fig. 12). Therefore, the anti-DβH experiments clearly indicate that exocytotic pits are preferentially present in plasma membrane areas devoid of F-actin (Vitale et al., 1991).

In conclusion, scinderin redistribution and actin filament disassembly seems to precede exocytosis. However, to consider this the only important phenomenon in secretion would be an oversimplified notion of what might be a fine regulation of exocytosis in which intervention of other messengers and modulators, such as calmodulin (Kenigsberg and Trifaró, 1985), cyclic AMP (Cheek and Burgoyne, 1987), G-proteins (Matter et al., 1989; Bader et al., 1989), protein kinase C (Burgoyne et al., 1989; Vitale et al., 1991b), and so on may occur.

Acknowledgments

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