THE CHROMAFFIN GRANULE SURFACE: THE PRESENCE OF ACTIN AND THE NATURE OF ITS INTERACTION WITH THE MEMBRANE

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1. Introduction

The involvement of the cytoskeleton in stimulus–secretion coupling has been implied through several lines of evidence (reviewed in [1]). Secretion can be inhibited by drugs known to disrupt cytoskeletal function [2], microfilamentous structures have been detected morphologically in secretory cells [3] and actin has been identified as a prominent component in such tissues [4,5]. Microfilaments and/or actin have been found in association with isolated secretory granules [6] and purified actin has been shown capable of 'reassociating' with membranes of secretory granules [7,8].

There has been a good deal of controversy surrounding the question of whether or not secretory granules from adrenal medulla (chromaffin granules) possess endogenous actin. Data has been presented [9] suggesting that actin is not present in isolated granules, while earlier reports have suggested that there is actin that associates with granule membranes [7]. While the reports in [7,9] relied solely on data obtained by gel electrophoresis (and fingerprint analysis [7] but not on granule membrane fractions), we used immunoprecipitation with monospecific antibodies to actin to test the presence of actin on granule membranes.

Due to the lack of information on secretory granule-associated actin, there is little available about the nature of this interaction. 'Reassociated' actin has been suggested [8] to interact hydrophobically with granule membranes. To further investigate this possibility we attempted to label the normally associated actin from within the membrane through the use of a lipophilic photoaffinity label.

Abbreviations: MES, morpholinoethanesulfonic acid; PBS, phosphate-buffered saline; EDTA, ethylenediamine tetraacetic acid

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2. Materials and methods

2.1. *Isolation of chromaffin granules and granule membranes*

Chromaffin granules were isolated as in [10] except that the homogenization medium consisted of 0.15 M KCl buffered to pH 7.0 with 10 mM MES, instead of unbuffered sucrose. All other solutions used throughout the isolation of either granules or membranes contained the aforementioned medium. Membranes were isolated from whole granules as in [7] except that the continuous sucrose gradient was replaced by a step gradient composed of a layer of 1.2 M sucrose overlaid with an equal volume of 0.3 M sucrose. Centrifugation was carried out for 3 h at 200 000 × *g* in an SW 41 rotor (Beckman). Granule membranes used for subsequent experiments were collected at the 1.2–0.3 M interface.

2.2. *Production of antibody*

Initial experiments were carried out using monospecific anti-actin generously donated by Dr B. Jockusch. Subsequently, all immunoprecipitations were made with antibody produced in our institute

as in [11]. Immunofluorescent labeling of stress fibers in 3T3 fibroblasts showed both sources of antibody (raised against smooth muscle actin) were identical in their affinity for non-muscle actin.

2.3. *Photoaffinity labeling*

5-[^{125}I]iodonaphthyl-1-azide (INA) was synthesized from 1,5-diaminonaphthylene as in [12]. Chromaffin granule membranes (1.0 mg protein/ml) were incubated for 15 min at 37°C in the dark with INA (10^6 cpm, added in 2 μl EtOH). Photolysis was carried out for 4 min using a 150 W xenon lamp. Membranes were then washed by centrifugation at 105 000 × *g* for 60 min in an SW 50.1 rotor (Beckman). Controls using pre-photolyzed INA showed little incorporation of radioactivity into protein as seen by autoradiography of polyacrylamide gels.

2.4. *Solubilization of membrane components*

Extraction of membranes in low ionic strength at high pH was as in [13]. Membrane samples (3.0 mg protein) were suspended in 0.5 ml 10 mM Tris–HCl, 1.0 mM EDTA and dialyzed overnight against 3 l distilled water adjusted to pH 9.5 with NH_4OH. The dialysis medium pH was checked once during the course of dialysis and readjusted to 9.5 if necessary. When total membranes were solubilized in Triton X-100 the following scheme was used. Membrane samples (3.0 mg protein) were suspended in PBS containing 1.0% (v/v) Triton X-100 and sonicated in a bath-type sonicator for 3 bursts of 5 s duration. This resulted in the total solubilization of the membranes such that no material was obtained by subsequent centrifugation at 105 000 × *g*. The solution was then diluted with PBS so that the final Triton X-100 concentration was 0.1%.

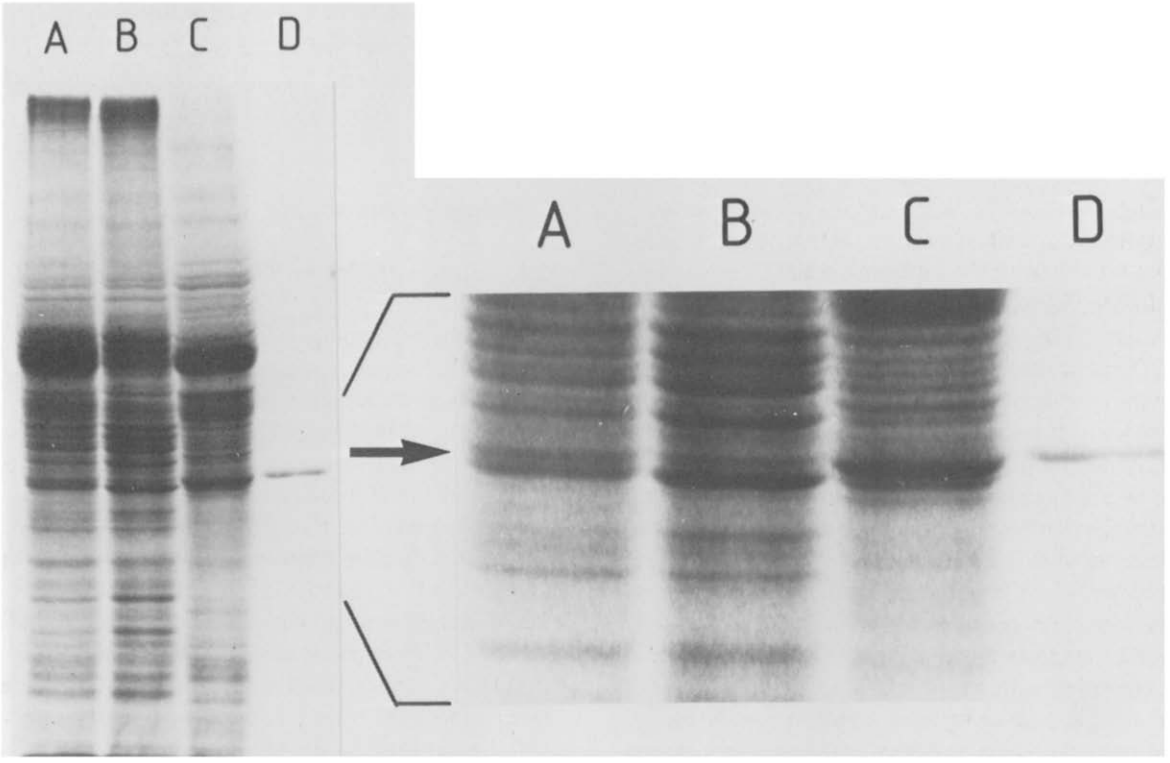

Fig.1. Selective solubilization of putative actin. (A) Membrane fraction of chromaffin granules isolated in the presence of 150 mM KCl as in section 2. (B) Membrane material insoluble at pH 9.5 (Subsequent treatment of the high pH-extracted membranes with high salt (0.5 M KCl) failed to remove any additional actin or significant amounts of other proteins.) (C) Membrane proteins solubilized by low ionic strength at pH 9.5. (D) Rabbit skeletal muscle actin. Enlargement: Note doublet (arrow) in A and the shifting of the upper band to the pH 9.5-soluble material (C), while the lower band remains associated with the 9.5-insoluble fraction.

2.5. *Antibody precipitation*

Immunoprecipitations were done in <1.0 ml at room temperature in Eppendorf centrifuge tubes rotated on an Eppendorf shaker. The initial incubation with antibody lasted from 45–60 min and was followed by the addition of 12 mg protein A–Sepharose (Pharmacia) to immobilize the antibody–antigen complex and remove it from solution. This was for 60 min and followed by 3–5 washes of the beads with PBS containing 1% Triton X-100 and 0.1 mM EDTA. The beads were ultimately brought up in a small volume of sample buffer for gel electrophoresis, and heated in a water bath for 5 min at 100°C. The beads were removed by centrifugation and the sample applied to a polyacrylamide gel.

Polyacrylamide gel electrophoresis was carried out on slab gels as in [14]. All samples were run in the presence of β-mercaptoethanol.

Protein was determined as in [15] using bovine serum albumin as a standard.

3. Results

Polyacrylamide gel electrophoresis of isolated chromaffin granule membranes shows two bands in close proximity in the molecular weight region of actin (fig.1). According to [13], membrane-associated actin can be extracted into solutions of high pH and low ionic strength. Dialysis of membranes against distilled water at pH 9.5 yielded a soluble fraction enriched significantly in the upper of the two bands in question (fig.1C). This treatment, where one of the bands in question is already purified by solubilization from the membrane, provides a convenient system for immune precipitation.

When the pH 9.5-soluble material is subjected to immunoprecipitation with monospecific anti-actin (fig.2), one protein is precipitated which corresponds in molecular weight to that band presumed to be actin (fig.2C). In order to investigate a possible hydrophobic interaction between actin and the membrane, a photoaffinity label, INA, was used which incorporates itself into the lipid bilayer and can covalently bind to molecules (lipids and proteins) in its proximity upon photolysis. When the membranes are labeled with the photoaffinity probe, prior to either high pH treatment or immune precipitation, a

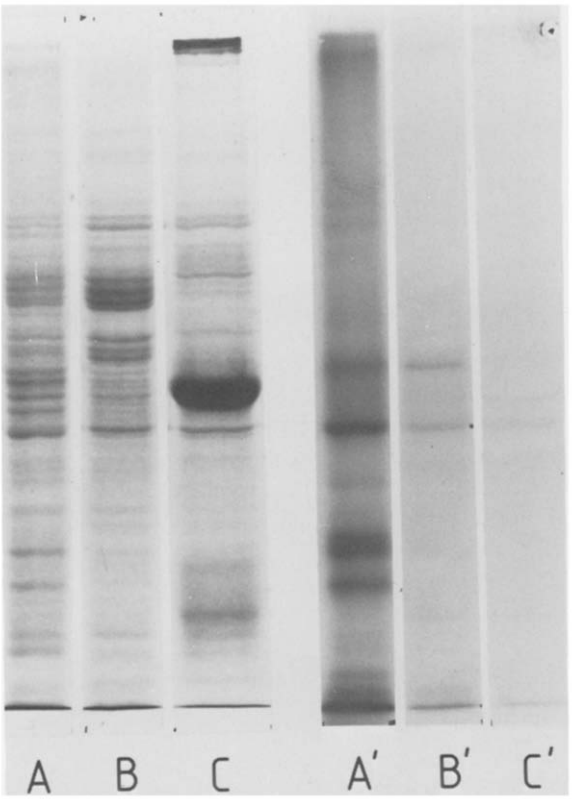

Fig.2. Immune precipitation of actin from pH 9.5-soluble chromaffin granule membrane proteins. (A) Membrane proteins prior to pH 9.5 treatment (labeled with INA). (A′) Autoradiogram. (B) pH 9.5-soluble material prior to immune precipitation. (B′) Autoradiogram. (C) Immunoprecipitation with monospecific anti-actin. (C′) Autoradiogram.

number of bands have incorporated radioactivity (fig.2A′), one rather strongly in the region of actin. This heavily labeled band does not, however, become soluble at pH 9.5 (fig.2B′). Furthermore, the actin which was immune precipitated (fig.2C) is not labeled by the photoprobe (fig.2C′). Thus it would appear that the upper band of the doublet in the actin region (fig.1A) is actin, and the lower a hydrophobic, probably integral, membrane protein.

To rule out the possibility that actin interacting hydrophobically with the membrane was present, and thus not solubilized at low ionic strength and high pH, the membranes were solubilized completely in Triton X-100. Figure 3 shows that the photolabeled proteins were all solubilized in detergent (slots A, B).

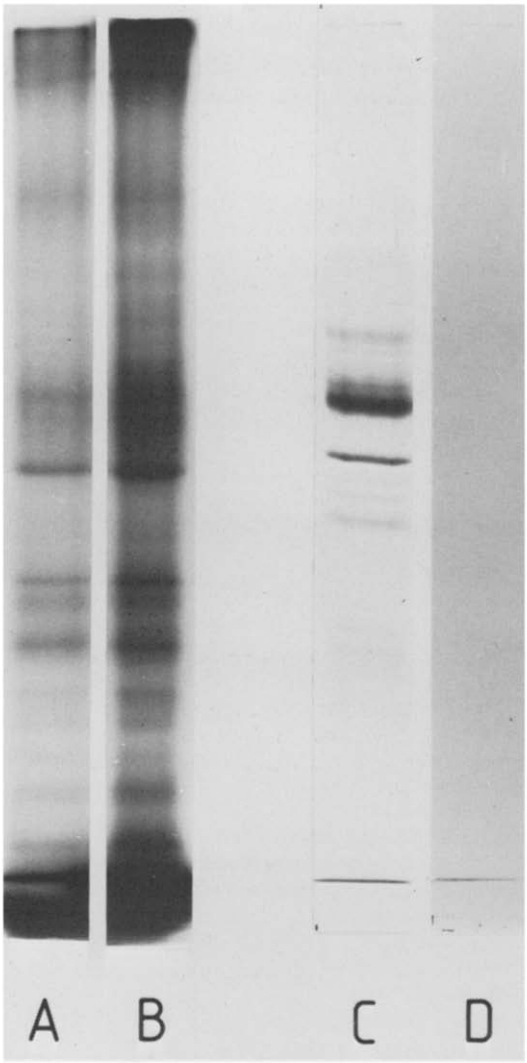

Fig.3. Immune precipitation of Triton X-100 extracts of chromaffin granule membranes. (A) Autoradiogram of membranes labeled with INA prior to solubilization. (B) Autoradiogram of Triton X-100-soluble membrane material. (C) Antibody precipitated material. Coomassie blue stained. (D) Autoradiogram of (C).

When precipitation with antibody was performed, a band corresponding to actin was brought down, but as can be seen, this band was not radioactively labeled (slots C, D). Taken together, these findings confirm the existence of actin associated with chromaffin granule membranes; the notion of a hydrophobic interaction is not supported in this system.

4. Discussion

The data presented here indicate that isolated membranes from chromaffin granules do contain actin. This agrees with the preliminary findings in [4]. In [4] actin was estimated to comprise some 2% of total membrane protein, but its identity could not be ascertained by fingerprint analysis due to a lack of sufficient starting material. Our approach, that of immunoprecipitation with monospecific antibody, unequivocally identifies this protein as actin.

It is maintained in [9] that actin is not associated with chromaffin granule membranes. This is probably a result of the harsh methods employed [9] to isolate membranes (repeated hypo- and hypertonic lyses). The need for high ionic strength (150 mM KCl), which supposedly preserves F-actin associated with the membranes, is not critical, as the identical experiments performed with 0.3 M sucrose instead of 150 mM KCl showed similar amounts of actin isolating with chromaffin granule membranes ([4], and D. M., unpublished). Therefore, in order to preserve the actin–membrane association, it seems that hypotonic conditions such as were used here to strip actin from the membrane prior to immune precipitation, must be avoided.

The nature of the actin–membrane interaction still remains unclear. α-Actinin was suggested [7] to be involved, as treatments known to remove α-actinin-like proteins from membranes (low ionic strength) diminish the binding capacity of these membranes for exogenously added actin. The presence of α-actinin in secretory granule membranes has been shown immunochemically [6], and it is known to be present in relatively large quantities in non-muscle tissues possessing prominent amounts of actin, such as platelets [16,17].

Actin has been intimated [8] to bind to secretory granules (in vitro) lipophilically. We have been able to rule out this possibility using the hydrophobic photoaffinity label INA which covalently attaches to molecules in its surroundings by forming the very reactive nitrene derivative upon photolysis. Since INA resides almost exclusively (>98%) within the lipid bilayer [12], it is safe to conclude that neither actin nor a portion of the actin molecule is to be found in this region. There exists the possibility that exogenously added actin interacts hydrophobically as suggested

[8]. Carrying out the same photolabeling experiments in a significant excess of F- or G-actin (50 μg/ actin/mg membrane protein) did not give rise to a population of photolabeled actin (D. M., unpublished). It would appear, then, that alternatives to direct (hydrophobic) actin–membrane interactions, such as those involving membrane-bound intermediates [18,19] must be given favor for the time being.

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