Binding of actin to chromaffin granules and mitochondrial fractions of adrenal medulla

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Received 29 May 1984

Binding of actin to chromaffin granules was confirmed and shown to be salt dependent and eliminated by prior trypsin treatment of the granules. However, purified granules bind less actin than do crude granules. A mitochondria-enriched fraction was found to bind substantially more actin per mg protein than did the secretory vesicle fraction. Binding of actin by the secretory vesicles therefore is not a good indication that actin plays an active role in exocytosis.

Chromaffin granule *Actin binding* *Mitochondrial fraction*

1. INTRODUCTION

The presence of actin in cells of the adrenal medulla [1] has led to the conjecture that actin may play a specific role in secretion by exocytosis. What that role might be, however, is not understood [2] and several roles may be considered. A direct role in the movement of secretory vesicles (chromaffin granules) to the plasma membrane has been suggested by the discovery that actin is associated with the granule membrane [3–8], but the specificity of the interaction between actin and the membrane is open to question [7,9]. We present here data casting further doubt on the specific roles suggested for actin in exocytosis since more actin is bound to mitochondrial membranes than to those of the secretory vesicles.

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From the PhD Thesis of L.-S.K., University of Massachusetts (1983), available from University Microfilms, Ann Arbor, MI 48108

2. MATERIALS AND METHODS

2.1. *Preparation of crude chromaffin granules*

Crude chromaffin granules are the 'large granule fraction' of [10] prepared with minor modifications described in [11]. Buffers used for the preparation were 0.27 M sucrose and 0.13 M potassium phosphate (pH 7.0) or 0.3 M sucrose and 10 mM Hepes (pH 7.0).

2.2. *Preparation of mitochondria-enriched and chromaffin granule-enriched fractions*

Crude granules were layered on top of Percoll density steps (3 ml 20%, 3.5 ml 40%, 3 ml 60% Percoll) and centrifuged for 30 min at 12000 × g in a Sorvall SS-34 rotor [12]. The interface between 20 and 40% Percoll was withdrawn by a pasteur pipette and is the mitochondria-enriched fraction based on enrichment of cytochrome oxidase. The interface between 40 and 60% Percoll is the chromaffin granule-enriched fraction based on catecholamine content. The cross-contamination of both fractions was calculated based on the marker assays of both fractions. About 3% of the protein in the purified granule fraction is due to mitochondria and 30% of the protein of the mitochondrial fraction to chromaffin granules.

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00145793/84/$3.00

2.3. *Purification and radiolabeling of bovine skeletal muscle actin*

Actin was purified as in [13] except that bovine skeletal muscle was used. Purified actin was labeled with [^{14}C]formaldehyde as follows [14]: The reaction was started by adding 5 μl of 0.25% H^{14}CHO (52 mCi/mmol, New England Nuclear) and 20 μl of 1% $NaCNBH_3$ to 2 mg dissolved actin in 1 ml buffer composed of 2 mM Tris–HCl (pH 8.0), 0.2 mM ATP, 0.5 mM β-mercaptoethanol and 0.2 mM $CaCl_2$. The reaction was carried out at room temperature for 1 h and then the mixture was dialyzed against the buffer to be used for binding until the radioactivity in the dialysate reached background. The specific activity of such a preparation was approx. 6.8×10^5 cpm/mg actin. A single band of radioactive protein was found upon electrophoresis of ^{14}C-methylated actin.

2.4. *Binding of unlabeled actin to chromaffin granules*

Granules (15–20 mg/ml) were incubated with or without trypsin (bovine pancreatic, 50 μg/ml, Sigma) at 0°C for 30 min. The reaction was stopped by adding soybean trypsin inhibitor (100 μg/ml) and 10 vols incubation buffer and then the solution was centrifuged at 17400 × g for 10 min. Binding was started by adding trypsin-treated or untreated granules to the actin solution, usually at room temperature, as described in section 3. The reaction mixture was then centrifuged in an Eppendorf microfuge for 10 min and the pellet was dissolved for SDS-gel electrophoresis in 50 mM Tris–HCl (pH 6.8), 2% SDS, 2 mM EDTA, 10% glycerol (v/v) and 143 mM β-mercaptoethanol (sample buffer).

When radiolabeled actin was used to quantitate the binding, the mixture of actin and 1 mg/ml of particulate fraction was incubated at room temperature for 10 min, then centrifuged through a cushion of 20% sucrose in a 400 μl microfuge vial. The vial was then quickly frozen in an ethanol–solid CO_2 mixture and the tip with the pellet was cut off and counted for radioactivity in 5 ml Scintiverse (Fisher Scientific) in a Beckman SC 100 scintillation counter. ^{3}H-labeled inulin was included in some mixtures to estimate the volume of extragranular liquid space.

2.5. *Analytical procedures*

SDS-acrylamide gel electrophoresis in 6–18% gel was used for protein analysis [15]. The marker for mitochondria (cytochrome c oxidase) was determined as in [16]. Catecholamines were determined fluorometrically as in [17]. Protein concentration was determined as in [18].

3. RESULTS AND DISCUSSION

It has been shown that actin filaments can associate with chromaffin granules, however, it is not clear to which side of chromaffin granule membranes actin filaments bind since either partially lysed granules [3] or granule membranes [7] were used for the studies. Here, granules were prepared both in hypertonic and in isotonic low ionic strength buffer and then actin binding ability was compared.

Fig.1 shows that when a preparation of intact chromaffin granules is subjected to electrophoresis, a band corresponding to the position of native actin is observed (lanes 1, 3) and that the amount of actin bound to the granules prepared in low-salt buffer (lane 3) is much less than to those prepared in a high-salt buffer (lane 1), although there is little effect on the rest of the protein patterns. Lanes 2 and 4 show that prior treatment of the granules with trypsin markedly reduces the amount of actin sedimenting with the granules. Incubation of the granules with purified skeletal muscle actin results in a substantial binding of the skeletal protein to the granules, particularly in the granules prepared in high salt buffer (lanes 5,7). Granules preincubated with trypsin show reduced binding of the muscle actin, suggesting that an intact binding protein is required for attachment of actin (lanes 6,8). This is in accord with a paper [6] showing that antibody to α-actinin will inhibit binding of actin to chromaffin granule preparations. It may be noted that muscle actin seems to have a slightly higher molecular mass than the endogenous secretory cell actin in accord with previous studies of the latter protein [1].

Although the above data agree with previous work by others, the organelle specificity of the actin binding was thrown into doubt when we compared the binding of actin to a crude granule fraction with binding to a purified granule fraction (fig.2). Incubation with actin over the same con-

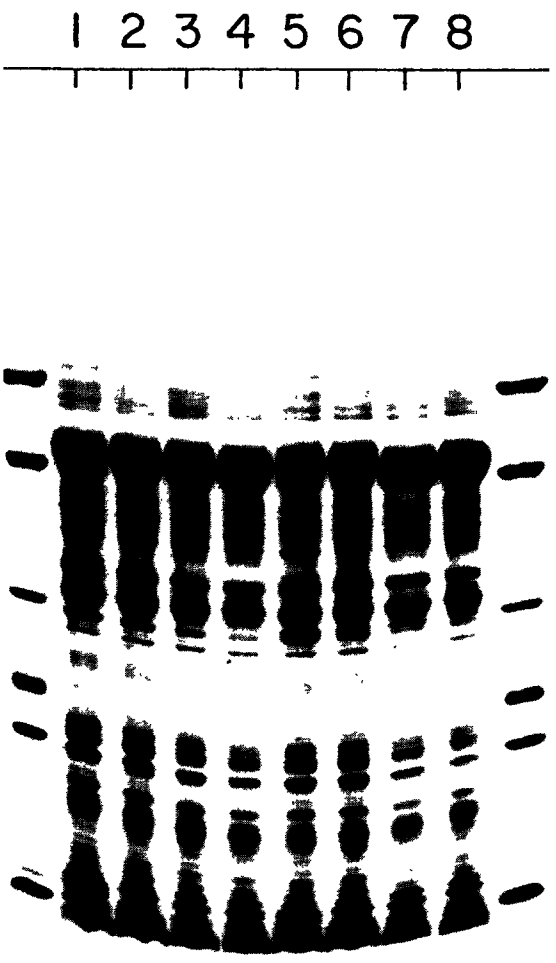

Fig.1. Actin binding to crude chromaffin granules. Crude granules were prepared in high-salt (0.27 M sucrose, 0.13 M potassium phosphate, pH 7.0) and low-salt (0.3 M sucrose, 10 mM Hepes, pH 7.0) buffers. Samples of both preparations were treated with trypsin as described in section 2. Half of the trypsin-treated and control samples were then incubated at a granule protein concentration of 1 mg/ml with actin at 70 μg/ml for 10 min in high-salt buffer and centrifuged. Pellets were then dissolved in SDS and run on the gradient gel. Lanes: (1) high-salt granules, (2) trypsin-treated high-salt granules, (3) low-salt granules, (4) trypsin-treated low-salt granules. Lanes 5–8: granules to which actin was added; samples are in the same order as lanes 1–4. M_r standards (top to bottom): phosphorylase *a* (92500), urease (70800), actin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (28000), lysozyme (14500).

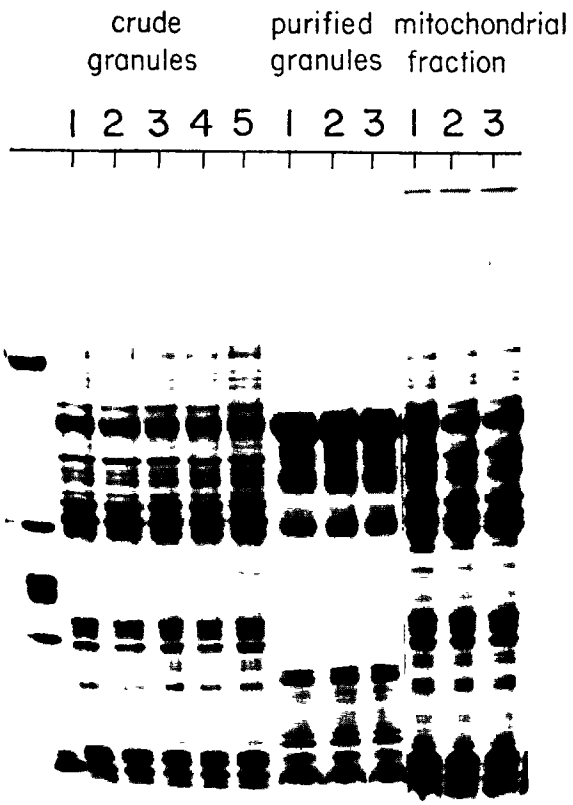

Fig.2. Actin binding to chromaffin granules, purified granules and mitochondrial fraction. Crude granules, purified granules and mitochondrial fraction were prepared in 0.27 M sucrose, 0.13 M potassium phosphate (pH 7.0) as described in section 2. One mg/ml of each preparation was then incubated with actin. The concentration of actin (μg/ml) incubated with crude granules for tracks 1–5 was 0, 17.5, 35, 52.5 and 66.5, respectively. For purified granules the concentrations were (tracks 1–3) 0, 53, 106; for the mitochondrial fraction (tracks 1–3) 0, 53, 106. After centrifugation each pellet was dissolved in sample buffer then run on the gradient gel (100 μg protein/track). M_r standards are as in fig.1 except that alcohol dehydrogenase (40000) was used and urease was not. Differences in appearance of crude granule tracks from fig.1 are due chiefly to differences in protein content. Purified granules lack proteins running with and just behind carbonic anhydrase; these are enriched in the mitochondrial fraction.

centration range produced far less incremental binding to purified granules than to the cruder fraction. The crude granule fraction is composed

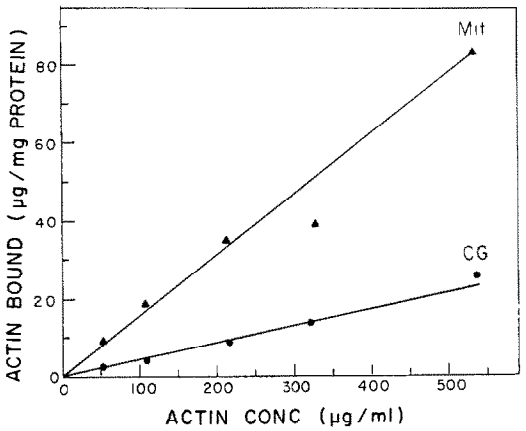

Fig.3. Binding of actin to purified chromaffin granules and to the mitochondrial fraction. The binding of actin was quantitated by using ^{14}C-labelled actin as described in section 2.

mainly of chromaffin granules; based on the assay of marker enzymes (see section 2) we calculate the crude granule fraction to be 60% chromaffin granules. If purified granules bind actin substantially less well than do crude granules, a contaminant of the crude fraction may be responsible for much of the actin binding capacity of the fraction.

To examine this apparent lack of specificity further we prepared a mitochondria-enriched fraction (see section 2) and found by electrophoresis that it bound large amounts of actin when treated in the same way as the chromaffin granule fraction (fig.2).

To put this observation on a quantitative basis, we measured the binding of ^{14}C-labeled actin to purified granules and to the mitochondria fraction. The results of the quantitative experiment are shown in fig.3 and table 1. The mitochondrial fraction binds roughly 4-times as much actin per mg protein as do chromaffin granules. The mitochondrial fraction we have used is certainly not pure, but it is enriched 1.8-fold over the crude granule fraction. The data of table 1 show that binding of actin correlates much better to the mitochondrial marker (cytochrome *c* oxidase) than to the chromaffin granule marker, catecholamine. If actin binding is not to the mitochondria of this fraction, then other particulate matter must bind actin with a very high capacity. The discrepancy between that binding and the binding exhibited by the chromaffin granule would become even greater. It may be noted that authors in [19] have shown the apparent binding of actin filaments to mitochondria and ribosomes by electron microscopy.

In conclusion, we feel that these data mean that binding of actin to secretory vesicle membranes is not evidence that the interaction is part of the process of exocytosis. Depolymerization of restricted regions of an actin network might play a permissive role in the movement of granules to the plasma membrane, as suggested by others (e.g., [20,21]) but our data suggest that the specificity of experimental phenomena should be tested by substituting membranes of other organelles such as mitochondria for granule fractions.

Table 1

Organelle marker distribution and actin binding in the crude granule fraction and two subfractions

	Cytochrome *c* oxidase (units/ mg protein)	Epinephrine (mg/mg protein)	Actin (µg/mg protein)
Crude granules	7.3	0.33	16.4
Chromaffin granule-enriched fraction	0.57	0.72	8.7
Mitochondria-enriched fraction	13.3	0.20	34.1

Preparations of chromaffin granule- and mitochondria-enriched fractions are described in the text

ACKNOWLEDGEMENTS

We thank Dr Bruce Jacobson for his advice on the work and Mrs Lois Havis for manuscript preparation. This work was supported in part by USPHS grant GM24197.

REFERENCES

[1] Lee, R.W.H., Mushynski, W.E. and Trifarò, J.M. (1979) Neuroscience 4, 843–852.
[2] Friedman, J.E., Lelkes, P.F., Rosenheck, K. and Oplatka, A. (1980) Biochem. Biophys. Res. Commun. 96, 1717–1721.
[3] Burridge, K. and Phillips, J.H. (1975) Nature 254, 526–529.
[4] Gabbiani, G., DaPrada, M., Richards, G. and Pletscher, A. (1976) Proc. Soc. Exp. Biol. Med. 152, 135–138.
[5] Meyer, D.I. and Burger, M.M. (1978) FEBS Lett. 101, 129–133.
[6] Aunis, D., Guerold, B., Bader, M.-F. and Cieselski-Treska, J. (1980) Neuroscience 5, 2261–2277.
[7] Wilkins, J.A. and Lin, S. (1981) Biochim. Biophys. Acta 642, 55–66.
[8] Bader, M.-F. and Aunis, D. (1983) Neuroscience 8, 165–181.
[9] Ostlund, R.E., Leung, J.T. and Kipnis, D.M. (1977) J. Cell Biol. 73, 78–87.
[10] Smith, A.D. and Winkler, H. (1967) Biochem. J. 103, 480–482.
[11] Tirrell, J.G. and Westhead, E.W. (1979) Neuroscience 4, 181–186.
[12] Meyer, D.I. and Burger, M.M. (1979) J. Biol. Chem. 254, 9854–9859.
[13] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[14] MacKeen, L.A., DiPeri, C. and Schwartz, I. (1979) FEBS Lett. 101, 387–390.
[15] Laemmli, U. (1970) Nature 227, 680–685.
[16] Wharton, D.C. and Griffiths, D.E. (1962) Arch. Biochem. Biophys. 96, 103–114.
[17] Campuzano, H.C., Wilkerson, J.E. and Horvath, S.M. (1975) Anal. Biochem. 64, 578–587.
[18] Bradford, M.M. (1976) Anal. Biochem. 73, 248–254.
[19] LeBeux, Y.J. and Willemot, J. (1975) Cell Tissue Res. 160, 1–36.
[20] Trifarò, J.M. (1978) Neuroscience 3, 1–24.