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Actin Associated with Membranes of Monoamine Storage Organelles (39345)

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Actomyosinlike proteins occur in various tissues other than muscle, e.g., in adrenal medulla (1, 2), neuronal elements (3), and blood platelets (4, 5). It has been suggested that in brain synaptosomes stenin (myosinlike) and neurin (actinlike) are localized in the presynaptic vesicles and the synaptic membranes respectively (3, 6), but this subcellular distribution remains to be demonstrated.

Recently, specific antiactin autoantibodies (AAA) binding to frozen sections of smooth and striated muscle as well as to platelets have been described in the serum of patients with chronic aggressive hepatitis (7). The use of such antibodies and the demonstration of filaments 40–80 Å in diameter by electron microscopy has been useful in demonstrating the presence of actin in several nonmuscular cells (8–10).

It was therefore of interest to investigate by means of immunofluoresence using AAA serum whether actin could be localized in isolated amine storage organelles of adrenal medulla and blood platelets.

Methods. Homogenates of bovine adrenal medulla and rabbit blood platelets were submitted to ultracentrifugation in a sucrose solution (11) and an Urografin gradient, respectively, as previously described (12, 13). The majority of the organelles storing catecholamines (adrenal medulla) or 5-hydroxytryptamine (5HT) (platelets) sedimented as a fine layer at the bottom of the tube (fraction 10). The supernatants were divided into nine equal portions, each of them was diluted 1:1 with 0.3 *M* sucrose, and ultracentrifuged for 30 min at 180,000 *g* in order to obtain the particulate matter (14). Furthermore, portions of the bottom layers (fraction 10) were lysed by twofold freezing and thawing in distilled water. The membranes of the lysed organelles were then sedimented by ultracentrifugation (15). Intact amine storage organelles and their membranes as well as the particulate matter of the other fractions were submitted to electron microscopy and processed for the immunofluorescence assay. Another portion of the chromaffin granules was lysed in hypotonic KCl (0.015 *M*) followed by dialysis against 4 liters of 0.15 *M* KCl (16). The membranes were sedimented by ultracentrifugation (180,000 *g* for 30 min) and also examined for immunofluorescence.

For electron microscopy, the material was fixed in an ice-cold 3% solution of glutaraldehyde in cacodylate buffer, pH 7.4, postfixed in 2% solution of osmium tetroxide in the same buffer at 4°, dehydrated in graded concentrations of alcohol, and embedded in Epon 812. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Philips 300 electron microscope.

The immunofluorescence reaction was performed on 4-μm sections obtained by cutting the frozen material on a cryostat. After treatment for 30 min with AAA serum, followed by washing in phosphate-buffered saline (PBS), the sections were stained for 30 min with a fluorescein-conjugated IgG fraction of goat antiserum to human IgG (Miles Seravac, Lausanne, Switzerland, Code 64.170). The intensity of fluoresence of the sections (rewashed with PBS and mounted in 90% glycerol in PBS) was compared with that of control sections treated with normal human serum. The AAA-containing sera were obtained from two patients with chronic aggressive hepatitis (7); their titers (judged by immunofluorescent staining of rat intestinal smooth muscle) amounted to 1/1280 and 1/640, respectively. Tests for antimitochondrial or anti-DNA antibodies as well as for antinuclear factor were negative (for details, see 7). Photographs were taken on Zeiss 50/65 barrier filter using Anscochrome color slide

film 500 daylight (Gaf Co., New York, N. Y.).

Results and discussion. In agreement with previous results, the electron micrographs of the bottom layers of the centrifugation tubes (as well as of the pellets from fractions 7, 8, 9 of the adrenal medullary homogenate) showed a population of virtually pure storage organelles with electron dense, osmiophilic cores due to the presence of high concentrations of catecholamines (Fig. 1c) and 5HT (14). After lysis of the organelles, vesicular membranes without or with little osmiophilic contents were seen (15). The particulate matter of the other fractions was very poor in amine storage organelles, but contained other subcellular elements, e.g., in adrenal medulla microsomes (fractions 1–2, Fig. 1a) and mitochondria (fraction 4, Fig. 1b) and in platelet α-granules (fractions 7, 8).

Treatment of sections of intact adrenal chromaffin storage vesicles (fraction 10) with AAA serum followed by staining with the fluorescein conjugated anti-human IgG fraction gave rise to an intense fluorescence throughout the preparation with a few bright spots probably due to irregular distribution of the organelles (Fig. 2b). A similar fluorescence pattern was seen with the vesicular membranes obtained by freezing and thawing the isolated storage organelles in

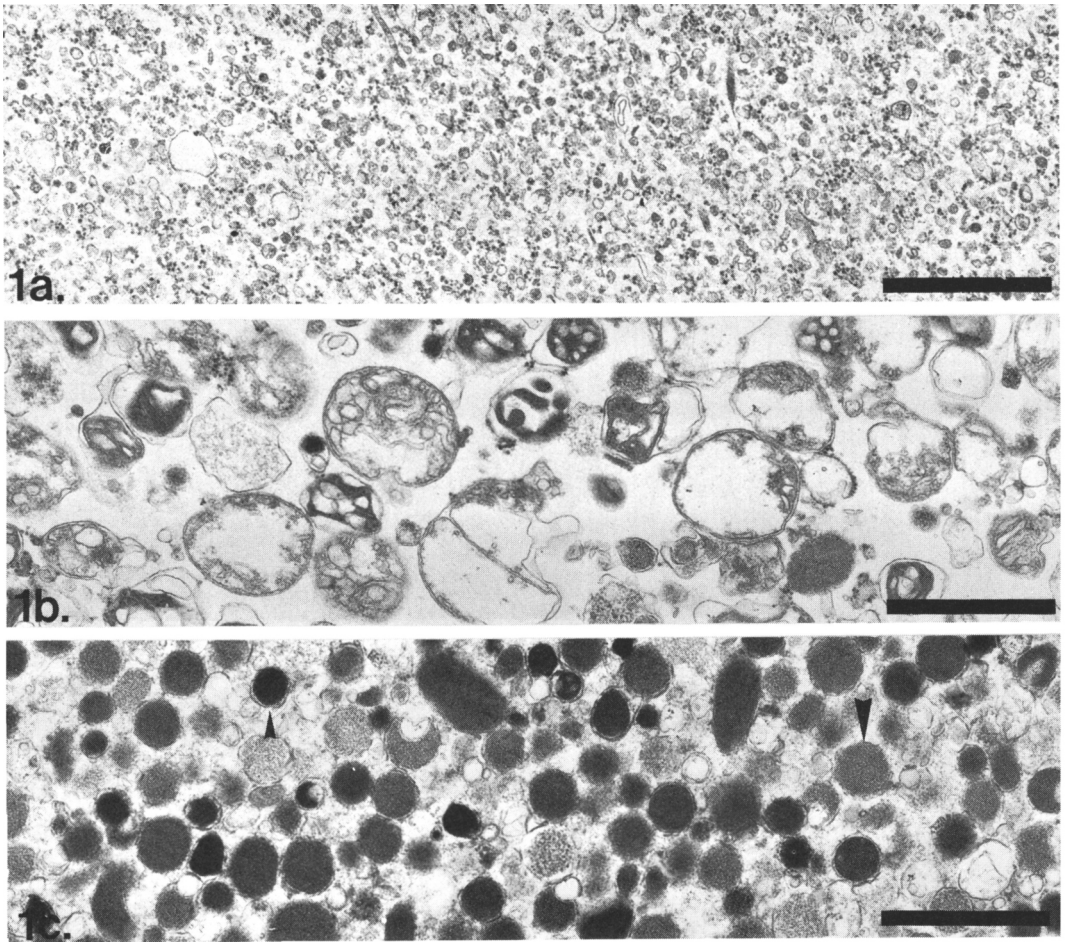

FIG. 1. Ultrastructural aspects of subcellular fractions of bovine adrenal medulla. (a) A microsomal pellet (fraction 2) is virtually pure in microvesicles and contains no chromaffin granules. (b) A crude mitochondrial pellet (fraction 4) is a mixed population of organelles containing very few chromaffin granules. (c) A chromaffin granule pellet (fraction 10) contains a high density of amine storage organelles. ►, Noradrenaline storage vesicle; ►, adrenaline storage vasicle. Bar = 1 μm. Magnification: × 22,000.

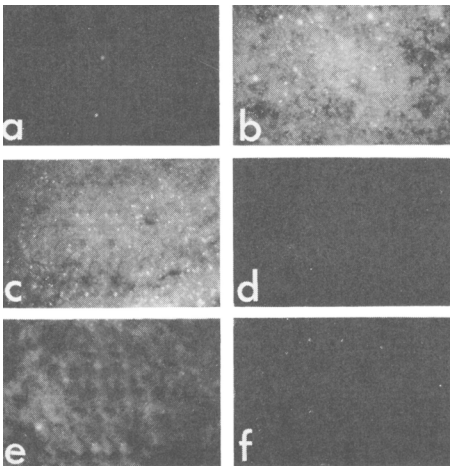

FIG. 2. Immunofluorescent staining of cryostat sections of different cellular fractions from adrenal medullary cells isolated by ultracentrifugation. (a) Intact storage organelles incubated first with normal human serum followed by goat anti-human IgG: no staining (× 400). (b) An adjacent section incubated first with AAA followed by fluorescent goat anti-human IgG: bright fluorescence is seen throughout the section with few brighter spots randomly distributed (× 400). (c) Storage organelles lysed after twofold freezing and thawing in distilled water stained as in (b). The intensity and the distribution of fluorescence are similar to that seen with intact granules (× 400). (d) Storage organelles lysed in 0.015 *M* KCl, treated as in (b): no staining (× 400). (e) Mitochondrial and microsomal fraction isolated from the adrenal medulla and treated as in (b). A very faint stain is present, much less intense than that seen in fractions of intact or frozen and thawed granules (× 400). (f) Intact storage granules incubated first with AAA absorbed with thrombostenin A, followed by goat anti-human IgG: no staining (× 400).

distilled water (Fig. 2c). Frozen sections of particulate matter from fractions containing microsomes and mitochondria (Fig. 1a, b) did not show relevant fluorescence after treatment with AAA (Fig. 2d).

The specificity of the immunofluorescence reaction is indicated by the following findings: (a) When sections of storage organelles were treated with AAA serum which had been preincubated with highly purified thrombostenin (17) (kindly supplied by Prof. E. F. Lüscher, Theodor-Kocher Institute, Berne, Switzerland) or rabbit skeletal muscle actin (18), no immunofluorescence was seen on exposure to the fluorescein conjugated IgG fractions (Fig. 2f). In contrast, sections treated with AAA serum preincubated in the buffers alone developed marked fluorescence. (b) Dialysis of the isolated storage organelles in 0.015 *M* KCl markedly decreased the immunofluorescence reaction of sections of the vesicular membranes (Fig. 2e). This is probably related to the previously demonstrated solubility of actin in KCl (19) leading to a washout of this protein from the membrane.

It has to be considered that during the homogenization and fractionation procedures actomyosin might have precipitated and cosedimented with the chromaffin granules at the bottom of the tube. This possibility was examined by putting an aqueous suspension of platelet actomyosin (isolated according to Probst *et al.* (17)) on a 1.6 *M* sucrose solution identical to that used for the isolation of the amine storage organelles (11). After centrifugation (45 min at 142,800 *g*), the actomyosin determined colorimetrically (20) equilibrated close to the top of the sucrose solution, and no sediment was found at the bottom of the tube.

Isolated 5HT organelles and their membranes from rabbit blood platelets also exhibited intense fluorescence on exposure to AAA. The immunofluorescence reaction showed the same specificity for actin as that observed with chromaffin granules. However, a marked fluorescence was also present in the fractions containing α-granules and mitochondria and, as observed earlier, in frozen sections of whole platelets throughout the cytoplasm (7). This indicates that in platelets actin is not only connected with membranes of 5HT organelles, but also with other subcellular structures.

It is noteworthy that, contrary to platelets (7), frozen sections of bovine adrenal medulla did not show significant immunofluorescent staining after treatment with AAA. This indicates that the overall amount of actin present in the cytoplasm of medullary cells is probably not sufficient in order to be visualized with the immunofluorescence technique. Isolation of the storage organelles by ultracentrifugation appeared to result in a concentration of actin, as shown by immunofluorescence. On the other hand, isolation of mitochondria or microsomes was not accompanied by concentration of the protein.

Recently, it has been reported that

chicken actin and myosin mixed with bovine adrenal chromaffin granules adhere to their membrane, having two independent attachment points (21). The present results indicate that, during the isolation procedure, endogenous actin remains firmly associated with the membranes of amine storage organelles. This association appears rather specific in the adrenal medulla, but not in the platelets, and may indicate a direct or indirect function of contractile proteins in catecholamine release and possibly in the release reaction of platelets.

The nature of the events leading to monoamine liberation, however, is not yet known. Actin, in relation with vesicular membranes, may interact with myosinlike proteins and, as suggested before (6, 22, 23), may lead to the fusion of vesicular and plasma membrane resulting in exocytosis. In addition, it has to be considered that the actomyosin system has a function in actively expelling the amines from the storage vesicles during the process of release (24).

Summary. A histo-immunofluorescence technique using anti-actin antibodies has been applied to various subcellular fractions of bovine adrenal medulla and rabbit blood platelets. In the adrenal medulla only the membranes of the chromaffin granules, but not the fractions containing other subcellular particles (microsomes, mitochondria) showed marked immunofluorescence. In platelets, considerable fluorescence was present in the membranes of the 5-hydroxytryptamine organelles as well as in other subcellular particles. It is concluded that in the adrenal medulla, actin is specifically associated with the membranes of the amine storage organelles, whereas in platelets the protein shows a rather general subcellular distribution.

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