Biochemical Pharmacology, Vol. 17, pp. 241–246. Pergamon Press. 1968. Printed in Great Britain

THE FATE OF THE CHROMAFFIN GRANULE DURING CATECHOLAMINE RELEASE FROM THE ADRENAL MEDULLA—III.

RECOVERY OF A PURIFIED FRACTION OF ELECTRON-TRANSLUCENT STRUCTURES*

S. MALAMED,† A. M. POISNER,‡ J. M. TRIFARÓ§ and W. W. DOUGLAS

Departments of Anatomy and Pharmacology, Albert Einstein College of Medicine, Yeshiva University, Bronx, N.Y., U.S.A.

(*Received* 8 *June* 1967; *accepted* 3 *August* 1967)

Abstract—Purified fractions of chromaffin granules from the adrenal medullae of cats have been prepared for an electron microscopic study of changes associated with the release of catecholamines. Fractions from unstimulated medullae consisted of electron-opaque, membrane-bounded spheroidal granules of about 4500 Å maximum diameter, similar to those seen *in situ*. Fractions from animals whose adrenal medullae had been stimulated with acetylcholine and depleted of catecholamines consisted almost entirely of electron-translucent structures. It is concluded that during release of catecholamines from the chromaffin granules, the granules discharge their electron-opaque contents, but not their membranes, which remain in the cell as discrete structures.

RECENT chemical evidence suggests that stimulation of the chromaffin cells of the adrenal medulla causes catecholamines to be released directly from the well known chromaffin granules. Thus, in addition to catecholamines, two other constituents of chromaffin granules have been found in the effluents from secreting glands in proportions similar to those found in the granules. These constituents are adenine nucleotides[1–3] and proteins[4, 5] immunologically characteristic of the granules.

Since phospholipid and cholesterol, lipids present in abundance in the membranes of chromaffin granules, do not escape into the effluents of perfused adrenal glands during catecholamine release,[6, 7] and since there is no fall in the lipids in subcellular fractions from catecholamine-depleted glands,[6, 8] it seems likely that after the granules discharge their contents their membranes are retained by the cells. Moreover, since density gradient centrifugation reveals that upon catecholamine depletion these lipids are found in a lighter layer, it has been suggested that the lipids are retained as discrete structures with reduced specific gravity, and further that these structures are probably the electron-translucent granules prominent in electron micrographs of stimulated chromaffin cells.[6, 8] The purpose of the present study was to examine this possibility.

* Supported by U.S.P.H.S. Grants GM-08900, NB-05219, 5K3-GM-25304, 5TI-GM-65-09 and 5 ROI-NB-4006.

† Present address: Department of Anatomy, Rutgers Medical School, New Brunswick, N.J., U.S.A.

‡ Research Career Development Awardee, United States Public Health Service.

§ Senior Postdoctoral Fellow, United States Public Health Service; present address: Department of Pharmacology, McGill University, Montreal, Canada.

Because representative sampling is a considerable problem in ultrastructural studies of changes *in situ*, we have adopted an alternative approach: we have compared the electron microscopic appearance of subcellular fractions containing chromaffin granules prepared from unstimulated and stimulated adrenal medullae of cats. This procedure permits rapid comparisons of granules derived from large populations of cells and reduces the sampling difficulties of electron microscopy involving tissue sections. A preliminary report of our results has appeared.[9]

METHODS

All experiments were performed on cats anesthetized with nembutal (45 mg/kg, i.p.) or with chloralose (80 mg/kg, i.v. after induction with ethyl chloride and ether). The animals that were used to provide unstimulated medullae were given hexamethonium (10 mg/kg) and atropine (1 mg/kg) i.p. along with the nembutal, or several minutes before inducing anesthesia with ethyl chloride. This was done to block the adrenal synapses.[10]

Perfusion of adrenal glands

The adrenal glands were perfused *in situ*[11] at room temperature (24–26°) through a cannula inserted into the abdominal aorta with Locke's solution of the following composition (mM):NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; $MgCl_2$, 1·0; $NaHCO_2$, 6·0; glucose, 10. The solution was equilibrated with 5% CO_2 in O_2. The venous effluent from the adrenal glands was collected, for assay of catecholamines, through cannulae inserted into the adrenal veins. To stimulate catecholamine secretion, perfusion was switched for periods of 2–3 min to an aliquot of this medium containing acetylcholine (10^{-6} to 2×10^{-5} g/ml). This process was repeated, with 4 min between stimulations, for a total of 60 min. In a single experiment, the total perfusion time was limited to 10 min and a higher concentration of acetylcholine (ACh) (10^{-4} g/ml) was present throughout.

Cell fractionation

After perfusion, both adrenal glands were rapidly removed, placed on ice, and the medullae dissected free from the cortices under a binocular microscope. The medullae (weighing together 10–25 mg) were homogenized in 1·5 ml ice-cold sucrose (0·3 M) with a teflon pestle. An aliquot of the homogenate was taken for catecholamine assay. The homogenate was then centrifuged at 600 *g* for 20 min and the supernatant suspension filtered through a succession of membrane filters (Millipore Filter Corp., Bedford, Mass.) in order to isolate the chromaffin granules. The pore sizes of the filters used successively were 1·2, 0·65, 0·45 (followed sometimes by 0·3 μ). The filtrate passing through the last filter was centrifuged at 20,000 *g* for 20 min and the resulting pellet (one from each cat) was used for electron microscopy. The isolation procedure is a modification of a method for obtaining bovine granules containing catecholamines recently mentioned in a preliminary report.[12]

Catecholamine determinations and drugs employed

Catecholamines in perfusates and homogenates of adrenal medullae were measured by the trihydroxyindole spectrophotofluorometric method.[18] To estimate the depletion of catecholamines from the perfused glands, the amount recovered in the perfusate was expressed as a per cent of the total catecholamines recovered in the perfusate

plus homogenate. Acetylcholine chloride (ACh), hexamethonium chloride, and atropine sulfate were the drugs employed. All doses are given in terms of the salts.

Electron microscopy

To select appropriate conditions of fixation and to provide a reference with which the isolated granules could be compared, blocks of medullary tissue (1 mm^3) were cut from two unstimulated adrenal glands perfused with Locke's solution containing hexamethonium and atropine as described above. The most satisfactory fixation was with the method described by Karnovsky,[14] slightly modified as follows: formaldehyde-glutaraldehyde, 4 to 5½ hr (cacodylate buffer, final pH 7·2, without calcium chloride), buffer wash, overnight, post fixed in 1% osmium tetroxide, 60 min (veronal–acetate buffer, pH 7·4). The following procedures were unsatisfactory, since they resulted in poor tissue preservation and shrinkage: 1% osmium tetroxide,[15] 2% osmium tetroxide,[15] 0·6% potassium permanganate[16] (each for 60 min, veronal–acetate buffer, pH 7·3–7·5) and 4% formaldehyde,[17] 60 min (phosphate buffer, pH 7·3–7·5), postfixed in 2% osmium tetroxide, 120 min (veronal–acetate buffer, pH 7·3–7·5).

Pellets of adrenal medullary granules were fixed by the modified method of Karnovsky[14] described above. Occasionally the pellets were repacked in conical microcentrifuge tubes[18] at some step during fixation or ethanol dehydration. When the original pellet from one cat was large enough (about half the experiments), it was broken into smaller fragments, each of which was prepared for electron microscopy. All the original pellets or fragments thereof were embedded with random orientation with regard to original centripetal-centrifugal axes. Epon[19] was used for embedding the tissue blocks, original pellets, and pellet fragments in BEEM capsules (BEEM, Inc., Bronx, N.Y.). Sections of the material were stained with uranyl acetate[20] and lead citrate[21] and studied in the electron microscope (RCA EMU 3G) at 100 kV. Two or three pellet fragments were examined in each experiment that permitted fragmentation of the original pellet. No significant difference was discerned in the appearance of different samples (pellet fragments) from one original pellet.

RESULTS

Adrena medulla in situ

To provide a standard with which the isolated fractions could be compared, sections were made of unstimulated adrenal medullae. The appearance of the chromaffin cells was similar to that of other mammalian chromaffin cells,[22, 23] including those of the cat.[24,25] Fig. 1 shows the variety of membrane-bounded granules seen in a section of a single chromaffin cell of such a control gland. Both oblong and circular profiles appear. (We have observed elongated chromaffin granules in unperfused, freshly fixed medullae, also.) The granules vary in the extent to which they are filled with electron-opaque material and also in the electron opacity of this material. The electron-opaque content or matrix itself shows a fine granularity. The maximum diameter of the granules is about 4100Å.

Purified granule fractions

Table 1 summarizes the data on catecholamine depletion of glands from which fractions were prepared for electron microscopy. In the unstimulated glands, the loss of catecholamines during perfusion was small (3 and 11 per cent). In contrast, depletions ranging from 45 to 97 per cent were achieved with ACh.

Unstimulated (control) glands

Electron micrographs of purified granule fractions prepared from the unstimulated adrenal medullae revealed, in each instance, a population of chromaffin granules resembling that present in chromaffin cells *in situ* with little, if any, contamination by

TABLE 1. CATECHOLAMINE DEPLETION FROM ADRENAL GLANDS USED TO PREPARE PURIFIED CHROMAFFIN GRANULES

Expt. no.*	Catecholamine depletion† (%)	Perfusion schedule		
		Total duration (min)	ACh	
			Time (min)	Conc. (g/ml)
1	3	60		
2	11	60		
3	45	60	20	10^{-6}
4	77	10	10‡	10^{-4}
5	83	60	20	10^{-5}
6	84	60	20	2×10^{-5}
7	97	60	27	2×10^{-5}

* One cat used in each experiment.

† Catecholamines in perfusate/Total catecholamines (perfusate + homogenate) × 100.

‡ ACh infused continuously; in all other experiments in which ACh was used, it was given intermittently at 4-min intervals.

other cell structures. A typical electron micrograph is presented in Fig. 2, which shows a section through a pellet of chromaffin granules from the adrenal medullae of a cat whose adrenal glands were perfused for 60 min with the synaptic blocking drugs, hexamethonium and atropine. In this experiment the loss of catecholamines during perfusion was only 3 per cent. Final filtration was through a filter with pores of 0·45 μ. The preparation consists principally of granules that are full of electron-opaque material. It contains membranous debris but is free of mitochondria and other recognizable cell structures. The spheroidal shape of the granules is inferred from the great preponderance of near-circular profiles. The matrices of the granules vary in electron opacity. Although the granularity of the matrices is enhanced by the double staining used for the sections illustrated in this paper, it was also apparent in other sections stained singly with either uranyl acetate or lead citrate. A narrow electron translucent space (40–140Å) separates the granule matrices from their membranes The largest diameter is about 4500 Å. When a filter with smaller pores (0·3 μ dia.) was. used for final filtration, the largest granule diameter was about 4100 Å; however, many of the granules showed blebs where the membrane was elevated from the matrix, presumably due to their being squeezed through the small pores.

Stimulated glands

Electron micrographs of purified chromaffin granule fractions prepared from the adrenal medullae of the 5 cats exposed to ACh were different from those obtained from the unstimulated glands in that many electron-translucent structures appeared. Fig. 3 is representative of the results obtained from the three experiments employing prolonged stimulation with ACh, 10^{-5} or 2×10^{-5} g/ml, where catecholamine

depletion ranged from 83 to 97 per cent (experiments 5, 6, and 7; Table 1). Although a few electron-opaque granules appear, the rest of the sample consists of electron-translucent spheroids ranging in diameter from 300–3700Å The larger of these structures is similar in size to the electron-opaque granules seen in the chromaffin cells *in situ* illustrated in Fig. 1.

The result obtained from an experiment (experiment 4, Table 1), where the total stimulation time was limited to 10 min and a higher dose of ACh was used resulting in catecholamine depletion of 77 per cent is illustrated in Fig. 4. Again there are few electron-opaque granules and many electron-translucent structures.

DISCUSSION

The morphological features of the electron-opaque granules obtained by membrane filtration from unstimulated adrenal medullae are similar to those of the classical chromaffin granules observed in medullary chromaffin cells *in situ*. The appearance of the isolated granules may therefore be used as a standard for comparison in the study of structural changes associated with catecholamine secretion. Fractions prepared in the same way from ACh-stimulated, catecholamine-depleted adrenal medullae show relatively few electron-opaque granules and many more electron-translucent structures. The coincidence of these two changes suggests that the empty structures are derived from the full granules. However, although the spectrum of sizes of the electron-opaque granules is similar to that observed in preparations from unstimulated glands, the electron-translucent structures range in size from a maximum that is about the same as that of the electron-opaque granules down to structures much smaller. Although it seems likely that the larger electron-translucent structures are emptied chromaffin granules, the origin of the abundant small electron-translucent structures is less obvious. One possibility is that they are fragments of emptied chromaffin granules. The fact that they are rare in preparations from unstimulated glands supports this. Possibly, emptied chromaffin granules are more fragile than full granules and are thus more subject to breakage during the preparative procedures. The resulting membrane shreds might then round up to form small empty spheroids or vesicles, as has been reported for certain biological membranes in suspension.[26,27] Alternatively, chromaffin granules that have been emptied of their contents may fragment within the chromaffin cell.

If these interpretations are correct, then the present results support the view that medullary stimulation causes chromaffin granules to release their contents to the cell exterior while the membranous components remain in the cell, for some time at least, as discrete structures.[6,8] Our results are in harmony both with the analyses of lipid efflux from and content of medullary tissue, to which reference was made earlier,[6–8] and with some electron microscopical results obtained from stimulated medullary tissue *in situ*, which show a loss of electron-opacity in the chromaffin granules.[24,28]

Two of the mechanisms that have been suggested for catecholamine release are consistent with retention of granule membranes in adrenal medullae depleted of their catecholamines. The first involves dispersion of the catecholamines from the granules to the cytoplasm and thence through the plasma membrane.[24,28,29] Basically this means that the catecholamines would move peripherally while the granules do not. This hypothesis, however, is difficult to reconcile with the chemical evidence that the catecholamines escape with stoichiometric amounts of adenine nucleotides and with

"granule" protein, a bulky molecule. The second hypothesis[22,28,30] accommodates the morphological and chemical results better. On this hypothesis a catecholamine-loaded granule would move through the cytoplasm to the plasma membrane where it would extrude its contents by exocytosis, that is, through a common aperture at the site of fusion of granule membrane and plasma membrane. De Robertis *et al.*[22] have suggested that after such a maneuver the granule membrane may be incorporated into the plasma membrane. However, the present results, showing what appear to be granule membranes after catecholamine depletion, and the recent evidence that "granule" lipid is retained[6-8] indicate that if exocytosis (reverse pinocytosis) does occur, then the attachment of the granule membranes to the plasma membrane does not involve long-term incorporation.

Acknowledgement—We thank Miss D. Norum, Miss A. Palazzolo and Mr. A. Hooper for technical assistance.

REFERENCES

1. W. W. DOUGLAS, A. M. POISNER and R. P. RUBIN, *J. Physiol., Lond.* **179**, 130 (1965).
2. W. W. DOUGLAS and A. M. POISNER, *J. Physiol., Lond.* **183**, 236 (1966).
3. W. W. DOUGLAS and A. M. POISNER, *J. Physiol., Lond.* **183**, 249 (1966).
4. P. BANKS and K. HELLE, *Biochem. J.* **97**, 40C (1965).
5. N. KIRSHNER, H. J. SAGE, W. J. SMITH and A. G. KIRSHNER, *Science* **154**, 529 (1966).
6. J. M. TRIFARÓ and A. M. POISNER, *Fedn. Proc.* **26**, 294 (1967).
7. J. M. TRIFARÓ, A. M. POISNER and W. W. DOUGLAS, *Biochem. Pharmac.* **16**, 2095 (1967).
8. A. M. POISNER, J. M. TRIFARÓ and W. W. DOUGLAS, *Biochem. Pharmac.* **16**, 2101 (1967).
9. S. MALAMED, A. M. POISNER and J. M. TRIFARÓ, *Anat. Rec.* **157**, 282 (1967).
10. W. W. DOUGLAS and A. M. POISNER, *Nature, Lond.* **208** 1102 (1965).
11. W. W. Douglas and R. P. Rubin, *J. Physiol., Lond.* **159**, 40 (1961).
12. M. OKA, T. OHUCHI, H. YOSHIDA and R. IMAIZUMI, *Life Sci.* **5**, 427 (1966).
13. A. H. ANTON and D. F. SAYRE, *J. Pharmac. exp. Ther.* **138**, 360 (1962).
14. M. J. KARNOVSKY, *J. Cell Biol.* **27**, 137A (1965).
15. G. E. PALADE, *J. exp. Med.* **95**, 285 (1952).
16. J. H. LUFT, *J. biophys. biochem. Cytol.* **2**, 799 (1956)
17. D. C. PEASE, *Histological Techniques for Electron Microscopy*, 2nd edn, p. 51. Academic Press, New York (1964).
18. S. MALAMED, *J. Cell Biol.* **18**, 696 (1963).
19. J. H. LUFT, *J. biophys. biochem. Cytol.* **9**, 409 (1961).
20. M. L. WATSON, *J. biophys. biochem. Cytol.* **4**, 475 (1958).
21. E. S. REYNOLDS, *J. Cell Biol.* **17**, 208 (1963).
22. E. D. P. De Robertis, W. W. NOWINSKI and F. A. SAEZ, *Cell Biology*, p. 432. W. B. Saunders, Philadelphia and London (1965).
23. R. E. COUPLAND, *The Natural History of the Chromaffin Cell.* Longmans, London (1965).
24. R. Wetzstein, *Z. Zellforsch. mikrosk. Anat.* **46**, 517 (1957).
25. D. E. WOLFE, in *The Hormones* (Ed. G. PINCUS, K. V. THIMANN and E. B. ASTWOOD), vol. IV. p. 427. Academic Press, New York (1964).
26. G. E. PALADE and P. SIEKEVITZ, *J. biophys. biochem. Cytol.* **2**, 671 (1956).
27. M. LÉVY, R. TOURY and J. ANDRÉ, *C.r. hebd. Séanc. Acad. Sci. Paris* **262**, 1593 (1966).
28. E. D. P. DE ROBERTIS and A. VAZ FERREIRA, *Expl Cell Res.* **12**, 568 (1957).
29. N.-Å. HILLARP, B. HOKFELT and B. NILSON. *Acta Anat.* **21**, 155 (1954).
30. E. D. P. DE ROBERTIS and D. D. SABATINI, *Fedn Proc. suppl.* **5**, 70 (1960).