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

LOSS OF PROTEIN AND RETENTION OF LIPID IN SUBCELLULAR FRACTIONS

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Abstract—Subcellular fractions obtained from bovine adrenal medullae stimulated to secrete catecholamines were compared with corresponding fractions from unstimulated medullae. Stimulation (with acetylcholine) caused a fall in the catecholamines and protein in the 'crude granule fraction', but no change in the phospholipid or cholesterol. Further resolution of the crude granule fraction by density gradient centrifugation revealed a redistribution of the phospholipid and cholesterol after stimulation from the denser to the lighter layers. The results are interpreted to mean that the membranes of the chromaffin granules remain in the chromaffin cells after catecholamine secretion as discrete particles with reduced specific gravity. Probably these particles are the electron-translucent granules observed by electron microscopy in stimulated chromaffin cells.

WHEN the adrenal medulla is stimulated, catecholamines appear in the effluent from the adrenal gland along with other substances known to be present in the chromaffin granules—adenine nucleotides[1–3] and protein.[4, 5] This has been taken to indicate that the chromaffin granules participate directly in catecholamine release. The granules, however, do not seem to be extruded intact into the vasculature since medullary stimulation causes no increase in the efflux of phospholipid and cholesterol,[6] the main components of the granule membranes. It thus appears that the chromaffin granules somehow discharge their contents of catecholamines, nucleotides, and protein without leaving the cell.

In the present experiments we have examined the lipid content of subcellular fractions from stimulated and unstimulated adrenal medullae with the aim of corroborating this conclusion and of learning something of the fate of the retained granule membranes. In addition, we have measured the protein content of these fractions, since earlier conclusions that protein in the chromaffin granules falls little, or not at all,[7, 8] upon catecholamine release seem to be in conflict with the recent evidence that such protein is discharged along with the catecholamines.

METHODS

Perfusion of adrenal glands in vitro. Bovine adrenal glands obtained from the slaughterhouse were perfused in retrograde fashion through the adrenal vein with

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Locke's solution at room temperature (22–25°) as described in the previous paper.[6] Acetylcholine (10^{-4} g/ml) was infused for 4-min periods at 9-min intervals until a total perfusion time of 180 min had elapsed. The total perfusate was assayed for catecholamines to estimate the efficacy of stimulation. Initially, glands perfused with Locke's solution (without ACh) served as controls, but in later experiments the control glands were not perfused and yielded qualitatively similar results. In each experiment, the control gland was the contralateral gland (right or left) from the same animal.

Perfusion of adrenal gland in situ. The left adrenal gland of a 4-week-old calf was perfused through its arteries with Locke's solution at room temperature (25°) as described in the previous paper.[6] Stimulation with acetylcholine (ACh) was performed as in the glands *in vitro* except that the concentration was 2×10^{-5} g/ml. Again, the perfusate was collected and assayed for catecholamines to assess the degree of stimulation. The right adrenal gland, which was removed before perfusion, served as the control.

Cell fractionation. Chromaffin granules and other subcellular fractions from stimulated and control adrenal glands were prepared by a method based on that of Banks.[9] The 600 *g* supernatant was centrifuged at 20,000 *g* for 20 min to recover all the chromaffin granules.[10] This sediment, which contains mitochondria and lysosomes in addition to chromaffin granules,[9, 10] was resuspended in 0·3 M sucrose and recentrifuged at 20,000 *g* for 20 min. The sediment thus obtained (which will be referred to as the crude granule fraction) was resuspended in 0·3 M sucrose and aliquots were taken for chemical determinations. In some experiments an aliquot of this crude granule fraction was further resolved by density gradient separation as described by Banks.[9] After centrifugation for 60 min at 145,000 *g*, six fractions were separated by aspiration and were used for chemical determinations. These fractions corresponded to the original sucrose layers of the gradients as follows: 0·3 M, 1·4 plus 1·5 M, 1·55 M, 1·6 M, 1·7 M, and 1·8 plus 2·5 M (see Fig. 1).

Chemical determinations. Catecholamines, phospholipids, cholesterol and protein were determined as described in the previous paper.[6]

Expression of results. Since the glands differed in size and catecholamine content, all values were normalized by relating the results to the weight of medulla used and correcting for the degree of homogenization. The latter was calculated from the fraction of the total catecholamines found in the low speed sediment. This fraction was assumed to represent unbroken cells and averaged about 20 per cent in both control and stimulated glands.

In a number of experiments the amount of phospholipid and cholesterol found in the crude granule fraction obtained from stimulated glands was compared with the amount of these substances which would be expected were whole chromaffin granules to be extruded from the glands. In calculating the expected values, the following formula was used: $S = 100[1 - (\Delta CA \times Rg/Sc)]$, where S = amount of phospholipid (or cholesterol) expected in the crude granule fraction from the stimulated gland as a per cent of that in the control gland, assuming whole granule extrusion; ΔCA = depletion of catecholamines in the crude granule fraction (control less stimulated in μg/g medulla); Rg = ratio of phospholipid (or cholesterol): catecholamines in pure chromaffin granules;[6] and S_c = amount of phospholipid (or cholesterol) in the crude granule fraction of the control gland (in μg/g medulla).

RESULTS

Effect of stimulation on the composition of the crude granule fraction

The mean output of catecholamines in response to stimulation with ACh in 10 glands was 8·94 ± 0·74 mg/gland. The crude granule fraction obtained from these stimulated glands contained 59·4 ± 4·7 per cent of the catecholamines found in the corresponding fractions from unstimulated glands. In contrast, stimulation with ACh caused no change in the phospholipid or cholesterol contents of the crude granule fractions. The fractions from stimulated glands contained 104·8 ± 7·3 per cent of the phospholipid and 103·8 ± 9·9 per cent of the cholesterol of the fractions from the control glands (Table 1).

TABLE 1. CATECHOLAMINES, PHOSPHOLIPIDS, AND CHOLESTEROL IN CRUDE GRANULE FRACTIONS FROM STIMULATED ADRENAL MEDULLAE

Substance	Value observed (% of control)	Significance of stimulated vs. control	Value calculated, assuming extrusion of whole granules (% of control)	Significance of observed vs. calculated
Catecholamines	59·4 ± 4·7	$P < 0.001$		
Phospholipids	104·8 ± 7·3	$P > 0.5$	64·3 ± 6·3	$P < 0.001$
Cholesterol	103·8 ± 9·9	$P > 0.6$	50·1 ± 6·7	$P < 0.001$

These experimentally determined values for phospholipid and cholesterol in the crude granule fraction may be compared with the values calculated on the hypothesis that whole chromaffin granules are released from the gland (see Methods). The calculated and observed values are significantly different (Table 1).

Although phospholipid and cholesterol in the crude granule fraction did not fall upon stimulation in any of the 10 experiments, there was a significant depletion of protein. The mean protein content of the crude granule fraction from the stimulated glands was 71·2 ± 5·0 per cent of that of the corresponding fraction from the unstimulated control glands ($P < 0.001$).

Effect of stimulation on the distribution of substances within the crude granule fraction

When the crude granule fraction was further resolved by density gradient centrifugation, it was found that the depletion of catecholamines was principally from the denser layers, which were shown by Banks[9] to contain the chromaffin granules. This effect was observed both in isolated glands (Fig. 1) and in the calf gland perfused *in situ* (a, Fig. 2). The loss of protein from the crude granule fraction in stimulated glands also seemed to be due mainly to loss from the denser layers of the sucrose gradient. One of four experiments is illustrated in Fig. 3. The results in the other three experiments were similar.

Although stimulation did not cause a fall in the phospholipid or cholesterol in the crude granule fraction, it did cause a change in the distribution of these lipids in the density gradient. Thus, in five experiments the phospholipid in fraction 6 from the stimulated glands was 58·4 ± 6·1 per cent of the corresponding control value. In four of these experiments the cholesterol content of fraction 6 was also measured and found to be 46·5 ± 9·8 per cent of the control value. The fall in both lipids after stimulation was significant ($P < 0.005$ and < 0.02 respectively). The fact that stimulation caused a fall in phospholipid and cholesterol in the densest fraction (which

contains pure chromaffin granules[9]), but no change in the phospholipid and cholesterol content of the whole crude granule fraction, indicates that some of these lipids that would otherwise have sedimented in the densest layers sedimented after stimulation in the lighter layers. The lighter layers contain much lipid from particles other than chromaffin granules, which obscures the pattern of redistribution. However, it was noted in each experiment that the phospholipid and cholesterol in fraction 2 from

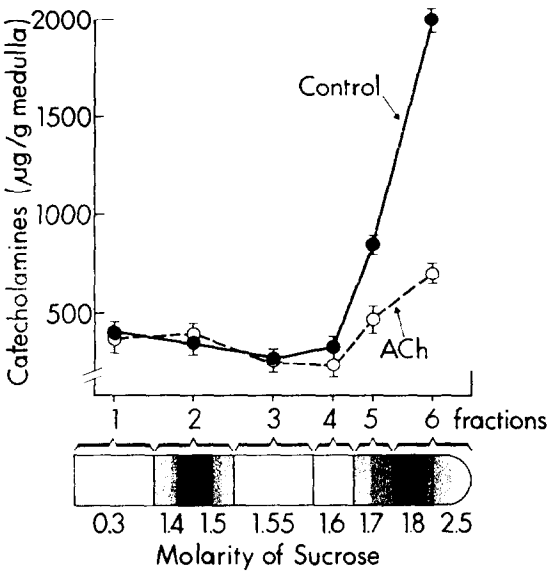

FIG. 1. Distribution of catecholamines in crude granule fractions obtained from unstimulated (control) and stimulated adrenal glands perfused *in vitro*. The crude granule fractions were resolved into six further fractions by density gradient centrifugation in sucrose as indicated by the diagram at the bottom of the figure. This diagram also shows the distribution of opaque material in the density gradient. The closed (●) and open (○) symbols represent the catecholamines in the fractions from control gland and glands stimulated with acetylcholine respectively. Each point represents the mean value (± S.E.) of three experiments.

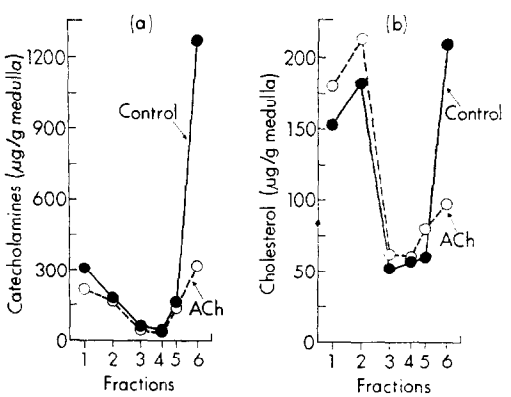

FIG. 2. Distribution of catecholamines and cholesterol in crude granule fractions obtained from an unstimulated (control) and a stimulated adrenal gland perfused *in situ*. The crude granule fractions were separated into six other fractions as in Fig. 1. The closed (●) and open (○) symbols represent the values from the control and ACh-stimulated glands respectively.

stimulated glands was greater than in the corresponding fraction from unstimulated glands, suggesting that some of the lipids lost from fraction 6 migrated to fraction 2. Although the increment was only about 15 per cent (fraction 2 contains the bulk of mitochondrial lipid) and was not statistically significant, the results obtained with

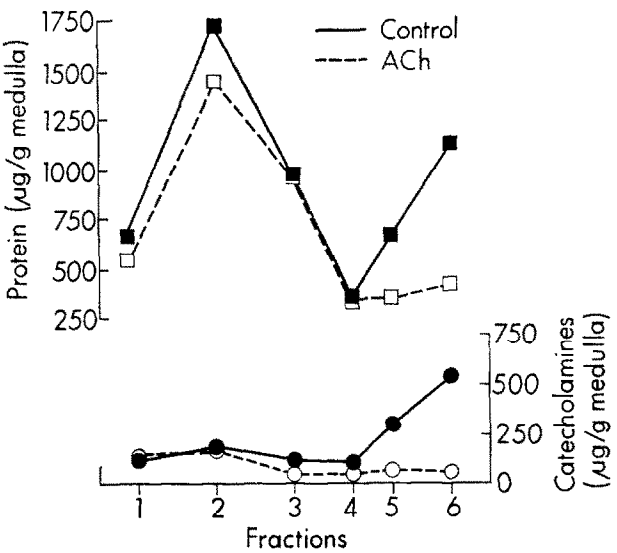

FIG. 3. Distribution of catecholamines and proteins in crude granule fractions obtained from an unstimulated (control) and a stimulated adrenal gland perfused *in vitro*. The crude granule fractions were separated into six other fractions as in Fig. 1. The closed symbols (● and ■) represent the values obtained from the control glands and the open symbols (○ and □) the values obtained from the gland stimulated with ACh.

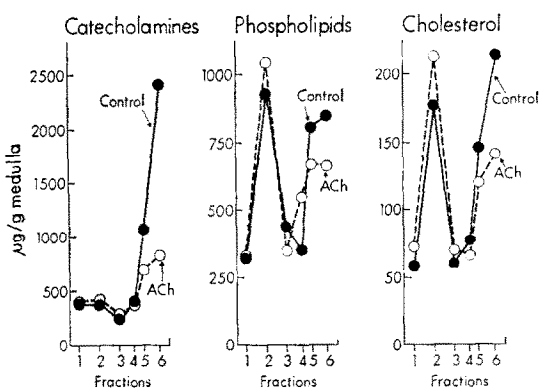

FIG. 4. Distribution of catecholamines, phospholipids, and cholesterol in crude granule fractions obtained from an unstimulated (control) and a stimulated adrenal gland perfused *in vitro*. The crude granule fractions were separated into six other fractions as in Fig. 1. (The closed (●) and open (○) symbols represent the values obtained from the control and ACh-stimulated glands respectively.

lysed granules (to be described below) are consistent with this interpretation. The results obtained in an experiment on an isolated gland are depicted in Fig. 4. In the gland perfused *in situ*, phospholipid was not measured, but the effect of stimulation on the distribution of cholesterol was the same as in the isolated glands (b, Fig. 2).

It seemed likely that the redistribution of lipid in the density gradient upon stimulation was due to a lowering of the specific gravity of chromaffin granules that had released catecholamines, nucleotides, and soluble protein. To test this, the following experiment was done. A purified fraction of chromaffin granules was prepared by using porous membrane filters.[11] One aliquot was layered directly on the density gradient, and after centrifugation most of the phospholipid and cholesterol was recovered in the 1·8 plus 2·5 M sucrose layer, as expected. A second aliquot was lysed in distilled water (which is known to release the water-soluble granule constituents*), sedimented, resuspended in 0·3 M sucrose, and then layered over the usual density gradient. After centrifugation, a sharp peak in phospholipids and cholesterol was found in the 1·4 M sucrose layer (i.e. fraction 2) with negligible amounts in the denser layers. The 1·4 M sucrose layer is precisely that in which the lipids increased in the experiments on the medullae stimulated with acetylcholine which are illustrated in Fig. 4 and in b, Fig. 2.

DISCUSSION

When the adrenal medulla is stimulated with ACh, there is clearly a fall in the protein content of the crude granule fraction, and most of the protein lost—to judge from the results obtained by density gradient centrifugation—is from the denser layers that have been shown by Banks[9] to contain highly purified chromaffin granules. This evidence is in harmony with recent demonstrations that soluble protein characteristic of the chromaffin granules appears in the effluent from adrenal medullae during catecholamine release.[4, 5] The earlier and contrary conclusion, that there is only a small and perhaps insignificant loss of protein from the chromaffin granules of stimulated adrenal medullary cells,[7, 8] can probably be explained by the fact that no allowance was made for other protein-containing particles in the granule fraction. Yet such contamination was doubtless present since the fraction was obtained with sedimentation forces comparable to those employed in the present experiments to prepare the crude granule fraction, which contains mitochondria and lysosomes in addition to chromaffin granules.[9, 10] In each of the earlier experiments a small fall in protein was in fact observed on stimulation[7, 8] and, if allowance is made for the presence of only a small amount of protein from sources other than chromaffin granules, the observed values can be reconciled with the view that soluble granule protein does escape on stimulation.

The present findings demonstrate, on the other hand, that the major lipid components of chromaffin granule membranes, phospholipids and cholesterol, remain in the medulla after catecholamine release. Unless there is some barrier interposed between the chromaffin cells and the venous sinuses that traps these lipids selectively while letting the nucleotides and protein escape, it must be concluded that the lipid components of the granules are retained by the chromaffin cells during secretory activity. The question that arises is what mechanism of release allows retention of granule lipid while letting the other granule constituents escape? Since ATP is a substance that would be expected to be rapidly hydrolyzed by the ATPases known to be present in the chromaffin cell[9] and, in addition, is generally believed to traverse membranes poorly, it was suggested that this mechanism of release might be reverse

* Dr. Sasha Malamed kindly prepared electron micrographs of the lysed chromaffin granules. These showed electron-translucent granules with intact membranes.

pinocytosis.[1, 3] The fact that the large molecules of the soluble protein of the chromaffin granules escape to the cell exterior may also be explained by such a mechanism. Some independent, morphological, evidence that reverse pinocytosis may be involved in catecholamine release from the chromaffin cell has been provided by electron micrographs.[12, 13]

There has been speculation that after reverse pinocytosis the membranes of emptied secretory granules may be incorporated into the plasma membrane of the cell. De Robertis *et al.* have applied this concept to the chromaffin cell and have suggested that the empty membranes of the chromaffin granules 'probably disappear within the surface membrane'.[14] The present results appear to be inconsistent with this conjecture. If the lipid membranes of the granules had been incorporated into the plasma membranes of the chromaffin cells, they should have sedimented along with these membranes in the low speed fraction and there should have been a corresponding fall in the lipid in the crude granule fraction. No such fall was observed. A further fact difficult to reconcile with the conjecture is that the phospholipid composition of the chromaffin granule membrane is quite unlike that of plasma membranes in having a high content of lysolecithin.[15, 16] Whittaker[17] has already pointed out that the lipid content of ACh-containing vesicles differs from that of the associated nerve membranes, and has advanced this as an argument against incorporation of vesicle membrane into nerve membrane during the release of ACh. And, finally, also counter to this view that granule membranes are incorporated into the plasma membrane, there is the electron microscopical evidence of electron-translucent granules, suggestive of emptied chromaffin granules, within the cytoplasm and subcellular fractions from stimulated medullary cells.[12, 18]

One alternative mechanism that would accommodate all the findings would be for the chromaffin granule to be attracted to the plasma membrane upon stimulation, to discharge some or all of its contents through an aperture in the fused membranes, and then return as a discrete particle to the cell cytoplasm. This would raise questions about the subsequent distribution of the granule membranes. It is possible that the chromaffin granules are cell organelles in the sense suggested by Wetzstein[19] and by Hillarp[20] and can be recharged with catecholamines. Alternatively, the emptied granules may be digested, perhaps by lysosomes, as has been proposed for intact secretory granules in the adenohypophysis.[21]

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