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EFFECT OF ACETYLCHOLINE ON THE SUBCELLULAR DISTRIBUTION OF ^{45}Ca IN BOVINE ADRENAL MEDULLA*

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Abstract—Bovine adrenals were perfused with ^{45}Ca in the presence and in the absence of acetylcholine. Extracellular ^{45}Ca was washed out by prolonged perfusion with ^{45}Ca-free medium. The ^{45}Ca (cpm/mg protein) remaining in medullary tissue after washout was proportional to the per cent of the total catecholamine store released during stimulation with acetylcholine. These data support the hypothesis that calcium may act at an intracellular site to initiate the release process.

Chromaffin granules present in the lower fractions of the density gradient of stimulated medullae contained relatively more ^{45}Ca than similar fractions of control glands. These results indicate that the exchange of calcium in heavy chromaffin granules with calcium in the environment increases during stimulation of adrenal medulla with acetylcholine.

ALTHOUGH it is known that ^{45}Ca is taken up by adrenal medulla during stimulation with acetylcholine,[1] there have been no previous reports of the subcellular disposition of the ^{45}Ca taken up. This study shows that the ^{45}Ca contents of nuclei, mitochondria, lysosomes and cell supernatant relative to the ^{45}Ca content of whole medulla are no different in stimulated as compared to control glands. The relative ^{45}Ca contents of chromaffin granules and microsomes, however, are altered by acetylcholine stimulation.

METHODS

Fresh bovine adrenal glands were obtained at a local abbatoir and placed on ice during transport. About 1–1½ hr post mortem, the glands were perfused through the adrenal vein with aerated Locke's solution[2] at room temperature at a rate of 5 ml/min with a multichannel metering pump (Harvard Apparatus Co.). After a period of equilibration of about 45 min, the glands were stimulated with 35 ml of Locke's solution containing 100 μg acetylcholine and 1 μc ^{45}Ca per ml. The acetylcholine was perfused through the adrenal vein in four portions, three of 10 ml and one of 5 ml. Each portion was separated by perfusion of 15 ml of acetylcholine-free and ^{45}Ca-free Locke's solution. This procedure was used in an effort to avoid the decreased response which occurs during prolonged stimulation.[2] Controls were treated in the same manner except that acetylcholine was omitted from the perfusing fluids. The rate of perfusion was not altered by acetylcholine treatment. The glands were then perfused with

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265 ml of Locke's solution free of acetylcholine and ^{45}Ca, in order to wash out the extracellular ^{45}Ca present in the tissue.[1]

Medullae were then separated from cortices and homogenized in cold (4°) 0·32 M sucrose in a glass homogenizer. Subcellular fractions were separated by differential and gradient density (1·25, 1·5, 1·75 and 2 M sucrose; 2 ml each) centrifugation as previously described,[3, 4] except that the density gradient was centrifuged at 120,000 *g* for 48 min. Fractions were removed by using a Pasteur pipette. Microsomes were separated from cell supernatant by centrifugation (110,000 *g* for 45 min) of the supernatant remaining after sedimentation of mitochondria, lysosomes and granules. The fractions were analyzed for protein by the biuret method[5] and for catecholamines by the colorimetric method of von Euler and Hamberg.[6] Small amounts of protein in the perfusates did not significantly interfere with the estimation of catecholamine, since recoveries of known amounts of epinephrine added to perfusates averaged 102 per cent with a coefficient of variation of 6·5 per cent. Changes in optical density of a solution of horse heart cytochrome *c* buffered to pH 7·5, measured at 550 mμ in the presence of succinate and cyanide, were taken as a measure of succinic cytochrome *c* reductase activity. Aliquots of the subcellular fractions were wet-digested with HNO_3 and H_2O_2. Aliquots of the digests were analyzed for calcium and magnesium by atomic absorption spectroscopy with a Perkin–Elmer model 290. Recoveries of known amounts added to medullary tissue averaged 89 per cent for calcium and 102 per cent for magnesium. The data given are not corrected for recovery. Digest aliquots were also dried on planchets and ^{45}Ca was measured in a gas flow counter with an efficiency of 20 per cent.

Cethepsin was assayed by the method of Anson[7] and acid phosphatase was measured at pH 5 (37°) with *p*-nitrophenyl phosphate as the substrate.

RESULTS

Purity of the fractions. The effectiveness of the separation of mitochondria, lysosomes and chromaffin granules was determined by measuring substances characteristic of these particles. Table 1 shows that mitochondria are most concentrated in fractions II and III, as reflected by the succinic cytochrome *c* reductase activities. Cathepsin and acid phosphatase activities are high in fraction III, indicating that this fraction

TABLE 1. ENZYME AND CATECHOLAMINE DISTRIBUTION IN SUCROSE DENSITY GRADIENT FRACTIONS OF BOVINE ADRENAL MEDULLA*

Fraction	Succinic cytochrome c reductase (μmoles cyt. *c* reduced/min/g protein) (n = 14)	Cathepsin (μmoles tyrosine/min/g protein) (n = 9)	Acid phosphatase (μmoles hydrolyzed/min/g protein (n = 13)	Epinephrine (μg/mg protein) (n = 7)	Norepinephrine (μg/mg protein) (n = 7)
II	31 ± 3·5	7·4 ± 2·7	16 ± 3	14·6 ± 1·9	7·1 ± 52
III	30 ± 3·3	56 ± 8·3	16 ± 1·8	87·4 ± 9·0	23 ± 2·6
IV	4·1 ± 0·46	34 ± 4	8·2 ± 4·8	205 ± 18	66·4 ± 10
V	1·1 ± 0·11	33 ± 19	3·7 ± 1·1	209 ± 37	305 ± 47
VI	2·8 ± 0·36	7·8 ± 5·9	6·6 ± 1·3	97 ± 16	125 ± 9·7

* Values given are means ± standard error. See text for methods.

is rich in lysosomes. A small microsomal component in fraction II very likely contributes to the acid phosphatase activity of this fraction. Catecholamines and therefore chromaffin granules are most concentrated in fraction V, although fractions IV and VI are also rich in chromaffin granules. In fraction IV epinephrine predominates, whereas in fraction VI norepinephrine is predominant. These results are generally in agreement with those of Smith and Winkler, who reported that mitochondria, lysosomes and chromaffin granules are separable on a sucrose density gradient.[8]

The distribution of subcellular particles in stimulated medullae was no different from that in non-stimulated medullae. Therefore, the relatively mild stimulation employed [average per cent of catecholamine (epinephrine plus norepinephrine) release during and between periods of acetylcholine stimulation = 6·7] did not produce any detectable changes in density of any of the subcellular particles in this tissue.

Correlation between ^{45}Ca uptake and catecholamine-release. Figure 1 shows that the per cent of the total catecholamine store which was released from bovine adrenals by acetylcholine is proportional to the ^{45}Ca remaining in the medulla after the perfusion.

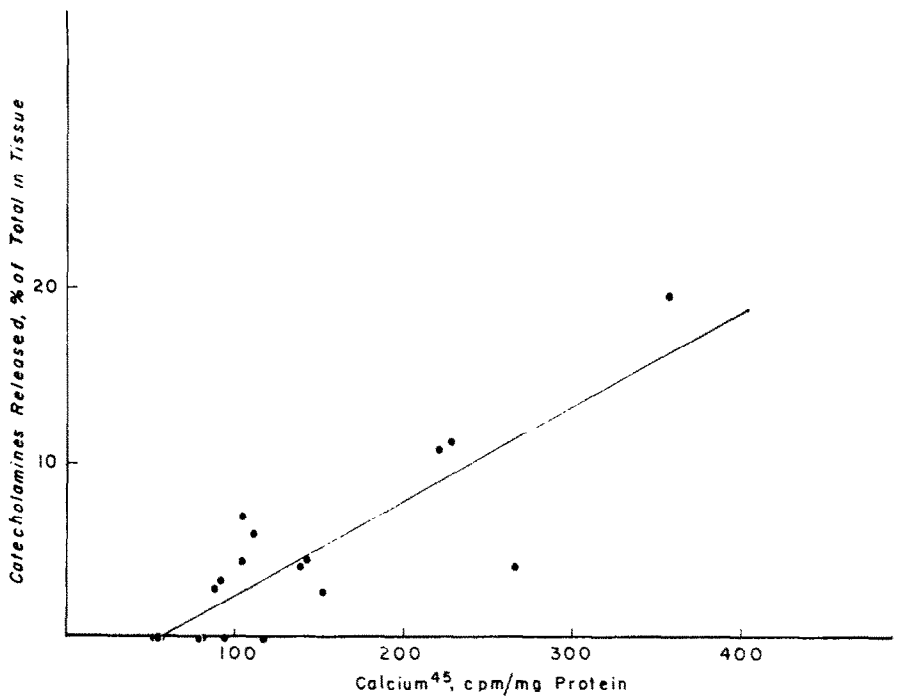

FIG. 1. Bovine adrenals were stimulated with acetylcholine in the presence of ^{45}Ca. Total catecholamines were measured in the effluent and in the medullary tissue after the perfusion. Per cent of total in tissue refers to the amount of catecholamine released by acetylcholine stimulation over the total catecholamines in the medulla before stimulation. $^{45}Calcium$ was measured in the tissue after washout with 265 ml of ^{45}Ca-free Locke's solution. The line was fixed by the method of least squares.

The glands in which no response was obtained were not treated with acetylcholine. The correlation is significant at the 0·1 per cent level.

Increased uptake of ^{45}Ca induced by acetylcholine may have been due to dilation of blood vessels by acetylcholine resulting in improved distribution to areas underperfused in controls. However, perfusion of bovine adrenals with 35 ml sodium nitrite

(1·0 mg/ml substituted for an isosmotic amount of sodium chloride in the Locke's solution) in the presence of ^{45}Ca (1 $\mu c/cm^3$), using the same procedure as with acetylcholine, produced no increase in ^{45}Ca uptake in medullae of two glands. Two control glands, perfused simultaneously with the nitrite-treated glands, both contained 67 cpm/mg protein of ^{45}Ca and corresponded to other nonstimulated controls shown in Fig. 1. The sodium nitrite-treated glands contained 63·5 and 72·7 cpm/mg protein of ^{45}Ca. This ^{45}Ca content was less than that of any of the acetylcholine-treated glands (Fig. 1). That the dose of sodium nitrite used was effective in dilating blood vessels of bovine adrenals was proven by perfusing four glands with 1 mg/ml of sodium nitrite during the 45-min equilibration period. In each of the four cases, the rate at which blood protein was washed out was increased by sodium nitrite treatment. In general these data show that dilation of the blood vessels of perfused bovine adrenals does not influence the uptake of ^{45}Ca by the adrenal medulla.

The correlation between per cent release and ^{45}Ca content was no better with any of the subcellular fractions than with the whole homogenate. For example, as seen in Fig. 2, the ^{45}Ca content of chromaffin granules is simply proportional to the ^{45}Ca content of the whole homogenate.

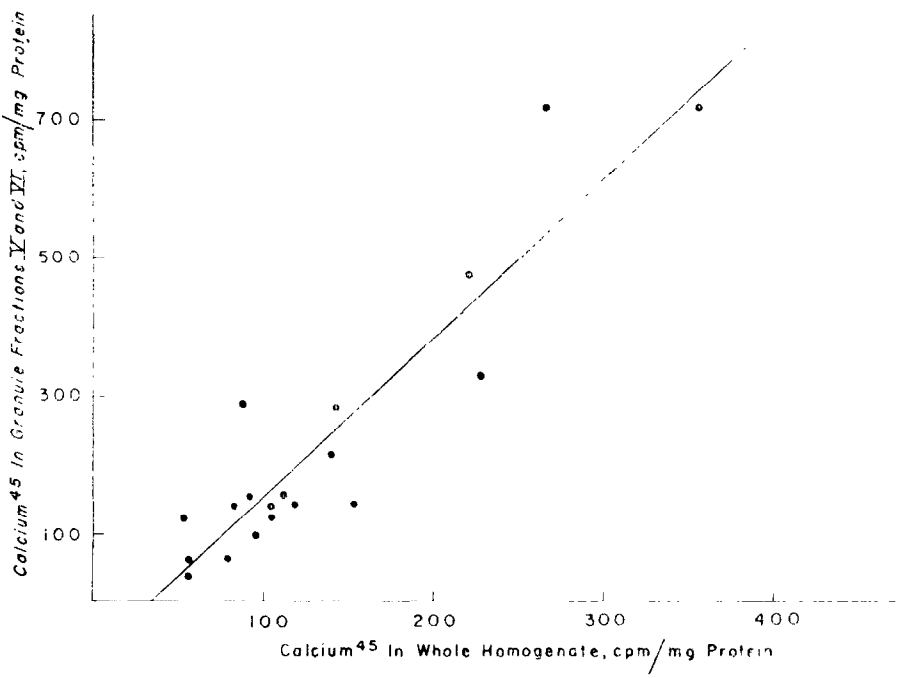

FIG. 2. The correlation between ^{45}Ca in chromaffin granule fractions V and VI and ^{45}Ca in whole homogenate of bovine adrenal medulla is significant at the 0·1 per cent level. The line was drawn by the method of least squares.

Calcium and magnesium in stimulated and control medullae. The release of enzymes from polymorphonuclear leukocytes is similar to the release of catecholamines from adrenal medulla in that both require calcium. Leukocytes have been shown to increase in calcium content 4-fold and decrease in magnesium content about 5-fold during

release of their granule-bound enzymes.[9] However, in bovine adrenal medulla, acetylcholine stimulation did not significantly change the calcium or magnesium contents per milligram of protein of the whole tissue homogenate or of any of the subcellular fractions (Table 2).

TABLE 2. CALCIUM AND MAGNESIUM DISTRIBUTION IN SUBCELLULAR FRACTIONS OF ACETYLCHOLINE-TREATED AND UNTREATED BOVINE ADRENAL MEDULLAE

Fraction and major type particle	Calcium (μmoles/g protein ± S.E.)		Magnesium (μmoles/g protein ± S.E.)	
	Treated (n = 10	Untreated (n = 5)	Treated (n = 10)	Untreated (n = 5)
I (Soluble material)	72 ± 18	31·5 ± 5·5	51 ± 12	54 ± 9·5
II (Mitochondria)	51 ± 4·8	51 ± 6·7	30 ± 4·5	32 ± 10
III (Mitochondria and lysosomes)	68 ± 8	57 ± 10	26 ± 4·1	21 ± 4·5
IV (Lysosomes and granules)	62 ± 9·8	61 ± 11	20 ± 4·1	19 ± 1·5
V (Granules)	120 ± 22	82 ± 21	52 ± 12	21 ± 5·8
VI (Granules)	93 ± 9	66 ± 13	30 ± 5·8	44 ± 14
Nuclei and cell debris	29 ± 4	17 ± 3	24 ± 7·8	15 ± 7·5
Microsomes	21 ± 3	26 ± 6	18 ± 4·1	23 ± 4·5
Cell supernatant	12 ± 1·5	12 ± 1·2	40 ± 5	45 ± 7·5

^{45}Calcium content of subcellular fractions of stimulated and control medullae. Although no significant differences in calcium content of subcellular fractions of stimulated versus control adrenal medullae were found, all subcellular fractions (except microsomes) of stimulated glands contained significantly more ^{45}Ca than corresponding fractions of controls (Fig. 3).

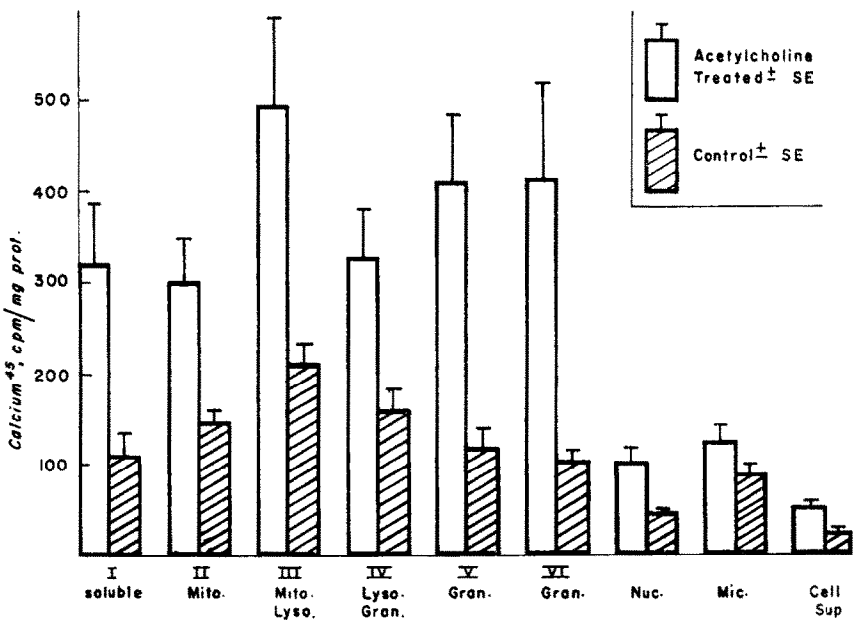

FIG. 3. The major type of particle present in the fractions is indicated on the graph. Soluble = soluble material; Mito. = mitochondria; Lyso. = lysosomes; Gran. = chromaffin granules; Nuc. = nuclei and cell debris; Mic. = microsomes; Cell Sup. = cell supernatant.

In order to compare more easily the ^{45}Ca contents of subcellular fractions of stimulated and nonstimulated medullae, the results of Fig. 3 were expressed as the following ratio:

$$\frac{^{45}Ca \text{ in each fraction/total } ^{45}Ca \text{ in whole homogenate}}{\text{protein in each fraction/total protein in whole homogenate.}}$$

These ratios are given in Table 3. By expressing the results in this manner, differences related to the larger amount of ^{45}Ca in stimulated medullae were eliminated. Table 3

TABLE 3. RATIO PER CENT TOTAL ^{45}Ca/PER CENT TOTAL PROTEIN (± S.E.) IN SUBCELLULAR FRACTIONS OF BOVINE ADRENAL MEDULLA*

Fraction and major type of particle	Perfused with ^{45}Ca		^{45}Ca added after perfusion	
	Acetylcholine treated	Control	Acetylcholine treated	Control
	(n = 12)	(n = 9)	(n = 6)	(n = 6)
I (Soluble material)	2·2 ± 0·42	1·29 ± 0·23	3·81 ± 0·80	2·26 ± 0·51
II (Mitochondria)	1·87 ± 0·16	1·87 ± 0·18	1·87 ± 0·27	2·32 ± 0·36
III (Mitochondria-lysosomes)	2·88 ± 0·16	2·59 ± 0·18	2·27 ± 0·28	3·13 ± 0·60
IV (Lysosomes-granules)	1·95 ± 0·16	1·87 ± 0·16	1·61 ± 0·37	1·36 ± 0·17
V (Granules)	2·39 ± 0·20	1·41 ± 0·21	1·77 ± 0·42	1·19 ± 0·22
VI (Granules)	2·30 ± 0·34	1·25 ± 0·17	1·12 ± 0·31	0·89 ± 0·11
Nuclei and cell debris	0·59 ± 0·027	0·64 ± 0·03	1·04 ± 0·28	0·95 ± 0·18
Microsomes	0·75 ± 0·053	1·11 ± 0·097	0·52 ± 0·083	9·46 ± 0·10
Cell supernatant	0·31 ± 0·025	0·28 ± 0·021	0·26 ± 0·036	0·26 ± 0·060

* The ratios given are the per cent of the total ^{45}Ca of the whole homogenate in each fraction divided by the per cent of the total protein of the whole homogenate in each fraction. In the glands perfused with ^{45}Ca, the ratios for granule fractions V and VI of stimulated glands are significantly different from those of controls ($P < 0·02$). Ratios for microsomes are also significantly different in ^{45}Ca perfused glands ($P < 0·01$). There are no significant differences in the ratios for acetylcholine-treated compared to control glands to which ^{45}Ca was added after the perfusion.

shows that the ratio is nearly the same for all subcellular fractions of the stimulated as compared to the control ^{45}Ca perfused glands, except for fractions I, V, VI and microsomes. The difference in the ratios of stimulated glands perfused with ^{45}Ca compared to those in controls is significantly greater ($P < 0·02$) in granule fractions V and VI and significantly less ($P < 0·01$) in microsomes. The ratios for fraction I of stimulated and control ^{45}Ca-perfused glands are not significantly different. Disruption of particles from fractions V and VI probably contributes in a variable manner to the elevated mean ratio of fraction I of stimulated glands.

Prolonged stimulation of bovine adrenal glands with acetylcholine causes a decrease in protein as well as catecholamine in granule fractions.[10] However, a decrease in protein was not involved in the observed increase in ^{45}Ca in granules, since the mild stimulation employed did not produce a significant decrease in protein content of chromaffin granules. Granule fractions V and VI of stimulated glands contained an average of 2·27 per cent of the protein of whole medulla, whereas the corresponding value for controls was 2·30 per cent.

The possibility that increased calcium exchange in heavy granules is related to increased synthesis of norepinephrine in stimulated glands cannot be ruled out. However, no catecholamine precursor was included in the fluid perfusing the glands

during the experiments showing increased calcium exchange in acetylcholine-stimulated medullae. Also, no significant difference in catecholamine per milligram of protein was found in acetylcholine-stimulated and control medullae, including resting secretion and secretion resulting from acetylcholine stimulation as well as the catecholamine remaining in the tissue. Therefore, no direct evidence is available indicating that synthesis of catecholamine is enhanced by acetylcholine stimulation of isolated perfused bovine adrenals.

Since fraction IV, which was rich in catecholamines, did not contain relatively more ^{45}Ca in stimulated glands, as was noted in the other granule fractions, it was thought that a subcellular particle different from the chromaffin granule might be responsible for the relative increase in ^{45}Ca content observed in granule fractions of stimulated glands. To test this possibility, granule fractions V and VI of four glands stimulated with acetylcholine in the presence of ^{45}Ca were homogenized in 2 M sucrose and separated by centrifugation for 1 hr at 120,000 *g* on a gradient density consisting of 2·1, 2·3 and 2·5 M sucrose (3 ml each). After centrifugation, the density gradients were separated into four fractions corresponding to the initial layers of sucrose. These fractions were analyzed as before for ^{45}Ca and total catecholamines. Protein was estimated by the method of Lowry *et al.*[11] Non-granule protein appeared in the 2·5 M sucrose, leaving relatively pure chromaffin granules in the upper layers of the density gradient. ^{45}Calcium per mg protein and catecholamine per mg protein paralleled one another in all the fractions of the density gradient (Fig. 4). These results

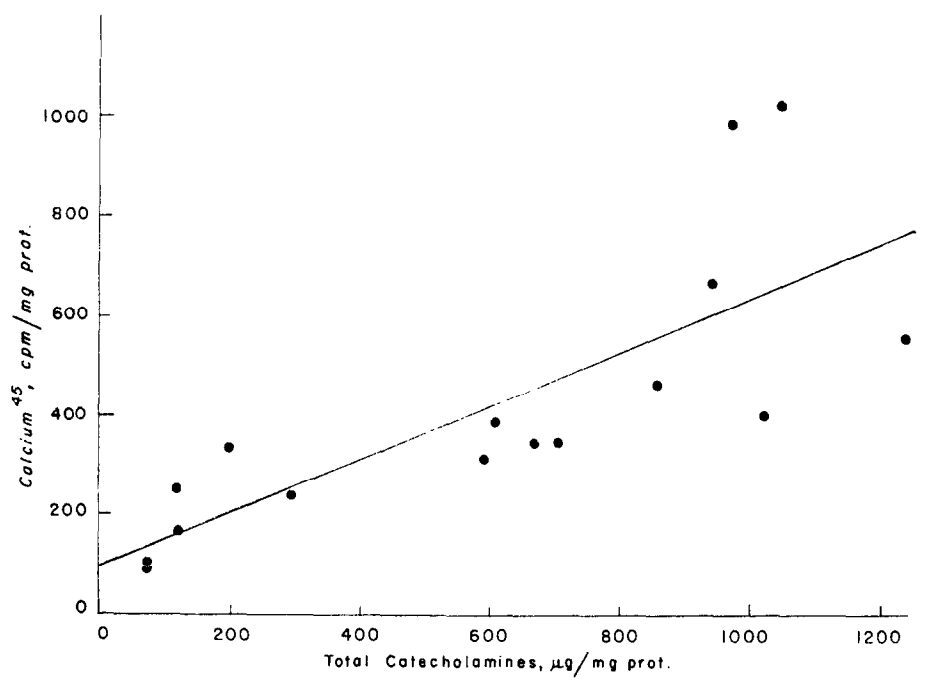

FIG. 4. Granule fractions V and VI (containing approximately 3 mg protein) from bovine adrenal medullae stimulated in the presence of ^{45}Ca, were separated on a sucrose density gradient and analyzed for ^{45}Ca catecholamines and protein The correlation is significant at the 0·1 per cent level The line was fixed by the method of least squares.

indicate that the ^{45}Ca found in fractions V and VI of stimulated glands is primarily associated with chromaffin granules.

The ^{45}Ca per mg protein of microsomes from glands stimulated in the presence of ^{45}Ca was not significantly different from the ^{45}Ca content of corresponding control medullae (Fig. 3). This is also reflected by the significantly lower ratio ($P < 0.01$), per cent total ^{45}Ca/per cent total protein, in medullary microsomes of glands stimulated in the presence of ^{45}Ca compared to their respective controls (Table 3).

To determine whether the presence of ^{45}Ca was necessary during stimulation with acetylcholine in order to obtain the observed ^{45}Ca distribution, experiments were done in which ^{45}Ca was added to adrenal medulla tissue just before homogenisation (Table 3). No significant differences in the ratios of acetylcholine-stimulated compared to control medullae were noted in these experiments and the ratios are analogous to those in nonstimulated glands perfused with ^{45}Ca, except for the nuclear and microsomal fractions and fraction I. Generally, these results indicate that ^{45}Ca must be present in the perfusing fluid at the time of acetylcholine stimulation or shortly thereafter in order to demonstrate an increased calcium exchange in granule fractions.

DISCUSSION

Douglas and Poisner have proposed that acetylcholine evokes secretion from adrenal medulla by promoting the uptake of calcium into adrenal medulla cells.[1] Calcium is thought to initiate catecholamine release by acting intracellularly.[12] The evidence obtained in the present study, showing a proportionality between catecholamine release and the ^{45}Ca remaining in adrenal medulla cells after washout, lends support to the hypothesis of Douglas and Poisner. Failure to find an increase in total calcium in stimulated glands is not evidence against the hypothesis, since small differences in intracellular calcium may have been masked by the relatively large extracellular calcium pool.

The results of the present study indicate that extracellular calcium enters the cells of the adrenal medulla during stimulation with acetylcholine and exchanges with available intracellular calcium pools. The data show that heavy chromaffin granules, containing relatively large amounts of norepinephrine, exchange calcium differently in acetylcholine-stimulated than in control medullae. This observation is interesting in view of the finding that calcium releases norepinephrine to a greater extent than epinephrine from isolated chromaffin granules.[13]

Increased ^{45}Ca exchange found in heavy granules during acetylcholine stimulation must involve a calcium pool which is normally inaccessible to extragranule calcium, since the ^{45}Ca content of heavy granules remained elevated compared to controls even after prolonged perfusion with ^{45}Ca-free Locke's solution and after isolation by centrifugation. It is known that all calcium binding sites of a disrupted chromaffin granule preparation are exchangeable.[3] It can be assumed, therefore, that the superficial calcium binding sites of chromaffin granules are not involved in the observed increase in granule calcium exchange induced by acetylcholine and that an intragranule calcium pool is involved.

Whether the increased penetration of ^{45}Ca into chromaffin granules during acetylcholine stimulation is related to changes in the granule membrane or to altered intragranule structure is not known. There is, however, some evidence to suggest that the granule membrane is normally permeable to calcium. Thus spontaneous loss

of calcium in parallel with catecholamines occurs from intact granules.[14] Also, intact granules increase in calcium content when incubated in a medium containing 5 mM $CaCl_2$.[15] This calcium binding is probably in excess of that which can occur on the external surface of the granules. Perhaps a rearrangement of intragranule structure occurs during acetylcholine stimulation and allows for the observed increase in calcium exchange.

It has been noted by several investigators that the lighter granules contain predominantly epinephrine, whereas heavy granules contain relatively more norepinephrine,[16–18]. If acetylcholine stimulation of bovine adrenals released only norepinephrine, it might be expected that only norepinephrine-containing granules would show the increased calcium exchange. However, we have shown in three experiments that the catecholamine released over a 4-min period by acetylcholine stimulation (10 cm^3 of a solution of 100 $\mu g/cm^3$, at a rate of 5 cm^3/min) of bovine adrenals is only 31·5% (S.E. = 2·4 per cent) norepinephrine. The evidence presented in this study indicates that chromaffin granules, which have a high density and high norepinephrine content, are functionally different from lighter epinephrine-containing granules with respect to calcium metabolism.

In glands perfused with ^{45}Ca, all subcellular fractions of acetylcholine-stimulated bovine adrenal medullae contained significantly more ^{45}Ca per mg protein than nonstimulated medullae, with the exception of the microsomal fraction. The calcium of endoplasmic reticulum apparently exchanges readily with extracellular calcium and ^{45}Ca is rapidly lost from this subcellular structure during the 53-min washout period.

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