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CATECHOLAMINE BINDING COMPLEX IN BOVINE ADRENAL MEDULLA

POSSIBLE INVOLVEMENT OF CALCIUM

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Abstract—Seventy per cent of the calcium of chromaffin granules of bovine adrenal medulla was estimated to be nonexchangeable by incubation of chromaffin granules with ^{45}Ca for 19 hr at 4°. By contrast, only 34 per cent of the calcium of a preparation from bovine adrenal medulla containing lysosomes and mitochondria, as well as chromaffin granules, was nonexchangeable. These results indicate that a relatively large amount of calcium is held in a nondiffusible form within chromaffin granules and may be part of the binding complex for catecholamines in adrenal medulla.

Stimulation of bovine adrenals with acetylcholine in the presence of DOPA and ^{45}Ca resulted in a greater accumulation of ^{45}Ca in granule fractions than stimulation with acetylcholine in the absence of DOPA. This finding suggests that calcium is incorporated into chromaffin granules during catecholamine synthesis and supports the hypothesis that calcium is part of the catecholamine storage complex in adrenal medulla.

CALCIUM is present in larger amounts in chromaffin granules of bovine adrenal medulla than any other metallic element.[1] The calcium present in these chromaffin granules is thought to be located inside the granules since it is released along with the catecholamines when the granules are exposed to a hypotonic medium.[2] Also, the sediment from disrupted granules binds no more calcium than the sediment from disrupted mitochondria, yet granules have more calcium per milligram of protein than mitochondria.[2] Furthermore, spontaneous release of catecholamines from isolated granules is associated with a parallel release of calcium.[3] The data of the present study provide further evidence that calcium is part of the binding complex for catecholamines in bovine adrenal medulla.

METHODS

Determination of nonexchangeable calcium in chromaffin granules. Nuclei and cell debris were separated from homogenates of fresh bovine adrenal medulla by centrifugation at 480 *g* for 10 min. Aliquots of the resulting supernatant were filtered through a series of millipore filters as described by Poisner and Trifaro.[4] The concentration of catecholamines per milligram of protein was increased from an average of 132 to 166 μg/mg protein by this procedure. The relatively pure granule material in the filtrates was separated from microsomes by centrifugation at 10,000 *g* for 30 min. The remaining unfiltered aliquots of the supernatant were centrifuged at 10,000 *g* for 30 min and this material is designated large granule fraction (LGF). The pellets were suspended in 0·32 M sucrose by gently homogenizing in a glass homogenizer. Aliquots of the

granule preparations containing on the average 5·6 mg of protein and LGF's containing an average of 8·2 mg of protein were incubated at 4° in 0·32 M sucrose containing 1·7 mM calcium, 0·1 μc of ^{45}Ca and 0·06 M acetate buffer, pH 6·0, (total volume = 6·5 ml). After incubation for 1 hr or 19 hr, particulate matter was isolated by centrifugation at 10,000 *g* for 30 min. The pellet was washed twice without resuspension with 0·32 M sucrose and then suspended in double distilled water. In some experiments the particles were washed by resuspension in 0·32 M sucrose and spun down again at 10,000 *g* for 30 min before suspension in distilled water. Aliquots of the suspensions were analyzed for protein by the biuret method[5] and for catecholamine by the method of vonEuler and Hamberg.[6] Aliquots were also wet digested with HNO_3 and H_2O_2. Aliquots of the digests were taken for measurement of calcium using a Perkin–Elmer, model 290, atomic absorption spectrometer. Additional aliquots of the digests were dried on plachets for determination of ^{45}Ca, using a Nuclear Chicago gas-flow counter with an efficiency of 40 per cent. ^{40}Ca was assumed to be maintained at 1·7 mM in the supernatants of the incubation mixtures. Exchangeable calcium was calculated using the following formula:

$$\text{Exchangeable } Ca^{2+} = \frac{(^{45}Ca \text{ in sediment})\,(^{40}Ca \text{ in supernatant})}{^{45}Ca \text{ in supernatant}}$$

Calcium in the granules in excess of the calculated exchangeable calcium was assumed to be bound in a nonexchangeable manner.

The above formula assumes no net gain or loss of calcium by the particles during the incubation. However, a small decrease in calcium content was noted in both LGF and granule fractions (14·7 and 17·7 per cent respectively) in one experiment. Such small changes in calcium content were not considered in the calculation and the estimate of percentage nonexchangeable calcium is based only on the calcium remaining in the sediment after the incubation.

Perfusion of bovine adrenals. Fresh bovine adrenals were perfused through venous openings with aerated Locke's solution, as previously described.[6] Acetylcholine, 100 μg/cc, 35 ml, was used as before to stimulate release of catecholamine in the presence of 1 μc/ml of ^{45}Ca.[7] The acetylcholine-^{45}Ca solution was divided into 4 portions, 3 of 10 and 1 of 5 ml. The 4 portions of acetylcholine solution were infused at intervals separated by perfusion with 15 ml of acetylcholine-free and ^{45}Ca-free Locke's solution. The glands were then perfused for 53 min with Locke's solution to wash out extracellular ^{45}Ca.

l-DOPA, (dihydroxyphenylalanine) 300 mg/l., was included in the perfusion medium 10 min prior to stimulation with acetylcholine, as well as during perfusion with acetylcholine. Vitamin C, 10^{-3} M, was present with DOPA to minimize oxidation of DOPA and was also included in controls. After washout of extracellular ^{45}Ca, subcellular fractions of adrenal medulla were separated by differential and gradient density centrifugation as before.[7] Aliquots of the fractions were analyzed for protein, catecholamines and ^{45}Ca.

RESULTS

Nonexchangeable calcium in chromaffin granules. Calcium exchange in purified granule preparations was compared to calcium exchange in LGF which contains lysosomes and mitochondria in addition to chromaffin granules. Incubation carried

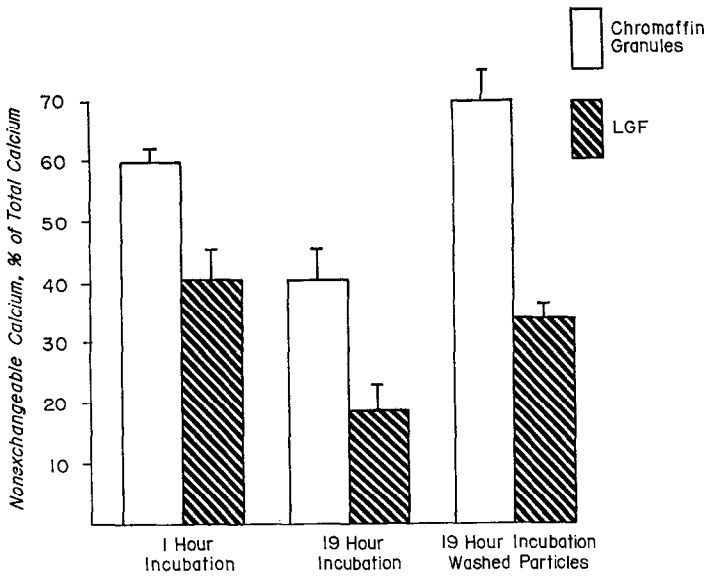

FIG. 1. Calcium exchange in subcellular fractions of bovine adrenal medulla. Relatively pure granule preparations were incubated at 4° with 1·7 mM calcium, 0·1 μc of ^{45}Ca, and 0·06 M acetate buffer, pH 6·0 (total volume = 6·5 ml). For comparison, a preparation containing mitochondria and lysosomes, as well as chromaffin granules from adrenal medulla (LGF), was used as a control. Means $\pm$ S. E. are given for four to six determinations. In each series of experiments, per cent non-exchangeable calcium was significantly greater in the chromaffin granule preparations than in the LGF's ($P < 0·01$). The mean calcium levels in granules and LGF in these experiments were found to be 2·55 and 1·98 μg/mg protein, respectively, and were not significantly altered by washing or by prolonging the incubation time. The mean ^{45}Ca levels in granules and LGF were 254 $\pm$ 24·5 (S. E.) and 342 $\pm$ 26·7 (S. E.) cpm/mg protein respectively. These values were decreased to 135 $\pm$ 7·5 (S. E.) and 244 $\pm$ 13 (S. E.) cpm/mg protein, respectively, after washing by resuspension, but were not significantly altered by prolonging the incubation time. The supernatants of the incubations contained 442 μg of ^{40}Ca and 247,000 $\pm$ 7000 (S. E.) dpm of ^{45}Ca.

out for 1 hr suggested that purified granule preparations contained more non-exchangeable calcium than LGF (Fig. 1).

To eliminate the possibility that equilibrium had not been reached between the calcium in the medium and the calcium in the particles, the incubation time was extended to 19 hr. The extended incubation time increased ($P < 0·05$) exchangeable calcium in granules from 40 to 60 per cent of the total calcium present. Exchangeable calcium in LGF was increased on the average from 63 to 81 per cent, but this difference was not significant. Thus, after the prolonged incubation, the purified granule preparation still exchanged less calcium than the LGF (Fig. 1).

Medium trapped between particles could have explained the estimated difference in exchangeable calcium between chromaffin granules and LGF. The larger particles in the LGF would be expected to trap more of the medium in the pellet than the smaller chromaffin granules. However, when the particles were washed by resuspension in 0·32 M sucrose, the difference in exchangeable calcium between LGF and granules was still evident (Fig. 1).

Lysis of the particles and loss of soluble material could have increased the percent

nonexchangeable calcium since the tonicity of the sucrose solution used to wash the particles after incubation was less than the tonicity of the incubation medium. However, when the particles were washed with a buffered sucrose solution with the same tonicity as the incubation medium, an increase in nonexchangeable calcium was still evident in both granules and LGF (86 and 42 per cent nonexchangeable calcium, respectively, means of two determinations).

These data indicate that a relatively large amount of the calcium of chromaffin granules from bovine adrenal medulla is bound in a nonexchangeable form.

Effect of DOPA on acetylcholine induced ^{45}Ca uptake into heavy chromaffin granules. It is possible that the reported uptake of ^{45}Ca into heavy chromaffin granules during acetylcholine induced catecholamine release from bovine adrenals was related to synthesis of catecholamine.[7] Accordingly, increasing synthesis by supplying the precursor, DOPA, might be expected to enhance ^{45}Ca uptake during acetylcholine-induced catecholamine release. Figure 2 shows that heavy chromaffin granules from

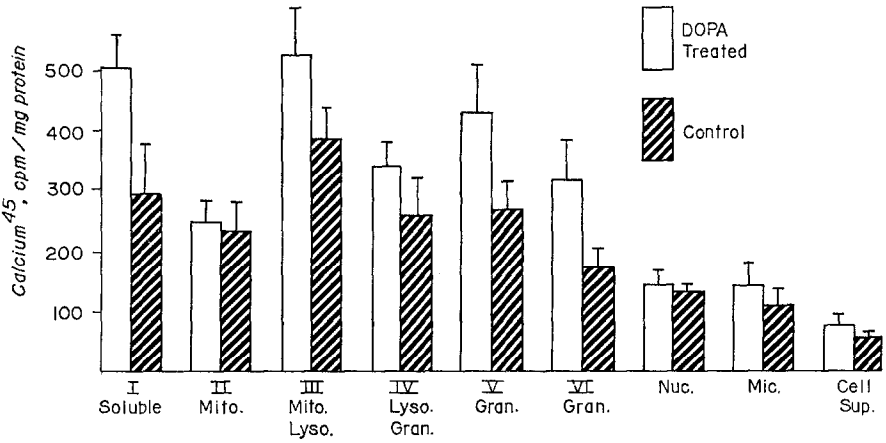

FIG. 2. Distribution of ^{45}Ca in subcellular fractions of bovine adrenal medulla stimulated with acetylcholine in the presence and in the absence of 1 DOPA (300 mg/l.). Each value given is the mean ± S. E. of five experiments. Fractions I through VI are fractions from the sucrose density gradients containing mitochondria (Mito.), lysosomes (Lyso.), chromaffin granules (Gran.), or soluble material (Soluble) from these particles. Other fractions contain primarily nuclear material (Nuc.), microsomes (Mic.), or cell supernatant (Cell Sup.).

glands stimulated with acetylcholine in the presence of 1 DOPA and ^{45}Ca, contained relatively more ^{45}Ca per milligram of protein than chromaffin granules from controls stimulated in the absence of 1 DOPA.

Figure 3 shows the ratio of the per cent 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 for Fractions V and VI of the density gradient. By expressing the results as a ratio in this manner, differences in total ^{45}Ca in the tissues of 1-DOPA treated and control glands are eliminated.

The ratios in Fraction V of DOPA treated glands were found to be significantly greater than the ratios in Fraction V of controls ($P < 0.05$) (Fig. 3) using a one-way

analysis of variance and Ducan's new multiple range test. The ratios of Fraction VI of DOPA-treated and control glands, however, were not significantly different by this analysis. Using a test of contrast, the overall effect of DOPA on the ratios in the granule fractions (V and VI) was found to be significant at the 2·5 per cent level (Fig. 3). Also, Fraction V was found to contain relatively more ^{45}Ca than Fraction VI in both DOPA-treated glands and controls ($P < 0{\cdot}01$). Thus, ^{45}Ca uptake into heavy granules of adrenal medulla is enhanced under conditions which promote synthesis of catecholamines, and the granules in Fraction V are more active in this regard than those in Fraction VI.

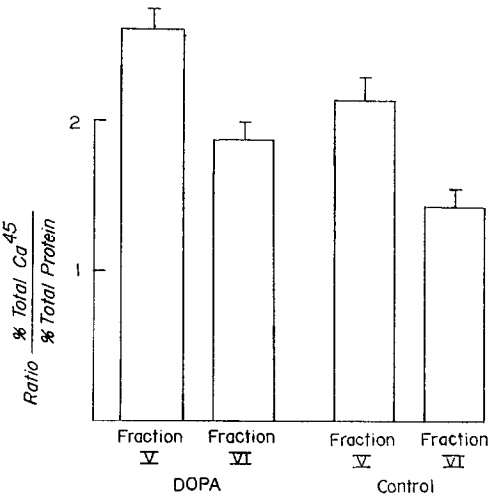

FIG. 3. The results of Fig. 2 from Fractions V and VI are shown here expressed as a ratio $\frac{^{45}Ca \text{ in each fraction/total } ^{45}Ca \text{ in whole homogenate}}{\text{protein in each fraction/total protein in whole homogenate}}$ to eliminate differences in total ^{45}Ca caused by DOPA treatment. ^{45}Ca is shown to accumulate in particles of Fraction V more than Fraction VI upon acetylcholine stimulation ($P < 0{\cdot}01$) in both DOPA-treated and control medullae. Stimulation with acetylcholine in the presence of 1 DOPA is shown to cause a greater accumulation of ^{45}Ca in granule Fractions V and VI than stimulation in the absence of 1 DOPA ($P < 0{\cdot}025$).

DISCUSSION

There is much evidence to indicate that catecholamines are combined with ATP in chromaffin granules of adrenal medula (see discussion by Hillarp).[8, 9] However, Weiner[10] stated that a complex between ATP and catecholamines may not be "the only means by which catecholamines are held within granules". According to the calculations of Hagen and Barrnett,[11] the catecholamines and ATP present in chromaffin granules would be approximately isotonic as a tetracatecholamine–ATP complex. Weiner[10] has pointed out, however, that no consideration was made for other solutes in the calculations of Hagen and Barrnett.[11] Furthermore, equivalence between cationic catecholamines and anionic phosphate of ATP is not always maintained in chromaffin granules; e.g. reserpine-induced depletion of catecholamines from fowl adrenals is not paralleled by loss of ATP.[12] Insulin, however, does cause equivalent decreases in ATP

and catecholamines in fowl[13] and rat[14] adrenals, but recovery of ATP levels after insulin depletion is more rapid than recovery of catecholamine levels.[14] Also, Schumann[3] has reported that exposure of isolated chromaffin granules to hypotonic media results in complete depletion of nucleotides while only 90 per cent of the catecholamines are released. Thus, it is possible that some of the ionic charges of the ATP of chromaffin granules are not associated with those of catecholamines and vice versa.

Calcium is present in adrenal chromaffin granules in relatively high concentrations.[1] It is estimated that for each calcium atom in chromaffin granules approximately 24 molecules of catecholamines are bound.[2] Assuming a 5:1 ratio of catecholamines to ATP,[14] there are about 5 molecules of ATP for each calcium atom present in granules of adrenal medulla. Since some granule calcium is very likely associated with the granule membrane, the actual intragranule ratio of ATP to calcium is probably in the order of 8 to 1. The results of the present study show that a substantial part of the granule calcium pool is nonexchangeable and therefore must be bound in some manner within the granule. Although it is not possible for calcium to interact with each ATP molecule present in the granules, it seems likely that a portion of the granule ATP store is calcium bound.

Hillarp[15] has shown that catecholamines and ATP are released in parallel both from the intact gland and from isolated granules. Kirshner *et al.*[16] have shown that release of vesicle protein and catecholamines by acetylcholine, barium or nicotine is also parallel within the limits of experimental error. Lastly, the release of calcium from isolated granules by exposure to hypotonic media occurs to about the same extent as release of catecholamines.[2] These results suggest that ATP, calcium, vesicle protein and the catecholamines combine to form the intragranule matrix seen in electron micrographs of chromaffin granules.

Magnesium is also associated with chromaffin granules, but to a lesser extent than calcium. There are 21 $\pm$ 5·8 S. E. μmoles of magnesium/g protein in a relatively pure granule fraction of bovine adrenal medulla.[7] By contrast, 82 $\pm$ 21 S. E. μmoles of calcium/g protein are contained in the same fraction. It is possible that any divalent metal ion may be used to form the catecholamine storage complex. However, some preference for calcium seems to exist since cell supernatant from bovine adrenal medulla contains about 3·5 times as much magnesium (on a μmole per gram of protein basis) as calcium, and yet calcium predominates in chromaffin granules.

The increase in ^{45}Ca in heavy chromaffin granules of adrenal medulla in the presence of DOPA suggests that calcium is incorporated into the granules during catecholamine synthesis. A similar observation was made by Burford and Gill[17] in cat salivary glands stimulated with acetylcholine. Stimulation not only caused release of salivary mucin but also increased mucin synthesis as reflected by increased hexosamine concentration in isolated granules. A large increase in calcium and ^{45}Ca was also noted after stimulation of cat submaxillary glands with acetylcholine. Thus, calcium may function as part of the storage form for salivary mucin as well as for catecholamines.

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