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UPTAKE OF CALCIUM IN CHROMAFFIN GRANULES OF BOVINE ADRENAL MEDULLA STIMULATED *IN VITRO*

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SUMMARY

The calcium content of bovine adrenal medulla perfused *in vitro* has been shown to increase about 30% in response to extensive acetylcholine stimulation. The calcium accumulated during secretion was mainly associated with the mitochondria and chromaffin granule fractions and to a lesser extent in the microsome fraction. While the calcium taken up by the mitochondria and microsomes was partly or totally removed by treatment with EDTA, the chelating agent had no effect on the granule content of calcium. The uptake of calcium in the mitochondria and microsomes during secretion is consistent with a function of these organelles in regulating the cellular calcium concentration. It is suggested that also the chromaffin granules may act as a "Ca-pump" in the chromaffin cell of the adrenal medulla.

INTRODUCTION

Calcium plays an important role in both storage and release of the catecholamines of the adrenal medulla. Thus calcium probably constitutes part of the storage complex binding the amines in a non-diffusable, osmotically inactive form in the chromaffin granule[1,2]. The apparent molecular weight of *in vitro* formed ATP–calcium aggregates increases by the addition of monoamines[3]. If similar reactions take place in the granules during maturation, the amines transported across the granule membrane[4] would subsequently be incorporated into a pre-existing complex of ATP, calcium and probably soluble proteins.

The essential role of calcium in the secretion of the catecholamines from the chromaffin cell was first demonstrated by Douglas and Rubin[5] but the intracellular action of calcium in triggering the secretory process is not known. It has been postulated that release of the amines is initiated by uptake of calcium in the granules[1], and calcium entering the cell in response to stimulation has been shown to exchange with the granular pool of calcium although no net increase in granular calcium was demonstrated[6].

Evidence has been presented indicating that chromaffin granules obtained from repeatedly stimulated bovine adrenal medulla represent immature granules[7]. These granules contained less catecholamines per unit protein and ATP than normally found for chromaffin granules. Further analysis of these granules revealed that they also contained a large excess of calcium.

Thus exhaustive secretion appears to give rise to chromaffin granules of a biochemical composition different from that of the granules from resting glands[7] and which in contrast to granules from moderately stimulated glands[6] seem to accumulate calcium during the secretory process. This apparent calcium-accumulating property of the granules from repeatedly stimulated glands has in the present paper been the subject for further investigation. The results presented were obtained from bovine adrenals perfused *in vitro* and stimulated with acetylcholine to release about 30% of their catecholamine content.

METHODS

Perfusion

The methods of perfusion and stimulation of the isolated bovine adrenals have been described previously[8,9]. Tyrode's buffer solution with a Ca^{2+} concentration of 1.80 mM was used as the perfusion medium[9]. Unless noted in the text both glands obtained from one animal were perfused, one serving as control. Total perfusion time was 195 min and secretion was maintained by injecting 4 ml of a 20 mM solution of acetylcholine chloride (Sigma) every 15 min giving 1 mmole acetylcholine in total.

Cell fractionation

Cell fractions were obtained from filtered homogenates[9], (1 g tissue wet wt per 5 vol. of 0.3 M sucrose of 0 °C) according to the scheme already described[8]. The homogenate was centrifuged at $800 \times g$ for 15 min and the supernatant was then centrifuged at $20000 \times g$ for 20 min. The $20000 \times g$ pellet, the large granule fraction, was further fractionated as described in the previous paper[9] using a sucrose density gradient made according to Banks[10]. Fraction 2 from the top (Fraction F2) contained mitochondria and Fraction 5, the layers of 1.7 + 1.8 M sucrose, contained most of the chromaffin granules (Fraction F5). Microsomes were isolated from the $20000 \times g$ supernatant by centrifugation at $105000 \times g$ for 60 min in the Beckman L2-65 ultracentrifuge, rotor Ti 50.

Analytical methods

Catecholamines were measured as the sum of adrenaline and noradrenaline as described by Bertler *et al.*[11] using a Farrand spectrofluorimeter, excitation at 390 nm and readings at 540 nm. Adrenaline bitartrate (Sigma) was used as standard.

Protein was estimated by the method of Lowry *et al.*[12]. Protein in cell fractions were precipitated with trichloroacetic acid, final concentration 5% (w/v). The precipitates were washed twice in 5% trichloroacetic acid and solubilized in 1 M NaOH. Granule protein was estimated after removal of the amines by dialysis against 2×500 vol. of 5 mM sodium succinate, pH 5.9.

Calcium was determined by atomic absorption spectrophotometry, using a Perkin Elmer spectrophotometer, model 290B. A 3-slot burner was applied and the gas mixture consisted of acetylene (4 l/min) and air (20.8 l/min). Cell fractions were extracted with $HClO_4$, final concentration 8% (w/v), and washed once with 8% $HClO_4$. The combined supernatants were used for determination of calcium after addition of $LaCl_3$ in a final concentration of 1% (w/v) (Lanthanoxide, purity grade A, Fluka AC, dissolved in conc. HCl). All standard solutions contained $LaCl_3$ and

$HClO_4$ in the same concentrations as the samples tested. In some experiments calcium in the cell fractions was determined after treatment with EDTA. Na_2–EDTA dissolved in a small volume of 0.3 M sucrose was added to aliquots of the homogenates to make a final concentration of 2 mM. Sucrose was added to control aliquots to make the same final volume and all samples were then gently shaken for 20 min at 4 °C before cell fractionation[1].

Statistics

Statistical significance was determined by Student's *t*-test for dependent groups.

RESULTS

The calcium content in cell fractions from non-perfused, perfused and perfused/stimulated bovine adrenal medullae

In the present study the efficiency of homogenization and the degree of lysis were about the same for the three types of glands studied[9]. Thus 87–98% (mean value 92%) of the catecholamines were recovered in the $800 \times g$ supernatant and 27–36% (mean value 31%) of the catecholamines were recovered in the $20000 \times g$ supernatant. The observed changes in protein and calcium content in various cell fractions from the stimulated glands as compared to the controls were thus not due to the homogenization procedure.

In glands repeatedly stimulated with acetylcholine the catecholamine content was reduced to 0.64 ± 0.06 μmole/mg protein ($n=5$) as compared to 0.90 ± 0.07 μmole/mg protein ($n=5$) for the perfused controls. The non-perfused controls contained 0.85 ± 0.02 μmole catecholamines/mg protein ($n=3$). Table I shows that the stimulation of the perfused glands produced a highly significant accumulation of calcium in the tissue. The increase in the concentration of calcium per mg protein in the perfused control glands as compared to the non-perfused glands can be explained partly by loss of cellular proteins[9] and partly by uptake of calcium during the spontaneous secretion[9,13] taking place during perfusion.

The greater part of the calcium in the tissue was associated with the large granule fraction, which also accumulated most of the calcium taken up in response to acetylcholine (Table I). About 70–80% of the calcium bound in the large granules was recovered in the fractions of mitochondria (Fraction F2) and chromaffin granules (Fraction F5) in both control and stimulated glands. Stimulation caused no significant changes in the calcium content of the $20000 \times g$ supernatant but increased it in the microsomes (Table I).

The content of protein in the various cell fractions was changed by the acetylcholine-induced secretion but not always in a parallel manner to that of calcium. The mitochondrial fractions of the stimulated glands contained nearly twice the amount of calcium as compared to those of control glands when related to total cell protein (Table I). The protein content in the mitochondrial fraction increased as a result of secretion (Tables I and II) due to the presence of the emptied granule vesicles[8,14,15]. The calcium content per mg protein in Fraction F2 of the stimulated glands was still significantly higher than the corresponding fractions from the control glands (Table II).

The fractions of chromaffin granules (Fraction F5) of the stimulated glands

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ONTENT OF CALCIUM AND PROTEIN IN CELL FRACTIONS FROM NON-PERFUSED, PERFUSED AND
LATED BOVINE ADRENAL MEDULLAE

isolated bovine adrenals were perfused *in vitro* in the retrograde manner, using Tyrode's buffer as perfusion medium, total time
Secretion was induced every 15 min by acetylcholine (12 injections of each 4 ml of 20 mM acetylcholine in Tyrode's buffer).
stimulated glands, the perfused collateral control glands and the non-perfused control glands were obtained as described un
unt of calcium in the fractions has been related to mg cell protein and the values are means ± S.E.

	No. of expts	*Homogenate*	*Large granules*	*Mitochondria (Fraction F2)*	*Chromaffin granules (Fraction F5)*	*20000 × g supernatant*	*Mi*
		nmoles Ca^{2+}/mg cell protein					
used	3	51 ± 6	31 ± 0	10 ± 1	15 ± 2	13 ± 4	3
	5	65 ± 2*	45 ± 3	13 ± 1	16 ± 2	14 ± 2	6
stimulated	5	86 ± 1*	59 ± 3	21 ± 2	21 ± 1	16 ± 5	9
		Protein in fraction as % of total cell protein					
used		100	32 ± 3	9 ± 2	12 ± 1	54 ± 0	8
		100	35 ± 1	13 ± 2	11 ± 1	49 ± 2	10
timulated		100	34 ± 1	14 ± 1	7 ± 1	53 ± 1	14

tistical significance for the difference between these figures $P < 0.001$.

TABLE II

THE EFFECT OF ACETYLCHOLINE-INDUCED SECRETION ON THE RELATION OF CALCIUM TO PROTEIN IN THE MITOCHONDRIAL AND CHROMAFFIN GRANULE FRACTIONS

The experimental conditions were as described in Table I. % LG protein and % LG Ca^{2+} refer to the percentage of total large granule protein and calcium recovered in the fractions of mitochondria and chromaffin granules. Values are means ± S.E.

Glands	*No. of expts*	*Large granules (nmoles Ca^{2+}/mg protein)*	*Mitochondria (Fraction F2)*			*Chromaffin granules (Fraction F5)*			
			% LG protein	*% LG Ca^{2+}*	*nmoles Ca^{2+}/mg protein*	*% LG protein*	*% LG Ca^{2+}*	*nmoles Ca^{2+}/mg protein*	*μmoles catechol-amines/mg protein*
Non-perfused	3	98 ± 9	27 ± 3	33 ± 1	113 ± 18	35 ± 3	53 ± 1	125 ± 15	2.49 ± 0.17
Perfused	5	129 ± 8*	35 ± 2	30 ± 1	114 ± 9**	29 ± 2	49 ± 2	156 ± 23***	2.55 ± 0.17†
Perfused/stimulated	5	178 ± 13*	42 ± 2	35 ± 3	154 ± 12**	22 ± 2	45 ± 3	242 ± 22***	2.14 ± 0.10†

* Statistical significance for the difference between these figures $P < 0.01$.

** Statistical significance for the difference between these figures $P < 0.01$.

*** Statistical significance for the difference between these figures $P < 0.02$.

† Statistical significance for the difference between these figures $P < 0.05$.

were also enriched in calcium although not to the same extent as the mitochondria (Table I). However, the chromaffin granules which have taken part in the secretory process do not sediment in Fraction F5 (ref. 9). Stimulation thus produced a decline in the protein content in the granule fraction (Tables I and II) and the uptake of calcium per mg protein was found to be higher in Fraction F5 than F2 (Table II). The chromaffin granules from stimulated glands contained less catecholamines per mg granule protein than those from control glands in agreement with earlier observations[8,9].

Smaller emptied vesicles seem partly to sediment with the microsomes[9,16] thus accounting for the increase in microsomal protein in the stimulated glands as compared to the perfused controls (Table I). The stimulation enhanced, although not significantly, the content of calcium per mg microsomal protein (Table III).

TABLE III

THE CALCIUM CONTENT IN MICROSOMES FROM NON-PERFUSED, PERFUSED AND PERFUSED/STIMULATED BOVINE ADRENAL MEDULLAE

The experimental conditions were as described in Table I. The microsomes were isolated as outlined under Methods. The microsomes from the non-perfused and perfused/stimulated glands were obtained from 4 pairs of glands. The homogenates were treated in presence and absence of 2 mM EDTA at 4 °C for 20 min before cell fractionation[1]. The number of experiments are given in brackets and values are means ± S.E.

Glands	*nmoles Ca^{2+}/mg protein*	
	−EDTA	*+EDTA*
Non-perfused	57 ± 6 (4)	61 ± 12 (4)
Perfused	58 ± 7 (5)	
Perfused/stimulated	109 ± 23 (4)	60 ± 5 (4)

The effect of EDTA on the content of calcium in mitochondria, chromaffin granules and microsomes from stimulated and control glands

The effect of EDTA on the content of calcium in sub-cellular fractions was tested using 4 pairs of adrenals. One gland of each pair was perfused and stimulated while the controls were homogenized without being perfused. The homogenates were treated with EDTA as described under Methods. The amount of catecholamines secreted and the amount of calcium taken up in response to acetylcholine were in good agreement with the above results, see Table I. Table IV shows that treatment with EDTA caused neither significant changes in the catecholamine–protein ratios of the large granule fractions of the control and stimulated glands nor altered the recovery of protein and catecholamines in the fractions of mitochondria and chromaffin granules. Mitochondria and chromaffin granules obtained from homogenates treated in the presence and absence of EDTA were therefore regarded equivalent.

Treatment with EDTA reduced significantly the calcium content in the mitochondria from both stimulated and control glands although not to the same level (Table IV). Thus after EDTA treatment the difference in calcium content in the mitochondria from stimulated and control glands was 8 nmoles, when related to

TABLE IV

THE EFFECT OF EDTA ON THE CALCIUM CONTENT IN MITOCHONDRIA AND CHROMAFFIN GRANULES FROM NON-PERFUSED AND PERFUSED/STIMULATED BOVINE ADRENAL MEDULLAE

The experimental conditions were as described in Table I. The homogenates of the perfused/stimulated glands and the collateral control glands were treated in the presence and absence of 2 mM EDTA at 4 °C for 20 min before cell fractionation[1]. Cell fractionation was carried out as described under Methods. % LG protein and % LG Ca^{2+} refer to the percentage of total large granule protein and calcium recovered in the fractions of mitochondria and chromaffin granules. Values are means ± S.E.

Glands (*n* = 4)	*EDTA* (*2 mM*)	*Large granules* (*μmoles catechol-amines/mg protein*)	*Mitochondria* (*Fraction F2*)			*Chromaffin granules* (*Fraction F5*)		
			% LG protein	*nmoles Ca^{2+}/mg cell protein*	*nmoles Ca^{2+}/mg protein*	*% LG protein*	*μmoles catechol-amines/mg protein*	*nmoles Ca^{2+}/mg protein*
Non-perfused	−	1.54 ± 0.08	30 ± 1	12 ± 2*	134 ± 16**	24 ± 1	2.20 ± 0.20	175 ± 12
	+	1.61 ± 0.07	28 ± 3	6 ± 2*	71 ± 12**	22 ± 2	2.39 ± 0.27	179 ± 8
Perfused/stimulated	−	1.16 ± 0.14	38 ± 2	22 ± 1***	212 ± 6†	19 ± 2	2.11 ± 0.30	257 ± 13
	+	1.15 ± 0.09	39 ± 2	14 ± 1***	137 ± 7†	18 ± 1	2.24 ± 0.34	288 ± 62

* Statistical significance for the difference between these figures $P < 0.02$.
** Statistical significance for the difference between these figures $P < 0.05$.
*** Statistical significance for the difference between these figures $P < 0.001$.
† Statistical significance for the difference between these figures $P < 0.001$.

total cell protein, *i.e.* expressed independently of the changes in protein in the fraction caused by secretion. The excess of EDTA-inaccessible calcium found in the mitochondrial fractions of the stimulated glands might have been partly or totally sequestered within the emptied chromaffin granules contaminating the fractions. Calcium has been shown to accumulate in the emptied storage vesicles of the polymorphonuclear leucocytes during the release of leucocyte protein[17] and a similar uptake of calcium may occur during the release of amines and protein from the chromaffin granule. The membrane of the chromaffin granules is impermeable to EDTA[18] and calcium within the vesicles may therefore be refractory to the action of EDTA.

The calcium of the chromaffin granules was not extracted by EDTA (Table IV) in agreement with the results of Borowitz *et al.*[1]. Similarly, the calcium accumulated in the granules in response to stimulation was also inaccessible to EDTA and thus probably enveloped by the granule membrane. As similar catecholamine–protein ratios were obtained for the control and stimulated glands in these experiments (Table IV) the ability to accumulate calcium seemed not to be restricted to granules of low catecholamine content.

Treatment with EDTA caused no change in the calcium content of the microsomes from non-perfused glands (Table III). In contrast to that observed for the chromaffin granules, the calcium accumulated in the microsomes in response to stimulation was removed by EDTA and was therefore present in a more loosely bound form.

DISCUSSION

The highest concentration of calcium in the adrenal medulla was found in the chromaffin granules in agreement with the results of Borowitz *et al.*[1]. Taking into account the catecholamines and the granule protein found in all subcellular fractions, it has previously been estimated that the granule protein constitutes about 30% of the total cell protein[9]. Thus granule-bound calcium accounts for a great part of the cellular calcium. (In the present experiments the stimulated medulla was found to release about 30% of its content of amines.) Thrombin induces a parallel secretion of nucleotides and calcium from blood platelets[19], and in view of the great similarity between the biochemical properties of the amine storage granules in the platelets and the chromaffin granules[20,21], calcium is probably also released from the chromaffin granule during secretion. However, according to Table I stimulation of the medulla caused an increase in the total amount of cellular calcium, indicative of a net influx of calcium across the depolarized membrane[22]. Apparently the tissue did not return to its normal resting level of calcium in the periods in between the successive stimulations, the effect being a gradual accumulation of calcium over the 3-h period of perfusion and stimulation. Such a gradual accumulation may explain the discrepancy between the marked increase in calcium content observed for the repetitively stimulated glands (present observations) and the lack of such an increase in glands exposed to moderate stimulation[6].

The similarity between the mechanism of secretion and muscle contraction was first pointed out by Douglas and Rubin[23]. In analogy to relaxation in muscle, secretion may also be terminated by a reduction of free calcium in the cytosol, caused by uptake of calcium in mitochondria and microsomal elements. An ATP-activated up-

take of calcium in isolated mitochondria and microsomes from adrenal medulla has been demonstrated by Poisner and Hava[24]. Furthermore, Tables II and III show that the calcium taken up by the perfused adrenal medulla in response to acetylcholine was partly bound to mitochondria and microsomes. These observations are thus compatible with a function of these organelles in regulating the concentration of free calcium in the cell, and thus the secretory activity of the gland.

The chromaffin granules were found to accumulate more calcium per unit protein than the mitochondria in response to stimulation. The inaccessibility to EDTA of the newly accumulated calcium indicated that the ion was probably retained within the vesicles either bound to some non-diffusable components of the granules or retained free in solution within the membrane. The calcium might also have been sequestered in the electron-light space between the membrane and the matrix seen in some of the granules isolated from the stimulated glands[7]. However, the chromaffin granules in the intact cell of perfused/stimulated glands have maintained their structure as revealed by electron microscopical examination (Helle, K. B. and Serck-Hanssen, G., unpublished). The deformation of the chromaffin granules thus seems to be a result of the isolation procedure. (The chromaffin granules are usually contaminated by microsomal elements[10], mitochondria and when isolated from stimulated glands also by emptied chromaffin granules[7].) The excess of calcium in the granule fractions of the stimulated glands might thus also have been bound to the membranes or retained free in solution within the membranes of these contaminating organelles. However, the possibilities of the microsomes, mitochondria or emptied vesicles being the main sites for uptake of calcium have been excluded for the following reasons: 1. In contrast to the calcium in the granule fractions the calcium in the microsomes was extractable with EDTA. 2. As the ratios of calcium to protein in the mitochondria of the stimulated glands were lower than that of the granules, it appears unlikely that contaminating mitochondria might account for the calcium uptake in the granule fractions. 3. The majority of the emptied granule vesicles sediment with the mitochondria[8,14,15]. On the assumption that all the EDTA-inaccessible calcium in the mitochondria of the stimulated glands was confined to the contaminating granule vesicles, these vesicles would account for 8 nmoles calcium per mg cell protein (Table IV). As approximately 5 nmoles of calcium per mg cell protein was accumulated in the chromaffin granules (Table I), it seems unlikely that this quantity of calcium could be ascribed to contaminating emptied granules. Accordingly, the chromaffin granules seem to possess the ability to accumulate calcium from the cytosol. When lysed suspensions of granules were studied by density gradient centrifugation, the calcium accumulated in response to stimulation was mainly recovered in the membrane-containing fraction (Serck-Hanssen, G. and Helle, K. B., unpublished).

The percentage of free amines found in the $20000 \times g$ supernatant was no greater for the stimulated glands than for the controls. Thus, the high concentration of calcium in the granules of the stimulated glands had no solubilizing effect on the intragranular storage complex. Uptake of free calcium in the granules from the cytoplasm therefore seems unlikely as a stage in the releasing mechanism.

The ability to accumulate calcium in response to stimulation was not restricted to granules of low catecholamine content (Table IV), and the question thus arises whether the granules participate in regulating the concentration of free calcium in

the cell, as also suggested by Rubin[25]. The amount of calcium taken up by the granules in the stimulated glands was about 100 nmoles per mg protein, which is of the same order of magnitude as that found for isolated sarcoplasmic reticulum[26]. It has been suggested that the relative importance of mitochondria and sarcoplasmic reticulum in regulating the calcium concentration depend on the quantitative distribution of these organelles in the muscular tissue in question (for review see ref. 27). If similar estimates are applied to the chromaffin cell, it can be seen from Table I that the mitochondria constitute about 10% and the microsomes about 8% of the total cell protein, whereas the chromaffin granules according to earlier estimates represent about 30% of the total cell protein[9]. Thus the population of chromaffin granules may be a quantitatively important recipient for calcium removed from the cytosol.

From the present experiments it cannot be decided whether calcium diffuses freely or is transported actively across the granule membrane. However, isolated granules exhibit an ATP-activated uptake of calcium, which differs from that of mitochondria[24] and a calcium-activated ATPase is located in the granule membrane[10,18]. Phosphorylation of the granule membrane in the presence of ATP is stimulated by magnesium and to a lesser degree by calcium[28]. The combined effect of these two ions, known to be the most potent in phosphorylating the sarcoplasmic reticulum[29,30], has not been tested. Nevertheless, these observations may be indicative of an energy-dependent mechanism for calcium transport in the chromaffin granule similar to that in the sarcoplasmic reticulum.

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