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EVIDENCE FOR A PLASMA MEMBRANE CALCIUM PUMP IN BOVINE ADRENAL MEDULLA BUT NOT ADRENAL CORTEX*

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SUMMARY

Continuous sucrose density gradient subfractions from bovine adrenal medullary microsomes were found to accumulate $^{45}Ca^{2+}$ in the presence of ATP and ammonium oxalate mainly in subfractions of intermediate density. $(Na^+ + K^+)$-ATPase (plasma membrane marker) and Ca^{2+}-ATPase activities were also concentrated in these intermediate subfractions but thiamine pyrophosphatase (Golgi apparatus marker) was not. NADH oxidase (endoplasmic reticulum marker) activity was distributed throughout all subfractions.

$^{45}Ca^{2+}$ accumulation in adrenal cortical microsomes was found to rise and fall in parallel with thiamine pyrophosphatase but not with $(Na^+ + K^+)$-ATPase or NADH oxidase activities.

Accumulation of $^{45}Ca^{2+}$ in membrane vesicles in these experiments suggests the existence of a calcium transfer mechanism in plasma membranes of the adrenal medulla but not adrenal cortex.

INTRODUCTION

The role of extracellular calcium in initiating acetylcholine- or potassium-induced release of catecholamines from the adrenal medulla has been well documented (for references, see ref. 1). Depolarization of the chromaffin cell membrane is known to result in calcium movement from extracellular to intracellular spaces where it then serves as a critical link in the process of stimulus-secretion coupling [2]. However, all the events responsible for removal of calcium from the medullary cytoplasm have not been clearly defined. Cytoplasmic calcium may be removed by mitochondria, microsomes or chromaffin granules [3–5]. Although intracellular organelles may play

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some role in removing cytoplasmic calcium to terminate secretion, it seems likely that they would rapidly become saturated. Extrusion of calcium to the cell exterior by a pump mechanism in the cell membrane might be a more efficient termination process. In the present investigation, the possibility that an energy-dependent calcium pump may be located within the chromaffin cell membrane was tested.

EXPERIMENTAL

Bovine adrenal glands were obtained from a local slaughterhouse and carried to the laboratory on ice and used within 1 h post mortem. Approx. 5–6 g of medullary tissue were dissected free from cortex and homogenized in 10 vols of 0.32 M sucrose using a loose-fitting conical all glass homogenizer (Duall tissue grinder, Kontes Glass Co.). When adrenal cortical tissue was used, the procedure was the same as that employed for preparation of the medulla. The homogenate was centrifuged at $800 \times g_{max}$ for 10 min to sediment nuclei and cell debris. The supernatant obtained was centrifuged at $27\,000 \times g_{max}$ for 10 min to sediment mitochondria and chromaffin granules. This supernatant was centrifuged at $105\,000 \times g_{max}$ for 60 min. The final pellet which represented purified microsomes was resuspended in 2 ml of 0.32 M sucrose and layered on a 20–40 % continuous sucrose density gradient containing 0.005 M Tris · HCl, pH 7.8. The gradient was then centrifuged at $153\,000 \times g_{max}$ for 16 h in a swinging bucket rotor (Spinco SW 41). All operations were carried out at 0–4 °C. The 2-ml volume of 0.32 M sucrose which was layered on the density gradient was first removed and seven subfractions were obtained from the density gradient with a Pasteur pipette. The volume of each subfraction was about 1.5 ml.

Adenosinetriphosphatase assays. Each of the seven subfractions obtained from the density gradient was made 0.1 % with respect to sodium deoxycholate. For Mg^{2+}-stimulated adenosinetriphosphatase (Mg^{2+}-ATPase) activity, a 0.2-ml aliquot of each subfraction was incubated for 20 min at 37 °C in a Dubnoff metabolic shaker with 4 mM ATP (di-magnesium salt) and 100 μmol of Tris · HCl buffer, pH 7.4, in a 3 ml volume. At the end of the incubation, the reaction was stopped with 5 % trichloroacetic acid and free inorganic phosphate was measured by the method of Lowry and Lopez [6].

For $(Na^+ + K^+)$-stimulated adenosinetriphosphatase ($(Na^+ + K^+)$-ATPase) activity, 67 mM NaCl and 3.3 mM KCl were added to the incubation media given above for Mg^{2+}-ATPase assay. The increment in activity is a measure of sodium-potassium activation of the enzyme.

In one experiment, strophanthin K (K and K Laboratories) 2.2 mg, was added to the incubation mixture prepared as above, including both potassium and sodium. The decrease in inorganic phosphate liberated in the presence of strophanthin K gave a measure of $(Na^+ + K^+)$-ATPase activity.

For Ca^{2+}-stimulated adenosinetriphosphatase (Ca^{2+}-ATPase) activity, a 2.0-ml aliquot of each subfraction was incubated for 20 min at 37 °C in a Dubnoff shaker with $CaCl_2 \cdot 2H_2O$, 2.5 mM; ATP (di-sodium salt), 4 mM; and 100 μmol of Tris · HCl, 7.4, in a 3-ml volume. Each sample was compared to a blank which contained all the above components except $CaCl_2$. Calcium-stimulated inorganic phosphate liberation was measured by the method of Lowry and Lopez [6].

Thiamine pyrophosphatase activity was determined using the method described for Mg^{2+}-ATPase except ATP was replaced by thiamine pyrophosphate (Sigma Chemical Co.). One ml of 0.02 M thiamine pyrophosphate was added to the incubation mixture and inorganic phosphate liberation was measured as before [6].

NADH oxidase assay. NADH oxidase was assayed by a modified method of Avruch and Wallach [7]. The oxidation of NADH was monitored at 340 nm using a Beckman DB Spectrometer. The sample cuvette contained 2 μmol NADH (K and K Laboratories), 32 μmol Tris · HCl, pH 7.4, 1.32 μmol $K_3Fe(CN)_6$ and 0.1 ml enzyme suspension in a 2-ml volume. The reference cuvette contained all the above components minus NADH.

$^{45}Ca^{2+}$ assay. $^{45}Ca^{2+}$ uptake was studied at 37 °C for 10 min in a 3-ml incubation mixture containing Tris · HCl (pH 7.4), 30 μmol; KCl, 300 μmol; ATP (dimagnesium salt), 9 μmol; ammonium oxalate, 15 μmol; sodium azide, 15 μmol; $^{45}Ca^{2+}$, 0.4 μCi; calcium, approx. 0.06 μmol; and 0.2 ml of each density gradient subfraction. Experiments were performed in the presence and absence of ammonium oxalate to show evidence for an uptake process rather than simple binding to tissue components. To determine ATP-mediated $^{45}Ca^{2+}$ uptake, all assays were performed with and without ATP. $^{45}Ca^{2+}$ uptake in the absence of ATP subtracted from that in its presence represented ATP-mediated $^{45}Ca^{2+}$ uptake.

Millipore filters (0.45 μm) were soaked in 250 mM KCl solution and then washed with 10 ml of 0.32 M sucrose. After incubation the samples were filtered through the 0.45-μm Millipore filters under suction. The particulate matter collected on each filter was washed with 10 ml of 0.32 M sucrose. The filters were then dried and placed in glass counting vials. 15 ml of scintillation fluid (0.6 percent POPOP, in 1 : 1 toluene and 2-ethoxyethanol) was added and the $^{45}Ca^{2+}$ counted in a Model 2002 Packard Tricarb Scintillation Counter. Counting efficiency was determined to be approx. 95 % using an internal standard.

Catecholamine assay. 1 ml of each density gradient subfraction was assayed colorimetrically for catecholamine content by the method of Von Euler and Hamberg [8].

Nucleic acid assay. 1 ml of each density gradient subfraction was dialyzed for 24 h in 0.9 % saline at 4 °C. After dialysis each sample was assayed for nucleic acid content by the method of Ceriotti [9].

Protein assay. Total protein was estimated colorimetrically by the biuret method [10].

Electronmicroscopy. The purified microsomal pellet was prepared for electronmicroscopy to show vesicle formation from broken membranes. The pellet was fixed with 1 % OsO_4 in phosphate buffer for 5 min. The pellet was dehydrated in a series of acetone concentrations (30, 60, 90, 100, 100 and 100, respectively), embedded in Epon 812 and polymerized at 60 °C for 48 h. Sections were cut with glass knives and stained with uranyl acetate and lead citrate.

RESULTS

$^{45}Ca^{2+}$ uptake by chromaffin cell membrane vesicles

The distribution of $(Na^+ + K^+)$-ATPase activity (marker for plasma membrane) (for references, see ref. 11) and NADH oxidase (marker for endoplasmic

reticulum) [7] were compared with the distribution of ATP-mediated $^{45}Ca^{2+}$ uptake in seven sucrose density gradient subfractions of adrenal medullary microsomes. Fig. 1 shows that $^{45}Ca^{2+}$ uptake correlates, in general, with $(Na^+ + K^+)$-ATPase distribution, but not with the distribution of NADH oxidase. Both $(Na^+ + K^+)$-ATPase and ATP-mediated $^{45}Ca^{2+}$ uptake were found to reach peak activities in subfractions 1 through 4, while NADH oxidase activity was distributed somewhat evenly throughout all seven subfractions. $(Na^+ + K^+)$-ATPase activity was significantly greater in subfractions 1 through 4 than in subfractions 5 through 7, using $P < 0.05$ as the limit for significance with the Student t test.

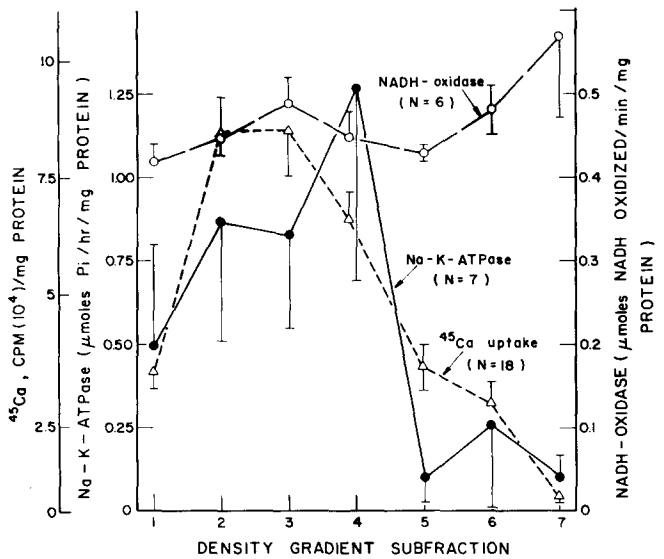

Fig. 1. Distribution of $(Na^+ + K^+)$-ATPase, NADH oxidase and ATP-mediated $^{45}Ca^{2+}$ uptake activities in continuous sucrose density gradient subfractions of adrenal medullary microsomes. ATPase assays in this and subsequent figures are represented as μmol of inorganic phosphate (P_i) liberated/h per mg protein. The sucrose concentration begins at 20 % in subfraction 1 and increases progressively to 40 % in subfraction 7.

One experiment was performed to determine if $(Na^+ + K^+)$-ATPase found in the present study was inhibited by cardiac glycosides. Strophanthin-K inhibited $(Na^+ + K^+)$-ATPase activity had the same distribution as that shown in Fig. 1, where activity was determined by incremental increase in ATPase caused by addition of sodium and potassium.

An attempt was made to compare the distribution of rough endoplasmic reticulum and $^{45}Ca^{2+}$ uptake. Distribution of pentose sugar within these subfractions was used as a measure of the location of RNA and therefore rough endoplasmic reticulum. Fig. 2 shows that RNA is low in concentration in the first two subfractions of the density gradient but progressively increases reaching peak levels in the last four subfractions. It is evident, therefore, that the distribution of rough endoplasmic reticulum does not parallel $^{45}Ca^{2+}$ accumulation in subfractions of adrenal medullary microsomes.

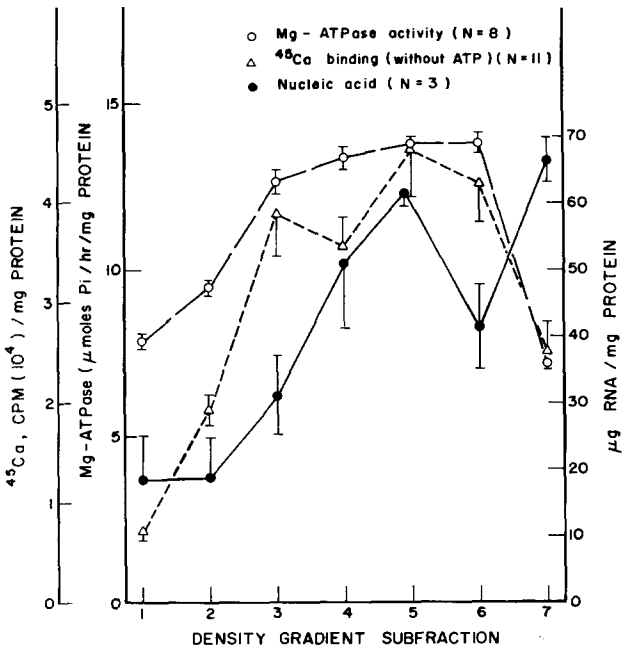

Fig. 2. Distribution of Mg^{2+}-ATPase activity, $^{45}Ca^{2+}$ nonspecific binding and nucleic acid in continuous sucrose density gradient subfractions of adrenal medullary microsomes.

Fig. 2 also indicates the location of Mg^{2+}-ATPase and ATP-independent $^{45}Ca^{2+}$ binding in subfractions of adrenal medullary microsomes. The distribution of Mg^{2+}-ATPase does not appear to correlate well with either plasma membrane or endoplasmic reticulum distributions. Mg^{2+}-ATPase is probably associated with both membrane systems. ATP-independent $^{45}Ca^{2+}$ binding, however, does correlate somewhat with rough endoplasmic reticulum distribution. Phospholipids and nucleoproteins are known to effectively bind calcium [12]. Therefore, $^{45}Ca^{2+}$ binding to nucleoproteins from ribosomal RNA probably explains the correlation between the distributions of RNA and $^{45}Ca^{2+}$ binding in the subfractions.

Electronmicrographs shown in Fig. 3 illustrate that vesicles of various sizes are formed within the microsomal fraction of bovine adrenal medulla. Very little mitochondrial or chromaffin granule contamination can be seen.

Fig. 4 illustrates that when oxalate is added to the incubation medium, $^{45}Ca^{2+}$ uptake into the vesicles is markedly increased. Addition of oxalate to each density gradient subfraction resulted in a significant increase in $^{45}Ca^{2+}$ uptake ($P < 0.05$ or better) in subfractions 1 through 5. $^{45}Ca^{2+}$ uptake was increased approx. 4–5-fold by addition of oxalate to assay mixtures of these subfractions.

The presence of Ca^{2+}-ATPase in adrenal medullary microsomes has been previously demonstrated [13, 14]. However, this Ca^{2+}-ATPase activity was attributed to endoplasmic reticulum. In the present study, the distribution of this enzyme in the density gradient subfractions was found to correlate, in general, with $^{45}Ca^{2+}$ uptake and $(Na^+ + K^+)$-ATPase activity (see Figs 1 and 5).

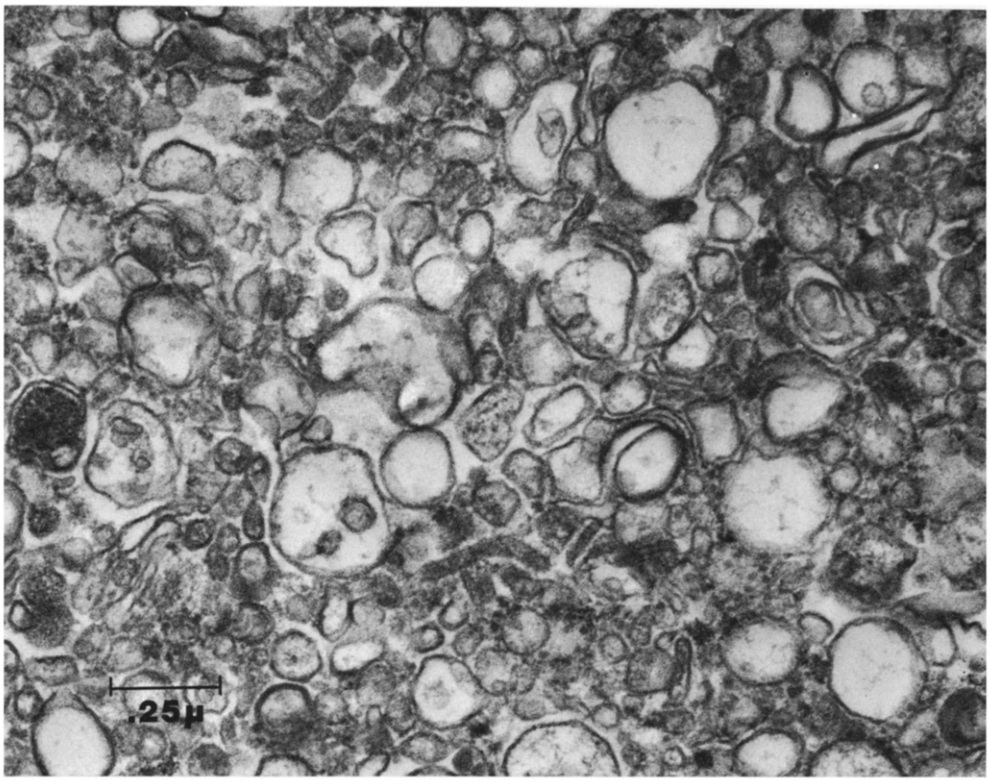

Fig. 3. Electronmicrographs of adrenal medullary microsomal pellet showing vesicle formation from broken membranes. Sections for electronmicroscopy were prepared from the 105 000 × g_{max} microsomal pellet (× 56 920).

Benedeczky et al. [15] have suggested that Ca^{2+}-ATPase activity is present in the Golgi apparatus in the hamster adrenal medulla as well as in the cell membrane. The distribution of Golgi apparatus within the density gradient subfractions was therefore determined and compared to the distribution of Ca^{2+}-ATPase activity (Fig. 5). Unlike Ca^{2+}-ATPase activity, thiamine pyrophosphatase (marker for Golgi apparatus) [16], was, in general, equally distributed throughout all of the subfractions. It appears that the distribution of Ca^{2+}-ATPase and thiamine pyrophosphatase activities do not correlate well. Therefore, although some Ca^{2+}-ATPase may be located in Golgi apparatus, the major portion of the Ca^{2+}-ATPase activity of medullary microsomes appears to be localized in plasma membrane. Also, thiamine pyrophosphatase activity found in the subfractions was very weak. Whereas peak Ca^{2+}-ATPase activation resulted in liberation of approx. 24 μmol of inorganic phosphate/h per mg protein, thiamine pyrophosphatase activation liberated only about 0.28 μmol of inorganic phosphate/h per mg protein, a 100-fold difference.

To rule out the possibility that chromaffin granule contamination was responsible for uptake of $^{45}Ca^{2+}$ within the density gradient, the distribution of catecholamines was determined. Catecholamine concentrations were found to average 3–4 μg/mg protein in subfractions 1 through 6, but increased in subfraction 7, reaching

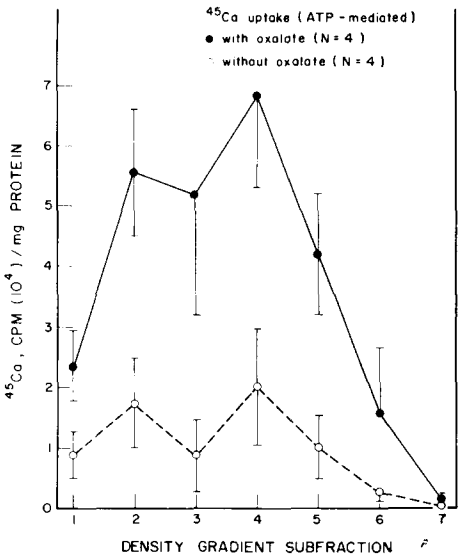

Fig. 4. Oxalate-mediated augmentation of ATP-induced $^{45}Ca^{2+}$ uptake in continuous sucrose density gradient subfractions of adrenal medullary microsomes. Addition of ammonium oxalate, 15 μmol to the assay mixture significantly increased ATP-mediated $^{45}Ca^{2+}$ uptake in subfractions 1 through 5 ($P < 0.05$ or better using Student t test). Ammonium oxalate caused an approx. 4–5-fold increase in $^{45}Ca^{2+}$ uptake as compared to controls in the seven density gradient subfractions.

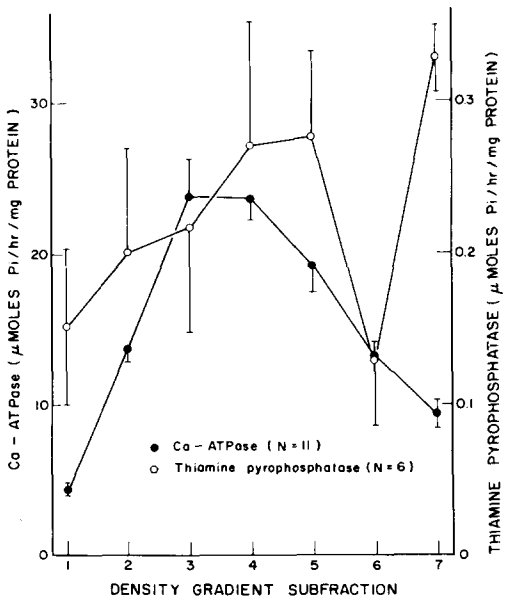

Fig. 5. Distributions of Ca^{2+}-ATPase and thiamine pyrophosphatase activities in continuous sucrose density gradient subfractions of adrenal medullary microsomes.

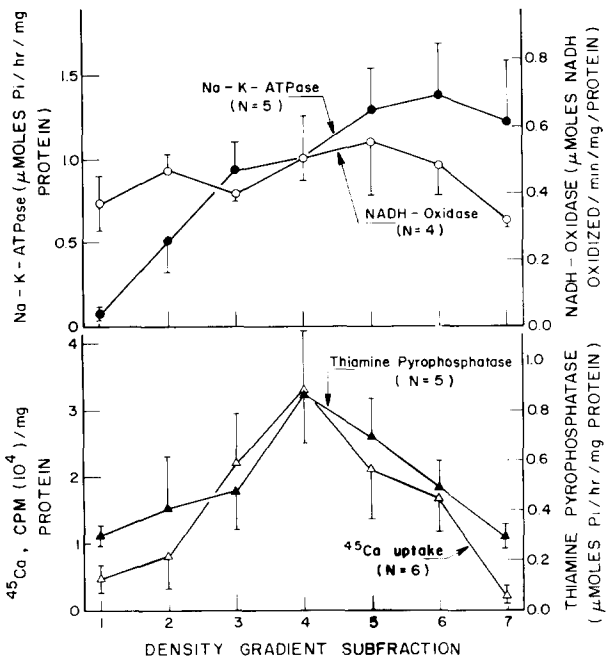

Fig. 6. Distribution of $^{45}Ca^{2+}$ uptake, thiamine pyrophosphatase, (Na^+ - K^+)-ATPase, and NADH oxidase activities in continuous sucrose density gradient subfractions of adrenal cortical microsomes. $^{45}Ca^{2+}$ uptake refers to accumulation of $^{45}Ca^{2+}$ within microsomal vesicles in the presence of ATP (di-magnesium salt).

a concentration of 17.6 μg catecholamine per mg protein. Since the distribution of catecholamines was found to be quite dissimilar to that of $^{45}Ca^{2+}$ uptake, it seems unlikely that the uptake process can be explained on the basis of chromaffin granule contamination.

Although no mitochondria were observed in the electronmicrographs, sodium azide was added to each incubation medium to rule out the possibility that mitochondrial contamination might be responsible for the $^{45}Ca^{2+}$ uptake process. Sodium azide is known to inhibit ATP-mediated mitochondrial calcium transport [4, 17].

$^{45}Ca^{2+}$ accumulation in adrenal cortical microsomes

Since the adrenal cortex is thought to utilize calcium only from an intracellular source to initiate corticosteroid secretion [18], the question arises as to whether a plasma membrane calcium pump mechanism exists in the adrenal cortex or whether cortical secretion may be terminated by way of an intracellular calcium uptake mechanism. Therefore, the occurrence of $^{45}Ca^{2+}$ uptake and the distributions of the various marker enzymes were compared in continuous sucrose density gradient subfractions of adrenal cortical microsomes. Fig. 6 shows the distributions of (Na^+ - K^+)-ATPase, NADH oxidase, thiamine pyrophosphatase, and $^{45}Ca^{2+}$ uptake within the density gradient. The data show that $^{45}Ca^{2+}$ uptake correlates, in general, with the distribution of thiamine pryophosphatase activity but not with the distributions

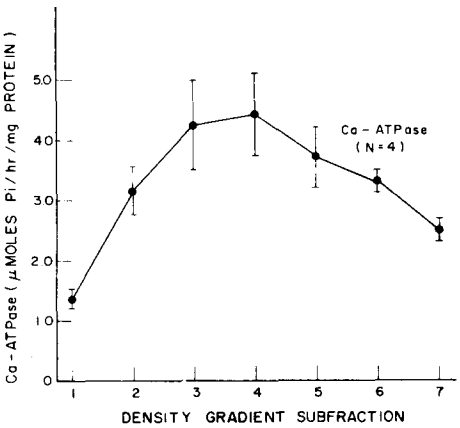

Fig. 7. Distribution of Ca^{2+}-ATPase activity in continuous sucrose density gradient subfractions of adrenal cortical microsomes.

of either $(Na^+ + K^+)$-ATPase or NADH oxidase activities. Likewise, Fig. 7 illustrates that Ca^{2+}-ATPase activity in the adrenal cortex is in good correlation with $^{45}Ca^{2+}$ uptake and thiamine pyrophosphatase activity.

Although $^{45}Ca^{2+}$ uptake and Ca^{2+}-ATPase activity correlated well in the adrenal cortex, it should be noted that levels of uptake of $^{45}Ca^{2+}$ and Ca^{2+}-ATPase activity are substantially lower in cortex compared to medulla. Peak Ca^{2+}-ATPase activity found in the cortex is only about 20 % of the activity found in the medulla. Likewise, $^{45}Ca^{2+}$ uptake in the cortex is approx. 30 % of the activity found in the medullary microsomes.

DISCUSSION

The results of the present investigation indicate that $^{45}Ca^{2+}$ uptake in the adrenal medullary microsomal fraction may be the result of an energy-dependent transport mechanism located in the cell membrane. The finding that plasma membrane is present in the microsomal fraction from the adrenal medulla is not surprising since plasma membrane has been found in microsomes from a number of tissue types [19–23]. Nijjar and Hawthorne [24] recently obtained a plasma membrane fraction from the post-mitochondrial supernatant fraction of bovine adrenal medulla. They also used a continuous sucrose gradient and found plasma membrane to exist in the intermediate subfractions. These data are in agreement with the present study.

That $^{45}Ca^{2+}$ accumulation in this study represents an uptake process rather than a binding phenomenon is supported by electronmicrographs which show that numerous vesicles are formed within the microsomal fraction into which calcium could be taken up, and from the biochemical evidence that $^{45}Ca^{2+}$ accumulation is markedly enhanced by the addition of oxalate.

While ATP-mediated $^{45}Ca^{2+}$ uptake was found to parallel $(Na^+ + K^+)$-ATPase, it did not appear to correlate with the distributions of endoplasmic reticulum or Golgi apparatus. Since the various membranes were not completely separated from one another on the density gradient, the possibility that endoplasmic reticulum and/or

Golgi apparatus may have some capability for active sequestration of calcium cannot be totally ruled out. Golgi apparatus may be capable of accumulating some $^{45}Ca^{2+}$ since histological evidence has shown Ca^{2+}-ATPase activity in Golgi apparatus and plasma membranes of the hamster adrenal medulla [15]. However, it is unlikely that rough endoplasmic reticulum is capable of taking up calcium to any appreciable extent, since the distribution of RNA, and therefore rough endoplasmic reticulum, differs substantially from that of $^{45}Ca^{2+}$ uptake throughout the density gradient. It is also unlikely that smooth endoplasmic reticulum is responsible for much of the noted $^{45}Ca^{2+}$ accumulation, since it probably exists largely in subfractions 1 and 2 and progressively decreases in amount in the more dense subfractions. This is compatible with an even distribution of NADH oxidase activity in all subfractions and the localization of RNA in the heavier subfractions. The suggestion that neither smooth nor rough endoplasmic reticulum is responsible for the major portion of the $^{45}Ca^{2+}$ uptake within the microsomes is supported by histological and biochemical evidence that Ca^{2+}-ATPase activity is not found in endoplasmic reticulum [15, 24].

The demonstration that plasma membrane vesicles from adrenal medullary microsomes actively take up calcium implicates the plasma membrane in termination of secretion. Such a calcium removal mechanism directed toward the cell exterior would provide an efficient means for removal of calcium to the extracellular spaces from which it enters to initiate catecholamine secretion. The fact that intracellular calcium must be maintained at low concentrations makes it very likely that a calcium pump located in adrenal medullary plasma membrane would be directed towards the cell exterior to maintain this normal calcium gradient.

Borowitz [25] has recently provided data which indicate that the half-life of catecholamine secretion is approx. 1.5–2.0 min in the isolated bovine adrenal medulla. Termination of the secretory process, however, is thought to be a rapid process occurring in a few seconds [25], since the above half-lives primarily represent the time required for washout of released catecholamines from the adrenal gland. It has been previously demonstrated that ATP-mediated $^{45}Ca^{2+}$ uptake in medullary microsomes reaches peak levels in approx. 5 min [4]. It is unlikely, therefore, that the plasma membrane calcium transfer mechanism found in the present study is totally responsible for the termination of secretion. More likely, this plasma membrane calcium extrusion system may be one of several systems responsible for removal of cytoplasmic calcium. Mitochondria and chromaffin granules may also play a role in calcium removal [3–5], as may a Na^{+}-Ca^{2+} exchange through plasma membrane as previously demonstrated in nerve cell membrane [26] and suggested in the adrenal medulla [27].

For comparison, $^{45}Ca^{2+}$ uptake studies were performed in microsomal density gradient subfractions of adrenal cortex. This tissue is thought to utilize intracellular calcium to initiate the secretory response [18]. The results obtained indicate that calcium uptake in adrenal cortical microsomes is not a function of the plasma membrane. Although there is a significant amount of plasma membrane found within the microsomal fraction of adrenal cortex, its distribution within continuous density gradient subfractions does not parallel the distribution of $^{45}Ca^{2+}$ uptake. Likewise, the distribution of endoplasmic reticulum within the subfractions does not correlate with the distribution of $^{45}Ca^{2+}$ uptake. On the other hand, Golgi apparatus was found to rise and fall very much in parallel with $^{45}Ca^{2+}$ uptake and Ca^{2+}-ATPase activity. These data indicate that neither plasma membrane nor endoplasmic reticulum is capable of

actively removing cytoplasmic calcium in the adrenal cortex. Removal of free calcium from the cytoplasm of the adrenal cortical cell may be a function of the Golgi apparatus.

Studies utilizing continuous sucrose density gradients for the partial separation of plasma membrane and endoplasmic reticulum have been performed in other tissues. Hurwitz et al. [22] have shown that in ileal smooth muscle, which requires extracellular calcium to maintain contraction, $^{45}Ca^{2+}$ uptake in microsomal subfractions parallels the distribution of plasma membrane but not endoplasmic reticulum. A calcium pump similar to that found in the present study in adrenal medulla was proposed. However, in other tissues $^{45}Ca^{2+}$ uptake did not correlate with plasma membrane distribution in microsomal subfractions. In rabbit aorta, which is thought to utilize intracellular calcium for contraction, $^{45}Ca^{2+}$ uptake in microsomal subfractions paralleled endoplasmic reticulum distribution rather than distribution of plasma membrane [22]. Also, in renal microsomes, the rise and fall of $^{45}Ca^{2+}$ uptake correlated with that of endoplasmic reticulum but not plasma membrane [23].

In summary, there are fundamental differences with regard to calcium uptake in the adrenal medulla and cortex. The plasma membrane appears to actively remove calcium from the adrenal medulla and probably other cells which require calcium from an extracellular source for initiation of activity. In tissues such as the adrenal cortex which utilize intracellular calcium for activity, calcium uptake by intracellular organelles appears to be responsible for termination of secretion.

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