

Adenosine Triphosphate–Dependent Calcium Uptake by Rat Submaxillary Gland Microsomes

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ABSTRACT Microsomes from rat submaxillary glands are able to take up calcium from the suspension media. Calcium uptake is greatly increased by the presence of ATP. This effect of ATP is not detected at 0°C. ADP cannot replace ATP to potentiate calcium uptake. ATP-dependent calcium uptake is not observed in the absence of magnesium. ATP-dependent calcium uptake is enhanced by oxalate and, to a lesser degree, by inorganic phosphate. Total calcium per milligram of microsomal protein observed when tests were performed without oxalate closely parallels the amounts for skeletal and cardiac muscles reported by several authors. Calcium uptake in salivary gland microsomes is slower than in muscle microsomes. Speculations are considered about the role of ATP-dependent calcium uptake. It is suggested that a decrease in intracellular free calcium levels returns these cells to the resting state after secretion.

INTRODUCTION

ATP-dependent Ca uptake by skeletal and cardiac muscle microsomes has been extensively studied during the last 10 years (1). A similar phenomenon has been described for microsomes from nervous tissues (2), liver (3), and kidney (4). It is generally believed that this microsomal Ca uptake reflects an active transport through the microsomal membrane. Energy for this transport is obtained from ATP hydrolysis. A Ca-dependent ATPase activity has been described in connection with the microsomal calcium pump (1). These facts have been related to normal physiological events. An increase in cytoplasmic free calcium concentration is critical for muscle contraction. Intracellular calcium must be reduced to bring about muscle fiber relaxation and nerve excitability and conductivity. The microsomal calcium pump has been identified with the mechanisms reducing cytoplasmic calcium.

Ionic calcium has been postulated as a vital link in excitation-secretion coupling in adrenal medulla and salivary glands. Calcium itself is a secret-

agogue (5) and the presence of this ion is necessary to obtain a secretory response in denervated cat submaxillary glands perfused with acetylcholine (6).

The experiments described in the present paper were carried out to explore the existence in rat submaxillary gland microsomes of a Ca-accumulating mechanism similar to those just described for other tissues.

MATERIALS AND METHODS

Drugs and Reagents

All reagents were analytical grade and deionized water was used in all cases. Tris-ATP was prepared from disodium ATP (Sigma Chemical Co., St. Louis, Mo.) by passage through a Dowex 50 column in the Tris form. ^{45}Ca was obtained from the New England Nuclear Corp., Boston, Mass., as chloride salt.

Preparation of the Microsomes

Rat submaxillary glands were obtained immediately after the animals were sacrificed by exsanguination under Nembutal anesthesia. The tissue was cut into small segments with scissors. Homogenization was performed in a glass homogenizer fitted with a Teflon pestle in 125 mM KCl and 5 mM histidine buffer (pH 6.5). The homogenate was centrifuged at 10,000 *g* for 30 min and the supernatant was decanted and re-centrifuged at 26,000 *g* for an hour. The resulting pellet was resuspended in the same solution. All the operations were performed at 0°C and the microsomal suspension was kept in ice. All the experiments were performed immediately after the preparation of the microsomes.

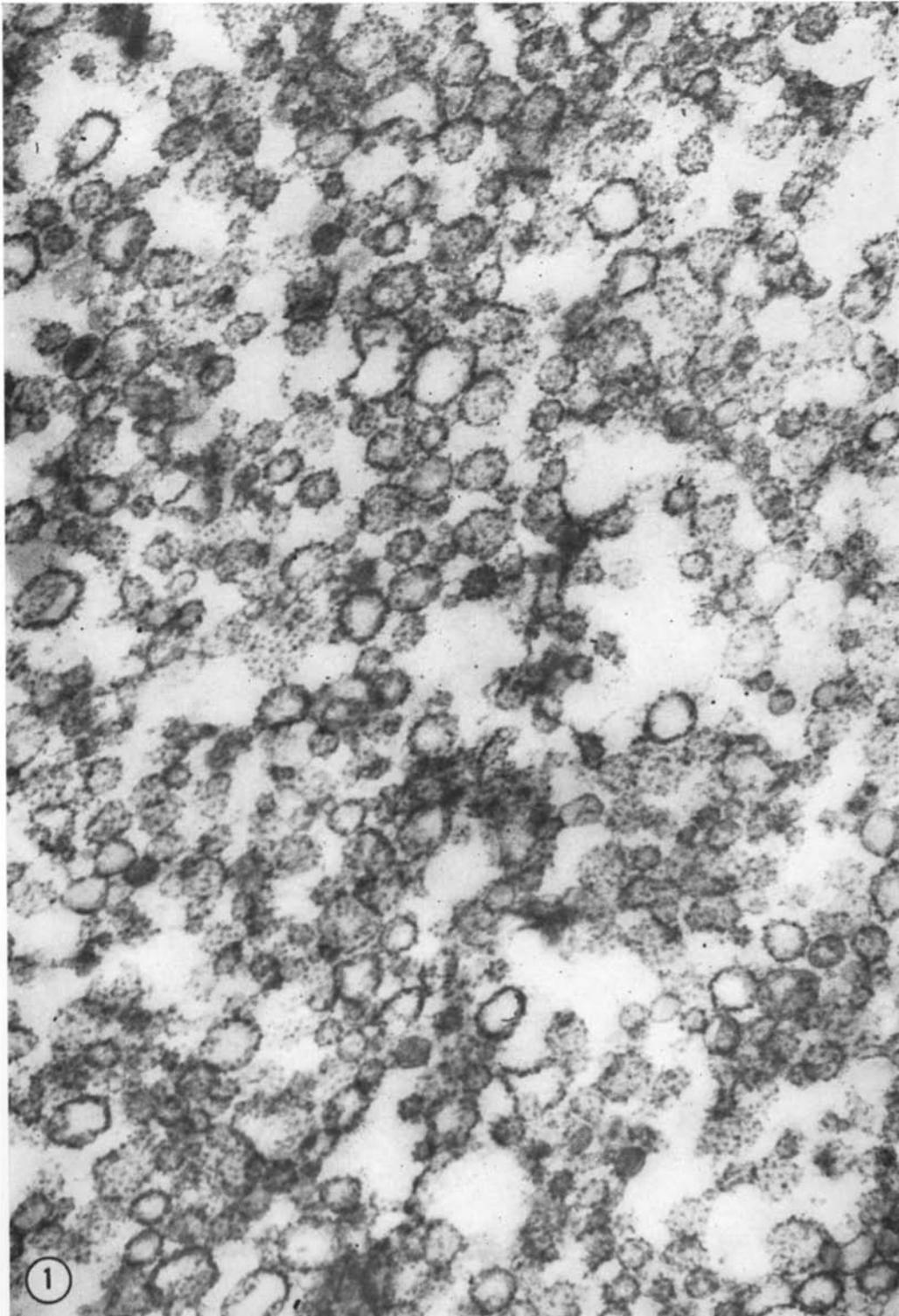
Protein concentration was measured by the method of Lowry et al. (7) using bovine serum albumin as standard. Intrinsic calcium in the preparation and incubation media was determined by the method of Toribara and Koval (8). Electron micrographs of the microsomal preparation were obtained from the pellets resulting from the last centrifugation using standard techniques

Determination of Calcium Uptake

Microsomes were incubated in a total volume of 2 ml under the conditions described below. After incubation, 1.5 ml were pipetted from the mixtures and run through a Millipore filter (0.45 μ average pore) by applying a slight negative pressure from the distal end. The filter was then washed by passage of 10 ml of 100 mM nonradioactive CaCl_2 . Both operations took less than half a minute. The filters with the microsomes were immediately dissolved in 10 ml of a scintillation solution (acetone, 3 volumes; methyl alcohol, 10 volumes; POPOP 0.2 g and PPO 4 g per liter of toluene, 20 volumes). Radioactivity was measured in a Beckman LS 200B liquid scintillation counter. Blanks without microsomes were prepared and always subtracted from all other values.

Determination of ATPase Activity

After incubation the reaction was stopped with a 5% final concentration of trichloroacetic acid and the denatured protein was precipitated by centrifugation.



Inorganic phosphate was measured in the supernatant following the method of Baginski et al. (9). Inorganic phosphate was taken as an index of ATPase activity.

RESULTS

Usually eight submaxillary glands from four rats yielded about 4 ml of microsomal suspension with a protein concentration of about 1 mg per ml. Intrinsic calcium in the preparation was of the order of 1 μg of Ca per mg of microsomal protein. Electronic micrographs of the preparation showed typical rough microsomes. Neither mitochondria nor mitochondrial fragments were present (Fig. 1).

ATP Dependence of Calcium Uptake

The microsomes of the rat submaxillary gland are able to take up Ca in the absence of ATP (nonspecific uptake). There was a great increase in Ca uptake when ATP was added to the incubation mixtures. This increase was almost absent at 0°C and when ATP was replaced by ADP (Table I). No significant ATP-dependent Ca uptake was observed in a fraction obtained by centrifugation of the last supernatant at 100,000 *g* for 2 hr. Nonsignificant amounts of radioactivity were lost by the washing procedure (Table II). A reduction in ATP-dependent Ca uptake was observed after washings with water or hypotonic CaCl₂ solutions.

Time Course of Calcium Uptake

The time course of ATP-dependent calcium uptake by rat submaxillary gland microsomes is shown in Fig. 2. A maximum value was not reached after a 10 min incubation. Results are in contrast with the high velocity observed for Ca uptake in skeletal muscle microsomes (10).

Dependence of Ca Uptake on ATP Concentration

Fig. 3 shows the dependence of Ca uptake on ATP concentration. ATP-dependent Ca uptake increases with ATP concentration. The K_m value for ATP was about 1 mM. This result is similar to that reported by Fanburg and Gergely (11) for cardiac microsomes.

Dependence of Calcium Uptake on the Ionic Composition of the Medium

Both nonspecific and ATP-dependent Ca uptake increased when Ca concentration was raised in the incubation medium (Table III). There was an almost

FIGURE 1. Electron micrograph of the microsomal preparation. Electron micrograph of a thin section of the microsome pellet from rat submaxillary glands. The pellets were fixed in 1% osmium tetroxide, embedded in Epon, stained with uranyl acetate and lead citrate, and observed in a Siemens Elmiskop 1 electron microscope. $\times 58,000$.

TABLE I
ATP-DEPENDENT Ca UPTAKE IN RAT
SUBMAXILLARY GLAND MICROSOMES

Additions	Incubation temperature	Ca uptake
	°C	nmoles/mg of protein
None	37	0.5
ATP 4.5 mM	37	21.4
ATP 4.5 mM	0	2.7
ADP 4.5 mM	37	2.2

Incubations were performed in a total volume of 2 ml for 5 min and at the temperatures shown. Incubation flasks contained 50 μ M $^{45}\text{CaCl}_2$, 5 mM MgCl_2 , 100 mM KCl, 45 mM Tris-buffer (pH 7.4), 0.27 mg of microsomal protein per ml, and nucleotides as specified.

TABLE II
 ^{45}Ca REMAINING IN THE FILTERS AFTER
WASHING WITH DIFFERENT SOLUTIONS

Washing procedure	^{45}Ca in the filter	
	With microsomes	Without microsomes
	<i>cpm</i>	<i>cpm</i>
Without washing	973	210
CaCl_2 100 mM, 5 ml	843	9
CaCl_2 100 mM, 15 ml	802	16
Nonradioactive incubation medium, 10 ml	852	10
CaCl_2 10 mM, 10 ml	655	9
H_2O , 10 ml	475	7

Incubations were performed at 37°C in the presence of ATP, with or without microsomes. Incubation media were as shown in Table I. After incubation the mixtures were filtered as described under Methods. Millipore filters were then washed with the solutions specified in the table.

linear relationship between total Ca uptake and Ca concentration up to 1 mM. Here again these results contrasted with those obtained under quite similar conditions in dog cardiac microsomes by Katz and Repke (12). Calcium concentration which gave half-maximum uptake was reported to be much lower for muscle microsomes in Katz and Repke's paper than the lowest value which could be derived from data shown in Table III.

Table IV shows the effect of different ionic composition of the incubation media. When K^+ was replaced by Na^+ , no significant difference in ATP-dependent Ca uptake was observed. Conflicting observations on this point have been reported by other authors when tests were performed in the absence of oxalate. While Palmer and Posey (13) observed a higher Ca uptake in cardiac microsomes with K^+ than with Na^+ , no differences were reported by Katz and

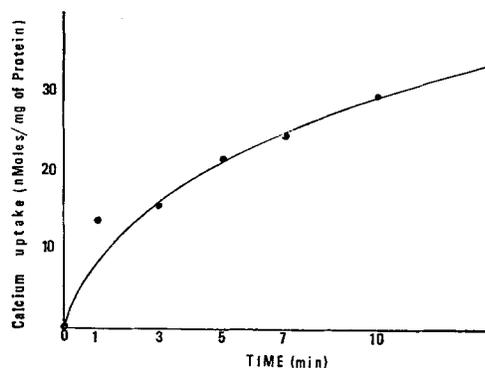


FIGURE 2. Time course of ATP-dependent calcium uptake. 0.27 mg of microsomal protein per ml was incubated in 50 μM $^{45}\text{CaCl}_2$, 100 mM KCl, 5 mM MgCl_2 , 45 mM Tris-buffer (pH 7.4), and 4.5 mM Tris-ATP. At the indicated times, 1.5 ml aliquots were processed for determination of calcium uptake. Nonspecific calcium uptake was subtracted from all other values.

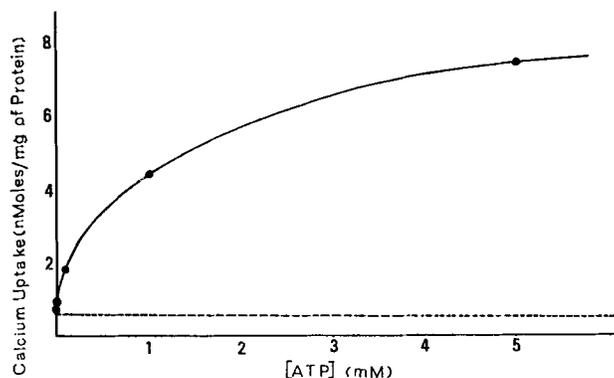


FIGURE 3. Dependence of calcium uptake on ATP concentration. 0.2 mg of microsomal protein per ml was incubated in different Tris-ATP concentrations at 37°C. All the other conditions were as in Table I. The dotted line shows nonspecific Ca uptake.

Repke (14). A marked increase in ATP-dependent Ca uptake by rat submaxillary gland microsomes was observed in the presence of oxalate. The addition of inorganic phosphate produced just a slight increase. There was almost no uptake when Mg was absent from the incubation medium (Fig. 4 and Table IV). ATP-dependent Ca uptake under experimental conditions such as those shown in Fig. 4 was detected only with concentrations of MgCl_2 higher than 0.1 mM.

ATPase Activity

Rat submaxillary gland microsomes showed a Mg^{++} -dependent ATPase activity. The rate of ATP splitting was over 100 μmoles of P_i per mg of micro-

TABLE III
DEPENDENCE OF CALCIUM UPTAKE ON CALCIUM
CONCENTRATION WITH AND WITHOUT ATP

[CaCl ₂]	Calcium uptake	
	With ATP	Without ATP
<i>M</i>	<i>nmoles/mg of protein</i>	<i>nmoles/mg of protein</i>
10 ⁻⁶	0.30	0.01
10 ⁻⁵	3.20	0.10
10 ⁻⁴	18.80	1.30
10 ⁻³	151.50	7.50

0.2 mg of microsomal protein per ml incubated at 37°C for 5 min, in the presence of the indicated ⁴⁵CaCl₂ concentrations. All the other conditions were as in Table I.

TABLE IV
DEPENDENCE OF CALCIUM UPTAKE ON THE
IONIC COMPOSITION OF THE MEDIA

Additions	Calcium uptake
	<i>nmoles/mg of protein</i>
KCl	0.79
MgCl ₂ and KCl	8.76
MgCl ₂ and NaCl	10.51
MgCl ₂ , KCl, and K-oxalate	46.69
MgCl ₂ , KCl, and K ₂ HPO ₄	13.51

All the incubation flasks contained 0.05 mg of microsomal protein per ml, 4 mM Tris-ATP, 45 mM Tris buffer (pH 7.4), and 50 μM ⁴⁵CaCl₂ plus the additions specified in the table at the following concentrations: KCl, 100 mM; MgCl₂, 5 mM; K-oxalate, 5 mM; NaCl, 100 mM; and K₂HPO₄, 5 mM. Experimental conditions were as in Table III.

somal protein per hr in the presence of 4 mM Tris-ATP, 100 mM KCl, 45 mM Tris-buffer (pH 7.4), and 5 mM MgCl₂. As a Mg⁺⁺-dependent and Ca⁺⁺-activated ATPase activity has been described in connection with other microsomal calcium pumps (1, 12), attempts were made to detect such an ATPase activity in salivary gland microsomes. Experiments were performed bringing Ca concentration up to 10 mM, increasing microsomal concentration, reducing incubation time and temperature, adding oxalate, replacing K⁺ by Na⁺, and combining all these variables. No Ca⁺⁺-dependent enhancement of Mg⁺⁺-ATPase activity could be obtained which might be related to the microsomal calcium pump. In some cases, 5 or 10 mM EGTA (ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid) was added to the homogenization suspension and/or incubation media. No reduction in Mg⁺⁺-ATPase activity was observed when this specific Ca chelator was added to the incubation

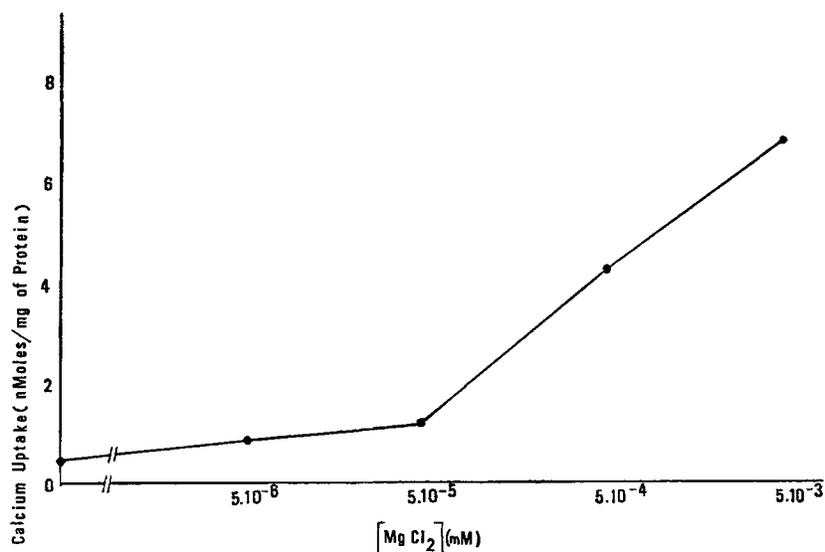


FIGURE 4. Mg influence on ATP-dependent calcium uptake. 0.05 mg of microsomal protein per ml was incubated in different MgCl_2 concentrations. Tris-ATP concentration was 4 mM and all the other conditions were as in Table I.

media, nor did EGTA-pretreated microsomes show a Ca enhancement of Mg^{++} -ATPase activity.

DISCUSSION

Rat salivary gland microsomes clearly showed an ATP-dependent ability to take up Ca from the medium. A similar property has been described for microsomes from other tissues (1-4). ATP-dependent Ca uptake by rat submaxillary gland microsomes, when observed in the absence of oxalate, was of the same order as that reported for skeletal (15) and cardiac (12) muscle and nervous tissue (16) microsomes.

Oxalate enhancement of ATP-dependent Ca uptake might be explained as in the case of muscle microsomes (1) as follows. While oxalate diffuses passively into the microsomal vesicle, Ca is actively transported. Both ions precipitate inside the vesicle when the solubility product is reached, keeping free Ca concentration low, allowing continuity of the transport process. Oxalate enhancement of Ca uptake is thus an indication of transport through the vesicular membrane rather than of binding to specific membrane sites. Additional evidence for this hypothesis is provided by the nonexchangeability of ATP-dependent Ca bound during the washing procedure, and also by the decrease in Ca uptake after washings with water or hypotonic CaCl_2 solutions, thought to be due to disruption of the vesicular membrane.

Two remarkable differences are found when the results presented in this

paper are compared with those obtained with muscle microsomes. First, while oxalate enhances Ca uptake by rat submaxillary gland microsomes only 6-fold, a 50-, or more, fold enhancement has been reported in experiments on muscle microsomes (1, 12). Our results are comparable to those obtained by Lieberman et al. (16) in crustacean nerves. Second, ATP-dependent Ca uptake by salivary gland microsomes is a relatively slow process when compared to the high velocity reported for Ca binding (uptake) to muscle microsomes (10). These two differences are probably related. Oxalate enhances total Ca uptake but its rate must be lower because of a decrease in extravascular Ca concentration. The smaller enhancement by oxalate of the microsomal uptake of Ca by the salivary gland could be explained either by a lower number of active sites or by lower Ca affinity. Compared with results for muscle microsomes (12) the lower affinity is supported by the slower uptake rate and the higher Ca concentration which is needed to give half-maximum uptake.

Even though we could not detect a Ca^{++} -activated ATPase as for muscle microsomes, a connection between such an ATPase activity and Ca uptake is suspected because: (a) ATP but not ADP is able to potentiate Ca uptake; (b) ATP-dependent Ca uptake is very low without Mg^{++} , as this cation is a co-factor for most ATPases; (c) ATP-dependent Ca uptake is not detected at 0°C ; (d) K_m for ATP in the uptake reaction is near the K_m range for many ATPases.

Ca^{++} -activated ATPase has been described in muscle microsomes in the presence of oxalate (1). A 1:1 or 1:2 molar relation for split ATP to transported Ca has been reported (1). Both a high uptake rate and a continuous transport process are thought to occur under these conditions in muscle preparations. Possibly we did not detect Ca-activated ATPase even in the presence of oxalate because of the low rate of our reaction. In fact, if a Ca transport to ATPase activity molar ratio of 1:1 or 2:1 is assumed, basic ATPase will be 10–50 times as great as Ca^{++} -activated ATPase. Therefore, the last one will not be detected.

Calcium ions seem to play a vital role in the secretion mechanisms in which cytoplasmic vesicles are involved; i.e., salivary secretion (6), catecholamine secretion by the adrenal medulla (5), acetylcholine (17) and norepinephrine (18) secretions by nerve endings, protein secretion by the exocrine pancreas (19), and histamine secretion by mast cells (20). Little is known about the mechanism by which calcium acts on these processes.

Prior to secretion, intracellular secretory granules accumulate on the cell secretory pole, and then they come in contact with the cell membrane, at which time the content of the vesicle is extruded (21). We suggest that the fusion of secretory granules with the cell membrane prior to secretion is a function of cytoplasmic free Ca concentration. This speculation is supported by the following facts: (a) An increase in cell Ca uptake has been reported

when secretion is elicited by different stimuli in salivary glands (22), adrenal medulla (5), and nerve endings (17); (b) Dallner and Nilsson (23) have reported that divalent cations produce aggregation of smooth microsomes. This type of microsome presumably contains the structures derived from the cell membrane and the membranes surrounding secretory granules (24); (c) Woodin et al. (25) have shown that the addition of calcium to leucocytes causes the enzyme-containing granules to become attached to the cell membrane. Calcium might be involved in this process either by changing the physicochemical state of the membranes or through a calcium-dependent chemical reaction.

As salivary gland secretion is not a continuous process, secretory cells must be equipped with a Ca-sequestering mechanism which would act to return the cell to the resting state. This action should be parallel to that taking place in striated and cardiac muscle fibers. A parallelism between several events occurring during secretion and contraction has been drawn by Douglas (26). We suggest that this Ca-sequestering mechanism, presumably occurring in vivo coincides with the vesicular preparation described in this paper.

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