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ATP-DEPENDENT CALCIUM UPTAKE BY MICROSOMAL PREPARATIONS FROM RAT PAROTID AND SUBMAXILLARY GLANDS

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SUMMARY

Microsomal fractions isolated from rat parotid and submaxillary glands readily demonstrate ATP-dependent calcium uptake. Concentrations of inhibitors which completely block calcium uptake in mitochondria do not appreciably inhibit calcium uptake in the gland microsomes. Ouabain at 0.1 mM concentration does not inhibit calcium uptake in gland microsomes. The reaction shows a high degree of specificity for ATP as the nucleoside triphosphate which has a K_m of 20 μM. The rate of calcium uptake per mg protein in the microsomal fraction is 25–30 nmoles per min at 30° and calcium storing capacity is 2.75 μmoles per mg protein. The indications that the microsomal vesicles are derived from the Golgi complex and the possible functions of calcium in this structure are discussed.

INTRODUCTION

Rat salivary glands contain a high concentration of calcium relative to other soft tissues[1,2]. Considerable movement of calcium, to and from the salivary glands, takes place in response to various stimulants[3]. It has also been demonstrated that a number of gland preparations require calcium for the process of secretion which is induced by specific hormones[4,5]. There is, however, little if any information on the subcellular structures participating in calcium transport in gland systems and the role of calcium in the secretory process is completely unknown[6].

The present communication describes a microsomal fraction from rat parotid gland which readily demonstrates ATP-dependent calcium uptake. A similar activity is also present in the submaxillary glands. The characteristics of calcium uptake by the salvary gland fraction differ from those of mitochondria and muscle microsomes. The possible role of calcium in the secretory process in glands is discussed.

METHODS

Preparation of subcellular fractions

Rat parotid gland homogenates were prepared essentially as described by SCHRAMM AND DANON[7] with the following modifications. Glands were collected and homogenized in a sucrose medium containing 0.3 M sucrose adjusted to pH 7.5 with

Na_2CO_3 and 1 μg/ml of diphenyl-*p*-phenylene diamine. A scheme of the isolation procedure is given in Fig. 1. Unless otherwise indicated all the experiments reported in this communication were conducted on Fraction P4. The various fractions were suspended in the homogenization medium at a concentration of 3–5 mg/ml and were kept frozen at −60° for a maximal period of 14 days.

Measurement of calcium accumulation

^{45}Ca uptake was measured with the aid of a Millipore filtration technique described by MARTONOSI AND FERETOS[8] for muscle microsomes. The assay system contained the following compounds: imidazole chloride buffer (pH 7.0), 5 mM; ATP, 1 mM; phosphoenolpyruvate, 4 mM; $MgCl_2$, 5 mM; KCl, 100 mM; oxalate, 5 mM; pyruvate kinase (EC 2.7.1.40), 60 μg/ml; $^{45}CaCl_2$, 20–100 μM. The reaction was initiated by the addition of the gland fraction to be tested, 25–300 μg protein per ml of assay system, and was terminated by filtration on a Millipore filter. Type HA filters with an average pore diameter of 0.45 μ were used and the amount of calcium uptake was calculated from the difference in radioactivity of the original solution and the particle-free filtrate. Radioactivity was measured in a liquid scintillation spectrometer using the scintillation solution described by BRAY[9].

Assay of enzymes

Nucleoside triphosphate phosphatase was determined as previously described[10]. Succinate dehydrogenase (EC 2.3.99.1) was measured according to GREEN *et al.*[11] and 5′-nucleotidase (EC 3.1.3.5) was determined as described by WIDNELL AND UNKELESS[12].

Na^+–K^+-stimulated ATPase

Liberation of inorganic phosphate from ATP was measured at 30° in the presence and absence of both 100 mM Na^+ and 5 mM K^+. The increment in ATPase activity caused by the presence of Na^+ and K^+ was taken as the Na^+–K^+-ATPase activity.

Protein determination

The method of LOWRY *et al.*[13] was used with crystalline bovine serum albumin as standard.

Materials

ATP, GTP and UTP were obtained from P-L Laboratories, CTP and ITP were products of Waldhoff. 3′,5′-Cyclic AMP, dibutyryl 3′,5′-cyclic AMP and pyruvate kinase were products of Boehringer Co. Monobutyryl 3′,5′-cyclic AMP was prepared by alkaline hydrolysis of dibutyryl cyclic AMP[14]. Phosphoenolpyruvate was synthesized according to the methods described by CLARK AND KIRBY[15] and was converted to the potassium salt before use. Oligomycin was a product of Sigma.

RESULTS

Calcium accumulation in rat parotid subcellular fractions

Calcium uptake and succinate dehydrogenase were both measured on the various subcellular fractions. It is shown that these two activities are distributed differently

among the various fractions (Table I). The highest calcium accumulation activity is obtained in the microsomal fractions sedimented at 100000 × *g* for 60 min whereas the highest succinate dehydrogenase activity is located mainly in the fractions sedimented at 10000 × *g* for 10 min. Since succinate dehydrogenase is confined to mitochondria it is evident that the calcium uptake resides mainly in non-mitochondrial fractions. Furthermore, when microsomes are removed from the mitochondrial fraction P'3 by resuspension and subsequent centrifugation at 5000 × *g* for 10 min only a very low activity of calcium uptake remains. Indeed mitochondria isolated from rat parotid gland in the absence of chelators are seriously damaged (H. FEINSTEIN AND M. SCHRAMM, to be published). Thus the calcium accumulation shown for unpurified fractions sedimented at 10000 × *g* (see Table I) is probably not a mitochondrial activity. The subcellular fractions suspended in the sucrose medium could be frozen once and still preserve their activity. When frozen in sucrose medium and stored at −20° for one week the microsomes loose about 50 % of their calcium uptake activity. Freezing in isotonic salt media caused complete loss of activity.

TABLE I

DISTRIBUTION OF CALCIUM UPTAKE AND SUCCINATE DEHYDROGENASE ACTIVITIES IN SUBCELLULAR FRACTIONS OF RAT PAROTID AND SUBMAXILLARY GLANDS

Calcium uptake and succinate dehydrogenase were measured on freshly prepared subcellular fractions as described under METHODS. The preparation of subcellular fractions is outlined in Fig. 1.

Subcellular fractions	*Succinate dehydrogenase in parotid gland (mequiv electrons/min per mg protein)*	*Calcium uptake in parotid gland*		*Calcium uptake in submaxillary gland; specific activity (nmoles/min per mg protein)*
		Specific activity (nmoles/min per mg protein)	*Total activity (% of homogenate)*	
P'3	375	12	6.8	8.8
P'4	30	14	46.0	9.4
P3	210	6	6.6	6.0
P4	32	25	22.0	13.0

Similar activities were found in fractions of rat submaxillary gland prepared by the procedure shown in Fig. 1. The distribution of calcium accumulation activity in submaxillary gland is shown in Table I.

Kinetic parameters of calcium accumulation

The course of calcium accumulation shows linear relationship with time (Fig. 2) and with the amount of membrane protein in the range of 25–300 μg protein per ml. It is noted that the curve connecting the experimental points at different time intervals does not intersect with the origin. This fact might be due to a very fast adsorption or exchange with unlabeled calcium in the membranes. The rate of calcium accumulation by the most active parotid gland membrane fraction is 25–30 nmoles calcium accumulated per min per mg protein at 30°. Calcium precipitating anions greatly promote calcium uptake (*cf.* ref. 16). Oxalate at 5 mM concentration or phosphate at 10 mM concentration are about equally effective (Fig. 2). Calcium accumulation in the absence of calcium precipitating anions is measured under conditions where

ATP splitting yielded less than 1 mM P_i. At this concentration P_i has essentially no effect on calcium uptake[17]. Calcium storing capacity in the presence of oxalate is 2.75 μmoles per mg protein (Fig. 3). This value is about half that reported by HASSELBACH[16] for purified muscle microsomes.

Under optimal conditions the parotid gland microsomes reduce the free calcium to a concentration of about 0.5 μM.

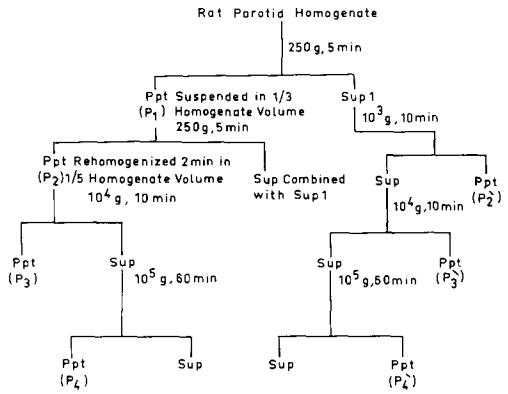

Fig. 1. Scheme for preparation of subcellular fractions from rat parotid gland homogenate. The homogenization procedure and the composition of homogenization medium are described under METHODS.

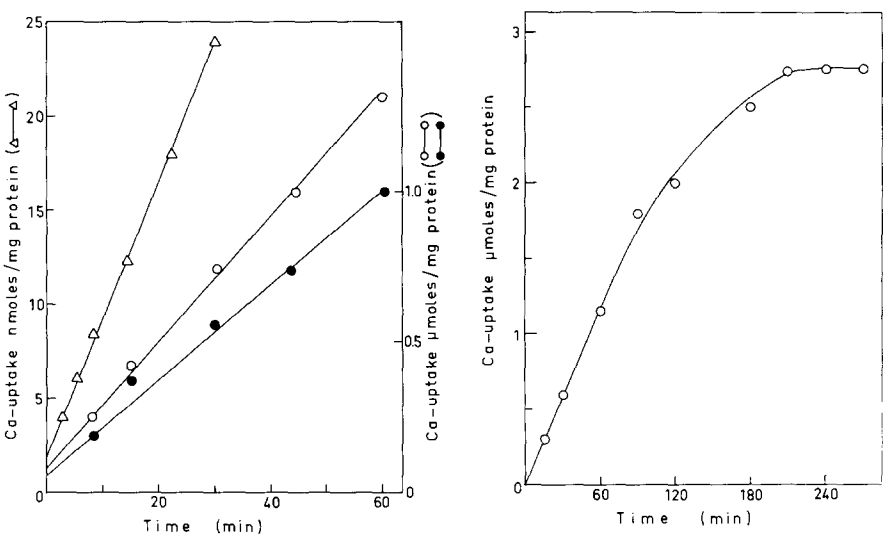

Fig. 2. The effect of anions on time course of calcium accumulation. Calcium uptake was measured in the standard assay for calcium accumulation. ○—○, oxalate 5 mM; ●—●, oxalate replaced by phosphate 10 mM. $^{45}CaCl_2$ concentration was 100 μM, membrane protein was 50 μg/ml. △--△, oxalate omitted, $^{45}CaCl_2$ concentration was 20 μM, membrane protein was 300 μg/ml.

Fig. 3. Calcium storing capacity of rat parotid microsomes. Calcium accumulation was measured under standard conditions for calcium uptake. $^{45}CaCl_2$ concentration 100 μM, membrane protein was 25 μg/ml. Aliquots were taken for Millipore filtration at intervals and the maximal calcium storing capacity was calculated after a steady state was reached.

Nucleoside triphosphate specificity of the calcium accumulation reaction

Calcium accumulation shows a high degree of specificity for ATP among the various nucleoside triphosphates tested (Table II). Succinate which readily supports calcium uptake in mitochondria did not replace ATP in the parotid system. When GTP or CTP in addition to ATP were included in the calcium accumulation assay at a concentration which was ten times that of ATP no inhibitory effect on calcium accumulation was observed. Thus calcium accumulation was neither supported nor

TABLE II

NUCLEOSIDE TRIPHOSPHATE SPECIFICITY OF THE CALCIUM ACCUMULATION SYSTEM

Calcium uptake was measured in the standard assay for calcium accumulation except that the ATP regeneration system was omitted. When indicated ATP was replaced by the corresponding nucleoside triphosphate at 1 mM concentration or by 20 mM succinate. $^{45}CaCl_2$ concentration was 50 μM, membrane protein was 85 μg/ml and the reaction time was 15 min. Calcium uptake is expressed as percent of the activity in the presence of ATP.

Nucleotide	*Calcium uptake* (% *of control*)
ATP	100
CTP	10
GTP	11
ITP	24
UTP	42
Succinate	10

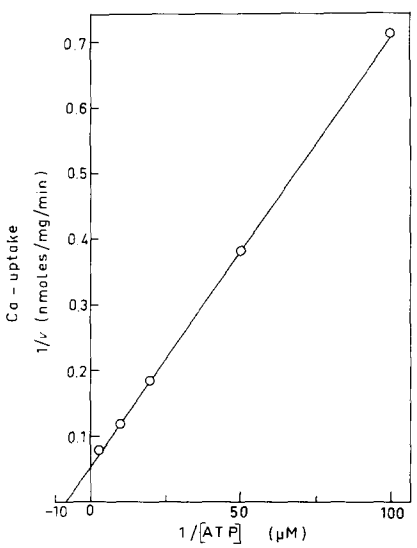

Fig. 4. Lineweaver–Burk plot for ATP in the calcium accumulation reaction. Incubation conditions were as described under METHODS except for varying the concentration of ATP. $^{45}CaCl_2$ concentration was 50 μM, membrane protein was 100 μg/ml.

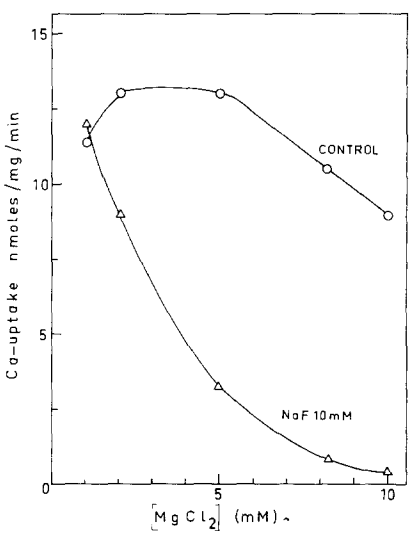

Fig. 5. The influence of magnesium on the inhibition of calcium uptake by NaF. Calcium uptake was measured in the standard assay for calcium accumulation containing the concentration of $MgCl_2$ indicated on the abscissa. O—O, standard assay for calcium uptake. △—△, standard assay for calcium uptake *plus* 10 mM NaF.

inhibited by GTP or CTP. Plotting the reciprocal values of ATP concentration and the rate of calcium uptake gave a Michaelis constant of $2 \cdot 10^{-5}$ M for ATP in the calcium accumulation reaction (Fig. 4). This value was 10 times lower than the K_m for ATP and other nucleoside triphosphates obtained by measuring the rate of liberation of inorganic phosphate. The discrepancy between the K_m for ATP measured by calcium accumulation and the K_m measured by ATP splitting probably reflects the existence of more than one type of ATPase in the preparation. Furthermore the preparation contained a high activity of an unspecific nucleoside triphosphatase. All nucleoside triphosphates shown in Table II were hydrolyzed at a rate of 0.5–0.7 μmole per min per mg protein at 30°. Because of the high unspecific ATP hydrolysis the stoichiometry between calcium uptake and ATP splitting could not be studied.

The rat parotid preparation was found to contain a highly active Na^+–K^+-ATPase. The possible participation of this enzyme in calcium uptake was tested. As shown in Table III calcium accumulation proceeded readily under conditions of complete inhibition of the Na^+–K^+-ATPase by ouabain.

Effect of various inhibitors on calcium accumulation

The extent of inhibition by various compounds is shown in Table III. Dinitrophenol inhibited calcium accumulation only slightly at a concentration at which this inhibitor completely blocks calcium uptake in mitochondria[18]. Oligomycin which inhibits ATP-driven calcium uptake in mitochondria inhibits ATP-dependent calcium uptake in parotid microsomes to a much smaller extent (*cf.* ref. 18). Azide, which at a concentration of 5 mM completely inhibited calcium uptake by mitochondria, had no effect on calcium accumulation by the parotid preparation. Fluoride which forms complexes with several metal-dependent enzymes inhibited calcium accumulation in the parotid system. As can be seen from Fig. 5 the inhibition by fluoride was influenced

TABLE III

EFFECT OF INHIBITORS ON CALCIUM UPTAKE BY RAT PAROTID MEMBRANES

The indicated concentrations of inhibitors were included in the standard assay for calcium uptake and the amount of calcium taken up was measured after 30 min. $^{45}CaCl_2$ concentration was 50 μM, membrane protein was 95 μg/ml.

Additions	*Calcium uptake (nmoles/min per mg protein)*	*Inhibition (% of control)*
None	24	0
2,4-Dinitrophenol (0.1 mM)	25	0
2,4-Dinitrophenol (0.5 mM)	16	36
Azide (5 mM)	24	0
Oligomycin (10 μg/mg protein)	24	0
Oligomycin (50 μg/mg protein)	19	23
Salygran (1.0 mM)	1	95
Ouabain* (0.1 mM)	23	5

* Ouabain does not inhibit Na^+–K^+-ATPase at the high KCl concentration usually employed in the calcium accumulation assay. Therefore the composition of the reaction mixture was modified to contain KCl, 5 mM and NaCl, 100 mM. All other components were those of the standard calcium uptake assay. Under these conditions 0.1 mM ouabain completely inhibits the Na^+–K^+-stimulated ATPase.

by the magnesium concentration suggesting a magnesium fluorophosphate as the active inhibitory compound.

TABLE IV

SUBFRACTIONATION OF THE MICROSOMES

Fraction P4 was suspended in concentrated sucrose solution at pH 7.5 (Na_2CO_3) to give a final sucrose concentration of 1.6 M. 1 ml of 0.3 M sucrose was carefully layered on top of 4 ml of the above suspension. Centrifugation for 60 min in the SW39 rotor at 173000 × *g* yielded a floating upper fraction and a lower sedimented fraction. The fractions were assayed for calcium uptake, 5′-nucleotidase activity and protein content.

Fraction	*Calcium uptake (nmoles/min per mg protein)*	*5′-Nucleotidase activity (μmoles/mg protein per 30 min)*
Upper fraction	17	10.1
Lower fraction	3.5	2.5
Unfractionated P4	13	4.2

Purification of the structural component involved in calcium uptake

Fraction P4 was resolved into two fractions by flotation in 1.6 M sucrose (*cf.* ref. 19). It is shown in Table IV that the upper fraction has a specific activity of calcium uptake which is five times higher than that of the lower fraction. The procedure seems to cause some inactivation of calcium uptake activity since purification over the original P4 fraction is only slight. The enzyme 5′-nucleotidase was also found to be enriched in the upper fraction. Electronmicroscopical studies which will be published elsewhere show that the upper fraction contains mainly smooth membrane vesicles while the lower fraction consists of rough microsomes.

DISCUSSION

To date there is little information about non-mitochondrial calcium pumps apart from that of muscle endoplasmic reticulum. The present paper describes for the first time the existence of a non-mitochondrial calcium pump in subcellular particles of exocrine glands. Two major lines of evidence indeed show that the calcium pump of the parotid fraction does not reside in the mitochondria. The distribution of calcium accumulation in various subcellular fractions was quite different from that of succinate dehydrogenase which served as a marker for the mitochondria. Also a number of inhibitors of calcium uptake in mitochondria were found to be either inactive or much less effective in the parotid system.

The properties of the parotid calcium pump differ from the well known calcium pump of muscle in the following aspects. Calcium accumulation is much slower in the parotid system than in muscle microsomes. It is not very likely that the slower rate of calcium uptake in parotid is due solely to contamination by inactive membranes since the maximal calcium storing capacity of the parotid preparation is almost as high as that of the muscle microsomes. Although the affinity for calcium seems to be lower in the parotid system than in muscle, it is quite considerable, since the free calcium concentration can be reduced to $5 \cdot 10^{-7}$ M. The salivary gland preparation

differs also from the muscle calcium pump in that the former shows a much higher specificity for ATP. In addition the two pump systems differ markedly with respect to stability towards freezing and thawing. More than one thawing completely inactivated the parotid calcium pump.

The present data do not permit a final identification of the cellular structure which demonstrates calcium uptake. However, there is good indication that this activity is localized in smooth membranes of the Golgi complex. Most of the calcium uptake activity was found in the microsomal fraction. When this fraction was subjected to centrifugation in 1.6 M sucrose the active structures were caused to float. Both our preliminary electronmicroscopical studies as well as previous work on other tissues[20] indicated that the floating fraction is composed predominantly of smooth membrane vesicles. It is not very likely that the fraction contains large amounts of plasma membranes since these are probably removed by sedimentation at a low centrifugal force[19]. The only other smooth cellular membranes present in considerable amounts in the parotid gland belong to the Golgi complex (*cf.* ref. 21).

The finding of a calcium pump in the parotid gland and submaxillary gland readily explains the high calcium concentrations present in these tissues (DREISBACH[1] and H. FEINSTEIN AND M. SCHRAMM, to be published). There are two phases in the function of the secreting cell in which calcium might participate. A number of reports indicate that calcium is somehow involved in the phase of enzyme secretion[4,5]. Calcium might also be involved in packing the proteins to be secreted within the zymogen granules[22] by forming a concentrated calcium–protein complex. The latter possibility is quite appealing since the granules are formed in the Golgi complex which may also be the location of the calcium pump.

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