Parotid microsomal Ca^{2+} transport

Subcellular localization and characterization

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Rat parotid gland homogenates were fractionated into mitochondrial, heavy microsomal and light microsomal fractions by differential centrifugation. ATP-dependent $^{45}\text{Ca}^{2+}$ uptake by the subcellular fractions paralleled the distribution of NADPH-cytochrome *c* reductase, an enzyme associated with the endoplasmic reticulum. The highest rate of Ca^{2+} uptake was found in the heavy microsomal fraction. Ca^{2+} uptake by this fraction was dependent on the presence of ATP and was sustained at a linear rate by 5 mM-oxalate. Inhibitors of mitochondrial Ca^{2+} transport had no effect on the rate of Ca^{2+} uptake. Na^{+} and K^{+} stimulated Ca^{2+} uptake. At optimal concentrations, Na^{+} stimulated Ca^{2+} uptake by 120% and K^{+} stimulated Ca^{2+} uptake by 260%. Decreasing the pH from 7.4 to 6.8 had little effect on Ca^{2+} uptake. The K_m for Ca^{2+} uptake was $3.7\text{ }\mu\text{M}$ free Ca^{2+} and 0.19 mM-ATP. Vanadate inhibited Ca^{2+} uptake; 60 μM -vanadate inhibited the rate of Ca^{2+} accumulation by 50%. It is concluded that the ATP-dependent Ca^{2+} transport system is located on the endoplasmic reticulum and may play a role in maintaining intracellular levels of free Ca^{2+} within a narrow range of concentration.

Many aspects of parotid gland metabolism are influenced by changes in the intracellular level of free Ca^{2+} (Butcher & Putney, 1980). These changes may be brought about by stimulation of the gland with adrenergic, cholinergic or peptidergic agonists. An increase in Ca^{2+} flux across the plasma membrane of parotid acinar cells stimulated with α -adrenergic, β -adrenergic and cholinergic agonists or with substance P has been demonstrated by several groups of workers (Kanagasuntheram & Randle, 1976; Miller & Nelson, 1977; Putney *et al.*, 1978). In addition, there is evidence suggesting that β -adrenergic stimulation may also mobilize intracellular Ca^{2+} associated with the mitochondria or other subcellular organelles (Kanagasuntheram & Randle, 1976). Since Ca^{2+} transport across the plasma membrane or intracellular organelles is likely to be of primary importance in controlling intracellular Ca^{2+} and Ca^{2+} -dependent processes, it is pertinent to study the properties and mechanism of regulation of membrane-associated Ca^{2+} -transport systems.

Selinger *et al.* (1970) demonstrated that rat parotid microsomes contain an ATP-dependent Ca^{2+} -transport system. These workers proposed that

this Ca^{2+} pump may be involved in sequestering Ca^{2+} for packaging within the secretory granules, which have been found to contain large amounts of Ca^{2+} (Feinstein & Schramm, 1970). Dormer & Ashcroft (1974), however, presented evidence suggesting that the microsomal Ca^{2+} pump may play a more dynamic role in regulating intracellular Ca^{2+} during stimulus–secretion coupling. Their studies showed that when parotid slices pre-loaded with $^{45}\text{Ca}^{2+}$ were stimulated with adrenaline, there was a decrease in the amount of $^{45}\text{Ca}^{2+}$ associated with the microsomal fraction.

To fully evaluate the role of the microsomal Ca^{2+} -transport system in intracellular Ca^{2+} homeostasis, it is necessary to delineate its subcellular localization and further characterize its properties. We report in the present paper studies showing that the microsomal Ca^{2+} -transport system of rat parotid glands is associated with the endoplasmic reticulum and displays properties similar to endoplasmic-reticulum-associated Ca^{2+} -transport systems from other non-muscle tissues such as the exocrine pancreas (Ponnappa *et al.*, 1981), liver (Moore *et al.*, 1975) and adipocyte (Bruns *et al.*, 1976).

Experimental

Materials

$^{45}\text{Ca}^{2+}$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of the highest purity available.

Methods

Subcellular fractionation. Parotid glands were removed from overnight-starved male Wistar rats weighing 200–250 g and placed in ice-cold medium containing 250 mM-sucrose, 0.1 mM-dithiothreitol and 5 mM-Tris/HCl, pH 7.4. All subsequent steps were carried out at 4°C. The glands were cleared of extraneous tissue, cut into small pieces and homogenized in the sucrose buffer to give a 10% (w/v) homogenate. This was carried out in a Potter–Elvehjem-type homogenizer (Arthur Thomas, size A) driven at 500 rev./min by an overhead motor. The homogenate was centrifuged at 1000 g for 10 min to remove nuclei and cell debris and the supernatant was centrifuged further at 10000 g for 10 min. The pellet consisting mainly of mitochondria was retained when necessary. The post-mitochondrial supernatant was centrifuged at 15000 g for 20 min to obtain a heavy microsomal fraction enriched in endoplasmic reticulum. Further centrifugation at 30000 g for 30 min yielded a light microsomal fraction enriched in plasma membranes.

$^{45}\text{Ca}^{2+}$ uptake. Unless otherwise stated, $^{45}\text{Ca}^{2+}$ uptake was measured in 1 ml of medium of the following composition: 100 mM-KCl, 10 mM-Tris/HCl, pH 7.4, 2.5 mM-MgCl₂, 2.5 mM-ATP, 5 mM-Tris/oxalate, 10 μM -CaCl₂, also containing 0.1 μCi of $^{45}\text{Ca}^{2+}$ /ml and about 100 μg of membrane protein. The reaction mixture lacking ATP and MgCl₂ was pre-incubated for 5 min at 30°C. $^{45}\text{Ca}^{2+}$ uptake was initiated by addition of ATP/MgCl₂ and portions of the reaction mixture were filtered through Millipore filters (0.45 μm pore diameter) at the given time periods. The filters were washed immediately with 5 ml of ice-cold medium containing 100 mM-KCl, 0.1 mM-EGTA and 5 mM-Tris/HCl, pH 7.4, dried and radioactivity was determined by liquid-scintillation counting in a toluene-based scintillation cocktail. Free $[\text{Ca}^{2+}]$ was calculated by using Ca-ATP and Mg-ATP association constants of $3.15 \times 10^4 \text{ M}^{-1}$ and $8.8 \times 10^4 \text{ M}^{-1}$ respectively at pH 6.8 as described by Katz *et al.* (1970).

Enzyme and other assays. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. 5'-Nucleotidase activity was determined as described by Widnell & Unkeless (1968). Succinate dehydrogenase was assayed at 37°C by monitoring the reduction of 2,6-dichloro-

indophenol (Green & Kohout, 1955). NADPH-cytochrome *c* reductase activity was determined as described by Strobel & Dignan (1978). For all enzymes, 1 unit is defined as the amount of enzyme that converts 1 μmol of substrate into product/min, under the assay conditions.

Results

Subcellular fractionation

The distribution of subcellular organelles in the 10000 g (mitochondrial), 15000 g (heavy microsomal) and 30000 g (light microsomal) pellets was determined by using succinate dehydrogenase, NADPH-cytochrome *c* reductase and 5'-nucleotidase as enzyme markers for mitochondria, endoplasmic reticulum and plasma membrane respectively. As shown in Fig. 1, the specific activity of succinate dehydrogenase was highest in the mitochondrial fraction, being about five times greater than in the heavy and light microsomes. 5'-Nucleotidase sedimented mainly in the light microsomal fraction. The mean specific activities of this enzyme in tissue homogenates and in the light microsomal fraction were 21.8 and 64.8 m-units/mg of protein respectively. High levels of NADPH-cytochrome *c* reductase activities were found in both the heavy and light microsomal fractions; the activity in the heavy microsomal fraction was, however, always greater than that in the light microsomal fraction. In four separate experiments, the mean specific activity of NADPH-cytochrome *c* reductase in the heavy microsomal fraction was 18.4 m-units/mg compared with 2.8 m-units/mg in tissue homogenates. This represents a 6.6-fold enrichment of endoplasmic reticulum in this fraction.

$^{45}\text{Ca}^{2+}$ uptake by the three subcellular fractions was measured in the presence of 5 mM-oxalate. As shown in Fig. 1, the distribution profile of Ca^{2+} transport activity in the three fractions paralleled that of NADPH-cytochrome *c* reductase activity, suggesting that this enzyme and the Ca^{2+} -transport system are located on the same membrane. Under the experimental conditions used for the fractionation, there appeared to be little or no interference from the mitochondrial Ca^{2+} -transport system as evidenced by the absence of any inhibition of $^{45}\text{Ca}^{2+}$ accumulation by all three fractions when 10 μM -Ruthenium Red or 2 μg of oligomycin/ml was included in the assay buffer (results not shown). This may be attributed to the omission of EGTA from the preparative medium, which results in uncoupling of parotid mitochondria (Feinstein & Schramm, 1970) and consequent loss of Ca^{2+} transport capacity. In separate experiments in which 1 mM-EGTA was included in the homogenizing buffer, the isolated mitochondrial fraction was found to retain an active

Fig. 1. Distribution of marker enzymes and ⁴⁵Ca²⁺ uptake in subcellular fractions

Parotid glands from four rats were homogenized and fractionated into mitochondrial (1), heavy microsomal (2) and light microsomal (3) fractions. The activities of 5'-nucleotidase (a), succinate dehydrogenase (b), NADPH-cytochrome c reductase (c) and ⁴⁵Ca²⁺ uptake in the presence of 2.5 mM-ATP and 5 mM-oxalate (d) were determined as described under 'Methods'. Results shown are representative of three experiments.

ATP-dependent Ca²⁺-transport system, which was inhibited by about 95% by 10 μ M-Ruthenium Red.

Time course of ⁴⁵Ca²⁺ uptake

The time course of ⁴⁵Ca²⁺ accumulation by the heavy microsomal fraction is shown in Fig. 2. In the absence of ATP, the microsomes bound 0.54 ± 0.04 nmol of Ca²⁺/mg of protein (mean $\pm$ S.E.M., $n = 6$). Binding was complete within 2 min and was not altered by the presence of oxalate. Addition of 2.5 mM-MgATP²⁻-stimulated ⁴⁵Ca²⁺ accumulation. In the presence of 5 mM-oxalate, there was an initial rapid uptake of ⁴⁵Ca²⁺ during the first 30 s of incubation, after which uptake proceeded at a slightly lower but linear rate for up to 10 min. About 50–60% of the medium Ca²⁺ was sequestered by the membrane vesicles during this time period. Whether the rapid uptake of ⁴⁵Ca²⁺ during the first 30 s of incubation with MgATP²⁻ is due to ATP-dependent ⁴⁵Ca²⁺ binding or due to a second Ca²⁺-transport system that becomes rapidly saturated even in the presence of oxalate is unclear. To avoid any interference from either of these possibilities, all subsequent determinations of Ca²⁺ uptake were made during the linear phase of uptake between the 1 and 5 min. The mean rate of ⁴⁵Ca²⁺ uptake during this phase was 5.60 ± 0.34 nmol/min per mg of protein (mean $\pm$ S.E.M., $n = 13$). When oxalate was omitted from the assay medium, the rate of ⁴⁵Ca²⁺ accumulation during the first 30 s after addition of MgATP²⁻ was almost as rapid as in the presence of 5 mM-oxalate. Thereafter, however, the rates declined rapidly until a steady state was established after about 10 min. From three separate experiments, ⁴⁵Ca²⁺ accumulated by the microsomes after 10 min of incubation in medium lacking oxalate was 11.09 ± 1.53 nmol/mg of protein.

Characterization of the Ca²⁺-transport system

Both Na⁺ and K⁺ influenced the rate of Ca²⁺ accumulation by the heavy microsomal fraction. To

Fig. 2. Time course of ⁴⁵Ca²⁺ uptake by the heavy microsomal fraction

⁴⁵Ca²⁺ uptake by the heavy microsomal fraction was determined as described under 'Methods', in the presence (●) and absence (○) of 5 mM-oxalate. Results shown are means of duplicate determinations in a representative experiment.

study the effects of these ions, $^{45}\text{Ca}^{2+}$ uptake was determined in medium containing 0–100 mM-NaCl or -KCl. The osmolality of the assay medium was maintained at a constant value by the addition of Tris/HCl. In the absence of Na^+ or K^+ , the average rate of $^{45}\text{Ca}^{2+}$ uptake was 1.57 ± 0.15 nmol/min per mg of protein. Addition of Na^+ or K^+ stimulated the rate of $^{45}\text{Ca}^{2+}$ uptake in a concentration-dependent manner (Fig. 3). At optimal concentrations of univalent cation, Ca^{2+} uptake was stimulated by about 260% by K^+ and 120% by Na^+ . When the Ca^{2+} -transport system was maximally stimulated with K^+ , further addition of Na^+ had no effect on the rate of transport. Thus, in a representative experiment, the rate of $^{45}\text{Ca}^{2+}$ accumulation by the microsomes incubated in medium containing 60 mM- K^+ , 0 mM- Na^+ and 60 mM- K^+ , 40 mM- Na^+ were 4.85 and 5.12 nmol/min per mg of protein.

Lowering the pH of the assay buffer from 7.4 to 6.8 had little effect on the rate of Ca^{2+} uptake. From three separate experiments, the mean rates of Ca^{2+} uptake were found to be 36.58 ± 2.6 and 37.4 ± 2.8 nmol/5 min per mg of protein at pH 7.4 and 6.8 respectively. The relationship between the rate of Ca^{2+} transport and the concentrations of Ca^{2+} and MgATP^{2-} in the assay medium was also studied. As shown in Fig. 4, increasing the Ca^{2+}

concentration from 5 to 50 μM increased the rate of $^{45}\text{Ca}^{2+}$ uptake. From a Lineweaver–Burk plot of the kinetic data, the K_m and $V_{\max}$ for Ca^{2+} transport were found to be $15.4 \pm 0.2 \mu\text{M}$ and 21.5 ± 0.2 nmol/min per mg of protein respectively. Correcting for Ca^{2+} binding to ATP as described by Katz *et al.* (1970), the K_m for Ca^{2+} was calculated to be $3.7 \pm 0.1 \mu\text{M}$ free Ca^{2+} . To determine the K_m for ATP, microsomes were incubated in medium containing 10 μM - Ca^{2+} and various concentrations of MgATP^{2-} . The K_m for ATP was found to be 0.19 mM; maximal rates of Ca^{2+} transport were sustained by ATP concentrations ranging from 1 to 5 mM. These values are in agreement with those reported previously by Selinger *et al.* (1970). Vanadate has been found to inhibit a variety of ATP-dependent ion-transporting systems, including the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase (Stekhoven & Bonting, 1981) and the Ca^{2+} -dependent ATPases of the erythrocyte and heart sarcolemmal membranes (Caroni & Carafoli, 1981). As shown in Fig. 5, the parotid endoplasmic-reticulum-associated Ca^{2+} -transport system was also inhibited by vanadate, although the sensitivity to inhibition was very much lower than that reported for the erythrocyte and sarcolemma Ca^{2+} -transport systems. Vanadate at 2 μM , which almost completely inhibits Ca^{2+} transport by the heart sarcolemma, had no effect on the parotid system. At the range of concentrations

Fig. 3. Effects of Na^+ and K^+ on $^{45}\text{Ca}^{2+}$ uptake by heavy microsomes

The heavy microsomal fraction from parotid glands was incubated in assay medium containing various concentrations of NaCl (O) or KCl (●). Constant osmolality was maintained by the addition of Tris/HCl. $^{45}\text{Ca}^{2+}$ uptake is expressed as a percentage of the rate obtained in medium containing 100 mM-KCl. Results shown are means of three experiments.

Fig. 4. Dependence of $^{45}\text{Ca}^{2+}$ uptake by microsomes on the total Ca^{2+} concentration

Microsomes were incubated in assay buffer, pH 6.8, containing 5–50 μM - $^{45}\text{CaCl}_2$. $^{45}\text{Ca}^{2+}$ accumulated between 1 and 5 min of incubation with ATP was determined as described under 'Methods'. Results shown are means of duplicate determinations in a representative experiment.

Fig. 5. Effect of vanadate on ⁴⁵Ca²⁺ uptake

Parotid microsomes were pre-incubated for 10 min in assay buffer containing 0–80 μM-sodium orthovanadate. Uptake of ⁴⁵Ca²⁺ was initiated by the addition of MgATP²⁻. Results are the means of three determinations.

tested, the maximal inhibition that could be achieved was about 50%.

Discussion

The present study indicates that the microsomal Ca²⁺-transport system identified by Selinger *et al.* (1970) in the rat parotid gland is associated with the endoplasmic reticulum. Subcellular fractions enriched in succinate dehydrogenase or 5'-nucleotidase activity showed lower rates of ⁴⁵Ca²⁺ accumulation than those enriched in NADPH-cytochrome *c* reductase. Moreover, there was a parallel distribution of NADPH-cytochrome *c* reductase, an enzyme found on the endoplasmic reticulum, and Ca²⁺ transport in the various subcellular fractions, suggesting that the two are components of the same membrane. The characteristics of the endoplasmic-reticulum Ca²⁺-transport system were quite distinct from those of the mitochondrial system. Neither oligomycin nor Ruthenium Red, both potent inhibitors of mitochondrial Ca²⁺ transport, had any effects on Ca²⁺ uptake by the endoplasmic-reticulum vesicles. These observations are consistent with those reported by Selinger *et al.* (1970) for parotid microsomes and by other workers for endoplasmic-reticulum-associated Ca²⁺-transport systems in other tissues (Moore *et al.*, 1975; Bruns *et al.*, 1976; Ponnappa *et al.*, 1981).

In the presence of a precipitating anion such as oxalate, the membrane vesicles sustained a linear rate of Ca²⁺ uptake. The rate of uptake in medium containing 5 mM-oxalate was higher than those

reported for other non-muscle tissues, such as the exocrine pancreas (Ponnappa *et al.*, 1981), liver (Moore *et al.*, 1975), adrenal cortex (Laychock *et al.*, 1978) and adipose tissue (Bruns *et al.*, 1976), determined under comparable conditions. This high capacity for Ca²⁺ accumulation by the parotid endoplasmic reticulum may relate to the gland's ability to accumulate and package large amounts of Ca²⁺ within its secretory granules.

Both Na⁺ and K⁺ stimulated the rate of Ca²⁺ uptake by the parotid microsomes. At equimolar concentrations, stimulation by K⁺ was always greater than that by Na⁺ and optimal rates were observed in medium containing 50–100 mM-K⁺. Similar observations on the effects of univalent cations on parotid microsomal Ca²⁺ accumulation have recently been reported by Bonis & Rossignol (1982). Activation by K⁺ may be a general feature of Ca²⁺-transport systems located on the endoplasmic reticulum since this characteristic is also displayed by liver and adipose tissue endoplasmic reticulum as well as by muscle sarcoplasmic reticulum (Moore *et al.*, 1975; Jones & Watanabe, 1977; Pershadsingh *et al.*, 1980a). In contrast, Ca²⁺-transport systems found on the plasma membrane of cells such as the adipocyte, heart or pancreatic islet display little if any sensitivity to K⁺ (Pershadsingh *et al.*, 1980a,b; Caroni & Carafoli, 1981). That changes in the intracellular concentration of Na⁺ or K⁺ may serve to regulate intracellular Ca²⁺ transport has been alluded to by several groups of workers (Crompton *et al.*, 1978; Sehlén, 1981; Bonis & Rossignol, 1982). Since the parotid gland is subject to relatively large fluctuations in its ionic composition, particularly with respect to K⁺, the possible implications of such changes on Ca²⁺ transport by the endoplasmic reticulum require consideration. On the basis of published data, the K⁺ concentration of parotid acinar cells is about 80–110 mM (Mangos *et al.*, 1975a; Kanagasuntheram & Randle, 1976; Putney *et al.*, 1978). Acute stimulation of the cell with secretagogues such as carbamoylcholine or adrenaline results in the transient release of about 30–40% of the cellular K⁺ (Mangos *et al.*, 1975b; Putney *et al.*, 1978). Despite this loss of K⁺, the intracellular K⁺ is not likely to fall below the threshold level of 40–50 mM necessary for sustaining optimal rates of Ca²⁺ transport. The assumption is made that there is little compartmentation of K⁺ within intracellular organelles. As such, the possibility of Ca²⁺ transport being influenced directly by changes in intracellular K⁺ appear to be small. Similarly, Na⁺ is also not likely to exert significant effects on endoplasmic-reticulum Ca²⁺ transport *in vivo*, since our studies indicate that changes in Na⁺ concentration have little or no effect on the transport properties if an optimal amount of K⁺ is present.

Most cellular Ca^{2+} -transport systems possess high affinity for Ca^{2+} , as expressed by K_m values in the micromolar or submicromolar range. For the parotid endoplasmic-reticulum Ca^{2+} -transport system, the K_m was estimated to be $3.7 \mu\text{M-Ca}^{2+}$. Any rise in the cytosolic free Ca^{2+} concentration to the micromolar range is therefore likely to result in Ca^{2+} accumulation by the endoplasmic reticulum. Such a process would offset increases in intracellular Ca^{2+} after stimulation with agonists and thereby enable the acinar cell to maintain Ca^{2+} homeostasis. The fate of the Ca^{2+} accumulated by the membrane vesicles is uncertain. It could either be concentrated and packaged within the secretory granules or released back into the cytosol in response to appropriate physiological stimuli. That the latter may hold true is supported by the investigations of Dormer & Ashcroft (1974), but more comprehensive studies on the mechanisms of regulation of Ca^{2+} transport by the parotid endoplasmic reticulum membrane are necessary before conclusions as to the exact physiological functions of this transport system can be arrived at.

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