Evidence that ATP-dependent Ca^{2+} transport in rat parotid microsomal membranes requires charge compensation

Bruce J. BAUM, Indu S. AMBUDKAR and Valerie J. HORN

Clinical Investigations and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892, U.S.A.

ATP-dependent Ca^{2+} transport was investigated in a rat parotid microsomal-membrane preparation enriched in endoplasmic reticulum. Ca^{2+} uptake, in KCl medium, was rapid, linear with time up to 20 s, and unaffected by the mitochondrial inhibitors NaN_3 and oligomycin. This Ca^{2+} uptake followed Michaelis–Menten kinetics, and was of high affinity ($K_m \sim 38$ nM) and high capacity (~ 30 nmol/min per mg of protein). In the presence of oxalate, Ca^{2+} uptake continued to increase for at least 5 min, reaching an intravesicular accumulation approx. 10 times higher than without oxalate. Ca^{2+} uptake was dependent on univalent cations in the order $\text{K}^+ = \text{Na}^+ > \text{trimethylammonium}^+ > \text{mannitol}$ and univalent anions in the order $\text{Cl}^- > \text{acetate}^- > \text{Br}^- = \text{gluconate}^- = \text{NO}_3^- > \text{SCN}^-$. Ca^{2+} uptake was not elevated if membranes were incubated in the presence of a lipophilic anion (NO_3^-) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Ca^{2+} transport was altered by changes in the K^+ -diffusion potential of the membranes. A relatively negative K^+ -diffusion potential increased the initial rate of Ca^{2+} accumulation, whereas a relatively positive potential decreased Ca^{2+} accumulation. In the presence of an outwardly directed K^+ gradient, nigericin had no effect on Ca^{2+} uptake. In aggregate, these studies suggest that the ATP-dependent Ca^{2+} -transport mechanism present in rat parotid microsomal membranes exhibits an electrogenic Ca^{2+} flux which requires the movement of other ions for charge compensation.

INTRODUCTION

Considerable effort has been focused on the maintenance of Ca^{2+} homeostasis in cells as well as on the signalling mechanisms involved in Ca^{2+} -mediated hormone/neurotransmitter-elicited responses. Currently it is considered that the signal Ca^{2+} trigger in eukaryotic cells results from inositol trisphosphate mobilization of Ca^{2+} from a non-mitochondrial intracellular pool, believed to be the endoplasmic reticulum (Berridge, 1987). Ca^{2+} is sequestered into this target pool through an ATP-dependent Ca^{2+} -transport system (see, e.g., Chueh & Gill, 1986; Henne & Söling, 1986). Several studies indicate that basal concentrations of cytosolic Ca^{2+} in non-excitable cells are primarily a result of the combined action of this Ca^{2+} -transport mechanism and another ATP-dependent Ca^{2+} transporter localized in the plasma membrane (Williamson *et al.*, 1981; Shamoo & Ambudkar, 1984; Bayerdorffer *et al.*, 1985; Carafoli, 1987; Enouf *et al.*, 1987). Consequently, a full understanding of the mechanistic nature of Ca^{2+} -coupled responses requires detailed knowledge of both membrane Ca^{2+} pumps as well as the inositol trisphosphate-elicited, and possibly other, Ca^{2+} -release steps (Chueh & Gill, 1986).

The rat parotid gland has been widely employed in studies attempting to elucidate steps in receptor-coupled Ca^{2+} signalling. Much of our knowledge of polyphosphoinositide metabolism and inositol-trisphosphate-induced Ca^{2+} release results from studies with this tissue (Irvine *et al.*, 1984; Putney *et al.*, 1986). Examination of Ca^{2+} -transport mechanisms in rat parotid membranes has been relatively limited. The existence of an ATP-dependent Ca^{2+} transporter in

endoplasmic-reticulum-enriched parotid microsomal membranes has been reported by several laboratories (Selinger *et al.*, 1970; Bonis & Rossignol, 1982; Kanagasuntheram & Teo, 1982; Immelman & Söling, 1983). However, the kinetic properties of this transporter have not been clearly assessed. In all studies, initial transport rates were not reported and oxalate was used during Ca^{2+} -transport measurements. Although oxalate is useful for demonstrating high Ca^{2+} -transport activity, it does not represent a physiological condition. In the present work, we demonstrate that parotid microsomal membranes display a very-high-affinity, high-capacity and rapidly saturating ATP-dependent Ca^{2+} -transport mechanism which requires the movement of other ions for charge compensation.

EXPERIMENTAL

Materials

Animals used in these studies were male (250 g) Wistar-strain rats purchased from Harlan/Sprague-Dawley. Rats were fed *ad libitum* and killed between 08:30 and 10:30 h. $^{45}\text{CaCl}_2$ was obtained from Amersham International. ATP (Tris salt), NaN_3 , oligomycin, NaVO_4 , dithiothreitol, nigericin and valinomycin were from Sigma. FCCP was from Aldrich and A23187 was from Calbiochem–Behring. All other reagents utilized were the highest grade commercially available.

Preparation of microsomal membranes

Parotid glands from 15–20 rats were homogenized, and membrane fractions obtained, as described previously (Takuma & Baum, 1985; Takuma *et al.*, 1985).

Abbreviations used: TMA, trimethylammonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Briefly, finely minced glands were placed in 0.3 M-sucrose containing 0.1 mM-phenylmethanesulphonyl fluoride, 1 mM-dithiothreitol and 10 mM-Tris/HCl, pH 7.5, and homogenized with a Brinkman Polytron. The homogenate (approx. 10 %, w/v) was centrifuged at 2600 *g* for 15 min, and the resulting supernatant was then centrifuged at 23 500 *g* for 20 min. The resulting pellet was discarded and the supernatant centrifuged at 41 700 *g* for 45 min. The resulting pellet was used as the microsomal membrane fraction and was resuspended in the sucrose buffer, frozen in liquid N₂ and stored at -70 °C until use. The RNA content of the membranes, measured by an assay described by Bayerdorffer *et al.* (1984), was enriched about 2-fold over that in the crude parotid homogenate (290 versus 157 µg/mg of protein). Frozen membrane samples were thawed once in cold water on ice before use in transport studies.

ATP-dependent Ca²⁺ transport

⁴⁵Ca²⁺ uptake into parotid microsomal-membrane vesicles was measured by rapid filtration through a Millipore filter (HA; 0.45 µm pore size). Typically reaction mixtures contained 12–18 µg of membrane protein, 1 mM-ATP, 150 mM-KCl, 10 mM-Tris/Hepes, pH 7.5, 1 mM-magnesium gluconate and ⁴⁵Ca²⁺ (10 µM total Ca²⁺ unbuffered, equal to ~3.3 µM free Ca²⁺) in a total volume of 100 µl. For kinetic experiments free Ca²⁺ was buffered with EGTA as described previously (Helman *et al.*, 1986). Transport usually was initiated by addition of protein (10 µl) to 90 µl of reaction mixture which had been preincubated at 37 °C for 30–60 s. For experiments in which membranes were preincubated with NaVO₄ or valinomycin, the experimental procedure was changed and transport was initiated by the addition of an ATP/⁴⁵Ca²⁺ stock solution. Transport experiments were carried out at 37 °C for 20 s (except time-course studies). Under these conditions, ⁴⁵Ca²⁺ transport was linear with respect to protein concentration between ~5 and 20 µg of protein/100 µl. Sucrose-loaded vesicles were routinely diluted with 300 mM-mannitol to this protein range before transport assays. Additional details of experimental conditions are given in Figure and Table legends. ⁴⁵Ca²⁺ uptake was terminated by adding 1.0 ml of ice-cold reaction buffer (stop solution; without Ca²⁺ or ATP) to reaction mixtures at appropriate times. This was rapidly passed over the Millipore filter and washed twice with ~2.5 ml of the same buffer. The stopping and washing procedures required < 10 s. In experiments in which ⁴⁵Ca²⁺ transport was measured in reaction mixtures containing major solutes other than KCl, the stop solutions also contained the same solute. The determinations reported were usually performed in triplicate, with a minimum of two separate membrane preparations. Background ⁴⁵Ca²⁺ uptake (zero time) was subtracted from all reported results. Zero time points were obtained by adding 1 ml of ice-cold stop solution to reaction mixtures before membrane addition, after which reaction mixtures were immediately filtered. Background values were typically < 5 % of that observed at 37 °C for 20 s. In initial experiments we evaluated whether any of the ⁴⁵Ca²⁺ accumulation observed with the parotid microsomal-membrane preparation used here was due to mitochondrial contamination. ATP-dependent ⁴⁵Ca²⁺ uptake was the same in the absence (12.9 ± 1.5 nmol/min per mg of protein) or presence of two mitochondrial inhibitors, NaN₃ (5 mM; 12.9 ± 1.7) and oligomycin

(2 µg/ml; 12.9 ± 0.3). Therefore mitochondrial inhibitors were not routinely included in reaction mixtures. Protein content of membranes was determined with the Bio-Rad assay kit, with bovine serum albumin as a standard.

RESULTS

General characteristics of ⁴⁵Ca²⁺ uptake in parotid microsomal membranes

ATP-dependent ⁴⁵Ca²⁺ uptake into parotid microsomal-membrane vesicles was very rapid (Fig. 1). Uptake was linear for only 20 s, at which time maximal transport was closely approximated. No significant further accumulation of ⁴⁵Ca²⁺ in vesicles was observed through 5 min incubation. In KCl-containing media, the initial rate (i.e. measured at 20 s) of ATP-dependent ⁴⁵Ca²⁺ transport was typically 12–18 nmol/min per mg of protein. ⁴⁵Ca²⁺ uptake in the presence of ATP was 10–20-fold greater than that found when ATP was omitted from reaction mixtures (Fig. 1). When Mg²⁺ was excluded from the reaction mixture, the initial rate of ⁴⁵Ca²⁺ uptake was decreased by ~50 % (results not shown). If oxalate (5 mM) was included in the Mg²⁺-free reaction mixture, the ATP-dependent ⁴⁵Ca²⁺ uptake measured at 20 s still remained 50 % lower. However, at 5 min, Mg²⁺-independent ⁴⁵Ca²⁺ uptake in the oxalate-containing reaction mixture was only 20 % of that observed with Mg²⁺ present. When a Ca²⁺ ionophore, A23187 (5 µM), was added to vesicles incubated for 5 min with ⁴⁵Ca²⁺ and ATP (in the absence of oxalate), there was a rapid release of all accumulated ⁴⁵Ca²⁺, to the values observed when the incubation was performed without ATP (Fig. 1). If A23187 was present in the reaction mixture from the start of incubation, no ⁴⁵Ca²⁺ was accumulated in vesicles beyond that observed without ATP (results not shown).

The anion oxalate is widely used in studies with microsomal membranes to increase maximal Ca²⁺-loading capacity. When we tested the effect of 5 mM-oxalate on the Ca²⁺-transport activity of parotid microsomal membranes, there was little difference in the

Fig. 1. Time course of ATP-dependent ⁴⁵Ca²⁺ uptake

Microsomal membranes were incubated at 37 °C in KCl reaction mixture, and ⁴⁵Ca²⁺ uptake was measured at the indicated times as described in the Experimental section. Data shown are means of triplicate determinations from a representative experiment of four performed: ●, ATP in reaction mixture; ○, no ATP in reaction mixture. Arrow indicates time of addition of A23187 (final concn. 5 µM).

Fig. 2. Effect of different $[\text{Ca}^{2+}]$ on ATP-dependent $^{45}\text{Ca}^{2+}$ uptake

Microsomal membranes were incubated at 37°C for 20 s with different free $[\text{Ca}^{2+}]$ (3–4320 nM), buffered by EGTA, 1 mM-ATP, 150 mM-KCl, 1 mM-magnesium gluconate and 10 mM-Tris/Hepes, pH 7.5, and $^{45}\text{Ca}^{2+}$ uptake was determined as described in the Experimental section. Data are means of triplicate determinations in two experiments performed with different membrane preparations and are presented in an Eadie-Hofstee plot. K_m and $V_{\max}$ were calculated to be 38.0 nM and 27.8 nmol/min per mg of protein respectively.

initial rate of $^{45}\text{Ca}^{2+}$ accumulation. However, these membranes continue to accumulate $^{45}\text{Ca}^{2+}$ after 20 s, unlike those incubated in non-oxalate-containing medium. After 5 min in a KCl medium with oxalate, $^{45}\text{Ca}^{2+}$ accumulation was more than 10-fold greater than that observed in the absence of oxalate (56.4 ± 3.3 nmol/mg of protein). Oxalate had no effect on $^{45}\text{Ca}^{2+}$ uptake measured in the absence of ATP.

Vanadate is known to inhibit ATP-dependent ion-transport proteins (Pedersen & Carafoli, 1987). We observed a dose-dependent inhibitory effect of sodium vanadate on ATP-dependent Ca^{2+} transport in our membrane preparation. ATP-dependent Ca^{2+} transport was decreased by $\sim 40\%$ with 100 μM - and by $\sim 70\%$ with 500 μM -sodium vanadate.

Ca^{2+} -dependence of ATP-dependent Ca^{2+} transport

ATP-dependent Ca^{2+} uptake into parotid microsomal membranes was dependent on the $[\text{Ca}^{2+}]$ present during incubations. In these studies the desired $[\text{Ca}^{2+}]$ was obtained by utilizing EGTA-buffered reaction mixtures. $^{45}\text{Ca}^{2+}$ uptake was a saturable process which obeyed Michaelis-Menten kinetics (Fig. 2). When these data were analysed by an Eadie-Hofstee plot, a K_m of 38 nM and a $V_{\max}$ of ~ 30 nmol/min per mg of protein were determined.

Effect of different cations on $^{45}\text{Ca}^{2+}$ uptake

Previously both Bonis & Rossignol (1982) and Kanagasuntheram & Teo (1982) reported that ATP-dependent Ca^{2+} transport in parotid microsomal membranes was significantly greater in reaction mixtures containing KCl than in those containing NaCl. We also examined the cation specificity of $^{45}\text{Ca}^{2+}$ uptake in these membranes. As shown in Table 1, there was no difference between the initial rate of $^{45}\text{Ca}^{2+}$ uptake in KCl or NaCl.

Table 1. Effect of different cations on $^{45}\text{Ca}^{2+}$ uptake by parotid microsomal membranes

Data are the means ($\pm$ S.E.M. where shown) of results from the numbers of experiments indicated in parentheses. At least two different membrane preparations were used for each condition, and all assays were performed in triplicate. The initial rate (i.e. measured at 20 s) of $^{45}\text{Ca}^{2+}$ uptake with KCl+ATP was set at 100% for each experiment (mean $\pm$ S.E.M., 15.1 ± 1.3 nmol of $^{45}\text{Ca}^{2+}$ /min per mg of protein; $n = 9$). The KCl medium contained 150 mM-KCl, 1 mM-ATP, 1 mM-magnesium gluconate, 10 mM-Tris/Hepes, pH 7.5, and 10 μM total Ca^{2+} . In the other media, KCl was replaced by the indicated solute so as to maintain a constant osmolarity, i.e. 150 mM-NaCl, 150 mM-TMACl, or 300 mM-mannitol.

Medium	$^{45}\text{Ca}^{2+}$ uptake (%)	
	–ATP	+ATP
KCl	8.3 ± 0.9 (7)	100 (9)
NaCl	7.4 ± 1.1 (5)	100.3 ± 6.0 (5)
TMACl	19.7 (2)	87.0 ± 9.5 (4)
Mannitol	11.9 ± 1.4 (4)	78.3 ± 4.5 (4)

Table 2. Effect of different anions on ATP-dependent $^{45}\text{Ca}^{2+}$ uptake by parotid microsomal membranes

Data are the means ($\pm$ S.E.M., where shown) of the results from the numbers of experiments indicated in parentheses. A different membrane preparation was used for each experiment, and all assays were performed in triplicate. The initial rate (i.e. that measured at 20 s) of $^{45}\text{Ca}^{2+}$ uptake for KCl was set at 100% for each experiment (mean $\pm$ S.E.M., 12.6 ± 1.7 nmol of $^{45}\text{Ca}^{2+}$ /min per mg of protein). The KCl medium contained 150 mM-KCl, 1 mM-ATP, 1 mM-magnesium gluconate, 10 mM-Tris/Hepes, pH 7.5, and 10 μM total Ca^{2+} . In the other media KCl was replaced by the indicated solute so as to maintain a constant osmolarity (i.e. each at 150 mM).

Medium	$^{45}\text{Ca}^{2+}$ uptake (%)
KCl	100 (3)
Potassium acetate	93.3 (2)
KBr	81.5 ± 7.1 (3)
Potassium gluconate	83.7 ± 3.2 (3)
KNO_3	84.4 (2)
KSCN	49.6 ± 2.4 (3)

ATP-dependent $^{45}\text{Ca}^{2+}$ transport was decreased, however, in the presence of the relatively impermeant cation TMA^+ , and in a non-ionic but iso-osmotic medium, mannitol. In addition, we measured $^{45}\text{Ca}^{2+}$ uptake in 5 mM-oxalate-containing medium (with KCl or NaCl) at a later time (5 min), i.e. conditions similar to those used by Bonis & Rossignol (1982) and Kanagasuntheram & Teo (1982). Under these incubation conditions we observed $\sim 25\%$ lower $^{45}\text{Ca}^{2+}$ accumulation in the NaCl/oxalate medium (results not shown).

Effect of different anions on $^{45}\text{Ca}^{2+}$ uptake

There has been considerable interest in the role of anions in the mechanism of ATP-dependent Ca^{2+}

Table 3. Effect of alterations in K⁺-diffusion potential on ATP-dependent ⁴⁵Ca²⁺ uptake

Data are means $\pm$ S.E.M. of results from three experiments, or the means of two experiments, each assayed in triplicate. In Expt. 1, membranes loaded with sucrose/mannitol were preincubated for 30 min at 23 °C with an equal volume of 150 mM-KCl/1 mM-magnesium gluconate/10 mM-Tris/Hepes, pH 7.5. Thereafter membranes were added to reaction mixtures containing 10 μ M-⁴⁵Ca²⁺, 1 mM-ATP, 10 mM-Tris/Hepes, pH 7.5, 1 mM-magnesium gluconate and either 150 mM-KCl or 300 mM-mannitol. After 20 s, 1 ml of ice-cold stop solution (KCl or mannitol) was added and the reaction mixtures were filtered, washed and assayed for radioactivity as described in the Experimental section. In Expt. 2, membranes were incubated for 60 min at 23 °C in an equal volume of diluent containing either 300 mM-mannitol or 300 mM-KCl, plus 1 mM-magnesium gluconate, 10 mM-Tris/Hepes, pH 7.5. Thereafter membranes were added to reaction mixtures as above for 20 s, stop solution was then added and membranes were filtered, washed and assayed for radioactivity. In Expt. 3, membranes were incubated for 60 min at 23 °C in an equal volume of diluent containing either 300 mM-TMACl or 300 mM-KCl, plus 1 mM-magnesium gluconate, 10 mM-Tris/Hepes, pH 7.5. Thereafter membranes were added to reaction mixtures, which included either 150 mM-KCl or 150 mM-TMACl, for 20 s, stop solution was then added and membranes were filtered, washed and assayed for radioactivity.

Conditions (intravesicular/ extravesicular)	<i>n</i>	⁴⁵ Ca ²⁺ uptake (nmol/min per mg of protein)
Expt. 1		
75 mM-KCl/ 150 mM-KCl	3	16.5 $\pm$ 1.5
75 mM-KCl/ 300 mM-mannitol	3	22.8 $\pm$ 0.3
Expt. 2		
300 mM-Mannitol/ 150 mM-KCl	2	14.1
150 mM-KCl/ 300 mM-mannitol	2	29.4
Expt. 3		
150 mM-TMACl/ 150 mM-KCl	2	15.9
150 mM-KCl/ 150 mM-KCl	2	17.4
150 mM-KCl/ 150 mM-TMACl	2	21.9

transport (see, e.g., Chu *et al.*, 1983; Smallwood *et al.*, 1983; Bayerdorffer *et al.*, 1984). We therefore examined the effect of different anions on the initial rate of ⁴⁵Ca²⁺ uptake in parotid microsomal membranes. These results are shown in Table 2. Maximum ATP-dependent ⁴⁵Ca²⁺ uptake was found in the presence of Cl⁻. Uptake decreased when Cl⁻ was replaced by other anions, in the following sequence: acetate⁻ > Br⁻ = gluconate⁻ = NO₃⁻ > SCN⁻.

Effect of K⁺-diffusion potential on ⁴⁵Ca²⁺ uptake

To assess the effect of alteration in K⁺-diffusion potential on ATP-dependent Ca²⁺ accumulation into

parotid microsomal-membrane vesicles, we measured the initial rate of ⁴⁵Ca²⁺ uptake into sucrose/mannitol-loaded vesicles in the presence of an inwardly directed KCl (150 mM) gradient in the absence and presence of the K⁺ ionophore, valinomycin (5 μ M). The initial rate of ⁴⁵Ca²⁺ uptake was decreased by ~25 % when valinomycin was present, from 16.3 $\pm$ 1.2 to 11.9 $\pm$ 0.8 nmol of ⁴⁵Ca²⁺/min per mg of protein, *n* = 4; *t* = 6.61, *P* = 0.0035. This did not appear to be due to a direct effect of valinomycin on the transporter, as control experiments, performed in mannitol-containing medium, showed minimal effect on ⁴⁵Ca²⁺ accumulation (~5 % difference).

Next, we further tested the effect of changing the K⁺-diffusion potential, without valinomycin, on ⁴⁵Ca²⁺ uptake (Table 3). In Expt. 1, we preincubated membranes with an equal volume of 150 mM-KCl for 30 min at 23 °C, which established an intravesicular [KCl] of 75 mM. Thereafter the initial rate of ⁴⁵Ca²⁺ accumulation into microsomal vesicles was measured in reaction mixtures containing either 150 mM-KCl or 300 mM-mannitol, which created, respectively, a relatively positive or relatively negative intravesicular K⁺-diffusion potential. As shown in Table 3, vesicles incubated with the mannitol-containing medium displayed ~35 % higher ATP-dependent ⁴⁵Ca²⁺ accumulation than did vesicles incubated in the KCl-containing medium. In Expt. 2, membranes were preincubated for 60 min at 23 °C with an equal volume of either 300 mM-mannitol or 300 mM-KCl. Thereafter ⁴⁵Ca²⁺ transport was measured in reaction mixtures containing either 150 mM-KCl or 300 mM-mannitol respectively, resulting in a relatively positive or relatively negative intravesicular K⁺ diffusion potential. ⁴⁵Ca²⁺ transport in the presence of an outwardly directed KCl gradient was more than twice that observed with an inwardly directed KCl gradient (Table 3). In each of the above experiments, significant ionic gradients were present during transport studies. Expt. 3 was therefore conducted in order to evaluate the effect of different K⁺ diffusion potentials, under conditions of equal intravesicular and extravesicular ionic strength, on ⁴⁵Ca²⁺ transport. Vesicles were preincubated for 60 min at 23 °C to create the desired intravesicular conditions before transport assays. In the presence of an outwardly directed K⁺ gradient (KCl_{in}/TMACl_{out}), ATP-dependent ⁴⁵Ca²⁺ transport was ~25 % greater than that at KCl equilibrium (KCl_{in}/KCl_{out}). In the presence of an inwardly directed K⁺ gradient (TMACl_{in}/KCl_{out}), ⁴⁵Ca²⁺ uptake was decreased by ~10 % from the equilibrium condition (Table 3).

Effect of proton permeability on ⁴⁵Ca²⁺ uptake

The above experiments strongly suggested that ATP-dependent Ca²⁺ transport in parotid microsomal membranes was electrogenic. Several studies have examined the involvement of protons in ATP-dependent Ca²⁺ transport (e.g. Niggli *et al.*, 1982; Kraus-Freedman *et al.*, 1982; Smallwood *et al.*, 1983; Kemmer *et al.*, 1987a). Accordingly, we have examined a possible role for proton movement in the Ca²⁺-uptake process studied here. As shown in Table 4, by using two different experimental designs, manipulations which might be predicted to enhance proton permeability in parotid microsomal membranes did not enhance ⁴⁵Ca²⁺ uptake. The relative Ca²⁺ uptake was not different when measured with KCl or KNO₃ in the presence or absence of FCCP

Table 4. Effect of changes in proton permeability on ATP-dependent $^{45}\text{Ca}^{2+}$ uptake

Data are means of results from two experiments, with different membrane preparations, each assayed in triplicate. Results are from measurements made at 20 s, i.e. initial rate. Qualitatively similar results were observed in both experiments when $^{45}\text{Ca}^{2+}$ uptake was measured at 60 s. In Expt. 1, membranes were incubated under the indicated conditions (plus 1 mM-magnesium gluconate, 1 mM-ATP, 10 mM-Tris/Hepes, pH 7.4, and 10 μM total Ca^{2+}) in the absence or presence of 20 μM -FCCP. An equivalent volume of ethanol served as the vehicle control. In Expt. 2, membranes were first incubated for 60 min at 23 °C in an equal volume of 300 mM-KCl (plus 1 mM-magnesium gluconate, 10 mM-Tris/Hepes, pH 7.5) as described in Table 3, Expt. 3. Thereafter membranes were added to reaction mixtures, which included either 150 mM-KCl or 150 mM-TMACl plus 0.185 μM -nigericin. At appropriate times stop solution was added, and membranes were filtered, washed and then assayed for radioactivity.

Conditions (intravesicular/extravesicular)	$^{45}\text{Ca}^{2+}$ uptake (nmol/min per mg of protein)	
	Vehicle control	+ FCCP
Expt. 1		
300 mM-Mannitol/ 150 mM-KCl	12.3	10.1
300 mM-Mannitol/ 150 mM-KNO ₃	9.4	8.7
Expt. 2		
150 mM-KCl/ 150 mM-TMACl + nigericin	10.6	
150 mM-KCl/ 150 mM-KCl + nigericin	9.7	

(see also Table 2) or when measured with an increased intravesicular $[\text{H}^+]$ created by the addition of nigericin in the presence of a K^+ gradient (see also Table 3).

DISCUSSION

These studies have considerably extended previous knowledge of the ATP-dependent Ca^{2+} -transport mechanism found in microsomal membranes from the rat exocrine parotid gland. Several lines of evidence suggest that the transporter studied here is located in a membrane fraction derived from the endoplasmic reticulum. The membranes employed are enriched ~2-fold in RNA and are separated from the other major membranous ATP-dependent Ca^{2+} -transport mechanism, that found in basolateral membranes, with the fractionation methods used (Takuma & Baum, 1985; Takuma *et al.*, 1985). Additionally, Ca^{2+} transport studied herein is unaffected by two mitochondrial inhibitors, NaN_3 and oligomycin, and is markedly enhanced when reaction mixtures included 5 mM-oxalate. Membranes from the endoplasmic reticulum, but not those of the plasma membrane, are permeable to oxalate (Moore *et al.*, 1975), and accordingly stimulate ATP-dependent Ca^{2+} accumulation.

This ATP-dependent Ca^{2+} transporter has both a very high affinity for Ca^{2+} ($K_m = 38 \text{ nM}$) and a high transport capacity ($V_{\text{max.}} \sim 30 \text{ nmol/min per mg of protein}$). Rest-

ing concentrations of free Ca^{2+} in rat parotid cells are 100–150 nM when measured with the intracellular fluorescent Ca^{2+} probes Quin 2 (Ambudkar *et al.*, 1988) or Fura (Merritt & Rink, 1987). Thus our findings suggest that the ATP-dependent Ca^{2+} transporter of rat parotid endoplasmic reticulum is likely to be substantially activated under basal conditions. Quite similar kinetic constants for an ATP-dependent Ca^{2+} -transport mechanism in rough endoplasmic reticulum, and resting intracellular Ca^{2+} concentrations, have been reported for rat pancreatic acini (Richardson & Dormer, 1984; Ochs *et al.*, 1983).

Unlike previous studies with parotid microsomal preparations, we have studied Ca^{2+} -transport activity in detail without the use of oxalate, because Ca^{2+} transport measured in oxalate media is markedly different with respect to time course and maximal accumulation. The inclusion of oxalate is clearly non-physiological, and our data suggest that oxalate modifies the effect of univalent cations on Ca^{2+} -transport activity. Previous studies have suggested that the optimal levels of ATP-dependent Ca^{2+} transport into parotid endoplasmic reticulum observed in KCl-containing reaction mixtures were decreased by 20–50 % when NaCl was substituted for KCl (Kanagasuntheram & Teo, 1982; Bonis & Rossignol, 1982). The potential physiological relevance of this differential effect of Na^+ and K^+ on the Ca^{2+} pump can be appreciated best by studying the events after neurotransmitter stimulation of parotid acinar cells. Neurotransmitter stimulation of α_1 -adrenergic and muscarinic-cholinergic receptors results in a rapid ~4–5-fold increase of parotid cytosolic Ca^{2+} (Ambudkar *et al.*, 1988; Merritt & Rink, 1987). Subsequently (within milliseconds) K^+ is lost from the parotid acinar cell via a Ca^{2+} -activated K^+ channel (Petersen & Maruyama, 1984), resulting in a hyperpolarization, and Na^+ enters the cell (Landis & Putney, 1979) primarily via a $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter (Turner *et al.*, 1986). The $[\text{Na}^+]$ within the cell is transiently quite high (~80 mM, according to Landis & Putney, 1979). Thus, if the above observations with oxalate were physiologically relevant, Na^+ would serve a regulatory function and act to inhibit partially ATP-dependent Ca^{2+} transport into the endoplasmic reticulum, resulting in a sustained increase in cytosolic Ca^{2+} . However, our results show that the Na^+ -dependent inhibition of Ca^{2+} transport can be only observed when oxalate is present. Quite similar findings have been reported with rough-endoplasmic-reticulum vesicles by Bayerdorffer *et al.* (1984). Therefore $[\text{Na}^+]$ probably cannot, under physiological conditions, regulate Ca^{2+} accumulation into the endoplasmic reticulum by modifying ATP-dependent transporter activity.

Additional observations where ATP-dependent Ca^{2+} transport is decreased when KCl (or NaCl) is replaced in reaction mixtures by a salt which contained an impermeant cation (TMACl) or by a non-ionic solute (mannitol) suggest that there may be specific ion requirements for optimal ATP-dependent Ca^{2+} -transport activity in parotid microsomal membranes. Studies examining the anion-dependence of $^{45}\text{Ca}^{2+}$ uptake in this preparation yielded results which are consistent with the ATP-dependent Ca^{2+} -uptake process requiring a charge compensation. Both the cation- and anion-dependence orders found here with rat parotid microsomal membranes are quite similar to those reported in rat pancreatic endoplasmic-reticulum membranes (Bayerdorffer *et al.*,

1984; Kemmer *et al.*, 1987b). Interestingly, in membranes from these exocrine glands, SCN^- is the anion least able to sustain $^{45}\text{Ca}^{2+}$ uptake. This might indicate that SCN^- is relatively impermeable to these membranes, or that it may have a chaotropic effect on the ATP-dependent Ca^{2+} transporter (Kemmer *et al.*, 1987b).

To gain further insight into the mechanistic nature of ATP-dependent Ca^{2+} transport in parotid microsomal membranes, we directly manipulated K^+ diffusion potential with or without valinomycin. In these experiments $^{45}\text{Ca}^{2+}$ uptake was elevated in the presence of a negative K^+ -diffusion potential and decreased with a positive potential. These findings were consistent with the ATP-dependent Ca^{2+} transporter of rat parotid endoplasmic reticulum being either an electrogenic uniport in nature, requiring permeant anions to compensate for positive charges generated by the flux of Ca^{2+} , or a $\text{Ca}^{2+}/n\text{H}^+$ exchange mechanism (Chiesi & Inesi, 1980; Ueno & Sekine, 1981; Niggli *et al.*, 1982; Smallwood *et al.*, 1983). Accordingly, we evaluated a possible effect of proton permeability on the parotid microsomal ATP-dependent Ca^{2+} transporter. Several lines of evidence suggest, but do not unequivocally demonstrate, that such involvement is unlikely, including: (1) the lipid-permeable organic acid acetate $^-$ failed to stimulate Ca^{2+} transport; (2) FCCP in the presence of a lipophilic anion (NO_3^-) did not increase Ca^{2+} transport; and (3) nigericin had no effect on Ca^{2+} transport under conditions in which it would be expected to promote K^+/H^+ exchange. Further studies, using a reconstituted transport system, are required to clarify the role of proton movements (Penniston, 1983).

We are aware of only two reports which demonstrate that an endoplasmic-reticulum-derived ATP-dependent Ca^{2+} transporter, from a non-excitabile tissue, is electrogenic [Muallem *et al.* (1985), rat liver; Kemmer *et al.* (1987b), rat pancreas]. It remains to be established what other ion-conductance pathways are necessary for optimal unidirectional Ca^{2+} uptake to occur via this ATP-dependent Ca^{2+} transporter and maintain electrical neutrality. Definitive proof of whether this Ca^{2+} transporter is electrogenic or coupled to other ions in an electroneutral way remains to be established (Kemmer *et al.* 1987b). However, it should now be recognized that the rapid intracellular changes in ionic composition which accompany neurotransmitter stimulation of parotid acinar cells may in part regulate the rate of Ca^{2+} re-accumulation into the endoplasmic reticulum, not in a Na^+ -dependent fashion, as has been previously suggested, but rather via potential changes across this membrane.

REFERENCES

- Ambudkar, I. S., Kuyatt, B. L., Roth, G. S. & Baum, B. J. (1988) *Mech. Ageing Develop.* **43**, 45–60
- Bayerdorffer, E., Streb, H., Eckhardt, L., Haase, W. & Schulz, I. (1984) *J. Membr. Biol.* **81**, 69–82
- Bayerdorffer, E., Eckhardt, L., Haase, W. & Schulz, I. (1985) *J. Membr. Biol.* **84**, 45–60
- Berridge, M. (1987) *Ann. N.Y. Acad. Sci.* **494**, 39–48
- Bonis, D. & Rossignol, B. (1982) *FEBS Lett.* **137**, 63–66
- Carafoli, E. (1987) *Annu. Rev. Biochem.* **56**, 395–433
- Chiesi, M. & Inesi, G. (1980) *Biochemistry* **19**, 2912–2918
- Chu, A., Bick, R. J., Tate, C. A., Van Winkle, W. B. & Entman, M. L. (1983) *J. Biol. Chem.* **258**, 10543–10550
- Chueh, S. H. & Gill, D. L. (1986) *J. Biol. Chem.* **261**, 13883–13886
- Enouf, J., Bredoux, R., Bourdeau, N. & Levy-Toledano, S. (1987) *J. Biol. Chem.* **262**, 9293–9297
- Helman, J., Kuyatt, B. L., Takuma, T., Seligmann, B. & Baum, B. J. (1986) *J. Biol. Chem.* **261**, 8919–8923
- Henne, V. & Söling, H.-D. (1986) *FEBS Lett.* **202**, 267–273
- Immelman, A. & Söling, H.-D. (1983) *FEBS Lett.* **162**, 406–410
- Irvine, R. F., Letcher, A. J., Lander, D. J. & Downes, C. P. (1984) *Biochem. J.* **223**, 237–243
- Kanagasuntheram, P. & Teo, T. S. (1982) *Biochem. J.* **208**, 789–794
- Kemmer, T. P., Bayerdorffer, E. & Schulz, I. (1987a) *Pflügers Arch.* **408** (Suppl.) R31
- Kemmer, T. P., Bayerdorffer, E., Will, H. & Schulz, I. (1987b) *J. Biol. Chem.* **262**, 13758–13764
- Kraus-Friedmann, N., Biber, J., Murer, H. & Carafoli, E. (1982) *Eur. J. Biochem.* **129**, 7–12
- Landis, C. A. & Putney, J. A., Jr. (1979) *J. Physiol. (London)* **297**, 369–377
- Merritt, J. E. & Rink, T. J. (1987) *J. Biol. Chem.* **262**, 4958–4960
- Moore, L., Chen, T., Knapp, H. R., Jr. & Landon, E. J. (1975) *J. Biol. Chem.* **250**, 4562–4568
- Muallem, S., Schoeffield, M., Pandol, S. & Sachs, G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4433–4437
- Niggli, V., Sigel, E. & Carafoli, E. (1982) *J. Biol. Chem.* **257**, 2350–2356
- Ochs, D. L., Korenbrot, J. I. & Williams, J. A. (1983) *Biochem. Biophys. Res. Commun.* **117**, 122–128
- Pedersen, P. L. & Carafoli, E. (1987) *Trends Biochem. Sci.* **12**, 146–150
- Penniston, J. T. (1983) in *Calcium and Cell Function* (Cheung, W. Y., ed.), vol. 4, pp. 99–149, Academic Press, New York
- Petersen, O. H. & Maruyama, Y. (1984) *Nature (London)* **307**, 693–696
- Putney, J. W., Jr., Aub, D. L., Taylor, C. W. & Merritt, J. E. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 2634–2638
- Richardson, A. E. & Dormer, R. L. (1984) *Biochem. J.* **219**, 679–685
- Selinger, Z., Naim, E. & Lasser, M. (1970) *Biochim. Biophys. Acta* **203**, 326–334
- Shamoo, A. E. & Ambudkar, I. S. (1984) *Can. J. Physiol. Pharmacol.* **62**, 9–22
- Smallwood, J., Waisman, D. M., Lafreniere, D. & Rasmussen, H. (1983) *J. Biol. Chem.* **258**, 11092–11097
- Takuma, T. & Baum, B. J. (1985) *Biochim. Biophys. Acta* **812**, 453–459
- Takuma, T., Kuyatt, B. L. & Baum, B. J. (1985) *Biochem. J.* **227**, 239–245
- Turner, R. J., George, J. N. & Baum, B. J. (1986) *J. Membr. Biol.* **94**, 143–152
- Ueno, T. & Sekine, T. (1981) *J. Biochem. (Tokyo)* **89**, 1239–1246
- Williamson, J. R., Cooper, R. H. & Hoek, J. B. (1981) *Biochim. Biophys. Acta* **639**, 243–295