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Ca^{2+} transport and Ca^{2+}-dependent ATP hydrolysis by Golgi vesicles from lactating rat mammary glands

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Ca^{2+} transport across mammary-gland Golgi membranes was measured after centrifugation of the membrane vesicles through silicone oil. In the presence of 2.3 μM free Ca^{2+} the vesicles accumulated 5.8 nmol of Ca^{2+}/mg of protein without added ATP, and this uptake was complete within 0.5 min. In the presence of 1 mM-ATP, Ca^{2+} was accumulated at a linear rate for 10 min after the precipitation of intravesicular Ca^{2+} with 10 mM-potassium oxalate. ATP-dependent Ca^{2+} uptake exhibited a K_m of 0.14 μM for Ca^{2+} and a $V_{max.}$ of 3.1 nmol of Ca^{2+}/min per mg of protein. Ca^{2+}-dependent ATP hydrolysis exhibited a K_m of 0.16 μM for Ca^{2+} and a $V_{max.}$ of 10.1 nmol of P_i/min per mg of protein. The stoichiometry between ATP-dependent Ca^{2+} uptake and Ca^{2+}-stimulated ATPase varied between 0.3 and 0.7 over the range 0.03–8.6 μM-Ca^{2+}. Both Ca^{2+} uptake and Ca^{2+}-stimulated ATPase were strongly inhibited by orthovanadate, which suggests that the major mechanism by which Golgi vesicles accumulate Ca^{2+} is through the action of the Ca^{2+}-stimulated ATPase. However, Ca^{2+} uptake was also decreased by the protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), indicating that it may occur by other mechanisms too. The effect of CCCP may be related to the existence of transmembrane pH gradients (ΔpH) in these vesicles: the addition of 30 μM-CCCP reduced ΔpH from a control value of 1.06 to 0.73 pH unit. Golgi vesicles also possess a Ca^{2+}-efflux pathway which operated at an initial rate of 0.5–0.57 nmol/min per mg of protein.

Golgi vesicles, isolated from lactating rat (West, 1981) and bovine (Baumrucker & Keenan, 1975) mammary glands, have been shown to accumulate Ca^{2+}. Two major consequences of this transport activity have been postulated. Firstly, Golgi apparatus may be important in concentrating Ca^{2+} into milk *in vivo* (Baumrucker, 1978; Holt, 1981; West, 1981). Secondly, accumulated Ca^{2+} may stimulate intravesicular lactose synthesis (Powell & Brew, 1976; Kuhn & White, 1977).

Despite the proposed importance of Ca^{2+} uptake by Golgi vesicles, certain aspects of this process remain poorly characterized. For example, the transport of 1–2 mol of Ca^{2+} across various membranes from other tissues is usually associated with the hydrolysis of 1 mol of ATP by the Ca^{2+}-stimulated ATPase enzyme (EC 3.6.1.3) (Hodson, 1978; de Meis & Vianna, 1979; Dawson & Fulton, 1983). However, mammary-gland Golgi vesicles were reported to exhibit a Ca^{2+}:ATP stoichiometry of around 0.02 (Baumrucker, 1978; West, 1981). Another puzzling aspect of these studies concerns the [Mg^{2+}]- and [Ca^{2+}]-dependence of ATP hydrolysis and Ca^{2+} uptake. West's group have not detected Ca^{2+}-dependent ATP hydrolysis in the presence of concentrations of both Mg^{2+} (1–5 mM) and Ca^{2+} (0.1–0.3 μM) similar to those found in the cytosol. Ca^{2+} was shown to stimulate ATPase activity only in the presence of 3 μM-Mg^{2+} (Easom & West, 1982; Easom *et al.*, 1982), or when 10 μM free Ca^{2+} was added with 5 mM-MgATP (West, 1981). Baumrucker & Keenan (1975) measured Ca^{2+} transport in the presence of 0.5 mM-Ca^{2+}. It is clear that an acceptance of Ca^{2+}-stimulated ATPase as a physiological mechanism

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ΔpH, transmembrane pH gradient; Ca^{2+} and Ca represent ionized and total calcium respectively.

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for Ca^{2+} transport must depend on such processes being observed in the presence of physiological cation concentrations.

We have re-investigated the relationship between ATP hydrolysis and Ca^{2+} uptake by rat mammary-gland Golgi vesicles. In doing so, we took note of a further observation made by West (1981). He found that protonophores inhibited Ca^{2+} uptake by up to 40%, and suggested that transmembrane pH gradients (ΔpH) could be involved in the mechanism of Ca^{2+} accumulation. This proposal is particularly intriguing in the light of a report that liver Golgi vesicles possess a ΔpH of 0.98 pH units (Zhang & Schneider, 1983). We have therefore performed experiments to determine if pH gradients exist across mammary-gland Golgi-vesicle membranes.

Zhang & Schneider (1983) assessed ΔpH by measuring the transmembrane distribution of methylamine, after rapidly separating Golgi vesicles from their incubation medium by gel filtration. We noted that the hyperosmotic media generally employed to isolate mammary-gland Golgi vesicles (Kuhn & White, 1977; West, 1981) would be expected to increase their density (see Pertoft *et al.*, 1978). We took advantage of this phenomenon and separated our vesicles from the incubation medium without any apparent loss of vesicle integrity by brief centrifugation through silicone oil. We also used this centrifugation technique as a means of measuring transmembrane Ca^{2+} transport.

Materials and methods

Preparation of Golgi vesicles

Female rats of a Wistar-derived strain, weighing between 280 and 350 g, were killed between days 12 and 16 of lactation after the birth of their first litters. Only those rats with litters of at least eight pups were used. Golgi vesicles were prepared from mammary tissue as described by Kuhn & White (1977), except that the medium employed for the final wash and resuspension was 225 mM-lactose plus 100 mM-Tes/KOH, pH 7.0.

Measurement of Ca^{2+} transport in Golgi vesicles

Vesicles (0.4–1 mg of protein/ml) were usually incubated at 37°C with 85 mM-lactose, 38 mM-Tes/KOH, pH 7.0, 50 mM-KCl, 3 mM-$MgCl_2$, 1 mM-ATP, 1 mM-EGTA, 5 mM-phosphocreatine and 3 units of creatine kinase/ml. Preliminary studies (results not shown) indicated that the initial rate of Ca^{2+} uptake could not be increased by altering the concentrations of Mg^{2+} and ATP. Where indicated, 10 mM-potassium oxalate was also present. To obtain the required concentrations of free Ca^{2+}, $^{45}CaCl_2$ (0.5 Ci/mol) was added at various concentrations, as calculated by the method of Storer & Cornish-Bowden (1976). Apparent stability constants were calculated for a pH of 7.0 and a temperature of 37°C, by the method of Burgess *et al.* (1983).

At appropriate times, 0.45 ml samples of the incubation medium were layered on 0.4 ml of silicone oil [AP100/AR20 (1:1, v/v); final sp.gr. 1.04; Wacker Chemical Co., Walton-upon-Thames, Surrey, U.K.], which itself rested above 0.1 ml of 2 M-$HClO_4$ in 1.5 ml plastic centrifuge tubes. These samples were centrifuged at 11600 *g* for 1 min in a MSE Microcentaur centrifuge. We estimated Ca^{2+} uptake in terms of that proportion of the protein added to the incubations which sedimented through the oil (80%; results not shown).

After centrifugation, 20 μl samples were taken from the supernatant above the oil in order to assess the specific radioactivity of Ca in the incubation medium. A micro-syringe was then inserted through the oil and 50 μl samples were removed from the $HClO_4$ layer. These samples were adjusted to 10 ml with xylene/Triton X-100/water (6:3:1, by vol.) containing 0.6% (w/v) diphenyloxazole and 0.015% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene. Radioactivity was measured in a liquid-scintillation spectrometer.

Ca^{2+} in the $HClO_4$ will be derived from several pools. These include the vesicle interior, Ca^{2+} in the extravesicular fluid which adheres to the membranes during centrifugation and non-transported Ca which binds to the external face of the vesicles. A minor proportion of Ca^{2+} uptake is independent of ATP (West, 1981; and see the Results section). Unless otherwise stated, our experiments measured ATP-dependent Ca^{2+} uptake into the vesicle interior. This was distinguished from both non-transported Ca and ATP-independent uptake in parallel incubations from which ATP was omitted.

In some experiments, Ca^{2+} uptake was also measured by membrane filtration. Here, cellulose nitrate filters (0.45 μm pore size, 2.5 cm diameter) were soaked for 1 h in ice-cold 'washing medium' containing 85 mM-lactose, 50 mM-KCl, 2 mM-EGTA, 38 mM-Tes/KOH, pH 7.0, before being placed on a sintered-glass filtration apparatus. A sample (0.4 ml) of the incubation was placed on each filter, and the suspending medium was removed with a Speedivac vacuum pump (Edwards High Vacuum, Crawley, Sussex, U.K.). The vesicles retained on the filters were washed twice with 5 ml of ice-cold 'washing medium'. These procedures took 25s to complete. Radioactivity trapped on the filters was measured after dissolving them in 10 ml of scintillation fluid (see above).

Enzyme assays

Galactosyltransferase (EC 2.4.1.22) was assayed as described by Kuhn & White (1977). β-Galactosidase (EC 3.2.1.23) was assayed by the method of Heyworth *et al.* (1981). Glutamate dehydrogenase (EC 1.4.1.2) was assayed as described by Shears & Kirk (1984). 5′-Nucleotidase (EC 3.1.3.5) was assayed as described by Michell & Hawthorne (1965). Arylesterase (EC 3.1.1.2) was assayed as indicated by Shephard & Hübscher (1969).

Mg^{2+}-stimulated ATPase activity was measured at 37°C with 0.1–0.2mg of Golgi protein in 0.5ml of 85mM-lactose, 38mM-Tes/KOH, pH7.0, 50mM-KCl, 3mM-$MgCl_2$, 2mM-EGTA, 10mM-potassium oxalate and 2mM-[γ-^{32}P]ATP (0.5–1Ci/mol). Ca^{2+} + Mg^{2+}-stimulated ATPase activity was measured in parallel incubations which included $CaCl_2$ to give the required free concentrations of Ca^{2+} as described above. Ca^{2+}-stimulated ATPase activity was calculated by difference. Reactions (in triplicate) were terminated after 12min by the addition of 0.6ml of 2% (v/v) $HClO_4$, containing 50mM-NaH_2PO_4 and 2.5% (w/v) activated charcoal (Norit GSX; Hopkin and Williams, Chadwell Heath, Essex, U.K.) to adsorb the ATP (Bais, 1975). After centrifugation for 2min at 11600*g*, 0.85ml portions of the supernatants were made up to 10ml with water, and [^{32}P]P_i release was estimated from the Čerenkov radiation in a liquid-scintillation spectrometer.

Protein was assayed by the method of Peterson (1977), with bovine serum albumin as a standard.

Estimation of transmembrane pH gradients

Incubations were performed as described for the estimation of Ca^{2+} uptake, except that $^{45}Ca^{2+}$ was replaced by 30μM-[^{14}C]methylamine (0.08μCi/ml), and oligomycin (2μg/ml) plus NaN_3 (5mM) were added. After 5min, 0.45ml samples were taken and the vesicles were centrifuged through silicone oil. After assay of ^{14}C above and below the oil (see above), ΔpH was calculated (Nicholls, 1974). Since methylamine is accumulated into an acidic environment, a positive value for ΔpH describes the extent to which the intravesicular pH is lower than that of the medium. The intra- and extra-vesicular volumes necessary for the calculations were estimated in parallel incubations containing non-radioactive methylamine plus 10μCi of ^3H_2O/ml and 1μCi of [U-^{14}C]sucrose/ml. The latter does not penetrate intact Golgi membranes (White *et al.*, 1980). Intravesicular and extravesicular volumes were calculated to be 1.97 ± 0.19 and 13.78 ± 1.4μl/mg of protein respectively ($n = 8$). The values of these parameters did not vary significantly during the course of the experiments. In similar experiments with mitochondria, intravesicular volume was greater than the extravesicular space (Werkheiser & Bartley, 1957). However, Golgi vesicles are smaller and less spherical in shape than isolated mitochondria (e.g. Keenan *et al.*, 1970). Both of these factors would tend to increase the ratio of extravesicular to intravesicular space.

Materials

All chemicals were of the highest purity available. The following were purchased from Sigma Chemical Co., Poole, Dorset, U.K.: A23187, ADP, bovine serum albumin, CCCP, EGTA, oligomycin, all enzymes and enzyme substrates. Stock solutions of CCCP (in ethanol) were made immediately before use. Cellulose nitrate filters were obtained from Sartorius, Belmont, Surrey, U.K. All radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Triton X-100 and scintillants were supplied by BDH, Poole, Dorset, U.K. Orthovanadate was generously given by Dr P. Mahi, Department of Minerals Engineering, University of Birmingham.

Results

Enzyme activities in preparations of Golgi vesicles

Galactosyltransferase was employed as an enzyme marker for the Golgi apparatus of rat mammary gland (Keenan *et al.*, 1970). Table 1 indicates that, compared with mammary-gland

Table 1. *Specific activities and recoveries of various enzymes in Golgi vesicles prepared from lactating rat mammary glands*
Results are means ± S.E.M. for the numbers of preparations in parentheses.

Enzyme	Specific activity (nmol/min per mg of protein)		
	Mammary-gland homogenate (H)	Golgi vesicles (G)	Relative specific activity (G/H)
Galactosyltransferase	5.9 ± 0.9 (7)	207.1 ± 21.6 (7)	35.1
Glutamate dehydrogenase	26.0 ± 6.4 (7)	17.0 ± 4.7 (7)	0.7
Arylesterase	42.7 ± 5.3 (3)	68.5 ± 1.2 (3)	1.6
5′-Nucleotidase	13.5 ± 1.0 (3)	10.2 ± 3.1 (3)	0.8
β-Galactosidase	0.9 ± 0.1 (4)	1.1 ± 0.2 (4)	1.2

homogenate, our preparations of Golgi vesicles were enriched 35-fold in galactosyltransferase activity. In contrast, there was no appreciable enrichment of marker enzymes for other subcellular fractions which carry out transmembrane Ca^{2+} transport and ATP hydrolysis (e.g. plasma membranes, mitochondria and endoplasmic reticulum). Table 1 also shows that our vesicle preparations were not substantially enriched in lysosomes, which are known to accumulate methylamine (Hoek *et al.*, 1980), a base which we used to measure ΔpH in Golgi vesicles (see below).

Time course of Ca^{2+} uptake by Golgi vesicles

Golgi vesicles were incubated as described in the Materials and methods section, with 1 mM-EGTA and 0.9 mM-$CaCl_2$, to give a free Ca^{2+} concentration of 2.3 μM. In the absence of ATP, and after correction for Ca in the extravesicular space (see the Materials and methods section), Golgi vesicles accumulated 5.8 ± 0.06 nmol of Ca^{2+}/mg of protein ($n = 8$). This uptake was faster than the resolution time of the assay, and was complete within 0.5 min. Potassium oxalate (10 mM) did not affect the extent of this ATP-independent uptake (results not shown).

In determining ATP-dependent Ca^{2+} uptake we subtracted the value for Ca^{2+} accumulation in the absence of ATP from that measured in its presence. This calculation also corrects for Ca bound to the outer face of the vesicular membranes (see the Materials and methods section), assuming that ATP does not itself affect Ca binding. This was verified by removing bound Ca by exposing the vesicles to 6 mM-EGTA for 0.5 min. This treatment decreased vesicular Ca by 3.7 ± 0.4 and 3.9 ± 0.3 nmol/mg of protein in the presence and absence of ATP respectively ($n = 3$).

The initial rate of ATP-dependent Ca^{2+} accumulation (Fig. 1) was 3.2 nmol/min per mg of protein. In the absence of oxalate, ATP-dependent Ca^{2+} uptake gradually diminished until, by 7 min, there was little further change in vesicle Ca content with time. In vesicles incubated with 10 mM-oxalate, the initial rate of Ca^{2+} uptake was linear for nearly 10 min. It is clear that precipitation of intravesicular Ca^{2+} by oxalate permits a more prolonged phase of ATP-dependent Ca^{2+} uptake.

In three further preparations of vesicles, we measured ATP-dependent oxalate-supported Ca^{2+} uptake, using both silicone-oil centrifugation and membrane filtration. Incubations were carried out as described in the legend to Fig. 1. Net Ca^{2+} uptake was 3.17 ± 0.46 and 3.22 ± 0.25 nmol/min per mg of protein (by centrifugation and filtration respectively). It is clear that the two techniques gave similar results.

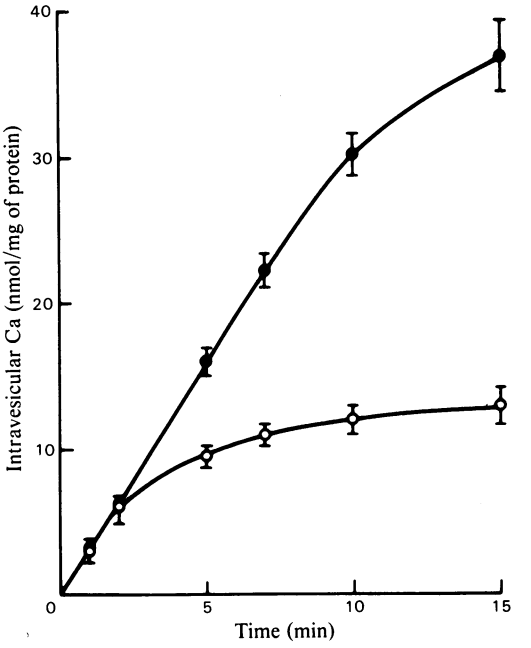

Fig. 1. *Time course of ATP-dependent Ca^{2+} uptake by Golgi vesicles from lactating rat mammary glands*
Vesicles were incubated and Ca^{2+} uptake was measured as described in the Materials and methods section, with 1 mM-ATP. Incubations were performed in the absence (○) or presence (●) of 10 mM-potassium oxalate. [EGTA] was 1 mM, and [$CaCl_2$] was 0.9 mM (2.3 μM free Ca^{2+}). Results are means ± S.E.M. for three preparations of Golgi vesicles.

Ca^{2+} efflux from Golgi vesicles

The decrease in the rate of vesicular Ca^{2+} uptake with time (Fig. 1) could be due to a diminution of influx, or an increase in the activity of a Ca^{2+} efflux pathway. We investigated these alternatives by rapidly depleting residual ATP in our incubations, with the addition of hexokinase, after the vesicles had accumulated Ca^{2+} in the presence of glucose for 15 min. Ca^{2+} uptake, which at this time is dependent on ATP, was thus inhibited. We observed that Ca^{2+} was lost from the vesicles at a linear rate of 0.5 nmol/min per mg of protein (Fig. 2*a*). In separate experiments we noted that the decrease of extravesicular [Ca^{2+}], from 2.0 to 0.02 μM by the addition of 4.6 mM-EGTA, induced a linear efflux of 0.57 nmol of Ca^{2+}/min per mg of protein (Fig. 2*b*).

Dependence on extravesicular [Ca^{2+}] of ATP-dependent Ca^{2+} uptake and Ca^{2+}-dependent ATPase

When extravesicular [Ca^{2+}] was varied with Ca^{2+}-EGTA buffers, the rate of ATP-dependent Ca^{2+} uptake was maximal above 1 μM-Ca^{2+} (Fig.

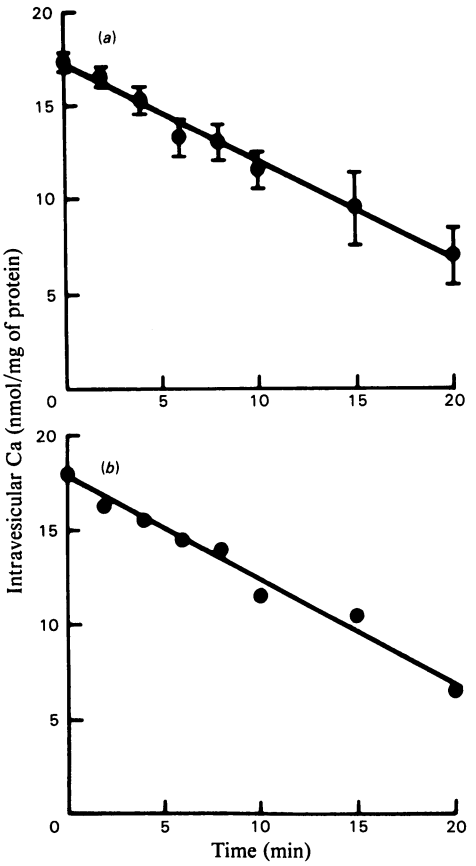

Fig. 2. *Ca^{2+} efflux from Golgi vesicles*
Vesicles (6mg of protein) were incubated at 37°C in 5ml of medium containing 85mM-lactose, 38mM-Tes/KOH, pH7.0, 50mM-KCl, 5mM-glucose, 3mM-$MgCl_2$, 2mM-ATP, 0.5mM-EGTA, 0.45mM-$^{45}CaCl_2$ (2.0μM free Ca^{2+}, 0.5Ci/mol). After 15min (zero time in the Figures) either (*a*) residual ATP was removed by the addition of 0.025ml of hexokinase suspension (final concn. 12units/ml) or (*b*) $[Ca^{2+}]$ was decreased to 0.02μM by addition of 0.8ml of 30mM-EGTA in 85mM-lactose (pH7.0). Intravesicular [Ca] was then measured as described in the Materials and methods section. Data in (*a*) and (*b*) were obtained from five and two vesicle preparations respectively, and vertical bars represent S.E.M.

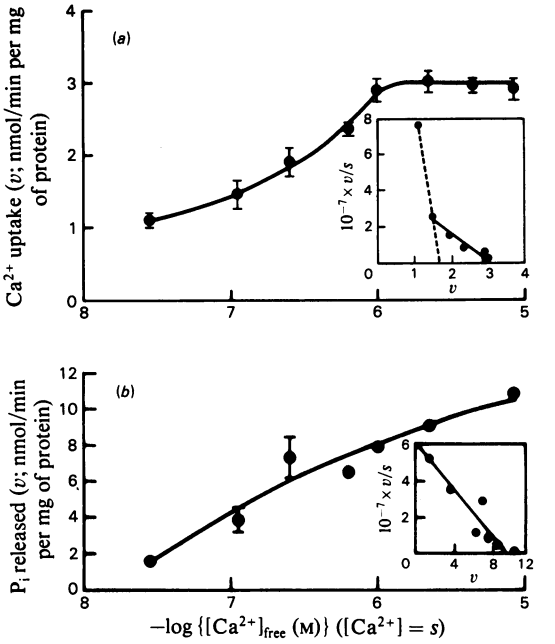

Fig. 3. *Dependence on $[Ca^{2+}]$ of ATP-dependent Ca^{2+} uptake and Ca^{2+}-dependent ATP hydrolysis*
Vesicles were incubated and assays were performed as described in the Materials and methods section. $[Ca^{2+}]$ was varied with Ca^{2+}-EGTA buffers. (*a*) ATP-dependent Ca^{2+} uptake was measured after 10min with 10mM-potassium oxalate present ($n = 3$); (*b*) Ca^{2+}-stimulated ATPase was measured after 12min ($n = 2$, except for the determinations at 0.11μM- and 0.26μM-Ca^{2+}, when $n = 5$; $P < 0.01$, by a paired t test, comparing incubations with and without Ca^{2+}). Vertical bars denote S.E.M. The inset in each Figure is an Eadie–Hofstee plot of the data.

3*a*). An Eadie–Hofstee plot of these data suggests that this process has a K_m of 0.14μM for Ca^{2+} and a $V_{max.}$ of 3.1nmol of Ca^{2+}/min per mg of protein. There was also an indication that a high-affinity uptake mechanism may exist (broken line, Fig. 3*a*), but we have insufficient data to clarify this possibility. In any case, since the putative K_m of such a mechanism (0.01μM) is well below cytosolic $[Ca^{2+}]$, it would not appear to be physiologically significant.

The rate of hydrolysis of ATP by Golgi vesicles varied substantially between preparations. In the absence of added Ca^{2+}, the Mg^{2+}-dependent ATPase released between 43.9 and 115.2nmol of P_i/min per mg of protein ($n = 12$; mean $\pm$S.E.M. $= 83.9 \pm 6.8$). Nevertheless, hydrolysis of 2mM-ATP was consistently stimulated by Ca^{2+} (Fig. 3*b*). This effect was significant ($P < 0.01$) at both 0.11μM- and 0.26μM-$[Ca^{2+}]$. These data represent the first demonstration of Ca^{2+}-dependent ATP hydrolysis by rat mammary-gland Golgi vesicles incubated with physiologically relevant concentrations of Ca^{2+}, Mg^{2+} and ATP. An Eadie–Hofstee plot (Fig. 3*b*) suggests that the Ca-dependent ATPase has a K_m of 0.16μM for Ca^{2+} and a $V_{max.}$ of 10.1nmol of P_i released/min per mg of protein.

The stoichiometry between ATP-dependent Ca^{2+} uptake and Ca^{2+}-stimulated ATPase activity was between 0.3 and 0.7mol of Ca^{2+} transported/

Table 2. *Effects of various inhibitors on ATP-dependent Ca^{2+} uptake, Mg^{2+}-dependent ATPase, Ca^{2+}-dependent ATPase and ΔpH in mammary-gland Golgi vesicles*

All incubations, carried out as described in the Materials and methods section, contained 1mM-EGTA, 0.9mM-$CaCl_2$ (2.3μM free Ca^{2+}), 10mM-potassium oxalate and 0.1% (v/v) ethanol. Other additions are listed in the Table. Data are means ± S.E.M. for the numbers of preparations in parentheses. * $P < 0.001$ (paired t test), ** $P < 0.01$ (unpaired t test), *** $P < 0.001$ (unpaired t test), all compared with controls.

	Ca^{2+} uptake (nmol/min per mg of protein)	Mg^{2+}-dependent ATPase (nmol of P_i released/min per mg of protein)	Ca^{2+}-dependent ATPase (nmol of P_i released/min per mg of protein)	ΔpH (pH units)
Control	3.40 ± 0.20 (6)	79.6 ± 5.7 (6)	9.5 ± 1.5 (5)	1.06 ± 0.19 (6)
CCCP (30μM)	2.50 ± 0.11 (3)**	80.4 (2)	9.8 (2)	0.73 ± 0.18 (6)*
Orthovanadate (0.1mM)	0.61 ± 0.14 (4)***	73.2 ± 5.4 (4)	−0.1 (2)	1.26 ± 0.17 (4)
Oligomycin (2μg/ml) plus 5mM-NaN_3	3.20 ± 0.27 (3)	62.6 ± 6.4 (3)	7.4 ± 1.6 (3)	

mol of ATP hydrolysed at external [Ca^{2+}] of 0.03–8.6μM. This is quite similar to the stoichiometry observed with other membrane-bound Ca^{2+}-stimulated ATPases (1–2: Hodson, 1978; de Meis & Vianna, 1979; Dawson & Fulton, 1983).

Effects of various inhibitors on Golgi-vesicle Ca^{2+} uptake, ATP hydrolysis and transmembrane pH gradients

West (1981) made the interesting observation that protonophores inhibit net Ca^{2+} uptake by Golgi vesicles, and he therefore suggested that transmembrane H^+ movements might be associated with Ca^{2+} accumulation. In investigating this putative involvement of H^+, we first confirmed that protonophores inhibited ATP-dependent Ca^{2+} uptake (by 26% in our experiments; Table 2). However, this effect was not associated with any change in the Ca^{2+}-stimulated ATPase activity (Table 2). We considered that mitochondria, which slightly contaminate our preparations (Table 1), might have contributed to the effect of CCCP on Ca^{2+} transport. However, we excluded this possibility by observing that oligomycin plus azide, which would inhibit mitochondrial Ca^{2+} transport, did not significantly affect ATP-dependent Ca^{2+} uptake by our vesicle preparations (Table 2).

The observation that CCCP partially inhibited ATP-dependent Ca^{2+} uptake, but not Ca^{2+}-stimulated ATPase, led us to investigate the effect of orthovanadate on these processes. Orthovanadate is a well-known inhibitor of Ca^{2+}-stimulated ATPase in various cell compartments (e.g. Chan & Junger, 1983) and we observed 100% inhibition in our experiments (Table 2). Orthovanadate inhibited ATP-dependent Ca^{2+} uptake by 80% (Table 2). The effects of orthovanadate and CCCP suggest that around 75–80% of ATP-dependent Ca^{2+} uptake is closely coupled to Ca^{2+}-stimulated ATP hydrolysis.

The inhibition of Ca^{2+} transport by CCCP may be correlated with an effect of the protonophore on transmembrane ΔpH. Vesicles in control incubations retained a pH gradient (1.06pH units, acid interior), which was significantly decreased by CCCP (Table 2). Orthovanadate did not significantly influence ΔpH (Table 2). It should be emphasized that we did not investigate the effects of CCCP at concentrations above 30μM, since, owing to the protonophore's limited solubility, it was necessary to add the compound as an ethanolic solution. Although 0.1% (v/v) ethanol did not significantly affect Ca^{2+} transport or Ca^{2+}-stimulated ATPase, higher concentrations did influence Golgi function (1% ethanol decreased ATP-dependent Ca^{2+} transport by 46%; results not shown).

Discussion

We have measured Ca^{2+} uptake into mammary-gland Golgi vesicles by centrifuging them through silicone oil. This separation procedure is facilitated by the increase in vesicle density which occurs during their preparation (see the introduction). As far as we know, Ca^{2+} uptake has not previously been measured in Golgi vesicles by this technique. The maximum velocity of ATP-dependent Ca^{2+} uptake in our incubations with 2.3μM-Ca^{2+} was 3.2nmol/min per mg of protein, and this rate was sustained for 10min (Figs. 1 and 3). This Ca^{2+} uptake exceeds that detected in previous work with mammary-gland Golgi vesicles incubated with higher concentrations of Ca^{2+} [7nmol of Ca^{2+}/mg of protein, during a 50min incubation with 5μM-Ca^{2+} (West, 1981), and 1.8nmol of Ca^{2+}/min per mg of protein in the presence of 0.5mM-Ca^{2+}

(Baumrucker & Keenan, 1975)]. These workers measured Ca^{2+} uptake by membrane filtration, but in our hands and in the presence of 2.3 μM-Ca^{2+}, this technique gave similar rates of ion transport to those observed by using silicone-oil centrifugation (see the Results section).

There was no significant vesicle lysis during our assays, as indicated by the linear rates of ATP-dependent Ca^{2+} uptake observed for nearly 10 min in the presence of oxalate (Fig. 1). Moreover, intravesicular volume, measured by membrane resistance to sucrose penetration (see the Materials and methods section), was constant throughout our incubations (results not shown). West (1981) assessed intravesicular volume with a centrifugation method (Casey *et al.*, 1977); this determination (2 μl/mg of protein) is almost identical with ours (1.97 μl/mg of protein).

Ca^{2+} uptake, under physiologically relevant conditions, was largely associated with Ca^{2+}-stimulated ATPase. Evidence in support of this association arises from several considerations. Firstly, Ca^{2+} uptake was largely dependent on ATP. Secondly, ATP-dependent Ca^{2+} uptake exhibited an affinity for Ca^{2+} (K_m 0.14 μM; Fig. 3*a*), which was similar to that seen for Ca^{2+}-stimulated ATPase (K_m 0.16 μM; Fig. 3*b*). These results are consistent with both activities being associated with the same enzyme. Thirdly, the ratio (mol of Ca^{2+} transported/min per mg of protein):(mol of ATP hydrolysed/min per mg of protein) was 0.3–0.7 (cf. Figs. 3*a* and 3*b*). This stoichiometry is much closer to that characteristic of other Ca^{2+}-transporting ATPases (1–2: Hodson, 1978; de Meis & Vianna, 1979; Dawson & Fulton, 1983) than the value of about 0.02 obtained in previous studies with mammary-gland Golgi vesicles prepared from the cow (Baumrucker, 1978) or the rat (West, 1981). These last studies did not employ physiological concentrations of Ca^{2+} and Mg^{2+} during the analysis of both Ca^{2+}-stimulated ATPase and Ca^{2+} uptake (see the introduction). We have also demonstrated that both Ca^{2+} uptake and Ca^{2+}-stimulated ATPase activity were greatly inhibited by orthovanadate (Table 2). However, 20% of ATP-dependent Ca^{2+} uptake remained insensitive to orthovanadate when Ca^{2+}-stimulated ATPase activity was completely eliminated (Table 2). Thus a proportion of the Ca^{2+} uptake may not be associated with Ca^{2+}-stimulated ATPase activity.

The protonophore CCCP, added to a concentration of 30 μM, inhibited ATP-dependent Ca^{2+} uptake by 26% (Table 2). This effect is similar to that observed by West (1981) with 0.1 mM-2,4-dinitrophenol (17% inhibition) or 0.25 mM-FCCP (40% inhibition). In the present study we used CCCP, which has a similar potency to FCCP (Heytler *et al.*, 1962), because the latter compound is no longer readily available. We did not add such high uncoupler concentrations as were used by West (1981) because the solvent (ethanol) would have inhibited Ca^{2+} uptake (see the Results section). The perturbation of Ca^{2+} transport by protonophores may be related to their effect on the transmembrane ΔpH, which we have discovered to exist across mammary-gland Golgi membranes (Table 2). The intravesicular space is slightly acid in relation to the extravesicular milieu, and it is possible that intravesicular Ca^{2+} binding may be optimized by this acidic environment. Hence the inhibition of Ca^{2+} uptake by CCCP may be due to a change in intravesicular Ca^{2+}-binding affinity. The size of the pH gradient across mammary-gland Golgi membranes (1.06 pH units; Table 2) is similar to that observed with rat liver Golgi vesicles (0.98 pH unit; Zhang & Schneider, 1983). We have not investigated how the pH gradient is formed, but it could arise from a Donnan distribution of H^+ or an ATP-driven H^+ pump. The former phenomenon presumably accounts for the persistence of ΔpH in the presence of CCCP (Table 2). However, it is still possible that, in the absence of protonophore, ΔpH is maintained by an ATP-driven H^+ pump operating across a membrane otherwise impermeable to H^+. It should also be noted that the small proportion of intravesicular Ca retained in the presence of the ionophore A23187 [West (1981); confirmed in the present study, but results not shown] could result from an equilibration of Ca^{2+} with the square of the pH gradient (Heaton & Nicholls, 1976).

In addition to the various mechanisms by which Ca^{2+} may be accumulated into Golgi vesicles, there is also a pathway for Ca^{2+} efflux (West, 1981). Our data do not indicate whether Ca^{2+} efflux occurs by non-specific diffusion, or through a specific carrier. We found efflux to occur at a linear rate for up to 20 min (Fig. 2), but, in contrast, West (1981) observed non-linear kinetics after the addition of EGTA. The reason for this discrepancy is unclear. A linear rate of Ca^{2+} efflux suggests that either efflux is independent of intravesicular [Ca^{2+}], or internal Ca^{2+}-binding sites buffer intravesicular [Ca^{2+}] to a constant concentration during these experiments. The rate of Ca^{2+} efflux was also very similar at 2.0 and 0.02 μM extravesicular [Ca^{2+}] (Fig. 2). The physiological role of this efflux pathway is unclear, but, since it apparently operates concomitantly with uptake and independently of extra- and intra-vesicular [Ca], our measurements of net Ca^{2+} uptake may underestimate total Ca^{2+} influx by up to 0.57 nmol/min per mg of protein.

Our observation that Golgi vesicles can retain transmembrane concentration gradients of H^+ and

Ca^{2+} is interesting in relation to other reports which indicate that Golgi vesicles have membrane pores which admit charged and uncharged monosaccharides up to an M_r of 300 (White *et al.*, 1980; 1984). It may transpire that Golgi vesicles fall into at least two distinct populations, with separate sugar- and ion-transport activities. In any case, our data are consistent with the hypothesis (Baumrucker, 1978; West, 1981) that Golgi vesicles have the capacity to contribute to the concentration of Ca^{2+} into milk.

Note added in proof (received 21 January 1985)

Since this paper was accepted for publication, Watters (1984) has reported studies on a Ca^{2+}-stimulated ATPase (K_m 0.8 μM-Ca^{2+}) in mammary Golgi vesicles prepared from the lactating mouse. In contrast with our study, Watters (1984) observed Ca^{2+}-stimulated ATPase activity only in the presence of sub-physiological concentrations of Mg^{2+} and ATP; in addition, he demonstrated that Ca^{2+}-stimulated ATPase activity was not influenced by the addition of calmodulin. This last finding is consistent with our unpublished results, which indicate that Ca^{2+} uptake into rat mammary Golgi vesicles was not influenced by: (*a*) 100nM-R24571 (a specific calmodulin antagonist; van Belle, 1981); (*b*) twice pre-washing the Golgi membranes with 5mM-EGTA to remove endogenous calmodulin; or (*c*) pre-washing our membranes as in (*b*), and then adding 5 μg of bovine brain calmodulin/ml.

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