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ENERGY-DEPENDENT CALCIUM SEQUESTRATION ACTIVITY IN A GOLGI APPARATUS FRACTION DERIVED FROM LACTATING RAT MAMMARY GLANDS

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Summary

A fraction isolated from lactating rat mammary glands was shown by marker enzyme assays to be rich in Golgi apparatus vesicles. This Golgi apparatus-rich fraction was shown to accumulate calcium in the presence of ATP but not in its absence. Other nucleoside triphosphates were only partially effective in promoting calcium transport. Mg^{2+} was required for the uptake which was also temperature and pH dependent. The uptake was sustained by the use of oxalate and phosphate as intravesicular trapping agents. In the presence of 10 mM oxalate the apparent K_m for calcium uptake was 0.24 μM ionized calcium. The V was 4.45 nmol calcium/min per mg protein. Preloaded calcium could be rapidly released by the addition of the ionophore A23187 indicating an intravesicular location for the sequestered ion. Addition of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid resulted in a slower release of preaccumulated calcium, indicating the existence of one or more efflux routes by which calcium leaves the vesicles in the presence of MgATP. Ruthenium red partially inhibited the uptake but lanthanum and particularly the sulphydryl inhibitor *p*-hydroxymercuribenzoate were much more effective. The properties of the calcium-sequestering system in the Golgi apparatus-rich fraction were similar to those reported for other non-muscular tissues and lend support to the hypothesis that calcium is secreted into milk via the Golgi apparatus of the mammary gland secretory cell.

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone; PCMB, sodium *p*-hydroxymercuribenzoate.

Introduction

During lactation the mammary gland secretory cell transports calcium into milk. Although the mechanism of this process is unknown, calcium is accumulated from blood where its concentration is 2—3 mM and secreted into milk where its concentration can be greater than 100 mM [1]. If the concentration of calcium in the cytoplasm of the mammary secretory cell is assumed to be of the same order as that measured in the cytoplasm of other cells, i.e. 10^{-7}—10^{-6} M [2], then, this implies the presence of an efficient system for accumulating calcium in an extra cytosolic subcellular compartment of the lactating mammary gland secretory cell.

Studies by electron microscopy [3] and radioactive labelling techniques [4,5] have suggested that the ion is secreted into milk in vesicles derived from the Golgi apparatus of the cell and Baumrucker and Keenan [6] have demonstrated that various subcellular fractions isolated from lactating bovine mammary glands, including a Golgi apparatus-rich fraction, had the ability to accumulate calcium against a concentration gradient. They, however, concentrated on the Ca^{2+}-ATPase activity of their fractions and did not further characterize the calcium-sequestering ability of the various subcellular organelles. Over the last 20 years research has shown that active transport of Ca^{2+} across biological membranes is a widespread phenomenon. An energy-dependent calcium-sequestering activity has been extensively characterized in microsomal membranes derived from the sarcoplasmic reticulum of skeletal muscle [7,8]. Similarly, ATP-dependent accumulation of calcium is shown by mitochondria [9] and at quantitatively much lower levels in membrane preparations from a variety of other tissues [10—15]. In all these systems the efficiency of calcium transport across the membrane is such that the intravesicular concentration of calcium can be maintained many times higher than in the extra vesicular fluid.

The present paper reports the results of an investigation into the accumulation of calcium by a Golgi apparatus fraction isolated from lactating rat mammary glands. This investigation was undertaken to determine how the characteristics of this Ca^{2+}-translocating system compared with those of the well-characterized systems mentioned above. In particular, evidence was sought to show that the properties of the system were compatible with the postulated role of the Golgi apparatus in the transport of Ca^{2+} into milk.

Experimental procedures

Preparation of Golgi vesicles. The rats were females of the Wistar strain undergoing their first lactation and weighed between 280 and 350 g. They were killed between the 10th and 14th days of lactation and their abdominal mammary glands were rapidly excised and weighed. The glands (9—12 g) were washed with an ice-cold solution (solution A) comprising 0.5 M sucrose, 5 mM $MgCl_2$, 1% dextran, 37.5 mM Tris/maleate buffer, pH 6.5, and then cut into small segments using the razor blade apparatus described by Greenbaum et al. [16]. All subsequent operations were performed at 0—4°C. The cut tissue was rapidly rinsed with several volumes of solution A to remove residual milk before resuspension in 2.5 vols. of the same solution. This suspension was

divided into two portions and each portion homogenised in a 25 ml glass beaker for 1 min using an Ultra Turrax TP 18/2K Homogeniser (The Scientific Instrument Centre Ltd., 1 Leeke Street, London, U.K.), with its thyristor control set at 60% of its maximum clockwise rotation. The homogenates were combined, squeezed rapidly through two layers of cheesecloth and then centrifuged for 10 min at $600 \times g_{av}$ (Sorval RC2B centrifuge, 8 × 50 ml angle rotor). The supernatant was collected and recentrifuged for 20 min at $8000 \times g_{av}$ (8 × 50 ml angle rotor). The supernatant from this centrifugation was discarded and the pellet resuspended in a small volume of solution A before layering onto a simple discontinuous sucrose gradient in each of three 12-ml tubes. The gradient consisted of 3.5 ml of solution A with the sucrose concentration increased to 1.22 M and 3.5 ml of solution A with sucrose concentration of 1.08 M. The tube contents were overlayered with 0.25 M sucrose and the tubes centrifuged at $105\,000 \times g_{av}$ for 50 min at 2°C. (MSE 65 centrifuge, 3 × 27 ml swingout rotor.) The membranes which collected at the two interfaces, viz. sample/1.08 M sucrose and 1.08 M sucrose/1.22 M sucrose were harvested in a minimum volume of fluid with the aid of a hypodermic syringe. After dilution with cold 0.25 M sucrose containing 10 mM Hepes/NaOH buffer (pH 7.0) to lower the density both lots of membranes were washed three or four times by sedimentation at $8000 \times g_{av}$ for 2—4 min and by resuspension in the sucrose/Hepes medium. The final pellets were resuspended in the same medium with the aid of a vortex mixer.

The vesicles were used on the same day as prepared.

Measurement of vesicle volume. The intravesicular water content of the Golgi vesicles was determined from the relative activities of ^3H_2O and [^{14}C]-sorbitol in the pellet and supernatant according to the method of Casay et al. [17].

Measurment of Ca^{2+} uptake. Accumulation of Ca^{2+} was measured by the addition of the Golgi membrane fraction (75—250 μg protein/ml final concentration) to a standard incubation medium of the following composition: 25 mM Hepes/NaOH buffer, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, and 5 mM ATP (pH adjusted to 7.0 with Tris), total volume 250 μl. The medium contained $CaCl_2$ at various concentrations with $^{45}CaCl_2$ at varying specific activity (see figure legends), and routinely contained 5 mM NaN_3 and 0.7 μg/ml oligomycin to inhibit mitochondrial calcium uptake. Further additions were also made to this medium as indicated in the figure legends. The reaction was terminated by filtering a 200 μl aliquot through membrane filters (0.45 μm) previously soaked in 0.25 M sucrose containing 2 mM EGTA/Tris, pH 7.0. The samples on the filters were then rapidly washed (15—20 s) with three 5-ml portions of the same sucrose solution maintained at 0°C. The retained radioactivity was then counted by standard liquid scintillation techniques.

Measurement of Ca^{2+} efflux. Efflux of calcium from preloaded vesicles was initiated by the addition of A23187 or EGTA as indicated in the figure legends. Aliquots removed at appropriate time intervals were filtered, washed and the retained radioactivity determined as described.

Enzyme assays. ATPase activity was measured by the isotopic assay method of Seals et al. [18] in the presence and absence of Ca^{2+}. The same assay mixture was used to measure Ca^{2+} uptake when uptake and ATPase activity were

being compared. Galactosyltransferase activity was assayed by the procedure of Kuhn and White [19] using *N*-acetylglucosamine as acceptor. 5'-Nucleotidase (EC 3.1.3.5) was assayed according to the method of Michell and Hawthorne [20]. Phosphodiesterase I (EC 3.1.4.1), NADPH-cytochrome *c* oxidoreductase (EC 1.6.99.3), and cytochrome *c* oxidase (EC 1.9.3.1) were assayed as described by Brown et al. [21].

Protein. Protein was determined by a modified Lowry method [22] standardized with bovine serum albumin.

Reagents. The ionophore A23187 was a gift from Dr. R.L. Hammill, Eli Lilly and Co., Indianapolis, U.S.A. UDP-D-[U-^{14}C]galactose (specific radioactivity 231 Ci/mol), D-[^{14}C]sorbitol (specific radioactivity 333 Ci/mol), [γ-^{32}P]ATP (specific radioactivity 3.2 Ci/mmol) and $^{3}H_2O$ were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. and $^{45}CaCl_2$ (specific radioactivity 26.8 Ci/g Ca^{2+}) from New England Nuclear Chemicals, F.R.G. The nucleotides were purchased from the Boehringer Corporation (London) Ltd., Lewes, U.K. and all other reagents from Sigma, Poole, U.K.

Results

The two membrane fractions obtained from the sucrose step gradient viz. sample/1.08 M sucrose interface (fraction 1) and the 1.08 M sucrose/1.25 M sucrose interface (fraction 2) were assayed for a number of enzyme activities. The results shown in Table I and in particular the enhancement of galactosyltransferase activity shown by fraction 1 in relation to the homogenate indicated that this fraction was the purer sample of Golgi membranes. Uptake of Ca^{2+} by fraction 1 was also substantially higher than that shown by fraction 2. In view of these results the membranes that concentrated above the 1.08 M sucrose layer were used throughout the investigation.

Calcium accumulation and efflux

Ca^{2+} uptake varied with the preparation but a typical course of Ca^{2+} accumulation is shown in Fig. 1*a* at a total calcium concentration of 25 μM (5 μM free

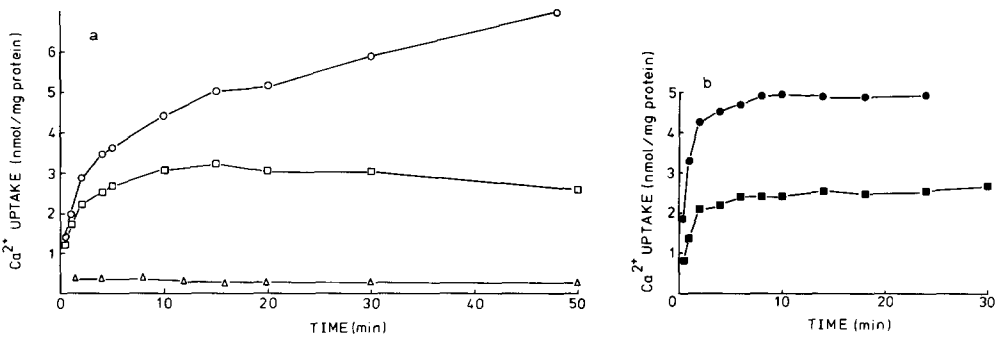


Fig. 1a. The $^{45}Ca^{2+}$ uptake by the Golgi membrane fraction expressed as nmol/mg protein for different experimental conditions as a function of time (min). □, standard assay medium; ○, plus 10 mM oxalate, and △, ATP-independent uptake. Total calcium was 25 μM (30 Ci $^{45}Ca^{2+}$/mol $CaCl_2$) and protein concentration (0.34 mg/ml). b. Incubation in standard assay medium. Isoosmotic wash solution contained 2 mM EGTA/Tris, pH 7.0 (■) or 2 mM Tris-HCl (●).

TABLE I

COMPARISON OF THE SPECIFIC ACTIVITIES OF ENZYMES IN GOLGI MEMBRANES AND WHOLE HOMOGENATES OF MAMMARY TISSUE

The number of experiments is in parentheses. Data for the specific activities are in nmol/min per mg protein, except for the calcium accumulation data which are in nmol/15 min per mg protein.

Substance assayed	Specific activities ± S.E.			Mean enrichment in Golgi fraction (-fold)	
	Homogenate	Interface 1	Interface 2	Interface 1	Interface 2
Galactosyltransferase	4.8 ± 1.8 (8)	116.0 ± 32 (8)	25.6 (3)	24	5.3
5'-Nucleotidase	124.0 ± 9.5 (12)	139.4 ± 22 (12)	129.0 (2)	1.12	1.04
NADPH-cytochrome *c* oxidoreductase	4.93 ± 0.66 (11)	4.54 ± 0.65 (12)	5.12 (2)	0.92	1.04
Cytochrome *c* oxidase	59.1 (2)	3.3 (2)	—	0.06	—
Calcium accumulation		36.8 (2)	3.79 (2)		

Ca^{2+} [23]). In the absence of MgATP little calcium accumulated. Continued incubation at 37°C for an extended period caused a gradual diminution in the activity of the preparation and accumulated calcium was slowly lost from the vesicles. This was not a reflection of the deterioration of the calcium-sequestering ability of the membranes because if $^{45}CaCl_2$ was added in tracer amounts after an initial incubation in the presence of non-radioactive calcium, uptake was vigorous (Fig. 2). Clearly, calcium was being released from the membranes and the maximum extent of uptake represented a balance between uptake and release.

That the calcium was accumulated into the vesicles and not simply bound by

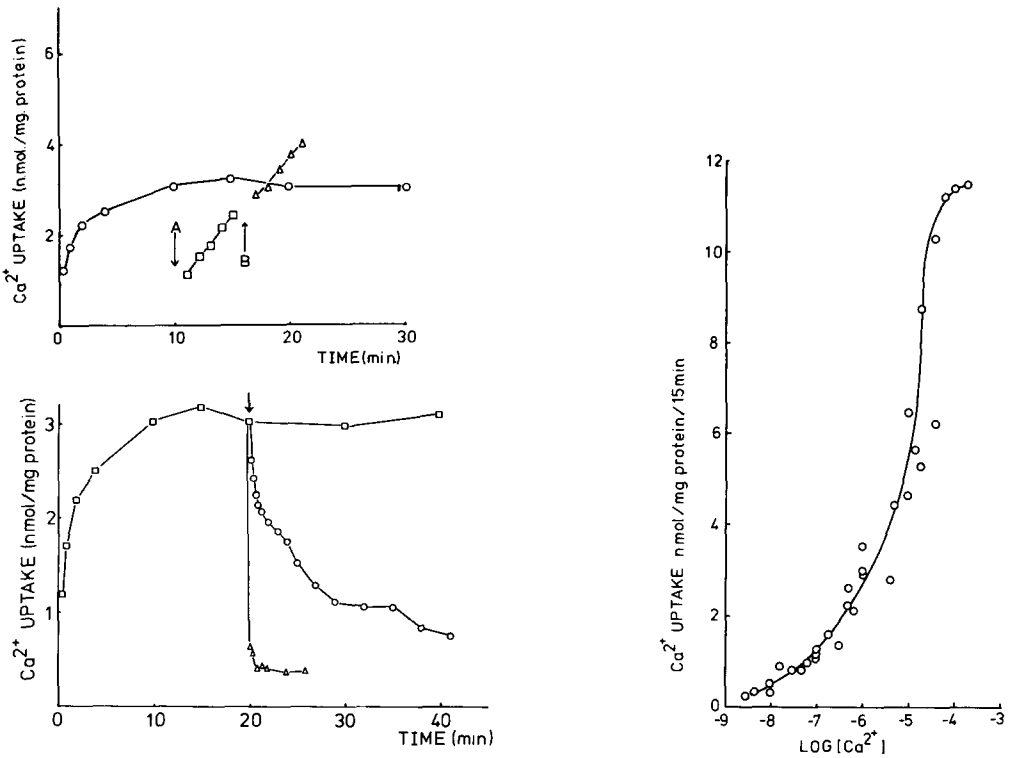


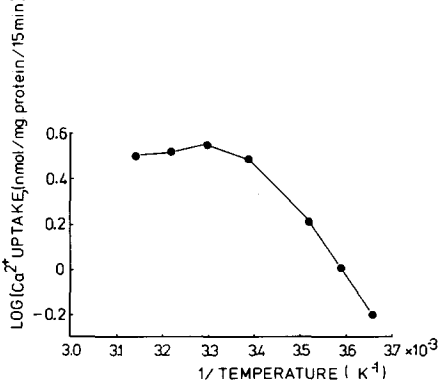
Fig. 2. (Upper left.) Uptake of $^{45}Ca^{2+}$ by the membrane fraction when preincubated with non-radioactive calcium. Standard assay medium containing 25 μM $CaCl_2$ and 0.1 mg/ml of membrane protein. Tracer $^{45}Ca^{2+}$ (400 Ci/mol) was added at (A) and calcium uptake was measured (□). At (B) oxalate (5 mM final concentration) was added and calcium uptake was again measured (△). ○, control; standard assay medium, $^{45}Ca^{2+}$ tracer added before initiation of reaction.

Fig. 3. (Lower left.) Release of accumulated $^{45}Ca^{2+}$ induced by A23187 (△) or EGTA (○). Standard assay medium containing 25 μM $CaCl_2$ (33 Ci $^{45}Ca^{2+}$/mol $CaCl_2$) and 0.29 mg/ml of membrane protein was used. A23187 (4 μM final concentration) or EGTA (6 mM final concentration) was added at the arrow and release of calcium determined (Experimental procedures). □, control reaction. Each point represents the mean of two membrane preparations each assayed in triplicate.

Fig. 4. (Right-hand figure.) Variation of $^{45}Ca^{2+}$ uptake with Ca^{2+} concentration. Standard assay medium used. At a Ca^{2+} concentration of 1 μM and below the Ca^{2+} was buffered to the required concentration by addition of EGTA with the $CaCl_2$ concentration constant at 25 μM. Above 1 μM $CaCl_2$ concentration was increased and ionized calcium concentrations calculated by the method of Katz et al. [23]. Incubation for 15 min at 37°C. Each point is the mean of quadruplicate determinations.

the membranes was shown by the following series of experiments. The ionophore A23187, which is known to increase the permeability of membranes for calcium [24,25], caused a rapid release of Ca^{2+} (Fig. 3) when added to the membrane fraction after it had been allowed to accumulate $^{45}Ca^{2+}$. Preloaded membrane vesicles also released calcium, although at a much slower rate when the external calcium concentration was reduced to less than 10^{-8} M by the addition of 6 mM EGTA (Fig. 3). By analogy with other calcium-transporting systems [26,27] no calcium would remain bound to the external membrane proteins at a concentration of 10^{-8} M and the slow release of Ca^{2+} must represent efflux of $^{45}Ca^{2+}$ accumulated within the membrane vesicles. A log plot of the data (not shown) is not a straight line, indicating that the efflux does not obey first-order kinetics and that more than one efflux mechanism is operating.

The slow rate of efflux noted on addition of EGTA also serves to validate the inclusion of EGTA in the solution used to wash the filters. At 37°C less than 5% of the accumulated Ca^{2+} had been released from the membranes in the first 30 s after the addition of EGTA. At 0°C the efflux process would be expected to be considerably slower so that by maintaining the wash solution in an ice bath and ensuring that the filters were washed as rapidly as possible (15–20 s total wash time) the loss of Ca^{2+} due to the washing procedure can be ignored. When EGTA was omitted from the wash solution the same pattern of uptake was observed but there was a considerable increase in the amount of $^{45}Ca^{2+}$ retained by the membranes (Fig. 1b). Control experiments indicated that the increase was not due to binding of $^{45}Ca^{2+}$ by the membrane filters. The apparent increase in uptake can thus be attributed to $^{45}Ca^{2+}$ bound to the exterior membrane surface and the washing procedure used here represents a useful technique for distinguishing between bound and translocated Ca^{2+}.



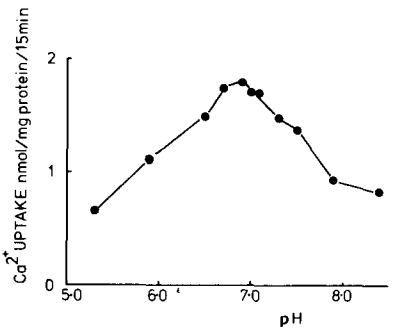


Fig. 5. Variation of calcium uptake with temperature: Arrhenius plot of the ATP-dependent Ca^{2+} uptake. The standard assay medium was used (see Experimental procedures); the solution also contained 25 μM $CaCl_2$ labelled with $^{45}Ca^{2+}$. The reaction was initiated by the addition of membrane preparation to a final protein concentration of 0.2 mg/ml. The samples were incubated for 3 min at 0, 6, 11, 22, 30, 37 or 45°C as indicated on the abcissa before the reaction was terminated. Each point represents the mean of quadruplicate determinations.

Fig. 6. Variation of calcium uptake with pH: Standard incubation medium containing 25 mM Mes/Tris or Hepes/Tris to cover the range of pH, 0.2 mg membrane protein/ml, 25 μM $CaCl_2$ (30 Ci $^{45}Ca^{2+}$/mol Ca^{2+}). Incubation for 15 min at 37°C. Each point represents the mean of quadruplicate determinations.

In the absence of permeant anions the initial rate of uptake was 2.52 ± 0.31 nmol $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ protein and total uptake averaged 4.72 nmol of Ca^{2+}/mg protein (seven preparations). The intravesicular volume was determined as approx. 2 μl/mg protein, indicating an intravesicular calcium concentration of $2.36 \cdot 10^{-3}$ M. At an extravesicular free Ca^{2+} concentration of $5 \cdot 10^{-6}$ M [23] this represents a 470 : 1 concentration gradient.

Dependency of calcium uptake on Ca^{2+} concentration

The rate of calcium uptake varied with the concentration of free Ca^{2+} in a sigmoidal manner (Fig. 4) with a marked increase in rate above 10^{-6} M Ca^{2+}. The apparent mean half-saturation constant for Ca^{2+} is approx. $5 \cdot 10^{-6}$ M.

Temperature and pH dependence

An Arrhenius plot (Fig. 5) of the variation of uptake with temperature shows that below 22°C the activation energy for uptake is about 6 kcal/mol of Ca^{2+} taken up, smaller than that shown by sarcoplasmic reticulum [28] but of the same order as that shown by presynaptic nerve terminals [29]. The ATP-dependent uptake was maximal at pH 6.9 (Fig. 6).

Inhibition of Ca^{2+} uptake

A number of different materials, known to inhibit Ca^{2+} uptake in other systems, were investigated to determine their influence on Ca^{2+} uptake in the Golgi membrane fraction (Table II). The sulphydryl reagents *p*-hydroxymercuribenzoate (PCMB) and *N*-ethylmaleimide inhibited Ca^{2+} accumulation, indicating the importance of -SH groups in the transport process, but inclusion of dithiothreitol throughout the isolation and assay procedure did not affect Ca^{2+} uptake. Lanthanum inhibited the uptake process as expected, since it is known to compete with Ca^{2+} for the binding sites on the membranes, but the transport process was only partially inhibited by ruthenium red at a concentration that completely inhibits Ca^{2+} uptake into mitochondria. The inhibition shown by carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) and dinitrophenol is interesting. These materials uncouple mitochondrial oxidative

TABLE II

THE INHIBITION OF CALCIUM UPTAKE INTO THE GOLGI MEMBRANE FRACTION

The reaction was initiated by the addition of the vesicular preparation to the standard incubation medium at 37°C containing the substance listed, and terminated after 15 min. The results represent the mean value obtained from a number of vesicle preparations (*n* in parenthesis) each assayed in quadruplicate.

Additions (mM)	Relative Ca^{2+} uptake (%)
None	100 (6)
— oligomycin	114.8 (3)
+ ruthenium red (0.005)	61.8 (6)
+ lanthanum chloride (0.5)	36.5 (5)
+ FCCP (0.25)	60.9 (2)
+ dinitrophenol (0.1)	83.2 (2)
+ A23187 (0.004)	14.8 (2)
+ PCMB (0.05)	6.1 (5)
+ *N*-ethylmaleimide (0.8)	58.7 (3)

TABLE III

ATPase ACTIVITY IN A GOLGI APPARATUS-RICH FRACTION FROM LACTATING RAT MAMMARY GLANDS

The accumulation of Ca^{2+} and hydrolysis of ATP were measured as outlined in Experimental procedures. The samples used were the combined Golgi fractions from 16 animals which were stored in liquid nitrogen before use. Representative results from two experiments.

Sample	nmol $^{32}P_i$/mg per min			Ca^{2+} uptake (nmol Ca^{2+}/mg per min)	ATPase/uptake ratio
	Mg^{2+}-ATPase	Total ATPase	Ca^{2+}-dependent ATPase		
A	15.4	24.4	9.0	0.144	62.5 : 1
B	18.3	26.1	7.8	0.168	45 : 1

phosphorylation by dissipating the potential generated by proton gradients across the mitochondrial membrane [30,31]. Although the mechanism by which Ca^{2+} is transported across the Golgi membrane is unknown, the inhibitory effect of these two compounds suggest that movement of protons across the membrane cannot be excluded as a possible mechanism.

ATPase activity

The Golgi vesicle fraction exhibited a 'basal' ATPase activity in the presence of Mg^{2+}, oxalate, oligomycin and ouabain (Table III). Addition of 10 μM free calcium to the incubation medium increased the total ATPase activity by approx. 50%. When oxalate was omitted from the reaction mixture the increase in activity on addition of calcium was not great enough to be measured with any accuracy. The average rate of ATP hydrolysis by the Golgi fraction, 25.5 nmol ^{32}P/min per mg, was comparable to the rate 41.7 nmol ^{32}P/min per mg observed for a Golgi membrane preparation isolated from bovine mammary tissue [6]. The rate of calcium uptake under these conditions was much slower and reference to the results shown in Table IV indicates that an average of over 50 mol of ATP were hydrolysed for each mol of Ca^{2+} translocated.

Nucleotide requirements for divalent cations

Calcium uptake was dependent on the presence of magnesium and ATP since

TABLE IV

EFFECT OF REPLACEMENT OF ATP BY OTHER NUCLEOTIDES ON $^{45}Ca^{2+}$ UPTAKE

The nucleotides listed were used at 5 mM concentration to replace ATP in the standard assay medium. Incubation for 15 min at 37°C. Protein concentration, 0.2 mg/ml; $CaCl_2$ 25 μM (25 Ci $^{45}Ca^{2+}$/mol Ca^{2+}).

Additions	Relative Ca^{2+} uptake (%)
None	10.5
ATP	100
ATP + ADP	50.7
GTP	44.9
ITP	33.1
UTP	28.8
CTP	13.7

omission of both Mg^{2+} and ATP from the mixture reduced uptake to a small percentage of the control values (Fig. 1). ATP was the only nucleotide that was really effective at sustaining Ca^{2+} uptake (Table IV) although GTP, UTP and ITP all showed a stimulation of uptake when compared with the nucleotide-free condition. ADP was not tested on its own but reduced uptake by 50% when added in equimolar proportions with ATP.

The ratio of Mg^{2+} to ATP also had a marked influence on uptake and the maximum amount of Ca^{2+} was sequestered at a Mg^{2+} : ATP ratio of 1 : 1. Reducing the Mg^{2+} concentration until the ratio was 1 : 5 reduced uptake to 58% of the maximum and a similar reduction was achieved when the Mg^{2+} : ATP ratio increased to 10 : 1. Manganese, which has been shown to be a requirement for the activity of lactose synthetase [32] was deleterious to Ca^{2+} uptake when added as a replacement for Mg^{2+}.

Effect of monovalent cations

Replacement of the 100 mM KCl by an equivalent amount of NaCl had little effect on Ca^{2+} accumulation whereas replacement by 200 mM sucrose reduced uptake by 37% (Table V). An attempt was made to compare the effect of cellular monovalent ion concentrations, including Cl^-, in comparison with the milk concentration of the same ions, using the figures given by Linzell and Peaker [33]. The measured uptakes were very variable with no clear result obtained in four experiments. Part of this variability was probably due to the use of acetate as a replacement for chloride since complete replacement of KCl by potassium acetate resulted in a 37% reduction of calcium accumulation.

Effect of oxalate, phosphate and citrate on calcium uptake

When 10 mM oxalate was included in the incubation medium calcium accumulation was sustained over a much longer period (Fig. 1) and a steady-state condition was not observed during the period of incubation. Furthermore in the experiment in which $^{45}Ca^{2+}$ tracer was added after preincubation with non-radioactive calcium (Fig. 2) the rate of influx of $^{45}Ca^{2+}$ was unaffected by the later addition of oxalate (Fig. 2). Oxalate was therefore included in the incubation for measurement of the kinetic parameters of the uptake reaction. Double-reciprocal analysis of data yielded an apparent K_m of 0.24 μM free cal-

TABLE V

VARIATION OF CALCIUM UPTAKE WITH MONOVALENT ION CONCENTRATION

Standard assay medium used in which KCl was replaced by the monovalent ions shown; incubation for 15 min at 37°C; 25 μM $CaCl_2$ (30 Ci $^{45}Ca^{2+}$/mol $CaCl_2$). The results are the mean value obtained from four vesicle preparations each assayed in quadruplicate.

Monovalent ion (mM)	Relative Ca^{2+} uptake (%)
KCl (100)	100
NaCl (100)	94 ± 1.3
Potassium acetate (100)	68 ± 1.5
Sucrose (200)	63 ± 1.6
Na^+ (43), K^+ (122), Cl^- (62), acetate (100)	92.1 ± 12.4
Na^+ (8), K^+ (24), Cl^- (12), acetate (20)	93.7 ± 11.1

TABLE VI

THE INFLUENCE OF PERMEANT ANIONS ON THE UPTAKE OF Ca^{2+} BY THE GOLGI MEMBRANE FRACTION

The assay was performed in the standard incubation medium containing 25 μM $CaCl_2$ including $^{45}Ca^{2+}$ (27 Ci/mol). The permeant anions were added in the concentrations shown and the pH was adjusted to 7.0 with NaOH before incubation. Assays were initiated by addition of membrane protein and were terminated, after 15 min at 37°C, as described in Experimental procedures. The figures in parentheses indicate the number of experiments performed each of which involved quadruplicate determinations.

Permeant anions	Concentration (mM)	Ca^{2+} uptake (% of control)
Oxalate	2.5	115 (6)
	3.0	123 (4)
	5.0	137 (4)
	10.0	186 (6)
Phosphate	2.5	126 (2)
	10.0	121 (4)
	20.0	154 (1)
	50.0	167 (1)
	100.0	284 (1)
Citrate	2.5	111 (2)
	4.0	97 (1)
	10.0	84 (5)

cium, calculated from the total calcium concentration according to the method of Katz et al. [23], and a V of 4.45 nmol calcium/mg protein per min.

Inclusion of phosphate, like oxalate, generally enhanced uptake (Table VI) but in the short time period of incubation used (15 min) neither anion increased uptake substantially. Citrate, which is known to complex the soluble calcium of bovine milk [34] and to be accumulated into Golgi vesicles [35] had little effect on uptake at low concentrations. Above 4.0 mM it was inhibitory.

Discussion

Previously reported preparative techniques for the isolation of Golgi membranes from lactating rat [19] and bovine [6] mammary glands have involved centrifugation over a cushion of 1.25 M sucrose (d = 1.164) as the final step. However, Hodson [36] and Fleischer and Kervina [37] prepared Golgi membranes from rat liver by harvesting membranes that collected at the interface with 1.0 M sucrose (d = 1.130). In the initial stages of this investigation a two step sucrose gradient of 1.08 M sucrose (d = 1.137) layered over 1.22 M sucrose (d = 1.160) was used as the final centrifugation step. Analysis of the two membrane fractions obtained indicated that centrifugation over a 1.08 M sucrose cushion produced the best sample of Golgi membranes.

This Golgi membrane fraction accumulated calcium against a concentration gradient when both Mg^{2+} and ATP were included in the medium. Uptake was both temperature and pH dependent and could be reversed by reducing the external calcium concentration. Total uptake could be increased by inclusion of oxalate and phosphate in the incubation although the increase was not as

great as that observed with skeletal muscle sarcoplasmic reticulum [38]. The values obtained for the apparent K_m and the V were comparable with values reported for other calcium-transporting systems particularly those isolated from non-muscular tissues [15,28,39—41]. In these systems the K_m approximates to the cytosolic calcium concentration suggesting that the lactating mammary gland has a cytosolic calcium concentration of the same order.

The properties of the calcium-transporting process in the Golgi membrane fraction thus show many similarities to calcium uptake by other transporting systems and although the stoicheiometry of the process cannot be determined due to the high ATPase activity present, the characteristics of the system are compatible with the proposed involvement of the Golgi apparatus of the mammary cell in the process of secretion of calcium into milk.

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References

1 Davis, J.S., Widdowson, E.M. and McCance, R.A. (1964) Br. J. Nutr. 18, 385—392
2 Blaustein, M. (1974) Rev. Physiol. Biochem. Pharmacol. 70, 33—82
3 Wooding, F.B.P. and Morgan, G. (1978) J. Ultrastruct. Res. 63, 323—333
4 Linzell, J.L., Mepham, T.B. and Peaker, M. (1976) J. Physiol. 260, 739—750
5 Neville, M.C. and Peaker, M. (1979) J. Physiol. 290, 59—67
6 Baumrucker, C.R. and Keenan, T.W. (1975) Exp. Cell Res. 90, 253—260
7 Hasselbach, W. (1977) Biophys. Struct. Mech. 3, 43—54
8 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1—79
9 Lehninger, A.L., Carafoli, E. and Rossi, C.S. (1967) Adv. Enzymol. 29, 259—321
10 De Meis, L., Rubin-Altschul, B.M. and Machado, R.D. (1970) J. Biol. Chem. 245, 1883—1889
11 Zellinger, Z., Naim, E. and Lasser, M. (1970) Biochim. Biophys. Acta 203, 326—334
12 Robblee, L.S., Shepro, D. and Belamarich, F.A. (1973) J. Gen. Physiol. 61, 462—481
13 Schatzman, H.J. and Vincenzi, F.F. (1969) J. Physiol. 201, 369—395
14 Sulakhe, P.V., Drummond, G.I. and Ng, D.C. (1973) J. Biol. Chem. 248, 4150—4157
15 Bruns, D.E., McDonald, J.M. and Jarett, L. (1976) J. Biol. Chem. 251, 7191—7197
16 Greenbaum, A.L., Salam, A., Sochor, M. and McLean, P. (1978) Eur. J. Biochem. 87, 505—516
17 Casay, R.P., Njus, D., Radda, G.K. and Sehr, P.A. (1977) Biochemistry 16, 972—977
18 Seals, J.R., McDonald, J.M., Bruns, D. and Jarett, L. (1978) Anal. Biochem. 90, 785—795
19 Kuhn, N.J. and White, A. (1977) Biochem. J. 168, 423—433
20 Michell, R.H. and Hawthorne, J.N. (1965) Biochem. Biophys. Res. Commun. 21, 333—338
21 Brown, A.E., Lok, M.P. and Elovson, J. (1976) Biochim. Biophys. Acta 426, 418—432
22 Peterson, G.L. (1977) Anal. Biochem. 83, 346—356
23 Katz, A.M., Repke, D.I., Upshaw, J.E. and Polascik, M.A. (1970) Biochim. Biophys. Acta 205, 473—490
24 Reed, P.W. and Lardy, H.A. (1972) in The Role of Membranes in Metabolic Regulation (Mehlman, M.A. and Hanson, R.W., eds.), pp. 111—131, Academic Press, New York
25 Pressman, B.C. (1973) Fed. Proc. Am. Soc. Exp. Biol. 32, 1698—1701
26 Shami, Y., Messer, H.H. and Copp, D.H. (1974) Biochim. Biophys. Acta 339, 323—332
27 Bruns, D.E., McDonald, J.M. and Jarett, L. (1977) J. Biol. Chem. 252, 927—932
28 Hasselbach, W. (1972) in The Enzymes (Boyer, P.D., ed.), Vol. 10, pp. 431—463, Academic Press Inc., New York
29 Blaustein, M.P., Ratzlaff, R.W. and Schweitzer, E.S. (1978) J. Gen. Physiol. 72, 43—66
30 Harold, F.M. and Altendorf, K. (1974) in Current Topics in Membranes and Transport (Bonner, F. and Kleinzeller, A., eds.), Vol. 5, pp. 1—50, Academic Press, New York
31 Harold, F.M. (1970) Adv. Microbiol. Physiol. 4, 45—104

32 Khatra, B.S., Herrles, D.G. and Brew, K.E. (1974) Eur. J. Biochem. 44, 537—560
33 Linzell, J.L. and Peaker, M. (1971) J. Physiol. 216, 683—701
34 Holt, C. and Muir, D.D. (1979) J. Dairy Res. 46, 433—439
35 Zulak, I.M., Eigel, W.N. and Keenan, T.W. (1978) J. Dairy Sci. 61, (Suppl. 1) 167
36 Hodson, S. (1978) J. Cell Sci. 30, 117—128
37 Fleischer, S. and Kervina, M. (1974) Methods Enzymol. 31, 6—41
38 Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329—369
39 Solaro, R.J. and Briggs, F.N. (1974) Circ. Res. 34, 531—539
40 Moore, L., Fitzpatrick, D.F., Chen, T.S. and Landon, E.J. (1974) Biochim. Biophys. Acta 345, 405-418
41 Moore, L., Chen, T., Knapp, H.R., Jr. and Landon, E.J. (1975) J. Biol. Chem. 250, 4562—4568