J. Membrane Biol. 61, 97–105 (1981)

The Journal of
Membrane Biology

ATP-Dependent Calcium Transport by a Golgi-Enriched Membrane Fraction from Mouse Mammary Gland

Margaret C. Neville*†, Frank Selker*, Kathleen Semple*, and Christopher Watters**
Department of Physiology, University of Colorado School of Medicine, Denver, Colorado 80262 and
Department of Biology, Middlebury College, Middlebury, Vermont 05753

Summary. Crude particulate preparations from the mammary glands of lactating mice were shown to transport calcium against a concentration gradient in the presence of ATP and mitochondrial inhibitors. Density gradient centrifugation with both sucrose and Percoll gradients indicated the presence of ATP-dependent transport in more than one membrane fraction. A Golgi-enriched membrane fraction possessed the highest specific activity of calcium transport. Digitonin, which increases the permeability of plasma membranes to calcium, did not affect this process. The Golgi fraction contained a 100,000 Dalton protein whose phosphorylation by γ-[^{32}P]-ATP was enhanced by a micromolar concentrations of free calcium. The phosphorylation was acid-stable and hydroxylamine-sensitive. These properties suggest that Golgi membranes in an actively secreting mammary epithelium possess a calcium transport system which resembles the calcium ATPase present in the sarcoplasmic reticulum of skeletal muscle.

Key words: Mammary gland, calcium transport, Golgi membrane, phosphorylated intermediate.

A calcium ATPase whose function it is to sequester calcium in the sarcoplasmic reticulum of skeletal muscle has been extensively characterized (Tada, Yamamoto & Tonomura, 1978). ATP-dependent calcium transport has been demonstrated in intracellular compartments from a wide variety of other tissues (Carafoli & Crompton, 1978). Examples include adipocytes (Black, McDonald & Jarett, 1980), hepatocytes (Moore, Chen, Knapp & Landon, 1975), chick embryo fibroblasts (Moore & Pastan, 1977), smooth muscle (Wuytack & Casteels, 1980) and such secretory tissues as the islets of Langerhans (Howell & Montague, 1975; Sehlin, 1976) and the adrenal medulla (Poisner & Hava, 1970). Blaustein and co-workers (1980) have defined the kinetic properties of an ATP-dependent transport system in internal compartments of synaptosomal vesicles (*see also* Blitz, Fine & Toselli, 1977). Morphological studies (McGraw, Somlyo & Blaustein, 1980) suggest that this compartment is in the smooth endoplasmic reticulum (*see also* Henkart, 1980). Thus, reports of ATP-dependent calcium transport by internal membranes other than mitochondria are becoming increasingly widespread.

The lactating mammary gland is a tissue of particular interest because it secretes large amounts of calcium, supplying this ion to milk at concentrations ranging between 10 and 125 mM (Jenness, 1979). The involvement of Golgi-derived secretory vesicles in the secretion of calcium into milk has been demonstrated morphologically (Saacke & Heald, 1974; Wooding, 1977) and physiologically (Neville & Peaker, 1979). Baumrucker and Keenan (1975) reported ATP-dependent calcium transport by a Golgi-enriched membrane fraction from this tissue. These findings suggest the presence of a mechanism of calcium transport in an intracellular compartment of mammary secretory cells. For this reason we have begun a careful study of calcium transport in subcellular fractions of mammary glands from lactating mice.

In these initial studies we have demonstrated ATP-dependent transport of calcium into membrane-limited subcellular vesicles in a crude particulate preparation from lactating mouse mammary gland. Fractionation of the crude preparation provided further evidence that at least part of the ATP-dependent transport activity was associated with Golgi membranes. We measured calcium-stimulated phosphorylation of the Golgi-enriched membrane fraction using γ-[^{32}P]-ATP and observed the molecular weight of the labeled

* University of Colorado.
** Middlebury College.
† For reprint requests.

0022-2631/81/0061-0097 $01.80

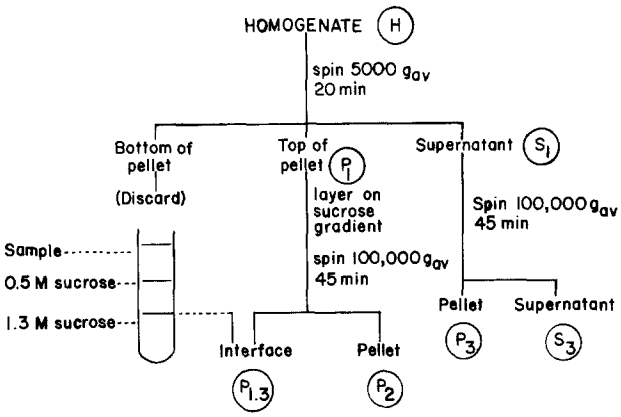

Fig. 1. Scheme for partial purification of mammary gland membranes (*see* Materials and Methods for details)

phosphoproteins with SDS-polyacrylamide gel electrophoresis. A preliminary report of this work has appeared (Neville, Selker, Semple & Watters, 1980).

Materials and Methods

Materials

CD1 mice were obtained from Charles Rivers Laboratories and bred in this laboratory. These mice routinely bear 10 to 16 pups in a litter; litter size was not adjusted. Lactating mice (9–15 days) were killed by cervical dislocation and the 4th and 5th mammary glands dissected and minced with sharp scissors. These glands contain no muscle and relatively small amounts of connective tissue. Unless stated otherwise all chemicals were reagent grade. ^{45}Calcium and γ-[^{32}P]-ATP were obtained from New England Nuclear.

Membrane Fractionation

A crude membrane pellet was prepared by homogenizing 2–5 g of minced mammary gland for 3–4 sec with a Polytron (Model PCU-2-110) run at the slowest speed in 2.5 volumes of homogenization medium (0.5 HM) containing 0.5 M sucrose, 5 mM $MgCl_2$, 1 mM HEPES and 1% Dextran (Sigma clinical grade, mol wt 200,000–275,000), pH 7.0. All preparations were maintained at 0 °C in an ice bath. The homogenate was spun for 10 min at $600 \times g$ to remove cellular debris and nuclei. The supernatant was spun at $20{,}000 \times g_{av}$ for 20 min in a Sorvall RC2B centrifuge to obtain a crude particulate fraction. Examination of this fraction with transmission electron microscopy revealed mitochondria, Golgi membranes, rough endoplasmic reticulum and a variety of unidentified membrane-bound particles (not shown). After washing, the pellet was resuspended in homogenization medium using not more than three strokes of a Teflon pestle.

The scheme used to obtain a *Golgi-enriched membrane fraction* is shown in Fig. 1 and is adapted from that used by Keenan, Huang and Morré (1972) to obtain Golgi membranes from rat and cow mammary glands. The homogenate *H*, obtained as described above, was spun at $5{,}000 \times g_{av}$ for 20 min in a Sorval RC2B centrifuge. When desired, the supernatant S_1, was removed and spun at $100{,}000 \times g_{av}$ for 45 min to obtain a microsomal pellet P_3. The white loose material at the top of the $5{,}000 \times g_{av}$ pellet P_1 was gently resuspended in 0.5–1.0 ml medium (0.25 HM) containing 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM EGTA, 37.5 mM HEPES and 1% dextran, pH 7.0. For larger yields we rehomogenized the pink lower portion of P_1 in 3 ml 0.5 HM using three strokes of a Teflon homogenizer. This homogenate was again spun at $5{,}000 \times g_{av}$ and the loose white material at the top of the pellet was combined with that obtained from the previous $5{,}000 \times g_{av}$ spin. Holding the P_1 on ice during the time necessary to execute this procedure resulted in no loss in either galactosyl transferase or calcium transport activity. The resulting suspension was layered on a discontinuous sucrose gradient consisting of 1.5 ml of medium 1.3 HM (containing 1.3 M sucrose, 5 mM $MgCl_2$, 1 mM EGTA, 37.5 mM HEPES and 1% dextran, pH 7.0) and 1.5 ml of 0.5 HM and spun at $100{,}000 \times g_{av}$ for 45 min. The material at the interface as well as pellet P_2 at the bottom of the tube were resuspended in 0.25 HM. All centrifugal separations were performed at 4 °C.

For membrane separation on Percoll (Pharmacia) gradients, mammary tissue from a lactating mouse was diced and homogenized as above using 0.25 HM throughout. After a 10-min spin at $100 \times g_{av}$ to remove nuclei and cellular debris, the supernatant was layered on 35% Percoll in 0.25 M sucrose and spun at $60{,}000 \times g_{av}$ for 45 min. The tube was pierced with a needle and 0.5 ml fractions were drawn off and analyzed for ATP-dependent calcium uptake, succinate dehydrogenase, galactosyl transferase and protein content as described below. Density marker beads (Pharmacia) were used to monitor the specific gravity of the fractions.

Enzyme Activity of Membrane Fractions

The activity of marker enzymes was assayed in the various fractions, using succinate dehydrogenase (Pennington, 1961) for mitochondria, galactosyl transferase (Kuhn & White, 1977) for Golgi membranes and cytochrome *c* oxido-reductase for rough endoplasmic reticulum. (Na + K)-ATPase, a marker for plasma membranes, was measured as the ouabain-dependent liberation of phosphate from ATP (Hegyvary, Kang & Bandi, 1979). Cryptic enzyme activity was released by prior dilution of the membrane fractions to 0.5–1.0 mg protein/ml and incubation for 30 min at 37 °C in the presence of 0.5 mg/ml sodium dodecylsulfate (Jorgenson, 1974). Protein was assayed by the Peterson (1977) modification of the Lowry method using bovine serum albumin as a standard.

^{45}Calcium Uptake by Membrane Fractions

Membrane fractions were resuspended in homogenization medium (0.25 HM) to a concentration of 1 to 5 mg protein/ml. Aliquots (100 µl) of this suspension were incubated at 37 °C with 900 µl of a solution containing 110 mM KCl, 1.3 mM EGTA, 0.7 mM $MgCl_2$, 27.5 mM imidazole, 0.11 mM DNP, 0.77 µg/ml oligomycin, 1.1 mM NaN_3 and 0.1 µCi ^{45}calcium/ml, pH 7.4. For a free calcium of 50 µM as calculated from the equilibrium constants given by Kerrick and Donaldson (1972), 1.25 mM calcium were added to the above solution; for a free calcium of 0.5 µM, 0.56 mM $CaCl_2$ were added to this solution. The reaction was started by adding ATP to a final concentration of 1.3 mM. At intervals, 100-µl aliquots of incubation solution were applied to 0.45 µ membrane filters under suction, the filters were washed three times with 5 ml of an ice-cold solution containing 0.25 µM sucrose, 2 mM EGTA and 20 mM Tris brought to pH 7.0 with HCl. The filters were placed in a scintillation vial with 5 ml Formula 963 (New England Nuclear) and counted in a Picker Liquimat β-scintillation counter. Aliquots (20 µl) of the incubation solution were counted similarly and used to calculate the specific activity of the incubation medium. ^{45}Calcium uptake was calculated as µmoles/mg protein from the following expression:

$$^{45}\text{Calcium uptake} = (\text{CPM}_s \times [\text{Ca}]_{tot})/(\text{CPM}_{inc} \times \text{PROT})$$

where CPM_s are the counts in the sample after subtracting the background, CPM_{inc} are the counts in 1 ml of incubation medium, $[\text{Ca}]_{tot}$ is the total concentration of calcium and PROT is the

amount of protein in 100 µl of membrane suspension. When used, digitonin and calcium ionophore A-23187 were present at final concentrations of 0.03% and 10^{-5} M, respectively.

Phosphorylation of the Golgi-Enriched Membrane Fraction

Fraction $P_{1,3}$ was washed once in solution containing 0.1 M KCl, 1.0 mM EGTA and 0.05 M Tris-maleate (pH 7.0, 0 °C) and then resuspended in the same solution to a final concentration of 1 to 4 mg protein/ml. In later experiments an extra hypotonic wash in the same solution without KCl was instituted. This resulted in more reproducible calcium stimulation. Membranes were phosphorylated by a procedure similar to that of Sumida and Tonomura (1974) in 0.5 or 0.2 ml of a solution containing, 0.1 M KCl, 1.0 mM EGTA, 0.0225 mM $MgCl_2$, 1.038 mM $CaCl_2$, 0.0108 mM Tris-ATP, 2–5 µCi γ-[^{32}P]-ATP (as the tetraethylammonium salt), 0.05 M Tris-maleate (pH 7.0, 0 °C), and 0.2 to 0.8 mg protein. From the dissociation constants of Kerrick and Donaldson (1972) this mixture contains 20 µM free magnesium, 50 µM free calcium and 2.5 µM Mg-ATP. The assay was performed on ice and was initated by the rapid addition of ATP and γ-[^{32}P]-ATP and terminated 20 sec later with an ice-cold solution (STOP) containing 6% trichloroacetic acid, 1.0 mM inorganic phosphate and 0.1 mM unlabeled Tris-ATP. The protein was recovered by centrifugation, washed 4× with the STOP and solubilized with a solution containing 2% SDS, 1.2% dithiothreitol, 2 mM EDTA, and 0.02 M Tris (pH 6.5, 20 °C). Aliquots of this resuspension were separated by SDS-polyacrylamide gel-electrophoresis (SDS-PAGE; *see below*) or assayed directly for radioactivity by liquid scintillation spectrometry.

The basal level of phosphorylation was measured in the absence of calcium, under conditions otherwise identical to those described above. The sensitivity of the phosphorylation to hydroxylamine was determined by resuspending washed samples in 0.6 N hydroxylamine in 0.08 M sodium acetate (pH 5.2) and incubating for 10 min at room temperature. As a control for nonspecific hydrolysis duplicate samples were incubated under identical conditions in the acetate buffer alone. Treated and control samples were washed once with STOP and once with ice-cold distilled water and solubilized as above.

Molecular Weight Determination of Phosphorylated Intermediate

SDS-PAGE of solubilized samples was performed by a modification (Rudenberg & Watters, 1978) of the method of Fairbanks, Steck and Wallach (1971), using 5.6, 6 or 7.5% gels, and a 0.1% SDS/0.04 M Tris-acetate buffer (pH 6.5, 10 °C). Immediately following electrophoresis, gels containing phosphorylated material were sliced into 2-mm sections; each section was placed in a separate scintillation vial with 0.5 ml of 0.1% SDS and incubated for 18 to 24 hr at 37 °C. Control gels, containing unlabeled material or a mixture of standard proteins (Biorad Corp.), were run in parallel and stained with Coommassie Blue.

Solubilized samples were mixed with Biofluor (New England Nuclear Corp.) and assayed in either a Beckman Liquimat or a Packard Tricarb 3330 liquid scintillation system.

Results and Discussion

ATP-Dependent Calcium Uptake by a Crude Particulate Fraction from Lactating Mouse Mammary Gland

Our initial experiments were carried out on crude particulate preparations to determine whether the reported ATP-dependent calcium transport by particulate fractions from lactating mammary gland (Baumrucker & Keenan, 1975) represented transport into a membrane-bound compartment or calcium binding to one or more intracellular fractions. The time course of ^{45}calcium uptake by this preparation in the presence and absence of ATP is shown in Fig. 2. In the absence of ATP we observed rapid uptake of ^{45}calcium which attained a peak at about 1 min then gradually declined. This initial rapid uptake phase was a consistent finding in the absence of ATP; it may represent exchange with previously accumulated calcium. In the presence of ATP the time course of uptake during the first minute was similar to that observed in the absence of ATP. However, the calcium content of the vesicles continued to increase reaching a plateau at 4 min. At 0 °C in both the presence and absence of ATP, entry was much slower eventually reaching a level similar to that achieved in the absence of ATP at 37 °C. The marked temperature-dependence of the uptake both in the presence and absence of ATP suggested entry of calcium into a membrane-limited compartment (or compartments) rather than binding. This interpretation was supported by the finding that the calcium ionophore, A23187, released 75% of the calcium accumulated during a 10-min incubation in the presence of ATP (Fig. 3). After a similar incubation in the absence of ATP about $^2/_3$ of the accumulated calcium was released by A23187, suggesting that part of the ATP-independent uptake also represents entry into membranous vesicles. Triton X-100 (1%) had an effect similar to that of A23187. Sodium oxalate (2 mM) stimulated uptake

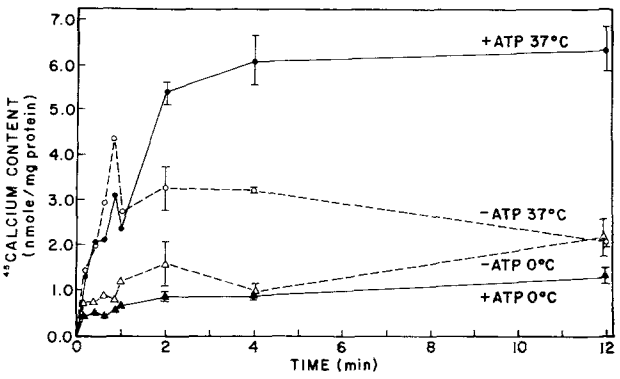

Fig. 2. Time course of ^{45}calcium uptake by a crude membrane preparation (20,000 × g pellet) in the presence and absence of ATP (1.3 mM). Incubation carried out as described in Materials and Methods in the presence of the mitochondrial inhibitors NaN_3 (1 mM), DNP (0.1 mM) and oligomycin (0.07 µg/ml). Free calcium concentration was 50 µM. The reaction was initiated by addition of ^{45}calcium. Points up to 1 min are single samples. After 1 min the points represent the mean of triplicate determinations; the distance between bars is two SEM

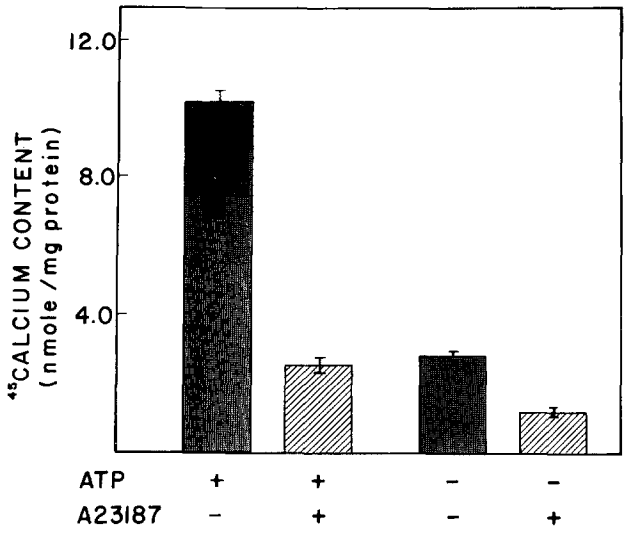

Fig. 3. Release of ^{45}calcium accumulated in the 20,000 × *g* pellet by the calcium ionophore, A23187. Incubation carried out for 10 min in the presence and absence of ATP as described in the legend to Fig. 2. A23187 (final concentration 10^{-5} M) was added to the incubation solution just prior to termination of incubation, where indicated

by only about 20% at 2 and 5 min in this preparation (data not shown). Because the enhancement of uptake was small the anion was not included in other experiments.

To provide additional evidence that ATP stimulates calcium entry against a concentration gradient we examined the time course of calcium accumulation over a longer period of time in the presence of varying amounts of membrane protein (Fig. 4). The amount of ATP remaining in the incubation medium was also assayed. The data indicate that there is rapid ATP hydrolysis by these preparations and that the rate of hydrolysis is proportional to the concentration of membrane protein. In all three samples calcium accumulation reached a maximum by 10 min; the level of calcium was maintained until the ATP concentration fell below about 0.25 mM at which point the ^{45}calcium began to exit from the vesicles. The finding that the continuous presence of ATP is required to maintain the steady-state level of intravesicular calcium is strong evidence that the observed ATP-dependent calcium uptake depends on a continuous supply of energy to maintain a concentration gradient.

Because high concentrations of mitochondrial inhibitors were present in the above experiments it was probable that nonmitochondrial vesicles were responsible for the calcium transport. To further clarify this point and to determine whether the compartment responsible for the ATP-dependent transport was the Golgi apparatus as suggested by the earlier work of Baumrucker and Keenan (1975), we examined calcium transport in more purified membrane preparations and correlated this activity with the activity of marker enzymes.

Calcium Transport by More Purified Membrane Fractions from Lactating Mouse Mammary Glands

The protocol followed for the separation of more purified membrane fractions from lactating mouse mammary glands is shown in Fig. 1 and described in detail in Materials and Methods. Table 1 shows enzyme activites from a representative experiment. Similar results were obtained in analyses of 5 different preparations. The highest concentrations of the mitochondrial marker were found in fractions P_1 and P_2. Electron microscopy of these pellets confirmed the presence of mitochondria. The Golgi marker enzyme, galactosyl transferase, attained the highest specific activity in fraction $P_{1,3}$ with a 14-fold purification over its activity in the homogenate. This fraction also showed the highest specific activity of ATP-dependent calcium transport and possessed minimal mitochondrial contamination (Fig. 5). The ATP-dependent uptake recovered in S_1+P_1 appeared to exceed that recovered in the homogenate. This was a consistent

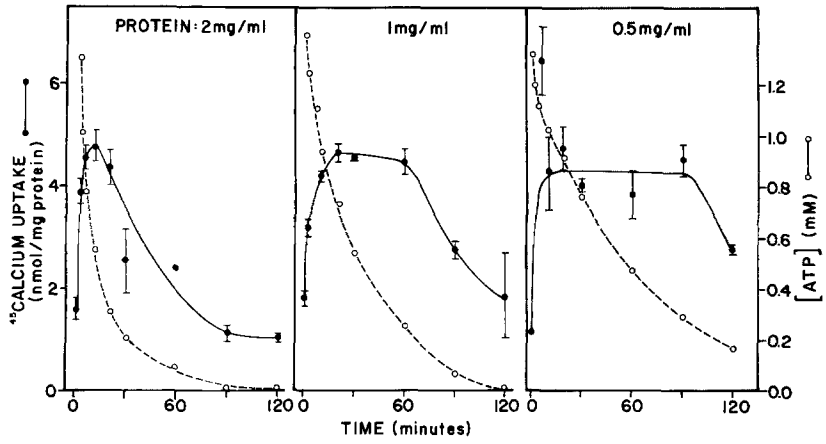

Fig. 4. The effect of ATP-depletion on retention of ^{45}calcium by the 20,000 × *g* pellet. Varying amounts of membranes were incubated as in Fig. 1 except that the reaction was started by ATP addition and ^{45}calcium content determined at intervals. The free calcium concentration was 0.5 μM. The ATP remaining in the solution was calculated from a concomitant measurement of free phosphate. ATP values represent the average of duplicate samples; the ^{45}calcium points represent the mean of triplicate determinations ± the SEM

Table 1. Enzyme activities of membrane fractions from mouse mammary gland

Fraction	Volume	Protein		Succinate dehydrogenase activity[a]		Galactosyl transferase activity[a]		ATP-dependent calcium uptake activity[a]	
	(ml)	mg/ml	Total	Specific	Total	Specific	Total	Specific	Total
H	9.7	28.4	277	9.5	2,631	9.6	2,660	0.2	60.4
S_1	7.0	16.4	115	0.6	69	3.0	339	0.3	33.8
P_1	3.0	21.6	65	10.6	689	18.9	1,229	0.9	60.3
$P_{1.3}$	0.3	4.7	1.4	0.3	0.5	130.4	184	3.4	4.8
P_2	1.9	16.4	37	9.7	363	19.7	737	1.5	55.3
P_3	0.5	22.0	11	0.9	10	16.3	178	1.1	11.8
S_3	6.0	15.8	95	0	0	1.0	96	0	0

[a] Specific activity in nmol·min^{-1}·mg prot^{-1}; total activity in nmol·min^{-1}.

finding which possibly relates to the very high background (ATP-independent) uptake observed in the homogenate. These data established the presence of extra-mitochondrial ATP-dependent calcium transport, possibly localized in Golgi membranes.

In a separate experiment, we determined the activity of the rough endoplasmic reticulum marker, cytochrome *c* oxidoreductase and the plasma membrane marker, (Na−K)-ATPase (Table 2) in the membrane fraction (Table 2). The results are representative of two separate experiments. The low activity of cytochrome *c* oxido-reductase in $P_{1.3}$ suggested little contamination by rough endoplasmic reticulum. The relatively high specific activity of (Na−K)-ATPase in the $P_{1.3}$ fraction suggested, however, that ATP-dependent calcium transport might be due to contamination by inside-out plasma membrane vesicles. To investigate this possibility we examined the effect of digitonin on our putative Golgi fraction since Doss, Caraway and Caraway (1979) used this detergent to separate Golgi membranes from plasma membranes in mammary particulate fractions. Their data and that of others (Murphy, Coll, Rich & Williamson, 1980) suggest that the detergent reacts mainly with plasma membrane. We have shown elsewhere (Evans, Jacobs & Neville, *unpublished*) that digitonin treatment renders mammary gland plasma membranes permeable to calcium. We reasoned that digitonin treatment should prevent net calcium uptake by plasma membrane vesicles but have less effect on transport by vesicles from internal membranes. As the data presented in Fig. 6 indicate, the presence of digitonin in the uptake solution had no significant effect on the time course of ATP-dependent calcium uptake by the Golgi-enriched membrane fraction. This was also true when the membrane was preincubated 20 min with digitonin prior to the uptake experiment (data not shown). These results provide evidence that plasma membrane vesicles are not responsible for the calcium transport in this fraction.

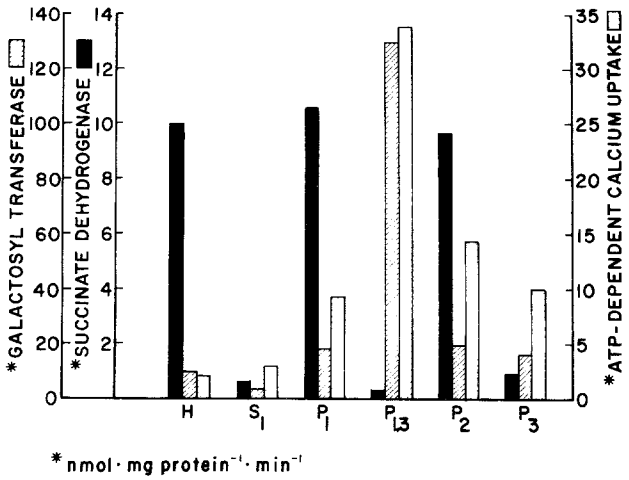

Fig. 5. Copurification of galactosyl transferase and ATP-dependent calcium transport activity. Plot of data from Table 1

Table 2. Plasma membrane and rough endoplasmic reticulum marker enzymes in membrane fraction

Membrane fraction	Protein (mg)	Cytochrome *c* oxidoreductase[a]		(Na + K)-ATPase[a]	
		Activity	Total	Activity	Total
H	396	24	9,504	1.3	514
P_1	39.4	60	2,364	2.4	95
$P_{1.3}$	2.8	4	11	1.7	4.8
P_2	32	31	992	0.9	29
P_3	34	44	1,496	1.7	58

[a] Specific activity in nmol·min^{-1}·mg prot^{-1}; total activity in nmol·min^{-1}

There is also substantial calcium transport activity in those fractions, P_1 and P_2, which contain large numbers of mitochondria and a disproportionate amount, compared to galactosyl transferase activity, in the microsomal pellet, P_3, as well (Fig. 5). It was not clear whether this activity could be ascribed to

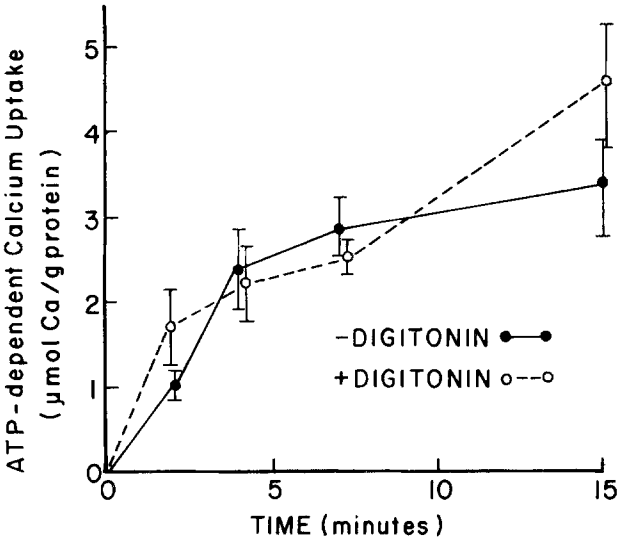

Fig. 6. The time course of ^{45}calcium accumulation in Golgi-enriched membranes in the presence and absence of 0.03% digitonin. Incubation as described in the legend to Fig. 2 using the $P_{1,3}$ pellet and starting the reaction with ATP addition. Free calcium concentration was 0.5 μM. Points are mean of dupicate samples; the distance between bars is two SEM

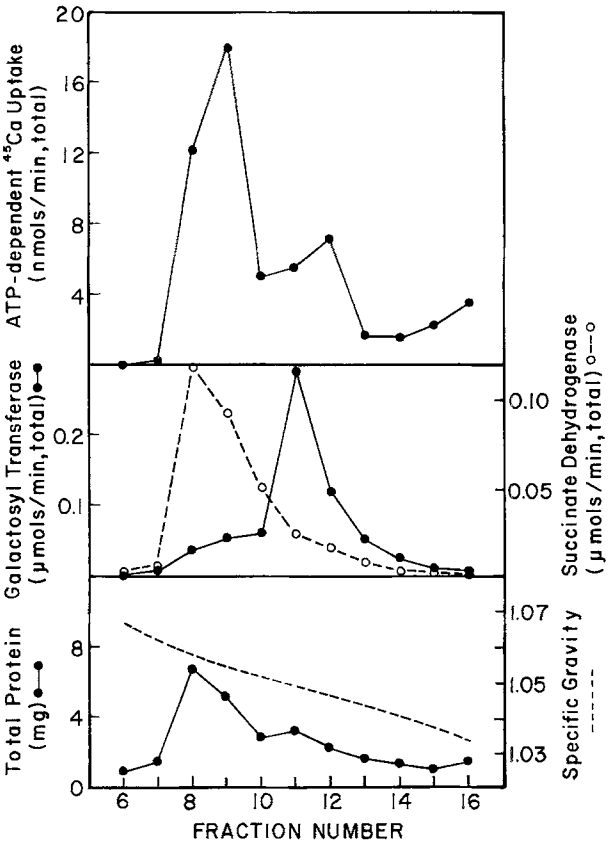

Fig. 7. Enzyme activity of mammary membranes separated on a Percoll gradient. After homogenization in 0.25 M HM the mammary tissue from one lactating mouse (~1.7 g) was centrifuged at $1{,}000 \times g_{av}$ for 10 min. The supernatant (3 ml) was layered on 8 ml of 35% Percoll in 0.25 M sucrose and spun at $60{,}000 \times g_{av}$ for 45 min. Fractions (0.5 ml) were drawn from the bottom of the tube and assayed for enzyme activity as described in the text

Golgi membranes or whether it represented uninhibited mitochondrial activity or ATP-dependent calcium transport arising from another source. In an attempt to clarify this point we separated a crude particulate fraction on a Percoll gradient. The results are plotted as total activity per fraction in Fig. 7; they are representative of three separate experiments. Three peaks of ATP-dependent calcium uptake were observed in this separation (Fig. 6, TOP). The first, at a specific gravity of 1.055 to 1.060 (Fractions 8–9), corresponded to the peak of activity of the mitochondrial marker, succinate dehydrogenase. We are currently investigating the possibility that this peak results from mitochondrial calcium transport which is not inhibited by our combination of inhibitors. The second peak (Fractions 11–12), at a specific gravity of 1.04–1.05, corresponded to the peak of galactosyl transferase activity and thus probably is representative of Golgi membranes. The highest specific activity for ATP-dependent calcium transport, found in fraction 12, was 3.1 $nmol \cdot min^{-1} \cdot mg^{-1}$, approximately equivalent to the specific activity of the $P_{1,3}$ obtained from the sucrose gradient. The third peak (Fraction 16) was seen in two of the three experiments. It appeared to correspond neither to the mitochondrial or Golgi fractions. This experiment suggests that there are multiple ATP-dependent calcium uptake systems in mammary membranes. Calcium transport by membrane fractions other than the Golgi-enriched fraction is currently under study in this laboratory.

Calcium-Stimulated Phosphorylation of the Golgi-Enriched Membrane Fraction

All characterized calcium transport ATPases are phosphorylated, in an intermediate step, by the γ-phosphate of ATP (Carafoli & Crompton, 1980). To determine whether the same mechanism operates in the Golgi-enriched fraction, we studied its phosphorylation under ionic conditions which favor formation of a stable phosphoprotein intermediate in the sarcoplasmic reticulum system (Yamada & Tonomura, 1972). Data from four experiments on two separate membrane preparations are presented in Table 3. In the presence of calcium there was a variable increase in phosphorylation averaging 6 nmol/g protein. Phosphorylation of the Golgi fraction was stable in the presence of 6% trichloroacetic acid, but decreased about 6 nmol/g protein following treatment with hydroxylamine.

Table 3. Calcium-dependent phosphorylation of Golgi-enriched membranes

Calcium	Post-treatments		Phosphorylation[c] (nmol/g protein)			
	NaAc[a]	Hydroxylamine[b]	Exp 1	Exp 2a	Exp 2b	Exp 2c
0	0	0	16.2	4.7	5.1	23.0
+	0	0	25.2	7.1	14.0	27.4
+	+	0	ND	ND	9.7	20.7
+	+	+	ND	ND	4.9	12.8
Calcium-dependent phosphorylation			9.0	2.4	8.9	4.4
Hydroxylamine-sensitive phosphorylation					4.8	7.9

[a] Incubated 10 min at room temp in 0.08 M sodium acetate after phosphorylation.
[b] Incubated 10 min at room temp in 0.08 M sodium acetate plus 0.6 M hydroxylamine after phosphorylation.
[c] Values represent the average of duplicate assays. Experiments 1 and 2 represent two different membrane preparations.

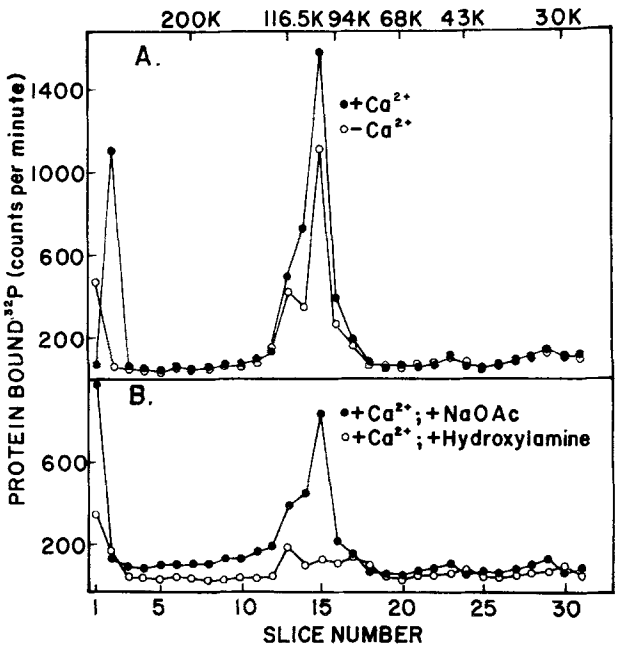

Fig. 8. Separation of phosphorylated protein by 5.6% polyacrylamide gel electrophoresis. *A.* Golgi-enriched membranes incubated as described in Materials and Methods with γ-[^{32}P]-ATP in the absence (closed circles) and presence (open circles) of 50 μM free calcium. *B.* Golgi membranes incubated in the presence of 50 μM added calcium. A 10-min incubation in 80 mM Na acetate, pH 5.2 with (open circles) or without (closed circles) 0.6 M hydroxylamine at room temperature followed the second trichloroacetic acid wash (*see* Materials and Methods)

To estimate the molecular weight of the phosphoprotein complex we dissolved the phosphorylated membranes in SDS and subjected the resulting solution to polyacrylamide gel electrophoresis. Figure 8 shows results from a typical experiment in which radioactivity is plotted as a function of gel slice; the position of proteins of known molecular weight, separated on duplicate gels, is indicated. The top curves of Fig. 8 show a distinct peak at about 100,00 Daltons. When 50 μM calcium was present in the incubation medium the peak height was significantly increased. Hydroxylamine entirely removed this peak. Incubation of the phosphoprotein in acetate buffer without hydroxylamine gave a similar peak, but of reduced height.

These data clearly show acid-stable, hydroxylamine-labile phosphorylation of a protein with a molecular weight of approximately 100,000 Daltons. The phosphorylation was enhanced by calcium at a free concentration of 50 μM. These characteristics suggest that the mammary Golgi membrane possesses a calcium transport protein similar to the one found in sarcoplasmic reticulum which has a molecular weight of approximately 100,000 Daltons (Tada et al., 1978). In this respect, the transport system differs from that present in the plasma membrane of red blood cells which has a molecular weight of about 150,000 Daltons (Schatzmann & Bürgin, 1978).

We have no explanation for our consistent finding of heavy phosphorylation of the 100,000 Dalton peak in the absence of added calcium. It is unlikely, however, that this incorporation represents the phosphorylated intermediate of the (Na+K)-ATPase, whose catalytic subunit has been shown to have a molecular weight of 100,000 Daltons (Knauf, Proverbio & Hoffman, 1974), because the conditions of our assay (zero sodium; 100 mM potassium) do not favor its formation. We are currently investigating the possibility that the enzyme exists in calcium-dependent and calcium-independent forms (Inesi, Cohen & Coan, 1976).

All the curves show a shoulder at about 116,000 Daltons. The identity of this peak is unknown, but it is acid stable and appears to be less sensitive to calcium and hydroxylamine than the peak at 100,000 Daltons.

Conclusions

The experiments presented here show that particulate fractions from the mammary gland of lactating mice transport calcium ion against a concentration gradient in the presence of ATP. The transport takes place in the presence of high concentrations of mitochondrial inhibitors, suggesting that nonmitochondrial membranes are involved. The crude particulate preparation was fractionated by differential and den-

sity-gradient centrifugation to obtain a Golgi-enriched component, identified by a 14-fold enhancement of galactosyl transferase activity and negligible succinate dehydrogenase activity. This component showed the highest specific activity of ATP-dependent calcium transport. Digitonin had no significant effect on calcium transport by this fraction suggesting that the Golgi apparatus and not contaminating plasma membranes are responsible for the observation.

Further insight into the nature of the transport molecule is provided by the finding that a 100,000 Dalton protein in the Golgi-enriched membrane fraction is phosphorylated in the presence of γ-[^{32}P]-ATP. Phosphorylation was enhanced by 50 μM calcium. The phosphoprotein intermediate was acid stable and hydroxylamine labile, indicating acylphosphate formation as in the other transport ATPases (Carafoli & Crompton, 1978). The calcium ATPase of the sarcoplasmic reticulum of skeletal muscle has a similar molecular weight as do the phosphoprotein transport intermediates of vesicles purified from synaptosomal membranes (Blitz et al., 1977; Papzian, Rahaminoff & Goldin, 1979). Evidence is beginning to appear in the literature that a Ca-ATPase like that which has been extensively characterized in sarcoplasmic reticulum is present in smooth endoplasmic reticulum of cell types other than muscle (Blaustein et al., 1980; Henkart, 1980). The present work represents the first firm evidence for the localization of an enzyme of similar molecular weight in the Golgi membrane.

This research was supported by Grant AM 15807 from the National Institute for Arthritis and Metabolic Disease to Margaret C. Neville and by faculty research grants from Middlebury College to Christopher Watters. Margaret C. Neville was the recipient of Research Career Development Award 5 K04-AM-0038. The authors wish to thank Tom Keenan for his assistance in applying Golgi membrane fractionation techniques to mouse tissues.

References

Baumrucker, C.W., Keenan, T.W. 1975. Membranes of mammary gland. X. Adenosine-triphosphate-dependent calcium accumulation by Golgi-apparatus-rich fractions from bovine mammary gland. *Exp. Cell Res.* **90**:253–260

Black, B.L., McDonald, J.M., Jarett, L. 1980. Characterization of Mg^{2+} and $(Ca^{2+}+Mg^{2+})$-ATPase activity in adipocyte endoplasmic reticulum. *Arch. Biochem. Biophys.* **199**:92–102

Blaustein, M.P., Ratzlaff, R.W., Schweitzer, E.S. 1980. Control of intracellular calcium in presynaptic nerve terminals. *Fed. Proc.* **39**:2790–2795

Blitz, A.L., Fine, R.E., Toselli, P.A. 1977. Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. *J. Cell Biol.* **75**:135–147

Carafoli, E., Crompton, M. 1978. The regulation of intercellular calcium. *Curr. Top. Membr. Transp.* **10**:151–216

Doss, R.C., Carraway, C.A.C., Carraway, K.L. 1979. Multiple forms of 5′-nucleotidase from lactating rat mammary gland resulting from the association of the enzyme with different membrane fractions. *Biochim. Biophys. Acta* **570**:96–106

Fairbanks, G., Steck, T.L., Wallach, D.F.H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606–2617

Hegyvary, C., Kang, K., Bandi, Z. 1979. Automated assay of phosphohydrolases by measuring the released phosphate without deproteinization. *Anal. Biochem.* **94**:397–401

Henkart, M. 1980. Identification and function of intracellular calcium stores in axons and cell bodies of neurons. *Fed. Proc.* **39**:2783–2789

Howell, S.L., Montague, W. 1975. Regulation by nucleotides of ^{45}calcium uptake in homogenates of rat islets of Langerhans. *FEBS Lett.* **52**:48–52

Inesi, G., Cohen, J.A., Coan, C.B. 1976. Two functional steps of sarcoplasmic reticulum ATPase. *Biochemistry* **15**:5293–5298

Jenness, R. 1979. Comparative aspects of milk proteins. *J. Dairy Res.* **46**:197–210

Jorgensen, P.L. 1974. Purification and characterization of $(Na^+ + K^+)$-ATPase. III. Purification from the outer medulla of mammalian kidney after removal of membrane components by sodium dodecyl sulfate. *Biochim. Biophys. Acta* **356**:36–52

Keenan, T.W., Huang, C.M., Morré, D.J. 1972. Membranes of the mammary gland. V. Isolation of Golgi apparatus and rough endoplasmic reticulum from bovine mammary gland. *J. Dairy Sci.* **55**:1577–1585

Kerrick, W.G.L., Donaldson, S.K.B. 1972. The effects of Mg^{2+} on submaximum Ca^{2+}-activated tension in skinned fibers of frog skeletal muscle. *Biochim. Biophys. Acta* **275**:117–122

Knauf, P.A., Proverbio, F., Hoffman, J.F. 1974. Electrophoretic separation of different phosphoproteins associated with Ca-ATPase and Na, K-ATPase in human red cell ghosts. *J. Gen. Physiol.* **63**:324–336

Kuhn, N.J., White, A. 1977. The role of nucleoside disphosphatase in a uridine nucleotide cycle associated with lactose synthesis in rat mammary-gland Golgi apparatus. *Biochem. J.* **168**:423–433

McGraw, C.F., Somlyo, A.V., Blaustein, M.P. 1980. Probing for calcium at presynaptic nerve terminals. *Fed. Proc.* **39**:2796–2801

Moore, L., Chen, T., Knapp, H.K., Jr., Landon, E.J. 1975. Energy-dependent calcium sequestration activity in rat liver microsomes. *J. Biol. Chem.* **250**:4562–4568

Moore, L., Pastan, I. 1977. Energy-dependent calcium uptake activity in cultured mouse fibroblast microsomes: Regulation of the uptake system by cell density. *J. Biol. Chem.* **252**:6304–6309

Murphy, E., Coll, K., Rich, J.L., Williamson, J.R. 1980. Hormonal effects on calcium homeostasis in isolated hepatocytes. *J. Biol. Chem.* **255**:6600–6608

Neville, M.C., Peaker, M. 1979. The secretion of calcium and phosphorus into milk. *J. Physiol.* **290**:59–67

Neville, M.C., Selker, F., Semple, K.S., Watters, C.D. 1980. ATP-dependent calcium transport by a Golgi membrane enriched fraction from mouse mammary gland. *J. Supramol. Struct. Suppl* **4**:101

Papazian, D., Rahaminoff, H., Goldin, S.M. 1979. Reconstitution and purification by "transport specificity fractionation" of an ATP-dependent calcium transport component from synaptosome-derived membranes. *Proc. Nat. Acad. Sci. USA* **76**:3708–3712

Pennington, R.S. 1961. Biochemistry of dystrophic muscle: Mitochondrial succinate-tetrazolium reductase and adenosine triphosphate. *Biochem. J.* **80**:648–654

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**:346–356

Poisner, A.M., Hava, M. 1970. The role of adenosine triphosphate and adenosine triphosphatase in the release of catecholamines from the adrenal medulla. IV. Adenosine triphosphate-activated uptake of calcium by microsomes and mitochondria. *Mol. Pharmacol.* **6**:407–415

Rudenberg, P., Watters, C.D. 1978. Changes in the protein composition of the bovine milk fat globule membrane during early lactation. *Physiologist* **21**:102

Saacke, R.G., Heald, C.W. 1974. Cytological aspects of milk formation and secretion. In: *Lactation, A Comprehensive Treatise.* B.L. Larson, editor. Vol. 2, pp. 147–189. Academic Press, N.Y.

Schatzmann, H.J., Bürgin, H. 1978. Calcium in red cells. *Ann. N.Y. Acad. Sci.* **307**:125–147

Sehlin, J. 1976. Calcium uptake by subcellular fraction of pancreatic islets. Effects of nucleotides and theophylline. *Biochem. J.* **156**:63–69

Sumida, M., Tonomura, Y. 1974. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. X. Direct evidence for Ca^{2+} translocation coupled with formation of a phosphorylated intermediate. *J. Biochem.* **75**:283–297

Tada, M., Yamamoto, T., Tonomura, Y. 1978. Molecular mechanism of active calcium transport by sarcoplasmic reticulum. *Physiol. Rev.* **58**:1–79

Wooding, F.B.P. 1977. Comparative mammary fine structure. *In*: Comparative Aspects of Lactation. M. Peaker, editor. pp. 1–42. Academic Press, London

Wuytack, F., Casteels, R. 1980. Demonstration of a (Ca^{++} and Mg^{++})-ATPase activity probably related to Ca^{++} transport in the microsomal fraction of porcine coronary artery smooth muscle. *Biochim. Biophys. Acta* **595**:257–263

Yamada, S., Tonomura, Y. 1972. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. VII. Recognition and release of Ca^{2+}ions. *J. Biochem.* **72**:417–425

Received 12 September 1980; revised 2 January 1981