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Casein kinase activity in rat mammary gland Golgi vesicles

Demonstration of latency and requirement for a transmembrane ATP carrier

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A Golgi vesicle-enriched preparation from mammary tissue of lactating rats has been used to investigate the phosphorylation of caseins *in vitro*. Casein kinase, together with its casein substrates, is enclosed within the lumen of Golgi membrane vesicles and has a requirement for Ca^{2+} and ATP. The permeability characteristics of the Golgi membrane to ATP and Ca^{2+} therefore have a possible regulatory influence on casein kinase activity. This influence has been investigated by alteration of the permeability characteristics by using several agents having differing degrees of selectivity. The ionophore A23187, which permits loss of Ca^{2+} from the vesicles, caused a decrease in casein phosphorylation which could be reversed by externally supplied Ca^{2+}. Alamethicin, an ionophore that creates larger transmembrane channels, caused an increase in casein phosphorylation. This increase showed a requirement for divalent metal ions which could be satisfied by either Ca^{2+} or Mn^{2+}. Under the same conditions, La^{3+} was inhibitory. Triton X-100 caused loss of intra-vesicular Ca^{2+}, yet this was accompanied by an increase in phosphate incorporation into the caseins. We conclude from these results that the binding site on casein kinase for ATP is within the Golgi membrane barrier and that they imply the presence of a transmembrane ATP-transport mechanism. Inhibition of casein phosphorylation by atractyloside and carboxyatractyloside lends support to this concept.

Golgi vesicles isolated from lactating rat mammary glands in such a manner as to maintain their osmotic integrity are able to synthesize lactose (Brodbeck & Ebner, 1966; Coffey & Reithel, 1968; Jones, 1972; Kuhn & White, 1977) when incubated with glucose and UDP-galactose. Both of these substrates undergo facilitated transport across the Golgi vesicle membrane (Kuhn & White, 1977, 1980) to reach the lactose synthetase which is situated within the lumen. The activity of this enzyme was found to be enhanced by the inclusion of low concentrations of Triton X-100 but was virtually abolished when the detergent concentration exceeded 0.025% (Kuhn *et al.*, 1980). Kuhn and co-workers postulated that the stimulation at low detergent concentrations was due to minor structural disorganization of the vesicle membrane which facilitated the transmembrane passage of substrates and other small solutes without allowing escape of macromolecules, in particular α-lactalbumin, which is a required constituent of the synthetase (Brew, 1969; Jones, 1972). At Triton X-100 concentrations above 0.025%, the membrane underwent sufficient disruption to allow loss of this protein, and therefore of lactose synthetase activity.

Abbreviation used: Tes, 2-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

We have shown that Golgi vesicles prepared in a similar manner also contain a casein kinase, situated probably on the luminal membrane surface, which incorporates additional phosphate groups into the caseins contained within the lumen when the vesicles are supplied with ATP (West & Clegg, 1983). This casein kinase bears a formal resemblance to lactose synthetase in that it requires both small molecules (Ca^{2+}, ATP) and a protein (one of the caseins) to function. The activity of this kinase undergoes an enhancement of similar magnitude to that experienced by lactose synthetase when these Golgi vesicles are exposed to low concentrations of Triton X-100 (West & Clegg, 1983). However, if the postulate advanced by Kuhn and colleagues (Kuhn *et al.*, 1980) to explain the detergent effect on lactose synthetase is also applicable to the casein kinase, then a diminution in casein phosphorylation would have

been expected rather than a stimulation. This is because minor structural disorganization of the Golgi membrane leading to an increase in its passive permeability to Ca^{2+} would cause a decrease in the intraluminal concentration of Ca^{2+} which would, as we have shown (West & Clegg, 1983), lead to a reduction in the activity of casein kinase.

Thus, either Ca^{2+} was not lost on detergent treatment, in contradiction to Kuhn's postulate, or its loss and the concomitant decrease in kinase activity was offset by other factors which stimulated casein phosphorylation. Based on the postulate of a less-organized Golgi membrane structure in the presence of Triton X-100, one possible mechanism for such a stimulation is that an increased diffusion of ATP across this membrane could lead to an increase in the velocity of casein kinase.

This paper describes the results of a series of experiments designed to investigate these possibilities. We demonstrate that Ca^{2+} is indeed lost from the Golgi vesicles when they are treated with low levels of Triton X-100 and have confirmed that in spite of this loss such treatment leads to an increase in casein phosphorylation. We have also made use of the ionophoretic properties of the polypeptide antibiotic alamethicin to study the influence of ATP accessibility on the activity of casein kinase. Alamethicin creates 'pores' or 'diffusion channels' across biological membranes large enough to permit unhindered passage of ATP (Jones *et al.*, 1980), so effectively removing any barrier between casein kinase and its nucleotide substrate in the Golgi vesicle system. In the presence of alamethicin, we report here that a much increased incorporation of ^{32}P into caseins is observed, indicating that increased permeation of ATP could indeed be responsible for the enhancement, by Triton X-100, of the activity of casein kinase. We interpret our results to indicate that the kinase has a binding site for ATP on the luminal side of the Golgi membrane, and we suggest, in the light of our findings with inhibitors of mitochondrial, and other organelle, adenosine nucleotide translocases, that permeation of this membrane by ATP is, at least in part, carrier-mediated.

Experimental

Animals and biological preparations

Golgi vesicles were prepared from the mammary tissue of lactating Wistar rats between 10 and 13 days after the birth of their litters. The maintenance of animals and methods for this preparation were as described previously (West, 1981; West & Clegg, 1983).

Chemicals and radiochemicals

A23187, atractyloside and carboxyatractyloside were purchased from BCL (Lewes, U.K.), and alamethicin was generously given by Dr. J. E. Grady of the Upjohn Company (Kalamazoo, MI, U.S.A.). $^{45}CaCl_2$ (0.48 Ci/mmol) and [γ-^{32}P]ATP (approx. 3000 Ci/mmol) were products of Amersham International (Amersham, Bucks., U.K.). All other chemicals and materials were purchased from Sigma except that EGTA was puriss grade from Fluka (Fluorochem Ltd., Glossop, Derby, U.K.). Free metal ion concentrations were calculated by means of an iterative computer program using the dissociation constants taken from Smith & Martell (1976) and Kerrick & Donaldson (1972).

Release of accumulated $^{45}Ca^{2+}$ by Triton X-100

Golgi vesicles (approx. 0.15 mg of protein) were incubated at 37°C in the following medium: 275 mM-lactose / 5 mM-MgATP / 0.1 mM-$^{45}CaCl_2$ (approx. 6.6 Ci/mol)/25 mM-Tes/NaOH buffer, pH 7.0, in a total volume of 0.65 ml. After 20 min the incubation was cooled to 0°C and a sample (0.1 ml) was filtered through a membrane filter (0.45 μm) previously soaked in 300 mM-sucrose containing 2 mM-$LaCl_3$ and 10 mM-Tris/HCl buffer, pH 7.0. To the remainder of the incubation was then added 2.75 μl of a solution of Triton X-100 in 275 mM-lactose/25 mM-Tes/NaOH buffer, pH 7.0, and further 0.1 ml samples were removed and filtered as above at the time intervals shown. Several incubations were treated, in parallel, in this way. The concentration of the Triton X-100 solution added was chosen to give the final concentrations shown in Fig. 1; for control incubations Triton X-100 concentration was zero. Sample remaining on each membrane filter was rapidly washed three times with 5 ml of the sucrose/$LaCl_3$/Tris/HCl solution, as above, at 0°C and its ^{45}Ca radioactivity was determined by liquid scintillation counting of the wet filters in 'Filtercount' scintillation cocktail (Packard).

Phosphorylation of caseins endogenous to the Golgi vesicle preparation

Generally, a sample of the Golgi preparation (0.5–2.0 mg of protein/ml) was added to an iso-osmotic lactose solution (275 mM-lactose/25 mM-Tes/NaOH buffer, pH 7.0) containing [γ-^{32}P]ATP (40 μM; approx. 1200 c.p.m./pmol) without added Mg^{2+} as described previously (West & Clegg, 1983). Variations in the composition of the medium are given in the Figure legends. When alamethicin or A23187 were used, the Golgi vesicles were preincubated with the ionophore for 20 min at 0°C before addition to the reaction mixture. Alamethicin was used in a 1 : 2 (w/w) ratio with Golgi protein; A23187, at a final concentra-

tion of 1 µM. Incubations were at 0°C unless otherwise indicated and phosphorylation was terminated after 40s by the addition of ice-cold 10% (w/v) trichloroacetic acid containing 20mM-NaH_2PO_4 and 1mM-ATP. Casein phosphorylation was linear over this period in the presence of ionophores or Triton X-100. The precipitated protein was collected by centrifugation, washed with acetone at 0°C, then disaggregated by boiling in a buffered solution of sodium dodecyl sulphate prior to electrophoresis in sodium dodecyl sulphate/polyacrylamide gels (Laemmli & Favre, 1973). Radioautography of dried gels at −80°C was used to locate the phosphorylated caseins. They were then cut from the gels and their content of ^{32}P radioactivity was determined by liquid-scintillation counting.

Results

Influence of Triton X-100 on Ca^{2+}-retention by Golgi vesicles

Treatment of Golgi vesicles, which were actively accumulating Ca^{2+}, with Triton X-100 caused a loss of the cation. The extent of this loss was, however, very dependent on the concentration of detergent used. At 1min after the addition of 0.01% Triton X-100, vesicles contained only 71% of the Ca^{2+} present in controls treated with buffer containing no detergent. When vesicles were similarly treated with 0.02% Triton X-100, this value was reduced to 58%, and was further reduced to 12% when the detergent concentration was 0.05%. The content of Ca^{2+} in these vesicles at subsequent time-points is documented in Fig. 1. It can be seen that at 0.01% Triton X-100, despite the initial loss of Ca^{2+}, accumulation of the ion continues thereafter at approximately the same rate as in controls. This accumulation does not occur in vesicles treated with higher concentrations of detergent.

This pattern of Ca^{2+} loss can be explained on the basis of the distribution of the ion within Golgi vesicles, where it exists either bound to the caseins and other proteins or in solution as soluble salts and the free ion (Baumrucker, 1978). In accordance with the proposals of Kuhn *et al.* (1980) the initial loss of Ca^{2+} on treatment with less than 0.025% Triton X-100 represents soluble salts whereas the retained calcium is that bound to the caseins. At greater concentrations of detergent, complete disruption of the membrane leads to almost total loss of the caseins, and hence of their associated calcium, as well as the soluble Ca^{2+}. An alternative explanation of the effects of Triton X-100 is that the detergent caused total lysis of a proportion of the vesicles and that this proportion increased with detergent concentration. This explanation carries the corollary that the remaining intact vesicles should then continue to accumulate Ca^{2+}, so that in the presence of detergent concentrations (<0.05%) that caused only partial loss of Ca^{2+}, accumulation of the ion should still be observed. Although such accumulation did persist in 0.01% Triton X-100-treated vesicles, its absence in preparations treated with 0.02% and 0.025% Triton X-100 (Fig. 1) argues against the generality of this proposal.

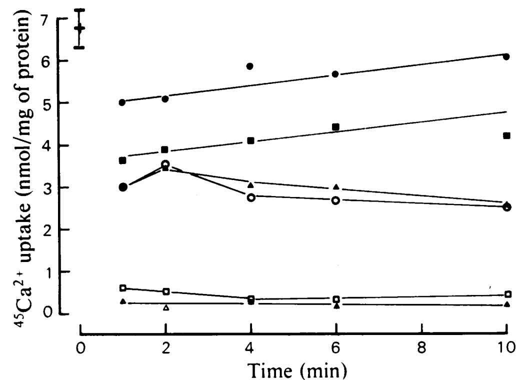

Fig. 1. *Effect of Triton X-100 on the retention of $^{45}Ca^{2+}$ previously accumulated into rat mammary gland Golgi vesicles and on their continued ability to accumulate Ca^{2+}*
Golgi vesicles, pre-loaded with $^{45}Ca^{2+}$ [+ ±S.E.M. (n = 6)], were treated with various concentrations of Triton X-100 as described in the Experimental section. ●, Control (no Triton X-100); ■, 0.01% Triton X-100; ○, 0.02% Triton X-100; ▲, 0.025% Triton X-100; □, 0.05% Triton X-100; △, 0.1% Triton X-100. All Triton X-100 concentrations are % (w/v). A representative experiment is shown.

Effects of alamethicin on casein phosphorylation

The ionophore alamethicin has been reported to activate membrane-enclosed ATP-dependent enzymes by facilitating transmembrane diffusion of ATP (Besch *et al.*, 1977). An effect of this type was noted with Golgi vesicles: when they were preincubated with alamethicin and then exposed to [γ-^{32}P]ATP, a 2–3-fold increase in casein phosphorylation relative to non-alamethicin-treated controls resulted (Table 1). The presence of the ionophore would also have resulted in enhanced permeability to Ca^{2+} (Besch *et al.*, 1977), the normal consequence of which is a loss of vesicle-associated calcium, resulting in a reduction in casein kinase activity (West & Clegg, 1983). The increased kinase activity observed in the presence of alamethicin thus indicates that enhanced access to ATP had a quantitatively greater influence on kinase activity than the opposing effect of the loss of intravesicular Ca^{2+}.

Treatment of Golgi vesicles with this ionophore

Table 1. *Effects of alamethicin, A23187 and Triton X-100 on the phosphorylation of endogenous caseins by rat mammary gland Golgi vesicles*

Golgi vesicles were incubated with [γ-^{32}P]ATP as described in the Experimental section. Alamethicin (0.5 mg/mg of Golgi protein), A23187 (1 μM) or Triton X-100 (0.05%) were added as indicated. N.D., not determined. The results shown are mean values from duplicate incubations in a single complete experiment. The individual effects of each incubation condition relative to a control incubation have been observed during at least two further experiments.

Additions to standard experimental conditions		^{32}P incorporation into caseins (pmol/mg of Golgi protein)		
Preincubation	Phosphorylation incubation	Casein M_r 42000	Casein M_r 27000	Casein M_r 23000
None	None	1.82	0.70	6.69
Alamethicin	None	4.61	1.63	8.05
None	1 mM-$CaCl_2$	6.91	1.11	7.78
Alamethicin	1 mM-$CaCl_2$	47.30	8.57	66.84
A23187	1 mM-$CaCl_2$	10.12	N.D.	11.41
Alamethicin/A23187	1 mM-$CaCl_2$	46.10	9.52	75.45
None	Triton X-100	7.48	1.33	10.46
None	Triton X-100/1 mM-$CaCl_2$	26.61	4.36	36.35

was, therefore, analogous in its effects to treatment with low concentrations of Triton X-100. The loss of intraluminal Ca^{2+} normally accompanying alamethicin treatment could be offset by addition of Ca^{2+} to the medium. When this was done, a massive increase in ^{32}P incorporation into caseins relative to controls was observed (Table 1). The same concentration of Ca^{2+} in the presence of the ionophore A23187 (which has no reported effects on adenosine nucleotide permeability in biological membranes) had comparatively little effect on casein phosphorylation when compared with the appropriate control, and inclusion of A23187 together with alamethicin and Ca^{2+} had no synergistic effect (Table 1). Evidently, the 6–8-fold increase in casein kinase activity following alamethicin treatment in the presence of Ca^{2+} was not due solely to the introduction of excess Ca^{2+}.

For comparison, Golgi vesicles were also incubated under phosphorylating conditions in the presence of 0.05% Triton X-100, the detergent concentration optimally effective for provoking an increase in casein phosphorylation (West & Clegg, 1983). As can be seen from the results in Table 1, while this concentration of Triton X-100 clearly increased ^{32}P incorporation relative to controls, the magnitude of its effect in the presence of Ca^{2+} was only half that of alamethicin treatment. As discussed above, this concentration of Triton X-100 would have disrupted the vesicles, allowing the previously entrapped caseins to be diluted by the bulk phase. In contrast, the alamethicin-treated vesicles would have retained their caseins, thereby ensuring an effectively higher concentration in the vicinity of casein kinase, of one of its substrates (casein) and allowing, as a result, a more rapid rate of casein phosphorylation than in detergent-treated Golgi vesicles.

Effects of Ca^{2+}, Mn^{2+} and La^{3+} on casein phosphorylation in the presence of alamethicin

When Golgi vesicles pretreated with alamethicin were incubated with [γ-^{32}P]ATP in the presence of EGTA, little casein phosphorylation resulted, indicating that unrestricted access of ATP to the interior of the vesicle is not itself a sufficient condition for casein phosphorylation, and confirming previous reports (Bingham & Groves, 1979; West & Clegg, 1981*a*,*b*, 1983; Szymanski & Farrell, 1982) that casein kinase

Table 2. *Effects of Ca^{2+} and Mn^{2+} in the presence of alamethicin on the phosphorylation of endogenous caseins by rat mammary gland Golgi vesicles*

Golgi vesicles were incubated with [γ-^{32}P]ATP as described in the Experimental section, after preincubation with alamethicin. Free Ca^{2+} concentrations of 10^{-6} and 10^{-5} M were achieved by use of a Ca^{2+}/EGTA buffer system; in all other cases, the values given are total metal ion concentrations (added as metal chlorides) in the absence of EGTA. N.D., not determined: additional phosphorylations, facilitated by Mn^{2+}, made determination of ^{32}P incorporation into the lower-M_r caseins ambiguous. Replication of results as in Table 1.

Additions to phosphorylation incubation	[Ca^{2+}] (M)	^{32}P incorporation into caseins (pmol/mg of Golgi protein) Casein M_r 42000	Casein M_r 27000	Casein M_r 23000
None	–	6.5	2.3	11.3
EGTA (2 mM)	$<10^{-7}$	1.0	0.4	1.6
Ca^{2+}/EGTA	10^{-6}	1.1	0.6	1.9
Ca^{2+}/EGTA	10^{-5}	1.0	0.5	1.7
Ca^{2+}	10^{-4}	17.3	5.3	27.4
Ca^{2+}	10^{-3}	66.4	12.0	93.8
Mn^{2+} (0.1 mM)	–	20.1	N.D.	N.D.
Mn^{2+} (1 mM)	–	32.7	N.D.	N.D.

requires divalent metal ions. This requirement was not satisfied by Ca^{2+} at 1 μM and 10 μM (Table 2). Raising the Ca^{2+} concentration to 100 μM (in the absence of other metal cations) resulted in an almost 3-fold increase in ^{32}P incorporation, whilst at 1 mM-Ca^{2+} casein phosphorylation was around 10 times that observed in controls. Mn^{2+} is normally present in Golgi vesicles from lactating mammary glands, where it is a required cofactor for lactose synthetase. It was more effective than Ca^{2+} at the 100 μM level, but this was reversed at higher concentrations such that it was less than one-half as stimulatory as Ca^{2+} at 1 mM (Table 2).

In the presence of Ca^{2+} and alamethicin, La^{3+} caused a 70% inhibition in casein phosphorylation (Table 3), indicating that La^{3+} could effectively compete for the intravesicular Ca^{2+}-binding sites of casein kinase. Surprisingly, La^{3+} also caused a substantial decrease in the activity of the kinase even when alamethicin was not included; addition of Ca^{2+} under these conditions was ineffective in preventing inhibition by La^{3+}.

Effects of atractyloside and carboxyatractyloside on casein phosphorylation

Since our experiments with alamethicin had suggested that some form of ATP-translocator may exist in the Golgi membrane, we investigated the effects of including atractyloside and carboxyatractyloside in phosphorylation incubations. Atractyloside, at a 1:2 molar ratio with respect to ATP, had little effect on casein phosphorylation when incubations were done in the normal way at 0°C. Phosphorylation was, however, inhibited (approx. 30%) when the inhibitor:ATP ratio was increased to 5:1 while maintaining the same

Table 3. *Effects of lanthanum on the phosphorylation of endogenous caseins by rat mammary gland Golgi vesicles*
Golgi vesicles were incubated with [γ-^{32}P]ATP as described in the Experimental section. Preincubation with alamethicin preceded the phosphorylation incubation where indicated. Replication of results as in Table 1.

Additions to standard experimental conditions		^{32}P incorporation into caseins (pmol/mg of Golgi protein)		
Preincubation	Phosphorylation incubation	Casein M_r 42000	Casein M_r 27000	Casein M_r 23000
None	None	2.6	1.0	9.4
None	1 mM-$CaCl_2$	9.7	1.6	10.9
None	1 mM-$LaCl_3$	0.7	0.4	1.5
None	1 mM-$CaCl_2$ + 1 mM-$LaCl_3$	1.2	0.9	1.7
Alamethicin	None	6.5	2.3	11.3
Alamethicin	1 mM-$CaCl_2$ + 1 mM-$LaCl_3$	2.3	1.2	3.1

Table 4. *Effects of atractyloside and carboxyatractyloside on the phosphorylation of endogenous caseins by rat mammary gland Golgi vesicles*
Golgi vesicles were incubated with [γ-^{32}P]ATP as described in the Experimental section, with modifications as below. Atractyloside and carboxyatractyloside were 200 μM. All incubations contained 1 mM-$CaCl_2$. N.A., not applicable. Replication of results as in Table 1.

Additions to standard experimental conditions				^{32}P incorporation into caseins (pmol/mg of Golgi protein)		
Preincubation	Phosphorylation incubation	Temperature of phosphorylation (°C)	Molar ratio inhibitor:ATP	Casein M_r 42000	Casein M_r 27000	Casein M_r 23000
None	None	0	N.A.	1.95	0.39	2.64
None	Atractyloside	0	1:2	1.95	0.40	2.09
None	Atractyloside	0	5:1	1.31	0.17	1.55
None	Carboxyatractyloside	0	5:1	1.66	0.22	1.93
None	A23187	0	N.A.	3.05	0.69	4.15
None	A23187 plus atractyloside	0	1:2	2.86	0.50	3.65
None	None	37	N.A.	7.35	1.95	13.27
None	Atractyloside	37	1:2	4.70	0.97	10.11
None	Atractyloside	37	5:1	3.23	0.78	5.48
None	Carboxyatractyloside	37	5:1	4.36	0.92	7.49
Alamethicin	None	37	N.A.	23.60	5.20	24.50
Alamethicin	Atractyloside	37	5:1	26.70	6.50	27.40

concentration of atractyloside (Table 4), suggesting that the glycoside was acting as a competitive inhibitor. Carboxyatractyloside was a less effective inhibitor of kinase activity. At an incubation temperature of 37°C, with consequently increased rates of casein phosphorylation, atractyloside was inhibitory at both 1:2 and 5:1 molar ratios with ATP. Either binding of the glucoside to the putative adenine nucleotide translocator occurred with increased efficiency at 37°C, or the rate of nucleotide transport into the Golgi lumen at this temperature exerted a greater degree of control on the overall pathway of casein phosphorylation, and therefore the pathway became more responsive to inhibition of the translocator.

In the presence of alamethicin, when the requirement for the ATP translocator was effectively bypassed, atractyloside had no inhibitory effect on casein phosphorylation (Table 4).

Discussion

The use of alamethicin to unmask the activities of a number of purine nucleotide-requiring enzymes has been reported (Besch *et al.*, 1977; Lad & White, 1979; Bonnafous *et al.*, 1982; Manalan & Jones, 1982) and in some cases it has been shown, by the persistence of functional coupling to hormone receptors, that such unmasking was achieved without generalized membrane disruption (Jones *et al.*, 1980). The activation of such latent enzymes has been attributed to increased access of nucleotide substrates across a biomembrane to their enzyme active sites. The results reported here show that when Golgi vesicles incubated in ATP-containing media are treated with alamethicin, an enhancement of casein kinase activity results. This indicates that, in intact Golgi vesicles, a membrane permeability barrier limits access of ATP in the bulk phase to casein kinase, and allows the conclusion that the ATP-binding site(s) of casein kinase is at a location luminal to the Golgi vesicle membrane. The question then arises as to how, under normal circumstances, the nucleotide crosses this membrane.

Kuhn's group (Kuhn & White, 1977) has presented evidence that the Golgi membrane is impermeable to UDP; it seems likely that the same will be true for ATP, and therefore that a specific carrier exists in the Golgi membrane. In support of this suggestion, we have demonstrated above that atractyloside (and to a lesser extent, carboxyatractyloside), the well-documented inhibitors of the mitochondrial adenine nucleotide translocator (for review see Stubbs, 1981), also inhibit casein kinase activity in intact Golgi vesicles, albeit at much higher concentrations than are effective against the mitochondrial carrier. However, since the primary function of the mitochondrial carrier is to exchange cytosolic ADP for intramitochondrial ATP, it is not a very suitable functional analogue to the putative Golgi transporter. The latter has more in common with a second mitochondrial carrier (Reynafarje & Lehninger, 1978) which allegedly catalyses entry of ATP from the cytosol in exchange for exit of ADP and inorganic phosphate. Kuhn & White (1977) have calculated that more inorganic phosphate is produced in the Golgi vesicles of rat mammary tissue as a consequence of lactose synthesis than is necessary to account for the phosphate content of rat milk: thus a requirement may exist to export this 'surplus' inorganic phosphate from the Golgi lumen into the cytosol. A nucleotide transporter of the Lehninger type described above could combine this function with that of ADP export in exchange for entry of ATP, the requisite phosphoryl donor in casein phosphorylation.

Regardless of the exact mechanism of ATP entry into Golgi vesicles, we have shown that, under certain circumstances, the rate of nucleotide delivery into the lumen can limit casein kinase activity. This being so, interpretation of some of our previous results (West & Clegg, 1981*a*, 1983) requires reappraisal. In that earlier work we had used the ionophore A23187 to equilibrate Ca^{2+} across the Golgi membrane; from a comparison of the extent of casein phosphorylation at various buffered concentrations of Ca^{2+} we had concluded that the vesicles as prepared contained between 0.1 mM and 1 mM free Ca^{2+}. In the present work we have repeated these experiments, but using alamethicin to prevent possible ATP limitation from obscuring the quantitative characteristics of Ca^{2+}-dependence of casein kinase. The results presented above (Table 2) indicate that the extents of casein phosphorylation obtained under these conditions with 0.01 mM- and 0.1 mM-Ca^{2+} bracket that observed in untreated vesicles (see Table 1). This could imply that the intravesicular free Ca^{2+} in untreated vesicles lies between these two concentrations, but the uncertainty surrounding the intravesicular concentration of ATP in such vesicles makes it unclear at present whether this estimate, or our previous higher value, gives a better indication of the free Ca^{2+} in this compartment.

The decrease in casein phosphorylation observed when La^{3+} was added to alamethicin-treated vesicles can be attributed to the competition between La^{3+} and Ca^{2+} for the intravesicular Ca^{2+}-binding site of casein kinase. However, the decrease in ^{32}P incorporation caused by La^{3+} when intact vesicles were used requires additional explanation. Although extravesicular Ca^{2+} is not an obligate requirement for casein phosphoryl-

ation (West & Clegg, 1983) the results presented above (Table 3) show that externally added Ca^{2+} does enhance ^{32}P incorporation. These Golgi vesicles accumulate Ca^{2+} when supplied with ATP (West, 1981), so this enhancement of phosphorylation can be attributed to an increase in intravesicular Ca^{2+} arising from the activity of the transporting system. La^{3+} is a well-documented inhibitor of Ca^{2+} transport (Schatzman & Tschabold, 1971; Weiner & Lee, 1972; Quist & Roufogalis, 1975) and we have previously demonstrated that it inhibits Ca^{2+} accumulation by these Golgi vesicles (West, 1981). In the present work, when La^{3+} was included with Ca^{2+} its presence prevented transport of the added Ca^{2+} and thereby prevented the enhancement of phosphorylation customarily caused by the additon of Ca^{2+} alone. However, La^{3+} also inhibited ^{32}P incorporation in the absence of added Ca^{2+}, a circumstance under which Ca^{2+} transport is not required for casein phosphorylation (West & Clegg, 1981*b*, 1983). Under these conditions, therefore, inhibition of casein phosphorylation by La^{3+} must be unrelated to its inhibition of Ca^{2+} transport. The possibility thus arises that La^{3+} may also act as a substrate for the Golgi Ca^{2+} translocator, thereby gaining access to the interior of the intact vesicle where it can bind to and inhibit casein kinase.

These results and those of previous studies (West & Clegg, 1983) demonstrate that mammary gland Golgi casein kinase functions within the Golgi lumen in an environment determined by the properties of the vesicle membrane, in particular by the activities of Ca^{2+}- and ATP-translocating systems. The use of ionophores of known specificity has allowed characterization of some of the properties of this casein kinase without requiring that it be physically removed from the Golgi vesicle. Access of ATP to the kinase has been shown to be a key regulator of the rate of casein phosphorylation. The kinetic properties and possible control of the Golgi membrane ATP carrier therefore assume particular importance since the rate of casein phosphorylation *in vivo* may be one of the determinants of the rate of milk production.

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