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TRANSIENT FORMATION OF A PHOSPHOPROTEIN DURING AUTOPHOSPHORYLATION OF RAT MAMMARY GLAND GOLGI VESICLES

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Incubation with [γ-^{32}P]ATP of Golgi vesicles, prepared from the mammary tissue of lactating rats, resulted in the phosphorylation of four of the proteins in the preparation which were resolvable by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Three of these had electrophoretic properties identical to the three major caseins of rat milk: their phosphorylation was approximately linear with respect to time during the course of the short (1 min) incubations analyzed. The fourth component ($M_{r,app.}$ 70000) behaved differently. It was very rapidly phosphorylated to a maximum level within 5 s at 0°C; its ^{32}P-content declined thereafter, with a $t_{1/2}$ for dephosphorylation of approx. 20 s. The extent of ^{32}P incorporation into this component, measured after incubation for 20 s at 0°C with [γ-^{32}P]ATP, was sensitive to the concentration of Ca^{2+} in the incubation medium, being enhanced at low concentrations ($<10^{-8}$ M) of Ca^{2+} and depressed at high (10^{-4} M) ones. Inclusion of ADP (100 μM) in such incubations also depressed ^{32}P incorporation into the 70 kDa component. This phosphoprotein was further distinguished from the other three by virtue of the lability of its incorporated phosphorus to treatment with hot trichloroacetic acid. The properties and possible function of this phosphoprotein are discussed in relation to the ATP-dependent Ca^{2+} transport that occurs in this Golgi vesicle preparation.

1. Introduction

Although calcium transport across biological membranes is a wide-spread phenomenon, the most thoroughly investigated Ca^{2+}-accumulation system remains that of the membranous vesicles of mammalian muscle sarcoplasmic reticulum [1]. Many of the details of the mechanism of Ca^{2+}-translocation first elucidated in this system have been shown to be applicable to other Ca^{2+}-transport systems: in particular, the reversible phosphorylaton of one or more enzyme protein components during Ca^{2+}-translocation has been shown to be a common feature of most ATP-dependent ion transport systems [2–8].

During lactation, mammary tissue secretes large quantities of calcium into milk and evidence is accumulating that the route of this calcium secretion is via the Golgi apparatus of the secretory cells [9]. We have previously characterised some aspects of the ATP-dependent accumulation of Ca^{2+} by a Golgi vesicle preparation isolated from the lactating rat mammary gland [10], but in an earlier study of the ATP-dependent phosphorylation of proteins present in this Golgi vesicle preparation we were unable to detect the presence of any transiently-phosphorylated protomers [11]. By analogy with the sarcoplasmic reticulum and other Ca^{2+}-transporting systems such unstable phosphoprotein components would be expected to be part of the Ca^{2+}-translocating enzyme system. We have therefore further investigated the ATP-dependent auto-phosphorylation of the proteins of the Golgi vesicle preparation, and in this com-

munication report the presence of a transiently-phosphorylated protein component the phosphorylation of which is dependent on the concentration of Ca^{2+} in the medium. However, unlike the enzyme-phosphate intermediate of the sarcoplasmic reticulum Ca^{2+}-ATPase, the transiently-phosphorylated component of the Golgi membranes is maximally phosphorylated in the absence of Ca^{2+}.

2. Materials and Methods

Golgi vesicles, prepared by a slight modification of the method of Kuhn and White [12], were incubated at 0°C in a total volume of 100 μl in 100 mM 2-(*N*-Morpholino)ethanesulphonic acid (Mes)-Tris, pH 6.8, containing 6 μM [γ-^{32}P]ATP ($9.4 \cdot 10^6$ cpm/nmol). The reaction was initiated by the addition of Golgi vesicles (final concn. approx. 1 mg protein/ml), and terminated by the addition of 500 μl 10% (w/v) ice-cold trichloroacetic acid containing 20 mM sodium phosphate and 1 mM ATP. The precipitated protein was recovered by centrifugation and washed once, by resuspension in the same trichloroacetic acid solution followed by re-sedimentation. The resulting pellets were either washed a further three times as above and subjected directly to scintillation counting for the determination of ^{32}P-radioactivity or disaggregated and analysed by SDS-polyacrylamide gel electrophoresis as described by Clegg and Skyrme [13], using 80 × 80 mm slab gels. Dried polyacrylamide gel slabs were autoradiographed onto 3M-R2 medical X-ray film. Densitometric scans were obtained by using the scanning accessory of a Gilford 250 spectrophotometer.

Caseins were isolated from rat milk as described by Rosen et al. [14].

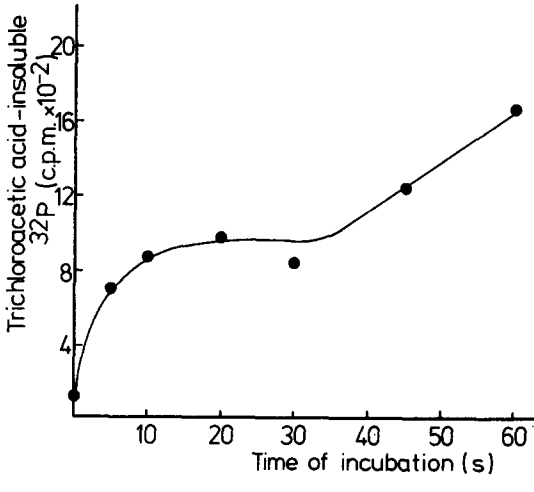

Fig. 1. Time-course of phosphorylation of Golgi vesicles in the presence of [γ-^{32}P]ATP. Conditions for incubation and precipitation with trichloroacetic acid were as described in the text. The result shown is of one experiment, representative of four that have been done. Total ^{32}P incorporation after 60 s was 8.3 pmol per mg protein.

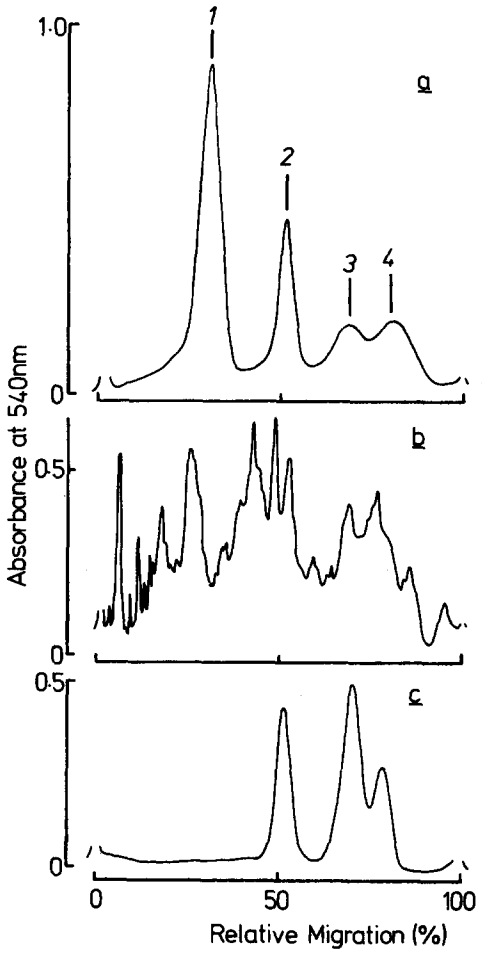

Fig. 2. Identification, by SDS-polyacrylamide gel electrophoresis, of Golgi vesicle proteins phosphorylated in the presence of [γ-^{32}P]ATP. Densitometric scans are shown above. (a) Autoradiogram exposed from a gel electrophoresis separation of Golgi vesicles phosphorylated, as described in the text, for 20 s; (b) Coomassie blue-stained gel separation of the same material; (c) Coomassie blue-stained separation of a mixture of the three major caseins isolated from rat milk.

3. Results and Discussion

Fig. 1 shows the kinetics of incorporation of radioactivity from [γ-^{32}P]ATP into trichloroacetic acid-insoluble material during incubation of the Golgi vesicle preparation at 0°C. Two distinct phases of phosphorylation were evident: during the first, a plateau of incorporation was rapidly reached (within the first 10 s of incubation) and was maintained over about 45 s. This is similar to the behaviour of sarcoplasmic reticulum vesicles incubated under comparable conditions [15]. The second phase began after 45 s when a slow increase in phosphorylation became evident.

To characterize further the phosphorylated protein components, trichloroacetic acid-insoluble material was recovered by centrifugation after 20 s of incubation (as in Fig. 1) and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The result of such an experiment is shown in Fig. 2a. For reference purpose densitometric profiles of co-electrophoresed Golgi vesicles and isolated rat caseins (both Coomassie blue-stained) are shown in Figs. 2b and 2c, respectively. Four distinct bands of ^{32}P-labelled protein (1–4 in Fig. 2a) can be distinguished in the autoradiogram with apparent molecular weights of 70000, 42000, 27000 and 23000, respectively. The kinetics of ^{32}P-incorporation into the individual phosphorylated components were determined by SDS-gel electrophoresis, autoradiography and quantitative densitometry of samples removed after various times of incubation between 0 and 60 s. In this quantitative analysis, the resolution between phosphoproteins 3 and 4 was incomplete and they were therefore treated as a single component. Phosphoproteins 2 and 3 + 4 showed a relatively slow and progressive increase in phosphorylation over this time-course (Fig. 3). The increase in ^{32}P-incorporation observed after 30 s in Fig. 1 and which we have previously reported to continue over a 10 min period [11] was evidently due to phosphorylation of these three proteins. We here propose a tentative identification of these with the three major caseins of rat milk on the basis of their similar mobilities to those of rat caseins electrophoresed in a parallel track on the same gel slab (Figs 2a and 2c).

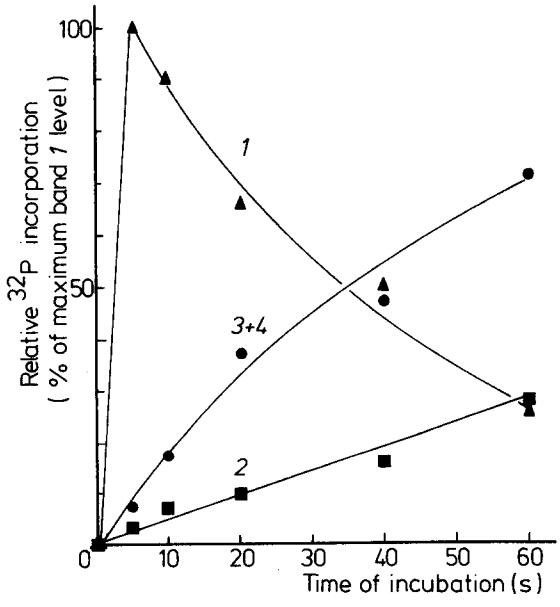

Fig. 3. Phosphorylation of Golgi vesicles in the presence of [γ-^{32}P]ATP: Kinetics of phosphorylation of individual phosphoproteins. Conditions of incubation and methods for the quantification of components 1, 2, 3 and 4 were as described in the text. Components 1, 2, 3 and 4 are identified in Fig. 2.

In contrast to the kinetic behaviour of phosphoproteins 2 and 3 + 4, the maximum detected phosphorylation of protein 1 occurred at the earliest time of sampling (5 s) and thereafter rapidly declined (see Fig. 3). The results presented in Fig. 1 are thus a composite of the phosphorylation behaviour of the four phosphoproteins (1–4) revealed by the type of analysis shown in Fig. 3.

The kinetic properties of phosphoprotein 1 suggested that it could be a phospho-enzyme intermediate in a process dependent on the hydrolysis of ATP. We have previously shown that this preparation contains an ATP-dependent Ca^{2+}-translocating system [10]. In order to determine whether phosphoprotein 1 was an intermediate in this particular process we investigated both the chemical stability of the protein phosphate bond and the influence of Ca^{2+}, Mg^{2+} and ADP on the phosphorylation process.

Stability of protein phosphate

Golgi membrane samples collected after 5 s incubation with [γ-^{32}P]ATP contained phosphoprotein 1 as the predominant ^{32}P-phosphorylated component (Fig. 1); such samples were

TABLE I

STABILITY OF PROTEIN PHOSPHATE BOND IN PHOSPHOPROTEIN 1

Golgi membranes were incubated with [γ-^{32}P]ATP as described in Materials and Methods and the reaction terminated after 5 s by the addition of ice-cold 10% trichloroacetic acid. The resultant precipitates were collected by centrifugation and resuspended under the conditions indicated above. Treatment with freshly prepared hydroxylamine was performed in 0.1 M sodium acetate buffer, pH 5.4 [17]. At the end of the incubation period specified, protein was again recovered by precipitation with trichloroacetic acid and the protein precipitate processed as described in the text for the quantitation of ^{32}P in phosphoprotein 1. The values given are the results of typical experiments. n.d., not detectable.

Treatment			Relative ^{32}P content phosphoprotein 1
	°C	Time	
10% trichloroacetic acid	0	60 min	100% (control)
10% trichloroacetic acid	100	12 min	n.d.
0.1 M NaOH	100	10 min	n.d.
0.8 M hydroxylamine	37	30 min	72%
0.8 M hydroxylamine	37	60 min	58%
0.8 M hydroxylamine	37	16 h	51%
0.8 M hydroxylamine, 3% SDS	37	30 min	30%

treated in a number of ways as summarized in Table I. The ^{32}P radioactivity was completely removed when the sample was heated at 100°C for 10 min in the presence of 0.1 M NaOH. Phosphotyrosine [16], phosphohistidine [17] and phospholysine [17] residues are stable under these conditions. The Golgi phosphoprotein 1 was stable to 10% trichloroacetic acid in the cold but was quantitatively dephosphorylated within 12 min in 10% trichloroacetic acid when the temperature was raised to 100°C. These properties are inconsistent with the presence of phosphoserine or phosphothreonine residues but suggest that the phosphate may be present as an acyl phosphate (phosphoaspartate or phosphoglutamate) [17]. However, the Golgi phosphoprotein 1 lost less than 30% of its [^{32}P]phosphate content when treated with hydroxylamine under conditions which are reported [18] to cause the complete dephosphorylation of authentic acyl phosphates. Dephosphorylation by hydroxylamine was increased to 70% if the acid-precipitated Golgi membranes were first treated with 3% sodium dodecyl sulphate prior to incubation, in order to facilitate accessibility of the phosphoprotein to hydroxylamine.

Therefore the identity of the phosphorylated residues in phosphoprotein 1 remains questionable: their reactivity towards hydrolyzing agents is different from that of the acyl phospho-enzyme intermediates in other well characterized systems such as the Ca^{2+}-ATPase of sarcoplasmic reticulum.

Influence of Mg^{2+}, Ca^{2+} and ADP

As can be seen from the results presented in Table II the inclusion of Ca^{2+}, Mg^{2+} or ADP in the standard incubation resulted in a decrease in the amount of ^{32}P incorporated into phosphoprotein 1 relative to control incubations with [γ-^{32}P]ATP alone. The decrease in phosphorylation which resulted when Ca^{2+} was included was opposite to that expected by analogy with the sarcoplasmic reticulum ATPase but was entirely consistent with the 3.5-fold increase in phosphorylation that was observed when the endogenous Ca^{2+} concentration was reduced to less than 10^{-8} M by the inclusion of EGTA.

The inclusion of ADP reduced the ^{32}P incorporation into phosphoprotein 1 to less than 25% of control values, an effect similar to that observed when ADP was added tot the [^{32}P]phospho-enzyme of sarcoplasmic reticulum vesicles [19] suggesting that the phosphoprotein could transfer its phosphorus to ADP. It was therefore surprising that when an ATP-regenerating system was included in the incubation to prevent accumulation

of ADP, no ^{32}P incorporation into phosphoprotein 1 could be detected at the 20 s time point. A possible explanation of this result is apparent when the effects of Mg^{2+}, added as a necessary component of the ATP-regenerating system, are considered. Since 1 mM Mg^{2+} markedly reduced ^{32}P incorporation (Table II), the 2 mM Mg^{2+} added with the phospho*enol*pyruvate-pyruvate kinase system would be expected to have had an equal or greater effect. However this level of Mg^{2+} would also have maximally stimulated the hydrolysis of ATP by the active ATPases known to be present in the mammary Golgi preparation [20,21] leading to a rapid decline in the specific radioactivity of the [γ-^{32}P]ATP added. These two effects thus combined to counteract the influence on component 1 phosphorylation of the removal of ADP from the system.

The results obtained in the presence of Ca^{2+}, Mg^{2+} and ADP also account for our inability to detect phosphoprotein 1 in earlier phosphorylation experiments [11]. In those experiments we had set out to study the phosphorylation of Golgi vesicle protein under the same conditions as were used for Ca^{2+} uptake studies [10] and the media contained both Ca^{2+} (125 μM) and Mg^{2+} (5 mM).

Influence of calmodulin

The effect of Ca^{2+} on phosphoprotein 1, observed in the present study, was the converse of its effect on the sarcoplasmic reticulum ATPase where the high affinity Ca^{2+}-binding sites of the enzyme require to be saturated in order that maximal phosphorylation by ATP (i.e., phospho-enzyme formation) can occur [2]. Thus, in sarcoplasmic reticulum vesicles, addition of EGTA before ATP prevented phospho-enzyme formation, and its addition after phospho-enzyme formation caused rapid dephosphorylation [2]. The opposite is true of the Golgi preparation since the greatest enhancement of ^{32}P incorporation occurred when EGTA was present prior to ATP addition. Although the addition of EGTA reduces the Ca^{2+} concentration of the incubation medium, its presence also results in the dissociation from the Golgi membranes of the Ca^{2+}-binding protein, calmodulin, which we have shown to be associated with this Golgi preparation [22]. The precise role of calmodulin in the Ca^{2+}-sensitive phosphorylation/dephosphorylation of phosphoprotein 1 is undefined at present but it is clear from the results shown in Table II that it does affect this process.

TABLE II

FORMATION OF PHOSPHOPROTEIN 1 IN GOLGI VESICLES: INFLUENCE OF VESICLE PRETREATMENT AND PHOSPHORYLATION CONDITIONS

Golgi vesicles were prepared either as described in the text ('non EGTA-washed vesicles') or with the addition of an incubation for 1 h at 0°C in buffer containing 250 mM lactose, 50 mM Na-Mes, 10 mM EGTA, pH 7.0, followed by re-isolation of the vesicles, washing three times, and resuspension as usual ('EGTA-washed vesicles'). Both preparations were incubated for 20 s under the phosphorylating conditions described in the text ('standard incubation') or modified as indicated above. Phosphorylation was terminated by the addition of ice-cold trichloroacetic acid and the protein precipitate processed, as described in the legend to Table I. The values given are averages of three experiments. The mean 100% values were, for non-EGTA-washed vesicles: 6.2 pmol ^{32}P/mg Golgi vesicle protein, and for EGTA-washed vesicles: 5.1 pmol ^{32}P/mg Golgi vesicle protein. n.t., not tested.

Additions to standard incubation	Relative ^{32}P-incorporation into phosphoprotein 1 (%)	
	EGTA-washed vesicles	non-EGTA-washed vesicles
None	100	100
Ca^{2+} (100 μM)	64	61
Mg^{2+} (100 μM)	37	n.t.
EGTA (2 mM)	226	348
ADP (100 μM)	n.t.	24

Calmodulin was removed from the preparation by washing the vesicles with isoosmotic buffer containing EGTA [22] and the ^{32}P incorporation into these membranes compared with the ^{32}P incorporation into non-washed vesicles when each was incubated with [γ-^{32}P]ATP. The addition of EGTA to the incubations resulted in an enhanced phosphorylation of phosphoprotein 1 in both washed and non-washed membranes in comparison with the standard incubation (Table II). The degree of enhancement was however, greater in the non-washed vesicles (i.e., in the presence of endogenous calmodulin). The reduction in phosphorylation of phosphoprotein 1 that was observed when excess Ca^{2+} was added to the incubations was, in

contrast, not dependent on the presence of calmodulin since ^{32}P incorporation was reduced to around 60% of control values in both washed and non-washed membranes. This was not an unexpected result since those Ca^{2+}-dependent effects that are mediated by calmodulin are generally expressed at Ca^{2+} concentrations in the micromolar range [23].

At present we can conclude only that the presence of Ca^{2+} in the incubation medium reduced the phosphorylation of component 1 but our observations do not indicate whether this, or indeed any of the experimentally-induced modulations in component 1 phosphorylation, were due to a change in the rate of the phosphorylation or of the dephosphorylation reaction.

The results presented in this communication indicate that in complete contrast to the sarcoplasmic reticulum ATPase the presence of Ca^{2+} is not a prerequisite for the phosphorylation of component 1 in the Golgi vesicle preparation.

The following mechanism could provide an explanation for the findings reported here:

$$E+ATP \rightleftharpoons ADP+E\text{-}P \overset{Ca^{2+}/Mg^{2+}}{\rightarrow} E+P_i$$

This mechanism is consistent with the observation that E–P formation does not require the presence of divalent metal ions but such ions are required for its further breakdown. This property does not preclude the involvement of this ATPase in the Ca^{2+}-transport system of the Golgi membrane although if component 1 is an intermediate in this transport process the mechanism must differ in considerable detail from that elucidated for the sarcoplasmic reticulum Ca^{2+}-ATPase.

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References

1 Hasselbach, W. (1977) Biophys. Struct. Mech. 3, 43–54
2 Yamamoto, T. and Tonomura, Y. (1968) J. Biochem. 64, 137–145
3 Bastide, F., Meissner, G., Fleischer, S. and Post, R.L. (1973) J. Biol. Chem. 248, 8385–8391
4 Sieghart, W., Forn, J. and Greengard, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2475–2479
5 Mackinose, M. (1969) Eur. J. Biochem. 10, 74–82
6 Nishikori, K., Takenaka, T. and Maeno, H. (1977) Mol. Pharmacol. 13, 471–478
7 Käser-Glanzmann, R., Gerber, E. and Lüscher, E.F. (1979) Biochim. Biophys. Acta 558, 344–347
8 Szasz, I., Hasitz, M., Sarcady, B. and Gardos, G. (1978) Mol. Cell. Biochem. 22, 147–152
9 Neville, M.C. and Peaker, M. (1979) J. Physiol. 290, 59–67
10 West, D.W. (1981) Biochim. Biophys. Acta 673, 374–386
11 West, D.W. and Clegg, R.A. (1980) Biochem. Soc. Trans. 8, 525–256
12 Kuhn, N.J. and White, A. (1977) Biochem. J. 168, 423–433
13 Clegg, R.A. and Skyrme, J.E. (1973) Biochem. J. 136, 1029–1037
14 Rosen, J.M., Woo, S.L.C. and Comstock, J.P. (1975) Biochemistry 14, 2895–2903
15 Kanazawa, T., Yomada, S., Yamamoto, T. and Tonomura, Y. (1971) J. Biochem. 70, 95–123
16 Plimmer, R.H.A. (1941) Biochem. J. 35, 461–469
17 Weller, M. (1979) in Protein Phosphorylation, Pion Ltd., London
18 Katz, S. and Blostein, R. (1975) Biochim. Biophys. Acta 389, 314–324
19 Takakawa, Y. and Kanazawa, T. (1979) Biochem. Biophys. Res. Commun. 88, 1209–1216
20 Baumrucker, C.R. and Keenan, T.W. (1975) Exp. Cell. Res. 90, 253–260
21 West, D.W. and Easom, R.A. (1982) Biochem. Soc. Trans., 10, 40
22 West, D.W. and Clegg, R.A. (1981) Biochem. Soc. Trans. 9, 77
23 Cheung, W.Y. (1980) Science 207, 19–27