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# Activation of sarcoplasmic reticular $Ca^{2+}$ transport ATPase by phosphorylation of an associated phosphatidylinositol

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**Approximately 1 mol phosphatidylinositol phosphate is formed per mol isolated $Ca^{2+}$ transport ATPase when the enzyme is incubated with ATP/$Mg^{2+}$. The phosphorylation of this enzyme-associated phosphatidylinositol represents the alkylphosphate formation described earlier. The phosphatidylinositol phosphate has been found in the hydrophobic core of the enzyme. A complex of phosphatidylinositol phosphate with protein can be extracted with acidic chloroform/methanol. The protein behaves like proteolipid during chromatography on Sephadex LH 60 and binds the radioactively labelled phosphatidylinositol phosphate. The phosphorylation of ~1 mol phosphatidylinositol per 100 000 g protein correlates with an enhancement of the $Ca^{2+}$ transport ATPase activity which is due to an ~7-fold enhanced affinity for $Ca^{2+}$ and an ~2-fold enhanced maximal turnover.**

*Key words:* $Ca^{2+}$ transport ATPase activation/fast skeletal muscle sarcoplasmic reticulum/phospatidylinositol phosphorylation/phosphatidylinositol phosphate

## Introduction

In addition to the well known acylphosphate, alkylphosphate is formed on the $Ca^{2+}$ transport ATPase of fast skeletal muscle sarcoplamsic reticulum (SR) when the enzyme is incubated with ATP/$Mg^{2+}$; however, this alkylphosphate formation can be observed only under special conditions, namely at high protein and high kinase concentrations (Varsanyi and Heilmeyer, 1981; cf., Varsanyi and Heilmeyer, 1979). By gel electrophoresis in the presence of SDS, the aklylphosphate can be shown to be present on the 100 000 mol. wt. $Ca^{2+}$ transport ATPase and on a component with an apparent mol. wt. of ~9000 (Varsanyi and Heilmeyer, 1981; Kurskii *et al.*, 1982; Kondratyuk *et al.*, 1982).

Phosphorylase kinase can stimulate $Ca^{2+}$ transport ATPase activity and $Ca^{2+}$ uptake into vesicles of fragmented SR (Hörl *et al.*, 1978; Hörl and Heilmeyer, 1978). Furthermore, phosphorylase kinase enhances both the steady-state level and the rate of alkylphosphate formation in SR vesicles which concomitantly reduces the steady-state level of the catalytic intermediate, the aspartyl phosphate (Varsanyi and Heilmeyer, 1981). Thus, changes in the activity of the $Ca^{2+}$ transport ATPase upon phosphorylation could be expected.

Neither the nature of the alkylphosphate nor the kinase which catalyzes this alkylphosphate formation has as yet been identified. Here we report that phosphatidylinositol phosphate is formed on the $Ca^{2+}$ transport ATPase and this correlates with an activation of the enzyme.

## Results

Figure 1 shows the time course of formation of TCA-precipitable alkylphosphate upon incubation of the isolated $Ca^{2+}$ transport ATPase with ATP/$Mg^{2+}$ at 1.6 nM and 0.45 $\mu$M free $Ca^{2+}$. This phosphate uptake is catalyzed by an endogenous kinase present in the isolated $Ca^{2+}$ transport ATPase. The maximal amount of precipitated radioactivity in the presence of nanomolar amounts of free $Ca^{2+}$ varied from 0.4 to 1.0 mol/100 000 g protein in different preparations. In the presence of micromolar concentrations of $Ca^{2+}$ the amount of alkylphosphate incorporated is significantly lowered and after reaching a maximum tends to decrease (Figure 1).

During this phosphorylation period the level of acylphosphate ranges from 0.01 to 0.05 mol/100 000 g protein, as tested by its hydroxylamine-sensitive release (not shown). The alkylphosphate content does not change during 30 min if the phosphorylation reaction is stopped by chelation of the free $Ca^{2+}$ and $Mg^{2+}$ with excess EGTA and EDTA (see Materials and methods). In the presence of free $Ca^{2+}$ and $Mg^{2+}$, as employed in the ATPase assay (see below), up to 30% of the radioactivity is released.

Partial hydrolysis of the $Ca^{2+}$ transport ATPase in 6 N HCl at 110°C for 2 h removes >95% of the radioactivity from the protein. The main radioactive material (~70% of the total) shows the paper chromatographic and ionophoretic properties of inositol bis-phosphate (Dawson and Dittmer, 1961). In addition, when run on Dowex 1 columns, it is eluted by increasing concentrations of ammonium formate in the position of inositol bis-phosphate (Richards *et al.*, 1979; Downes and Michell, 1981). A small amount of radioactive material, although not positively identified, is probably inositol monophosphate arising by acid dephosphorylation of the inositol bis-phosphate produced initially. Thus, the radioactive phosphate might be present in the $Ca^{2+}$ transport ATPase as an acidic phosphoinositide.

Neither chloroform/methanol (2/1 v/v) nor ether/ethanol (1/1 v/v) extract any radioactive label from the TCA-precipitated material. However, ~95% of the radioactivity is extracted with chloroform/methanol/concentrated HCl (40/20/1 v/v). T.l.c. on oxalate-impregnated silica plates identifies the predominant radioactive material (86%) as phosphatidylinositol phosphate (spot B, Figure 2).

This was confirmed by isolation of the main radioactive phospholipid (B) by preparative t.l.c. followed by acid hydrolysis (5 N HCl, 8 min, 100°C) and alkaline ethanolysis (0.03 M NaOH in ethanol/$H_2O$ 9:1 v/v, 37°C, 20 min). Two-dimensional separation of the released phosphate esters on paper using phenol/ethanol/acetic acid/water solvent followed by ionophoresis at pH 3.6 gave the typical decomposition patterns of phosphatidylinositol phosphate with inositol bis-phosphate (acid hydrolysis) and glycerophosphoinositol phosphate (alkaline ethanolysis) predominating (Dawson and Dittmer, 1961; Dawson, 1976). The ex-

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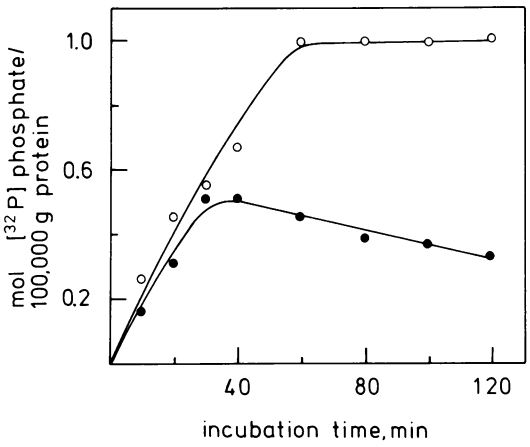


**Fig. 1.** 2.45 mg/ml isolated $Ca^{2+}$ transport ATPase was phosphorylated in the presence of 1.6 nM (○——○) and 0.45 μM (●——●) free $Ca^{2+}$. Alkylphosphate was determined as described in Materials and methods.

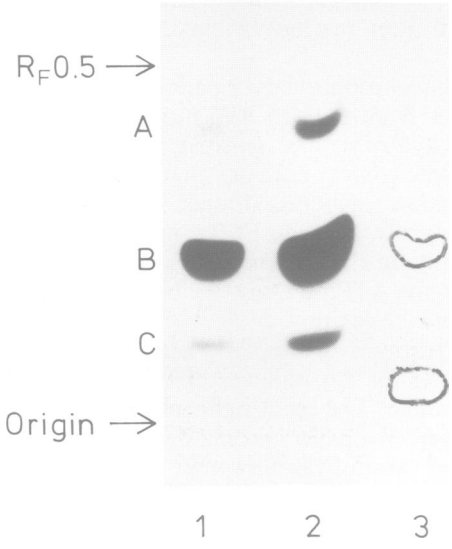


**Fig. 2.** Silica gel thin layer plates were sprayed lightly with 1% $K_2$ oxalate and dried for 1 h at 100°C. The protein-depleted acidic chloroform/methanol extract containing the $^{32}P$-labelled lipids was prepared as described in Materials and methods. Portions (15 000 and 40 000 d.p.m.) of extract were applied to the thin layer plates (**lanes 1** and **2**) and the chromatogram was developed in chloroform/methanol/concentrated ammonium hydroxide/water (45/35/2.5/7.5, v/v). Radioactive [$^{32}P$]lipids were visualized by autoradiography; the diphosphoinositide (faster running) and triphosphoinositide (slower running) markers in the third channel were visualized by spraying with P-detecting reagent and their outlines traced on the negative.

tract contained a number of minor radioactive substances, two of which (A and C, Figure 2) each contained ~5% of the radioactivity, the other 1% or less. Spot A ran identically to phosphatidic acid and gave the expected glycerophosphate after deacylation by alkaline ethanolysis. C is believed to be lysophosphatidylinositol phosphate because of its position on the chromatogram and since it produced identical breakdown products to phosphatidylinositol phosphate on acid and alkaline degradation as above. Staining with iodine vapour shows the presence of a wide variety of unlabelled phospholipids.

The phosphatidylinositol phosphate which is associated with the native ATPase can neither by hydrolyzed with alkaline or acidic phosphatases nor with phospholipases $A_2$,

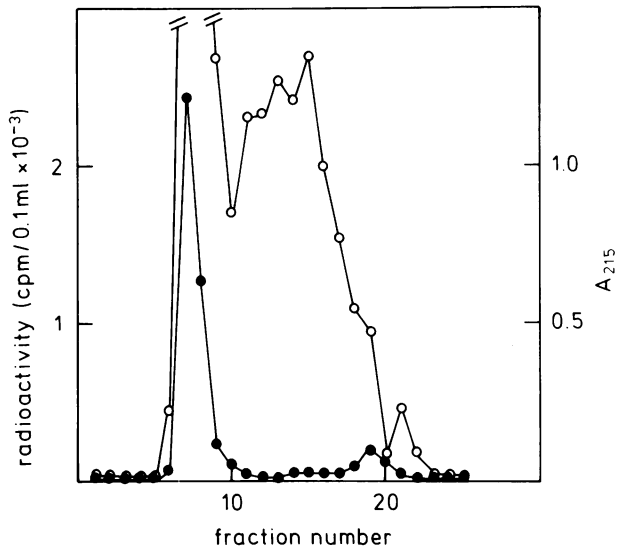


**Fig. 3.** The tryptic digest of the $Ca^{2+}$ transport ATPase containing radioactively labelled phosphatidylinositol phosphate was prepared as described in Materials and methods. 500 μl tryptic digest was applied to a 0.9 x 55 cm column of Sephadex G50 superfine pre-equilibrated in 50 mM $NH_4HCO_3$, pH 7.8. The flow rate was 12 ml/h, 2 ml fractions were collected. In each fraction absorbance at 215 nm (○——○) and radioactivity (●——●) were assayed.

C or D. This indicates a tight association of this acidic phospholipid with the native protein.

Trypsin releases maximally 10% of the radioactively labelled phosphatidylinositol phosphate from the phosphorylated $Ca^{2+}$ transport ATPase. To increase the digestion of the protein, the ATPase was succinylated and carboxymethylated. Thereafter, it was digested by trypsin according to Allen (1980). The digest was subjected to gel filtration over Sephadex G 50 superfine. Figure 3 shows that the radioactively labelled phosphatidylinositol phosphate elutes together with the hydrophobic core of the enzyme; only a minor amount of radioactivity is found in the fraction of the solubilized tryptic peptides.

Denaturation of the enzyme with TCA and immediate extraction with chloroform/methanol/HCl, without preextraction of the neutral lipids with chloroform/methanol, solubilizes a complex of protein with the radioactively labelled phosphatidylinositol phosphate. This complex elutes from Sephadex LH 60 in chloroform/methanol/750 mM $NH_4HCO_3$ as one peak; (Figure 4) the ratio of ~0.5 mol phosphatidylinositol phosphate/25 000 g protein is approximately constant over the whole profile. Electrophoresis of this complex on polyacrylamide in the presence of SDS-urea shows that the main fraction of the radioactivity, which is present as phosphatidylinositol phosphate, co-migrates with a protein of apparent mol. wt. 25 000 (Figure 4, inset). Two minor fractions migrate with apparent mol. wts. of 10 000 and ~1600, respectively.

To study the effect of formation of phosphatidylinositol phosphate on the ATPase, the enzymatic activity was assayed in parallel with the phosphate uptake after adapting the ATPase activity assay to the phosphorylation conditions. This results in a test mixture similar to that used for the assay of the ATPase when present in SR vesicles (See Hasselbach, 1966 as modified by Hörl *et al.*, 1978). Under these conditions, the isolated ATPase expresses nearly the same activity as SR vesicles and it is 5- to 10-fold lower than that determin-

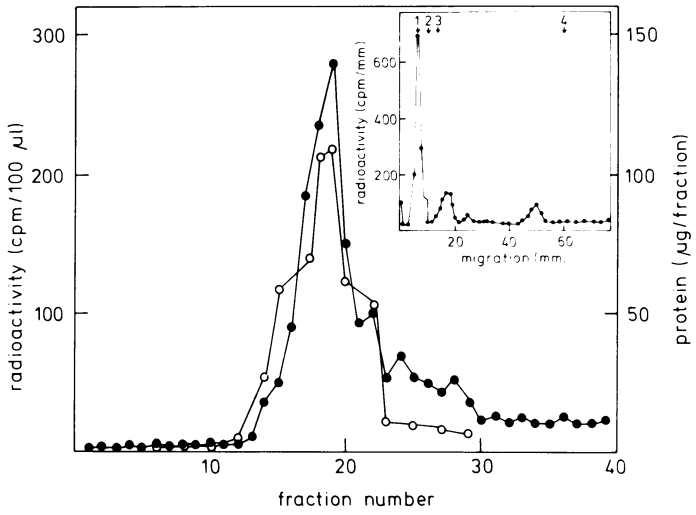


**Fig. 4.** The complex of protein with phosphatidylinositol phosphate was extracted as described in Materials and methods. The material containing 60 nmol phosphatidylinositol phosphate was dissolved in 500 µl elution buffer and applied to a 1.6 x 25 cm column of Sephadex LH 60 pre-equilibrated in chloroform/methanol/750 mM $NH_4HCO_3$ (46/46/8, v/v). The flow rate was 8 ml/h; 1.5 ml fractions were collected. In each fraction the amount of protein (○——○) and radioactivity (●——●) were determined. The protein was obtained after evaporation of the solvent. **Inset:** a portion of extract containing 20 nmol phosphatidylinositol phosphate and 10 µg each of the standards was applied to a 12.5% acrylamide gel and run as described by Swank and Munkres (1971). Gels were run for 20 min with 1 mA/gel and 3 h with 5 mA/gel. Bromophenol-blue was used as front marker; **1**, soybean trypsin inhibitor (mol. wt. 21 000); **2**, myoglobin (17 800); **3**, cytochrome c (12 400); **4**, bromphenol-blue.

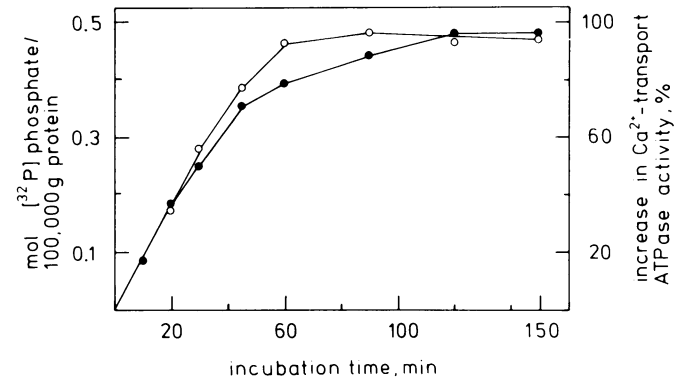


**Fig. 5.** 2.46 mg/ml isolated $Ca^{2+}$ transport ATPase was phosphorylated in the presence of 1.6 nM free $Ca^{2+}$ as described in Materials and methods. During the phosphorylation at each time point two samples of 10 µl were removed. The first one was employed for determination of the TCA-precipitable radioactivity (●——●). The second one was diluted in 500 µl 10 mM EDTA, 0.2 mM EGTA, 20 mM imidazole, pH 7.0 and ATPase activity (○——○) was determined at 69.6 µg/ml protein. At time 0 the ATPase activity was 2.1 µmol/min/mg.

ed according to MacLennan (1970) (for comparision of the assays, see Materials and methods).

The phosphorylation of the phosphatidylinositol is accompanied by an activation of the $Ca^{2+}$ transport ATPase activity (Figure 5). When phosphate uptake and ATPase activity, as determined in our standard assay, are measured in parallel, the degree of the activation varies from 30 to ~120% and seems to depend on the preparation of the isolated $Ca^{2+}$ transport ATPase. The same amount of alkylphoshate formed in different ATPase preparations causes different degrees of activation.

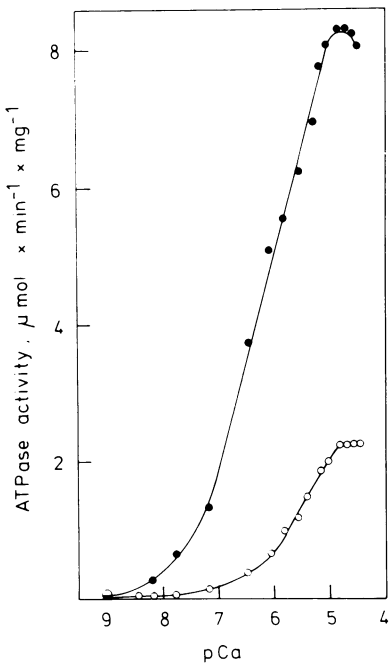


**Fig. 6.** Activity assays were carried out with 41.2 µg/ml control (○——○) and 40.5 µg/ml (●——●) $Ca^{2+}$ transport ATPase containing 0.84 mol radioactively labelled phosphatidylinositol phosphate/100 000 g protein. Free $Ca^{2+}$ concentrations were established with varying ratios of $Ca^{2+}$ and EGTA. The ATPase activities were calculated from the initial phosphate liberation.

The isolated $Ca^{2+}$ transport ATPase was phosphorylated preparatively and thereafter isolated by precipitation with ammonium acetate as described in Materials and methods; it contained 0.84 mol phosphatidylinositol phosphate/ 100 000 g protein. A non-phosphorylated control ATPase was subjected to the same procedure. Figure 6 shows that the control ATPase is essentially inactive up to ~10 nM free $Ca^{2+}$; then its activity increases with rising free $Ca^{2+}$ concentrations. The curve can be fitted to a hyperbola (Table I). The radioactively labelled phosphatidylinositol phosphate-containing $Ca^{2+}$ transport ATPase shows higher activity in the range $10^{-9}-10^{-7}$ M free $Ca^{2+}$ than the control enzyme (Figure 6). The whole curve can be fitted in two ways. One hyperbola results in an ~4-fold enhanced $V_{max}$ value with a slight increase in the affinity for $Ca^{2+}$ and a reduced Hill coefficient (Table I). It can be assumed that the curve represents the sum of two enzymatic activities. According to the optimal fit the first one shows very similar characteristics to the non-phosphorylated enzyme; the second activity might represent the phosphatidylinositol phosphate-containing enzyme which is characterized by an ~2-fold enhanced $V_{max}$ value and an ~7-fold higher affinity for $Ca^{2+}$ (Table I).

The activities of both forms of ATPase show essentially identical $K_M$ values for ATP/$Mg^{2+}$, the maximal activities differ by a factor of approximately three (Table II).

## Discussion

The amount of alkylphosphate formed on the isolated $Ca^{2+}$ transport ATPase is a function of the free $Ca^{2+}$ concentration; in the presence of micromolar concentrations of $Ca^{2+}$ it is 2- to 3-fold lower than at nanomolar concentrations. This relationship was also observed in the intact SR vesicles (Varsanyi and Heilmeyer, 1981). Previously, we employed phosphorylase kinase, which has been shown to be

**Table I.** Kinetic parameters on $Ca^{2+}$ activation of the control and of the $Ca^{2+}$ transport ATPase containing 0.84 mol labelled phosphatidylinositol phosphate/100 000 g protein

| $Ca^{2+}$ transport ATPase | $K_a$ ($Ca^{2+}$) (M) | $V_{max}$ (μmol/min/mg) | h | *r* |
|---|---|---|---|---|
| Control | $2.8 \times 10^{-6}$ | 2.6 | 0.97 | 0.083 |
| Phosphatidylinositol phosphate-containing | [a] $1.0 \times 10^{-6}$ | 9.8 | 0.63 | 0.213 |
| | [b] $2.1 \times 10^{-6}$ | 2.5 | 1.0 | |
| | $3.2 \times 10^{-7}$ | 5.8 | 1.0 | 0.327 |

Experimental conditions for the assay of both $Ca^{2+}$ transport ATPase activities were the same as described in Materials and methods.
*r* represents the standard deviation of the data.
[a]Fit of the data to one hyperbola, [b]to two hyperbolas.

**Table II.** Comparison of kinetic parameters of control and of the $Ca^{2+}$ transport ATPase containing 0.84 mol labelled phosphtidylinositol phosphate/100 000 g protein

| $Ca^{2+}$ transport ATPase | $K_m$ (ATP/$Mg^{2+}$) (mM) | $V_{max}$ (μmol/min/mg) |
|---|---|---|
| Control | 1.04 ± 0.15 | 26. ± 0.10 |
| Phosphatidyl inositol phosphate-containing | 0.96 ± 0.06 | 7.7 ± 0.11 |

The activities of 31.2 μg/ml control and 40.5 μg/ml phosphatidylinositol phosphate-containing $Ca^{2+}$ transport ATPase were assayed between 100 μM and 10 mM ATP/$Mg^{2+}$, at 18.5 μM $Ca^{2+}$, 3 mM free $Mg^{2+}$ and pH 7.5 (26°C).
The rates of ATP hydrolysis were calculated from the initial phosphate liberation, the $V_{max}$ and $K_m$ values from a fit to a hyperbola.

present in SR membranes (Varsanyi *et al.*, 1978; Hörl *et al.*, 1975, 1978) to catalyze this alkylphosphate formation. Since then we have shown that phosphatidylinositol kinase activity is present in the phosphorylase kinase preparation (Georgoussi and Heilmeyer, unpublished data). In the experiments described here the endogenous phosphatidylinositol kinase present in the $Ca^{2+}$ transport ATPase preparation catalyzed phosphatidylinositol phosphate formation. The relationship of these two kinases is at present unknown.

The endogenous kinase present in SR membranes or in the isolated $Ca^{2+}$ transport ATPase incorporates, into the phosphatidylinositol, 0.7 – 0.9 mol phosphate per 100 000 g protein (cf. Varsanyi and Heilmeyer, 1981; Figure 1). Thus, only a part of the phosphatidylinositol present in SR membranes (9 – 10 mol/mol ATPase (Meissner and Fleischer, 1971; Swoboda *et al.*, 1979) is phosphorylated under the conditons employed. It could also result from a rapid formation and decomposition of phosphatidylinositol phosphate which could explain the somewhat lower degree of phsphorylation in the SR membranes than in the isolated $Ca^{2+}$ transport ATPase (compare Varsanyi and Heilmeyer, 1981, and Figure 1). As the data in Figure 1 indicate, this turnover seems to occur faster at micromolar concentrations of free $Ca^{2+}$.

The main reaction product is phosphatidylinositol phosphate; no phosphatidylinositol bis-phosphate was detected. SDS is apparently not able to disrupt the linkage between phosphatidylinositol phosphate and protein since a part of this acidic phospholipid co-migrates during electrophoresis with the $Ca^{2+}$ transport ATPase and another part with a 9000 – 10 000 mol. wt. component (compare Varsanyi and Heilmeyer, 1981, and Figure 6). This inositol derivative seems to be associated with the hydrophobic core of the enzyme (see Figure 3). The core might contain a proteolipid to which the phosphatidylinositol phosphate is associated as judged from the extractability of this complex with an acidic organic solvent and its behaviour during chromatography on Sephadex LH 60 (see Figure 4). This complex seems to exist in several associated forms as revealed by the gel electrophoresis pattern in SDS (Figure 4, inset).

The existence of a proteolipid in SR membranes extractable with acidic organic solvents in a molar ratio of probably 1:1 to the 100 000 mol. wt. $Ca^{2+}$ transport ATPase has been suggested by MacLennan *et al.* (1972). The ratio of phosphatidylinositol phosphate to $Ca^{2+}$ transport ATPase is preserved in the extracted proteolipid containing this acidic phospholipid. A maximal incorporation of ~1 mol phosphate as phosphatidylinositol phosphate also implies that the proteolipid is present in a 1:1 molar ratio to the $Ca^{2+}$ transport ATPase. The presence of phosphatidylinositol phosphate on the isolated proteolipid, however, does not allow the conclusion that it is bound to the $Ca^{2+}$ transport ATPase since complex formation during extraction cannot be excluded.

The proteolipid extracted from SR binds phospholipids (MacLennan *et al.*, 1972); a similar proteolipid from erythrocyte membranes binds preferentially the acidic phospholipids, phosphatidylserine and phosphatidylinositol. Phosphatidylinositol phosphate formation on the proteolipid has been described (Redman, 1972). Finally, a heat stable factor extracted during the purification of the SR $Ca^{2+}$ transport ATPase can stimulate the $Ca^{2+}$ translocating activity (Racker and Eytan, 1975). These authors concluded that the heat stable factor is the proteolipid characterized by McLennan *et al.* (1972). However, the proteolipid could contain bound phosphatidylinositol phosphate which might be responsible for the observed $Ca^{2+}$ transport ATPase activation shown here. The correlation between the formation of the labelled phosphatidylinositol phosphate and the increase in ATPase activity suggests that the $Ca^{2+}$ transport ATPase activity may be regulated by this acidic phospholipid, but this remains to be shown in intact muscle.

In erythrocyte plasma membranes the formation of di- and triphosphoinositides correlates with an activation of the $Ca^{2+}$ transport ATPase (Buckley and Hawthorne, 1972). The SR $Ca^{2+}$ transport ATPase is activated by the diphosphoinositide as shown here. Even though the plasma membrane and the SR $Ca^{2+}$ transport ATPases are different molecular entitites (Niggli *et al.*, 1979) they might be activated by the same polyphosphoinositide, namely phosphatidylinositol phosphate. The phosphatidylinositol phosphate could be involved directly in the $Ca^{2+}$ translocation through the membrane (Redman, 1972). However, the rate of formation of phosphatidylinositol phosphate is orders of magnitude slower than the acylphosphate formation (Varsanyi and Heilmeyer, 1981). Furthermore, upon depletion of ATP in a SR phosphorylation mixture the acylphosphate disappears immediately whereas the phosphatidylinositol phosphate decreases very slowly (Varsanyi and Heilmeyer, unpublished data). Alternatively, the phosphatidylinositol phosphate could serve as a lipophilic allosteric effector of the $Ca^{2+}$ transport ATPase which can regulate the ATPase activity by

changing its affinity for $Ca^{2+}$ and its maximal turnover. Thus, the phosphatidylinositol phosphate could determine the final intracellular steady-state $Ca^{2+}$ concentation as well as the duration of a $Ca^{2+}$ transient.

## Materials and methods

Ortho-[$^{32}$P]phosphate (carrier free) was obtained from New England Nuclear. Reagents for [$\gamma$-$^{32}$P]ATP synthesis (Glynn and Chappel, 1964) were obtained from Boehringer-Mannheim. Sephadex G50 superfine and LH 60 were obtained from Pharmacia.

Inositol phospholipids used as standards were isolated from ox brain white matter (Dittmer and Dawson, 1961). Phosphatidic acid was prepared by phospholipase D digestion of phosphatidylcholine. T.l.c. cellulose plates (20 cm x 20 cm, 0.1 mm thickness, POLIGRAM Cel 300) were obtained from Machery-Nagel and Silicagel plates (20 cm x 5 cm, 0.1 mm thickness, F 1500) from Merck. Two-dimensional thin layer electrophoresis was carried out on a Pharmacia Flat Bed Apparatus FBE 3000 connected to a Pharmacia Power Supply ECPS 2000/300.

The following enzymes were used: *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) Sigma; potato acidic phosphatase (EC 3.1.3.2) Boehringer-Mannheim; crude dried venom phosphodiesterase (EC 3.1.4.1) Sigma; bee venom phospholipase $A_2$ (EC 3.1.1.4), *Bacillus cereus* phospholipase C (EC 3.1.4.3) and cabbage phospholipase D (EC 3.1.4.4) Boehringer-Mannheim; 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (TPCK) treated trypsin was purchased from Merck.

SR membranes were isolated according to De Meis and Hasselbach (1971) and the $Ca^{2+}$ transport ATPase as described by MacLennan (1970). Protein concentration was measured by the method of Lowry *et al.* (1951). Protein-bound radioactivity was assayed according to Mans and Novelli (1961). Radioactivity was assayed as described in Varsanyi and Heilmeyer (1979). Kinetic data were fitted using a computer program developed by Kalbitzer and Stehlik (1979). Free $Ca^{2+}$ concentrations were calculated as described in Jahnke and Heilmeyer (1980).

*Activity assay of the $Ca^{2+}$ transport ATPase*

1 ml reaction mixture contained 50 $\mu$mol Tris/HCl, pH 7.5, 100 $\mu$mol KCl, 15 $\mu$mol $MgCl_2$, 350 nmol $CaCl_2$, 5 $\mu$mol EDTA, 0.1 $\mu$mol EGTA and 40 – 60 $\mu$g protein. The mixture was preincubated at 26°C and the reaction was started by addition of 10 $\mu$mol ATP. Aliquots were removed and diluted ~10-fold in 10% SDS. Inorganic phosphate was determined on an automatic analyzer according to Fiske and Subbarow (1925). Free concentrations of the following species were calculated with a program fit according to Jahnke and Heilmeyer (1980); $Ca^{2+}$ 0.73 $\mu$M, $Mg^{2+}$ 1.02 mM, ATP/$Mg^{2+}$ 9.23 mM, ATP/$Ca^{2+}$ 3.4 $\mu$M.

Under these conditions the specific activity of the isolated $Ca^{2+}$ transport ATPase ranges from 0.8 to 2.2 $\mu$mol/min/mg. This specific activity is considerably lower than that determined according to MacLennan (1970). In the latter assay, our preparations showed specific activities of 7 – 13 $\mu$mol/min/mg which is in good agreement with the values reported by De Meis and Tume (1977), Watanabe *et al.* (1981), Epstein *et al.* (1980) and MacLennan (1970). The main difference, apart from temperature, is the free $Mg^{2+}$ concentration. For the activity assay of MacLennan (1970) calculation of the free species yields: $Ca^{2+}$ 1.1 $\mu$M, $Mg^{2+}$ 0.065 mM, ATP/$Mg^{2+}$ 4.9 mM and ATP/$Ca^{2+}$ 48.8 $\mu$M. No Ca/EGTA buffer is used; the $Ca^{2+}$ added is in the range of contaminating $Ca^{2+}$. Therefore, the free $Ca^{2+}$ in the assay may vary considerably.

*Analytical procedure for the phosphorylation of the isolated $Ca^{2+}$ transport ATPase with endogenous kinase*

Isolated $Ca^{2+}$ transport ATPase (~2 mg protein/ml reaction mixture) was preincubated for 1.5 min in a total volume of 600 $\mu$l at pH 7.5, at 26°C, containing 100 mM KCl, 100 mM Tris-HCl, 10 mM EDTA, 1 mM EGTA, 10 mM [$\gamma$-$^{32}$P]ATP. Aliquots of 25 $\mu$l were taken after 0.5, 1 and 1.5 min to determine the radioactivity bound unspecifically to the protein. At the second minute the phosphorylation was started by addition of 20 $\mu$l 25 mM $CaCl_2$ + 500 mM $MgCl_2$ or by adding only 20 $\mu$l of 500 mM $MgCl_2$. The presence of EGTA and EDTA yields free concentrations of 0.45 $\mu$M $Ca^{2+}$ and 5.1 mM $Mg^{2+}$ or 1.6 nM $Ca^{2+}$ and 5.1 mM $Mg^{2+}$, respectively.

Simultaneously, two aliquots of 15 $\mu$l were withdrawn and applied to Whatman GF/C filter paper discs. In the first series the total protein-bound radioactivity was determined as described above. The filter papers of the second series were analogously treated with TCA and thereafter incubated for ~6 h in 0.1 M $NH_2OH$ (pH 7.5) (Makinose, 1969). Thereafter, the remaining radioactivity on the filter disc was counted.

*Preparation of phosphorylated ATPase*

18 mg isolated $Ca^{2+}$ transport ATPase was phosphorylated exactly as described above for the analytical procedure. Phosphorylation was stopped by addition of chelators to a final concentration of 1 mM EGTA and 10.5 mM EDTA, pH 7.0. The ATPase was precipitated from the phosphorylation mixture by addition of 0.2 volumes of 50% saturated ammonium acetate. The precipitated protein was collected by centrifugation at 80 000 *g* for 20 min, dissolved in 0.66 M sucrose, 50 mM Tris-HCl, 1 mM histidine, 10.3 mM EDTA, 1.03 mM EGTA, pH 8.0 and stored at −20°C.

*Tryptic digestion of the phosphorylated ATPase according to Allen (1980)*

After stopping the phosphorylation reaction with the two chelators (see above) 50 $\mu$l 100 mM 1,4-dithioerythritol (DTE) and then stepwise 5 mg succinic anhydride was added to the 2 ml incubation mixture. During the succinylation, the pH was adjusted to 6.0 – 7.5 by repetitive addition of 2 $\mu$l 5 N NaOH. After 30 min, 200 $\mu$l 500 mM iodoacetate was added and the mixture was incubated for a further 30 min at 35°C in the dark. The protein was precipitated by addition of 4 ml 10% TCA and centrifuged at 4000 r.p.m. for 20 min. The pellet was washed four times with water, homogenized in 500 $\mu$l 50 mM $NH_4HCO_3$, pH 7.8 and digested with 20 $\mu$g TPCK-treated trypsin (added in two portions of 10 $\mu$g each) for 60 min at 37°C. The pH was readjusted to 7.8 by addition of 0.1 N NaOH.

*Two-dimensional thin layer electrophoresis of acid hydrolysate of ATPase*

3.8 mg phosphorylated $Ca^{2+}$ transport ATPase (after dialysis against 50 mM triethanolamine, pH 7.5 for 16 h) was hydrolyzed in 6 N HCl in a sealed tube for 2 h at 110°C. The sample was filtered, dried by evaporation and solubilized in 100 $\mu$l electrophoresis buffer, pH 1.9. 25 $\mu$l were applied to cellulose thin layer plates together with 10 $\mu$g phosphothreonine, 10 $\mu$g phosphoserine and 10 $\mu$g phosphotyrosine as standards. The electrophoresis buffer in the first dimension was acetic acid/formic acid/water pH 1.9, (78/25/897, v/v) and that in the second dimension pyridine/acetic acid/water pH 3.5 (50/5/945, v/v). The voltage used in both directions was 1000 V, the running time 50 min in the first and 45 min in the second direction.

*Extraction of a protein-phosphatidylinositol phosphate complex from the phosphorylated $Ca^{2+}$ transport ATPase*

25 mg phosphorylated $Ca^{2+}$ transport ATPase (1.5 ml) was precipitated by the addition of 5 ml 15% TCA and centrifuged for 10 min at 5000 r.p.m. The precipitate was washed six times with water until the supernatants contained no detectable radioactivity. The precipitate was dried *in vacuo* and then extracted with 3 ml chloroform/methanol/concentrated HCl (40/20/1, v/v). This extract was filtered through a Whatman GF/C filter disc. The organic phase obtained was washed with 2 ml of water; it contained 90% of the TCA-precipitated radioactivity and up to 1 mg/ml of protein. The organic solvent was evaporated *in vacuo*.

*Extraction of phosphatidylinositol phosphate from the phosphorylated $Ca^{2+}$ transport ATPase*

Phosphorylated ATPase was precipitated by TCA and washed with water as described above. In order to remove neutral lipids and the bulk of the phospholipids, the dried precipitate was pre-extracted with 3 ml of chloroform/methanol (2/1, v/v) and centrifuged for 15 min at 5000 r.p.m. Then, the precipitate was extracted with 3 ml chloroform/methanol/concentrated HCl (40/20/1 v/v) and filtered as above. The filtrate was washed with 0.6 ml of 1% NaCl solution and the lower chloroform-rich phase was evaporated *in vacuo*. The residue contained 90% of the TCA-precipitated radioactivity and only traces of protein.

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