# PHOSPHORYLATION OF THE 100 000 $M_r$ $Ca^{2+}$-TRANSPORT ATPase BY $Ca^{2+}$ OR CYCLIC AMP-DEPENDENT AND -INDEPENDENT PROTEIN KINASES

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## 1. Introduction

Isolated rabbit skeletal muscle sarcoplasmic reticulum (SR) contains, as an intrinsic membrane component, the 100 000 $M_r$ $Ca^{2+}$-transport ATPase (~70% of the total protein) (review [1]). The peripheral proteins in this membrane are calsequestrin, $Ca^{2+}$ high affinity binding protein [1], calmodulin [2] as well as protein-kinases and -phosphatases [3–7]. During $Ca^{2+}$ transport the 100 000 $M_r$ ATPase becomes phosphorylated at an aspartyl residue, and the product is a high energy acylphosphate intermediate of the catalytic cycle (review [1]). Characteristically, it reacts with hydroxylamine to yield the corresponding hydroxamate [8].

Protein-kinases and protein-phosphatases associate with these membranes in a $Ca^{2+}$-dependent manner [9,10], and seem to be involved in the regulation of the $Ca^{2+}$-transport ATPase activity [3,4]. Earlier studies on SR, isolated from phosphorylase kinase deficient I-strain mice, indicate that more than one protein kinase might be involved in this regulatory process [11,13]. From the specificity of these protein kinases one would expect that seryl- or threonyl-residues of membrane components can be phosphorylated. Indeed, as will be shown here, incorporation of $^{32}P$ into hydroxylamine stable alkylphosphate can be observed if, in contrast to the earlier experiments, high SR concentrations nearly physiological (~7 mg/ml) are employed. In the presence of $\mu$M $Ca^{2+}$ a total of ~0.8 mol phosphate is incorporated into the 100 000 g SR protein. A fraction of this phosphate, ~0.3 mol/100 000 g protein, is insensitive to the hydroxylamine treatment, i.e., it represents alkylphosphate. Addition of phosphorylase kinase has two effects:

1. It accelerates ~3-fold the alkylphosphate incorporation rate;
2. It enhances the final level of incorporated phosphate up to 0.7 mol/100 000 g protein.

At nM $Ca^{2+}$, alkylphosphate incorporation occurs at high SR-concentration to a maximal extent of 0.5 mol/100 000 g protein. Under these conditions the acylphosphate level is low, but can be enhanced to ~0.7 mol/100 000 g SR protein by the catalytic subunit of the cAMP-dependent protein kinase.

The incorporated alkylphosphate is localized in two proteins of $M_r$ 100 000 and 9000. The 100 000 $M_r$ protein represents the $Ca^{2+}$-transport ATPase; phosphorylase kinase incorporates 0.7 mol alkylphosphate/mol protein into the purified ATPase.

Dedicated to Professor Dr H. Holzer on the occasion of his 60th birthday

## 2. Materials and methods

SR membranes, enzyme preparations and assays were done as in [3,13]. ATPase was isolated from SR and assayed as in [14,15]. Its specific activity was 20 $\mu$mol ATP hydrolyzed . $min^{-1}$ . $mg^{-1}$. Heat-stable inhibitor of the cyclic AMP-dependent protein kinase was obtained from Sigma Chemical Co. (St Louis MO). Phosphorylation assay: SR membranes or the isolated ATPase were incubated for 1.5 min in a total volume of 600 $\mu$l at pH 7.5, 26°C, containing 100 mM KCl, 100 mM Tris–HCl, 10 mM EDTA, 1 mM EGTA and 10 mM [$\gamma$-$^{32}P$]ATP. An aliquot of 25 $\mu$l was used to determine the radioactivity bound unspecifically to the membranes. After 2 min the phosphorylation was started by addition of 20 $\mu$l 25 mM $CaCl_2$ + 500 mM $MgCl_2$ or 500 mM $MgCl_2$ only. Chelation with EGTA and EDTA yields free concentrations of 0.45 $\mu$M $Ca^{2+}$

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and 5.1 mM $Mg^{2+}$ or 1.6 nM $Ca^{2+}$ and 5.1 mM $Mg^{2+}$, respectively. Simultaneously, 2 aliquots of 25 μl were withdrawn and applied to paper discs (Whatman GF/C diam. 25 mm). In one series the total protein-bound radioactivity was determined [16]. The aliquots of the second series were analogously treated with trichloracetic acid and thereafter incubated for ~6 h in 0.1 M $NH_2OH$ (pH 7.5). The recovery of alkylphosphate after such treatment was determined with radioactively labelled phosphorylase *a* as control, to be ~97%. The phosphate which is incorporated into the SR protein and which is not removed by hydroxylamine treatment behaves like ester in that it is stable in 0.25 M HCl and cleaved by 0.25 M NaOH. It does not consist of phospholipid, as it is not extractable with ethanol or ether. Free $Ca^{2+}$ concentrations were calculated as in [17].

## 3. Results

### 3.1. *SR phosphorylation at low and high protein concentrations*

At 0.1 mg protein/ml and μM $Ca^{2+}$, 1 mol trichloracetic acid-precipitable phosphate is incorporated into 100 000 g SR protein. This phosphate is almost completely released upon treatment with hydroxylamine, which characterizes it as acylphosphate (fig.1A). When an identical experiment is carried out at ~7 mg protein/ml, a slightly lower amount of total phosphate (~0.8 mol/100 000 g protein) is incorporated. However, in this case the amount of hydroxylamine-stable phosphate increases rapidly during ~3 min and reaches after 23 min a level of ~0.3 mol/100 000 g protein (fig.1B). Initially almost exclusively acylphosphate is formed; upon further incubation its steady state level decreases in two phases. In presence of μM $Ca^{2+}$ alone, without addition of mM $Mg^{2+}$, no phosphate incorporation can be observed; thus under these conditions phosphorylation requires the addition of $Mg^{2+}$ (fig.1B, insert).

Between 5–10 mg protein/ml the initial $P_i$ liberation rate by the $Ca^{2+}$-transport ATPase amounts to ~0.1 μmol . $min^{-1}$ . $mg^{-1}$. The total amount of liberated $P_i$ is equivalent to ~2 mol/mol ATP, indicating the presence of adenylate kinase activity and the progress curve of $P_i$-formation with time curves downwards (not shown). Under these conditions protein phosphorylation can proceed at least 40 min. To further characterize phosphate incorporation all the following experiments were carried out at ~7 mg SR/ml.

### 3.2. *Effect of phosphorylase kinase on the SR phosphorylation at μM $Ca^{2+}$*

Addition of 0.3 mg/ml phosphorylase kinase accelerates the initial incorporation rate of alkylphosphate

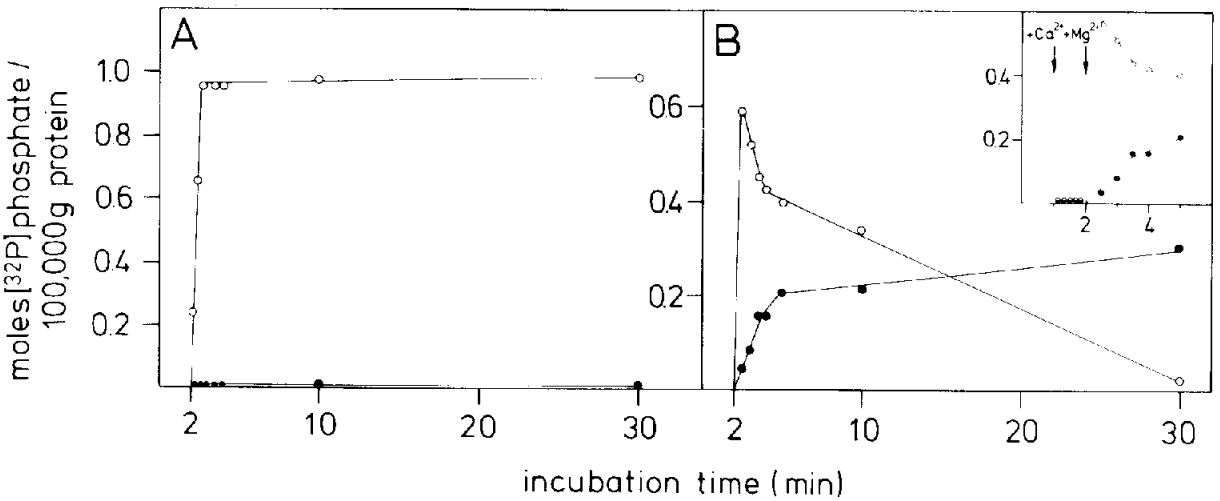


Fig.1. SR at 0.1 mg/ml (A) and 7.2 mg/ml (B) was preincubated for 1 min in the assay mixture as in section 2. After 1 min $Ca^{2+}$ was added (free $Ca^{2+}$ 0.45 μM). At the times indicated samples were removed for determination of protein-bound radioactivity. After 2 min the phosphorylation was started by addition of $MgCl_2$ (free $Mg^{2+}$ 5.1 mM). Acyl (○) and alkyl (●) phosphates were determined as in section 2. The decrease of the acylphosphate level in two phases (B) varies with the SR preparation and may remain at a higher level in the second phase. The insert shows in an enlarged scale the initial part of (B).

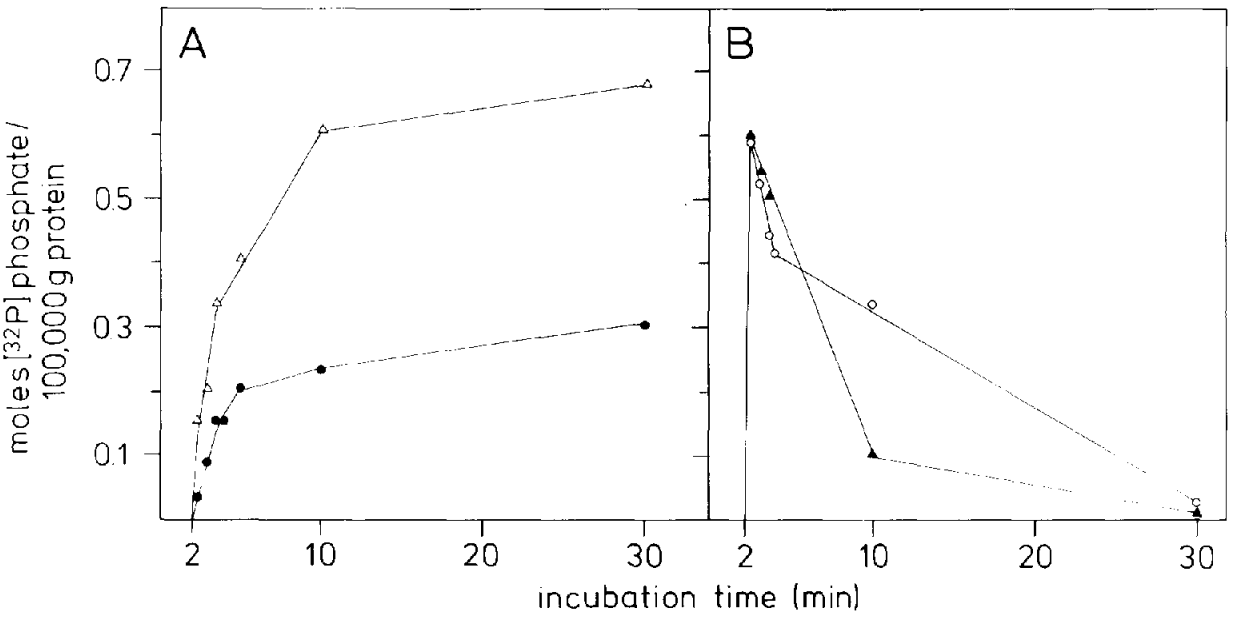


Fig.2. SR (7.2 mg/ml) was phosphorylated in presence of 0.45 μM free $Ca^{2+}$ and the formed phosphates were differentiated as in section 2. (A) Represents the incorporated alkylphosphate, (B) the acylphosphate. The phosphorylation was carried out in absence (●,○) and presence of 300 μg/ml phosphorylase kinase (△,▲).

~3-fold and approximately doubles the total amount of incorporated phosphate (fig.2A). Antibodies against phosphorylase kinase inhibit these stimulations; they have no effect on the alkylphosphate incorporation in absence of phosphorylase kinase (not shown). The most pronounced effect of phosphorylase kinase addition on the acylphosphate steady state level is an accelerated decrease in the first phase (fig.2B).

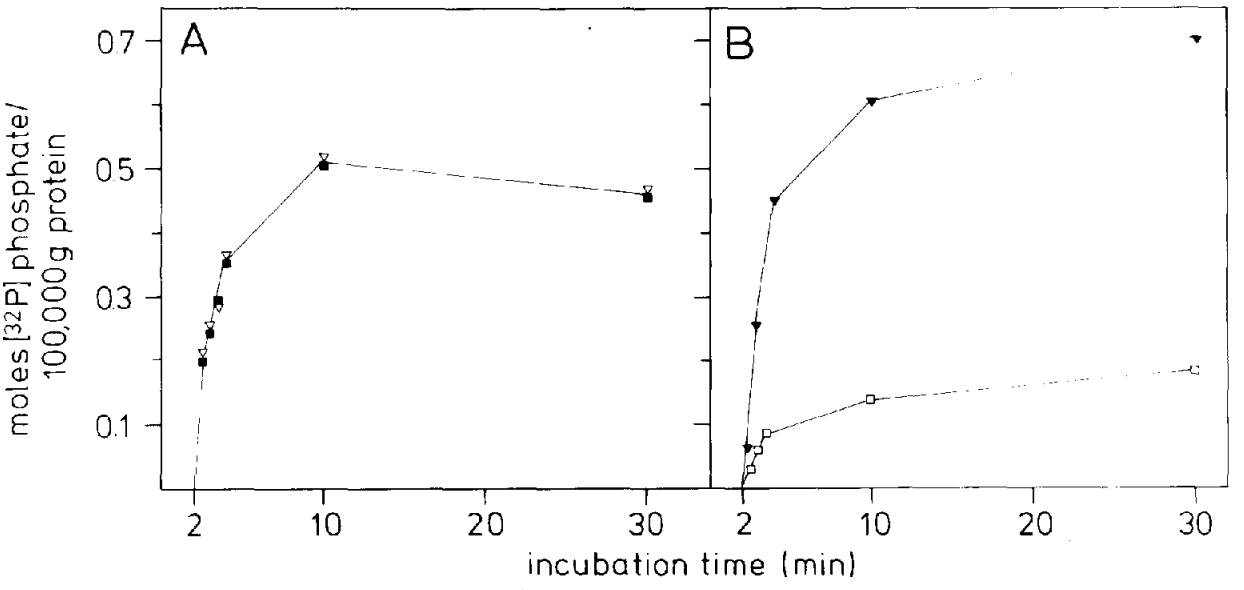


Fig.3. SR (7.2 mg/ml) was phosphorylated in presence of 1.6 nM $Ca^{2+}$ and the formed phosphates were differentiated as in section 2. (A) Alkyl (▼,■); (B) acylphosphate(▼,□)). The phosphorylation was carried out in absence (■,□) or presence (▽,▽) of 74 μg/ml catalytic subunit of the cyclic AMP-dependent protein kinase.

### 3.3. *Effect of the catalytic subunit of the cAMP-dependent protein kinase on the SR phosphorylation at nM $Ca^{2+}$*

Lowering the free $Ca^{2+}$ from μM to nM level, leads to ~2-fold enhancement of the incorporated alkylphosphate level, as can be seen from a comparison of fig.2A,

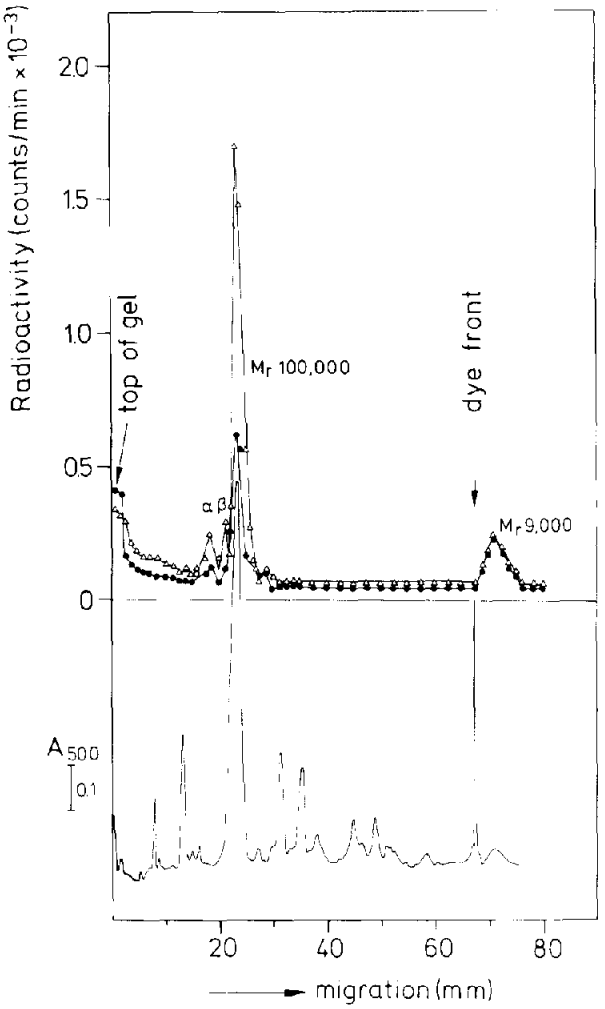


Fig.4. SR at 7.2 mg/ml was phosphorylated in presence of $Ca^{2+}$ with and without added phosphorylase kinase (0.3 mg/ml) as in section 2. After 30 min aliquots were withdrawn and the protein was precipitated with 10% trichloracetic acid. After 10 min at 0°C the precipitate was removed by centrifugation for 20 min at 8000 × *g*. The precipitate was resuspended in 0.1 M $NH_2OH$ (pH 7.5) and incubated for 6 h at 20°C. Protein was collected after repeated trichloracetic acid precipitation and 100 μg were prepared for polyacrylamide gel-electrophoresis. The lower part shows the densitogram of the stained protein; the upper part the radioactivity distribution without (•) and with added phosphorylase kinase (○), after cutting the gel into 1 mm slices as in [11].

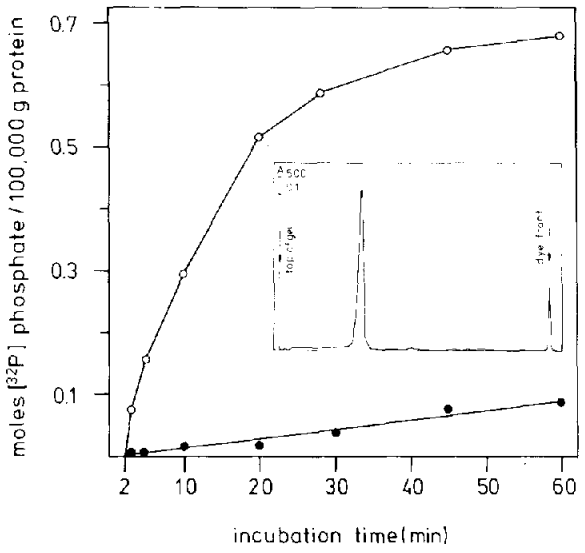


Fig.5. Purified SR $Ca^{2+}$-transport ATPase (4.2 mg/ml) was phosphorylated as in section 2. The phosphorylation was carried out at 0.45 μM $Ca^{2+}$, without (•) or with 300 μg/ml added phosphorylase kinase (○). The insert shows a densitogram of 7 μg purified $Ca^{2+}$-transport ATPase on an SDS–polyacrylamide gel.

3A. This $Ca^{2+}$-independent alkylphosphate incorporation is neither influenced by the heat-stable cyclic AMP-dependent protein kinase inhibitor nor by antibodies against phosphorylase kinase. Furthermore, this alkylphosphate incorporation is unaffected by addition of phosphorylase kinase which is essentially inactive under these conditions [27] or by the catalytic subunit of the cAMP-dependent protein kinase (not shown). Surprisingly, the latter enzyme increases the acylphosphate level from a low level of ~0.1 up to ~0.7 mol/100 000 g protein (fig.3B).

### 3.4. *Localization of the alkylphosphate*

The alkylphosphate which remains after treatment with hydroxylamine can be localized in two proteins after separation of the SR peptides by gel electrophoresis (fig.4). About 50% of the radioactivity co-migrates with a 100 000 $M_r$ band, the other half with a 9000 $M_r$ polypeptide. Addition of phosphorylase kinase increases exclusively the radioactivity incorporation into the 100 000 $M_r$ protein. A low amount of phosphate is incorporated autocatalytically into the α and β subunits of phosphorylase kinase itself (fig.4). The radioactivity distribution between the 100 000 and 9000 $M_r$ proteins (compare fig.4) is the same at nM and μM free $Ca^{2+}$ (not shown).

3.5. *Phosphorylation of purified 100 000* $M_r$ *$Ca^{2+}$-transport ATPase*

The 100 000 $M_r$ $Ca^{2+}$-transport ATPase was isolated as homogeneous protein (fig.5, insert). Similarly to the observations in the intact SR at μM $Ca^{2+}$ and 4 mg protein/ml, the ATPase incorporates alkylphosphate, but at a much slower rate (fig.5). This rate is greatly stimulated by addition of phosphorylase kinase; a final level of 0.7 mol/mol protein is obtained (fig.5). It is not shown here, but the acylphosphate level increases in a very similar manner as observed in intact SR (compare with fig.1B).

## 4. Discussion

We conclude that alkylphosphate incorporation into the 100 000 $M_r$ polypeptide of intact SR represents phosphorylation of the $Ca^{2+}$-transport ATPase. This follows from:

(i) The maximum of incorporated alkylphosphate which amounts to ~0.7 mol/100 000 g protein (compare fig.2) corresponds to a nearly stoichiometric phosphorylation of the $Ca^{2+}$-transport ATPase, since this protein represents ~70% of the total SR protein.

(ii) Phosphorylase kinase catalyzes an incorporation of alkylphosphate into the purified $Ca^{2+}$-transport ATPase to a nearly indentical extent (compare fig.5).

An alkylphosphate incorporation into a polypeptide in the 9000 $M_r$ range has never been observed before in skeletal muscle membranes. It could be similar to phospholamban ($M_r$ 11 000) which is phosphorylated in cardiac microsomes ([18], review [19]).

Previously only very low levels of alkylphosphate incorporation (~0.02 mol/100 000 g SR protein) were observed [20,21], which may be explained by phosphorylation of contaminants, present in isolated SR, like glycogen synthase (0.02%), cyclic AMP-dependent protein kinase (0.01%) or phosphorylase *b* (6%) ([7] cf. [22]). These earlier experiments were carried out at SR levels of 0.1–1 mg protein/ml, i.e., at 0.7–7 μM $Ca^{2+}$-transport ATPase. The $K_m$-value of phosphorylase kinase for phosphorylase *b* at pH 7.5 is ~100 μM [23] and for the alternate substrates glycogen synthase, 50–100 μM [24] or holotroponin, 25–35 μM [25]. If phosphorylase kinase possesses a similar $K_m$-value for the $Ca^{2+}$-transport ATPase the low protein concentrations employed in earlier studies would represent only 1/10–1/100th of the half-saturating concentration resulting in very low reaction rates. In muscle, the SR concentration is ~10 mg/g, i.e., that of the $Ca^{2+}$-transport ATPase ~70 μM [26], which is comparable to that of phosphorylase *b* (~100 μM) [23]. The concentration of phosphorylase kinase in white muscle fibres is ~0.3 μM [7]. Therefore, the SR phosphorylations as reported here were carried out near physiological protein–substrate and kinase concentrations.

Alkylphosphate incorporation into the 100 000 $M_r$ $Ca^{2+}$-transport ATPase can be observed under two conditions: (i) at nM free $Ca^{2+}$; and (ii) at μM $Ca^{2+}$ in the absence and presence of phosphorylase kinase. The incorporation at nM free $Ca^{2+}$ may be catalyzed by an endogeneous membrane-bound $Ca^{2+}$-independent protein kinase. If it exists, this protein kinase is not identical with phosphorylase kinase of cAMP-dependent protein kinase since the phosphorylation observed under these conditions is neither inhibited by antibodies against phosphorylase kinase or the heat-stable inhibitor of the cyclic AMP-dependent protein kinase nor can it be stimulated by addition of phosphorylase kinase itself (not shown). At μM $Ca^{2+}$ without addition of phosphorylase kinase ~50% the amount of alkylphosphate is incorporated in comparison to that at nM $Ca^{2+}$. This phosphorylation might also be catalyzed by the $Ca^{2+}$-independent endogeneous protein kinase since it is, as well, not inhibited by antibodies against phosphorylase kinase (not shown). Addition of phosphorylase kinase enhances the amount of incorporated alkylphosphate (see fig.2). Therefore, the observed phosphorylations might possibly occur on two sites: one phosphorylated by the endogeneous protein kinase; the other by the exogeneously added phosphorylase kinase. During purification of the $Ca^{2+}$-transport ATPase the endogeneous protein kinase has been probably removed since without added phosphorylase kinase only very slow alkylphosphate incorporation can be observed at μM (see fig.5) and nM $Ca^{2+}$.

The initial alkylphosphate incorporation rate into the $Ca^{2+}$-transport ATPase present in SR, which is catalyzed by phosphorylase kinase, amounts to 42 nmol phosphate transferred . $min^{-1}$ . $mg^{-1}$ (see fig.2). Under comparable conditions phosphorylase kinase transfers ~50 nmol phosphate . $min^{-1}$ . $mg^{-1}$ to phosphorylase *b* [27]. This consistency suggests that phosphorylase kinase is competent in phosphorylating the $Ca^{2+}$-transport ATPase in the muscle cell.

$Ca^{2+}$ activates phosphorylase kinase by binding to the fourth subunit, δ, which is identical to calmodulin [27–29]. Addition of purified calmodulin has no effect on the $Ca^{2+}$-transport ATPase activity or SR (M. V., L. M. G. H., unpublished), probably since already ~1% of the SR protein consists of calmodulin [2]. The reported calmodulin activation of erythrocyte $Ca^{2+}$-transport ATPase might also be mediated by a $Ca^{2+}$-dependent calmodulin-stimulated phosphorylation of the ATPase [30,31]. Direct phosphorylase kinase stimulation of the muscle SR $Ca^{2+}$-transport ATPase activity [4], as well as cardiac sarcolemma $Ca^{2+}$ accumulation has been observed [32].

Generally, an inverse relationship seems to exist between the incorporated amount of alkylphosphate, incorporated either by the endogeneous $Ca^{2+}$-independent protein kinase or the added phosphorylase kinase, and the acylphosphate content (compare fig.2,3). It is possible that the stimulation of acylphosphate incorporation at nM $Ca^{2+}$ by the catalytic subunit of the cyclic AMP-dependent protein kinase represents direct phosphate transfer from ATP to the aspartyl residue. It would be a novel type of reaction catalyzed by this kinase; however, at this time intermediates can not be excluded.

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