CALCIUM-DEPENDENT ADSORPTION AND DESORPTION OF PHOSPHORYLASE KINASE ON MEMBRANE FRACTIONS OF SARCOPLASMIC RETICULUM

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1. Introduction

Phosphorylase kinase is a Ca^{2+}-dependent protein kinase obtained in soluble form from rabbit skeletal muscle [1–3]. Phosphorylase kinase activity is detectable in preparations of sarcoplasmic reticulum (SR) [4] and addition of purified phosphorylase kinase to such membrane suspensions is associated with functional changes of the Ca^{2+}-transport ATPase [5]. Evidence obtained from phosphorylase kinase-deficient mice [6] led to the conclusion that SR membranes contain a Ca^{2+}-stimulated protein kinase distinct from phosphorylase kinase. This kinase activity may in part be attributable to calsequestrin [7]. However, there has been no direct biochemical evidence for a physical association of phosphorylase kinase with these membrane fractions which could be of physiological significance.

During experiments directed towards the analysis of adsorption phenomena of proteins on interfaces [8,9] it was found that purified non-activated phosphorylase kinase, which appears to be more hydrophobic than phosphorylase *b* [10], can be adsorbed to SR vesicles in the presence of calcium [10]. This paper describes details of the adsorption and desorption of a Ca^{2+}-dependent protein kinase on biological membrane fractions as a function of the Ca^{2+} signal.

2. Materials and methods

Phosphorylase *b* (1.3 μkat/mg) [11], phosphorylase kinase (115–133 nkat/mg at pH 8.2; activity ratio pH 6.8/pH 8.2 ~ 0.02–0.05) [1,12] and sarcoplasmic reticulum (Ca^{2+}-transport ATPase in the presence of oxalate: 20–30 nkat/mg) [13] isolated at pH 7.0 [5] were prepared from rabbit skeletal muscle. SR prepared by the above method [13] is practically free of nuclei, sarcolemma, mitochondria and myofibrils [14,15]. However these preparations (crude vesicles) may be contaminated with endogenous enzymes of glycogen metabolism together with glycogen [16]. The approximate composition and some endogenous enzyme activities of SR as employed in this work were as follows: protein 40–50 mg/ml; Ca^{2+}-transport ATPase in the presence of oxalate 0.8–1.5 μkat/ml (activity ratio [4] + oxalate/–oxalate ~2.5); phosphorylase *b* 0.6–4.2 μkat/ml; phosphorylase kinase (pH 8.2) 1–3 nkat/ml (activity ratio ~0.13); glycogen 2–4 mg/ml.

Anti-phosphorylase kinase [4,16] was partially purified by $(NH_4)_2SO_4$ precipitation (40% saturation) followed by dialysis against H_2O (removal of euglobulins). Before use, shell-fish glycogen (Merck, Darmstadt) was purified according to [17]. α-Amylase (*Bacillus subtilis*) was obtained from Boehringer, Mannheim.

Phosphorylase *b* [18], phosphorylase kinase (final conc. Ca^{++} in assay: 0.1 mM) [19] and the Ca^{2+}-transport ATPase [4,13] were measured as described. Protein [20] was determined on an AutoAnalyzer (Technicon) employing bovine serum albumin as standard. Antibody inhibition of phosphorylase kinase [4] was measured in the automated assay [19]. Glycogen was determined [21] after removal of protein with 5% Trichloroacetic acid and ethanol precipitation (66%) of the glycogen.

Analytical gel filtration on Sepharose 2B (Pharmacia) at 5°C overnight on a column (1 cm i.d. × 60 cm, bed ht 40–50 cm, sample vol. 0.9–1.2 ml, fraction vol. 1.2–1.4 ml, flowrate 5 ml/h) was performed as in [10,22]. The gel was either equilibrated with

buffer A (50 mM sodium β-glycerophosphate, 0.5 mM ethylene glycol-bis (2-aminoethyl ether)-*N,N,N',N'* tetraacetic acid (EGTA), pH 7.0 or with buffer B (buffer A + 0.5 mM $CaCl_2$). The free [Ca^{2+}] of buffers A and B was ~10 nM and ~10 μM, respectively. [Ca^{2+}] of the solution were checked by calcium determinations [23].

For control and reconstitution experiments crude vesicles were either digested with trypsin (0.1 mg/ml, 30 s, 20°C) followed by addition of trypsin inhibitor as in [24] or with α-amylase (0.1–0.15 mg/ml, 10 min, 30°C). The amylase digested crude vesicles (= 1 vol.) were further purified by dilution in 14 vol. 0.1 M Tris–HCl (pH 7.0) 0°C and gentle homogenization in a Potter-Elvehjem homogenizer. The SR vesicles were then centrifuged at 5°C for 35 min 170 000 × *g* into a cushion of 0.5 vol. 2 M sucrose (= purified vesicles).

SDS–polyacrylamide (10%) gel electrophoresis was performed according to [25].

3. Results and discussion

3.1. *Calcium-dependent adsorption of phosphorylase kinase*

The paired analytical gel filtration experiment in fig.1 shows that in the presence of ~10 nM free Ca^{2+} (fig.1A) only ~7% of the exogenously added purified phosphorylase kinase elutes together with the Ca^{2+}-transport ATPase ($V_e/V_0 \sim 1.2$) and SR protein; the major portion (85–90%) elutes in soluble form ($V_e/V_0 \sim 2.1$). Endogenous phosphorylase *b* elutes with a major activity peak together with soluble phosphorylase kinase and tails forward into the region of the Ca^{2+}-transport ATPase.

In contrast at ~10 μM free Ca^{2+} in the buffer (fig.1B) >50% of the exogenously added purified phosphorylase kinase elutes ($V_e/V_0 \sim 1.2$) complexed to the vesicles of SR (SR-kinase complex) as indicated by the Ca^{2+}-transport ATPase activity and vesicle protein. The ratio of phosphorylase kinase protein (calculated from a specific activity of 133 nkat/mg) to SR protein is ~0.032. About 60% of the endogenous phosphorylase *b* activity now elutes together with the Ca^{2+}-transport ATPase activity, the remainder tailing backwards to the soluble phosphorylase kinase peak ($V_e/V_0 \sim 2.1$).

The chromatographic distribution of endogenous phosphorylase *b* correlates with the distribution of

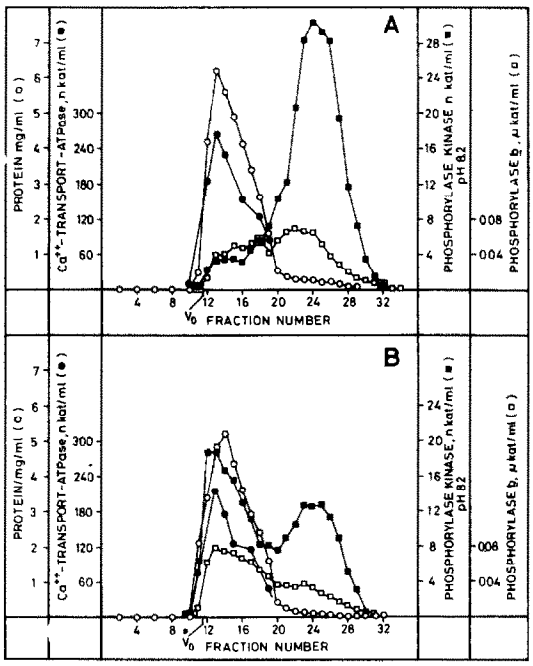

Fig.1. Adsorption of phosphorylase kinase to vesicles of sarcoplasmic reticulum at ~10 nM free Ca^{2+} (A) and ~10 μM free Ca^{2+} (B). (A) Analytical gel filtration of a mixture of purified phosphorylase kinase and crude vesicles of SR on Sepharose 2B in buffer A. (B) Analytical gel filtration of an identical sample (see below) in buffer B. The sample (1.2 ml) consisted of: 34 mg/ml crude vesicles, 1.4 mg/ml purified phosphorylase kinase, 16 mM sodium β-glycerophosphate, 0.3 mM EDTA, 0.3 mM dithioerythritol, 0.5 mM EGTA, 66 mM Tris–HCl (pH 7.0) and in (B) additionally 0.5 mM $CaCl_2$. The activity of the Ca^{2+}-transport ATPase was determined in the presence of oxalate [4,13]. For further details see text.

endogenous glycogen and not with the elution of the purified enzyme ($V_e/V_0 \sim 2.6$) (cf. [26]). In the fractions containing the SR-kinase complex values for glycogen ranged from 0–10 and 5–20 μg/ml SR protein at ~10 nM and ~10 μM free Ca^{2+}, respectively, depending on the run (for further details on the effect of glycogen see section 3.4.).

SDS–Polyacrylamide gel electrophoresis (fig.2) demonstrates that the intact subunits (especially α, α' and β) of phosphorylase kinase are detectable in the SR–kinase complex obtained at ~10 μM free Ca^{2+} (see fig.1B). From densitometer tracings (not shown) of the gels (fig.2A) the molar ratio of $\alpha\alpha'/\beta$ is 0.76 ± 0.1 in the fractions 23–25. This value is not significantly different from the subunit ratio (~0.8) of the enzyme prior to incubation with SR [27].

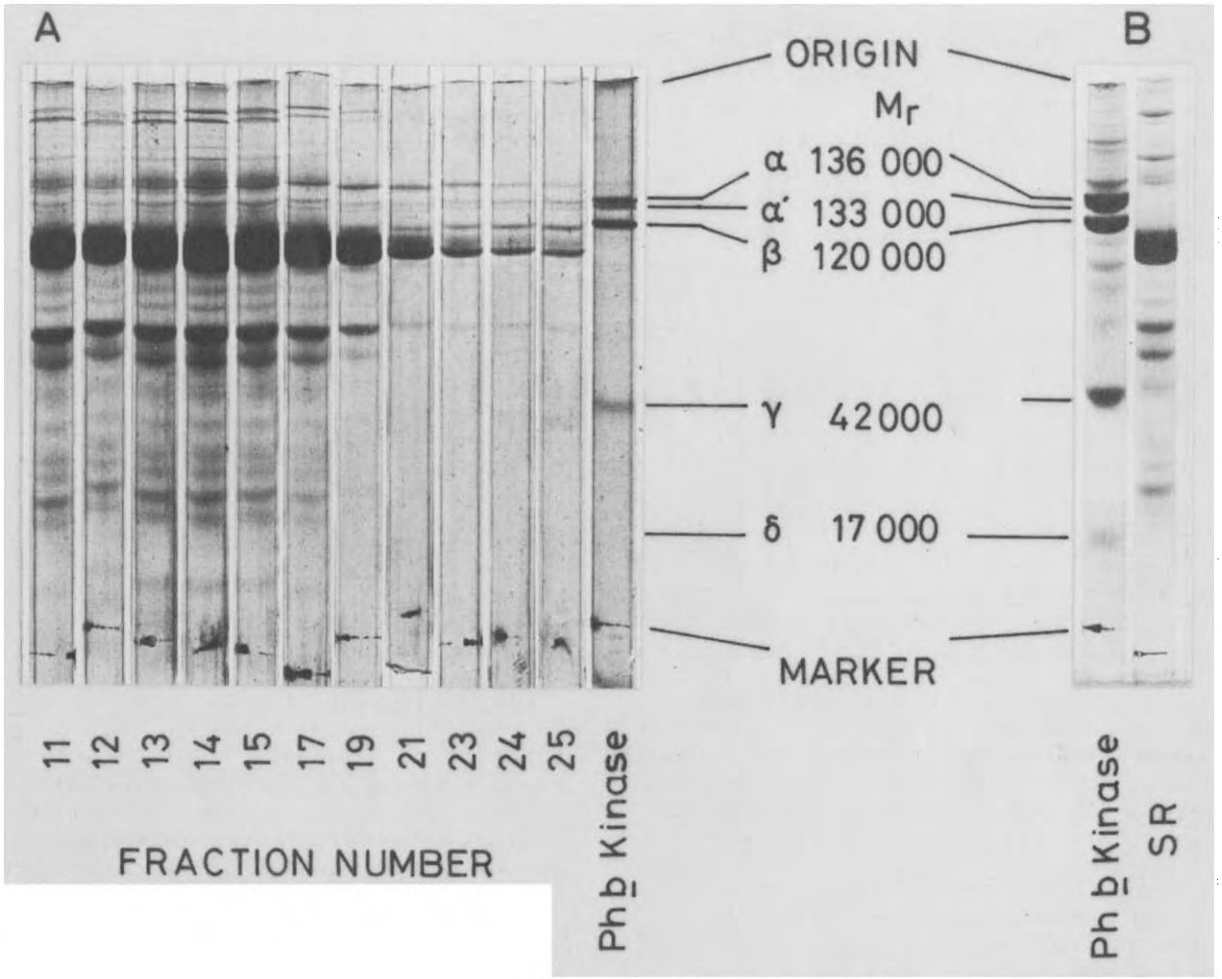

Fig.2. Identification of adsorbed phosphorylase kinase in vesicle fractions by SDS gel electrophoresis. (A) 20 μl of fractions obtained from a run under the conditions of fig.1B (~10 μM free Ca^{2+}) was applied to the top of a gel with sample buffer in 70 μl total vol. This corresponds to 80–110 μg protein in the SR peak fractions 11–15. Phosphorylase kinase (Ph *b* kinase) as control was applied at 7 μg. (B) As control phosphorylase kinase was applied at 32 μg to demonstrate the δ-subunit and crude vesicles (SR) were applied at 60 μg/gd. M_r indicates the apparent relative molecular mass [3,27] of the subunits of phosphorylase kinase. The total polyacrylamide concentration was 10%. For further details see text.

3.2. *Calcium-dependent desorption of phosphorylase kinase*

If the peak fractions of a chromatographic run at ~10 μM free Ca^{2+} (e.g., fig.1B) containing the SR–kinase complex are pooled and aliquots are immediately rechromatographed at ~10 nM and ~10 μM free Ca^{2+} the profiles of fig.3 are obtained. At ~10 nM free Ca^{2+} (fig.3A) up to 30% of the pre-adsorbed phosphorylase kinase can be desorbed. However only ~7% of the adsorbed activity is released at ~10 μM free Ca^{2+} (fig.3B).

3.3. *Effect of adsorption on enzyme activities*

As shown in table 1 the activity ratio of phosphorylase kinase does not change significantly during the adsorption run at ~10 nM free Ca^{2+} (see fig.1A); the Ca^{2+}-transport ATPase however loses its capability to be stimulated by oxalate (activity ratio ~1.0). During adsorption at ~10 μM free Ca^{2+} (see fig.1B) the activity ratio of adsorbed phosphorylase kinase increases ~2-fold to 0.113 in comparison to the ratio of soluble enzyme; the Ca^{2+}-transport ATPase retains its oxalate sensitivity. During the desorption runs

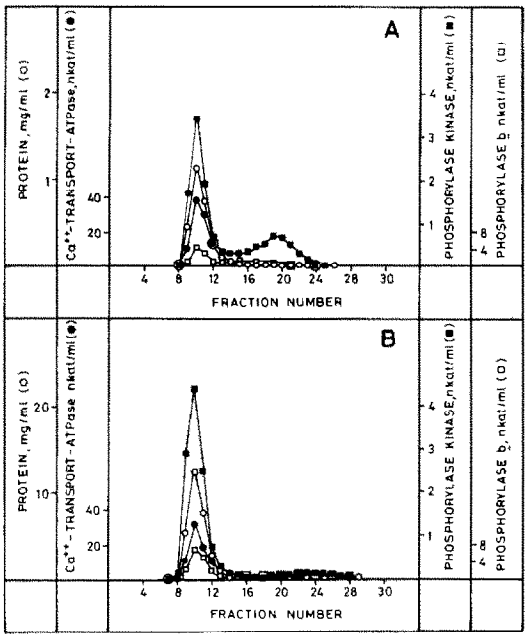

Fig.3. Desorption of phosphorylase kinase from vesicles of sarcoplasmic reticulum at ~10 nM free Ca^{2+} (A) and ~10 μM free Ca^{2+} (B). (A) Analytical gel filtration of the SR–kinase complex in buffer A on Sepharose 2B. (B) Analytical gel filtration of identical SR–kinase complex in buffer B. The sample (0.9 ml) obtained from a run under the conditions of fig.1B contained: 4.7 mg protein/ml, phosphorylase kinase activity (pH 8.2) 24 nkat/ml (activity ratio: 0.078), Ca^{2+}-transport ATPase activity (+ oxalate 200 nkat/ml (activity ratio: 7.0). To the sample run in buffer A 5 μl EGTA (0.2 M) was added. For further details see text and fig.1.

(table 1, fig.3) the Ca^{2+}-transport ATPase loses its sensitivity towards oxalate at both ~10 nM and ~10 μM free Ca^{2+}. The activity ratio of soluble phosphorylase kinase released from SR at ~10 nM free Ca^{2+} (fig.3A) corresponds to the ratio observed for the free native enzyme. Thus the increase in activity ratio of phosphorylase kinase absorbed to SR is reversed by desorption and could be explained by the action of lipid membrane components [28].

Both the adsorbed and soluble form of phosphorylase kinase (fig.1) can be inhibited to ~97% by anti-phosphorylase kinase (not shown); half maximal inhibition of the enzyme (100 pkat/ml, pH 8.2) occurs at 23 and 34 μg antibody/ml for the two forms, respectively. Thus the catalytically significant segments of the adsorbed enzyme appear to be in a state readily available to the antibodies and indistinguishable from the soluble enzyme. The effective inhibition

Table 1
Activity ratios of phosphorylase kinase (pH 6.8/pH 8.2) and of the Ca^{2+}-transport ATPase (+oxalate/–oxalate) after analytical gel filtration under various conditions

Peak fraction	Phosphorylase kinase act. pH 6.8/pH 8.2 ratio	ATPase act. +oxalate/ –oxalate ratio
Adsorption		
SR + Kinase–Ca^{2+}		
ATPase (V_e/V_o ~ 1.2)	0.042	1.1
sol. Kinase (V_e/V_o ~ 2.1)	0.052	–
SR + Kinase + Ca^{2+}		
ATPase (V_e/V_o ~ 1.2)	0.113	4.5
sol. Kinase (V_e/V_o ~ 2.1)	0.054	–
Desorption		
SR · Kinase–Ca^{2+}		
ATPase (V_e/V_o ~ 1.2)	0.081	0.9
sol. Kinase (V_e/V_o ~ 2.1)	0.021	–
SR · Kinase + Ca^{2+}		
ATPase (V_e/V_o ~ 1.2)	0.180	1.0
sol. Kinase (V_e/V_o ~ 2.1)	0.219	–

The data are taken from the peak fractions of fig.1 and 3. Buffer A is denoted by $-Ca^{2+}$ and buffer B by $+Ca^{2+}$. The non-adsorbed, soluble phosphorylase kinase is denoted by 'sol. kinase'. For further details see fig.1,3 and text

of the adsorbed enzyme by antibodies again indicates a native state of phosphorylase kinase [6].

3.4. *Digestion and reconstitution experiments*

Incubation of crude SR vesicles with trypsin (see section 2) reduces the capacity of the vesicles to bind phosphorylase kinase by 40–80% (not shown). This indicates that the integrity of sarcoplasmic reticulum proteins is required for the Ca^{2+}-dependent adsorption of phosphorylase kinase.

Amylase digestion of crude vesicles (see section 2) decreases the endogenous glycogen content by ~80% to ~15–20 μg/mg SR protein. Further purification of amylase digested SR (see section 2) reduces the endogenous glycogen content to ~1–2 μg/mg SR protein. This value (see method of glycogen assay) is not corrected for possible glycoproteins which are not precipitated by acid protein denaturants [29,30] but precipitate with 66% ethanol [30]. Endogenous phosphorylase *b* and phosphorylase kinase (pH 8.2) specific activity are reduced to 590 and 7 pkat/mg, respectively.

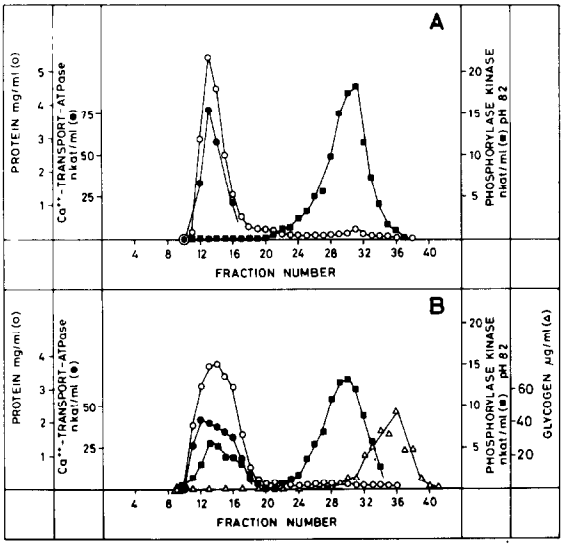

Fig.4. Reconstitution of purified vesicles of sarcoplasmic reticulum with glycogen at ~10 µM free Ca^{2+}. (A) Analytical gel filtration of a mixture of purified phosphorylase kinase and purified (amylase digested) vesicles of sarcoplasmic reticulum on Sepharose 2B in buffer B. (B) Analytical gel filtration of an identical sample to which 0.5 mg/ml of purified shell-fish glycogen has been added (Sepharose 2B, buffer B). The sample (1.1 ml) consisted of 26 mg/ml of purified SR protein, 1.1 mg/ml of purified phosphorylase kinase and the other constituents listed in the legend to fig.1 (A) and in addition glycogen (B). For further details see fig.1 and the text.

Purified SR vesicles have lost the capacity to bind phosphorylase kinase at ~10 µM free Ca^{2+} (fig.4A). The binding capacity can however be restored by reconstitution of purified SR with purified exogenous glycogen (fig.4B). Phosphorylase *b* (no activity detectable in the fractions) is without effect (not shown). In the peak fractions (fig.4A,B) the activity ratio of the Ca^{2+}-transport ATPase is ~2.5. The activity ratio of phosphorylase kinase is 0.093 and 0.049 in the respective peak fractions of the adsorbed and soluble enzyme forms (fig.4B). Since soluble phosphorylase kinase does not form complexes with glycogen at $V_e/V_o \sim 1.2$ or at $V_e/V_o > 1.2$ (fig.4B) [31], a simple aggregation mechanism can be excluded. In fig.4B the carbohydrate (glycogen) concentration in the fractions of the SR–kinase complex is very low (<0.3 µg/ml SR protein; mean value of 3 runs, ~0.6 µg/mg SR protein). The amount of carbohydrate present in SR after reconstitution with glycogen and gel filtration is therefore not significantly higher than the initial endogenous carbohydrate content of purified SR (see above). Thus the mechanism of action of glycogen in restoring the binding capacity of SR for phosphorylase kinase remains to be elucidated. Possibly the induction of a certain conformation of the phosphorylase kinase molecule may play a role.

The results of the binding experiments described in fig.1–4 are in agreement with localization studies. Immunofluorescence analysis of skeletal muscle sections with antibodies against the subunits of phosphorylase kinase [32] indicate that this enzyme is localized in close proximity to SR and glycogen [33]. The results in fig.3 also offer an explanation for the difficulty of eliminating endogenous phosphorylase kinase in preparations of SR.

From the data it may be concluded that 3 macromolecular components (ternary system) must be present for the Ca^{2+}-dependent formation of the SR–kinase complex: (1) recognition protein (on SR); (2) carbohydrate moiety (glycogen); and (3). Ca^{2+}-dependent protein kinase (phosphorylase kinase). From the change in the activity ratio (table 1) of adsorbed phosphorylase kinase an interaction with membrane lipids [28] is also probable. Thus it appears that during evolution the cell has devised a very complex mechanism for the binding and regulation of a Ca^{2+}-dependent protein kinase on biomembranes.

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