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Ca^{2+}- and Mg^{2+}-dependent association of phosphorylase kinase with human erythrocyte membranes

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The interaction of rabbit muscle phosphorylase kinase (EC 2.7.1.38) with human erythrocyte membranes was investigated. It was found that at pH 7.0 the kinase binds to the inner face of the erythrocyte membrane (inside-out vesicles) and that this binding is Ca^{2+}- and Mg^{2+}-dependent. The sharpest increase in the binding reaction occurs at concentrations between 70 and 550 nM free Ca^{2+}. Erythrocyte ghost or right-side out erythrocyte vesicles showed a significantly lower capacity to interact with phosphorylase kinase. Autophosphorylated phosphorylase kinase shows a similar Ca^{2+}-dependent binding profile, while trypsin activation of the kinase and calmodulin decrease the original binding capacity by about 50%. Heparin (200 μg/ml) and high ionic strength (50 mM NaCl) almost completely blocks enzyme–membrane interaction; glycogen does not affect the interaction.

Rabbit skeletal muscle phosphorylase kinase (EC 2.7.1.38), the key enzyme involved in the regulation of glycogen metabolism is a multisubunit calcium/calmodulin-dependent enzyme composed of four different subunits with a stoichiometry $(\alpha\beta\gamma\delta)_4$ (for reviews see Refs. 1–3). The γ-subunit contains a catalytic site [4] and belongs to the family of protein kinases [5]. The smallest δ-subunit is calmodulin [6] and serves two functions, namely, as activator in its Ca^{2+}-saturated and as inhibitor in its Ca^{2+}-free form [7]. Although a cytosolic enzyme, phosphorylase kinase activity is associated with sarcoplasmic reticulum or plasma membranes [8,9]. Recently, Thieleczek et al. [10] have localized molecular structures related to phosphorylase kinase at the sarcoplasmic reticulum of rabbit skeletal muscle employing polyclonal antibodies directed against the holoenzyme as well as monoclonal antibodies specific for its α-, β- or γ-subunits. In addition, we found that certain membrane-component acidic phospholipids and the neutral phospholipid lysophosphatidylcholine greatly stimulated the activity of non-activated phosphorylase kinase at neutral pH [11].

In the present work, we demonstrate the ability of cytoplasmic phosphorylase kinase to interact with inside-out erythrocyte vesicles in a Ca^{2+}- and Mg^{2+}-dependent manner and we further characterize the influence of several factors upon this membrane–kinase association process.

Rabbit muscle phosphorylase *b* [12], phosphorylase kinase [13], calmodulin [14] and white ghosts [15] were prepared by established procedures. Sealed inside-out and right-side-out vesicles were prepared as described in Ref. 16 using Tris-

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HCl instead of sodium phosphate. Sarcoplasmic reticulum vesicles were prepared from skeletal muscle of male, 2- month-old starved (20 h) rabbits according to DeMeis and Hasselbach [17]. The approximate content of sarcoplasmic reticulum in glycogen and endogenous phosphorylase kinase activity was as follows: glycogen, 1.7 μg/mg and 0.7 μg/mg of sarcoplasmic reticulum protein before and after amylase digestion, respectively, phosphorylase kinase (pH 8.2), $(4–5) \cdot 10^{-3}$ units/mg of sarcoplasmic reticulum protein. Autophosphorylation of phosphorylase kinase with [γ-^{32}P]ATP was performed according to Ref. 18 and partial proteolysis with trypsin was performed according to Ref. 19. Phosphorylase kinase was assayed at pH 8.2 by measuring phosphorylase *a* activity [13]. Protein concentrations were determined as in Ref. 20 using bovine serum albumin as the standard.The glycogen content of membrane preparations was determined [21] after removal of protein with 5% trichloroacetic acid and ethanol precipitation of the glycogen. Treatment of the sarcoplasmic reticulum membranes with α-amylase (EC 3.2.1.1) was performed according to Ref. 22. Free Ca^{2+} in EGTA-buffered incubation media was calculated using a computer programme [23]. Several phosphorylase kinase preparations, with slightly varying specific activity, were used for the binding experiments. The reaction mixture was incubated at room temperature for 15 min and then centrifuged for 20 min in an Eppedorf (type 5414 S) centrifuge at 4° C. The supernatant was removed, the membranes were suspended in 70 μl 0.5 mM Tris-HCl/1mM dithiothreitol (pH 8.0) and the amount of phosphorylase kinase bound to the membranes or remaining in the supernatant was determined. A control of phosphorylase kinase was always centrifuged under the same experimental conditions, in the absence of membranes, but in the presence of bovine serum albumin (at similar concentrations to those of membrane protein) and other effectors (when present). The amount of phosphorylase kinase precipitated under these conditions was never higher than 3% of the total kinase applied for centrifugation. The binding assay of nonactivated phosphorylase kinase with sarcoplasmic reticulum membranes was the same as that with erythrocyte vesicles, except that the incubation mixture contained also 45 mM NaCl and the centrifugation was performed at 40000 × *g* for 45 min (at 4° C) [24]. Under the above conditions, about 60–70% of the sarcoplasmic reticulum protein was recovered in the pellet after centrifugation.

TABLE I

SYNERGISTIC EFFECT OF Ca^{2+} AND Mg^{2+} ON THE ASSOCIATION OF NONACTIVATED PHOSPHORYLASE KINASE (PK) WITH ERYTHROCYTE INSIDE-OUT VESICLES

The binding assay was performed in 0.125 ml of 10 mM Tris/HCl buffer (pH 7.0) containing glycerol 2-phosphate (0.8 mM)/dithiothreitol (1 mM)/phenylmethylsulphonyl fluoride (0.1 mM)/benzamidine (2 mM)/glycerol (1.6%, v/v) EGTA (0.5 mM) in the presence of 80 μg/ml of phosphorylase kinase and 1.1 mg/ml of membrane protein. $CaCl_2$ was added to give the indicated free-Ca^{2+} concentration. The amount of phosphorylase kinase bound to the membranes was determined by measuring phosphorylase kinase activity at pH 8.2.

Metal concentration		Units ($\times 10^3$) of PK bound	% Binding
Ca^{2+} (μM)	Mg^{2+} (mM)		
–	–	1.7	3
–	3	10.8	19.5
0.09	–	2.3	4.1
0.09	3	16.9	30.4
13.4	–	8	14.4
13.4	3	25	45

Initially, we surveyed the extent of binding of nonactivated phosphorylase kinase by several preparations of erythrocyte ghosts and vesicles, in the presence of 3 mM Mg^{2+} and 13 μM free Ca^{2+} (conditions as in Table I). Less than 5% of the total kinase activity was recovered with the white ghost membrane fraction. On the other hand, 15% of the kinase activity was retained by the right-side-out vesicles, while about 45% of the enzyme activity precipitated with the inside-out membrane pellet. Enzyme association with erythrocyte inside-out vesicles was found to be Ca^{2+}- and Mg^{2+}-dependent. As shown in Table I, although in the absence of both metals phosphorylase kinase binding was insignificant, the presence of either Ca^{2+} or Mg^{2+} significantly increased enzyme–membrane interaction. In addition, Ca^{2+} and Mg^{2+}, when added together, induced a synergistic effect. At this point, it must be noted that incubation of phosphorylase kinase in the presence of both ions

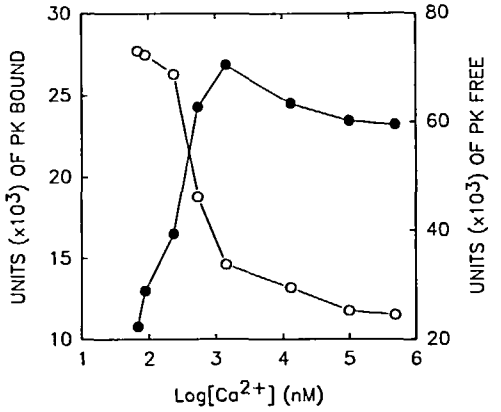

Fig. 1. Ca^{2+}-dependent binding of non-activated phosphorylase kinase (PK) to erythrocyte inside-out vesicles. (●) bound enzyme; (○) free enzyme. The binding procedure was performed as described in Table I in the presence of 3 mM Mg^{2+} and various free-Ca^{2+} concentrations as indicated. The amount of phosphorylase kinase activity remaining in the supernatant (free enzyme) was also determined by measuring phosphorylase kinase activity at pH 8.2.

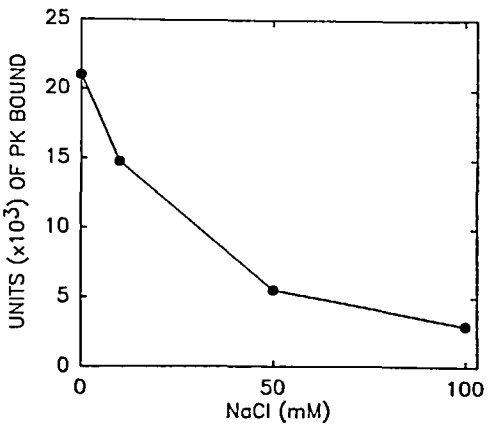

Fig. 2. The effect of NaCl on the binding of nonactivated phosphorylase kinase (PK) to erythrocyte inside-out vesicles. The binding assay was performed in the presence of 13.4 μM free Ca^{2+} and 3 mM Mg^{2+}. Other conditions were as in Table I.

(in the absence of membranes) did not result in kinase activation under our experimental conditions (pH 8.2), in accordance with the observations of King and Carlson [25]. In the presence of 3 mM Mg^{2+}, the sharpest increase in the binding reaction occured at concentrations between 70 and 550 nM free Ca^{2+} (Fig. 1), agreeing favourably with the reported levels of intracellular Ca^{2+} in basal and in various stimulated conditions, respectively [26]. At 1.45 μM free Ca^{2+}, 44.3 ± 3.5% (mean ± S.D. for five binding experiments) of the total applied enzyme activity was obtained bound with erythrocyte vesicles after centrifugation.

In the presence of increasing concentrations of NaCl, the association of the kinase with inside-out vesicles is gradually inhibited (Fig. 2). 10 mM glycerol 2-phosphate, under the same conditions, induced a similar inhibition to that of 50 mM NaCl.

In order to characterize the association of non-activated phosphorylase kinase with erythrocyte inside-out vesicles, we followed the binding reaction as a function of membrane protein (Fig. 3) or enzyme (Fig. 4) concentration. The profile of the association reaction in the presence of increasing concentrations of membrane protein reached a maximum at about 1.1 mg/ml of membrane protein. On the other hand, the linear Scatchard plot of Fig. 4 suggests a single class of homogenous noninteracting binding sites.

Calmodulin at a concentration of 20 μg/ml inhibited about 45% of the binding of non-activated phosphorylase kinase to inside-out vesicles (Table II). Melittin, an amphiphilic peptide, which is well known to bind to calmodulin with a binding constant in the nanomolar

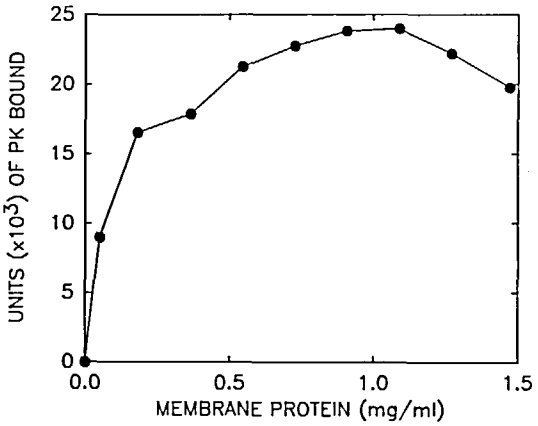

Fig. 3. Dependence of the binding reaction on erythrocyte membrane concentration. The binding assay was performed in the presence of 13.4 μM free Ca^{2+} and 3 mM Mg^{2+}. The membrane protein concentration was varied as indicated. Other conditions were as described in Table I. PK, protein kinase.

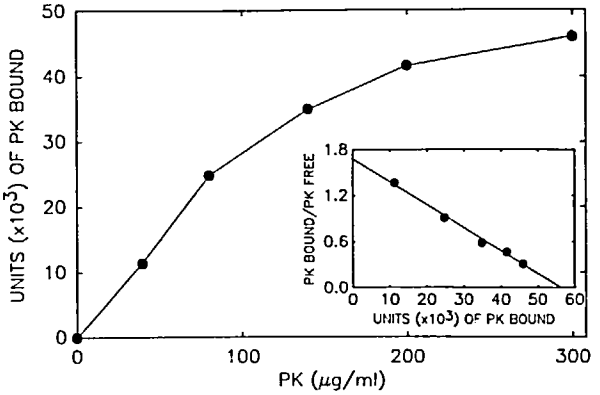

Fig. 4. Dependence of the binding reaction on phosphorylase kinase (PK) concentration. The binding assay was performed in the presence of 13.4 μM free Ca^{2+} and 3 mM Mg^{2+}. The phosphorylase kinase concentration was varied as indicated. Other conditions were as described in Table I. Inset: a Scatchard plot of nonactivated phosphorylase kinase binding to inside out vesicles.

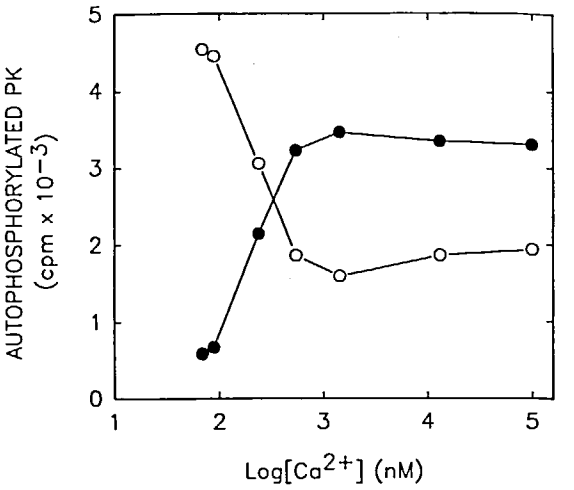

Fig. 5. Ca^{2+}-dependent binding of autophosphorylated (^{32}P-labelled) phosphorylase kinase (PK). (●) bound enzyme; (○) free enzyme. The amount of autophosphorylated phosphorylase kinase bound to the membranes or remaining in the supernatant was determined by quantitation of ^{32}P radioactivity by liquid scintillation counting. The binding assay mixture also contained 10 mM NaF. Other conditions were as in Fig. 1.

range and disrupts calmodulin-dependent processes [27,28] had no significant effect on the membrane–kinase interaction (Table II). In contrast, heparin, which has been shown to affect all three activities of phosphorylase kinase (A_0, A_1, A_2) and to change the aggregation state of the enzyme by dissociating the δ-subunit [7], almost completely inhibited Ca^{2+}-dependent binding of the kinase to erythrocyte membranes (Table II).

When autophosphorylated (^{32}P-labelled) phosphorylase kinase was incubated with inside-out vesicles, a Ca^{2+}- and Mg^{2+}-dependent enzyme association was observed (Fig. 5). This association occurred mostly at concentrations between 90 and 550 nM free Ca^{2+}, and the amount of ^{32}P-labelled phosphorylase kinase remaining in the supernatant gradually decreases with increasing amounts of ^{32}P radioactivity in the pellet. At 1.45 μM free Ca^{2+} and 3 mM Mg^{2+}, about 68.5% of the total radioactivity applied precipitated with the membrane pellet (Fig. 5), while in the absence of Mg^{2+}, only 35.3% of the total ^{32}P was recovered with the membrane vesicles (not shown).

When phosphorylase kinase was activated by limited proteolysis, another well-known mechanism of phosphorylase kinase activation [29], the enzyme's ability to interact with the membranes was reduced significantly. At 13.4 μM free Ca^{2+} and 3 mM Mg^{2+}, the trypsinolyzed kinase retained 51% of its initial capacity for membrane association.

It has been reported [22] that nonactivated phosphorylase kinase can be adsorbed to sarcoplasmic reticulum vesicles in a Ca^{2+}-dependent manner and that glycogen is needed for the formation of sarcoplasmic reticulum-kinase complex; in that study, high concentrations of both kinase

TABLE II

EFFECT OF CALMODULIN, MELITTIN AND HEPARIN ON THE ASSOCIATION OF NONACTIVATED PHOSPHORYLASE KINASE (PK) WITH ERYTHROCYTE INSIDE-OUT VESICLES

The binding procedure was performed as described in Table I, in the presence of 3 mM Mg^{2+} and 13.4 μM free Ca^{2+}.

Addition	Units ($\times 10^3$) of PK bound	% Binding
None	24.2	45
Calmodulin (5 μg/ml)	16.3	30.6
Calmodulin (20 μ/ml)	13.2	24.6
Melittin (15 μM)	23.8	44.6
Melittin (30 μM)	23	43.1
Heparin (50 μg/ml)	10.2	19.1
Heparin (200 μg/ml)	4.2	7.8
Heparin (400 μg/ml)	3.6	6.8

(1.1–1.4 mg/ml) and sarcoplasmic reticulum membrane protein (26–34 mg/ml) were used for the binding experiments. Using sarcoplasmic reticulum preparations with a low glycogen content and varying the membrane protein concentration from 0.5 to 5 mg/ml in the presence of 80 μg/ml phosphorylase kinase, 3 mM Mg^{2+} and 12 μM free Ca^{2+}, we could not obtain association of nonactivated kinase higher than 15–17% (100% was the total exogenous kinase activity). The highest binding was observed at 3 mg/ml sarcoplasmic reticulum protein and, under our experimental conditions, we were unable to find any significant Ca^{2+} or Mg^{2+} stimulation of sarcoplasmic reticulum–kinase association. In addition, the presence of exogenous glycogen (up to 3 mg/ml) or the use of amylase-treated sarcoplasmic reticulum membranes could not significantly affect the above sarcoplasmic reticulum–phosphorylase kinase interaction. Under the centrifugation conditions used in the above experiments, we were unable to use phosphorylase kinase concentrations significantly above 1 mg/ml, especially at low ionic strength, due to the tendency of the protein to aggregate in the presence of Ca^{2+} and Mg^{2+} [30]. On the other hand, it has been found [31] that the formation of molecular complexes between glycogen and phosphorylase kinase when present at concentrations higher than 1 mg/ml was greatly enhanced by Ca^{2+} and Mg^{2+}.

In this investigation, we used human erythrocyte membranes as the model of choice for studying phosphorylase kinase–membrane interaction. This membrane system is well defined, and lacks endogenous glycogen and phosphorylase kinase activity. We have shown that phosphorylase kinase preferentially binds to the inner surface of the human erythrocyte membrane in a Ca^{2+} and Mg^{2+}-dependent manner and that the sharpest increase of this association occurs at concentrations between 70 nM and 550 nM free Ca^{2+} (Fig. 1) when 3 mM Mg^{2+} is present. Ca^{2+} concentrations in the same range are also known to induce a rapid, reversible membrane association of the protein kinase C [26,32]. The similarity of binding curves for both nonactivated (Fig. 1) and autophosphorylated (Fig. 5) phosphorylase kinase, where the enzyme binding was determined either by catalytic activity (after 400-fold dilution) or radioactivity measurements, implies that the enzyme activity measured in the pellet is not significantly affected by the presence of membrane components [11]. Of course we cannot exclude the possibility that even after such a large dilution, the non-phosphorylated kinase activity was still affected by the membrane. The sharp decrease of phosphorylase kinase–erythrocyte vesicles interaction with increasing ionic strength (Fig. 2) suggests that electrostatic interactions play an important role in the binding reaction. Similarly, the association of some glycolytic enzymes with erythrocyte ghosts is also negatively affected by ionic strength [33,34]. Taking into account the model concerning the spatial arrangement of phosphorylase kinase subunits [35,36], we can suggest that the inhibition of the membrane–kinase interaction by calmodulin (Table II) is kinase directed. Namely, exogenous calmodulin may block some sites on exposed (susceptible to proteolysis) areas of the α- and/or β-subunits, which are responsible for the association. The inability of sarcoplasmic reticulum membranes to bind exogenous phosphorylase kinase specifically in a Ca^{2+}-dependent manner may result from the fact that endogenous phosphorylase kinase molecules are already associated with sarcoplasmic reticulum very tightly [10]. Thus, the occupation of specific membrane-binding sites by the endogenous kinase leads added phosphorylase kinase to Ca^{2+}-independent, unspecific adsorption. We suggest that the difference between our results and those of Jennissen and Lahr [22] are possibly due to the high concentrations of kinase and sarcoplasmic reticulum used in their study.

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