CALCIUM AND CALMODULIN ACTIVATION OF MUSCLE PHOSPHORYLASE KINASE

Effect of tryptic proteolysis

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Received 16 July 1979

1. Introduction

Phosphorylase kinase (EC 2.7.1.38) occupies a central position in the regulation of glycogen metabolism in various tissues [1,2]. The muscle enzyme was shown to be composed of three different subunit types, A, B, and C, arranged in a probable stoichiometry of $(ABC)_4$ [3,4]; recent studies [5] have also demonstrated the presence of a fourth component, mol. wt 17 000, that appears to be identical to the Ca^{2+}-dependent regulator protein or calmodulin. Calmodulin is thought to be present at a stoichiometry of close to 1:1 with respect to the other subunit types [6]. Phosphorylase kinase as purified is relatively inactive at pH 6.8 compared with pH 8.2 [3,4]. A well-known means of activating the enzyme, which is most evident at pH 6.8, involves the proteolytic degradation of the A and B subunits such as by limited trypsin action [3,4,7,8]; during proteolytic activation, the proportions of the C subunit and calmodulin remain essentially unchanged [3,4,8]. Another important property of phosphorylase kinase is its reversible activation by Ca^{2+} [3,4]. The additional presence of calmodulin leads to a further activation of the kinase. Here we present evidence that this latter activation correlates with the ability of the enzyme to bind added calmodulin, and that this process is distinguishable from the activation occurring in the presence of Ca^{2+} alone.

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid; SDS, sodium dodecyl sulfate

2. Materials and methods

2.1. *Purification of enzymes*

Phosphorylase kinase was purified from rabbit skeletal muscle as in [4] except that 0.5 mM phenylmethylsulfonyl fluoride and 0.05 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl were present in all buffers. The characteristics of the resulting enzyme were as in [8,9]. Recrystallized (3X) rabbit-muscle phosphorylase was stored at $-20^{\circ}C$ as a lyophilized powder, and was recrystallized and treated with charcoal to remove AMP before use as substrate.

2.2. *Assay of phosphorylase kinase activity*

The measurement of the incorporation of ^{32}P from [γ-^{32}P]ATP into protein by the chromatographic method [10] has been detailed in [8]. Phosphorylase was 2 mg/ml and the pH was 8.2 unless noted.

2.3. *Preparation of calmodulin-coupled Sepharose 4B*

Calmodulin for this purpose was prepared by heating purified phosphorylase kinase for 2 min at $100^{\circ}C$ followed by centrifugation at 10 000 × *g*. The supernatant contained calmodulin of ~90% purity as judged by polyacrylamide gel electrophoresis. Calmodulin (1.7 mg) was coupled to 0.35 g CNBr-activated Sepharose 4B (Sigma) by the method in [11] except that Ca^{2+} was 0.5 mM rather than 0.02 mM. In this way, 86% of the calmodulin was coupled to the Sepharose. Material for a control column (ethanolamine–Sepharose 4B) was processed identically except that no calmodulin was included.

2.4. *Other materials and methods*

Protein was estimated by the Lowry method [12] using bovine serum albumin as standard. Polyacrylamide gel electrophoresis for the analysis of calmodulin followed the method in [13] and for the analysis of the other subunits, the method in [14] with 7.5% acrylamide.

3. Results

3.1. *Activation of native phosphorylase kinase by calmodulin*

The activity of phosphorylase kinase, at pH 6.8 or pH 8.2, is shown as a function of the added calmodulin concentration in fig.1. At pH 6.8, no stimulation of activity was observed; at pH 8.2, a saturable increase in activity was found with increasing calmodulin concentration. This activation required the presence of Ca^{2+} (not shown). The extent of stimulation was variable over a series of experiments, ranging from 1.5–8-times the basal kinase activity. Half-maximal activation was found at molar ratios, calmodulin to phosphorylase kinase, of 1:3 (referred to an ABC-calmodulin unit of kinase).

3.2. *Binding of phosphorylase kinase to calmodulin–Sepharose 4B*

Muscle phosphorylase kinase bound quantitatively to an affinity column of calmodulin in the presence of Ca^{2+} (fig.2). Washing with buffer containing 0.5 M KCl (not shown) did not elute the phosphorylase kinase but did lead to the specific elution of a peptide of ~95 000 daltons. Quantitative recovery of phosphorylase kinase activity was obtained by eluting the column with an excess of EGTA over Ca^{2+}. Other than the removal of the polypeptide noted above, the phosphorylase kinase eluted from the column was of identical subunit composition to the enzyme applied as judged by SDS–polyacrylamide electrophoresis. Phosphorylase kinase did not bind to a control column (fig.2) nor to a column to which phosphorylase *b* had been coupled (not shown). Therefore, the binding of phosphorylase kinase to the calmodulin–Sepharose 4B column was not the result of non-specific adsorption.

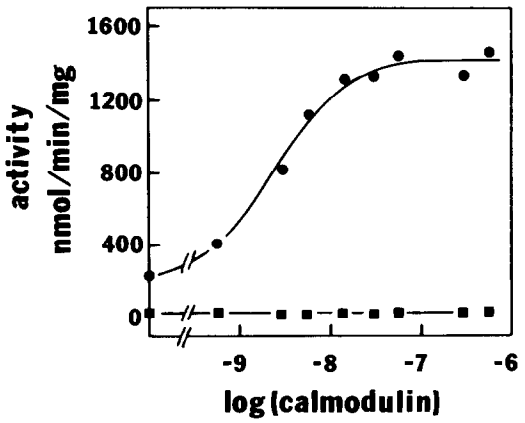

Fig.1. Activation of phosphorylase kinase by added calmodulin. Enzyme activity, at pH 6.8 (▪) and pH 8.2 (•), is plotted against the logarithm of calmodulin concentration (Molar). Phosphorylase kinase was 0.25 μg/ml (pH 8 2) or 6.25 μg/ml (pH 6.8).

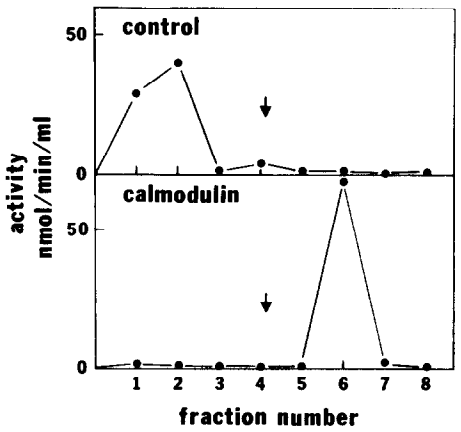

Fig.2. Affinity chromatography of native phosphorylase kinase. Phosphorylase kinase (175 μg in 70 μl) was applied to columns (0.5 × 6.5 cm) of either calmodulin–Sepharose 4B or ethanolamine–Sepharose 4B (control) which had been equilibrated with 50 mM Tris–HCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$, 5% (v/v) glycerol (pH 7.5). The flow rate was 2 ml/h and 1 ml fractions were collected. After collection of fraction 4 (see arrow), elution was continued with buffer containing 5 mM EGTA.

3.3. *Effect of tryptic proteolysis*

Partial tryptic proteolysis was effected by incubating 2.5 mg/ml of phosphorylase kinase with 0.5 μg/ml of trypsin for 15 min at 30°C (see also [8]). At this time, 4 μg/ml of the soybean trypsin inhibitor were added. The pattern of polypeptides as determined by

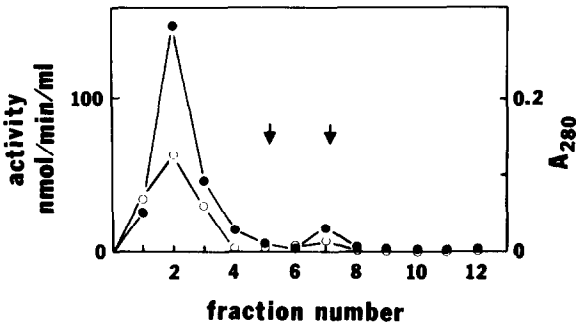

Fig.3. Chromatography of trypsin-treated phosphorylase kinase. Trypsin-treated enzyme (250 μg in 100 μl) was applied to a calmodulin–Sepharose 4B column equilibrated as in fig.2. The flow rate was 2 ml/h and 1 ml fractions were collected. After collecting fraction 5 (first arrow), elution was continued with buffer containing 0.3 M KCl and after fraction 7 (second arrow), EGTA was substituted for KCl. Activity (•) and A_{280} (○) were determined for each fraction.

Table 1
Effect of proteolysis on the activation of phosphorylase kinase by Ca^{2+} and calmodulin

Phosphorylase kinase	Activity (nmol.min^{-1}.ml^{-1})		
	No addition	+Ca^{2+}	+Ca^{2+} and calmodulin
Native	41	1714	3912
After trypsin	423	1821	1423
Column pass-through	8	45	48
Eluted with KCl	1.7	6	6

Phosphorylase kinase was treated with trypsin as described in the text and applied to a calmodulin–Sepharose 4B affinity column. Most activity did not bind to the column (pass-through fraction) and a small amount eluted with 0.3 M KCl (see text)

SDS–polyacrylamide gel electrophoresis was very similar to an earlier report (fig.6 in [8]). Almost complete loss of the A and B subunits had occurred at this time with no apparent changes in the proportion of the C subunit or calmodulin. The proteolytically degraded enzyme no longer bound to the calmodulin affinity column (fig.3), and most of the protein and activity were recovered in the pass-through fractions. Washing the column with buffer containing 0.3 M KCl eluted <10% of the total kinase activity. Elution with EGTA caused no further removal of activity. Analysis by polyacrylamide gel electrophoresis (not shown) indicated no differences between the subunit compositions of the applied enzyme and that eluted from the column.

Proteolysis of phosphorylase kinase also led to a loss of stimulation by added calmodulin (table 1). In the experiment shown, native kinase was activated 4-fold by Ca^{2+} alone and a further 2-fold by the additional presence of calmodulin. Proteolysis did cause some reduction in the Ca^{2+} activation of the enzyme; however, the effect of added calmodulin was completely abolished. This loss of calmodulin-activation was found for trypsin-treated enzyme both before and after passage over the calmodulin column, as well as for the small amount of activity eluting at 0.3 M KCl. In other experiments, it had been shown that binding of native phosphorylase kinase to the calmodulin column followed by elution with EGTA did not abolish stimulation by calmodulin.

4. Discussion

The involvement of Ca^{2+} in determining the activity of muscle phosphorylase kinase is thought to have important physiological implications [1,2]. Not only does Ca^{2+} alone activate the enzyme as purified from muscle but the additional presence of calmodulin causes a further stimulation. The native enzyme will also bind quantitatively to a calmodulin–Sepharose 4B column.

Limited tryptic proteolysis of phosphorylase kinase is known to cause extensive structural changes, both in terms of cleaving preferentially the A and B subunits [3,4,7,8] as well as generating enzyme molecules of lower molecular weight [7]. However, basic catalytic activity can be preserved following this treatment. We report here that proteolytic modification of phosphorylase kinase also leads to a loss of stimulation by added calmodulin as well as the ability to bind to calmodulin–Sepharose 4B. By contrast, the enzyme is still markedly stimulated by Ca^{2+} alone, although the ratio of activity $-Ca^{2+}/+Ca^{2+}$ has been increased.

We suggest, then, that:

(i) The stimulation by added calmodulin correlates

with the ability of the kinase to bind to this component;

(ii) This activation, lost on proteolysis, is a distinct process from activation by Ca^{2+} alone, which is largely retained after proteolysis.

This is generally in agreement with the suggestion [6] that the effect of added calmodulin reflects the binding of a second mole of calmodulin beyond that present intrinsically in phosphorylase kinase. An obvious hypothesis is that the intrinsic calmodulin mediates the activation by Ca^{2+} alone but, to our knowledge, this contention has yet to be proven rigorously.

In conclusion, Ca^{2+} can stimulate phosphorylase kinase:

(i) Directly, by interaction with subunit(s) not yet conclusively identified;

(ii) Through the mediation of added calmodulin.

Acknowledgements

We express our gratitude to Dr Joseph Larner for his encouragement and for providing the facilities for this work. Supported by a grant from the American Diabetes Association, and by United States Public Health Service Grants AM 22125 and AM 14334 (to Joseph Larner), and AM 25939-01 (to P.J.R.). A.A. DeP.-R. is a recipient of a Juvenile Diabetes Foundation fellowship. J. B. G. is a predoctoral fellow in the Department of Pharmacology, University of Virginia School of Medicine.

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