Calcium–calmodulin-dependent activation of porcine liver phosphorylase kinase

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Porcine liver phosphorylase kinase was activated about 1.5-fold by calmodulin in a calcium-dependent manner. Half-maximal stimulation was observed at about 80 nM calmodulin and the activation was almost pH-independent. The specific binding of procine liver phosphorylase kinase to calmodulin–Sepharose affinity column exhibited an absolute dependence upon the presence of calcium. The physiological role of the calmodulin-dependent activation for liver phosphorylase kinase is discussed.

Phosphorylase kinase *Calcium* *Calmodulin* *Porcine liver* *Epinephrine* *Glycogen metabolism*

1. INTRODUCTION

Liver glycogen phosphorylase kinase (EC 2.7.1.38) is known to be one of the key enzymes for glycogen metabolism in controlling blood sugar level. Liver glycogenolysis is mediated by various hormones including α-adrenergic agents [1–3]. The α-adrenergic activation of liver glycogenolysis is mediated by Ca^{2+} [4–6]. However, the detailed mechanism of this α-adrenergic activation remains unexplored. In [7] it was established that calmodulin is involved in many metabolic processes which are controlled by Ca^{2+} [7]. Authors in [8] have first described that the δ subunit of muscle phosphorylase kinase is identical with calmodulin and that this subunit apparently confers the Ca^{2+} sensitivity to muscle phosphorylase kinase. They have also proposed that exogenous calmodulin (δ') stimulates phosphorylase kinase activity [9]. It was subsequently reported [10] that in contrast to the white muscle enzyme, the red muscle phosphorylase kinase does not appear to be regulated by exogenous calmodulin. Here we observed that porcine liver phosphorylase kinase is activated 1.5–2-fold by exogenous calmodulin in a calcium-dependent manner and its possible physiological functions are discussed.

Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride

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2. MATERIALS AND METHODS

Glycogen phosphorylase kinase [11] and phosphorylase *b* [12] from rabbit skeletal muscle, and calmodulin from bovine brain [13] were homogenous preparations. Heparin–Sepharose CL-6B was purchased from Pharmacia (Uppsala). Calmodulin–Sepharose 4B was prepared as in [14]. [U-^{14}C]Glucose-1-phosphate was purchased from New England Nuclear (Boston MA). Other experimental materials were obtained from commercial sources. Phosphorylase kinase activity was determined by measuring the conversion of phosphorylase *b* to phosphorylase *a* as in [15]. Protein was determined using Protein Assay (Bio-Rad) with ovalbumin as a standard. Porcine liver was obtained at a slaughter house and partially

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purified phosphorylase kinase was prepared as in [16]. Further purification was done by using heparin–Sepharose CL-6B (0.5 × 3.5 cm) as in [17]. The specific activity of the phosphorylase kinase fraction after this procedure was 4.5–9.4 units/mg protein. All experiments were carried out with at least 4 preparations and the assays were carried out in duplicate.

3. RESULTS

3.1. *Activation of liver phosphorylase kinase by calmodulin*

The activity of liver phosphorylase kinase is shown as a function of added calmodulin concentration (fig. 1). The stimulation was about 1.5-fold over the basal activity. The concentration of calmodulin required for half-maximal enzymatic activity was 8×10^{-8} M and the maximal activity was obtained at 4×10^{-7} M.

3.2. *Calcium-dependent activity by calmodulin*

The activity of phosphorylase kinase was measured as a function of Ca^{2+} in the presence or absence of exogenous calmodulin (fig. 2). It can be seen that half-maximal stimulation was obtained at about 5×10^{-7} M Ca^{2+} both in the presence and absence of calmodulin. At saturating concentrations of Ca^{2+} (10^{-6} – 10^{-5} M), phosphorylase kinase was activated about 1.5-fold in the presence of calmodulin.

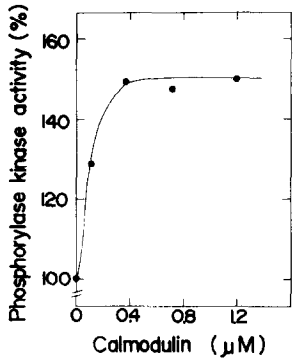

Fig. 1. Activation of liver phosphorylase kinase by exogenous calmodulin. The enzyme activity was determined as in [15] except that various concentrations of calmodulin were added to the initial reaction mixture (phosphorylase *b* to phosphorylase *a* reaction). Activity of phosphorylase kinase in the absence of calmodulin is taken as 100%.

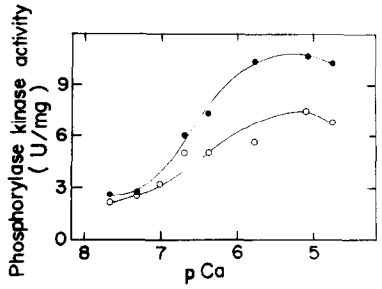

Fig. 2. Effect of calmodulin at various concentrations of calcium. Activity of the enzyme was determined at different pCa using Ca–EGTA buffers with (●) or without 1.2 μM calmodulin (○); 1.2 μM calmodulin was added to the initial reaction mixture. pCa was calculated [22], using the stability constant for the Ca–EGTA complex at pH 8.0 as follows: $K_{pH\ 8.0}^{Ca-EGTA} = 1.54 \times 10^7\ M^{-1}$ in 25 mM Tris–glycerophosphate buffer. Other conditions were as in [15].

3.3 *Effect of pH on calmodulin-dependent activation*

It has been demonstrated that the activation of skeletal muscle phosphorylase kinase by exogenous calmodulin is dependent on pH [14,18,19]. It was therefore of interest to see whether over a wide range of pH values calmodulin was effective in activating liver phosphorylase kinase. Fig. 3 shows

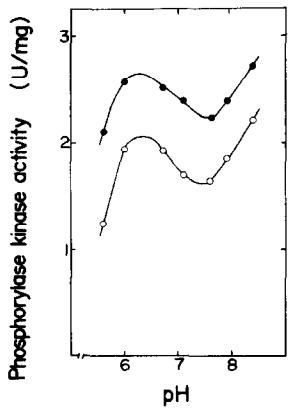

Fig. 3. Effect of pH on liver phosphorylase kinase in the presence (●) or absence (○) of calmodulin. The enzyme activity was assayed as in [15] except that 50 mM Tris–glycerophosphate buffers were employed as the indicated pH.

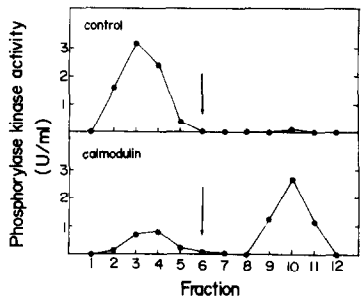

Fig. 4. Calmodulin affinity column chromatography. A calmodulin–Sepharose 4B column or glycine–Sepharose 4B (control) column (0.5 × 5.5 cm) was equilibrated with 25 mM Tris–HCl, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, pH 7.5 (buffer A), containing 2 mM $CaCl_2$. Then the enzyme was applied to these columns and they were washed with the same buffer. The flow rate was 2 ml/h and fractions of 1 ml each were collected. After collection of fraction 5 (see arrow), elution was continued with buffer A containing 2 mM EGTA.

the effect of pH on the activity of liver phosphorylase kinase. Fig. 3 shows the effect of pH on the activity of liver phosphorylase kinase with or without exogenous calmodulin. Liver phosphorylase kinase was stimulated by the addition of exogenous calmodulin about 1.3–1.7-fold at various pH-values.

3.4 *Binding of phosphorylase kinase to calmodulin–Sepharose 4B column*

Next, we examined whether liver phorphorylase kinase could bind to an affinity column of calmodulin (fig. 4). Most of the phosphorylase kinase bound to the calmodulin–Sepharose 4B column in the presence of 2 mM Ca^{2+} in buffer A, and could be eluted with 2 mM EGTA in buffer A. In addition, phosphorylase kinase did not bind to the control column (fig. 4). Therefore, the binding of phosphorylase kinase to the calmodulin–Sepharose 4B column was not the result of non-specific absorption.

4. DISCUSSION

These results demonstrate that like the skeletal muscle enzyme, porcine liver phosphorylase kinase is also activated by the addition of exogenous calmodulin. The activation is Ca^{2+}-dependent and is almost independent of pH. Although most of the liver phosphorylase kinase bound to the calmodulin–Sepharose 4B column with 2 mM Ca^{2+}, a small amount of the enzyme never bound to the column. The reason for this elution profile of the liver enzyme must await further studies.

There have been many protein kinases thus far reported which are controlled by calmodulin including skeletal muscle phosphorylase kinase. With respect to other calmodulin-dependent protein kinases, no data were available that calmodulin-dependent enzymes phosphorylate inactive forms of phosphorylase kinase or phosphorylase *b* (for review see [20]). Furthermore, to rule out the possibility of the participation of another unknown calmodulin-dependent protein kinase, the time course of liver phosphorylase kinase was studied in the presence of calmodulin. We obtained a linearity in the initial velocity (not shown). Although the evidence is not yet conclusive, these results seem to suggest that this calcium-calmodulin-dependent activation of liver phosphorylase kinase is a direct stimulation and that no other calmodulin-dependent reaction is involved.

It has been shown that skeletal muscle phosphorylase kinase is activated 2–7-fold by adding exogenous calmodulin [14,18,19]. In the present studies we observed that liver phosphorylase kinase is activated about 1.5-fold by exogenous calmodulin. This activation seems to be slightly lower than that of muscle enzyme. Several possible explanations for this lower activation are that: (i) the enzyme could be partially phosphorylated; (ii) the enzyme could be partially degraded; (iii) the enzyme could contain endogenous calmodulin; (iv) the 1.5 fold activation is the true characteristic of liver phosphorylase kinase.

Proteolytic degradation seems unlikely to be the cause of low activation by calmodulin, since gel filtration analysis revealed the apparent M_r-value of liver phosphorylase kinase employed was about 1 300 000 with skeletal muscle phosphorylase kinase as a marker. In addition, several protease inhibitors were employed throughout the early purification stages [16]. The biphasic pattern of the pH curve (fig. 3) suggests that the enzyme could be partially phosphorylated [21]. Even if the low activation by calmodulin is correct such a small stimulation of glycogen breakdown through the activation of phosphorylase kinase could still

have a profound effect in vivo under conditions where glycogen breakdown and synthesis are well balanced.

It is very attractive to suggest that a small elevation of intracellular calcium concentration in response to α-adrenergic stimulation remarkably enhances the activity of liver phosphorylase kinase through the action of calmodulin and corresponds to many physiological and biological demands.

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