# ACTIVATION OF PHOSPHORYLASE KINASE FROM RABBIT SKELETAL MUSCLE BY CALMODULIN AND TROPONIN

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## 1. Introduction

Phosphorylase kinase is of central importance in the nervous and hormonal control of glycogenolysis in mammalian skeletal muscle, since its activity is dependent on $Ca^{2+}$ and stimulated by a phosphorylation reaction catalysed by cyclic AMP-dependent protein kinase [1].

Phosphorylase kinase from mammalian skeletal muscle has mol. wt 1 280 000 and possesses the structure $(\alpha\beta\gamma\delta)_4$ where the $\alpha$- and $\beta$-subunits are the components phosphorylated by cyclic AMP-dependent protein kinase [1] and the $\delta$-subunit is identical to the calcium binding protein termed calmodulin [2,3]. Calmodulin, formerly termed the modulator protein or calcium-dependent regulator protein, was originally identified as a factor which stimulated the activity of the high $K_m$ cyclic nucleotide phosphodiesterase of brain tissue [4,5] but it has subsequently been implicated in the control of a variety of intracellular processes which are regulated by $Ca^{2+}$ [6–11].

Calmodulin binds 4 $Ca^{2+}$/mol [12] and the calcium binding properties of phosphorylase kinase [13] support the view that all the high affinity binding sites for calcium are located on the $\delta$-subunit [3]. This in turn suggests that the $\delta$-subunit is the component which confers calcium sensitivity to phosphorylase kinase.

Although calmodulin is present in stoichiometric proportions with the $\alpha$-, $\beta$- and $\gamma$-subunits, the activity of phosphorylase kinase is increased up to 7-fold by the addition of further calmodulin to the assay [3]. Several lines of evidence have demonstrated that this additional activation is caused by the interaction of a second molecule of calmodulin with each $\alpha\beta\gamma\delta$ unit [3]. Thus the antipsychotic drug trifluoperazine, or troponin-I, which form complexes with calmodulin in the presence of $Ca^{2+}$, prevent the activation of phosphorylase kinase by the second molecule of calmodulin, but do not have a significant effect on the calcium-dependent activity in the absence of calmodulin [3]. These compounds can therefore distinguish between a more weakly bound molecule of calmodulin which activates the enzyme, and the tightly bound molecule of calmodulin (the $\delta$-subunit), which is an integral component of the enzyme, and which is responsible for the dependence of phosphorylase kinase activity on $Ca^{2+}$.

Troponin-C shows 50% identity in amino acid sequence with calmodulin [14], and is reported to activate cyclic nucleotide phosphodiesterase to the same extent as calmodulin, although a 600-fold higher concentration is required [15]. Since troponin-C is present at very high concentrations in skeletal muscle, it therefore seemed important to test whether troponin-C could substitute for the second molecule of calmodulin in the activation of phosphorylase kinase. The results of these experiments are described in this communication.

## 2. Materials and methods

Phosphorylase *b* [16], phosphorylase kinase [17],

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calmodulin [3], troponin-C [18] and the troponin complex [19] were isolated from rabbit skeletal muscle. The troponin complex and one preparation of troponin-C were provided by Professor T. C. Vanaman, Duke University, NC. A second preparation of troponin-C was a gift from Dr Paul Leavis, National Institutes of Health, MD. The calmodulin binding protein, termed the inhibitory protein, was isolated from pig brain [20]. The homogeneity of each of the preparations was established by polyacrylamide gel electrophoresis in the presence of sodium dodecyl-sulphate. Phosphorylase kinase showed equimolar proportions of the four subunits $\alpha, \beta\ \gamma$ and $\delta$ [2,3], the troponin complex was composed of equimolar proportions of the troponin-T, troponin-I and troponin-C components [21] and the inhibitory protein was composed of two subunits of mol. wt 61 000 and 15 000, respectively [20]. The other proteins showed single bands. The concentrations of phosphorylase *b* and phosphorylase kinase were measured spectrophotometrically, assuming $A^{1\%}_{280}$ values of 13.1 and 12.4, respectively [14]. The concentrations of the other proteins were measured by the procedure [22] using bovine serum albumin ($A^{1\%}_{280} = 6.50$) as a standard. The mol. wt of calmodulin, troponin-C, the troponin complex, the inhibitory protein and phosphorylase kinase ($\alpha\beta\gamma\delta$ unit) were taken as 16 700, 18 000, 69 000, 76 000 and 335 000, respectively. The activity of phosphorylase kinase was measured at pH 8.2 and 30°C in the presence of 0.2 mM $CaCl_2$ [3]. The enzyme was almost completely inactive in the absence of $Ca^{2+}$ (> 97% inhibition) in the presence or absence of added calmodulin. One unit of activity was that amount which catalysed the phosphorylation of 1.0 μmol phosphorylase *b*/min. Glycogen phosphorylase [23], glycogen synthase [24] and phosphorylase phosphatase [25] were assayed by standard procedures. The last two assays were carried out by Mr Noor Embi and Mr Alex Stewart, respectively, in this laboratory.

## 3. Results

### 3.1. *Activation of phosphorylase kinase by the second molecule of calmodulin*

All preparations of phosphorylase kinase have spec. act. 14 ± 1 U/mg when assayed in the presence of saturating levels of calmodulin. In the absence of calmodulin the specific activity ranges from 2–7 U/mg, so that the stimulation by calmodulin varies from 2–7-fold with different preparations of phosphorylase kinase [13]. Preparations stimulated 4–5-fold by calmodulin were used in the present work.

In the standard assays which contain 0.45 nM phosphorylase kinase (0.15 μg/ml), half maximal activation occurs at 9 ± 3 nM calmodulin (0.15 ± 0.05 μg/ml). Maximal activation occurs at ~200 nM calmodulin (fig.1).

### 3.2. *Activation of phosphorylase kinase by troponin-C and the troponin complex*

The effect of troponin-C on the activity of phosphorylase kinase is illustrated in fig.1. Troponin-C activates phosphorylase kinase to the same extent as calmodulin, but a 200-fold higher concentration is required. Half-maximal activation is observed at 2.0 μM troponin-C (36 μg/ml). Identical results were obtained with two different preparations of troponin-C.

Troponin-C exists as a complex with troponin-T and troponin-I in skeletal muscle, and the effect of the troponin complex on the activity of phosphorylase kinase is shown in fig.1. The troponin complex could

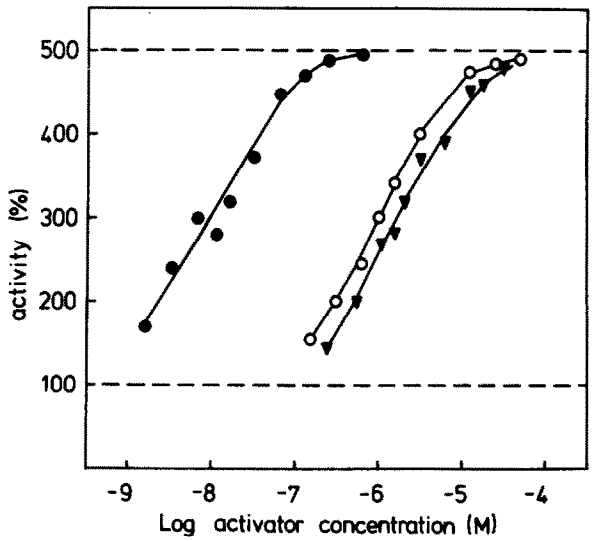


Fig.1. Influence of calmodulin and troponin on the activity of phosphorylase kinase. The broken lines show the level of activity in the presence (500) and absence (100) of saturating amounts of calmodulin. Symbols: calmodulin (•); troponin-C (▾); troponin complex (○).

also substitute for calmodulin in the activation of phosphorylase kinase, and on a molar basis it was 2-fold more effective than troponin-C. Half maximal activation occurred at 1.0 μM troponin (68 μg/ml). The finding that the troponin complex was slightly more effective as an activator could be related to the method used to purify troponin-C, which involves disruption of the troponin complex in 8 M urea, followed by ion-exchange chromatography on DEAE-cellulose [18].

### 3.3. *The activation of phosphorylase kinase by troponin-C and the troponin complex is not due to contamination with calmodulin*

The troponin complex and troponin-C were 100–200-fold less effective than calmodulin in the activation of phosphorylase kinase (fig.1) and this raised the possibility that the activation by these proteins was really caused by trace contamination with calmodulin. In order to test this possibility, use was made of a calmodulin binding protein isolated from brain, which is termed the inhibitory protein, since it inhibits the activation of cyclic nucleotide phosphodiesterase by calmodulin [19]. Four conclusions can be drawn from these experiments which are illustrated in fig.2.

1. The inhibitory protein has no effect on the calcium-dependent activity of phosphorylase kinase in the absence of calmodulin (fig.2, channels 1, 2).

2. The inhibitory protein can completely prevent the activation of phosphorylase kinase by the second molecule of calmodulin (fig.2, channels 3, 4).

3. The inhibitory protein does not prevent the activation of phosphorylase kinase by either troponin-C or the troponin complex (fig.2, channels 5, 6).

4. Troponin-C and the troponin complex do not prevent the inhibitory protein from inhibiting the

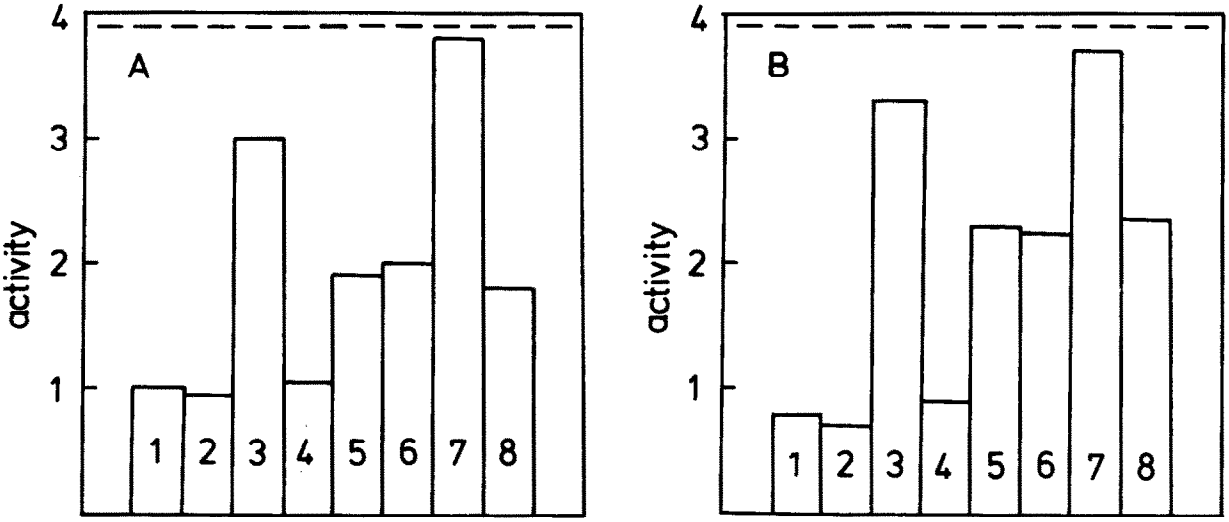


Fig.2. Influence of the inhibitory protein on the activation of phosphorylase kinase by troponin-C (A) and the troponin complex (B). The ordinate shows the activity in arbitrary units and the broken line the activity in the presence of saturating concentrations of calmodulin. Channel 1, no additions; channel 2, activity in the presence of 0.3 μM inhibitory protein; channel 3, activity in the presence of 0.06 μM calmodulin; channel 4, activity in the presence of 0.06 μM calmodulin + 0.3 μM inhibitory protein; channel 5, activity in the presence of 1.2 μM troponin-C (A) or 1.2 μM troponin complex (B); channel 6, activity in the presence of 0.3 μM inhibitory protein and either 1.2 μM troponin-C (A) or 1.2 μM troponin complex (B); channel 7, activity in the presence of 0.06 μM calmodulin and either 1.2 μM troponin-C (A) or 1.2 μM troponin complex (B); channel 8, activity in the presence of 0.06 μM calmodulin, 0.3 μM inhibitory protein and either 1.2 μM troponin-C (A) or 1.2 μM troponin complex (B). Different preparations of phosphorylase kinase were used in the experiments with troponin-C (A) and the troponin complex (B); phosphorylase kinase was 0.45 nM (0.15 μg/ml) in the assays.

activation of phosphorylase kinase by calmodulin (fig.2, channels 7, 8).

These experiments show conclusively that the activation of phosphorylase kinase by troponin-C and the troponin complex is not due to trace contamination with calmodulin. The finding that the inhibitory protein abolishes the activation of phosphorylase kinase by calmodulin, but does not affect the activity in the absence of calmodulin, is very similar to the effects of trifluoperazine and troponin-I ([3] and see section 1).

### 3.4. *Activation of phosphorylase kinase by calmodulin and troponin are not additive*

The effect of combining both calmodulin and troponin-C, or calmodulin and the troponin complex, on the activity of phosphorylase kinase is illustrated in table 1. It can be seen that the activation produced by saturating levels of calmodulin and troponin in combination, is very similar to that produced by each activator molecule alone. The activations produced by calmodulin and troponin are therefore not additive.

Table 1
Effects of calmodulin and troponin on the activity of phosphorylase kinase are not additive

| Additions to assay | Activity (%) |
|---|---|
| (1) None | 100 |
| (2) Troponin-C | 400 |
| (3) Troponin complex | 470 |
| (4) Calmodulin | 490 |
| (5) Calmodulin + troponin-C | 490 |
| (6) Calmodulin + troponin complex | 530 |

Calmodulin, troponin-C and troponin complex were 0.2 μM, 6.0 μM and 6.0 μM, respectively, in all experiments

### 3.5. *Evidence for an interaction of the enzymes of glycogen metabolism with myofibrillar components in the presence of $Ca^{2+}$*

Rabbit skeletal muscle was homogenized in the presence of either EDTA or $CaCl_2$, and the myofibrillar proteins were removed by centrifugation. Both extracts were found to possess identical lactate dehydrogenase activity, and the protein concentration in the $CaCl_2$ extract was only 10–15% lower than that of the EDTA extract (table 2). In contrast, the activities of

Table 2
Effect of EDTA and calcium chloride on the extraction of the enzymes of glycogen metabolism for rabbit skeletal muscle

| Activity measurement | Relative activities | |
|---|---|---|
| | EDTA extraction | $CaCl_2$ extraction |
| Protein (mg/ml) | 16.5 | 14.8 |
| Lactate dehydrogenase | 100 | 104 |
| Phosphorylase kinase | 100 | 42 |
| Phosphorylase phosphatase | 100 | 54 |
| Glycogen phosphorylase | 100 | 57 |
| Glycogen synthase | 100 | 50 |

Minced rabbit muscle (500 g) was divided into two portions. One half was homogenized in 600 ml 4.0 mM EDTA–0.1% (v/v) mercaptoethanol (pH 7.0) and the other half in 600 ml 2.0 mM $CaCl_2$–0.1% (v/v) mercaptoethanol. Homogenisations were for 30 s at low speed in a Waring blender at 0°C. The homogenates were centrifuged at 10 000 × *g* for 10 min and the supernatants decanted through glass wool. The extract prepared by homogenisation in $CaCl_2$ was immediately made 5 mM in EDTA. Protein (mg/ml) and activity measurements (U/ml) were carried out as in section 2. The pH 6.8/8.2 activity ratio of phosphorylase kinase was identical in the $CaCl_2$ and EDTA extracts (0.07–0.08) showing that no significant proteolytic activation of the enzyme had taken place during the 15 min exposure to high concentrations of $CaCl_2$

the enzymes of glycogen metabolism were much lower in the $CaCl_2$ extract and the activity of phosphorylase kinase was 2.5-fold lower (table 2). These experiments indicate that a considerable proportion of the enzymes of glycogen metabolism remain attached to the myofibrils in the presence of $CaCl_2$.

## 4. Discussion

This paper describes a simple general method for detecting traces of calmodulin in troponin-C and other proteins, such as parvalbumin, which appear to mimic the biological activity of calmodulin [26]. It is based on the observation that the calmodulin binding protein, termed the inhibitory protein, inhibits the activation of phosphorylase kinase by calmodulin, but does not inhibit the activation of phosphorylase kinase by troponin-C. This method has been used to demonstrate that troponin-C can activate phosphorylase kinase to the same extent as calmodulin, although a 200-fold higher concentration is necessary in order to achieve half-maximal activation.

These findings raise the question of whether troponin-C or the second molecule of calmodulin would activate phosphorylase kinase in vivo, and 2 lines of evidence suggest that troponin-C cannot be discounted as a physiological regulator of phosphorylase kinase:

(1) The troponin complex (i.e., the form in which troponin-C exists in vivo) is at least as effective as troponin-C in the activation of phosphorylase kinase (fig.1).
(2) Although the activation of phosphorylase kinase requires 100-times more troponin than calmodulin, large amounts of troponin are present in rabbit skeletal muscle.

The average concentration is 100 $\mu$M [27], which is clearly sufficient to maximally activate phosphorylase kinase (fig.1). The total amount of calmodulin in rabbit skeletal muscle is 6 $\mu$M [3], but since the concentration of phosphorylase kinase is 2.5 $\mu$M [1], ~40% of the total calmodulin is already bound to phosphorylase kinase (as the $\delta$-subunit). Maximal activation by the second molecule of calmodulin (fig.1) would therefore require most of the remaining calmodulin of the tissue, and much of this protein may be complexed with other enzymes, such as myosin light chain kinase [10].

The enzymes of glycogen metabolism appear to be linked together in vivo on protein–glycogen particles [1,28], which are known to be localized in skeletal muscle at the level of the thin filaments [29]. The present work has shown that phosphorylase kinase associates with a myofibrillar component (troponin) in vitro in a calcium-dependent manner. It is therefore attractive to speculate that the interaction of phosphorylase kinase with troponin-C may be responsible for the relatively selective association of the enzymes of glycogen metabolism with the myofibrils. This interaction would not only activate phosphorylase kinase, but also serve to anchor the glycogen particles to the contractile apparatus.

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