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PLATELET PHOSPHORYLASE KINASE ACTIVITY AND ITS REGULATION BY THE CALCIUM-DEPENDENT REGULATORY PROTEIN, CALMODULIN

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Summary

Platelet phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38) was found to be a Ca^{2+}-sensitive enzyme. It has two K_a values for Ca^{2+} viz. 0.25 and 2.6 μM, respectively. The "calcium-dependent regulator" or calmodulin can enhance the activity of phosphorylase kinase, increasing its affinity for Ca^{2+}. In the presence of calmodulin phosphorylase kinase has only one, high affinity binding site for Ca^{2+} (K_a = 0.27 μM).

Platelet phosphorylase kinase can be phosphorylated by endogenous cyclic AMP-dependent protein kinase increasing its catalytic activity and this activation process is reversed by dephosphorylation. The changing level of intracellular Ca^{2+} and cyclic AMP may control the activity of phosphorylase kinase, regulating the mobilization of glycogen.

Introduction

Phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38) is a component of the enzyme cascade which controls the metabolism of glycogen in skeletal muscle. It catalyses the activation of phosphorylase *b* by phosphorylation of a serine hydroxyl group in each of the subunits of phosphory-

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Abbreviations: ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); EGTA, ethylene glycol bis(β-aminoethyl ester)-*N,N,N′,N′*-tetraacetic acid; Mes, 2-(*N*-morpholinoethanesulphonic) acid.

lase. Phosphorylase kinase has been reported as a dodecamer of three different subunits [1,2] and has an absolute Ca^{2+}-requirement for its catalytic activity [3]. Recently, Cohen et al. [4] have found a further subunit in the phosphorylase kinase, which is similar to calmodulin, the calcium-dependent regulatory protein, and is responsible for the Ca^{2+}-sensitivity of the enzyme. Protein phosphorylation-dephosphorylation reactions also play a regulatory role in the activity of phosphorylase kinase itself; the enzyme is phosphorylated by cyclic AMP-dependent protein kinase and this activation process is reversible by the action of a protein phosphatase [5].

Glycogen metabolism is important in the platelet providing the energy required for the different functions of the cell [6]. The glycogenolytic enzymes have been studied, including phosphorylase [7—9], protein phosphatase [10], cyclic AMP-dependent protein kinase [11,12], and show considerable similarities with the respective muscle enzymes. It has been demonstrated that phosphorylase kinase controls the conversion of platelet phosphorylase *b* into *a* [8,13] and the reaction requires micromolar concentrations of Ca^{2+}. The nonlinearity of the double reciprocal plots [13] prevented the calculation of a correct K_a for Ca^{2+}. The authors also suggested that the activation of phosphorylase kinase by cyclic AMP-dependent protein kinase does not appear to be involved in the regulation of its activity [13].

In this communication we have examined the phosphorylase kinase activity of human platelets and the effects of calmodulin on this activity at low Ca^{2+} concentrations.

Materials and Methods

Platelets were isolated from human blood as described by Baenziger and Majerus [14] and washed twice in 140 mM NaCl/15 mM Tris/5.5 mM glucose (pH 7.4) and resuspended in 2 volumes of 40 mM MES/4 mM EDTA/2 mM dithiothreitol (pH 6.8, buffer A). The suspension was homogenised with a Waring blendor (5 times 1 min) and then centrifuged at 10 000 × *g* for 10 min. The supernatant solution was brought to 35% saturation with solid ammonium sulphate. After 30 min the ammonium sulphate precipitate was collected (10 000 × *g*, 20 min), dissolved in a minimal volume of buffer A and dialysed overnight against buffer A. After dialysis the precipitated proteins were removed by centrifugation and the clear supernatant used as the phosphorylase kinase sample. All operations were carried out at 4°C.

Crystalline rabbit skeletal muscle phosphorylase *b* was prepared according to the method of Fischer and Krebs [15] and calmodulin from bovine brain by the method of Watterson et al. [16]. The calmodulin appeared as a single polypeptide band of molecular weight 16 500 on SDS polyacrylamide Laemlli gels.

The activation of platelet phosphorylase kinase was studied as follows: the dialysed platelet sample was incubated at 30°C in 40 mM Mes (pH 6.7) in the presence of 5 mM Mg $(CH_3COO)_2$, 1 mM ATP or ATP-γ-S and 5 μM dibutyryl cyclic AMP, with or without calmodulin at different concentrations and low and high levels of calcium. Aliquots were withdrawn at intervals and assayed immediately for phosphorylase kinase activity as described below.

Phosphorylase kinase activity of the sample was assayed using exogenous

rabbit muscle phosphorylase *b* as a substrate and measuring the formed phosphorylase *a*. Phosphorylase kinase activity was determined at 30° C in an incubation medium containing 40 mM Mes, 10 mM $(CH_3COO)_2$, 3 mM ATP, 1.5 mg (80 units) per ml phosphorylase *b* and the dialysed platelet sample (final volume 0.15 ml). We found that the pH optimum of the kinase assay was 6.7 and therefore all assays were carried out at this pH value. The Ca^{2+} concentration of the medium was adjusted with Ca^{2+}-EGTA described by Portzehl et al. [17]. Aliquots were removed from the incubation mixture, diluted in 80 mM NaF/40 mM glycerophosphate/5 mM EDTA (pH 6.8) and assayed for phosphorylase *a* activity [18]. The kinase assay was performed so that the enzyme did not convert more than 15% of phosphorylase *b*. The initial reaction rates were calculated and expressed as formed phosphorylase *a* units/min per ml platelet sample. The endogenous (platelet) phosphorylase *b* content did not interfere with the assay because it was less than 1% of the exogenous (muscle) phosphorylase.

Results and Discussion

As shown in Fig. 1 calmodulin increased the activity of phosphorylase kinase in the presence of Ca^{2+}. The increase was the highest at low levels of Ca^{2+} up to 146% increase in phosphorylase kinase activity at 1 μM Ca^{2+} from a base value of 0.64 units/min per ml of platelet sample in the absence of calmodulin. Calmodulin had no effect in the presence of a high concentration of Ca^{2+} (20 μM). This suggests that calmodulin enhances the affinity of phosphorylase kinase toward Ca^{2+}. The data of Fig. 2, plotted in double reciprocal form, are evidence that platelet phosphorylase kinase has two K_a values for Ca^{2+} in the absence of calmodulin. The K_a for the high affinity site is 0.25 μM and for the low affinity site 2.6 μM. In the presence of calmodulin phosphorylase kinase was also found to require Ca^{2+} but the low affinity site disappeared yielding a value of 0.27 μM. Gear and Schneider reported a nonlinear plot for the Ca^{2+}-sensitivity of platelet phosphorylase kinase which gave a straight line only after storage or by

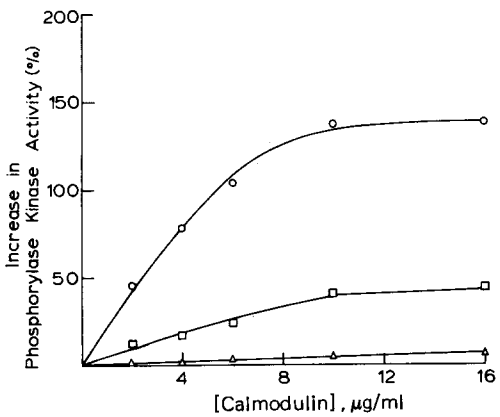

Fig. 1. Effect of calmodulin on the phosphorylase kinase activity. The dialysed platelet samples were assayed as described in the text in the presence of various amounts of calmodulin and 1 μM Ca^{2+} (○), 5 μM Ca^{2+} (□), 20 μM Ca^{2+} (△).

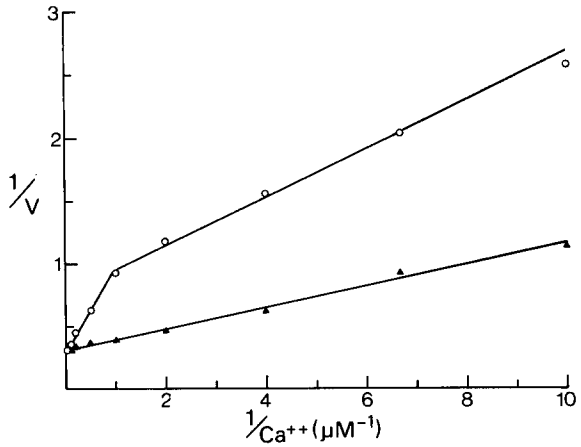

Fig. 2. Reciprocal plots of reaction velocity versus Ca^{2+} concentration. Data are presented for platelet phosphorylase kinase in the absence of calmodulin (○) and in the presence of 12 μg/ml calmodulin (▲).

freeze-drying the preparation. These authors reported an affinity constant of 0.33 μM Ca^{2+} [13]. It is possible that both these processes resulted in the liberation of calmodulin causing the increase in affinity for Ca^{2+} and the linearity of the double reciprocal plot.

It is known that platelets contain calmodulin [19] in appreciable amounts (10 mg calmodulin/100 g wet weight platelets; Young, N.J., personal communication). Thus calmodulin could affect the activity of phosphorylase kinase in vivo increasing its affinity toward Ca^{2+}. This would be important when the intracellular Ca^{2+} concentration is low (1 μM or less).

Fig. 3 shows the activation of phosphorylase kinase by endogenous protein kinase. In the presence of ATP the activation process is reversible due to the protein phosphatase content of the sample. An irreversible activation was observed with ATP-γ-S. ATP-γ-S is the thiophosphate analogue of ATP incorporating a thiophosphate group into the proteins. The formed thiophosphate-activated proteins are resistant to the action of phosphatase. This has been demonstrated with several proteins including muscle phosphorylase kinase [20]. The activation process is not entirely cyclic AMP dependent since an increase of phosphorylase kinase activity could be observed in the absence of the cyclic nucleotide. The platelet sample might contain some endogenous cyclic AMP and therefore a small amount of activated protein kinase would activate the phosphorylase kinase in the absence of added cyclic AMP.

It is known that the platelet release reaction and aggregation are inhibited by agents which enhance the intracellular cyclic AMP concentration resulting in the activation of cyclic AMP-dependent protein kinase. The activation (phosphorylation) of phosphorylase kinase regulates glycogen breakdown as part of the energetic requirements of these platelet functions. There is also a close relationship between the levels of Ca^{2+} and cyclic AMP in platelets [11] and the precise role of calmodulin in regulating platelet intracellular events which depend upon these agents warrants further study.

After this manuscript was submitted for publication similar results have been

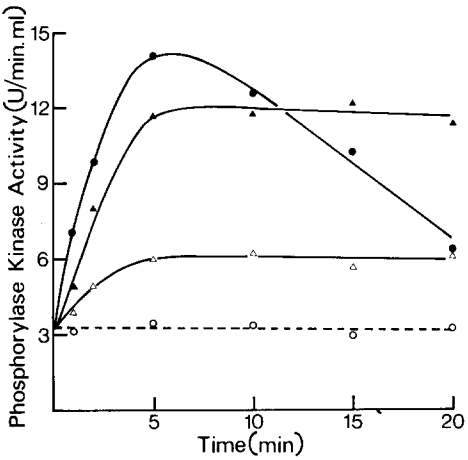

Fig. 3. Activation of phosphorylase kinase in the dialysed sample. Conditions of the activation process and the phosphorylase kinase assay are given in the text. Activation with 1 mM ATP + 5 μM dibutyryl cyclic AMP (●), 1 mM ATP-γ-S + 5 μM dibutyryl cyclic AMP (▲), 1 mM ATP-γ-S without cyclic AMP (△). The dotted line (○- - - - -○) represents the control incubation without nucleotide and Mg^{2+}.

demonstrated [21] in which they found that calmodulin from pig brain activated phosphorylase kinase from rabbit skeletal muscle by up to 7-fold.

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