PROTEIN PHOSPHATASES FROM CANINE HEART: EVIDENCE FOR FOUR DIFFERENT FRACTIONS OF THE ENZYME

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1. Introduction

It seems generally accepted that adenosine 3′,5′-monophosphate (cyclic AMP) exerts its diverse effects by stimulation of protein kinases. Protein phosphatases which catalyze the removal of the phosphate group from the phosphorylated proteins can be regarded as a means of terminating the action of cyclic AMP. Evidence from several laboratories indicates the existence of 2 protein phosphatase fractions in rabbit skeletal muscle [1], 3 fractions in rabbit liver [2] and rat cerebral cortex [3], and 6 fractions from rat liver [4]. On the other hand, studies on the partially purified glycogen synthetase phosphatase of rabbit skeletal muscle [5,6] and bovine heart [7,8] have revealed that a single phosphatase exhibits activities toward several phosphoproteins. This communication reports that the protein phosphatase activity of canine heart was resolved into four fractions by diethylaminoethyl (DEAE)-cellulose chromatography. The activity of these four fractions differs from one another in several respects: they are modulated by various metabolites in different ways.

2. Experimental

^{32}P-labeled-phosphohistone (^{32}P-histone) was prepared from calf thymus histone (histone II, Sigma) according to Meisher and Langan [9]. The specific activity of ^{32}P-histone used in assay varied from 100 to 550 cpm per pmol of alkali-labile phosphate (P_i). Protein phosphatase activity was measured at 30°C in an incubation volume of 0.1 ml containing 50 mM Tris–Cl, pH 7.6, 1 mM dithiothreitol, 2 μM ^{32}P-histone (in terms of P_i content) and the enzyme. After 10 min incubation, $^{32}P_i$ product was separated from ^{32}P-histone by a paper chromatographic method [10] for determination of radioactivity. Protein kinase activity was assayed according to Li and Felmly [11].

Protein phosphatases were separated from dog heart as follows. The cytosol fraction of dog heart prepared according to Harigaya and Schwartz [12] was fractionated by the addition of solid $(NH_4)_2SO_4$. The 0–55% $(NH_4)_2SO_4$ fraction was dissolved in a small volume of Buffer A (20 mM Tris–Cl, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 1 mM EDTA and 50 mM KCl, pH 7.4), dialyzed overnight against this buffer and applied to a DEAE-cellulose (Whatman DE-52) column (1.5 × 60 cm) previously equilibrated with same buffer. After sample application, the column was rinsed with Buffer A. Proteins that absorbed to the DEAE-cellulose were eluted by an increasing salt gradient which was formed as follows: Three equal size chambers were connected in series and placed on the same level. The first chamber contained 330 ml of Buffer A, the second contained 330 ml of Buffer A plus 0.15 M KCl, and the third contained 330 ml of Buffer A plus 0.35 M KCl. The gradient was withdrawn from the first chamber to the column. 5.7 ml per fraction was collected. The phosphatase A, B, and C were the pool of fraction numbers 76 to 88, 105 to 145, and 155 to 185, respectively, as indicated in fig.1. Each of the pooled fractions was then concentrated, by ultrafiltration with a Diaflo membrane (PM 10, Amicon Corporation), to approx. 5 ml. The concentrated fractions were dialyzed overnight against 10 mM Tris–Cl buffer, pH 7.4, containing 50 mM β-mercaptoethanol. All operations were carried out at 4°C, except where otherwise indicated.

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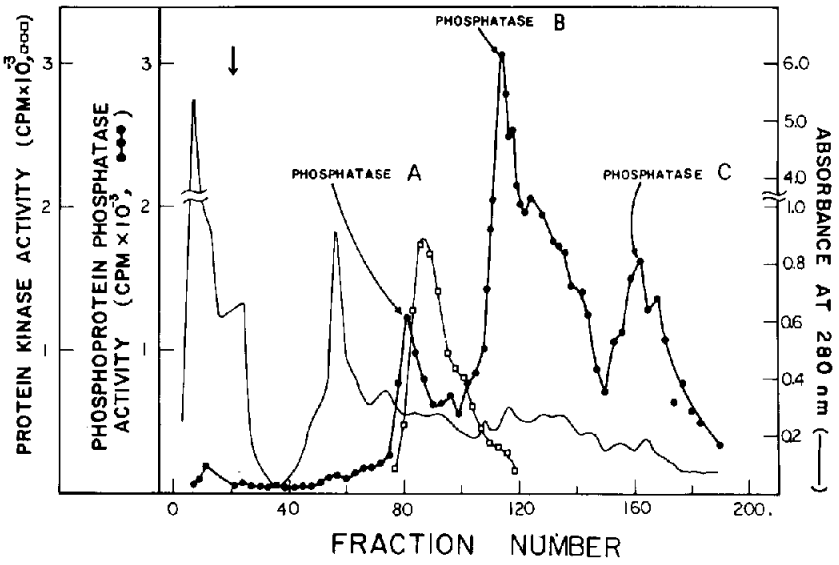

Fig.1. Column chromatography of enzymes on DEAE-cellulose. The 55% $(NH_4)_2SO_4$ fraction was applied on a DEAE-cellulose column (1.5 × 60 cm) and enzymes were eluted with a KCl gradient as described under Experimental. The arrow indicates the start of gradient elution. Protein phosphatase activity (o—o—o) and protein kinase activity (□—□—□) were determined with 10 μl aliquots from the indicated fractions. Absorbance at 280 nm was measured against a buffer blank.

3. Results and discussion

As shown in fig.1, phosphoprotein phosphatase activity in the 0–55% $(NH_4)_2SO_4$ fraction was separated into three major peaks. When the enzyme was assayed in the presence of 5 mM $MnCl_2$, an additional major peak of protein phosphatase appeared in the elution profile between peaks A and B (data not shown). This enzyme, whose activity was totally dependent on the presence of Mn^{2+} or Co^{2+}, was designated as phosphatase U. Mg^{2+} and Ca^{2+} were ineffective. The majority of the protein kinase activity overlapped with phosphatases A and U.

As shown in fig.2, phosphatase A was progressively inhibited by an increase of either ATP (fig.2a) or GTP (fig.2b). Fifty percent inhibition of phosphatase A activity was observed at 2.5 mM ATP or 1.5 mM GTP. By contrast, phosphatase B was stimulated by either ATP or GTP. At optimum concentration of ATP (3 mM), phosphatase B was stimulated more than 4-fold. Phosphatase C was slightly stimulated by either ATP or GTP at concentrations below 2 mM. Higher concentrations of the nucleoside triphosphates caused inhibition. As shown in fig.3, phosphatase A was slightly stimulated by $MgCl_2$ and $MnCl_2$ at the concentrations indicated. Both phosphatase B and C, however, were markedly stimulated by divalent cations. It is clearly shown in fig.3 that phosphatase C required Mg^{2+} while B required Mn^{2+} for maximum stimulation. At 20 mM of $MnCl_2$ or $MgCl_2$, phosphatase C was stimulated 5-fold and 7-fold, respectively. At 20 mM $MgCl_2$ or $MnCl_2$, phosphatase B was stimulated 3-fold and 10-fold, respectively. As indicated in fig.4, phosphatase A is only slightly stimulated at comparatively low concentrations of KCl, but at concentrations higher than 40 mM, it is inhibitory. Phosphatase B and C, on the other hand, are stimulated by KCl at a higher and wider concentration range, and the stimulations are to a greater extent before the onset of inhibition. NaCl exhibited essentially the same effects as did KCl on the phosphatases. All four fractions of the protein phosphatase were inhibited by NaF, P_i and pyrophosphate at mM concentrations.

These results show that at least four major fractions of protein phosphatase can be physically separated from the cytosol of canine heart by DEAE-cellulose column chromatography. Each of these four fractions shows different catalytic properties, namely, phospha-

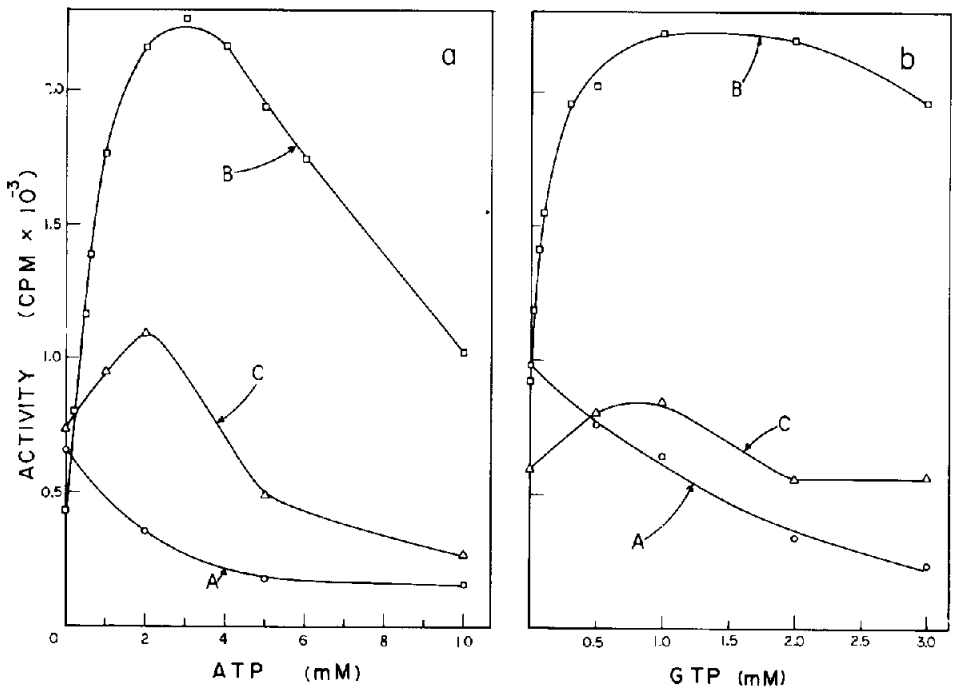

Fig.2. Effects of ATP or GTP concentrations on protein phosphatases. Phosphatase A, B, or C was assayed as described under Experimental except ATP (2a) or GTP (2b) was included in the reaction mixture at the concentrations indicated.

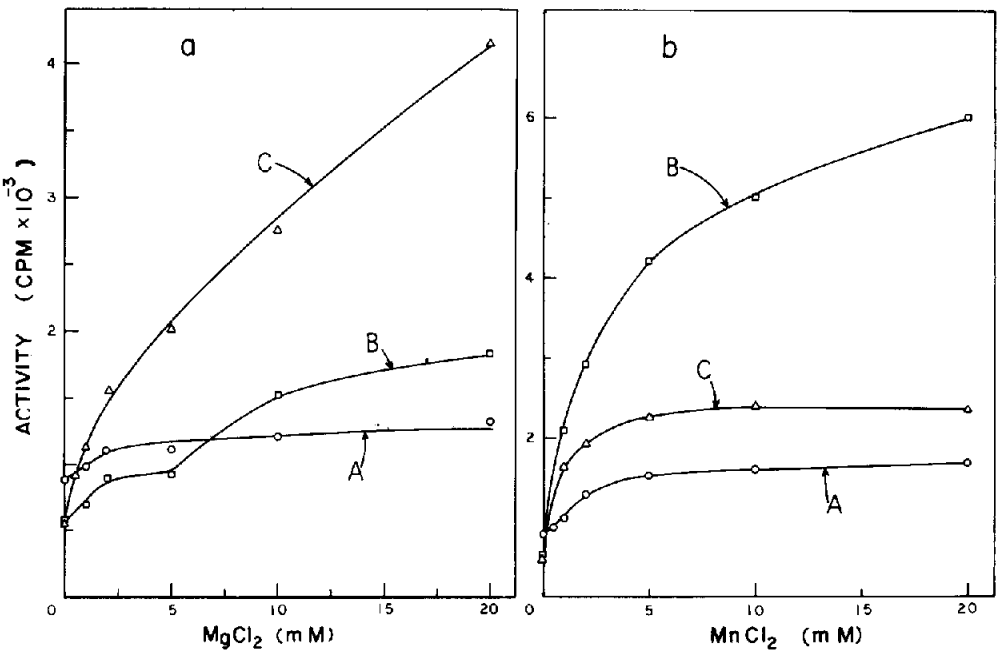

Fig.3. Effects of $MgCl_2$ and $MnCl_2$ on protein phosphatases. Phosphatase A, B, or C was assayed as described under Experimental except $MgCl_2$ (3a) or $MnCl_2$ (3b) was included in the reaction mixture at the concentrations indicated.

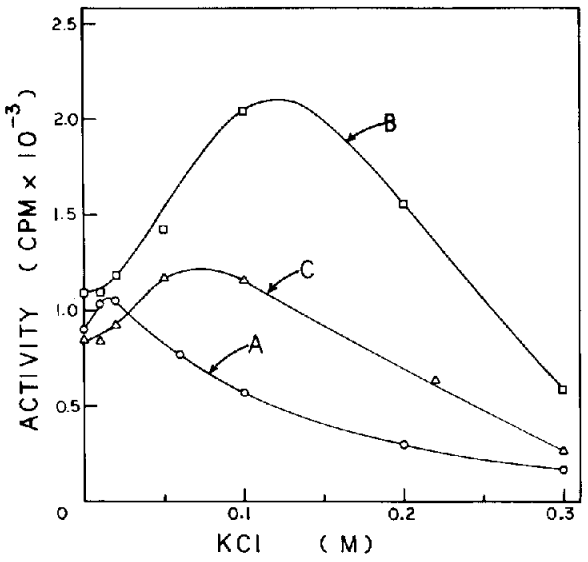

Fig.4. Effects of KCl on protein phosphatases. Phosphatase A, B, or C was assayed as described under Experimental except KCl was included in the reaction mixture at the concentrations indicated.

tase A is inhibited by ATP, phosphatase B is stimulated by ATP, phosphatase C requires Mg^{2+} for maximum activity and phosphatase U is totally dependent on Mn^{2+} or Co^{2+} for activity. Such differential regulatory properties of the different protein phosphatases might be important physiologically. Recently, protein phosphatase has been proposed to consist of an enzyme-inhibitory protein complex [13]. It is possible that these four fractions may derive from the combination of different kinds of enzyme and regulatory protein.

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