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Mg^{2+} COUNTERACTS THE INHIBITORY EFFECT OF SPERMINE ON LIVER PHOSPHORYLASE KINASE*

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SUMMARY: When the effect of polyamines on pig liver phosphorylase kinase was examined, spermine was found to be the most inhibitory. Although putrescine stimulated the reaction slightly, the spermidine effect was dependent on the phosphorylase b concentration. The inhibitory effect of spermine was counteracted by increasing the Mg^{2+} concentration. At 0.3 mM Mg^{2+}, the apparent Km for phosphorylase b was increased 9-fold by the addition of 5 mM spermine. However, increasing Mg^{2+} to 3 mM decreased the value to the initial level obtained at 0.3 mM Mg^{2+} alone. These results suggest that a possible role of Mg^{2+} in the regulation of liver phosphorylase kinase is to protect the enzyme from the inhibitory action of a polyamine such as spermine.

There have been several reports indicating a regulatory role of free Mg^{2+} on the activities of skeletal muscle and liver phosphorylase kinases (1-6). Kilimann and Heilmeyer (7) indicated that Mg^{2+} has the ability to regulate Ca^{2+}-binding properties of the skeletal muscle enzyme in addition to providing the substrate, ATP-Mg^{2+}. Recent reports from several laboratories have shown that polyamines are involved in controlling various types of protein kinase activities through direct effects on the enzymes or by modifying substrate properties (8-11). When the effect of polyamines on liver phosphorylase kinase were examined, spermine was shown to be most inhibitory. However, this effect was counteracted by increasing the Mg^{2+} concentration to 2-3 mM. These results may suggest that a role for Mg^{2+} in the regulation of liver phosphorylase kinase

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is to protect the enzyme from inhibitory action by polyamine such as spermine.

EXPERIMENTAL PROCEDURES

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride and polyethylene glycol (# 6,000) were obtained from Nakarai Chemicals, Kyoto. Phosphocreatine and creatine phosphokinase were purchased from Sigma. (U-^{14}C)Glucose 1-phosphate was purchased from New England Nuclear. Other experimental materials were obtained from commercial sources. Glycogen phosphorylase b was prepared as described by Fischer and Krebs (12). For the determination of activity, phosphorylase b was diluted in the buffer containing 20 mM Tris-HCl (pH 7.0), 30 mM 2-mercaptoethanol and 1 mg/ml bovine serum albumin. The suitably diluted enzyme solution (50 µl) containing 2 mM 5'-AMP was mixed with the phosphorylase assay mixture (50 µl), which contained 100 mM glycerophosphate (pH 6.1), 20 mg/ml oyster glycogen and 49 mM (^{14}C)glucose 1-phosphate (24,000-28,000 cpm). After incubation at 30° for an appropriate time, the reaction was stopped by the addition of 75 % ethanol and the insoluble materials were treated as described previously (13). One unit of phosphorylase b was the amount of enzyme that transferred 1 µmol of glucose into glycogen per min under the conditions mentioned above. Enzyme with a specific activity of 55-95 units/mg was employed. Pig liver phosphorylase kinase was partially purified as described before (6) with slight modifications as indicated below. To the supernatant obtained by ultracentrifugation of the crude extract, 50 % (w/v) polyethylene glycol, dissolved in the homogenizing buffer (14) (0.25 M sucrose was replaced to 20 % (w/v) glycerol), was added to the final concentration of 5 % and the precipitated materials were dissolved in the homogenizing buffer. The insoluble materials were removed by ultracentrifugation at 105,000 g for 1 h. The supernatant solution (8.3 ml, 457 mg protein) was applied to two Sepharose CL-4B columns equilibrated with 25 mM Tris-HCl (pH 7.4), 10 mM 2-mercaptoethanol, 0.1 mM PMSF[1]/ and 20 % (w/v) glycerol. The gel filtration was performed with the same buffer. Phosphorylase kinase activity was determined as follows except otherwise noted. The first incubation mixture (50 µl) contained 25 mM Tris-glycerophosphate (pH 8.5), 20 mM 2-mercaptoethanol, 4 mg/ml phosphorylase b, 10 mM $MgCl_2$, 1 mM ATP, 24 mM phosphocreatine, 16 units/ml creatine phosphokinase and enzyme preparation. After incubation at 30° for an appropriate time, 5 µl of reaction mixture was transferred to the 50 µl of the stopping solution containing 10 mM glycerophosphate (pH 6.1), 20 mM NaF, 40 mM 2-mercaptoethanol and 2 mM EDTA. To this solution, phosphorylase assay mixture (50 µl) was added and phosphorylase a activity was determined as described above without 5'-AMP. In parallel with the complete system, the reaction was also performed in the absence of phosphorylase kinase and this activity was subtracted. After the purification by Sepharose CL-4B, phosphocreatine and creatine phosphokinase were omitted from the assay mixture. Definition of one unit of phosphorylase kinase and phosphorylase a was the same as described previously (13). The specific activity of the enzyme employed in this study was 75-94 units/mg. Protein was determined using Protein Assay (Bio-Rad) with bovine serum albumin as a standard.

RESULTS

As shown in Fig. 1, the effect of polyamines on liver phosphorylase kinase was variable, depending on the species of amine and the

1/ The abbreviation used is: PMSF, phenyl methylsulfonyl fluoride.

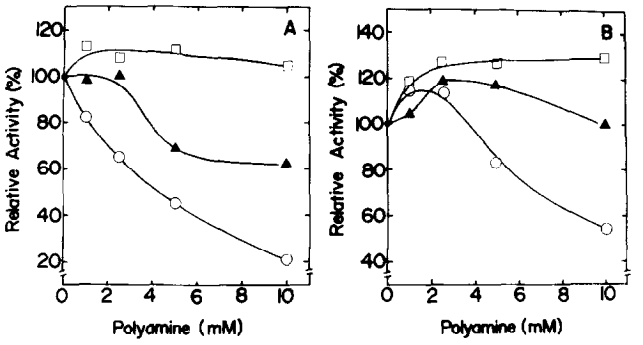

Fig. 1. Effect of polyamines on liver phosphorylase kinase activity. The first incubation mixture (50 µl) contained 25 mM Tris-glycerophosphate (pH 6.8), 20 mM 2-mercaptoethanol, 80 units/ml (A) or 320 units/ml (B) phosphorylase b, 0.1 mM ATP, 0.3 mM $MgCl_2$, phosphorylase kinase preparation and various concentrations of polyamines. Other assay conditions were as described in "EXPERIMENTAL PROCEDURES". Phosphorylase kinase activities taken as 100 % were 0.42, 0.45 and 0.46 units/ml (A) or 1.2, 1.3 and 1.3 units/ml (B), respectively, for the series of experiments to which putrescine (□), spermidine (▲) and spermine (○) were included.

phosphorylase b concentrations. Although putrescine stimulated the reaction irrespective of the substrate concentrations, the extent of this effect was only 1.1-1.3 fold. In contrast, spermidine at concentrations above 2.5 mM was inhibitory at lower concentration of phosphorylase b (80 units/ml). When the substrate concentration was increased to 320 units/ml, only slight stimulation was observed with spermidine. Among the polyamines tested, spermine was shown to be most inhibitory. At the lower phosphorylase b concentration, the activity was inhibited 80 % at 10 mM spermine. However, this polyamine showed a biphasic (stimulatory and inhibitory) effect at the higher concentration of phosphorylase b. The results described in Fig. 1 was obtained in the presence of 0.1 mM ATP and 0.3 mM $MgCl_2$. When the $MgCl_2$ concentrations were increased from 0.3 mM to 3 mM, the inhibitory effect of spermine was completely counteracted (Fig. 2). Although the reactions were performed in the presence of 80 units/ml phosphorylase b and 5 mM spermine, this Mg^{2+} effect was also observed when 320 units/ml phosphorylase b and 10 mM spermine were present.

In order to understand the mode of action of spermine and Mg^{2+}, kinetic analyses were performed as summarized in Table I. In the

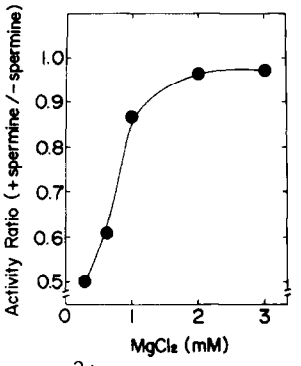

Fig. 2. Counteraction by Mg^{2+} of spermine effect on liver phosphorylase kinase. The enzyme activity was determined as described in the legend to Fig. 1 except that the reaction mixture was modified as indicated below. (1) phosphorylase b concentration was 80 units/ml. (2) $MgCl_2$ concentrations were varied as indicated. (3) The reactions were performed in the absence and presence of 5 mM spermine. Phosphorylase kinase activities changed from 0.22-0.80 unit/ml and 0.43-0.82 unit/ml in the presence and absence of 5 mM spermine, respectively, by increasing the Mg^{2+} concentration.

presence of 0.3 mM Mg^{2+}, spermine increased the apparent Km for phosphorylase b 9-fold although the apparent Vmax was also increased 3.4-fold. However, the presence of both 3 mM Mg^{2+} and 5 mM spermine returned the elevated Km value to the initial low level. When the ATP concentrations were changed, both the apparent Km and Vmax were

TABLE I

Effect of spermine and Mg^{2+} on kinetic properties of liver phosphorylase kinase.

Effector		Phosphorylase b		ATP	
Mg^{2+} (mM)	Spermine (mM)	Km (U*/ml)	Vmax (U**/mg)	Km (μM)	Vmax (U**/mg)
0.3	0	119±4	135±6	93±5	159±7
0.3	5	1,055±78	465±21	69±3	153±13
3	5	109±8	207±4	49±7	290±30

* unit of phosphorylase b
** unit of phosphorylase kinase

The enzyme activity was determined as described in the legend to Fig. 1 except that the reaction mixture was modified as indicated below. (1) $MgCl_2$ and spermine were added as indicated. (2) When phosphorylase b concentration was changed, the concentrations of this substrate were varied from 22 units/ml to 440 units/ml and bovine serum albumin (4 mg/ml) was included for maintaining the total protein concentration. (3) When the ATP concentration was changed, the concentrations of this substrate were varied from 30 μM to 150 μM and phosphorylase b concentration was 220 units/ml.

not markedly influenced by the addition of spermine or increasing Mg^{2+} concentration. These results suggest that the inhibitory effect of spermine on liver phosphorylase kinase is mainly due to modifying the affinity of this enzyme for phosphorylase b.

DISCUSSION

Recently, experimental results have accumulated indicating that the effects of Mg^{2+} are closely related to the Ca^{2+}-binding property of skeletal muscle phosphorylase kinase (7). In addition,it was also shown that the affinity of the skeletal muscle enzyme for glycogen phosphorylase b is increased greatly in the presence of enough amount of Ca^{2+} (15). These results suggest that Mg^{2+} and Ca^{2+} have related effects on the expression of skeletal muscle phosphorylase kinase activity.

In the case of liver phosphorylase kinase, more recent reports from Exton's and our laboratories have indicated a role of free Mg^{2+} in the control of this enzyme under various conditions (5,6). The results obtained in this study propose the role for Mg^{2+} as a protector of the enzyme from the inhibitory action of spermine. Although the Km value for glycogen phosphorylase b was greatly enhanced in the presence of spermine, increasing the Mg^{2+} concentration was effective in decreasing the value to the initial low level in spite of the presence of the polyamine. These results may be compatible with the supposition that a Mg^{2+}-binding site may exist on liver phosphorylase kinase and proper binding of this cation may influence the enzyme with respect to both Ca^{2+}-binding capacity and the affinity for phosphorylase b. These effects of Mg^{2+} may be blocked by spermine through occupation of the Mg^{2+}-binding site on this enzyme, especially at low Mg^{2+} concentrations.

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