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EFFECTS OF POLYAMINE HYDROCHLORIDES AND SALTS ON PHOSPHOPROTEIN PHOSPHATASE

HIROFUMI USUI, MICHINORI IMAZU, TEIJI IMAOKA and MASAO TAKEDA

Department of Biochemistry, Hiroshima University School of Medicine, Hiroshima (Japan)

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Summary

Polyamine hydrochlorides, NaCl and magnesium acetate stimulated the enzymatic dephosphorylation of phosphorylated H2B histone by two forms (large form, mol. wt. 250 000; small form, mol. wt. 30 000) of a pig heart phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16). These ionic compounds stimulated the large form of the enzyme 5—9-fold but stimulated the small form of the enzyme only 2-fold. With phosphorylated H2B histone as substrate, these effectors caused an increase in both K_m and V values of the two forms of the enzyme. On the other hand, when a tryptic phosphodecapeptide derived from phosphorylated H2B histone was used as substrate, these effectors were always inhibitory apparently non-competitively with respect to the substrate. Using phosphorylated H1 histone as substrate, these effectors stimulated the large form of the enzyme 2-fold but inhibited the small form. With phosphorylase *a* as substrate, the reactions were also inhibited by these effectors irrespective of the enzyme employed. With respect to phosphorylase *a*, this inhibition was apparently of a competitive type for the large form and a non-competitive type for the small form of the enzyme.

Introduction

In eukaryotic cells, cyclic AMP produces its effects through the phosphorylation of specific cellular proteins catalyzed by cyclic AMP-dependent protein kinase. The action of cyclic AMP is shut off through the dephosphorylation of the phosphorylated protein catalyzed by phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16). Although the regulation mechanism of cyclic AMP-dependent protein kinase has been well documented [1],

Abbreviations: cyclic AMP, adenosine 3′,5′-monophosphate; H2B histone-P, H2B histone phosphorylated with cyclic AMP-dependent protein kinase; H1 histone-P, H1 histone phosphorylated with cyclic AMP-dependent protein kinase.

the regulation mechanism of phosphoprotein phosphatase is not well understood. It has been reported that various ionic compounds such as AMP and metal salts could either stimulate or inhibit the reactions catalyzed by phosphoprotein phosphatases [2—9]. Since polyamines are ionic compounds which may regulate various biological processes, especially during the early stage of cell proliferation [10], we initiated studies on the effect of polyamine hydrochlorides on the phosphoprotein phosphatase.

In a previous report [11], we described the partial purification of a high molecular weight form (large form, mol. wt. 250 000) of the phosphoprotein phosphatase from pig heart and its conversion to a smaller form (small form, mol. wt. 30 000) by ethanol treatment. H2B histone-P, tryptic phosphopeptides derived from H2B histone-P, H1 histone-P and phosphorylase *a* served as substrates for the two forms of the enzyme [11].

In this paper, we will report the effects of polyamine hydrochlorides, NaCl and magnesium acetate on the dephosphorylation of these substrates catalyzed by the two forms of the enzyme. The data indicate that the activity of the enzyme was influenced by these ionic compounds dependent upon the substrate employed. The present findings also indicate that dissociation of the large form to the small form of the enzyme was accompanied by a pronounced change in response of the enzyme to these ionic compounds.

Materials and Methods

Materials. H2B histone and H1 histone were prepared from calf thymus as described previously [12]. Cyclic AMP-dependent protein kinase was purified from pig heart by the method described by Rubin et al. [13]. [γ-^{32}P]ATP with a specific activity of 2—50 cpm/pmol was prepared by the method of Glynn and Chappell [14]. Rabbit muscle phosphorylase *b* (3 × crystallized) was prepared by the method of Fischer and Krebs [15]. Phosphorylase *b* kinase was purified as described by Cohen [16]. Bovine serum albumin, spermine · 4HCl, spermidine · 3HCl and putrescine · 2HCl were obtained from Sigma. Trypsin was obtained from Worthington. 3-Phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase were purchased from Boehringer Mannheim. Other chemicals were obtained from commercial sources.

Preparation of substrates. ^{32}P-labelled H2B and H1 histones were prepared with [γ-^{32}P]ATP and pig heart cyclic AMP-dependent protein kinase as described before [12]. ^{32}P-labelled H2B histone, used in the present studies, contained 53% of total alkali-labile phosphate (63 nmol/mg of H2B histone) at Ser-36 and 43% at Ser-32. ^{32}P-labelled H1 histone contained 17 nmol alkali-labile phosphate at Ser-38 per mg of H1 histone. A radioactive tryptic phosphodecapeptide, Lys-Glu-Ser(P)-Tyr-Ser-Val-Tyr-Val-Tyr-Lys was prepared from ^{32}P-labelled H2B histone as described previously [17] and identified by amino acid analysis with a Nihondenshi amino acid analyzer, Model 5AH. ^{32}P-labelled phosphorylase *a* was prepared by phosphorylating phosphorylase *b* with phosphorylase *b* kinase as described by Krebs et al. [18]. ^{32}P-labelled phosphorylase *a* was crystallized three times by the method of Brandt et al. [19] and contained 8 nmol $^{32}P_i$ and 5 units per mg of phosphorylase *a*. All substrate concentrations represent the concentration of the alkali-labile phosphate moiety of the substrate protein.

Enzyme assay. Phosphoprotein phosphatase was routinely assayed by measuring [^{32}P]orthophosphate which was released from ^{32}P-labelled H2B histone. The reaction mixture (0.1 ml), containing 50 μM ^{32}P-labelled H2B histone, 50 mM Tris · HCl at pH 7.2, 50 mM magnesium acetate and 0.5 mM dithiothreitol (standard assay conditions), was incubated for 10 min at 30°C and [^{32}P]orthophosphate release was determined as described previously [11]. One unit of the enzyme was defined as the amount of enzyme which catalyzed the release of 1 nmol of [^{32}P]orthophosphate per min. Phosphorylase *a* was assayed by the method of Brandt et al. [19]. Protein was determined by the method of Lowry et al. [20] with bovine serum albumin as a standard.

Purification of phosphoprotein phosphatase. The large form of phosphoprotein phosphatase was purified from pig heart as described previously [11] except that the first ammonium sulfate precipitation and subsequent dialysis were omitted. The large form of the enzyme had a molecular weight of approx. 250 000 and a specific activity of 75 units/mg protein. The small form of phosphoprotein phosphatase was prepared by ethanol treatment of the large form as described before [11]. The small form of the enzyme had a molecular weight of approx. 30 000 and a specific activity of 4300 units/mg protein.

Results and Discussion

The effects of polyamine hydrochlorides on the rate of dephosphorylation of H2B histone-P by the large and small forms of phosphoprotein phosphatase is shown in Fig. 1. With the large form of the enzyme, the reaction rate was stimulated 5—9-fold by the addition of spermine · 4HCl, spermidine · 3HCl and putrescine · 2HCl with optimal concentrations of 30, 60 and 125 mM, respectively (Fig. 1A). With the small form of the enzyme, the rate of P_i release was stimulated about 2-fold by spermine · 4HCl, spermidine · 3HCl and putrescine · 2HCl with optimal concentrations of 20, 40 and 75 mM, respectively, and the rate was inhibited by higher concentrations (Fig. 1B).

The effects of various metal chlorides on the dephosphorylation of H2B histone-P by the two forms of phosphoprotein phosphatase were also investigated (Table I). Chlorides of NH_4^+, Li^+, Na^+, K^+, Rb^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} and Mn^{2+} at the indicated concentrations stimulated the reactions of both forms of the enzyme. The large form of the enzyme was much more sensitive to the stimulatory effects of these salts. Chlorides of Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Sn^{2+} and Pb^{2+} at 1—5 mM, strongly inhibited the reactions of both forms.

Putrescine · 2HCl could replace NaCl or magnesium acetate and thereby cause a shift in the optimal concentration of these salts in the dephosphorylation of H2B histone-P catalyzed by the two forms of the enzyme (Figs. 2 and 3). The magnitude of stimulation by optimal concentrations of NaCl or magnesium acetate, in the presence of putrescine · 2HCl, was larger than those by NaCl or magnesium acetate alone, so that the effects of putrescine · 2HCl and these salts were partially additive and not completely identical. The ionic strengths of the polyamine hydrochlorides NaCl and magnesium acetate at the optimal concentration for stimulating the activity of the large form of the enzyme toward H2B histone-P were all about the same (0.3—0.4 M). For the

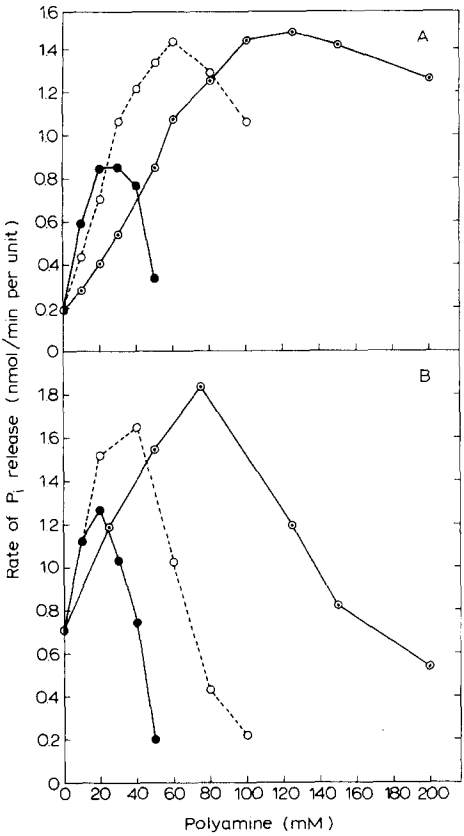

Fig. 1. Effect of polyamine hydrochlorides on phosphoprotein phosphatase activity. The rate of P_i release from H2B histone-P was measured with 0.58 unit/ml of the large form (A) or 0.75 unit/ml of the small form (B) of the enzyme under standard assay conditions except that magnesium acetate was replaced by spermine · 4HCl (— • —), spermidine · 3HCl (- - ○ - -) or putrescine · 2HCl (— · —) at the indicated concentration.

stimulation of the small form of the enzyme, however, the optimal ionic strengths of the polyamine hydrochlorides were more than twice (0.2—0.24 M) as much as that of NaCl and magnesium acetate (0.9—1.0 M). Although the effectiveness of these ionic compounds were found to be intimately correlated to their ionic strengths, the magnitude of the stimulation at the optimal ionic strength was dependent upon their ionic species.

Polyamine hydrochlorides, NaCl and magnesium acetate caused similar changes in the K_m and V values for H2B histone-P of the large and small forms of the enzyme (Table II). In the absence of ionic compounds, apparent K_m values for H2B histone-P of the large and small forms of the enzyme were 0.9 and 8.7 μM, respectively. In the presence of ionic compounds at the indicated concentrations, K_m values increased 3—10-fold and V values also increased 2—5-fold with either the large or small form of the enzyme. Both K_m and V values were increased progressively by increasing amounts of putrescine · 2HCl from 20 to 120 mM.

When a tryptic decapeptide containing the phosphorylated Ser-36 in H2B

TABLE I

EFFECTS OF VARIOUS SALTS ON TWO FORMS OF PHOSPHOPROTEIN PHOSPHATASE

The rate of P_i release from H2B histone-P was measured under standard assay conditions except that various salts at the indicated concentrations were added instead of magnesium acetate with 0.75 unit/ml of phosphoprotein phosphatase as described under Materials and Methods. The rate without salts was taken as 100.

Salts	Concentration (mM)	Rate of P_i release	
		Large form	Small form
None	0	100	100
NH_4Cl	100	422	216
LiCl	100	322	191
NaCl	100	286	200
KCl	100	227	201
RbCl	100	300	162
CsCl	100	95	13
$MgCl_2$	20	314	213
$CaCl_2$	20	336	220
$SrCl_2$	20	288	232
$BaCl_2$	20	313	136
$MnCl_2$	20	778	211
$ZnCl_2$	5	3	0
$CdCl_2$	5	0	1
$HgCl_2$	5	3	0
$SnCl_2$	1	22	4
$PbCl_2$	1	48	5
$CuCl_2$	5	0	0
$FeCl_2$	5	8	1
$FeCl_3$	1	42	61
$CoCl_2$	5	26	34
$NiCl_2$	5	64	29

histone-P, Lys-Glu-Ser(P)-Tyr-Ser-Val-Tyr-Val-Tyr-Lys, was utilized under identical conditions, no activation but inhibition of the dephosphorylation was observed with putrescine · 2HCl, NaCl and magnesium acetate (Fig. 4). These results strongly suggest that the effectors exert their stimulatory effect by binding to H2B histone-P.

The apparent $s_{0.5}$ * values for the tryptic phosphopeptide of the large and small forms of the enzyme were 34.6 and 38.4 μM, respectively (Table III). Putrescine · 2HCl, NaCl and magnesium acetate at the indicated concentrations did not change the apparent $s_{0.5}$ value but significantly reduced the V value of either the large or small form of the enzyme (Table III). The inhibition of the two forms of the enzyme by these ionic compounds was of an apparently non-competitive type with respect to the tryptic phosphopeptide. It is therefore assumed that both forms of the enzyme were inhibited by binding of the effectors to a site on the enzyme other than the catalytic site. The extent of

* Although the Lineweaver-Burk plot for both forms of the enzyme with the tryptic phosphopeptide was curved, the plot of $1/v$ vs. $1/s^2$ was linear. Therefore $s_{0.5}$, the substrate concentration that yields $V/2$, for the tryptic phosphopeptide was estimated instead of determining K_m values as described in the legends to Table III.

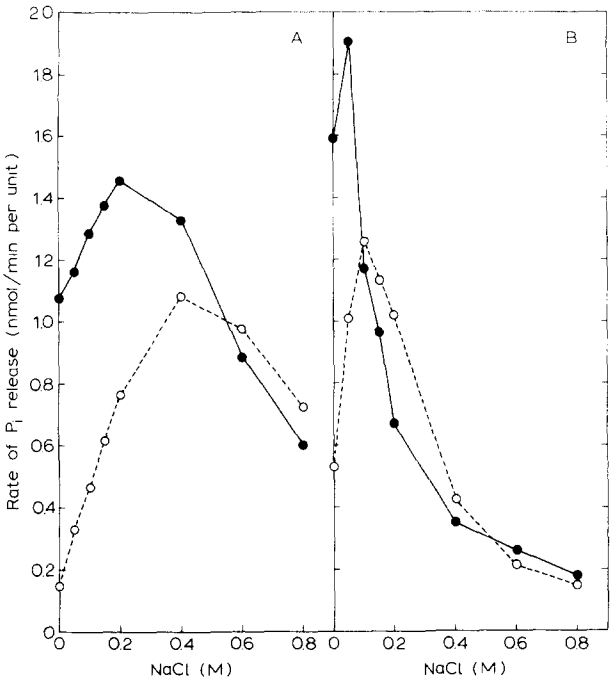

Fig. 2. Combined effect of NaCl and putrescine · 2HCl. The rate of P_i release from H2B histone-P was measured with 0.37 unit/ml of the large form (A) or 0.20 unit/ml of the small form (B) of the enzyme under standard assay conditions except that magnesium acetate was replaced by NaCl at the indicated concentration in the presence (— ● —) or absence (- - ○ - -) of 60 mM putrescine · 2HCl.

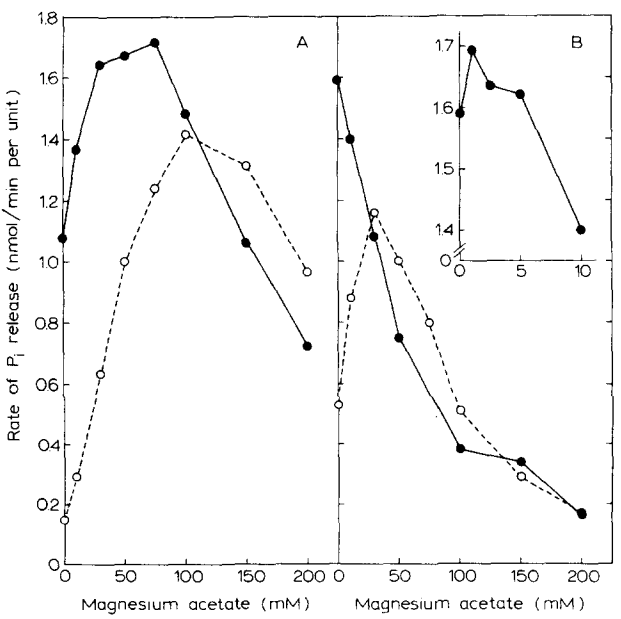

Fig. 3. Combined effect of magnesium acetate and putrescine · 2HCl. The rate of P_i release from H2B histone-P was measured with 0.37 unit/ml of the large form (A) or 0.20 unit/ml of the small form (B) of the enzyme under standard assay conditions except that magnesium acetate at the indicated concentration was included in the presence (— ● —) or absence (- - ○ - -) of 60 mM putrescine · 2HCl.

TABLE II

KINETIC CONSTANTS FOR H2B HISTONE-P OF TWO FORMS OF PHOSPHOPROTEIN PHOSPHATASE IN THE PRESENCE OR ABSENCE OF POLYAMINE HYDROCHLORIDES AND OTHER SALTS

The rate of P_i release from H2B histone-P was measured with 0.1—0.5 unit/ml of either the large or small forms of phosphoprotein phosphatase under standard assay conditions as described under Materials and Methods except that magnesium acetate was replaced by polyamine hydrochlorides or other salts at the indicated concentrations. Kinetic constants were estimated by fitting the data to Michaelis—Menten equations using the method of least squares [21]. Values are averages (with the range) of values for two separate experiments.

Additions	Concentration (mM)	Large form		Small form	
		K_m (μM)	V (pmol/min per unit)	K_m (μM)	V (pmol/min per unit)
None	—	0.9 ± 0.1	458 ± 42	8.7 ± 0.3	910 ± 73
Putrescine · 2HCl	20	—	—	22.2 ± 6.7	1274 ± 289
	40	1.5 ± 0.1	684 ± 55	39.1 ± 5.5	2202 ± 73
	60	5.0 ± 0.9	1743 ± 227	57.8 ± 1.5	3034 ± 59
	120	11.7 ± 0.4	2499 ± 195	—	—
Spermidine · 3HCl	30	10.8 ± 1.6	1325 ± 137	38.1 ± 13.0	1712 ± 426
Spermine · 4HCl	20	12.0 ± 3.0	1279 ± 45	42.0 ± 11.8	1847 ± 476
NaCl	200	11.6 ± 3.6	1299 ± 242	53.2 ± 6.3	2587 ± 273
Magnesium acetate	50	23.7 ± 2.1 *	1410 ± 82 *	81.4 ± 0.8 *	2604 ± 64 *

* Values were taken from the previous report [11].

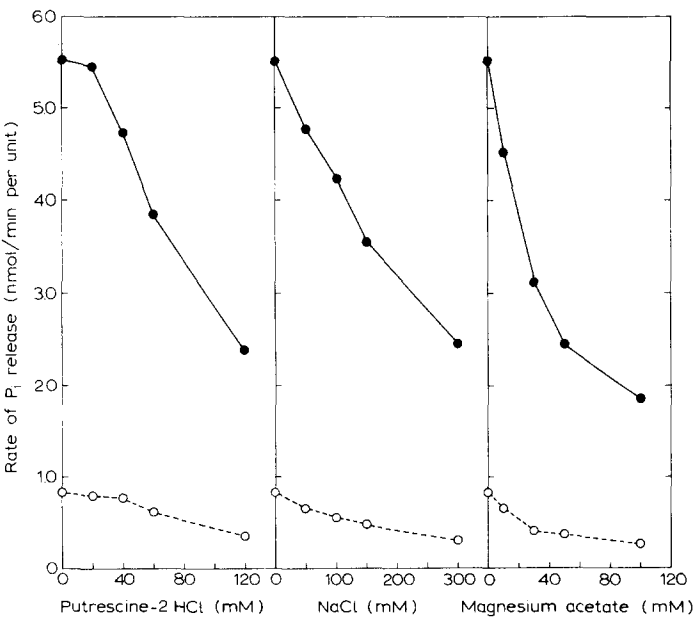

Fig. 4. Effect of putrescine · 2HCl, NaCl and magnesium acetate on the dephosphorylation of a tryptic phosphopeptide by phosphoprotein phosphatase. The rate of P_i release from a tryptic phosphopeptide (50 μM), Lys-Glu-Ser(P)-Tyr-Ser-Val-Tyr-Val-Tyr-Lys, was measured with 0.64 unit/ml of the large form (— ● —) or 1.02 units/ml of the small form (- - ○ - -) of the enzyme under standard assay conditions except that putrescine · 2HCl, NaCl or magnesium acetate at the indicated concentration was included instead of 50 mM magnesium acetate.

TABLE III

KINETIC CONSTANTS FOR A TRYPTIC PHOSPHOPEPTIDE OF TWO FORMS OF PHOSPHOPROTEIN PHOSPHATASE IN THE PRESENCE OR ABSENCE OF PUTRESCINE HYDROCHLORIDE AND OTHER SALTS

The rate of P_i release from a tryptic phosphopeptide, Lys-Glu-Ser-(P)-Tyr-Ser-Val-Tyr-Val-Tyr-Lys, was measured with 0.6—0.9 unit/ml of either the large or small forms of phosphoprotein phosphatase under standard assay conditions as described under Materials and Methods except that magnesium acetate was replaced by putrescine · 2HCl or other salts at the indicated concentrations. Since the double reciprocal plots were not linear, the V values were estimated by extrapolation of the curve in the plot of $1/v$ versus $1/[s]^2$. The half-saturation concentration values ($s_{0.5}$, the substrate concentration that yields $V/2$) for the tryptic phosphopeptide were estimated from the curve in the plot of v versus s. Values are averages (with the range) of values for two separate experiments.

Additions	Concentration (mM)	Large form		Small form	
		$s_{0.5}$ (μM)	V (pmol/min per unit)	$s_{0.5}$ (μM)	V (pmol/min per unit)
None	—	34.6 ± 2.9	5097 ± 14	38.4 ± 0.1	851 ± 33
Putrescine · 2HCl	60	35.4 ± 0.3	3614 ± 403	41.4 ± 10.3	726 ± 4
NaCl	200	34.6 ± 3.0	3699 ± 280	28.1 ± 2.6	764 ± 66
Magnesium acetate	50	32.8 ± 2.8	2632 ± 28	34.0 ± 0.9	447 ± 73

the stimulatory effect of the ionic compounds on the dephosphorylation of H2B histone-P may reflect the net effect of their interactions with the substrate resulting in a better substrate and with the enzyme causing an inactivation.

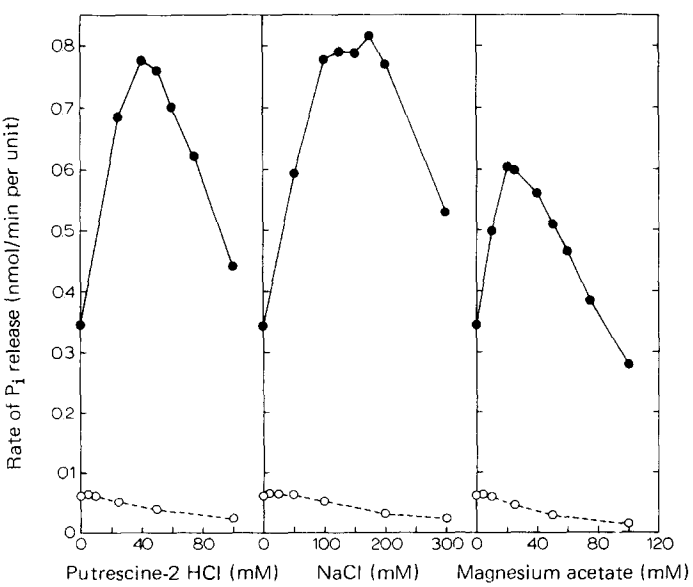

Fig. 5. Effect of putrescine · 2HCl, NaCl and magnesium acetate on the dephosphorylation of H1 histone-P by phosphoprotein phosphatase. The rate of P_i release from H1 histone-P (25 μM) was measured with 0.65 unit/ml of the large form (— ● —) or 0.99 unit/ml of the small form (- - ○ - -) of the enzyme under standard assay conditions except that putrescine · 2HCl, NaCl or magnesium acetate at the indicated concentration was included instead of 50 mM magnesium acetate.

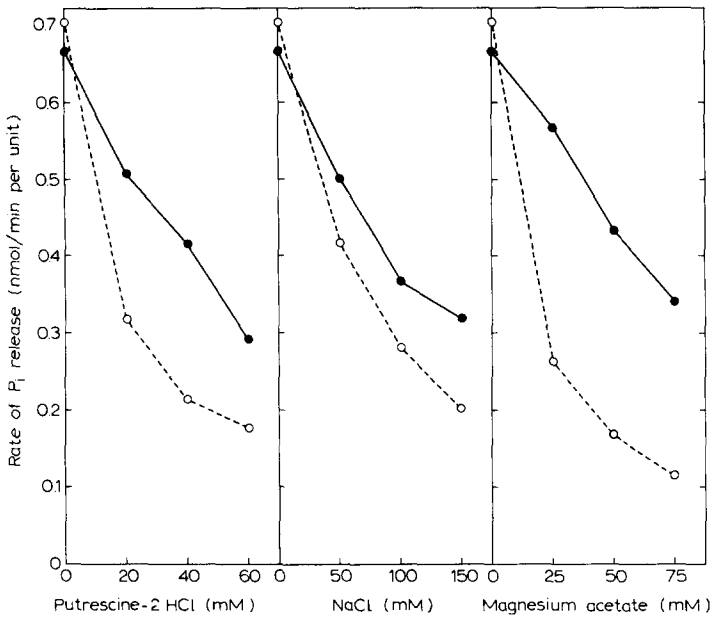

Fig. 6. Effect of putrescine · 2HCl, NaCl and magnesium acetate on the dephosphorylation of phosphorylase *a* by phosphoprotein phosphatase. The rate of P_i release from phosphorylase *a* (8.75 μM) was measured with 0.52 unit/ml of the large form (— • —) or 0.57 unit/ml of the small form (- - ○ - -) of the enzyme under standard assay conditions except that putrescine · 2HCl, NaCl or magnesium acetate at the indicated concentration was included instead of 50 mM magnesium acetate.

The activity of the large form of the phosphoprotein phosphatase toward H1 histone-P was stimulated about 2-fold by putrescine · 2HCl, NaCl and magnesium acetate with optimal concentrations of 40, 150 and 20 mM, respectively (Fig. 5). Whereas, the activity of the small form of the enzyme toward H1 histone-P was not significantly stimulated but inhibited by these ionic compounds (Fig. 5). These observations indicate that following the transformation of the large form of the enzyme to the small form, the properties of the large form responsive to the stimulatory effect of the ionic compounds were lost.

Using phosphorylase *a* as substrate, both large and small forms of the enzyme were inhibited by polyamine hydrochlorides, NaCl and magnesium acetate (Fig. 6). The inhibition was more significant with the small form of the enzyme. Spermine · 4HCl, spermidine · 3HCl and putrescine · 2HCl at 20 mM inhibited the reaction of the large form of the enzyme by 51, 34 and 23% and of the small form of the enzyme by 83, 74 and 58%, respectively, under conditions employed in Fig. 6.

Apparent K_m values for phosphorylase *a* of the large and small forms were 12.3 and 33.2 μM, respectively (Table IV). Putrescine · 2HCl, NaCl and magnesium acetate caused an increase in the K_m value but no significant change in the V value of the large form of the enzyme indicating that the inhibition of the effectors was apparently competitive with respect to phosphorylase *a* (Table IV). On the other hand, these effectors greatly reduced the V value without affecting the K_m value of the small form of the enzyme indicating that

TABLE IV

KINETIC CONSTANTS FOR PHOSPHORYLASE *a* OF TWO FORMS OF PHOSPHOPROTEIN PHOSPHATASE IN THE PRESENCE OR ABSENCE OF PUTRESCINE HYDROCHLORIDE AND OTHER SALTS

The rate of P_i release from phosphorylase *a* was measured with 0.4—1.9 units/ml of either the large or small forms of phosphoprotein phosphatase under standard assay conditions as described under Materials and Methods except that magnesium acetate was replaced by putrescine · 2HCl, NaCl or magnesium acetate at the indicated concentrations. Kinetic constants were estimated by fitting the data to Michaelis-Menten equations using the method of least squares [21]. The mean ± S.D. of data of experiments with four separate analyses are given.

Additions	Concentration (mM)	Large form		Small form	
		K_m (μM)	V (pmol/min per unit)	K_m (μM)	V (pmol/min per unit)
None	—	12.3 ± 0.6	1068 ± 230	33.2 ± 1.0	5398 ± 848
Putrescine · 2HCl	60	30.4 ± 5.7	1026 ± 47	30.3 ± 2.2	472 ± 125
	120	50.6 ± 20.5	1033 ± 321	—	—
NaCl	200	42.6 ± 18.1	905 ± 349	35.8 ± 8.9	668 ± 85
Magnesium acetate	50	35.9 ± 7.4	1290 ± 195	35.2 ± 3.9	499 ± 77

the inhibition of the small form of the enzyme was non-competitive with respect to phosphorylase *a* (Table IV).

The inhibition of a phosphoprotein phosphatase by $MgCl_2$ has been reported by Martensen et al. [6] to be competitive and by Khandelwal [9] to be non-competitive, with respect to phosphorylase *a*. The discrepancy could arise because of the use of different enzyme preparations since Martensen et al. [6] used a rabbit skeletal muscle enzyme which was not treated with ethanol, and Khandelwal [9] employed an ethanol-treated homogeneous form (mol. wt. 30 000) of rat liver enzyme. Nakai and Glinsmann [22] reported that spermine · 4HCl at 0.5 mM inhibited rabbit skeletal muscle phosphoprotein phosphatase which was treated by 30% ethanol and the inhibition was non-competitive with respect to phosphorylase *a*.

Polyamine hydrochlorides, NaCl and magnesium acetate had similar effects on the activities of the two forms of the enzyme toward each substrate employed and caused similar changes in kinetic parameters for each substrate of both forms of the enzyme. These results may imply that the ionic compounds might exert their effects on common sites of the enzymic reactions.

The different effects of the ionic compounds on each dephosphorylation of different substrates by the same phosphoprotein phosphatase indicate that substrates play an important role in determining the effects of the ionic compounds. These results support the notion that phosphoprotein phosphatases might be controlled in vivo by either allosteric [8,23—25] or covalent [26] modification of a substrate.

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