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POLYAMINES ALTER THE PHOSPHORYLATION PATTERN OF CHROMATIN PROTEINS BY ENDOGENOUS PROTEIN KINASES

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The effect of polyamines on the chromatin phosphorylation by endogenous protein kinases was investigated. Polyamines not only selectively stimulated the phosphorylation of chromatin proteins but also concurrently inhibited the phosphorylation of a number of polypeptides. In particular, a 11,000-dalton polypeptide with pI 4.5-5.0 was highly phosphorylated in the absence of polyamines, despite being a minor component whereas the phosphorylation was strongly inhibited in the presence of polyamines.

INTRODUCTION

The aliphatic polyamines, putrescine, spermidine and spermine are natural constituents of eukaryotic cells and these amines affect various enzyme activities involved in nuclear function [1,2]. With respect to nuclear phosphorylation, polyamines have a stimulatory effect on nuclear protein kinase activities [3-5] and on the endogenous phosphorylation of non-histone chromatin proteins in isolated nuclei [6,7]. Thus, much attention has been directed to the stimulatory effect of polyamines on the phosphorylation of nuclear proteins. In a previous paper, we demonstrated that polyamines have both stimulatory and inhibitory effects on the nuclear protein kinase NII, depending upon the substrates used, albeit non-physiological [8]. Therefore, chromatin together with endogenous protein kinases was employed to investigate whether or not polyamines have inhibitory as well as stimulatory effects on the phosphorylation of nuclear proteins

MATERIALS AND METHODS

Ampholines were purchased from LKB. Protein assay dye reagent was from Bio-Rad and sodium molybdate was from Sigma. Chymostatin, leupeptin and pepstatin were gifts from Dr. M.Ishizawa, Kyushu University, Fukuoka. The sources of other biochemicals were as outlined elsewhere [9].

Abbreviation: PMSF, phenylmethylsulfonyl fluoride

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Preparation of chromatin : Nuclei were isolated from rat liver [8] and washed twice in 75mM NaCl, 25mM EDTA pH 8.0 and 0.5mM PMSF by homogenization and centrifugation at 1000xg for 7 min. The resulting pellet was resuspended and centrifuged twice in 10mM Tris-HCl pH 8.0 and 0.5mM PMSF at 1000xg for 7 min. The final pellet served as the chromatin preparation.

Endogenous phosphorylation of chromatin : In vitro phosphorylation experiments were carried out by incubating 150 μg of chromatin proteins in a 100 μl of reaction mixture containing 50mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 10mM β-mercapto-ethanol, 5% glycerol, 20μM [γ-^{32}P]ATP, 5mM PMSF, 10μg/ml of chymostatin, leupeptin and pepstatin and 1mM EDTA. The specific activity of [γ-^{32}P]ATP were 0.2 Ci/mmol for experiments in Fig.1 and 2 Ci/mmol for electrophoresis. The in vitro reaction was performed at 30° for an indicated time and stopped by transferring all the mixture onto a Whatman 3MM filter and the radioactivity was measured as previously described [9].

In the presence of proteolytic inhibitors such as 5mM PMSF, 10μg/ml of chymostatin, leupeptin and pepstatin and 1mM EDTA [10], the effect of polyamines was more definitely and reproducibly demonstrated. Therefore, these proteolytic inhibitors were added to all incubation mixtures.

One dimensional SDS polyacrylamide gel electrophoresis :

Samples were incubated for 10 min as described above and the reaction was terminated by the addition of 100μl of double-strength sample buffer containing 0.25M Tris-HCl pH 6.8, 4% SDS, 20% glycerol and 10% β-mercaptoethanol. The samples were then heated at 100° for 3 min and subjected to 15% polyacrylamide gels in the presence of 0.1% SDS [11]. Gels were stained with Coomassie brilliant blue, destained with methanol-acetic acid and then rinsed with water. For autoradiography, vacuum-dried gels were exposed to Kodak X-OMAT S films for about one week. Molecular weight determinations were made by the position of known reference markers.

Two dimensional gel electrophoresis :

In vitro phosphorylation reaction was terminated by the addition of ten volumes of ice-cold solution containing 3M NaCl, 7M urea and 0.5mM PMSF. After 12-hour of shaking at 4°, the solution was centrifuged at 40,000rpm for 20h in a Beckman 50 Ti rotor. The supernatant was dialysed against 40mM acetic acid and lyophilized. The lyophilized proteins were dissolved in a solution containing 1% SDS and 5% β-mercaptoethanol and heated at 100° for 1 min. Then, the solutions were mixed with the same volume of 8% NP-40, 10M urea and 4% Ampholines (3.2% pH5-7 and 0.8% pH3-10 Ampholines) and subjected to two dimensional polyacrylamide gel electrophoresis according to the method of O'Farrell [12]. The first dimension isoelectric focusing gels contained 1.6% pH5-7 and 0.4% pH3-10 Ampholines. The second dimension was performed on exponential acrylamide gradient gel slabs (10-20% acrylamide). Protein concentrations were determined using Bio-Rad protein assay reagent [13].

RESULTS

When investigating the effects of polyamines on the phosphorylation of chromatin proteins by endogenous protein kinases, the time course of the phosphorylation reaction was first studied in the presence or absence of spermine (Fig.1-A). The phosphorylation of chromatin proteins increased linearly with incubation up to 10 min, and thereafter showed a gradual increase with or without spermine. In the presence of 2mM spermine, the phosphorylation of chromatin was significantly enhanced, at any incubation time. The effect

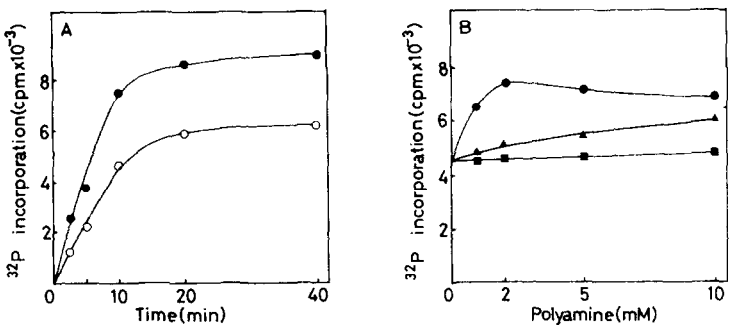

Fig.1 Effects of polyamines on the endogenous phosphorylation of chromatin
A) Time course of the chromatin phosphorylation was studied in the presence (●) or absence (○) of 2mM spermine as described in Materials and Methods.
B) Assays were carried out for 10 min in the presence of various concentrations of spermine (●), spermidine (▲) or putrescine (■).

of various concentrations of polyamines on the chromatin phosphorylation is shown in Fig.1-B. Spermine was the most effective compound in eliciting increased phosphorylation of chromatin. Two mM spermine activated the chromatin phosphorylation 1.6-fold. Spermidine also stimulated the phosphorylation reaction 1.4-fold at 10mM, while putrescine was all but ineffective, even at 10mM.

SDS polyacrylamide gel electrophoresis was performed for the analysis of chromatin proteins phosphorylated in vitro, in the presence of various concentrations of polyamines (Fig.2). The stimulation of chromatin phosphorylation by polyamines was not due to a general increase in the specific radioactivity of all proteins to be phosphorylated, but several polypeptides were selectively more highly labeled in the presence of spermine or spermidine. Concurrently, the phosphorylation of polypeptides 10K, 11K and 43K, named according to their molecular weights, decreased with increases in polyamine concentrations. Spermine and spermidine had a potent inhibitory effect on the phosphorylation of these polypeptides. Putrescine had only a weak inhibitory effect at 12mM. The polypeptides 10K and 11K must be minor components as they were not detectable with the Coomassie staining method. The Coomassie staining pattern was virtually identical for preparations either in the presence or absence of polyamine.

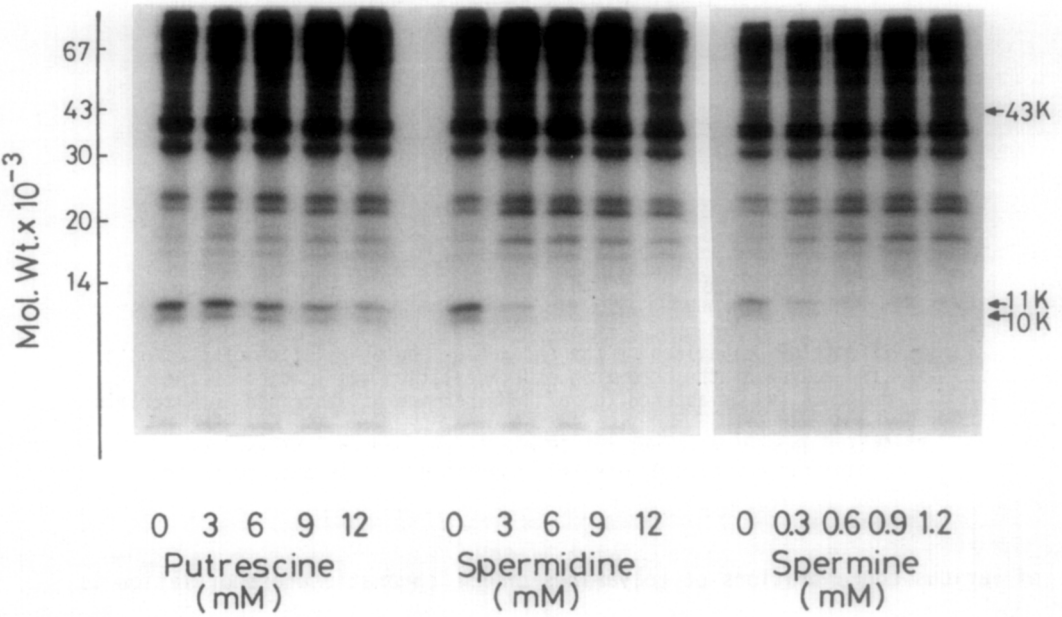

Fig.2 Autoradiographic pattern of chromatin proteins phosphorylated *in vitro* in the presence of various concentrations of polyamine.
Chromatin proteins were phosphorylated with [γ-^{32}P]ATP in the presence of various concentrations of polyamine and labeled proteins were separated by SDS polyacrylamide gel electrophoresis and subjected to autoradiography, as described in Materials and Methods. Arrows indicate the three polypeptides in which the endogenous phosphorylation was inhibited by polyamines.

With special interest directed to the polyamine-inhibitable polypeptides, kinetic examinations of the phosphorylation and dephosphorylation of the polypeptides 10K, 11K and 43K were carried out in the presence or absence of polyamine, as shown in Fig.3. In the presence of polyamine, the phosphorylation of these polypeptides was inhibited, at any incubation time tested (Fig.3-A). Then, the effects of polyamines on the dephosphorylation of these polypeptides were investigated with chromatin proteins prelabeled *in vitro* with [γ-^{32}P]ATP for 15 min in the absence of polyamine. Subsequent incubation with 5mM spermine did not alter the phosphorylation of these polypeptides, as demonstrated in Fig.3-B. Furthermore, the phosphorylation of these polypeptides was not affected in the presence of 1mM sodium molybdate, a phosphatase inhibitor (data not shown). Therefore, these polyamines probably do not exert effects on the dephosphorylation process but rather effect the phosphorylation process.

The phosphorylation of chromatin proteins in the presence or absence of polyamine was analysed by two dimensional gel electrophoresis and autoradio-

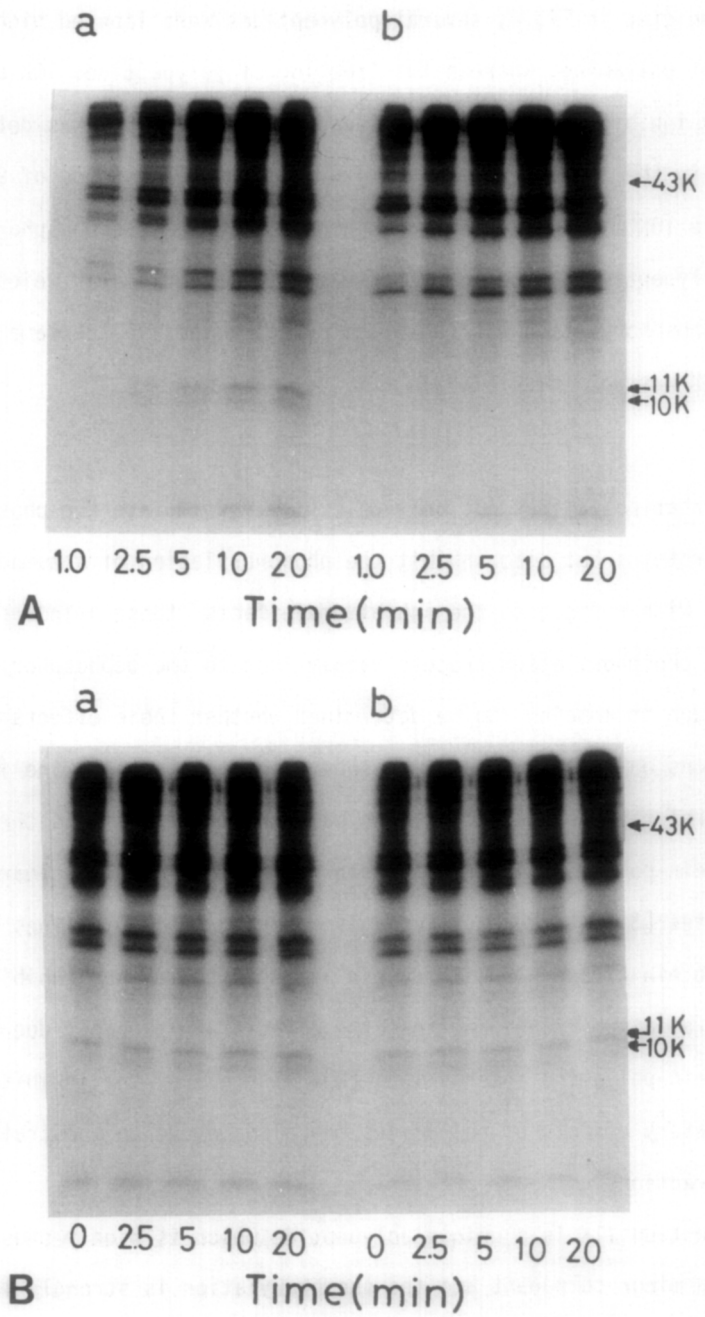

Fig.3 Autoradiograph showing the kinetics of the phosphorylation and dephosphorylation of the polypeptides 10K, 11K and 43K in the absence (a) or presence (b) of 5mM spermine.

A) Each reaction mixture was incubated with or without 5mM spermine for the various times indicated and was then analysed by electrophoresis in polyacrylamide gels, as described in Materials and Methods.
B) Chromatin proteins were prelabeled with [γ-^{32}P]ATP for 15 min in the absence of polyamine. Further incubation was carried out in the absence or presence of 5mM spermine for the indicated time and the preparation then analysed using electrophoresis.

graphy. As depicted in Fig.4, several polypeptides were labeled higher in the presence of polyamine, whereas the labeling of polypeptides 10K and 11K was completely inhibited. The pI of polypeptides 10K and 11K was determined to be 4.5-5.0 in the isoelectric focusing system in the presence of 9M urea. The polypeptide 10K might be a degradation product or a less phosphorylated form of the polypeptide 11K because they have similar molecular weights and pI and their phosphorylation was inhibited by polyamines in the same manner. Polypeptide 43K was not identified in this gel system.

DISCUSSION

We found that polyamines not only selectively stimulate the phosphorylation of chromatin proteins but also inhibit the phosphorylation of a number of polypeptides. With respect to the inhibitory effects, these amines probably related to the phosphorylation process rather than to the dephosphorylation process, although it remains to be determined whether these effects are due to direct actions on protein kinases or substrate protein-polyamine interactions There are several reports that polyamines may exert their effects through substrate protein-polyamine interactions in the activation of nuclear protein kinase activities [3,14]. We have recently reported that polyamines stimulate the nuclear protein kinase NII with casein as a substrate, but inhibit it with phosvitin as a substrate, and that the effects are probably due to substrate protein-polyamine interactions [8]. Therefore, the inhibitory as well as stimulatory effects of polyamines may also be due to substrate protein-polyamine interactions.

The polypeptide 11K is a unique polypeptide which is highly phosphorylated despite being a minor component and the phosphorylation is strongly inhibited by polyamines. This polypeptide has characteristics similar to component 10, a low-molecular-weight and highly-phosphorylated non-histone chromatin protein with pI near 4.5, which was isolated from mouse liver nuclei and characterized by MacGillivray et al.[15]. Since there is evidence that the phosphorylation of non-histone chromatin proteins might be closely involved in gene activation [16,17], the polypeptide 11K might have an important function in the cell

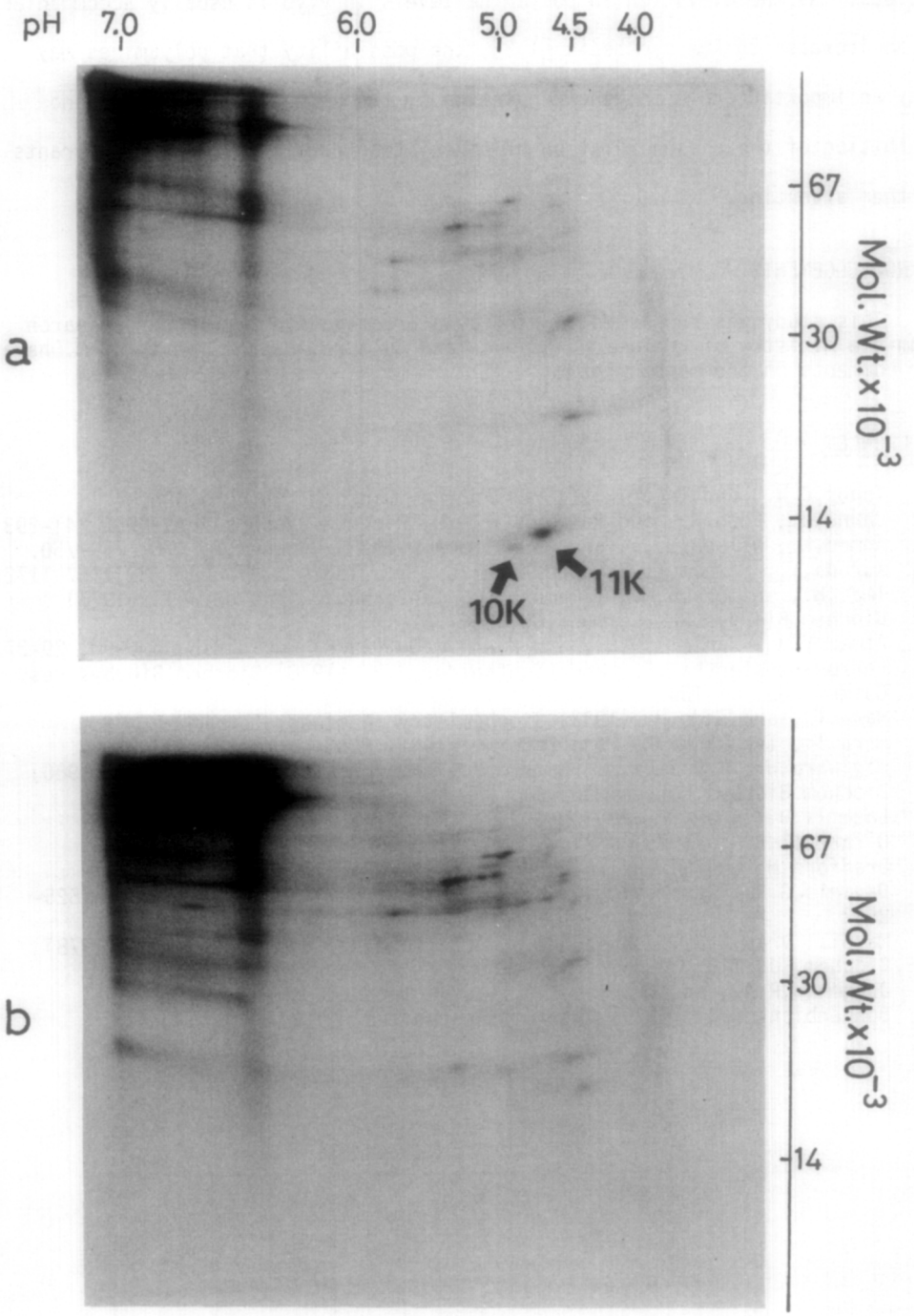

Fig.4 Autoradiograph of the two dimensional gels of chromatin proteins labeled in vitro in the absence (a) or presence (b) of 5mM spermine.

Chromatin proteins were phosphorylated in vitro for 10 min with or without 5mM spermine and then analysed by two dimensional gel electrophoresis and autoradiography, as described in Materials and Methods. The pH gradient was determined in a parallel gel as described in [11].

nucleus. As the elevation in polyamine levels *in vivo* is usually accompanied by an increase in RNA synthesis [1,2], the possibility that polyamines may play an important role in gene regulation by the selective activation and inhibition of the phosphorylation of non-histone chromatin proteins warrants further attention.

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