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POLYAMINE-LIKE EFFECTS OF COBALT(III) HEXAAMMINE ON VARIOUS CYCLIC NUCLEOTIDE-INDEPENDENT PROTEIN PHOSPHOKINASE REACTIONS

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SUMMARY: The chemically inert trivalent ion cobalt(III) hexaammine, $Co^{3+}(NH_3)_6$, was found to exert polyamine-like effects in enhancing certain cyclic nucleotide-independent protein kinase reactions catalyzed by nuclear enzyme preparations from rat ventral prostate or liver. At 1 mM, $Co^{3+}(NH_3)_6$ stimulated chromatin- and also non-histone-protein- associated kinase activities with partially dephosphorylated phosvitin as substrate by 38% and 72% respectively, whereas chromatin-associated kinase-catalyzed phosphorylation of lysine-rich histones was not affected under the same conditions. ^{32}P incorporation (from γ-^{32}P-ATP) into endogenous protein substrates of chromatin or non-histone protein fractions catalyzed by their endogenous kinase activity was increased by 47% and 153%, respectively. These effects of $Co^{3+}(NH_3)_6$ were similar to those produced by 1 mM spermine. Autoradiographic analysis of endogenous ^{32}P-labelled nonhistone proteins revealed similar enhancements of the phosphorylation of several of the same proteins, induced by 1 mM spermine or 1 mM $Co^{3+}(NH_3)_6$ or 2mM spermidine. The stimulatory actions of polyamines or $Co^{3+}(NH_3)_6$ were not mimicked by raising the ionic strength by addition of comparable concentrations of NaCl. The effects of 1 mM spermine and of 1 mM $Co^{3+}(NH_3)_6$ tested separately were not additive. Phosphorylation of lysine-rich histones by beef heart cyclic AMP-dependent protein kinase was not affected by polyamines or $Co^{3+}(NH_3)_6$. Various findings hint that the enhancement of cyclic nucleotide-independent kinase-catalyzed phosphorylation of certain protein substrates by spermidine, spermine and $Co^{3+}(NH_3)_6$ is primarily due to interaction of these cations with appropriate protein substrates affecting their conformational status. Further, these effects of polyamines may be a reflection of their cationic charge properties rather than being dependent on any particular conformations assumed by the polyamines.

Physiological concentrations of spermidine and spermine can directly enhance certain reactions catalyzed by cyclic nucleotide-independent protein phosphokinases from several sources(1-7). In our earlier studies on protein kinase preparations from rat ventral prostate cell nuclei (2,3) we observed

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that the stimulatory effects of polyamines depended on a variety of factors, including the types of enzyme preparations, the nature of the protein substrates, the total ionic strength of the incubation mixtures and the concentrations of Mg^{2+}. With partially dephosphorylated phosvitin (DPV)[1] as substrate, polyamine stimulation of protein kinase activities promoted by prostatic chromatin, or non-histone protein preparations (NHP) derived therefrom, was observed only with optimal levels of $MgCl_2$ (2-4 mM) and at concentrations of NaCl or KCl or NH_4Cl that were far below the optimal value (160-200 mM). Marked enhancement of these reactions by spermidine or spermine at 1-2 mM could not be mimicked by raising the ionic strength to equivalent levels by addition of NaCl. When phosphorylation of endogenous non-histone proteins of the chromatin was determined, spermine enhanced both the initial rates and final extents of transphosphorylation from [γ-^{32}P]ATP to proteins, even in the presence of optimal concentrations of NaCl. By contrast, spermidine or spermine had no effect on phosphorylation of lysine-rich histones by the prostatic nuclear kinase preparations. Several lines of evidence hint that polyamine enhancement of various cyclic nucleotide-independent protein kinase reactions primarily reflects infuences of the polyamines on the conformational status of the protein substrates rather than on the catalytic activity of the enzymes (2,7).

The aforementioned findings pose questions concerning the extent to which the effects of polyamines on certain protein kinase reactions are related to the structural and particularly the conformational features of spermidine and spermine, rather than to their charge properties as trivalent or quadrivalent cations. This is reminiscent of considerations given to the effects of polyamines on nucleic acids, especially in the context of modulation by spermidine or spermine of various reactions catalyzed by polynucleotide polymerases and toposiomerases, various tRNA aminoacyl synthetases, and

1. List of abbreviations used: DPV, partially dephosphorylated phosvitin; NHP, non-histone protein preparation derived from chromatin.

so forth (8-13). In this regard, it is noteworthy that compaction of DNA molecules by spermidine and spermine does not seem to involve chemical specificity, since similar effects are induced by chemically inert cation cobalt(III) hexaammine, when added at concentrations expected to evoke 89% or greater neutralization of polynucleotide charge, calculated according to counterion condensation theory as applied to linear poly-electrolytes (3,14). It was therefore of interest to determine the action of $Co^{3+}(NH_3)_6$ on the protein kinase reactions we had previously shown to be stimulated by polyamines (2-4). $Co^{3+}(NH_3)_6$ is chemically inert since its amino groups are uncharged and very firmly bound. The experiments to be presented show that $Co^{3+}(NH_3)_6$ stimulates certain cyclic AMP-independent protein kinase reactions, in a manner similar to spermine or spermidine.

EXPERIMENTAL PROCEDURES

Materials: Spermine tetrahydrochloride and spermidine trihydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, and were adjusted to pH 7.4 by adding an appropriate amount of Tris base before use. $Co^{3+}(NH_3)_6.3HCl$ was purchased from Pfaltz and Bauer (Stamford, CT). It was dissolved in H_2O and allowed to crystallize by adding C_2H_5OH to a final concentration of 70-80% (v/v) at 25°C. Solutions of the recrystallized material had the following characteristics: λ max=474nm; $\varepsilon=56M^{-1}cm^{-1}$.

Methods: Chromatin, and non-histone protein fraction (NHP) derived from it, were prepared from ventral prostate or liver of male Sprague-Dawley rats (weighing 295-325 g, from Harlan Co., Indianapolis, IN) according to procedures detailed previously (2,15). Chromatin- and NHP-associated protein kinase activity towards partially (30%) dephosphorylated phophosvitin (DPV) as substrate (16), as well as the rate of incorporation of ^{32}P from [γ-^{32}P]-ATP into chromatin-associated proteins and NHP-associated proteins was determined as detailed previously (2,3). The reaction medium for chromatin- and NHP-associated kinase activity towards DPV consisted of 30mM Tris/HCl, pH 7.45 (measured at 37°C), 4 mM $MgCl_2$, 3mM ATP, 1 mM dithiothreitol, and 2 mg/ml of DPV, in the presence of chromatin (equivalent to 8 µg of DNA) or NHP (equivalent to 9 µg of protein) in a final reaction vol of 0.5 ml. The reaction medium for phosphorylation of endogenous chromatin- and NHP-associated phosphoproteins consisted of 30 mM Tris/HCl, pH 7.45 (at 37°C), 4mM $MgCl_2$, 0.1mM [γ-^{32}P]ATP (specific radioactivity 1-8X10^8 dpm/µmol of ATP), 1 mM dithiothreitol, and chromatin or NHP each equivalent to 30-100 µg of protein in a final reaction vol of 0.5 ml. All reactions were initiated by the addition of chromatin or NHP as the case may be, and were measured under conditions where the reaction rates were linear with time at 37°C. Bovine heart cyclic AMP-dependent protein kinase (Sigma, P-5511) was assayed with mixed histone (Worthington Biochemical Corp., Freehold, NJ) as substrate, as described previously (17). ^{32}P-labelled NHP-associated phosphoproteins were subjected to SDS/polyacrylamide gradient gel electrophoresis according to the procedure of MacGillivray et al. (18). The gels were dried and radioactivity incorporated in various protein bands was visualized by autoradiography using a No-Screen Kodak X-ray film.

RESULTS AND DISCUSSION

Our previous studies (2) established optimal conditions for the enhancement by spermidine or spermine of the phosphorylation of partially dephosphorylated phosvitin (DPV) by protein kinases associated with isolated prostatic nuclear chromatin, or by non-histone protein preparations (NHP) isolated therefrom. Under very similar experimental conditions we have observed that, with either type of enzyme preparation, $Co^{3+}(NH_3)_6$ at the optimal level of 1-2 mM exerted maximal stimulatory effects that were quantitatively similar to those evoked by 1 mM spermine or 2 mM spermidine. With the chromatin-associated kinase as enzyme, 2 mM $Co^{3+}(NH_3)_6$ was less active than at 1 mM, whereas this was not the case with the NHP preparations for which maximal stimulation by $Co^{3+}(NH_3)_6$ occurred at roughly 2 mM. A representative experiment is depicted in Table 1. Raising the total ionic strength of the reaction mixtures to levels comparable to 1mM polyamine or $Co^{3+}(NH_3)_6$ by addition of NaCl (10mM) had very little effect. It is also worth noting that: (i) these results were obtained when $MgCl_2$ was present at the optimal level (4 mM) [note that spermine cannot replace the Mg^{2+} requirement (2)]; and (ii) the lack of reactivity of $Co^{3+}(NH_3)_6$ makes it very unlikely that under these circumstances formation of complexes of ATP or ADP with cobalt(III) ammines (19,20) would take place. At higher concentrations

Table 1

Effects of Cobalt(III) hexaammine and spermine on chromatin- and non-histone-protein-associated protein kinase reactions towards dephosphophosvitin (DPV) as substrate.

Additions	Protein Kinase Activity Associated With			
	Chromatin		Non-histone Proteins (NHP)	
	Activity*	% Increase	Activity*	% Increase
None	164	-	141	-
10mM NaCl	176	7	155	10
1mM spermine	228	39	254	80
0.1mM $Co^{3+}(NH_3)_6$	164	0		
0.5mM $Co^{3+}(NH_3)_6$	212	29	205	46
1.0mM $Co^{3+}(NH_3)_6$	226	38	250	77
2.0mM $Co^{3+}(NH_3)_6$	212	29	277	97

*nmol ^{32}P incorporated into protein substrate (DPV) per hr per mg of DNA in chromatin or per mg of protein in NHP as enzyme preparation. The experimental details are given in the text.

of $Co^{3+}(NH_3)_6$ or polyamines (3 mM or greater), a precipitation of the protein substrate was observed with resultant decrease in the extent of stimulation of the kinase reaction (unpublished results). Neither $Co^{3+}(NH_3)_6$ nor spermine or spermidine enhanced chromatin- or NHP-stimulated kinase reactions with lysine-rich histone as substrates. Comparably, neither the $Co^{3+}(NH_3)_6$ or the polyamines at 1-2 mM significantly influenced the activity of beef heart cAMP-dependent protein kinase determined as described above under Methods (results not shown). In other experiments no presented, very similar stimulations of DPV phosphorylation by $Co^{3+}(NH_3)_6$ and spermidine or spermine were found to be catalyzed by protein kinases associated with isolated liver chromatin preparations. Likewise, both $Co^{3+}(NH_3)_6$ and spermine caused similar stimulations of chromatin-associated protein kinase- catalyzed reactions with non-dephosphorylated phosvitin or casein as substrate. The action of $Co^{3+}(NH_3)_6$ on transfer of ^{32}P from [γ-^{32}P]ATP to endogenous proteins of chromatin or NHP preparations incubated in vitro is illustrated in Table 2. Again, the enhancement by $Co^{3+}(NH_3)_6$ of these endogenous protein kinase reactions was concentration-dependent, maximal effects being observed at 1mM. We have previously demonstrated (4) that the most marked stimulation by spermine or spermidine of the phosphorylation of endogenous chromatin-

Table 2

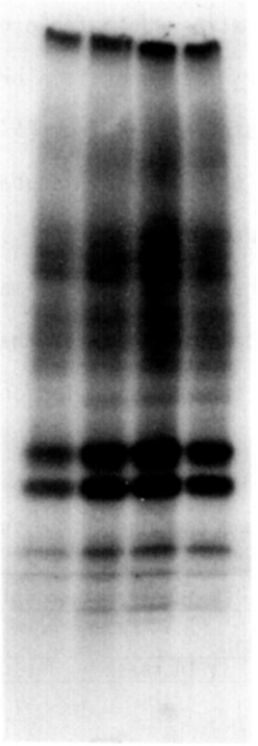
Effect of Cobalt(III) hexaammine and spermine on the incorporation of ^{32}P from [γ-^{32}P]ATP into endogenous phosphoproteins of chromatin and NHP fractions.

Additions	^{32}P incorporation into phosphoproteins of			
	Chromatin		NHP	
	Activity	% Increase	Activity	% Increase
None	14.9	-	11.6	-
10mM NaCl	14.9	0	11.6	0
1mM spermine	22.5	51	30.3	161
0.5mM $Co^{3+}(NH_3)_6$	19.4	30	26.8	131
1.0mM $Co^{3+}(NH_3)_6$	21.9	47	29.3	153
2.0mM $Co^{3+}(NH_3)_6$	18.7	26	28.2	143

The activity units are nmol ^{32}P incorporated/hr into endogenous phosphoproteins of chromatin or NHP enzyme preparations per mg of protein therein. Experimental details are provided in the text.

associated proteins was apparent in two fractions, one of which contained basic and neutral non-histone proteins, and the other a class of acidic non-histone proteins. To determine whether the $Co^{3+}(NH_3)_6$, spermine and spermidine stimulated the phosphorylation of the same types of proteins associated with chromatin or NHP preparations, the labelled proteins of NHP were separated by gel electrophoresis and then examined autoradiographically. Fig. 1 shows that 1 mM $Co^{3+}(NH_3)_6$, 1 mM spermine, or 2 mM spermidine caused very similar changes in the profiles of ^{32}P-labelled proteins.

It appears from the foregoing results that the cationic properties of spermidine and spermine rather than their conformational status may be mainly



1 2 3 4

Figure 1 - Autoradiography of non-histone-protein-associated ^{32}P-labelled phosphoproteins: Effect of $Co^{3+}(NH_3)_6$, spermine, and spermidine. Rat ventral prostate NHP-associated phosphoproteins were labelled with ^{32}P from [γ-^{32}P]ATP in the presence of 1.0 mM spermine or 1.0 mM $Co^{3+}(NH_3)_6$ or 2.0 mM spermidine. The proteins were separated by gel electrophoresis (SDS/4.5-21% acrylamide gradient gel; the direction of the gradient and migration were from top to bottom). Gel was subjected to autoradiography as described under Experimental Procedures. Lane 1, normal control; lane 2, +1.0 mM spermine; lane 3, + 2.0 mM spermidine; Lane 4, +1 mM $Co^{3+}(NH_3)_6$.

responsible for their capacity to augment the cAMP-independent protein kinase reactions examined, since $Co^{3+}(NH_3)_6$ produced remarkably similar effects. The fact that the actions of $Co^{3+}(NH_3)_6$ on the protein kinase reactions examined are entirely similar to those of polyamines tends to discount the possibility, at least in rat prostatic and liver nuclear preparations employed by us, of the presence of specific polyamine-dependent protein kinase(s). Taken together, our findings are consistent with the view (2) that other types of stimulatory cations influence the phosphorylation of certain proteins more by affecting the conformations of the protein substrates than via interactions with the kinase enzyme proteins. Of potential relevance are reports (7,21) that phosphorylation of casein by a nuclear cyclic nucleotide-independent protein kinase from Morris hepatoma 3924A, named NII kinase, was markedly enhanced by spermine in concentrations of a few millimolar. The enzyme phosphorylated both serine and threonine residues in casein, and spermine preferentially stimulated transphosphorylation from ATP to threonine residues. A similar highly purified enzyme preparation from rat liver (Goueli and Ahmed, unpublished) tested with DPV as substrate was also stimulated equally by spermine or $Co^{3+}(NH_3)_6$ (results not shown). It would be interesting to determine, under the conditions of the experiments reported here, whether $Co^{3+}(NH_3)_6$ and polyamines differentially influence the phosphorylation of serine and threonine, and even perhaps of tyrosine (22) residues in phosvitin and other substrates utilized by the protein kinase preparations we have examined.

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