Effects of Polyamines on Prostatic Chromatin- and Non-Histone-Protein-Associated Protein Kinase Reactions

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(Received 22 May 1978)

Studies are presented on the influence of polyamines on prostatic chromatin- and non-histone-protein-associated protein kinase reactions involving both exogenous and endogenous substrates. The activities toward the model acidic protein substrate, dephosphophosvitin, were maximal at 160–200 mM-NaCl (or -KCl or -NH₄Cl). Under these conditions, spermidine and spermine added in concentrations up to 2 mM were essentially without effect. However, without addition of NaCl to the medium, marked stimulation of these reactions was elicited by these polyamines at 1–2 mM concentrations. The stimulatory effects were not due to non-specific changes in the ionic strength or to substitution of spermine for Mg²⁺, as maximal stimulation by 1 mM-spermine was observed only at optimal (2–4 mM) Mg²⁺ concentrations. Qualitatively similar effects of polyamines were observed with enzyme preparations from the prostates of castrated rats, and with chromatin and non-histone-protein preparations from other tissues besides ventral prostate. When phosphorylation of endogenous non-histone proteins of the chromatin was measured, spermine stimulated both the initial rates and the final extent of transphosphorylation, even in the presence of optimal concentration of NaCl. By contrast, spermine or spermidine had no effect on the chromatin- and non-histone-protein-associated protein kinase reactions determined with lysine-rich histones as substrates. Chemically *NN*-dimethylated dephosphophosvitin was a less active substrate for the chromatin-associated protein kinase, but its phosphorylation was more markedly stimulated by spermine in comparison with unmodified dephosphophosvitin. These observations hint that the polyamine stimulations of the various protein kinase reactions may be due to effects on the conformations of the non-histone protein substrates rather than on the kinases themselves.

Much recent work has suggested that chromosomal proteins may play a regulatory role in the control of transcription. There is considerable evidence that non-histone proteins and their phosphorylated derivatives may serve as positive regulatory agents (for reviews see, e.g., Busch *et al.*, 1963; Stellwagen & Cole, 1969; MacGillivray *et al.*, 1972; Allfrey, 1974; Baserga, 1974; Kleinsmith, 1974; Ahmed, 1975). It has also been documented that phosphorylation of chromosomal proteins (particularly the non-histone proteins) is mediated by protein kinases endogenous to the nucleus (Ahmed & Ishida, 1971; Takeda *et al.*, 1971; Teng *et al.*, 1971; Ruddon &

Anderson, 1972; Ishida & Ahmed, 1974; Kish & Kleinsmith, 1974; Keller *et al.*, 1975). That the control of chromosomal non-histone-protein phosphorylations may be germane to androgen action on the prostate is suggested by a number of observations. Nuclear-associated protein kinase reactions of rat ventral prostate show varying androgen sensitivity. In particular, it appears that nucleolar and chromatin-associated protein kinase reactions involving acidic protein substrates (rather than histones) are greatly influenced by the androgenic status of the animals (Ahmed & Ishida, 1971; Ahmed & Wilson, 1975; Wilson & Ahmed, 1975, 1977). By contrast, the prostatic cytosol cyclic AMP-dependent protein kinase is relatively insensitive to androgen administration or withdrawal (Reddi *et al.*, 1971; Fuller *et al.*, 1978). Further, it appears that non-histone proteins in prostate chromatin may be involved as acceptors in

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nuclear binding of complexes between 5 α -dihydrotestosterone and specific androgen-receptor proteins (Mainwaring & Peterken, 1971; Tymoczko & Liao, 1971; Mainwaring *et al.*, 1976; Nyberg & Wang, 1976), and also that phosphorylation of chromatin non-histone proteins may stimulate such acceptor functions (Klyzsejko-Stefanowicz *et al.*, 1976).

The aliphatic polyamines spermine and spermidine and the diamine putrescine are present in substantial concentrations in all nucleated animal cells. Although the precise function of polyamines remains unclear, they appear to be involved in growth-related activities of cells. A rapid increase in their concentrations and their biosynthetic decarboxylases accompanies the induced growth of many tissues; these changes usually precede or are concomitant with increases in RNA, DNA and protein contents in the cell (for reviews see, e.g., Williams-Ashman *et al.*, 1969, 1976; Stevens, 1970; Cohen, 1971; Bachrach, 1973; Raina & Jänne, 1975; Tabor & Tabor, 1976).

Imai *et al.* (1975) have reported that spermine and spermidine stimulate the incorporation of ^{32}P *in vitro* into a non-histone-protein fraction derived from pig liver nuclei. In view of the androgen dependence of prostatic nuclear protein kinase reactions, and considering that adult-rat ventral prostate and its secretions are strikingly rich in spermidine and spermine, and that polyamine synthesis in this organ is strictly controlled by androgenic hormones (Williams-Ashman *et al.*, 1969; Pegg *et al.*, 1970), it was decided to examine the effects of polyamines on various prostatic chromatin-associated protein kinases. It is noteworthy that the prostatic cytosol cyclic AMP-dependent protein kinase reactions are uninfluenced by polyamines *in vitro* (Reddi *et al.*, 1971).

In the following studies on the effects of polyamines on prostatic nuclear protein kinase reactions with a variety of substrates, we found that these aliphatic amines stimulate chromatin- and non-histone-protein-associated protein kinase reactions that utilize exogenous acidic proteins such as dephosphophosvitin, and also endogenous non-histone proteins of chromatin, but not with histones as substrates. Chemical methylation of dephosphophosvitin alters its activity as substrate as well as its response to polyamines. We suggest that the effect of polyamines in these reactions is mediated, at least in part, by their influencing the conformation of the phosphoprotein acceptor. A preliminary account of this work has been presented (Ahmed *et al.*, 1976).

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 295–325 g (from ARS/Sprague-Dawley Co., Madison, WI,

U.S.A.) were used as the source of ventral prostate (and other tissues when needed). They were given standard laboratory diet and water *ad libitum* while being maintained on a constant photoperiod of 12.5 h of light and 11.5 h of darkness. Castration was carried out via the scrotal route under light ether anaesthesia.

Chemicals

Polyamines (spermine and spermidine hydrochlorides) and other aliphatic amines were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. They were adjusted to pH 7.4 before use by adding an appropriate amount of Tris base. Lysine-rich histone was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Highly polymerized yeast RNA was obtained from Schwartz-Mann, East Orange, NJ, U.S.A. Purified tRNA and (16S+23S) rRNA from *Escherichia coli* were from Miles Laboratories, Elkhart, IN, U.S.A. Poly(X), poly(I), poly(G) and poly(A) (as potassium or sodium salts) were obtained from Sigma. All other reagents used were of the highest purity available.

Preparation of chromatin and non-histone-protein fractions

The details of preparation and properties of purified rat ventral-prostate nuclei and water-soluble chromatin material derived therefrom have been given earlier (Ahmed, 1971; Ahmed & Wilson, 1975). The water-soluble chromatin was used further as the source of non-histone-protein fraction, which was obtained by the procedure described by Kish & Kleinsmith (1974) with certain modifications. In brief, to the chromatin solution at 4°C was added an equal volume of 0.02 M-Tris/HCl (pH 8.1 at 4°C)/2.0 M-NaCl, with gentle stirring. This was then vigorously sheared by using a Tissuemizer (Brinkman and Co., Westbury, NY, U.S.A.). With dial settings of 8, two 30 s mixing periods were used, with a short cooling period on ice in between. All these operations were carried out at 4°C. To the mixture, 3 vol. of 0.02 M-Tris/HCl, pH 8.1 at 4°C, was added slowly with stirring, to give a final concentration of 0.4 M-NaCl. After centrifugation at 80000g for 60 min, the supernatant was carefully removed and the residue (nucleohistone) was discarded. To the clear supernatant phase was added 0.5 ml (100 mg) of a suspension of Bio-Rex 70 (Bio-Rad Co., Richmond, CA, U.S.A.)/30 ml portion of the supernatant. The mixture was left for 10 min on ice with occasional mixing and was then centrifuged for 10 min at 10000g. The supernatant phase was carefully pipetted off and dialysed against 40 vol. of 0.05 M-Tris/HCl, pH 8.1 at 4°C, containing 0.025 M-NaCl and 1 mM-2-mercaptoethanol. The diffusate was then concentrated severalfold overnight with the aid of a Millipore molecular separator, and was stored in small batches

at -20°C , at which temperature the protein kinase activities were stable for fairly long periods.

Preparation of dephosphophosvitin

Partially (30–45%) dephosphorylated phosvitin was used as the model acidic-protein substrate for the chromatin- or non-histone-protein-associated protein kinase assays. The details of its preparation and use as substrate have been given previously (Ahmed *et al.*, 1975; Ahmed & Wilson, 1975).

Chemically *N,N*-dimethylated dephosphophosvitin was prepared by adapting the procedure described by Lin *et al.* (1969). To a solution of dephosphophosvitin at 0°C (1.5 g dissolved in 150 ml of 0.1 M-sodium borate buffer, pH 9.0) were added 300 mg of NaBH_4 and a few drops of octan-2-ol with rapid stirring. This was followed by the addition of 3 ml of 37% formaldehyde (in 0.1 ml increments) over a period of 30 min with continuous mixing. After a few additional minutes, the solution was adjusted to pH 7.0 by the addition of 50% (v/v) acetic acid, and was then dialysed overnight against several changes of water. The diffusate was freeze-dried and stored at -20°C dissolved in a small volume of water. An analysis of free amino groups in the methylated dephosphophosvitin by the procedure of Habeeb (1966) indicated that more than 90% of the free amino groups in dephosphophosvitin were methylated.

Protein kinase assays

The procedure for measuring chromatin-associated protein kinase activity toward dephosphophosvitin or lysine-rich histone has been given in detail previously (Ahmed & Wilson, 1975; Wilson & Ahmed, 1977). The standard reaction medium for measuring chromatin-associated protein kinase activity toward dephosphophosvitin consisted of 200 mM-NaCl, 5 mM-MgCl₂, 1 mM-dithiothreitol, 3 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 3000 d.p.m./nmol), 30 mM-Tris/HCl, pH 7.4 at 37°C , and saturating concentrations of dephosphophosvitin, determined for each batch of dephosphophosvitin as described by Ahmed *et al.* (1975). The reaction was initiated by adding an amount of chromatin containing 5–20 μg of DNA and was generally carried out for 30 min at 37°C . The same procedure was used when methylated dephosphophosvitin was the substrate.

For measuring chromatin-associated protein kinase activity toward lysine-rich histone, the standard reaction medium consisted of lysine-rich histone (3 mg/ml), 6 mM-MgCl₂, 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 60000 d.p.m./nmol), 1 mM-dithiothreitol and 30 mM-Tris/HCl, pH 7.45 at 37°C .

The standard reaction medium for measuring non-histone-protein-associated protein kinase activity toward dephosphophosvitin or methylated dephosphophosvitin consisted of 160 mM-NaCl, 4 mM-MgCl₂, 1 mM-dithiothreitol, 3 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp.

radioactivity 3000 d.p.m./nmol), 30 mM-Tris/HCl, pH 7.45 at 37°C , albumin (100 μg /ml) and saturating amounts of dephosphophosvitin or methylated dephosphophosvitin. The reaction was started by adding non-histone-protein fraction (2–10 μg of protein) in 0.25 M-NaCl, and this contributed about 5–10 mM-NaCl in the final reaction medium.

For measuring non-histone-protein-associated protein kinase activity toward lysine-rich histone, the reaction medium consisted of lysine-rich histone (3 mg/ml), 4 mM-MgCl₂, 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 60000 d.p.m./nmol), 1 mM-dithiothreitol, 30 mM-Tris/HCl, pH 7.45 at 37°C , and albumin (100 μg /ml). The procedures for assaying the ^{32}P radioactivity incorporated in each of the substrates used have been detailed previously (Ishida & Ahmed, 1974; Ahmed & Wilson, 1975; Ahmed *et al.*, 1975; Wilson & Ahmed, 1975, 1977). Suitable zero-time and blank controls (in the absence of added substrates) were routinely included in each protocol.

To establish that chromatin-associated protein kinase activity toward lysine-rich histone was indeed due to incorporation of ^{32}P into lysine-rich histone and not to contamination from radioactive non-histone proteins, the following experiment was carried out. The above described protein kinase reactions toward lysine-rich histone were terminated by the addition of 1 ml of a mixture of 9.0 M-urea and 0.6 M-guanidine hydrochloride, in 0.15 M-potassium phosphate buffer, pH 7.0. This mixture was then passed through a column containing Bio-Rex 70 (2 ml; 1 g) previously equilibrated with a medium consisting of 6 M-urea and 0.4 M-guanidine hydrochloride, in 0.1 M-potassium phosphate buffer, pH 7.0. This amount of resin is enough to exchange 10 mg of protein (van den Broek *et al.*, 1973). The column was washed out with 6 M-urea/0.4 M-guanidine hydrochloride/0.1 M-phosphate buffer, pH 7.0, until the A_{280} of the effluent decreased to the baseline determined in the presence of the wash medium alone. The column was then eluted with 5 ml of 6 M-urea/4 M-guanidine hydrochloride/0.1 M-potassium phosphate buffer, pH 7.0. This procedure quantitatively removed the histone fraction bound to the column. The eluate was then dialysed overnight against two changes of water (2 litres each time). To the diffusate were added 5 mg of phosvitin (as a carrier protein) and trichloroacetic acid to a final concentration of 25%. The mixture was left for 60 min on ice, and the precipitate was removed by centrifugation at 10000 g for 15 min. It was then washed with 5 ml each of ethanol, acetone and ether. The final precipitate was solubilized and its radioactivity measured as described earlier (Wilson & Ahmed, 1975, 1977). Control experiments utilizing non-histone-protein fraction (which is devoid of histones) in the absence and presence of exogenous lysine-rich histone demonstrated that in the absence of the exogenous histone substrate no detectable

radioactivity was bound to the Bio-Rex column, indicating that non-histone proteins did not contaminate the histone fraction isolated by the above procedure.

When the phosphorylation of endogenous chromatin proteins was measured, the reaction mixtures contained 30mM-Tris/HCl, pH 7.6 at 37°C, 5mM-MgCl₂, 1mM-dithiothreitol, 0.1mM-[γ -³²P]ATP (sp. radioactivity 30000 d.p.m./nmol), approx. 50 μ g of prostatic chromatin protein, and NaCl as indicated in the protocols. The radioactivity incorporated into non-histone proteins was determined as described previously (Ahmed & Ishida, 1971; Ishida & Ahmed, 1973). Under these conditions, the amount of radioactivity in the endogenous histone fractions of chromatin was less than 10% of that incorporated in the non-histone fraction.

Other procedures

All pH values refer to final reaction pH at the given temperature, and were measured with pH meter 26 (Radiometer, Copenhagen, Denmark) by using a glass and Ag/AgCl combination electrode (A. H. Thomas Co., Philadelphia, PA, U.S.A.) standardized

at the appropriate temperature. The procedure for preparing [γ -³²P]ATP was the same as given previously (Ahmed & Wilson, 1975). Protein was assayed by the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard. DNA was analysed by the method of Burton with calf thymus DNA as standard (Burton, 1956).

All protein phosphorylations were measured under conditions where the reaction rates were linear with respect to time at 37°C.

Results

Characteristics of prostatic non-histone-protein-associated protein kinase activity

In previous work (Ahmed & Wilson, 1975) we established that the optimal experimental conditions for assaying prostatic chromatin-associated protein kinase activity with the model acidic protein substrate dephosphophosvitin included 200mM-NaCl, 5mM-MgCl₂, 3mM-ATP and 1mM-dithiothreitol. Similar studies were undertaken by using non-histone-protein fraction derived from purified chromatin that, with dephosphophosvitin as the

Fig. 1. General characteristics of non-histone-protein-associated protein kinase toward dephosphophosvitin as substrate. (a) Effect of NaCl concentration on non-histone-protein-associated protein kinase activity toward dephosphophosvitin as substrate. All experimental details were the same as described in the Materials and Methods section except that the NaCl concentration was varied as shown. Final reaction volume was 0.5 ml. (b) Effect of Mg²⁺ concentration on non-histone-protein-associated protein kinase activity toward dephosphophosvitin as substrate. Optimal experimental conditions were used except for Mg²⁺ concentration, which was altered as shown. (c) Time course of the non-histone-protein-associated protein kinase reaction toward dephosphophosvitin as substrate. Symbols: ●, + bovine serum albumin (100 μ g/ml); ○, control with no albumin.

substrate, gave maximal rates of enzyme activity at 160mM-NaCl (Fig. 1a), and 4mM-Mg²⁺ in the presence of 3mM-ATP (Fig. 1b). The reaction was not linear with time beyond 10–15min; however, when bovine serum albumin (final concentration 100µg/ml) was added, linear rates of reaction were obtained over 60min (Fig. 1c).

The effects of univalent salts other than NaCl on the rates of reaction were also investigated. At 160mM both NH₄Cl and KCl were as effective as NaCl, whereas in the presence of LiCl the rate was decreased by some 50%.

Effect of spermine and spermidine on chromatin- and non-histone-protein-associated protein kinase reactions toward dephosphophosvitin substrate

When various concentrations of spermine or spermidine were added to the protein kinase assay medium with chromatin as the source of enzyme activity, it was observed that under optimal cationic conditions (200mM-NaCl) there was essentially no effect of spermine at 0.4–2.0mM or of spermidine at 1.0–3.0mM on the reaction (result shown in Table 1 only for 1mM-spermine or -spermidine). However, when the NaCl concentration was 10mM or less, very significant stimulation of the protein kinase activity was observed. Thus a stimulation of 39, 65 and 133% respectively was observed in the presence of 0.4, 1.0 and 2mM-spermine, whereas it was 48, 62 and 95% respectively in the presence of 1.0, 2.0 and 3.0mM-spermidine (results for 0.4mM-spermine and 3mM-spermidine not shown in Table 1). Since these polyamines are cationic compounds, their stimulatory effects might simply reflect non-specific alterations of the ionic strength of the incubation media. Therefore, respective controls containing equivalent amounts of NaCl to compensate for the increase in ionic strength due to addition of spermine or spermidine were included in the reaction mixtures. Thus, at 8mM- and 4.5mM-NaCl, the increase in protein kinase activity (compared with that with no added NaCl) was only 15 and 8% respectively. At 16mM-NaCl, providing an equivalent increment in total ionic strength to that given by 2mM-spermine at pH 7.4 and 37°C (Hirschman *et al.*, 1967), the stimulation of protein kinase activity was only 31%, compared with 133% in the presence of 2mM-spermine with no added NaCl. Thus the remarkable stimulatory effects of polyamines on chromatin-associated protein kinase reactions cannot be attributed to simple cation replacement. It is also noteworthy that the rate of the enzyme reaction in the presence of 1mM-spermine is 65% of that observed under optimal cationic conditions (i.e. 200mM-NaCl), whereas it was essentially maximal in the presence of 2mM-spermine (94% of the value in the presence of 200mM-NaCl).

The effects of spermine on protein kinase activity

associated with the non-histone-protein fraction derived from purified prostatic chromatin were also examined (Table 1). Spermine at 1mM stimulated the non-histone-protein-associated protein kinase (under suboptimal ionic conditions) by about 70% compared with the corresponding NaCl-compensated control. Again, it is noteworthy that in the presence of 1mM-spermine the rate of enzyme activity was almost 90% of that obtained under optimal ionic conditions (160mM-NaCl), a result similar to that observed with chromatin as the source of enzyme activity. Table 1 also illustrates the effects of spermidine on non-histone-protein-associated protein kinase toward dephosphophosvitin as substrate; these were comparable with those obtained with spermine.

Effect of spermine on chromatin- and non-histone-protein-associated protein kinase toward lysine-rich histone substrate

Purified rat ventral-prostate chromatin exhibits low but definite protein kinase activity toward the basic lysine-rich histone as a substrate (Wilson & Ahmed, 1977). It was therefore decided to examine the effect of spermine on chromatin-associated and non-histone-protein-associated protein kinase activities toward this substrate. Table 1 shows that, compared with the corresponding NaCl-containing controls, spermine has no effect on the protein kinase activity toward lysine-rich histone as substrate. Thus it appears that, under the experimental conditions described above, polyamines stimulate the chromatin- and non-histone-protein-associated protein kinase reactions in the presence of exogenous acidic phospho-protein substrate such as dephosphophosvitin, but not the basic histone substrate.

Effect of varying the Mg²⁺ concentration on spermine stimulation of non-histone-protein-associated protein kinase

Polyamines have been shown to substitute for Mg²⁺ in certain enzyme transphosphorylations (Fuchs, 1976; Tabor & Tabor, 1976). This was not observed in the present studies; rather, we found that there was a requirement for optimal Mg²⁺ to achieve maximal stimulatory effect of protein kinase activity by 1 or 2mM-spermine (Table 2).

Effect of spermine on non-histone-protein-associated protein kinase of other tissues

As shown in Table 3, 1mM-spermine also stimulated the rat liver non-histone-protein-associated protein kinase toward dephosphophosvitin by 45%, whereas the equivalent control value in the presence of 8mM-NaCl was only 7% greater than the control value without salt. The rate of protein kinase activity in the presence of 1mM-spermine was fairly close to the optimal rate achieved in the presence of 160mM-NaCl. Similar results were obtained when chromatin

Table 1. *Effects of spermine, spermidine and NaCl on prostatic chromatin- and non-histone-protein-associated protein kinase reactions toward dephosphophosvitin and lysine-rich histone as substrates*

The reaction was initiated by the addition of chromatin (equivalent to 10 μ g of DNA) or non-histone protein (5 μ g of protein), and continued for 10 min at 37°C. In the latter case, some 8 mM-NaCl was contributed by the enzyme solution added to the reaction. Tubes containing 4.5–16 mM-NaCl provided control ionic-strength values corresponding to various concentrations of polyamines: 160 mM-, 200 mM- and 80 mM-NaCl for optimal salt requirement for non-histone-protein-associated kinase toward dephosphophosvitin, chromatin-associated kinase toward dephosphophosvitin and chromatin- or non-histone-protein-associated kinase toward lysine-rich histone respectively. All other experimental details are given in the Materials and Methods section.

[NaCl] (mM)	[Polyamine](mM)		Protein kinase activity associated with:			
			Chromatin (nmol of 32 P/h per mg of DNA)		Non-histone proteins (nmol of 32 P/h per mg of protein)	
	Spermine	Spermidine	Dephosphophosvitin substrate	Lysine-rich histone substrate	Dephosphophosvitin substrate	Lysine-rich histone substrate
—	—	—	130	5.0	—	—
4.5	—	—	140	—	—	—
8.0	—	—	150	6.2	407	7.6
16.0	—	—	170	—	476	8.3
80	—	—	—	6.9	—	—
160	—	—	—	—	827	—
200	—	—	321	—	—	—
—	1.0	—	215	6.0	810	7.8
—	2.0	—	303	6.0	855	8.1
—	—	1.0	193	—	642	—
—	—	2.0	211	—	763	—
160	1.0	—	—	—	873	—
160	—	1.0	—	—	887	—
200	1.0	—	324	—	—	—
200	—	1.0	300	—	—	—

Table 2. *Effect of MgCl₂ concentration on spermine stimulation of non-histone-protein-associated protein phosphokinase toward dephosphophosvitin as substrate*

MgCl₂ concentration was varied as shown. The reaction mixture contained only about 8 mM-NaCl, which was contributed by the enzyme solution. In the absence of added MgCl₂ (and in the presence of 20 mM-EDTA), the protein kinase activity was less than 2% with or without 2 mM-spermine compared with values at optimal [MgCl₂]. All other experimental details were the same as for Table 1.

[MgCl ₂] (mM)	Protein kinase activity (nmol of 32 P/h per mg of protein)	
	No spermine	1 mM-Spermine
1.0	151	211
2.0	293	484
4.0	285	453
6.0	292	320
10.0	186	226

Table 3. *Effect of spermine on rat liver non-histone-protein-associated protein kinase activity toward dephosphophosvitin as substrate*

The reaction was initiated by adding non-histone protein (3 μ g of protein), which contributed about 4 mM-NaCl in the reaction mixture. Other additions were as shown. The tube containing 8 mM-NaCl provided the equivalent control for that with 1 mM-spermine. For comparison, a tube containing optimal NaCl (160 mM) was also included.

Additions	Protein kinase activity (nmol of 32 P/h per mg of protein)
—	443
8 mM-NaCl	474
160 mM-NaCl	691
0.01 mM-Spermine	447
0.10 mM-Spermine	558
1.0 mM-Spermine	639

Effect of castration on the spermine stimulation of the prostatic chromatin-associated protein kinase activity

The rat ventral-prostate chromatin-associated protein kinase activity toward dephosphophosvitin declines rapidly as a result of castration (Ahmed & Wilson, 1975). It was therefore decided to examine

preparations from rat submandibular gland and from human benign prostatic hyperplasia tissue were examined (K. Ahmed & M. J. Wilson, unpublished work).

Table 4. *Effect of concentrations of NaCl and spermine on chromatin-associated protein kinase activity towards dephosphovitin with chromatin from prostates of 96 h-castrated rats*

Chromatin was prepared from pooled prostates of 24 adult rats castrated 96 h previously. The reaction was started by adding chromatin (equivalent to 20 μ g of DNA) and carried out for 30 min at 37°C. NaCl concentrations as shown (2–16 mM) provided the equivalent of ionic strength for the corresponding spermine-containing (0.25–2 mM) tubes. For comparison, the tube containing 200 mM-NaCl gives the maximal rate of reaction.

[NaCl] (mM)	[Spermine] (mM)	Protein kinase activity (nmol of 32 P/h per mg of DNA)	Increase (%)
—	—	33.9	—
2	—	34.2	1
4	—	35.0	3
8	—	37.3	10
16	—	41.6	23
—	0.25	43.1	27
—	0.50	44.7	32
—	1.0	49.2	45
—	2.0	54.6	61
200	—	83.0	144

Table 5. *Effects of various amines on prostatic non-histone-protein-associated protein kinase activity towards dephosphovitin*

Various amines were added at 1 mM as shown. The relative activity is based on comparison with the corresponding NaCl-containing controls (100%). All other details are as given for Table 3.

Amine	Relative activity (%)
1 mM-Putrescine	111
1 mM-Hexane-1,6-diamine	110
1 mM-Methylglyoxal bis(guanylhydrazone)	110
1 mM-Spermine	140

whether the loss of prostatic chromatin-associated protein kinase activity in castrated rats could be reversed *in vitro* by the addition of spermine. Table 4 shows that significant stimulation was caused by spermine (0.25–2.0 mM) on protein kinase activity of chromatin from prostates of 96 h-castrated rats. However, the effects were somewhat lower than that observed when chromatin from rats of normal androgenic status was used. This relative loss of sensitivity to spermine in the prostatic chromatin preparations from castrated animals may possibly reflect altered association of protein kinases with chromatin constituents, or may be due to loss of certain spermine-sensitive protein kinase(s) after castration.

Comparison of the effects of various aliphatic amines on non-histone-protein-associated protein kinase

The results in Table 5 show that aliphatic diamines such as putrescine (butane-1,4-diamine) and hexane-1,6-diamine are considerably less active than spermine in stimulating non-histone-protein-associated protein kinase activity. The even more highly ionized poly-

Fig. 2. *Effect of spermine on the time course of incorporation of 32 P into chromatin-associated phosphoproteins*

The reaction mixture in a final volume of 0.5 ml consisted of NaCl as shown, 30 mM-Tris/HCl (pH 7.45 at 37°C), 5 mM-MgCl₂, 1 mM-dithiothreitol, 0.1 mM-[γ - 32 P]ATP (sp. radioactivity 60000 d.p.m./nmol) and an amount of chromatin equivalent to 50 μ g of protein. The reaction temperature was 37°C and time as shown. Symbols: ○, no NaCl added; □, +8 mM-NaCl; △, +30 mM-NaCl; ●, no NaCl, but 1 mM-spermine added. Incorporation of 32 P into the phosphoproteins was measured as described in the Materials and Methods section.

amine analogue methylglyoxal bisguanylhydrazone {1,1' - [(methylethanediyldine)dinitrilo]diguanidine}, which is also a specific inhibitor of putrescine-

activated *S*-adenosylmethionine decarboxylase (Williams-Ashman & Schenone, 1972), did not stimulate the protein kinase reactions.

Effect of spermine on the time course of phosphorylation of chromosomal proteins

The above data relate primarily to the effects of polyamines on the initial rate of protein kinase reactions with exogenous substrates. Therefore experiments were undertaken to examine if these amines had a similar effect on the phosphorylation of endogenous chromosomal proteins. Fig. 2 clearly shows that spermine markedly increased not only the initial rate, but also the extent of phosphorylation, of these proteins. This stimulatory effect of spermine was even more marked than that of the optimal concentrations of NaCl in the reaction. For example, 1 mM-spermine stimulated the initial rate of phosphorylation of chromosomal phosphoproteins by at least 60% compared with the control values obtained in the presence of 0, 8 or 30 mM-NaCl. This stimulation was, however, apparent even when the transphosphorylation had plateaued in the controls (at 30 min), so that in the presence of 1 mM-spermine it was 48% greater than that with 0, 8 or 30 mM-NaCl-containing controls. Furthermore, 1 mM-spermine stimulated the phosphorylation of endogenous

chromosomal non-histone proteins even in the presence of optimal concentrations of NaCl (Table 6). This contrasts with the above studies with dephosphophosvitin as substrate, in which the stimulation by polyamines was observed only at suboptimal salt concentrations.

Interaction of polynucleotides with non-histone-protein-associated protein kinase toward dephosphophosvitin

The effects of highly polymerized yeast RNA, purified tRNA and rRNA from *E. coli* (at concentrations of 20 µg/ml) were investigated on non-histone-protein-associated protein kinase toward dephosphophosvitin. Under the present experimental conditions and at concentrations of these nucleic acids 2–4 times that of non-histone-protein, there was no effect on the kinase reaction (results not shown). It is possible that the substrate (dephosphophosvitin) caused a dissociation of the non-histone-protein–nucleic acid interaction under these conditions *in vitro*. Mäenpää (1977) observed an inhibition of a cytosol (cyclic AMP-independent) phosphitin kinase by RNA, which he interpreted in terms of a possible competition between RNA and phosphitin.

Among other nucleotides tested, poly(A) was without effect, poly(G) and poly(I) were somewhat

Table 6. *Effect of spermine on the rate of incorporation of ^{32}P into rat ventral-prostate chromatin non-histone phosphoproteins in the presence or absence of NaCl*

The reaction contained chromatin (about 25 µg of DNA) and was carried out for 1 min at 37°C. All other details were as described in the Materials and Methods section.

[NaCl] (mM)	[Spermine] (1 mM)	^{32}P incorporated (nmol of ^{32}P /h per mg of protein)	Stimulation (%)
—	—	13.3	—
12	—	14.1	6
24	—	14.3	8
—	+	21.3	60
24	+	20.2	52

Table 7. *Effect of spermine and NaCl on polynucleotide inhibition of non-histone-protein-associated protein kinase toward dephosphophosvitin*

Various additions were as shown. The reaction contained 4 µg of non-histone protein that contributed some 3 mM-NaCl in the reaction mixture. The reaction was started by adding non-histone protein to the system; however, in separate experiments non-histone protein was also preincubated with various polynucleotides to see if the inhibitory effect increased with time. This was not the case, and the order of addition of non-histone protein or polynucleotide gave the same result.

Polynucleotide (20 µg/ml)	Protein kinase activity (nmol of ^{32}P /h per mg of protein)		
	Control	With 1 mM-spermine	With 170 mM-NaCl
None	288	476	600
Poly(A)	285	—	—
Poly(G)	197	—	—
Poly(I)	203	—	—
Poly(X)	67	489	258

inhibitory, whereas poly(X) was a potent inhibitor of the non-histone-protein-associated phosphokinase reaction toward dephosphophosvitin (Table 7). Addition of 1 mM-spermine before or after treatment with poly(X) fully reversed the inhibitory effect (Table 7). On the other hand, only partial reversal of this inhibition was obtained at optimal concentrations of NaCl in the reaction. The result in Fig. 3 shows that with increasing concentrations of NaCl the inhibition by poly(X) decreased, although even at 330 mM-NaCl (which is itself inhibitory in the controls) complete reversal of inhibition was not achieved.

Fig. 3. Effect of NaCl concentration on poly(X) inhibition of non-histone-protein-associated protein kinase toward dephosphophosvitin
The amounts of poly(X) and non-histone protein present in the reaction mixture were 10 µg and 6.4 µg respectively. Final reaction volume was 0.5 ml. Symbols: ○, controls; ●, + poly(X) (20 µg/ml).

Effect of substrate modification on spermine stimulation

The above results suggest that polyamine effects on chromatin- and non-histone-protein-associated protein kinase reactions were most probably mediated via interactions with the substrate rather than the enzyme itself. It is known that *NN*-dimethylation of protein substrates alters their activity toward proteolytic enzymes (Lin *et al.*, 1969). By analogy, we considered that alteration in the structure of dephosphophosvitin, e.g., by *NN*-dimethylation, should modify spermine action on the protein kinase reaction if the polyamine effect was primarily mediated through conformational changes of the acceptor phospho-protein. The results in Table 8 show that *NN*-dimethylation of dephosphophosvitin resulted in a significant loss of activity as a substrate. This was not due to change in the apparent *K_m* for methylated dephosphophosvitin compared with the original dephosphophosvitin. The data in Table 8 clearly show that the *NN*-dimethylation of dephosphophosvitin resulted in a greater degree of stimulation by spermine.

Discussion

The above results clearly demonstrate that, at concentrations in the millimolar range, spermine and spermidine markedly stimulated the phosphorylation by ATP of exogenous partially dephosphorylated phosvitin catalysed by prostatic chromatin preparations or by non-histone proteins obtained therefrom. With both types of prostatic nuclear protein kinase preparations, whose activities are not enhanced by cyclic AMP (Ahmed & Wilson, 1975), stimulatory effects of polyamines on dephosphophosvitin phosphorylation were observed only at suboptimal concentrations (5–10 mM-NaCl or lower) of univalent salt. However, the enhancement of dephosphophosvitin phosphorylation catalysed by both types

Table 8. Effect of methylation of dephosphophosvitin substrate with respect to spermine stimulation of chromatin-associated protein kinase reaction

All substrates were present in the reaction at saturating concentrations. Methylation of dephosphophosvitin did not result in a change in the apparent *K_m*. Analysis of free nitrogen in methylated dephosphophosvitin showed that over 90% of it was methylated. The values given in parentheses are percentage stimulations caused by various additions compared with corresponding control values. All other experimental details were as given in the Materials and Methods section.

Additions	Protein kinase activity (nmol of ³² P/h per mg of DNA)		Inhibition of protein kinase due to methylation of dephosphophosvitin (%)
	Dephosphophosvitin substrate	Methylated dephosphophosvitin substrate	
None	110 (0)	21 (0)	81
8.0 mM-NaCl	139 (26%)	28 (33%)	80
1.0 mM-Spermine	181 (65%)	57 (170%)	68
200 mM-NaCl	231 (110%)	46 (119%)	80

of nuclear protein kinase preparations by 1–2 mM-spermine or -spermidine was much greater than the small stimulations evoked by 16 mM-NaCl (or less) that provided an equivalent increment in total ionic strength to that given by 2 mM-spermine at pH 7.4 and 37°C (Hirschman *et al.*, 1967). Omission of Mg^{2+} from the control reaction mixtures diminished the rate of dephosphophosvitin phosphorylation by more than 98% and the Mg^{2+} requirement was not fulfilled by 1–2 mM-spermine or -spermidine. Neither can the stimulatory effects of polyamines on the phosphorylation of dephosphophosvitin be ascribed to inhibition of a nuclear-associated phosphatase; these reactions were found to be uninfluenced by polyamines (M. J. Wilson & K. Ahmed, unpublished work).

Spermine and spermidine also enhanced the transfer of phosphate from ATP to endogenous non-histone proteins associated with prostatic chromatin preparations. The stimulation was apparent with respect to both the initial rates of reaction and the extent of phosphorylation of chromatin proteins. Under these conditions, enhancement of this reaction by spermine was similar in the absence or presence of optimal concentrations (24 mM) of NaCl in the reaction, which contrasts with the studies with dephosphophosvitin as substrate, where the stimulation by polyamines was observed only at suboptimal salt concentrations.

Neither spermine nor spermidine at 1–2 mM caused any significant change in the much lower rates of phosphorylation of lysine-rich histone. Kuroda *et al.* (1977) suggested that histones did not act as a substrate for two purified nuclear protein kinases. However, by using chromatin and non-histone proteins derived therefrom, we observed some direct phosphorylation of lysine-rich histone (see also Wilson & Ahmed, 1977). Prostatic chromatin contains several protein kinases (K. Ahmed, S. A. Goueli & M. J. Wilson, unpublished work) and it is likely that only some of these enzymes are active toward lysine-rich histone as substrate. Kish & Kleinsmith (1974) also observed varying degrees of phosphorylation of histone by nuclear enzyme fractions separated with a phosphocellulose column. Thus it appears that polyamines stimulate phosphorylation of acidic-protein-type substrates, but not basic histone. It seems likely that the basic polyamines can enhance the phosphorylation of the former type of substrates more by affecting the conformational status of these acceptor proteins than by a direct activation of the pertinent protein kinase(s).

It also appears improbable that the stimulatory effects of polyamines on the chromatin-associated protein kinase reactions are due to any interaction of these molecules with nucleic acids in the chromatin preparations that might influence the enzyme activity. The stimulation of chromatin- and non-histone-protein-associated protein kinase reactions by 1 mM-

spermine is of the same order of magnitude, even though the amount of DNA in the latter fraction is only 5–10% of that in the original chromatin. Some of the non-histone-protein preparations used in the present work contained very low amounts of DNA, but still responded to polyamines, as did the other preparations that had higher nucleic acid content.

Comparable stimulatory effects of polyamines on various nuclear protein kinase reactions were also observed with chromatin preparations from other tissues besides rat ventral prostate, including rat liver and human benign hypertrophic prostate (results for the latter not shown). The actions of spermidine and spermine described in the present paper are similar to those reported by Imai *et al.* (1975) with respect to stimulation of phosphorylation to the endogenous proteins of pig liver chromatin. Murray *et al.* (1976) and Mäenpää (1977) have reported stimulation of cyclic AMP-independent cytosol kinases by polyamines with casein and phosphovitin as substrates. However, it is worth noting that spermine and spermidine were without influence on the soluble cyclic AMP-dependent phosphokinase(s) of soluble rat testis extracts that phosphorylate histones and related basic proteins, and could not replace the Mg^{2+} requirement for these reactions (Reddi *et al.*, 1971).

The physiological significance of the enhancement of various rat prostate nuclear protein kinase reactions by polyamines *in vitro* is problematic. The activity of rat ventral-prostate protein kinases with dephosphophosvitin as substrate falls dramatically within 24 h after castration, and declines to even lower activities at 72 h after androgen withdrawal under conditions where the reactions are completely restored by concurrent treatment of the castrated animals with testosterone (Ahmed & Wilson, 1975; Wilson & Ahmed, 1976, 1977). However, the decline of spermine and spermidine contents in this tissue after castration, and the restoration of polyamine concentrations by subsequent androgen treatment, are considerably more sluggish (Williams-Ashman *et al.*, 1969; Pegg *et al.*, 1970). This suggests that the effects of androgens *in vivo* on the prostatic nuclear protein phosphorylations may relate more to hormonal regulation of the synthesis and/or degradation of the appropriate nuclear protein kinases (or on the association of these enzymes with chromatin constituents) than to any prior androgen-induced changes in prostatic polyamine concentrations. The diminished phosphorylation of dephosphophosvitin by chromatin isolated from the prostates of rats that were castrated 96 h previously was somewhat less enhanced by spermine (0.25–2 mM) when the reactions were measured at suboptimal NaCl concentrations. Nevertheless, such interpretative conclusions are complicated by a lack of reliable data about the concentrations of polyamines in cell

nuclei or other intracellular compartments of the prostate gland (Williams-Ashman *et al.*, 1969, 1976; Tabor & Tabor, 1976).

Germane to the stimulation by spermine and spermidine of the aforementioned chromatin-associated non-cyclic AMP-dependent protein kinases are reports that polyamines can directly enhance certain nucleoside diphosphate kinase reactions (Nakai & Glinsmann, 1977) and choline kinase (Fukuyama & Yamashita, 1976), although in these instances it seems unlikely that the polyamines are acting via interaction with the appropriate low-molecular-weight substrates. By contrast, the aforementioned hypothesis that spermidine and spermine stimulate phosphorylation of dephosphophosvitin and endogenous non-histone proteins by chromatin-associated protein kinases by complexing with the acidic protein substrate is pertinent to evidence that polyamine enhancement of various nucleic acid polymerases and tRNA methyltransferases (see, e.g., Williams-Ashman *et al.*, 1969, 1976; Tabor & Tabor, 1976) may be due to the polyamines primarily affecting the conformational status of the polynucleotide templates or substrates for these enzymes. The stimulation by poly-L-lysine of the phosphorylation by ATP of chromatin acidic proteins (Kaplowitz *et al.*, 1971) may also be analogous to these considerations.

The investigations were supported in part by N.I.H. research grants CA 15062 and HD-04592. We thank Alan Davis, Thomas Fischbach, Mary Norvitch, Kathleen Matteson and Rolv Slungaard for their excellent technical assistance during the course of this work.

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