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Spermine-Enhanced Protein Phosphorylation in Human Placenta (41877)

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Abstract. Polyamines are known to have a role in cell proliferation, differentiation, and protein synthesis. During pregnancy, major changes in polyamine levels occur in maternal serum, amniotic fluid, and placental tissue. Polyamine-activated phosphorylation has recently been proposed as a mechanism by which polyamines may regulate metabolic processes in target tissues. Polyamine-activated protein phosphorylation has not been studied in placenta. Homogenate membrane and cytosol fractions from human placenta were subjected to an endogenous protein phosphorylation assay using [γ-^{32}P]ATP in the presence and absence of the polyamines, spermine and spermidine, and the diamine, putrescine. Protein phosphorylation was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. When compared to basal levels, spermine (10^{-3} *M*) significantly ($P < 0.001$) stimulated ^{32}P incorporation into phosphoproteins having molecular weights of 55,000 and 105,000. At this concentration spermidine and putrescine failed to stimulate phosphorylation. Half-maximal ^{32}P incorporation was observed with $3.7 \pm 1.25 \times 10^{-4}$ *M* spermine. Polylysine enhanced the phosphorylation of phosphoproteins of the same molecular weight as those enhanced by spermine. Heparin and high Mg^{2+} inhibited spermine-induced phosphorylation. cAMP and Ca^{2+} did not stimulate phosphorylation of the spermine-dependent phosphoproteins. Spermine, however, acted as an antagonist for cAMP-dependent phosphorylation of a M_r 45,000 phosphoprotein.

The biochemistry and physiology of the polyamines, spermine and spermidine, and their biosynthetic precursor putrescine have been extensively reviewed (1–4). Requirement for these aliphatic amines for growth and differentiation has been established in several systems, and data supporting their roles in DNA, RNA, and protein biosynthesis and/or regulation are extensive. In addition, numerous hormones increase polyamine biosynthesis in specific target tissues, suggesting that polyamines may in part function as mediators of hormone action.

Polyamine concentrations increase during human pregnancy in both plasma (5, 6) and urine (5) as well as fluctuate in placenta (7) and amniotic fluid (5) as a function of gestational age (8, 9). However, the specific targets of enhanced polyamine biosynthesis during pregnancy remain to be established. Since the placenta plays a central role in fetal growth and development, this tissue could be a primary target for the increased polyamine biosynthesis seen in pregnancy.

Recent studies indicate that polyamines stimulate the phosphorylation of specific proteins and could thereby act as regulators of intracellular metabolism (10, 11). Accordingly, we have examined the ability of polyamines to alter phosphorylation of placental proteins. In this study we describe for the first time that spermine selectively stimulates the phosphorylation of several placental proteins.

Materials and Methods. *Materials.* [γ-^{32}P]ATP (35 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). Spermine, spermidine, putrescine, polylysine, cAMP, and other reagents were purchased from Sigma Chemical Company (St. Louis, Mo.). All other reagents were obtained from Fisher Scientific Company.

Tissue preparation. Placentas from uncomplicated pregnancies were obtained at delivery and processed immediately. Cotyledons to be used were perfused free of blood with normal saline. Decidual tissue was removed by sharp dissection. The remaining tissue was then washed in iced buffer containing 0.25 *M* sucrose and 10 m*M* Tris–HCl (pH 7.2). Throughout the remainder of the preparation procedure the tissue was maintained at 4°C. Approximately 30 g of washed tissue was then homogenized with a Tekmar Tissuemizer (Tekmar Co., Cincinnati, Ohio) in 300 ml of

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the sucrose buffer (3 × 10 sec at high setting). The homogenate was poured through four layers of cheesecloth. Aliquots were then removed and used as the crude homogenate preparation. The remaining material was centrifuged at 5000*g* for 10 min. The pellet was discarded and aliquots of the supernatant served as the 5000*g* supernatant fraction. The rest of the supernatant was centrifuged at 100,000*g* for 60 min. The supernatant of this centrifugation was used as the placental cytosol preparation. The 100,000*g* pellet was washed twice by resuspension in equal amounts of the sucrose buffer and finally resuspended in sucrose buffer to give a protein concentration of 2–3 mg/ml. Placental fractions were assayed on the day of preparation or after freezing and storage at −70°C. No differences were seen in fresh and frozen preparations under these conditions. Protein concentrations were determined by the method of Lowry *et al.* (12).

Gel electrophoresis and autoradiography. Phosphorylated substrates were identified by incubation of placental fractions with [γ-^{32}P]ATP followed by gel electrophoresis and autoradiography. The reaction mixture (total volume 0.2 ml) consisted of the protein sample (5–10 μg), 5 μM [γ-^{32}P]ATP (5–20 cpm/pmole), 20 mM Tris–HCl (pH 7.2), 5 mM $MgCl_2$, 10 mM EGTA, and 10 mM NaF. Potential effector compounds were added as indicated. In some assays cAMP or $CaCl_2$ was added. The amount of $CaCl_2$ required to give specific free Ca^{2+} concentrations was calculated by a computer program according to the method of Sillin and Martell (13). Assays were initiated by addition of [γ-^{32}P]ATP and incubated for 6 min at 30°C in a water bath. The reaction was terminated by the addition of 100 μl of ice-cold 12% trichloroacetic acid (TCA). The protein was then precipitated by centrifugation in a Beckman B microfuge for 5 min and the pellet washed twice with 350 μl H_2O. The pellet was dissolved in a solution (60 μl) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue and boiled for 5 min. Samples were then loaded on an SDS–polyacrylamide gel (4.3% stacking, 10% running) for electrophoresis according to the method of Laemmli (14). The molecular weight standards used included myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,400), BSA (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000). The gels were fixed overnight in 50% methanol and 7.5% acetic acid, stained with Coomassie blue, and destained. Gels were dryed on Whatman Grade 3 filter paper, autoradiographed on Kodak XR film, and developed for the desired length of time (24 hr to 2 weeks) to demonstrate the phosphorylated proteins. The autoradiographs were then scanned at 550 nm with a Beckman Du-8 spectrophotometer with slab gel scanner attachment.

Where indicated, after destaining, the gels were cut into 1-mm slices, dissolved in 3% Protosol (New England Nuclear), and counted by standard scintillation technique.

Phosphate bond analysis. To demonstrate that phosphate was incorporated into proteins and to establish the type of phosphate bond formed, the following experiments were performed: Protein phosphorylation was performed exactly as described in the preceding paragraph except that two tubes each containing 50 μg of cytosol protein in total incubation volumes of 2 ml were used. One contained 10^{-3} M spermine and the other contained no spermine. After protein precipitation with a comparable amount of 12% TCA, the 2.0-ml samples were each divided into seven 200-μl aliquots (a–g) and treated as follows: (a) The first aliquot was centrifuged and the pellet extracted three times with acetone (350 μl). The resulting pellet was then placed in a 60-μl solution (Laemmli) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue and boiled for 5 min. (b) The second tube was treated identically as (a) except that the pellet was extracted three times with chloroform/methanol (2/1) immediately following the acetone step. (c) The third tube was treated like (a) except that following the initial centrifugation and prior to the acetone extraction, the pellet was resuspended in ice-cold 0.5 M NaOH for 5 min and then precipitated in 5% TCA (350 μl). (d) The fourth was exactly as (c) except that the NaOH solution was boiled for one of the 5 min. (e) The fifth was as in (a) except that following the 12% TCA addition, the solution was boiled for 1 min prior to centrifugation. (f, g) These were treated as in (a) except that following addition of the Laemmli solution they were exposed to Pro-

nase (0.01 mg) and ribonuclease A (0.01 mg), respectively, for 10 min at 37°C. All of the boiled samples were then loaded on gels and processed exactly as in the preceding paragraph.

Results. Spermine (10^{-3} *M*) activated protein phosphorylation of several endogenous proteins in human placental cytosol. Phosphoproteins of M_r 55,000 and 105,000 consistently demonstrated polyamine-dependent phosphorylation in ten different placental preparations (Fig. 1). Densitometer scans of autoradiographs of SDS–polyacrylamide gels of placental cytosol in the presence or absence of spermine (10^{-3} *M*) demonstrated significant differences ($P < 0.001$) in each of these polyamine-activated phosphoproteins. Densitometer readings were 0.504 ± 0.19 and 0.868 ± 0.22 (arbitrary unit) for the 105,000 and 55,000 M_r phosphoproteins, respectively, in the absence of spermine, and 1.459 ± 0.30 and 1.059 ± 0.31 in the presence of 10^{-3} *M* spermine. Gels which were sliced into 1-mm sections also demonstrated spermine-induced incorporation of ^{32}P into proteins of the same molecular weight (Table I). In many preparations phosphoproteins of M_r 205,000, 150,000, 116,000 99,000, 85,000, 64,000, 52,000, and 46,000 also appeared to incorporate additional ^{32}P in the presence of spermine (10^{-3} *M*). However, augmented phosphorylation of these proteins was not as pronounced and/or consistent as that observed for the M_r 55,000 and 105,000 proteins. In membrane containing crude placental homogenate and 5000*g* supernatant fractions spermine (10^{-3} *M*) increased phosphorylation of the proteins of the same molecular weight as those seen in placental cytosol. Phosphorylation in these fractions was not as pronounced nor were additional spermine-induced phosphoproteins detected in the membrane containing fractions (data not shown).

The nature of the phosphate bond was studied by exposure of the phosphorylated cy-

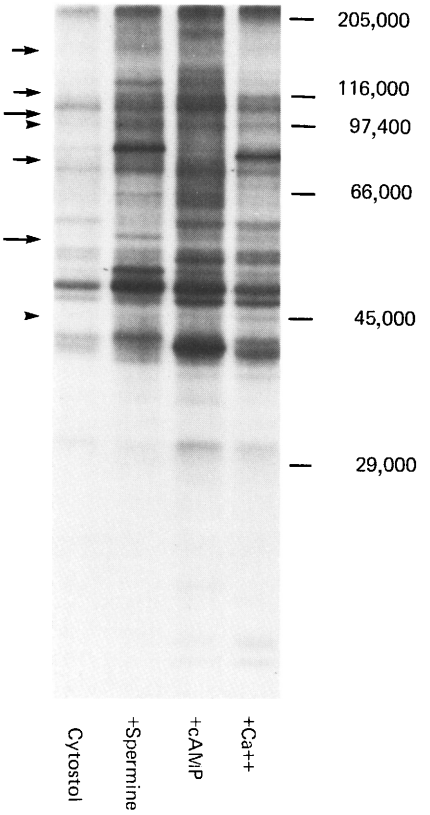

FIG. 1. Autoradiograph of phosphorylated proteins in placental cytosol. Placental cytosol was incubated as described under Materials and Methods with no effector, cAMP (10^{-6} *M*), spermine (10^{-3} *M*), or calcium (10^{-5} *M*) as indicated. The arrows (to the left) indicate proteins differentially phosphorylated by spermine (large arrows indicate phosphoproteins seen in all preparations; small arrows indicate those seen in many preparations). Arrowheads alone indicate those proteins stimulated by cAMP or calcium. The positions of the marker proteins are indicated at the right. The results shown are representative of gels from placental cytosol of ten placentas ($n = 10$).

TABLE I. ^{32}P INCORPORATION INTO SPERMINE-DEPENDENT PHOSPHOPROTEINS

	^{32}P Incorporation (pmole PO_4/mg cytosol protein)	
Spermine-dependent phosphoprotein (M_r)	+ Spermine	− Spermine
105,000	0.96 ± 0.1*	0.28 ± 0.2
55,000	1.45 ± 0.2*	0.51 ± 0.2

Note. Phosphorylation and gel electrophoresis of placental cytosol were performed as described under Materials and Methods. The gels were then cut into sequential 1-mm slices which were dissolved in 3% Protosol (New England Nuclear) at 37°C overnight and then counted by standard scintillation technique. For each of the spermine-dependent phosphoproteins, the increase in ^{32}P incorporation induced by spermine (10^{-3} *M*) is listed ($n = 3$). Values are means ± SD.

* $P < 0.001$ by a paired Student *t* test.

tosol to hot and cold TCA, hot and cold 0.5 *N* NaOH, Pronase, ribonuclease A, acetone, and chloroform/methanol (see Material and Methods). As illustrated in Table II, none of these treatments had major effects on the incorporation of ^{32}P into polyamine-dependent phosphorylated proteins except hot NaOH and Pronase. Treatment with hot NaOH or Pronase removed the proteins as well as the ^{32}P label from the gel. The results of these experiments indicate that the ^{32}P associated with the two proteins whose phosphorylation is stimulated by spermine was incorporated into protein rather than lipid or nucleic acid. In addition the stability of these phosphorylated compounds in base at low temperatures, and in trichloroacetic acid at elevated temperatures, indicates that the bonds are of the phosphoester rather than acylphosphate type (15).

Spermine, at concentrations above 10^{-5} *M*, induced dose-dependent increases in the phosphorylation of specific phosphoproteins. Dose–responsive phosphorylation analysis of the M_r 105,000 protein showed that half-maximal phosphorylation occurred at approximately $3.7 \pm 1.3 \times 10^{-4}$ *M* spermine (Fig. 2). In contrast to spermine, neither spermidine nor putrescine at 10^{-3} *M* caused detectable enhancement of protein phosphorylation. Spermidine, however, at nonphysiological concentrations (10^{-2} *M*) did enhance phosphorylation of the spermine-activated phosphoproteins comparable to that seen with 10^{-4} *M* spermine (Fig. 2). In contrast, the diamine putrescine did not enhance protein phosphorylation even at 10^{-2} *M* concentrations. Putrescine, however, inhibited spermine-induced phosphorylation in a dose-dependent manner (Figs. 3a, b). The synthetic polycation, polylysine, stimulated phosphorylation of the spermine-dependent phosphoproteins at concentrations as low as 10^{-6} *M* (data not shown).

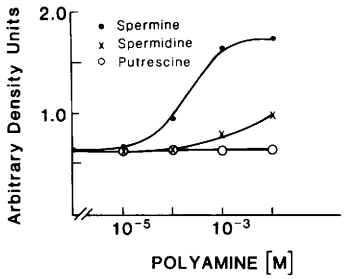

FIG. 2. Dose response of phosphorylation of the M_r 105,000 protein. The degree of phosphorylation of the M_r 105,000 protein (arbitrary units) as determined by densitometer scanning of autoradiographs (vertical axis) is shown versus concentration (*M*) of added spermine, spermidine, or putrescine. The results are representative of three experiments ($n = 3$).

TABLE II. PHOSPHATE BOND ANALYSIS EXPERIMENTS

Treatment	Arbitrary density units	
	+ Spermine	− Spermine
1. Acetone	1.237	0.596
2. Chloroform/methanol	1.469	0.673
3. NaOH (cold)	1.225	0.505
4. NaOH (boiling)	—	—
5. TCA (boiling)	1.292	0.548
6. Pronase	—	—
7. Ribonuclease A	1.425	0.608

Note. Phosphorylation of placental cytosol was performed with the after treatment listed in the table as described under Materials and Methods. For each treatment the densitometer scanner reading in arbitrary units of the M_r 105,000 spermine-dependent phosphoprotein is given ($n = 2$).

The effect of polyamines on cAMP and Ca^{2+}-activated phosphorylation in placental cytosol was also examined. The cAMP and Ca^{2+}-dependent phosphoproteins were distinct from those whose phosphorylation was augmented by polyamines (Fig. 1). Spermine, however, caused a dose-dependent inhibition of cAMP-dependent phosphorylation. Several cAMP-dependent phosphoproteins including the major M_r 45,000 phosphoprotein were inhibited by spermine (Figs. 4a, b). Inhibition of cAMP-induced phosphorylation occurred at concentrations of spermine 10-fold lower than that required for detectable spermine-activated phosphorylation. At higher spermine concentrations, inhibition of Ca^{2+}-dependent phosphorylation was also seen (data not shown).

Magnesium at low concentrations (>1 m*M*) was necessary for polyamine-enhanced phosphorylation. At higher, nonphysiological concentrations (Mg >20 m*M*), magnesium alone caused phosphorylation of proteins which comigrated with the polyamine-induced proteins. Addition of 10^{-3} *M* spermine resulted in less phosphorylation of these proteins than with the high magnesium alone (Fig. 5). Sodium chloride (>60 m*M*) and heparin inhib-

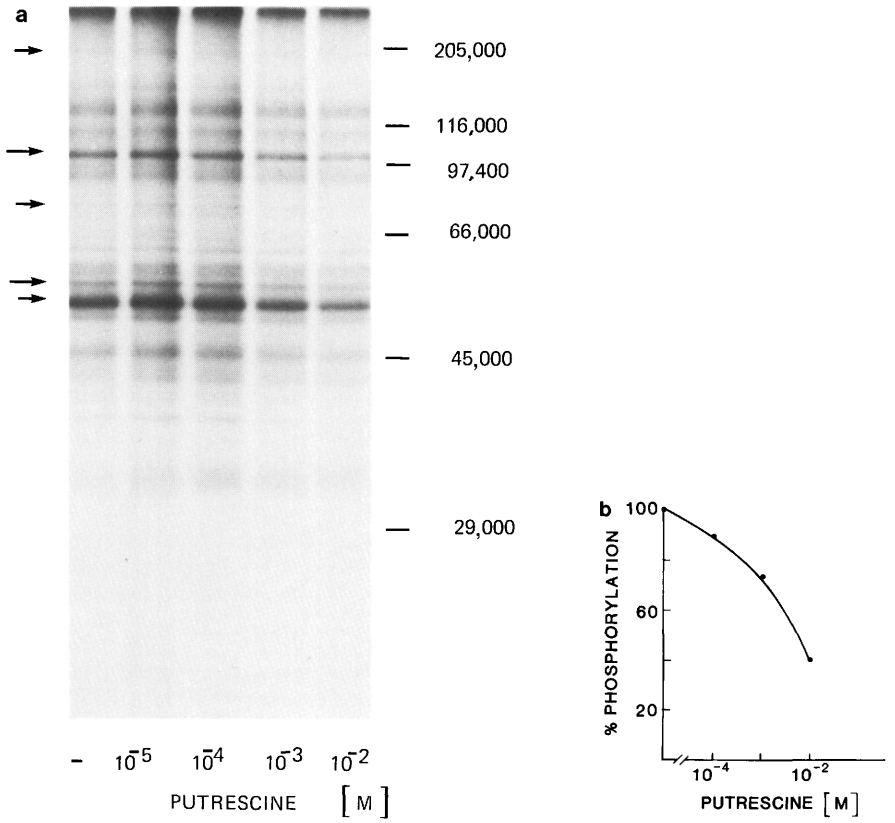

FIG. 3. Inhibition of spermine-dependent phosphorylation by putrescine. A representative autoradiograph demonstrating the effect of increasing concentrations of putrescine (10^{-5} to 10^{-2} M) upon phosphorylation of proteins by spermine (10^{-3} M). Phosphorylated proteins (left) and standards (right) are indicated as in Fig. 1. (b) Densitometer readings (arbitrary units) of the degree of phosphorylation of the spermine-dependent 105,000 M_r protein (vertical axis) are plotted against the spermine concentration (M). These results are representative of three experiments (n = 3).

ited spermine-dependent phosphorylation in placental cytosol as well as many non-spermine-dependent phosphoproteins (data not shown).

Discussion. The central finding in this study is that the phosphorylation of a select group of proteins in human placenta extracts is influenced by polyamines. These observations raise the possibility that polyamines, or their metabolic products, are primary effector compounds for specific cascade systems and, thereby, participate in the regulation of one or more metabolic processes associated with pregnancy. Polyamine biosynthesis shows marked changes during pregnancy: In human pregnancy, polyamine levels increase significantly in maternal blood (5) and urine (5, 6), as well as fluctuate in placenta (7) and amniotic fluid (5) as a function of gestational age. Amniotic fluid polyamine concentrations in some types of high-risk pregnancies (e.g., intrauterine growth retardation) deviate significantly from concentrations observed in normal pregnancies (16). Polyamine fluctuation in the pregnant rat has been more extensively studied: Serum polyamine concentrations are elevated (17, 18) and placental tissue and fetal tissue polyamine levels change with gestational age (19, 20). Further, the specific activity of ornithine decarboxylase, the rate-limiting step in polyamine biosynthesis, changes in placenta and fetal tissue during gestation (19, 20) and is elevated in maternal liver (17). As polyamines are recognized as important metab-

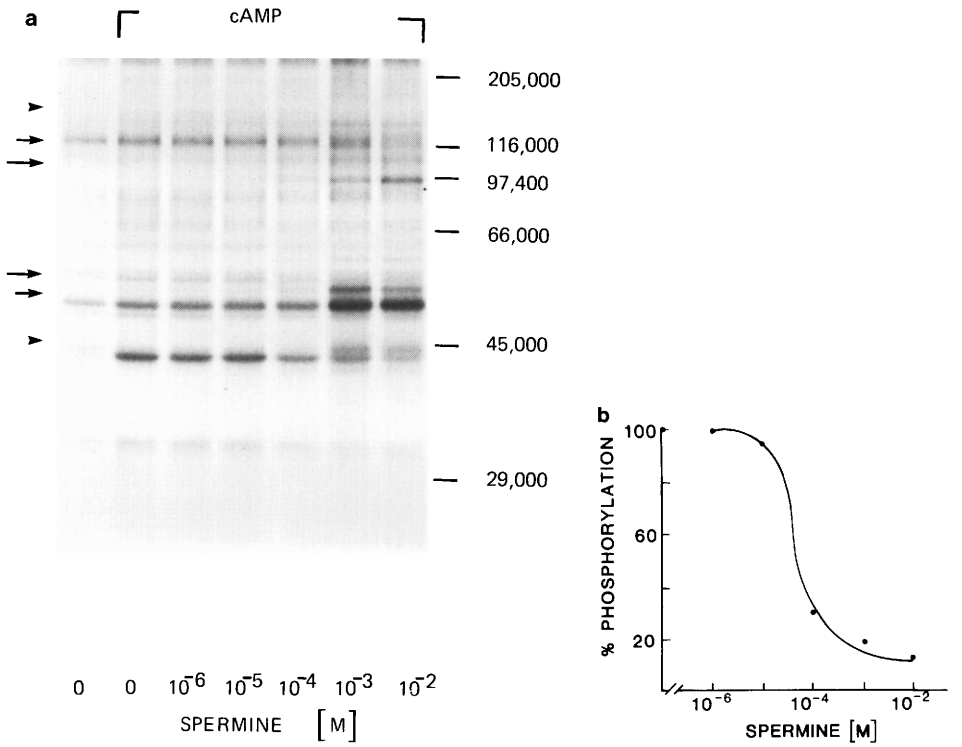

FIG. 4. Inhibition of cAMP-dependent phosphorylation by spermine. A representative autoradiograph demonstrating the effect of increasing concentrations of spermine (10^{-6} to 10^{-2} M) upon phosphorylation of proteins by cAMP (10^{-6} M). The major cAMP-dependent phosphoprotein (M_r = 45,000) is indicated by an arrowhead on the left; spermine-enhanced phosphoproteins (left) and standards (right) are indicated as in Fig. 1. (b) Densitometer readings (arbitrary units) of the degree of phosphorylation of the cAMP-dependent 45,000 M_r protein (vertical axis) are plotted against the spermine concentration (M). These results are representative of three experiments (n = 3).

olites in growth and differentiation (1–4), the increased polyamine concentrations observed during pregnancy are, at least in part, a reflection of the rapidly growing fetus. Nevertheless specific targets of the elevated polyamine concentrations during pregnancy remain to be determined.

Placental cytosol contains a protein kinase which is activated by spermine to phosphorylate proteins of M_r 55,000 and 105,000. However, spermine also activates phoshorylation of eight other proteins in many preparations. In addition, the resolving ability of the gel system used in these studies is limited. Therefore, although the M_r 55,000 and 105,000 proteins are discussed here as single proteins, they may represent several proteins of similar size. A final listing of spermine-activated phosphoproteins in placenta must await further studies in which individual proteins are identified.

The effects of four polyamines upon placental cytosol phosphorylation was examined. The potency order of the three naturally occurring polyamines, spermine ≫ spermidine ≫ putrescine, was similar to that seen in rat adrenocortical tissue extracts (21, 22). This potency order parallels the placental tissue levels of these polyamines at term gestation (7). The spermine concentration required for half-maximal ^{32}P incorporation into the M_r 105,000 phosphoprotein was demonstrated to be $3.7 \pm 1.3 \times 10^{-4}$ M. This concentration is within the physiological range of many human and animal tissues (16). More specifically, human placental spermine concentrations have been reported to vary from a low of 210 ± 9.8 nmol/g wet wt at 8–10 weeks pregnancy to

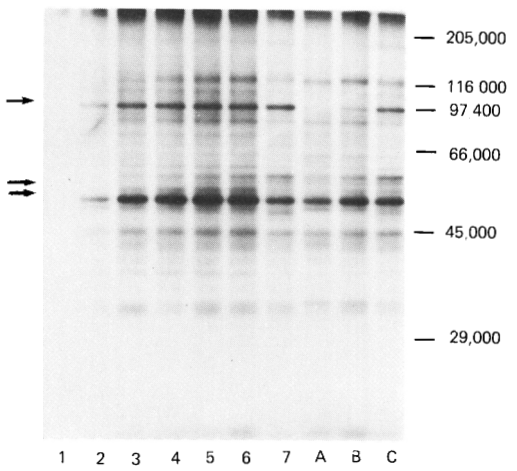

FIG. 5. Effect of Mg^{2+} upon spermine-dependent phosphorylation. Effect of increasing concentrations of Mg^{2+} (no added Mg^{2+} to 50 mM) upon spermine (10^{-3} M)-dependent phosphorylation is shown in the first seven (1–7) lanes. The three remaining lanes (A–C) show the effect of Mg^{2+} without spermine. The spermine-dependent proteins are designated by arrows to the left (as in Fig. 1) and marker proteins are shown on the right. The results are representative of three experiments (n = 3). The components of individual wells are shown below.

	1	2	3	4	5
Cytosol	+	+	+	+	+
$[Mg^{2+}]$ (mM)	–	0.5	1.5	5.0	10
Spermine	+	+	+	+	+
	6	7	A	B	C
Cytosol	+	+	+	+	+
$[Mg^{2+}]$ (mM)	20	50	5.0	20	50
Spermine	+	+	–	–	–

356 ± 17.2 nmol/g wet wt at term gestation (7).

The spermine-induced phosphoproteins in human placenta are distinct from both cAMP (M_r 25,000, 27,000, 39,000, 45,000, 52,000, 58,000, and 73,000) and Ca^{2+}-induced (M_r 97,000, 90,000, 20,000, 19,000) phosphoproteins (23) in placental cytosol. Although spermine does not activate phosphorylation of cAMP or Ca^{2+}-dependent phosphoproteins, spermine inhibits cAMP and Ca^{2+}-dependent phosphorylation in the placenta. This is consistent with reports that spermine inhibits the catalytic subunit of cAMP-dependent protein kinase and Ca^{2+}/calmodulin kinases in other systems (24–26). The evidence for cAMP-mediated effects on placental metabolism is abundant (27–31). The complete cascade for such effects including β-adrenergic receptors, catecholamine-sensitive adenylate cyclase, cAMP-dependent protein kinase, and specific cAMP-dependent phosphoproteins has also been described in human placenta (32–35). At this time, there is no evidence for a Ca^{2+}-mediated pathway in placenta; however, specific Ca^{2+}-dependent phosphoproteins have been demonstrated (23). Polyamines may therefore act both directly, by mediating phosphorylation of specific polyamine-dependent proteins, and indirectly, by inhibiting phosphorylation induced by other known secondary messengers.

The effects of magnesium, sodium, heparin, and polylysine upon polyamine-enhanced phosphorylation in placental cytosol were examined to determine whether they would modulate phosphorylation of placental cytosolic proteins similarly to polyamine-dependent casein G kinase seen in other tissues (36–38). Mg^{2+} at low levels was required for kinase activity but at higher levels (>20 mM) it inhibited polyamine-induced phosphorylation. Nonphysiological concentrations of Mg^{2+} (>50 mM) resulted in phosphorylation of the two spermine-induced phosphoproteins in the absence of the polyamines. Heparin and sodium chloride resulted in inhibition of polyamine-induced phosphorylation while polylysine was able to substitute for spermine. All are consistent with the reported characteristics of the casein G kinase I (11).

Results in this study reinforce previous suggestions that polyamines may exert some of their effects during growth and differentiation by mediating protein phosphorylation in one or more cascade systems. Confirmation of the physiological significance of these observations, however, must await identification of specific proteins whose phosphorylation is enhanced and demonstration that increases in the phosphorylation of these proteins are paralleled by increases in polyamine biosynthesis in an *in vivo* system and are accompanied by specific metabolic changes *in vivo*. Phosphorylation studies in human placental cells in culture are now in progress to examine these criteria.

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