Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 354-360

POLYAMINES STIMULATE ENDOGENOUS PROTEIN PHOSPHORYLATION IN THYROID CYTOSOL

Steven Levasseur, Thomas Poleck, and Gerald Burke

Division of Endocrinology
Department of Medicine
Cook County Hospital
Chicago, Illinois 60612

Received October 21, 1985

Summary: The polyamine, spermine (1-5 mM), when added to rat thyroid cytosol, increases the phosphorylation of a 107 kDa protein 4-fold as analyzed by sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis (SDS-PAGE) and autoradiography; spermidine was less effective and putrescine was without effect. Sodium chloride, when tested at equivalent ionic strengths (4-40 mM), did not reproduce the effects of spermine. In addition to stimulating the phosphorylation of a 107 kDa protein, spermine had an apparent biphasic effect on the phosphorylation of 88 and 65 kDa proteins; maximum stimulation of approximately 60-70% was observed at 0.5-2 mM. Both basal and spermine-stimulated protein phosphorylation patterns were identical whether [γ-^{32}P] ATP or [γ-^{32}P] GTP was used as phosphate donors. Heparin (1 μg/ml) reduced spermine-stimulated phosphorylation of the 107 kDa protein by 64%. Phosphorylation of a 107 kDa protein was not restricted to rat thyroid as spermine was found to augment the phosphorylation of 107 kDa protein(s) in mouse and beef thyroid cytosol preparations.

Thyrotropin has been shown to augment thyroid ornithine decarboxylase activity and, consequently, polyamine levels both *in vitro* and *in vivo* (1-7). Although the proximal effects of such regulation in thyroid are not known, Cochet and Chambaz have, in a recent review (8), suggested that polyamines may augment protein phosphorylation via substrate interaction which they have termed polyamine-mediated phosphorylation.

We have recently shown that polyamines exert differential effects on rat thyroid protein kinase activities (9). Polyamines were shown to stimulate casein kinase activities in rat thyroid cytosol and nuclear preparations whereas they inhibited histone H_1 kinase A activity in both crude cytosol and partially purified cytosol

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; protein kinase A, cyclic-AMP-dependent protein kinase; portein kinase C, diacylglycerol activated calcium/phospholipid-dependent protein kinase.

0006-291X/85 $1.50

preparations. Further, among the polyamines tested, spermine was found to be a specific inhibitor of protein kinase C (histone H_1) activity partially purified by DE-52 chromatography from rat thyroid cytosol.

Although several studies have identified polyamine-responsive casein kinase activity from nuclear preparations (10-11) as well as polyamine-altered levels of phosphorylation of chromatin proteins (12-16), there remains a paucity of information available on the potential physiologic role of polyamines in the regulation of extranuclear kinase activity. In order to further assess the potential physiologic role of polyamines in the regulation of thyroid cell function, we determined the effect of polyamines on the phosphorylation of endogenous proteins in rat thyroid cytosol.

MATERIALS AND METHODS

Chemicals: Putrescine, spermidine, spermine, ATP, GTP, molecular weight markers (SDS-6H) and protein kinase A inhibitor (rabbit skeletal muscle; the "Walsh inhibitor") were purchased from Sigma Chemical Co., St. Louis, MO. [γ-^{32}P] ATP (650 Ci/mmole) and [γ-^{32}P] GTP (650 Ci/mmole) were obtained from ICN, Irvine, CA. Heparin was purchase from Elkins-Sinn, Cherry, NJ.

Animals: Male Sprague Dawley rats (150-300 g) and female Swiss Webster mice were maintained on standard laboratory chow and tap water. Beef thyroid glands were obtained from a local slaughter house and transported to the laboratory on ice.

Cytosol Preparation: Thyroid cytosol fractions were prepared at 4°C as recently described (9). Briefly, 30-60 mg thyroid tissue was homogenized 1:60 [wt/vol] in 0.25M sucrose/5mM $MgCl_2$/6mM 2-mercaptoethanol/1mM phenylmethylsulfonyl-fluoride/5mM potassium phosphate buffer (pH 7.0 at 20°C) using a Polytron (Brinkmann Instruments) at speed 4 for 10 sec. The homogenates were centrifuged 100,000 x g x 60 min. and the supernatant (cytosol) fractions were used immediately. Protein was determined using the method of Lowry *et al.*

Phosphorylation of Endogenous Proteins: Incubation mixtures contained 60-100µg cytosol protein, 50mM potassium phosphate buffer (pH 7.4 at 20°C), 10mM Mg Cl_2 0.5µM ATP, 2-3 x 10^6 dpm [γ-^{32}P] ATP, and test substances as indicated in a final volume of 200µl. In some experiments [γ-^{32}P] ATP was replaced with [γ-^{32}P] GTP; unlabeled ATP and GTP was added as indicated. After 10 min. at 30°C the reactions were terminated with the addition of 50µl of 3% SDS/3% 2-mercaptoethanol/0.002% bromophenol blue/10% glycerol/63mM tris-HCl (pH 6.8 at 20°C) and placed in a boiling water bath for 3 min.

SDS-PAGE And Autoradiography: Samples of the reaction mixtures were analyzed by SDS-PAGE (18) by loading 35-45µg protein of the sample preparations onto 4-12% gradient slab gels. Following electrophoresis (3mA per lane; 4 hrs.) the gels were equilibrated overnight at 20°C in 25mM tris, 192mM glycine, and 20% methanol. Proteins were transferred to nitrocellulose paper with a Trans Blot apparatus (BioRad) using methodology outlined by the vendor. The nitrocellulose blots were stained with Coomassie blue and exposed to x-ray film for 2-3 days at -20°C. Zones of radioactivity were cut from the nitrocellulose blots and counted in 7 mls 3A70B (RPI).

Data Analyses: All data were normalized as percent of control values and analyzed for statistical significance using Student's t test (two-tailed) for unpaired samples.

RESULTS

Using [γ-^{32}P] ATP as phosphate donor, the phosphorylation of at least 4 proteins of MW 107, 88, 65, and 41 kDa could be detected in rat thyroid cytosol. The effects of putrescine, spermidine, and spermine on endogenous phosphorylation were tested at 1mM and 5mM (Figure 1). Spermine stimulated phosphorylation of the 107 kDa protein by 111±17% and 388±63% at 1mM and 5mM, respectively ($\bar{x}$ + SEM, n =7; P<0.001). 5mM spermidine increased phosphorylation of the 107 kDa protein by 40±8% (n = 3; P<0.01); putrescine at 1mM or 5mM and 1mM spermidine were without effect (Figure 1). Possible non-specific ionic effects were ruled out by our findings that sodium chloride (4-40mM) failed to reproduce the effects of 0.5-5.0mM spermine on phosphorylation of the 107 kDa protein (Figure 2). The effect of spermine was reversible. Overnight dialysis of cytosol preincubated with 20mM spermine restored the capacity of the cytosol to respond to 5mM spermine in the phosphorylation assay (data not shown).

In addition to stimulating the phosphorylation of the 107 kDA protein, spermine effected a moderate (~65%) biphasic effect on 88 and 65 kDa proteins; increased phosphorylation of both proteins was maximal in the 0.5-2mM range (Figure 3).

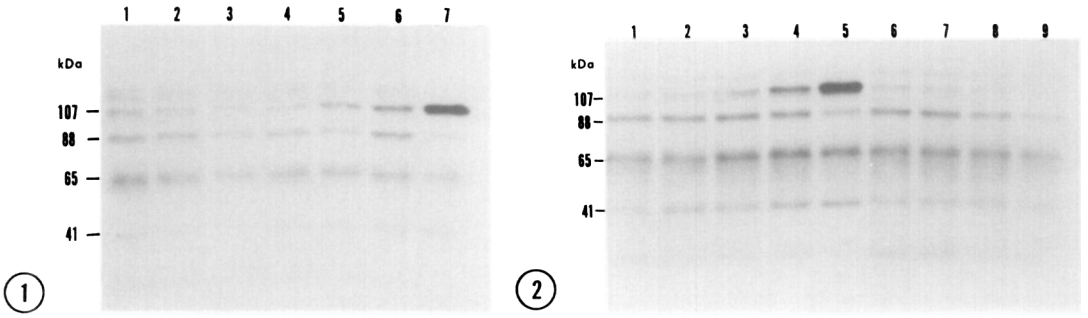
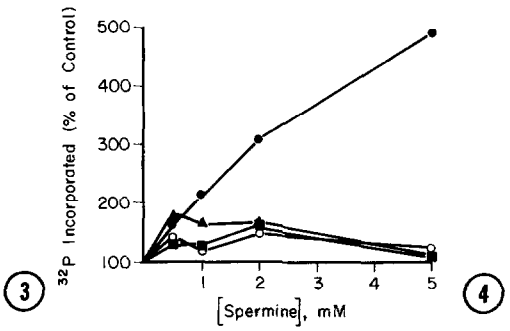


Figure 1 The effect of polyamines on endogenous protein phosphorylation in rat thyroid cytosol. Rat thyroid cytosol was prepared and incubated with 0.5μM [γ-^{32}P] ATP, polyamines, and analyzed by SDS-PAGE and autoadiography as described in Methods. Lanes: 1, control; 2-3, 1 and 5mM putrescine; 4-5, 1 and 5mM spermidine; 6-7, 1 and 5mM spermine. The autoradiogram is representative of 3 experiments performed using 3 different cytosol preparations.

Figure 2 The effect of spermine and sodium chloride on endogenous protein phosphorylation in rat thyroid cytosol. Rat thyroid cytosol was prepared and incubated with 0.5μM [γ-^{32}P] ATP, ± 0.5-5mM spermine, ± 4-40mM NaCℓ, and the samples analyzed by SDS-PAGE and autoradiography as described in Methods. Lanes: 1, control; 2-5, 0.5, 1.0, 2.0, and 5.0mM spermine, respectively; 6-9, 4, 8, 16, and 40mM NaCℓ, respectively. The autoradiogram is representative of 3 experiments performed using 3 different cytosol preparations.



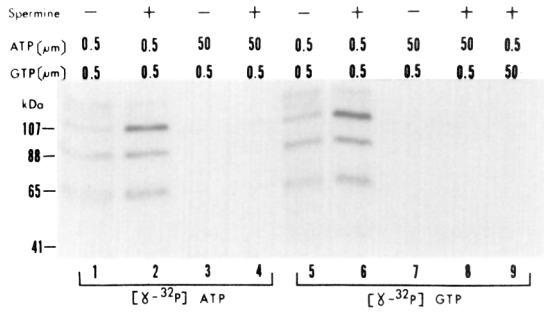


Figure 3 The effect of spermine on the phosphorylation of 107 (●), 88 (▲), 65 (■), and 41 (o) kDa proteins. Rat thyroid cytosol preparations were incubated with 0.5μM [γ-^{32}P] ATP and 0.5, 1.0, 2.0, or 5.0mM spermine and samples analyzed as described in Methods. Each data point represents the mean of 3-7 experiments using different cytosol preparations and are derived from experiments described in Figures 1 and 2.

Figure 4 The effect of [γ-^{32}P] ATP and [γ-^{32}P] GTP dilution with ATP on ^{32}P incorporation into rat thyroid cytosol proteins. Rat thyroid cytosol was incubated with either [γ-^{32}P] ATP (lanes 1-4) or [γ-^{32}P] GTP (lanes 5-9) and indicated concentrations of unlabeled ATP, GTP, and spermine (5mM). The chromatogram is representative of two experiments.

Since polyamine-mediated phosphorylation is characteristic or protein kinase activity with the capacity to utilize both GTP and ATP as phosphate donor (8), we tested the effect of spermine on endogenous phosphorylation using 0.5μM [γ-^{32}P] GTP as a phosphate donor. The patterns of both controls and 5mM spermine-stimulated protein phosphorylation were found to be identical whether 0.5μM [γ-^{32}P] ATP or 0.5μM [γ-^{32}P] GTP was used as phosphate donor (data not shown). Further, 100-fold dilution of radiolabeled ATP and radiolabeled GTP with unlabeled ATP virtually eliminated ^{32}P incorporation into all protein bands (Figure 4).

Polyamine-mediated phosphorylation has been shown to be inhibited by heparin (8). Heparin (1μg/ml) reduced 5mM spermine-stimulated phosphorylation of the 107 kDa protein by 64±5% ($\bar{x}$ + SEM; n = 3).

It should be noted that, under the assay conditions used, 10μM cyclic-AMP/ 0.5mM 3-isobutyl-1-methylxanthine did not stimulate the phosphorylation of endogenous proteins in the rat cytosol preparation; rat thyroid cytosol prepared in an identical manner has previously been shown to contain protein kinase A using exogenous histone H1 substrate (9). Further, the Walsh inhibitor (50μg/ml) did not reduce the ^{32}P content of the phosphorylated proteins (data not shown).

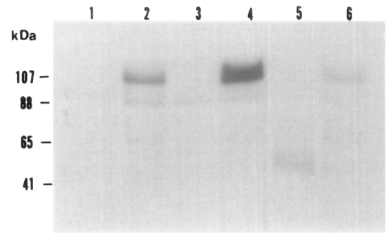


Figure 5 The effect of spermine on endogenous protein phosphorylation in rat, mouse, and beef thyroid cytosol. Thyroid cytosol preparations (100µg protein) were incubated with 0.5µM [γ-^{32}P] ATP, ± 5mM spermine and the samples analyzed by SDS-PAGE and autoradiography. Lanes: 1-2, rat thyroid cytosol, control and 5mM spermine; 3-4, mouse thyroid cytosol, control and 5mM spermine. The data shown are representative of those obtained with at least 3 different rat, mouse, and beef thyroid cytosol preparations.

In the next series of experiments, we addressed the question of species specificity. Figure 5 shows that 5mM spermine stimulated ^{32}P incorporation into mouse and beef cytosol proteins which co-migrated with the 107 kDa protein in rat thyroid cytosol. Although both the magnitude of ^{32}P incorporation and spermine stimulation were similar in rat and mouse cytosol, phosphorylation of the corresponding protein was notably lower in beef thyroid cytosol.

DISCUSSION

Elevated ornithine decarboxylase activity, the rate-limiting enzyme in polyamine biosynthesis and consequently, polyamine levels, are characteristics of embryonic, tumor, and hormone stimulated tissues (19). In general, polyamines have been shown to augment messenger-independent casein kinase (G) activities in both cytosolic and nuclear preparations and inhibit protein kinase A and C (histone) activities in cytosol preparations (20, 9). Further, polyamines have been shown to exert differential effects on phosphorylation patterns in nuclear preparations and extracts corroborating work which describes the presence of messenger independent (10-11), protein kinase A (21-22), and protein kinase C (23) activities in the nucleus.

The present study shows that elevated polyamine levels may also exert stimulatory effects on the phosphorylation of cytosolic proteins in thyroid. Although the interconversion of ATP and GTP in cytosol remains a possiblity, our finding that [γ-^{32}P] GTP serves as phosphate donor and that heparin inhibits spermine-

stimulated phosphorylation strongly argues that the phosphorylation of the 107 kDa protein is mediated by a casein kinase G (8). The biphasic effect of spermine on both the 88 kDa and 65 kDa proteins remains an enigma. However, it should be noted that glycogen synthetase (MW = 88 kDa) is phosphorylated by a variety of protein kinases including a casein kinase G (glycogen synthetase kinase 5 (24)) in rabbit skeletal muscle and that polyamines stimulate phosphoprotein phosphatase (25). Whether these mechanisms are operative in thyroid remains to be shown.

It is interesting to note that spermine stimulation of phosphorylation of the 107 kDa protein was observed not only in rat but also mouse and beef thyroid. These data indicate that not only the mechanism of phosphorylation but the protein substrate as well may be well conserved in thyroid.

ACKNOWLEDGEMENTS

We would like to thank Ms. Jan LaShea for typing the manuscript. We would also like to thank Dr. Maija Mednieks and the late Mr. Philip Murphy of the National Institute of Dental Research for helpful discussions regarding the SDS-PAGE, blotting, and autoradiography procedures. This work was supported by Grant AM 17561 of the United States Public Health Service.

REFERENCES

1. Richman, R., Park, S., Akbar, M., Yu, S., and Burke, G. (1975) Endocrinology 96, 1403-1412.
2. Zusman, D.R., and Burrow, G.N. (1975) Endocrinology 97, 1089-1095.
3. Matsuzaki, S., and Suzuki, M. (1975) Endocrinol. Japon. 22, 339-345.
4. Friedman, Y., Park, S., Levasseur, S., and Burke, G. (1977) Biochem. Biophys. Res. Commun. 77, 57-64.
5. Friedman, Y., Lang, M., Levasseur, S., and Burke, G. (1979) Endocrinology 104, 467-475.
6. Matsuzaki, S., Kakegawa, T., Suzuki, M., and Hamana, K. (1978) Endocrinol. Japon. 25, 129-139.
7. Mockel, J., Decaux, G., Unger, J., and Dumont, J.E. (1980) Endocrinology 107, 2069-2075.
8. Cochet, C., and Chambaz, E.M. (1983) Mol. Cell. Endocrin. 30, 247-266.
9. Levasseur, S., Henricks, L., Poleck, T., Friedman, Y., and Burke, G. (1985) J. Cell. Biochem. 28, 299-306
10. Rose, K.M., Bell, L.E., Siefken, D.A., and Jacob, S.T. (1981) J. Biol. Chem. 256, 7468-7477.
11. Dahmus, M.E. (1981) J. Biol. Chem. 256, 3319-3325.
12. Martelo, O.J., and Hirsch, J. (1974) Biochem. Biophys. Res. Commun. 58, 1008-1015.
13. Imai, H., Shimoyama, M., Yamamoto, S., Tanigawa, Y., and Ueda, I. (1975) Biochem. Biophys. Res. Commun. 66, 856-862.
14. Farron-Furstenthal, F., and Lightholder, J.R. (1978) Biochem. Biophys. Res. Commun. 83, 94-100.
15. Hara, T., Takakashi, K., Yamamoto, M., Kisaki, H., and Endo, H. (1982) Biochem. Biophys. Res. Commun. 106, 131-138.

16. Chen, K.Y., and Verma, R. (1984) Biochem. Biophys. Res. Commun. 118, 710-716.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
18. Laemmli, U.K., and Favre, M. (1973) J. Mol. Biol. 80, 575-599.
19. Jänne, J., Poso, H., and Raina, A. (1978) Biochem. Biophys. Acta 473, 241-293.
20. Murray, A.W., Froscio, M., and Rogers, A. (1976) Biochem. Biophys. Res. Commun. 71, 1175-1181.
21. Squinto, S.P., Kelley-Geraghty, D.C., Kuettel, M.R., and Jungmann, R.A. (1985) J. Cycl. Nucleotide and Protein Phosphor. Res. 10, 65-73.
22. Byus, C.V., and Fletcher, W.H. (1985) J. Cycl. Nucleotide and Protein Phosphor. Res. 10, 9-22.
23. Kimura, K., Katok, N., Sakurada, K., and Kubo, S. (1984) Endocrinology 115, 2391-2399.
24. Cohen, P., Yellowlees, D., Aitken, A., Donella-Deana, A., Hemmings, B.A., and Parker, P.J. (1982) Eur. J. Biochem. 124, 21-35.
25. Tung, H.Y.L., Peleck, S., Fisher, M.J., Pogson, C.I., and Cohen, P. (1985) Eur. J. Biochem. 149, 305-313.