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Differential Effects of Polyamines on Rat Thyroid Protein Kinase Activities

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Ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, has been shown to be regulated in thyroid by thyrotropin both in vivo and in vitro. Little, however, is known of the role of polyamines in thyroid cell function. Since studies in other tissues suggest that polyamines may influence protein phosphorylation, we studied the effect of the polyamines on various protein kinase activities in rat thyroid. Putrescine, spermidine, and spermine inhibit cyclic-AMP-dependent histone H_1 kinase activity when measured in the cytosol fraction of rat thyroid; this effect is largely reproduced by NaCl concentrations of equivalent ionic strength. Both spermidine and spermine effect a 1.6–2.4-fold increase in cytosolic cyclic-AMP-independent (messenger-independent) casein kinase activity; stimulation by both polyamines is maximal at 5mM. A similar profile of stimulation is observed for messenger-independent casein kinase activity in crude nuclear preparations. Sodium chloride fails to stimulate both cytosolic and nuclear messenger-independent casein kinase activities at ionic strength equivalent to the spermine concentrations used. Spermine, but not putrescine, spermidine, or sodium chloride, inhibits calcium/phospholipid-dependent protein kinase C activity in cytosol extracts partially purified by DEAE chromatography. These findings suggest that regulation of protein kinase(s) by polyamines may represent a proximal locus (i) of action of thyrotropin-regulated ornithine decarboxylase activity in thyroid.

Key words: polyamines, cyclic-AMP-dependent protein kinase, calcium/phospholipid-dependent protein kinase C, messenger-independent protein kinase, ornithine decarboxylase, rat thyroid

Ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, has been shown to increase in activity in response to thyrotropin (TSH) in vivo and in vitro in murine thyroid [1–5] with concomitant increases in polyamine concentrations reported following chronic goitrogen administration [3,6]. Although a variety of roles have been postulated for polyamines in cell function [7–9], several reports have implicated protein kinase(s) as a proximal locus (i) of polyamine action [9–16].

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As cited above, the effect of acute and chronic TSH regulation of ornithine decarboxylase activity are well documented, however, the role of polyamine metabolism in thyroid function has not been studied. In light of reports that suggest polyamines may contribute to the regulation of protein phosphorylation, we inititated studies designed to test the effect of polyamines on various protein kinase activities. Our data show that: (1) spermine, and to a lesser extent, spermidine and putrescine, stimulates both cytosolic and nuclear casein kinase activities (messenger-independent protein kinase [17]; (2) spermine inhibits calcium/phospholipid-dependent protein kinase C activity in partially purified cytosolic extract; and (3) the polyamines inhibit cyclic-AMP-dependent protein kinase; however, the effect was shown to be largely a nonspecific ionic effect.

MATERIALS AND METHODS

Male Holtzman rats weighing 150–200 g were maintained on standard laboratory chow and tap water. Histone H_1 (F_1), phosphatidylserine, ATP, recrystallized bovine serum albumin, partially dephosphorylated casein, putrescine, spermidine, and spermine were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclic-AMP and [8-^{3}H]-cyclic-AMP (20 Ci/mmol) were obtained from Schwartz-Mann (Orangeburg, NY) [γ-^{32}P]-ATP (149 Ci/mmol) was supplied by ICN (Irvine, CA). 3-isobutyl-1-methylxanthine was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Tissue Preparation

All tissue preparations were carried out at 4°C. For the determination of cyclic-AMP-dependent and messenger-independent protein kinase activities, thyroids were homogenized 1:60 (wt/vol) in Buffer A (5 mM potassium phosphate/5 mM $MgCl_2$/6 mM 2-mercaptoethanol, pH 7.0, at 20°C) containing 0.25 M sucrose using a Polytron® (Brinkmann) at speed 5 for 10 sec. Homogenates were filtered through a fine mesh screen and crude nuclear fractions were prepared by centrifuging the homogenate at 600g for 20 min. The pellet was resuspended in one-half of the starting volume of Buffer A containing 0.14 M NaCl and gently homogenized with four strokes in a Dounce homogenizer fitted with a loose fitting pestle. The suspension was recentrifuged at 1,000g for 20 min and the final pellet resuspended with Buffer A to a final protein concentration of 1.5 mg/ml. A high-speed supernatant fraction (cytosol) was prepared by centrifuging the homogenate at 10,000g for 10 min and centrifuging the resulting supernatant fraction at 100,000g for 1 hr. When protein kinase C activity was determined, rat thyroids were homogenized 1:8 (wt/vol) in Buffer B (20 mM Tris-HCl/2 mM EGTA/1 mM phenylmethylsulfonylfluoride, pH 7.4 at 20°C) using the Polytron® at speed 5 for 10 sec. The homogenate was centrifuged 100,000g for 60 min and the supernatant fraction further resolved by DEAE chromatography as described below.

DEAE Chromatography

Cyclic-AMP-dependent protein kinase activity was further resolved by loading 8 mg of 100,000g supernatant protein onto a 1 × 8-cm DEAE (DE-52, Whatman) column pre-equilibrated with Buffer A, washed with 15 ml of Buffer A, and eluted with a linear gradient of KCl (0–350 mM) in Buffer A. Fractions (2-ml) were

collected at a flow rate of 15 ml/hr. Aliquots (50-μl) were assayed for protein kinase activity and [^{3}H]-cyclic-AMP binding. Peaks of activity were concentrated using an Amicon ultrafiltration apparatus with a Diaflo® PM 10 membrane and dialyzed overnight against 500 volumes of Buffer A. Protein kinase C activity was partially purified by loading 5–7 mg protein of the supernatant fraction that was centrifuged at 100,000g for 60 min onto a 0.7 × 1.5-cm DE-52 column pre-equilibrated with Buffer B. The sample was washed with 3 ml of Buffer B and the protein kinase C activity eluted with 3 ml Buffer B containing 70 mM NaCl [18]. The fraction was dialyzed overnight at 4°C against 500 vol of 20 mM Tris-HCl/2 mM EGTA (pH 7.4 at 20°C).

Protein Kinase Assays

Cyclic-AMP-dependent protein kinase was assayed as previously described [5] with minor modifications. Briefly, the mixture contained 2 μmol $MgCl_2$, 10 μmol potassium phosphate buffer (pH 6.5 at 20°C), 4 nmol ATP, 200,000–400,000 dpm [γ-^{32}P]-ATP, 100 μg histone H_1, 50 nmol 3-isobutyl-1-methylxanthine, 400 pmol cyclic-AMP, 25–50 μg cytosol protein, or 50 μl DEAE fraction or peak, and indicated amounts of putrescine, spermidine, or spermine in a final volume of 200 μl. Following incubation for 5 min at 37°C, the reaction was terminated and the samples filtered and counted as described in [5].

Messenger-independent protein kinase was assayed in a mixture containing 2 μmol $MgCl_2$, 10 μmol potassium phosphate buffer (pH 6.5 at 20°C), 4 nmol ATP, 800,000–1,200,000 dpm [γ-^{32}P]-ATP, 500 μg partially dephosphorylated casein, 25–50 μg cytosol or crude nuclear protein, and indicated amounts of putrescine, spermidine, or spermine in a final volume of 200 μl. The mixture was incubated, the reaction terminated, and the samples filtered as described [5].

Calcium/phospholipid-dependent protein kinase C activity was assayed in a mixture containing 2 μmol $MgCl_2$, 10 μmol Tris-HCl (pH 7.4 at 20°C), 4 nmol ATP, 0.4 μmol dithiothreitol, 400,000–600,000 dpm [γ-^{32}P]-ATP, 80 μg histone H_1, 40 nmol $CaCl_2$, 4 μg phosphatidylserine, 20 μg protein from the enzyme preparation, and indicated amounts of polyamines or sodium chloride in a final volume of 200 μl. After incubation for 10 min at 30°C, the reaction was terminated and the samples filtered as described [5].

Other Procedures

[^{3}H]-cyclic-AMP binding was measured using the procedure of Menon and Azhar [19]. DNA was measured using the method of Burton [20] and protein using the method of Lowry [21] using recrystallized bovine serum albumin as standard.

RESULTS

The cytosol fraction of rat thyroid glands contain cyclic-AMP-dependent histone kinase and messenger-independent casein kinase [5,22–27]. As shown in Figure 1, putrescine, spermidine, and spermine inhibit cyclic-AMP-dependent histone H_1 kinase activity when the high-speed supernatant fraction is used as an enzyme source. NaCl inhibits the cytosol cyclic AMP-dependent enzyme by 33 ± 3% and 42 ± 5% at 80 mM and 100 mM, respectively ($\bar{x}$ + SEM; n = 3). The polyamines have no apparent effect on [^{3}H]-cyclic-AMP binding (data not shown). In order to investigate any potential differential effects that polyamines may exert on cyclic-AMP-dependent

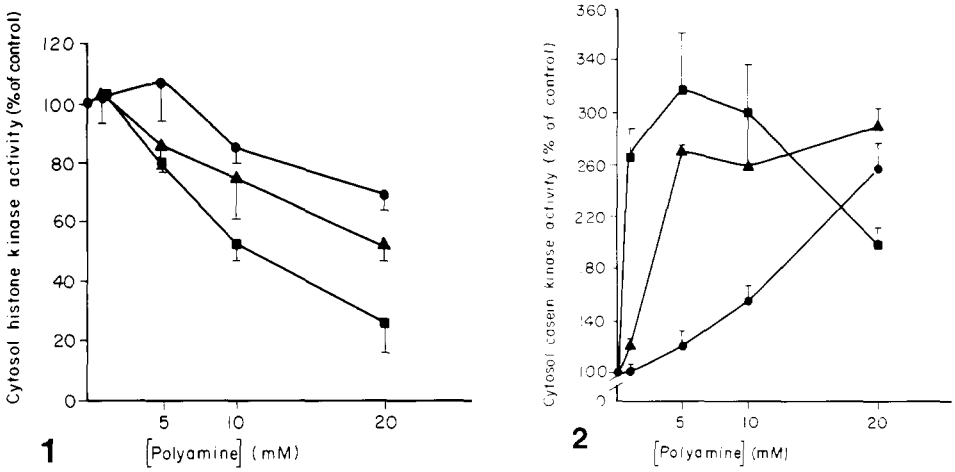

Fig. 1. Effects of polyamines on cytosolic cyclic-AMP-dependent histone H_1 kinase activity. Rat thyroid cytosol was assayed for cyclic-AMP-dependent histone H_1 kinase activity as described in Methods in the presence of 2 μM cyclic-AMP and indicated concentrations (0, 1, 5, 10, 20 mM) of putrescine (●), spermidine (▲), and spermine (■). Each data point is the mean + SEM of three independent observations performed in duplicate. The specific activities of the preparations assayed in the absence and presence of 2 μM cyclic-AMP were 195 $\pm$ 50 and 705 $\pm$ 115 pmol P transferred per mg cytosol protein min^{-1} and reflects an activity ratio of 0.25 $\pm$ 0.03.

Fig. 2. Effects of polyamines on cytosolic cyclic-AMP-independent casein kinase activity. Polyamines (0, 1, 5, 10, 20 mM) were incubated with rat thyroid cytosol and 500 μg of partially dephosphorylated casein in the absence of exogenous cyclic-AMP as described in Methods. The basal specific activity of the three preparations was 90 $\pm$ 6 pmol P transferred per mg cytosol protein min^{-1} (●), putrescine; (▲), spermidine; (■), spermine.

protein kinase isoenzymes, the cytosol was resolved by DEAE chromatography. Histone H_1 kinase activity measured in the presence of 2 μM cyclic-AMP and [^{3}H]-cyclic-AMP binding occurs predominantly as a single peak at 155 mM KCl (range 145–163 mM; n = 3). This finding is in agreement with Leonard et al [24] and Combest et al [27], but is in some disagreement with other findings [25]. Polyamine inhibition of the partially purified enzyme is qualitatively similar to studies performed using the crude supernatant fraction (data not shown).

Both spermidine and spermine stimulate messenger-independent casein kinase activity in cytosol approximately 2 fold (Fig. 2). The stimulation by both polyamines is maximal at 5 mM; putrescine is considerably less potent. Sodium chloride (40 mM), equivalent in ionic strength to 5 mM spermine, has an insignificant effect on the cytosolic cyclic-AMP-independent enzyme activity (106 $\pm$ 7% of control; $\bar{x}$ + SEM; n = 3). When the casein substrate was replaced with histone H_1, 5 mM spermine failed to have any significant effect on protein kinase activity; protein kinase activity measured in the absence and presence of 5 mM spermine was 98 $\pm$ 24 and 98 $\pm$ 45 pmol P transferred per mg cytosol protein min^{-1} ($\bar{x}$ + SEM; n = 3).

Rat thyroid cytosol also contains calcium/phospholipid protein kinase C activity [28]. The partially purified enzyme is unaffected by either calcium (0.2 mM) or

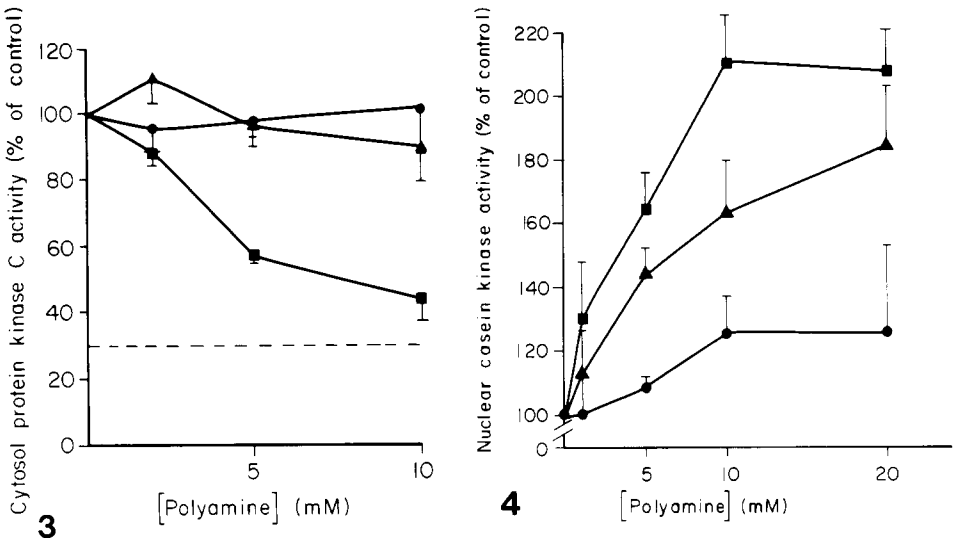

Fig. 3. The effects of polyamines on partially purified calcium/phospholipid-dependent protein kinase C activity from rat thyroid cytosol. Rat thyroid cytosol was prepared, and protein kinase C activity was resolved by DEAE chromatography as described in Methods. Assay mixtures contained 0.2 mM calcium, 4 μg phosphatidylserine, 50 μl histone H_1 and 0, 2, 5, or 10 mM polyamines. The horizontal broken line indicates protein kinase activity assayed in the absence of phosphatidylserine. Each data point represents the $\bar{x}$ + SEM of three triplicate determinations using three different enzyme preparations. The three preparations exhibited specific activities of 44 ± 11 and 153 ± 45 pmol P transferred per mg protein min^{-1} when measured in the presence of calcium and calcium plus phosphatidylserine, respectively. (●), putrescine; (▲), spermidine; (■), spermine.

Fig. 4. Effects of polyamines on nuclear cyclic-AMP-independent casein kinase activity. Rat thyroid nuclei were prepared and assayed in the absence or presence of polyamines (1, 5, 10, 20 mM) as described in Methods. Each data point is the $\bar{x}$ + SEM of five independent determinations performed in duplicate. The casein kinase activity of the five nuclear preparations was 59.0 ± 10.4 pmol P transferred per mg nuclear protein min^{-1}. (●), putrescine; (▲), spermidine; (■), spermine.

phosphatidylserine (20 μg/ml) alone (data not shown). Calcium/phospholipid-dependent protein kinase C, partially purified by DEAE chromatography, is inhibited 17–80% by 2–10 mM spermine (Fig. 3). Neither putrescine nor spermidine have a significant effect on protein kinase C activity. Sodium chloride reduces protein kinase activity 7% and 17% when added at 40 mM and 80 mM, respectively.

Partially purified rat thyroid nuclei prepared as described in Methods exhibit a specific activity of 59.0 ± 10.4 pmol P transferred per mg protein min^{-1} ($\bar{x}$ + SEM; n = 5). The DNA/protein ratio of the preparation was 0.30 ± 0.01 ($\bar{x}$ + SEM; n = 5). Figure 4 shows that spermidine and spermine augment messenger-independent casein kinase from 5–20 mM in crude nuclear preparations. Putrescine is less effective. NaCl, at 80 mM, effects a 27 ± 14% increase in activity ($\bar{x}$ + SEM; n = 5).

DISCUSSION

The present study demonstrates that polyamines exert differential effects on rat thyroid protein kinase activities. Based upon the endogenous polyamine concentrations reported in thyroid [6], the potent stimulation of messenger-independent casein kinase activity by polyamines in both the cytosol and crude nuclear fractions, as well as the demonstration that this effect is not due to nonspecific ionic effects, suggests that this observation may be physiologically important. Additionally, several studies by Matsuzaki's group [3,6,29] have shown concomitant increases in ornithine decarboxylase activity/elevated polyamine levels and RNA accumulation in thyroid. Polyamines have been shown to augment incorporation of ^{32}P into nonhistone chromatin proteins in several systems [30–31], and there are several lines of evidence that strongly support reversible phosphorylation as an important mechanism in the regulation of transcription. The reported alterations in the number of initiation sites [32], as well as RNA polymerase I [24,33,34] and RNA polymerase II [35] activities, suggest that control of transcription by reversible phosphorylation may be operative at several levels. Although our data suggests that the term polyamine-dependent protein kinase may be appropriate to describe, at least in part, messenger-independent protein kinase activity, Cochet and Chambaz have, in a recent review [9], suggested that, because polyamines may interact primarily with substrate, the term "polyamine-mediated phosphorylation" may be more appropriate. Such a mechanism in rat thyroid cytosol is supported by our finding that, while polyamines stimulate casein kinase activity, spermine fails to augment histone H_1 phosphorylation.

The inhibitory effect of polyamines on cyclic-AMP-dependent protein kinase activity observed in the present study confirms similar findings reported in other tissues [10–13]. However, a physiologic role for the inhibitory effect of polyamines on cyclic-AMP-dependent protein kinase activity appears to be more speculative. Arguing against such a role is our finding that sodium chloride reproduces the inhibition and, therefore, strongly suggests a non-specific ionic effect. On the other hand, two important observations should be noted. First, polyamines appear to inhibit the catalytic activity as opposed to interfering with the dissociation and activation of the holoenzyme, since [^{3}H]-cyclic-AMP binding was unaffected by the polyamines. Secondly, Spaulding's group has reported [36,37] that cyclic-AMP-mediated histone H_1 phosphorylation increased linearly in calf thyroid slices beginning at 10 min and continued through the last time tested (2 hr). Further, Lamy et al [38] have shown that thyrotropin-stimulated phosphorylation remains elevated at 3 hr in dog thyroid slices. Therefore, the temporal requirement of continued cyclic-AMP-dependent protein-kinase-mediated histone H_1 phosphorylation and the onset of increased ornithine decarboxylase activity/polyamine levels (2–4 hr) appears to be fulfilled. We have recently reported that rat thyroid cytosol contains calcium/phospholipid-dependent protein kinase C activity [28]. The inhibition by spermine of protein kinase C activity differs somewhat with the findings of Wise et al [39] and Qi et al [40]. In the latter study all of the polyamines tested were inhibitory as low as 1–2 mM, whereas in the present study, spermine was a rather specific inhibitor of thyroid protein kinase C activity. The physiological significance, if any, of this observation is presently unclear, since all of the work of which we are aware on thyroid protein kinase C has been published in the form of preliminary reports. Protein kinase C activity has been detected in both rat and beef thyroid cytosol [28,41,42] and in beef thyroid plasma membrane [42]. Endogenous substrates of greater than 200, 60, 38, 33, and 38 kDa

have been reported in beef thyroid cytosol and plasma membrane fractions, respectively [41–42]. Time studies have not yet been reported.

It should be noted that we have not been able to detect calcium-activated calmodulin-dependent protein kinase activity in rat thyroid cytosol, whereas the activity is demonstrable in beef thyroid cytosol [41].

In summary, we have shown that polyamines stimulate messenger-independent protein kinase activity in the cytosol and nuclear fractions of rat thyroid. Previous studies that show a temporal correlation between an increase in ornithine decarboxylase activity/polyamine levels and RNA synthesis suggest that these observations may be physiologically important. The polyamines inhibit cyclic-AMP-dependent protein kinase activity; our data show that the polyamines inhibit catalytic activity rather than activation of the enzyme. Among the polyamines tested, only spermine inhibited calcium/phospholipid-dependent protein kinase activity. The physiological role of the inhibitory effects of polyamines on thyroid protein kinases remains speculative and awaits further study.

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