PARALLEL REGULATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE AND PHOSPHOPROTEIN PHOSPHATASE IN RAT THYROID

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1. Introduction

The reversible phosphorylation and dephosphorylation of enzymes and regulatory proteins is an important cellular control mechanism [1]. While there are several reports characterizing thyroidal protein kinases and their regulation by TSH [2–6], little is known about the enzymes that dephosphorylate (inactivate) thyroid phosphoproteins. The presence of phosphoprotein phosphatase (PP) was shown in calf thyroid [7] but there was no examination of the effects thereon of TSH. Changes in PP activity could be of importance in limiting, and thus regulating the magnitude and duration of cAMP effects in thyroid. It was of interest therefore to see if rat thyroid contains this enzyme, and if so, whether or not it is regulated by TSH. The data presented here confirm the presence of PP activity in rat thyroid, and demonstrate parallel regulation of thyroid cAMP-dependent protein kinase (PK) and PP by TSH.

2. Materials and methods

Male Holtzman rats (~200 g) were maintained on standard laboratory chow and tap water. Hypophysectomized male Holtzman rats (~200 g) were obtained from Hormone Assay Labs and maintained on standard laboratory chow and tap water supplemented only by orange slices. Animals 4–6 were assigned to each treatment group. At the time of sacrifice, the rats were anesthetized with ether and exsanguinated by cardiac puncture. The serum was removed and frozen at −10°C for hormone measurement. The thyroid glands were removed and trimmed of excess fatty material. Trimmed thyroid glands from each treatment group were pooled, weighed and homogenized in 25 vol. 0.25 M sucrose with polytron (Brinkmann Instruments). The homogenates were centrifuged at 100 000 × g for 50 min at 4°C and the supernates were used for enzyme assays after protein determinations [8].

PK activity was assayed in triplicate as in [9] with minor modifications. The incubation mixture (final vol. 200 μl) consisted of 100 μg enzyme protein, 180 μg mixed calf thymus histone (Calbiochem), 50 mM potassium phosphate buffer (pH 6.5), 10 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP (3–5 × 10^5 cpm) (ICN), ± 1 μm cAMP (Schwarz-Mann). The reaction, run at 37°C, was initiated by the addition of [γ-^{32}P]ATP and terminated after 10 min by addition of ~2 ml 5% trichloroacetic acid (TCA) followed by 50 μl of (625 mg %) bovine serum albumin, fraction V, as carrier protein. The precipitated protein was collected on Whatman GF/A glass fiber paper, washed extensively with 5% TCA and counted in 5 ml Bray's solution [10] in a Searle Mark (R) II liquid scintillation system. Activity is expressed as pmol P incorporated.mg enzyme protein^{-1}. min^{-1}.

^{32}P-labeled histone was prepared as a substrate for PK assay. The reaction mixture (final vol. 1 ml each tube) contained ~100 μg purified bovine thyroid PK (purified as in [11]), 900 μg mixed calf thymus histone, 50 mM potassium phosphate buffer (pH 6.5), 10 mM $MgCl_2$, 1 μM cAMP and 1 μM [γ-^{32}P]ATP (~3–5 μCi). The tubes were incubated at 37°C for 30 min after which 0.25 ml 100% TCA was added to terminate the reaction. Precipitates were recovered by centrifugation (1200 × g, 10 min), resuspended in 1 ml water and reprecipitated by addition of 0.25 ml

100% TCA. This washing step was repeated once more and the final precipitate was taken up in ~1 ml 2 mM Hepes buffer (pH 7.0) and dialyzed overnight in the cold against the same buffer. The mixture was then centrifuged at 20 000 × *g* for 10 min and the supernatant was used as a substrate for PP assay.

PP activity was assayed in triplicate as in [12] with minor modifications. The incubation mixture (final vol. 200 μl) consisted of 100 μg enzyme protein, 20–50 μg ^{32}P-labeled histone (2–5 × 10^4 cpm) and 10 mM Hepes (Sigma), 0.1 mM DTT (Sigma) buffer (pH 7.0). The reaction, run at 30°C, was initiated by the addition of [^{32}P]histone and terminated after 10 min by addition of 300 μl 33% TCA followed by 100 μl (625 mg%) bovine serum albumin, fraction V, as carrier protein. The precipitate was removed by centrifugation (1200 × *g*, 10 min). To 400 μl supernate, 50 μl 10 mM KH_2PO_4 followed by 150 μl ammonium molybdate (5 g/100 ml) were added and the phosphomolybdate was extracted with 1 ml isobutanol. After separating the two phases by centrifugation (1200 × *g*, 10 min), 0.8 ml organic phase was pipetted into a vial and counted in 2 ml NCS (Amersham) and 10 ml Econofluor TM (New England Nuclear). Activity is expressed as pmol P released.mg enzyme protein^{-1}. min^{-1}.

Serum TSH was measured by radioimmunoassay [13] using reagents supplied by NIAMDD rat pituitary hormone distribution program. Serum T_4 was measured by radioimmunoassay, using reagents from Abbott Labs and serum T_3 was measured by modification of the radioimmunoassay in [14]. Data were analyzed for statistical significance by Student's *t*-test.

3. Results

Initial experiments demonstrated PP activity in rat thyroid nuclear, particulate, and soluble (100 000 × *g* supernates) fractions (data not shown here). All studies reported herein were carried out with the soluble enzyme. PP activity was enhanced in vitro by 100% with 2 mM $MgCl_2$ and 60% by 2 mM $MnCl_2$, whereas 2 mM $CoCl_2$ and NaF had no effect. However, higher concentrations of $CoCl_2$ (5–10 mM) and NaF (5–20 mM) inhibited enzyme activity by 50–75% and 10–30%, respectively (data not shown here). Addition of up to 1 U TSH, 5 μM cAMP, or 250 μM T_3 or T_4 directly into the reaction mixture had no effect on PP activity (data not shown here).

3.1. *Effect of thyroid stimulation on enzyme activities*

In these experiments, rats were given 'goitrogen' (4-hydroxy-6-methyl-2-thiopyrimidine) (Sigma) 100 mg/l in drinking water for a period of 0–6 days. Though the magnitude of change in enzyme activities differed slightly from experiment to experiment (4 expts), the results were qualitatively uniform. Results of a representative experiment are summarized in fig.1. Circulating TSH levels rose slightly on day 1, increased sharply on day 2, and thereafter steadily increased. Serum T_3 (and T_4 (not shown)) levels fell sharply on day 1 and thereafter remained well below normal levels. PK activity increased by 60% over con-

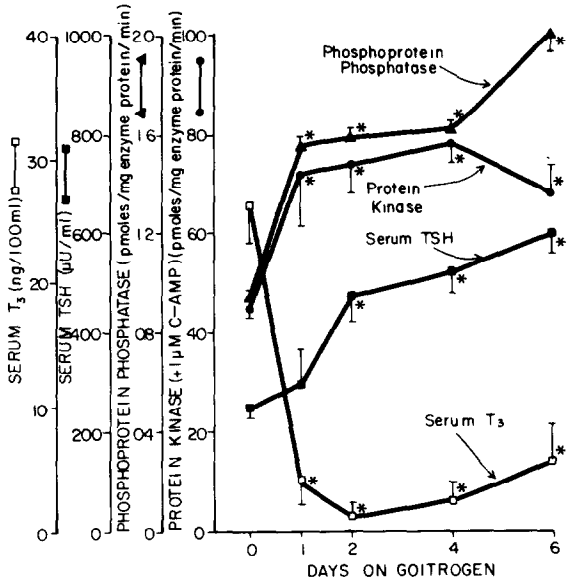

Fig.1. Effect of goitrogen on rat thyroid cAMP-dependent protein kinase and phosphoprotein phosphatase activities and serum TSH and T_3. Groups of 4 animals were given a goitrogen (4-hydroxy-6-methyl-2-thiopyrimidine) in their drinking water for 0–6 days. The animals were exsanguinated and extirpated thyroids assayed for enzyme activities. Each serum sample was assayed for TSH and T_3. Enzyme activities are expressed as pmol P incorporated (PK) or released (PP).mg enzyme protein^{-1}. min^{-1}. Serum TSH levels are expressed as μU/ml and T_3 levels as ng/100 ml. Each point represents a mean of 3 determinations for enzymes and 4 determinations for TSH and T_3 and the vertical bars show standard error of the mean. *Significantly ($p < 0.025$) greater than control.

trol ($p < 0.025$) on day 1 and remained significantly elevated thereafter; –cAMP/+cAMP PK activity ratios remained constant (within limits of experimental error) throughout. PP activity increased by 65% over control ($p < 0.025$) on day 1 and remained significantly elevated thereafter. [Acute administration (i.p.) of 1–2 U of TSH (Armour) did not change enzyme activities over 1–24 h (data not shown here).]

3.2. *Effect of suppresssion of thyroid function on enzyme activities*

3.2.1. Thyroid hormone administration

In this series of experiments, endogenous TSH secretion and, in consequence, the activity of the thyroid gland were suppressed by administration of T_4 (L-thyroxine, Sigma) or T_3 (3,5,3'-L-triiodothyronine, Sigma) either in drinking water (5 mg/l) for a period of 0–6 days or by daily intraperitoneal injections of 20 μg for a period of 0–3 days. When T_4 was given in drinking water (fig.2), circulating levels of TSH fell sharply on day 1 and remained significantly depressed thereafter. PK activity declined by 10% below control on day 1 and by 20% below control ($p < 0.05$) on day 2 and thereafter remained significantly below control levels; –cAMP/+cAMP PK activity ratios remained constant (within limits of experimental error) throughout. PP activity declined by 30% below control ($p < 0.025$) on day 1 and thereafter remained significantly below control levels. Though the magnitude of change in enzyme activities differed from experiment to experiment (4 expts), the results were qualitatively similar. Similar results were obtained when the rats were either maintained on T_3 in drinking water or were given T_3 or T_4 by intraperitoneal injections (data not shown here).

3.2.2. Hypophysectomy

The effect of hypophysectomy was studied over a period of 0–4 days (fig.3). Serum TSH levels fell precipitously on day 1 and thereafter remained at these levels. PK activity decreased by 20% below control ($p < 0.05$) on day 1 and thereafter showed no significant further change; –cAMP/+cAMP PK activity ratios remained unaltered (within limits of experimental error) throughout. PP activity decreased by 25% below control ($p < 0.025$) on day 1 and declined further on days 2 and 3.

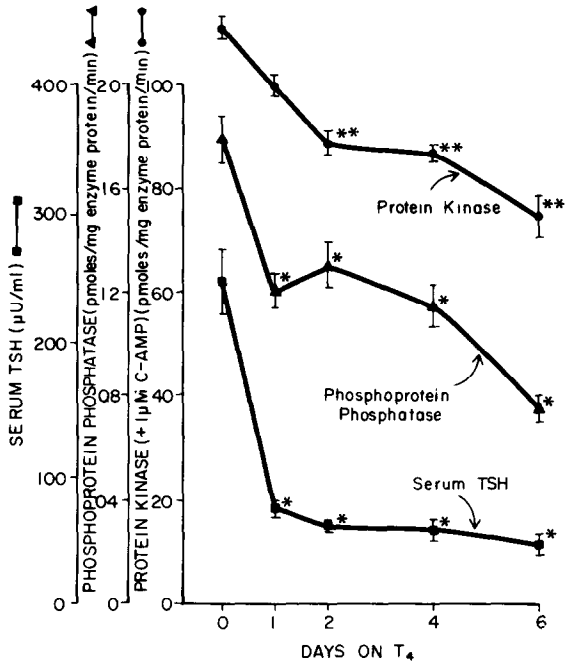

Fig.2. Effect of T_4 on rat thyroid cAMP-dependent protein kinase and phosphoprotein phosphatase activities and serum TSH. Groups of 4 animals were given T_4 (L-thyroxine) in their drinking water for 0–6 days. The animals were exsanguinated and extirpated thyroids assayed for enzyme activities. Each serum sample was assayed for TSH. Enzyme activities are expressed as pmol P incorporated (PK) or released (PP).mg enzyme protein^{-1}. min^{-1}. Serum TSH levels are expressed as μU/ml. Each point represents a mean of 3 determinations for enzymes and 4 determinations for TSH and the vertical bars show standard error of the mean. *Significantly ($p < 0.025$) lower than control. **Significantly ($p < 0.05$) lower than control.

4. Discussion

The results presented herein confirm the presence in rat thyroid of PP activity. The enzyme shares many features with PPs from other sources (see [15]) including calf thyroid [7] in that it has multiple subcellular localizations and its activity in vitro is enhanced by Mg^{2+} and Mn^{2+} and inhibited by Co^{2+} and F^-. The data presented here clearly show that thyroid PK activity (with and without cAMP) is elevated in the presence of high endogenous TSH (fig.1) and depressed by TSH lack (fig.2,3), thus confirming the findings [5,6]. What is of particular significance is the finding

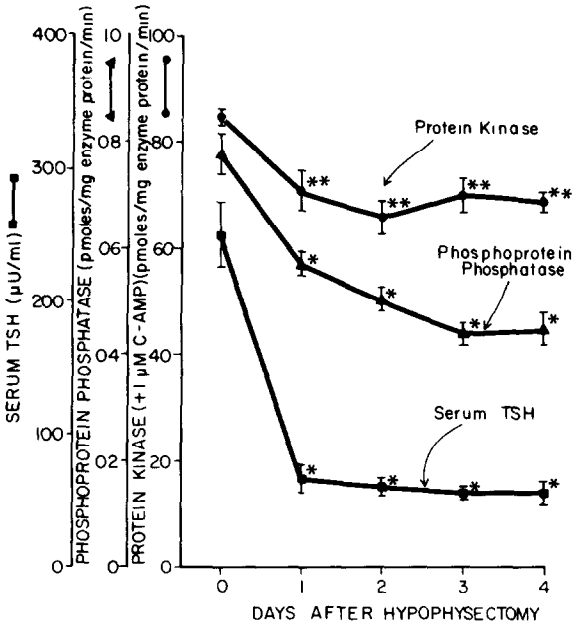

Fig.3. Time-related effect of hypophysectomy (as measured daily, up to 4 days, after pituitary extirpation) on rat thyroid cAMP-dependent protein kinase and phosphoprotein phosphatase activities and serum TSH. Groups of 6 animals were exsanguinated and extirpated thyroids assayed for enzyme activities. Each serum sample was assayed for TSH. Enzyme activities are expressed as pmol P incorporated (PK) or released (PP).mg enzyme protein^{-1}. min^{-1}. Serum TSH levels are expressed as μU/ml. Each point represents a mean of 3 determinations for enzymes and 6 determinations for TSH and the vertical bars show standard error of the mean. *Significantly ($p < 0.025$) lower than control. **Significantly ($p < 0.05$) lower than control.

that, under these conditions, changes in PP activity parallel those in PK activity. These changes in PP activities cannot be explained as a direct effect of TSH and/or T_3/T_4 on the enzyme, since these hormones did not influence PP activity in vitro. It is apparent, therefore, that PP in rat thyroid is also regulated by TSH and, further, that this regulation parallels regulation of PK. While there is some precedent for stimulation of PP by hormones in their target tissues [16,17], we are not aware of any studies in which the activities of PK and PP were shown to be subject to parallel regulation. Similar findings with respect to TSH effects on thyroid adenylate cyclase and phosphodiesterase activities have been reported in [18]. They showed parallel regulation of these two 'opposing' enzymes under conditions of chronic TSH stimulation or depletion. There is, therefore, substantive precedent for this type of autoregulatory mechanism in thyroid.

Obviously, it would be premature to assign physiological significance to our findings until one can find the endogenous substrate(s) that is(are) phosphorylated and dephosphorylated. However, the findings [19,20] indicate that phosphohistone (phosphoprotamine) phosphatases may be representative of physiologic (as opposed to non-specific) PPs. Additionally, the findings [21,22] that TSH induced the phosphorylation of histone suggests that our findings may have physiological significance.

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