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CYCLIC AMP BINDING PROTEINS IN THE RAT THYROID CYTOSOL

EFFECTS OF SUPPRESSION AND STIMULATION OF THYROID ACTIVITY

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Summary

Suppression of thyroid activity by treatment of rats with thyroxine in vivo significantly decreases the relative concentration of protein kinase regulatory subunits in the thyroid cytosol. Stimulation by thyroid-stimulating hormone (TSH) in rats previously treated by thyroxine for 5 days significantly increases the relative concentration of regulatory subunits. Two species of regulatory subunits of protein kinases were separated by ion exchange chromatography. The observed modifications after thyroxine and TSH treatment affect both species of regulatory subunits to the same extent, whereas chronic stimulation by propylthiouracil for 30 days preferentially increases the type II regulatory subunit.

Introduction

Many studies have demonstrated that cyclic AMP is involved in the functional activation of the thyroid by thyroid-stimulating hormone (TSH) and probably in the trophic action of this hormone (see Ref. 1 for review).

It is generally believed that cyclic AMP is able to activate cyclic AMP-dependent protein kinases after coupling with a regulatory subunit. It has also been

shown that the regulation of thyroid activity during supressive treatments (hypophysectomy or thyroxine administration) or stimulation (administration of propylthiouracil) may involve variations in the concentration of protein kinases [2—3]. Furthermore, thyroid tissue is known to contain type I and type II cyclic AMP-dependent protein kinases [4—7] which can be separated by ion exchange chromatography.

Type I and II protein kinases have been described in many other types of tissue and it has been shown that the relative amounts of the two enzymes can vary during different events affecting cell life [8—12]. In vivo studies of the thyroid have demonstrated that supressive treatment of animals with thyroxine [7] or stimulation with propylthiouracil [13] may also induce changes in the relative levels of type I and preferentially type II protein kinase. In these studies, enzyme concentrations were estimated after measuring the protein kinase activity. Study of the variations in the concentration of the regulatory subunits is also of interest for the following reasons. (1) Measurement of the activity of the holoenzymes may be influenced by different factors, especially the degree of dissociation of the holoenzyme as well as the presence of inhibitors which might also regulate protein kinases [14]. (2) It is generally agreed that the two cyclic AMP dependent protein kinases differ with respect to their regulatory and not their catalytic subunits [15,16]. Consequently, the action of TSH on the protein kinases might preferentially involve the cyclic AMP binding proteins. (3) Variations in the concentration of cyclic AMP binding proteins might be specifically involved in hormone action. In this respect Majumder and Turkington [17] showed that under certain experimental conditions in mammary cells, prolactin induces an increase in cyclic AMP binding proteins without changes in protein kinases [17].

The present study shows that in the thyroid cytosol the concentration of regulatory subunits of cyclic AMP dependent protein kinases is significantly modulated by suppressive (thyroxine administration) or stimulating (TSH) treatment. Separation of the two types of subunits isolated by ion exchange chromatography is also shown and the relative amounts of these subunits are compared under different experimental conditions.

Material and Methods

Chemicals. TSH was purchased from Organon, France and propylthiouracil from Serlabo. L-Thyroxine, calf thymus histone IIA, dithiothreitol, EDTA disodium salt were purchased from Sigma. Cyclic [^{3}H]AMP (32—37 Ci/mmol) and γ-[^{32}P]ATP (0.5—3 Ci/mmol) were from the Radiochemical Centre, Amersham, England; DEAE-Sephadex A-25 from Pharmacia Fine chemicals. Other chemicals were from Merck. Cellulose ester filters (HAWP 02500) were from Millipore corporation and columns from Pharmacia.

Treatment of rats. Male Sprague-Dawley rats (150—200 g) were used in this study. Supression of the thyroid activity was obtained by adding thyroxine in drinking water (3 mg/l dissolved in 0.05% bovine serum albumin). Stimulation of the gland was achieved by injecting 200 mU TSH per animal 6 h before they were killed, or by adding propylthiouracil in drinking water (1 g/l dissolved in 1% sucrose).

Cytosol preparation. Glands were homogenized in 20 vol. (v/w) of 10 mM Tris-HCl/4 mM EDTA Na_2/1 mM dithiothreitol pH 7.0 (buffer H) with an Ultra-Turrax blendor for 10 s (3 times) at max. speed. The homogenate was centrifuged at 900 × *g* 10 min and the resulting supernatant at 105 000 × *g* 1 h. The last supernatant (cytosol) was collected and stored at −20°C and the protein determined using an aliquot. For each experiment, cytosol was prepared from the pooled glands of at least 3 animals of the same lot.

Assay for regulatory subunits of protein kinases. Cytosol proteins (1 mg/ml) were incubated in buffer H containing $2 \cdot 10^{-7}$ M cyclic [^{3}H]AMP for 6—10 h at 2°C. These conditions offered a satisfactory compromise ensuring both the stability of the cyclic AMP binding proteins and maximum exchange between the exogenous cyclic [^{3}H]AMP and endogenous cold cyclic AMP bound to proteins. In these conditions cyclic [^{3}H]AMP was a saturating concentration with more than 90% of sites being labelled. It was not necessary to take into account any eventual variations in total endogenous cold cyclic AMP resulting from the different treatments of the animals. Indeed under our experimental conditions the concentration of endogenous cyclic AMP varied from 2—5 nM i.e. a negligible amount compared to the quantity of cyclic [^{3}H]AMP introduced into the incubation medium. The possibility that adenosine containing metabolites other than cyclic AMP (mainly adenosine in the thyroid) might interfere was also ruled out. We confirmed that adenosine 10^{-5} M does not compete with cyclic AMP for its binding proteins separated by gel filtration, and Orgiazzi et al. [25] demonstrated that AMP itself does not compete. It is also known [26, 27] that neither AMP nor adenosine inhibit the stimulation of thyroidal protein kinases by cyclic AMP. Consequently, under these conditions the concentration of cyclic AMP binding proteins can be assayed as the radioactivity retained on the Millipore filter after filtration of the incubation medium and counting of the radioactivity on the dried filters in a toluene Permafluor I mixture.

Other methods. Protein kinase activity was assayed as previously described [6], protein measured according to Lowry et al. [18] and DNA according to Burton [19].

Comparative separations of cyclic AMP binding proteins by DEAE-Sephadex chromatography were performed in parallel using a multichannel gradient former, a multichannel pump and multichannel collector. Volume of gels and flow rates were rigorously controlled to be identical from one column to another and it was previously verified that in our experimental conditions identical elution profiles of cyclic AMP binding proteins were obtained in the same experiment with two identical samples.

Results

Effect of treatment on the concentration of regulatory subunits

The results of a typical experiment (Table I) show that the quantity of cyclic AMP binding protein per mg total protein was significantly decreased after treatment of rats with thyroxine for 5 days. Conversely, when TSH was injected 6 h prior to killing in rats pretreated with thyroxine for 5 days, the amount of cyclic AMP binding protein was increased. Similar results were ob-

TABLE I

EFFECTS OF IN VIVO ADMINISTRATION OF THYROXINE AND TSH ON AMOUNTS OF CYCLIC AMP BINDING PROTEINS IN THYROID CYTOSOL

Thyroxine and TSH were administered as described in Methods. 0.2 mg of cytosol proteins was incubated with cyclic [^{3}H]AMP and the bound radioactivity measured. In the thyroxine treatment study, the control consists of animals from the same lot which have not been treated. In the TSH treatment experiment, the control value was obtained in animals from the same lot pretreated with thyroxine only.

Treatment	Activity per mg protein			Activity per μg DNA		
	pmol cyclic AMP bound	percent of control		pmol cyclic AMP bound	percent of control	
Normal	1.10	100		33	100	
Thyroxine (5 days)	0.67	61	100	26	80	100
TSH (5 days after thyroxine)	1.07		163	40		152

tained in all experiments although the degree of response varied. The mean response after treatment with thyroxine was a 37% decrease (5 groups of animals) and after TSH injection was a 35% increase (4 groups of rats) in cyclic AMP binding protein. It should be noted that the above treatments may have induced a variation in extracellular thyroglobulin content thus rendering the observed variations of little significance. Nevertheless, Table I shows that when the results are expressed per μg DNA the quantity of cyclic AMP bound protein was consistently decreased after thyroxine treatment and increased after TSH injection. Similar results were obtained when cyclic AMP binding protein was expressed per mg fresh tissue (44% decrease after thyroxine treatment and 69% increase after administration of TSH) or per whole thyroid gland (25% decrease and 52% increase after thyroxine and TSH respectively).

Separation of the regulatory subunits

It was first verified that the incubation of cytosol with cyclic [^{3}H]AMP in the above conditions (see methods) led to the dissociation of the regulatory subunits of the protein kinases yielding a form of homogeneous molecular weight. Indeed when cytosol was filtered on a 1.5m Bio-Gel column (results not shown) several cyclic AMP binding species were identified whereas filtration of the cytosol preincubated with cyclic [^{3}H]AMP showed that all the bound cyclic [^{3}H]AMP was eluted as a single peak corresponding to a species of lower M_r.

Fig. 1 illustrates the separation of cyclic AMP-binding regulatory subunits during column chromatography on DEAE-Sephadex A-25 of the cytosol preincubated in the presence of cyclic [^{3}H]AMP. Free cyclic [^{3}H]AMP was slightly retarded and was eluted by extensive washing. Fig. 1 shows that the bound cyclic [^{3}H]AMP was entirely retained on the column and eluted as 2 peaks at 0.1 M and 0.3 M on a NaCl concentration gradient. The figure also demonstrates that the protein kinases were completely dissociated during the preincubation since the histone kinase activity was almost totally excluded and was no longer cyclic AMP dependent. A correct estimate of the qualitative distribution of the two species binding to cyclic AMP was obtained since reincubation of

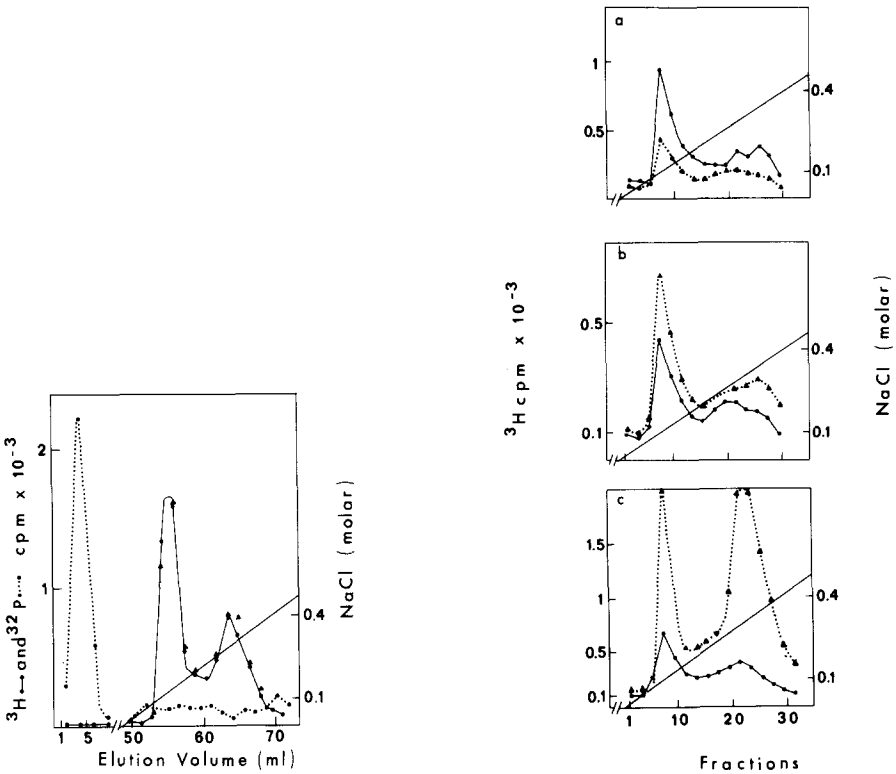

Fig. 1. DEAE-Sephadex A-25 elution pattern of cyclic AMP binding proteins and histone kinases in rat thyroid cytosol. 3 mg proteins were preincubated in the presence of cyclic [^{3}H]AMP and then chromatographed on a DEAE-Sephadex A-25 column (0.9 × 3 cm) previously equilibrated with buffer H. The column was washed with the same buffer until radioactivity was near 0. Bound cyclic [^{3}H]AMP was eluted with a linear gradient from 0—0.45 M NaCl in the buffer H. Flow rate was 10 ml/h. Fractions were assayed as follows: radioactivity retained on Millipore filters after filtration of an aliquot (160 µl) of each fraction (●———●); histone kinase activity (●- - - - -●); radioactivity retained on Millipore filters after reincubation 160 µl of each fraction in the presence of $2 \cdot 10^{-7}$ M cyclic [^{3}H]AMP (▲———▲).

Fig. 2. DEAE-Sephadex A-25 elution patterns of cyclic AMP binding proteins in the thyroid cytosol after supressive or stimulating treatments: Conditions are as described in Fig. 1 except that 2 mg proteins only were loaded on the columns. Volume of collected fractions is 0.75 ml. Cytosol from (a) control rats (●———●) or from rats treated with thyroxine for 5 days (▲- - - -▲). (b) Rats treated for 5 days with thyroxine (●———●); same treatment and injection of 200 mU TSH 6 h before killing (▲- - - - -▲). (c) Control rats (●———●) or rats treated for 30 days with propylthiouracil (▲- - - - -▲).

each fraction eluted on the gradient in the presence of $2 \cdot 10^{-7}$ M cyclic [^{3}H]-AMP did not increase the radioactivity retained on the Millipore filter.

Specificity of action after treatment of rats

It was of interest to know whether supressive and stimulating treatment preferentially affected one of the two species identified after chromatography on DEAE-Sephadex.

(a) Effects of thyroxine. Comparison of the elution profiles of cyclic AMP binding proteins of the thyroid cytosol from control rats with those of the thyroid from rats of the same lot pretreated with thyroxine (Fig. 2a) showed that thyroxine treatment caused a decrease (about 50% in this case) in the rela-

tive amount of cyclic AMP binding proteins. A similar comparison was made in three lots of three animals. In all cases, the decrease in cyclic AMP binding proteins involved both species to the same extent.

(b) Effects of TSH. Fig. 2b shows that when TSH was injected 6 h before killing in rats pretreated with thyroxine for 5 days, the total amount of cyclic AMP binding proteins increased (about 50% in this case). Separation of the proteins by DEAE-Sephadex chromatography demonstrated that both species of cyclic AMP binding proteins increased to the same extent. Identical results were obtained in two other lots of animals. Conversely, when TSH was injected in rats after 30 days pretreatment with thyroxine (not shown) both peaks of cyclic AMP binding protein remained unchanged.

(c) Effects of chronic stimulation. After chronic stimulation of the thyroid by treatment with propylthiouracil for 30 days a marked increase in cyclic AMP binding proteins was observed. The elution profile obtained by DEAE-Sephadex chromatography clearly differed from that obtained in control thyroids from rats of the same lot (Fig. 2c). The increase preferentially involved the second peak displaying a 4-fold increase over the control value, whereas the first peak only showed a 2-fold increase. This preferential increase of the second peak was reproducible since in two other experiments peak II displayed a 2.5-fold increase whereas peak I increased 1.5-fold.

Discussion

The results of this study show that the two regulatory subunits of the cyclic AMP dependent protein kinases can be separated after dissociation of the holoenzymes of the thyroid cytosol and chromatography on DEAE-Sephadex A-25. Similar results have been obtained by Evain et al. [20] from the cytosol of the adrenal gland. Optimum conditions for estimation of the amount of cyclic AMP-binding proteins in the thyroid cytosol are also presented in this study. In other tissues, cyclic AMP binding proteins other than regulatory subunits of protein kinases have been demonstrated [28,29]. However they are not present in all tissues [29]. In our conditions they are not found in the thyroid cytosol. In any case, they would not interfere in the present study since it has been shown that, in the thyroid, protein bound cyclic AMP resides almost uniquely in association with the regulatory subunits of the protein kinases [30].

Our results demonstrate that the amount of cyclic AMP-binding proteins varies according to the functional state of the gland. Supression of the thyroid activity due to thyroxine treatment for 5 days is accompanied by a significant decrease in the relative amount of cyclic AMP-binding proteins; the decrease involved both protein species separated on DEAE-Sephadex to the same extent. TSH injection after pretreatment with thyroxine induced a significant increase in both protein species. Under certain experimental conditions, the relative amount of type I to type II regulatory proteins can be significantly modified, i.e. chronic stimulation with propylthiouracil for 30 days yielded a preferential increase in the type II protein.

Due to possible variations in the extracellular level of thyroglobulin, the choice of an appropriate reference for estimates of changes in cytosol protein concentration is problematic in the type of experiment reported in the present

study. Most authors express their results per mg protein or per a given weight of wet tissue [2,3,7,13] and in some cases per μg DNA [21]. The whole thyroid gland may also be used as a reference. The conclusions given above remain valid regardless of the reference mode employed. Furthermore, in one experiment we measured the variations in cyclic AMP binding proteins in the 105 000 $\times g$ pellet after treatment with thyroxine for 5 days or chronic stimulation for 30 days. Data being expressed per mg protein; the observed variations in the cytosol were also found in the 105 000 $\times g$ pellet.

The effect of supressive (thyroxine) or chronic stimulating treatment (propylthiouracil for 30 days) on cyclic AMP dependent protein kinase activities has been studied by many workers [2,3,7,13]. Data from these studies generally show that after treatment of animals with thyroxine or propylthiouracil, the changes in protein kinase activities are similar to our results involving the regulatory subunits. According to Delbauffe et al. [7] the decrease in type I and type II protein kinases is only found after 10 days of treatment with thyroxine. Accordingly, there may exist a dissociation in time between the observed effects involving protein kinase activity and those involving the regulatory subunits. Conversely, the specific increase of the type II regulatory subunit after chronic stimulation by propylthiouracil in this study is in agreement with the specific increase in type II protein kinase reported by Habhab et al. [13] after similar treatment. Thus, one can envisage the coordinated regulation of the levels of the regulatory and catalytic subunits, however it cannot be rigorously assumed that their regulation is identical. Differences have in fact been suggested in the mammary gland by work of Majumder and Turkington [17] and in neuroblastoma-glyoma hybrid cells by Walter et al. [31].

To our knowledge, the partial or total recovery of cyclic AMP dependent protein kinases occuring after TSH injection in rats pretreated with thyroxine and over a relatively short but sufficiently long period to allow stimulation of synthesis of RNA and proteins has not been studied previously. The lack of effect of TSH after long term thyroxine administration may be similar to the results of other workers who have reported that various responses of the thyroid to TSH are altered after treatment with thyroid hormones [22—24].

In conclusion, our results may at least partially be taken as evidence for the operation of complex regulatory mechanisms through which the cell is able to adapt its capacity to receive a stimulatory signal at a basal level of stimulation. Further studies are needed to identify the kinetic properties and molecular basis of these regulatory mechanisms.

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