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EFFECT OF THYROID STATUS ON β-ADRENERGIC RECEPTOR, ADENYLATE CYCLASE ACTIVITY AND GUANINE NUCLEOTIDE REGULATORY UNIT IN RAT CARDIAC AND ERYTHROCYTE MEMBRANES

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Abstract—The effect of thyroid hormone on the β-receptor coupled adenylate cyclase in rat crude cardiac membranes was analysed by measuring the number of DHA-binding sites, adenylate cyclase activity and the amount of cholera toxin catalysed ADP-ribosylation of a protein with a molecular weight of 42,000 in cardiac and erythrocyte membranes. In crude rat cardiac membranes, the number of DHA-binding sites (78 ± 15 fmole/mg protein in the euthyroid state) is increased to 158 ± 20 fmole/mg protein in the hyperthyroid state and decreased to 51 ± 6 fmole/mg protein in the hypothyroid state; the affinity of the binding sites remained unchanged (K_D 2.9–4.3 nM). L-Isoprenaline (10^{-4} M)-stimulated adenylate cyclase activity varied in parallel to the number of DHA-binding sites in hyper- and euthyroidism. Thyroid hormone, however, did not influence GppNHp (10^{-4} M)-stimulated adenylate cyclase activity. Cholera toxin catalysed ADP-ribosylation of normal crude cardiac membranes resulted in a 1.8 fold increase in adenylate cyclase activity in the presence of GTP (10^{-4} M) and L-isoprenaline (10^{-4} M), presumably as a result of inhibition of GTPase. In crude cardiac membranes cholera toxin catalyses the ADP-ribosylation of one major protein, which comigrates on sodium dodecylsulfate–polyacrylamide gel electrophoresis with the putative regulatory component of adenylate cyclase (mol. wt 42,000). In different thyroid states the amount of the regulatory component (as determined by cholera toxin dependent labelling) was equal (112 fmole/mg protein in euthyroid crude cardiac membranes). Basal activity of adenylate cyclase showed a significant difference between activity in euthyroid (3.7 ± 0.2 pmole cAMP/mg protein/min) and hypothyroid (5.4 ± 0.2 pmole cAMP/mg protein/min), but not in hyperthyroid crude cardiac membranes (3.4 ± 0.2 pmole cAMP/mg protein/min). Our results indicate, that thyroid hormone regulates the number of DHA-binding sites and basal activity (in hypothyroidism) in crude cardiac membranes and thereby causes different results in L-isoprenaline-induced adenylate cyclase activity.

Hyperthyroidism leads to a state of apparent increased β-adrenergic activity whereas hypothyroidism causes the opposite effect [1]. It has been shown in rat heart tissue [2–6] that these changes of increased β-adrenergic effect correlate with different numbers of β-adrenergic receptors. Moreover the thyroid state influences the stimulatory effect of catecholamines on adenylate cyclase activity in the same way [3, 6].

These data can be explained with a thyroid hormone induced *de novo* protein synthesis of receptor- and enzyme-molecules [4]. Contrary to this parallel relationship between increase of β-receptors and adenylate cyclase activity. Bilezikian *et al.* [7] showed in hyperthyroidism an unchanged number of β-receptors, but a catecholamine dependent amplification of the enzyme activity. Malbon *et al.* [8] found no detectable change in the number of β-receptors but changes in the activity of adenylate cyclase from isolated fat cell-membranes depending directly on the thyroid state. The authors discussed a thyroid hormone dependent modulation of the coupling mechanism between β-adrenergic receptor and adenylate cyclase as an explanation for their results.

To elucidate this modulating role of thyroid hormone, it is necessary to characterize each component of the β-receptor coupled adenylate cyclase system in different thyroid states. In recent years genetic and biochemical evidence has indicated that hormone sensitive adenylate cyclase consists of at least three separate components: a hormone-receptor, a catalytic unit and a regulatory component [9]. Cassel and Pfeuffer [10], as well as Gill and Meren [11], have described conditions which permit identification of cholera toxin specific ADP-ribosylation in pigeon erythrocyte membranes. A major cholera toxin specific target of ADP-ribosylation is a membrane protein (mol. wt 42,000), experimentally identified as the adenylate cyclase associated GTP-binding protein described by Pfeuffer [12] and Spiegel *et al.* [13].

We, therefore, investigated whether the amount of toxin specific incorporated ADP-ribose in the regulatory component of crude cardiac- and erythrocyte membranes is changed in different thyroid

Abbreviations used: GppNHp, guanosine 5′-(β,γ-imino) triphosphate; GTP, guanosine triphosphate; DHA, −dihydroalprenolol; ADP, adenosine diphosphate; NAD, nicotinamide adenosine diphosphate; regulatory component is used as a term for guanine-nucleotide binding protein, as it is not yet standardized, it is the same as N-protein, G-unit, GN-unit.

states, thereby altering the coupling mechanism between β-receptor and adenylate cyclase.

MATERIALS AND METHODS

Materials. Radiolabelled nucleotides were obtained from ICN, Chemicals and Radioisotope Division (Irvine, CA). Cholera toxin was from Schwarz/Mann (Orangeburg, NY). Other chemicals were the best grade commercially available.

Treatment of animals. Male rats (250–400 g) of the same age and weight before treatment were divided into three groups. One group was thyroidectomized 65 days before preparing the membranes and additionally given 1% calcium gluconate with drinking water. These animals were used for determination of ADP-ribosylated erythrocyte and cardiac membranes and adenylate cyclase activity in crude cardiac membranes. One additional group of rats was treated with 0.1% propylthiouracil (w/v) for 21 days. These rats were used for measurement of adenylate cyclase activity as well as [^{3}H]DHA-binding assays. The values for adenylate cyclase activity were identical in both groups.

Rats were made hyperthyroid by subcutaneous injection of triiodothyronine (0.5 mg/kg body wt). A stock solution of triiodothyronine was dissolved in 0.1 M NaOH, stored in a freezer, protected against light, and diluted shortly before use. Rats were injected once daily for 5 days and used 24 hr after the last treatment. These and the normal group were kept under identical conditions with free access to water, normal laboratory meal, with the exception of iodine-poor meal for the thyroidectomized rats.

Triiodothyronine and thyroxine serum levels. Compared to values in euthyroid rats ($T_3 = 1.2 \pm 0.14$ ng/ml, $T_4 = 4.0 \pm 0.7$ μg/100 ml) triiodothyronine serum level in T_3-treated rats was 9.0 ± 1.4 ng/ml (T_3-RIA) and thyroxine serum level in throidectomized or propylthiouracil-treated rats was below the level of detection (2.0 μg/100 ml; T_4-Emit. Merck, Darmstadt, F.R.G).

Crude cardiac membrane preparation. Rats were decapitated and their hearts quickly removed and freed from connective tissue, atria and valves. The ventricles were dissected and washed free from blood. The washed ventricular tissue was minced and a crude membrane fraction was prepared [14]. For the adenylate cyclase assay the membranes were quickly frozen at −70°.

Preparation of red cell membranes. Membranes were prepared by hypotonic lysis of red cells in 5 mM sodium phosphate buffer, pH 8.0 [15]. The membrane preparation was washed extensively in the same buffer, and stored in aliquots; equivalent to 2.0 mg protein in liquid nitrogen.

Cholera toxin treatment of crude cardiac and erythrocyte membranes. This was performed according to Kaslow *et al.* [16]. Membranes (1.5–4.0 mg per ml) were incubated with 12 mM potassium phosphate buffer, pH 7.5, 20 mM thymidine, 5 mM ADP-ribose, 20 mM arginine–HCl, 100 U/ml Trasylol, 0.1 mM GTP, 100 μg/ml cholera toxin (activated with 20 mM DTT at 30° for 10 min) and NAD [for labelling experiments [^{32}P]NAD$^+$ was 10 μM (5–30 Ci/nmole), for other experiments NAD$^+$ was 1 mM].

After addition of membranes, the mixture was incubated for 20 min at 30°. The reaction was terminated by addition of 10 vol. of ice cold potassium phosphate buffer 15 mM, pH 7.5, and washed three times by centrifugation. The final pellet was resuspended in 10 mM Tris–HCl, pH 7.5, containing 1.0 mM $MgCl_2$ and assayed as indicated at 30° for 20 min.

Enzyme assay. Adenylate cyclase activity was determined as previously described [17], using 0.125 mM ATP as substrate. Results are expressed as cAMP generated in pmole/mg protein/min or as indicated and are the mean of triplicate determinations.

Binding assay. Membrane suspensions, freshly prepared (0.4–0.8 mg protein) were incubated at 37° in 7.5 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$ with [^{3}H](−)dihydroalprenolol and unlabelled (−)alprenolol (final volume 1 ml). At the given time (usually 15 min) membrane bound and free ligands were separated by rapid filtration followed by two washes (10 ml each) on Whatman fiberglass filters (GF/C). Radioactivity on filters was determined by liquid scintillation counting (Insta Fluor, Fa. Zinsser, Frankfurt, F.R.G.).

Unspecific binding was determined in the presence of unlabelled (−) and (+)alprenolol, 10^{-5} M. It amounted to about 50% of maximal binding, as described by Krawietz and Erdmann [18].

SDS polyacrylamide gel electrophoresis. With membranes (2–4 mg/ml) discontinuous SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [19] as described by O'Farrell [20] using 10% acrylamide gels and electrophoresed at 400 V for 7 hr. After staining, destaining and drying, X-ray film was exposed to the gels. In order to identify the 42K-band, the cholera toxin specific target for ADP-ribosylation, a short exposure was performed [16] with the disadvantage of a faint autoradiogram.

Measurement of incorporated labelled ADP-ribose. Crude cardiac and erythrocyte membranes were incubated with 10 μM [^{32}P]NAD in the presence and absence of 100 μg/ml activated cholera toxin under conditions described previously under cholera toxin treatment of membranes. Radioactivity incorporated in mol. wt 42,000 protein was determined following separation by electrophoresis on polyacrylamide slab gels in the presence of sodium dodecyl sulfate. After staining, destaining, drying and autoradiography, the band corresponding to mol. wt 42,000 was excised and counted by liquid scintillation. The radioactivity in the membranes corresponding to mol. wt 42,000 protein bound was corrected for incorporation without toxin. The bands above and below the 42K band showed at least 38–61% less radioactivity incorporated. The amount of protein electrophoresed in each track was 0.33 mg.

Protein. Protein was measured according to the method of Lowry *et al.* [21].

RESULTS

The number of β-adrenergic receptors increases in hyperthyroidism and decreases in hypothyroidism. Table 1 shows our results, demonstrating the changes

Table 1. Effects of triiodothyronine and propylthiouracil treatment on DHA binding sites in rat crude cardiac membranes

Group	(−)DHA binding capacity (fmole/mg protein)	Dissociation constant (K_D) (nM)
Euthyroid ($n = 6$)	78 ± 15	3.0
Hyperthyroid ($n = 6$)	158 ± 20*	2.9
Hypothyroid ($n = 6$)	51 ± 6†	4.3

Binding experiments were carried out as described under Materials and Methods.
Values represent the mean ± S.E.M. of the indicated number of experiments done in triplicate.
*$P < 0.05$, †$P < 0.5$ compared to euthyroid.

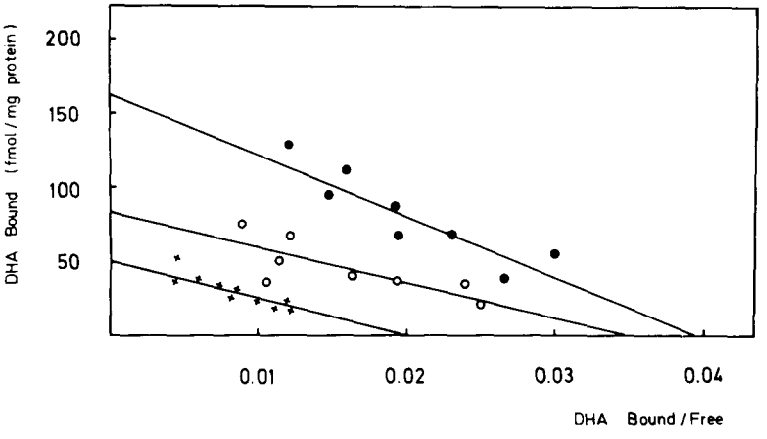

Fig. 1. Equilibrium binding of DHA to crude cardiac membranes from rats of different thyroid states: hyperthyroid (●——●), euthyroid (○——○), hypothyroid (×——×). Incubation was performed as described under Materials and Methods in the presence of increasing concentrations of unlabelled (−)alprenolol at 37° for 15 min. Each value was determined in triplicate. The data were plotted according to Scatchard. The binding capacity was calculated from the intercept of the plot with the ordinate, the dissociation constant (K_D) from the slopes of single plots (2.9–4.3 nM). Data were plotted and calculated on Hewlett Packard HP 9830.

Table 2. Influence of the thyroid hormone state on L-isoprenaline, and GppNHp dependent-stimulation of adenylate cyclase activity in rat crude cardiac membranes

Adenylate cyclase assay conditions	Adenylate cyclase activity in rat crude cardiac membranes					
	Hyperthyroid		Euthyroid		Hypothyroid	
($n = 6$)	pmoles cAMP / mg × min	Percent stimulation	pmoles cAMP / mg × min	Percent stimulation	pmoles cAMP / mg × min	Percent stimulation
Basal	3.4 ± 0.2	100	3.7 ± 0.2	100	5.4 ± 0.2*	100
L-isoprenaline (10^{-4} M)	7.4 ± 0.3*	217	5.7 ± 0.4	154	7.0 ± 0.4†	129
GppNHp (10^{-4} M)	19.6 ± 1.6		16.6 ± 1.8		15.4 ± 1.9	

Adenylate cyclase assay was performed as described under Materials and Methods.
*$P < 0.05$, †$P < 0.1$ compared to euthyroid.

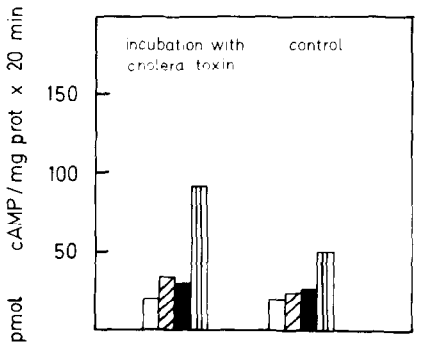

Fig. 2. Crude cardiac membranes (2–3 mg/ml) were preincubated with and without cholera toxin as described under Materials and Methods with L-isoprenaline (10^{-4} M), GTP (10^{-4} M); L-isoprenaline and GTP together demonstrate a more than additive stimulatory effect on adenylate cyclase activity in the cholera toxin treated membranes. Basal activity (□), L-isoprenaline (10^{-4} M) (▨), GTP (10^{-4} M) (■), L-isoprenaline (10^{-4} M) and GTP (10^{-4} M) together (▥).

of the number of DHA-binding sites in crude cardiac membranes of different thyroid states. The increase of DHA-binding sites in hyperthyroidism is about 2.0 fold and the decrease 0.3 fold in hypothyroidism compared to euthyroidism. The binding affinity of the receptor is not significantly altered (2.9–4.3 nM). The data of one representative experiment are shown in Fig. 1, in which binding capacity and receptor affinity in crude cardiac membranes of different thyroid states are calculated according to Scatchard [22]. Unspecific binding is defined and subtracted according to Krawietz and Erdmann [18].

Basal adenylate cyclase activity is almost equal in hyperthyroidism and euthyroidism (Table 2), whereas in hypothyroidism a significantly higher value of basal activity is measured.

Absolute values of catecholamine-stimulated adenylate cyclase activity in hypothyroidism are higher than in euthyroidism. Calculating these values in per cent over basal, a smaller catecholamine-induced effect on adenylate cyclase activity in hypothyroidism (129% over basal) than in euthyroidism (154% over basal) is observed, correlating with a 35% decrease of DHA-binding sites in hypothyroidism. Similarly, the absolute values of the difference between basal- and catecholamine-induced adenylate cyclase activity demonstrate decreasing amounts of cAMP produced, when euthyroidism is compared with hypothyroidism. GppNHp (10^{-4} M) increased adenylate cyclase activity independently of the thyroid state, resulting in absolute values between 16.6 and 19.6 pmole cAMP/mg protein/min.

The increased isoprenaline-stimulated adenylate cyclase activity in hyperthyroidism might be modulated additionally by a different amount of regulatory components. Therefore, the specific ADP-ribosylated protein in the presence of cholera toxin in crude cardiac and erythrocyte membranes was characterized.

Membranes were incubated with and without cholera toxin to measure the specific effect of cholera toxin catalysed ADP-ribosylation on adenylate cyclase activity. In crude cardiac membranes basal activity is identical in cholera toxin treated and control membranes (Fig. 2). Stimulation by L-isoprenaline (0.1 mM) or GTP (0.1 mM) shows a small increase over basal activity, but resulting in higher values of stimulated activity in cholera toxin treated membranes. Incubation of L-isoprenaline together with GTP induced only in cholera toxin treated membranes an increase of adenylate cyclase activity, which is more than additive. This result indicates a cholera toxin catalysed increase of activation of adenylate cyclase activity in rat crude cardiac membranes. In order to show that this specific effect on adenylate cyclase activity correlates with a cholera toxin catalysed labelling of a distinct membrane protein (mol. wt 42,000) by ADP ribose in crude cardiac membranes and erythrocyte membranes of rats, we compared the result with the proved labelling of human erythrocyte membranes [16] (Fig. 3). Each membrane preparation shows in the autoradiography a predominant protein band (mol. wt 42,000) in the presence of cholera toxin in crude cardiac and erythrocyte membranes of rats.

The autoradiography of the same cholera toxin catalysed ADP-ribosylated membranes does not allow any conclusion about different amounts of incorporated [^{32}P]NAD. Therefore, the amount of incorporated [^{32}P]NAD in the excised mol. wt 42,000 band was measured by counting the radioactivity. The amount of incorporated labelled ADP-ribose in the 42K band in crude cardiac membranes and in erythrocyte membranes of different thyroid states showed no difference (crude cardiac membranes: hyperthyroid 126, hypothyroid 116; erythrocyte membranes: hyperthyroid 107, hypothyroid 105—numbers in fmole/mg protein).

DISCUSSION

The increased catecholamine sensitivity of the cardiovascular system in hyperthyroidism and decreased sensitivity in hypothyroidism have been widely investigated [2–6]. Nevertheless the data in the literature are contradictory [7, 8, 25] in respect to the number of the β-receptors and the correlating change in the adenylate cyclase activity in different thyroid states. A changed catecholamine-induced adenylate cyclase activity in hyperthyroidism without any change in the number or affinity of β-receptors is reported by Bilezikian *et al.* [7] and Malbon *et al.* [8]. In order to explain their results, both authors postulate a mechanism, modulating the coupling between β-adrenergic receptor and enzyme. This mechanism is now supposed to be due to the function of the recently characterized regulatory unit coupling β-receptor and catalytic unit [9].

In order to elucidate the biochemical basis for the effects of thyroid hormone on the β-receptor coupled adenylate cyclase system, each of these components have to be characterized.

Influence of thyroid hormone on [^{3}H]*DHA binding capacity*

In rat cardiac membranes our results clearly show, that thyroid hormone influences the number of β-receptors (Table 1), according to the results of Ciar-

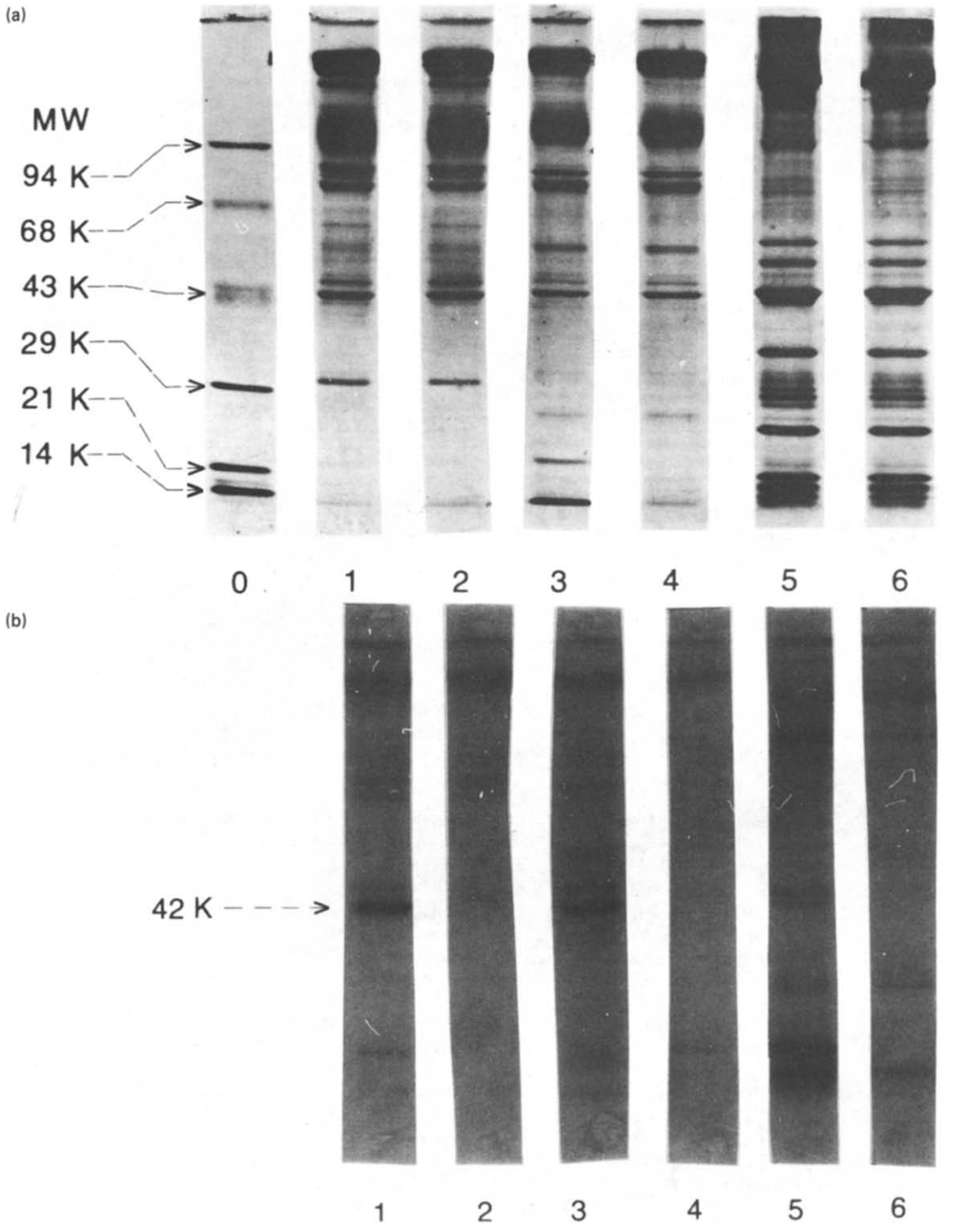

Fig. 3. (a) Sodium dodecylsulfate–polyacrylamide gel electrophoresis of human erythrocyte membranes, rat erythrocyte membranes and rat crude cardiac membranes. Membranes were prepared and treated as described under Materials and Methods and electrophoresed in a polyacrylamide slab gel at 300 V for 7 hr. The gels were stained with Coomassie blue, destained and dried as described under Materials and Methods. 0, molecular weight standard; 1, human erythrocytes with cholera toxin; 2, human erythrocytes without cholera toxin; 3, rat erythrocytes with cholera toxin; 4, rat erythrocytes without cholera toxin; 5, crude cardiac membranes with cholera toxin; 6, crude cardiac membranes without cholera toxin. (b) Polyacrylamide gel analysis (autoradiogram) of radioactive products resulting from cholera toxin-catalysed reaction of human erythrocyte membranes, rat erythrocyte membranes and rat crude cardiac membranes with [^{32}P]NAD^+. Freshly prepared membranes were pelleted, incubated, washed and electrophoresed as described under Materials and Methods. For explanation for 1–6, see Fig. 3a.

aldi and Marinetti [6]. Bilezikian *et al.* [7] measured no change of β-receptors in turkey erythrocyte membranes in hyperthyroidism, but a decrease in hypothyroidism; data of Malbon *et al.* [8] in rat fat tissue show no change in hypo- or hyperthyroidism.

The increase of receptors (Table 1) after triiodothyronine treatment may occur by immigration of pre-existing receptors into the membrane. This has been found in heart slices after brief exposure to triiodothyronine where the increase of receptors is not blocked by inhibition of protein synthesis [4]. On the other hand, the observed cardiac hypertrophy after 5 days *in vivo* treatment with triiodothyronine and cardiac hypotrophy in hypothyroidism would favour a mechanism including a change in protein synthesis [6]. The conflicting data which have been obtained in turkey erythrocyte membranes and in rat fat cells compared to rat cardiac tissue may be tissue and/or species related.

Influence of thyroid hormone on the amount of specifically ADP-ribosylated proteins

The increased number of β-receptors may involve a change in the amount of regulatory components. Therefore, we established a method to quantify the amount of regulatory units in crude cardiac membranes.

Cholera toxin specific ADP-ribosylation of a membrane protein (mol. wt 42,000) in pigeon erythrocyte membranes induces an increased adenylate cyclase activity by GTP together with L-isoprenaline compared to control [10]. In further experiments Cassel and Pfeuffer [10] showed the identity of the regulatory unit with the specific labelled protein. They explained the increased adenylate cyclase activity by inhibition of the GTPase due to ADP-ribosylation of the regulatory component [23, 24]. In rat crude cardiac membranes our experiments also show by autoradiography the specific band of the cholera toxin catalysed ADP-ribosylation of the membrane protein with the molecular weight of 42,000. We measured also an increase in adenylate cyclase activity by GTP together with L-isoprenaline (Fig. 2). In the crude cardiac membrane preparation for the autoradiography (Fig. 3a and 3b), additional pigeon erythrocyte cytosol was not added, in contrast to the experimental procedure as performed by Malbon *et al.* [25] in rat fat cell preparation. The reasons for this is the very small overall effect of cytosol on adenylate cyclase activity (unpublished data) over the effects shown in Fig. 2. Moreover, pigeon cytosol could also have blurred differences in the amount of regulatory components in the investigated thyroid states, as it increased specific ADP-ribosylation [11]. Another disadvantage arises in obtaining only faint autoradiograms [16] of the 42K band, one major target of ADP-ribosylation [16]. A second protein in cardiac membranes with a molecular weight about 46,000 is labelled. The significance of this protein remains unclear. Radioactivity incorporated in the 46K band was at least 38% less than in the 42K band. Membranes of S49 lymphona cells (wild type) and rat hepatoma (HTC-4) cells exhibit also an additional band (mol. wt 52,000–53,000) [16] and also human fibroblasts (mol. wt 47,000) [26]. Recently, the regulatory component has been purified from rabbit liver plasma membranes and shown to consist of three polypeptides with approximate molecular weights of 52,000, 45,000 and 35,000 [27]. But, until now, no data were available on rat crude cardiac tissue. If thyroid hormone influences the amount of regulatory components besides the number of β-receptors, 65 days after thyroidectomy should be sufficient to change the amount of radioactivity incorporated in the 42K band of erythrocyte membranes of hypothyroid animals compared to euthyroid animals (half-life of rat erythrocytes 32 days) [28]. Our results, however, reveal no differences in erythrocyte or crude cardiac membranes for the amount of regulatory components in different thyroid states. A similar result was obtained by Malbon *et al.* [25] in fat cell ghosts. The measured incorporated radioactivity in our study does not show differentiation between any subunits of the regulatory component.

Influence of thyroid hormone on adenylate cyclase activity

Basal activity in euthyroidism and hyperthyroidism have the same absolute values, whereas in hypothyroidism (Table 2) the basal value is increased. Basal activity of adenylate cyclase indicates the separation of receptor and enzyme, with the stimulating guanine nucleotide GTP bound to the regulatory component, reflecting a state of the rate of GTP-binding, GTP-induced activity, GTP-hydrolysis and release [29], and thereby GTPase activity. Higher absolute basal activity in hypothyroidism may therefore indicate a reduced GTPase activity. This explanation remains to be proved by measuring GTPase activity in different thyroid states, which is complicated by high unspecific nucleosidetriphosphatase activity in mammalian tissue [30]. The same result of an increased basal activity in hypothyroid crude cardiac membranes has been presented by Ciaraldi *et al.* [6] without any explanation. A similar mechanism but of an increased GTPase activity is discussed by Bhalla *et al.* [31] in cardiac tissue to explain a decreased cardiac basal adenylate cyclase activity in hypertensive compared to normotensive rats. In hyperthyroidism compared to euthyroidism, the L-isoprenaline-induced adenylate cyclase activity is significantly increased (absolute values and per cent stimulation), due to an increased number of β-receptors. In hypothyroidism the L-isoprenaline-induced adenylate cyclase activity in absolute values is nearly as high as in hyperthyroidism. The per cent stimulation over basal in hypothyroidism is only 129%. This result indicates a reduced effect of L-isoprenaline, which could be explained by the decreased number of β-receptors in hypothyroidism.

The stimulatory effect of the non-hydrolysable GppNHp shows similar absolute values. Per cent stimulation over basal activity is not calculated, because GppNHp binds to the same nucleotide binding site, which in basal state is occupied by the intracellularly available GTP. GppNHp is a non-hydrolysable nucleotide and its activation potency is not influenced by GTPase activity.

So far our results indicate, that thyroid hormone influences in rat cardiac tissue the number of β-receptors and basal activity (in hypothyroidism) and

thereby the changes of adenylate cyclase activity. We cannot exclude a qualitative alteration in the regulatory unit as an explanation for the effects of thyroid hormone.

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