UNCOUPLED β-ADRENERGIC RECEPTORS AND ADENYLATE CYCLASE CAN BE RECOUPLED BY A GTP-DEPENDENT CYTOSOLIC FACTOR

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1. Introduction

The adenylate cyclase system (ATP pyrophosphate lyase (cyclizing) EC 4.6.1.1) is a key factor in the action of many different hormones. This system is an intrinsic component of the cell membrane in eukariotic cells, consisting at least of a receptor and of a catalytic unit; these are apparently connected within the membrane since binding of an effector to the receptor results in changes in the activity of the adenylate cyclase [1]. Little is known of the mechanism of the coupling process which occurs between the interaction of the hormone with the receptor and the stimulation of the enzyme itself. In particular, the existence of a specific component ('transducer') connecting the receptor and the catalytic unit remains hypothetical. Moreover, the exact role of guanine nucleotides in the modulation of this coupling process remains to be elucidated [2–4].

In the present study, we show that repeated washing of a particulate fraction from rat liver (1000 × *g* pellet) resulted in an impairment in the response of adenylate cyclase to epinephrine. Functional coupling between the β-adrenergic receptors and adenylate cyclase was restored upon addition of a cytosolic factor and GTP to the adenylate cyclase assay medium. These findings strongly argue for the existence of a specific transducing component, which may interact with guanine nucleotides, and which is responsible for the coupling of the hormone receptor with adenylate cyclase.

2. Materials and methods

2.1. *Materials*

Phentolamine, (±)alprenolol (Ciba Geity), (+)epinephrine (Sterling Winthrop), (±)propranolol (Imperial Chemical Industries) were obtained as gifts. (−)Epinephrine, (−)isoproterenol and nucleotides were from Sigma. Cyclic AMP and phosphocreatine were purchased from Calbiochem. Creatine phosphokinase was obtained from Boehringer. [α-^{32}P]ATP (6–29 Ci/mmol) and (−)[^{3}H]dihydroalprenolol (33 Ci/mmol) were obtained from the New England Nuclear Corp. and cyclic [8-^{3}H]AMP (13 Ci/mmol) from the CEA (Saclay France).

2.2. *Preparation of rat liver fractions*

All steps were performed at 0–4°C. Livers from female, albino Wistar rats (about 100 g body wt) were homogenized in 5 vol. 1 mM $NaHCO_3$ with a Dounce homogenizer (loose pestle). The homogenate was filtered through 2 layers of cheesecloth and diluted twice after a sample was retained to serve as a source of homogenate. The particulate fraction refers to the pellet obtained after centrifugation at 1000 × *g* for 15 min. This pellet was repeatedly washed by centrifugations at 1000 × *g* in 20 vol. 1 mM $NaHCO_3$, suspended in 2–3 vol. 1 mM $NaHCO_3$ (so as to contain 1–2.5 mg protein/ml) and finally homogenized in a Teflon–glass homogenizer. Fresh preparations were used for each experiment.

For preparation of the 100 000 × *g* supernatant

fraction, minced livers were homogenized in a Dounce homogenizer in 1.5 vol. 1 mM $NaHCO_3$. The filtered homogenate was subsequently centrifuged at 100 000 × *g* for 1 h. The supernatant fraction, containing 30–45 mg protein/ml, was stored in liquid N_2 until use. No change in the activity was observed up to 3 months of storage. Several batches of frozen supernatant fractions gave similar results.

2.3. *Adenylate cyclase assay*

Adenylate cyclase activity was measured as reported [5,6]. Results are expressed in nmol cyclic AMP formed/10 min/mg protein at 30°C unless otherwise indicated. The results, obtained from triplicate determinations, agreed within 5%.

2.4. *Binding assay of (−)[^{3}H]dihydroalprenolol*

The homogenate (2.5 mg protein/ml) or particulate fraction (1–2.5 mg protein/ml) was incubated at 30°C in an assay medium consisting of 50 mM Tris–HCl (pH 7.6) containing 10 mM $MgCl_2$ (unless otherwise stated), and the indicated concentrations of (−)-[^{3}H] dihydroalprenolol in final vol. 600 μl. At the end of the incubation, four 100 μl aliquots were rapidly diluted with 4 ml cold 50 mM Tris–HCl (pH 7.6) containing 10 mM $MgCl_2$ (wash buffer) and filtered through Whatman GF/F glass fiber under vacuum. The filters were washed twice with 4 ml cold wash buffer, placed in scintillation vials, eluted with 1 ml methanol and finally counted by mixing with 10 ml Unisolve (Koch-Light-Laboratory) in an Intertechnique SL 31 liquid scintillation counter. In each experiment, nonspecific binding was determined by measuring the amount of radioactivity retained on filters when incubations were performed in the presence of 0.1 mM (±)alprenolol; such binding generally amounted to approx. 25% of the binding observed in incubations performed in the absence of (±)alprenolol. Similar results were obtained when only 5 μM (±) alprenolol was used instead of 0.1 mM. Specific binding was defined as the total amount of (−)[^{3}H]dihydroalprenolol bound by the homogenate or by particulate fraction protein minus nonspecific binding. The results obtained from four determinations agreed within 10%.

3. Results

3.1. *Increase in adenylate cyclase activity and (−)-[^{3}H]dihydroalprenolol binding in washed particulate fractions*

Samples of the homogenate and 1000 × *g* pellet repeatedly washed in 1 mM $NaHCO_3$ were analyzed for adenylate cyclase activity and (−)[^{3}H]dihydroalprenolol binding (fig.1). Repeated washing resulted in progressive purification of basal adenylate cyclase activity and of alprenolol binding sites assayed at 0.9 nM tritiated ligand; a 2.0–2.5-fold enrichment as compared to homogenate was observed after 4 washes. The use of (−)[^{3}H]dihydroalprenolol as a specific marker of β-adrenergic receptors has been substantiated in several systems [7]. Binding of this ligand to the liver particulate fraction reached equilibrium within 5 min and was saturable. Maximal binding corresponded to 0.32 pmol bound/mg protein at 30°C,

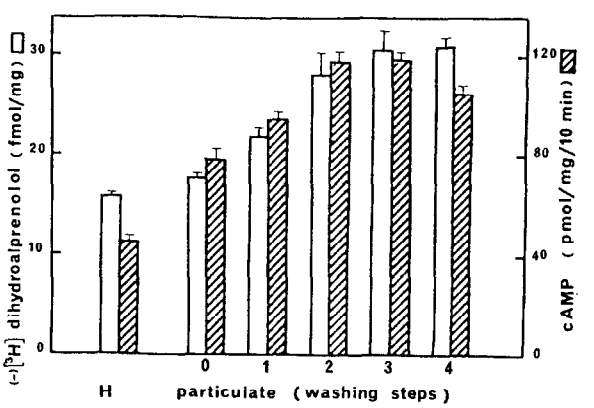

Fig.1. Basal adenylate cyclase activity and (−)[^{3}H]dihydroalprenolol binding in the homogenate and in the washed particulate fractions from rat liver. Assay conditions are described under Materials and methods. The protein content per assay for binding and determination of adenylate cyclase activity was 1355 μg and 510 μg, respectively, when total liver homogenate was used (H). These values were 1485–170 μg, 900–90 μg, 550–60 μg, 860–60 μg, 1050–80 μg when particulate fractions washed 0, 1, 2, 3, 4 times, respectively, were used. Adenylate cyclase activity is expressed in pmol cyclic AMP formed/10 min/mg protein and is represented by the hatched bars. The specific binding (−)[^{3}H]dihydroalprenolol, represented by the open bars, is expressed in fmol bound/mg protein after 15 min incubation at 0.9 nM (−)[^{3}H]dihydroalprenolol (13 000 cpm). The results are the mean ± SE of four determinations in fractions from two animals.

pH 7.6. As determined by Scatchard analysis, (−)-[^{3}H]dihydroalprenolol interacted with a single class of non-cooperative binding sites with an apparent dissociation constant (K_d) 9.0 nM. Kinetic analysis gave a first-order rate constant for the dissociation k_2 0.37 min^{-1} and a second-order rate constant k_1 for the association $28 \cdot 10^6$ M^{-1} min^{-1}. The ratio k_2/k_1 = 13.2 nM confirmed the value of K_d obtained by Scatchard analysis. Displacement of (−)[^{3}H]dihydroalprenolol from membrane binding sites permitted estimation of the K_d values of various other drugs. They clearly indicate that (−)[^{3}H]dihydroalprenolol binds to the physiological β-adrenergic receptors of rat liver. Adrenergic β agonists competed for the binding sites with the following order of potencies: (−)isoproterenol (K_d 4.5 nM) > (−)epinephrine (K_d 450 nM) > (+) epinephrine (K_d 4500 nM). Adrenergic β antagonists such as (±) propranolol and (±) alprenolol were strong competitors with K_d 90 nM and 9 nM respectively, whereas the α antagonist, phentolamine, had only a weak affinity for the binding sites (K_d 27 μM).

3.2. *Effect of washing on the catecholamine-sensitive adenylate cyclase*

Repeated washing of the particulate fraction rendered the adenylate cyclase unresponsive to epinephrine (fig.2.): a 50% reduction in the stimulation of the adenylate cyclase by epinephrine occurred after the first centrifugation step. After 4 washes, the particulate fraction exhibited a very low and non-reproducible sensitivity to epinephrine.

3.3. *Reversal of the effect of washing by addition of 100 000 × g supernatant and GTP*

The reduction in the stimulation of adenylate cyclase activity by epinephrine which occurred after repeated washing could not be correlated with either a loss of adenylate cyclase itself or a depletion of the β-adrenergic receptors: this is clearly shown by the results depicted in fig.1. We therefore considered the possibility that the change in the properties of the adenylate cyclase could be due to the loss of soluble factors. The epinephrine sensitivity of the washed particulate fraction was examined as a function of increasing amounts of 100 000 × *g* supernatant protein, or of GTP, or of both added together to the incubation medium (fig.3). Addition of supernatant

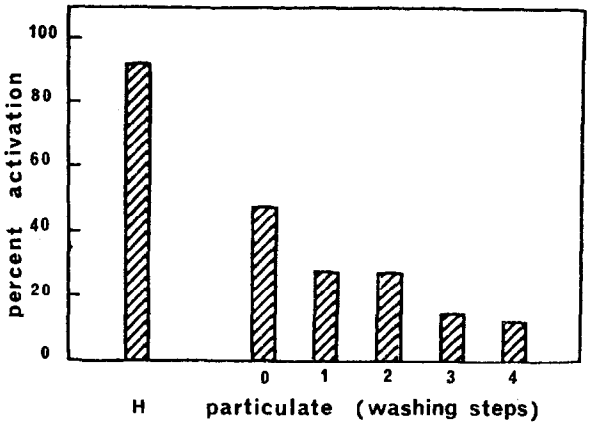

Fig.2. Catecholamine-sensitive adenylate cyclase in homogenate and in washed particulate fractions from rat liver. Adenylate cyclase from homogenate (H) and from particulate fractions washed repeatedly was measured in the presence and absence of 50 μM epinephrine in the assay medium described under Materials and methods. The protein content per assay is reported in the legend to fig.1. The results are expressed as percent activition over basal activity represented in fig.1 and are the mean of four determinations.

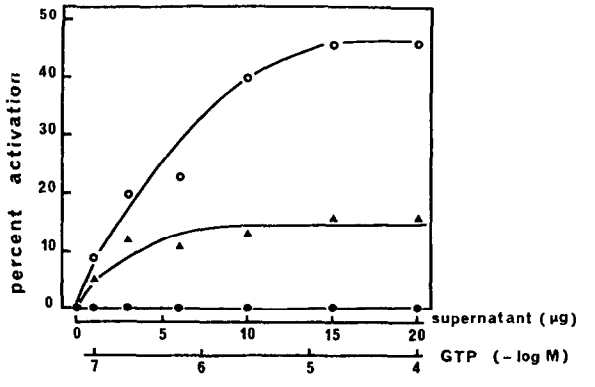

Fig.3. The effect of increasing concentrations of GTP and of the 100 000 × *g* supernatant on epinephrine responsiveness. The adenylate cyclase activity in a particulate fraction washed four times (69 μg protein/assay) was measured as described under Materials and methods, in the presence of varying amounts of cytosol protein (•), or GTP (▲), added separately or together (○) to the assay medium. The experiment was performed in the presence and absence of 50 μM epinephrine. Adenylate cyclase activity is expressed as percent activation due to epinephrine over basal level. The results are the mean of triplicate determinations; the experiment was repeated three times.

alone resulted in a 4-fold increase in the basal adenylate cyclase activity (not shown) but did not reveal any sensitivity to epinephrine. In the presence of GTP, a 4-fold increase in the basal level was also obtained, and was accompanied with a weak response to epinephrine at 100 μM GTP (16% activation over basal level). Finally, addition of both supernatant and GTP restored the response to epinephrine: stimulation of the adenylate cyclase from the washed particulate fraction by 50 μM epinephrine was readily detectable at 1 μg supernatant protein plus 0.1 μM GTP; addition of maximally effective concentrations of both effectors (20 μg supernatant protein, 0.1 mM GTP) resulted in a 45% activation of epinephrine over basal activity. This restored sensitivity to epinephrine was confirmed when adenylate cyclase activity was tested as a function of hormone concentration (fig.4). Epinephrine had no effect upon cyclase in the absence of effectors; in contrast, in the presence of 20 μg supernatant protein and 0.1 mM GTP, epinephrine activated the particulate adenylate cyclase over the concentration range 0.1–100 μM, producing half-maximal activation at 2 μM. Similar values were obtained under other experimental conditions using membranes from adrenalectomized rats [5] or using non-washed particulate fractions in the presence of GTP [8]. This effect of epinephrine was abolished by 0.1 μM (±)propranolol (data not shown).

3.4. *Lack of effect of GTP and 100 000 × g supernatant on epinephrine binding*

Competition experiments of epinephrine for (–)[^{3}H]dihydroalprenolol binding sites were performed in the presence and absence of 100 000 × *g* supernatant protein and GTP at concentrations maximally effective in promoting resensitization of adenylate cyclase to epinephrine. These experiments were performed in the medium used for adenylate cyclase assay: thus, the assay conditions for both experiments reported in figs.4. and 5 were identical.

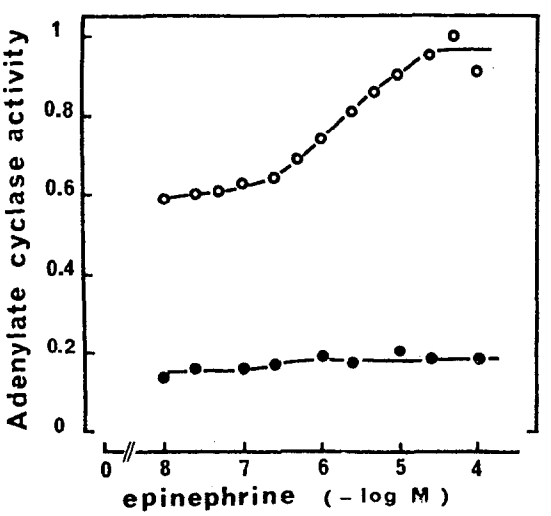

Fig.4. Effect of 100 000 × *g* supernatant plus GTP on epinephrine sensitivity of adenylate cyclase from washed particulate fraction. The adenylate cyclase activity in a particulate fraction washed four times (21 μg protein/assay) was measured in the assay medium described under Materials and methods in the presence of varying concentrations of epinephrine and in the absence (•) or presence (○) of 20 μg cytosol protein plus 0.1 mM GTP. Adenylate cyclase activity is expressed as nmol cyclic AMP formed/10 min/mg protein. The data are the mean of triplicate determinations; the experiment was repeated three times.

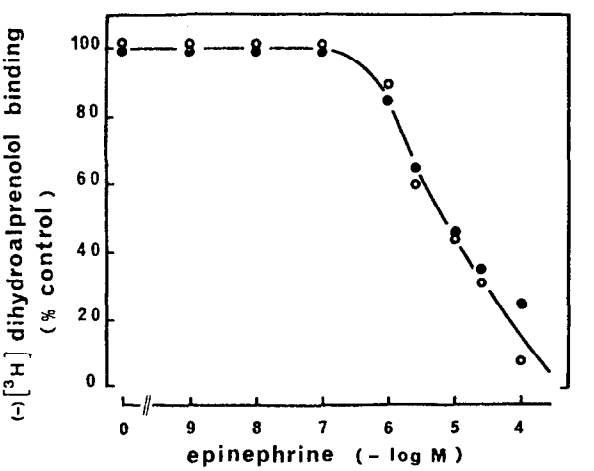

Fig.5. Lack of effect of 100 000 × *g* supernatant plus GTP on competition of epinephrine for (–)[^{3}H]dihydroalprenolol binding sites in the washed particulate fraction. (–)[^{3}H]dihydroalprenolol (13 000 cpm) at a concentration of 0.9 nM was incubated with particulate fraction washed four times (1.5 mg protein/ml) for 15 min at 30°C in the medium for adenylate cyclase assay, in the presence of increasing concentrations of epinephrine. The experiment was performed in the absence (•) or in the presence of 20 μg supernatant protein plus 0.1 mM GTP (○). The results are expressed as percent of maximal binding (20 fmol (–)[^{3}H]dihydroalprenolol bound/mg protein under both experimental conditions) and are the mean of four determinations from two experiments.

Cytosol plus GTP did not affect the amount of (–)[^{3}H]dihydroalprenolol bound in the absence of cold ligand; the displacement curve by epinephrine was unchanged (fig.5.).

4. Discussion

Direct evidence to demonstrate that the β-adrenergic receptor and the catalytic unit of the adenylate cyclase are two separable macromolecules has recently been provided [9,10]. The remaining question concerns the mechanism of the coupling process which occurs following hormone–receptor interaction and which results in stimulation of adenylate cyclase. In fact, the available experimental data strongly argue for the existence of a component modulating the efficacy of receptor–enzyme coupling. Thus, in rat kidney medulla, it was suggested that adrenal steroids were implicated in the control of synthesis of such a coupling component [11]. Uncoupling of the catecholamine activation of adenylate cyclase was also observed in pigeon erythrocyte membranes treated with filipin with no modification neither in hormone binding to the receptor nor in the activity of the catalytic unit of the adenylate cyclase system [12,13]. The observation by Haga et al. [14] that a mutant clone of S 49 mouse lymphoma cell line retained β-adrenergic receptors but lacked hormone-responsive adenylate cyclase activity further supports the suggestion that components distinct from hormone receptor and catalytic unit, regulate the hormone responsiveness of the cyclase.

Guanine nucleotides are well known to stimulate adenylate cyclase activity and to alter hormone binding [2,5,15]; this role has been strengthened by the description of binding sites for these nucleotides associated with adenylate cyclase in various tissues [16,20]. Thus it is generally accepted, but not demonstrated, that guanine nucleotides are involved in the coupling mechanism. A further indication that this indeed might occur is the observation that desensitization of β-adrenergic receptors by β-adrenergic agonists could be reversed by guanine nucleotides in frog erythrocyte membranes [21]. As a working hypothesis, it can be admitted that the various hormone receptors would have a common attachment site by which they couple to the nucleotide binding unit [9].

It is well known that adenylate cyclase from purified plasma membranes of normal rat liver is not sensitive to epinephrine in vitro [22–25] unless either GTP [26] or a cytosolic protein-nucleotide factor [6] are added to the assay mixture. In the light of the data reported here, it is clear that this unresponsiveness is due to the loss of a coupling factor during the purification procedure. Repeated washing in 1 mM $NaHCO_3$ of a crude particulate fraction from rat liver results in a loss of sensitivity of the adenylate cyclase to epinephrine. This occurs independently of any impairment in binding capacity and in adenylate cyclase activity which were even increased after repeated washing (figs 1. and 2.). The coupling process is restored by a cytosolic factor interacting with GTP in vitro. It is tempting to assume that the coupling factor lost during the washing at low ionic strength, is the GTP protein factor described [6]. We suggest that GTP protein complex present in the cytosol, might easily recombine with the uncoupled receptor and catalytic unit of the adenylate cyclase.

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