HIGH AND LOW AFFINITY STATES OF β-ADRENERGIC RECEPTORS AND THEIR COUPLING WITH THE ADENYLATE CYCLASE IN A MUSCLE CELL LINE

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1. Introduction

The two main effects of guanyl nucleotides on receptor–adenylate cyclase system are:

(i) To permit stimulation of the enzyme by the hormone [1]; and

(ii) To modulate the affinity of the receptor for the agonist [2].

It has been shown that [^{3}H]antagonist/agonist competition curves are shallow with 'pseudo' Hill slope of <1 [3,4]. In the adrenergic sensitive adenylate cyclase of frog erythrocytes [5,6] these curves can be fitted with a model assuming that the β-adrenergic receptors exist in two discrete and interconvertible states with a high and low affinity for the agonist, respectively. In the presence of GTP, the competition curves are steeper with 'pseudo' Hill slope ~1 [5,6] indicating that all the receptors are under the low affinity state. Although a great number of studies has been done on these different states of the receptor using binding experiments, no attempt has been made to find experimental conditions in which it would be possible to compare both the characteristics of the two states of the receptor and those of the agonist–adenylate cyclase activation in the same preparation and under the same experimental conditions. We found such a study was possible in a crude membrane preparation of a muscle cell line, BC_3H_1 [7].

2. Materials and methods

BC_3H_1 muscle cell line was a gift from Dr J. Patrick. They were grown as in [8]. For preparation of particulate fractions, cells were washed twice with 0.150 M NaCl and scraped off with a rubber probe in 5 ml 0.15 M NaCl/dish. They were centrifuged for 3 min at 400 × *g* and the pellet was homogenized at 0°C in 5 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$ and 0.4 mM EDTA (~5 × 10^6 cells/ml) with a Teflon–glass homogenizer. The homogenates was diluted at ~1.5 × 10^6 cells/ml with the homogenization buffer filtered through a silk screen (150 μm pore diam.) and centrifuged for 5 min at 500 × *g*. The supernatant was then centrifuged for 15 min at 12 000 × *g* at 4°C. The pellet was resuspended in 50 mM Tris–HCl (pH 7.6), 25 mM $MgCl_2$ and 10 mM EDTA at ~4.5 mg protein/ml. This particulate fraction was kept at 0°C and used for experiments within 2 h. For (–)[^{3}H]-dihydroalprenolol binding ([^{3}H]DHA) the particulate fractions (450 μg/ml in the final assay) were incubated for 10 min at 30°C in 200 μl of a medium containing (50 mM Tris–HCl (pH 7.6), 2.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ATP, 0.4 IU/ml of adenosine deaminase, 10 mM phosphocreatine, 0.2 mg creatine kinase/ml, 0.1 mM papaverine and 1–2 nM [^{3}H]DHA). Incubations were terminated by addition of 1 ml ice-cold medium (50 mM Tris–HCl (pH 7.6) and 10 mM $MgCl_2$) followed by rapid filtration through Whatman GF/B glass fiber filters. The latter were washed with 15 ml of the same medium. Specific binding was defined as the binding which is inhibited by 30 μM isoproterenol (60% of total binding). Adenylate cyclase assays were done in the same medium except that trace amounts of c[^{3}H]AMP (0.001 μCi) and [α-^{32}P]ATP (1.5 μCi) were added. The reaction was stopped and c[^{32}P]AMP purified as in [9]. Computer analysis was done by a non-linear least square curve fitting procedure as in [10].

Using the extra sum of square principle as applied in [6], the goodness of the fit was evaluated in binding studies assuming that the receptor has either 1 or

2 affinity states and in adenylate cyclase studies assuming that the adenylate cyclase activation has either 1 or 2 components.

3. Results

3.1. *Binding experiments*

They were done in an adenylate cyclase medium. Scatchard analysis indicated that [^{3}H]DHA interacted with one category of binding sites (K_d 1.2 nM, B_{max} 50 fmol/mg protein). [^{3}H]DHA binding properties have been described [8]. As in other systems in the absence of added GTP, the [^{3}H]antagonist/agonist curves were shallow (fig.1). Computer analysis indicated that the best fit was obtained with a model that involved [^{3}H]DHA labelling 2 populations of sites with the same K_d for the labelled antagonist and different affinities for the agonist ($F = 12, p < 0.01$ when compared to a 1 site model). For isoproterenol 45% of the receptors have high affinity (R_H) with dissociation constant ($K_{d,H}$) of 10.7 nM and 55% have a low affinity ($K_{d,L}$) of 256 nM (mean values in table 1). Similar results were obtained with salbutamol, epinephrine, norepinephrine. The percentage of R_H was not statistically different for these different agonists (table 1). In the presence of GTP (10^{-4} M) the competition curve for isoproterenol shifted to the right and steepened. Computer modeling indicated that only one state was present having a low affinity component ($K_{d,L}$ = 220 nM) (fig.1). This affinity was nearly identical to that of the low affinity component ($K_{d,L}$) determined in the absence of GTP (fig.1). Mean values confirmed that $K_{d,L}$ were not statistically different when determined in the absence or the presence of GTP for both isoproterenol and salbutamol (table 1).

3.2. *Adenylate cyclase experiments*

In the absence of agonist, the basal adenylate cyclase was highly stimulated by GTP (15–20-fold). Stimulation was apparent at 10^{-7} M and maximal at 5×10^{-5} M. When no GTP was added, isoproterenol enhanced basal adenylate cyclase activity (2.4 ± 0.1-

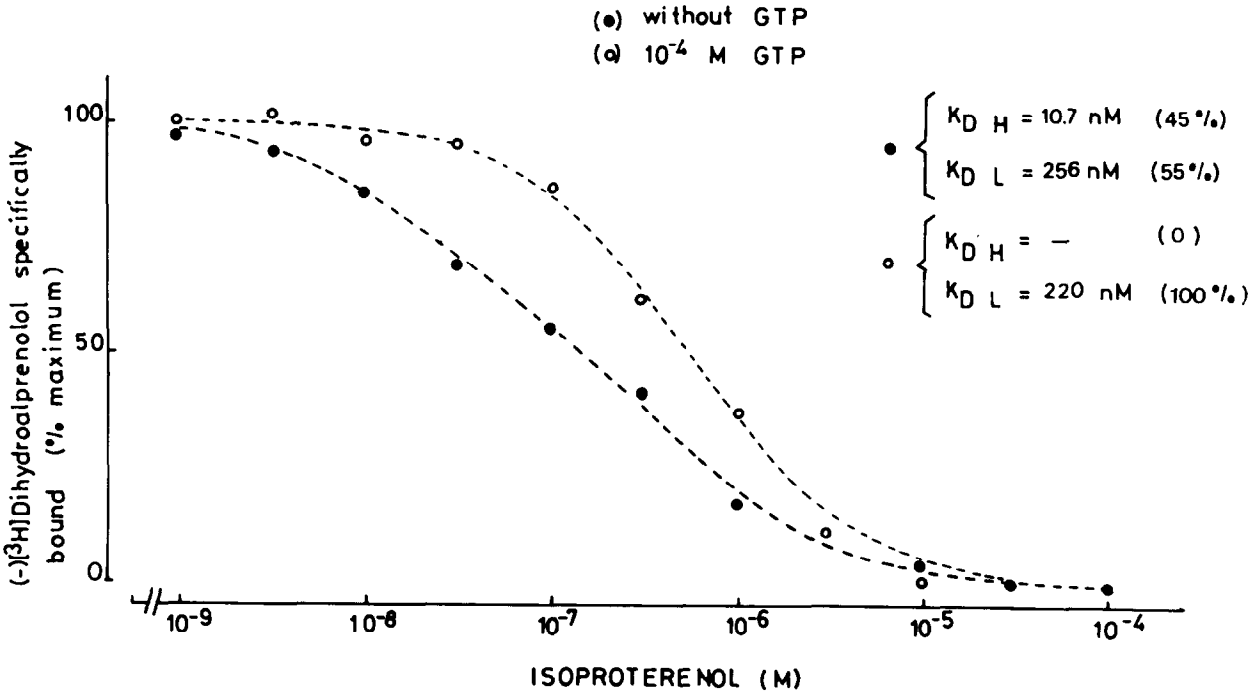

Fig.1. Computerized fitting data obtained from the displacement of [^{3}H]DHA by isoproterenol in the presence and absence of GTP. [^{3}H]DHA was 1.7 nM. The specific binding was 32 fmol/mg protein in the absence (•) and the presence (○) of GTP (10^{-4} M). Experimental points are the means of triplicate determination. Dashed lines represent the computer-generated curves fitting the observed data.

Table 1
Computer-model parameters of agonist interaction with [^{3}H]DHA binding sites

Agonist	% RH	$K_{d,H}$ (nM)	$K_{d,L}$ (nM)
(−) Isoproterenol (4)	51 ± 7	19.2 ± 5.9	205 ± 45[a,c]
(−) Isoproterenol + 10^{-4} M GTP (5)	0	–	273 ± 84[d] n.s.
(±) Salbutamol (3)	48 ± 9	240 ± 75	2290 ± 370[c,a]
(±) Salbutamol + 10^{-4} M GTP (3)	0	–	2560 ± 1100[d] n.s.
(−) Epinephrine (3)	42 ± 11	11 ± 3	427 ± 20[a,c]
(−) Norepinephrine (3)	42 ± 5	523 ± 98	13 300 ± 4000[b,c]

% RH is the percentage of total receptors in the high affinity state. $K_{d,H}$ and $K_{d,L}$ are the respective dissociation constants for the high and low affinity states of the receptor. The number of experiments is given in parentheses. The results are expressed as means ± SEM. Statistical analysis was done using Student's t-test: [a] $p < 0.01$; [b] $p < 0.05$; n.s. not significant; [c] comparison between $K_{d,L}$ and $K_{d,H}$; [d] comparison between $K_{d,L}$ determined in the absence and the presence of GTP

fold, $n = 6$). This stimulation is probably due to a low GTP contamination of our preparation. In the presence of increasing concentration of GTP the accumulation of AMP due to the agonist increased. This GTP effect was already significant at 3×10^{-8} M and maximal at 3×10^{-6} M. In the absence of added GTP, the dose–response for adenylate cyclase activation by isoproterenol was shallowed (fig.2A). Stimulation was clearly significant at 10^{-9} M and maximal at 10^{-6} M. The Hofstee plot of this dose–activation curve was curvilinear (fig.2B) indicating that it cannot be described by simple mass-action kinetics. Computer modeling indicated that the best fit was obtained assuming that the dose–activation curve was the sum of two Michaelis-Menten components ($F = 29$, $p < 0.001$ when compared with a one component model). The high affinity one had a K_a (concentration giving half-maximal activation of 2.9 nM) and was responsible for 59% of the total adenylate cyclase activation. The low affinity one had a K_a of 140 nM and was responsible for 41% of the total activation (mean values in table 2). Similar results were obtained for several agonists and the proportions of high and low affinity components were not statistically different except for norepinephrine. To make sure that proportions of high and low affinity components were determined at steady state, the dose–activation curves were examined either from 0–5 min or from 5–10 min. Identical results were obtained (not shown). In the presence of GTP (10^{-4} M) the dose–activation curve for agonists shifted to the right (fig.2A). Only one component was detected by computer modeling and by Hofstee plot (fig.2C). Its K_a ($K_{a,CL}$) was very close to that of the low affinity component determined in the absence of added GTP (fig.2A). Statistical analysis on isoproterenol and salbutamol dose–activation curves done in the presence or the absence of GTP confirmed this result.

4. Discussion

Binding experiments showing 2 states of the β-adrenergic receptor were conducted in simple Tris buffer conditions[5,6]. In our system, these 2 states were detectable by binding experiments conducted in a medium allowing measurement of adenylate cyclase activity and its stimulation by agonists. The % of the total receptor population in the high affinity state was very similar, irrespective of the agonist tested (table 1). In [5] there was a correlation between the intrinsic activity of the agonist and the proportion of the high affinity component. However in our system the intrinsic activity of the agonists only

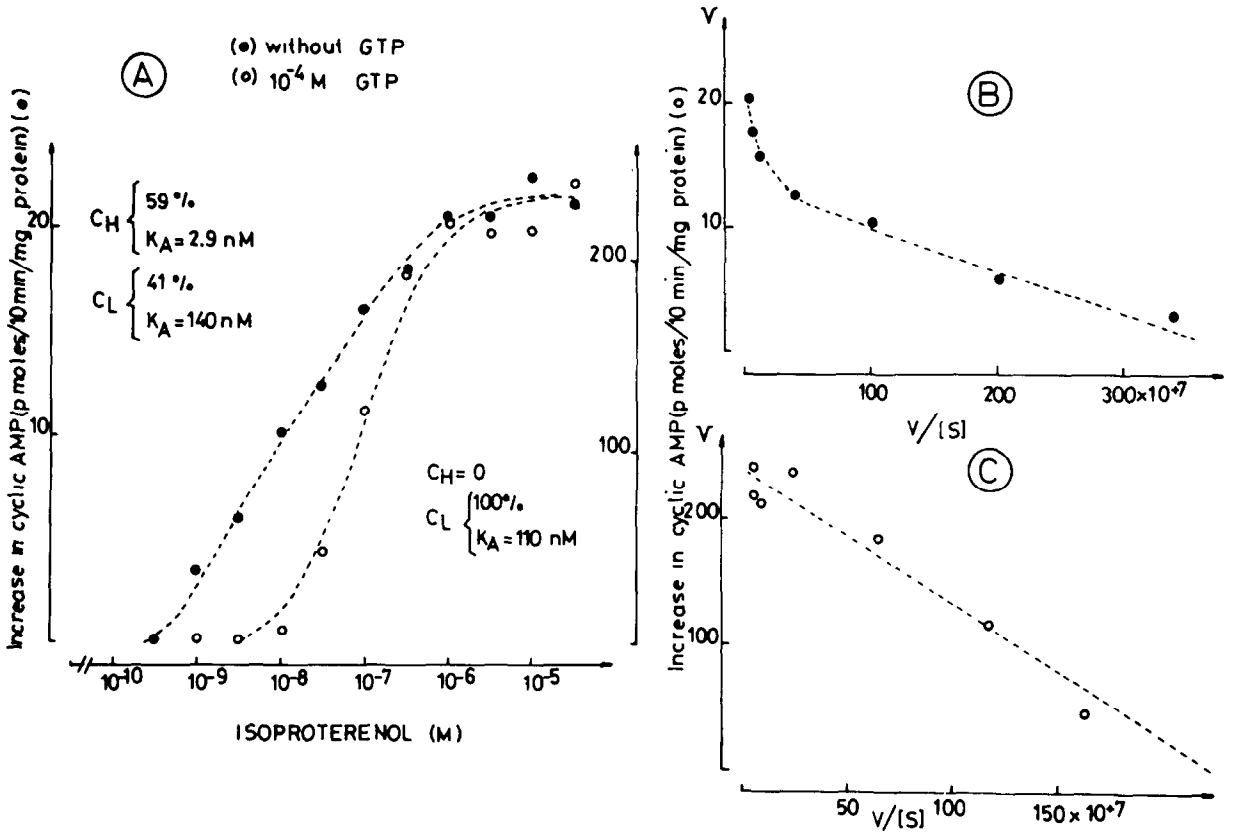

Fig.2. Effect of increasing concentration of isoproterenol on the adenylate cyclase activity in the presence and absence of GTP. (A) Increase in cAMP production by isoproterenol in the absence (•) and presence (○) of 10^{-4} M GTP. Experimental points are the means of a triplicate determination. Dashed lines represent the computer-generated curves fitting the observed data. Here, the basal adenylate cyclase activities were 24 pmol . 10 min^{-1} . mg $protein^{-1}$ in the absence of GTP and 496 pmol . 10 min^{-1} . mg $protein^{-1}$ in the presence of 10^{-4} M GTP. (B,C) Hofstee plots for the dose–response curves obtained for isoproterenol in the absence of GTP (B) and in the presence of 10^{-4} M GTP (C). V is the adenylate cyclase activity in the presence of isoproterenol, minus basal activity. S = isoproterenol concentration (in mol/l).

varied from 80–100% (table 2) and therefore such a correlation is difficult to detect. In the presence of GTP the number of β-adrenergic receptors did not change and all the receptors are under a low affinity state. The fact that the affinity of this state did not statistically differ from the affinity of the low affinity state detected in the absence of added GTP suggests that in the presence of GTP the high affinity state has been converted to a low affinity state.

Similarly, the dose–response curves for adenylate cyclase activation by agonists were biphasic in the absence of added GTP and could be resolved in two Michaelis-Menten components with high and low affinity for agonists. The % of high affinity component did not statistically differ (see table 2) from the % of high affinity state detected in binding experiments except for norepinephrine (tables 1,2). In the presence of GTP only 1 component was detected having a K_a which did not statistically differ from the K_a of the low affinity component detected without added GTP (fig.2, table 2). The shift in K_a to the lower values seen here in the presence of GTP has been also been described in [3] in S_{49} murine lymphoma cell membranes. The K_a of the high affinity components (K_aCH) was 2.8 ± 0.2 nM (n = 6). To our knowledge such a high affinity component has never been reported for an isoproterenol-sensitive adenylate cyclase in an in vitro system. This is probably

Table 2
Computer-modeled parameters of agonist activation of adenylate cyclase

Agonist	Intrinsic activity	% CH	$K_{a,CH}$ (nM)	$K_{a,CL}$ (nM)
(−) Isoproterenol (6)	1	39 ± 8[c] n.s.	2.8 ± 0.8	72 ± 23[a,d]
(−) Isoproterenol + 10^{-4} M GTP (4)	–	0	–	68 ± 11[b] n.s.
(±) Salbutamol (4)	0.80 ± 0.04	47.5 ± 13[c] n.s.	56 ± 12	500 ± 90[a,d]
(±) Salbutamol + 10^{-4} M GTP	–	0	–	571 ± 55[b] n.s.
(−) Epinephrine (3)	1.05 ± 0.02	39 ± 6[c] n.s.	20 ± 2	358 ± 119[a,e]
(−) Epinephrine + 10^{-4} M GTP (1)	–	0	–	360
(−) Norepinephrine (3)	0.93 ± 0.01	75 ± 6[c,e]	565 ± 83	30 500 ± 3400[a,d]

Intrinsic activity was defined as the ratio of the maximal activation of adenylate cyclase achieved by the agonist tested with no added GTP to the maximal activation achieved by (−) isoproterenol. % CH is the percentage of total activity in the high affinity component. $K_{a,CH}$ and $K_{a,CL}$ are the respective K_a for the high and low affinity components. [a] Comparison between $K_{a,CH}$ and $K_{a,CL}$; [b] comparison between $K_{a,CL}$ determined in the absence and the presence of GTP; [c] comparison between % CH (table 2) and % RH (table 1) for each agonist; [d] $p < 0.01$; [e] $p < 0.05$; n.s. not significant

because most of the studies on β-adrenergic sensitive adenylate cyclase have been done with high GTP concentrations.

Our data clearly demonstrated that two states of β-adrenergic receptors could be detected both by binding experiments and by analysis of the adenylate cyclase dose–activation curves in the absence of added GTP. The main question arising from our results is whether or not the 2 components of adenylate cyclase are directly related to the 2 states of the β-adrenergic receptors. We have no direct experimental proof allowing a positive answer. However, the disappearance of both the high affinity component of the adenylate cyclase and the high affinity state of binding in the presence of a high concentration of GTP constitutes a good indication in favor of a direct relationship between them. There is still a debate to whether 1 or 2 nucleotide-binding proteins are involved in the 2 main effects of guanyl nucleotides, i.e., hormonal stimulation and the decrease in agonist affinity for the receptor [11,12]. A recent model [6] implies only 1 nucleotide binding protein. These authors suggested that the high affinity state of the receptor is a ternary complex RHN (N, nucleotide binding protein) in which N is occupied by GDP (RHN_{GDP}). In the presence of GTP and hormone, this complex rapidly dissociates and the N unit loaded with GTP activates the catalytic unit of the adenylate cyclase. The dissociation of N also leads to the formation of an RH complex of low affinity.

If we take this model into consideration our results could only be explained if one admits that the high affinity state (RHN_{GDP}) can be coupled with the catalytic unit. Indeed in S_{49} [12], NS 20 neuroblastoma cell and corpora latea membranes, GDP was able to promote coupling under experimental conditions in which the proportion of GDP converted to GTP was negligible.

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