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RELATIONSHIP BETWEEN THE (−)-[^{3}H]-DIHYDROALPRENOLOL BINDING TO β-ADRENOCEPTORS AND TRANSMEMBRANE ^{86}Rb EFFLUX OF THE BC_3H1 NONFUSING MUSCLE CELL LINE

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1 We have studied the binding properties of the β-adrenoceptor antagonist [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) on a membrane preparation of the non-fusing muscle cells BC_3H1.

2 [^{3}H]-DHA appears to bind to two classes of sites. The first site has a high affinity (K_D = 0.53 nM) and a low capacity (B_{max} = 58 fmol/mg of protein). The second site has a low affinity (K_D = 110 nM) and a high capacity (B_{max} = 1100 fmol/mg of protein).

3 The pharmacological properties of the high affinity low capacity site correspond to the known properties of the β_2-adrenoceptors since the agonists inhibit [^{3}H]-DHA binding following the series isoprenaline > adrenaline > noradrenaline > phenylephrine and the antagonists following the series alprenolol ≃ propranolol > butoxamine > practolol > phentolamine.

4 The binding properties of the β-adrenoceptors were correlated with the effect of β-adrenoceptor agonists and antagonists on ^{86}Rb efflux rate from BC_3H1 cells.

5 There is very good correlation between the dissociation constants obtained by inhibition of [^{3}H]-DHA binding by the antagonists alprenolol and propranolol, and the inhibition constants calculated from their antagonism of the ^{86}Rb efflux rate stimulation by adrenaline. The ratio of the dissociation constants obtained by inhibition of [^{3}H]-DHA binding by agonists and their EC_{50}, calculated from ^{86}Rb efflux curves, is higher than 1. This high K_D/EC_{50} ratio indicates a high coupling efficiency between receptor occupancy by agonists and the biological effect measured.

Introduction

The clonal cell line BC_3H1 isolated by Schubert, Harris, Devine & Heinemann (1974) was shown to possess many characteristics of muscle. The cell membrane is excitable, being depolarized by acetylcholine, and hyperpolarized by noradrenaline. We have shown that catecholamines and carbachol increase ^{86}Rb efflux rate in a dose-dependent manner in these cells (Mauger, Moura & Worcel, 1978). It has been possible to characterize pharmacologically the receptors involved in this membrane response. The action of catecholamines appears to be due to the stimulation of both α- and β-adrenoceptors. It has been repeatedly shown that the labelled blocking agent (−)-[^{3}H]-dihydroalprenolol ([^{3}H]-DHA) interacts with specific binding sites on particulate cell fractions containing β-adrenoceptors (Lefkowitz, Limbird, Mukherjee & Caron, 1976; Wolfe, Harden & Molinoff, 1977; Maguire, Ross & Gilman, 1977). The aim of this work was to try to correlate the action of β-adrenoceptor agonists and antagonists on ^{86}Rb efflux rate, with their binding properties on the β-adrenoceptors of the BC_3H1 cell line.

The results obtained indicate that [^{3}H]-DHA binds specifically to a particulate fraction on two classes of site. The high affinity site appears to correspond to the β_2-adrenoceptor responsible for the biological response studied.

An account of some of these results has been given to the British Pharmacological Society (Mauger & Worcel, 1979).

Methods

Tissue culture

BC_3H1 muscle cell line was a gift from Dr J. Patrick. Cells were grown in Dulbecco modified Eagle's

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medium (Vogt & Dulbecco, 1963) containing 10% foetal calf serum, at 37°C in an atmosphere of 12% CO_2 and 88% air. The line was maintained in the exponential phase of growth by passage every 4 days. The cells were resuspended in 0.25% Viokase in Dulbecco modified Eagle's medium, centrifuged and resuspended in the growth medium. They were plated in plastic tissue culture dishes at 10^3 cells/cm^2. For radioligand binding studies they were seeded in 90 mm diameter dishes. The cells used for ^{86}Rb efflux studies were grown on 10.5 ± 50 mm glass slides in 90 mm diameter dishes. The medium was changed twice a week. For all the experiments the cells were used 12 to 15 days after seeding, that is about 1 week after reaching confluence.

Preparation of the particulate fraction

Usually, 4 dishes were used amounting to about 3×10^7 cells. Cells were washed twice at room temperature with NaCl 0.15 M and scraped off with a rubber probe into 20 ml NaCl 0.15 M. They were centrifuged for 3 min at 400 *g* and the pellet was homogenized at 0°C in 6 ml of 5 mM Tris-HCl pH 7.6, 1 mM $MgCl_2$ with a Thomas tissue grinder. The homogenate was centrifuged for 2 min at 400 *g* at 4°C to eliminate nuclei and unbroken cells and the supernatant was centrifuged for 10 min at 30,000 *g* at 4°C. The pellet was resuspended in 6 ml of 50 mM Tris HCl pH 7.6, 10 mM $MgCl_2$ and centrifuged for 10 min at 30,000 *g* at 4°C and washed once more. The final pellet was suspended in 2 ml Tris-HCl 50 mM, $MgCl_2$ 10 mM and ascorbic acid 0.1% at a protein concentration of about 2.5 mg/ml. This particulate fraction was kept at 0°C and used for binding studies within 1 h. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

[^{3}H]-dihydroalprenolol binding

A small volume (40 µl) of the crude membrane fraction (about 100 µg protein) was incubated for 15 min at 30°C in a total volume of 150 µl of a solution containing 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 0.1% ascorbic acid and 0.2 to 10 nM [^{3}H]-DHA. Incubations were terminated by addition of 1 ml ice cold washing medium containing 50 mM Tris-HCl pH 7.6 and 10 mM $MgCl_2$, followed by rapid filtration through Whatman GF/C glass fibre filters and by washing with 20 ml of ice cold medium. Filters were dried and counted in Permafluor scintillation fluid. Specific binding was defined as the difference between the total binding observed and the non specific binding measured in the presence of 20 µM isoprenaline or 0.1 mM alprenolol (see Results section).

Measurement of ^{86}Rb efflux rate

The details of the method have been described previously (Mauger *et al.*, 1978). Briefly, the slide with the cells attached to one side was removed from the culture dish, rinsed once and incubated for at least 120 min in a physiological saline solution (PSS) containing 10 to 20 µCi/ml of ^{86}Rb and gassed with a 95% O_2 and 5% CO_2 mixture. The PSS had the following composition (mM): Na^+ 136.9, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 133.5 and glucose 11.5. After loading, the slide was perfused continuously with the PSS at 37°C. The doses of the drug tested were administered over 6 min and the slide was washed for 20 min before being superfused with another dose of the drug. When antagonists were used, they were present in the PSS during all the washing procedure. To avoid any interactions with α-adrenoceptors, all the experiments were carried out in the presence of 1 µM phentolamine. The entire effluent was collected every 2 min and counted in a well γ-counter. At the end of the experiment the radioactivity remaining in the cells was counted. A desaturation curve was calculated by adding in a reverse order the washout curve to radioactivity remaining in the cells at the end of the experiment.

Drugs

All salts were reagent grade. (−)-[^{3}H]-dihydroalprenolol was obtained from NEN, (−)-alprenolol tartrate, (±)-propranolol hydrochloride, (−)-isoprenaline bitartrate, (−)-adrenaline, (−)-noradrenaline hydrochloride, (−)-phenylephrine hydrochloride, from Sigma; (+)-alprenolol tartrate, from Hässle; phentolamine hydrochloride from Ciba-Geigy; (±)-butoxamine hydrochloride from Burroughs-Wellcome, (±)-practolol hydrochloride from ICI Pharmaceuticals; Viokase, Dulbecco modified Eagle's medium and foetal calf serum from Gibco.

Results

Binding properties of [^{3}H]-dihydroalprenolol on cell membranes

The binding of [^{3}H]-DHA to BC_3H1 cell membranes reaches equilibrium within 15 min at 30°C. A concentration-binding curve was obtained in equilibrium conditions in the presence and in the absence of 0.1 mM non radioactive alprenolol. Under these conditions, the binding to displaceable sites does not reach a plateau even with a concentration of [^{3}H]-DHA as high as 100 nM (Figure 1a). A Scatchard analysis of these data (Figure 1b) clearly indi-

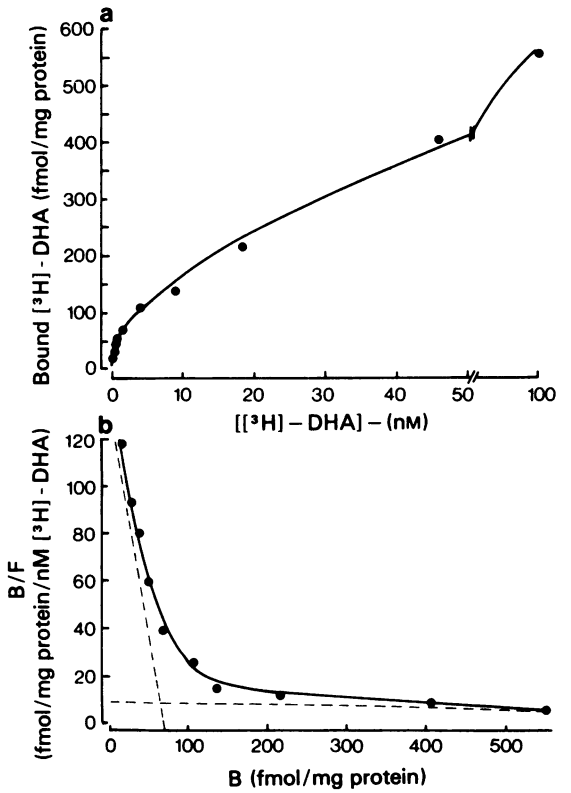

Figure 1 Binding of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) to BC_3H1 cell membranes. (a) Binding was assayed over a range of 0.2 to 100 nM [^{3}H]-DHA under the conditions described in Methods. Specific binding is defined as the difference between binding observed in the absence and the presence of 0.1 mM alprenolol. (b) Scatchard plot of [^{3}H]-DHA binding to BC_3H1 cell membranes. The curve was calculated assuming that [^{3}H]-DHA binds to two classes of site characterized in Figure 3b and 3c and indicated here by dashed lines.

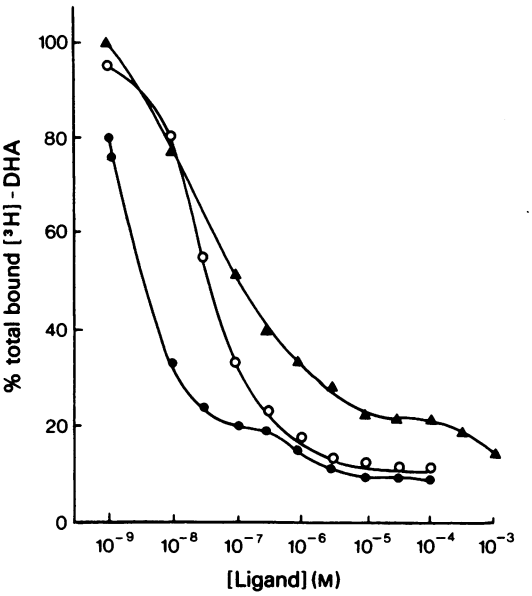

Figure 2 Inhibition of total [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding to BC_3H1 cell membranes by (−)-alprenolol (●), (+)-alprenolol (○) and (−)-isoprenaline (▲). [^{3}H]-DHA concentration was 0.5 nM, incubations were performed as described in Methods. Each curve is the mean of two duplicate experiments.

cates that [^{3}H]-DHA does not bind to a homogeneous class of sites. This behaviour is further confirmed by the inhibition of [^{3}H]-DHA binding by different adrenoceptor agonists and antogonists. The occupation of [^{3}H]-DHA binding sites by isoprenaline (Figure 2), noradrenaline and adrenaline (not shown) is bimodal. The radioligand cannot be totally displaced from the low affinity site by the agonists. Their EC_{50} for this site is higher than 10^{-4} M. The same biphasic curve was observed when [^{3}H]-DHA binding was displaced by alprenolol (Figure 2) or propranolol (results not shown). It can be seen that the second low affinity site is totally occupied by a concentration of alprenolol below 10^{-4} M. This effect of alprenolol is not stereospecific.

On the basis of the previous experiments it appeared to be possible to evaluate separately the binding of [^{3}H]-DHA to each of the two components. Accordingly, in the experiment described in Figure 1 'specific binding 1' (Sp 1) was defined as the radioactivity displaced by 20 μM isoprenaline. 'Specific binding 2' (Sp 2) is the difference between the binding observed in the presence of 20 μM isoprenaline and the value obtained in the presence of 0.1 mM alprenolol. The saturation curve for the first site shows a clear-cut plateau (Figure 3a). The Scatchard analysis of these data (Figure 3b) indicates that binding of the radioligand measured under these conditions is saturable to a homogeneous class of sites. The K_D of this site is 0.54 nM and the maximal binding 71 fmol/mg of protein. The Scatchard analysis of the [^{3}H]-DHA binding site displaced by 0.1 mM alprenolol and not displaced by 20 μM isoprenaline is shown in Figure 3c. This site seems to be saturable and homogeneous having a K_D of 94 nM and a maximal binding capacity of 944 fmol/mg of protein.

If binding of [^{3}H]-DHA to each of these sites follows mass-action behaviour, we can calculate a saturation curve for the total specific binding (Sp 1 + Sp 2) displaced by 0.1 mM alprenolol by the expression:

$$B_T = B_1 + B_2 = \frac{B_{1max}F}{K_{D_1} + F} + \frac{B_{2max}F}{K_{D_2} + F}$$

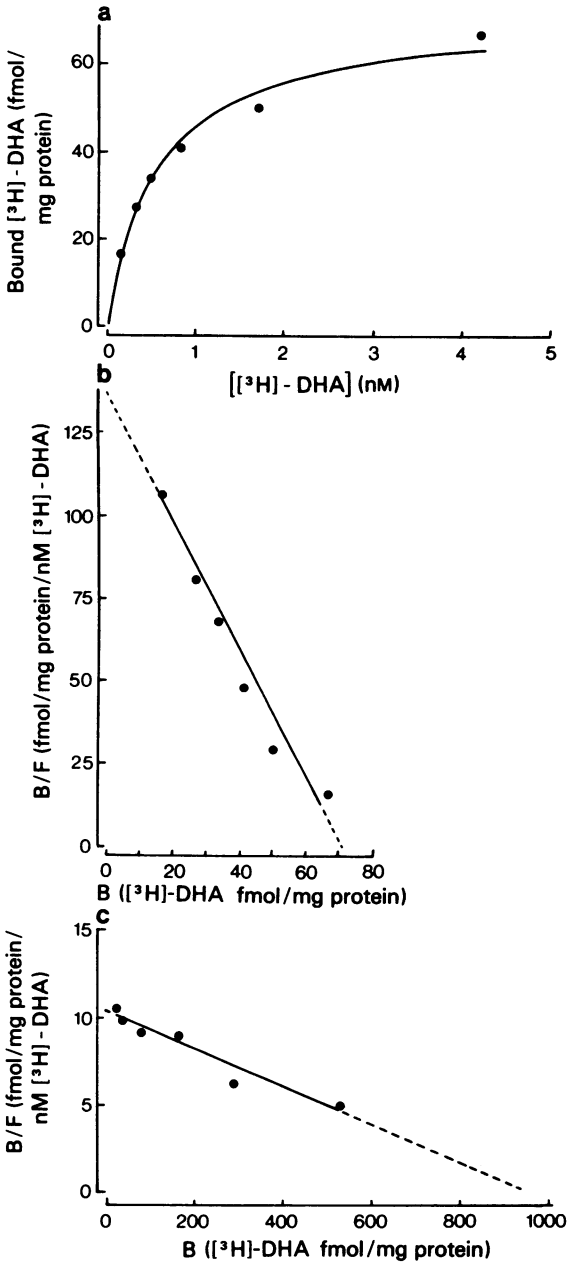

Figure 3 Binding of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) to two different sites on BC_3H1 cell membranes. The results described in this figure were obtained during the same experiment as shown in Figure 1. (a) Saturation curve of binding of [^{3}H]-DHA to BC_3H1 cell membranes. Specific binding 1 is defined as the difference between binding observed in the absence and the presence of 20 μM isoprenaline. (b) Scatchard plot of specific binding 1 of [^{3}H]-DHA to BC_3H1 cell membranes. The regression line gives a K_{D_1} of 0.54 nM and a B_{max_1} of 71 fmol/mg of protein. (c) Scatchard plot of specific binding 2 of [^{3}H]-DHA to BC_3H1 cell membrane. Specific binding 2 is defined as the difference between binding observed in the presence of 20 μM isoprenaline and in the presence of 0.1 mM alprenolol, with [^{3}H]-DHA concentrations ranging from 2 to 100 nM. The regression line gives a K_{D_2} of 94 nM and B_{max_2} of 944 fmol/mg of protein.

B_T is the bound radioligand, F is the free radioligand concentration, $B_{1\,max}$ and $B_{2\,max}$ being the maximal capacity of each site.

It can be seen that the curve, obtained by the calculation of this total specific binding and by use of the parameters of curves 3b and 3c, fits the experimental data of Figure 1a excellently.

It can be concluded from these experiments that [^{3}H]-DHA binds to two classes of sites. The first site has a high affinity ($K_{D1} = 0.53 \pm 0.04$ nM; $n = 14$) and a low capacity ($B_{1\,max} = 58 \pm 2.4$ fmol/mg of protein; $n = 14$). The second site has a low affinity ($K_{D2} = 110$ nM; $n = 3$) and a high capacity ($B_{2\,max} = 1100$ fmol/mg of protein, $n = 3$).

In the following experiments we have tried to characterize further the binding to the first class of sites (displaced by 20 μM isoprenaline) which appears to behave like a classical β-receptor.

Properties of the high affinity, low capacity [^{3}H]-dihydroalprenolol binding site

The specific binding to the BC_3H1 cell membrane, defined now by the difference between total binding and the binding observed in the presence of 20 μM isoprenaline, represents 70 to 80% of the total binding. Association and dissociation kinetic experiments were performed at 3 concentrations of free [^{3}H]-DHA: 0.2, 0.7 and 1 nM. A dissociation rate constant of 0.104 ± 0.015 min^{-1} ($n = 3$) was determined and an association rate constant of 0.375 ± 0.073 nM min^{-1} ($n = 3$) was calculated following the relation

$$k_1 = \frac{k_{obs} - k_2}{[[^3\text{H}]\text{-DHA}]}$$

where k_1 is the association rate constant; k_{obs}, the pseudo first order association rate constant, k_2 the dissociation rate constant and [[^{3}H]-DHA] the free concentration of radioligand. A K_D value of 0.28 nM was calculated from the relation

$$K_D = \frac{k_2}{k_1}$$

which is close to the value obtained by equilibrium binding experiments.

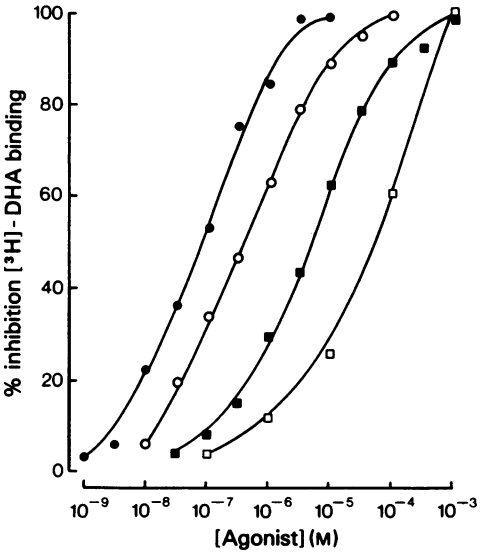

Figure 4 Inhibition of specific [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding to BC_3H1 cell membranes by the following adrenoceptor agonists: (●) isoprenaline, (○) adrenaline, (■) noradrenaline, (□) phenylephrine. [^{3}H]-DHA concentration was 0.5 nM, incubations were performed as described in Methods. Each curve is the mean of three duplicate experiments.

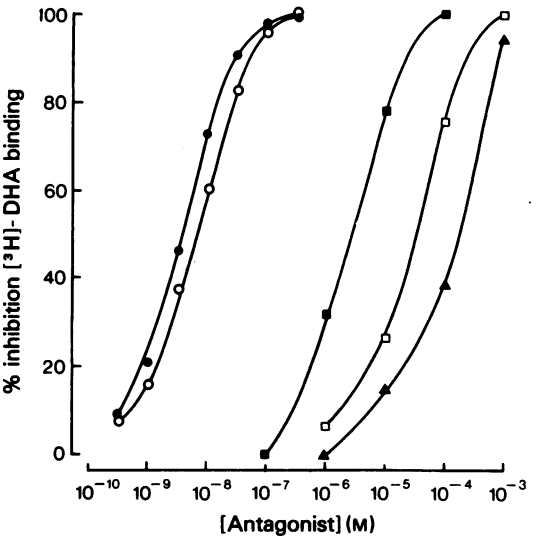

Figure 5 Inhibition of specific [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding to BC_3H1 cell membranes by the following adrenoceptor antagonists: (●) alprenolol, (○) propranolol, (■) butoxamine, (□) practolol, (△) phentolamine, [^{3}H]-DHA concentration was 0.5 nM, incubations were performed as described in Methods. Each curve is the mean of three duplicate experiments.

The ability of several adrenoceptor agonists and antagonists to displace [^{3}H]-DHA from its binding site was tested. The potency of the agonists in displacing [^{3}H]-DHA was: isoprenaline > adrenaline > noradrenaline > phenylephrine (Fig. 4). The order of potency of agonists is typical of β_2-adrenoceptors (Lands, Arnold, McAuliff, Luduena & Brown, 1967). Confirming these results the order of potency for the antagonists was: alprenolol ≃ propranolol > butoxamine > practolol > phentolamine (Figure 5), consistent again with the β_2-adrenoceptor properties.

It is noticeable that the agonist displacement curves are flatter than the antagonist curves. A Hill plot of these data shows that the slope is close to 1 for the antagonists alprenolol and propranolol but is less than 1 for the agonists (Table 1).

Table 1 Binding properties of different adrenoceptor agonists and antagonists and correlation with their effect on ^{86}Rb efflux rate

β-Adrenoceptor agent	K_D(nM)*	nH†	EC_{50}(nM)‡	K_B(nM)§	$\frac{K_D}{EC_{50}}$ or K_B
Isoprenaline	38	0.74	16	—	2.37
Adrenaline	180	0.75	20	—	9
Noradrenaline	2,100	0.63	1,100	—	1.91
Alprenolol	1.9	1.05	—	2.1	0.90
Propranolol	2.8	0.96	—	3	0.93
Butoxamine	1,000	—	—	—	—
Practolol	19,000	—	—	—	—

*The dissociation constants are calculated from the IC_{50} determined from Figures 4 and 5. †The Hill coefficient was determined from the Hill plot of the data from Figures 4 and 5. ‡The EC_{50} of stimulation of ^{86}Rb efflux rate was determined from Figure 6. § K_B was calculated from the pA_2 value determined by a Schild plot for antagonism of the adrenaline dose-effect curve.

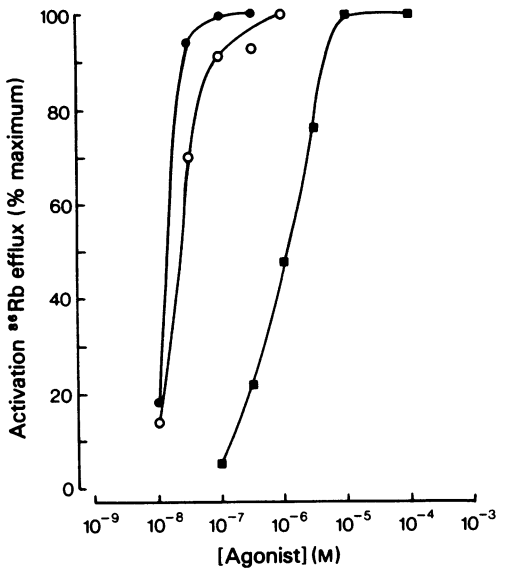

Figure 6 Log dose-effect curve of ^{86}Rb efflux activation from BC_3H1 cells by (●) isoprenaline, (○) adrenaline and (■) noradrenaline. ^{86}Rb efflux was analysed in terms of the rate coefficient (r) as defined in the text. Maximal $100\,\Delta r$ was equal to $0.96 \pm 0.10\,min^{-1}$ ($n = 10$). Each curve is the mean of at least three experiments.

A K_D can be calculated for these agonists and antagonists from the relationship:

$$IC_{50} = K_D\left(1 + \frac{(DHA)}{K_{D(DHA)}}\right)$$

where (DHA) is the concentration of [^{3}H]-DHA, $K_{D\,(DHA)}$ is the dissociation constant of [^{3}H]-DHA calculated from the Scatchard plot, K_D is the dissociation constant of unlabelled agonists and antagonists for the [^{3}H]-DHA binding site, and IC_{50} is the concentration of these ligands that reduced the binding of [^{3}H]-DHA by 50% (Cheng & Prusoff, 1973). The values obtained for different agonists and antagonists are presented in Table 1.

Effects of some adrenoceptor agonists and antagonists on ^{86}Rb efflux rate

We have shown previously that it is possible to gauge the membrane response of the BC_3H1 cells to different agonists and antagonists by studying their effect on ^{86}Rb efflux (Mauger, *et al.*, 1978). ^{86}Rb effluxes were evaluated by calculating the rate coefficient:

$$r = \frac{A_1 - A_2}{\frac{A_1 + A_2}{2}\Delta t}$$

where A_1 and A_2 are two points in the desaturation curve separated by the period Δt. Dose-response curves were obtained in the presence of 1 μM phentolamine in order to block α-adrenoceptors. Under these conditions, the order of potency of agonists (isoprenaline > adrenaline > noradrenaline) was identical to the order obtained by studying the displacement of [^{3}H]-DHA binding (Fig. 6). The β-adrenoceptor antagonists, alprenolol and propranolol, displaced agonists dose-efflux curves to the right. Figure 7a shows the effect of increasing concentrations of alprenolol on the adrenaline dose-response curve. A Schild plot (Schild, 1947) of these data gives a straight line with a slope of 1.18 which is compatible with a competitive antagonism (Figure 7b). Similar results (not shown) were obtained when adrenaline dose-efflux curves were displaced by propranolol. The K_B values for these antagonists, calculated from the pA_2 are 2.1×10^{-9} M for alprenolol and 3×10^{-9} M for propranolol.

We have compared the EC_{50} or K_B values for the agonists and antagonists obtained from the ^{86}Rb efflux curves and the K_D obtained by displacement of [^{3}H]-DHA from its binding site. There is a very good correlation for the constants obtained by the two methods for the antagonists, alprenolol and propranolol (Figure 8). On the other hand, the constants for the agonists do not coincide at all. The ratio between the K_D obtained by [^{3}H]-DHA displacement and the EC_{50} of ^{86}Rb efflux for isoprenaline adrenaline and noradrenaline is respectively 2.4, 9 and 2. It must be stressed that the dose-effective curves for ^{86}Rb efflux are much steeper than the curves of inhibition of [^{3}H]-DHA binding. This discrepancy indicates a non linear coupling between the receptor occupancy and the biological effect. The maximal biological effect is obtained before all the receptors are occupied by the agonist. The occupancy-concentration curve was calculated assuming that the [^{3}H]-DHA binding inhibition curve is displaced by a factor of

$$1 + \frac{DHA}{K_{D(DHA)}}.$$

In these conditions, maximal response is obtained with isoprenaline, adrenaline and noradrenaline when 67%, 65% and 71% respectively of the receptors are occupied.

Discussion

It has been shown previously by Schubert *et al.* (1974) that the BC_3H1 cells are excitable. The hyperpolarization induced by noradrenaline is associated with an increase in the K^+ or Rb^+ efflux rate (Mauger *et al.*, 1978). The study of the pharmacological character-

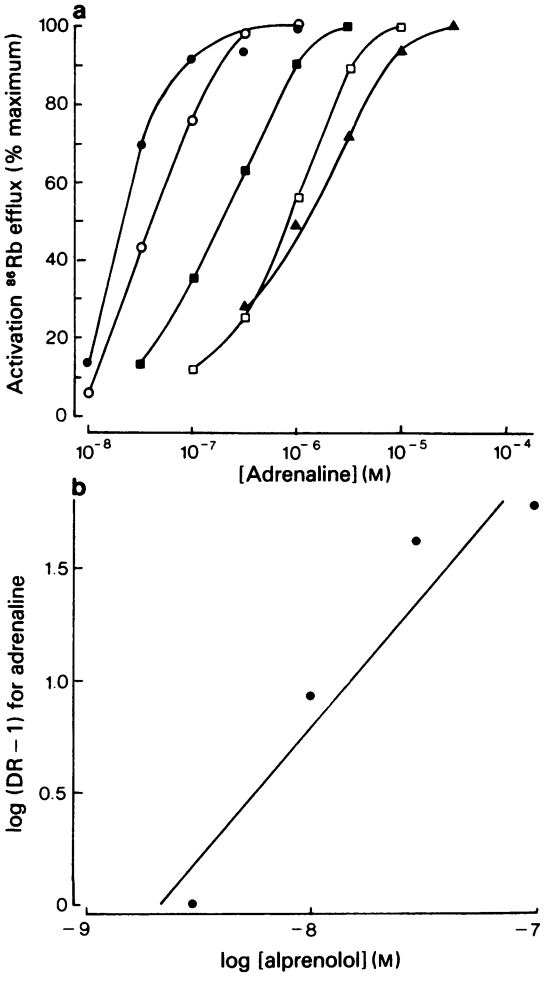

Figure 7 (a) Inhibitory effect of the β-adrenoceptor antagonist, alprenolol, on ^{86}Rb efflux stimulated by adrenaline. Dose-effect curves were obtained in the absence (●) or in the presence of alprenolol 3×10^{-9} M (○), 10^{-8} M (■), 3×10^{-8} M (□) and 10^{-7} M (▲). Each point is the mean of three experiments. (b) Schild plot of the dose-response curves obtained in the presence of alprenolol. The dose ratio (DR) is the ratio of the concentration of adrenaline causing half maximal stimulation of ^{86}Rb efflux in the presence and absence of a given concentration of the antagonist. A slope of 1.18 was determined by linear regression analysis ($r = 0.96$), the pA_2 being 8.7.

istics of the ^{86}Rb efflux response has made possible the identification of α and β-adrenoceptors in this cell line.

The purpose of the present work has been to correlate the binding properties of the β-adrenoceptor antagonist [^{3}H]-DHA on the BC_3H1 membranes, with

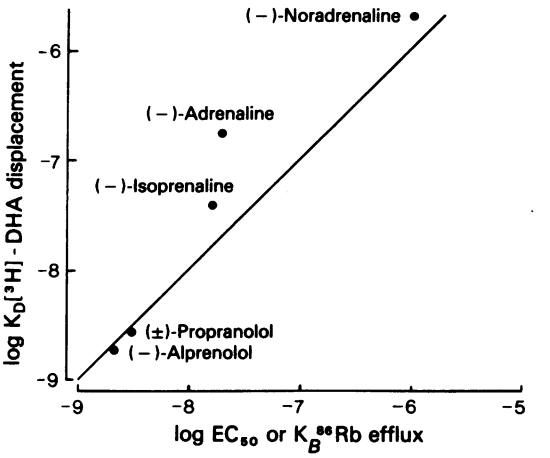

Figure 8 Comparison between the K_D obtained by inhibition of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding to BC_3H1 cell membranes and the EC_{50} or K_B of stimulation of ^{86}Rb efflux from BC_3H1 cells for different β-adrenoceptor agonists and antagonists. Values were calculated from Table 1. The line drawn is the line of identity.

the biological response induced by the stimulation of the β-receptors.

The Scatchard analysis of [^{3}H]-DHA binding indicates that the radioligand binds specifically to at least two classes of site. This heterogeneity is confirmed by the study of the inhibition of [^{3}H]-DHA binding by different adrenoceptor agonists and antagonists. The characteristics of the high affinity [^{3}H]-DHA binding site correspond to that described for the β-adrenoceptors. The K_D value of 0.53 nM is close to that found in different tissues such as rat lung (Barnett, Nahorski & Rugg, 1977), calf cerebellum and cortex and rat lung and heart (U'Prichard, Bylund & Snyder, 1978) and cultured S49 lymphoma cells (Insel & Stoolman, 1978).

The maximal binding capacity of 58 fmol/mg protein corresponds to at least 6000 sites per cell. Since we do not know the recovery of binding sites during the membrane preparation, this value is probably underestimated.

The properties of the high affinity [^{3}H]-DHA binding site correspond to the β_2-adrenoceptors as described by Lands *et al.* (1967) since the agonists compete with [^{3}H]-DHA binding following the series isoprenaline > adrenaline > noradrenaline > phenylephrine and the antagonists the series alprenolol ≃ propranolol > butoxamine > practolol > phentolamine (Levy, 1966; Dunlop & Shanks, 1968).

The slope of the displacement curves obtained by competition of antagonists with [^{3}H]-DHA are steeper than the agonist curves. The Hill plot of the agonist curves gives a straight line with a slope much

lower than 1, as opposed to the antagonist curves which have a slope very close to 1. We have observed that guanosine triphosphate (GTP) steepens isoprenaline binding curves shifting them to the left and increasing 3 times its K_D (results not shown). The low Hill coefficient observed with agonists could be due to the desensitization of the β-adrenoceptors during the incubation. This possibility seems excluded, since the inhibition of [^{3}H]-DHA binding by isoprenaline at concentrations of the agonist varying between 10^{-8} to 10^{-5} M remains constant during incubation times of 10 to 30 min (data not shown). A similar difference in the Hill constants for agonists and antagonists has been found by other workers. Low Hill coefficients with β-adrenoceptor agonists have been observed by Ross, Maguire, Sturgill, Biltonen & Gilman (1977) with the membrane preparation from S49 lymphoma cells. These authors have shown that in the absence of purine nucleotides, agonists' binding yields Hill coefficients near to 0.5. In the presence of nucleotides the slope of the Hill plot approaches a value of 1. They interpreted the results obtained with S49 cells on the basis of the existence of states of the receptor having different affinity for agonists and interconverting in the presence of the nucleotides.

The pharmacological characteristics of the β-adrenoceptor mediated activation of ^{86}Rb efflux, are similar to some of the properties of the [^{3}H]-DHA high affinity site. β-Adrenoceptor agonists increase ^{86}Rb efflux rate in a dose-dependent manner, their relative potencies being isoprenaline > adrenaline > noradrenaline, corresponding to a β_2-receptor type activation. Alprenolol and propranolol behave as typical competitive antagonists. The K_B values for these compounds, calculated from the pA_2 value, are very close to those found in [^{3}H]-DHA binding competition experiments. The dissociation constants of agonists, determined by competition of radioligand binding are higher than the ED_{50} determined from the ^{86}Rb efflux curves. The K_D/EC_{50} ratios for isoprenaline, adrenaline and noradrenaline are 2.4, 9.0 and 2.0, respectively. The calculation of the K_D/EC_{50} values, on the basis of displacement experiments performed in the presence of GTP might have given even bigger ratios. These results indicate a non linear relationship between the agonist occupation of the β_2 receptor site and the activation of the ^{86}Rb efflux. In fact, the maximal biological effect is obtained at concentrations of the agonists which partially occupy the receptors, suggesting the existence of spare receptors in this preparation. Alternatively, the occupation of the receptors at higher concentrations of the agonists might lead to just a small, non detectable, increase in the biological effect. A similar discrepancy between agonists binding and dose-effect curves has been observed in other cell systems in which the biological response studied was the stimulation of adenylcyclase: broken C_6 glioma cells (Lucas & Bockaert, 1977) or S49 lymphoma cells (Ross *et al.*, 1977), and intact glioma cells (Terasaki & Brooker, 1978) or S49 lymphoma cells (Insel & Stoolman, 1978).

We have not pursued the study of the properties of the low affinity site having a K_D for [^{3}H]-DHA of 1.1×10^{-7} M. This site appears to have a negligible affinity for agonists. Isoprenaline, adrenaline and noradrenaline displace [^{3}H]-DHA from the low affinity site with an EC_{50} higher than 10^{-4} M. These characteristics are not compatible with the properties of the ^{86}Rb efflux response. Several laboratories have described a similar low affinity binding site of antagonists, on turkey erythrocyte membranes (Brown, Fedak, Woodard, Aurbach & Rodbard, 1976), as well as on intact cultured S49 cells (Insel & Stoolman, 1978). This low affinity binding site could be located intracellularly and so be detected in crude membrane preparations or intact cells. Indeed, Schneck, Pritchard & Hayes (1977), have found that propranolol binds to microsomal and mitochondrial fractions from several tissues, following intravenous administration of the ligand and *in vitro* incubation with homogenates.

In conclusion, [^{3}H]-DHA binds to a crude membrane fraction from BC_3H1 non fusing muscle cells, at two classes of site. The high affinity site has binding properties characteristic of the β_2-adrenoceptor type. The coupling between the receptor occupancy and the membrane events responsible for the ^{86}Rb response is not linear. The occupation of the low affinity [^{3}H]-DHA site does not appear to be related to the biological response studied.

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