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β-ADRENERGIC RECEPTORS OF BRAIN CELLS

MEMBRANE INTEGRITY IMPLIES APPARENT POSITIVE COOPERATIVITY AND HIGHER AFFINITY

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β-Adrenergic receptors were studied in intact cells of chick, rat and mouse embryo brain in primary cultures, by the specific binding of [^{3}H]dihydro-L-alprenolol ([^{3}H]DHA). The results were compared to the receptor binding of broken cell preparations derived from the cell cultures or from the forebrain tissues used for the preparation of the cultures. Detailed analysis of [^{3}H]DHA binding to living chick brain cells revealed a high-affinity, stereoselective, β-adrenergic-type binding site. Equilibrium measurements indicated the apparent positive cooperativity of the binding reaction. By direct fitting of the Hill equation to the measured data, values of $B_{max} = 12.01$ fmol/10^6 cells (7200 sites/cell), $K_d = 60.23$ pM and the Hill coefficient $n = 2.78$ were found. The apparent cooperative character of the binding was confirmed by the kinetics of competition with L-alprenolol, resulting in maximum curves at low ligand concentrations. The rate constants of the binding reaction were estimated as $k_+ = 8.31 \cdot 10^7$ $M^{-1} \cdot min^{-1}$ and $k_- = 0.28$ min^{-1} from the association results, and $k_- = 0.24$ min^{-1} from the dissociation data. The association kinetics supported the cooperativity of the binding, providing a Hill coefficient $n = 1.76$; K_d, as $(k_-/k_+)^{1/n}$ was found to be 101 pM. Analysis of the equilibrium binding of [^{3}H]DHA to rat and mouse living brain cells resulted in values of $B_{max} = 13.04$ fmol/10^6 cells (7800 sites/cell), $K_d = 43.85$ pM and $n = 2.52$, and $B_{max} = 8.08$ fmol/10^6 cells (4800 sites/cell), $K_d = 46.70$ pM and $n = 1.63$, respectively, confirming the apparent cooperativity of the β-receptor in mammalian objects, too. The [^{3}H]DHA equilibrium binding to broken cell preparations of either chick, rat or mouse brain cultures or forebrain tissues was found to be non-cooperative, with a Hill coefficient $n = 1$, K_d in the range 1–2 nM, and a B_{max} of 10^3–10^4 sites/cell. Our findings demonstrate that cell disruption causes marked changes in the kinetics of the β-receptor binding and in the affinity of the binding site, although the number of receptors remains unchanged.

Introduction

Kinetic analysis of hormone-receptor complex formation may provide information about the characteristics and function of the receptor. β-Adrenergic receptors are commonly studied by means of the labelled antagonist [^{3}H]DHA ([^{3}H]dihydro-L-alprenolol) or ^{125}I-labelled hydroxybenzylpindolol. The ligand-receptor specific binding has been found to be stereoselective, rapid and reversible. In most cases it has been described as a simple bimolecular reaction. Steady-state analyses by Scatchard and Hill plots revealed one homogeneous class of binding sites and a lack of cooperative interactions [1–4].

In some studies, however, the specific binding

of the β-adrenergic ligand proved to be more complex; a curvilinear Scatchard plot and a Hill coefficient less than unity were found. This can be explained by the heterogeneity of the binding sites or by the apparent negative cooperativity of the receptors, or both. Negative cooperativity was confirmed in guinea-pig cardiac and lung membranes [5], in rat kidney membranes [6], in frog erythrocytes [7] and in human leucocytes [8]. The possibility of conformational changes of the receptor as the origin of the apparent cooperativity was supposed by Krawietz et al. [5]. The cooperativity of β-receptor binding has been challenged, however, by some authors [9].

The goal of the present work was to examine the possibility of cooperative phenomena in β-adrenergic receptors by kinetic methods, using primary cultures of chick, rat and mouse brain. As far as we know, intact, normal brain cells have not previously been the object of β-receptor binding studies.

The results of [^{3}H]DHA binding to intact cells were compared to those for broken cell preparations. This was of interest, because it is still an open question as to whether the results obtained on membrane particles can be extrapolated to the receptors functioning in the living cells [10]. Some recent studies on cell lines revealed that, besides basic similarities, the parameters of β-receptor binding of intact and disrupted cells are not identical. Differences were found in the receptor number in S49 lymphoma cells [11], in the affinity to β-agonists in C6 glioma cells [12] and in lymphocytes [13], in the affinity to the β-antagonist ligand and in the value of the Hill coefficient in leucocytes [8]. Differences were also found in the adenylate cyclase responsiveness [14]. The evaluation of these results, however, is problematical, since the parameters of the β-receptor ligand-binding are not immutable characteristics of the cells. The receptor concentration can be influenced by metabolic inhibition (starvation) [15], or by the presence of a high concentration of β-agonists (desenzitization) [12,14,16]; it also shows cell-cycle-specific changes [17] and variations during development [16,18,19], while the affinity of the binding site changes under the influence of cytoplasmic factors [13], on disintegration of the microtubules (colchicine treatment) [8] or on homogenization of the cells [8].

These data emphasize the importance of the careful choice of experimental arrangement if parameters of receptor binding are to be compared. In our work the β-receptor binding parameters of intact, cultured brain cells were compared to those of membrane particles derived from the cultured cells or from the brain tissues used for the preparation of the cultures. We presumed that the differences in the binding kinetics of these preparations might provide information on the role of the intact membrane in the β-receptor function. Studies were performed on avian and mammalian objects, to expose the variations due to the genetic differences.

Materials and Methods

Materials

Chemicals used for cell cultivation were from GIBCO, and other chemicals from Sigma. GF/C glass fibre filters (2.5 cm diameter) were Whatman products. [^{3}H]Dihydro-L-alprenolol ([^{3}H]DHA) was prepared in our institute, with a specific radioactivity of 69.7 Ci/mmol.

Preparation of primary cultures

Chick brain cultures were prepared from cortical hemispheres of 8-day-old chick embryos by either mechanical or enzymatic dissociation, which provided similar cultures, as previously described [20,21]. Approx. 10^6 cells were seeded in Falcon plastic Petri dishes (6 cm diameter) and cultivated in Eagle's minimum essential medium supplemented with 20% fetal calf serum and antibiotics, in a standard incubator (37°C, humidified air flow containing 5% CO_2). Experiments were performed on 8–10-day-old cultures containing a well-developed neuronal network on top of a glial sheet.

Rat and mouse brain cultures were prepared from forebrains of newborn animals. The tissue, free of meninges, was dissociated by the enzymatic method [21]. $2 \cdot 10^6$ cells were placed in 3 cm diameter Falcon plastic Petri dishes and cultivated for 8–10 days as in the case of the chick. These cultures consisted of non-neuronal (glial) cells, mainly astroblasts, and only a few neurons.

The cell number was determined at the time of the experiments from parallel dishes, after secondary dissociation of the cell layer, by counting in a Buerker chamber. The variation of the cell number within a series of cultures was around 5%.

Measurement of [^{3}H]DHA binding

(1) Primary cultures. Binding studies were always performed on living cells of intact cultures. Incubations were carried out in a standard CO_2 incubator.

Cultures were washed free of the growth medium with 3×2 ml Tyrode solution at 37°C, and preincubated in 1 ml serum-free Eagle's minimum essential medium for 20 min. The binding reaction was started by adding the [^{3}H]DHA, in concentrations of 10–180 pM. Nonspecific binding was measured in the simultaneous presence of 1–10 μM L-alprenolol. During the time of the experimental incubation (15 min in the case of the equilibrium experiments) the culture dishes were gently moved for continuous mixing. At the end of the binding reaction the radioactive medium was discarded and a rapid and intensive washing was performed by filling the Petri dishes three times with physiological saline at room temperature, within 10 s. Longer or more washings did not influence the specific and nonspecific binding values. The bound radioactivity was quantitatively transferred to Bray's solution either by dissolving the cells in 1 M NaOH and neutralizing the solution with 4 M HCl, or by treating the wet cell layer with 2×500 μl methanol [22]. The two methods provided similar results. Radioactivity was measured in a liquid scintillation counter.

The specific binding was obtained as the difference of the total and nonspecific bindings. Results are given as bound fmol/10^6 cells. The nonspecific binding did not exceed 30% of the total binding, and increased linearly with the cell number of the cultures and with the [^{3}H]DHA concentration of the medium in the studied range.

Association of the labelled ligand to the specific binding sites was followed in time between 0.5 and 15 min at [^{3}H]DHA concentrations of 40, 70 and 110 pM. Nonspecific binding was independent of the incubation time; this value was subtracted from the association total binding results.

Dissociation was measured by an isotope dilution method. After a 15 min incubation with 70 pM [^{3}H]DHA, the cultures received 1 μM L-alprenolol without change in the medium. The decrease in the specific binding with time was followed. The simple dilution method weas omitted owing to technical difficulties [23].

Displacement experiments were performed at 30, 45 and 80 pM [^{3}H]DHA, by adding increasing amounts of unlabelled L-alprenolol simultaneously with the labelled one, and incubating the samples for 15 min.

The competition of L- and D-propranolol, L-norepinephrine and DL-phenoxybenzamine for the specific binding sites was measured at increasing drug concentrations at a fixed [^{3}H]DHA concentration of 80 pM. The incubation time was 15 min.

(2). Homogenized cultures and forebrains. Chick, rat and mouse cell cultures of the same age as in the intact-cell experiments were washed with 3×2 ml Tyrode solution at room temperature. The cells were pooled by scraping with a silicone rubber and homogenized by hand in ice-cold 50 mM Tris-HCl (pH 8.0), using a glass homogenizer. Cell disruption was controlled under the microscope.

Forebrains of 10-day-old chick embryos or newborn rats or mice were homogenized in the same buffer.

The binding of [^{3}H]DHA to homogenates was measured according to the method of Bylund and Snyder [3]. 50–100 μg protein was incubated with 0.1–10 nM [^{3}H]DHA in the presence or absence of 10 μM L-alprenolol in 1 ml 50 mM Tris-HCl (pH 8.0) at room temperature. After 10 min the samples were diluted and filtered through GF/C filters. Washing was performed with 4×5 ml of the same buffer. Filters were dried and counted in toluene-based scintillation fluid.

The results were calculated as bound fmol/10^6 cells in the case of the homogenized cultures, and as bound fmol/100 μg total protein for homogenized brains. These values are comparable, since the average protein content of 10^6 cells in different types of culture is around 80–120 μg. Protein was determined by the method of Lowry et al. [24].

Calculations

The equilibrium binding of [^{3}H]DHA was interpreted on the basis of the model:

$$nF + R \underset{k_-}{\overset{k_+}{\rightleftarrows}} B \qquad (1)$$

The corresponding differential equation is:

$$\frac{dB}{dt} = k_+ F^n \cdot R - k_- B \qquad (2)$$

The steady-state solution gives the Hill equation:

$$B_{eq} = \frac{B_{max} \cdot F^n}{K_d^n + F^n} \quad (3)$$

where B is the number of receptors occupied by the ligand; R corresponds to the free receptors; B_{max} is the total number of receptors; F is the amount of the free ligand, which is nearly equal to the total ligand concentration, T, if the ligand is in large excess; K_d^n is the dissociation constant; and n is the Hill coefficient.

The values of B_{max}, K_d and n were determined by computer fitting of Eqn. 3 to the measured data. The kinetics of inhibition of specific [^{3}H]DHA binding with unlabelled L-alprenolol were analysed by the modified Hill equation [26]:

$$B^* = \frac{B_{max}(T+C)^n}{K_d^n + (T+C)^n} \cdot \frac{T}{T+C} \quad (4)$$

where C is the unlabelled ligand concentration, T is the labelled ligand concentration and B^* corresponds to the bound radioactivity.

Kinetic rate constants were estimated by the pseudo-first-order approximation of Eqn. 2, which describes association other than the simple bimolecular model:

$$\frac{dB}{dt} = k_+ T^n R - k_- B \quad (5)$$

Since $R = B_{max} - B$:

$$\frac{dB}{dt} = k_+ T^n B_{max} - B(k_+ T^n + k_-) \quad (6)$$

which on integration yields:

$$B = B_{eq}(1 - e^{-\lambda t}) \quad (7)$$

where B_{eq} is the equilibrium concentration of B, as given by Eqn. 3 with F nearly equal to T. From this formula:

$$\lambda = k_+ T^n + k_- \quad (8)$$

Since:

$$\lambda B_{eq} = k_+ B_{max} T^n \quad (9)$$

from the plot of log λB_{eq} vs. log T the values of n and $k_+ B_{max}$ can be estimated. Knowing n, the plot λ vs. T^n yields k_+ and k_-. Then B_{max} and $K_d = (k_-/k_+)^{1/n}$ may also be computed.

Results

[^{3}H]DHA binding to intact, primary brain cells

The specific [^{3}H]DHA binding to living brain cells was found to be stereoselective as measured by the competition of the β-adrenergic antagonists D- and L-propranolol. Fig. 1 shows the results obtained on chick brain cultures. The IC_{50} values were estimated as $1.2 \cdot 10^{-9}$ M and $2.5 \cdot 10^{-11}$ M, respectively, demonstrating a difference of about two orders of magnitude in the effectivities of the stereoisomers. Fig. 1 also shows that the β-agonist L-norepinephrine proved to be a more effective

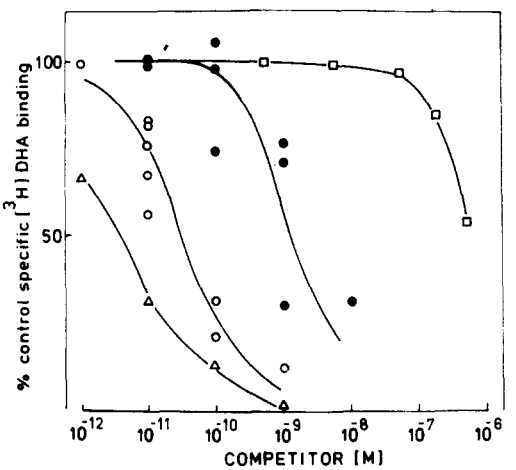

Fig. 1. Inhibition of specific [^{3}H]DHA binding to living, cultured chick brain cells by L-norepinephrine (△———△), L-propranolol (○———○), D-propranolol (●———●) or DL-phenoxybenzamine (□———□). 50% inhibition values (IC_{50}) were estimated as:

Drug	IC_{50} (M)
L-Norepinephrine	$3.5 \cdot 10^{-12}$
L-Propranolol	$2.5 \cdot 10^{-11}$
D-Propranolol	$1.2 \cdot 10^{-9}$
DL-Phenoxybenzamine	$4 \cdot 10^{-7}$

Specific [^{3}H]DHA binding was measured by incubating the cultures with 80 pM [^{3}H]DHA, simultaneously with increasing concentrations of competitors, in 1 ml Eagle's minimum essential medium for 15 min at 37°C. 100% binding is equal to 7.8 fmol/10^6 cells. Points represent individual experiments.

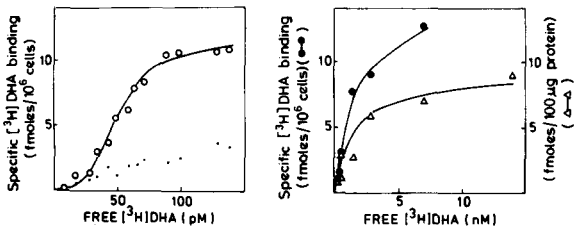

Fig. 2. Equilibrium binding of [^{3}H]DHA to intact, cultured chick brain cells (○——○) and to broken membranes obtained from these cultures (●——●) or from the forebrain tissue used for the cultivation (△——△). Intact cells of 8-day-old cultures were incubated in serum-free Eagle's minimum essential medium for 15 min at 37°C with 10–180 pM [^{3}H]DHA. Nonspecific binding was measured in the simultaneous presence of 1–10 µM L-alprenolol. [^{3}H]DHA binding of broken membranes was measured in 50 mM Tris-HCl (pH 8.0) by the method of Bylund and Snyder [3]. Nonspecific binding was determined in the presence of 10 µM L-alprenolol. Points represent the averages of 3–4 independent determinations. Curves were obtained by direct fitting of the Hill equation to the points. Parameters of the binding reaction estimated in this way are: $B_{max} = 12.01$ fmol/10^6 cells, $K_d = 60.23$ pM, $n = 2.78$ for intact cells; $B_{max} = 9.24$ fmol/10^6 cells; $K_d = 1.41$ nM, $n = 1$ for broken cultured cells; and $B_{max} = 16.42$ fmol/100 µg protein, $K_d = 1.99$ nM, $n = 1$ for forebrain homogenate. Dots show the non-specific binding.

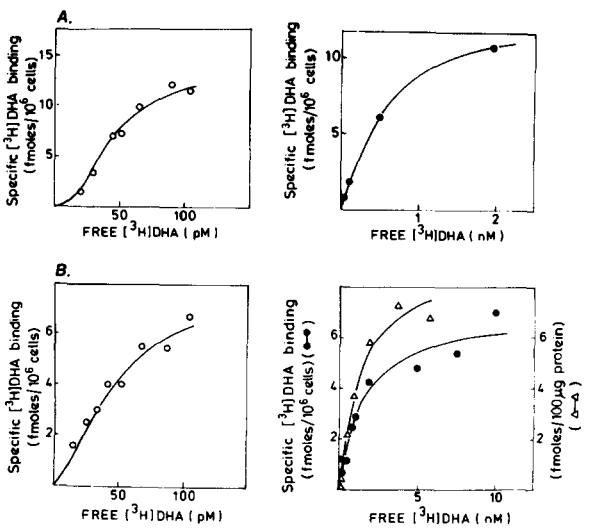

Fig. 3. Equilibrium binding of [^{3}H]DHA to living, cultured brain cells (○——○) or fragmented membranes of cultured cells (●——●) of rat (panel A) or mouse (panel B), as well as to membrane fragments of forebrain tissue of mouse (△——△, panel B). Intact cells of 10-day-old cultures were incubated in serum-free Eagle's minimum essential medium for 15 min at 37°C with 10–150 pM [^{3}H]DHA. Nonspecific binding was measured in the simultaneous presence of 1–10 µM L-alprenolol. [^{3}H]DHA binding to broken membranes was measured in 50 mM Tris-HCl (pH 8.0) by the method of Bylund and Snyder [3]. Nonspecific binding was measured in the presence of 10 µM L-alprenolol. Points represent the average of 2–3 determinations. Curves were obtained by direct fitting of the Hill equation to the measured values. Parameters of the binding reaction estimated by this method are given in Table I.

competitor ($IC_{50} \approx 3.5 \cdot 10^{-12}$ M) than the antagonist L-alprenolol (Fig. 4: $IC_{50} \approx 1.05 \cdot 10^{-10}$ M) or L-propranolol ($IC_{50} \approx 2.5 \cdot 10^{-11}$ M). The α-antagonist DL-phenoxybenzamine interacted with the [^{3}H]DHA binding sites only at very high concentrations ($IC_{50} \approx 4 \cdot 10^{-7}$ M). These results confirm the β-adrenergic character of the binding site.

The equilibrium [^{3}H]DHA binding to intact cells followed sigmoidal saturation kinetics between ligand concentrations of 10 and 180 pM (Figs. 2 and 3). The parameters of the binding reaction were estimated by direct fitting of the Hill equation (Eqn. 3) to the measured data (Table I). This method resulted in values of $B_{max} = 12.01$ fmol/10^6 cells, $K_d = 60.23$ pM and $n = 2.78$ for the specific [^{3}H]DHA binding to chick brain cells (Fig. 2). In the case of mammalian brain cultures (Fig. 3), the following parameters were estimated by the same method for the binding reaction: $B_{max} = 13.04$ fmol/10^6 cells, $K_d = 43.85$ pM and $n = 2.52$ for rat brain cells, and $B_{max} = 8.08$ fmol/10^6 cells, $K_d = 46.70$ pM and $n = 1.63$ for mouse brain cells. The parameters obtained for the different studied species are of the same order of magnitude.

The competition of L-alprenolol for the [^{3}H]DHA binding sites of chick brain cells was measured at labelled ligand concentrations of 30, 45 and 80 pM as well. The results are presented in Fig. 4. The curves were calculated on the basis of Eqn. 4 with parameters of $K_d = 60$ pM, $B_{max} = 12$ fmol/10^6 cells and $n = 2.5$. The measured data confirm the validity of the calculations within the error of determinations. The competition curve steadily decreases at [^{3}H]DHA concentrations near to K_d or higher than it (80 pM) (curve I), while at ligand concentrations lower than K_d (45 and 30 pM) the specific binding changes according to a maximum curve with increasing amounts of non-

TABLE I

PARAMETERS OF SPECIFIC [^{3}H]DHA BINDING TO LIVING CELLS AND TO MEMBRANE FRAGMENTS OF CHICK, RAT AND MOUSE BRAIN

Intact-cell specific [^{3}H]DHA binding was measured by incubating the primary cultures with 10–180 pM [^{3}H]DHA in serum-free Eagle's minimum essential medium for 15 min at 37°C. Nonspecific binding was determined in the simultaneous presence of 1–10 μM L-alprenolol. The binding reaction was stopped by the removal of the medium, followed by a rapid and intensive washing (see Materials and Methods). Bound radioactivity was transferred to Bray's solution and measured by liquid scintillation. Broken membranes were obtained by homogenization of either the cultured cells or the brain tissues used for cultivation using 50 mM Tris-HCl (pH 8.0). [^{3}H]DHA binding was measured by the method of Bylund and Snyder [3] at 0.1–15 nM [^{3}H]DHA. Nonspecific binding was determined in the simultaneous presence of 10 μM L-alprenolol. Parameters given here were obtained by direct fitting of the Hill equation (Eqn. 3) to the measured values presented in Figs. 2 and 3.

	B_{max}	K_d	n	Residual variance [a]
Living, cultured brain cells:	fmol/10^6 cells	pM		
chick	12.01	60.23	2.78	0.02
rat	13.04	43.85	2.52	0.02
mouse	8.08	46.70	1.63	0.04
Homogenate of cultured cells:	fmol/10^6 cells	nM		
chick	9.24	1.41	1	0.03
rat	17.14	1.16	1	0.02
mouse	7.13	1.52	1	0.04
Homogenate of forebrains:	fmol/100 μg protein	nM		
chick	16.42	1.99	1	0.02
mouse	9.14	1.31	1	0.03

[a] Characteristic for accuracy of fitting.

labelled L-alprenolol (curves II and III).

The association of [^{3}H]DHA to intact brain cells reached equilibrium after 5 min at the studied ligand concentrations. Fig. 5 shows the data for chick brain cells. The kinetic rate constants and binding parameters were calculated by applying the theoretical considerations given in the Materials and Methods (Eqns. 5–9). The association rate constant was estimated as $k_+ = 8.31 \cdot 10^7$ $M^{-1} \cdot min^{-1}$, the dissociation rate constant as $k_- = 0.28$ min^{-1}, the Hill coefficient $n = 1.76$ and $K_d = 101$ pM. These data are similar to the results of equilibrium measurements.

The dissociation, performed by isotopic dilution after equilibrium was reached at 70 pM [^{3}H]DHA, was rapid (Fig. 6), and may be evaluated as a first-order reaction. The dissociation rate constant was calculated as $k_- = 0.24$ min^{-1}, in accordance with that estimated from the association experiments. With measurement at a [^{3}H]DHA concentration of 110 pM, the dissociation rate constant proved to be very similar (not shown).

[^{3}H]DHA binding to homogenates of brain cultures and brain tissues

The [^{3}H]DHA equilibrium binding to both disrupted cultured cells and homogenized forebrains of either chick, rat or mouse followed a simple saturation curve (Figs. 2 and 3). The Hill equation was fitted to the measured data, and the best fitting was attained for the Hill coefficient $n = 1$.

The parameters calculated for the binding reaction are given in Table I. The K_d values were estimated as 1.41, 1.16 and 1.52 nM for broken cultured cells of chick, rat and mouse, and 1.99 and 1.31 nM for tissue homogenates of chick and mouse brain, respectively, while in intact cultured cells the corresponding values were 60.2, 43.8 and 46.7 pM in chick, rat and mouse, respectively. This demonstrates that homogenization causes an in-

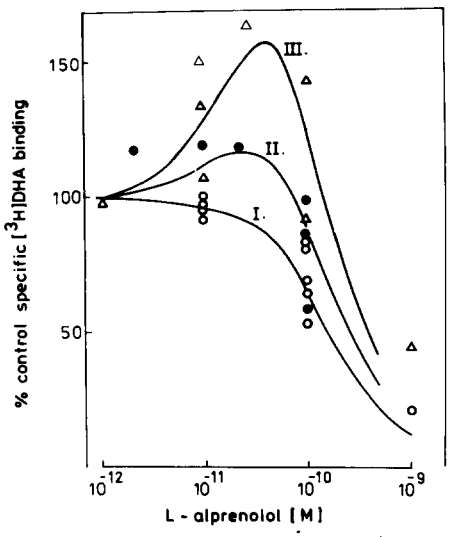

Fig. 4. Inhibition of specific [^{3}H]DHA binding of living, cultured chick brain cells by unlabelled L-alprenolol. Primary cultures were incubated with 30 pM (curve III), 45 pM (curve II) or 80 pM (curve I) [^{3}H]DHA for 15 min at 37°C in the simultaneous presence of increasing concentrations of unlabelled L-alprenolol. 100% binding was equal to 1.9, 3.4 and 7.8 fmol/10^6 cells, respectively. Points represent individual experiments. Theoretical curves were calculated with the following parameters: $B_{max} = 12$ fmol/10^6 cells, $K_d = 60$ pM and $n = 2.5$. The IC_{50} value of L-alprenolol at 80 pM [^{3}H]DHA was estimated as $1.05 \cdot 10^{-10}$ M.

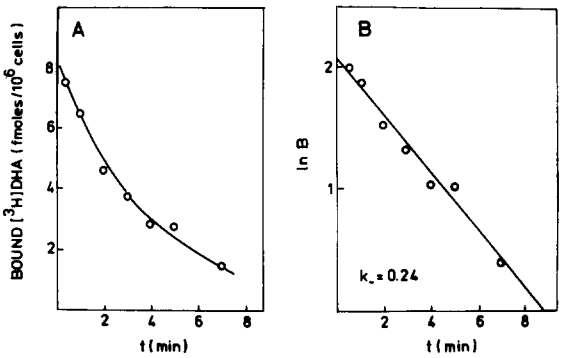

Fig. 6. Dissociation kinetics of specific [^{3}H]DHA binding to living, cultured brain cells of chick. Cells were incubated in serum-free Eagle's minimum essential medium at 37°C in the presence of 70 pM [^{3}H]DHA for 15 min, when 1 μM L-alprenolol was added without medium change, and incubation continued for the appropriate time. Points are the averages of two determinations. Panel B shows the graphical estimation of the dissociation rate constant as $k_- = 0.24$ min^{-1}.

crease of about two orders of magnitude in the K_d value.

The binding capacities (B_{max}) of intact and homogenized cultured cells were very similar. Some differences were found in the binding capacities of homogenized cultured cells and brain tissues, perhaps owing to the different cell compositions.

Discussion

β-Adrenergic receptor studies by specific ligand binding in membrane fragments of the brain tissue [1,3,27] or the aggregate culture [16,28] of various

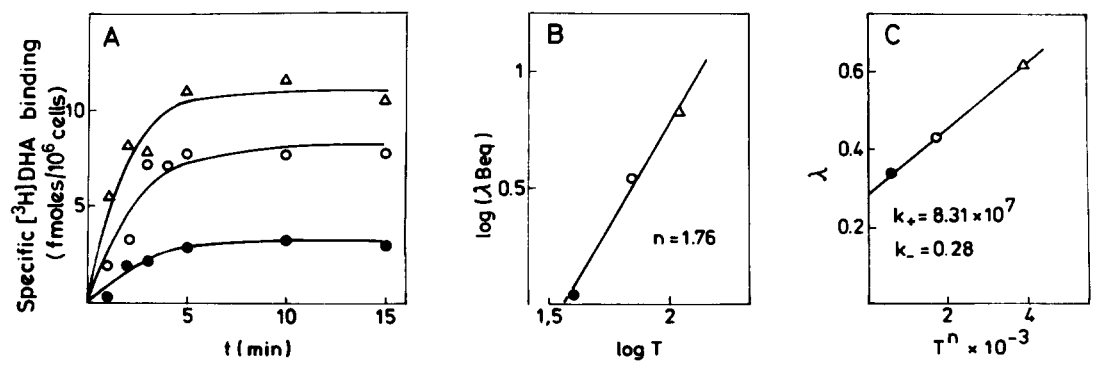

Fig. 5. Panel A. Association kinetics of specific [^{3}H]DHA binding of living, cultured chick brain cells, measured at 40 (●———●), 70 (○———○) or 110 (△———△) pM [^{3}H]DHA. Cells were incubated with the radioligand in serum-free Eagle's minimum essential medium at 37°C for the appropriate time, when a rapid and intensive washing was performed. Points represent the averages of three or four determinations. Panel B: Graphical solution of Eqn. 9 gives a Hill coefficient $n = 1.76$. Panel C: Graphical estimation of the rate constants: $k_+ = 8.31 \cdot 10^7$ M$^{-1} \cdot$min^{-1}, $k_- = 0.28$ min^{-1}. From these values $K_d = 101$ pM.

species revealed a homogeneous population of receptors and a lack of cooperative interactions.

Our experiments on homogenates of chick and mouse embryonic brain provided results in accordance with these data. The specific [^{3}H]DHA binding followed non-cooperative kinetics, with the dissociation constant varying between 1 and 2 nM and B_{max} being around 10^3–10^4 sites/cell.

The cultivation did not influence the kinetics and the parameters of the binding reaction. Our studies on membrane particles derived from cultured brain cells of chick, rat or mouse also revealed non-cooperative ligand binding, with K_d between 1 and 2 nM. The binding capacities were somewhat different, owing to the different cell compositions of the culture and the brain tissue [19].

The application of ligand-binding techniques to study membrane receptors in intact cells involves some methodological difficulties. Examples are the high nonspecific association of the ligand to the cell membrane, or the possibility of its uptake [10,23]. In our investigations on brain cells we developed a procedure resulting in a nonspecific binding amounting to not more than 30% of the total. The uptake of the ligand can be neglected because of the very low ligand concentrations even when saturation equilibrium is attained.

The specific [^{3}H]DHA binding to intact brain cells of chick, rat or mouse revealed a high-affinity, stereoselective, β-adrenergic-type binding site. In contrast to the results obtained on membrane particles, the equilibrium binding followed sigmoid saturation kinetics. This indicates apparent cooperative interactions in the β-receptor-ligand binding reaction. Scatchard transformation of the data resulted in curvilinear plots (not shown), confirming the apparent cooperativity.

If the possibility of cooperative interactions exists, the Hill approximation may be applied for analysis of the results. Thus, we calculated a Hill coefficient $n = 2.78$ for chick cells, $n = 2.52$ for rat cells and $n = 1.63$ for mouse cells.

Further support for the apparent cooperativity of the binding reaction may be provided by the competition kinetics of the unlabelled ligand [29]. In our experiments on chicken brain cells the increasing amounts of L-alprenolol resulted in biphasic competition curves if measured at [^{3}H]DHA concentrations less than K_d. Analysis of the data with a modified Hill equation (Eqn. 4) confirmed a Hill coefficient of $n = 2.5$.

The apparent cooperativity of the binding reaction must be reflected in the association kinetics as well. The association is usually considered to be a simple bimolecular reaction [30]. With our data on intact chick cells this treatment resulted in a negative value for the dissociation rate constant. Therefore, a more complex approximation was needed. This is given in Eqns. 5–9 and yielded $k_+ = 8.31 \cdot 10^7\ M^{-1} \cdot min^{-1}$, $k_- = 0.28\ min^{-1}$ and a Hill coefficient $n = 1.76$.

Thus, different kinetic approaches support the apparent positive cooperativity of the β-receptor binding reaction in intact brain cells.

The affinity of the binding site of living, cultured cells was markedly higher than that of the membrane fragments of either primary cultured cells or forebrain tissues. The K_d values were estimated to be between 40 and 100 pM, instead of 1 and 2 nM, in all three studied species. This result is in accordance with our earlier finding that adenylate cyclase stimulation occurs at much lower β-agonist concentrations in intact chick brain cells [31] than in broken cells [32].

Comparison of the [^{3}H]DHA binding capacities of different samples revealed that the cell disruption did not influence the number of measurable β-receptors. The differences found between the chick, rat and mouse brain cells may originate not only from the genetic variations, but also from the different neuron : glia ratios of these cultures. The effect of the cell composition of the culture on the number of β-receptors is analysed in another paper [19].

The cooperative character of intact brain cell β-receptors can also be demonstrated by using another β-antagonist ligand, ^{125}I-labelled hydroxybenzylpindolol (data not shown). Our results on chick and rat intact brain cells revealed a Hill coefficient $n > 1$ and a K_d around 80 pM.

The apparent positive cooperativity of the β-receptor in intact cells, and the lack of cooperativity in membrane fragments, considering also the affinity differences, allows several interpretations. It may imply interactions between the receptors, leading to an increasing affinity with increasing receptor occupancy. These can be weak interactions, perhaps induced by the hormone, but some

recent data do not exclude the oligomeric nature of the β-receptor [33,34]. Our results can also be explained by the ligand-induced conformational isomerization of the receptor [35]. Conformational changes, as the origin of the negative cooperativity of β-receptors of rat kidney membranes, have already been suggested by Krawietz et al. [5]. We assume that in the intact membrane a lower- and a higher-affinity receptor conformation may exist simultaneously, in a ligand concentration-dependent equilibrium. This is manifested in the ligand-binding reaction as an apparent positive cooperativity and a higher measurable affinity. On homogenization, this equilibrium may be shifted towards the lower-affinity conformation, resulting in non-cooperative binding kinetics and a lower apparent affinity. The equilibrium shift may be caused by cellular factors released on cell disruption, including intracellular β-agonists [36] and different protein factors [13]. Modification of the membrane-lipid surroundings of the receptor [37], as well as disintegration of the cytoskeleton [8], the lack of an energy supply, and several other factors may also explain why the cooperativity of the β-receptors is not maintained in membrane fragments. Discrimination between these possibilities necessitates further investigation.

The change of the cooperative receptor-binding kinetics to non-cooperative on cell disruption has already been described for leucocyte β-receptor [8] and brain opiate receptors [1]. We ourselves demonstrated the apparent positive cooperativity of muscarinergic acetylcholine receptors in intact glial cells [22], in contrast to the non-cooperative binding of membrane fragments [38]. Previously we revealed [19] apparent positive cooperativity of β-receptors in homogenous neuronal and glial cultures as well. Since we obtained similar results for the three species studied here, we presume that the apparent positive cooperativity may be a general phenomenon of β-receptors in intact developing brain cells.

As far as the physiological significance of the positive cooperativity is concerned, we suppose that the mechanism involving increasing receptor affinity with increasing occupancy may serve for the perception of weak, modulatory stimuli. This theory agrees with our result [19] that β-adrenergic receptors may function in the glia-neuron communication in a developing culture of chick brain.

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