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IDENTIFICATION AND CHEMICAL CHARACTERISATION OF β-ADRENERGIC RECEPTORS IN INTACT TURKEY ERYTHROCYTES

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Abstract—The radiolabelled β-adrenergic antagonist (−)-[^{3}H]dihydroalprenolol bound to two independent classes of sites on intact turkey erythrocytes. About 1300 high affinity and 18,000 low affinity sites per cell were observed. The low affinity sites (K_D = 210 nM) were different from the functional β-adrenergic receptors since the binding of (−)-[^{3}H]dihydroalprenolol could be displaced by β-adrenergic antagonists but not by agonists and since, in marked contrast with the β-adrenergic receptors in membranes, the sites were sensitive to the alkylating agent *N*-ethylmaleimide and resistant to the reducing agent dithiothreitol.

Agonist-displaceable binding of the tracer only occurred to the high affinity sites. The radiolabelled binding properties of these sites (K_D = 2.7 nM) were in close agreement with those earlier reported for the functional receptors in purified membranes. Bound tracer could be stereospecifically displaced from these sites by both β-adrenergic agonists and antagonists. The specific order of potencies for agonists to displace bound tracer (isoproterenol > norepinephrine > epinephrine), as well as the low affinity for α-adrenergic ligands and non-bioactive catechol compounds confirmed the β_1-adrenergic nature of these high affinity sites.

DTT§ caused a time- and concentration-dependent decline in the number of receptors of intact erythrocytes as well as of purified and solubilised membranes. Binding of β-adrenergic agonists and antagonists protected the receptors against the action of DTT. These findings indicate that essential disulphide bond(s) of the receptor are located at the extracellular face of the membrane, probably at, or near, the hormone binding site. In contrast with earlier findings on membranes, β-adrenergic agonists did not appear to sensitize the intact cell receptors to inactivation by *N*-ethylmaleimide.

INTRODUCTION

The direct identification of hormone receptors by binding of radiolabelled ligands has considerably increased our knowledge about their functional and chemical properties. In our laboratory much effort has been spent to characterise the turkey erythrocyte β-adrenergic receptors: which are multicomponent membrane proteins with molecular weights of 30,000–60,000 daltons [1, 2] separable from both guanine nucleotide binding components and the adenylate cyclase enzyme [3–6]. Investigation of the effect of group-specific reagents has revealed that these receptors contain one or more essential disulphide bonds probably located in the vicinity of the hormone binding site [7], and that they undergo a conformational change upon agonist binding [8, 9]. Evidence for the mutual modulation between the turkey erythrocyte β-adrenergic receptors and one or more guanine nucleotide binding membrane components has arisen from the recent observations that catecholamines stimulate a membrane GTP-ase activity [10] and that GTP affects the agonist–receptor interaction [11].

Most of the information concerning the structure and function of β-adrenergic receptors has been obtained by investigating the receptors from purified or solubilised cell membranes, thus in the absence of intracellular substances and structures such as soluble and loosely membrane-associated proteins, microtubuli and microfilaments which all may affect the receptor properties under normal or pathophysiological conditions. A striking example of an intact cell dependent regulation is the loss of ability of agonists to cause receptor desensitisation upon purification of the cell membranes [12]. Readdition of these intracellular structures and substances to purified membranes may only partially restore the native conditions, due to the loss of vectoriality of the interaction with the membranes.

The above considerations have led us to investigate β-adrenergic receptors present on intact cell preparations, in which the spatial organisation of the plasma membranes and the physiological intracellular milieu are maintained. In this report, we present a convenient assay for the identification of β-adrenergic receptors present on intact turkey erythrocytes by binding of the tritiated antagonist (−)-[^{3}H]dihydroalprenolol. We further compare the action of the reducing agent dithiothreitol and of the

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§ Abbreviations: (−)-[^{3}H]DHA, (−)-[^{3}H]dihydroalprenolol; NEM, *N*-ethylmaleimide; and DTT, dithiothreitol.

alkylating reagent *N*-ethylmaleimide upon free and ligand-bound cell receptors with previously reported results obtained with purified and solubilised membrane preparations.

MATERIALS AND METHODS

Materials. The following were obtained as kind gifts: (+) and (−)-propranolol (Imperial Chemical Industries, U.K.), (+) and (−)-epinephrine bitartrate and (−)-isoproterenol bitartrate (Sterling Winthrop), (±)-protokylol (Lakeside Laboratory), phentolamine and (±)-alprenolol hydrochloride (Ciba-Geigy). *N*-ethylmaleimide (NEM) and (±)-isoproterenol were purchased from Aldrich Europe, dithiothreitol (DTT) from Merck, (−)-norepinephrine tartrate from Sigma, pyrocatechol from Carlo Erba, and (−)-[^{3}H]dihydroalprenolol (42 Ci/mmole) was purchased from New England Nuclear.

Turkey erythrocytes. Turkey blood (75 ml) was drawn from a wing vein into a receptacle containing 25 ml of 75 mM sodium citrate (pH 5.0)/75 mM D-glucose, and diluted with 100 ml of 145 mM NaCl. All subsequent steps were performed at 4°. Cells were centrifuged for 15 min at 800 *g*. The supernatant and the lymphocyte layer were discarded and the packed cells resuspended in 200 ml of 145 mM NaCl. Washing of the cells was repeated twice, after which they were finally packed by centrifugation for 15 sec at 12,000 rpm in an Eppendorf centrifuge. Packed cells (5×10^9 cells/ml) could be stored for 48 hr at 0° in the dark without noticeable lysis and alteration of the properties of the β-adrenergic receptors. When required, the cells were lysed immediately before use by 64-fold dilution in hypotonic medium [7.5 mM Tris–HCl (pH 7.4)/2.5 mM $MgCl_2$] and centrifuged for 1 min in an Eppendorf centrifuge. Lysis was repeated twice. The resulting nucleated ghosts were washed three more times in 75 mM Tris–HCl (pH 7.4)/25 mM $MgCl_2$/145 mM NaCl (buffer A).

Turkey erythrocyte membranes. Membranes were prepared as described previously [11], and stored in liquid nitrogen prior to use. The protein concentration was determined by the method of Lowry *et al.* [13] using bovine serum albumin as the standard.

Cell and membrane preincubations. Unless otherwise stated, membranes (2 mg/ml), intact or lysed cells (4×10^7 cells/ml, corresponding to a 128-fold dilution of packed cells) were preincubated for 15 min at 30° with the indicated compounds in buffer A in a total volume of 1 ml. The preincubations were performed in 1.5 ml conic polyethylene tubes, which allowed their immediate centrifugation (for 1 min) in an Eppendorf centrifuge. After initial centrifugation, the packed cells (or membranes) were resuspended in 1 ml of fresh buffer A. Unless otherwise stated, washing was repeated four times.

Cell and membrane incubations with (−)-[^{3}H]DHA. Membranes (2 mg/ml), intact or lysed cells (4×10^7 cells/ml) were incubated for 10 min at 30° with 1–100 nM of (−)-[^{3}H]DHA in buffer A in a total volume of 400 μl. At the end of the incubation, triplicate 100 μl aliquots were diluted in 4 ml of ice-cold buffer A, and immediately filtered under reduced pressure upon glass fibre filters (Whatmann, GF/F, Ø 2.5 cm). The filters were rapidly washed twice with 4 ml of ice-cold buffer A, placed in 20 ml polyethylene scintillation vials containing 1 ml 0.1 N NaOH and 9 ml of Aqualuma (Lumac), and counted. Application of the intact cells upon the filters and subsequent washing caused their complete lysis. Non-specific binding to the cells was obtained by incubation of the cells with tracer in the presence of 10 μM (±)-propranolol, and amounted for 10–14% of total binding in presence of 10 nM of tracer (Table 1).

Specific (i.e. (±)-propranolol displaceable) binding of (−)-[^{3}H]DHA occurred to both high and low affinity sites. Specific binding to the high affinity sites corresponded to binding that could be displaced by the agonist (42–48% of total binding at 10 nM of tracer). Specific binding to the low affinity sites cor-

Table 1. Effect of DTT, NEM and (−)-isoproterenol upon high and low affinity (−)-[^{3}H]DHA binding sites

Cells preincubated with:	(-)-[^{3}H]-DHA bound (in c.p.m.) in presence of: buffer	isoproterenol	propranolol	Binding to sites with: high affinity	low affinity
Buffer only	1934±39	189± 8	1122±23	812	933
DTT (1mM)	1354±30	260±19	1183±30	171	923
NEM (50μM)	1057±43	198±11	324±22	733	126
IPR¶ (0.5μM)	1823±55	198± 6	1014±45	809	816
NEM+IPR (0.5μM)	1069±43	203±12	311±14	758	108
NEM+GTP (1mM)	1217±37	221±23	375±32	742	154

Turkey erythrocytes (128-fold dilution of packed cells) were preincubated in the presence of the indicated compounds for 15 min at 30°, washed five times with buffer, and incubated for 10 min with 10 nM (−)-[^{3}H]DHA either in the absence or presence of 10 μM (±)-propranolol or 200 μM (±)-isoproterenol. Values are means ± S.D. of three individual experiments. Binding to high affinity sites refers to total binding (i.e. binding in the absence of competitor) minus binding in presence of (±)-isoproterenol. Binding to low affinity sites refers to binding in presence of (±)-isoproterenol minus binding in presence of (±)-propranolol.

¶ IPR: (−)-isoproterenol.

responded to (±)-propranolol-displaceable minus (±)-isoproterenol-displaceable binding. The incubation medium that was used for previous studies on membranes [i.e. 75 mM Tris–HCl (pH 7.4)/25 mM $MgCl_2$] was brought to hypertonicity by addition of 145 mM NaCl to prevent lysis and to reduce the extent of non-specific binding.

Binding of the tracer to lysed cells and membranes was determined as above, except that non-specific binding was measured in the presence of 2 μM (±)-alprenolol. Specific binding, defined by total binding minus non-specific binding, occurred to a single class of sites with high affinity: K_D for (−)-[^{3}H]DHA equalled 5.5 ± 1.5 and 8 ± 2 nM for lysed cells and membranes [4, 7, 11], respectively.

Data shown are means of values from individual experiments (each value being means of three determinations) using cell suspensions from different bleedings and different, freshly prepared drug solutions.

RESULTS

Characteristics of (−)-[^{3}H]DHA binding to intact turkey erythrocytes. (−)-[^{3}H]DHA saturation binding was performed by incubating the cells with 1–100 nM of tracer. Scatchard analysis of the binding that could be displaced with 10 μM (±)-propranolol yielded a curvilinear plot (Fig. 1). This suggested the existence of either multiple classes of binding sites with different affinities for (−)-[^{3}H]DHA or the presence of a single class of sites showing negative cooperativity for the tracer [14]. The latter assumption could be discarded since dissociation of bound (−)-[^{3}H]DHA (10 nM) in function of the time revealed no discrepancy between 200-fold dilution in the presence and absence of an excess of (±)-propranolol (10 μM) (Fig. 2). Propranolol displaceable binding of (−)-[^{3}H]DHA thus occurs to two or more classes of binding sites. Assuming the simplest case, wherein the tracer binds with different affinities to two classes of sites, we have analysed the Scatchard plot by a computer based iterative method, described by Minneman, Hegstrandt and Molinoff [15]. According to this method, (−)-[^{3}H]DHA binds with high affinity (K_D = 2,7 ± 4 nM) to 1300 ± 280 sites/cell and with low affinity (K_D = 210 ± 65 nM) to 18,000 ± 5600 sites/cell (values are means ± standard deviation from five experiments).

Scatchard analysis [16] of (−)-[^{3}H]DHA saturation binding that can be displaced by the agonist (±)-isoproterenol, reveals the presence of only one class of sites (Fig. 1). The K_D for binding (2.6 ± 5 nM) and the number of sites (1400 ± 120 sites/cell), (values are means ± standard deviation of three experiments) suggests that β-adrenergic agonists only compete with (−)-[^{3}H]DHA for binding to the high affinity sites. This contention is further supported by the net decline of the fraction of (±)-isoproterenol-displaceable (−)-[^{3}H]DHA binding sites when the concentration of tracer is increased from 10 to 100 nM, compared to the much smaller effect for (±)-propranolol (Fig. 3).

The (−)-[^{3}H]DHA binding characteristics of the high affinity sites are very close to those earlier observed for the β-adrenergic receptors in purified turkey erythrocyte membranes [4]. These observations strongly suggest that, in intact cells, only the

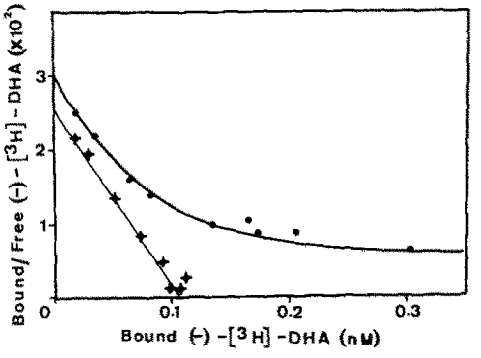

Fig. 1. (−)-[^{3}H]DHA saturation binding to intact turkey erythrocytes. Turkey erythrocytes (128-fold dilution of packed cells, 4 × 10^7 cells/ml) were incubated with increasing concentrations of (−)-[^{3}H]DHA (1–100 nM) in the absence or presence of either 10 μM (±)-propranolol or 200 μM (±)-isoproterenol for 10 min at 30°, and filtered. The resulting saturation binding data are represented by the method of Scatchard [16]. (●——●): (±)-propranolol displaceable binding yields a curvilinear plot. This curve was dissected into its two original components by the computer-based iterative procedure described by Minneman, Hegstrandt and Molinoff [15], to yield high affinity binding (K_D = 2.7 ± 4 nM) to 1300 ± 280 sites/cell and low affinity binding (K_D = 210 ± 65 nM) to 18,000 ± 5600 sites/cell. Values are means of five experiments. (+——+): (±)-isoproterenol displaceable binding gives a straight Scatchard plot (r = 0.991), indicating that binding occurs to a single class of sites: K_D = 2.6 ± 5 nM for 1400 ± 120 sites/cell. Values are means of three experiments.

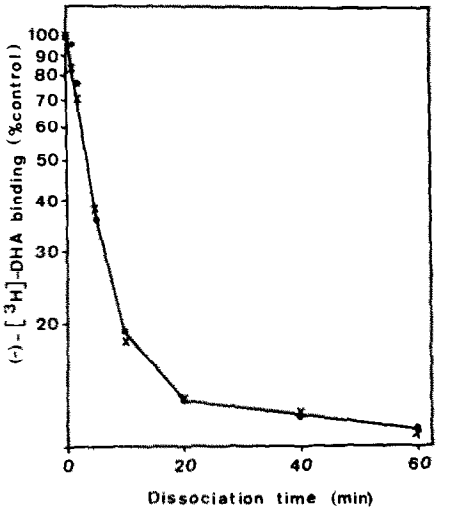

Fig. 2. Dissociation of bound (−)-[^{3}H]DHA in the absence or presence of an excess of unlabelled propranolol. Turkey erythrocytes (128-fold dilution of packed cells) were incubated with 10 nM (−)-[^{3}H]DHA for 10 min at 30°. Then 100 μm aliquots were diluted at 30° in 20 ml of buffer only (●——●), or buffer containing 10 μM (±)-propranolol (×——×). The diluted samples were further incubated at 30° for the indicated periods of time and filtered. Total binding of the tracer is expressed on a logarithmic scale as per cent control, i.e. equilibrium binding to undiluted samples. Values are means of two experiments.

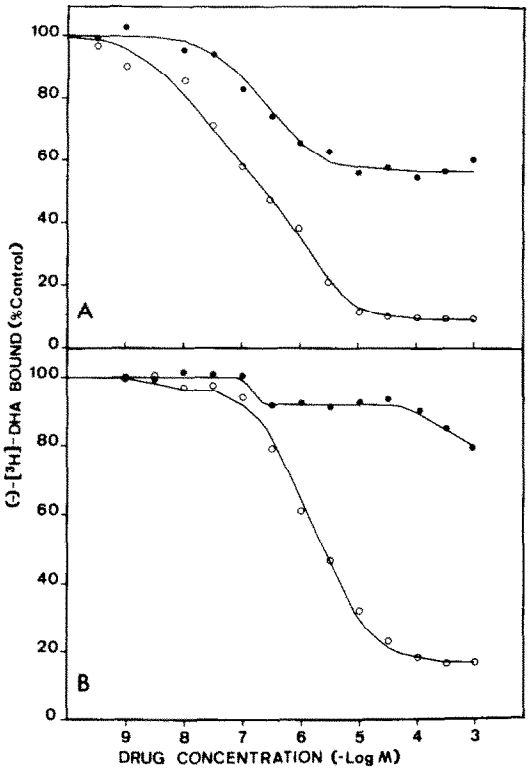

Fig. 3. Competition between (−)-[^{3}H]DHA and (±)-isoproterenol/(±)-propranolol for binding: dependence of the (−)-[^{3}H]DHA concentration. Turkey erythrocytes were incubated for 10 min at 30° with 10 nM (A) or 100 nM (B) (−)-[^{3}H]DHA in the absence or presence of increasing concentrations of (±)-isoproterenol (●——●) or (±)-propranolol (○——○). Total binding of the tracer is expressed as per cent of control, i.e. binding in the absence of competitor, 2076 and 11,024 cpm for 10 and 100 nM of tracer, respectively. Values are means of two experiments.

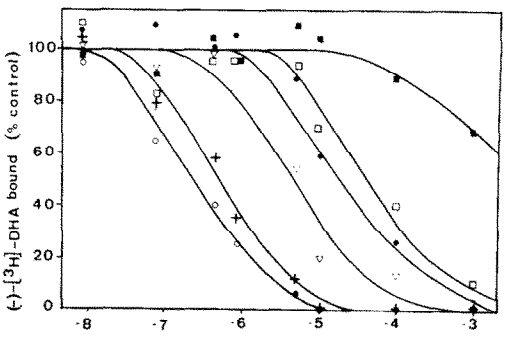

Fig. 4. Competition between (−)-[^{3}H]DHA and β-adrenergic agonists for binding to the high affinity sites. Turkey erythrocytes were incubated for 10 min at 30° with 10 nM (−)-[^{3}H]DHA in the absence or presence of increasing concentrations of (−)-isoproterenol (○——○), (±)-protokylol (+——+), (±)-norepinephrine (▽——▽), (−)-epinephrine (●——●), phentolamine (□——□), and pyrocatechol (■——■). Only the portion of (−)-[^{3}H]DHA binding that can be displaced by 200 μM (±)-isoproterenol (i.e. binding to high affinity sites) is taken into consideration. Control binding refers to (−)-[^{3}H]DHA binding to the cells in the absence of competing ligand. Values are means of two experiments.

high affinity (−)-[^{3}H]DHA binding sites correspond to the functional β-adrenergic receptors.

As shown in Fig. 4, β-adrenergic agonists compete with (−)-[^{3}H]DHA for binding to the high affinity (isoproterenol-displaceable) sites with the order of potencies: (−)-isoproterenol > (±)-protokylol > (−)-norepinephrine > (−)-epinephrine, typical for binding to β_1-adrenergic receptors [17]. Binding is stereoselective: (−)-epinephrine is about 10-fold more potent than its dextrorotary isomer (Table 2). The α-adrenergic antagonist phentolamine and the non-bioactive compound pyrocatechol showed only weak affinity (Fig. 4). The equilibrium-dissociation constants (K_D values) for agonist binding to the high affinity sites was calculated from their concentration that caused half-maximal displacement of (−)-[^{3}H]DHA binding by the method of Cheng and Prusoff [18]. The K_D values, presented in Table 2, are in close agreement with the corresponding data for purified membranes [4, 7, 11], which supports our identification of the high affinity (−)-[^{3}H]DHA binding sites in intact cells as the β_1-adrenergic receptors. In contrast, binding of the tracer to the low affinity sites (i.e. binding that can be displaced by propranolol but not by isoproterenol) is not affected by 1 mM of the β-adrenergic agonists, phentolamine and pyrocatechol (data not shown).

Inactivation of the β-adrenergic receptors on intact erythrocytes by DTT. We have recently reported that membrane bound, solubilised and purified β-adrenergic receptors bear one or more essential disulphide bonds [4, 7]. The following observations indicate that these bonds are exposed at the outer face of the cell membrane. Pretreatment of intact erythrocytes with 1 mM DTT for 15 min at 30° caused a net decline of subsequent (−)-[^{3}H]DHA binding to the high affinity sites (Table 1). As depicted in Fig. 5A, the number of sites decreased with increasing DTT concentrations, whereas the affinity of the remaining sites for the tracer was unchanged. Binding to the low affinity sites and non-specific binding were not affected (Table 1).

Inactivation of the high affinity sites by DTT was a time-dependent process. As shown in Fig. 6, there was an exponential decrease in the number of sites upon increasing the preincubation time. The pseudo first-order rate constant for the inactivation process (K_{ob}) equals 0.0019 s^{-1} at a DTT concentration of 1 mM.

Agonists as well as antagonists protected the high affinity sites against the action of DTT. As depicted in Table 2 protection was (1) dose dependent, (2) proportional to the affinity of the β-adrenergic agonists for binding to the receptor, (3) more pronounced for laevorotary ligands than for their dextrorotary analogs, and (4) not significant in the presence of 1 mM phentolamine or pyrocatechol. These observations are in accordance with our previous findings obtained on purified membranes [7] and indicate that β-adrenergic ligands protect the receptor against the action of DTT by binding to the active site of the receptor.

Effect of NEM upon (−)-[^{3}H]DHA binding to intact erythrocytes. In contrast with DTT, the alkyl-

Table 2. Protection of high affinity (−)-[^{3}H]DHA binding sites against DTT inactivation by β-adrenergic agonists and antagonists

Cells preincubated with DTT plus:	Ligand added concentration (in μM)	(K_D¶¶ for receptor) (in μM)	(−)-[^{3}H]-DHA bound (% control)
none			13±2
β-agonists			
(−)-isoproterenol	0.5	0.05	74±8
(±)-protokylol	0.5	0.11	50±2
(−)-norepinephrine	0.5	1.4	41±8
(−)-epinephrine	0.5	5.5	29±3
	5.0		53±4
(+)-epinephrine	5.0	48	28±6
β-antagonists			
(−)-propranolol	0.04	0.005	45±7
(+)-propranolol	0.04	0.220	13±3
(−)-[^{3}H]-DHA	0.004	0.003	20±7
	0.04		71±4
α-antagonist			
phentolamine	100	>500	16±4
nonbioactive			
pyrocatechol	100	>500	11±3

Cells were incubated with 1 mM DTT in the absence or presence of different ligands at the indicated concentrations for 15 min at 30°, washed five times with buffer, and incubated for 10 min with 10 nM (−)-[^{3}H]DHA. Only binding to the high affinity sites, as defined in the legend of Fig. 4, is taken into consideration. Binding is expressed as per cent of control, i.e. binding to cells preincubated with buffer only. Values are means ± ranges of two individual experiments.

¶¶ The K_D for (−)-[^{3}H]DHA was obtained from Fig. 1, and K_D values for unlabelled ligands were calculated from their concentration that caused half-maximal displacement of bound tracer (Fig. 4) by the method of Cheng and Prusoff [18].

ating reagent NEM did not affect (−)-[^{3}H]DHA binding to the high affinity sites at concentrations as high as 1 mM (Fig. 5B). However, binding to the low affinity sites was greatly reduced at 50 μM NEM (Table 1) and completely abolished at 1 mM of the reagent. As indicated in Fig. 5B, Scatchard plots of isoproterenol and propranolol displaceable (−)-[^{3}H]DHA saturation binding were superimposable when the cells were pretreated with 1 mM NEM. Inactivation of the low affinity sites by NEM was not affected by agonists nor GTP (Table 1). Nonspecific binding was not affected by any of these conditions either (Table 1).

We have recently reported that β-adrenergic agonists but not antagonists cause a rapid and reversible conformational change of approximately 50% of the β-adrenergic receptors in turkey erythrocyte membranes, resulting in their susceptibility to inactivation by NEM [8, 9, 11, 27, 28]. The data presented below indicate that this phenomenon is not demonstrable on intact cells.

Preincubation of the cells for 15 min at 30° with 1 μM (−)-isoproterenol or with 0.5 mM NEM did not affect subsequent binding of (−)-[^{3}H]DHA to the high affinity sites (Tables 1 and 3). The inability of (−)-isoproterenol alone to affect subsequent binding of the tracer is in agreement with the earlier observed lack of desensitisation of turkey erythrocyte β-adrenergic receptors upon prolonged exposure to β-adrenergic agonists [19]. The lack of effect of NEM alone is in agreement with the earlier observed resistance of the free receptors against inactivation by alkylating reagents [9]. However, preincubation of the cells with a combination of both compounds did not result in a decrease in subsequent (−)-[^{3}H]DHA binding (Tables 1 and 3). Under the same experimental conditions, a near 50% decrease of binding activity was readily observed after pretreatment of the lysed erythrocytes or the purified membranes with (−)-isoproterenol plus NEM, even at NEM concentrations as low as 50 μM (Table 3). Two possible causes for the lack of isoproterenol/NEM effect on the cells were studied in more detail.

First, intact cells might cause rapid destruction of either the agonist, the alkylator, or both. This assumption was validated by the observation that

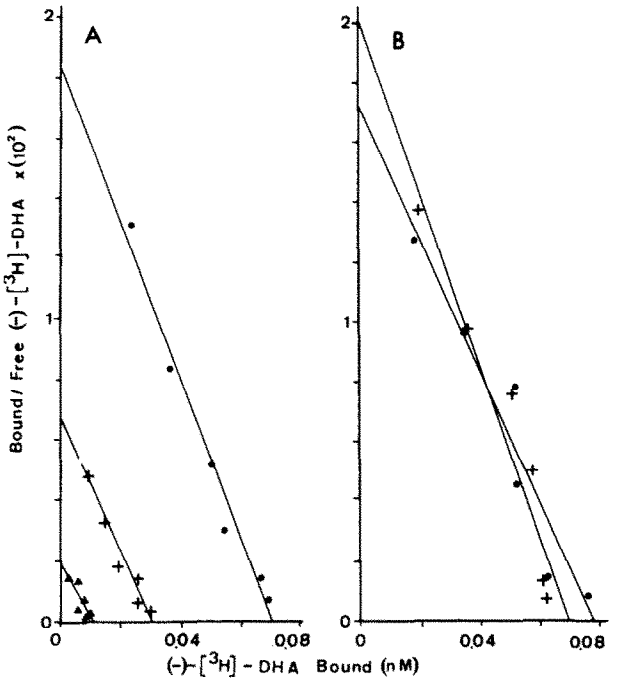

Fig. 5. Effect of DTT and NEM upon (−)-[^{3}H]DHA saturation binding to intact turkey erythrocytes. (A) Effect of DTT. Turkey erythrocytes (128-fold dilution of packed cells) were preincubated for 20 min at 30° with buffer only (●——●), with 0.33 mM DTT (+——+), or with 1 mM DTT (▲——▲); washed with buffer once, and incubated with increasing concentrations of (−)-[^{3}H]DHA (1–100 nM) for 10 min at 30°. Bound tracer is represented by the method of Scatchard. Only (−)-[^{3}H]DHA binding to the high affinity sites, as defined in the legend of Fig. 4, is taken into consideration.

(B) Effect of NEM. Turkey erythrocytes were preincubated for 20 min at 30° with 1 mM NEM, washed three times with buffer, and incubated with increasing concentrations of (−)-[^{3}H]DHA as described above. Bound tracer is represented by the method of Scatchard. (●——●): propranolol-displaceable binding, (+——+): isoproterenol-displaceable binding. Values are means of two experiments.

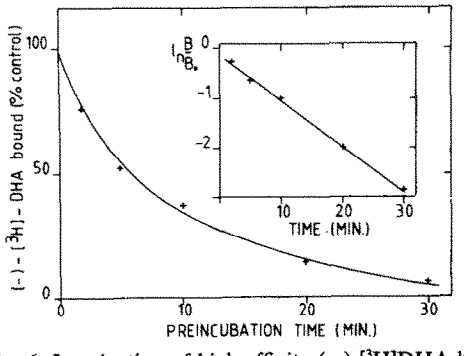

Fig. 6. Inactivation of high affinity (−)-[^{3}H]DHA binding sites by DTT as a function of the time. Turkey erythrocytes (128-fold dilution of packed cells) were preincubated with 1 mM DTT for different periods of time at 30°, washed three times with buffer containing 2 mM NEM and incubated with 10 nM (−)-[^{3}H]DHA. Only (−)-[^{3}H]DHA binding to the high affinity sites, as defined in the legend of Fig, 4, is taken into consideration. Bound tracer is represented in function of the preincubation time. Control binding refers to binding of tracer to cells pretreated with DTT for less than 10 sec. Values are means of two experiments. Inset: semilogarithmic representation of the same binding data. B_0 is control binding and B is binding after pretreatment of the cells with DTT for the corresponding time. The pseudo first-order rate constant (K_{ob}) is given by the absolute value of the slope: $K_{ob} = 0.0019\ sec^{-1}$.

the supernatant of cells pretreated with 1 μM (−)-isoproterenol plus 50 μM NEM caused no appreciable inactivation of the β-adrenergic receptors of purified membranes, whereas a fresh isoproterenol/NEM solution caused the expected 50% inactivation (Tables 3 and 4). Supplementation of the cell supernatant with fresh NEM (1 mM) restored the ability to inactivate the receptors in membranes, whereas supplementation with 10 μM (−)-isoproterenol was without effect (Table 4). These data indicate that the cells cause rapid inactivation or disappearance of NEM from the medium. However, when the initial NEM concentration was raised to 0.5 or 1 mM the resulting supernatant was still able to inactivate 40% of the β-adrenergic receptors in purified membranes (Table 4). Under these conditions, still no decrease in the receptor number was observed on the intact cells (Table 3). Accordingly, the resistance of the cell receptors to agonist/NEM is not only dependent on a limited supply of either compound.

Addition of GTP to purified turkey erythrocyte membranes confers full protection of the β-adrenergic receptors against agonist/NEM inactivation [11]. As depicted in Table 3, protection of the receptors by the nucleotide is also apparent when investigating lysed cells. Accordingly, a more likely reason for the lack of (−)-isoproterenol induced NEM effect

Table 3. Effect of (−)-isoproterenol plus NEM upon β-adrenergic receptors on intact erythrocytes, nucleated ghosts and purified membranes

Preincubation in presence of:	(−)-[^{3}H]-DHA bound (in % control) to		
	intact cells	nucleated ghosts	membranes
Buffer only	100±3	100±2	100±6
IPR^{++} (1μM)	96±7	106±7	104±2
NEM (50μM)	96±3	104±3	102±3
NEM (0.5mM)	102±6	99±7	95±3
IPR (1μM) + NEM (50μM)	104±7	48±6	49±4
IPR (1μM) + NEM (0.5mM)	101±8	56±4	55±7
IPR (1μM) + NEM (50μM) + GTP (1mM)	105±2	96±6	105±5
IPR (1μM) + NEM (0.5mM) + GTP (1mM)	99±5	108±2	96±1

Cells, nucleated ghosts (lysed cells) and purified membranes were preincubated for 15 min at 30° in the presence of the indicated ligands, washed five times with buffer, and incubated for 10 min with 10 nM (−)-[^{3}H]DHA. For the intact cells, only binding to the high, agonist-displaceable sites was taken into consideration. Only specific binding of (−)-[^{3}H]DHA (i.e. displaceable by 2 μM (±)-alprenolol) was taken into consideration for lysed cells and membranes. Specific binding (> 85% of total binding at 10 nM of tracer) occurred to a single class of non-cooperative sites with respective K_D values of 5.5 ± 1.5 and 8 ± 2 nM for ghosts and membranes [4, 7, 11]; specific binding was completely displaced in a non-cooperative manner ($n_H = 0.96 \pm 0.08$) by both (±)-isoproterenol ($K_D = 0.20$ and 0.15 μM, respectively) and (±)-propranolol ($K_D = 12$ and 15 nM, respectively). For each preparation, binding is expressed as per cent of control, i.e. binding after preincubation with buffer only. Values are means ± ranges of two individual experiments. None of the pretreatments caused significant variations of non-specific binding in cells, ghosts and membranes (data not shown).

†† IPR: (−)-isoproterenol.

Table 4. Effect of supernatants of (−)-isoproterenol plus NEM-treated erythrocytes upon purified membranes

Membrane preincubation in presence of:		(−)-[^{3}H]-DHA bound
supernatant of cells preincubated with	other additions	(in % control)
buffer only	– †††	100±6
IPR ††† (1μM)	–	101±5
NEM (50μM)	–	100±6
NEM (0.5mM)	–	101±7
IPR (1μM) + NEM (50μM)	–	92±3
IPR (1μM) + NEM (50μM)	IPR (10μM)	90±4
IPR (1μM) + NEM (50μM)	NEM (1mM)	46±5
IPR (1μM) + NEM (0.5mM)	IPR (10μM) + NEM (1mM)	55±2
IPR (1μM) + NEM (0.5mM)	–	65±8
IPR (1μM) + NEM (1mM)	–	61±1

Intact cells were preincubated for 15 min at 30° in the presence of the indicated ligands, and centrifuged for 1 min at 12,000 rpm in an Eppendorff centrifuge. Fresh, purified membranes were suspended in the recovered supernatants and preincubated for 15 min at 30° in the absence or presence of a fresh supply of the indicated ligands. The supernatants represented 80% of the volume. The membranes were then washed five times with buffer, and incubated with 10 nM (−)-[^{3}H]DHA for 10 min at 30°. Binding is expressed as per cent of control, i.e. binding after preincubation with the supernatant of buffer-treated cells. Values are means ± ranges of two individual experiments.

††† −: no additions; IPR = (−)-isoproterenol.

on intact cell receptors might be the presence of intracellular GTP or GDP.

DISCUSSION

β-Adrenergic receptors have been identified in a large number of tissues by binding of radiolabelled ligands such as (−)-[^{3}H]DHA and [^{125}I]-iodohydroxybenzyl pindolol [20]. Most of the reported studies have dealt with the pharmacological characterisation of the receptors, the effect of normal and pathophysiological conditions on their density and the effect of external factors (ions, nucleotides) which regulate the activity of the catecholamine-sensitive adenylate cyclase system by modulating the binding affinity for agonist molecules. Only a limited number of reports have been devoted to the chemical characterisation of the β-adrenergic receptors as well as to their investigation in their natural environment, i.e. in the intact cell. In the present study, we have extended these studies and have investigated the effect of group-specific reagents upon the β-adrenergic receptors present on intact turkey erythrocytes.

A convenient method was first developed for the labelling of the intact cell receptors by binding of the antagonist (−)-[^{3}H]DHA. The assay, described in the experimental section, consisted in incubation of the cells with the tracer in a hypertonic medium. Under these conditions, no lysis of the cells was observed, and the extent of non-specific binding was much reduced compared to similar incubation in isotonic or slightly hypotonic media. Separation of bound and free tracer by the filtration method was rapid, and allowed concomitant disruption of the cells so that, in contrast to the centrifugation method earlier described by Malchoff and Marinetti [21], no decolouration of the samples was required prior to radioactivity determination.

(−)-[^{3}H]DHA saturation binding to the intact cells was non-Michaelian when non-specific binding was determined in the presence of an excess of the unlabelled antagonist (±)-propranolol (Fig. 1). The possibility of negative cooperativity between the binding sites could be discarded since 200-fold dilution in the presence or absence of an excess of (±)-propranolol gave rise to the same dissociation profile of bound tracer (Fig. 2). The curvilinear Scatchard plot of the saturation binding suggests therefore the presence of two distinct classes of binding sites: 1300 and 18,000 sites/cell with respective K_D values for (−)-[^{3}H]DHA of 2.7 and 210 nM.

The non-identity of the low affinity sites with the functional β-adrenergic receptors is evidenced by the following findings: (1) binding of the tracer is not displaceable by β-adrenergic agonists, and (2) in contrast with membrane-bound or solubilised receptors [4, 7], the low affinity sites in intact cells are not sensitive to the reducing agent dithiothreitol and are rapidly destroyed by the alkylator NEM.

Low-affinity (−)-[^{3}H]DHA binding sites have been reported to be present in murine lymphocyte membrane fragments [22] and rat peritoneal mast cells [23]. They were not detected in intact erythrocytes [24, 25] and in no case on purified turkey erythrocyte membranes. These differences might be related to (1) small variations in the binding assays, and (2) loss or inactivation of these sites during the membrane preparation. The marked sensitivity of the low affinity sites to NEM, observed in the present study, suggests the involvement of protein structures. However, their exact nature is still unknown, and further investigation will be required to find out whether they have any pharmacological significance.

The (−)-[^{3}H]DHA binding characteristics of the high affinity binding sites on intact cells were very close to those previously reported for β-adrenergic receptors in purified and solubilised turkey erythrocyte membranes (i.e. 600–900 sites/cell and K_D values between 6 and 10 nM [4, 7, 11]). Their identity as functional β-receptors was further confirmed by (1) the ability of both β-adrenergic agonists and antagonists to cause stereospecific displacement of (−)-[^{3}H]DHA binding, (2) the very low affinity of α-adrenergic ligands and non-bioactive catechol derivatives, and (3) the order of potencies of β-adrenergic agonists to displace bound tracer (i.e. (−)-isoproterenol > (−)-norepinephrine > (−)-epinephrine, Fig. 4) which is typical for receptors of the β_1-adrenergic subclass. Based on the inability of the low affinity sites to recognise agonist molecules, the β-adrenergic receptors could be directly identified on intact erythrocytes as agonist.displaceable (−)-[^{3}H]DHA binding sites.

As for the membrane-bound and solubilised receptors, inactivation of the cell receptors by DTT was complete and time dependent (Fig. 5). These observations indicate that one or more essential disulphide bonds of the receptor are located at the outer face of the cell membrane. This interpretation is further supported by the reported ability of DTT to affect β-adrenergic receptor-mediated physiological responses in intact tissue preparations [26]. β-Adrenergic agonists and antagonists also caused very effective protection of the receptors against the action of DTT. The protection was concentration-dependent and, at the same concentration, proportional to the affinity of the different ligands for binding to the receptor (Table 2). We have previously explained the protective effect of the β-adrenergic ligands by either the ability of both agonist and antagonist molecules to induce a conformational change of the receptor, resulting in the removal of the disulphide bond(s) from the receptor surface, or by the location of the disulphide bond(s) at the proximity of the ligand binding site of the receptor [7]. The latter hypothesis is strongly supported by our subsequent findings that only β-adrenergic agonists can cause a conformational change of the receptor in purified membranes [8, 9], and by the present evidence for the external location of the dilsulphide bonds.

We have previously observed that approximately 50% of the β-adrenergic receptors in turkey erythrocyte membranes become sensitive to inactivation by NEM upon binding of agonist but not of antagonist molecules [8, 9]. This phenomenon was also observed in human adipocytes [27] and S49 lymphoma cell membranes [28], and was explained by the ability of agonists to cause a conformational transition of the receptor, resulting in the uncovering (and exposure to NEM) of essential alkylatable

groups. Inactivation of agonist-bound receptors in intact cells was not observed in the present study (Table 3). At low NEM concentrations (i.e. 50 μM) the lack of a (−)-isoproterenol induced NEM effect could be explained by the rapid inactivation of the latter compound. This phenomenon could, however, be neglected when the NEM concentration was raised to 0.5 mM: the supernatant of cells treated with 1 μM (−)-isoproterenol plus 0.5 mM NEM still caused 40% inactivation of the receptors in fresh membranes (Table 4).

The lack of a (−)-isoproterenol induced NEM effect in intact cells might be due to three alternative reasons. (1) The ability of agonists to cause a conformational change of the receptors might only be observed after alteration or modification of part of the receptors (50%) during the membrane preparation. (2) Agonists might cause exposure of the alkylatable sites of the receptor at the cytoplasmic side of the membrane. (3) Endogenous substances might confer protection of the receptors against inactivation by (−)-isoproterenol/NEM. The first explanation is less likely since (−)-isoproterenol/NEM can already inactivate 50% of the receptors in lysed cells, wherein the membranes underwent a minimum of manipulations (Table 3). Moreover, the extent of receptor inactivation ranged between 45 and 60%, for all preparations of lysed cells and purified membranes. Finally, the agonist/NEM-sensitive receptor subpopulation (50%) in human adipose membranes was found to be of physiological significance since only this subpopulation underwent desensitisation upon prolonged incubation of the intact adipocytes with β-adrenergic agonists [27]. The second explanation is weakened by preliminary findings that (−)-isoproterenol/NEM can still cause inactivation of the β-adrenergic receptors present on closed, right side out vesicles of S49 lymphoma cells [29]. A definitive proof for external exposure of the alkylatable groups of the turkey erythrocyte receptors will, however, have to await further investigation by use of sealed ghosts.

The absence of a (−)-isoproterenol induced NEM effect on intact erythrocytes can be best explained by the presence of intracellular substances. Likely candidates are guanyl nucleotides such as GTP which have been shown to protect the receptors against agonist induced NEM inactivation in purified membranes [11], and in this study, also in lysed cells (Table 3).

The results, presented in this and former reports, clearly show that β-adrenergic receptors can be directly identified on intact cells by means of radioligand binding studies, and that these receptors contain essential disulphide bonds facing the outer side of the membrane. The absence of agonist-induced NEM inactivation of the intact cell receptors contrasts with earlier findings on purified membranes, and might be due to the presence of intracellular GTP. Our results clearly illustrate the necessity of comparing studies performed on intact cells and purified membranes for the analysis of the ligand–receptor interactions.

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