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The influence of filtrability on β-adrenergic ligand binding to membrane fragments from human erythrocytes and mononuclear leucocytes

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β-Adrenergic receptors have been identified on nucleated erythrocytes from frog [1] and turkey [2]. Also non-nucleated rat erythrocytes contain β-adrenergic receptors [3]. Saturable binding sites with affinity similar to that of β-adrenergic receptors have been detected on intact human erythrocytes by centrifugation [4], but not on broken cells by ultrafiltration [5–7]. Before accepting the absence of β-adrenergic receptor binding sites on human erythrocytes, we evaluated the application of ultrafiltration for membrane fragments from these cells in order to substantiate the hypothesis that human erythrocyte membrane fragments may deform and pass through the glass fibre filters during the ultrafiltration. Human mononuclear leucocytes were applied as the reference system since the existence of β-adrenergic receptors has been established for these cells [5, 8]. The radioactivity of [^{3}H]-(−)-dihydroalprenolol on the glass fibre filter was used to evaluate binding, and the presence of proteins in the filtrate to evaluate filter retention efficacy.

Materials and methods

[^{3}H]-(−)-Dihydroalprenolol ([^{3}H]DHA) with a specific activity of 49.1 Ci/mmole was obtained from New England Nuclear (Dreieich, F.R.G.); (±)-propranolol hydrochloride was obtained from Imperial Chemical Industries Ltd., Pharmaceutical Division (Cheshire, U.K.). Other chemicals were of analytical grade.

Erythrocytes and mononuclear leucocytes were obtained from heparinized human blood (10 IU/ml). The erythrocytes were prepared according to [9]. After three washes with 110 mM NaCl/10 mM Tris–HCl, pH 7.4 (hematrocrit = 0.40) and removal of buffy coat, the cells were lysed in distilled water (1 ml packed cells/10 ml ice-cold water). Membrane fragments of human erythrocytes (HEM) were obtained by homogenization of the lysate in a Potter–Elvehjem glass homogenisator with three gentle strokes by the motor driven Teflon pestle. The homogenate was adjusted to 5 mM Tris–HCl/2 mM $MgCl_2$, pH 7.4 and centrifuged at 30,000 *g* for 15 min. The pellet from 1 ml packed cells was washed twice in this buffer and finally resuspended in 75 mM Tris–HCl/25 mM $MgCl_2$, pH 7.4 Packed cells (1 ml) yielded 1 ml of HEM suspension. Total protein concentrations were determined according to Lowry [10] and residual haemoglobin concentrations according to [11]. Membrane protein concentration was 5 mg/ml, defined as the difference in concentration between total protein and residual haemoglobin. All procedures were performed at 0–4°. Mononuclear leucocytes were obtained according to [12] and prepared according to [5]. The cells (0.5–1.0 × 10^8) were washed twice in 20 ml 50 mM Tris–HCl/10 mM $MgCl_2$, pH 8.1, and homogenized like the erythrocytes. The homogenate was centrifuged at 30,000 *g* for 15 min and the pellet was washed twice in 15 ml 50 mM Tris–HCl/10 mM $MgCl_2$, pH 8.1. Finally, membrane fragments from human mononuclear leucocytes (HLM) were resuspended in 3 ml 50 mM Tris–HCl/10 mM $MgCl_2$, pH 7.4, with a protein concentration of 2 mg/ml determined by the method of Lowry [10]. All procedures were performed at 0–4°.

Results

When HEM were incubated, diluted, filtered and the filters washed at 22°, approximately 90% of the applied membrane proteins were recovered in the filtrate (Table 1). The use of ice-cold dilution buffer with increasing equilibration time at 0–4° before filtration and washing the filters with ice-cold buffer reduced the filtrability of HEM. When HEM were incubated, diluted, filtered and the filters washed at 0–4°, approximately 25% of the applied membrane proteins were present in the filtrate (Table 1).

In parallel incubations with 10 nM [^{3}H]DHA in the absence or presence of 10 μM (±)-propranolol, a significant negative correlation between filter radioactivity and the fraction of applied membrane protein recovered in the filtrate was found (Fig. 1). Specific binding became similar for the different incubation and filtration conditions when filter radioactivity was correlated to filter protein content and not to the incubation mixture protein content (Table 1).

In order to identify saturable β-adrenergic ligand binding to HEM, different concentrations of [^{3}H]DHA were incubated in the absence or presence of 10 μM (±)-propranolol. For incubations at 22°, with dilution and immediate filtration/washing of filters at 0–4°, without any correction for the amount filtered protein small differences were found between radioligand binding in the absence and presence of propranolol (data not shown). When all procedures were performed at 0–4°, saturable [^{3}H]DHA binding was found (Fig. 2). When plotted according to Scatchard [14], a dissociation constant of 1.7 ± 0.6 nM (mean ± S.D., $n = 3$) and a maximum binding capacity (B_{max}) of 21 ± 5 fmole/mg (mean ± S.D., $n = 3$) were found (Fig. 2, inset).

In contrast, saturable radioligand binding to HLM was obtained after incubation at 22° with dilution, filtration and washing of the filters at 0–4° (Fig. 3) with no detectable protein in the filtrate [12]. When plotted according to Scatchard [14], a dissociation constant of 3.5 ± 0.5 nM

Table 1. Presence of membrane proteins in filtrate and specific [^{3}H]DHA-binding on filter after ultrafiltration.*

Incubation Duration (min)/ temperature	Dilution Buffer temperature/ equilibration time (min)	Filtrability Protein concentration in filtrate as per cent of incubation mixture	Specific [^{3}H]DHA binding (fmol/filter)	Specific [^{3}H]DHA binding (fmol/mg protein/ filter)
30/22°	22°/0	92	0.30	18.8
30/22°	0–4°/0	86	0.55	19.6
30/22°	0–4°/1	52	1.8	18.8
30/22°	0–4°/2	38	2.4	19.4
30/22°	0–4°/5	32	2.7	19.9
60/0–4°	0–4°/0	26	3.0	20.3

* HEM (200 μg) were incubated in 250 μl 75 mM Tris–HCl/25 mM $MgCl_2$. [^{3}H]DHA (10 nM) in the absence or presence of 10 μM (±)-propranolol was present. The protein mixture was incubated at the indicated temperature and time. The incubations were terminated by dilution and equilibration with 2 ml of the incubation buffer at the indicated temperature and time. The samples were then rapidly filtered through a single Whatman GF/C glass fibre filter and the filters washed with 10 ml of incubation buffer at the indicated temperature. Relative protein concentrations were determined by a single radial immunodiffusion technique [13] in agarose gel (Indubiose A37, L'Industrie Biologique Français) using 1.5% rabbit antiserum against human erythrocyte membrane (DAKO-immunoglobulins, Copenhagen, Denmark). Membrane protein concentrations were defined as the difference in concentration of total protein determined according to Lowry [10] and of residual haemoglobin determined according to [11]. After filtration the filters were transferred to counting vials containing 1 ml 0.5 M HCl and 9 ml scintillation liquid (Hydrosolve®, Lumac Systems AG, Basel, Switzerland). Radioactivity was washed out of the filters by gentle shaking for about 20 hr and measured in a Packard Tri-Carb scintillation spectrometer, Model 3300. The results are given as the mean value of two separate experiments.

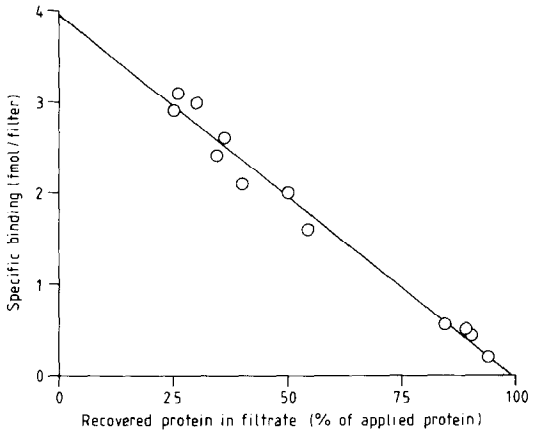

Fig. 1. Specific binding of [^{3}H]DHA to HEM defined as the difference between radioligand binding in the absence or presence of (±)-propranolol for experimental conditions described in Table 1. The binding is presented as the function of the protein fraction in the filtrate. The results represent two different experiments. The least-square regression line is given ($r = -0.99$, $P < 0.001$, $n = 2$).

(mean ± S.D., $n = 6$) and B_{max} of 72 ± 16 fmole/mg (mean ± S.D., $n = 6$) was found (Fig. 3, inset).

Discussion

The present study shows that membrane fragments of the human erythrocyte, as represented by membrane protein content in the filtrate, are able to pass through glass fibre filters with a pore size of 1.4 μm, supported by previous *in vitro* studies of erythrocyte deformability using micropore filters [15, 16]. It was demonstrated that intact normal erythrocytes passed through channels with diameters of less than 3 μm, and that the membrane was deformed upon application of only 4 mm water negative pressure. Standard incubation and filtration conditions associated with a minimum amount of ligand binding material on the glass fibre filters gave no significant saturable [^{3}H]DHA binding in the present study. This observation is in accordance with former ultrafiltration studies on human erythrocyte membrane fragments [5–7].

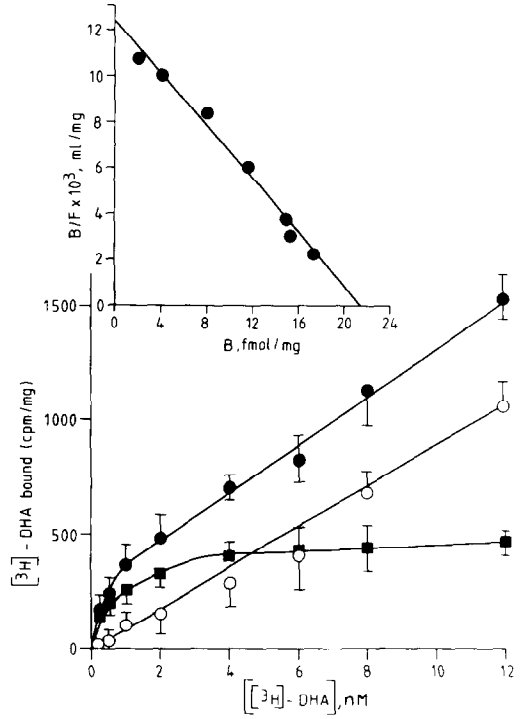

Fig. 2. Binding of [^{3}H]DHA to HEM determined by ultrafiltration. Protein (200 μg) was incubated at 0–4° with different concentrations of [^{3}H]DHA in the absence or presence of 10 μM (±)-propranolol in a total volume of 250 μl 75 mM Tris–HCl/25 mM $MgCl_2$, pH 7.4, for 30 min. The incubations were terminated by the addition of 2 ml ice-cold incubation buffer immediately before filtration through a single Whatman GF/C glass fibre filter in a 1225 Sampling Manifold, Millipore® (Millipore Corp., MA) at 0–4°. The filtration rate was 45 ml/min. The filters were washed once with 10 ml ice-cold buffer. Total (only radioligand: ●), unspecific (radioligand in presence of propranolol: ○) and specific binding (difference between total and unspecific binding; ■). The binding of three separate experiments are presented as mean ± S.E.M. Inset: Specific binding plotted according to Scatchard [14], with the least-square regression line ($r = -0.99$, $P < 0.001$, $n = 3$).

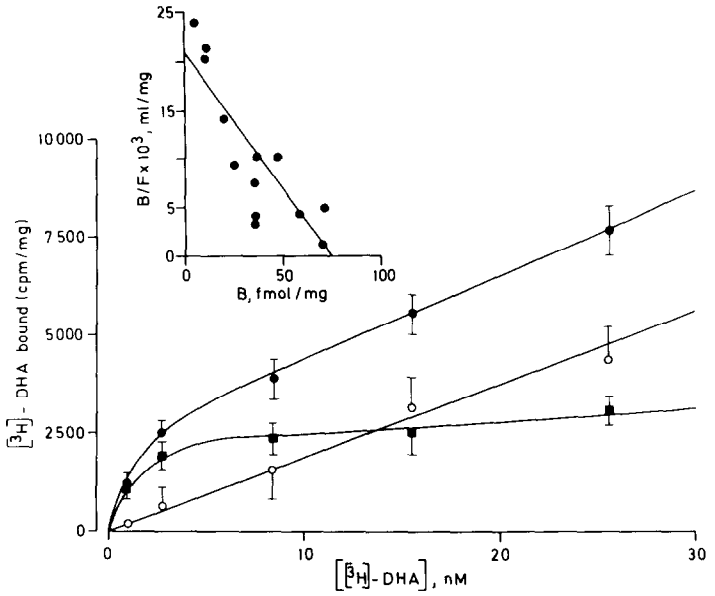

Fig. 3. Binding of [^{3}H]DHA to HLM determined by ultrafiltration. The binding experiments were performed as described in the legend of Fig. 2 with the exceptions that 100 μg HLM was incubated with different concentrations of [^{3}H]DHA in the absence or presence of propranolol in a total volume of 175 μl 50 mM Tris–HCl/10 mM $MgCl_2$, pH 7.4 for 30 min at 22°. Total (●), unspecific (○), and specific binding (■) are presented as mean ± S.E.M. Inset: Specific binding plotted according to Scatchard [14] with the least-square regression line ($r = -0.80$, $P < 0.001$, $n = 6$).

We found that equilibration of HEM with ice-cold buffer reduced filtrability of the applied membrane fragments, probably due to a temperature-dependent decrease of erythrocyte membrane flexibility. Accordingly, when specific radioligand binding was correlated to filter protein content, a similar binding capacity was obtained for pre-filtration equilibrium times up to 5 min.

The concentration binding curves of [^{3}H]DHA to HEM at 0–4° could be decomposed into saturable binding with small binding capacity and non-saturable binding with large binding capacity. The affinity of the saturable binding sites was similar to that of established β-adrenergic receptors [5, 8]. The present results are also in accordance with recent reports of β-receptor binding sites on broken human erythrocytes [17, 18]. However, maximum binding capacity was considerably lower than that found for human mononuclear leucocytes in a previous study [5]. It is possible that human erythrocyte plasma membranes are vulnerable to washing and homogenization with removal of the membrane-bound receptor binding sites. In a recent study it has been found that washing of intact human erythrocytes reduces the number of saturable binding sites for propranolol and alprenolol [4]. Loss of catecholamine-sensitive protein kinase activity has been reported as a result of extensive preparation of these cells [19]. The present study adds further evidence to the existence of a small, but significant, population of β-adrenergic receptor binding sites on human erythrocytes [17, 18].

Membrane fragments from human mononuclear leucocytes behaved entirely different from that of human erythrocytes in the filtration binding studies. No significant filtrability was observed after incubation at 22°, indicating the absence of membrane deformability in these cells under the applied conditions. The presence of specific radioligand binding with affinity and binding capacity similar to that of a previous study [5] demonstrates that the ultrafiltration technique is reproducible and useful for ligand-binding studies of these cells.

The inborn ability of the human erythrocyte to deform and pass through narrow channels may explain the apparent absence of β-adrenergic receptors using the ultrafiltration techniques [5–7]. The present study shows the importance of establishing the retention of biological material on the filters under the applied experimental conditions and the use of different binding techniques for the identification of receptor binding sites.

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β-2 adrenergic receptors on intact human erythrocytes

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Binding sites for (±)-propranolol and (−)-alprenolol have been identified on intact human erythrocytes [1]. The binding was saturable with affinity comparable to that of established β-adrenergic receptors in other human blood cells [2–4]. It was also shown [1] that β-adrenergic agonists were able to inhibit propranolol and alprenolol binding by competition. The present investigation was undertaken to substantiate the hypothesis that these binding sites on the intact human red cell have binding characteristics and functional properties similar to those of β-adrenergic receptors.

Materials and methods

The following chemicals were employed in the study: [^{3}H]-(−)-dihydroalprenolol hydrochloride (specific activity 48.6 Ci/mmole) and [^{3}H]adenosine 3′,5′-cyclic monophosphate (specific activity 37 Ci/mmole) from New England Nuclear (Dreieich, F.R.G.); unlabelled (−)-, and (+)-propranolol hydrochloride from Radiochemical and Pharmaceutical Division of Imperial Chemical Industries Ltd. (Cheshire, U.K.); (−)- and (+)-alprenolol tartrate from Hassle (Mölndalen, Sweden); (−)-isoproterenol hydrochloride, (+)-isoproterenol bitartrate, (−)-epinephrine bitartrate. (−)-norepinephrine bitartrate (−)-phenylephrine hydrochloride, ascorbic acid, theophylline and adenosine 3′,5′-cyclic monophosphate from Sigma Chemical Corp. (St. Louis, MO); sodium heparin from Novo Industries (Copenhagen, Denmark). Other chemicals were of analytical grade. Washing and incubation buffer: NaCl 122 mM, KCl 4.9 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM, NaH_2PO_4 15.9 mM. The buffer contained 10 mM D-glucose and 10 IU/ml heparin, and pH was adjusted to 7.40.

Blood was obtained from young, healthy individuals without any medication after an overnight fast, and collected in plain glass tubes containing heparin to achieve a final concentration of 10 IU/ml. The cells were separated from plasma by centrifugation at 1000 *g* for 15 min and plasma including buffy coat was aspirated. After one washing with buffer at 22°, the cells were resuspended to original hematocrit in buffer at pH 7.4. The number of blood cells and the number of platelets were determined in a Coulter Counter®, Model S5, Coulter Electronics Ltd., U.K. The following results were obtained before and after washing, respectively: erythrocytes: 4.5 ± 0.3 and 4.2 ± 0.2 × 0.2 10^{12}/l., leucocytes: 7.5 ± 0.5 and 0.6 ± 0.2 × 10^9/l., platelets: 250 ± 50 and 40 ± 20 × 10^9/l. (mean ± S.D., $n = 5$).

Binding of alprenolol was determined at thermodynamic equilibrium at 22° as described by Sager and Jacobsen [1]. In the studies of human erythrocyte cAMP* levels, incubations were performed as previously described [5] and the cAMP levels were determined by radioimmunoassay [6].

Results

After removal of plasma including buffy coat and one wash, (−)-alprenolol binding to intact human erythrocytes could be decomposed into two classes of saturable binding sites and one component of non-saturable binding. The non-saturable binding was estimated for an (−)-alprenolol concentration of 100 μM. Saturable (−)-alprenolol binding was obtained according to the method described by Chamness and McGuire [7], using the equation

$$B_{sat} = B_{tot} - \left(\frac{\lim B/F}{B \to \infty}\right) \times F$$

and defining $B \to \infty$ when $F \to 100\,\mu M$. When plotted according to Scatchard [8], a curvilinear plot was obtained (Fig. 1). Assuming the simplest case, wherein the ligand binds with different affinities to two classes of sites, the Scatchard plot was analyzed by the computer-based iterative method described by Minneman *et al.* [9]. According to this method, (−)-alprenolol binds with high affinity ($K_d = 1.8 \pm 0.5$ nM) to 900 ± 300 sites per cell and with lower affinity ($K_d = 190 \pm 80$ nM) to 18,500 ± 6500 sites per cell.

In the intact human erythrocytes [^{3}H]DHA inhibition characteristics of isoproterenol and alprenolol differed (Fig. 2). The maximal difference between inhibition by the two agents was observed at 100 μM. (−)-Alprenolol was markedly more potent in the inhibition of radiologand binding. Since binding inhibited by isoproterenol has been shown to be receptor binding [10], high affinity binding in the present study was defined as the difference in radiologand binding in the absence and presence of 500 μM (−)-isoproterenol.

As shown in Fig. 3A, β-adrenergic agonists compete with [^{3}H]DHA high affinity binding sites with the order of potency: Ipr > Epi > NE, typical for β-2 adrenergic receptors [11]. Binding was stereospecific with the laevoforms considerably more potent than the dextroforms (Fig. 3B). The equilibrium dissociation constants (K_d values) for agonists and antagonists binding to the high affinity binding sites were calculated from the concentration that caused half-maximal inhibition of [^{3}H]DHA binding by the method of Cheng and Prusoff [12]. The values are given in Table 1. The equilibrium dissociation constants for (−)-alprenolol binding to the high affinity binding sites obtained from the Scatchard plot and from the competition binding studies

* Abbreviations used: [^{3}H]DHA, [^{3}H]-(−)-dihydroalprenolol; cAMP, adenosine 3′,5′-cyclic monophosphate.