7. T. B. Nielsen, P. M. Lad, S. Preston and M. Rodbell, *Biochim. biophys. Acta* **629**, 143 (1980).
8. J. F. Krall, M. Connelly and M. L. Tuck, *J. Pharmac. exp. Ther.* **214**, 554 (1980).
9. M. G. Caron and R. J. Lefkowitz, *J. biol. Chem.* **251**, 2374 (1976).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. E. J. van Kampen and W. G. Zijlstra, *Clinica chim. Acta* **6**, 538 (1961).
12. A. Boyum, *Tissue Antigens* **4**, 269 (1974).
13. G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemistry* **2**, 235 (1965).
14. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
15. M. I. Gregersen, C. A. Bryant, W. E. Hanerle, S. Usami and S. Chien, *Science* **157**, 825 (1967).
16. S. Chien, S. Usami, R. S. Dellenback and M. I. Gregersen, *Science* **157**, 827 (1967).
17. T. Tsukamoto and M. Sonenberg, *Adv. cyclic Nucleotide Res.* **14**, 658 (1981).
18. G. Sager, *Biochem. Pharmac.* **31**, 99 (1982).
19. T. Tsukamoto and M. Sonenberg, *J. clin. Invest.* **64**, 534 (1979).

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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.

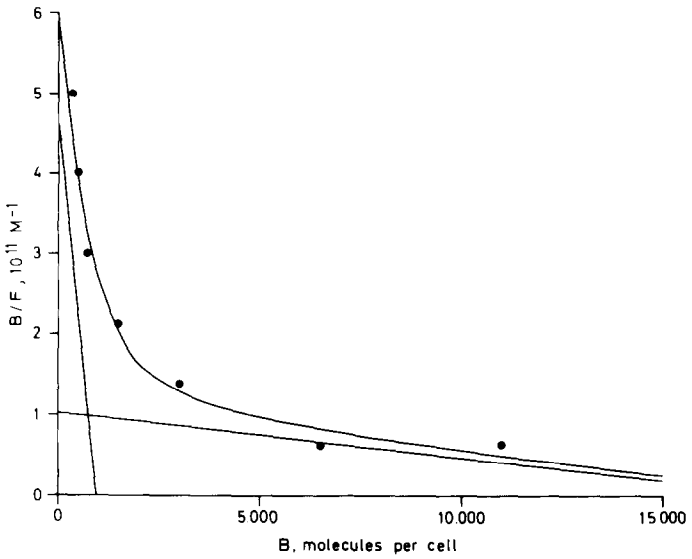


Fig. 1. (−)-Alprenolol saturation binding to intact human erythrocytes. After removal of plasma including buffy coat, [$^3$H]DHA and (−)-alprenolol were added simultaneously to achieve concentrations from 0.25 nM to 100 $\mu$M in the cell suspensions which were incubated for 1 hr at 22°. The cells were separated from the incubation medium by centrifugation, and binding was determined as described before [1]. (−)-Alprenolol saturation binding was curvilinear when plotted according to Scatchard [8]. The curve was dissected into its two original binding components, represented by the binding lines for the high and low affinity saturable (−)-alprenolol binding sites. Values are means of three separate experiments.

were in close agreement. It was found that the $\alpha$-adrenergic ligand phenylephrine had a very low affinity for the [$^3$H]DHA high affinity binding sites.

Human erythrocyte cAMP levels were detectable, but extremely low. (−)-Isoproterenol caused a concentration-dependent increase of cAMP levels (Table 2) with a maximum at 10 $\mu$M. A considerable inter-individual variation was found in both basal and stimulated cAMP levels. Propranolol inhibited the (−)-isoproterenol-induced cAMP accumulation in a concentration-dependent way (Fig. 4). The ability of other adrenergic agonists to raise cAMP levels was tested for an agonist concentration of 10 $\mu$M. As shown in Table 2, the $\beta$-adrenergic agonists elevated the cAMP levels by an order of potency: (−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine, typical for $\beta$-2 adrenergic receptors. The dextroform of isoproterenol was markedly less potent in cAMP elevation compared to the laevoform. In accordance to this, it was found that propranolol inhibited (−)-isoproterenol-induced cAMP elevation stereospecifically (Fig. 4).

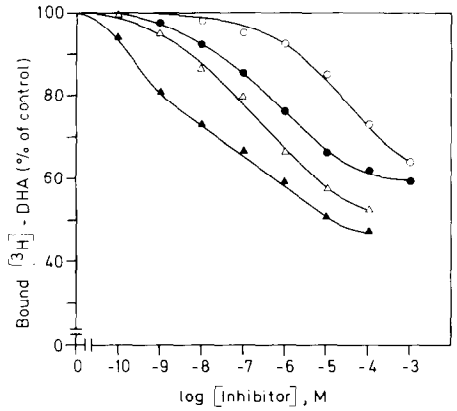


Fig. 2. Inhibition of 0.75 nM [$^3$H]DHA in intact human erythrocytes by alprenolol and isoproterenol. The incubations were performed at 22° in buffer after the removal of plasma including buffy coat and one wash. The cells were pre-incubated with inhibitors in the presence of 0.1 mM ascorbic acid before the addition of [$^3$H]DHA. After 1 hr the cells were separated from the incubation medium by centrifugation, and binding was determined as described before [1]. (−)-Alprenolol (▲),(+)-alprenolol(△),(−)-isoproterenol(●) and (+)-isoproterenol(○). The control binding refers to total radioligand binding in the absence of competitor. Values are means of three separate experiments.

*Discussion*

The present results show that (−)-alprenolol binding to intact human erythrocytes can be decomposed into three

Table 1. Equilibrium dissociation constants for adrenergic ligands determined by inhibition of high affinity [$^3$H]DHA binding, calculated from the concentrations that caused half-maximal inhibition of radioligand as described by Cheng and Prusoff [12]*

| Competitive ligand | $K_d$ ($\mu$M) |
|---|---|
| (−)-Alprenolol | 0.0012 ± 0.0006 |
| (+)-Alprenolol | 0.047 ± 0.040 |
| (−)-Propranolol | 0.00086 ± 0.00043 |
| (+)-Propranolol | 0.032 ± 0.019 |
| (−)-Isoproterenol | 0.120 ± 0.064 |
| (+)-Isoproterenol | 3.6 ± 1.4 |
| (−)-Epinephrine | 3.0 ± 1.5 |
| (−)-Norepinephrine | 48 ± 18 |
| (−)-Phenylephrine | 100 ± 60 |

* The incubations were performed as described in the legend of Fig. 2. High affinity binding refers to the difference in [$^3$H]DHA binding for the absence and presence of 0.5 mM (−)-isoproterenol. The results are presented as mean ± S.D. of three separate experiments.

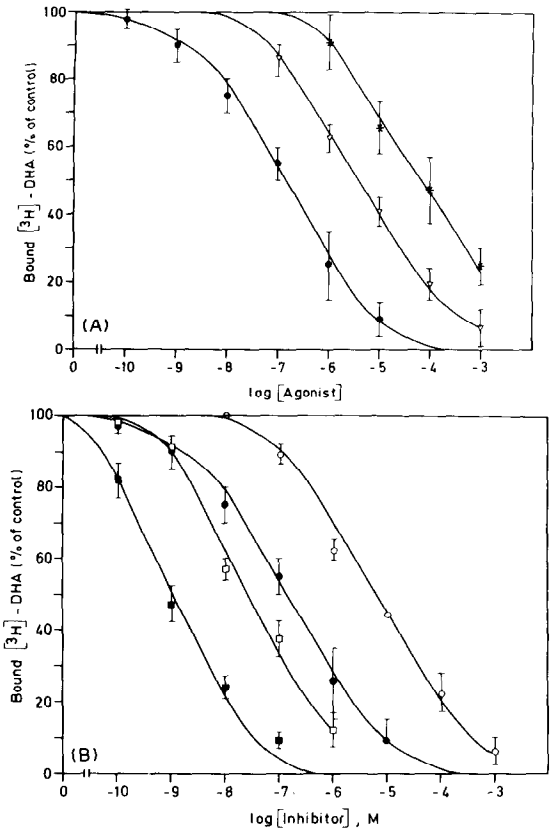


Fig. 3. Competition between [$^3$H]DHA and adrenergic agents for the high affinity binding sites. Human erythrocytes were incubated (as described in the legend of Fig. 2) with radioligand in the absence or presence of increasing concentrations of the competitors. (A)(−)-Isoproterenol (●), (−)-epinephrine (▽), (−)-norepinephrine (*). (B) (−)-Propranolol (■), (+)-propranolol (□), (−)-isoproterenol (●), (+)-isoproterenol (○). Only the portion of [$^3$H]DHA binding that could be displaced by 0.5 mM (−)-isoproterenol was taken into consideration. Control binding (300–400 molecules per cell) refers to radioligand binding in the absence of competitive ligand. Values are mean ± S.E.M. from three separate experiments.

different binding components. The non-saturable binding was determined by an excess of (−)-alprenolol. The Scatchard plot of the saturable (−)-alprenolol binding was curvilinear and could be decomposed into 900 high affinity and 18,500 low affinity binding sites per cell. This observation is in accordance with a recent study on intact turkey erythrocytes [13].

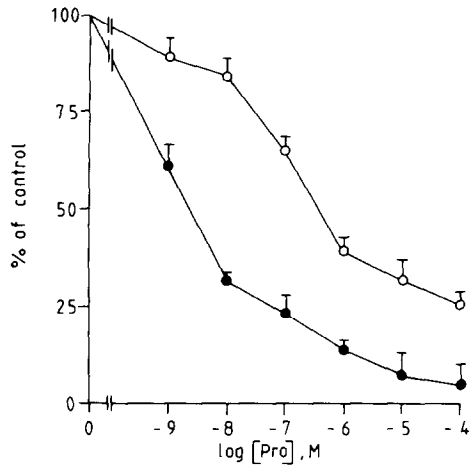


Fig. 4. Inhibition of cAMP formation by propranolol. The cells were prepared and pre-incubated as described before [5] with the exception that various concentrations of (−)- and (+)-propranolol were present during pre-incubation and that the cells were incubated with 1 mM isoproterenol for 5 min. Basal cAMP levels were obtained in parallel incubations without isoproterenol. The control value refers to the difference between stimulated (8.3 ± 2.5 pmole/$10^8$ cells per 5 min) and basal level (3.2 ± 1.5 pmole/$10^8$ cells per 5 min) in the absence of propranolol. The results are given as mean value ± S.E.M. for three separate experiments. (−)-Pro (●) and (+)-Pro (○).

The competition binding curves also supported the existence of more than one type of saturable binding sites. It was found that (−)-isoproterenol and (−)-alprenolol differed markedly in displacement of totally bound [$^3$H]DHA. In agreement with earlier studies [10, 14], the antagonist was far more potent than the agonist. Assuming the simplest case wherein [$^3$H]DHA is associated to saturable high and low affinity binding sites, the present results can be explained by the inability of the low affinity binding site to recognize the agonist molecule. In this way, the high affinity binding sites could directly be characterized using a suitable concentration of (−)-isoproterenol.

The binding characteristics of the high affinity binding site where in good agreement to those reported for β-adrenergic receptors on human erythrocytes [5], lymphocytes [2, 3] and granulocytes [4]. In the present study a close agreement between the equilibrium dissociation constant for (−)-alprenolol determined by the Scatchard plot

Table 2. cAMP levels before and after adrenergic stimulation*

| Agonist | Concentration (μm) | cAMP levels (pmole/$10^8$ cells per 2.5 min) |
|---|---|---|
| None | — | 2.4 ± 1.2 |
| (−)-Isoproterenol | 0.001 | 3.6 ± 1.4 |
| | 0.1 | 5.3 ± 1.6 |
| | 10 | 7.7 ± 2.4 |
| | 1000 | 4.6 ± 1.6 |
| (+)-Isoproterenol | 10 | 4.3 ± 1.4 |
| (−)-Epinephrine | 10 | 6.5 ± 1.9 |
| (−)-Norepinephrine | 10 | 5.3 ± 1.7 |

* Intact erythrocytes were prepared, pre-incubated and incubated and cAMP levels were assayed as described before [5, 6]. The results are presented as mean value ± S.D. of four separate experiments.

($K_d$ = 1.8 nM) and that from the competition binding curves ($K_d$ = 1.2 nM) was also found.

The identity of the high affinity binding sites as β-adrenergic receptors was further supported by (1) the ability of both agonist and antagonist to cause stereospecific displacement of [$^3$H]DHA binding, (2) the very low affinity of the α-adrenergic agonist, phenylephrine, (3) the order of potency of β-adrenergic agonists to displace bound radioligand [(−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine] typical for β-2 adrenergic receptors [11].

The presence of adenylate cyclase activity has been demonstrated in human erythrocytes, but with extremely low catecholamine sensitivity [15]. However, these cells seem to be relatively enriched in guanine regulatory protein [16–18]. The present study demonstrates the existence of β-adrenergic receptors functionally coupled to adenylate cyclase in human erythrocytes by (1) the concentration-dependent cAMP elevation by (−)-isoproterenol, (2) the concentration-dependent and stereospecific inhibition of (−)-isoproterenol stimulation by propranolol, (3) stereospecificity of isoproterenol stimulation and (4) the order of agonist potencies [(−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine] in cAMP elevation, typical for β-2 adrenergic receptors [11].

Human leucocytes have β-adrenergic receptors [2–4]. The stimulation of these receptors could have contributed to the observed cAMP levels, but only to a minor degree, since the ratio between the number of erythrocytes and the number of leucocytes in the incubation mixture was approximately 7000:1. The ratio between cAMP levels in erythrocytes and leucocytes is approximately 1:25 under comparable incubation conditions [19].

Even if the reported cAMP levels in the human erythrocytes are low, the basal and maximal levels represent an accumulation of about 90 and 300 molecules cAMP per sec per cell, respectively. Such changes in cAMP levels are probably enough to exert biological effects. Cyclic nucleotides have been proposed to play a role in the deformability of the human red cell [20]. The effect of adrenergic amines on human red cell haemolysis exhibited a β-2 adrenergic profile [21]. It was also observed that the effect of catecholamines was blocked by propranolol, but mimicked by exogenous cAMP.

The present study shows that intact human erythrocytes possess a population of functional β-2 adrenergic receptors. However, further studies are necessary to establish the physiological role of these receptors.

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REFERENCES

1. G. Sager and S. Jacobsen, *Biochem. Pharmac.* **28**, 2167 (1979).
2. L. T. Williams, R. Snyderman and R. J. Lefkowitz, *J. clin. Invest.* **57**, 149 (1976).
3. J. F. Tohmeh and P. E. Cryer, *J. clin. Invest.* **65**, 836 (1980).
4. B. H. Dulis and I. B. Wilson, *J. biol. Chem.* **255**, 1043 (1980).
5. G. Sager, *Biochem. Pharmac.* **31**, 99 (1982).
6. T. Skomedal, B. Grynne, J. B. Osnes, A. E. Sjetnan and I. Øye, *Acta pharmac. tox.* **46**, 200 (1980).
7. G. C. Chamness and W. L. McGuire, *Steroids* **26**, 538 (1975).
8. G. Scatchard, Ann. N.Y. Acad. Sci. **51**, 660 (1949).
9. K. P. Minneman, L. R. Hegstrand and P. B. Molinoff, *Molec Pharmac.* **16**, 34 (1979).
10. S. R. Nahorski and A. Richardson, *Br. J. Pharmac.* **66**, 469P (1979).
11. A. M. Lands, A. Arnold, J. P. McAuliff, F. P. Luduena and T. G. Brown, Jr., *Nature, Lond.* **214**, 597 (1967).
12. Y.-C. Cheng and W. H. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1973).
13. C. André, G. Vauquelin, J.-P. de Backer and A. D. Strosberg, *Biochem. Pharmac.* **30**, 2787 (1981).
14. E. M. Dax and J. S. Partilla, *Molec. Pharmac.* **22**, 5 (1982).
15. J.-P. Piau, J. Delaunay, S. Fischer, M. Tortolero and G. Schapira, *Blood* **56**, 963 (1980).
16. Z. Farfel, H. R. Kaslow and H. R. Bourne, *Biochem. biophys. Res. Commun.* **90**, 1237 (1979).
17. T. B. Nielsen, P. M. Lad, S. Preston and M. Rodebell, *Biochim. biophys. Acta* **629**, 143 (1980).
18. H. R. Kaslow, G. L. Johnson, V. M. Brothers and H. R. Bourne, *J. biol. Chem.* **255**, 3736 (1980).
19. F. Wisløff and T. Christoffersen, *Int. Archs Allergy appl. Immun.* **53**, 42 (1977).
20. Y. Yawata, H. S. Jacob, N. Matsumoto and J. White, *J. Lab. clin. Med.* **88**, 555 (1976).
21. H. Rasmussen, W. Lake and J. E. Allen, *Biochim. biophys. Acta* **411**, 63 (1975).

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## Adriamycin-iron catalysed phospholipid peroxidation: a reaction not involving reduced adriamycin or hydroxyl radicals

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The anthracycline antitumour antibiotic adriamycin is widely used in modern cancer therapy. Its mode of action can be related to its ability to intercalate with cell DNA and it is thought to cause strand scissions in the DNA molecule [1]. Strand scissions occur after the drug has undergone a reductive cycle in which electrons are transferred to dioxygen, resulting in the formation of oxygen radicals [1, 2].