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BETA-ADRENERGIC RECEPTORS OF HUMAN LEUKOCYTES

STUDIES WITH INTACT MONONUCLEAR AND POLYMORPHONUCLEAR CELLS AND MEMBRANES COMPARING TWO RADIOLIGANDS IN THE PRESENCE AND ABSENCE OF CHLOROQUINE*

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Abstract—Owing to the large differences in reported values for β-adrenergic receptor numbers and binding affinity in normal leukocytes, we undertook a systematic re-examination of the binding of two widely used beta antagonists, (−)-[^{3}H]dihydroalprenolol (DHA) and (±)-[^{125}I]iodohydroxybenzylpindolol (HYP), to intact normal mononuclear (MN) leukocytes and polymorphonuclear (PMN) leukocytes and membrane preparations. Assays were conducted in the presence and absence of chloroquine, which has been proposed recently to eliminate ligand uptake into a non-receptor cell compartment such as lysosomes. The binding curves relating radioligand concentration to specific sitesper intact cell were biphasic. At high (10–24 nM) (−)-DHA ligand concentration in the absence of chloroquine, a large number (20,000–60,000 sites/cell) of low affinity (K_d 12–15 nM) stereospecific binding sites were detected in both cell types. This class of binding sites was eliminated by 10 μM chloroquine not only in PMN cells but also in the lysome-poor MN cells (≥ 90% lymphocytes), leaving 2000–3000 specific high affinity (−)-DHA sites/cell. In the absence of chloroquine, comparably low numbers of specific high affinity binding sites/cell were also obtained by the use of appropriately low concentrations of (−)-DHA or (±)-HYP (800 pM or less). However, even at these low radioligand concentrations chosen to measure high affinity specific binding, the addition of 10 μM chloroquine produced a moderate reduction in the number of sites/cell, without a detectable change in the apparent K_d. Mean (± S.E.M.) site numbers obtained in the presence of chloroquine were: 1331 ± 100 sites/MN cell and 1135 ± 129 sites/PMN cell (K_d 143–153 pM) using (−)-DHA; and 1487 ± 210 sites/MN cell and 1065 ± 69 sites/PMN cell [avg. $K_{d(\pm)}$ 224–274 pM] using (±)-HYP. Chloroquine had no effect on agonist-stimulated cAMP production but produced an apparent increase in the effectiveness of (−)-propranolol as an inhibitor of DHA binding. Competition studies on the binding of DHA and HYP with zinterol and practolol confirmed that the receptor was of the β_2-subtype for both MN and PMN cells. The detection of a moderately larger number of high affinity binding sites at saturation (Scatchard analysis) by (±)-HYP than by (−)-DHA was a consistent finding with either intact cells or membranes, with or without chloroquine. The possible overestimation of receptor numbers by a racemic ligand such as (±)-HYP is discussed and leads us to favor the use of a pure stereoisomer such as (−)-DHA. A system employing 800 pM (−)-[^{3}H]DHA, 1 μM (−)-propranolol and 10 μM chloroquine with intact MN and PMN cells yielded reproducible and plausible results. Our values for β-adrenergic receptor numbers of intact MN and PMN cells and membranes are compared to others in the literature.

Many studies on the numbers or functional status of adrenergic receptors in man have been stimulated by the hypothesis that a primary abnormality in adrenergic balance may underlie certain diseases such as bronchial asthma [1, 2]. Peripheral blood leukocytes or platelets have been employed as test cells for the study of human β- or α-adrenergic receptors, respectively, on the assumption that these cells should be representitive of a putative systemic defect in patients.

The literature reveals considerable variability in the reported *normal* values for β-adrenergic receptor number or binding affinity in human leukocytes [3–23]. In studies of the leukocytes of asthmatic patients, there are conflicting conclusions concerning β-receptor number or catecholamine-dependent cAMP responses and on the degree to which drug therapy has affected the results [4, 24–40]. In these studies, conditions for radioligand binding have varied widely, and in some instances did not distinguish between high and low affinity binding sites. Most laboratories have employed membrane preparations

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from mononuclear (MN)* or polymorphonuclear (PMN) leukocytes, whereas relatively few studies have used intact cells [11, 14, 20, 38, 39]. Mechanical disruption of cells followed by centrifugation and washing has been reported to cause a partial loss of β-receptors [38] and adenylate cyclase activity [38, 40].

In the present report, we have sought to resolve some of these apparent discrepancies and to define optimal conditions for measuring β-adrenergic receptors in intact human MN and PMN leukocytes, using two different radioligands. Comparative binding data on leukocyte membrane preparations are also provided. Attention has been paid to high versus low affinity binding, receptor subtype, correlations with agonist-stimulated cAMP production, and both technical and biological contributions to variability. Furthermore, based on the study of Dulis and Wilson [20] indicating that PMN leukocytes may exhibit β-adrenergic ligand uptake into a chloroquine-inhibitable compartment, we investigated in some detail the effect of chloroquine on the binding of β-adrenergic ligands to both intact MN and PMN cells and their derived membranes, and its effect on basal and isoproterenol-stimulated cAMP levels.

MATERIALS AND METHODS

The reagents and their sources are as follows: theophylline, (−)-isoproterenol-D-bitartrate, (+)-propranolol, and (−)- and (+)-isoproterenol from the Sigma Chemical Co., St. Louis, MO; (−)- and (+)-alprenolol from Labkemi AB, 3421 31 Vastra Frolunda, Sweden; (−)-propranolol from Ayerst Laboratories, New York, NY; zinterol from Mead Johnson, Evansville, IN; practolol from Imperial Chemical, Macclesfield, England; ACS mixture from Amersham-Searle, Arlington Heights, IL; (±)-[^{125}I]iodohydroxybenzylpindolol (HYP) (sp. act. 2200 Ci/mmole) and (−)-dihydroalprenolol (DHA) hydrochloride[propyl-1,2,3-^{3}H] (sp. act. 45-60 Ci/mmole) from the New England Nuclear Corp., Boston, MA; and a cAMP radioimmunoassay kit from Collaborative Research, Inc., Waltham, MA. The purity of the labeled ligands was verified by thin-layer chromatography (TLC) on Merck silica gel 60 plates (VWR Scientific, Rochester, NY) using methanol–water–acetic acid (80:20:0.5) as solvent.

Preparation of MN and PMN leukocytes and membrane fragments. Whole blood (50–100 ml) was drawn between 7:30 and 9:00 from healthy volunteers, ages 20–45, into heparinized tubes. The volunteers had no allergic or asthmatic symptoms by history, although a few had two or three positive immediate intradermal skin tests with a battery of ten inhalant allergens. Their values did not differ from those with totally negative skin tests. None of the donors was taking adrenergic drugs, theophylline or antihistamines. Persons with active viral respiratory infections were excluded.

MN cells were separated from the red cells and PMN cells by centrifugation through a Ficoll-Hypaque gradient [41]. MN cells were washed three times in ice-cold Dulbecco's phosphate buffer, pH 7.4, and once in cold calcium-free Krebs–Ringer bicarbonate (KRB) buffer, pH 7.4, containing 5 mM glucose. The MN cells were resuspended in KRB buffer, counted, and held in an ice bath until used (1–4 hr). PMN cells were separated from red cells by dextran sedimentation [42] at 4° and washed once in cold Dulbecco's buffer. The cell pellet was then subjected to one or two hypotonic/hypertonic washes to lyse red cells [43]. Following two more washes in cold Dulbecco's buffer, the cells were washed and resuspended in ice-cold KRB buffer, counted, and held at 4°. Calcium was omitted in the KRB buffer to minimize release of lysosomal enzymes.

"MN cells" comprised 90% or more lymphocytes and had less than 1% contamination with PMN cells. "PMN cells" were primarily granulocytes and had 4–6% contamination with MN cells. Red cell contamination in the MN and PMN cell preparations was not significant in that human red cells showed no specific binding of HYP or DHA. MN cell preparations contained 5–12 platelets/MN cell, whereas PMN cell preparations had less than 1–2 platelets/PMN cell. Platelets contain α-adrenergic receptors but have not been reported to contain β-adrenergic receptors [43–45]. We have found no specific binding of HYP or DHA to pure preparations of human platelets.

Membranes were prepared by the method of Williams *et al.* [3]. Protein was assayed according to Lowry *et al.* [46].

Ligand binding by rapid filtration. The ligands and propranolol were made up in 20% ethanol in KRB buffer. The final ethanol concentration in the reaction mixtures was 2%. *n*-Butanol (final concentration 0.3%) can substitute for ethanol. The use of dimethyl sulfoxide as a solvent for the ligands did not improve binding. Radioligand binding increased linearly in proportion to the number of cells over a range of 0.25–3.0 × 10^6 cells. In our standard assay, 10^6 cells were incubated in polypropylene tubes at 37° for different times (2–60 min) in 500 μl of KRB buffer containing radioactive ligand and various amounts of (−) and (+) cold antagonist or agonist. Membranes (200–300 μg protein) were incubated with ligands in 500 μl of 50 mM Tris buffer, pH 7.4, containing 10 mM $MgCl_2$. At the end of the incubation, the cells or membranes were diluted with 2.0 ml of ice-cold KRB buffer (for cells) or 50 mM Tris buffer (for membranes). The cells or membranes were filtered immediately through 24 mm GF/C glass fiber filters (Whatman) using a Hoefer stainless steel suction filtration unit. The incubation tubes were rinsed three times with 2.5 ml of either ice-cold KRB buffer or ice-cold Tris buffer which were also run through the filters. The filters were washed twice with 5-ml aliquots of ice-cold Tris buffer, dried, and counted in 10 ml of Amersham-Searle ACS mixture using a Searle Delta 300 scintillation spectrometer. In the case of [^{125}I]HYP, counting was done on a Packard gamma counter. Specific binding of HYP and DHA is empirically defined as the binding which is inhibited by 1–10 μM (−)-propranolol. Specific bind-

* Abbreviations: MN cells, human mononuclear leukocytes; PMN cells, human polymorphonuclear leukocytes; HYP, hydroxybenzylpindolol; and DHA, dihydroalprenolol.

ing varied from 40 to 70% of total binding for cells and 40 to 80% for membranes, depending on the concentration of (−)-propranolol and on the presence and concentration of chloroquine. Stereospecific binding was more pronounced at 0.1 and 1 μM (−)-propranolol than at 10 μM. All samples were analyzed in quadruplicate. K_d values were calculated by Scatchard analysis [47] and K_i values by the method of Cheng and Prusoff [48]. Very little or no degradation of either DHA or HYP, as assessed by TLC, occurred in the presence of cells during the 30-min incubation used to measure binding.

Other modifications of the binding conditions. We have tried different buffers including 100 mM Tris, 50 mM Tris, 100 mM mannitol, and Krebs–Ringer phosphate buffer with and without 5–10% glycerol for ligand binding to intact cells. These have given results similar to our standard KRB buffer. We have also used 50 μM (−)-isoproterenol to measure specific binding of DHA and HYP to MN cells. This agonist has given a similar number of binding sites as 1 μM (−)-propranolol on the same cell preparation. Phentolamine (100 μM) and catechol (1 mM) were also tested as agents to inhibit uptake of ligand and to possibly enhance specific binding. In our hands, these agents did not improve significantly the binding over that obtained with 10 μM chloroquine.

cAMP assay. For basal cAMP levels, 1–2 × 10^6 cells in 0.4 ml KRB buffer, pH 7.4, containing 5 mM theophylline were placed in polypropylene tubes in a shaking water bath. For hormone-stimulated cAMP levels, another 1–2 × 10^6 cells were incubated in 0.4 ml KRB buffer containing both 5 mM theophylline and 10 μM (−)-isoproterenol. The cells were incubated for 5 min at 37°, then heated at 90° for 5 min, and spun at 1200 *g* for 15 min at 4°. The supernatant fractions (0.25 ml) were added to 0.25 ml of 50 mM acetate buffer, pH 6.2. The samples were acetylated, and cAMP was measured by radioimmunoassay [49]. All samples were analyzed in triplicate.

RESULTS

Studies with membrane preparations. We first measured β-adrenergic receptor levels in membrane preparations from MN and PMN cells, to permit comparison with the results of others. (±)-HYP or (−)-DHA binding to MN or PMN membranes reached equilibrium by 30 min at 37°, reached saturation at 800 pM for HYP and at 1 nM for DHA, was reversible, and was stereospecific for both ligands, i.e. the K_i values for (−)-propranolol and (−)-isoproterenol were much lower than the K_i values for the (+) isomers. The K_d values for high affinity binding to MN and PMN membranes varied from 150 to 340 pM for (±)-HYP and 300 to 500 pM for (−)-DHA in the absence of chloroquine (Table 1), consistent with high affinity receptors. Because of the prominent use of chloroquine in our studies on intact cells (below), ligand binding to membranes was also carried out in the presence of 10 μM chloroquine (Table 1). A significant change in K_d values was not found. Our overall range for high affinity

Table 1. Dissociation constants for binding of (−)-[^{3}H]DHA and (±)-[^{125}I]HYP to MN and PMN membranes in the presence and absence of chloroquine

Ligand	Preparation	K_d* (pM) Chloroquine absent	K_d* (pM) Chloroquine present
DHA	MN membranes	300, 500†	360
	PMN membranes	350	310
HYP‡	MN membranes	150	160, 200†
	PMN membranes	300, 340†	300

* The K_d values represent the high affinity sites and were determined by linear least squares regression analysis of Scatchard plots. The chloroquine concentration was 10 μM and the propranolol concentration was either 1 or 10 μM. The DHA and HYP concentrations varied from 300 to 1000 pM. Pooled cells from three donors were used for each determination recorded in the table.

† Represents values from two experiments.

‡ The K_d values shown for the racemic (±)-HYP are average values, i.e. $K_{d(\pm)}$. The $K_{d(-)}$ is presumed to be considerably smaller (see Discussion).

DHA and HYP binding to either MN or PMN membranes in the absence of chloroquine was 10–40 fmoles/mg membrane protein. Higher levels of binding were generally observed with (±)-HYP than with (−)-DHA, as in Table 2. The use of either 1 or 10 μM (−)-propranolol to measure specific binding gave similar results.

The analytical reproducibility of high affinity binding of HYP and DHA was tested by studying aliquots of a single preparation of frozen, pooled MN membranes as three separate unknowns on the same day (Table 2). Specific HYP binding displayed a 3.6% variability; for specific DHA binding, a 7.5% variability was observed.

Studies with intact cells. As illustrated in the dose–response study with MN cells in Fig. 1, specific binding of (−)-DHA followed a biphasic curve, with a change in slope at approximately 1 nM. A similar binding profile was seen with PMN cells. When (−)-DHA concentrations greater than 1 nM were employed, binding was rapid, saturable, and stereospecific; however, Scatchard analysis yielded very high estimates of specific binding sites/cell. For example, using 24 nM (−)-DHA, which approached apparent saturating conditions according to Fig. 1, values of 28,700 ± 19,000 (mean ± S.D.) sites/MN cell and 43,900 ± 18,200 sites/PMN cell were obtained. The K_d for binding under these conditions was 12–15 nM, indicating sites with low average affinity.

In contrast, in the presence of 10 μM chloroquine, 24 nM DHA yielded only 2930 ± 880 specific binding sites/MN cell in three experiments. The K_d value for binding was 100–170 pM. Similarly, based on the lower range of the binding curve (Fig. 1, inset), the use of 800 pM (−)-DHA with the same numbers of cells, in the absence of chloroquine, yielded 4260 ± 642 sites/MN cell and 2740 ± 190 sites/PMN cell in three experiments. These sites also had K_d values indicative of high affinity (approximately 200 pM, see below). These findings clearly implied that a large number of low affinity sites were being

Table 2. Reproducibility of binding of (±)-[^{125}I]HYP and (−)-[^{3}H]DHA to membranes from frozen MN leukocytes*

Expt. No.	Ligand binding (fmoles) Total	With 1 μM (−)-propranolol	Specific binding
DHA			
I	4.64 ± 0.16	2.31 ± 0.09	2.33 ± 0.18
II	5.16 ± 0.21	2.41 ± 0.08	2.75 ± 0.22
III	6.14 ± 0.07	3.25 ± 0.10	2.87 ± 0.11
Mean ± S.E.M.	5.31 ± 0.17	2.66 ± 0.10	2.65 ± 0.21†
% variability	3.2	3.8	7.5
HYP			
I	10.6 ± 0.16	6.0 ± 0.05	4.60 ± 0.16
II	11.1 ± 0.12	6.8 ± 0.11	4.24 ± 0.16
III	11.1 ± 0.06	7.1 ± 0.07	4.04 ± 0.14
Mean ± S.E.M.	10.9 ± 0.11	6.63 ± 0.08	4.29 ± 0.15‡
% variability	1.0	1.2	3.6

* Binding of 300 pM [^{125}I]HYP and 1 nM [^{3}H]DHA to membranes (230 μg protein) in 1 ml of 50 mM Tris buffer, pH 7.4, containing 10 mM $MgCl_2$. Specific binding was 40% of total binding for HYP and 50% for DHA. Values are the mean ± S.E.M. of samples analyzed in quadruplicate on the same batch of membranes studied as the three separate unknowns on the same day. Chloroquine was omitted in these experiments. The percent variability is the S.E.M./mean × 100.

† This equals 11.5 ± 0.9 fmoles/mg protein.

‡ This equals 18.6 ± 0.7 fmoles/mg protein.

detected at the higher DHA concentrations unless chloroquine were present. A similar finding was observed with HYP. Thus, at a 1 nM concentration, (±)-HYP detected 10,900 sites/PMN cell in the absence of chloroquine.

Recognizing that low concentrations of DHA alone or higher concentrations of DHA plus chloroquine yielded similar but apparently not identical numbers of high affinity sites, we undertook more detailed studies of ligand binding in the presence of chloroquine. Saturation of (−)-DHA binding was attained at approximately 800 pM (Fig. 2A). Scatchard plots of DHA binding were linear (Fig. 2B) and in this experiment gave K_d values of 160 and 178 pM for MN and PMN cells, respectively, and 2444 and 1250 sites per cell at saturation. The mean K_d values for DHA binding in three experiments were 143–153 pM in the presence of chloroquine and 210—220 pM in its absence (Table 3).

Parallel studies with (±)-[^{125}I]HYP depicted in Fig. 3 also gave linear Scatchard plots which yielded 3650 sites/MN cell and 1760 sites/PMN cell. In three experiments, the observed average K_d values (mean ± S.D.) for (±)-HYP binding to MN and PMN cells in the presence of chloroquine were 224 ± 58 and 274 ± 46 pM respectively (Table 3). K_d values in the absence of chloroquine were not significantly different (Table 3).

The observation that *at saturation* (determined by Scatchard analysis) (±)-HYP detects a somewhat larger number of high affinity sites on both MN and PMN cells than does (−)-DHA (Figs. 2 and 3) was a consistant finding. The likelihood that some degree of overestimation of binding sites may result from conventional Scatchard analysis of the binding of a racemic ligand such as (±)-HYP is discussed below in relation to the findings of Bürgisser *et al.* [50]. In most of our subsequent studies with (±)-HYP, this

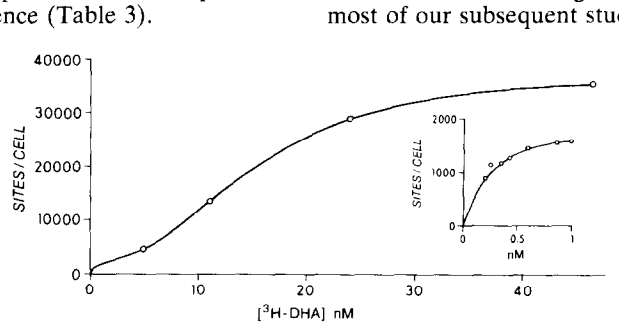

Fig. 1. Concentration profile for the binding of (−)-[^{3}H]DHA to intact MN cells. MN cells (10^6) were incubated for 30 min at 37° in KRB buffer, pH 7.4, containing different concentrations of (−)-[^{3}H]DHA (100 pM–48 nM) with and without 10 μM propranolol. The insert shows the expanded concentration profile from 0.1 to 1.0 nM DHA. Chloroquine was omitted in these experiments in order to show the large number of sites at high DHA levels. Similar results were obtained with PMN cells. Pooled cells from two normal donors were used.

Table 3. Dissociation constants for binding of (−)-[^{3}H]DHA and (±)-[^{125}I]HYP to MN and PMN cells in the presence and absence of chloroquine

Ligand	Preparation	K_d* (pM) Chloroquine absent	K_d* (pM) Chloroquine present
DHA	MN cells	220†	143 ± 38‡
	PMN cells	210†	153 ± 46‡
HYP§	MN cells	300†	224 ± 58‡
	PMN cells	280†	274 ± 46‡

* The K_d values represent the high affinity site and were determined by linear least squares regression analysis by Scatchard plots. The chloroquine concentration was 10 μM and the propranolol concentration was either 1 or 10 μM.

† Determinations were carried out on pooled cells of two to three donors.

‡ Values are mean ± S.D. for cell preparations from three normal donors.

§ The K_d values shown for the racemic (±)-HYP are average $K_{d(\pm)}$ values. The $K_{d(-)}$ for (−)-HYP is presumed to be considerably smaller (see Discussion).

racemic ligand was used at 300 pM which, on face value, seems too close to the observed $K_{d(\pm)}$ to allow receptor saturation. However, since the true $K_{d(-)}$ for (−)-HYP may be in the range of 10–50 pM [50], 300 pM (±)-HYP should saturate the high affinity stereospecific sites.

The stereospecificity* of (±)-HYP and (−)-DHA binding in the presence of chloroquine is demonstrated in Figs. 4 and 5. Stereospecificity was more pronounced at concentrations of (−)-propranolol between 0.01 and 1 μM than at 10 μM (see Discussion).

The effects of various concentrations of chloroquine on specific binding of 300 pM (±)-HYP are presented in Fig. 6. In agreement with the data of Dulis and Wilson [20] on DHA binding to PMN cells, the optimal range of chloroquine concentration

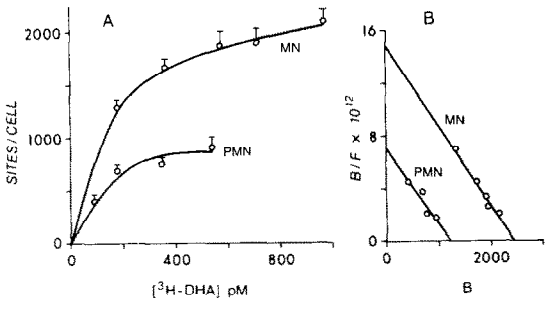

Fig. 2. Concentration profiles (A) and Scatchard plots (B) for the binding of (−)-[^{3}H]DHA to MN and PMN cells. MN or PMN cells (10^6) were incubated for 30 min at 37° in KRB buffer, pH 7.4 containing 10 μM chloroquine and different concentrations (90–1000 pM) of (−)-[^{3}H]DHA. A 1 μM concentration of (−)-propranolol was used to measure specific binding. Bars represent the S.E.M. of quadruplicate analyses. The lines in Fig. 2B are fitted by computerized least squares linear regression analysis. Pooled cells from two normal donors were used.

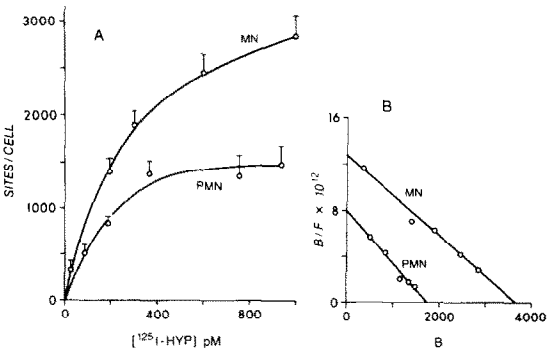

Fig. 3. Concentration profiles (A) and Scatchard plots (B) for the binding of (±)-[^{125}I]HYP to MN and PMN cells. Experimental design and analysis are the same as in Fig. 2. The HYP concentrations are for the racemic mixture.

for minimizing the presumed lysosomal uptake of HYP was 10–50 μM for MN cells and possibly also for PMN cells, although with PMN cells a plateau was not reached by 50 μM chloroquine. Even though 300 pM (±)-HYP was chosen to measure selectively the high affinity receptors, chloroquine still produced a moderate reduction in the number of sites with respect to the values derived in the absence of chloroquine. Chloroquine appeared to have no significant effect on the K_d for HYP of DHA binding to intact cells when the ligand concentration was 300–1000 pM (Table 3). Given these results and the data illustrated

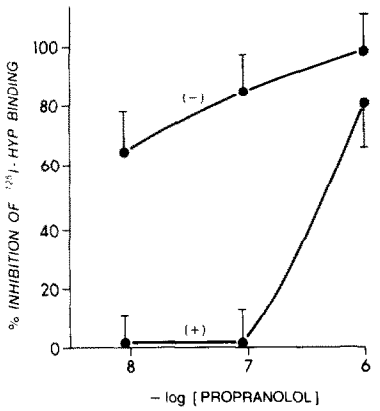

Fig. 4. Inhibition of (±)-[^{125}I]HYP binding to MN cells by (−)- and (+)-propranolol in the presence of chloroquine. Cells (10^6) were incubated in KRB buffer containing 300 pM (±)-[^{125}I]HYP at 37° for 30 min in the presence of 10 μM chloroquine with and without different concentrations of (−)- and (+)-propranolol. Inhibition of binding by 1 μM (−)-propranolol represents maximal specific binding (3420 sites/cell) and is set at 100%. Values are the mean ± S.E.M. of quadruplicate analyses. Pooled cells from two normal donors were used.

* Specific binding, empirically defined as the fraction of total binding inhibitable by (−)-propranolol, is dependent on the concentration of propranolol used and should more appropriately be called apparent specific binding. Stereospecific binding, distinguished by the use of (−)- and (+)-propranolol, is seen only at concentrations at or below 1 μM (−)-propranolol in our system.

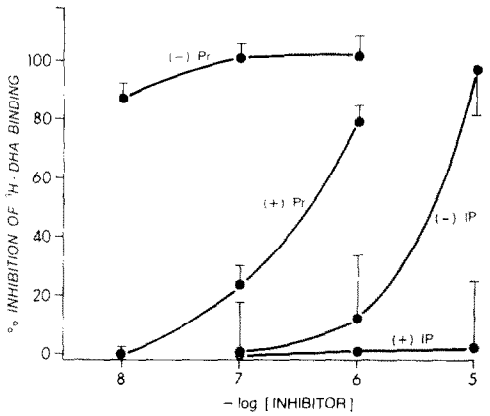

Fig. 5. Inhibition of binding of (−)-[^{3}H]DHA to MN cells by (−)- and (+)-propranolol and (−)- and (+)-isoproterenol in the presence of chloroquine. Cells (10^6) were incubated in KRB buffer for 30 min at 37° with 800 pM (−)-[^{3}H]DHA in the presence of 10 μM chloroquine and with and without different concentrations of (−)- and (+)-propranolol and (−)- and (+)-isoproterenol. Values are the mean ± S.D. of quadruplicate analyses. Inhibition of binding by 1 μM (−)-propranolol represents maximal specific binding (1644 sites/cell) and is set at 100%. Pooled cells from two normal donors were used.

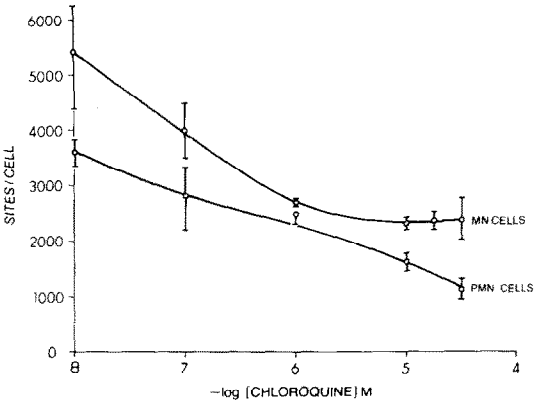

Fig. 6. Dose–response of chloroquine concentration in relation to the binding of (±)-[^{125}I]HYP to intact MN and PMN cells. Cells (10^6) were incubated for 30 min at 37° in KRB buffer, pH 7.4, containing 300 pM (±)-[^{125}I]HYP and different concentrations of chloroquine. Values are the mean ± S.E.M. of quadruplicate analysis. A 10 μM concentration of (−)-propranolol was used to measure specific binding. Pooled cells from two normal donors were used.

in Fig. 6, it would appear that chloroquine not only can eliminate specific low affinity sites but may also suppress a portion of the specific high affintiy sites measurable in intact cells (see Discussion).

The β_2-antagonist, zinterol [51], was more potent than the β_1-antagonist, practolol [51], in inhibiting binding of HYP or DHA to both MN and PMN cells. These results were comparable in the presence or absence of chloroquine. Thus, β_2 subtype selectivity is not affected by chloroquine.

As summarized in Table 4 (Expt. I), 10 μM chloroquine had no effect on the basal or isoproterenol-stimulated cAMP levels in intact MN cells. Thus, the large number of low affinity, stereospecific DHA binding sites which are eliminated by chloroquine are not essential for agonist-induced cAMP synthesis. On the other hand, 400 pM (−)-alprenolol (the dehydro analogue of DHA), which should saturate nearly all high affinity sites, inhibited the isoproterenol-stimulated cAMP levels by 80%. Chloroquine did not alter this inhibition. As also shown in Table 4 (Expt. II), zinterol was clearly a more effective inhibitor than practolol in this functional assay, in agreement with their effects on ligand binding.

The effect of 10 μM chloroquine on binding of DHA to MN cells was studied at different concentrations of (−)- and (+)-propranolol (Fig. 7). Chloroquine caused a shift to the left in the inhibition

Table 4. Effects of chloroquine, zinterol and practolol on basal and isoproterenol-stimulated cAMP levels in MN leukocytes

	cAMP [pmoles · min^{-1} · (10^8 cells)$^{-1}$]	
	No chloroquine	10 μM Chloroquine
Expt. I*		
Basal	11.2	12.8
(−)-Isoproterenol (10 μM)	235 ± 72	300 ± 87
(−)-Isoproterenol + 400 pM (−)-alprenolol	49 ± 14	54 ± 13
(−)-Isoproterenol + 5 nM (−)-alprenolol	5 ± 1.2	11 ± 2.9
Expt. II†		
Basal		14 ± 1.0
(−)-Isoproterenol (10 μM)		141 ± 3.3
(−)-Isoproterenol + zinterol (100 μM)		60 ± 5.8
(−)-Isoproterenol + practolol (100 μM)		132 ± 16

* Cells (10^6) were incubated with and without isoproterenol, alprenolol, and chloroquine in KRB buffer for 6 min at 37°. cAMP was analyzed by radioimmunoassay as described in the text. All analyses were done on the same leukocyte preparation. Each sample was analyzed in triplicate and represents cells from one normal donor. Values are the mean ± S.E.M.

† Cells (10^6) were incubated for 6 min at 37° with and without 10 μM isoproterenol, 100 μM zinterol and 100 μM practolol in KRB buffer containing 10 μM chloroquine. cAMP was assayed as described in the text. Each sample was analyzed in triplicate and represents cells from one normal donor. Values are the mean ± S.E.M.

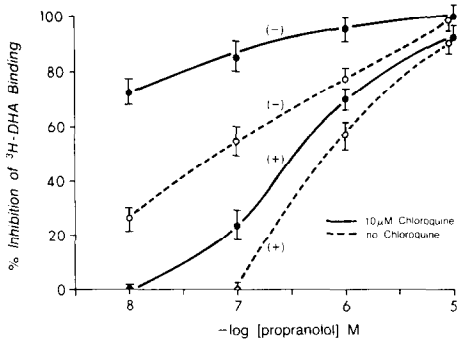

Fig. 7. Stereospecific inhibition of binding of (−)-[^{3}H]DHA to MN cells by (−)- and (+)-propranolol in the presence and absence of chloroquine. MN cells (10^6) were incubated for 30 min at 37° in KRB buffer, pH 7.4, containing 1 nM (−)-[^{3}H]DHA, different concentrations of (−)- and (+)-propranolol, and with and without 10 μM chloroquine. Values are the mean ± S.E.M. of quadruplicate analyses. Inhibition of binding by 10 μM (−)-propranolol represents maximal specific binding and is set at 100%. The maximal values as sites/cell are as follows: in the absence of chloroquine, 2343 ± 174 for (−)-propranolol and 2153 ± 166 for (+)-propranolol; in the presence of chloroquine, 1262 ± 102 for (−)-propranolol and 1189 ± 228 for (+)-propranolol. Pooled cells from three normal donors were used.

curves. Chloroquine reduced the total binding by about 60% and reduced the number of specific sites/cell by 20% at 1 μM (−)-propranolol and by 46% (from 2343 to 1262 sites per cell) at 10 μM (−)-propranolol. When the (−)-propranolol concentration was increased from 0.1 to 10 μM, the apparent specific binding (as defined in the previous footnote) increased from 45 to 60% in the presence of chloroquine and from 20 to 70% in its absence. As was the case with HYP binding (Fig. 4), stereospecific binding (as defined in the previous footnote) of DHA was seen only at or below 1 μM propranolol (Fig. 7).

β-Adrenergic receptor levels in normal human leukocytes. A summary of our measurements of high affinity (±)-HYP and (−)-DHA binding sites in the presence of chloroquine on intact MN and PMN cells from seven normal human donors is presented in Table 5. The intersubject variability on the β-receptor number was 7–14%. Based on the data in Fig. 7, the (−)-propranolol concentration used was 1 μM rather than 10 μM. Although at 10 μM (−)-propranolol we measured a larger number of binding sites, at 1 μM propranolol we expected to obtain a closer estimate of the stereospecific binding sites which we believe are more respresentitive of the physiologically relevant β-adrenergic receptors. We did not use 0.1 μM propranolol which yields more pronounced stereospecific binding, since the number of counts actually bound stereospecifically was smaller and more variable and receptor numbers might be underestimated. The observed number of binding sites for DHA and HYP agree closely (Table 5), even though binding of 800 pM (−)-DHA and

Table 5. Binding of (±)-[^{125}I]HYP and (−)-[^{3}H]DHA to intact MN and PMN leukocytes in the presence of chloroquine*

	Sites/Cell	
Ligand	MN cells	PMN cells
[^{3}H]DHA	1331 ± 100 (7)	1155 ± 129 (7)
% variability	7.5	11.2
[^{125}I]HYP	1487 ± 210 (7)	1065 ± 69 (7)
% variability	14.1	6.5

* Cells (10^6) were incubated for 30 min at 37° in 500 μl of KRB buffer, pH 7.4, with 10 μM chloroquine and with either 300 pM (±)-[^{125}I]HYP or 800 pM (−)-[^{3}H]DHA. (−)-Propranolol (1 μM) was used to determine specific-binding. Results are the mean ± S.E.M. of each sample analyzed in quadruplicate. The number of different individuals is given in parentheses. With six individuals the average of two to four separate cell preparations was used and included as one value for each. The percent variability is the S.E.M./mean × 100.

Table 6. Intrasubject daily variation in β-adrenergic receptor levels in MN and PMN cells*

	Sites/Cell				
Date	Donor P. T.		Date	Donor B. L.	
	MN	PMN		MN	PMN
6/15	1285 ± 46†	948 ± 136†	6/18	1219 ± 69†	1855 ± 107†
6/22	1723 ± 77	1190 ± 178	7/1	1304 ± 171	1229 ± 88
6/23	2404 ± 143	855 ± 85	7/2	1820 ± 195	1190 ± 41
6/24	1404 ± 34	965 ± 61	7/8	1236 ± 81	1278 ± 92
7/8	2196 ± 111	898 ± 33			
Mean ± S.E.M.	1802 ± 243‡	971 ± 65‡		1407 ± 155‡	1388 ± 156‡
Day-to-day variability (%)	13.5	6.7		11.1	11.3

* Binding of 800 pM (−)-[^{3}H]DHA to intact cells was carried out as described in Table 5.
† Mean ± S.E.M. of quadruplicate determinations on a given cell sample.
‡ Mean ± S.EM. of the dated values. The percent variability is this S.E.M./mean × 100.

300 pM (±)-HYP are being compared. This aspect will be elaborated in the Discussion. β-Receptor measurements with these same radioligands were also made in the *absence* of chloroquine. These results, only some of which were concurrent with those in Table 3, were as follows: with 300 pM (±)-HYP, 3155 ± 243 sites/MN cell (N = 46) and 4213 ± 437/PMN cell (N = 2), and with 800 pM (−)-DHA 4260 ± 642 sites/MN cell (N = 3) and 2740 ± 190/PMN cell (N = 2).

The day-to-day variability in β-receptor levels measured in intact cells, which is highly pertinent to clinical studies, was assessed over a period of 3 weeks in two healthy volunteers and found to be 7–14% (Table 6), although the range can be as much as 2-fold. Part of this variability was presumably due to technical aspects of the binding assay (see Table 2), while the remainder may reflect *in vivo* modulation or shifts in sub-populations of cells.

DISCUSSION

We have re-examined the measurement of β-adrenergic receptors in intact, normal human leukocytes by parallel binding studies with MN cells (90% or more lymphocytes) and PMN leukocytes (95% granulocytes) employing two commonly used radioligands, enantiomeric (−)-[^{3}H]DHA and racemic (±)-[^{125}I]HYP, together with a fairly detailed analysis of the effects of chloroquine. Use of the latter drug had been proposed to eliminate radioligand uptake into "non-receptor" compartments such as lysosomes [20]. We confirmed that 10 μM chloroquine eliminated a large component of ligand binding to human PMN cells [20]. This chloroquine-suppressible binding was not attributable solely to nonspecific uptake, since it appeared to reflect stereospecific binding sites with low average affinity (K_d 12–15 nM). Furthermore, a comparable chloroquine-induced reduction in low affinity specific binding sites was observed with MN cells (predominantly lymphocytes). Since lymphocytes possess few cytoplasmic granules [52], it seems unlikely that this effect of chloroquine can be ascribed solely to its "lysosomotropic" action. From the current data it is not possible to distinguish whether or to what extent these chloroquine-suppressible, low affinity sites are on the cell surface, are in intracellular organelles, or represent nascent uncoupled intracellular receptors.

In addition to suppressing the large number of low affinity binding sites, the same concentration of chloroquine appeared to induce a moderate reduction in binding sites apparently possessing high affinity for β-adrenergic ligands. That is, at low concentrations of labeled antagonist (800 pM or less) chosen to selectively measure high affinity sites, binding of the radioligand was reduced moderately in the presence of 10 μM chloroquine, without a significant change in K_d (Table 3). Agonist-stimulated cAMP production, however, was not affected. Admittedly, the latter finding was not unexpected in the light of evidence that only a fraction of PMN leukocyte β-receptors need to be operative for effective cyclase stimulation [11]. Again, we do not know the cellular location of these apparently high affinity binding sites that are suppressed by chloroquine. It is of interest that 10 μM chloroquine also reduced the binding of 800 pM DHA to PMN and MN *membrane* preparations by 12–15%, also under conditions of high affinity ligand binding (K_d 150–360 pM; see Table 1). We think it is quite possible that at least some of these chloroquine-suppressible sites are intracellular, e.g. in nascent but not yet exteriorized membrane protein containing adrenergic receptors. It is also conceivable that the hydrophobic nature of chloroquine might allow it to act as a weak competitive inhibitor at the β-receptor site. Such a property could explain chloroquine's potentiation of propranolol inhibition of DHA binding shown in Fig. 7.

A comparison of the binding of racemic (±)-HYP with enantiomeric (−)-DHA shows that, at saturation, (±)-HYP consistently detects a greater number of high affinity specific sites than does (−)-DHA. Bürgisser *et al.* [50] have presented evidence that the traditional assumption that the binding of the (+) isomer of a racemic ligand such as (±)-HYP is negligible [53, 54] is incorrect and can lead to overestimation of the number of the physiologically relevant receptors. Based on this newer work [50], we believe we can assume with some assurance that the $K_{d(-)}$ for (−)-HYP in our systems is significantly lower (i.e. 10–50 pM) than the observed average $K_{d(\pm)}$ of 249 pM (mean of six values from MN and PMN cells, Table 3). Thus, our choice of 300 pM (±)-HYP for measurement of β-adrenergic receptor levels in intact cells from normal humans (Table 5) should detect all the high affinity sites with minimum risk of overestimating sites by Scatchard analysis. The latter method requires data based on (±)-HYP concentrations at and above 1000 pM wherein the contribution of the (+)-HYP to the binding can be significant. Indeed, 300 pM (±)-HYP gave values for β-adrenergic receptor numbers in close agreement with those obtained with saturating levels of 800 pM (−)-DHA. We believe that β-receptor measurement by a pure enantiomorph such as (−)-DHA is generally preferable.

The question whether intact cells or membrane preparations represent the best system to measure α- and β-adrenergic receptors is complex and obviously varies with the objective of the particular study. In the process of isolating membranes, it may not be possible to prevent some alteration of the plasma membrane or its receptors. On the other hand, the fact that membranes can be stored frozen and studied at the convenience of the investigator is an asset that has contributed to their widespread use. Moreover, for investigation of molecular mechanisms of ligand:receptor interactions, the use of *purified* receptors would be ideally suited. For surveys of clinical diseases, however, we prefer the use of intact cells because ligand binding is not only reproducible (see Results) but can be correlated in the same cell sample both with cAMP production and with metabolic events controlled by cyclic nucleotides. This would potentially permit detection of abnormalities "distal" to the receptor under comparable experimental conditions. Intact leukocytes, however, are subject to clumping and also could theoretically take up ligand into non-receptor compartments more readily than membranes. For that reason, at our present state of knowledge, we

Table 7. Summary of literature values for (±)-[^{125}I]HYP binding to human leukocytes

Authors	Ref. No.	System	Ligand concn (nM)	K_d (nM)	Ligand binding (fmoles/mg protein)	Sites/Cell
Aarons *et al.*	37	MN memb.	0.01–0.23		15.3	
Soman *et al.*	17	MN homog.	2.5–50		810–890	
Sano *et al.*	6	MN memb.				1188§
Sano *et al.*	6	PMN memb.				1716§
Ruoho *et al.*	22	PMN homog.	0.03–0.6	0.16	14	1200–1600*
Marinetti *et al.*	†	MN memb.	0.3	0.15	18.6	
Krawietz *et al.*‡	14	MN cells (normal)	0.05–50	0.14–0.18		255–575
Pochet *et al.*	39	F1-T cells	0.1–0.4			0
Pochet *et al.*	39	F2-T cells	0.1–0.4	0.25		650 ± 100
Pochet *et al.*	39	B-cells	0.1–0.4			600 ± 100
Pochet *et al.*	39	MN cells	0.1–0.4			500 ± 100
Marinetti *et al.*	§	MN cells	0.3	0.224 ± 0.058		1487 ± 210
Marinetti *et al.*	§	PMN cells	0.3	0.274 ± 0.046		1065 ± 69

* Represent extrapolated values which may be subject to considerable error.
† Data from this paper (Tables 1 and 2) in the absence of chloroquine.
‡ The ligand used was [^{125}I]CYP.
§ Data from this paper (Tables 3 and 5) in the presence of 10 μM chloroquine.

favor the use of chloroquine with intact leukocytes although we cannot defend this idea rigorously without more information on the location and nature of the relatively high affinity sites that chloroquine appears to eliminate. Incidentally, no significant degradation of DHA or HYP (by thin-layer chromatography) has been observed with intact leukocytes under our experimental conditions.

A final point on this topic relates to the fact that binding of radioligands to membranes is properly expressed as fmoles/mg membrane protein. (Extrapolation to sites/cell involves questionable

Table 8. Summary of literature values for (−)-[^{3}H]DHA binding to human leukocytes

Authors	Ref. No.	System	Ligand concn (nM)	K_d (nM)	Ligand binding (fmoles/mg protein)	Sites/Cell*
Kariman	13	MN memb.	12		76.9 ± 6.8	
Brooks *et al.*	5	MN memb.	12		263 ± 391	
Gullner *et al.*	7	MN memb.	5		236 ± 56	
Schocken and Roth	23	MN memb.	1–100	20	150–630	
Lee	8	MN memb.	100		100–160	
Galant *et al.*	9	PMN memb.	30		25.1 ± 1.8	863 ± 60
Tohmeh and Cryer	16	MN homog.	0.1–10	0.1–2.0	25–60	810–1,710
Davies and Lefkowitz	18	MN memb.	0.3–3	0.57	66 ± 2.4	
Davies and Lefkowitz	18	PMN memb.	0.3–3	0.58	48 ± 2.3	
Williams *et al.*	3	MN memb.	10		75 ± 12	2,000
Bishopric *et al.*	21	B-cell memb.	1–10	1.4	51.8 ± 10	
Bishopric *et al.*	21	T-cell memb.	1–10	1.5	53.8 ± 9.2	
Szentivanyi *et al.*	19	MN memb.	4		48–79	
Ruoho *et al.*	22	PMN homog.	0.2–1.0	2.0		1,200–1,600
Ruoho *et al.*	22	PMN homog.	10–100	10		15,000–20,000
Sheppard *et al.*	12	MN memb.	50		682 ± 114	
Galant *et al.*	10	PMN memb.	1–5	1.1	24.6 ± 3.6	818 ± 129
Marinetti *et al.*	†	MN memb.	1	0.3–0.5	11.5	
Tashkin *et al.*	25	MN cells	0–3.5	1.5		1,035 ± 452
Krall *et al.*	4	MN cells	0.1–7	1.5		2,000
Dulis and Wilson	20‡	PMN cells	0.36–5.1			10,000–60,000
Dulis and Wilson	20§	PMN cells	0.36–5.1	0.41		1,770 ± 270
Galant and Allred	11	PMN cells	0.1–10	0.66		864 ± 114
Marinetti *et al.*	‖	MN cells	0.8	0.143 ± 0.038		1,331 ± 100
Marinetti *et al.*	‖	PMN cells	0.8	0.153 ± 0.046		1,155 ± 129

* Values listed here as sites per cell using membrane preparations are extrapolated estimates which may be subject to considerable error.
† Data from this paper (Tables 1 and 2) in the absence of chloroquine.
‡ No chloroquine.
§ A 50 μM concentration of chloroquine.
‖ Data from this paper (Tables 3 and 5) in the presence of 10 μM chloroquine.

assumptions.) Accurate estimation of protein in a membrane preparation is therefore critical. We have found that the standard methods for separation of human leukocytes [41] regularly leaves a substantial contamination of platelets in the mononuclear cell fraction (4–16 platelets/MN cell; 1–1.5 platelets/PMN cell).* Although contaminating platelets would not contribute β-receptors of their own, their protein content could introduce significant errors and variability in protein measurements on mononuclear (lymphocyte) membrane preparations. In contrast, platelet contamination can readily be assessed (or excluded) by electronic or manual counting in intact leukocyte preparations, and the results can be expressed directly as sites/cell. Based on routine electronic platelet counting in our laboratory, platelet contamination does not demonstrably interfere with estimation of β-receptors in intact MN cells except in persons with extemely high platelet counts (e.g. post-splenectomy).

In order to compare our results with HYP and DHA binding to human leukocytes with those from other laboratories, we have tabulated our data and data from the literature (Tables 7 and 8). It is evident that differing experimental conditions have led to large differences in the measured number of adrenergic receptors in human MN and PMN cells. The number of sites/cell using (±)-HYP varies from 255 to 1716 (Table 7). Some of these values were extrapolated by the authors from membrane preparations. Reported values for HYP binding to MN cell membranes varies from 15 to 890 fmoles/mg protein. The average K_d values for (±)-HYP binding are in reasonable agreement and vary from 140 to 274 pM. A greater variability has been recorded for DHA binding (Table 8). With MN membranes (lymphocyte membranes), DHA binding varies from 25 to 682 fmoles/mg protein. By extrapolation this would denote a spread of 175 to 200,000 sites/cell. The high values were obtained when very high concentrations of DHA were used and undoubtedly include the low affinity sites described above (Results). Accordingly, the K_d values for DHA binding vary from 100 to 20,000 pM. It was against this background that we undertook our systematic re-evaluation of β-receptor estimation in human leukocytes.

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