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EFFECTS OF CELL ISOLATION PROCEDURES AND RADIOLIGAND SELECTION ON THE CHARACTERIZATION OF HUMAN LEUKOCYTE β-ADRENERGIC RECEPTORS*

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Abstract—Radioligand binding techniques are commonly used in the characterization of β-adrenergic receptors on human peripheral leukocytes. Accurate interpretation of receptor binding parameters necessitates appropriate radioligand selection. In addition, cell isolation techniques should have minimal effect on the binding parameters of receptors. Our observation of curvilinear Scatchard plots with (−)-[^{125}I]iodocyanopindolol (ICYP) resulted in a re-evaluation of this radioligand and the influence of cell isolation techniques on leukocyte β-adrenergic receptor binding parameters. Membranes from mononuclear (MN) and polymorphonuclear (PMN) cells isolated by a standard procedure (Ficoll–Hypaque) resulted in biphasic Scatchard plots with ICYP in three of four subjects. In contrast, linear Scatchard plots were observed for ICYP binding to membranes from MN and PMN cells isolated from the same four subjects with an alternative procedure utilizing plasma Percoll. Competition and saturation binding assays with ICYP identified a high degree of nonspecific binding. Decreased stereoselectivity with (−)- and (+)-propranolol was observed with membranes from Ficoll–Hypaque cells as compared to plasma Percoll cells. Kinetic analysis with ICYP demonstrated apparent irreversible binding whether displacement was initiated with a β-adrenergic receptor antagonist or agonist. These problems with ICYP prompted evaluation of an alternative radioligand, (−)-[^{125}I]iodopindolol (IPIN); this radioligand demonstrated rapid and completely reversible binding, improved stereoselectivity, and low nonspecific binding. Using IPIN, Scatchard plots from three additional subjects were linear for both cell isolation procedures. Based on these observations, the preferred method of human leukocyte β-adrenergic receptor analysis incorporates the plasma Percoll cell isolation technique and the radioligand IPIN.

The β-adrenergic receptor is an essential transducer between specific chemical stimuli and physiologic responses. Several clinical conditions, for example, asthma and hypertension, may manifest changes in β-adrenergic receptor density and affinity due to either the nature of the disease process or the treatment rendered. Peripheral blood leukocytes, which possess measurable β-adrenergic receptors, have been utilized to investigate alterations in receptor presentation in relation to disease and drug therapy [1–16].

Recently, Marinetti *et al.* [17] commented on the wide variability in reported leukocyte β-adrenergic receptor density and binding affinity from control subjects. This variability was attributed to differences in experimental techniques, such as the use of intact cells versus membrane preparations, a variety of radioligands, and the analysis of curvilinear Scatchard plots. It was suggested that the biphasic binding plot was secondary to ligand uptake into non-receptor cell compartments such as lysosomes. As a result, chloroquine was recommended as a potential inhibitor of lysosomal uptake for intact cells [17, 18]. Our own observations regarding biphasic Scatchard plots in membrane preparations prompted an investigation into the etiology of this potential artifact in receptor analysis.

A basic assumption underlying the application of leukocyte receptor analysis is that receptor binding parameters are not affected by the preparative procedures essential to cell isolation. Our recent experience with the effect of isolation techniques on polymorphonuclear (PMN‖) cell function raised concern regarding potential alterations in β-adrenergic receptor binding parameters [19]. In the present investigation, we analyzed the effects of two cell isolation techniques on leukocyte β-adrenergic receptors. The first procedure incorporated Ficoll–Hypaque for cell separation similar to the standard methodology for leukocyte isolation [20]. The alternative procedure utilized a plasma Percoll technique which decreases

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‖ Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ICYP, (−)-[^{125}I]iodocyanopindolol; IPIN, (−)-[^{125}I]iodopindolol; MN, mononuclear; and PMN, polymorphonuclear.

exposure to bacterial lipopolysaccharide and apparently minimizes disturbances of cell function [19]. In addition, observations of a high percent nonspecific binding and biphasic Scatchard plots with the β-adrenergic receptor antagonist (−)-[^{125}]iodocyanopindolol (ICYP) prompted evaluation of an alternative radioligand, (−)-[^{125}I]iodopindolol (IPIN). Both have advantages of high specific activity and β-adrenergic receptor selectivity, beneficial in reducing the tissue requirement for adequate receptor characterization.

MATERIALS AND METHODS

Cell isolation

Ficoll–Hypaque procedure. To minimize platelet contamination and to isolate individually both PMN and MN leukocytes, the beneficial features of two cell isolation techniques were combined [20–22].

Whole blood was drawn, anticoagulated with 3.8% citrate (Fisher Scientific Co., Pittsburgh, PA) using a sterile non-pyrogenic 19-gauge Butterfly (Abbott Hospital Products; North Chicago, IL) and 50-ml Plastipak disposable polyethylene syringes (Becton Dickinson, Rutherford, NJ), and centrifuged at 300 *g* for 20 min at room temperature (RT). After removing the platelet-rich plasma, the lower layer was resuspended in 0.6% dextran (M_r 500,000, Pharmacia Fine Chemicals, Piscataway, NJ) with sterile saline and allowed to stand for 30 min at RT. The supernatant fraction which contained leukocytes was separated and centrifuged at 300 *g* for 10 min at RT. The resultant pellet was then resuspended in phosphate-buffered saline (pH 7.4), layered over prepared 10.2% Hypaque solution (density 1.060 g/ml, Winthrop Laboratories, Sterling Drug Inc., New York, NY), and centrifuged for 10 min at 250 *g* at RT.

The supernatant fraction was removed, and the Hypaque-containing pellet was resuspended in 4 ml of phosphate-buffered saline (pH 7.4). Aliquots of the cell suspension were underlayered with Ficoll–Paque (density 1.077 g/ml, Pharmacia) and centrifuged at 750 *g* for 25 min at RT. The interface layer containing MN leukocytes was washed twice with 20 mM HEPES/154 mM NaCl (HEPES-isosaline), pH 7.3, at 4°. The pellet containing PMN leukocytes was reconstituted with 0.2% NaCl for less than 3 min to lyse erythrocytes; then 1.6% NaCl was added to restore to 0.9% NaCl. The PMN leukocyte suspension was centrifuged and washed twice with 20 mM HEPES-isosaline. The yield from a 200-ml blood sample was approximately 3.5×10^8 total PMN leukocytes and 2.0×10^8 total MN leukocytes. Viability with this preparation was greater than 95% as demonstrated by trypan blue exclusion.

Plasma Percoll procedure. This method is a modification of a procedure described by Danpure *et al.* [23] with slightly different densities and a two-step discontinuous gradient to pellet erythrocytes at the base of the tube [19]. A significant advantage of this procedure is that it minimizes exposure of neutrophils to lipopolysaccharide (LPS) during the cell isolation, since LPS may activate PMN and macrophage cells [24, 25]. All reagents and serum used in this method were negative for endotoxin as determined by the limulus amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO) with a sensitivity level of 0.1 ng/ml endotoxin.

Similar to the previous procedure, blood was drawn, mixed with 3.8% citrate, and centrifuged at 300 *g* for 20 min at RT. The platelet-rich plasma layer was carefully aspirated and centrifuged at 2500 *g* for 15 min at 25° to produce platelet-poor plasma (PPP). Five milliliters of 6% dextran (M_r 500,000, Pharmacia) was added to the remaining contents of each tube, and the volume was made up to 50 ml with 0.9% saline, mixed gently and thoroughly, and allowed to stand for 30 min for erythrocyte sedimentation to occur.

The leukocyte-rich plasma was aspirated and centrifuged at 275 *g* for 6 min, and the pellet was resuspended in 2–3 ml PPP and transferred to a 15-ml polystyrene tube where it was underlayered with 2 ml of freshly prepared 42% Percoll in PPP, which was, in turn, underlayered with 2 ml of freshly prepared 51% Percoll in PPP using a siliconized Pasteur pipette (sterilized by baking at 240° for 4 hr). The Percoll solutions were prepared from a stock solution of Percoll (100% fine grade, Pharmacia) diluted in 0.9% saline (Abbott Hospital Products, North Chicago, IL) in a ratio of 9:1 (v/v) Percoll–saline.

The gradients were centrifuged for 10 min at 275 *g*. MN cells and some platelets remained at the top interface between plasma and the 42% Percoll layer, with PMNs in a wider band at the interface of the 42% and 51% Percoll layers and extended into the 51% Percoll layer to a few millimeters above the erythrocyte pellet. Platelets were removed from the MN cells either by a further 5 min 275 *g* centrifugation step through 25% Percoll in PPP or by adding a third 25% Percoll in the PPP step to the original gradient. Each band was aspirated using a polyethylene transfer pipette (Fisher Scientific Co., St. Louis, MO).

The neutrophils from each gradient were washed once in PPP and once in Krebs–Ringer phosphate buffer, pH 7.23, with 0.2% dextrose (KRPD) prepared with dextrose in 0.9% sodium chloride solution. The efficiency of neutrophil recovery from the gradient was >80%, and the neutrophil band was >95% pure with 1–2% erythrocytes, 3–5% eosinophils and <0.5% mononuclear cells. The yield from this isolation procedure was comparable to that of the Ficoll–Hypaque procedure. There were no platelets visible in the neutrophil preparation. MN cell purity was greater than 90% with minimal platelet contamination. Cell viability was greater than 99% by the trypan blue exclusion technique. The MN leukocyte fraction for thirty separate trials consisted of 60 ± 9% (SD) lymphocytes, 33 ± 8% monocytes, and 7 ± 4% PMNs.

Membrane preparation

With each cell preparation, the resultant PMN and MN leukocyte fractions were counted with a hemocytometer, divided into aliquots containing 50×10^6 cells, and stored frozen in pellet form at −70°. Frozen cell pellets were thawed in an ice bath, resuspended in 10 ml of cold 20 mM HEPES-isosaline and homogenized with a Brinkmann Polytron for 10 sec. The resultant homogenate was centri-

fuged at 39,000 *g* for 30 min at 4°. The pellet was then resuspended with an appropriate volume of 20 mM HEPES-isosaline to yield the desired concentration of homogenized cell preparation (1×10^6 cells/100 μl). The protein yield in these preparations averaged 1 mg/3×10^7 cells. All experiments were performed within 3 days after cell isolation.

Binding studies

Radioligand binding studies were performed with (−)-[^{125}I]iodocyanopindolol (ICYP) (sp. act 2200 Ci/mmol) and (−)-[^{125}I]iodopindolol (IPIN) (sp. act 2200 Ci/mmol) purchased from the New England Nuclear Corp., Boston, MA. Binding assays were routinely conducted in new disposable polypropylene tubes (Walter Sarstedt Co., No. 55.538, Princeton, NJ). The total assay volume was 250 μl. Assays contained 50 μl ICYP or IPIN in 1 mM HCl, 50 μl of 1 mM HCl or drugs in 1 mM HCl, 50 μl of 1 mM HCl or 1.5 mM GTP (final concentration 0.3 mM) in 1 mM HCl and 100 μl membrane suspension in 20 mM HEPES-isosaline (pH 7.5). Aliquots of the membrane suspension representing 1×10^6 cells (MN or PMN leukocytes) were incubated with ICYP or IPIN for 120 or 30 min, respectively, at 37°. Specific binding was defined as the difference in radioligand bound in the absence and presence of 1 μM (−)-propranolol. Incubations were terminated by adding 5 ml of cold wash buffer (10 mM Tris–HCl/154 mM NaCl, pH 7.5) followed by rapid vacuum filtration, through glass fiber filters (Schleicher & Schuell No. 30, Keene, NH). The filters were rinsed once again with 3×5 ml cold buffer washes and counted in a Beckman Biogamma II counter.

Kinetics. The total period of evaluation for association extended to 6 hr for ICYP and 2 hr for IPIN, at 37°. After a suitable time to reach equilibrium (120 min for ICYP and 30 min for IPIN), the dissociation time course was initiated by adding (1 μM (−)-propranolol or 10 μM (−)-isoproterenol. At designated times after the addition of the competitive ligand, individual incubations were terminated as previously described. The kinetic association (k_1) and dissociation (k_{-1}) constants were determined as described by Weiland and Molinoff [26].

Competition binding assays. To determine the potency of radioligand binding by various competing ligands, IPIN or ICYP was incubated at 37° with various concentrations (10^{-11} to 10^{-3} M) of the competing agent. The Hill coefficient, n_H, and the concentration of inhibitor which results in 50% inhibition of specific binding, IC_{50}, were derived for each plot. The inhibition constants, K_i, were determined from the IC_{50} value corrected by the method of Cheng and Prusoff [27].

To examine the effect of cell isolation on stereoselectivity, whole blood was obtained from three separate donors. Each sample was divided into equal portions, and cells were isolated by the plasma Percoll and Ficoll–Hypaque procedures. MN and PMN cells were incubated with ICYP (50 or 200 pM) or IPIN (140 pM) in the presence of (−)- or (+)-propranolol.

To characterize the β-adrenergic receptor subtype, competition assays were conducted on MN and PMN cell homogenates prepared with the plasma Percoll procedure. Membrane preparations were incubated with 100 pM IPIN with: (−)- or (+)-isoproterenol, (−)-epinephrine, (−)-norepinephrine, ICI 118551 (β_2-receptor antagonist) or ICI 89406 (β_1-receptor antagonist) in the presence of 0.3 mM GTP.

Saturation binding assays. Whole blood was obtained from seven separate donors. Each sample was divided into equal parts, and cells were isolated simultaneously by the plasma Percoll and Ficoll–Hypaque procedures. MN and PMN cell membranes were utilized individually for measurements of receptor density (B_{max}) and affinity (K_d) with ICYP or IPIN. Measurements with ICYP included four donors and IPIN three other donors. Aliquots of cell membrane homogenates were incubated with various concentrations of ICYP (1–700 pM) or IPIN (10–700 pM) at 37°.

For data analysis, the weighted, nonlinear, least-squares, curve-fitting program of Munson and Rodbard [28] was used. This program, LIGAND, is based on a general model for complex ligand–receptor interactions [29]. Saturation curves were analyzed based on the model for mass action binding of the radioligand and competing drugs to one or more binding sites. This program derived the affinities and the number of binding sites from the saturation binding data. Receptor density was normalized per mg protein. Protein was measured by the method of Bradford [30] using bovine serum albumin as the standard. Deviations of the observed data points from the theoretically predicted values were weighted according to the reciprocal of the variance. The statistical significance of the fits generated by the different models was assessed using an F test analysis of the residual variances of the fits of the data.

Drugs

(−)- and (+)-Propranolol were obtained from Ayerst Laboratories, Inc., New York, NY; (−)- and (+)-isoproterenol from the Sigma Chemical Co.; (−)-epinephrine and (−)-norepinephrine from Sterling–Winthrop Research Institute, Rensellaer, NY; and ICI 118551 and ICI 89406 from Imperial Chemical Industries, PLC, Macclesfield, Cheshire, England.

RESULTS

Association and dissociation binding assays

Incubation of MN cell membranes with ICYP and IPIN demonstrated rapid onset of binding (Fig. 1). The time to reach steady state was slowest for ICYP binding to PMN leukocyte membranes. A concentration of 12 pM ICYP reached steady state at approximately 60 min (data not shown); therefore, 120 min of incubation time was chosen for further studies for ICYP. The IPIN steady state was attained by 20 min for both cell types (Fig. 1B). Therefore, 30 min was selected as the time period for incubation.

Upon addition of 1 μM (−)-propranolol to MN cell membranes incubated with ICYP, minimal displacement was observed (Fig. 1A). Similar results were observed for PMN cell membranes. With essen-

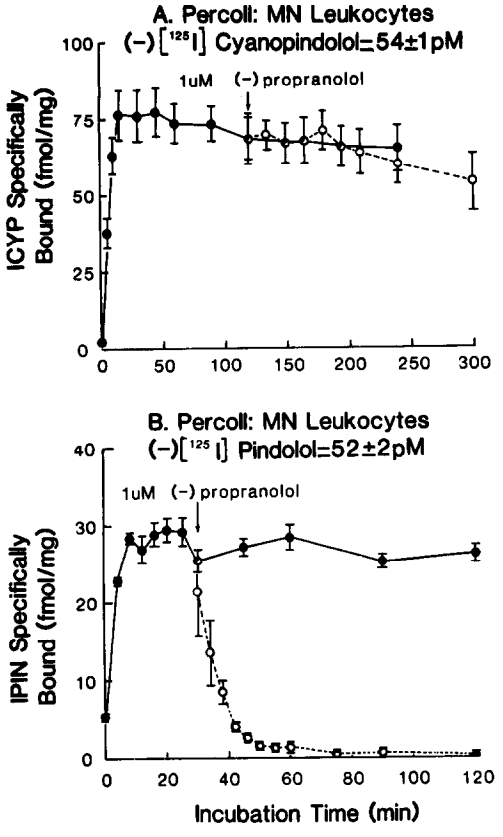

Fig. 1. Time course of (−)-[^{125}I]iodocyanopindolol (A) and (−)-[^{125}I]iodopindolol (B) association with (closed symbols) and dissociation from (open symbols) β-adrenergic receptors on mononuclear leukocyte membranes prepared with the plasma Percoll technique. Dissociation was measured following the addition of 1 μM (−)-propranolol as indicated by the arrow. Binding experiments were conducted at 37°. Values shown are mean ± SEM for three determinations.

tially no dissociation, it was impossible to derive kinetic K_d values for ICYP binding to MN and PMN cell membranes. A similar low degree of displacement occurred in another set of MN and PMN cell membranes when agonist [10 μM(−)-isoproterenol] rather than antagonist was added to induce dissociation (data not shown). In contrast to the kinetic data with ICYP, for MN and PMN membranes incubated with IPIN, binding was rapidly and completely displaced with the addition of 1 μM (−)-propranolol (Fig. 1B). The dissociation data plotted as a semi-log plot was linear. The kinetic K_d values calculated from these data for both cell types were 20–85 pM. A similar pattern of rapid and completely reversible displacement was observed after adding 10 μM (−)-isoproterenol to MN and PMN cell membranes incubated with IPIN for 30 min (data not presented).

Competition binding assays to evaluate stereoselective binding

Incubations with (−)- or (+)-propranolol and PMN or MN leukocyte membranes were conducted with two different concentrations of ICYP, 47 pM or 216 pM. These concentrations were within the range of concentrations used in saturation binding assays. At 47 pM ICYP, the stereoselectivity ratio as determined by the ratio of affinities of the two isomers of propranolol ($K_i(+)/K_i(-)$) was 67 for MN cells separated by the Ficoll–Hypaque method and 110 for the plasma Percoll technique (Fig. 2, A and B). At higher concentrations of ICYP (216 pM), however, it was much more difficult to determine what constituted nonspecific binding. Using 1 μM (−)-propranolol to define specific binding, with the Ficoll–Hypaque isolated MN cells, the propranolol stereoisomer affinity ratio with the higher concentration of ICYP decreased to 7 (Fig. 2C). For the plasma Percoll separation, the ratio was 320 with the apparent nonspecific binding being 60% of the total in the presence of 1 μM (−)-propranolol (Fig. 2D). Therefore, a significant difference in the degree of stereoselectivity was observed with the two cell isolation techniques, using a high concentration of ICYP and membranes isolated from MN cells.

For MN cell membranes incubated with a concentration of IPIN slightly higher than its K_d value (142 pM), the stereoselectivity ratio was high (350–600) regardless of the cell isolation technique used (Fig. 2E and 2F). Specific IPIN binding was approximately 75% of the total in each case using 1 μM (−)propranolol as the definition of specific binding.

Similar results were obtained for PMN cell membranes; however, the propranolol stereoselectivity ratio was significantly lower for both cell isolation techniques compared to MN cells at the respective ICYP and IPIN concentrations. The mean propranolol stereoselectivity ratio for three determinations using IPIN as the radioligand was 74 for Ficoll–Hypaque isolated cell membranes, and 56 for plasma Percoll cell membranes.

Saturation binding assays

Incubation of MN or PMN cell membranes with various concentrations of ICYP and IPIN in the presence and absence of 1 μM (−)-propranolol identified different saturation isotherms for each radioligand, dependent on the cell isolation procedures. MN cells isolated by the Ficoll–Hypaque technique revealed biphasic Scatchard plots with ICYP as the radioligand for three of the four subjects evaluated (Fig. 3A and Table 1). For MN cells isolated simultaneously by the plasma Percoll procedure, the ICYP binding curve was linear for all four subjects (Fig. 3B and Table 1).

Specific binding reached a plateau for plasma Percoll isolated MN cell membranes at ICYP concentrations above 80 pM (inset, Fig. 3B). In comparison, specific ICYP binding to membranes from MN cells isolated with Ficoll–Hypaque increased with concentrations exceeding 100 pM ICYP. With both cell isolation procedures nonspecific binding increased linearly over the entire range of radioligand concentrations used. The percent nonspecific binding exceeded specific binding with incubation ICYP concentrations above 80 pM for Ficoll–Hypaque cells and 180 pM for plasma Percoll MN cells (inset, Fig. 3A and 3B).

Using IPIN, the binding parameters were similar for cells isolated by both techniques (Fig. 3C and

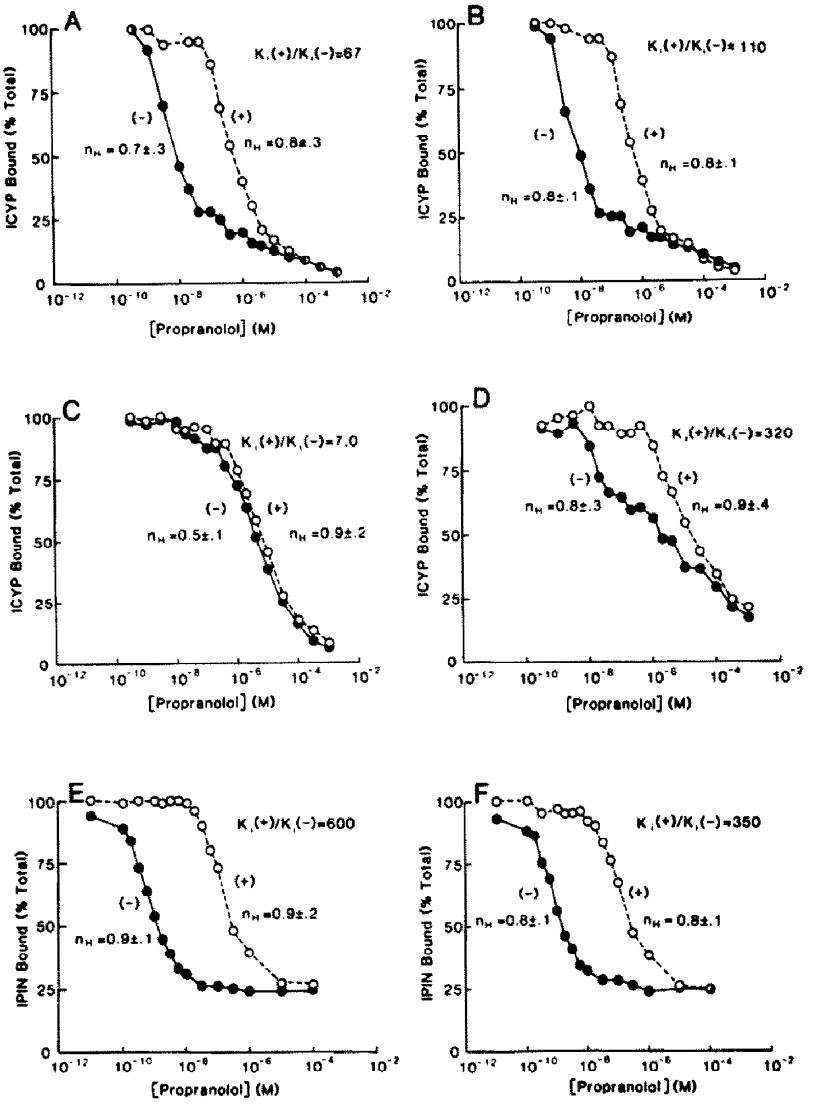

Fig. 2. Comparison of the inhibition of (−)-[^{125}I]ICYP (A–D) and (−)-[^{125}I]IPIN (E and F) binding, at 37°, by the isomers of propranolol to β-adrenergic receptors on MN cell membranes prepared with either the Ficoll–Hypaque (A,C,E) or the plasma Percoll (B,D,F) technique. Inhibition was measured by radioligand displacement in the presence of (−)- and (+)-propranolol (closed and open circles, respectively) and are shown as log dose–response curves. Concentrations of radioligand used were 47 pM ICYP (A,B), 216 pM ICYP (C,D) and 142 pM IPIN (E,F). Values represent the means for three subjects providing leukocytes for both cell isolation techniques. $K_i(+)/K_i(-)$ represents the ratio of the binding constants for the propranolol stereoisomers and n_H is the Hill coefficient.

3D). Nonspecific binding remained less than 50% of the total in the critical range of the saturation binding curves. Only one of three trials (Subject 5, Table 1) demonstrated a difference in receptor density for the Ficoll–Hypaque isolated cells as compared to plasma Percoll MN cells.

Similar results were obtained with PMN cells isolated by the two techniques. Ficoll–Hypaque PMN cell Scatchard plots with ICYP were biphasic in three of four trials (Table 1), as compared to the linear ICYP binding curve for plasma Percoll cells. For plasma Percoll PMN cells, ICYP specific binding reached a plateau with concentrations exceeding 100 pM. For Ficoll–Hypaque PMN cells, a specific binding plateau was not identifiable until concentrations were greater than 250 pM. The percent ICYP nonspecific binding exceeded specific binding with concentrations greater than 100 pM for Ficoll–Hypaque PMN cells and 150 pM for plasma Percoll cells.

For PMN cells incubated with IPIN, the saturation binding curves were similar for both cell isolation methods (Table 1). Specific binding exceeded 70% of the total for all concentrations within the saturation binding curve.

β-Adrenergic receptor subtype analysis

Competition binding assays were performed to

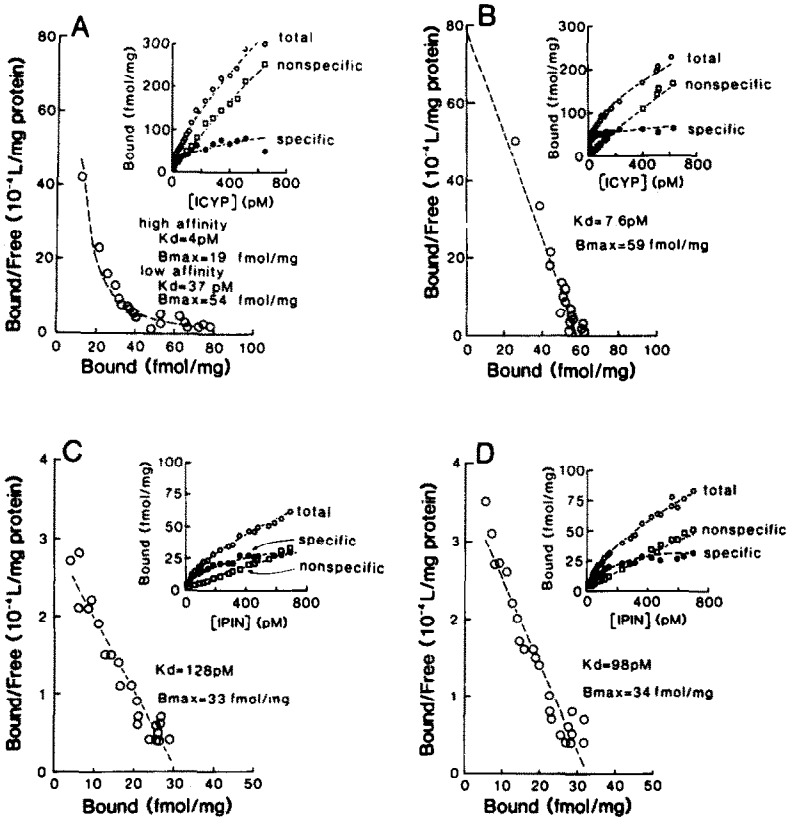

Fig. 3. Saturation binding curves for β-adrenergic receptors measured with (−)-[^{125}I]ICYP (A,B) and (−)-[^{125}I]IPIN (C,D), at 37°, on MN cell membranes. Data shown are representative from a blood sample from a single donor that was divided, and MN cells were isolated by the Ficoll–Hypaque (A,C) and plasma Percoll (B,D) techniques. Separate subjects were utilized to evaluate each radioligand. Nonspecific binding was measured in the presence of 1 μM (−)propranolol. Saturation binding data (inset) is shown transformed as a Scatchard plot. Binding parameters shown were derived from untransformed data using the LIGAND program [28].

confirm previous observations of leukocyte β-adrenergic receptors as β_2 in binding characteristics. Consistent with a β_2-receptor subtype, incubation of MN leukocyte membranes obtained with the plasma Percoll method revealed the following order of potency (K_i value in μM): (−)-isoproterenol, 0.15; (−)-epinephrine, 1.5; and (−)-norepinephrine, 13. The specific β_2-receptor antagonist ICI 181551 was approximately 100-fold more potent than the specific β_1-receptor antagonist ICI 89406 (Fig. 4). Similar rank order results were obtained for membranes from the plasma Percoll isolated PMN cells (data not shown).

DISCUSSION

The presence of β_2-adrenergic receptors on MN and PMN leukocytes provides an opportunity to evaluate receptor changes as a result of *in vivo* or *in vitro* experimental conditions. It is often assumed that measurements of leukocyte β-adrenergic receptors reflect systemic receptor binding parameters; however, this has been demonstrated only in a single study [31]. A basic assumption is that leukocyte β-adrenergic receptor binding is minimally affected by cell preparative procedures. In addition, use of leukocytes for *in vitro* assessment of conditions affecting β-adrenergic receptor function assumes that the cells are in a resting state. Information obtained from this investigation, suggests that the cell isolation procedure and the radioligand may influence significantly the experimental observations.

Beneficial characteristics of a radioligand include its selectivity for the designated receptor. Previous investigations identified the leukocyte β-adrenergic receptor as a β_2 subtype [1, 2] with no evidence of coexisting β_1 and β_2 receptors on these cells, and our results agree with this conclusion (Fig. 4). While it would be important to select a radioligand that specifically identifies the subtype of β-adrenergic receptor being studied, no radioligands selective for β_2 receptors are yet available. Additional desirable properties include high specific activity and relatively high affinity to minimize tissue requirement, simple kinetics and a minimal degree of nonspecific binding.

ICYP has been proposed as a useful radioligand for the measurement of lymphocyte β-adrenergic receptors [32]. Using this radioligand, however, saturation binding data with cell membranes transformed as Scatchard plots were biphasic (Fig. 3A), similar

Table 1. Summary of leukocyte β-adrenergic receptor binding parameters in relation to radioligand and cell isolation technique

A. (−)-[^{125}I]Iodocyanopindolol

	Mononuclear cells				Polymorphonuclear leukocytes			
	Ficoll–Hypaque		Plasma Percoll		Ficoll–Hypaque		Plasma Percoll	
Subject	K_d*	B_{max}†	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
1	5(48)‡	50(70)	1.3	44	7.3(33)	20(50)	35	53
2	3.7(37)	19(54)	7.6	59	28	77	34	71
3	1.6(34)	15(50)	6.3	63	9.9(135)	21(66)	68	64
4	22	155	5.0	120	9.4(18)	61(215)	33	89

B. (−)-[^{125}I]Iodopindolol

	Mononuclear cells				Polymorphonuclear leukocytes			
	Ficoll–Hypaque		Plasma Percoll		Ficoll–Hypaque		Plasma Percoll	
Subject	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
5	115	52	185	140	150	41	140	47
6	130	33	98	34	250	21	140	37
7	57	115	41	110	67	31	67	27

* K_d, pM.
† B_{max}, fmol/mg protein.
‡ Parentheses indicate low-affinity site for analysis indicating two-site binding; single value indicates one site fit for binding.

to those presented by Marinetti *et al.* [17], with intact cells. These investigators recommended chloroquine as a method to resolve this problem. Linear plots did not occur with the addition of chloroquine in our investigations (unpublished observations). Competition binding assays with propranolol showed poor stereoselectivity and a high degree of nonspecific binding as the ICYP concentration was increased to the higher limits of concentrations incorporated in saturation binding assays (Fig. 2C). At these high ICYP concentrations, it was difficult to define nonspecific binding accurately; however, nonspecific binding remained linear throughout the concentration range included. This high degree of nonspecific binding was evident in saturation binding analysis (inset, Fig. 3A), particularly at ICYP concentrations exceeding 100 pM. To adequately characterize leukocyte β-adrenergic receptor binding density, it was considered essential to include individual concentrations in the range of 1–700 pM.

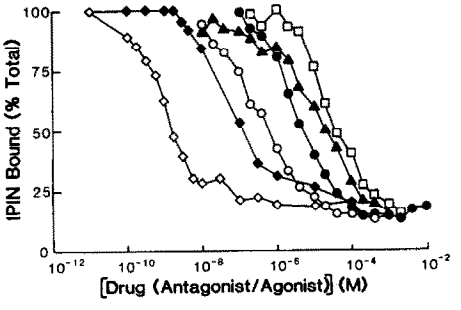

Fig. 4. Inhibition of (−)-[^{125}I]IPIN binding to MN cell membranes isolated with plasma Percoll gradients by agonists and antagonists. Membranes were incubated with IPIN (100 pM), at 37°, and various concentrations of ICI 118551 (◇), a β_2 antagonist; ICI 89406 (◆), a β_1 antagonist; (−)-isoproterenol (○); (+)-isoproterenol (●); (−)-epinephrine (▲) and (−)-norepinephrine (□) in the presence of 0.3 mM GTP. Data shown are either from a single experiment or are the mean values for three determinations.

Biphasic patterns of saturation binding assays may not be detected with an inadequate range or number of concentrations. Data provided in the original reference [32], although linear, included only six points within a limited range of the saturation binding curve. Our experiments, including eighteen or more ICYP concentrations, incorporate a wider range. The analysis of curvilinear binding data necessitates the use of nonlinear computer-fitting programs, such as that provided by Munson and Rodbard [28], to derive accurate binding parameters. A two-site analysis was usually more appropriate for describing binding parameters for β-adrenergic receptors for leukocytes isolated via the Ficoll–Hypaque procedure (Table 1).

The two binding sites on Ficoll–Hypaque-isolated MN and PMN cell membranes were characterized as a high-affinity state with an approximate K_d of 6 pM and a B_{max} of 30 fmol/mg protein, and a lower affinity site with a K_d of 50 pM and a B_{max} of 85 fmol/mg protein. Complex binding of an antagonist to β-adrenergic receptors may be related to the presence of poorly defined nonspecific binding, the influence of negative cooperativity of ligand binding, the presence of more than one site for specific binding,

ligand–ligand interactions, or ligand hetereogeneity [33, 34].

The most likely etiology of the apparently complex binding is the high degree of nonspecific binding, especially at the higher concentrations of ICYP. This is confirmed by the competition binding assay at 216 pM ICYP (Fig. 2C) and the degree of nonspecific binding observed during saturation binding analysis (inset, Fig. 3A). These observations could be related to the extremely high affinity of the ligand, inherent characteristics of the cells, or a property induced by the cell isolation technique. Support for the latter hypothesis was the reported effect of cell isolation procedures on other receptors, specifically the Fc receptor, and altered cell function [19, 35]. A comparison of human neutrophils isolated by different cell separation techniques demonstrated that the use of a Ficoll–Hypaque method resulted in the spontaneous change of cell shape, enhanced formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated release of superoxide anion, increased release of lysosomal enzymes upon subsequent FMLP stimulation, and reduced chemotactic responsiveness, by comparison with other methods including a plasma Percoll gradient [19]. Furthermore, recent studies indicate that activated macrophages may induce deterioration of guinea pig tracheal β-adrenergic function, possibly through the release of oxygen radicals [36]. Corresponding measurements of smooth muscle β-adrenergic receptors were not obtained.

Differences in β-adrenergic receptor binding parameters from MN and PMN cells isolated by the plasma Percoll technique were observed in comparison to those isolated with the Ficoll–Hypaque method. In general, competition binding assays with plasma Percoll cells demonstrated improved stereoselectivity at high ICYP concentrations (Fig. 2D vs 2C) and linear saturation binding plots (Fig. 3B vs 3A) with plasma Percoll MN and PMN leukocyte β-adrenergic receptors. Possible explanations include an alteration in ICYP binding or β-adrenergic receptor presentation induced by the Ficoll–Hypaque technique. This could represent enhanced nonspecific binding, reduced receptor density, or the formation of a previously unrecognized lower affinity β-adrenergic receptor binding site. ICYP has been reported to be actively transported into intact cells [37]; it is possible that membranes isolated with the Ficoll–Hypaque technique preferentially form vesicles under our assay conditions and retain ICYP. This could result in apparently high nonspecific binding. We also examined the effect of adding a protease inhibitor, bacitracin, to the membrane preparation. These experiments showed no differences in the levels of specific binding to PMN membranes in the absence and presence of bacitracin with concentrations as high as 0.4% bacitracin.

Despite the improved binding characteristics of ICYP in plasma Percoll isolated MN and PMN cells, examination of the kinetic properties of ICYP in these cells identified the inability of 1 μM (−)-propranolol and 10 μM (−)-isoproterenol to reverse effectively ICYP binding (Fig. 1A). This may be a specific problem with leukocytes since we have observed slowly dissociable binding in brain tissue (unpublished observations). This prompted investigation of an alternative radioligand, IPIN [38].

As compared to ICYP, IPIN binding to plasma Percoll MN and PMN cell membrane was rapidly and completely reversible with the addition of 1 μM (−)-propranolol or 10 μM (−)-isoproterenol (Fig. 1B). Excellent stereoselectivity was observed in competition binding assays (Fig. 2E and 2F) with a distinct description of nonspecific binding at 1 μM (−)-propranolol for IPIN. Saturation binding parameters were linear and similar for cells isolated from the two binding techniques with a tendency for decreased receptor density on cells prepared by Ficoll–Hypaque as compared to the plasma Percoll technique.

Potential explanations for the differences in observations with the two cell isolation techniques could be related to components in the individual preparations. Although there is no significant difference between the cellular composition resulting from each isolation technique, previous investigations suggest that lipopolysaccharide may be a contaminant in the Ficoll–Hypaque isolation procedure, and it is possible that this is an important factor inducing alterations in neutrophil function; however, other etiologies are also possible [19]. Nevertheless, it appears from these observations that measurement of leukocyte β-adrenergic receptors should incorporate the plasma Percoll isolation technique, with advantages of minimal effect on cell characteristics, and the radioligand (−)-[^{125}I]iodopindolol.

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