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DEMONSTRATION OF HUMAN PLATELET β-ADRENERGIC RECEPTORS USING ^{125}I-LABELED CYANOPINDOLOL AND ^{125}I-LABELED HYDROXYBENZYLPINDOLOL

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The radioiodinated pindolol analogs ^{125}I-labeled cyanopindolol ([^{125}I]CYP) and ^{125}I-labeled hydroxybenzylpindolol ([^{125}I]HBP) have been used to study binding to human platelet β-adrenergic receptors. [^{125}I]CYP binds to a saturable class of binding sites on platelet membranes with a dissociation constant (K_d) of 14±3 pM and maximal binding capacity (B_{max}) of 18±4 fmol/mg protein. Binding of [^{125}I]CYP is reversible and is characterized by forward and reverse rate constants of $1.8 \cdot 10^7$ s$^{-1} \cdot$ M^{-1} and $3.8 \cdot 10^{-4}$ s^{-1}, respectively. [^{125}I]HBP binds to a saturable class of platelet membrane sites with a K_d of 50±10 pM and B_{max} of 32±6 fmol/mg protein. [^{125}I]HBP also binds to a saturable class of sites on intact platelets with a K_d of 58±14 pM and B_{max} of 24±4 molecules per platelet. Binding of [^{125}I]CYP and [^{125}I]HBP is stereospecifically inhibited by propranolol and epinephrine; the (−) stereoisomers are at least 50-times more potent than the (+) stereoisomers. Binding of both radioligands is inhibited by adrenergic ligands with a potency order of propranolol ≫ isoproterenol > epinephrine > practolol > norepinephrine > phenylephrine. These observations indicate that [^{125}I]CYP and [^{125}I]HBP bind to platelet sites which have the pharmacological characteristics of β-adrenergic receptors but which are not typical of either the β_1 or β_2 sub-type.

Introduction

Aggregation of human platelets induced by agents such as adenosine diphosphate, collagen, and α-adrenergic hormones can be inhibited by drugs which elevate intraplatelet levels of cyclic AMP. Isoproterenol is believed to inhibit in vitro platelet aggregation by stimulating adenylate cyclase [1]. Blockade of this phenomenon by propranolol but not phentolamine has suggested that it is mediated by β-adrenergic receptors, although direct binding studies demonstrating the presence of β-adrenergic receptors on human platelets have not been reported. In this communication, we report studies which have employed the β-antagonists iodocyanopindolol ([^{125}I]CYP) and iodohydroxybenzylpindolol ([^{125}I]HBP) to quantitate and characterize β-receptors on intact human platelets and membranes isolated from human platelets.

Materials and Methods

(−)-Norepinephrine, (−)-epinephrine, (−)-isoproterenol, and (±)-propranolol were purchased from Sigma. Phentolamine, cyanopindolol, hydroxybenzylpindolol, and practolol were kind gifts

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Abbreviations: CYP, cyanopindolol; HBP, hydroxybenzylpindolol; [^{125}I]CYP, ^{125}I-labeled cyanopindolol; [^{125}I]HBP, ^{125}I-labeled hydroxybenzylpindolol.

of Ciba-Geigy, Drs. G. Engel and D. Hauser of Sandoz Pharmaceuticals, and Ayerst Labs, respectively. (+)-Propranolol was purchased from Adams Co. and ^{125}I (approx. 2300 Ci/mmol) from the Radiochemical Centre, Amersham, U.K. Other chemicals were of reagent grade quality.

Experimental procedures

Preparation of human platelets and platelet membranes. Platelet membranes were prepared as described previously [2] from outdated platelets obtained from the Hadassah-Hebrew University Medical Center Blood Bank. The membranes were frozen in liquid N_2 and used not more than three weeks after purification. Platelet membrane protein was measured according to the method of Lowry et al. [3] using bovine serum albumin as the standard. Intact platelets were prepared from blood taken from donors who had ingested no aspirin-containing drugs for at least two weeks prior to blood donation. Platelet-rich plasma was prepared from freshly obtained whole blood by centrifugation ($2000 \times g$, 2.5 min). The platelets were pelleted by another centrifugation ($4300 \times g$, 10 min), washed twice by repeated centrifugation ($4300 \times g$, 10 min) and resuspension in buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6), and used immediately to study binding to intact platelets.

[^{125}I]CYP and [^{125}I]HBP preparation. These radioligands were prepared according to the iodination procedure described by Maguire et al. [4].

Binding sites. Binding of [^{125}I]CYP and of [^{125}]HBP to platelet membranes was studied using a reaction volume of 0.4 ml of 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 μM phentolamine and 30 to 200 μg membrane protein per ml. Unless otherwise stated, incubations were for 50 to 60 min at 37°C ([^{125}I]CYP) or 30°C ([^{125}I]HBP). Binding of [^{125}I]HBP to intact platelets was studied using a reaction volume of 0.4 ml of 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, 150 mM NaCl and $1.2 \cdot 10^8$ platelets per ml. These incubations were for 1 h at 30°C. Binding reactions were terminated by diluting the samples with 4 ml of 10 mM Tris-HCl (pH 7.5), filtering the samples through Whatman GF/C filters, and washing each filter four times with 4 ml of 10 mM Tris-HCl (pH 7.5) buffer. The time required to dilute, filter, and wash the samples did not exceed 20 s. Non-specific binding of radioligands was defined as that which occurred in the presence of 1 μM (−)-propranolol. Specific binding was determined by subtracting this value from the total binding obtained in the absence of propranolol. Specific binding was 30 to 40% ([^{125}I]CYP) or 20 to 30% ([^{125}I]HBP) of total binding. Using the conditions described above, specific binding reached a constant value after 40 min and remained unchanged during the next hour, and was linearly related to the concentration of protein over the range of 30 to 200 μg per ml used.

Displacement studies. Binding of [^{125}I]CYP (60 pM) and [^{125}I]HBP (250 pM) in the presence of varying concentrations of β-adrenergic ligands was measured as described above. Dissociation constants for the competing ligands were determined according to the method of Cheng and Prusoff [5] using the following equation:

$$K_d = \frac{IC_{50}}{1 + (S/K_S)}$$

where IC_{50} is the concentration of the unlabeled ligand which causes 50% inhibition of [^{125}I]CYP or [^{125}I]HBP binding, S is the [^{125}I]CYP or [^{125}I]HBP concentration, and K_S is the K_d for [^{125}I]CYP or [^{125}I]HBP measured in saturation studies using these radioligands.

Presentation of results. Data reported in this communication, unless otherwise stated, are from single experiments which are representative of two or more such studies. Mean values of replicates are shown in the figures, and the S.E. of replicates was generally ±10%. Scatchard plots [6] of binding data were evaluated by linear regression analysis.

Results

[^{125}I]CYP binds to a saturable class of sites with a K_d of 14 ± 3 pM (Fig. 1). Maximal specific binding is 18 ± 4 fmol per mg protein. Binding of [^{125}I]CYP proceeds along a roughly exponential time course, with a half-time of approx. 5 min (Fig. 2A). At a concentration of 69 pM [^{125}I]CYP, the observed rate constant (k_{ob}) for the association reaction, obtained by measuring the slope of the plot shown in Fig. 2B, is $1.6 \cdot 10^{-3}$ s^{-1}. As

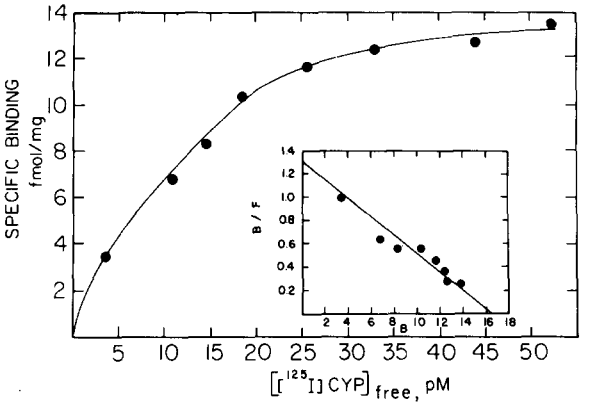

Fig. 1. Binding of [^{125}I]CYP to platelet membranes. Platelet membranes (38 μg/ml) were incubated for 50 min at 37°C with varying concentrations of [^{125}I]CYP in the presence or absence of 1 μM propranolol, and specifically bound [^{125}I]CYP determined as described in the text. Results represent mean of triplicate determinations. Inset shows Scatchard plot of data were B represents specifically bound [^{125}I]CYP expressed as fmol/mg protein and F represents the free concentration of [^{125}I]CYP in pM.

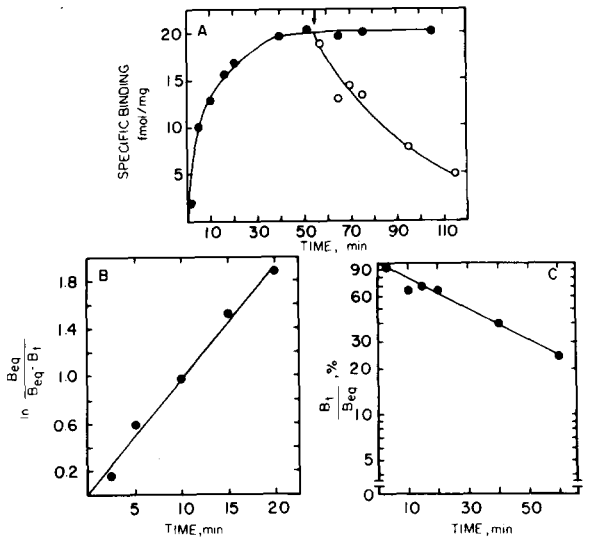

Fig. 2. Time dependence of [^{125}I]CYP binding and dissociation. (A) Platelet membranes (180 μg/ml) were incubated with 69 pM [^{125}I]CYP at 37°C in the presence or absence of 1 μM propranolol for varying periods, and specifically bound [^{125}I]CYP determined as described in the text. At the time indicated by the arrow (55 min), propranolol was added to a final concentration of 1 μM in some of the samples previously incubated without propranolol, and dissociation of [^{125}I]CYP was monitored (O——O). Results represent mean of triplicate determinations. (B) Semilogarithmic plot of [^{125}I]CYP binding data taken from (A). B_{eq} represents maximal specific binding and B_t represents specific binding at time t. (C) Semilogarithmic plot of [^{125}I]CYP dissociation data taken from (A).

noted in Fig. 2A, [^{125}I]CYP is reversibly bound. Dissociation is slower, with a half-time of approx. 30 min and a rate constant (k_{-1}) of $3.8 \cdot 10^{-4}$ s^{-1} (Fig. 2C). The second order association rate constant (k_{+1}), calculated from the equation $k_{+1} = (k_{ob} - k_{-1})([^{125}\text{I}]\text{CYP})^{-1}$, where [^{125}I]CYP is the concentration of [^{125}I]CYP used (69 pM), is $1.8 \cdot 10^7$ s$^{-1} \cdot$ M^{-1}. The ratio of the dissociation and association rate constants (k_{-1}/k_{+1}) equals the dissociation constant for [^{125}I]CYP. Using these values, the kinetically derived value for the K_d of [^{125}I]CYP was found to be 22 pM which agrees well with the value (14 ± 3 pM) obtained by measuring the concentration dependence of [^{125}I]CYP binding (Fig. 1).

[^{125}I]HBP also binds to a saturable class of sites with a K_d of 50 ± 10 pM (Fig. 3). Maximal specific binding using [^{125}I]HBP is 32 ± 6 fmol per mg protein. The concentration dependence of [^{125}I]HBP binding to intact platelets is shown in Fig. 4. Using intact platelets, the K_d for [^{125}I]HBP was found to be 58 ± 14 pM and each platelet possesses 24 ± 4 [^{125}I]HBP binding sites (Fig. 4).

The ability of various adrenergic ligands to inhibit both [^{125}I]CYP and [^{125}I]HBP binding was evaluated. These studies permitted calculation of K_d values for the non-radioactive ligands. As noted

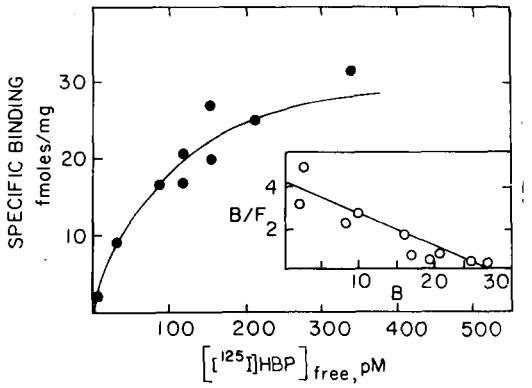

Fig. 3. Binding of [^{125}I]HBP to platelet membranes. Platelet membranes (100 μg/ml) were incubated for 1 h at 30°C with varying concentrations of [^{125}I]HBP in the presence or absence of 1 μM propranolol, and specifically bound [^{125}I]HBP determined as described in the text. Results represent mean of duplicate determinations. Inset shows Scatchard plot of data where B represents specifically bound [^{125}]HBP expressed as fmol/mg protein and F represents free concentration of [^{125}I]HBP in pM.

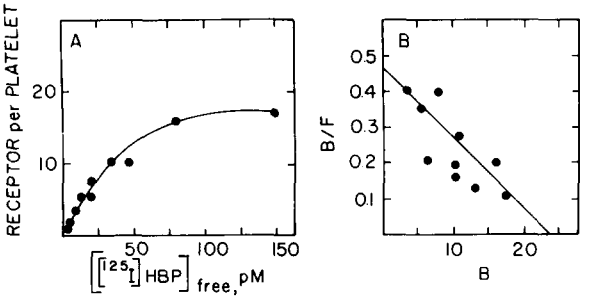

Fig. 4. Binding of [^{125}I]HBP to intact platelets. Platelets ($1.2 \cdot 10^8$ ml) were incubated for 1 h at 30°C with varying concentrations of [^{125}I]HBP in the presence or absence of 1 μM propranolol, and specifically bound [^{125}I]HBP measured as described in the text. (B) shows Scatchard plot of data in (A), where B represents specifically bound [^{125}I]HBP expressed as molecules per platelet and F represents the free [^{125}I]HBP concentration in pM.

in Table I, the order of potency for agonists is isoproterenol > (−)-epinephrine > (−)-norepinephrine > (+)-epinephrine, and for antagonists is (−)-propranolol > practolol > (+)-propranolol. Results obtained using either radioligand are similar in terms of the order of potency for the various β-agents, although some differences in the calculated values for their K_d are noted.

TABLE I

BINDING OF ADRENERGIC LIGANDS TO PLATELET β-RECEPTORS

Platelet membranes (40 μg/ml and 100 μg/ml) were incubated for 1 h with [^{125}I]CYP or [^{125}I]HBP (60 pM or 250 pM, respectively) in the presence of varying concentrations of nonradioactive adrenergic ligands, and specifically bound [^{125}I]CYP or [^{125}I]HBP determined as described in the text. Dissociation constants were calculated according to the method of Cheng and Prusoff [5]. n.d. indicates no inhibition of radioligand binding at a concentration of 1 μM ((+)-propranolol) or 1 mM ((+)-epinephrine, phenylephrine).

Ligand	K_d based on inhibition of specific binding (μM)	
	[^{125}I]CYP	[^{125}I]HBP
Antagonists		
(−)-Propranolol	0.007 ±0.004	0.002 ±0.001
(+)-Propranolol	n.d.	n.d.
Practolol	29 ±15	29 ±10
Agonists		
(−)-Isoproterenol	0.7 ± 0.4	2.8 ± 1.4
(−)-Epinephrine	2.9 ± 1.5	10 ± 5
(−)-Norepinephrine	60 ±30	150 ±50
(+)-Epinephrine	n.d.	n.d.
Phenylephrine	n.d.	n.d.

Discussion

[^{125}I]HBP has been successfully employed to characterize β-adrenergic receptors in a number of types of intact cells as well as in membranes prepared from these cells. Because of its relatively high affinity for β-receptors and its high specific radioactivity, [^{125}I]HBP is particularly well suited for studies probing binding sites which are present in only small amounts. Recently, Engel has reported the development of another pindolol analog which, after radioiodination, can also be used to study β-adrenergic receptors [6]. This ligand, [^{125}I]CYP, like HBP, has high affinity for β-receptors but appears to have slightly less nonspecific binding to membranes when compared with [^{125}I]HBP.

Both [^{125}I]CYP and [^{125}I]HBP bind to a saturable class of platelet membrane sites. Saturation studies using [^{125}I]CYP indicate that this ligand binds with a K_d of 14 ± 3 pM which is similar to the value (22 pM) obtained from kinetic studies evaluating the processes of [^{125}I]CYP association and dissociation. The K_d measured in saturation studies using [^{125}I]HBP is 50 ± 10 pM. Maximal binding, B_{max}, for the two ligands to platelet membranes is similar: 18 ± 4 fmol per mg for [^{125}I]CYP and 32 ± 6 fmol per mg for [^{125}I]HBP. [^{125}I]HBP also binds to a saturable class of sites on intact platelets with a K_d of 58 ± 14 pM and a B_{max} of 24 ± 4 molecules per platelet.

The binding sites for [^{125}I]CYP and [^{125}I]HBP have been characterized by evaluating the ability of various adrenergic agents to inhibit radioligand binding. Both propranolol and epinephrine stereospecifically inhibit [^{125}I]CYP and [^{125}I]HBP binding, and the (−)-stereoisomers are at least 50 times more potent than the (+)-stereoisomers. The potency order with which adrenergic ligands inhibit [^{125}I]CYP and [^{125}I]HBP binding is similar and is as follows: (−)-propranolol ≫ (−)-isoproterenol > (−)-epinephrine > practolol > (−)-

norepinephrine ≫ phenylephrine. These observations indicate, that both radioligands bind to platelet membrane sites having the pharmacological characteristics of β-adrenergic receptors. Due to the small amount of [^{125}I]HBP specifically bound to intact platelets, further studies characterizing these sites could not be performed. Although it is likely that the sites identified on intact platelets are β-receptors, the validity of this conclusion must remain in doubt until stereospecific inhibition of [^{125}I]HBP binding by β-adrenergic ligands can be demonstrated.

Reports by Mills and Roberts in 1967 [7], Abdulla in 1969 [8], and Haslam and Taylor in 1971 [9] indicated that human platelet aggregation could be inhibited or reversed by β-adrenergic agents. Stimulation of platelet adenylate cyclase by β-agents was also noted by Abdulla and, more recently, pharmacologically characterized by Jakobs et al. [1]. Presumably, β-adrenergic agents inhibit aggregation as a result of β-receptor mediated stimulation of adenylate cyclase and a rise in platelet cyclic AMP levels. In each of these studies, however, β-adrenergic stimulation caused only a limited (30 to 50%) increase in adenylate cyclase activity and only a weak inhibition of platelet aggregation. One might speculate that the limited nature of these phenomena reflect the fact that platelets possess only a small number of β-receptors, as noted in the present communication.

The potency order with which β-agonists and antagonists inhibit the binding of [^{125}I]CYP and [^{125}I]HBP corresponds well with that noted by Jakobs et al. for the effects of these agents on platelet adenylate cyclase. The β_2-agonist norepinephrine and the β_1-antagonist practolol demonstrate very low affinity for these β-receptors. These observations support the suggestion originally made by Jakobs et al. [1], that platelet β-receptors do not have characteristics typical of either β_1- or β_2-receptors.

The present communication adds another to the growing list of surface membrane binding sites on human platelets, which have been identified and characterized by direct radioligand binding studies. Some of the other receptor type studies have included those for adenosine diphosphate, serotonin, thrombin, collagen, fibrinogen, and α-adrenergic receptors. Future studies may document changes in the number and/or behavior of these receptors which accompany disease states and alterations in platelet function.

Acknowledgments

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