0026-895X/90/020214-08$02.00/0

MOLECULAR PHARMACOLOGY, 38:214-221

p-[^{125}I]Iodoclonidine Is a Partial Agonist at the α_2-Adrenergic Receptor

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Received February 5, 1990; Accepted May 11, 1990

SUMMARY

The binding properties of p-[^{125}I]iodoclonidine ([^{125}I]PIC) to human platelet membranes and the functional characteristics of PIC are reported. [^{125}I]PIC bound rapidly and reversibly to platelet membranes, with a first-order association rate constant (k_{on}) at room temperature of $8.0 \pm 2.7 \times 10^6$ M^{-1} sec^{-1} and a dissociation rate constant (k_{off}) of $2.0 \pm 0.8 \times 10^{-3}$ sec^{-1}. Scatchard plots of specific [^{125}I]PIC binding (0.1–5 nM) were linear, with a K_d of 1.2 ± 0.1 nM. [^{125}I]PIC bound to the same number of high affinity sites as the α_2-adrenergic receptor (α_2-AR) full agonist [^{3}H]bromoxidine (UK14,304), which represented approximately 40% of the sites bound by the antagonist [^{3}H]yohimbine. Guanosine 5′-(β,γ-imido)triphosphate greatly reduced the amount of [^{125}I]PIC bound (>80%), without changing the K_d of the residual binding. In competition experiments, the α_2-AR-selective ligands yohimbine, bromoxidine, oxymetazoline, clonidine, p-aminoclonidine, (−)-epinephrine, and idazoxan all had K_i values in the low nanomolar range, whereas prazosin, propranolol, and serotonin yielded K_i values in the micromolar range. Epinephrine competition for [^{125}I]PIC binding was stereoselective. Competition for [^{3}H]bromoxidine binding by PIC gave a K_i of 1.0 nM (n_H = 1.0), whereas competition for [^{3}H]yohimbine could be resolved into high and low affinity components, with K_i values of 3.7 and 84 nM, respectively. PIC had minimal agonist activity in inhibiting adenylate cyclase in platelet membranes, but it potentiated platelet aggregation induced by ADP with an EC_{50} of 1.5 μM. PIC also inhibited epinephrine-induced aggregation, with an IC_{50} of 5.1 μM. Thus, PIC behaves as a partial agonist in a human platelet aggregation assay. [^{125}I]PIC binds to the α_{2B}-AR in NG-10815 cell membranes with a K_d of 0.5 ± 0.1 nM. [^{125}I]PIC should prove useful in binding assays involving tissues with a low receptor density or in small tissue samples and in studies of cloned and expressed α_2-AR.

AR are a family of proteins that are coupled to their effector systems via G proteins. The α_2-AR is coupled to a class of G proteins (G_{i1-3},G_o) whose members can be labeled with pertussis toxin (1). Platelets possess at least four G proteins, G_s, G_{i2}, G_{i3}, $G_{x/x}$ (2). G_{i2} is the predominant species in human platelets (2, 3) and appears to be responsible for the inhibition of adenylate cyclase (2). Inhibition of adenylate cyclase is the best characterized biochemical effect of α_2-AR. In platelet membranes, α_2-AR agonists can attenuate up to 40% of forskolin-stimulated adenylate cyclase activity (4). Other effector pathways might also be activated by the α_2-AR (5). Platelet α_2-AR are reported to couple also to the Na^+/H^+ antiporter, but the importance of this effector pathway has not been fully elucidated (6). The α_2-AR is distinguished from the other adrenergic receptors based on its relatively high affinity for yohimbine, as compared with prazosin (α_1-AR) and propranolol (β-AR). There are at least three subtypes of α_2-AR, based on pharmacological and receptor cloning data (7–11). One model for the study of the α_2-AR is the human platelet, which is reported to possess only the α_{2A}-AR subtype (7). The α_{2A}-AR has a relatively high affinity for oxymetazoline and a lower affinity for prazosin, when compared with the α_{2B}-AR subtype (10, 12). NG-10815 cells are reported to possess only the α_{2B}-AR subtype (7).

A model for the interaction of G proteins and receptors is the ternary complex model (13). A modified version of this model has been used to account for experimental data in which antagonists bind with a single affinity but agonist binding displays multiple affinities (14). The ligation state of the coupled G protein is believed to influence the receptor's affinity for agonist. When either GTP or GDP is bound to the G protein, the receptor has a low affinity for agonist. However, the ternary complex with no nucleotide bound has a high affinity for agonist. The high affinity form of the α_2-AR is a requisite step in the activation of the G protein and the effector

This work was supported by Grants HL37551 and GM39561 and funds from the office of the Vice President for Research at the University of Michigan. M.A.G. is supported by a Hartford Foundation Geriatric Fellowship (87399-3G) and a Medical Scientist Training Program Fellowship (GM97863). R.R.N. is an American Heart Association/Genentech Inc. Established Investigator.

ABBREVIATIONS: AR, adrenergic receptor(s); bromoxidine, UK14,304 [5-bromo-6-*N*-(2,4,5-dihydroimidazolyl)quinoxaline]; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; PAC, p-aminoclonidine; PIC, p-iodoclonidine; PRP, platelet-rich plasma; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; G protein, GTP-binding protein.

system (15). G protein activation with the associated GTP binding coverts the receptor from its high affinity form to its low affinity form.

To gain a better understanding of the α_2-AR, it is useful to develop better ligands for binding studies. Radioligand binding is a rapid and useful technique to study receptors, but it requires ligands that possess selectivity and high affinity. Ligands that also have high specific radioactivity offer several advantages that make their development and characterization important in receptor studies. There are several tritiated ligands that have high affinity and good selectivity for the α_2-AR. The only ^{125}I-labeled ligands reported for the α_2-AR are the antagonists ^{125}I-17α-hydroxy-20α-yohimban-16β-[*N*-4-amino-phenethyl]carboxamide and 17α-hydroxy-20α-yohimban-16β-[*N*-(4-azido-3-[^{125}I]iodo)phenyl]carboxamide, a photoaffinity compound (16, 17). Due to a lack of suitable iodinated agonist ligands, we previously synthesized an ^{125}I-analog of clonidine that has high specific radioactivity (18). We report here its binding and functional characteristics. Some parts of this work have been previously described in abstract form (19, 20).

Materials and Methods

Radiochemicals. [^{125}I]PIC (2200 Ci/mmol), [^{3}H]yohimbine (72.5–79.1 Ci/mmol), and [^{3}H]UK 14,304 (bromoxidine; 82.7 Ci/mmol) were obtained from New England Nuclear. 3′,5′-Cyclic [^{3}H]AMP (34 Ci/mmol) and [α-^{32}P]ATP (30 Ci/mmol) were obtained from Amersham.

Competing drugs. (−)-Epinephrine bitartrate and yohimbine were obtained from Sigma, bromoxidine (UK 14,304) and prazosin from Pfizer, idazoxan from Reckitt and Colman, oxymetazoline from Schering, guanabenz from Wyeth, phentolamine from Ciba-Geigy, dihydroergocryptine from Sandoz, propranolol from Ayerst, and PAC and clonidine from Boehringer-Ingelheim. PIC was from Research Biochemicals (Natick, MA) and a gift from Drs. M. Van Dort and R. Counsell (University of Michigan).

Human platelet plasma membranes. Plasma membranes enriched in the α_2-AR were prepared as previously described (21). Aliquots were frozen at −70° until used (within 2 months). Five different membrane preparations were used and displayed similar binding properties.

Kinetic binding studies. Kinetic binding assays were performed as previously described (4), except that all experiments were performed at room temperature. Briefly, membranes (0.05–0.35 mg/ml) were added to TME buffer (50 mM Tris·HCl, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.6), which contained 0.1, 0.25, or 0.5 nM [^{125}I]PIC. Nonspecific binding was defined using 10 μM yohimbine in a separate aliquot. At the appropriate time, a 100-μl sample was diluted with 3 ml of iced TM buffer (50 mM Tris·HCl, 10 mM $MgCl_2$, pH 7.6), immediately filtered over MFS GC50 glass fiber filters on a Hoefer binding apparatus, and washed twice with 10 ml of TM buffer at room temperature. Filters were counted in a Beckman LS-5500 γ-counter at 78% efficiency.

Equilibrium binding studies. Equilibrium binding studies were performed in TME buffer as previously described (4), with minor modifications. Briefly, membranes were added to the reaction tubes containing radioligand, other drugs, and buffer, vortexed, and allowed to incubate at room temperature for 30–60 min. Nonspecific binding was defined using 10 μM yohimbine in all experiments. Rapid filtration was performed immediately following dilution on either a Hoefer or a Brandel filtration apparatus. In experiments employing the Hoefer binding apparatus, dilution with 3 ml of iced TM buffer was followed by two 10-ml washes. In experiments with the Brandel apparatus, dilution with 5 ml of iced TM buffer was followed by two 5-ml iced TM washes. Saturation experiments were performed with [^{3}H]bromoxidine (0.05–2 nM; 0.1 mg of protein in 1 ml), [^{3}H]yohimbine (1–25 nM; 0.04–0.075 mg of protein in 100 μl), and [^{125}I]PIC (0.05–5 nM; 0.015–0.025 mg of protein in 100–250 μl). Competition for 1 nM [^{3}H]bromoxidine was performed in 1 ml of TME buffer (0.1 mg of protein). Competition for 0.5 nM [^{125}I]PIC (0.02–0.025 mg of protein) was performed in 100 μl, and competition for 10 nM [^{3}H]yohimbine (0.025–0.05 mg of protein) was performed in 100–250 μl. Filters with [^{3}H]yohimbine or [^{3}H]bromoxidine were dried 15 min under a heat lamp before counting in 4 ml of ScintiVerse liquid scintillation cocktail, whereas filters for [^{125}I]PIC were counted as described above.

Platelet aggregation. Blood was obtained by venipuncture from donors who denied taking any drugs in the previous 10 days. PRP was prepared by centrifugation at 180 × *g* for 7 min in 9:1 (v/v) sodium citrate anticoagulant (2.47% trisodium citrate, 0.44% citric acid). Aggregation experiments were performed in a Chronolog aggregometer at 37° with stirring. PIC was added 30 sec before ADP or epinephrine addition. In some experiments, the PRP was filtered over a Biogel A50 agarose column (Bio-Rad) to remove plasma proteins. In these experiments, fibrinogen was added (1 mg/ml) before the aggregation experiments were performed.

Adenylate cyclase. Adenylate cyclase assays were performed as previously described (4). Assays were conducted for 15 min at 30° in the presence of 10 μM forskolin. The 5-ml aqueous fraction was counted in 15 ml of Ultima Gold liquid scintillation cocktail. Two separate membrane preparations were employed in adenylate cyclase experiments.

NG-10815 membranes. NG-10815 cells (passage 14) were received as a gift from Dr. M. Nirenberg (National Institutes of Health, Bethesda, MD). They were grown as previously described (22), and cells of passage 27 and 28 were used. After reaching confluency, the cells were incubated in serum-free medium for 16–20 hr. Cells were harvested, centrifuged at 800 × *g* for 5 min, washed once, and homogenized in a glass-Teflon homogenizer in hypotonic Tris buffer (5 mM Tris, 5 mM $MgCl_2$, pH 7.5). The homogenate was centrifuged at 1000 × *g* for 5 min, and the supernatant was saved. The pellet was washed, homogenized, and centrifuged again, and the resulting supernatant was added to the first supernatant. The supernatants were centrifuged for 30 min at 100,000 × *g*, resuspended in TME buffer, frozen in liquid nitrogen, and stored at −70° until used. After thawing, membranes were pelleted at 100,000 × *g* for 30 min and then resuspended in TME buffer. The binding assay was the same as described for platelet membranes.

Thin layer chromatography. [^{125}I]PIC was spotted onto Kodak 13181 silica gel with fluorescent indicator, developed with ethanol, cut, and counted. The R_F of authentic PIC was 0.72. [^{125}I]PIC (1 or 10 nM) was incubated with platelet membranes for 60 min at room temperature, as described for equilibrium binding. Aliquots of the mixture were then separated with thin layer chromatography.

Data analysis. Data were fit using the nonlinear least squares fitting features of the computer program InPlot (GraphPAD Software, San Diego, CA). In platelet aggregation experiments, the IC_{50} values were calculated from the initial slope of primary aggregation, whereas the EC_{50} values were calculated from the secondary phase plateau (similar results were obtained by performing the analysis with the primary phase peak).

Results

Association and dissociation kinetics of [^{125}I]PIC. Experiments were performed to determine the kinetic binding parameters of [^{125}I]PIC and appropriate conditions for equilibrium binding studies. Association of [^{125}I]PIC to the α_2-AR in platelet membranes (Fig. 1) at room temperature was rapid, reached equilibrium within 30 min, and remained stable for at least 90 min. Binding of [^{125}I]PIC was reversible. Addition of 10 μM yohimbine resulted in complete dissociation of all specifically bound [^{125}I]PIC (Fig. 1). The calculated first-order association rate constant (k_{on}) was 8.0 ± 2.7 × 10^6 M^{-1} sec^{-1} (mean ± SE; three experiments). The dissociation rate constant was 2.0 ± 0.8 × 10^{-3} sec^{-1} (mean ± SE; three experiments),

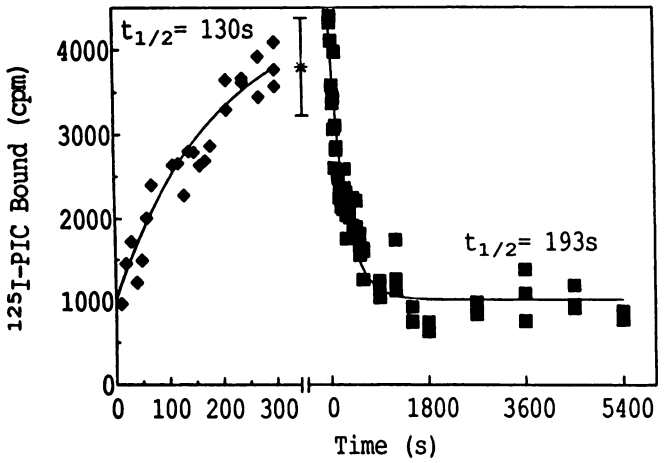

Fig. 1. Association and dissociation kinetics of [^{125}I]PIC. ♦, The time course of binding of 0.5 nM [^{125}I]PIC to platelet membranes (35 μg of protein in 200 μl of TME buffer) at room temperature. *, The 90-min time point (mean ± standard deviation of triplicate measurements), demonstrating that equilibrium has been achieved. Dissociation was initiated at 95 min by addition of yohimbine to a final concentration of 10 μM and binding was determined at the indicated times after addition (■). Results shown are from a single experiment but are similar to three other experiments. ——, Nonlinear least square fits of the data, determined as described in Materials and Methods.

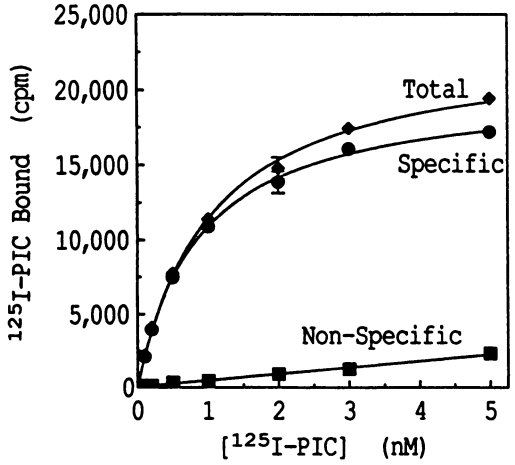

Fig. 2. Saturation binding of [^{125}I]PIC. Equilibrium binding of [^{125}I]PIC to platelet membranes (17.5 μg of protein) is shown as the average of triplicates ± standard deviation; nonspecific binding is shown as the average of duplicates ± standard deviation, determined in the presence of 10 μM yohimbine. Specific binding was calculated by subtracting values of the nonspecific binding determined by linear regression from the total binding. ——, Nonlinear least squares fits, determined as described in Materials and Methods. The K_d determined from this experiment was 0.85 nM. Results shown are from a single experiment but are similar to 14 other experiments (see Table 1).

corresponding to half-time of 5.8 min. The average kinetically derived K_d was 0.44 ± 0.28 nM (mean ± SE; three experiments).

Saturation binding of [^{125}I]PIC. Equilibrium binding of [^{125}I]PIC was saturable (0.01–5 nM), and nonspecific binding was less than 20% at the highest concentration tested (Fig. 2). One advantage of [^{125}I]PIC is its very high specific activity. This is clear from Fig. 2, in which binding to low amounts of protein (17.5 μg) resulted in tens of thousands of counts specifically bound. Tritiated agonists would give approximately 60-fold fewer counts under the conditions depicted.

Scatchard transformation of specific [^{125}I]PIC binding produces a straight line, consistent with the conclusion that [^{125}I]

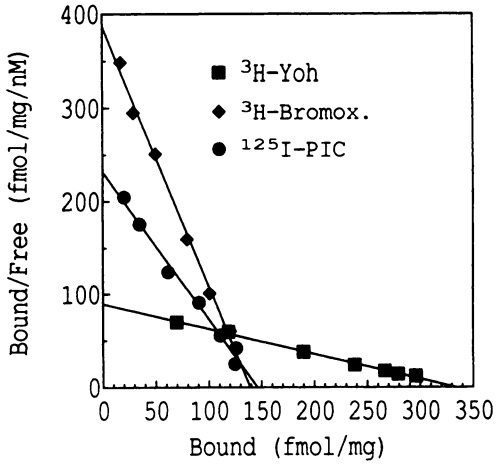

Fig. 3. Scatchard plots of specific [^{125}I]PIC, [^{3}H]bromoxidine, and [^{3}H]yohimbine binding. Specific binding of [^{125}I]PIC (●), [^{3}H]bromoxidine (♦), and [^{3}H]yohimbine (■) is shown. Results shown are from a single experiment but are similar to four other experiments (see text and Table 1).

TABLE 1

Equilibrium binding parameters for α_2 radioligands

The K_d and B_{max} values determined from experiments such as those in Figs. 2 and 3 are summarized. Values shown are the mean ± standard error. The individual K_d and B_{max} values were obtained from an unweighted nonlinear least squares fit of the specific binding data to a hyperbolic binding function. The B_{max} for [^{125}I]PIC as a fraction of the [^{3}H]yohimbine B_{max} was calculated from either a [^{3}H]yohimbine B_{max} determined in the same experiment or from the average [^{3}H]yohimbine B_{max} for that membrane preparation in the experiments where [^{3}H]yohimbine binding was not performed in the same experiment. The average [^{3}H]yohimbine B_{max} was 361 fmol/mg of protein for five membrane preparations. See also Materials and Methods. *n* is the number of experiments.

	K_d	B_{max}
	nM	% of [^{3}H]yohimbine B_{max}
[^{125}I]PIC	1.2 ± 0.1 (*n* = 15)	41.4 ± 2.7 (*n* = 6)
[^{3}H]Bromoxidine	0.70 ± 0.09 (*n* = 11)	45.0 ± 1.3 (*n* = 4)
[^{3}H]Yohimbine	6.9 ± 1.0 (*n* = 11)	

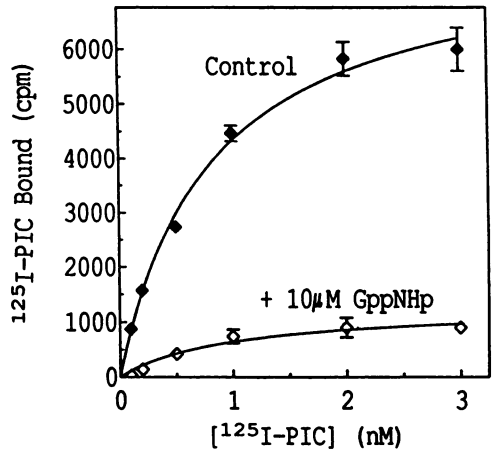

Fig. 4. Effect of Gpp(NH)p on [^{125}I]PIC binding. Specific binding of 0.1–3 nM [^{125}I]PIC to platelet membranes (20 μg of protein) with (◇) and without (♦) 10 μM Gpp(NH)p is shown. Results shown are from a single experiment (average of triplicates ± standard deviation) but are similar to five other experiments. ——, Nonlinear least squares fits, determined as described in Materials and Methods.

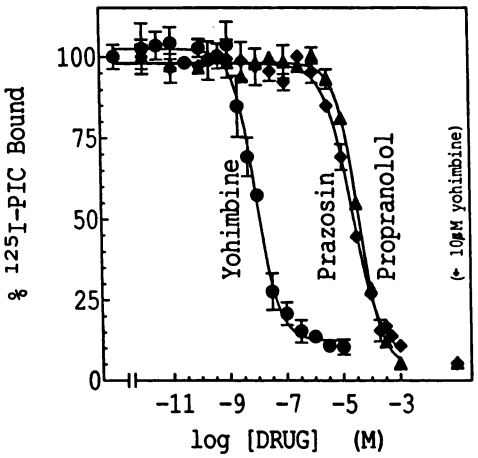

Fig. 5. Competition for [^{125}I]PIC binding. Competition for 0.5 nM [^{125}I]PIC binding by yohimbine (●), prazosin (♦), and propranolol (▲) is shown. In the prazosin and propranolol experiments, one data point with 10 μM yohimbine alone was included to determine the extent of nonspecific binding. Results shown are the average of triplicates ± standard deviation from a single experiment. ——, Nonlinear least squares fits, determined as described in Materials and Methods.

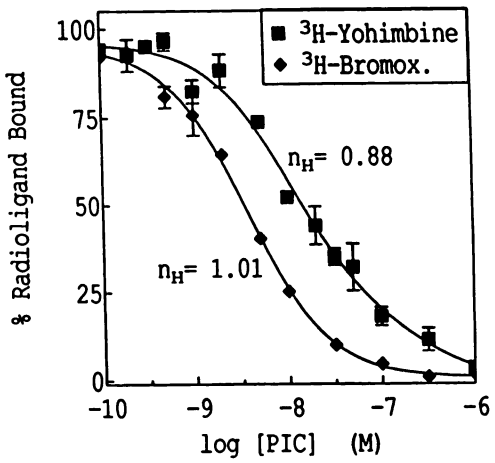

Fig. 6. PIC competition for α_2-AR agonist and antagonist binding. PIC competition for 1 nM [^{3}H]bromoxidine (♦) and 10 nM [^{3}H]yohimbine (■) binding is shown. [^{3}H]Bromoxidine results (average of triplicates ± standard deviation) are from a single experiment, repeated three times. [^{3}H]Yohimbine results are the pooled resulted (average of triplicate determinations from four separate experiments) expressed as average ± standard error. ——, Nonlinear least squares fits, determined as described in Materials and Methods. The two-site of the [^{3}H]yohimbine data is shown. See Materials and Methods for details. Approximately 200 fmol/mg was bound by 1 nM [^{3}H]bromoxidine and 350 fmol/mg was bound by 10 nM [^{3}H]yohimbine.

TABLE 2

Pharmacologic specificity of [^{125}I]PIC binding

K_i and n_H values were determined from competition experiments such as those shown in Fig. 5. Values shown are the mean ± range or the mean ± standard error (for $n > 2$). Individual experiments were performed in triplicate. The directly measured K_d of [^{125}I]PIC (of that membrane preparation used in the experiment) was used to calculate the K_i. n is the number of experiments.

	K_i	n_H	n
	nM		
Bromoxidine	1.0 ± 0.4	0.94	2
PIC	1.4 ± 0.3	0.92	2
Oxymetazoline	2.3 ± 0.09	0.88	3
(−)-Epinephrine	4.6 ± 0.9	0.79	4
Yohimbine	6.0 ± 0.5	1.07	2
PAC	6.1 ± 0.5	0.89	2
Clonidine	17.4 ± 7.9	0.94	3
Idazoxan	21.6 ± 4.9	0.91	4
(+)-Epinephrine	43.0 ± 1.6	0.94	2
Serotonin	5,200 ± 2,300	0.80	2
Prazosin	12,000 ± 2,000	0.92	2
Propranolol	23,000 ± 7,000	1.3	2

PIC binds to a single high affinity site in platelet membranes (Fig. 3). Furthermore, [^{125}I]PIC binds to the same number of sites as the full α_2-AR agonist [^{3}H]bromoxidine (Fig. 3). These high affinity binding sites represent approximately 40% of the binding sites of the α_2-AR antagonist [^{3}H]yohimbine, which binds to the entire α_2-AR population with a single affinity. Table 1 summarizes the equilibrium binding parameters of [^{125}I]PIC, [^{3}H]bromoxidine, and [^{3}H]yohimbine. The directly measured K_d (1.2 ± 0.1 nM) for [^{125}I]PIC agrees with the K_d obtained from kinetic experiments (0.44 nM).

Functional coupling of [^{125}I]PIC binding to G proteins. Saturation experiments were performed in the presence or absence of 10 μM Gpp(NH)p (Fig. 4), to test whether [^{125}I]PIC binding was sensitive to the functional state of coupled G protein(s). Inclusion of Gpp(NH)p in the assay greatly decreased total binding (≥80% decrease in B_{max}) but did not alter the K_d of the residual binding. The K_d in the presence of Gpp(NH)p was 1.5 ± 0.4 nM (mean ± SE; five experiments). Therefore, in the binding assay, [^{125}I]PIC exhibits the profound guanine nucleotide sensitivity characteristic of α_2-AR agonists.

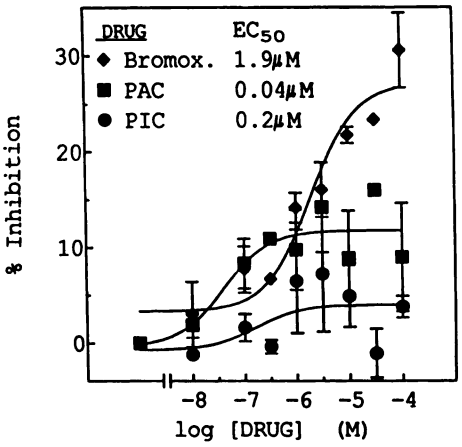

Fig. 7. Inhibition of forskolin-stimulated adenylate cyclase. Inhibition of forskolin-stimulated adenylate cyclase in platelet membranes by bromoxidine (♦), PAC (■), and PIC (●) is shown. Activity in the absence of inhibitors was 638 pmol/min/mg of protein. Results from four experiments, performed in duplicate or triplicate were averaged and are expressed as percentage of inhibition ± standard error. ——, Nonlinear least squares fit, determined as described in Materials and Methods.

[^{125}I]PIC binding to the α_2-AR. Competition experiments were performed to determine the pharmacological specificity of [^{125}I]PIC binding to human platelet plasma membranes. Yohimbine competed for [^{125}I]PIC binding with a single high affinity (n_H = 1.07), compared with the α_1-AR antagonist prazosin and the β-AR antagonist propranolol (Fig. 5 and Table 2). Competition for [^{125}I]PIC binding was examined with several other ligands (Table 2) to provide further evidence for α_2-AR specificity. The α_2-AR-specific ligands yohimbine, bromoxidine, oxymetazoline, clonidine, PAC, (−)-epinephrine, and idazoxan had K_i values in the low nanomolar range. Propranolol, prazosin, and serotonin had K_i values in the micromolar range. Epinephrine displayed stereoselectivity in competing for [^{125}I]PIC binding (Table 2). (−)-Epinephrine had nearly a 10-fold

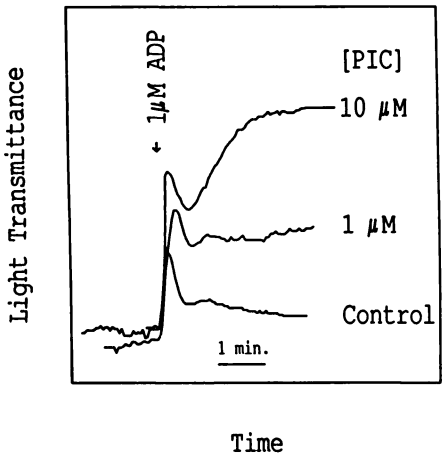

Fig. 8. PIC potentiates ADP-induced platelet aggregation. PRP (450 μl) was aliquotted into a stirred cuvette at 37°, and ADP (1 μM final concentration) was added to initiate platelet aggregation. PIC at the indicated concentrations was added 30 sec before 1 μM ADP. Results shown are from a single experiment but are similar to two other experiments.

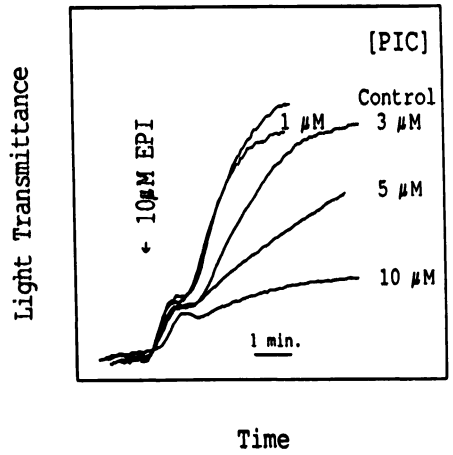

Fig. 9. PIC inhibits epinephrine-induced platelet aggregation. PRP (450 μl) was aliquotted into a stirred cuvette at 37°, and epinephrine (10 μM final concentration) was added to initiate platelet aggregation. PIC at the indicated concentrations was added 30 sec before 10 μM epinephrine. Results shown are from a single experiment but are similar to two other experiments.

greater affinity than the (+)-epinephrine stereoisomer. The n_H values for most ligands were close to unity, ranging from 0.88 to 1.07.[1] Furthermore, the same amount of nonspecific binding was observed in the presence of a 10 μM concentration of the following α-AR and α_2-AR ligands when competing for 0.5 nM [^{125}I]PIC binding: guanabenz, phentolamine, and dihydroergocryptine. The relative affinities and K_i values for yohimbine, prazosin, propranolol, and other ligands demonstrate that [^{125}I]PIC is binding to the α_2-AR.

We performed experiments to determine the characteristics of PIC competition for the binding of a full α_2-AR agonist and an α_2-AR antagonist. [^{3}H]Bromoxidine is a full α_2-AR agonist that binds to the high affinity state of the α_2-AR. PIC competes for all of the [^{3}H]bromoxidine binding (Fig. 6) with a K_i of 1.0 nM, which is identical to its directly measured K_d. The Hill slope (n_H) is 1.01. Therefore, PIC appears to bind with a single affinity to the full population of [^{3}H]bromoxidine sites.

PIC competition for binding of the antagonist [^{3}H]yohimbine (Fig. 6) does not conform to a single-site binding model. Data from four experiments were fit by a two-site competition curve (Fig. 6). The high affinity K_i is 3.7 nM (representing 80% of the sites) and the low affinity K_i is 84 nM. When fit to a Hill equation, the K_i was 5.9 ± 0.4 nM and the n_H was 0.79. We repeated the competition experiment with the inclusion of 10 μM Gpp(NH)p. This resulted in a 2-fold increase in the K_i value and an increase in the n_H to 1.07 (data not shown).

[^{125}I]PIC binding to NG-10815 cell membranes. We performed saturation binding experiments on NG-10185 cell membranes to determine the K_d of [^{125}I]PIC at the α_{2B}-AR. [^{125}I]PIC bound to approximately 60% of the [^{3}H]yohimbine sites with a K_d of 0.5 ± 0.1 nM (three experiments; data not shown).

Ligand stability during incubation. Incubation of 1 or 10 nM [^{125}I]PIC with platelet membranes for 1 hr at room temperature does not result in degradation. Thin layer chromatography of [^{125}I]PIC from the binding assay incubation did not reveal any change in the thin layer chromatography profile of the radioactivity, as compared with nonincubated controls (data not shown).

Adenylate cyclase experiments. We tested whether PIC could inhibit forskolin-stimulated adenylate cyclase and how that inhibition compared with inhibition produced by a full agonist (bromoxidine) and a partial agonist (PAC). At 100 μM, bromoxidine inhibited 25% and PAC inhibited 12% of the forskolin-stimulated adenylate cyclase (Fig. 7). However, PIC had minimal agonist properties in attenuation of adenylate cyclase (Fig. 7), with a maximal inhibition of ≈4%.

Platelet aggregation experiments. Platelet aggregation was employed as another functional assay to determine the properties of PIC *in vitro*. Activation of the α_2-AR either can directly produce aggregation or can potentiate aggregation induced by another agent. ADP-stimulated platelet aggregation was potentiated in a concentration-dependent manner by PIC (Fig. 8), as is observed for clonidine and low concentrations of epinephrine. The EC_{50} for potentiation by PIC was 1.7 ± 0.3 μM (three experiments). Clonidine produced similar results (EC_{50} = 0.3 ± 0.2 μM; data not shown). Epinephrine alone produces platelet aggregation via the α_2-AR. PIC produced a concentration-dependent inhibition of epinephrine-induced aggregation, with an IC_{50} of 5.3 ± 0.1 μM (three experiments) (Fig. 9). PIC alone failed to initiate platelet aggregation. Clonidine produced very similar results (IC_{50} = 1.6 ± 0.2 μM; data not shown).

The lower potency of PIC compared with clonidine as a potentiator and as an antagonist in the standard aggregation assay may be due to increased binding of PIC to the plasma proteins. We tested this hypothesis by removing plasma proteins by gel filtration and repeating the measurements of epinephrine-induced aggregation. Using gel-filtered platelets, the IC_{50} values were 1.4 ± 0.1 μM and 0.4 ± 0.07 μM for clonidine and PIC, respectively (three experiments). Thus, protein binding of PIC may account for the lower potency of PIC compared with clonidine in the aggregation assay with PRP.

[1] Because the Hill coefficient of (−)-epinephrine competition for [^{125}I]PIC binding was low, we combined the data from all the competition experiments and analyzed the combined data. When fit to the Hill equation, the n_H was 0.87 and the K_i was 4.4 nM. Two-site competition analysis of the combined data gave a high affinity K_i of 3.0 nM (81% of the sites) and a low affinity K_i of 37 nM.

Discussion

We report here the binding properties of the first radioiodinated α_2-AR agonist ligand, [^{125}I]PIC. It possesses very high specific radioactivity without sacrificing either specificity or affinity. [^{125}I]PIC binds rapidly and reversibly. [^{125}I]PIC binding is inhibited by α_2-AR-specific ligands in a competitive manner. Binding of [^{125}I]PIC is highly sensitive to guanine nucleotides, and [^{125}I]PIC binds to both the α_{2A}- and α_{2B}-AR subtypes.

Several imidazoline α_2-AR ligands also bind to distinct non-α_2-adrenergic sites in platelets and other tissues (23–26). It has been suggested that there are three types of nonadrenergic imidazoline binding sites (23). The imidazoline/guanidinium binding site in human kidney binds idazoxan but not clonidine. A binding site in central nervous system tissue recognizes imidazole compounds (e.g., cimetidine) in addition to clonidine and other imidazoline compounds. The third type is found in rabbit kidney and may represent a species-specific form of the first type of site. [^{125}I]PIC is an imidazoline, so it could potentially bind to these non-α_2 sites. Preliminary data (27) suggest that [^{125}I]PIC does bind to an imidazole site in brain and carotid body. However, significant binding of [^{125}I]PIC to a platelet imidazoline binding site in our experiments is unlikely for three reasons. First, the yohimbine data argue against interactions at a non-α_2-AR binding site in our binding assay. Yohimbine competes for all of the [^{125}I]PIC binding with a single high affinity. If there was substantial binding of [^{125}I]PIC to imidazoline/guanidinium binding sites (K_i for yohimbine > 10 μM) or to imidazole binding sites (K_i for yohimbine ≈ 1 μM), this would have been detected in the competition curves. Also, yohimbine, which does not compete with [^{3}H]idazoxan for binding to the non-α_2 imidazoline/guanidinium binding site (23), was used to determine nonspecific binding in all of our experiments. The same amount of nonspecific binding was observed with idazoxan and yohimbine, ligands that do and do not recognize the non-α_2 imidazoline/guanidinium binding site, respectively (23). Second, the binding studies were performed with platelet plasma membranes. The plasma membrane fraction of human platelets has few if any imidazoline/guanidinium binding sites (26). Third, epinephrine competes for all of the [^{125}I]PIC binding with high affinity. Catecholamines do not bind to the nonadrenergic imidazoline/guanidinium or imidazole binding sites (23). These observations suggest that there is little if any binding to non-α_2 imidazoline binding sites in our platelet membrane assays. Further studies will be necessary to characterize the binding affinity of PIC at the nonadrenergic imidazoline binding site in tissues other than platelets.

PIC is a partial agonist in functional assays. Although PIC binds to the functionally active high affinity state of the receptor, it has little if any agonist activity in attenuating adenylate cyclase. PAC and bromoxidine acted as partial and full agonists, respectively, in all experiments. The degree of inhibition by the latter compounds was similar to that seen previously in human platelet membranes (4). Agonist activation of the α_2-AR can induce platelet aggregation or potentiate aggregation induced by another agent. PIC blocked epinephrine-induced aggregation but potentiated ADP-induced aggregation. We conclude that PIC is a partial agonist in the human platelet aggregation assay.

Inhibition of adenylate cyclase and probably platelet aggregation are mediated by G protein(s) (28, 29). Although an attractive hypothesis, it is now clear that inhibition of adenylate cyclase does not induce platelet aggregation (30–32). Our data support this contention. The mechanism of α_2-AR-mediated aggregation is not known, but the Na^+/H^+ antiporter may play a role in α_2-mediated platelet aggregation by leading to activation of phospholipase A_2 (6, 33). Furthermore, in Chinese hamster ovary cells with expressed α_{2A}-AR, it appears that inhibition of adenylate cyclase and activation of phospholipase A_2 are separate events (34).

One of the most intriguing aspects of our data concerns the partial agonist activity of PIC in platelet aggregation but the virtual lack of activity in inhibiting adenylate cyclase. Clonidine and PAC have effects on aggregation similar to those of PIC but are clearly partial agonists in adenylate cyclase assays. This discrepancy argues against a single high affinity receptor activating unique signaling pathway(s). Three possibilities could account for this observation. First, two α_2-AR subtypes that are coupled to separate effector pathways could explain the results. Based on binding studies, human platelets are reported to possess α_{2A}- but not α_{2B}-AR (7). A third α_2-AR subtype has been cloned recently (11) that is different from the two previously cloned (8). The function and distribution of all three subtypes is not known at this time. Second, a receptor that can assume two or more distinct active conformations could explain the data. In this model, the separate conformations could have different affinities for G protein/effector pathways and full agonists could induce all possible conformations, whereas the partial agonists could induce only one or the other of the distinct conformations. Third, a non-α_2 receptor could be playing a role. This seems unlikely, because the binding specificity and competition data indicate that PIC is binding to only the α_2-AR in platelet membrane preparations. Furthermore, the aggregation and adenylate cyclase responses are blocked by yohimbine (28), which is specific for the α_2-AR.

The functional properties of PIC are not unique. Clare *et al.* (29) described the effects on aggregation and the ability to attenuate adenylate cyclase of a series of imidazoline compounds. The compounds in that study were divided into four functional groups: full agonists, two types of partial agonists, and antagonists. One group of partial agonists displayed agonist activity in adenylate cyclase assays and antagonist properties in the aggregation assay. The other group of partial agonists (including oxymetazoline and guanabenz) potentiated aggregation but were antagonists in the adenylate cyclase assay. Thus, PIC is functionally similar to oxymetazoline and guanabenz. Those authors also concluded that a model where a single α_2-AR mediated both responses was not consistent with the data.

[^{125}I]PIC and [^{3}H]bromoxidine bind the same number of high affinity sites but do not possess the same intrinsic activity. This observation has been seen for many partial agonists, including clonidine (28) and PAC (4), at the α_2-AR. At the α_2-AR, a positive correlation between intrinsic activity (inhibition of adenylate cyclase) and the ratio of the high affinity K_d and low affinity K_d has been made (4, 28, 35). In these studies, the ratio of affinities was greater for the full agonists. This correlation is seen in [^{3}H]yohimbine competition studies, where the ratio of the low to high affinity for bromoxidine is 65 (4), whereas the corresponding ratio of low and high affinities for PIC is 22. Furthermore, the percentage of high affinity sites does not correlate with the ability of α_2-AR agonists to inhibit

adenylate cyclase (4, 28, 35). At the α_2-AR, all agonists appear to bind the same percentage of receptors (those in the high affinity state). Wreggett and De Lean (36) also saw a positive correlation between the ratio of affinities and the ability to inhibit adenylate cyclase with the D_2-dopamine receptor. This correlation was not seen with the β-AR (13), which stimulates adenylate cyclase. Wreggett and De Lean (36) suggest that the molecular mechanism of receptor-effector interaction for the inhibition and stimulation of adenylate cyclase may be responsible for these differences. Furthermore, our laboratory has extensively modeled and tested the ternary complex model (4, 14). We have demonstrated that this model is not sufficient to explain high and low affinity binding to the α_2-AR. The amount of low affinity binding appears to be determined by receptor heterogeneity or compartmentation rather than a simple ternary complex mechanism. In our modified ternary complex model, the amount of receptor uncoupled from G protein (i.e., low affinity) would be the same for full and partial agonists, whereas relative affinities would vary, as is observed.

The large difference between the K_d for binding of [^{125}I]PIC and the EC_{50}/IC_{50} in functional assays is an artifact. The difference in these values is due to the very different conditions of the binding and functional assays. The functional assays include high concentrations of Na^+ and GTP, whereas the binding assay does not. Both Na^+ (37, 38) and GTP (15, 38) cause large decreases in affinity of agonists at the α_2-AR. A similar discrepancy between the K_d and EC_{50} for the full agonist bromoxidine has been examined in detail in our laboratory (38).

The binding and functional properties of the full α_2-AR agonist [^{3}H]bromoxidine were previously characterized by our laboratory (4). PIC competes for all of the binding sites of [^{3}H] bromoxidine with high affinity. In contrast, the data suggest that PIC is not competing for [^{3}H]yohimbine binding with a single affinity. Our interpretation of the [^{3}H]yohimbine competition curve is that PIC competes for both the high and low affinity binding states of the α_2-AR. There is approximately a 22-fold difference between the high and low affinity K_i values for PIC. There is a discrepancy between the high affinity K_i in the [^{3}H]yohimbine competition studies and the directly measured K_d. Also, there is a substantially greater percentage of high affinity sites (80%) in the competition studies (Table 1). To examine these differences, we fit competition data with the parameters predicted from the Scatchard analysis. That is, we constrained the high affinity K_i to 1.2 nM and/or the percentage of high affinity sites at 41.4%. In the constrained fit of the [^{3}H] yohimbine competition data, the sum of the squared residuals was not statistically worse (F test; $p > 0.05$) than the fit where all of the parameters were free to vary (Fig. 6). Thus, the binding parameters obtained from direct measurements (K_d of 1.2 nM, 41% of yohimbine sites) provide a statistically adequate fit of the yohimbine competition data. Indeed, the directly measured parameters for the high affinity binding are probably more accurate. The n_H value of PIC competition for [^{3}H] yohimbine binding is relatively high (0.79). The effects of Gpp(NH)p on the high affinity component of this competition support the interpretation that there are two sites, one of which is sensitive to Gpp(NH)p.

In previous studies from this laboratory, competition for [^{3}H] yohimbine binding by the partial agonist PAC was best fit by a model with three separate affinities (4). The functional significance of these multiple affinity states is not known. However, it is possible that the two high affinity states reflect either discrimination of two α_2-AR subtypes or discrimination of distinct high affinity receptor conformations. Again, the data are not consistent with a single high affinity receptor model. We do not observe three separate affinities with PIC. The relationship between the functional data with PIC and the binding data with PAC is not understood at this time.

In summary, [^{125}I]PIC is a high affinity, α_2-AR-specific partial agonist that is ideal for radioligand binding studies. It offers clear advantages over other α_2 radioligands and should prove useful in the analysis of cloned and expressed receptors, as well as in experiments with small tissue samples or tissues with low receptor density. [^{125}I]PIC has the potential to be used in *in vivo* studies of the α_2-AR. Finally, because PIC has functional effects that are different from those of other partial agonists, it may prove useful as a tool to probe the coupling of the α_2-AR to differing effector systems.

Acknowledgments

We thank Dr. M. Van Dort and Dr. R. Counsell for the synthesis of the PIC used in some of the experiments. We also thank Drs. R. Garlick, J. Miller, and P. Atkocius at New England Nuclear for providing the [^{125}I]PIC used in these studies. We also thank Ms. C. Graeser for preparation of the NG-10815 membranes.

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