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Solubilization and Characterization of Putative *Alpha*-2 Adrenergic Isoceptors from the Human Platelet and the Rat Cerebral Cortex[1]

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ABSTRACT

Alpha-2 adrenergic receptor isotypes have been proposed to explain several pharmacologic differences between rodent and nonrodent species. In support of this hypothesis, we found that the differences in the pharmacologic properties of rat cerebral cortex and human platelet *alpha*-2 adrenergic receptors were not due to 1) differential proteolysis of the receptor, 2) degradation of the ligand, 3) the detection of different affinity states or 4) the presence of different quantities of various affinity modulators. In an effort to test the hypothesis further we have characterized these prototype isoceptors in soluble form. Soluble receptors from both species showed the appropriate rank order of potencies for various adrenergic agonists and antagonists expected for an *alpha*-2 adrenergic receptor. The K_D values for soluble human platelet and rat cerebral cortical *alpha*-2 receptors were 4.2 and 5.9 nM, respectively. The species differences in the affinities of prazosin and oxymetazoline were retained upon solubilization. Both soluble receptors were insensitive to modulation by guanine nucleotides, indicating uncoupling from the inhibitory regulatory subunit N_i. Sucrose density gradient centrifugation of soluble receptors did not indicate any significant molecular size differences.

The classification of adrenergic receptors is based on the rank order of potencies of various agonists as originally proposed by Ahlquist (1948). With the development of more specific drugs, it became apparent that there were subdivisions in the original *alpha* and *beta* scheme. *Alpha* receptor subtypes are currently identified pharmacologically by the order of potencies of subtype selective agonists and antagonists (Bylund and U'Prichard, 1983). For *alpha*-2 receptors, the antagonist yohimbine is more potent than the antagonist prazosin.

Recent work by Cheung *et al.* (1982), Latifpour *et al.* (1982) and Feller and Bylund (1984) as reviewed by Bylund (1985) suggest *alpha*-2 adrenergic receptor heterogeneity between rodent and nonrodent mammalian species. This hypothesis has been proposed to explain the observations that 1) the affinity of [^{3}H]yohimbine is 5 to 10 times lower in rodent tissues compared to nonrodent tissues; 2) prazosin is relatively more potent in inhibiting ^{3}H-agonist and ^{3}H-antagonist binding at the *alpha*-2 receptors of rodents as compared to nonrodents and 3) oxymetazoline is more potent in nonrodent tissues as compared to rodent tissues. The term *isoceptors* is used here to indicate the *alpha*-2 adrenergic receptor heterogeneity between different species whereas the term *subtype* is reserved to indicate heterogeneity of receptors within the same animal.

Our working hypothesis is that the pharmacologic differences in rodent and nonrodent *alpha*-2 adrenergic receptors are due to the existance of *alpha*-2 isoceptors. Alternatively, these differences could be due to 1) differential proteolysis of the receptors, 2) degradation of the ligand, 3) the detection of different affinity states in the different tissues and 4) the presence of different quantities of various affinity modulators (primarily monovalent and divalent cations). In this paper, we describe experiments which help to eliminate these alternate hypotheses and also describe the solubilization and characterization of putative *alpha*-2 adrenergic isoceptors from the rat cerebral cortex and the human platelet. The *alpha*-2 receptor has been solubilized previously from human platelets using the detergent digitonin (Michel *et al.*, 1981; Smith and Limbird, 1981), from the adrenocortical carcinoma 494 using CHAPS (Nambi *et al.*, 1982) and, during the course of this study, from the calf cerebral cortex using CHAPS (Sladeczek *et al.*, 1984).

Materials and Methods

[^{3}H]Yohimbine (specific activity, 81–84 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Digitonin was obtained

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ABBREVIATIONS: CHAPS, 3-[3-cholamidopropyldimethylammonio]-1-propane sulfate; UC-14,304, 5-bromo-6-[2-imidazolin-2-ylamine]-quinoxaline; PEG, polyethylene glycol.

from J. T. Baker Co. (Phillipsburg, NJ), Calbiochem (San Diego, CA) and Fisher Scientific Company (Fair Lawn, NJ). Azocoll, Tris, glycylglycine, EDTA, Sephadex G50 (100–300 μm), bovine γ-globulin, bovine serum albumin, polyethylene glycol (mw 8000), (−)-epinephrine, (−)-norepinephrine and (−)-isoproterenol were purchased from the Sigma Chemical Company (St. Louis, MO). The following drugs were kindly donated by the indicated companies: (−)-isoproterenol and (+)-norepinephrine, Sterling-Winthrop Research Institute (Rensselaer, NY); prazosin, Pfizer Chemical Division, Pfizer, Inc. (New York, NY); oxymetazoline, Schering Corporation (Kenilworth, NJ); and UK-14304, Pfizer Central Research (Sandwich, Kent, England).

Tissue preparation. Female Sprague-Dawley rats (Sasco, Omaha, NE) were decapitated, and their brains were removed immediately and dissected on ice. The tissue was either stored at −20°C or used fresh. There was no significant difference in the parameters measured in fresh *vs.* frozen tissue. Generally, 0.5 g of cortex was homogenized in 35 ml of ice-cold 50 mM Tris-HCl (pH 8.0 at 25°C) using a Tekmar Tissumizer at maximum speed for 20 sec. The homogenate was centrifuged at 48,000 × *g* for 10 min. The supernatant was discarded and the pellet was resuspended and centrifuged twice. Outdated platelets obtained from the blood bank were prepared as described previously (Jones *et al.*, 1983).

Membrane binding assay. The washed particulate fraction was resuspended at 100 volumes (1 g wet weight/100 ml of buffer) in 25 mM glycylglycine (pH 7.4). For saturation experiments, 970 μl of tissue homogenate was incubated with increasing concentrations of [^{3}H] yohimbine (20 μl) with or without norepinephrine (10 μl). Specific binding was defined as the difference of binding in absence or presence of 10 μM norepinephrine. The data were transformed by the method of Rosenthal (1967) and the maximum binding and K_D values were calculated using unweighted linear regression analysis. Inhibition experiments were conducted by incubating the tissue preparation with 8 to 10 concentrations of an unlabeled drug and a fixed concentration of [^{3}H]yohimbine. After 30 min at 23°C, the suspensions were filtered through GF/B glass fiber filters (Whatman, Clifton, NJ) and washed with 10 ml of ice-cold 50 mM Tris-HCl (pH 8.0). Radioactivity retained by the G/B filters was determined by scintillation spectroscopy using 2a70 (Research Products International, Mount Prospect, IL) at 38 to 41% counting efficiency. The concentration of drug which inhibits 50% of the specific binding (IC_{50}) was determined by logit transformation (Robard and Frazier, 1975). The inhibitory dissociation constant was calculated from $K_i = IC_{50}/(1 + F/K_D)$ where F is the free radioligand concentration and K_D is the equilibrium dissociation constant.

Supernatant transfer experiments. One gram of rat cerebral cortex was homogenized in 35 ml of platelet lysing buffer (5 mM Tris-HCl and 5 mM EDTA, pH 7.5), using the Tissumizer at maximum speed for 20 sec and centrifuged at 48,000 × *g* for 10 min. The resultant supernatant was saved on ice. The membrane pellet was resuspended in 50 mM Tris-HCl, pH 8.0, and recentrifuged as before. Human platelets were prepared as described by Jones *et al.* (1983) and treated in a similar manner. Washed rat cerebral cortical membranes were resuspended in 35 ml of either human serum or homogenized human platelet supernatant. Similarly, washed human platelet membranes were resuspended in 35 ml of homogenized rat cerebral cortex supernatant. Both preparations were incubated for 20 min at 37°C and washed once with 50 mM Tris-HCl, pH 8.0. Prazosin inhibition of [^{3}H] yohimbine binding was then conducted as described previously.

Ligand rebinding experiments. Prazosin inhibition of [^{3}H]yohimbine binding experiments were set up in the usual manner for both human platelets and the rat cerebral cortex. Instead of separating bound from free by filtration, the tubes were centrifuged at 48,000 × *g* for 10 min at 4°C. Nine hundred microliters of supernatant containing the unbound [^{3}H]yohimbine and prazosin from human platelets were transferred to tubes containing rat cerebral cortical membranes in 100 μl of 25 mM glycylglycine, prepared as usual but at 10 times the concentration (1 g wet weight/10 ml of buffer). The reciprocal experiment was conducted with rat cortical supernatants and human platelet membranes. After a 30-min incubation at 23°C the assay was filtered and counted as usual. New ligand concentrations were calculated assuming a negligible change in the free ligand concentration.

Solubilization and binding assay of *alpha*-2 adrenergic receptors. The washed particulate fraction from 0.5 g of rat cortex or platelet membranes (25–30 mg/ml of protein) was homogenized (Teflon-glass homogenizer, 30 strokes) in 10 ml of 1% (w/v) digitonin, 25 mM glycylglycine and 5 mM EDTA (pH 7.4). After 30 min on ice, the preparation was centrifuged at 100,000 × *g* for 60 min at 4°C and the pellet discarded. The transparent supernatant was diluted with 5 ml of ice-cold 25 mM glycylglycine and 5 mM EDTA, pH 7.4.

The binding assay contained the same volume of radioligand and unlabeled drugs as the membrane assay, except 470 μl of digitonin receptor extract was substituted for 970 μl of membrane suspension for a final volume of 0.5 ml. Nonspecific binding was determined in the presence of 200 μM norepinephrine for saturation experiments. After 2 to 3 hr at 5°C, [^{3}H]yohimbine bound was separated from free by placing each 0.5-ml sample onto a 1 × 18.5 cm Sephadex G-50 column equilibrated and subsequently developed with 25 mM glycylglycine and 5 mM EDTA, pH 7.4 at 4°C. An aliquot of 0.1% (w/v) digitonin in equilibration buffer was added before sample application to reduce sample adsorption to the column. Two milliliters of effluent containing the void volume of the column was collected and counted in 10 ml of Budget Solve (Research Products International, Mount Prospect, IL). Alternatively, 200 to 250 μl of 15 mg/ml bovine γ-globulin and 1 ml of 30% (w/v) PEG at 4°C were added to precipitate the radioligand-receptor complex. After a 10-min incubation at 5°C, the mixture was filtered through GF/B filters and washed with 10 ml of 8.5% (w/v) PEG. The retained radioactivity was detected using scintillation spectroscopy.

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. One percent digitonin, 25 mM glyclyglycine and 5 mM EDTA were included in the standards where indicated.

Sucrose density gradient centrifugation. Membrane particulate homogenates were prepared with 10 μg/ml of soybean trypsin inhibitor, 30 μM phenylmethylsulfonyl fluoride (PMSF) and 100 μg/ml of bacitracin. After solubilizing the receptor and subjecting the preparation to centrifugation at 100,000 × *g* as described previously, the sample was concentrated 10-fold using a CF25 Centriflo membrane cone (Amicon, Danvers, MA) and then centrifuged at 480 × *g* for 35 min. The concentrated sample was carefully layered over a 10 ml, 5 to 20% linear sucrose gradient containing 0.1% digitonin and buffered with 25 mM glycylglycine and 5 mM EDTA. The gradient was centrifuged at 200,000 × *g* for 12 to 13 hr. Catalase and yeast alcohol dehydrogenase were used as markers in separate gradients. Each gradient was fractionated into 700- to 800-μl fractions. Enzyme markers were detected by their ability to absorb at 280 nm whereas receptor fractions were diluted with 300 μl of 25 mM glycylglycine, 5 mM EDTA and were assayed for [^{3}H]yohimbine binding as described above.

Results

Human platelet and rat cerebral cortical homogenate supernatant transfer experiments. The hypothesis that differences in the *alpha*-2 receptors of rodents and nonrodents are due to differential proteolytic activity in various tissues was examined by transferring the supernatant from rat cerebral cortical homogenates to human platelet membranes. The analogous experiment, incubating human serum and platelet lysate with rat cerebral cortical membranes, was also conducted. Control and experimental resuspensions were incubated for 20 min at 37°C to allow proteolytic activity to occur. The inhibition of [^{3}H]yohimbine binding by unlabeled prazosin demonstrated that the relative pharmacological differences between rodent and nonrodent receptors were not affected by incubation of the receptors in the supernatant of the complementary species (fig. 1).

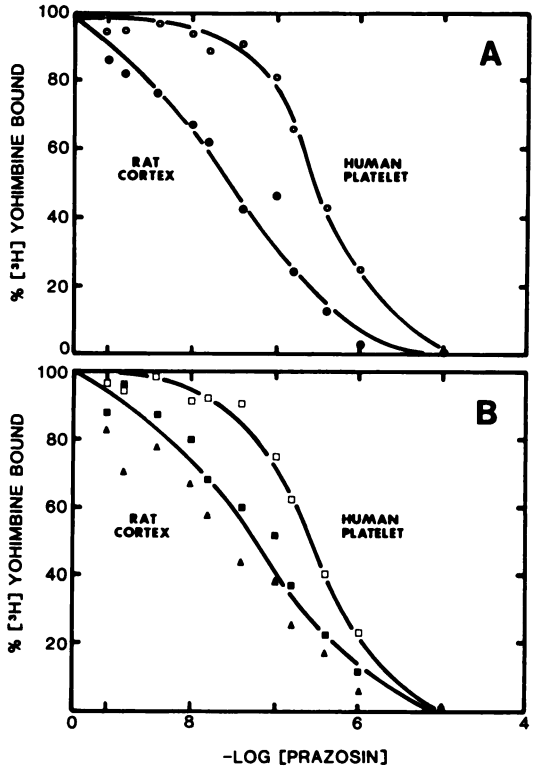

Fig. 1. Supernatant transfer experiments. Human platelet and rat cerebral cortical homogenates were prepared and centifuged at 48,000 × *g* for 10 min. One set of tubes containing human platelet (○) and rat cerebral cortex (●) was resuspended in fresh assay buffer (A) whereas in the other set (B) the rat membrane preparation was resuspended in serum (▲) or platelet lysate (■). Similarly, human platelet membranes were resuspended in rat cortical homogenate supernatants (□). All sets were incubated at 37°C for 20 min, washed and [^{3}H]yohimbine *vs.* prazosin inhibition experiments were conducted.

TABLE 1

Effect of proteolytic inhibitors on the prazosin inhibition of [^{3}H] yohimbine binding

	K_i	n_H
	nM	
Human platelet[a]		
10 μM STI, 30 μM PMSF and 100 μg/ml of bacitracin	120 ± 3	0.94 ± 0.17
1 mM pAPMSF	150 ± 17	0.85 ± 0.03
Rat cerebral cortex		
10 μg of STI, 30 μM PMSF and 100 μg/ml of Bacitracin	22 ± 3	0.50 ± 0.01
1 mM pAPMSF	25 ± 4	0.56 ± 0.05
5 mM EDTA	41 ± 6	0.82 ± 0.02

[a] The buffer used in the preparation of platelet membranes already contains 5 mM EDTA. STI, soybean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; pAPMSF, *p*-amidinophenylmethylsulfonyl fluoride.

Azocoll is an azodye-coupled, general proteolytic substrate for the detection of most types of proteolytic activity. Incubation of 10 mg of washed Azocoll in 25 times the usual concentration of human platelet or rat cerebral cortex homogenates (200 μl total volume) showed minimal proteolytic activity when incubated at 37°C for 34 min. These activities are equivalent to 0.05 benzoyl-arginine ethyl ester units (rat cortex) and 0.13 benzoyl-arginine ethyl ester units (human platelet) of trypsin activity. Inclusion of 10 μg/ml of soybean trypsin inhibitor, 30 μM phenylmethylsulfonyl fluoride and 100 μg/ml of bacitracin, 1 mM *p*-amidinophenylmethylsulfonyl fluoride or 5 mM EDTA in the preparation buffer did not abolish the rodent *vs.* nonrodent differences as determined by the inhibition of [^{3}H]yohimbine binding by prazosin (table 1). The preparation of human platelets routinely included 5 mM EDTA (Jones *et al.*, 1983).

Rebinding of [^{3}H]yohimbine and unlabeled prazosin after incubation with human platelet and rat cerebral cortical membranes. To determine if the rodent and nonrodent differences are due to changes in the labeled or unlabeled ligand, rebinding experiments were performed. Washed human platelet or rat cerebral cortical membranes were incubated with [^{3}H]yohimbine and various concentrations of prazosin. The membranes were sedimented by centrifugation and supernatants containing unbound ligands were transferred to fresh membranes of the same species (fig. 2A) or to the opposing species (fig. 2B). The results indicate that previous incubation

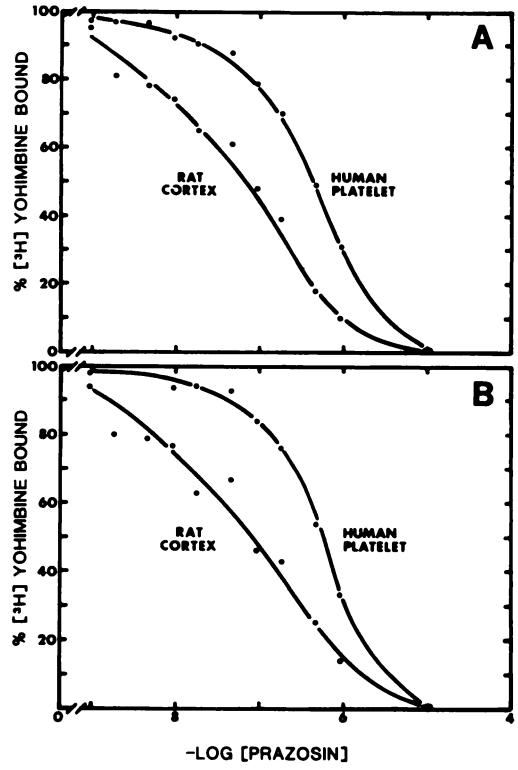

Fig. 2. Ligand rebinding in human platelet and rat cerebral cortex membranes. Human platelet (●) and rat cerebral cortical (○) membranes were incubated with a fixed concentration of [^{3}H]yohimbine and various concentrations of unlabeled prazosin. After 30 min at 23°C the tubes were centrifuged for 10 min at 48,000 × *g* and a 900-μl aliquot containing the unbound [^{3}H]yohimbine and prazosin was transferred back onto fresh tissue of the same species (A) or onto tissue of the other species (B). Tubes were incubated at room temperature for 30 min and bound separated from free using vacuum filtration.

TABLE 2

Prazosin inhibition of [^{3}H]yohimbine binding: effects of $MgCl_2$, GppNHp and NaCl

	K_i	n_H
	nM	
Human platelet		
Control	217 ± 53	0.77 ± 0.06
1 mM $MgCl_2$	266 ± 30	0.97 ± 0.19
Rat cerebral cortex		
Control	55 ± 15	0.61 ± 0.08
100 μM GppNHp	71 ± 10	0.72 ± 0.07
40 μM NaCl	36 ± 6	0.75 ± 0.04

of the ligands with tissue of the opposing species did not affect the relative pharmacological differences between rodent and nonrodent species.

The effect of GTP and $MgCl_2$ on the inhibition of [^{3}H] yohimbine binding by prazosin. An alternative hypothesis states that the rodent and nonrodent differences are due to the labeling of different receptor affinity states (this hypothesis assumes that prazosin would be an agonist at *alpha*-2 receptors, see "Discussion") or to the presence of various amounts of *alpha*-2 adrenergic receptor affinity modulators (GTP, $MgCl_2$ or NaCl). We tested this hypothesis by attempting to shift the K_i of prazosin of one species toward the other by adding maximal concentrations of guanine nucleotides or other affinity modulators. Table 2 summarizes the data from inhibition experiments using [^{3}H]yohimbine and unlabeled prazosin in the presence or absence of 100 μM GppNHp or 1 mM $MgCl_2$. Inasmuch the rodent and nonrodent differences were originally observed in a buffer with approximately 10 mM Na^+ (NaOH was used to adjust the pH of the glycylglycine buffer), sodium did not appear to be the modulator responsible for the rodent and nonrodent affinity differences. Furthermore, neither addition of 40 mM NaCl nor the addition of 100 μM GppNHp or 1 mM $MgCl_2$ was able to abolish the ability of prazosin to discriminate between the putative *alpha*-2 isoceptors (table 2).

Soluble *alpha*-2 adrenergic receptor saturation experiments. In an effort to characterize further the *alpha*-2 adrenergic isoceptors, prototype receptors were solubilized with 1% digitonin from the human platelet and rat cerebral cortex. After centrifugation at 100,000 × *g* for 60 min the supernatant was diluted and incubated with increasing concentrations of [^{3}H] yohimbine in the absence and presence of 200 μM (−)-norepinephrine. Soluble receptors from both the human platelet and rat cerebral cortex were saturable and retained high affinity when bound ligand was separated from free ligand using the gel filtration technique (figs. 3 and 4). The PEG separation technique gave comparable results for the soluble *alpha*-2 receptor from the rat cerebral cortex (K_D = 5.9 ± 0.8 nM, B_{max} = 68 ± 11 fmol/mg of protein). Table 3 summarizes and compares membrane and soluble *alpha*-2 adrenergic receptor dissociation constants and densities. In general, soluble receptors showed both a lower affinity (an increase in K_D) and a lower density. In platelet experiments reported here, 33% of the protein and 28% of the receptors were solubilized. For the rat cerebral cortex 54% of the membrane protein and 21% of the *alpha*-2 receptors were solubilized.

Soluble *alpha*-2 adrenergic receptor inhibition experiments. Table 4 summarizes the results of inhibition experiments in which the indicated unlabeled drugs were added in increasing concentrations to fixed concentrations of [^{3}H]yohimbine and membrane homogenate or solubilized *alpha*-2 adrenergic receptor. Human platelet and rat cerebral cortex soluble *alpha*-2 receptor preparations displayed the relative potencies of (−)-epinephrine > (−)-norepinephrine ≫ (−)-isoproterenol, suggesting an *alpha* rather than *beta* character. In addition, stereoselectivity of the (−)- and (+)-enantiomers of norepinephrine was retained for both soluble receptors. The selective *alpha*-2 antagonist, yohimbine, was more potent than the *alpha*-1 antagonist, prazosin, thereby establishing the *alpha*-2 nature of the soluble receptor from both human platelets and rat cerebral cortex. The Hill coefficients (n_H) of agonists are generally lower than those measured for antagonists in membranes. This property was retained when the receptor was solubilized.

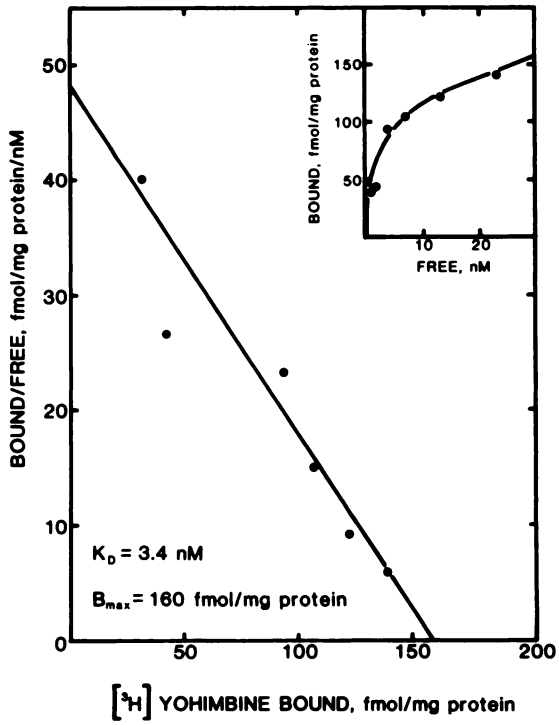

Fig. 3. [^{3}H]Yohimbine binding to solubilized *alpha*-2 receptors from the human platelet. Soluble receptor preparations were incubated with increasing concentrations of [^{3}H]yohimbine. Specific binding was defined as the difference in binding in the absence and presence of 200 μM (−)-norepinephrine. Bound was separated from free ligand using the gel filtration method. Specifically bound [^{3}H]yohimbine is plotted against free [^{3}H]yohimbine concentration in the insert. These data were transformed by the method of Rosenthal (1967) and the maximum binding (B_{max}) and K_D values were calculated using unweighted linear regression analysis.

The K_i values of both soluble receptors were up to 40-fold larger than the values measured in the membrane but correlated well with the rank order of potency established in membrane homogenates (fig. 5). The K_i of yohimbine measured in inhibition studies with the soluble receptor agrees well with the K_D determined from saturation studies.

Figure 6 compares the inhibition by oxymetazoline and prazosin of [^{3}H]yohimbine binding. The relative pharmacological differences between rodent and nonrodent isoceptors are retained upon solubilization.

Effect of GTP and GppNHp on the soluble human platelet and rat cerebral cortex *Alpha*-2 receptors. UK-14304 is putative full *alpha*-2 adrenergic agonist (Loftus *et al.*, 1984). UK-14304 inhibition of [^{3}H]yohimbine binding showed the expected 3- and 4-fold decrease in affinity in the presence of 100 μM GTP or GppNHp when examined in membrane preparations of human platelet and rat cerebral cortex, respectively. However, when these receptors were solubilized, guanine nucleotide sensitivities could not be demonstrated (table 5).

Sucrose density gradient centrifugation of the solubilized receptors. Solubilized receptors were concentrated by ultrafiltration and centrifuged for 12 to 13 hr on a 5 to 20% linear sucrose gradient. The gradient was fractionated and assayed for specific [^{3}H]yohimbine binding (fig. 7). No significant differences were observed in the sedimentation profiles of soluble human platelet and rat cerebral cortical *alpha*-2 receptor.

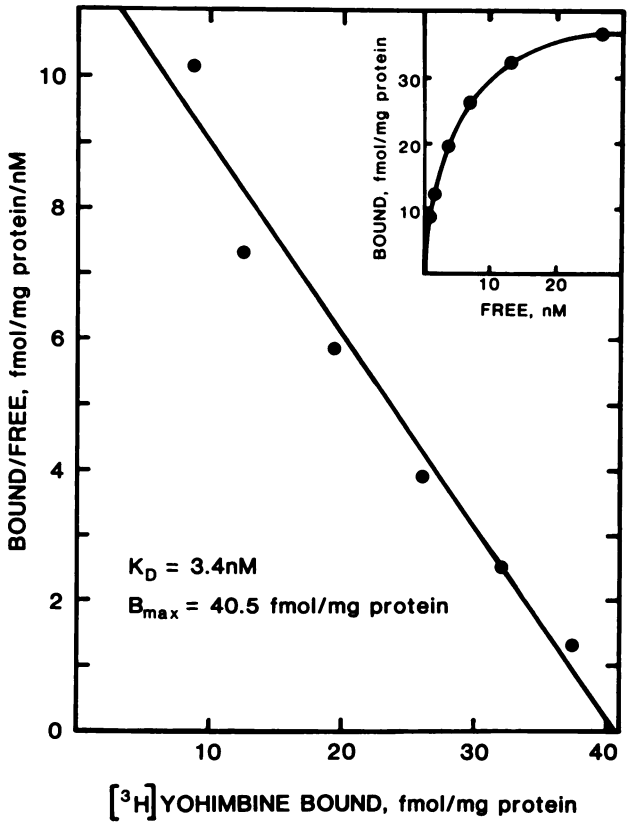

Fig. 4. [^{3}H]Yohimbine binding to solubilized *alpha*-2 receptors from the rat cerebral cortex. Increasing concentrations of [^{3}H]yohimbine (0.9–27 nM) were incubated with soluble rat cerebral cortex preparation. Bound was separated from free using the gel filtration method. B, max, maximum binding.

TABLE 3

Affinities and densities of [^{3}H]yohimbine binding for membranes and solubilized *alpha*-2 receptors

	Membranes	Solubilized
Human platelets		
K_D, nM	0.35 ± 0.02[a]	4.2 ± 1.2
B_{max},[b] fmol/mg of protein	461 ± 24[a]	169 ± 9
Rat cerebral cortex		
K_D, nM	2.2 ± 0.3	5.9 ± 1.6
B_{max}, fmol/mg of protein	121 ± 16	47 ± 6

[a] Data from Jones *et al.* (1983).
[b] B_{max}, maximum binding.

Discussion

Alpha-2 adrenergic receptor heterogeneity has been suggested previously by a number of investigators. Functional studies suggest *alpha*-2 receptor differences may occur between species (Turner *et al.*, 1984; Bylund, 1985) or within the same tissue (Raiteri *et al.*, 1983). Radioligand binding studies (Cheung *et al.*, 1982; Randall *et al.*, 1983; Feller and Bylund, 1984) have generally led various investigators to the same hypothesis. We propose the term isoceptor to be used to indicate pharmacological differences which occur between species and reserve the term subtype to indicate heterogeneity within the same organism.

Alternative hypotheses do not explain differences between rodents and nonrodents. Previously reported differences between rodent and nonrodent *alpha*-2 adrenergic receptors (Cheung *et al.*, 1982; Feller and Bylund, 1984) have been confirmed using the rat cerebral cortex and human platelet. The results of this study indicate that these differences are not due to 1) differential proteolysis of the receptor, 2) degradation of the ligand, 3) the detection of different affinity states or 4) the presence of different amounts of monovalent and divalent cations. The elimination of these alternative hypotheses gives support to the original hypothesis of *alpha*-2 isoceptors.

If the observed receptor differences were due to differential proteolytic cleavage of the same receptor, one might expect to abolish those differences by exposing the receptor to the proteases. However, incubating supernatant fractions from tissue homogenates from one species with the membranes of the other or with proteolytic inhibitors did not alter the observed differences. Proteolytic activity appeared to be minimal even in preparations 25 times more concentrated than usual. Thus, soluble proteases cannot account for these differences.

Recent evidence in the study of the *alpha*-1 receptor (Leeb-Lundberg *et al.*, 1984) and *beta* adrenergic receptors (Stiles *et al.*, 1984) using photoaffinity labeling indicates that lower molecular weight fragments appear to be generated from the action of metaloproteases. Although 2 mM EDTA appeared to inhibit the appearance of these lower molecular weight products, no substantial evidence was provided to rule out the alternative suggestion that this effect was due to the alteration of post-translational modifications including phosphorylation and glycosylation or the possibility that these larger forms are the precursors for normal processing of the adrenergic receptor. In any case, inclusion of 5 mM EDTA or various serine protease inhibitors and bacitracin did not affect the observed affinity differences for prazosin between the rat cerebral cortex and the human platelet (table 1). Although it may still be argued that the right inhibitor may have been overlooked, these results make it less likely that proteolytic modification of the same receptor can fully account for the differences in the affinity of prazosin.

The hypothesis that the affinity differences are due to the metabolism of the ligand rather than differences in the receptor was tested. Degradation of the ligands did not account for these pharmacological differences as demonstrated in the rebinding experiments (fig. 2). These results also indicate that the *alpha*-2 receptor differences are inherent in the particulate fraction and not in the surrounding soluble environment or ligands.

Drugs such as BHT-920 acting as an *alpha*-2 agonist (van Meel *et al.*, 1981) and an *alpha*-1 antagonist (Kobinger and Pichler, 1981) coupled with the observation that, in the rat, prazosin is more potent and consistently displays Hill coefficients less than that found in the human platelet led to the hypothesis that, in the rat, the greater affinity of prazosin is due to the detection of different agonist affinity states. A critical assumption of this hypothesis is that prazosin acts as an *alpha*-2 agonist. The failure of guanine nucleotides to modulate the rat cerebral cortical *alpha*-2 receptor affinity in the usual manner indicated that this hypothesis was not likely to be correct (table 2). Neither is there any direct functional evidence that prazosin acts as an *alpha*-2 agonist in the rat cortex.

The effect of affinity modulators, generally monovalent and divalent cations, on platelet *alpha*-2 adrenergic receptor affinity has been documented (Limbird *et al.*, 1982; Bylund and U'Prichard, 1983). Sodium appears to slightly increase the apparent affinity for antagonists in the human platelet. The inclusion of 10 mM Na^+ in the glycylglycine buffer and the magnitude of the sodium effect makes it unlikely that differences in sodium

TABLE 4

Inhibition of [^{3}H]yohimbine binding by various agonists and antagonists in the rat cortex and human platelets

IC_{50} values (concentration of drug which inhibits 50% of the specific binding) were determined by logit transformation. The inhibitory dissociation constant (K_i ± S.E.M.) was calculated from $K_i = IC_{50}/(1 + F/K_D)$ where F is the free radioligand concentration (membranes: 0.2 nM; soluble: 2.4–5.3 nM). The Hill coefficient is represented by n_H.

Drug	Human Platelets				Rat Cerebral Cortex			
	Membranes		Soluble receptor		Membranes		Soluble receptor	
	K_i	n_H	K_i	n_H	K_i	n_H	K_i	n_H
	nM		nM		nM		nM	
1. Oxymetazoline	0.8 ± 0.1	0.91 ± 0.01	11 ± 5	0.58 ± 0.11	9 ± 1	0.61 ± 0.03	241 ± 124	0.39 ± 0.10
2. UK 14304	8 ± 2	0.76 ± 0.04	44 ± 27	0.66 ± 0.06	20 ± 4	0.82 ± 0.04	279 ± 166	0.77 ± 0.33
3. (−)-Epinephrine	156 ± 62	0.78 ± 0.06	347 ± 22	0.79 ± 0.14	15 ± 6	0.58 ± 0.10	147 ± 53	0.45 ± 0.12
4. (−)-Norepinephrine	314 ± 107	0.83 ± 0.06	591 ± 42	0.85 ± 0.19	84 ± 11	0.58 ± 0.03	564 ± 275	0.43 ± 0.10
5. (+)-Norepinephrine	3,190 ± 110	0.78 ± 0.02	2,290 ± 260	1.03 ± 0.14	1,100 ± 300	0.64 ± 0.05	3,560 ± 400	0.37 ± 0.07
6. (−)-Isoproterenol	22,500 ± 4,100	0.79 ± 0.10	259,000 ± 33,000	1.06 ± 0.09	6,900 ± 500	0.56 ± 0.09	124,000 ± 75,000	0.48 ± 0.03
7. Phentolamine	7 ± 1	1.12 ± 0.12	3 ± 1	1.01 ± 0.10	9 ± 2	1.00 ± 0.5	20 ± 2	0.66 ± 0.13
8. Yohimbine	0.6 ± 0.2	1.04 ± 0.06	3 ± 1	0.93 ± 0.09	5 ± 1	0.87 ± 0.01	9 ± 5	0.83 ± 0.06
9. Prazosin	217 ± 53	0.97 ± 0.06	8,700 ± 300	1.40 ± 0.25	55 ± 15	0.61 ± 0.08	470 ± 100	0.62 ± 0.09

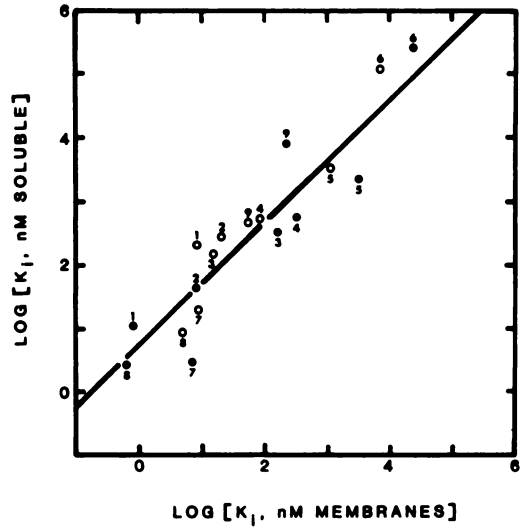

Fig. 5. Comparison of the affinities of adrenergic drugs for membrane and solubilized *alpha*-2 receptors. K_i values for membrane and soluble human platelet (●) and rat cerebral cortex (○) *alpha*-2 receptors were determined from inhibition experiments using [^{3}H]yohimbine. Drugs are identified numerically as listed in table 4. Linear regression of the data resulted in a slope of 1.00 and $r^2 = 0.84$.

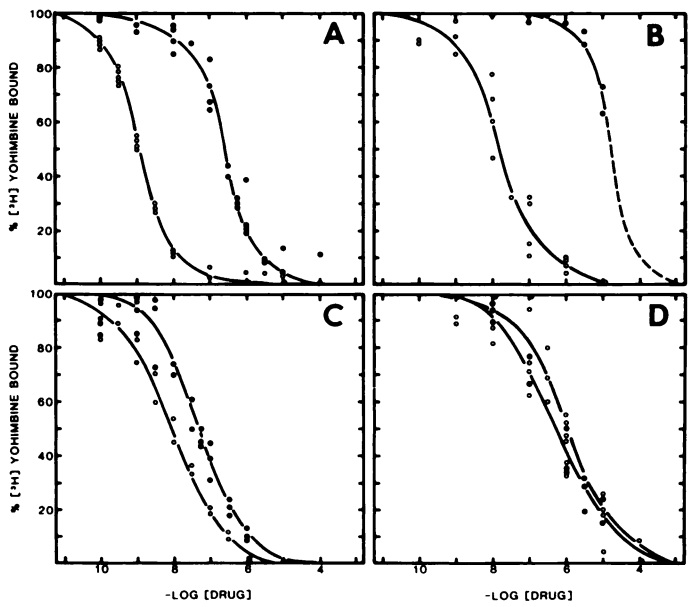

Fig. 6. Inhibition of [^{3}H]yohimbine binding to solubilized human platelet and rat cerebral cortex *alpha*-2 receptors. Human platelet membranes (A), soluble human platelet *alpha*-2 receptors (B), rat cortex membranes (C) and soluble rat cerebral cortex *alpha*-2 receptors (D) were incubated with [^{3}H]yohimbine and the indicated concentrations of unlabeled oxymetazoline (○) or prazosin (●).

could fully account for the rodent *vs.* nonrodent differences. Direct addition of 40 mM NaCl did not abolish or change the relative receptor differences in the affinity for prazosin. The inclusion of 1 mM $MgCl_2$ (table 1) also did not affect the relative differences in potencies. With the observation that the magnesium effect for antagonists is generally small (2- to 3-fold, Bylund and U'Prichard, 1983), it appears that the differential effects of magnesium cannot explain the *alpha*-2 receptor differences. By using the affinity differences displayed by prazosin as an index for the relative differences observed between rodent and nonrodent species, it appears unlikely that any of the proposed alternative hypotheses are viable.

Soluble *alpha*-2 receptors were saturable and retained high affinity. In an effort to provide direct evidence of *alpha*-2 adrenergic receptor heterogeneity, we solubilized receptors from both human platelet and rat cerebral cortex. The binding of [^{3}H]yohimbine to both receptors was saturable and retained high affinity (table 3). Comparison with the data obtained in membranes indicated a small decrease of affinity in the rat cortex but a larger decrease of affinity in the human platelet. This difference in the two receptors resulted in the loss of the 5- to 10-fold difference in affinity that distinguishes rodent and nonrodent species. The K_D reported here for the soluble *alpha*-2 receptor from the human platelet agrees well with the K_D reported previously by Smith and Limbird (1981).

Generally, 21% (rat cortex) and 28% (human platelet) of the *alpha*-2 adrenergic receptors were solubilized. This yield compares well with the solubilization of brain muscarinic, dopaminergic and serotonergic receptors where yields vary between 5 to 20% (Laduron and Ilien, 1982). Human platelet *alpha*-2 adrenergic receptors have been solubilized with a reported yield of 85% (Smith and Limbird, 1981). However, in an independent determination under slightly different detergent and buffer conditions (Michel *et al.*, 1981), the maximum number of receptor sites was 20% of that reported by Smith and Limbird (1981). Nambi *et al.* (1982), using the dipolar detergent CHAPS in the adrenocortical carcinoma 494, reported that [^{3}H]dihy-

TABLE 5
Inhibition of [^{3}H]yohimbine binding by UK-14304 in the presence or absence of GTP or GppNHp

	Human Platelet				Rat Cortex			
	Membranes		Soluble		Membranes		Soluble	
	K_i	n_H	K_i	n_H	K_i	n_H	K_i	n_H
UK-14304	8 ± 2	0.76 ± 0.04	43 ± 26	0.66 ± 0.06	20 ± 4	0.82 ± 0.04	279 ± 166	0.77 ± 0.33
+100 μM GTP	21 ± 2	0.82 ± 0.10	59 ± 31	0.84 ± 0.09				
+100 μM GppNHp	21 ± 3	0.93 ± 0.06			89 ± 27	0.75 ± 0.16	309 ± 110	0.68 ± 0.04

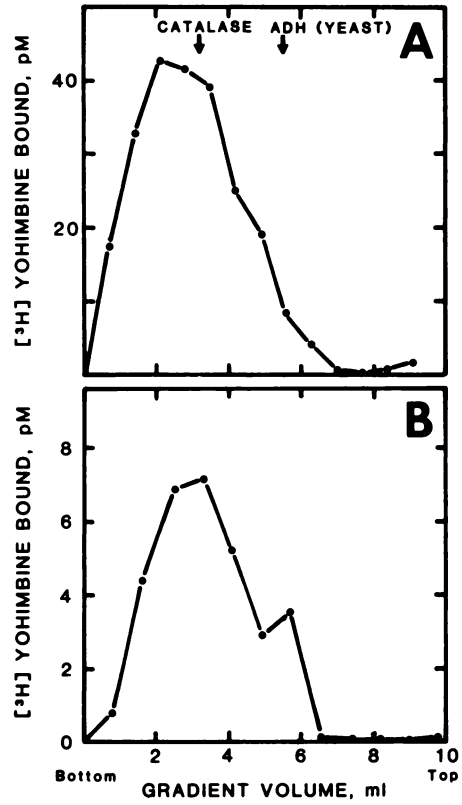

Fig. 7. Density gradient centrifugation of solubilized human platelet and rat cerebral cortex *alpha*-2 adrenergic receptors. Solubilized *alpha*-2 receptors from the human platelet (A) and rat cerebral cortex (B) were concentrated approximately 10 times by ultrafiltration and centrifuged for 13 hr at 200,000 × *g* on a linear 5 to 20% sucrose gradient. Approximately 0.5-ml fractions were then analyzed for specific [^{3}H] yohimbine binding in the presence or absence of 200 μM (−)-norepinephrine. Catalase and yeast alcohol dehydrogenase were used as standards.

droergotamine binding to the soluble and membrane bound receptors was identical and that 30 to 45% of the protein was solubilized.

The solubilized *alpha*-2 receptor retained the appropriate pharmacology. The pharmacological profile of both solubilized receptors paralleled that found in membranes (fig. 5). The relative potencies of (−)-epinephrine > (−)-norepinephrine ≫ (−)-isoproterenol, and steroselectivity of the enantiomers of norepinephrine and the greater potency of yohimbine when compared to prazosin established the *alpha*-2 nature of the solubilized receptors. The K_i values for the soluble receptors ranged from the same to 40-fold higher than the values measured in the membranes. Similar observations of lowered affinity have been made previously by Smith and Limbird (1981) and Sladeczek *et al.* (1984). In agreement with saturation experiments (table 3), the 8-fold difference in yohimbine affinity between the rat and human tissue was reduced upon solubilization to only a 3-fold difference. However, the relative rodent *vs.* nonrodent affinities for prazosin and oxymetazoline were retained after solubilization (fig. 6). In the human platelet oxymetazoline is generally 200- to 800-fold more potent than prazosin in competing for [^{3}H]yohimbine binding to the *alpha*-2 receptor. This difference was retained when the membranes were solubilized. In contrast, oxymetazoline was only 2- to 6-fold more potent than prazosin at the *alpha*-2 receptors in membranes from the rat cerebral cortex when measured under similar conditions. Prazosin was more potent at the soluble receptors from the rat cortex as compared to the human platelet, whereas oxymetazoline was more potent at the soluble receptor from the human platelet than the rat cortex. Thus, the relative affinity differences for oxymetazoline and prazosin which characterize rodent and nonrodent *alpha*-2 isotypes were retained upon solubilization.

Solubilized *alpha*-2 receptors are insensitive to guanine nucleotides. The Hill coefficients (n_H) of agonists are generally lower than those of antagonists. This property was retained after receptor solubilization. Previous work in the human indicates that solubilization abolished the ability of guanine nucleotides to regulate the *alpha*-2 receptor. However, when the receptor was preoccupied with an agonist and solubilized regulation by guanine nucleotides was retained (Smith and Limbird, 1981). On sucrose density gradients, the agonist prelabeled receptor appeared to be larger than the unoccupied receptor, suggesting that it was associated with the inhibitory guanine nucleotide regulatory subunit (N_i) (Smith and Limbird, 1981, 1982).

Sladeczek *et al.* (1984) working with the calf cerebral cortex demonstrated a retention of guanine nucleotide sensitivity in their solubilized preparations using the detergent CHAPS. If, in the rat cerebral cortex, the solubilized receptor is still coupled to the inhibitory guanine nucleotide regulatory subunit (N_i), then guanine nucleotide sensitivity similar to the soluble calf cerebral cortex should be observed. This would also explain the apparent low Hill coefficients for agonists. In a direct test of this hypothesis (table 5) with the *alpha*-2 agonist UK-14304, guanine nucleotide sensitivity could not be demonstrated. Alternative hypotheses for the low Hill coefficient include the presence of negative cooperativity or the existence of subtypes within the rat cerebral cortical *alpha*-2 receptors. Current work is being directed toward elucidating this point (Bylund, 1985).

The solubilized *alpha*-2 receptors have similar sedimentation profiles. Sucrose density gradient centrifugation of the soluble human platelet and rat cortex receptors was in good agreement with values obtained previously by Michel *et al.* (1981) and Smith and Limbird (1981). No difference in the molecular size of the two isoceptors was observed using this technique. Although the exact contribution of aggregation and detergent binding could not be assessed, these results are con-

sistent with the data obtained using radiation inactivation (Venter *et al.*, 1983) in which the size of the receptor was estimated to be fairly large (160,000 daltons).

The results presented in this paper are consistent with the hypothesis that there exists rodent and nonrodent *alpha*-2 adrenergic isoceptors. The definitive evidence, however, will require the discovery of additional selective drugs and the eventual purification and sequencing of the putative *alpha*-2 adrenergic isoceptors.

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