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Synthesis and Characterization of a High Affinity Radioiodinated Probe for the α_2-Adrenergic Receptor

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SUMMARY

The availability of radioiodinated probes has facilitated the localization and molecular characterization of cell membrane receptors for hormones and neurotransmitters. However, such probes are not available for the study of the α_2-adrenergic receptor. This report describes the synthesis and characterization of functionalized derivatives of the selective α_2-adrenergic antagonists, rauwolscine and yohimbine, which can be radiolabeled to high specific activity with ^{125}I. Following demethylation of rauwolscine or yohimbine, the resultant carboxylic acid derivatives were reacted with 4-aminophenethylamine to yield the respective 4-aminophenethyl carboxamides, 17α-hydroxy-20α-yohimban-16β-[*N*-4-amino-phenethyl]carboxamide (rau-pAPC) and 17α-hydroxy-20β-yohimban-16α-[*N*-4-aminophenethyl]carboxamide. In competitive inhibition studies using rat renal membranes and the radioligand [^{3}H]rauwolscine, rau-pAPC (K_i = 11 ± 1 nM) exhibited a 14-fold greater affinity than the corresponding yohimbine derivative (K_i = 136 ± 45 nM). The higher affinity compound, rau-pAPC, was radioiodinated by the chloramine T method, and the product, ^{125}I-rau-pAPC [17α-hydroxy-20α-yohimban-16β-(*N*-4-amino-3-[^{125}I]iodophenethyl)carboxamide], was purified by reverse phase HPLC to high specific activity (2175 Ci/mmol) and its binding characteristics were investigated in rat kidney membranes. Specific binding of ^{125}I-rau-pAPC was saturable and of high affinity as determined by Scatchard analysis (K_D = 1.8 ± 0.3 nM) or from kinetic studies ($K_D = k_2/k_1$ = 0.056 ± 0.013 min^{-1}/4.3 ± 0.2 × 10^7 M^{-1} min^{-1} = 1.3 ± 0.3 nM). In competition studies, α-adrenergic antagonists and agonists inhibited the binding of ^{125}I-rau-pAPC with a potency order consistent with an interaction at α_2-adrenergic receptors (rauwolscine > phentolamine > prazosin; clonidine > (−)-epinephrine > (−)-norepinephrine > dopamine > (+)-epinephrine). In rat liver and human platelet membranes, high affinity binding of ^{125}I-rau-pAPC was also observed (liver, K_D = 1.2 ± 0.4 nM; platelet, K_D = 3.2 ± 1.5 nM). In addition, the density of α_2-adrenergic receptors identified from binding studies with ^{125}I-rau-pAPC in kidney, liver, and platelet membranes was similar to that observed in parallel studies with [^{3}H]rauwolscine. These findings indicate that ^{125}I-rau-pAPC is a high affinity probe that selectively identifies α_2-adrenergic binding sites. Availability of this radioligand should facilitate the localization and biochemical characterization of this α-adrenergic receptor subtype.

α- and β-Adrenergic receptors have been classified on the basis of their relative affinities for a variety of synthetic compounds, both in functional studies and using radioligand binding techniques (1–3). In addition to a proposed prejunctional location of α_2-adrenergic receptors on sympathetic nerve terminals (4), this α-adrenergic receptor subtype has also been identified at postjunctional sites in several organs as well as in noninnervated tissue (5, 6).

The molecular characterization of adrenergic receptors in terms of subunit composition and receptor-effector coupling has been limited by the minute quantities of these receptors present in most tissues. Nevertheless, much progress has been made in the purification and structural characterization of β_1-, β_2-, and α_1-adrenergic receptors from a number of tissues (7). Similar studies with the α_2-receptor have not advanced as rapidly, although some success has now been reported with the α_2-adrenergic receptor in human platelets and rat adrenocortical 494 tumor cells (8–12). The progress that has been achieved with the molecular characterization of other adrenergic receptor subtypes has been made possible, primarily by the development of subtype-selective, high affinity radioiodinated

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ABBREVIATIONS: ^{125}I-rau-pAPC, 17α-hydroxy-20α-yohimban-16β-(*N*-4-amino-3-[^{125}I]iodophenethyl)carboxamide; EDTA, ethylenediaminetetraacetic acid; rau-pAPC, 17α-hydroxy-20α-yohimban-16β-[*N*-4-aminophenethyl]carboxamide; yoh-pAPC, 17α-hydroxy-20β-yohimban-16α-[*N*-4-aminophenethyl]carboxamide; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography; DMF, dimethyl formamide.

probes, which can be used in receptor binding assays or as adducts for affinity and photoaffinity labeling of the ligand-binding site (13–18).

To facilitate the identification and characterization of α_2-adrenergic receptors, the development of radioiodinated probes with high affinity and selectivity for this adrenergic receptor subtype was undertaken. The synthesis and binding properties of a radioiodinated derivative of rauwolscine *p*-aminophenethylcarboxamide, ^{125}I-rau-pAPC, are described.

Experimental Procedures[1]

Materials

Carrier-free Na^{125}I was purchased from Amersham Corp. (Arlington Heights, IL). [^{3}H]Rauwolscine (80 Ci/mmol) and [^{3}H]prazosin (15 Ci/mmol) were obtained from New England Nuclear (Boston, MA); Hydrofluor was from National Diagnostics (Somerville, NJ); 24-mm glass fiber filters (No. 32 glass) were from Schleicher and Schuell (Keene, NH); and ammonium bicarbonate and sucrose were from Fisher Scientific Co. (Pittsburgh, PA). Phenylmethanesulfonyl fluoride, sodium azide, soybean trypsin inhibitor, bovine serum albumin, (−)-epinephrine, (−)-norepinephrine, (−)-isoproterenol, dopamine, EDTA, Tris-HCl, and yohimbine were purchased from Sigma Chemical Co. (St. Louis, MO); guanyl 5′-imidodiphosphate was from Boehringer-Ingelheim Ltd. (Indianapolis, IN); Percoll was from Pharmacia (Piscataway, NJ); and rauwolscine was from Roth (Karlsruhe, Federal Republic of Germany). Leupeptin and pepstatin were obtained from Transformation Research (Framingham, MA). The following were gifts: phentolamine (Ciba-Geigy, Basel, Switzerland), prazosin (Pfizer, Groton, CT), (+)-epinephrine and (+)-norepinephrine (Winthrop, Rensselaer, NY), (−)-alprenolol (Hassle, Molndal, Sweden), clonidine (Boehringer-Ingelheim), methysergide (Sandoz Inc., East Hanover, NJ), and haloperidol (McNeil Pharmaceuticals, Spring House, PA). All other organic chemicals were purchased from Aldrich (highest grade possible). Rats (Sprague-Dawley) were obtained from Charles River Breeding Laboratories (Wilmington, MA).

Membrane Preparations

Rat kidney. Purified rat renal membranes were made by modifications of methods described previously (19, 20). Male Sprague-Dawley rats (200–250 g) were sacrificed, and both kidneys were rapidly excised, decapsulated, and placed in ice-cold buffer A (250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). All subsequent steps were carried out in a cold room on ice and all buffers used contained the following protease inhibitors: leupeptin 1 μM; pepstatin, 1.4 μM; phenylmethanesulfonyl fluoride; 100 μM; soybean trypsin inhibitor, 10 BAEE[2] units/ml; and benzamidine, 10 μM. The renal cortex was dissected from the medulla and papilla, minced, diluted with buffer A (20:1, v/w), and homogenized with 20 strokes of a Teflon-glass homogenizer (setting 10, Wheaton Instruments, Millville, NJ). The membrane preparation was further disrupted with a Polytron tissue homogenizer (once for 20 sec, setting 9; Brinkmann Instruments, Westbury, NY), after which the nuclear material and undisrupted membrane aggregates were pelleted by centrifugation for 10 min at 2,400 × *g* (Sorvall RC5B, type SS34 rotor). The supernatant was decanted and centrifuged at 24,000 × *g* to obtain a pellet which consisted of a dense dark core overlayed by a loosely packed fluffy white layer. After discarding the 24,000 × *g* supernatant, the fluffy white layer was harvested by gently swirling two times with 5.5 ml of buffer A.

For preparation of purified membranes by density gradient centrifugation, 13.8 ml of the fluffy white layer was resuspended with 10 strokes of the Wheaton homogenizer and mixed with 1.2 ml of Percoll (self-orienting colloidol silica medium) and centrifuged at 48,000 × *g* for 30 min. Visual inspection of the gradient indicated the majority of membrane protein to be in the bottom 5 ml and in a thin white band overlaying the bottom membrane layer, representing, respectively, the brush-border and basolateral membranes of the renal tubular epithelial cells (20). The gradient was divided into 5-ml fractions, diluted 1:5 with buffer B (100 mM Tris-HCl, 5 mM EDTA, pH 7.4), and centrifuged at 100,000 × *g* for 60 min (Beckmann ultracentrifuge, model L5-50B) to achieve separation of Percoll from the membranes which layered on top of the glassy Percoll pellet. Fraction I (top 5 ml) contained no visible protein and was discarded. After aspiration of the supernatant, fraction II (basolateral membranes) was resuspended in 4–6 ml of buffer B.

Human platelets. Outdated human platelets were obtained from the blood bank at Massachusetts General Hospital or from New England Red Cross within 1–2 days of the expiration date. Purified human platelet membranes were prepared as described by Neubig and Szamraj (21) and resuspended in buffer B at a protein concentration of 1–2 mg/ml, prior to use in the binding studies.

Rat Liver. Purified rat liver membranes were prepared as described by Prpic *et al.* (22) and resuspended in buffer B at a protein concentration of 1–2 mg/ml, prior to use in the binding studies.

Binding Studies

Membrane binding assays were conducted at 24° as described previously (23). Briefly, membranes (50–100 μg of protein/50 μl of buffer B) were added to polypropylene test tubes containing 25 μl of radioligand and 25 μl of buffer B or competing ligand. After a 45-min incubation, the reaction mixture was diluted with 4 ml of buffer B, poured onto glass fiber filters, and rapidly washed under vacuum three times with 4-ml aliquots of buffer B (24°). The radioactivity retained by the filters was determined in a gamma spectrometer (Micromedic) at 80% efficiency for ^{125}I samples. For ^{3}H samples, 10 ml of Hydrofluor scintillation fluid were added to the filters before counting in a liquid scintillation spectrometer (Beckmann, model LS1800) with a counting efficiency of 50%. Nonspecific binding was determined with 10 μM yohimbine or 10 μM phentolamine for [^{3}H]rauwolscine and ^{125}I-rau-pAPC or with 10 μM prazosin for [^{3}H]prazosin. For binding assays with the radioiodinated probe, the glass fiber filters were presoaked with 10% polyethylene glycol-8000 for 15 min to reduce nonspecific retention of the radioligand. A blank containing radioligand and buffer without membranes was included in each experiment with the radioiodinated probe. These filtration blanks ranged from 0.4 to 0.7% of the total amount of ligand in each tube.

All binding studies were performed in fresh membrane preparations following determination of the protein concentration by the method of Lowry *et al.* (26). In competition studies the dissociation constant (K_i) of the competing ligand was calculated as previously described (25). Saturation binding isotherms were analyzed by the method of Scatchard (26) as modified by Rosenthal (27). Values of the derived affinity constants and receptor densities are expressed as the mean ± standard error.

Results

Synthesis and binding properties of rauwolscine and yohimbine 4-aminophenethyl carboxamides. The structures of rau-pAPC and yoh-pAPC are shown in Fig. 1. These two compounds, as is the case with the parent carboxylic acid derivatives, are diastereoisomers differing in stereochemistry at carbons 16 and 20.

The effect of structural modification on the binding properties of these compounds was investigated in competitive inhibition studies with [^{3}H]rauwolscine. As shown in Fig. 2, rau-pAPC retained high affinity (K_i = 11 ± 1 nM; n = 4) for the renal membrane α_2-adrenergic receptor, whereas the *p*-aminophenethyl carboxamide derivative of yohimbine (yoh-pAPC)

[1] Portions of Experimental Procedures related to synthetic and chemical characterization data are presented in the Supplement at the end of this paper.

[2] BAEE, Nα-benzoyl-L-arginine ethyl ester.

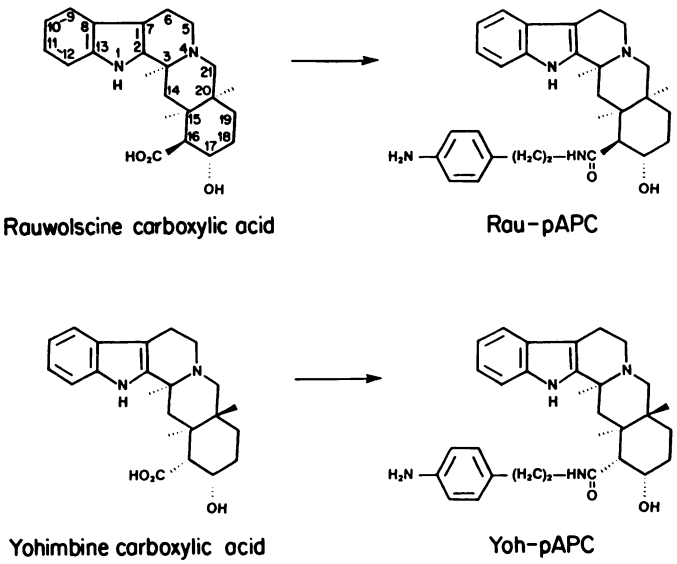

Fig. 1. Synthesis and structure of 17α-hydroxy-20α-yohimban-16β-(*N*-4-aminophenethyl)carboxamide (*Rau-pAPC*) and 17α-hydroxy-20β-yohimban-16α-(*N*-4-aminophenethyl)carboxamide (*Yoh-pAPC*).

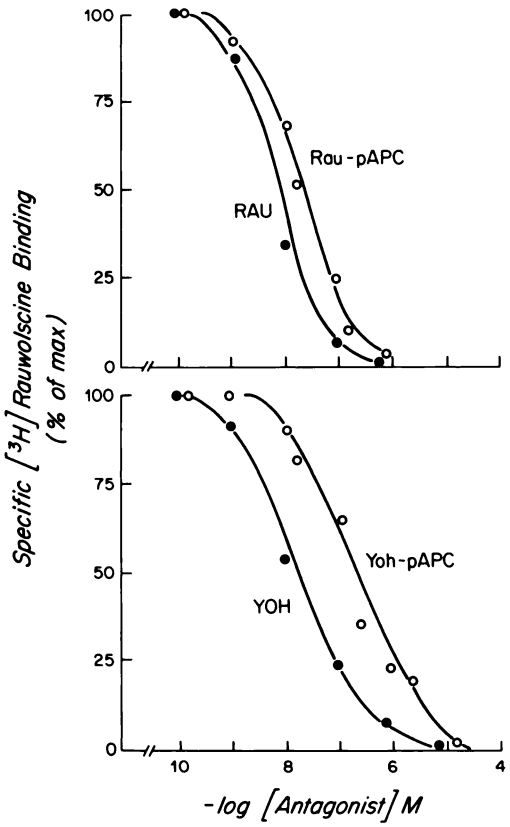

Fig. 2. Competition by rau-pAPC and yoh-pAPC for [^{3}H]rauwolscine (6 nM) binding to rat kidney membranes. Experiments were performed as described in Experimental Procedures (Methods). In these studies, specific binding (inhibited by 10 μM rauwolscine) represented 70% of total counts bound (~1800 cpm). The data are the average of triplicate determinations and are representative of four different experiments. For abbreviations and chemical structure, see Fig. 1.

exhibited a 14-fold lower affinity (K_i = 136 ± 45 nM; n = 4). These values contrast to affinities of 3.8 ± 2.4 and 13 ± 3 nM (n = 5) for the parent compounds, rauwolscine and yohimbine, respectively (Fig. 2). In competition studies with the selective α_1-radioligand, [^{3}H]prazosin, rau-pAPC and yoh-pAPC exhibited dissociation constants >1 μM.

Synthesis and characterization of an iodinated analog of rauwolscine 4-aminophenethyl carboxamide. As rau-pAPC exhibited a higher affinity for α_2-adrenergic receptor-binding sites than did the corresponding yohimbine derivative, it was selected for subsequent radioiodination. Prior to radioiodination, nonradioactive ^{127}I-rau-pAPC was synthesized and its structure characterized (Fig. S1, cf. Supplement).

The reaction sequence leading to the synthesis of the radioiodinated analog of rau-pAPC is shown in Fig. 3. Iodine was incorporated directly into the phenyl ring of rau-pAPC after initial protection of the hydroxyl group by acetylation. Reverse phase HPLC allowed complete resolution of the radiolabeled product from the starting compound (Fig. 4). Based on the use of carrier-free Na^{125}I, a specific activity of 2175 Ci/mmol was thus assumed for ^{125}I-rau-pAPC. ^{127}I-rau-pAPC and the radioiodinated analog, ^{125}I-rau-pAPC, eluted with identical retention times by HPLC (Fig. 4) and also comigrated with TLC (cf. Supplement). In rat kidney membranes, the nonradioactive ^{127}I-rau-pAPC exhibited a dissociation constant of 3.3 ± 0.4 nM (n = 3) in competition studies with [^{3}H]rauwolscine. This contrasts with the 1000-fold lower potency (K_i = 4.6 ± 2 μM; n = 3) observed in competition studies with [^{3}H]prazosin in rat hepatic membranes.

Binding characteristics of ^{125}I-rau-pAPC. Specific binding of ^{125}I-rau-pAPC to rat kidney membranes increased linearly with membrane protein concentrations ranging from 0.2 to 4 mg/ml. Studies were routinely performed with 50–100 μg of membrane protein/tube; however, specific ^{125}I-rau-pAPC binding could be detected with as little as 10 μg of membrane protein.

Saturation binding studies with ^{125}I-rau-pAPC demonstrated that specific binding with this radioligand was saturable and typically accounted for 80–85% of total binding at a ligand concentration of 1 nM (Fig. 5). Scatchard analysis (Fig. 5, *inset*) indicated that ^{125}I-rau-pAPC binds to an apparent homogeneous class of sites with a dissociation constant of 1.8 ± 0.3 nM (n = 5).

Kinetic studies with ^{125}I-rau-pAPC demonstrated that, at a concentration of 2 nM, specific binding of the radioiodinated probe required 15–20 min to reach equilibrium at 24° (Fig. 6) and remained stable for up to 90 min. The apparent association rate constant (k_{ap}) determined from these studies was 0.14 ± 0.018 min^{-1} (n = 3).

The dissociation rate constant was determined after elimination of the forward reaction by dilution with 4 ml of buffer B at 24°. Specific binding was reversible with a $t_{1/2}$ of 9 min. A dissociation rate constant of 0.056 ± 0.013 min^{-1} (n = 3) was determined and used to calculate k_1 ($k_1 = k_{ap} - k_2/[^{125}$I-rau-pAPC]) resulting in a $K_D = k_2/k_1$ = 1.3 ± 0.3 nM (n = 3) (Fig. 6).

Specificity of ^{125}I-rau-pAPC binding. α-Adrenergic antagonists inhibited specific ^{125}I-rau-pAPC binding to rat renal membranes with a potency order consistent with binding to α_2-adrenergic receptors. Rauwolscine competed with a 10-fold greater potency than the nonselective α-receptor antagonist phentolamine, and with a 50-fold greater potency than the α_1-selective antagonist, prazosin (Fig. 7A). As shown in Table 1, the K_i values for these competing ligands are similar to corresponding values obtained in parallel studies with [^{3}H]rauwol-

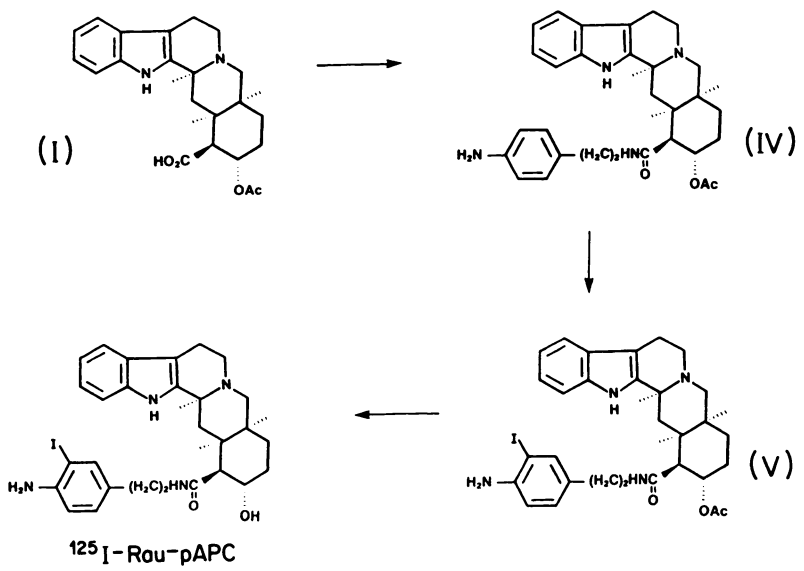

Fig. 3. Reaction sequence leading to the synthesis of 17α-hydroxy-20α-yohimban-16β-(4-amino-3-[^{125}I]iodophenethyl)carboxamide (^{125}*I-Rau-pAPC*).

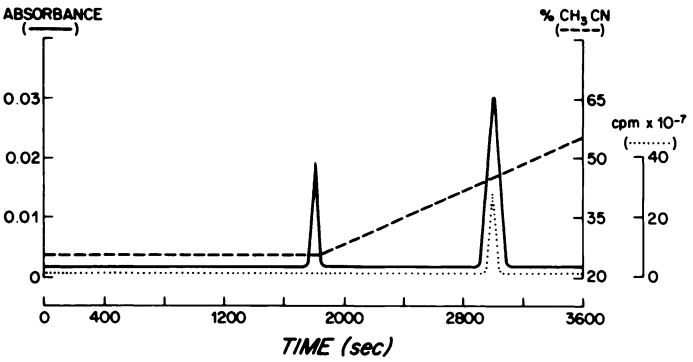

Fig. 4. HPLC separation of (*left* to *right*) rau-pAPC and ^{127}I-rau-pAPC. The *dashed line* represents the elution of ^{125}I-rau-pAPC. Absorbance was measured at 220 nm.

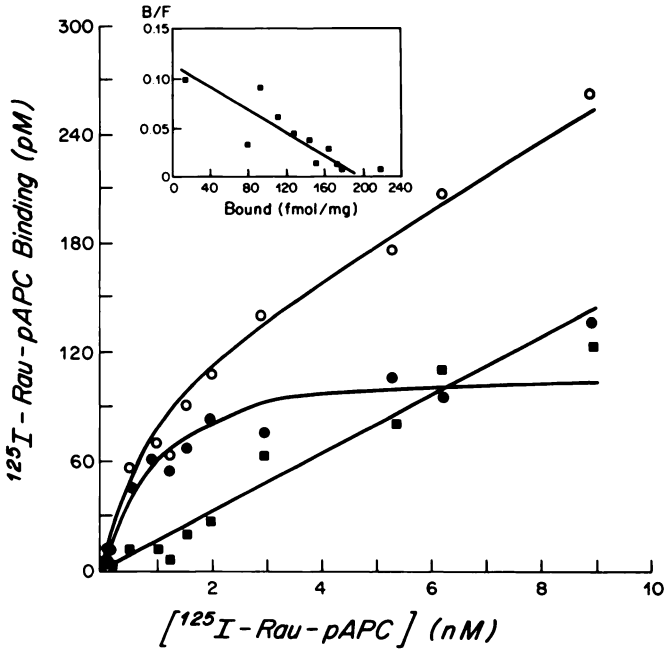

Fig. 5. Equilibrium binding studies with ^{125}I-rau-pAPC in rat kidney membranes. Studies were performed as detailed in Experimental Procedures (Methods), using increasing concentrations of the radioiodinated probe from 0.05 to 10 nM. *Inset*, Scatchard analysis of specific ^{125}I-rau-pAPC binding.

scine. Reserpine, an alkaloid structurally related to rauwolscine and rau-pAPC which does not bind to α-receptors, also did not inhibit the binding of ^{125}I-rau-pAPC in concentrations as high as 100 μM.

^{125}I-rau-pAPC binding was also inhibited by adrenergic agonists, with clonidine exhibiting greater potency than (−)-epinephrine and (−)-norepinephrine (Fig. 7B, Table 1). (+)-Epinephrine was approximately 50-fold less potent than (−)-epinephrine in competition studies with ^{125}I-rau-pAPC, indicating that the radioligand binds stereoselectively to the membrane site (Fig. 7B, Table 1). In contrast to competition curves with antagonists, which were steep and displayed pseudo-Hill coefficients not significantly different from unity, agonist inhibition curves with (−)-epinephrine or (−)-norepinephrine were shallow with pseudo-Hill coefficients of 0.7–0.8, and could be resolved into two components of high and low affinity. In the presence of the nonhydrolyzable GTP analog, guanyl 5′-imidodiphosphate, and magnesium, the binding could be fitted best to a one-site model for low affinity sites (28) (data not shown).

To investigate further the α_2-adrenergic specificity of ^{125}I-rau-pAPC binding, competition experiments were also conducted with the dopamine antagonist haloperidol, the serotonin antagonist methysergide, and the β-adrenergic antagonist, (−)-alprenolol. Haloperidol and methysergide competed with a 1000-fold lower potency than the selective α_2-receptor antagonist, rauwolscine. Similar affinities were observed in competition studies with [^{3}H]rauwolscine (Table 1). (−)-Alprenolol and the calcium channel antagonist, nifedipine, did not inhibit ^{125}I-rau-pAPC binding at concentrations as high as 10 μM. A structurally dissimilar calcium channel antagonist, verapamil (K_i = 20 μM), effectively competed with ^{125}I-rau-pAPC binding, consistent with the results of Motulsky et al. (29), that verapamil but not nifedipine competes for α_2-adrenergic receptor binding sites identified with [^{3}H]yohimbine.

Binding of ^{125}I-rau-pAPC to rat liver and human platelet membranes. To investigate the binding characteristics of ^{125}I-rau-pAPC in other tissues known to contain α_2-adrenergic receptors, saturation binding isotherms were con-

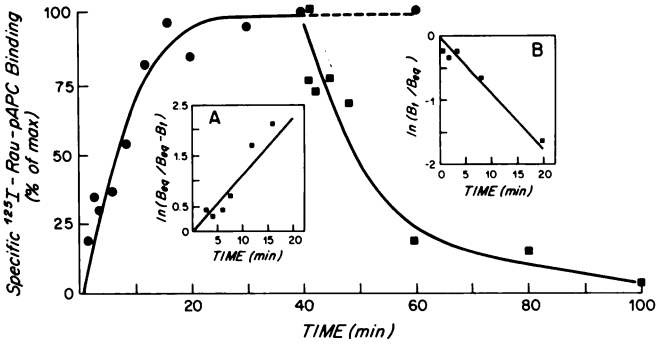

Fig. 6. Kinetics of ^{125}I-rau-pAPC binding to rat kidney membranes. For the association reaction, 50 μg of rat kidney membranes were added to test tubes containing ^{125}I-rau-pAPC (2 nM, final) and either 10 μM yohimbine or buffer B at 24°. At the times indicated, the incubation was terminated by vacuum filtration as described in Experimental Procedures (Methods). For the dissociation reaction, after a 45-min preincubation the reaction mixture described above was diluted with 4 ml of buffer B and the incubation continued at 24° for the times indicated. The apparent rate constant (k_{ap}) for the pseudo-first order association reaction was calculated from the slope of the line (*inset A*) relating $\ln[B_{eq}/(B_{eq}-B_t)]$ and time (determined by linear regression analysis, $r = 0.97$), where B_{eq} is the amount of ^{125}I-rau-pAPC bound at equilibrium and B_t is the amount bound at each time, t. The second order rate constant (k_1) was calculated according to the equation $k_1 = (k_{ap} - k_2)/[^{125}\text{I-rau-pAPC}]$, where k_2 is the dissociation rate constant. This latter constant (k_2) was determined from the dissociation reaction, where k_2 is the slope of the line (*inset B*) relating $\ln(B_t/B_{eq})$ and time (determined by linear regression analysis, $r = 0.98$), where B_t is the amount of specific binding at each time t and B_0 is the amount bound at equilibrium prior to dilution.

structed in membrane preparations from rat liver and human platelets. Specific ^{125}I-rau-pAPC binding was saturable and of high affinity and, at a ligand concentration of 1 nM, represented 70–75% of total binding. The density of α_2-adrenergic receptors determined in these membranes, as well as in renal membranes, was similar to the number of binding sites identified with [^{3}H] rauwolscine (Fig. 8, Table 2).

Discussion

The major goal of this study was to develop high affinity radioiodinated probes for the α_2-adrenergic receptor. Derivatives of the selective α_2-adrenergic receptor antagonist, yohimbine, and its diastereoisomer, rauwolscine, were synthesized, and their binding properties were characterized in rat kidney membranes. Yohimbine and rauwolscine were selected for structural modification since they are potent antagonists at α_2-adrenergic receptors. Furthermore, both compounds have been radiolabeled with tritium and their utility as specific α_2-radioligands has been confirmed (2).

As the *p*-aminophenethyl carboxamide derivative of rauwolscine retains high affinity for the α_2-adrenergic receptor, it was radioiodinated and the binding properties of the ^{125}I-derivative were characterized in rat kidney membranes. The binding of this probe is saturable, reversible, stereoselective, and inhibited by a variety of agonists and antagonists with a rank order of potency expected for binding to the α_2-adrenergic receptor. ^{125}I-rau-pAPC binding is of high affinity as determined by both kinetic analysis and equilibrium binding studies. These results, together with the observation that nonradiolabeled ^{127}I-rau-pAPC exhibits low affinity in competition studies with the α_1-selective ligand [^{3}H]prazosin, indicate that ^{125}I-rau-pAPC is a selective radioligand for rat renal membrane α_2-adrenergic receptors. Preliminary observations with ^{127}I-rau-pAPC in the isolated perfused rat kidney also indicate that this compound is a potent α_2-adrenergic antagonist as it produced a 70% increase in the release of norepinephrine elicited by adrenergic nerve stimulation,[2] as would be expected of a prejunctional α_2-receptor blocking agent (30).

The utility of ^{125}I-rau-pAPC as a specific radioligand for the identification of α_2-adrenergic receptors is also apparent from the studies carried out with human platelet and rat hepatic membranes. The α_2-adrenergic receptor of human platelets has been studied extensively in terms of its molecular properties and the mechanisms involved in receptor-effector coupling (8–11, 31). In both tissues, saturable and high affinity ^{125}I-rau-pAPC binding was observed. Furthermore, in membranes from these tissues as well as rat kidney, [^{3}H]rauwolscine and ^{125}I-rau-pAPC identified comparable numbers of binding sites.

Our studies with the *p*-aminophenethyl carboxamide derivative of yohimbine suggest that the spatial orientation of the *p*-aminophenethyl substituent influences the ligand's interaction with the receptor's binding site. Specifically, yoh-pAPC was 10-fold less potent than yohimbine in competition studies

[3] K. U. Malik (Department of Pharmacology, University of Tennessee Center for Health Sciences), personal communication. ^{127}I-rau-pAPC did not effect the basal release of norepinephrine but increased the release of norepinephrine elicited by adrenergic nerve stimulation. Similar results were obtained with rauwolscine and yohimbine. The relative potency of these compounds in facilitating the nerve stimulation-induced release of norepinephrine is currently under investigation.

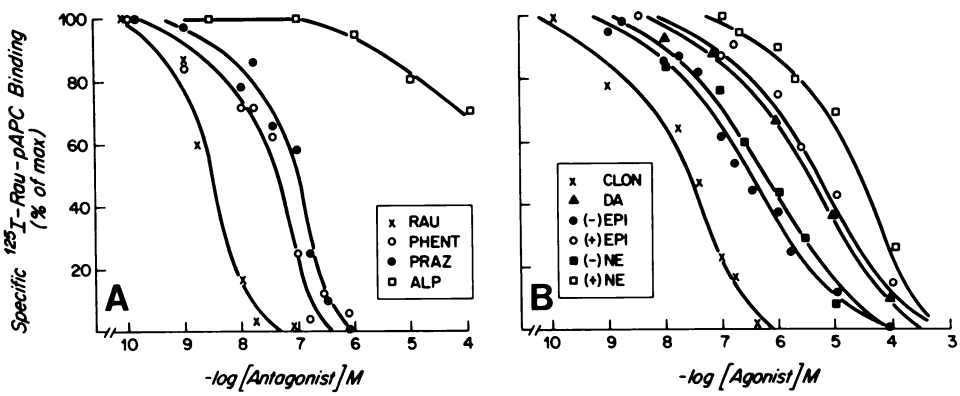

Fig. 7. Inhibition of ^{125}I-rau-pAPC binding to rat kidney membranes by various adrenergic antagonists (A) and agonists (B). Rat kidney membranes were incubated with increasing concentrations of competing antagonists as described in Experimental Procedures (Methods). In these studies specific binding (inhibited by 10 μM rauwolscine) represented 75% of total counts bound (~25,000 cpm). The data represent the average of duplicate determinations and are representative of three to six different experiments (cf. Table 1). *RAU*, rauwolscine; *PHENT*, phentolamine; PRAZ, prazosin; *ALP*, (−)-alprenolol; *CLON*, clonidine; *DA*, dopamine; *(−)EPI*, (−)-epinephrine; *(+)EPI*, (+)-epinephrine; *(−)NE*, (−)-norepinephrine; *(+)NE*, (+)-norepinephrine.

TABLE 1

Comparison of inhibitory binding constants (K_i) for various drugs at rat renal membrane α_2-adrenergic receptors identified with [^{3}H]rauwolscine and ^{125}I-rau-pAPC

Each value represents the mean ± SE determined from three to five different experiments for each compound. K_i values were calculated by Cheng-Prusoff analysis (25).

Competing ligand	[^{3}H]Rauwolscine K_i	^{125}I-rau-pAPC K_i
	nM	nM
Rauwolscine	3.8 ± 2.4	3.2 ± 0.5
Phentolamine	38 ± 13	40 ± 8.1
Prazosin	88 ± 13	172 ± 83
Clonidine	73 ± 38	123 ± 47
(−)-Epinephrine	225 ± 80	375 ± 72
(−)-Norepinephrine	NT[a]	1,190 ± 380
(+)-Epinephrine	NT	25,700
(+)-Norepinephrine	NT	25,000
Dopamine	5,410 ± 2,500	9,850 ± 3,251
Methysergide	4,560	2,800
Haloperidol	2,100	4,900

[a] NT, not tested.

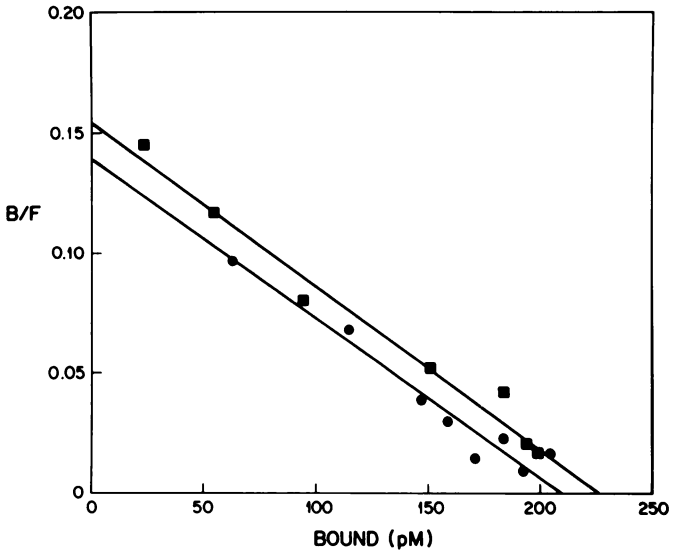

Fig. 8. Scatchard analysis of equilibrium binding studies with [^{3}H]rauwolscine and ^{125}I-rau-pAPC in rat kidney membranes. Studies were performed as detailed in Experimental Procedures (Methods), using concentrations of the radiolabeled probes from 0.1 to 20 nM. The data are the average of triplicate determinations and are representative of three different experiments. ●, [^{3}H]rauwolscine (K_D = 1.5 nM, B_{max} = 308 fmol/mg); ■, ^{125}I-rau-pAPC (K_D = 1.5 nM, B_{max} = 333 fmol/mg).

with [^{3}H]rauwolscine, whereas only a 3-fold decrease in affinity was observed after similar derivatization of rauwolscine. These results may reflect the difference in the stereochemistry at carbon 16 of rau-pAPC and yoh-pAPC, where the *p*-aminophenethyl substituent has been introduced axially in rauwolscine and equatorially in yohimbine. To investigate these observations further, we have synthesized additional carboxamide derivatives in which the phenyl ring is separated from carbon 16 of the parent molecule by zero, one, or four atoms. The binding characteristics of these compounds are currently being determined and, in combination with molecular modeling techniques, these studies should provide insight into the topography of the receptor's binding site.

The availability of high affinity radioiodinated probes, which have been chemically modified for use as affinity adducts, have proven invaluable in structural studies of receptors for a variety

TABLE 2

Comparison of [^{3}H]rauwolscine and ^{125}I-rau-pAPC binding to α_2-adrenergic receptors in rat kidney, rat liver, and human platelet membrane preparations

K_D and B_{max} values of equilibrium binding studies were calculated from nontransformed radioligand binding data (triplicate determinations) with the mass action law-based, weighted nonlinear curve fitting program, LIGAND (30).

Tissue[a]	[^{3}H]Rauwolscine		^{125}I-rau-pAPC	
	K_D	B_{max}	K_D	B_{max}
	nM	fmol/mg	nM	fmol/mg
Rat kidney (n = 3)	1.8 ± 0.2	276 ± 24	1.8 ± 0.3	274 ± 48
Rat liver (n = 3)	1.2 ± 0.4	191 ± 40	1.2 ± 0.4	161 ± 39
Human platelet (n = 3)	1.8 ± 1.0	224 ± 26	3.2 ± 1.5	213 ± 22

[a] In each tissue the K_D and B_{max} values for [^{3}H]rauwolscine and ^{125}I-rau-pAPC were not significantly different as determined by the extra sum of squares principle (30).

of hormones and neurotransmitters (7, 32–34). Similar probes with specificity for α_2-adrenergic receptors are not yet available. The radioiodinated derivative of rauwolscine described herein can be readily converted to a photolabile arylazide analog which should prove useful as a photoaffinity adduct for the identification of the hormone-binding subunit of the α_2-adrenergic receptor protein. Studies aimed at investigating this potential are currently in progress.

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Supplement: Experimental Procedures

Methods and Syntheses

Synthesis of rauwolscine and yohimbine 4-aminophenethylamine carboxamides. The carboxylic acid derivatives of rauwolscine and yohimbine were prepared by alkaline hydrolysis of the parent compound, as previously described (35, 36). The carboxamide derivatives shown in Fig. 1 were then prepared as follows.

17α-Hydroxy-20α-yohimban-16β-(N-4-aminophenethyl)carboxamide (rau-pAPC). To a stirred solution of 4-aminophenethylamine (0.059 g, 0.433 mmol) in 10 ml of CH_2Cl_2 under an N_2 atmosphere was added triethylamine (0.053 ml, 0.423 mmol). 17α-Hydroxy-20α-yohimban-16β-carboxylic acid hydrochloride monohydrate (0.15 g, 0.38 mmol) was added, followed by 1-hydroxybenzotriazole (0.077 g, 0.57 mmol). The resulting mixture was stirred for 15 min and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (0.169 g, 0.38 mmol) was added, followed by 2.0 ml of DMF. The reaction was stirred for 18 hr and evaporated to dryness. The residue was dissolved in 20 ml of $CHCl_3$/EtOH (7:3), washed with H_2O (twice in 10 ml), dried over anhydrous $MgSO_4$, filtered, and evaporated to give 0.217 g of crude product. Chromatography of the crude product on Brinkmann silica gel (230–400 mesh), eluting with a gradient of CH_2Cl_2/MeOH, gave the desired material (0.07 g, 40.0%). For analysis, a sample was further purified on a 15-cm C_{18} reverse phase silica gel column eluting with a 15/85 gradient of CH_3CN/pH 3.0 potassium phosphate buffer (0.01 M); m.p. 283° (dec).

$C_{28}H_{34}N_4O_2 \cdot 1/2H_2O$

% Calculated: 71.92% C, 7.54% H, 11.98% N
% Found: 72.17% C, 7.67% H, 11.68% N

IR (KBr): 1660 cm^{-1} (C = 0), 3520 cm^{-1} (OH)
MS *m/z*: 458.2656 (M^+) Calculated 458.2678
UV (MeOH): λ_{226} (ϵ = 37,942), λ_{282} (ϵ = 8,069), λ_{289} (ϵ = 7,127)

NMR (CD_3OD): 7.37 (*d*,1, C_9H), 7.3 (*d*,1, $C_{12}H$), 7.05 (*d*,2, *p*-aminophenyl H), 7.02 (*s*,1, NH), 6.97 (*m*,2, C_{10}, $C_{11}H$), 6.68 (*d*,2, *p*-aminophenyl H), 3.95 (*m*,1, $C_{17}H$), 3.1 (*m*,1, C_3H), 3.08 (*d*,1, OH), 2.35 (*m*,1, $C_{16}H$), 2.2 (*m*,1, $C_{15}H$), 1.9, 1.6 (*m*,2, $C_{14}H$).
TLC (E. Merck Silica Gel 60): toluene/isopropanol/NH_4OH (85:15:1) R_f = 0.16
$[\alpha]_D^{22^\circ}$ = +41.0 (*c* = 0.4, MeOH)

17α-Hydroxy-20β-yohimban-16α-(N-4-aminophenethyl)carboxamide (yoh-pAPC). yoh-pAPC was synthesized by the same reaction scheme described for rau-pAPC utilizing yohimbine carboxylic acid (35, 36) as the starting material (Fig. 1) with a final yield of 58%.

$C_{28}H_{34}N_4O_2 \cdot 1/4CH_3OH$

% Calculated: 72.71% C, 7.56% H, 12.01% N
% Found: 72.68% C, 7.44% H, 11.97% N

m.p. 229–232° (dec)
UV (MeOH): λ_{220}(ϵ = 37,849), λ_{273} (ϵ = 7,108), λ_{281} (ϵ = 7,368), λ_{289} (ϵ = 6,528)

MS *m/z*: 458.2659 (M^+) Calculated 458.2682
TLC (E. Merck Silica Gel 60): toluene/isopropanol/NH_4OH (85:15:1) R_f = 0.14
$[\alpha]_D^{22^\circ}$ = +4.46 (*c* = 0.41, MeOH)

Synthesis of rauwolscine 4-amino-3-iodophenethyl carboxamide. The four-step reaction sequence leading to the synthesis of 17α-hydroxy-20α-yohimban-16β-[*N*-4-amino-3-iodophenethyl]carboxamide (^{127}I-rau-pAPC) is shown in Fig. S1.

The first step involved acetylation of the hydroxy group at carbon 17 to parallel the reaction sequence leading to the radioiodinated analog (see below). The next step entailed iodination of 4-aminophenethylamine which was then coupled (step 3) to the protected derivative of rauwolscine carboxylic acid (**I**) to yield acetylated rauwolscine 4-amino-3-iodophenethylamine carboxamide (**III**). The presence of an acetate group at carbon 17 reduced the affinity of this series of compounds, necessitating deacetylation (step 4) as described below.

Step 1: 17α-Acetoxy-20α-yohimban-16β-carboxylic acid (**I**). To a stirred solution of 17α-hydroxy-20α-yohimban-16β-carboxylic acid hydrochloride monohydrate (0.5 g, 1.27 mmol) in 5 ml of DMF was added triethylamine (0.18 ml, 1.27 mmol) followed by acetic anhydride (0.13 g, 1.27 mmol). The reaction was stirred for 18 hr and then evaporated to dryness. Water (12.5 ml) was added to the residue and the precipitated product was filtered to give 0.408 g (84%). A portion of the crude product (0.259 g) was recrystallized from isopropanol to give 0.171 g, m.p. 232–235° (dec).

$C_{22}H_{26}O_4N_2$

% Calculated: 69.09% C, 6.85% H, 7.32% N
% Found: 69.23% C, 7.01% H, 7.14% N

MS *m/z*: 382.1897(M^+) Calculated 382.1910
UV (MeOH): λ_{222}(ϵ = 33,142), λ_{273}(ϵ = 6,981), λ_{281}(ϵ = 7,131), λ_{289}(ϵ = 5,968)
TLC (E. Merck Silica Gel): *n*-butanol/water/acetic acid (9:1:1) R_f = 0.2
$[\alpha]_D^{22^\circ}$ = −8.02 (*c* = 0.44,MeOH)

Step 2: 4-Amino-3-iodophenethylamine hydrochloride (**II**). To a stirred solution of 4-aminophenethylamine (2.0 g, 14.68 mmol) in 1 N hydrochloric acid (35.2 ml, 35.2 mmol) was added 0.5 M sodium acetate buffer (pH 5.6) (293.6 ml, 146.8 mmol). The pH of the solution was adjusted to 5.6 with 1.0 M sodium hydroxide, and then solid sodium iodide (5.04 g, 33.6 mmol) was added. To the resulting solution was added, dropwise, a solution of chloramine T (7.6 g, 33.4 mmol) in 10 ml of water.

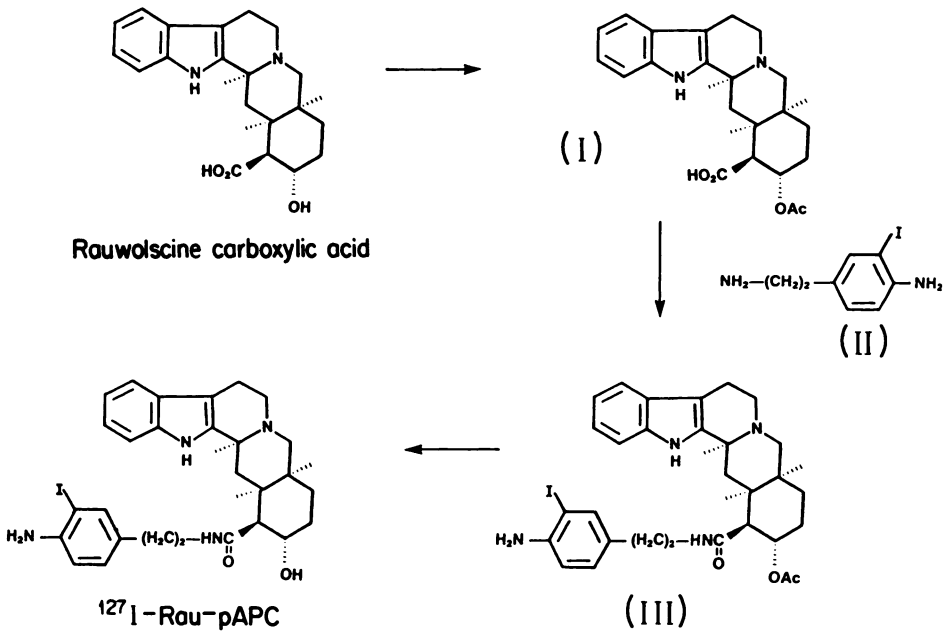

Fig. S1. Reaction sequence leading to the synthesis of 17α-hydroxy-20α-yohimban-16β-(*N*-4-amino-3-iodophenethyl)carboxamide (^{127}*I-rau-pAPC*).

During the chloramine T addition, the pH of the reaction was kept at 5.6 by the addition of 1 N hydrochloric acid or 1 M sodium hydroxide as needed. The reaction was stirred for 2 min after completion of the chloramine T addition, and then solid sodium metabisulfite (6.4 g, 33.6 mmol) was added. The reaction pH was adjusted to 8.5 with 1 M sodium hydroxide and the reaction was extracted with chloroform/ethanol (7:3) (three times for 10 ml). The combined extracts were dried over anhydrous $MgSO_4$, filtered, and evaporated to give 4.63 g of crude product. The crude product was purified on an Amberlite CG-50 ion-exchange resin column eluting with a gradient of methanol/dilute hydrochloric acid. The fractions containing product were combined and evaporated to give 1.2 g of product (27%), m.p. 202–204° (dec).

$C_8H_{11}N_2I \cdot HCl$

% Calculated: 32.18% C, 4.05% H, 9.38% N
% Found: 31.99% C, 4.08% H, 9.30% N

MS *m/z:* 261.9955 (M^+) Calculated 261.9967
NMR (CD_3OD): 7.53 (*d*,1, C_2 aromatic), 7.04 (*m*,1, C_6 aromatic) 6.78 (*d*,1, C_5 aromatic), 3.08 (*m*,2, methylene) 2.78 (*m*,2, methylene)
TLC (E. Merck Silica Gel): toluene/isopropanol/ammonium hydroxide (85:15:1) $R_f = 0.1$

Step 3: 17α-Acetoxy-20α-yohimban-16β-(*N*-4-amino-3-iodophenethyl)carboxamide (**III**). To a stirred suspension of 4-amino-3-iodophenethylamine hydrochloride (0.078 g, 0.261 mmol) in 2.3 ml of methylene chloride under a nitrogen atmosphere was added triethylamine (0.036 ml, 0.26 mmol) followed by 2.3 ml of DMF. The mixture was stirred for 15 min until a complete solution was obtained. 17-α-Acetoxy-20α-yohimban-16β-carboxylic acid (0.089 g, 0.233 mmol) was added, followed by 1-hydroxybenzotriazole (0.047 g, 0.35 mmol). The resulting solution was stirred for 15 min and then a solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.99 g, 0.234 mmol) in 1.0 ml of methylene chloride was added. The reaction was stirred for 18 hr and then diluted to 25 ml with methylene chloride. The resulting solution was washed with water (twice for 10 ml) dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness to give 0.181 g of crude product. The crude product was chromatographed twice on Brinkmann silica gel (230–400 mesh) eluting with a gradient of methylene chloride/methanol. The fractions containing product were collected and evaporated to give 0.049 g (34%). Further purification on a 15-cm C_{18} reverse phase silica gel column, eluting with a 40/60 gradient of acetonitrile/pH 3.0 potassium phosphate buffer (0.01 M), afforded 0.019 g of product, m.p. 175° (dec).

$C_{30}H_{35}O_3N_3I$

% Calculated: 57.51% C, 5.63% H, 8.94% N
% Found: 57.12% C, 5.65% H, 8.77% N

MS *m/z:* 626.1793 (M^+) Calculated 626.1832
UV (MeOH): $\lambda_{218}(\epsilon = 52{,}257)$, $\lambda_{283}(\epsilon = 8{,}950)$, $\lambda_{290}(\epsilon = 8{,}423)$
TLC (E. Merck Silica Gel 60): toluene/isopropanol/ammonium hydroxide, (85:15:1) $R_f = 0.55$, methylene chloride/methanol (20:1) $R_f = 0.13$
$[\alpha]_D^{22^\circ} = +19.4$ ($c = 0.21$, MeOH)

Step 4: 17α-Hydroxy-20α-yohimban-16β-(*N*-4-amino-3-iodophenethyl)carboxamide (^{127}I-rau-pAPC). To a solution of 17α-acetoxy-20α-yohimban-16β-(*N*-4-amino-3-iodophenethyl)carboxamide (0.0635 g, 0.1 mmol) in 10 ml of methanol at room temperature under a nitrogen atmosphere was added a 1 M solution of potassium hydroxide in methanol (0.2 ml, 0.2 mmol). The reaction was stirred for 18 hr and then evaporated to dryness. To the residue was added 15 ml of saturated sodium chloride, and the resulting solution was extracted with ethyl acetate (three times in 10 ml). The organic extract was dried over anhydrous $MgSO_4$, filtered, and evaporated to give 0.055 g of crude product. The crude product was purified on a 15-cm C_{18} reverse phase silica gel column, eluting with a 40/60 gradient of acetonitrile/pH 3.0 potassium phosphate buffer (0.01 M) to give 0.031 g of (53%) product, m.p. 150° (dec).

$C_{28}H_{33}O_2N_4I$

% Calculated: 57.54% C, 5.69% H, 9.58% N
% Found: 57.57% C, 5.87% H, 9.83% N

MS *m/z:* 584.1569 (M^+) Calculated 584.1644
UV (MeOH): $\lambda_{217}(\epsilon = 52{,}744)$, $\lambda_{283}(\epsilon = 8{,}768)$, $\lambda_{290}(\epsilon = 8{,}268)$
TLC (E. Merck Silica Gel 60): methylene chloride/methanol (17:3) $R_f = 0.35$
$[\alpha]_D^{22^\circ} = +43.0$ ($c = 0.26$, MeOH)

Synthesis of ^{125}I-rau-pAPC. The three-step reaction sequence leading to the synthesis of 17α-hydroxy-20α-yohimban-16β-(*N*-4-amino-3-[^{125}I]iodophenethyl)carboxamide (^{125}I-rau-pAPC) is shown in Fig. 2. The hydroxy group at carbon-17 of rau-pAPC was first acetylated to avoid oxidation during the chloramine T reaction. In the next step, the protected derivative of rau-pAPC was radiolabeled using chloramine T and carrier free Na^{125}I. The radiolabeled product was deacetylated (step 3) prior to purification by reverse phase HPLC.

Step 1: 17α-Acetoxy-20α-yohimban-16β-(*N*-4-aminophenethyl)carboxamide (**IV**). Compound **IV** was synthesized by the same reaction scheme described above for the synthesis of compound **III** except that 4-aminophenethylamine hydrochloride was used in place of 4-amino-3-iodophenethylamine hydrochloride, m.p. 272–275° (dec).

$C_{30}H_{36}O_3N_4 \cdot H_2O$

% Calculated: 69.47% C, 7.38% H, 10.80% N
% Found: 69.68% C, 7.21% H, 10.75% N

UV (MeOH): $\lambda_{226}(\epsilon = 43{,}309)$, $\lambda_{280}(\epsilon = 9{,}094)$, $\lambda_{288}(\epsilon = 8{,}012)$
MS *m/z:* 500.2767 (M^+) Calculated 500.2787
TLC (E. Merck Silica Gel 60): toluene/isopropanol/ammonium hydroxide (85:15:1) $R_f = 0.3$
$[\alpha]_D^{22^\circ} = 27.1$ ($c = 0.1$, MeOH)

Step 2: Radioiodination of 17α-acetoxy-20α-yohimban-16β-(*N*-4-aminophenethyl)carboxamide (**IV**). Carrier-free Na^{125}I (3 mCi in 30 μl of dilute NaOH) was added to 17α-acetoxy-20α-yohimban-16β-(*N*-4-aminophenethyl)carboxamide (42 nmol) dissolved in 30μl of 0.5 M sodium acetate buffer (pH 5.2). The reaction was initiated by the addition of 6 μl of chloramine T (1 mg/ml of H_2O, 21.5 nmol). After 1 min the reaction was quenched by the addition of 8 μl of sodium metabisulfite (1 mg/ml of H_2O, 42 nmol). The radiolabeled products as well as

the starting material were extracted into ethyl acetate (three times in 300 μl), concentrated, and applied to a TLC plate (Silica Gel 60, 5 × 20 cm, EM Reagents). The plate was developed in toluene/isopropanol/ammonium hydroxide (85:15:1), and the radiolabeled product (V), which comigrated with nonradioactive acetylated ^{127}I-rau-pAPC, was scraped from the TLC plate, eluted from the silica with methanol (three times in 300 μl), and subsequently concentrated under nitrogen. Separation of compound V from unreacted starting material by TLC was not complete and thus further purification utilized reverse phase HPLC as described below.

Step 3: 17α-Hydroxy-20α-yohimban-16β-(*N*-4-amino-3-[^{125}I] iodophenethyl)carboxamide (^{125}I-rau-pAPC). Acetylated ^{125}I-rau-pAPC (V) was deprotected in 1 N NaOH in methanol for 3 hr at room temperature. The deacetylated product was extracted into ethyl acetate, concentrated, and then purified by HPLC on an ODS 5-μm C_{18} reverse phase column (Altex), eluting with a 25/55 gradient of acetonitrile/0.1 M ammonium bicarbonate (Fig. 4). Fractions of the eluate from HPLC containing the radiolabeled product were concentrated by lyophilization, and ^{125}I-rau-pAPC was subsequently stored under vacuum in methanol containing 0.1 mM phenol at 4°. The final yield of ^{125}I-rau-pAPC was 200–300 μCi. ^{125}I-rau-pAPC and nonradioactive ^{127}I-rau-pAPC comigrated by TLC analysis in two different developing solvent systems [toluene/isopropanol/ammonium hydroxide (85:15:1) R_f = 0.4; methylene chloride/methanol (17:3) R_f = 0.35].

Instrumentation

NMR spectra were recorded on a Bruker WM250 spectrometer, IR spectra were recorded on a Perkin-Elmer 283B recording infrared spectrometer, and UV spectra were recorded on a Hewlett-Packard 8450A array spectrometer. The Beckmann model 420 system was attached to a variable wavelength spectrophotometer (Hewlett-Packard model 8450A), computer (Hewlett-Packard model 85), and data plotter (Hewlett-Packard model 7225B).

References

1. Minneman, K. P., R. N. Pittman, and P. B. Molinoff. Beta-adrenergic-receptor-subtypes, properties, distribution and regulation. *Annu. Rev. Neurosci.* **4**:419–461 (1981).
2. Bylund, D. B., and D. C. U'Prichard. Characterization of alpha$_1$- and alpha$_2$-adrenergic receptors. *Int. Rev. Neurobiol.* **24**:343–431 (1983).
3. Langer, S. Z., and P. E. Hicks. Alpha-adrenoceptor subtypes in blood vessels: physiology and pharmacology. *J. Cardiovasc. Pharmacol.* **6**:S547–S558 (1984).
4. Langer, S. Z. Presynaptic regulation of catecholamine release. *Biochem. Pharmacol.* **23**:1793–1800 (1974).
5. Timmermans, P. B. M. W. M., and P. A. van Zweiten. The postsynaptic alpha$_2$-adrenoceptor. *J. Auton. Pharmacol.* **1**:171–183 (1981).
6. Timmermans, P. B. M. W. M., and P.A. van Zweiten. Alpha$_2$-adrenoceptors: classification, localization, mechanisms and targets for drugs. *J. Med. Chem.* **25**:1389–1401 (1982).
7. Homcy, C. J., and R. M. Graham. Molecular characterization of adrenergic receptors. *Circ. Res.* **56**:635–650 (1985).
8. Smith, S. K., and L. E. Limbird. Solubilization of human platelet alpha-adrenergic receptors: evidence that agonist occupancy of the receptor stabilizes receptor-effector interactions. *Proc. Natl. Acad. Sci. USA* **78**:4026–4030 (1981).
9. Smith, S. K., and L. E. Limbird. Evidence that human platelet alpha-adrenergic receptors coupled to inhibition of adenylate cyclase are not associated with the subunit of adenylate cyclase ADP-ribosylated by cholera toxin. *J. Biol. Chem.* **257**:10471–10478 (1982).
10. Regan, J. W., N. Barden, R. J. Lefkowitz, M. G. Caron, R. M. DeMarinis, A. J. Kroy, K. G. Holden, W. D. Mathews, and J. P. Hieble. Affinity chromatography of human platelet alpha$_2$-adrenergic receptors. *Proc. Natl. Acad. Sci. USA* **79**:7223–7227 (1982).
11. Regan, J. W., R. M. DeMarinis, M. G. Caron, and F. J. Lefkowitz. Identification of the subunit-binding site of alpha$_2$-adrenergic receptors using [^{3}H] phenoxybenzamine. *J. Biol. Chem.* **259**:7864–7869 (1984).
12. Jaiswal, R. K., and R. K. Sharma. Purification and biochemical characterization of alpha$_2$-adrenergic receptor from the rat adrenocortical carcinoma. *Biochem. Biophys. Res. Commun.* **130**:58–64 (1985).
13. Maguire, M. E., R. A. Wiklund, H. S. Anderson, and A. G. Gilman. Binding of [^{125}I]iodohydroxybenzylpindolol to putative beta-adrenergic receptors of rat glioma cells and other cell clones. *J. Biol. Chem.* **254**:1221–1231 (1976).
14. Rashidbaigi, A., and A. E. Ruoho. Iodoazidobenzylpindolol, a photoaffinity probe for the beta-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **78**:1609–1613 (1981).
15. Shorr, R. G. L., S. L. Heald, P. W. Jeffs, T. N. Lavin, M. W. Strohsacker, R. J. Lefkowitz, and M. G. Caron. The beta-adrenergic receptor: rapid purification and covalent labeling by photoaffinity crosslinking. *Proc. Natl. Acad. Sci. USA* **79**:2778–2782 (1982).
16. Seidman, C. E., H. J. Hess, C. J. Homcy, and R. M. Graham. Photoaffinity labeling of the alpha$_1$-adrenergic receptor using an ^{125}I-labeled aryl azide analogue of prazosin. *Biochemistry* **23**:3765–3770 (1984).
17. Leeb-Lundberg, L. M. F., K. E. J. Dickinson, S. L. Heald, J. E. S. Wikberg, P. O. Hagen, J. F. DeBernardis, M. Winn, D. L. Arendsen, R. J. Lefkowitz, and M. G. Caron. Photoaffinity labeling of mammalian alpha$_1$-adrenergic receptors: identification of the ligand binding subunit with a high affinity radioiodinated probe. *J. Biol. Chem.* **259**:2579–2587 (1984).
18. Dickinson, K. E. J., L. M. F. Leeb-Lundberg, S. L. Heald, J. E. S. Wikberg, J. F. Debernardis, M. G. Caron, and R. J. Lefkowitz. Photoaffinity cross-linking of a radioiodinated probe, ^{125}I-A55453, into alpha$_1$-adrenergic receptors. *Mol. Pharmacol.* **26**:187–195 (1984).
19. Liang, C. T., and B. Sacktor. Preparation of renal cortex basal-lateral and brush border membranes. Localization of adenylate cyclase and guanylate cyclase activities. *Biochim. Biophys. Acta* **466**:474–487 (1977).
20. Lash, L. H., and D. P. Jones. Localization of the membrane associated thiol oxidase of rat kidney to the basal-lateral plasma membrane. *Biochem. J.* **203**:371–376 (1982).
21. Neubig, R. R., and O. Szamraj. Large-scale purification of α_2-adrenergic receptor-enriched membranes from human platelets. Persistent accumulation of guanine nucleotides with nonpurified membranes. *Biochim. Biophys. Acta* **854**:67–76 (1984).
22. Prpic, V., K. C. Green, P. F. Blackmore, and J. H. Exton. Vasopressin-, angiotensin II-, and alpha$_1$-adrenergic-induced inhibition of Ca^{2+} transport by rat liver plasma membrane vesicles. *J. Biol. Chem.* **259**:1382–1385 (1984).
23. Graham, R. M., H. J. Hess, and C. J. Homcy. Solubilization and purification of the alpha$_1$-adrenergic receptor using a novel affinity resin. *Proc. Natl. Acad. Sci. USA* **79**:2186–2190 (1981).
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
25. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitors which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
26. Scatchard, G. The attractions of protein for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
27. Rosenthal, H. E. A graphic method for the determination of binding parameters in a complex system. *Anal. Biochem.* **20**:525–532 (1967).
28. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
29. Motulsky, H. J., M. D. Snavely, R. J. Hughes, and P. A. Insel. Interaction of verapamil and other calcium channel blockers with alpha$_1$- and alpha$_2$-adrenergic receptors. *Circ. Res.* **52**:226–231 (1983).
30. Starke, K., T. Endo, and H. D. Taube. Relative pre- and post-synaptic potencies of alpha-adrenoceptor agonists in the rabbit pulmonary artery. *Naunyn Schmiedebergs Arch. Pharmacol.* **291**:55–78 (1975).
31. Limbird, L. E., T. M. Connolly, J. D. Sweatt, and H. D. Uderman. Human platelet alpha$_2$-adrenergic receptors: effect of Na^+ on interaction with the adenylate cyclase system and on epinephrine-stimulated platelet secretion. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**:235–242 (1985).
32. Johnson, G. L., V. I. MacAndrew, and P. F. Pilch. Identification of the glucagon receptor in rat liver membranes by photoaffinity cross linking. *Proc. Natl. Acad. Sci. USA* **78**:875–878 (1981).
33. Massague, J., B. J. Guillette, M. P. Czech, C. S. Morgan, and R. A. Bradshaw. Identification of a nerve growth factor receptor protein in sympathetic ganglion membranes by affinity labeling. *J. Biol. Chem.* **256**:9419–9424 (1981).
34. Yip, C. C., M. L. Moule, and C. W. T. Yeung. Subunit structure of insulin receptor of rat adipocytes as demonstrated by photoaffinity labeling. *Biochemistry* **21**:2940–2945 (1982).
35. Le Hir, A., M. M. Janot, and R. Goutarel. Stereoisomeres de la yohimbine. III. Allo-yohimbine et α-yohimbine. *Bull. Soc. Chim. Fr.* 1027–1032 (1953).
36. Graham, R. M., H. J. Hess, E. H. Haber, and C. J. Homcy. Antibodies to the alpha$_1$- and alpha$_2$-selective antagonists prazosin and yohimbine as probes of the alpha-adrenergic binding site. *Hypertension* **4**:SII183–SII187 (1982).

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