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Biochemistry

Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype

(G protein/photoaffinity labeling/[^{3}H]rauwolscine binding/α_2-adrenoceptor/α_2B-adrenergic receptor)

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ABSTRACT **An α_2-adrenergic receptor subtype has been cloned from a human kidney cDNA library using the gene for the human platelet α_2-adrenergic receptor as a probe. The deduced amino acid sequence resembles the human platelet α_2-adrenergic receptor and is consistent with the structure of other members of the family of guanine nucleotide-binding protein-coupled receptors. The cDNA was expressed in a mammalian cell line (COS-7), and the α_2-adrenergic ligand [^{3}H]rauwolscine was bound. Competition curve analysis with a variety of adrenergic ligands suggests that this cDNA clone represents the α_2B-adrenergic receptor. The gene for this receptor is on human chromosome 4, whereas the gene for the human platelet α_2-adrenergic receptor (α_2A) lies on chromosome 10. This ability to express the receptor in mammalian cells, free of other adrenergic receptor subtypes, should help in developing more selective α-adrenergic ligands.**

The adrenergic receptors (subtypes α_1, α_2, β_1, and β_2), which bind epinephrine and norepinephrine, are encoded by separate genes (1). Although these genes are distinct, they appear to be homologous and are members of a large family of guanine nucleotide-binding protein (G protein)-coupled receptors. This family includes the muscarinic cholinergic receptors (2, 3), the substance K receptor (4), and even rhodopsin, the receptor for light (5). Interestingly, the results of molecular cloning studies show even greater heterogeneity of receptors than heretofore appreciated. For example, until recently the muscarinic cholinergic receptors were classified into two subtypes based largely on results from pharmacologic studies. From cloning and expression studies, however, at least four subtypes exist (6, 7).

Recently we cloned and expressed the gene for the human platelet α_2-adrenergic receptor (8). Southern blot analysis of DNA from somatic cell hybrids revealed that a probe made from the human platelet α_2-adrenergic receptor gene recognized three different genes. The gene for the human platelet α_2-adrenergic receptor was localized to chromosome 10. The other two genes, localized to human chromosomes 2 and 4, may code for related receptor proteins. We now report the molecular cloning and expression of a cDNA containing the gene localized to chromosome 4.‡ Expression of this cDNA shows that it binds α_2-adrenergic ligands and represents an α_2-adrenergic receptor subtype.

METHODS

Cloning and Sequencing. A human kidney λGT10 cDNA library, provided by S. Orkin (Children's Hospital Medical Center, Boston, MA), was screened using the 0.95-kilobase (kb) *Pst* I fragment from the human platelet α_2-adrenergic receptor as a probe (8). The probe was labeled with ^{32}P by the method of random priming. Duplicate filters were hybridized in 6× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/10× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.1% sodium pyrophosphate/0.1% NaDodSO$_4$/sheared salmon sperm DNA at 50 μg/ml at 60°C for 18 hr. Filters were washed in 0.5× SSC at 65°C. λ phage hybridizing to the probe were plaque purified, and λDNA was prepared (9). For Southern blot analysis and subcloning, cDNA was inserted into pSP65. Nucleotide sequence analysis was done by the Sanger dideoxy nucleotide chain-termination method (10) on denatured double-stranded plasmid templates by use of either the Klenow fragment of DNA polymerase I or avian reverse transcriptase (Promega Biotec, Madison, WI).

Expression. Eukaryotic expression vectors were made for the DNA corresponding to α_2-C4 (see abbreviations) and to α_2-C10 as follows. *Eco*RI-digested α_2-C4 cDNA was blunt-ended with the Klenow fragment of DNA polymerase and was then cleaved with *Nco* I. The 1.4-kb *Nco* I–*Eco*RI fragment containing the α_2-C4 coding sequence was then ligated into pSPNar (11), which had been digested with *Nco* I–*Eco*RV to remove the β_2-adrenergic receptor coding sequence. The resulting plasmid (pSPα_2-C4) contained the coding region of α_2-C4 and 40 bp of the 5′-, and all of the 3′-, untranslated region of the β_2-adrenergic receptor. Similarly, *Hin*dIII-digested α_2-C10 DNA was blunt-ended and cleaved with *Nco* I, and the 1.4-kb *Nco* I–*Hin*dIII fragment containing the α_2-C10 coding sequence was ligated to the *Nco* I–*Eco*RV site of pSPNar to give pSPα_2-C10. The 2.0-kb *Nar* I–*Eco*RI restriction fragment of the human β_2-adrenergic receptor (12) was blunt-ended and was ligated to the *Hin*dIII–*Bam*HI fragment of the expression vector pBC12MI (13), which had been blunt-ended and previously modified by removal of its *Nco* I site. The resulting plasmid was digested with *Nco* I and *Sal* I to yield a restriction fragment (pBCβ-5′) containing 40 base pairs (bp) of the 5′-untranslated region of the β_2-adrenergic receptor adjacent to the Rous sarcoma virus promoter. *Nco* I–*Sal* I restriction fragments of pSPα_2-C4 and of pSPα_2-C10 (containing the coding regions of α_2-C4 and of α_2-C10, as well as the 3′-untranslated region of the β_2-adrenergic receptor) were then ligated to pBCβ-5′ to give the plasmids pBCα_2-C4 and pBCα_2-C10, respectively. These plasmids were then used to transfect COS-7 cells (13).

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Abbreviations: G protein, guanine nucleotide-binding protein; α_2-C4, the α_2-adrenergic receptor the gene for which is located on chromosome 4; α_2-C10, the α_2-adrenergic receptor the gene for which is located on chromosome 10.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03853).

Binding Studies. Culture flasks (75 cm^2) were rinsed with phosphate-buffered saline (2.7 mM KCl/1.5 mM KH_2PO_4/0.5 mM $MgCl_2$/137 mM NaCl/8.1 mM Na_2HPO_4, pH 7.3), and the cells were scraped into 6 ml of TME solution (50 mM Tris·HCl/10 mM $MgCl_2$/1 mM EDTA, pH 7.4). A lysate was prepared with a Brinkmann homogenizer (model PT10/35) and was separated into 1-ml aliquots, which were frozen and stored at −80°C. Individual aliquots were thawed at room temperature and were diluted with TME solution to a final volume of 10 ml for cells transfected with pBCα_2-C4 and 20 ml for cells transfected with pBCα_2-C10. Binding assays contained 400 μl of diluted membrane preparation in a final volume of 500 μl. Assays were started by the addition of membranes, were incubated at 25°C for 60 min, and were concluded by filtration through Whatman GF/C filters followed by four separate 4-ml rinses with cold (4°C) phosphate-buffered saline. For competition curve analysis, assays contained a final concentration of either 1 nM or 5 nM [^{3}H]rauwolscine (74.1 Ci/mmol; 1 Ci = 37 GBq) depending upon whether the assay was for α_2-C4 or α_2-C10, respectively. For saturation curve analysis, nonspecific binding was measured in the presence of 10 μM rauwolscine. Data were analyzed by computer on an iterative nonlinear regression program (14).

Drugs. Sources of drugs were as follows: idazoxan (Reckitt and Colman), phentolamine (CIBA–Geigy), phenoxybenzamine and SKF 104078 (Smith Kline & French), prazosin and UK 14304 (Pfizer), BE 2254 (Beiersdorf), WY 26392 (Wyeth), L 654284 (Merck Sharp & Dohme), RS 21361 (Syntex), BHT 920 and BHT 933 (Boehringer Ingelheim), rauwolscine (Roth, Karlsruhe, F.R.G.), yohimbine (Aldrich), WB 4101, *p*-aminoclonidine and dopamine (Research Biochemicals, Wayland, MA), corynanthine, guanabenz, oxymethazoline, phenylephrine, epinephrine, and norepinephrine (Sigma).

RESULTS

A human kidney λGT10 cDNA library was screened with the 0.95-kb *Pst* I restriction fragment derived from the coding block of the gene for the human platelet α_2-adrenergic receptor. Two incomplete, but overlapping, clones were isolated and were characterized by restriction endonuclease mapping and DNA sequence analysis. Complementary portions of the clones were then ligated to give a full-length clone. Somatic cell hybridization analysis showed that the gene corresponding to this cDNA was localized to human chromosome 4 (data not shown). Accordingly this cDNA was referred to as α_2-C4, whereas the designation α_2-C10 refers to the gene for the human platelet α_2-adrenergic receptor.

Fig. 1 shows the nucleotide and deduced amino acid sequence of α_2-C4. This cDNA codes for a protein of 461 amino acids. Hydropathy analysis (15) of the amino acid sequence indicates that there are seven hydrophobic regions, each consisting of 20–25 amino acid residues, separated by hydrophilic stretches of various length (data not shown). This pattern is essentially the same as that seen for other G protein-coupled receptors and supports the model wherein the receptor, an integral membrane protein, contains seven transmembrane-spanning segments (16).

Fig. 2 shows this model as applied to α_2-C4. Solid circles indicate residues in α_2-C4 identical with α_2-C10; clearly, regions of greatest similarity span the membrane (≈75%). The least similar regions are the amino terminus (14%), third cytoplasmic loop (21%), and third extracellular loop (11%). Although lacking amino acid-sequence identity, the third cytoplasmic loops of α_2-C4 and α_2-C10 are alike in length (≈150 amino acids) and in the high content of charged residues. In this respect, these loops also resemble the muscarinic cholinergic receptors but differ from the β-

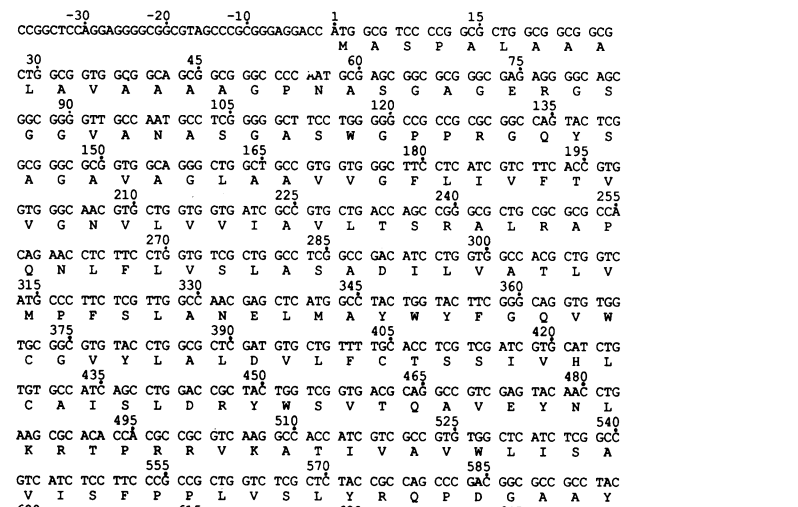

FIG. 1. Nucleotide and deduced amino acid sequence of the α_2-C4 human kidney cDNA clone.

adrenergic receptors, which have relatively short third cytoplasmic loops (≈60 residues).

To compare ligand-binding characteristics, we inserted α_2-C4 and α_2-C10 into the mammalian expression vector pBC12MI (13). Two days after transfection of COS-7 cells, membranes were prepared, and binding of the α_2-adrenergic antagonist [^{3}H]rauwolscine was examined. Fig. 3 is a Scatchard plot that shows [^{3}H]rauwolscine to bind with ≈5-fold higher affinity to α_2-C4 as compared with α_2-C10. Membranes from nontransfected COS-7 cells exhibited no specific binding of [^{3}H]rauwolscine. The specific binding activity for α_2-C10 in this crude membrane preparation was ≈30 pmol per mg of protein, more than two orders of magnitude greater than for washed human platelet membranes.

Fig. 4*A* shows results from the competition of either prazosin or idazoxan for the binding of [^{3}H]rauwolscine to membranes prepared from COS-7 cells transfected with either pBCα_2-C4 or pBCα_2-C10. The most striking feature is that prazosin, a traditional α_1-adrenergic selective antagonist, shows much higher affinity for α_2-C4 as compared with α_2-C10. On the other hand, idazoxan, an α_2-selective antagonist, binds with nearly equal affinity to both receptors. At both α_2-C4 and α_2-C10 idazoxan is more potent than prazo-

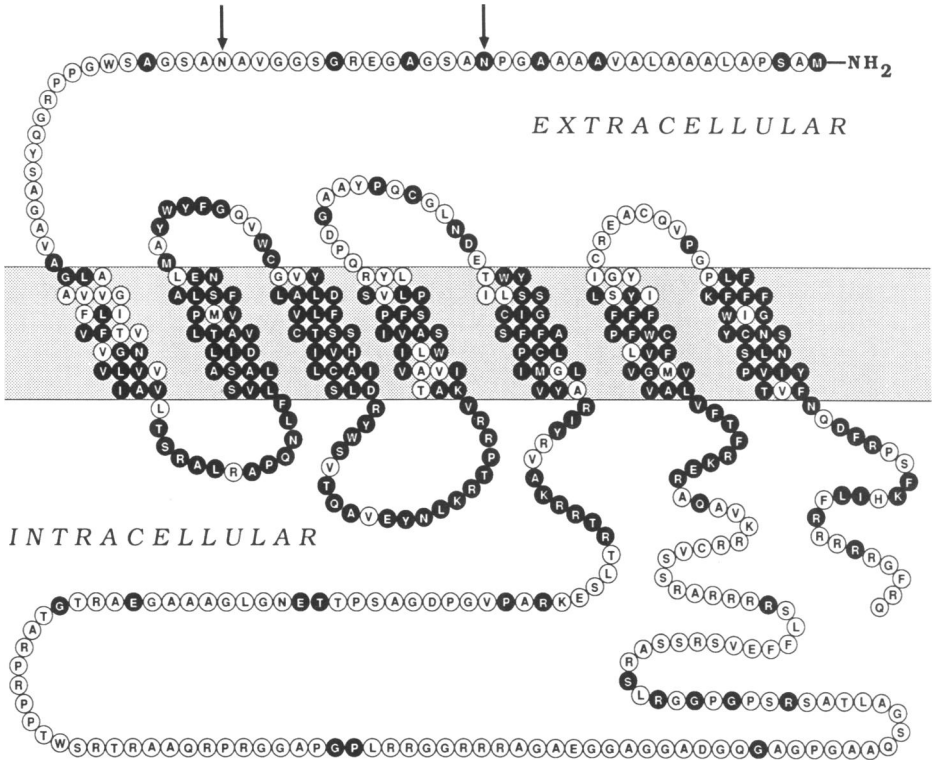

FIG. 2. Seven transmembrane-spanning model of α_2-C4 showing amino acid identity with the human platelet α_2-adrenergic receptor. Solid circles indicate amino acids common to the corresponding position in the human platelet α_2-adrenergic receptor (8). The arrows indicate potential sites of N-linked glycosylation.

sin. These antagonist competition curves are steep and monophasic with slope factors of ≈1.

Fig. 4*B* shows similar competition curve data for norepinephrine and oxymetazoline. Norepinephrine, a physiological neurotransmitter, binds with nearly 10-fold higher affinity to α_2-C4 as compared with α_2-C10. By contrast, oxymetazoline, an α_2-selective agonist, has much lower affinity for α_2-C4 as compared with α_2-C10. Agonist competition curves were more shallow than the antagonist curves—with slope factors ranging between 0.8 and 0.95.

K_i values for the competition data shown in Fig. 4 as well as for data obtained using a variety of other adrenergic ligands are listed in Table 1. Aside from rauwolscine, most traditional (yohimbine, idazoxan) and recently developed (SKF 104078, WY 26392, L 654284, RS 21361) α_2-selective

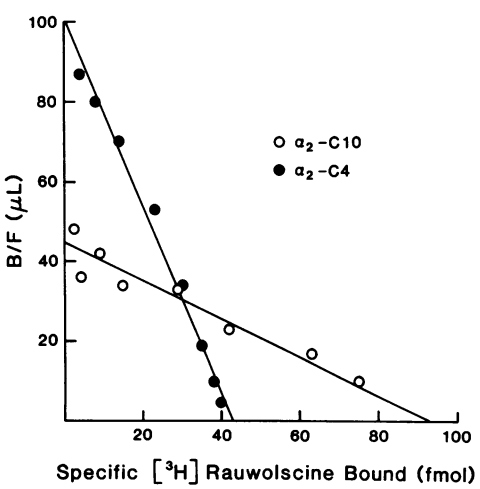

FIG. 3. Scatchard plots of the specific binding of [^{3}H]-rauwolscine to membranes prepared from COS-7 cells transfected with either pBCα_2-C4 (●) or pBCα_2-C10 (○). The parameter estimates (linear-regression analysis) are as follows: α_2-C4, K_d = 0.43 nM, B_{max} = 43 fmol per assay (6.9 pmol per mg of protein); and α_2-C10, K_d = 2.1 nM, B_{max} = 93 fmol per assay (25 pmol per mg of protein). This experiment was repeated three times, and the average K_d values (nonlinear-regression analysis) for α_2-C4 and α_2-C10 were 0.38 ± 0.01 nM and 2.7 ± 0.3 nM, respectively ($\bar{x}$ ± SEM).

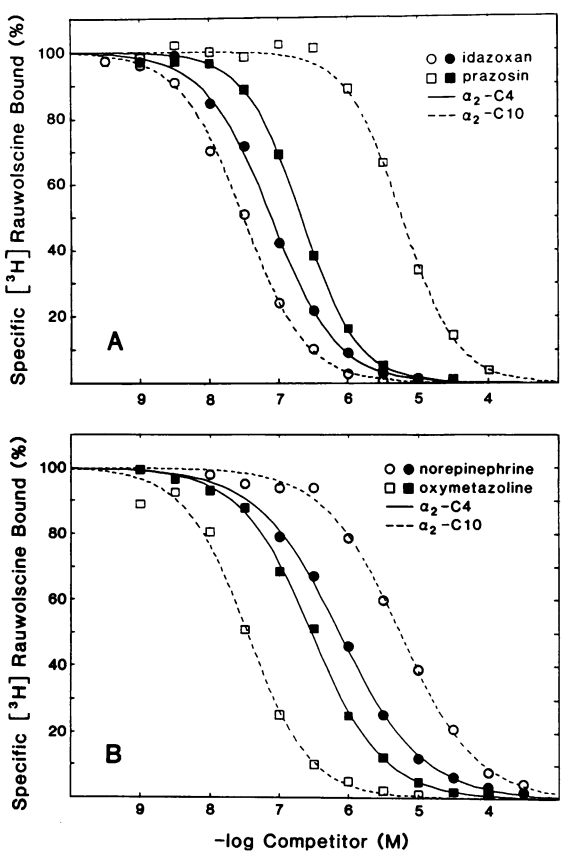

FIG. 4. Antagonist (*A*) and agonist (*B*) competition for the binding of [^{3}H]rauwolscine to membranes prepared from COS-7 cells transfected with either pBCα_2-C2-C4 (solid symbols and lines) or pBCα_2-C10 (open symbols and dashed lines). (*A*) Circles, idazoxan; squares, prazosin. (*B*) Circles, norepinephrine; squares, oxymetazoline. K_i estimates for these and other adrenergic compounds are listed in Table 1.

Table 1. Competition by α-adrenergic compounds for the binding of [^{3}H]rauwolscine to membranes prepared from COS-7 cells transfected with pBCα_2-C4 or pBCα_2-C10

	K_i, nM		
	α_2-C4	α_2-C10	Ratio
Antagonists			
Yohimbine	0.93	1.6	2
Idazoxan	17	10	0.6
SKF 104078	33	86	3
WY 26392	2.9	8.1	3
L 654284	0.74	1.2	2
RS 21361	200	400	2
Phentolamine	33	10	0.3
Phenoxybenzamine	41	70	2
Prazosin	41	1800	40
WB 4101	0.94	7.8	8
BE 2254	0.94	9.3	10
Corynanthine	73	710	10
Agonists			
p-Aminoclonidine	81	74	0.9
UK 14304	210	72	0.3
Oxymetazoline	62	11	0.2
Guanabenz	59	14	0.2
BHT 920	140	194	1
BHT 933	2200	3100	1
Epinephrine	170	1000	6
Norepinephrine	240	2400	10
Dopamine	1000	4500	4
Phenylephrine	2900	1500	0.5

These data result from single determinations done simultaneously for both α_2-C4 and α_2-C10. SEs of the K_i values are <10%. Ratios (α_2-C10/α_2-C4) were rounded to the nearest digit.

antagonists bind with comparable affinity to both α_2-C4 and α_2-C10. Interestingly, most traditional α_1-selective antagonists (prazosin, WB 4101, BE 2254, and corynanthine) bind with higher affinity to α_2-C4: in the case of WB 4101 and BE 2254, they bind with very high affinity. The affinity of prazosin for α_2-C4, however, is still significantly less than for an α_1-adrenergic receptor; e.g., the K_i of prazosin for α_1-adrenergic receptors in DDT$_1$MF-2 smooth muscle cells is ≈0.5 nM (17).

The endogenous catecholamines (epinephrine, norepinephrine, and dopamine) all show higher affinity for α_2-C4 as compared with α_2-C10. The imidazoline agonists (*p*-aminoclonidine, UK 14304, and oxymetazoline) range widely in relative affinity for these receptors; from little (or no) selectivity for *p*-aminoclonidine and UK 14304 to a markedly higher affinity (6-fold) at α_2-C10 for oxymetazoline. Although the affinity of oxymetazoline is lower for α_2-C4 than for α_2-C10, it is not as low as that for the α_1-adrenergic receptors in DTT$_1$MF-2 cells (K_i ≈ 400 nM; ref. 17). A related α_2-adrenergic agonist, guanabenz, also shows higher affinity for α_2-C10 as compared with α_2-C4. The azepine derivatives BHT 920 and BHT 933, which are α_2-selective agonists, have similar affinities for both α_2-C4 and α_2-C10. It should be underscored that whether or not these compounds are, in fact, agonists at α_2-C4 is yet unknown.

The M_r of the human platelet α_2-adrenergic receptor (α_2-C10) is ≈64,000 as determined from NaDodSO$_4$/PAGE of purified (18) and photoaffinity-labeled receptors (19). To determine the M_r of α_2-C4, as expressed in COS-7 cells, membranes from cells transfected with either pBCα_2-C4 or pBCα_2-C10 were photoaffinity-labeled with [^{3}H]SKF 102229. Fig. 5 shows an autoradiograph after NaDodSO$_4$/PAGE of crude-membrane preparations photoaffinity-labeled with [^{3}H]SKF 102229 either without or with 10 μM phentolamine. For cells transfected with α_2-C10, a labeled band was seen

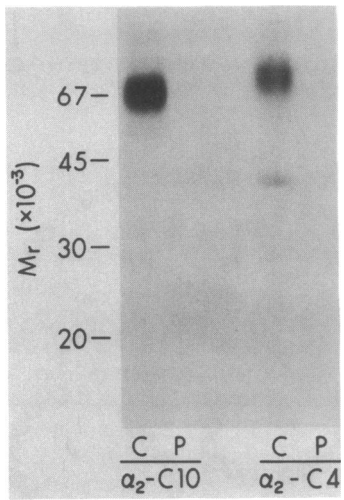

FIG. 5. [^{3}H]SKF 102229-photoaffinity labeling of α_2-C10 and α_2-C4 expressed in COS-7 cells. Labeling was done as described (19). Cell lysates were thawed and used without further dilution. Controls (C) contained 500 μl of lysate and a final concentration of 50 nM [^{3}H]SKF 102229 and 5 mM dithiothreitol. Samples used to assess nonspecific labeling (P) also contained 10 μM phentolamine. After photolysis the samples were centrifuged, and the pellets were resuspended with 200 μl of NaDodSO$_4$/PAGE sample buffer (20). The samples (100 μl) were loaded onto a 12% polyacrylamide gel and were electrophoresed. The gels were dried and prepared for fluorography; exposures were made for 1 week at −80°C. Positions of M_r standards are at left.

with an M_r of ≈67,000. For cells transfected with α_2-C4, however, a labeled band with an M_r of ≈75,000 was obtained; in addition, a labeled band of ≈42,000 was also present.

DISCUSSION

α-Adrenergic receptors were first subclassified based on anatomic location, either on the pre- or postsynaptic sides of nerve junctions (21). Subsequently this anatomic classification evolved into a pharmacologic one when presynaptic receptors were also found at postsynaptic locations (22, 23). Because of the latter realization the pre- versus postsynaptic designations were eventually replaced in favor of the terms α_1 (postsynaptic) and α_2 (presynaptic) (21–24). More recent evidence indicates further heterogeneity of the α_2-adrenergic receptors.

One line of evidence, obtained from binding studies, shows that prazosin has significantly higher affinity for rat brain α_2-adrenergic receptors as compared with human platelet α_2-receptors (25). It was not clear, however, whether this affinity difference simply reflected species differences or true receptor heterogeneity with the different subtypes exhibiting specific tissue distributions. A classification based upon the higher affinity of prazosin for rodent versus nonrodent α_2-adrenergic receptors was also proposed (26). Besides prazosin, oxymetazoline was said to differentiate between these α_2-adrenergic receptors by showing higher affinity for the nonrodent subtype.

α_2-Adrenergic receptor heterogeneity can also be found within a single species (27). In human cerebral cortex prazosin competes for the binding of [^{3}H]yohimbine monophasically and with a K_i of 240 nM, whereas in the human caudate nucleus the data can be resolved into high- and low-affinity sites with K_i values of 8 and 330 nM, respectively. Likewise oxymetazoline has a K_i of 1 nM in the human cerebral cortex and K_i values of 4 and 580 nM in the caudate nucleus. The binding of [^{3}H]yohimbine itself is monophasic in both the cortex and caudate nucleus. It was proposed that

the site with high affinity for oxymetazoline and low affinity for prazosin be called α_2A, and the sites with low affinity for oxymetazoline and high affinity for prazosin be called α_2B (26).

A second line of investigation has found that rauwolscine can be used to resolve α_2-adrenergic receptor subtypes. By the use of quantitative autoradiography [^{3}H]rauwolscine was found to label a subset of all α_2-adrenergic receptors that are labeled by [^{3}H]idazoxan (28). The two classes of receptors were defined as α_2-R_s, for rauwolscine sensitive, and α_2-R_i, for rauwolscine insensitive (29). Support for this classification came from binding studies (29). Thus, in the rat septum, rauwolscine competes for the binding of [^{3}H]idazoxan with a slope factor of 1.2 and a K_i of 40 nM. In the caudate nucleus, however, rauwolscine competes for the binding of [^{3}H]-idazoxan with a slope factor of 0.5, and the binding could be resolved into high- and low-affinity components with K_i values of 1 and 100 nM, respectively. The binding of [^{3}H]-idazoxan itself was monophasic, indicating no discrimination between these receptor subtypes.

A third line of investigation suggests that pre- versus postsynaptic α_2-adrenergic receptors can be differentiated (30). SKF 104078, an α_2-adrenergic antagonist, blocks agonist-induced inhibition of neurotransmission (a presynaptic response) and agonist-induced vasoconstriction (a postsynaptic response) to different extents. For the postsynaptic response, SKF 104078 shows high affinity (K_d = 80 nM), whereas for the presynaptic response SKF 104078 is virtually inactive ($K_d > 10$ μM).

The binding data obtained for α_2-C4 in this study clarify some previous findings regarding α_2-receptor heterogeneity. α_2-C4 has high affinity for prazosin and low affinity for oxymetazoline as compared with α_2-C10. This finding is consistent with the results of Petrash and Bylund (27) with regard to pharmacologic characteristics of the α_2B adrenergic receptor. α_2-C4 also shows significantly higher affinity for rauwolscine as compared with α_2-C10—data agreeing with the results of Boyajian and Leslie (29) concerning the pharmacologic characteristics of α_2-R_s subtype. That the α_2B and α_2-R_s subtypes have both been localized to the caudate nucleus suggests that they are identical and equivalent to α_2-C4. The α_2A subtype, on the other hand, would be the same as α_2-R_i subtype and equivalent to α_2-C10. The latter contention is supported by our results and by earlier data (27, 29), which indicate that neither yohimbine nor idazoxan are selective for these α_2-adrenergic receptor subtypes. Additionally, our data show that SKF 104078 cannot discriminate significantly between α_2-C4 and α_2-C10 subtypes, having moderately high affinity for both receptors. This fact indicates that α_2-C4 is not the presynaptic α_2-receptor, as defined by Hieble *et al.* (30) and that at least three α_2-adrenergic receptor subtypes must exist. We suggest that the presynaptic α_2-adrenergic receptor be classified as α_2C subtype after the α_2A and α_2B nomenclature. Whether or not the α_2C receptor is also found postsynaptically or the α_2A and α_2B subtypes are found presynaptically needs to be answered.

The results of our photoaffinity-labeling studies indicate that α_2-C4 has a higher apparent molecular weight, as compared with α_2-C10, when expressed in COS-7 cells. Thus, even though both α_2-C10 and α_2-C4 have nearly identical M_r values, as deduced from their nucleotide sequences (49,382 and 49,534, respectively), the effects of posttranslational modifications on their mobility in $NaDodSO_4$/PAGE are different. The most likely factor affecting their mobility in $NaDodSO_4$/PAGE is glycosylation, although differential covalent modification with fatty acids is another possibility.

Activation of human platelet α_2-adrenergic receptors (α_2-C10) inhibits the activity of adenylyl cyclase. The biochemical effects of activation of α_2-C4 are presently unknown. Additionally, tissue distribution and physiological effects of α_2-C4 activation await determination; α_2-C4 was cloned from a human kidney cDNA library and thus must be present in human kidney. Furthermore, from studies of the α_2B adrenergic receptor α_2-C4 is probably also present in the caudate nucleus of the human brain. The presence of α_2-C4 in these critical organs offers a prospective target for drugs yet to be developed.

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