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Heterologous Expression of the Cloned Guinea Pig α_2A, α_2B, and α_2C Adrenoceptor Subtypes

RADIOLIGAND BINDING AND FUNCTIONAL COUPLING TO A cAMP-RESPONSIVE REPORTER GENE

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ABSTRACT. Functional studies have shown that 6-chloro-9-[(3-methyl-2-butenyl)oxy]-3-methyl-1*H*-2,3,4,5-tetrahydro-3-benzazepine (SKF 104078) has very low affinity for prejunctional α_2-adrenoceptors (α_2-AR) in the guinea pig atrium. In this study, we have cloned guinea pig homologues of the human α_2-C10, α_2-C2 and α_2-C4 AR subtypes and have studied them in isolation by heterologous expression in cultured mammalian cells. Oligonucleotide primers, designed from conserved areas of the human α_2-ARs were used in a polymerase chain reaction (PCR) with template cDNA synthesized from guinea pig atrial mRNA. Three PCR products were obtained that shared identity with the three human α_2-AR subtypes. A guinea pig (gp) genomic library was screened with a cDNA clone encoding a portion of the gp-α_2A, and genes containing the complete coding sequences of the guinea pig α_2A, α_2B, and α_2C AR subtypes were obtained. These guinea pig genes were subcloned into a eukaryotic expression plasmid and were expressed transiently in COS-7 cells. The binding of the α_2-selective antagonist [^{3}H]MK-912 to membranes prepared from these cells was specific and of high affinity with K_d values of 810 pM for gp-α_2A, 2700 pM for gp-α_2B and 110 pM for gp-α_2C. Competition for the binding of [^{3}H]MK-912 by SKF 104078 indicated that it was of moderately high affinity (~100 nM) but that it was not selective for any of the guinea pig α_2-AR subtypes. Co-expression of guinea pig α_2-AR subtypes with a cyclicAMP-responsive chloramphenicol acetyltransferase (CAT) reporter gene resulted in agonist-dependent modulation of CAT activity. For the gp-α_2A, a biphasic response was obtained with low concentrations of noradrenaline (NE) decreasing forskolin-stimulated CAT activity and high concentrations causing a reversal. For the gp-α_2B, NE produced mostly potentiation of forskolin-stimulated activity, and for the gp-α_2C, NE caused mainly inhibition. Overall, the pharmacology of the cloned guinea pig α_2-AR subtypes was in agreement with data obtained for the native guinea pig receptors and was functionally similar to that of the cloned human α_2-AR subtypes. BIOCHEM PHARMACOL 51;3:291–300, 1996.

KEY WORDS. pharmacology; G-protein coupled; adenylyl cyclase; reporter gene

Molecular cloning studies have identified three human genes that encode unique α_2-AR† subtypes termed α_2-C10, α_2-C2, and α_2-C4, where C denotes the chromosomal location of the gene [1–3]. The native α_2-AR subtypes have also been characterized pharmacologically as α_2A, α_2B and α_2C and have been shown to correspond, respectively, to the cloned α_2-C10, α_2-C2, and α_2-C4 [4]. Homologues of the human α_2-ARs have also been obtained from several other animal species including rat, mouse, porcine and fish [reviewed in Ref. 5]. Interestingly, significant pharmacological differences have been shown to occur between subtype homologues from different animal species. For example, an α_2D subtype that corresponds to the rat homologue of the human α_2-C10 has been defined pharmacologically. Understanding these pharmacological differences is important if we are to make sense of prior studies with nonhuman tissues and if one is going to use an appropriate animal model for future drug development.

The guinea pig ileum was one of the first tissues where the α_2-ARs were classified [6]. Other guinea pig tissues, such as the trachea, have been used extensively to study the pharmacology of smooth muscle contraction [7]. In addition, the guinea pig atrium is a model for the pharmacology of the prejunctional α_2-AR [8]. A potentially significant finding with respect to this model is that SKF 104078, an α_2-AR antagonist, has 100-fold lower potency in the guinea pig atrium as compared with its potency in dog and rabbit saphenous vein, models for the postjunctional α_2-AR [9]. Thus, SKF 104078 may differ-

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† *Abbreviations:* AR, adrenergic receptor; SKF 104078, 6-chloro-9-[(3-methyl-2-butenyl)oxy]-3-methyl-1*H*-2,3,4,5-tetrahydro-3-benzazepine; PCR, polymerase chain reaction; TM, transmembrane; gp, guinea pig; CAT, chloramphenicol acetyltransferase; NE, noradrenaline; RT, reverse transcription; MK-912, (2*S*,12b*S*)1′3′-dimethylspiro(1,3,4,5′,6,6′,7,12b-octahydro-2*H*-benzo(b)furo(2,3-*a*)quinazoline)-2,4′-pyrimidin-2′-one; CRE, cAMP response element; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

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entiate between pre- and postjunctional α_2-ARs. Radioligand binding studies with the cloned human and rat α_2-ARs, however, show no significant differences between subtypes in their affinity for SKF 104078 [10, 11]. It would seem that the α_2-AR in the guinea pig atrium is either a unique subtype, a pharmacological variant of a previously cloned human homologue, or there are further aspects of the chemistry of SKF 104078 or the biology of the guinea pig atrium that contribute to this apparent lower affinity. To test the two former possibilities, we hypothesized that one of the guinea pig α_2-AR subtypes would have low affinity for SKF 104078. We, therefore, screened guinea pig atrial cDNA and genomic libraries and cloned the guinea pig homologues of α_2A, α_2B, and α_2C ARs. We studied the pharmacology of these α_2-AR subtypes and found that SKF 104078 had high affinity for all of them, suggesting that an additional subtype may still exist or that there is additional complexity with respect to the functional activation of the guinea pig atrial α_2-ARs.

MATERIALS AND METHODS

RT-PCR

Polyadenylated mRNA was prepared from the left and right guinea pig (Sasco Hartley) atria using the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). Reverse transcription coupled with the polymerase chain reaction (RT-PCR) was performed as previously described [12]. cDNA was synthesized from 1.5 μg of atrial mRNA in a reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 40 U RNase inhibitor (Invitrogen), 0.5 mM dNTPs (Pharmacia, Piscataway, NJ), 2 μg random primers (Invitrogen) and 24 U of AMV reverse transcriptase (United States Biochemical, Cleveland, OH). The reaction was incubated for 10 min at room temperature, then for 60 min at 42° and was stopped by heating at 95°. A PCR mixture containing a 1 μM concentration of the sense (#958) and antisense (#956) primers, 5% DMSO and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT) was added to the RT reaction. The primers were as follows:

#958: 5′-CGCCGAATTCGCTGTTGCAGTAGC3′
#956: 5′-CTGCGCATCCGC$^{G}/_{T}$CC$^{C}/_{T}$TGCCTCA-TCAT3′

Sixty cycles of the following PCR conditions were used: 95°, 1 min; 50°, 2 min; 72°, 3 min. Controls using either RNase or DNase were performed to establish that the PCR products originated from mRNA. PCR products representing the guinea pig α_2A, α_2B, and α_2C subtypes were obtained and were subcloned into pBluescript KS$^+$ (Stratagene, La Jolla, CA) and sequenced by the di-deoxy chain termination method using Sequenase (United States Biochemical) [13].

cDNA Library Screening

Total RNA from guinea pig atrium was prepared using extraction with guanidinium isothiocyanate and CsCl centrifugation [14]. A cDNA library in λgt10 was prepared by Clontech Laboratories (Palo Alto, CA), and 500,000 recombinants were screened using the cloned RT-PCR products (above) labeled with ^{32}P by nick translation. Nylon filters (Plaquescreen, NEN) were prehybridized at 37° for 2.5 hr in 50% formamide, 1% SDS, 1 M NaCl, and 100 μg herring sperm DNA. The probes were added (49×10^6 cpm) and hybridized overnight at 37°. The filters were washed at 50° for 1 hr in 1 × SSC/0.1% SDS, and overnight exposures were made at −70° using Kodak XAR film. A positive clone (gp29) was identified and isolated following two additional rounds of plaque hybridization. Phage DNA was prepared using LambdaSorb (Promega, Madison, WI) and was subcloned into pBluescript to yield KS/gp29. Nucleotide sequencing showed that gp29 was an α_2A subtype containing 900 bp of coding sequence and 893 bp of 3′-untranslated sequence, and was not full length.

Genomic Library Screening

A 1.1 kb *Hin*dIII fragment of KS/gp29 was labeled with ^{32}P by nick translation and was used to screen 450,000 recombinants of a guinea pig genomic library in λ-EMBL3 (average insert size ~15 kb, range 8–20 kb; Clontech). Hybridization and wash conditions were the same as described above and yielded eleven positive clones that were isolated by two additional rounds of plaque hybridization. Restriction enzyme analysis of the purified phage DNA, and Southern blotting, established that among the clones were three that encoded the guinea pig α_2A subtype and four each that encoded the guinea pig α_2B and α_2C subtypes. Restriction enzyme fragments that appeared large enough to contain complete coding sequences were identified and subcloned into pBluescript for nucleotide sequencing. These fragments were as follows: α_2A, 3.3 kb *Bam*HI; α_2B, 2.3 kb *Apa*I; and α_2C, 2.3 kb *Pst*I, and they were found to be intron-less in their coding domains.

gp-α_2A Expression Construct

The 3.3 kb *Bam*HI fragment of the genomic clone encoding the pg-α_2A lacked nine bases of the 3′-end of the open reading frame, and a full-length clone was prepared as follows (Fig. 1). The genomic clone was cleaved immediately upstream of the start codon with *Nco*I, was blunt-ended with Klenow, and was cleaved 701 bp downstream of the start codon with *Mro*I. This genomic fragment was isolated and was ligated with the large fragment obtained from the cleavage of KS/gp29 with *Mro*I and *Eco*R5. The resulting plasmid, KS/gp-α_2A, contained the complete open reading frame of the gp-α_2A. KS/gp-α_2A was digested *Hin*dIII, and the resulting 1466 bp fragment was isolated, was blunt-ended with Klenow, and was ligated into the blunted *Hin*dIII/*Bam*HI sites of pBC12BI as previously described [15]. The resulting expression construct, pBC/gp-α_2A, contained the complete coding sequence, 3 bp of additional 5′-sequence, and 113 bp of additional 3′-sequence.

gp-α_2B Expression Construct

The 2.3 kb *Apa*I genomic fragment was digested with *Sac*I and *Sac*II, was blunt-ended with Klenow, and the 1519 bp frag-

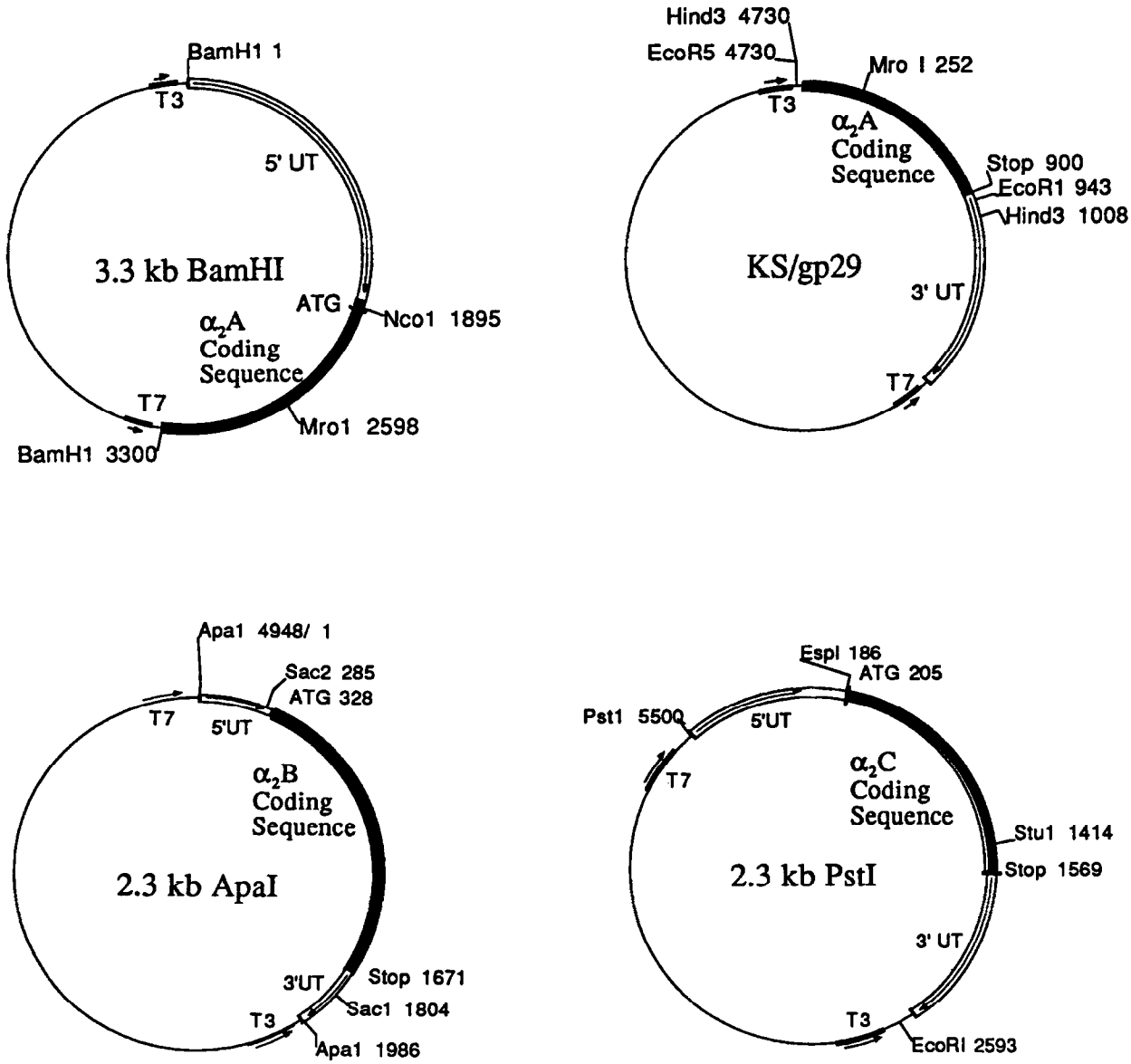

FIG. 1. Plasmid maps of the cloned guinea pig α_2-adrenergic receptor subtypes. Shown are pBluescript KS$^+$ plasmids containing the genomic clones encoding guinea pig α_2A (3.3 kb *Bam*HI), α_2B (2.3 kb *Apa*I), α_2C (2.3 *Pst*I) and the partial cDNA clone of the guinea pig α_2A (KS/gp29) used to create the expression constructs as described in Materials and Methods.

ment was isolated and was ligated into the blunted *Hin*dIII/*Bam*HI sites of pBC12BI (Fig. 1). The resulting expression construct, pBC/gp-α_2B, contained the complete coding sequence of the gp-α_2B, 43 bp of additional 5′-sequence, and 133 bp of additional 3′-sequence.

gp-α_2C Expression Construct

Convenient restriction sites were not present in the 3′-untranslated region of the genomic fragment encoding the gp-α_2C and so they were introduced as follows (Fig. 1): The 2.3 kb *Pst*I genomic fragment was used as the template in a PCR reaction with a sense primer (#958) and an antisense primer corresponding to nucleotides −10 to −27 of the 3′-untranslated sequence and containing additional restriction sites for *Eco*RI and *Bam*HI. A 770 bp product was obtained and was cleaved in the primer sequence with *Eco*RI and with *Stu*I, which cleaves 155 bp upstream the stop codon. The original pBluescript clone containing the 2.3 kb *Pst*I genomic fragment was digested with *Eco*RI and *Stu*I, and the resulting large fragment was ligated to the cleaved 770 bp PCR product. The resulting plasmid was digested with *Esp*I, was blunt-ended with Klenow, and was cleaved with *Bam*HI. The 1416 bp fragment was isolated and was ligated to the plasmid backbone of pBC12BI, which had been cleaved with *Hin*dIII, blunted, and cleaved with *Bam*HI. The resulting expression construct, pBC/gp-α_2C, contained the complete coding sequence of the gp-α_2C, 21 bp of additional 5′-sequence, and 30 bp of additional 3′-sequence.

Expression and Radioligand Binding

pBC/gp-α_2A, pBC/gp-α_2B, and pBC/gp-α_2C were transiently expressed in COS cells, and radioligand binding to membranes was studied as previously described [2] using [^{3}H]MK-912. For saturation experiments, nonspecific binding was determined in the presence of 10 μM phentolamine or 10 μM atipamezole. For competition curve analysis, a final concentration of 1 nM [^{3}H]MK-912 was used. The radioligand binding data were analyzed using Prism™ software (GraphPAD Software Inc., San Diego, CA). K_i values were calculated using the Cheng-Prusoff conversion [16]. Protein concentrations were determined by the Bradford assay (Bio-Rad).

Functional Expression in JEG-3 Cells

pBC/gp-α_2A, pBC/gp-α_2B, and pBC/gp-α_2C were transiently expressed in JEG-3 cells, and the expression of a CAT reporter gene linked to a CRE was studied as previously described [17].

Drugs

The following drugs were used: (-)-noradrenanaline HCl, clonidine HCl, oxymetazoline HCl, yohimbine HCl, chlorpromazine HCl (Sigma Chemical Co., St. Louis, MO, U.S.A.); atipamezole, dexmedetomidine (gift from Dr. J. M. Savola, Orion Corp./Farmos, Turku, Finland); SKF 104078 (6-chloro-9-[(3-methyl-2-butenyl)oxy]-3-methyl-1*H*-2,3,4,5-tetrahydro-3-benzazepine) (gift from Dr. J. P. Hieble, SmithKline Beecham Pharmaceuticals, King of Prussia, PA, U.S.A.); Prazocin (Pfizer Central Research, Sandwich, UK); [^{3}H]MK-912 [(2*S*,12b*S*)1′,3′-dimethylspiro(1,3,4,5′,6,6′,7,12b-octahydro-2*H*-benzo(b)furo(2,3-*a*)quinazoline)-2,4′-pyrimidin-2′-one] (82.5 Ci/mmol) (NEN, Wilmington, DE); and phentolamine HCl (CIBA-Geigy, Basel, Switzerland).

RESULTS

Cloning and Sequence of the Guinea Pig α_2ARs

Reverse transcription of guinea pig atrial mRNA, followed by PCR using primers designed from the fifth and the seventh transmembrane domains of the human α_2-ARs, resulted in three products with approximate sizes of 700, 725, and 750 bp (results not shown). When these products were subcloned and sequenced, the data revealed that they encoded the 5–7 TM fragments of the guinea pig homologues of the human α_2A, α_2B, and α_2C subtypes. Control experiments with RNase (not shown) indicated that the products arose from mRNA, as opposed to genomic DNA, suggesting that all three subtypes are normally expressed in guinea pig atrium.

A cDNA library prepared from guinea pig atrial RNA was screened with the ^{32}P-labeled PCR products, and one clone was obtained that encoded part of the gp-α_2A. This clone was labeled with ^{32}P and was used to screen a guinea pig genomic library; clones encoding all three guinea pig α_2-ARs were obtained. The average insert size of the genomic fragments in this library was ~15 kb, and restriction enzyme analysis and Southern blotting were used to identify smaller fragments likely to contain complete coding sequences. For the α_2A, a 3.3 kb *Bam*HI fragment was subcloned and sequenced and was found to have a 1341 bp incomplete open reading frame; however, overlapping sequence of the previously cloned cDNA indicated that it was only 9 bp short of the stop codon. For the α_2B and α_2C, 2.3 kb *Apa*I and 2.3 kb *Pst*I fragments were subcloned and sequenced and were found to contain complete open reading frames of 1344 and 1365 bp, respectively. As with the human sequences, the guinea pig sequences were intron-less in their coding domains. The nucleotide sequences of these clones have been submitted to GenBank under the following accession numbers: gp-α_2A, U25722; gp-α_2B, U25723; and gp-α_2C, U25724.

Figure 2 shows an alignment of the deduced amino acid sequences of the three guinea pig α_2-AR subtypes according to the seven-transmembrane model for G-protein coupled receptors. The deduced amino acid sequence of the gp-α_2A encodes a protein of 450 amino acids; however, a second in-frame methionine was present 14 amino acids upstream of the indicated start site, which has the potential to encode a protein of 464 amino acids. The second methionine was chosen as the start site because of its match with the consensus sequence for start codons [18] and because of the lack of any significant identity of the potential upstream amino acids with the previously cloned α_2A receptors. The gp-α_2B and gp-α_2C subtypes encode proteins of 448 and 455 amino acids, respectively. As indicated in Fig. 2, there were consensus sites for N-linked glycosylation in the amino termini of the gp-α_2A and gp-α_2C subtypes but not for the gp-α_2B. This is consistent with the cloned human, rat, and mouse gp-α_2Bs, which also lack N-linked glycosylation sites. The number of completely conserved amino acid residues between the different guinea pig subtypes was 186, and the average overall sequence identity was 41%. The amino acid sequence identity between the guinea pig α_2-ARs and their corresponding human homologues was 87% for gp-α_2A; 85% for gp-α_2B; and 89% for gp-α_2C.

The deduced amino acid sequences of all the cloned α_2-ARs were compared and were used to develop the phylogenetic relationship shown in Fig. 3. This analysis, which compares the relative evolutionary distances between the amino acid sequences, demonstrated that there are two main branches of the α_2-AR family. One branch consists of the α_2B subtypes, whereas the second branch includes both the α_2A and α_2C subtypes. Bootstrap analysis was used as a statistical test of the assigned grouping, which showed a high level of confidence (96 or greater) for all the major branches. A slightly lower level of confidence (85) was obtained for the minor branch consisting of the guinea pig/domestic pig and the rat/mouse α_2A receptors. Interestingly, based on the affinity of these α_2-receptors for oxymetazoline and prazosin, one might assume that the α_2B would be more closely related to the α_2C than to α_2A. However, this phylogenetic comparison suggests a closer relationship between α_2A and α_2C rather than between α_2B and α_2C.

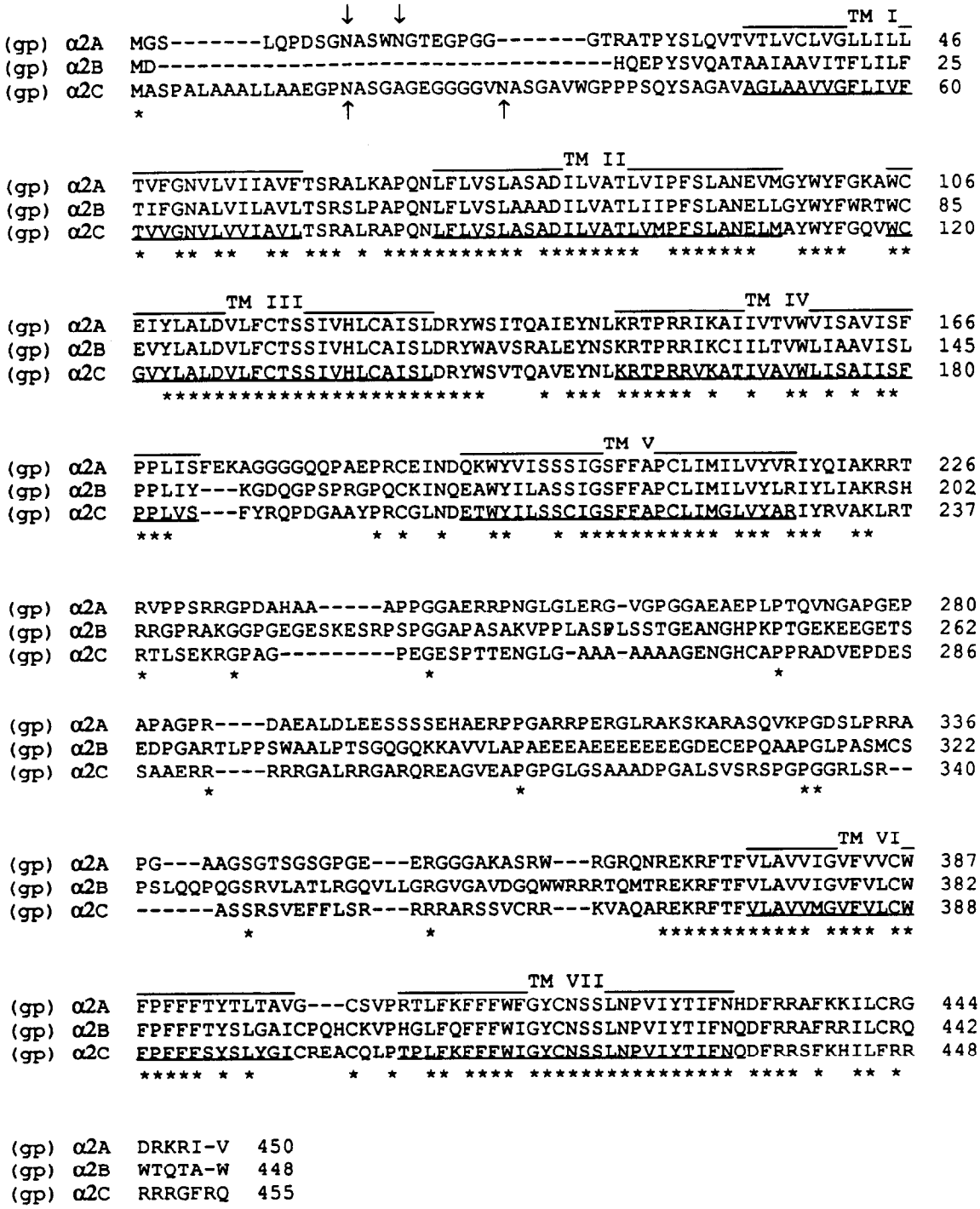

FIG. 2. Alignment of the cloned guinea pig α_2-adrenergic receptor subtypes. The deduced amino acid sequences of the cloned guinea pig α_2A, α_2B and α_2C adrenergic receptor subtypes were aligned by the on-line computer at the National Institute of Genetics (Japan) using the FASTA algorithm in ODEN (a software package for the analysis of protein sequence). Asterisks (*) denote conserved amino acids. Bars mark putative transmembrane domains, and arrows indicate consensus sites for N-linked glycosylation.

Expression of the Guinea Pig α_2-ARs

The coding sequences of the guinea pig α_2-ARs were subcloned into a mammalian expression vector and were expressed in COS cells to study the pharmacology of these receptors. Figure 4 shows that the binding of the α_2-adrenoceptor antagonist [^{3}H]MK-912 to membranes prepared from these transfected cells was specific and saturable for each of the subtypes. The gp-α_2C was found to have the highest affinity for [^{3}H]MK-912 (K_d = 110 ± 20 pM) followed by the gp-α_2A (K_d = 810 ± 250 pM) and gp-α_2B (K_d = 2700 ± 500 pM).

Competition for the binding of [^{3}H]MK-912 with com-

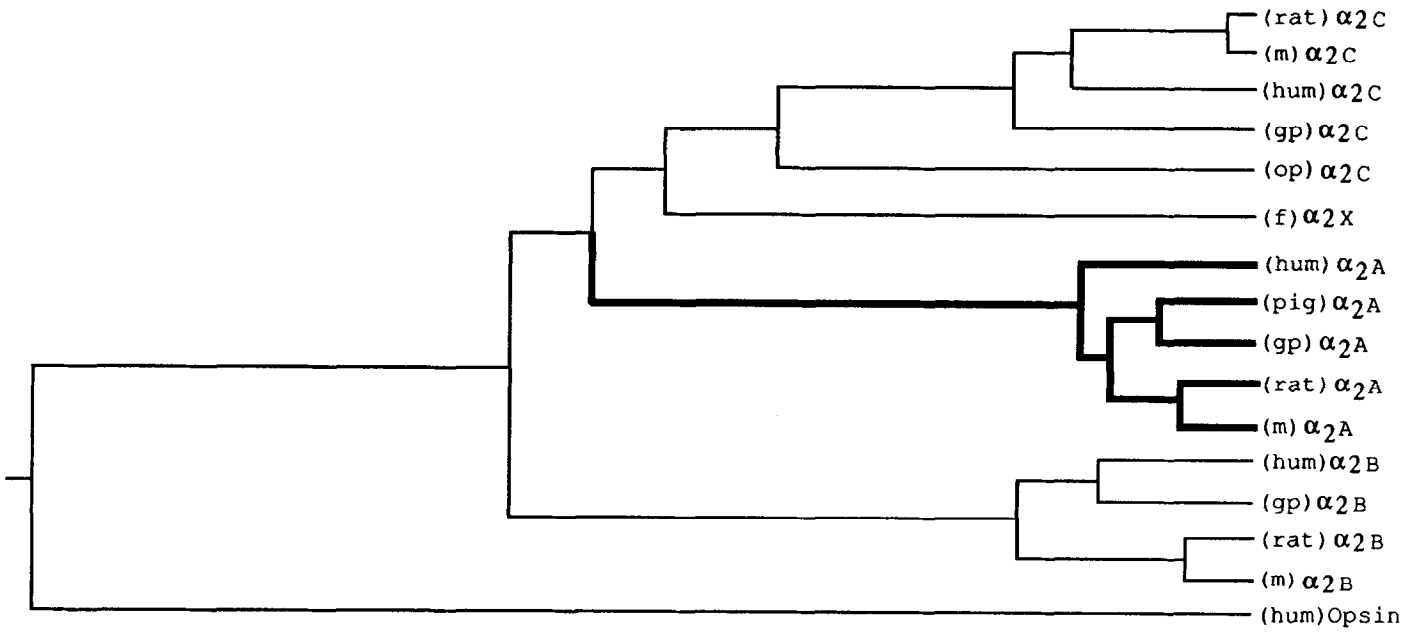

FIG. 3. Phylogenetic relationships between the cloned α_2-adrenergic receptor subtypes. Phylogenetic inference using the unweighted pair-group method with arithmetic means (UPGMA) was done using the MEGA program [19], which compares the relative evolutionary distance between related amino acid sequences. This method assumes a constant rate of amino acid substitution for each lineage and is therefore rooted. The cloned α_2-AR sequences were obtained from GenBank (for references, see Ref. 5); the human opsin receptor sequence was included as a distantly related outgroup. Abbreviations are: m, mouse; hum, human; gp, guinea pig; op, opossum; and f, fish.

pounds previously characterized as agonists and antagonists was also done with membranes from COS cells transfected with the guinea pig α_2-ARs. The data for dexmedetomidine, yohimbine and prazosin are shown in Fig. 5, and their K_i values are listed in Table 1 along with some additional compounds. Prazosin, an important α-adrenoceptor antagonist for the original classification of α_2-AR subtypes, had characteristically low affinity (K_i = 1440 nM) for the gp-α_2A and higher affinities for the B and C subtypes (K_i ~150 nM). The α_2-selective antagonist, yohimbine, had high affinity for the gp-α_2C subtype (K_i = 3.4 nM) and lower affinities for the A and B subtypes (K_i values 20–80 nM). As compared with the human α_2-ARs, which have uniformly high affinity for yohimbine, the guinea pig α_2-ARs are like those of the rat and mouse, which also have low affinity for yohimbine. Calculating the ratio of the K_i values of prazosin versus yohimbine also illustrates this difference. Thus, for the guinea pig α_2B this ratio is 1.9, whereas for the human α_2B it is 25 [11]. Among the agonists tested, dexmedetomidine had the highest affinity for all the subtypes with K_i values for gp-α_2A and B of ~0.7 nM and ~5 nM for α_2C (Table 1).

Functional Coupling of the Guinea Pig α_2-ARs

The ability of the guinea pig α_2-AR subtypes to activate second messenger pathways was examined by co-expression of the cloned genes with a cyclicAMP responsive reporter gene in human JEG-3 cells. The reporter gene, consisting of a CRE linked to the gene encoding CAT has been characterized previously with the human α_2-ARs, and has been shown to respond appropriately to α_2-adrenoceptor stimulation and blockade [17]. Figure 6 shows the effects of NE on forskolin-stimulated CAT activity for each of the guinea pig α_2-AR subtypes. For gp-α_2A the response was biphasic with low concentrations of NE (10–100 nM) inhibiting CAT activity by ~50% and high concentrations (1–10 μM) of NE causing a reversal. Biphasic responses were also obtained for gp-α_2B and gp-α_2C. However, stimulation of CAT activity was predominant for gp-α_2B, whereas inhibition of CAT activity was more pronounced with gp-α_2C.

DISCUSSION

Using a combination of PCR and screening guinea pig cDNA and genomic libraries, we have cloned and expressed three guinea pig α_2-AR subtypes that are homologues of the cloned human α_2-C10, α_2-C2 and α_2-C4 subtypes. We have designated these subtypes, respectively, as gp-α_2A, gp-α_2B, and gp-α_2C, although gp-α_2A and gp-α_2B have the pharmacological characteristics of the so-called α_2D subtype. The α_2D, which has been characterized in rats and mice, is a biochemical homologue of the human α_2-C10 that has relatively low affinity for the α_2-selective antagonist yohimbine (Table 1) [1, 20, 21]. The molecular basis of this pharmacological species difference appears to be the result of a change in one amino acid in which cysteine^{201} in transmembrane-5 of the human α_2A is a serine at the equivalent position of the mouse receptor [20]. Both gp-α_2A and gp-α_2B also have a serine in this position, which is consistent with their low affinity for yohim-

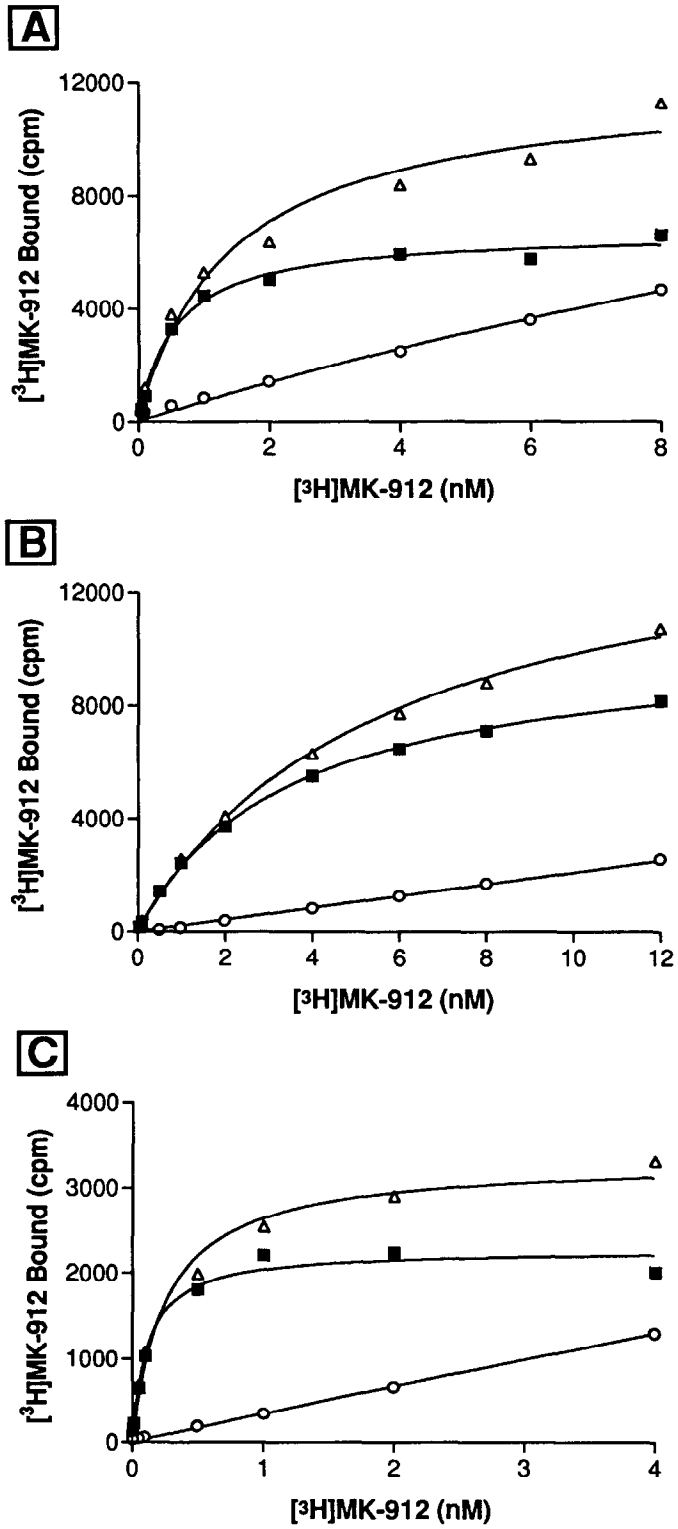

FIG. 4. Saturation binding of [^{3}H]MK-912 to membranes prepared from COS cells transfected with the cloned guinea pig α_2A (panel A), α_2B (panel B), and α_2C (panel C) adrenergic receptor subtypes. Representative experiments show saturation curves for the binding of [^{3}H]MK-912 performed as described in Materials and Methods to membranes prepared from COS cells transiently transfected with plasmid DNA encoding either guinea pig α_2A, α_2B, or α_2C adrenoceptor subtypes. Key: total binding (△), nonspecific binding (○), and specific binding (■).

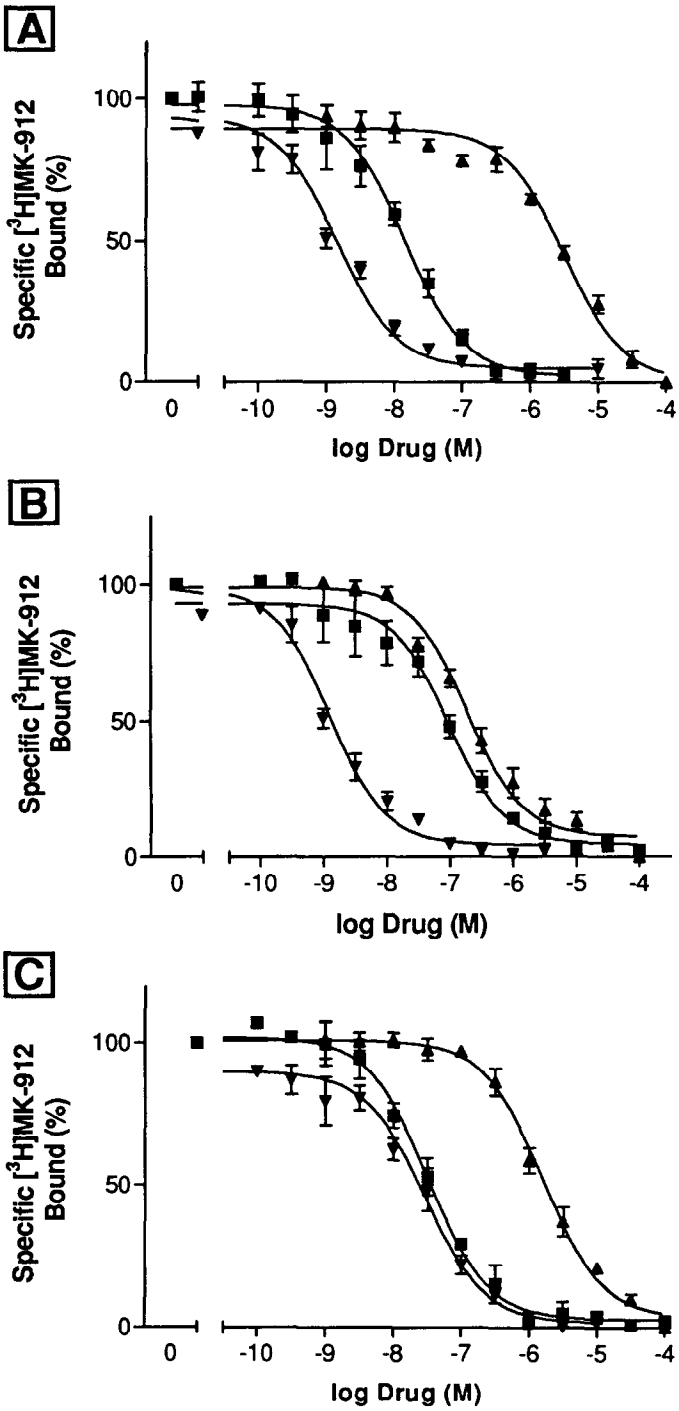

FIG. 5. Competition for the binding of [^{3}H]MK-912 by α-adrenoceptor ligands to the cloned guinea pig α_2A (panel A), α_2B (panel B), and α_2C (panel C) adrenergic receptor subtypes. Competition curves for dexmedetomidine (▼), yohimbine (■), and prazosin (▲) were obtained as described in Materials and Methods using membranes prepared from COS cells transiently transfected with plasmid DNA encoding either guinea pig α_2A, α_2B, or α_2C adrenoceptor subtypes. The final concentration of [^{3}H]MK-912 was 1 nM. Data are the means ± SEM for 3 experiments. One hundred percent is the specific [^{3}H]MK-912 bound in the absence of competitor, which was 2170 ± 320 cpm for α_2A, 1580 ± 120 cpm for α_2B, and 1730 ± 200 cpm for α_2C.

TABLE 1. Binding Affinities of Adrenoceptor Ligands at the Cloned Guinea Pig α_2-Adrenoceptor Subtypes*

	K_i (nM)		
	α_{2A}	α_{2B}	α_{2C}
Agonists			
Noradrenaline	783 ± 82	86 ± 14.5	254 ± 49
Clonidine	9.5 ± 0.6	35 ± 4.5	95 ± 16
Oxymetazoline	25 ± 3	250 ± 16	106 ± 43
Dexmedetomidine	0.55 ± 0.10	0.83 ± 0.31	4.6 ± 0.8
Antagonists			
Yohimbine	20.5 ± 4.4	81 ± 6	3.4 ± 0.2
SKF 104078	79 ± 70	132 ± 52	104 ± 26
Atipamezole	0.04 ± 0.02	1.8 ± 0.4	0.6 ± 0.1
Prazosin	1440 ± 201	152 ± 42	163 ± 40
Chlorpromazine	157 ± 76	13 ± 5.5	10 ± 2.5

* K_i values were determined by nonlinear regression analysis with data obtained from radioligand binding competition studies using [^{3}H]MK-912. Data are the means ± SEM of three separate experiments.

bine. The gp-α_2C, on the other hand, has a cysteine at this position and has higher affinity for yohimbine.

Radioligand binding studies with COS cells transfected with the α_2-AR subtypes showed that the α_2-selective antagonist [^{3}H]MK-912 had its highest affinity for the gp-α_2C subtype, a finding consistent with pharmacological studies of native guinea pig α_2-ARs [22] and recombinant human α_2-AR subtypes [23]. When the affinities of other compounds for the cloned guinea pig α_2-AR subtypes were determined by competition for the binding of [^{3}H]MK-912, it was found that affinities for the cloned receptors were consistently lower as compared with the native receptors. This may be related to the assay conditions [22]; a notable exception, however, was chlorpromazine, which had much higher affinity for the cloned guinea pig α_2-ARs than for the native receptors (6 to 300 fold). In addition, for the cloned receptors, chlorpromazine had its highest affinity for the B and C subtypes, whereas for the native receptors it had its highest affinity for the A subtype. Reasons for these differences are unknown, although, they do suggest that caution should be exercised when using chlorpromazine as a selective ligand for the α_2B subtype [cf. Ref. 11].

Of particular interest, in light of previous work, was the affinity of SKF 104078 for the cloned and expressed guinea pig α_2-AR subtypes. Thus, functional evidence has been obtained with isolated guinea pig atrium which indicates that SKF 104078 has low affinity for the prejunctional α_2-receptor (K_b > 3000 nM), whereas for several types of postjunctional α_2-receptors it has high affinity (~100 nM; [24, 25]). Furthermore, radioligand binding studies with the cloned human α_2-ARs have shown that all the subtypes have relatively high affinity for SKF 104078 (K_i values ~100 nM), suggesting that, functionally, the guinea pig atrial α_2 represents a novel subtype or that it is a homologue of a previously cloned subtype with a different pharmacology, vis-à-vis α_2D. The functional response mediated by α_2-ARs in the guinea pig atrium does not seem to represent a homologue of a previously cloned subtype since the results obtained for the cloned guinea pig α_2s were

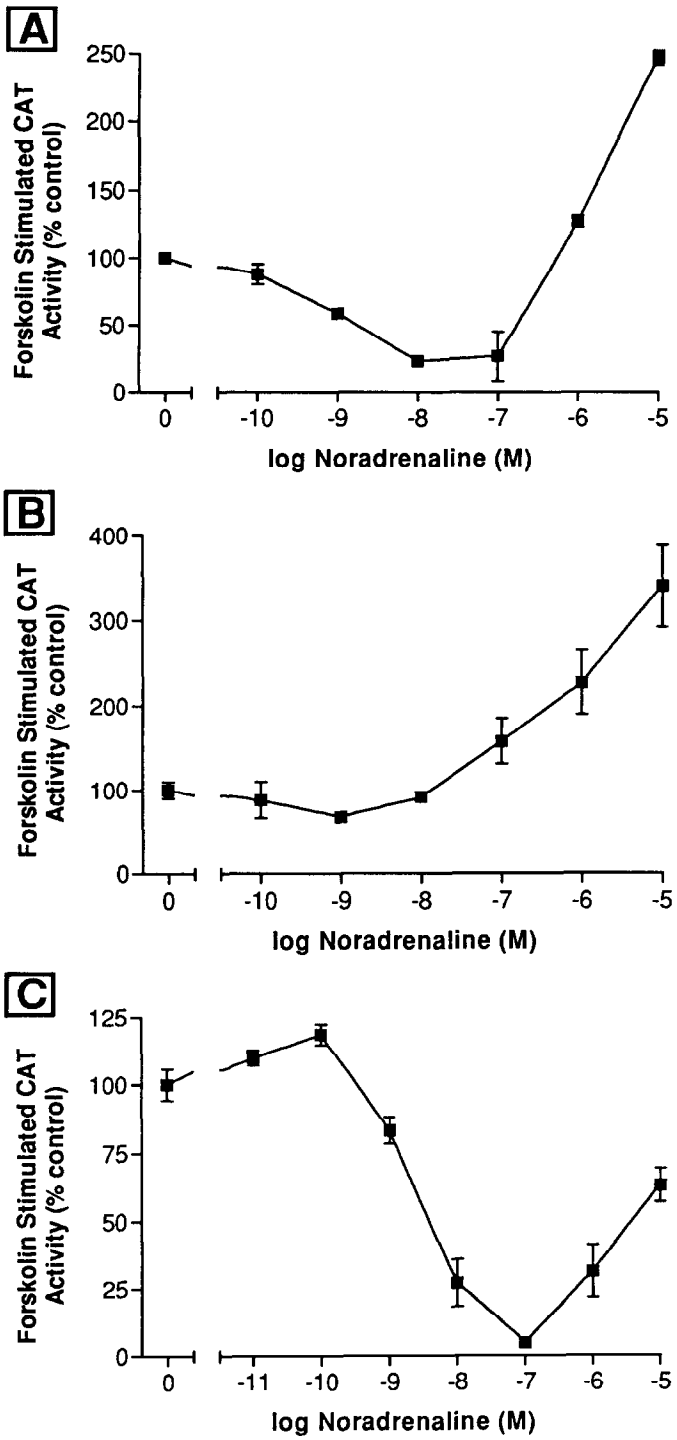

FIG. 6. Functional interactions of the cloned guinea pig α_2A (panel A), α_2B (panel B), and α_2C (panel C) adrenergic receptor subtypes with a cAMP-responsive reporter gene in transiently transfected JEG-3 cells. cAMP-dependent reporter gene activity was monitored using a plasmid containing a CRE linked to the gene encoding CAT essentially as described [17]. CAT activity was initially stimulated with 1 μM forskolin (FSK), and then concentration-response curves were generated for each of the subtypes using noradrenaline. Data are the means ± SEM for duplicate plates of cells and are representative of 3 separate experiments. One hundred percent is the forskolin-stimulated CAT activity in the absence of noradrenaline, which was 1460 ± 10 cpm for α_2A; 1090 ± 60 cpm for α_2B and 1520 ± 220 cpm for α_2C.

similar to those of the human in which SKF 104078 was not selective and had relatively high affinity (K_i values ~100 nM) for all of the subtypes. Based upon our results of screening and PCR with both guinea pig atrial cDNA and genomic libraries, we also do not have evidence for a novel, previously uncloned, subtype.

There are other possibilities that might explain the lack of a functional response of the guinea pig atrium to SKF 104078. For example, prejunctional inhibition in the atrium could be mediated by an imidazoline-preferring receptor or by a guinea pig homologue of a cloned human α_2-AR that has undergone further posttranslational modification. Although, recent data [26] suggest that inhibitory prejunctional α_2-ARs and putative imidazoline-preferring sites co-exist on postganglionic sympathetic nerves in the rabbit heart, previous pharmacologic data with the guinea pig atrium do not support the involvement of imidazoline-preferring sites with the prejunctional inhibition of sympathetic neurotransmitter release [25]. It is possible that a posttranslational modification could change the pharmacology of the guinea pig atrial α_2-receptor, but there is no evidence to date of this occurring for any adrenoceptor, and future studies will be needed to assess this possibility. A further consideration is that in other functional models, SKF 104078 does not differentiate between pre- and postjunctional α_2-receptors. Thus, Connaughton and Docherty [27] report that SKF 104078 had similar affinity for the prejunctional inhibition of [^{3}H]NE release in isolated rat atria as it did for the postjunctional inhibition of smooth muscle contraction in isolated human saphenous vein. Taken together, the effects of SKF 104078 on the guinea pig atrium are complex and appear to reflect the species, tissue, and perhaps the ligand itself [9, 28].

There is a developing interest in the possibility of using α_2-AR antagonists for the treatment of angina pectoris or ischemia [29]. The use of subtype-specific drugs in this situation might be advantageous, requiring a knowledge of the expression of α_2-AR subtypes in the heart. Our initial RT-PCR studies of guinea pig atrial RNA identified all three subtypes, which if true for the atria and the rest of the heart would be interesting. Interpretation of these results, however, is difficult since products may arise from genomic DNA, non-translated mRNA, or the RNA of nonresident cells such as macrophages or blood cells. While our results diminish the possibility of genomic contamination, they do not address these other questions. The availability of the cloned guinea pig α_2-ARs and the knowledge that they may all be expressed in the heart will help towards understanding the effects of α_2-adrenoceptor agents on the heart and their role in cardiac function.

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