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Molecular cloning and functional expression of the guinea pig α_{1a}-adrenoceptor

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Abstract

In the present paper, the cloning and expression of the guinea pig α_{1A}-adrenoceptor is presented. The nucleotide sequence had an open reading frame of 1401 bp that encoded a 466 amino-acid protein with an estimated molecular mass of $\approx$ 51.5 kDa. When the clone was expressed in Cos-1 cells, specific high-affinity binding of [^{3}H]prazosin and [^{3}H]tamsulosin was observed. Chloroethylclonidine treatment of membranes slightly decreased the total binding with both radioligands. Binding competition experiments using [^{3}H]tamsulosin showed the following potency order: (a) for agonists: oxymetazoline $\gg$ epinephrine > norepinephrine > methoxamine, and (b) for antagonists: prazosin $\geq$ 5-methyl-urapidil = benoxathian > phentolamine $\gg$ BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione). Photoaffinity labeling using [^{125}I-aryl]azido-prazosin revealed a major broad band with a molecular mass between 70 and 80 kDa. The receptor was functional, as evidenced by an epinephrine-increased production of [^{3}H]inositol phosphates that was blocked by prazosin. © 2001 Published by Elsevier Science B.V.

Keywords: α_1-Adrenoceptor; α_{1a}-Adrenoceptor; Cloning

1. Introduction

The natural adrenergic catecholamines, epinephrine and norepinephrine, are among the major modulators of the general metabolism of vertebrates. Their actions are mediated through three families of G protein-coupled receptors, the α_1-, α_2- and β-adrenoceptors; each family has three members, i.e., nine adrenoceptors are presently known (Hieble et al., 1995). The liver plays a very general role in homeostasis and particularly in the control of metabolism. Receptor subtypes of the α_1- and β-adrenoceptor families mediate the main actions of catecholamines in this organ (Exton, 1980; Sulakhe et al., 1988).

β_2-Adrenoceptors mediate the effects of catecholamines in the liver of different species under normal conditions (Sulakhe et al., 1988; García-Sáinz et al., 1996). β_1-Adrenoceptors have been observed in hepatoma cells (Lacombe et al., 1976; García-Sáinz et al., 1989; Sanae et al., 1989). Considerable variation exists in the α_1-adrenoceptor subtypes expressed in the liver of different species. Thus, α_{1A}-adrenoceptors are predominantly expressed in the liver of rabbits, dogs, cats and humans. α_{1B}-Adrenoceptors, on the other hand, are the main subtypes expressed in the liver of fish, domestic fowl and rodents, such as rats, mice and hamsters; coexpression of these subtypes has been observed in the liver of monkeys (reviewed in García-Sáinz and Macías-Silva, 1995).

The α_1-adrenoceptor subtype expressed in the guinea pig liver has been particularly difficult to define. Pharmacological characterization indicated the presence of α_{1A}-adrenoceptors (García-Sáinz and Romero-Avila, 1993; García-Sáinz et al., 1992a,b, 1995). However, Northern blot analysis showed the expression of α_{1D}-adrenoceptor mRNA in guinea pig hepatocyes (García-Sáinz et al., 1992a). It was recently shown that there is a change in the expression of α_1-adrenoceptor subtype mRNA during liver cell isolation, i.e., the liver mainly expresses α_{1A}-adrenoceptor mRNA, whereas isolated hepatocytes express α_{1D}-adrenoceptor mRNA (González-Espinosa et al., 1999). This change in expression seems to explain the paradox. However, in order to define the receptor subtype more precisely, we continued our studies and in this manuscript we report the cloning and expression of the guinea pig α_{1A}-

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adrenoceptor. The cloned receptor has the pharmacological characteristics of the receptor detected in the guinea pig liver, which led us to conclude that the receptor expressed in this organ is of the α_{1A} subtype.

2. Materials and methods

2.1. Materials

(−)-Epinephrine, (−)-norepinephrine, oxymetazoline, prazosin, reagents used for polyacrylamide gel electrophoresis and protease inhibitors were obtained from Sigma. Phentolamine and methoxamine were generous gifts from Ciba-Geigy and Burroughs-Wellcome, respectively. Benoxathian, 5-methyl-urapidil, chloroethylclonidine and BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione) were from Research Biochemicals International. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, antibiotics and other reagents used for cell culture were from Gibco BRL. [^{3}H]Prazosin (74.4 Ci/mmol), [^{125}I-aryl]-azido-prazosin (2200 Ci/mmol), [2,3-^{3}H]myo-inositol (22.9 Ci/mmol) and labeled nucleotides were from New England Nuclear. Yamanouchi Europe generously provided [^{3}H]tamsulosin (56.3 Ci/mmol). DNA purification kits were from Qiagen. Restriction and modification enzymes were from Biolabs and Boehringer. Superscript Preamplification System for first strand synthesis and 5′ and 3′ RACE (rapid amplification of cDNA ends) kits were from Life Technologies, PCR-Script™ Amp Cloning Kits were from Stratagene and nylon membranes were from Amersham. Oligonucleotides were synthesized and automatic sequencing was performed in the Molecular Biology Unit of our Institute.

2.2. Cloning strategy

Total liver RNA was isolated as described (Chirgwin et al., 1979) with some modifications (Le Huërou et al., 1990), and repurified (Chomczynski and Sacchi, 1987). The integrity of RNA was checked by electrophoresis on formaldehyde-containing agarose gels and visualization under UV light using ethidium bromide staining. Total RNA (5 μg) was subjected to reverse transcription and this cDNA was used to perform a polymerase chain reaction (PCR) using the following primers: 5′ TCTGGGCGGCGGTGGACGTCCTGTGCT 3′ (coding sense, primer A) and 5′ ACGTAGACCCGGCAGTACATGACCAGGATG 3′ (anticoding sense), corresponding to bases 302–329 and 597–627 of the bovine α_{1A}-adrenoceptor cDNA (Schwinn et al., 1990). This fragment was cloned on the *Srf* I site of the pPCR-Script Amp plasmid, sequenced and used to generate the nested primers necessary for the subsequent steps. 3′ RACE (Frohman et al., 1988) was performed as recommended by the manufacturer. Briefly, a new batch of cDNA was synthesized from 5μg of total RNA using the AP primer contained in the kit; the RNA template was degraded using RNAse H. The first PCR step was performed using primer A and primer UAP (included in the kit) during 35 cycles at 94 °C for 1 min, 55 °C for 50 s, and 72 °C for 3 min. Fragments from 850 to 1500 bp were purified from a 1.5% agarose gel and reamplified using primers 5′ ACGAGGATCCCAGCTACGTGCTCTT 3′ (coding sense) and primer AUAP (from the kit) during 35 cycles at 94 °C for 1.5 min, 55 °C for 1.0 min and 72 °C during 3 min. A product of approximately 920 bp was obtained and cloned on the *Srf* I site of the pPCR Script Amp plasmid. The sequence of this fragment contained the 3′ end of the cDNA. 5′ RACE was done following the manufacturer's instructions, using primers derived from the 3′ clone. For 5′ cloning, cDNA was synthesized using the primer 5′ GAGCTGGCCACCCCACTGCCTCCTA 3′ (anticoding sense), RNA was degraded with RNAse H, first strand was purified using a Qiagen column, and the tailing reaction was done as recommended. First PCR amplification was performed using the Ambridged Anchor Primer from the kit and the primer 5′ GCGTGACTTGCTCTGAATCCGACTT 3′ (anticoding sense) during 35 cycles at 94 °C for 1 min, 55 °C for 50 s and 72 °C for 3 min. Fragments from 650 to 900 bp were extracted from a 1.5% agarose gel and reamplified using the primers AUAP from the kit and 5′ ACCACGTAGACCCGGCAGTACATGA 3′ (anticoding sense) from the 3′ clone. A product of approximately 650 bp was obtained and cloned on the pPCR Script plasmid. A full-length cDNA of the receptor was obtained by ligation of the 5′ and 3′ sequences digested with *Srf* I (in the vector) and *Acc*I, a site that was localized at the base 620 of the open reading-frame sequence and was contained in both 5′ and 3′ clones. Finally, the complete cDNA was amplified using oligonucleotides containing sites for *Eco*RI in the 5′ and *Not*I in the 3′ sequences; these sites were used in order to clone the full-length open reading frame into digested and dephosphorylated pCDNA3. Total sequencing, in both directions, of two identical full-length clones was performed using overlapping templates and a DNA sequencer.

2.3. Cell culture and transfection

Cos-1 cells were cultured in glutamine-containing high-glucose DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin B at 37 °C under a 95% air/5% CO_2 atmosphere as described previously (Vázquez-Prado et al., 2000). Cells at 80–90% confluence (seeded the previous day) were transfected with 2 μg of plasmid for each 10-cm Petri dish. Experiments with transfected cells were performed 48–60 h after transfection. Transfection

efficiency (50–70%) was determined in parallel dishes transfected with plasmid pCH110 and evaluated by the activity of β-galactosidase.

2.4. Membrane preparation, radioligand-binding and photoaffinity-labeling studies

Membranes were prepared as described previously (Vázquez-Prado et al., 2000). In brief, cells were scraped with a rubber policeman into 1 ml of ice-cold buffer (20 mM HEPES, pH 7.5, 5 mM EDTA, 100 μM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, plus protease inhibitors (20 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 500 μg/ml bacitracin, 50 μg/ml soybean trypsin inhibitor)) and homogenized with a tight-fitting Dounce homogenizer. The homogenate was centrifuged at $1500 \times g$ for 10 min at 4 °C, the pellet was discarded and the supernatant was centrifuged at $20{,}000 \times g$ for 20 min at 4 °C. The resulting pellet was resuspended in binding buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.5). Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Binding studies were performed by incubating the radioligand ([^{3}H]prazosin, 0.05–10 nM; [^{3}H]tamsulosin 0.01–4 nM in saturation experiments and 0.3–0–6 nM in binding competition studies) with the membranes (10–20 μg of protein) in a final volume of 0.25 ml of binding buffer for 60 min at 30 °C in a water bath shaker. The incubation was terminated by addition of 5 ml of ice-cold buffer and filtration through GF/C filters using a Brandel harvester. Filters were washed three times, dried and radioactivity was measured in a liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM phentolamine; specific binding represented $> 90\%$ of total binding at the K_D for [^{3}H]tamsulosin and ≈ 60–70% for [^{3}H]prazosin. Curves were analyzed by the EBDA program (Biosoft-Elsevier) and K_i values were calculated according to Cheng and Prusoff (1973).

Receptor photoaffinity labeling was performed as described (Vázquez-Prado et al., 2000). In brief, membranes (15 μg protein in 0.25 ml) were incubated with 6 nM of [^{125}I-aryl]azido-prazosin in the dark. After 1 h at room temperature, 1 ml of 50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 5 mM EDTA and protease inhibitors (leupeptin 20 μg/ml, aprotinin 20 μg/ml, bacitracin 500 μg/ml and soybean trypsin inhibitor 50 μg/ml) was added. Open tubes were exposed to UV light for 3 min (Stratalinker, Stratagene). Membranes were centrifuged at $12{,}700 \times g$ for 15 min, washed and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Gels were fixed, dried and exposed to X-OMAT X-ray films (Eastman Kodak) at −80 °C with an intensifying screen. The specificity of

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ATGGTGTTTC TCTCTGGAAA TGCTTCCGAC AGCTCCAACT GCACCCAACC
GCCGGCACCG GTGAACATTC CCAAAGCCAT TCTGCTCGGA GTGATCTTAG  100
GGGTCCTCAT CCTTTTCGGG GTGCCGGGGA ACATCTTAGT GATCCTCTCC
GTGGCCTGTC ACCGTCATCT GCACTCGGTC ACGCACTACT ACATCGTCAA  200
CCTGGCGGTG GCCGACCTAC TACTCACCTC CACGGTGCTG CCCTTCTCAG
CCATCTTTGA GATCCTGGGC TACTGGGCCT TCGGCAGGGT CTTCTGCAAC  300
ATCTGGGCGG CGGTGGACGT CCTGTGCTGC ACCGCGTCCA TCATGAGCCT
CTGCATCATC TCCATCGACC GCTACATCGG CGTGAGCTAC CCGCTACGCT  400
ACCCCACCAT CGTCACCCAG AGGCGGGGGC TCCGGGCTCT GCTCTGCCTC
TGGGCGCTGT CCCTGGTCAT CTCCATCGGG CCGCTGTTTG GCTGGAGGCA  500
GCCGGCCCCC CAGGACGAGA CCATCTGCCA GATCAACGAG GATCCCAGCT
ACGTGCTCTT CTCCGCGCTG GGATCCTTCT ACGTGCCGCT GGCCATCATC  600
CTGGTCATGT ACTGCCGGGT CTACGTGGTG GCCAAAAGGG AGAGCCGGGG
CCTCACATCC GGCCTCAAGA CCGACAAGTC GGATTCAGAG CAAGTCACGC  700
TGCGCATCCA CCGGAAAAAT GCCCCGCTAG GAGGCAGTGG GGTGGCCAGC
TCCAAGAACA AGACGCACTT CTCTGTGCGG CTCCTCAAGT TTTCCCGGGA  800
GAAGAAAGCG GCCAAAACGC TGGGCATCGT GGTCGGCTGC TTCGTCCTCT
GCTGGCTGCC CTTCTTCTTA GTGATGCCCA TTGGGTCTTT CTTCCCTGAT  900
TTCAAGCCCT CGGAAACAGT TTTTAAAATA GTATTTTGGC TCGGATACCT
AAACAGCTGC ATCAACCCCA TCATATACCC ATGCTCCAGT CAAGAGTTCA 1000
AAAAGGCCTT TCAGAATGTC TTGAAAATCC AGTGTCTTCG CAGAAAGCAG
TCTTCCAAAC ATGCCCTGGG CTACACTCTG CACCCGCCCA GCCAGGCCGT 1100
GGAAGGGCAG CACAAGGACA TGGTGCGCAT CCCAGTGGGA TCTAGAGAGA
CCTTCTATAA GATCTCCAAG ACGGATGGCG TTTGTGAATG GAAGTTTTTC 1200
TCTTCCATGC CCCGTGGATC TGCCAGGATT ACAGTGCCCA AAGACCAATC
AGCCTGCACC ACAGCCCGGG TGAGAAGTAA AAGCTTTTTG CAGGTCTGCT 1300
GCTGTGTAGG GCCCTCAACC CCCAACCCCG GAGAGAACCA TCAAGTTCCA
ACCATTAAGA TCCACACCAT CTCCCTCAGT GAAAATGGGG AGGAAGTCTA 1400
G                                                       1401
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Fig. 1. Nucleotide sequence of the guinea pig α_{1A}-adrenoceptor.

labeling was determined using 10 μM phentolamine as competitor.

2.5. [^{3}H]inositol phosphate production

Cells were transfected as described above and 48 h later were labeled with [^{3}H]inositol (5 μCi/ml) for 18–24 h in inositol-free DMEM containing 1% fetal bovine serum. On the day of the experiment, cells were washed twice with Krebs-Ringer-HEPES buffer containing 1.3 mM $CaCl_2$ and preincubated for 20 min in 2 ml of the same buffer containing 20 mM LiCl, at 37 °C in a 5% CO_2 atmosphere. Incubations were for 15 min and were terminated by the addition of 0.4 ml of 30% ice-cold perchloric acid. Supernatants were neutralized and [^{3}H]inositol phosphates (IP_1, inositol monophosphate; IP_2, inositol bisphosphate; and IP_3, inositol trisphosphate) were separated by Dowex AG1-X8 chromatography (Berridge et al., 1983).

3. Results

With the cloning strategy indicated in Materials and Methods, a clone was isolated containing an open reading frame of 1401 bp (Fig. 1) that encoded a 466 amino-acid protein whose sequence is shown in Fig. 2. The isolated

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Clone   MVFLSGNASDSSNCTQPPAPVNIPKAILLGVILGVLILFGVPGNILVILSVACHRHLHSV 60
Rabbit  MVFLSGNASDSSNCTHPPAPVNISKAILLGVILGGLILFGVLGNILVILSVACHRHLHSV 60
Rat     MVLLSENASEGSNCTHPPAPVNISKAILLGVILGGLIIFGVLGNILVILSVACHRHLHSV 60
Mouse   MVLLSENASEGSNCTHPPAQVNISKAILLGVILGGLIIFGVLGNILVILSVACHRHLHSV 60
Human   MVFLSGNASDSSNCTQPPAPVNISKAILLGVILGGLILFGVLGNILVILSVACHRHLHSV 60
Bovine  MVFLSGNASDSSNCTHPPPPVNISKAILLGVILGGLILFGVLGNILVILSVACHRHLHSV 60

Clone   THYYIVNLAVADLLLTSTVLPFSAIFEILGYWAFGRVFCNIWAAVDWLCCTASIMSLCII 120
Rabbit  THYYIVNLAVADLLLTSTVLPFSAIFEILGYWAFGRVFCNIWAAVDVLCCTASIISLCVI 120
Rat     THYYIVNLAVADLLLTSTVLPFSAIFEILGYWAFGRVFCNIWAAVDVLCCTASIMGLCII 120
Mouse   THYYIVNLAVADLLLTSTVLPFSAIFEILGYWAFGRVFCNIWAAVDVLCCTASIMGLCII 120
Human   THYYIVNLAVADLLLTSTVLPFSAIFEVLGYWAFGRVFCNIWAAVDVLCCTASIMGLCII 120
Bovine  THYYIVNLAVADLLLTSTVLPFSAIFEILGYWAFGRVFCNVWAAVDVLCCTASIMGLCII 120

Clone   SIDRYIGVSYPLRYPTIVTQRRGLRALLCLWALSLDISIGPLSGWRQPAPQDETICQINE 180
Rabbit  SIDRYIGVSYPLRYPTIVTQRRGLRALLCVWAFSLVISVGPLFGWRQPAPDDETICQINE 180
Rat     SIDRYIGVSYPLRYPTIVTQRRGVRALLCVWVLSLVISIGPLFGWRQPAPEDETICQINE 180
Mouse   SIDRYIGVSYPLRYPTIVTQRRGVRALLCVWALSLVISIGPLFGWRQQAPEDETICQINE 180
Human   SIDRYIGVSYPLRYPTIVTQRRGLMALLCVWALSLVISIGPLFGWRQPAPEDETICQINE 180
Bovine  SIDRYIGVSYPLRYPTIVTQKRGLMALLCVWALSLVISIGPLFGWRQPAPEDETICQINE 180

Clone   DPSYVLFSALGSFYVPLAIILVMYCRVYVVAKRESRGLTSGLKTDKSDSEQVTLRIHRKN 240
Rabbit  EPGYVLFSALGSFYVPLTIILAMYCRVYVVAKRESRGLKSGLKTDKSDSEQVTLRIHRKN 240
Rat     EPGYVLFSALGSFYVPLAIILVMYCRVYVVAKRESRGLKSGLKTDKSDSEQVTLRIHRKN 240
Mouse   EPGYVLFSALGSFYVPLTIILVMYCRVYVVAKRESRGLKSGLKTDKSDSEQVTLRIHRKN 240
Human   EPGYVLFSALGSFYLPLAIILVMYCRVYVVAKRESRGLKSGLKTDKSDSEQVTLRIHRKN 240
Bovine  EPGYVLFSALGSFYVPLTIILVMYCRVYVVAKRESRGLKSGLKTDKSDSEQVTLRIHRKN 240

Clone   APLGGSGVASSKNKTHFFVRLLKFSREKKAAKTLGIVVGCFVLCWLPFFLVMPIGSFFPD 300
Rabbit  APAGGSGVASAKNKTHFSVRLLKFSREKKAAKTLGIVVGCFVLCWLPFFLVMPIGSFFPD 300
Rat     VPAEGGGVSSAKNKTHFSVRLLKFSREKKAAKTLGIVVGCFVLCWLPFFLVMPIGSFFPD 300
Mouse   VPAEGSGVSSAKNKTHFSVRLLKFSREKKAAKTLGIVVGCFVLCWLPFFLVMPIGSFFPN 300
Human   APAGGSGMASAKTKTHFSVRLLKFSREKKAAKTLGIVVGCFVLCWLPFFLVMPIGSFFPD 300
Bovine  AQVGGSGVTSAKNKTHFSVRLLKFSREKKAAKTLGIVVGCFVLCWLPFFLVMPIGSFFPD 300

Clone   FKPSETVFKIVSWLGYLNSCINPIIYPCSSQEFKKAFQNVLKIQCLRRKQSSKHALGYTL 360
Rabbit  FKPPETVFKIVFWLGYLNSCINPIIYPCSSQEFKKAFQNVLKIQCLRRKQSSKHALGYTL 360
Rat     FKPSETVFKIVFWLGYLNSCINPIIYPCSSQEFKKAFQNVLRIQCLRRRQSSKHALGYTL 360
Mouse   FKPPETVFKIVFWLGYLNSCINPIIYPCSSQEFKKAFQNVLRIQCLRRRQSSKHALGYTL 360
Human   FKPSETVFKIVFWLGYLNSCINPIIYPCSSQEFKKAFQNVLRIQCLCRKQSSKHALGYTL 360
Bovine  FRPSETVFKIAFWLGYLNSCINPIIYPCSSQEFKKAFQNVLRIQCLRRKQSSKHTLGYTL 360

Clone   HPPFQAVEGQHKDMVRIPVGSRETFYKISKTDGVCEWKFFSSMPRGSARITVPKDQSACT 420
Rabbit  HAPSQALEGQHKDMVRIPVGSGETFYKISKTDGVCEWKFFSSMPRGSARITVPKDQSACT 420
Rat     HPPSQALEGQHRDMVRIPVGSGETFYKISKTDGVCEWKFFSSMPQGSARITVPKDQSACT 420
Mouse   HPPSQAVEEQHRGMVRIPVGSGETFYKISKTDGVCEWKFFSSMPQGSARITMPKDQSACT 420
Human   HPPSQAVEGQHKDMVRIPVGSRETFYRISKTDGVCEWKFFSSMPRGSARITVSKDQSSCT 420
Bovine  HAPSHVLEGQHKDLVRIPVGSAETFYKISKTDGVCEWKIFSSLPRGSARMAVARDPSACT 420

Clone   TARVRSKSFLQVCCCVGPSTPNPGENHQVPTIKIHTISLSENGEEV 466
Rabbit  TARVRSKSFLQVCCCVGPSTPNPGENHQVPTIKIHTISLSENGEEV 466
Rat     TARVRSKSFLQVCCCVGSSAPRPEENHQVPTIKIHTISLGENGEEV 466
Mouse   TARVRSKSFLQVCCCVGSSTPRPEENHQVPTIKIHTISLGENGEEV 466
Human   TARVRSKSFLQVCCCVGPSTPSLDKNHQVPTIKVHTISLSENGEEV 466
Bovine  TARVRSKSFLQVCCCLGPSTPSHGENHQIPTIKIHTISLSENGEEV 466
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Fig. 2. Amino acid sequence of the guinea pig α_{1A}-adrenoceptor and comparison with the sequence of α_{1A}-adrenoceptors of other species. Rabbit (SwissProt O02824), rat (SwissProt P43140), mouse (EMBL O54913), human (SwissProt P35348), bovine (SwissProt P181310). Putative transmembrane domains are underlined, sequence difference are indicated (bold).

Table 1
Membrane expression of [^{3}H]prazosin and [^{3}H]tamsulosin binding sites

Treatment	K_d (nM)	B_{max} (fmol/mg protein)
[^{3}H]Prazosin		
Control	0.60 ± 0.15	1080 ± 290
Chloroethylclonidine	1.00 ± 0.20	730 ± 225
[^{3}H]Tamsulosin		
Control	0.12 ± 0.02	980 ± 190
Chloroethylclonidine	0.15 ± 0.03	720 ± 130

Data are the means ± S.E.M. of 4–5 determinations, using different membrane preparations for [^{3}H]prazosin and [^{3}H]tamsulosin, respectively.

clone showed a remarkable similarity to α_{1a}-adrenoceptors already cloned from other species (Fig. 2), which led us to conclude that the obtained clone encodes the guinea pig α_{1a}-adrenoceptor. The nucleotide sequence was submitted to GenBank with an accession number AF108016.

When the guinea pig clone was expressed in Cos-1 cells, specific binding of [^{3}H]prazosin and [^{3}H]tamsulosin was observed (Table 1 and Fig. 3). No specific binding was detected in cells transfected exclusively with the vector (pCDNA3) data not shown). The binding of these radioligands was rapid, reversible and saturable, and the Rosenthal transformation of the saturation isotherms resulted in straight lines consistent with the radiolingand binding to homogeneous populations of receptors (Fig. 3). The number of sites was similar using the two radioligands. Nevertheless, we observed that the K_d for [^{3}H]prazosin was relatively high (0.5–1 nM) and that the nonspecific binding was high (≈ 40% of total binding at the K_d). In contrast, the binding of [^{3}H]tamsulosin was very specific (< 95% of the total binding was specific), and the K_d was low (≈ 0.1 nM) and very similar to that observed in guinea pig liver membranes (García-Sáinz et al., 1995).

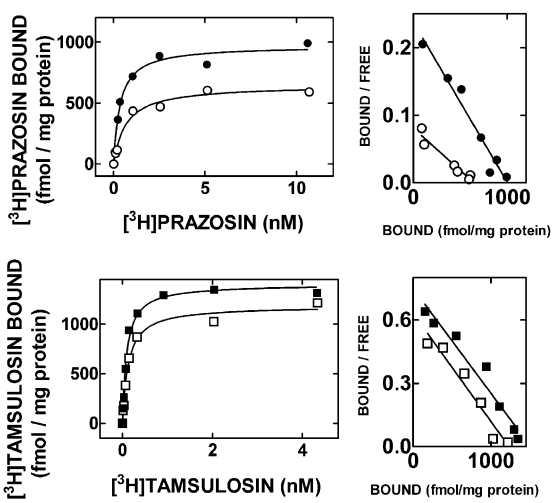

Fig. 3. Binding saturation isotherms for [^{3}H]prazosin (upper panels) or [^{3}H]tamsulosin (lower panels). Membranes were incubated in the absence of any agent (filled symbols) or in the presence of 100 μM chloroethylclonidine (open symbols) and were washed extensively by centrifugation/resuspension before the radioligand-binding study. Rosenthal transformations of the saturation data are presented in the right panels. Data are representative of 4–5 determinations using different membrane preparations.

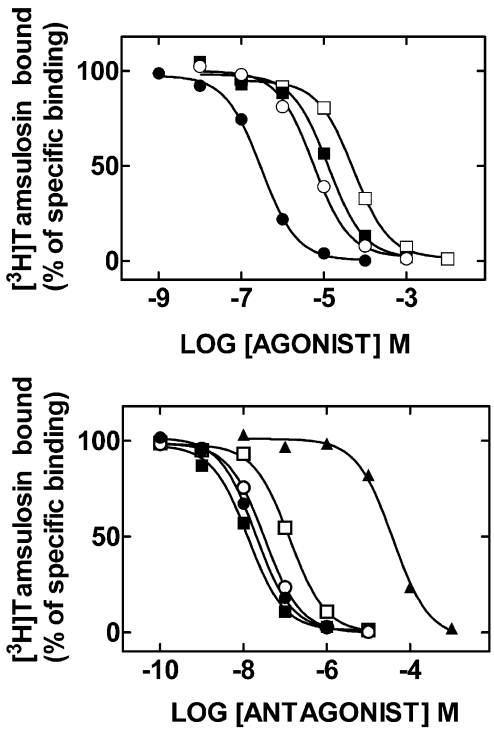

Fig. 4. [^{3}H]Tamsulosin-binding competition experiments. Upper panel, agonist competition curves: oxymetazoline (filled circles), epinephrine (open circles), norepinephrine (filled squares) and methoxamine (open squares). Lower panel, antagonist competition curves: prazosin (filled circles), 5-methyl-urapidil (filled squares), benoxathian (open circles), phentolamine (open squares) and BMY 7378 (filled triangles). Data are representative of 4–6 determinations using different membranes preparations.

Chloroethylclonidine is an irreversible antagonist that has been used to differentiate receptor subtypes (Han et al., 1987). The α_1-adrenoceptor expressed in the guinea pig liver is particularly resistant to inactivation by this antago-

Table 2
Pharmacological profile of [^{3}H]tamsulosin binding sites

Agent	K_i (nM)	Slope
Oxymetazoline	125 ± 50	0.82 ± 0.08
Epinephrine	1355 ± 375	0.84 ± 0.07
Norepinephrine	3250 ± 575	0.85 ± 0.06
Methoxamine	6535 ± 1635	0.72 ± 0.08
Prazosin	0.80 ± 0.15	0.93 ± 0.18
5-Methyl-urapidil	1.85 ± 0.35	1.00 ± 0.06
Benoxathian	1.93 ± 0.64	0.91 ± 0.13
Phentolamine	7.16 ± 1.35	0.85 ± 0.06
BMY 7378	1155 ± 70.0	1.08 ± 0.07

Data are the means ± S.E.M. of 4–6 determinations using different membrane preparations.

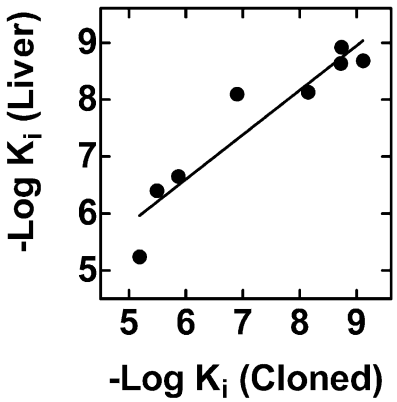

Fig. 5. Correlation plot. Data from guinea pig liver were taken from García-Sáinz et al. (1995).

nist (García-Sáinz and Romero-Avila, 1993; García-Sáinz et al., 1992a, 1995). Therefore, the effect of membrane preincubation with 100 μM chloroethylclonidine for 15 min at 37 °C on radioligand binding was tested. As seen in Fig. 3 and Table 1, treatment with the antagonist slightly decreased the total binding with both radioligands. Nevertheless, the decrease in B_{max}, as compared to that of membranes incubated in the absence of chloroethylclonidine, was greater when the radioligand was [^{3}H]prazosin (Fig. 3). In addition, a decrease in affinity for [^{3}H]prazosin, but not for [^{3}H]tamsulosin, was observed in some experiments (Fig. 3) after treatment with the irreversible antagonist. Nevertheless, such changes were not statistically significant.

Because of the difficulties with [^{3}H]prazosin and the fact that the previously reported characterization of the guinea pig liver receptor was performed with [^{3}H]tamsulosin, all additional experiments were performed with the latter radioligand. Binding competition experiments were performed with agonists and antagonists. It can be observed in Fig. 4 and Table 2 that the potency order for agonists was oxymetazoline ≫ epinephrine > norepinephrine > methoxamine. For antagonists, the potency order was prazosin ≥ 5-methyl-urapidil = benoxathian > phentolamine ≫ BMY 7378. A good correlation was observed (Fig. 5, slope = 0.78, $r^2 = 0.89$) between the pK_i values obtained with the cloned receptor in the present study and those previously observed using guinea pig liver membranes (García-Sáinz et al., 1995).

The cloned receptor was further characterized by photoaffinity labeling using [^{125}I-aryl]azido-prazosin. A major

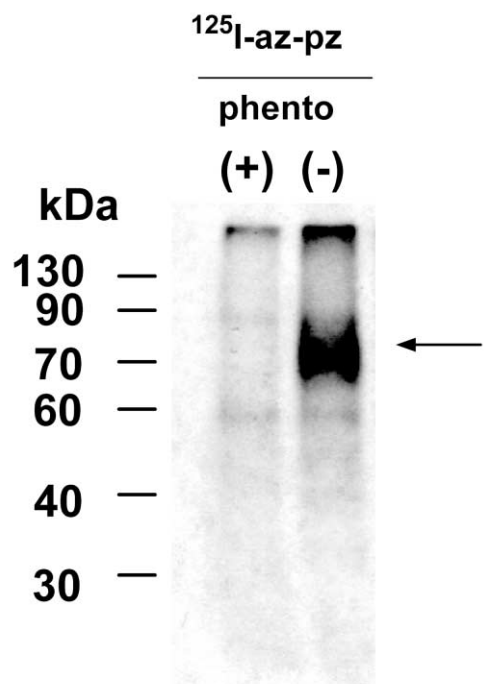

Fig. 6. Photoaffinity labeling of the guinea pig α_{1A}-adrenoceptor. Membranes from transfected Cos-1 cells were labeled with [^{125}I-aryl]azidoprazosin (^{125}I-az-pz) in the absence (−) or presence (+) of 10 μM phentolamine (phento). A representative autoradiograph of at least three independent experiments is shown; molecular mass markers are indicated at the left.

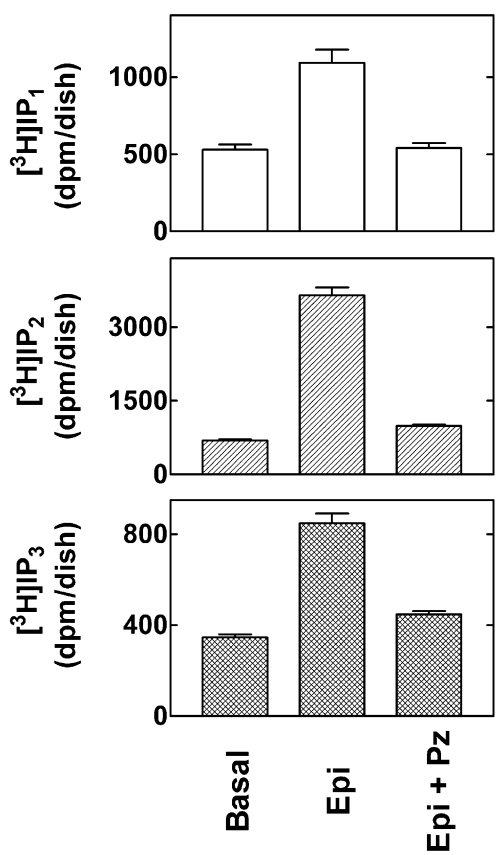

Fig. 7. Effect of adrenergic stimulation on the production of inositol phosphates. Cos-1 cells transfected with the cloned guinea pig α_{1A}-adrenoceptor and labeled with [^{3}H]inositol were stimulated with no agent (Basal), 10 μM epinephrine (Epi) or 10 μM epinephrine plus 1 μM prazosin (Epi + Pz). Plotted are the means and vertical lines represent the S.E.M. of 8–10 determinations using four different cell transfections.

broad band with M_r between 70 and 80 kDa was photolabeled. The specificity of the band was evidenced by the competition with phentolamine (Fig. 6). Other bands were also labeled, albeit to a much lesser extent and did not correspond to the receptor, as evidenced by the absence of competition by phentolamine (Fig. 6).

The ability of an adrenergic agonist to stimulate inositol phosphate production showed the functionality of the cloned receptor. In [^{3}H]inositol-labeled cells transfected with the cloned receptor, epinephrine markedly increased the production of [^{3}H]inositol phosphates and this effect was blocked by prazosin (Fig. 7). In Cos-1 cells transfected only with the vector, no effect of epinephrine was observed (data not shown).

4. Discussion

In the present paper, the cloning and expression of the guinea pig α_{1A}-adrenoceptor is presented. The nucleotide sequence of the open reading frame was very similar to that of rabbit (93%), human (93%), bovine (88%), rat (87%) and mouse (87%) α_{1A}-adrenoceptors (Schwinn et al., 1990, 1995; Forray et al., 1994; Laz et al., 1994; Miyamoto et al., 1997; Xiao et al., 1998). Comparison of the protein sequences also indicated a great similarity with α_{1A}-adrenoceptors from other species, i.e., rabbit (94%), human (93%), bovine (90%), rat (90%) and mouse (91%) α_{1A}-adrenoceptors (Schwinn et al., 1990, 1995; Forray et al., 1994; Laz et al., 1994; Miyamoto et al., 1997; Xiao et al., 1998). As expected, the sequence indicated that the cloned receptor has all the main characteristics of G protein-coupled receptors of the α_{1A}-adrenoceptor subfamily (Schwinn et al., 1990, 1995; Forray et al., 1994). During the process of cloning, other clones were observed which might represent truncated or splice variants of the receptor. Such variants seem to be similar to those observed for the human and rabbit α_{1A}-adrenoceptor, and it is possible that they play a regulatory function (Chang et al., 1998; Cogé et al., 1999; Suzuki et al., 2000). The abundance of such variants was not very high in our samples and no further effort was made to characterize them in the present work.

Expression of the cloned receptor in Cos-1 cells resulted in the emergence of specific [^{3}H]prazosin and [^{3}H]tamsulosin binding sites in the cell membranes. As indicated, the affinity for [^{3}H]prazosin was relatively low, which is consistent with the K_i values obtained in the binding competition experiments (García-Sáinz and Romero-Avila, 1993; García-Sáinz et al., 1992a, 1995). We had great difficulty in detecting specific [^{3}H]prazosin binding using guinea pig liver membranes and, for this reason, [^{3}H]bunazosin (García-Sáinz et al., 1992a), [^{3}H]5-methyl-urapidil (García-Sáinz and Romero-Avila, 1993) or [^{3}H]tamsulosin (García-Sáinz et al., 1995) was used. It is possible that the low abundance of sites, the hydrophobic nature of prazosin and the relatively low affinity of the receptor contributed to this difficulty. Other authors have observed differences in the density of receptors estimated with [^{3}H]tamsulosin or [^{3}H]prazosin (Michel and Goepel, 1998; Hein et al., 2000); however, in the present study, no clear difference was detected.

A characteristic of the liver guinea pig α_1-adrenoceptor is its resistance to chloroethylclonidine inactivation (García-Sáinz and Romero-Avila, 1993; García-Sáinz et al., 1992a, 1995). We observed a low sensitivity to the akylating antagonist with [^{3}H]tamsulosin and a larger effect with [^{3}H]prazosin. Nevertheless, the effect of chloroethylclonidine was much smaller than that observed with rat liver (α_{1B}-adrenoceptors) or rabbit liver (α_{1A}-adrenoceptors) membranes (García-Sáinz et al., 1995), emphasizing the resistance of the cloned receptor to inactivation by this antagonist. The action of chloroethylclonidine is complex: the agent can act as a weak partial agonist in rat-1 cells stably expressing the bovine α_{1A}-adrenoceptor (Villalobos-Molina et al., 1997). Chloroethylclonidine is known to undergo intramolecular cyclization to a reactive aziridinium ion before irreversible receptor inactivation; the aziridinium ion presumably forms a covalent bond with free SH-groups of exposed cysteine residues (Vargas et al., 1993; Marjamäki et al., 1998). Chloroethylclonidine binds to α_{2A}-adrenoceptors by irreversibly interacting with exposed cysteines in the fifth membrane-spanning domain (Marjamäki et al., 1998). The sites of interaction of chloroethylclonidine with α_1-adrenoceptors are not known.

The results of the binding competition experiments were in agreement with what was observed in the guinea pig liver membranes. Similarly, the functional studies indicated that the cloned receptor, when activated by epinephrine, can stimulate phosphoinositide turnover, as shown for the guinea pig liver receptor (García-Sáinz et al., 1992a,b).

Finally, the photoaffinity-labeling experiments indicated that the cloned receptor is expressed as a protein with a molecular mass of $\approx$ 70–80 kDa. This is higher than the molecular mass observed for the photolabeled bovine α_{1A}-adrenoceptor ($\approx$ 60 kDa expressed in rat-1 cells) (Vázquez-Prado et al., 2000). The estimated molecular mass of the guinea pig cloned receptor is $\approx$ 51.5 kDa on the basis of the amino-acid sequence. Therefore, the data suggest that this receptor is also extensively glycosylated as has been observed for other adrenoceptors. This is consistent with the presence of conserved asparagines residues, which are potential sites for N-linked glycosylation.

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