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Research Report

Cloning and characterization of a pharmacologically distinct A_1 adenosine receptor from guinea pig brain

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Abstract

Three full-length cDNA clones encoding the guinea pig A_1 adenosine receptor have been isolated by polymerase chain reaction (PCR) and low-stringency hybridization screening of a guinea pig brain cDNA library. These three cDNAs, though differing in their 5′ untranslated regions, contain the same open reading frame encoding a 326 amino acid residue protein with seven hydrophobic α-helices long enough to form the transmembrane domains, suggesting that it belongs to the G protein-coupled receptor superfamily. This protein is highly homologous to the A_1 adenosine receptors previously cloned from other species. Pharmacological characterization of this receptor transiently expressed in mammalian cells demonstrates that, despite its high homology to A_1 adenosine receptors of other species, the guinea pig A_1 adenosine receptor displays a unique pharmacological profile: high affinity for the A_1-selective antagonist CPX, yet very low affinity for some A_1-selective agonists such as CCPA, CHA and R-PIA. Northern blotting for different guinea pig tissues and in situ hybridization for guinea pig brain sections reveal an abundant and broad distribution of mRNA of this A_1 subtype receptor in the brain.

Keywords: G protein-coupled receptor; Polymerase chain reaction; Radioligand binding; Mammalian cell expression; In situ hybridization

1. Introduction

Adenosine not only plays important roles in energy metabolism and in the cellular synthesis of nucleic acids, but also acts as a neurotransmitter to mediate a variety of central physiological functions, such as sedation, pain modulation, and pre-synaptic inhibition of neurotransmitter release. Peripherally, it has been implicated in the inhibition of cardiovascular activity, in the regulation of renal glomerular filtration, and in the modulation of the immune system and of white blood cell and platelet functions [24,36]. Adenosine binds to its specific membrane receptors and activates signal transduction pathways through second messengers. Based on their effects on adenylyl cyclase, adenosine receptors have been classified into two main types: the A_1 type which inhibits adenylyl cyclase and decreases intracellular cAMP levels, and the A_2 type which stimulates adenylyl cyclase and increases cAMP [32]. The cloning of A_1, A_2 and other types of adenosine receptors from several species [13–17,8,19,25,26,28,31,35,37] has facilitated the molecular study and understanding of this subfamily of G protein-coupled receptors.

Species differences among the adenosine receptors in their interactions with agonists and antagonists have been reported based on receptor binding studies using mammalian brain membrane preparations [7,33]. Therefore, cloning and characterization of the adenosine receptors from commonly used laboratory-animal species would be of importance and interest for the study of structure-activity relationship of receptors. Here we report the cloning of an A_1 adenosine receptor from guinea pig brain, the pharmacological and functional characterization of this receptor in transiently transfected cells, and its mRNA expression in guinea pig brain and other tissues.

2. Materials and methods

2.1. mRNA preparation and cDNA library construction

Total RNA was isolated from male Hartley guinea pig brains by guanidinium thiocyanate extraction followed by centrifugation in

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cesium chloride solution [29]. Poly(A)$^+$ RNA was purified using oligo-dT cellulose spun column (Pharmacia). The construction of a cDNA library was principally based on the method developed by Okayama and Berg [22] and modified by Gubler and Hoffman [10]. The first strand cDNA was synthesized by AMV reverse transcriptase using oligo(dT)-*Not* I Primer-Adaptor [oligo(dT)$_{15}$ containing *Not* I site on its 5′ end, Promega], the second strand cDNA was synthesized using *E. coli* DNA polymerase I and RNase H. After treatment with T_4 DNA polymerase to blunt their ends, the double-stranded cDNAs were ligated with *Bst* XI linker (Invitrogen) by T_4 ligase at 14°C for 24 h. The cDNAs were digested by restriction enzyme *Not* I to create *Not* I sticky ends, then loaded on 1% agarose gel for size fractionation. The cDNAs with size >1.5 kb were electroeluted from the gel onto DE81 ion-exchange paper (Whatman) and recovered by eluting with 1 M NaCl. The cDNAs were unidirectionally inserted into the *Bst* XI–*Not* I sites of pME18S expression vector [18,34] by T_4 ligase, and transformed into host DH5α competent cells by electroporation (Bio-Rad). The resulting cDNA library contains 1.8×10^6 independent clones with insert sizes from 1.5 to 5.0 kb.

2.2. Oligonucleotide primer design and PCR amplification

Six degenerate oligonucleotides were synthesized based on published G protein-coupled receptor cDNA sequences.

Three forward:

(1) 5′-AAC(T)C(T)A(T)ITTC(T)A(C)TIATIA(T)CICTIGCITC(G)IGCIGA-3′
(2) 5′-A(C)GITTIC(T)TIATGTGC(T)AAC(T)CTIA(T)C(G)C(T)TTC(T)GCIGA-3′
(3) 5′-ACIGTITAC(T)ATIACICAC(T)C(T)TIA(T)C(G)IATIGCIGA-3′,

and three reverse:

(4) 5′-GCC(T)TTIGTA(G)AAIATIGCA(G)TAIAGA(G)AAIGGA(G)TT-3′
(5) 5′-AAA(G)TCIGGA(G)C(G)A(T)ICGIC(G)AA(G)TAIATC(G)AIIGGA(G)TT-3′
(6) 5′-C(G)A(T)IC(G)A(T)ICCIACA(G)AAA(G)TAIATA(G)AAIGGA(G)TT-3′.

Every possible combination of one forward and one reverse oligonucleotides was employed as one set of primers for polymerase chain reaction (PCR) [27].

Five fmol of guinea pig brain cDNAs were used as PCR templates, 1 μM of each primer, 200 μM of each dNTP and 5 units of *Taq* DNA Polymerase (Perkin-Elmer) were used in each 100 μl volume reaction. The PCR temperature cycle on the DNA Thermal Cycler (Coy Laboratory) was set as follows: for the first five cycles, denaturation at 94°C for 1 min, annealing at 37°C for 2 min, extension at 50°C for 5 min; for the next 30 cycles, denaturation at 94°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 3 min; the last five cycles had an extension time of 10 min at 72°C in each cycle.

Aliquots of the PCR products were directly loaded onto 1.2% agarose gel to analyze their sizes. DNAs from bands of 0.4–1.2 kb were recovered by electroelution, ligated into the *Eco*RV site of pBluescript KS II (Stratagene) for sequencing and for preparation of ^{32}P-labeled probes for colony hybridization.

2.3. Colony hybridization and DNA sequencing

Approximately 3×10^5 *E. coli* colonies from the guinea pig brain cDNA library were lifted onto Nytran filters (S&S). Each PCR product probe was labeled with ^{32}P by random priming [6]. The conditions of low-stringency hybridization were: 35% formamide, 5×SSPE, 5×Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA (Pharmacia), 1×10^6 cpm probe/ml, 42°C, 24 h. Washing was conducted at 37°C three times with 2×SSPE, 0.2% SDS for 30 min each. Positive colonies were picked and further purified to single clones by second round screening and southern hybridization with ^{32}P-probes.

Both strands of cloned cDNA were sequenced by dideoxynucleotide chain termination method [30] with Sequenase (USB). DNA and deduced protein sequences were analyzed by Genetics Computer Group programs (Version 7).

2.4. Mammalian cell transfection and receptor binding assay

COS-7 cells were transfected by electroporation [21] with plasmids purified by CsCl gradient centrifugation. Exponentially growing COS-7 cells were plated (5×10^5 cells/10 cm dish) in DMEM, 10% FCS. After one day in culture at 37°C, 5% CO_2, cells were harvested and washed twice with D-PBS, then resuspended in 1 ml D-PBS containing 10 μg of the plasmid DNA encoding the guinea pig A_1 receptor (gpA_1R) inserted in the pME18S vector. The cell suspensions were kept in a sterile electroporation cuvette on ice for 5 min, and electroporated in a Cell-Porator (BRL) at 330 μF, 300 V, 4°C, then placed on ice again for 5 min. They were subsequently suspended in 10 ml DMEM containing 10% FCS, and cultured at 37°C, 5% CO_2.

After 3 days in culture, transfected cells were harvested by treatment with D-PBS (without calcium and magnesium) containing 0.5 mM EDTA and 0.01% sodium azide, washed three times with KHB [115 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM HEPES, pH adjusted to 7.4 with NaOH), and resuspended in KHB. Receptor binding assays were performed in 1 ml KHB with 1×10^5 cells.

In order to distinguish different types of adenosine receptors, the tritiated A_1 agonists 2-chloro-N^6-[cyclopentyl-2,3,4,5-^{3}H]cyclopentyladenosine ([^{3}H]CCPA, spec. act. 35.4 Ci/mmol), and cyclohydroxyadenosine ([^{3}H]CHA, 30.2 Ci/mmol), the A_1 antagonist 8-[dipropyl-2,8-^{3}H(N)]cyclopentyl-1,3-dipropylxanthine ([^{3}H]CPX, 108.0 Ci/mmol), and the A_2 agonist [carboxyethyl-^{3}H(N)]CGS 21680 (39.6 Ci/mmol) (all from NEN) were used to label the binding sites. Unlabeled ligands used in binding assays included: the A_1-selective agonists CCPA, CPA, and CHA; the relatively A_1-selective agonists ADAC, R-PIA, and BA; the A_1-selective antagonists CPX and DPX; the relatively A_1-selective antagonists XAC and IBMX; the A_2-selective agonist CGS 21680; relatively A_2-selective agonist MECA; relatively A_2-selective antagonists DMPX, DPMX; and the non-selective agonist NECA, as well as adenosine and ATP. For the saturation studies, various concentrations of radiolabeled ligands were used, and nonspecific binding was determined in the presence of 10 μM unlabeled R-PIA for A_1 and 10 μM CGS 21680 for A_2. In one parallel experiment, 5 units/ml of adenosine deaminase were included in the binding assay buffer to determine the influence of endogenously released adenosine; in another parallel experiment, transfected cell membrane preparations in 50 mM Tris buffer (pH 7.4) were used to assess the influence of salts in KHB on binding affinities. For competition studies, minimum amounts of radiolabeled ligands were used to give about 2000 cpm specific binding per tube, and various concentration of unlabeled ligands were used to compete. The assay reactions were carried out at room temperature for 1.5 h and terminated by placement on ice for 5 min. Bound and free radioligands were separated by filtration through glass fiber filters (#32, S&S) on a multi-channel cell harvester (M-24S, Brandel). The filters were counted for radioactivity in scintillation solution. Binding assay data were analyzed by the Ligand program [20] and are presented as the mean value of three experiments.

2.5. GTPase assay

The experimental procedure for the GTPase assay was based on the method described by Cassel and Selinger [2]. Briefly, plasmid

pME18S-gpA$_1$R transfected COS cell membranes were prepared by homogenization and centrifugation in 10 mM Tris-HCl buffer containing 0.1 mM EDTA, pH 7.5. Ten micrograms of membrane protein suspended in 10 μl were added to an 85 μl reaction mixture containing 0.5 mM [γ-^{32}P]GTP, 1 mM AppNHp, 1 mM ATP, 1 mM Ouabain, 10 mM creatine phosphate, 5 units of creatine phosphokinase, 2 mM dithiothreitol, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA, 12.5 mM Tris-HCl, pH 7.5. This mixture was kept on ice before the addition of adenosine receptor agonists. Immediately after the addition of the A_1 agonist CPA or R-PIA (10 μM and 1 μM) or the A_2 agonist CGS 21680 (10 μM), the reaction tubes were transferred to 37°C for 20 min. The reaction was terminated by placing on ice, and adding 0.9 ml of stop solution containing 20 mM phosphoric acid and 5% activated charcoal, pH 2.3. After centrifugation in an Eppendorf microcentrifuge at 4°C for 10 min, the radioactivities in the supernatants were measured.

2.6. cAMP assay

COS-7 cells transfected with plasmid pME18S-gpA$_1$R were cultured in DMEM, 10%FCS for two days. After washing twice with IMDM, the cells were cultured in IMDM, 10% Hyclone FCS. Forskolin (1 μM) was added to raise the intracellular cAMP levels, and then various adenosine ligands were added to the cell culture and incubated at 37°C for 30 min. To terminate the reaction, 30% trichloroacetic acid (ice cold, equal volume to the cell culture media) was added to the dish. The cells were scraped into a glass test tube and put on ice for 30 min for a complete precipitation. After centrifugation at 2000×g, 4°C for 10 min, the supernatant was ether extracted, lyophilized in a Speed Vac and resuspended in 50 mM Tris-HCl, 2 mM EDTA, pH 7.5. Cyclic AMP levels were measured with a cAMP Assay Kit (TRK 432, Amersham).

2.7. Northern blot analysis

Total RNA was extracted from guinea pig whole brain and peripheral tissues with 5 M GITC solution (5 M guanididium isothiocyanate, 10 mM EDTA, 50 mM Tris, and 8% β-mercaptoethanol) and 5 M LiCl. The RNA samples (25 mg for each tissue) were electrophoresed on a 1% agarose gel in 1×MOPS containing 6.6% formaldehyde, and then transferred onto a Nytran filter. The filter was hybridized with 7×10^5 cpm/ml [α-^{32}P]UTP-labeled gpA$_1$R cRNA probe under high-stringency conditions: 50% Formamide, 5×SSPE, 5×Denhardt's reagent, 0.5% SDS, 70°C, for 24 h. The filter was washed once with 1×SSPE, 0.5% SDS at 70°C for 30 min and three times with 0.1×SSPE, 0.5% SDS at 70°C for 30 min and then exposed to an X-ray film (XAR-5, Kodak) with two intensifying screens for 6 days at −80°C.

2.8. In situ hybridization

All in situ hybridizations were conducted with [^{35}S]UTP-labeled cRNA riboprobes. A_1 cRNA riboprobe was produced from an *Xba*I–*Sac*I fragment of the gpA$_1$R cDNA ligated into pBluescript KS II using either the T_3 or T_7 RNA polymerases. This fragment (500 bp) encompasses the entire sequence of the third cytoplasmic loop, a region of least homology among G protein-coupled receptors. Labeled probe was separated from free nucleotides over a Sephadex G-50 column. Its specificity was confirmed by absence of signal both in sections hybridized with sense A_1 probe and in sections treated with RNase prior to hybridization with the antisense (cRNA) A_1 probe.

To examine major brain structures of the guinea pig, brain horizontal sections (15 μm thick) were prepared, and placed into 4% buffered paraformaldehyde at room temperature. After 1 h, slides were rinsed in PBS and treated with proteinase K (1 μg/ml in 100 mM Tris-HCl, pH 8.0) for 10 min at 37°C. Subsequently, sections underwent successive washes in water for 1 min, in 0.1 M triethanolamine (pH 8.0, plus 0.25% acetic anhydride) for 10 min, and in 2×SSC (0.3 mM NaCl, 0.03 mM sodium citrate, pH 7.2) for 5 min. Sections were then dehydrated through graded ethanol solutions and air dried.

1.0×10^6 dpm ^{35}S-labeled riboprobes were diluted in hybridization buffer containing 75% formamide, 10% dextran sulfate, 3×SSC, 50 mM sodium phosphate buffer (pH 7.4), 1×Denhardt's solution, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol in a total volume of 25 ml. The diluted probe was applied to post-fixed sections on a glass coverslip which was sealed into place with rubber cement, and hybridized overnight at 55°C in a humid environment.

After hybridization, the rubber cement was removed and sections were washed in 2×SSC for 5 min and then treated with RNase A (200 μg/ml in 10 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl) for 60 min at 37°C. Subsequently, sections were washed in 2×SSC for 5 min, 1×SSC for 5 min, 0.5×SSC for 60 min at 55°C, 0.5×SSC at room temperature for 5 min, and then dehydrated in graded ethanol solutions and air dried. Sections were exposed to an X-ray film for 2 days at room temperature.

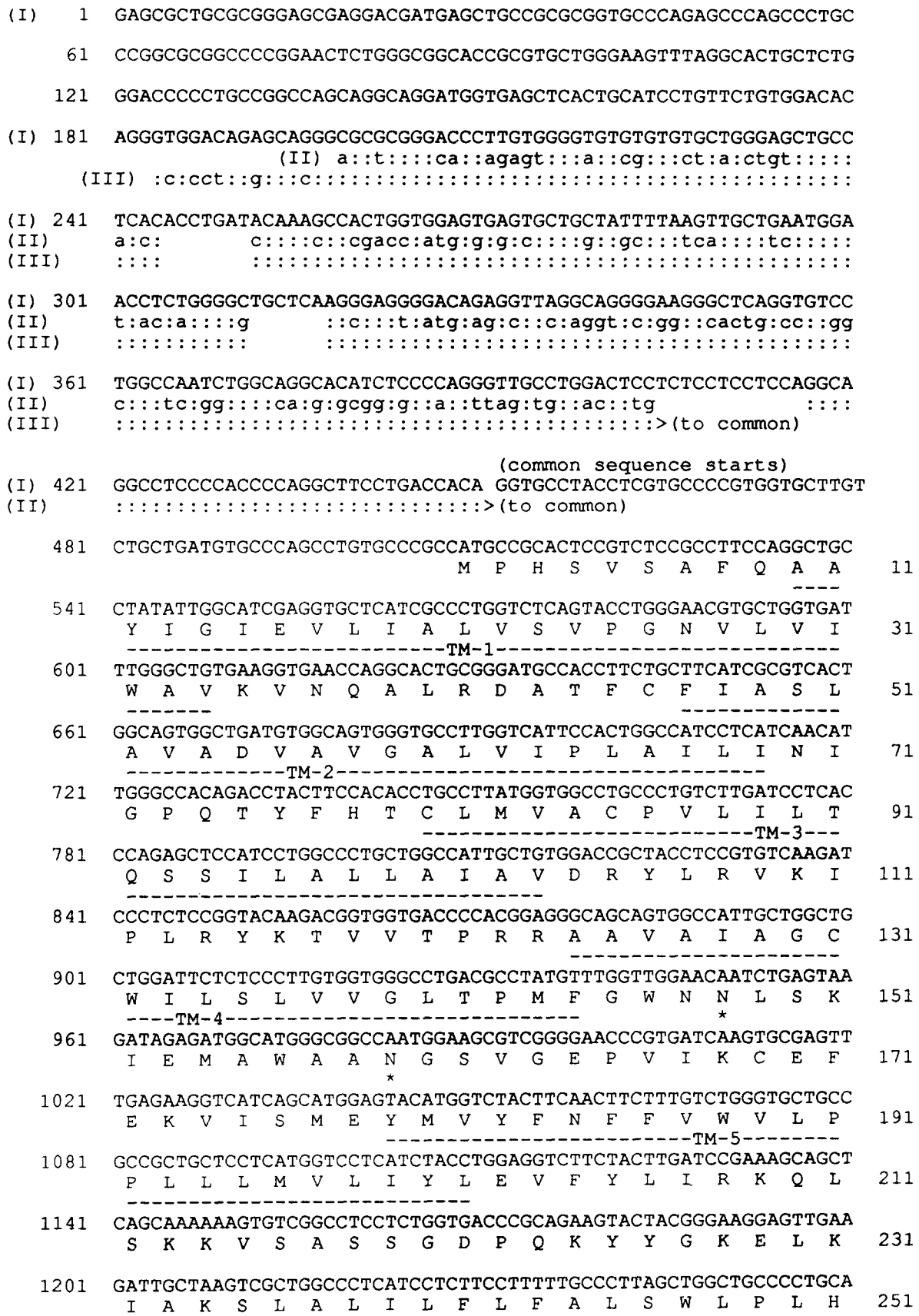
2.9. In vitro receptor autoradiography

[^{3}H]CPX, [^{3}H]CHA and [^{3}H]CCPA binding studies on guinea pig brain horizontal sections (15 mm thick) were performed according to published methods [5]. Slide-mounted sections were incubated in 0.17 M Tris-HCl with 0.5 units/ml adenosine deaminase (pH 7.4) for 20 min, and then incubated with 2 nM [^{3}H]CPX, or 10 nM [^{3}H]CHA, or 10 nM [^{3}H]CCPA for 90 min at room temperature. Slides were washed twice in incubation buffer for 5 min, and dried in a stream of cold air. Non-specific binding was determined in the presence of 2 μM CPX. Sections were apposed to tritium-sensitive Hyperfilm (Amersham) at room temperature for 14 days.

3. Results

3.1. Cloning of gpA$_1$R cDNAs

Sequence analysis of several PCR-amplified fragments revealed different sequences with various degrees of homology (from 62% to 88%) to cDNAs encoding the A_1 and A_2 adenosine receptors of the dog and rat. These PCR products were labeled with ^{32}P and used as the probes to screen the guinea pig brain pME18S cDNA library to obtain full-length clones. Some of the clones we isolated were found to encode the guinea pig A_2 adenosine receptor, which we have described elsewhere [19]. Twenty-one positive clones were found to be highly homologous to A_1 adenosine receptor. Although they all contained an identical long open reading frame and an identical 3'-untranslated region (3'UT), the 21 clones exhibited three variant sequences in their 5'UT (Fig. 1). Variant I has the longest 5'UT (508 bp); the 5'UT of variant II is shorter and exhibits about 60% homology to that of variant I; the 5'UT region of variant III is identical to that of variant II except for a 34 bp deletion. It is not known whether this 5'UT variation has any significance in the regulation of the expression, localization and


(I) 1 GAGCGCTGCGCGGGAGCGAGGACGATGAGCTGCCGCGCGGTGCCCAGAGCCCAGCCCTGC
61 CCGGCGCGGCCCCGGAACTCTGGGCGGCACCGCGTGCTGGGAAGTTTAGGCACTGCTCTG
121 GGACCCCCTGCCGGCCAGCAGGCAGGATGGTGAGCTCACTGCATCCTGTTCTGTGGACAC
(I) 181 AGGGTGGACAGAGCAGGGCGCGCGGGACCCTTGTGGGGTGTGTGTGTGCTGGGAGCTGCC
(II) a::t::::ca::agagt:::a::cg:::ct:a:ctgt:::::
(III) :c:cct::g:::c::
(I) 241 TCACACCTGATACAAAGCCACTGGTGGAGTGAGTGCTGCTATTTTAAGTTGCTGAATGGA
(II) a:c: c::::c::cgacc:atg:g:g:c::::g::gc:::tca::::tc:::::
(III) :::: ::
(I) 301 ACCTCTGGGGCTGCTCAAGGGAGGGGACAGAGGTTAGGCAGGGGAAGGGCTCAGGTGTCC
(II) t:ac:a::::g ::c:::t:atg:ag:c::c:aggt:c:gg::cactg:cc::gg
(III) :::::::::::: ::
(I) 361 TGGCCAATCTGGCAGGCACATCTCCCCAGGGTTGCCTGGACTCCTCTCCTCCTCCAGGCA
(II) c:::tc:gg::::ca:g:gcgg:g::a::ttag:tg::ac::tg ::::
(III) ::>(to common)
(common sequence starts)
(I) 421 GGCCTCCCCACCCCAGGCTTCCTGACCACA GGTGCCTACCTCGTGCCCCGTGGTGCTTGT
(II) ::::::::::::::::::::::::::::::>(to common)
481 CTGCTGATGTGCCCAGCCTGTGCCCGCCATGCCGCACTCCGTCTCCGCCTTCCAGGCTGC
M P H S V S A F Q A A 11
----
541 CTATATTGGCATCGAGGTGCTCATCGCCCTGGTCTCAGTACCTGGGAACGTGCTGGTGAT
Y I G I E V L I A L V S V P G N V L V I 31
----------TM-1----------
601 TTGGGCTGTGAAGGTGAACCAGGCACTGCGGGATGCCACCTTCTGCTTCATCGCGTCACT
W A V K V N Q A L R D A T F C F I A S L 51
------- -------------
661 GGCAGTGGCTGATGTGGCAGTGGGTGCCTTGGTCATTCCACTGGCCATCCTCATCAACAT
A V A D V A V G A L V I P L A I L I N I 71
----------TM-2----------
721 TGGGCCACAGACCTACTTCCACACCTGCCTTATGGTGGCCTGCCCTGTCTTGATCCTCAC
G P Q T Y F H T C L M V A C P V L I L T 91
----------TM-3---
781 CCAGAGCTCCATCCTGGCCCTGCTGGCCATTGCTGTGGACCGCTACCTCCGTGTCAAGAT
Q S S I L A L L A I A V D R Y L R V K I 111
----
841 CCCTCTCCGGTACAAGACGGTGGTGACCCCACGGAGGGCAGCAGTGGCCATTGCTGGCTG
P L R Y K T V V T P R R A A V A I A G C 131
----
901 CTGGATTCTCTCCCTTGTGGTGGGCCTGACGCCTATGTTTGGTTGGAACAATCTGAGTAA
W I L S L V V G L T P M F G W N N L S K 151
----TM-4---------- *
961 GATAGAGATGGCATGGGCGGCCAATGGAAGCGTCGGGGAACCCGTGATCAAGTGCGAGTT
I E M A W A A N G S V G E P V I K C E F 171
*
1021 TGAGAAGGTCATCAGCATGGAGTACATGGTCTACTTCAACTTCTTTGTCTGGGTGCTGCC
E K V I S M E Y M V Y F N F F V W V L P 191
----------TM-5--------
1081 GCCGCTGCTCCTCATGGTCCTCATCTACCTGGAGGTCTTCTACTTGATCCGAAAGCAGCT
P L L L M V L I Y L E V F Y L I R K Q L 211

1141 CAGCAAAAAAGTGTCGGCCTCCTCTGGTGACCCGCAGAAGTACTACGGGAAGGAGTTGAA
S K K V S A S S G D P Q K Y Y G K E L K 231
1201 GATTGCTAAGTCGCTGGCCCTCATCCTCTTCCTTTTTGCCCTTAGCTGGCTGCCCCTGCA
I A K S L A L I L F L F A L S W L P L H 251

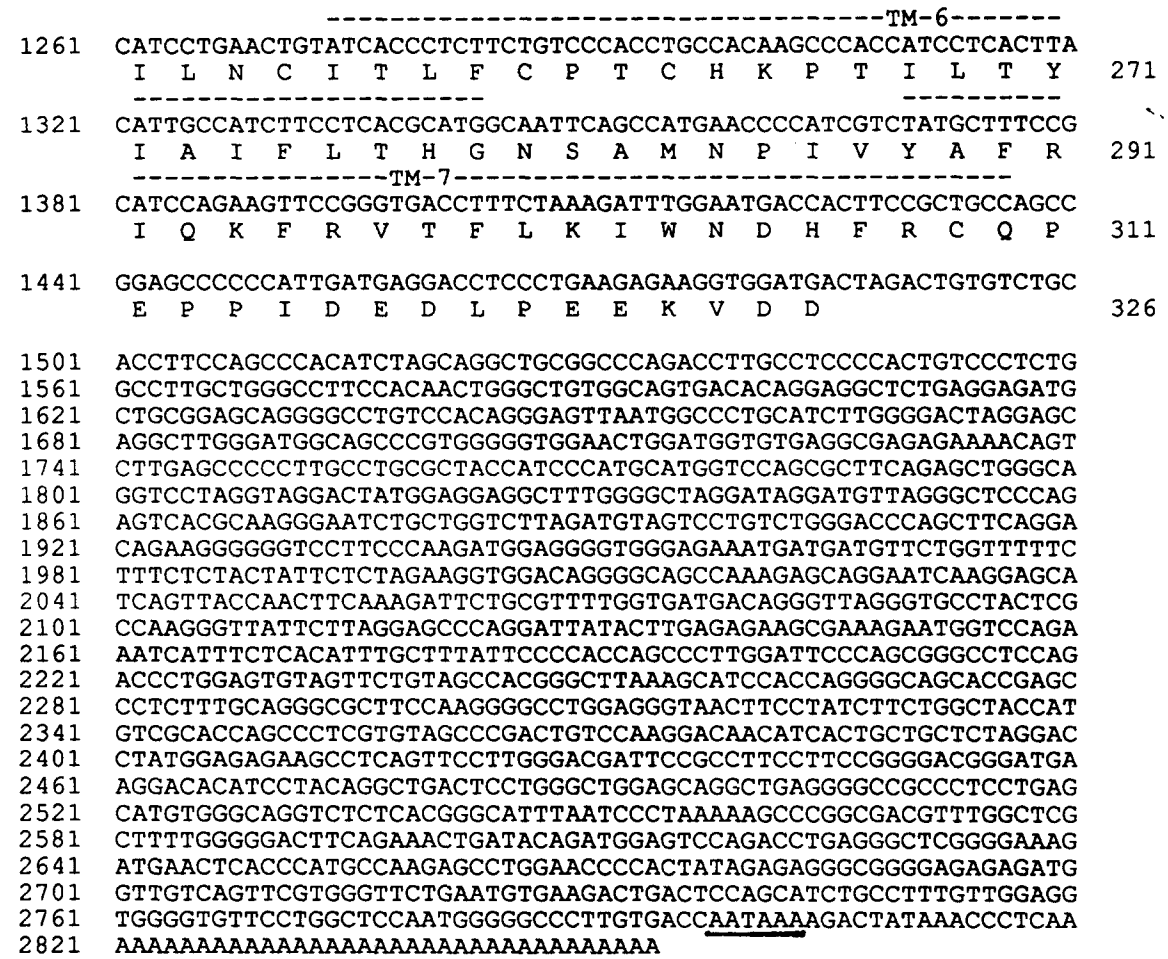


Fig. 1. Nucleotide and deduced amino acid sequence of gpA_1R. I, II, and III are the three variants. Nucleotides of Variant I are numbered on the left of the rows and amino acids on the right. Colons (:) indicate identities with sequences immediately above. Possible N-glycosylation sites (*) are marked, and polyadenylation signal sequence underlined. Putative hydrophobic transmembrane domains TM-1 through TM-7 are identified according to hydrophobisity analysis [12] and are underlined with broken lines. The sequence has been deposited in the GenBank database (accession no. U04279).

stability of the mRNAs, or is due to recombination, or is even an artifact of the cDNA library construction, though the high sequence-similarity of the three variants makes the last possibility very unlikely. Since variation in the untranslated regions does not alter the protein-coding sequence, it is unlikely to affect the properties of the encoded receptor protein itself, including the ligand binding profile. Therefore, we chosen variant I (named gpA_1R) for all subsequent studies.

3.2. Sequence analysis of gpA_1R

Clone gpA_1R contains an insert of 2,837 bp including a poly(A) tail. A long open reading frame is found after an ATG (methionine) at position 509–511. The sequences around this ATG fit the Kozak consensus sequence for an eukaryotic translation initiation site [11]. Several in-frame stop codons are located upstream of this ATG. This open reading frame encodes a protein composed of 326 amino acids (including the first methionine) (Fig. 1), with a calculated molecular mass of 36,551 Da. The other two variants encode the same protein.

The deduced amino acid sequence of gpA_1R shows high homology to the previously cloned A_1 adenosine receptors from other species: 91% identity to dog A_1, 93% to rat A_1, 91% to bovine A_1, and 95% to human A_1 (Fig. 2). It also shares a substantial degree of homology with other types of adenosine receptors: 49% identity to dog A_{2a}, 50% to human A_{2a}, 50% to rat A_{2a}, 49% to guinea pig A_{2a}, 47% to rat and human A_{2b}, and 45% to rat A_3. Its similarities with other members of the G protein-coupled receptor superfamily are lower but significant (for example, within the transmembrane domains, 28% identity with human β_2-adrenergic receptor, 27% with human H_2 histamine receptor, 25% with human D_1 dopamine receptor, 27%

with rat 5-HT_{1C} receptor, 23% with rat substance P receptor, 23% with rat cannabiniod receptor, and 18% with mouse thyrotropin-releasing hormone receptor).

Hydrophobicity analysis (method of Kyte & Doolittle) [12] of gpA_1R revealed the existence of seven hydrophobic segments, each consisting of about 23–27

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          1                                                          60
GPA1      MPHSVSAFQAAYIGIEVLIALVSVPGNVLVIWAVKVNQALRDATFCFIASLAVADVAVGA
HumanA1   --P-I-----------------------------------------V----------
RatA1     --PYI----------------------------------------V----------
DogA1     --PAI----------------------------------------V----------
CowA1     --P-I----------------------------------------V----------
RabbitA1  --P-I----------------------------------------V----------

                                                                    120
GPA1      LVIPLAILINIGPQTYFHTCLMVACPVLILTQSSILALLAIAVDRYLRRQDPLRYKTVVT
HumanA1   ----------------------------------------------VKI-----M---
RatA1     ----------------------------------------------VKI---------
DogA1     -------------R-------------------------------VKI---------
CowA1     -------------R-------K--------------------------VKI---------
RabbitA1  -------------E-------------------------------VKI-----A---

                                                                    180
GPA1      PRRAAVAIAGCWILSLVVGLTPMFGWNNLSKIEMAWAANGSVGEPVIKCEFEKVISMEYM
HumanA1   ---------------F--------------AV-R-------M------------------
RatA1     Q-----------------------------VV-QD-R----------------------
DogA1     ---------------F------L----R-GEAQR-------G------------------
CowA1     ----V---T------F--------------AV-RD-L----------E-Q----------
RabbitA1  ----------------------------REVQR-------------------------

                                                                    240
GPA1      VYFNFFVWVLPPLLLMVLIYLEVFYLIRKQLSKKVSASSGDPQKYYGKELKIAKSLALIL
HumanA1   --------------------------------N----------------------------
RatA1     --------------------------------N----------------------------
DogA1     ----------------------------R--G----------------------------
CowA1     --------------------M-------------------------------------
RabbitA1  ----------------------------R-----A-------H-----------------

                                                                    300
GPA1      FLFALSWLPLHILNCITLFCPTCHKPTILTYIAIFLTHGNSAMNPIVYAFRIQKFRVTFL
HumanA1   --------------------S----S--------------------------------
RatA1     ----------------------Q--S--I----------------------H-------
DogA1     --------------------S-R--S--M----------------------------
CowA1     --------------------S--M-R--I------S----------------------
RabbitA1  ---------------V-----S-Q--S--V-T------------------H-------

                                      328
GPA1      KIWNDHFRCQPEPP..IDEDLPEEKVDD
HumanA1   -----------A--..--------RP--
RatA1     -----------K--..---------AE-
DogA1     -----------T--..V---P---APH-
CowA1     -----------A--..----A-A-RP--
RabbitA1  ---------R-A-AGDG--------PN-
```

Fig. 2. Amino acid sequence comparison of cloned A1 adenosine receptors. The deduced amino acid sequence of guinea pig A1 (GPA1) is in single-letter form and numbered on the top. Dashed lines in the A1 sequences of human [15], rat [26], dog [13], cow [35] and rabbit (1a) represent amino-acid residues identical to those in GPA1. Dots represent the gaps of alignment.

amino-acid residues, sufficiently long to form an α-helix which could span the cell membrane. These hydrophobic regions possess the highest similarities among G protein-coupled receptors. The gpA1R does not contain a hydrophobic signal sequence at its N-terminus.

Several features of the primary structure of gpA_1R are noteworthy and are also conserved in all other cloned A_1 receptors: Being 326 amino acids in length, it is one of the smallest members of the G protein-coupled receptor superfamily cloned to date; its N-terminal extracellular region before the TM I is very short (< 10 residues), and does not contain any potential N-glycosylation sites; on the other hand, there are two potential N-glycosylation sites in the second extracellu-

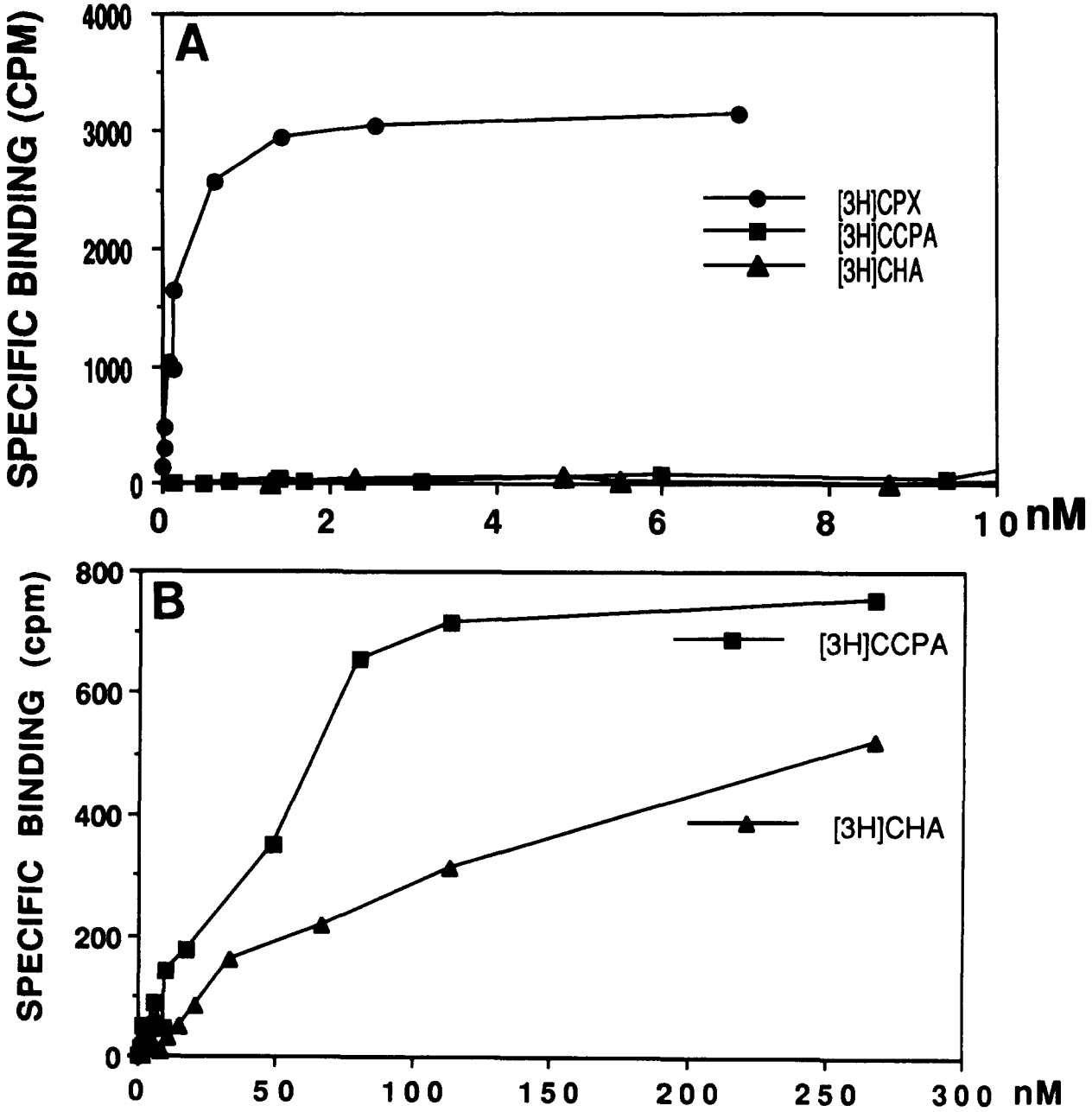

Fig. 3. Radio-ligand receptor binding assays on gpA_1R transfected COS-7 cells. A,B: saturation curves of [^{3}H]CPX, [^{3}H]CCPA and [^{3}H]CHA specific binding. Ten μM of unlabeled R-PIA are used to produce total displacement and define the background. Note wide range of concentrations tested and different x-axis scale in B. C: competition of [^{3}H]CPX specific binding by CPX (▲), XAC (△), CPA (*), CCPA (■), R-PIA (◆), IBMX (●), CHA (□), NECA (◘), DPX (⧅), DPMX (×), ADAC (▪), adenosine (◙), BA (–), DMPX (⊞), ATP (⊠), MECA (+), CGS21680 (◇).

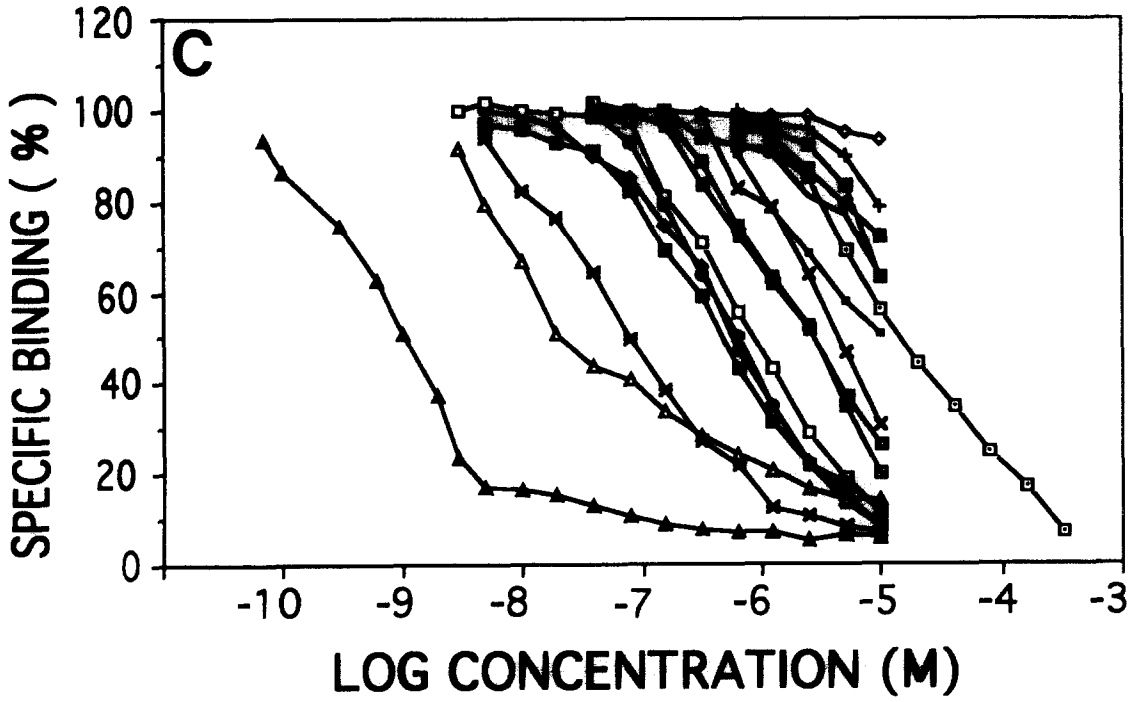

Fig. 3 (continued).

lar loop; though it couples to Gi protein to inhibit adenylyl cyclase (data shown below), gpA_1R has a very short third intracellular loop (only 34 amino acids), which is distinct from more prototypical Gi-coupled receptors (e.g. D_2 dopamine receptor) which contain a long third intracellular loop of about 130–140 residues; the short C-terminal intracellular tail (36 amino acids) contains only a single threonine residue and no serine residue, possibly indicating a minor role of phosphorylation in modifying this domain. However, in the third intracellular loop, there are five serines and three tyrosines, which may participate in mechanisms of receptor regulation via phosphorylation.

3.3. Receptor binding assay of transfected COS-7 cells

Untransfected COS-7 cells, do not express either A_1 or A_2 adenosine receptor mRNA, nor do they demonstrate detectable [^{3}H] CPX and [^{3}H]CGS 21680 binding. Thus, COS-7 cells were transfected with plasmid gpA_1R, and binding assays (both saturation and competition studies) were performed on whole cells. Cells transfected with plasmid gpA_1R expressed high-affinity, saturable specific binding for the selective A_1 antagonist [^{3}H]CPX ($K_d = 0.13$ nM), with specific binding representing about 75–80% of total binding (Fig. 3A). However, the selective A_1 agonists [^{3}H]CCPA and [^{3}H]CHA showed only low affinity binding to gpA_1R: [^{3}H]CCPA K_d: > 50 nM; [^{3}H]CHA K_d: > 100 nM (Fig. 3B). Several experiments were conducted in an attempt to improve these binding affinities: binding assays were performed with transfected COS-7 cell membrane preparations in Tris buffer in contrast to whole cells in KHB; studies were carried out in the presence of adenosine deaminase; different concentrations of Mg^{2+} were employed; finally, co-transfection of gpA_1R with either rat G_i 1α, 2α or 3α subunits was attempted. All these studies yielded similar results, with low affinity for those A_1 agonists (data not shown). The selective A_2 agonist [^{3}H]CGS 21680 did not bind to gpA_1R (not shown). However, in parallel experiment using a cloned guinea pig A_2 receptor cDNA (gpA_2R) and the same expression system, both A_2-selective agonist CGS21680 and A_2 antagonist DMPX bound well, and all the relatively A_1-selective and non-selective adenosine agonists displayed appreciable binding affinities toward this guinea pig A_2 receptor, which in good agreement with the literature-reported values [19]; in another control experiment using guinea pig brain membrane preparations, [^{3}H]CCPA and [^{3}H]CHA did show high-affinity binding (K_d 0.67 nM and 13.84 nM, respectively), ruling out the possibility of technical problems in our transfection system and binding assays.

In competition studies, the selective A_1 antagonist CPX competed very well for [^{3}H]CPX binding in gpA_1R transfected COS-7 cells, and the non-selective adenosine antagonist XAC and the selective A_1 agonist CPA were moderate competitors; however, the selective A_1 agonists CCPA, CHA and R-PIA com-

peted very poorly. Other ligands such as the A_1 agonists BA and ADAC, and non-selective agonists NECA, and the A_1 antagonist DPX and IBMX were all poor competitors. The A_2 agonist CGS 21680 and antagonist DMPX, as well as ATP and ADP did not compete [^{3}H]CPX binding at all. The rank order of potency of these ligands in displacing [^{3}H]CPX binding to gpA_1R-transfected cells is as follows: CPX (K_i 0.17 nM) ≫ XAC (3.54 nM) > CPA (13.1 nM) ≫ CCPA (81.0 nM) > R-PIA (98.3 nM) ≈ IBMX (102 nM) > CHA (138 nM) ≫ NECA = DPX (492 nM) > DPMX (736 nM) > ADAC (> 1400 nM) > adenosine (> 1800 nM) > BA, ATP, ADP, DMPX (> 2000 nM) > CGS 21680 (no binding) (Fig. 3C).

3.4. The effects of A_1 ligands on GTPase activity and cAMP levels in gpA_1R-transfected COS cells

The A_1 agonists CPA and R-PIA stimulated GTPase activity up to more than 32% from control level, and reduced forskolin-induced cAMP level by about 40% in gpA1R transfected cells. These effects could be blocked by the A_1 antagonist CPX (Table 1). The A_2 agonist CGS 21680 had little effect on GTPase activity and cAMP levels in the same transfectants. The A_1-selective agonist CPA did not significantly affect the GTPase activity and did not decrease the cAMP levels in COS-7 cells transfected with plasmid pME18S-gpA_2R [19].

3.5. Northern blot analysis

Northern blot analysis of total RNA from guinea pig brain, heart, lung, spleen, kidney, liver, stomach, and intestine with gpA_1R cRNA probe showed a hybridized broad band centered at 3.0 kb in the brain. After longer exposure, a faint broad band of same size was also found in heart, spleen and kidney (Fig. 4).

3.6. In situ hybridization and receptor autoradiography

As can be seen from Fig. 5, the highest levels of gpA_1R mRNA expression were evident within the cerebellum, the hippocampal formation and neocortex. In the hippocampus, the distribution of gpA_1R mRNA

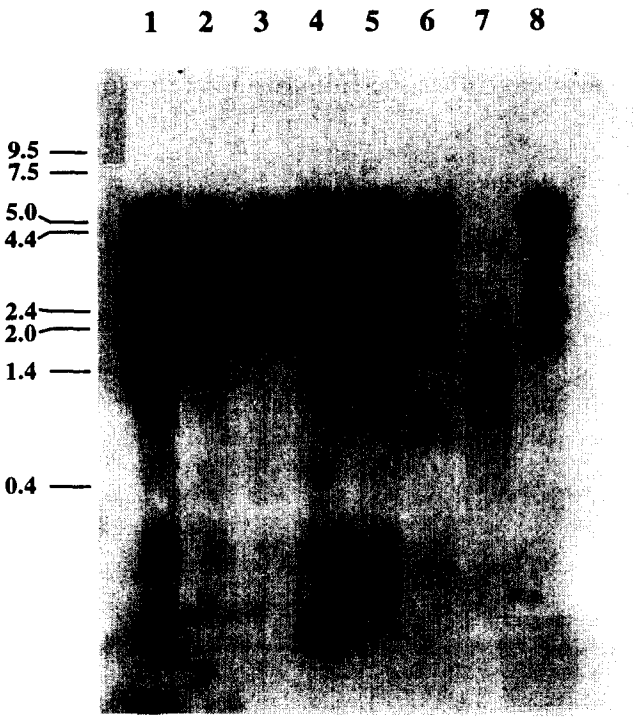

Fig. 4. Northern hybridization of RNAs from different guinea pig tissues. Lanes are: (1) brain, (2) heart, (3) lung, (4) spleen, (5) kidney, (6) liver, (7) stomach, and (8) intestine. The molecular weight estimate was based on an RNA ladder (BRL) and a Ribosomal RNA marker (Pharmacia).

was heterogeneous: high levels of expression were found in the pyramidal cell layer of CA subfields and lower levels in the dentate gyrus and subiculum. This subfield distribution is comparable to the hippocampal distribution of A_1 binding sites as labeled with [^{3}H]CPX (Fig. 5). Within the cortex, gpA_1R mRNA expression was most prominent in intermediate and deep cortical layers. Again, this is in agreement with the cortical distribution of [^{3}H]CPX binding. Lower levels of both gpA_1R mRNA and A_1 binding were found in the caudate nucleus, septal nuclei and thalamic regions. The distribution of gpA_1R mRNA and [^{3}H]CPX binding were generally in agreement, except that the binding of [^{3}H]CPX in cerebellum was significantly low. Autoradiographic examination of both [^{3}H]CHA and [^{3}H]CCPA binding revealed an anatomical distribution

Table 1
GTPase and cAMP assays on gpA_1R-transfected COS-7 cells

	Control	R-PIA (μM)			CPA (μM)			R-PIA (10 μM) + CPX (10 μM)	CGS21680 (10 μM)
		10	1	0.1	10	1	0.1		
GTPase activity (%)	100 ± 4	132 ± 4*	118 ± 5	104 ± 3	135 ± 7*	120 ± 6	101 ± 4	102 ± 3	98 ± 5
Cyclic AMP level (%)	100 ± 3	61 ± 5*	83 ± 6	94 ± 4	59 ± 8*	85 ± 5	97 ± 7	99 ± 6	102 ± 4

Data are presented as mean ± S.E. of triplicates. GTPase activities without the application of ligands and forskolin-raised cAMP levels in transfected COS-7 cells were measured as control. *$P < 0.05$, as compared with control (Student t-test). In the same experiments in COS-7 cells transfected with plasmid pME18S-gpA_2R, upon the application of 10 μM CPA, GTPase activity was only slightly increased to 107 ± 4, and forskolin-raised cAMP level was unchanged as compared with the control [19].

almost identical to that obtained using [^{3}H]CPX, except that in cerebellum the signals of the former two were stronger than the latter (data not shown).

4. Discussion

In this report, we describe a guinea pig cDNA encoding a novel protein with the following features:
(a) The deduced protein gpA_1R sequence shows a topology of seven hydrophobic α-helices, characteristic of the G protein-coupled receptor superfamily; it is most homologous to adenosine receptors cloned from other species, with a higher degree of sequence identity to A_1 (91–95%) than to A_2 (47–51%).
(b) The gpA_1R, when expressed in COS cells, binds the A_1-selective antagonist CPX with high affinity, and also generally prefers most A_1-selective ligands over A_2-selective ligands, although it exhibits low affinity toward several classical A_1-agonists.
(c) Agonist activation of expressed gpA_1R in COS cells increases GTPase activity and decreases intracellular cAMP levels.
(d)The gpA_1R mRNA exhibits a distinctive distribution in brain, which parallels the pattern of A_1 receptor autoradiography.

These results are generally consistent with the characteristics of an A_1 type adenosine receptor. Therefore, we conclude that the cloned gpA_1R encodes a guinea pig A_1 adenosine receptor.

However, despite its high sequence-similarity (91–95%) to A_1 adenosine receptors from other species (dog, rat, bovine, and human), the cloned gpA_1R displayed a unique pharmacological profile: the most interesting feature is its low-affinity binding to a group of A_1-selective agonists such as CCPA and CHA which

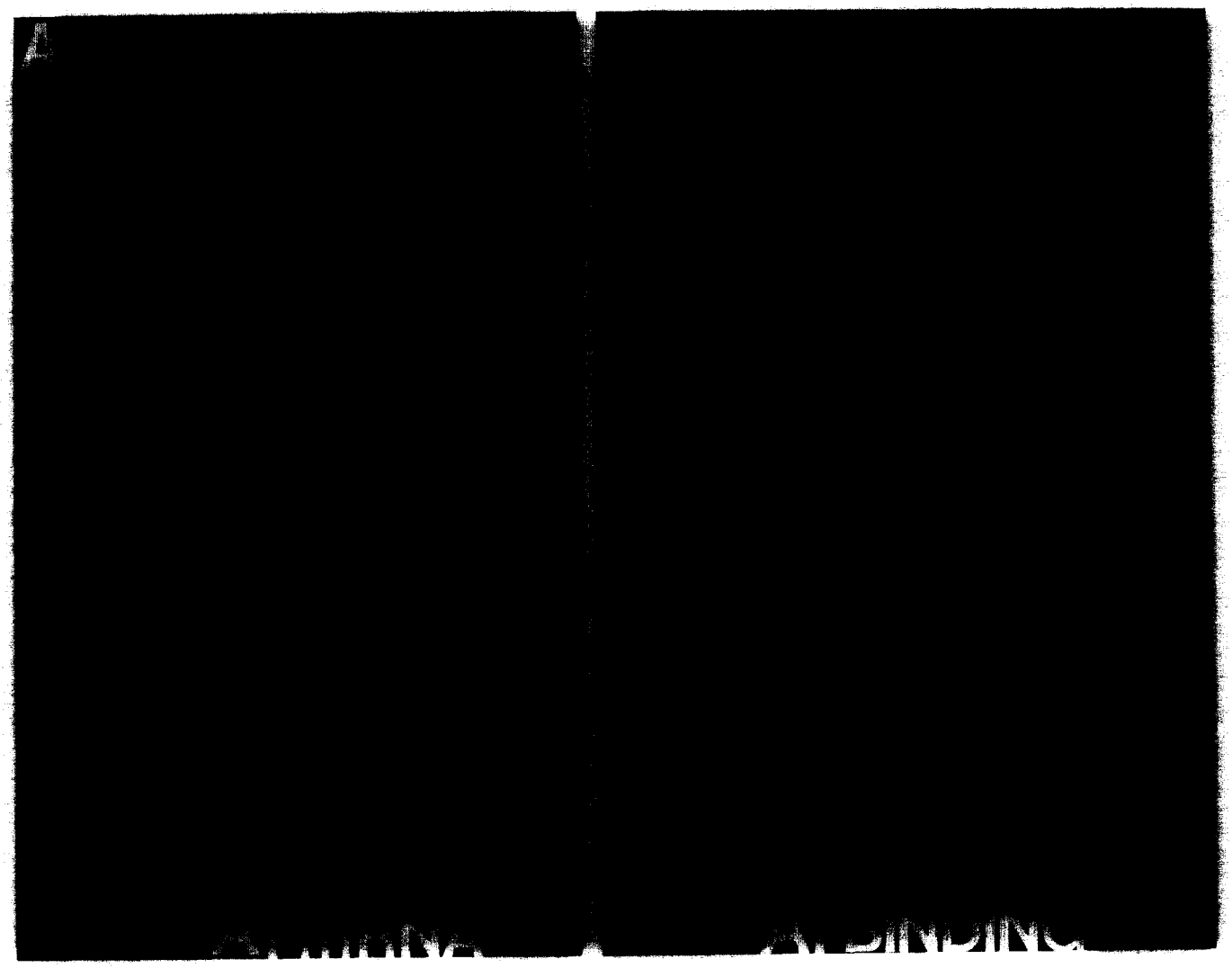

Fig. 5. Specific expression of gpA_1R mRNA in brain. Comparative in situ hybridization with gpA_1R cRNA probe (A) and autoradiographic localization of A_1 receptor sites (B) (antagonist [^{3}H]CPX, 2 nM) in horizontal sections of guinea pig brain. CTx, neocortex; CPu, caudate/putamen; $CA_{3/4}$, field CA_3 and CA_4 (Ammon's horn); CBL, cerebellum.

are reported to have high-affinity binding for A_1 receptors cloned from other species [14,15,17,26,35].

We first examined the possibility of whether the low-affinity binding was a result of technical problems. The quality of the tritiated ligands [^{3}H]CCPA and [^{3}H]CHA was confirmed in receptor-autoradiographic experiments using rat and guinea pig brains, where studies at low nanomolar concentrations of ligands indicated similar levels of agonist binding in the two species; in a side-by-side binding experiment using both guinea pig brain membrane and transfected COS cell membrane preparations, we actually obtained high affinities (K_d 0.67 nM for [^{3}H]CCPA, 13.84 nM for [^{3}H]CHA) with the brain membranes, which agreed with reported values in the literature, while still low affinities with transfected cell membranes. In addition, NEN confirmed the chemical purity (> 99%) of the ligands upon our request. We also demonstrated that the K_i values for unlabeled CCPA, CHA and other agonists obtained in competition studies agreed well with K_d values from the saturation binding studies utilizing the tritiated agonists. Therefore, we conclude that the low-affinity binding of CCPA, CHA and several other agonists is due to an intrinsic property of the cloned receptor (at least in our transient expression system) rather than the quality of the ligands we used.

This being the case, there are several possibilities to consider: (i) The gpA_1R may not have been properly coupled to the appropriate G protein in the transiently transfected host COS cells; (ii) gpA_1R may actually be the guinea pig counterpart of the same A_1 subtype cloned from other species, but the divergent residues confer to it a unique binding profile distinctive from others, which was previously unrecognized; (iii) gpA_1R may be a new subtype (low-affinity) of A_1 adenosine receptors, raising the possibility that there may exist another A_1 subtype in guinea pig with a profile identical to that of the high-affinity A_1 receptors cloned from other species. We shall discuss each of these possibilities in turn.

Is it possible that the expressed receptor may not couple to the right G protein, since the host cells are from different species? At least, it is not true for other cloned A_1 adenosine receptors. The A_1 receptors cloned from dog, rat, bovine and human [14,15,26,35], have been reported by other laboratories to have high affinity for A_1-agonists CCPA and CHA when expressed in monkey COS cells. In contrast, gpA_1R exhibited low-affinity binding for CCPA and CHA in COS cells, the affinities being about 1–2 orders of magnitude lower than those for other species. We have shown that A_1 agonists CPA and R-PIA increased GTPase activity and decreased forskolin-induced cAMP levels in gpA_1R-transfected COS-7 cells, suggesting that gpA_1R expressed in COS cells did, in fact, functionally couple to G protein(s). It should also be noted that in side-by-side experiments the same COS cells transfected with a guinea pig A_2 receptor cDNA, under the same conditions, have displayed very high affinity for A_2-selective agonists and appreciable affinities for A_1- and non-selective agonists [19]. Thus, it is unlikely that the observed low-affinity binding is due to the improper coupling of guinea pig A_1 receptor with monkey G protein in COS cells as compared to the natural coupling in guinea pig brain. A side-by-side binding comparison of this gpA_1R with an cloned A_1 receptor from other species, using the same expression vector and the same host cells under same conditions, is needed to further address this issue.

It is possible that gpA_1R represents the guinea pig counterpart of the main known A_1 adenosine receptor subtype described for other species, but with quite different properties. There has been evidence that same receptor subtypes from different species, can exhibit distinct pharmacological properties. In the case of serotonin receptors, human 5-HT_{1B} and rat 5-HT_{1B} are 93% identical, but their pharmacological profiles differ in terms of their binding for several key ligands. When residue Thr^{355} in the human 5-HT_{1B} receptor was changed by site-directed mutagenesis to Asn which was the corresponding residue in the rat 5-HT_{1B}, the mutated human 5-HT_{1B} displayed a pharmacological profile identical to that of the rat 5-HT_{1B} [23]. A similar situation may apply to the cloned guinea pig A_1 receptor. Although gpA_1R has > 90% amino acid sequence identity to other A_1 receptors from different species, it is possible that specific changes in key amino acids may cause substantial differences in interaction with the agonists tested.

However, it is also possible that another A_1 adenosine receptor subtypes exist in the guinea pig brain which would have the same property of those from other species, and that the cDNA we have identified codes for a novel receptor subtype with low affinity for agonists but high affinity for antagonists. Results from side-by-side experiments indicate that endogenous A_1 receptors with high affinity for CCPA and CHA do exist in guinea pig brain, as evidenced in the sub- and low-nanomolar binding of the same agonists in brain membrane preparations and in receptor autoradioagraphic studies. This possibility needs to be confirmed by molecular cloning.

If gpA_1R is a unique subtype, one would expect that it may exhibit a distinct distribution. However, this does not appear to be the case. In the autoradiographic study, we have observed that the high-affinity [^{3}H]CCPA and [^{3}H]CHA binding distribution were very similar to the high-affinity [^{3}H]CPX binding (except in the cerebellum). The binding of both agonists and antagonist, in turn, paralleled the in situ hybridization distribution revealed by using the gpA_1R probe. We do not know whether the high-affinity binding sites

are encoded by the same gpA_1R gene, or by a different gene with parallel expression. The possibility that two different receptor subtypes with a shared endogenous ligand may be co-localized is not without precedent. In the case of the melanocortin receptor family, several receptors have been cloned and their distributions in brain appeared identical as revealed by in situ hybridization [9].

Of the possibilities we have considered above, we tend to favor the idea that we have cloned a low-affinity A_1 receptor subtype, which may either exist uniquely in guinea pig, or may have counterparts in other species. However, we cannot eliminate other possibilities, such as the existence in the guinea pig brain of unique factors or modification mechanisms which may endow this receptor with a high affinity for agonists.

By Northern blot analysis, we have determined that gpA_1R mRNA is expressed in the brain, heart, kidney, and spleen. Compared to the expression level of the guinea pig A_2 adenosine receptor [19], the present results indicate that A_1 adenosine receptor mRNA is more abundant in guinea pig brain than is A_2 receptor mRNA [19]. In the periphery, adenosine, via stimulation of A_1 adenosine receptors, can regulate myocardial, renal, and immune functions [36]. In particular, the stimulation of A_1 adenosine receptors decreases heart rate and myocardial contractility [4]. In the kidney, adenosine can decrease cortical tubular production of cAMP and hormone-induced production of cAMP [1]. Evidence also indicates that adenosine is involved in the immune system, where activation of A_1 adenosine receptors promotes neutrophil chemotaxis in response to tissue injury and bacterial infection [3]. Presumably, adenosine receptors are present in spleen because older neutrophils and other lymphocytes are harvested by the spleen. Given these reported functions of adenosine, the peripheral distribution of gpA_1R mRNA is consistent with the idea that A_1 adenosine receptors may mediate some of the effects in the cardiovascular, renal, and immune systems, as well as the CNS.

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Abbreviations

ADAC	adenosine amine congener
BA	N^6-benzyladenosine
bp	nucleotide base pair
CCPA	2-chloro-N^6-cyclopentyladenosine
CGS 21680	2-p-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamido adenosine hydrochloride; CHA, N^6-cyclohexyladenosine; CPA, N^6-cyclopentyladenosine
CPX	8-cyclopentyl-1,3-dipropylxanthine
DME	Dulbecco's modified Eagle's medium
DMPX	3,7-dimethyl-1-propargylxanthine
DPMX	1,3-dipropyl-7-methylxanthine
DPX	1,3-dipropyl-8-(2-amono-4-chlorophenyl)-xanthine
FCS	fetal calf serum
G protein	guanine nucleotide-binding protein
IBMX	3-isobutyl-1-methylxanthine
IMDM	Iscove's modified Dulbecco's medium
KHB	Kreb-HEPES buffer
MECA	5′-*N*-methylcarboxamido-adenosine
NECA	5′-*N*-ethylcarboxamido-adenosine
D-PBS	Dulbecco's phosphate-buffered saline
R-PIA	R(−)-N^6-(2-phenyl-isopropyl)adenosine
SSPE	sodium chloride, sodium phosphate EDTA buffer
TM	transmembrane domain
XAC	xanthine amine congener

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