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Cloning and Expression of the A_{2a} Adenosine Receptor from Guinea Pig Brain*

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A full-length complementary DNA (cDNA) clone encoding the guinea pig brain A_2 adenosine receptor has been isolated by polymerase chain reaction (PCR) and low-stringency-hybridization screening of a guinea pig brain cDNA library. This cDNA contains a long open reading frame encoding a 409-amino acid-residue protein which is highly homologous to the A_2 adenosine receptors previously cloned from other species. Hydrophobicity analysis of the deduced protein sequence reveals seven hydrophobic regions, characteristic of a member of the G-protein-coupled receptor superfamily. Radioligand binding assay and functional (GTPase and cAMP) assays of the receptor, transiently expressed in mammalian cells, demonstrate typical characteristics of the A_2 type adenosine receptor. The messenger RNA (mRNA) of this A_2 receptor is found in the brain, heart, kidney and spleen. Receptor autoradiography with [^{3}H]CGS21680, a specific A_2 agonist, and in situ hybridization with A_2 cRNA probe in guinea pig brain indicate that the receptor is expressed exclusively in the caudate nucleus. The pharmacological profile and anatomical distribution of this receptor indicate that it is of the A_{2a} subtype. This work represents the first cloning of an A_{2a} receptor in a rodent species, offers a complete pharmacological characterization of the receptor and provides an anatomical comparison between binding profile and gene expression of the receptor.

KEY WORDS: Gs-coupled receptor; adenylyl cyclase; radioligand binding; mammalian cell expression; in situ hybridization; caudate/putamen.

INTRODUCTION

Adenosine, a member of the purinergic neurotransmitter system, mediates a variety of important physiological functions, such as sedation, pain modulation, presynaptic inhibition of neurotransmitter release, inhibition of cardiovascular activity, regulation of renal glomerular filtration, and regulation of immune system, white blood cell and platelet functions (24,40). Adenosine initiates

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Abbreviations: ADAC, adenosine amine congener; BA, N^6-benzyladenosine; bp, nucleotide base pair; cAMP, cyclic adenosine 3′,5′-monophosphate; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS 21680, 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamido adenosine hydrochloride; CHA, N^6-cyclohexyladenosine; CNS, central nervous system; 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; IBMX, 3-isobutyl-1-methylxanthine; KHB, Kreb-HEPES buffer; MECA, 5′-N-methylcarboxamidoadenosine; NECA, 5′-N-ethylcarboxamidoadenosine; D-PBS, Dulbecco's phosphate buffered saline; PCR, polymerase chain reaction; R-PIA, R(−)-N^6-(2-phenylisopropyl)adenosine; SSPE, sodium chloride-sodium phosphate-EDTA buffer; TM, transmembrane domain; XAC, xanthine amine congener.

these actions by binding to its specific receptors in the membranes of target cells. Adenosine receptors are pharmacologically classified into two main types, A_1 and A_2. The A_1 type adenosine receptor inhibits adenylyl cyclase and therefore decreases cAMP levels, while A_2 stimulates adenylyl cyclase (35). Species differences in high-affinity A_1 and A_2 binding have been reported based on the binding studies with mammalian brain membranes (6,36). A_1 (16,17,19,26,38) and A_2 (16,18,25,34) adenosine receptors have been cloned from several species. This has established a new subfamily within the superfamily of the G-protein-coupled receptors; new adenosine receptor subtypes (42), which had not been discerned by binding studies, have emerged from molecular cloning including the A_{2a} and A_{2b} subtypes. However, the A_{2a} subtype has only been cloned from dog and human, making it difficult to characterize its anatomy and pharmacology in smaller experimental animals. Here we report the cloning of a cDNA, named gpA_2R, encoding the A_{2a} adenosine receptor from guinea pig brain; we describe the pharmacological characterization of this receptor in transiently transfected cells, and its expression in guinea pig brain and other tissues.

EXPERIMENTAL PROCEDURE

Poly(A) RNA preparation and cDNA library construction. Total RNA was isolated from male Hartley guinea pig brains by guanidinium thiocyanate extraction followed by centrifugation in cesium chloride solution (28). Poly(A)$^+$ RNA was purified using an oligo-dT cellulose spun column (Pharmacia). The construction of cDNA library was principally based on the method developed by Okayama and Berg (23) and modified by Gubler and Hoffman (12). 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′ side, 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 and electrophoresed for size fractionation. The cDNAs with sizes > 1.5 kb were electroeluted from the gel to DE81 ion exchange paper (Whatman) and recovered by eluting with 1 M NaCl. After ethanol precipitation and washing, the cDNAs were unidirectionally inserted into the Bst XI-Not I sites of pME18S expression vector by T_4 ligase to construct the cDNA library. This size-fractionated (>1.5 kb) guinea pig brain cDNA library was composed of 1.8 × 10^6 independent colonies transformed into host $DH_{5\alpha}$ competent cells.

The pME18S vector(20), containing the Hae II-Aat-II fragment from pUC18, the stuffer region from plasmid CDM8 and the SR_α promoter (37), with stop codon in each frame immediately after the cDNA insert, is a high-copy numbered (~5–10 mg plasmid/L culture), small vector (3.4 kb) plasmid with a strong promoter, suitable for constructing size-selected, unidirectional cDNA libraries and for expression in different mammalian cell systems.

Oligonucleotide Primer Design and PCR Amplification. Six 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) CIC-TIGCITC(G)IGCI GA-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) IA-TIGCIGA-3′.

Three reversed:

(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 reversed oligonucleotides was employed as one set of primers for polymerase chain reaction (PCR) (27).

Taq polymerase and all other PCR reagents were purchased from Perkin Elmer. Guinea pig brain cDNAs were used as PCR templates. Five fmol of DNA templates, 1 μM of each primer, 200 μM of each dNTP and 5 units of Taq Polymerase were used in each 100 μl-volume reaction. The PCR temperature cycle on the DNA Tempcycler (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 have an extension time of 10 min at 72°C in each cycle.

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

Colony Hybridization and DNA Sequencing. About 3 × 10^5 E.coli colonies from the guinea pig brain cDNA library were lifted onto Nytran filters (S&S). PCR product probe was labeled with ^{32}P by the random primer method (5). The conditions for 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 GCG programs (Wisconcin).

Mammalian Cell Transfection and Receptor Binding Assay. Simian COS-7 cells were transfected with CsCl-gradient-centrifugation-purified plasmids by electroporation (22). Exponentially growing COS-7 cells were plated (5 × 10^5 cells/10 cm dish) in DME medium with 10% FCS and cultured in a 37°C, 5% CO_2 incubator. After 24h, cells were harvested and washed twice with D-PBS, then resuspended in 1 ml D-PBS containing 10 μg of plasmid DNA in a sterile electroporation cuvette. After sitting on ice for 5 min, the cells were electroporated (330 μF, 300 V, 4° C) in a Cell-Porator (BRL), then sat on ice for 5 min, followed by suspension in 10 ml DMEM containing 10% FCS in a cell culture dish and incubated at 37°C, 5% CO_2

After three days of culturing, transfected cells were harvested by treatment with D-PBS (without calcium and magnesium) containing

0.5 mM EDTA, 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 1×10^6 cells/ml in KHB. Receptor binding assays were performed in 1 ml KHB with 1×10^5 cells, competitors and radiolabeled ligands. In order to label different types of adenosine receptors, tritiated A_1 agonists 2-chloro-N^6-[cyclopentyl-2,3,4,5-^{3}H]-Cyclopentyladenosine ([^{3}H]CCPA, specific activity: 35.4 Ci/mmole, NEN) and Cyclohexyladenosine ([^{3}H]CHA, 30.2 Ci/mmole, NEN), tritiated A_1 antagonist 8-[dipropyl-2,8-^{3}H(N)]Cyclopentyl-1,3-dipropylxant hine ([^{3}H]CPX, 108.0 Ci/mmole, NEN), and tritiated A_2 agonist [carboxyethyl-^{3}H(N)]-CGS 21680 (39.6 Ci/mmole, NEN) were used. Unlabeled ligands (all from Research Biochemicals Incorporated, MA) used for competition were: the A_1 selective agonists CCPA, CPA and CHA, 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 selective A_2 agonist CGS 21680 HCl, the relatively A_2 selective agonist MECA, the relatively selective A_2 antagonists DMPX and DPMX, and the non-selective agonist NECA, as well as adenosine and ATP. For saturation studies, various concentrations of radiolabeled ligands were used, nonspecific binding was determined in the presence of 20 μM unlabeled R-PIA for A_1 and 20 μM unlabeled CGS 21680 HCl for A_2; in a parallel experiment, 5 units/ml of adenosine deaminase was included in the binding assay buffer to determine the influence of endogenously released adenosine in this binding system. For competition studies, minimum amounts of radiolabeled ligands were used to give about 3000 cpm specific binding per tube, and various concentrations of unlabeled ligands were used as competitors. The assay reaction mixtures were incubated at room temperature for 1.5 h and stopped by sitting on ice for 5 min. Cells bound with radioligands and free ligands were separated by filtering through glass fiber filters (#32, S & S) on a multi-channel cell harvester (M-24S, Brandel). The filter membranes were counted for radioactivity in scintillation solution. Binding assay data were presented as the mean value of three experiments, and analyzed by the Ligand program (21).

GTPase Assay. GTPase assays (2) were performed with transiently transfected COS-7 cells. Briefly, plasmid pME18S-gpA_2R 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 mg of membrane protein in a 10 μl volume was added to 85 μl reaction mixture containing 0.5 μM [γ-^{32}P]GTP, 1 mM AppNHp, 1 mM ATP, 1 mM Ouabain, 10 mM creatine phosphate, 5 units of creatine phosphokinase, 2 mM dithiothteitol, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA, 12.5 mM Tris-HCl, pH 7.5, then placed on ice before the addition of adenosine receptor agonists. Immediately after the addition of A_1 agonist CPA (final concentration: 10 μM and 1 μM) or A_2 agonist CGS 21680 (final concentration: 10 μM and 1 μM), the reaction tubes were transferred to 37°C for 20 min. The reaction was terminated on ice with the addition of 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 radioactivity was measured from the supernatants.

Cyclic AMP Assay. COS-7 cells transfected with plasmid pME18S-gpA_2R were cultured in DMEM, 10%FCS for two days, washed twice with IMDM, and then cultured in IMDM, 10% Hyclone FCS. Different concentration of the A_1 agonist CPA, the A_2 agonist CGS 21680, or the A2 antagonist DMPX 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* at 4° C for 10 min, the supernatant was ether extracted, lyophilized on 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).

Northern Blot Analysis. All the Northern and in situ hybridizations were conducted with ^{32}P or ^{35}S-labeled cRNA riboprobes. A_2 cRNA riboprobe was produced from a 900bp Pst I fragment of the gpA_2R cDNA ligated into pBluescript KS II(−) (Stratagene). [^{32}P]UTP or [^{35}S]UTP-labeled Riboprobes were produced using either T3 or T7 transcription systems in a standard labeling reaction mixture. The reaction was incubated at 37°C for 90 minutes, labelled probe being separated from free nucleotides over a Sephadex G-50 column.

Total RNA was extracted from brain and peripheral tissues of a male Hartley guinea pig using GITC and LiCl. The RNA samples were fractionated on a 1% agarose-formaldehyde gel, electrophoresed and then transferred onto a nylon filter (Nytran). The filter was hybridized with 5 X 10^5 cpm/ml ^{32}P-labeled RNA probe from gpA_2R under high stringency conditions (50% Formamide, 5 X SSPE, 5 X Denhardt's reagent, 0.5% SDS, 70°C, 24 h). The filter was washed once with 1 X SSPE, 0.5% SDS at 70°C for 30 min and twice with 0.1 X SSPE, 0.5% SDS at 70°C for 30 min. and then exposed to an x-ray film (Kodak) with two intensifying screens for 7 days at −80°C.

In Situ Hybridization. Guinea pig brain sections were removed from storage at −80°C and placed directly into 4% buffered paraformaldehyde at room temperature. After 60 minutes, slides were rinsed in isotonic phosphate buffered saline (10 minutes) and treated with proteinase K (1 μg/ml in 100 mM Tris/HCL, pH8.0) for 10 minutes at 37°C. Subsequently, sections underwent successive washes in water (1 minute), 0.1 M triethanolamine (pH 8.0, plus 0.25% acetic anhydride) for 10 minutes and 2X SSC (0.3 mM NaCl, 0.03 mM sodium citrate, pH 7.2) for 5 minutes. Sections were then dehydrated through graded alcohols and air dried.

Post-fixed sections were hybridized with 1.0×10^6 dpm [^{35}S]UTP-labeled riboprobes in hybridization buffer containing 75% formamide, 10% dextran sulphate, 3X SSC, 50 mM sodium phosphate buffer (pH7.4), 1X Denhardts solution, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol in a total volume of 25 ml. The diluted probe was applied to sections on a glass coverslip which was sealed into place with rubber cement. Sections were hybridized overnight at 55°C in a humid environment. A_2 probe specificity was confirmed by absence of signal in both sections labeled with sense A_2 probe and sections pre-treated with RNase prior to hybridization with antisense (cRNA) A_2 probe.

Post-hybridization the rubber cement was removed and sections were washed in 2X SSC for 5 minutes and then treated with RNase A (200 μg/ml in 10mM Tris/HCL, pH8.0, containing 0.5M NaCl) for 60 minutes at 37°C. Subsequently, sections were washed in 2X SSC for 5 minutes, 1 × SSC for 5 minutes, 0.5 × SSC for 60 minutes at hybridization temperature, 0.5 × SSC at room temperature for 5 minutes and then dehydrated in graded alcohols and air dried. For signal detection, sections were placed on Kodak XAR-5 X-ray film and exposed for 2 days at room temperature.

In Vitro Receptor Autoradiography. [^{3}H]CGS 21860 binding was performed according to previously published autoradiographic methods (4). Slide-mounted tissue sections were preincubated in 0.17 M Tris/HCl, pH 7.4, for 20 minutes at room temperature. Subsequently, sections were incubated with 10 nM [^{3}H]CGS 21860 (+0.5 i.u./ml adenosine deaminase) for 90 minutes at room temperature. Post-incubation, slides were washed in incubation buffer (2 × 5 minutes) and dried in a stream of cold air. Non-specific binding was determined in the presence of 10 μM adenosine. Sections were apposed to tritium-sensitive Hyperfilm (Amersham) and exposed at room temperature for 14 days.

RESULTS

Cloning of gpA_2R cDNA. PCR using guinea pig brain cDNA as templates amplified several bands with sizes compatible with the G-protein-coupled receptor fragments flanked by the corresponding primers. Sequence analysis of these PCR fragments revealed different sequences with various homologies (from 62%–88%) to cDNA sequences encoding dog and rat A_1 and A_2 adenosine receptors. The mixture of these PCR products was directly labeled with ^{32}P and used as probe to screen guinea pig brain pME18S cDNA library by low-stringency hybridization to get full-length clones. Twenty-one positive clones were obtained in 3×10^5 colonies screened. One of these full-length clones, gpA_2R, showed highest homology to A_2 adenosine receptors.

Sequence Analysis of gpA_2R. Clone gpA_2R contained an insert of 2,085 base pairs (bp) followed by a poly(A) tail. A long open reading frame was found after an ATG (Methionine) at position 16–18. The sequences around this ATG met the Kozak consensus sequence criteria for a translation initiation site (14). This open reading frame encodes a protein with 409 amino acids (including the first methionine) (Fig. 1) and a calculated molecular mass of 44,831 Daltons.

Genebank search showed that the deduced amino acid sequence of gpA_2R is highly homologous to the previously cloned A_2 adenosine receptors from other species: 88% identity with dog A_{2a} receptor, 87% with human A_{2a}, 61% identity with rat A_{2b}, and 58% with human A_{2b}. The protein sequence of gpA_2R also shares 51% identity with dog, rat and bovine A_1 adenosine receptors. Its similarities with other members of the G-protein coupled receptor superfamily are lower but significant (for example, in transmembrane regions, 34% identity with rat 5-HT_{1A} receptor, 30% with human and rat α_1-adrenergic receptor, 29% with human histamine H_2 receptor, and 29% with human D_2 dopamine receptor).

Computer-aided hydrophobicity analysis (method of Kyte & Doolittle) (15) of gpA_2R revealed that it contains seven hydrophobic segments, each consisting of about 23–27 amino acid residues, long enough to form a α-helix to span the cell membrane. These hydrophobic regions possess the highest similarities to many other G-protein-coupled receptors. Several features of the amino-acid sequence of gpA_2R are noteworthy, and are also conserved in all other cloned A_2 receptors: The N-terminal hydrophilic region before the TM I is very short (4 residues), and there is no potential N-glycosylation site on this N-terminal extracellular end; two potential N-glycosylation sites can be found in the second extracellular loop; there are 18 serine and threonine residues after TM VII, which may participate in the mechanism of receptor phosphorylation. The deduced protein sequence of gpA_2R does not have a signal sequence on its N-terminus.

Receptor Binding Assay on Transfected COS-7 Cells. Simian COS-7 cells, which endogenously expressed neither gpA_1R or gpA_2R positively-hybridizing mRNAs nor detectable [^{3}H] CPX or [^{3}H]CGS 21680 binding sites, were transfected with plasmid gpA_2R. Binding assays (both saturation and competition studies) were performed on whole cells.

Cells transfected with plasmid gpA_2R expressed saturable, high affinity binding sites for selective A_2 agonist [^{3}H]CGS 21680 (K_d = 5.7 nM), specific binding was about 75–80% of total binding (Fig. 2A). A_1-selective agonists [^{3}H]CCPA, [^{3}H]CHA and antagonist [^{3}H]CPX showed very little binding to gpA_2R (data not shown). Binding assays performed with cell membrane preparations gave similar results.

In competition studies, the selective A_2 agonist CGS21680 competed well with [^{3}H]CGS21680 binding on gpA_2R transfected COS-7 cells, the non-selective adenosine agonist NECA and antagonist XAC were also good competitors, while the relatively selective A_2 agonist MECA and the A_2 antagonists DMPX and DPMX exhibited moderate competitions. In contrast, the selective A_1 agonists CCPA, CHA and the A_1 antagonist CPX competed very poorly. ATP did not compete [^{3}H]CGS21680 binding. The rank order of these ligands to compete with [^{3}H]CGS21680 binding on gpA_2R transfected cells was as follows: CGS21680 >> XAC = NECA >> adenosine = MECA = DMPX = DPMX > R-PIA = CPA >CHA > CPX >> ATP (Fig. 2B).

The Effects of A_2 Ligands on GTPase Activity and cAMP Level in gpA_2R Transfected COS Cells. The A_2 agonist CGS 21680 stimulated GTPase activity up to 37% above control level and increased cAMP level by two-fold in gpA_2R transfected cells. These effects could be partially blocked by the A_2 antagonist DMPX. A_1 agonists had very little effect on GTPase activity and cAMP levels in the same transfectants (Table I).

Northern Hybridization in Different Guinea Pig Tissues. Northern blot analysis of total RNAs from 8 tissues of the guinea pig probed with gpA_2R RNA probe showed a single hybridized transcript of approximately 3 kb in brain, heart, spleen and kidney (Fig. 3).

In Situ Hybridization. Within the brain, gpA_2R mRNA appeared to be exclusively localized in the caudate/putamen (Fig. 4). After a two-week exposure time,

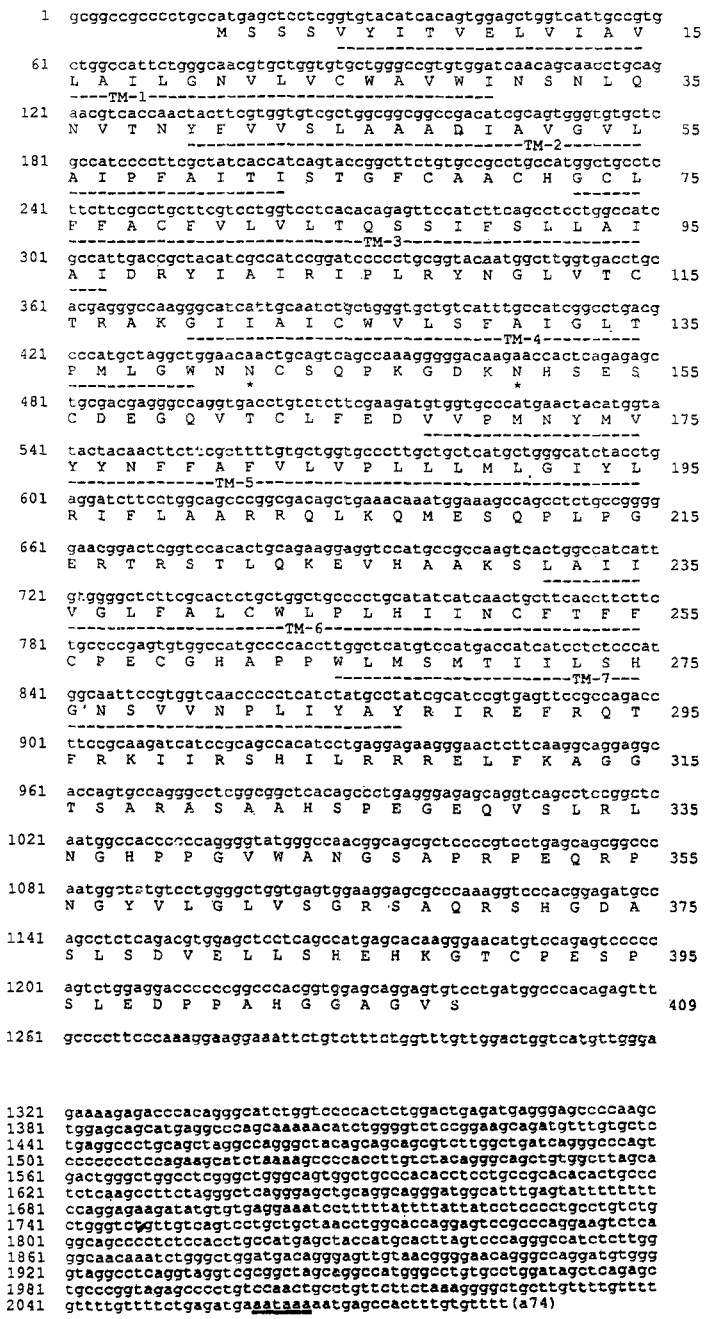

Fig. 1. Nucleotide and deduced amino acid sequence of gpA_2R.
Nucleotides are numbered on the left of the rows and amino acids on the right. Possible N-glycosylation sites (*) are marked and polyadenylation signal sequence underlined. Putative hydrophobic transmembrane domains TM-1 through TM-7 are underlined with broken lines. The sequence has been deposited to GenBank data base (accession no. U04201).

the level of gpA_2R mRNA expression in the caudate was 8-fold greater (as measured by mean O.D.) than that in adjacent cortical areas. The complementary distribution of A_2 receptor binding sites in this region indicates that A_2 receptors are intrinsically localized within the striatum. A_2 mRNA was undetectable within the cerebral cortex, hippocampus, substantia nigra and cerebellum (Fig. 4).

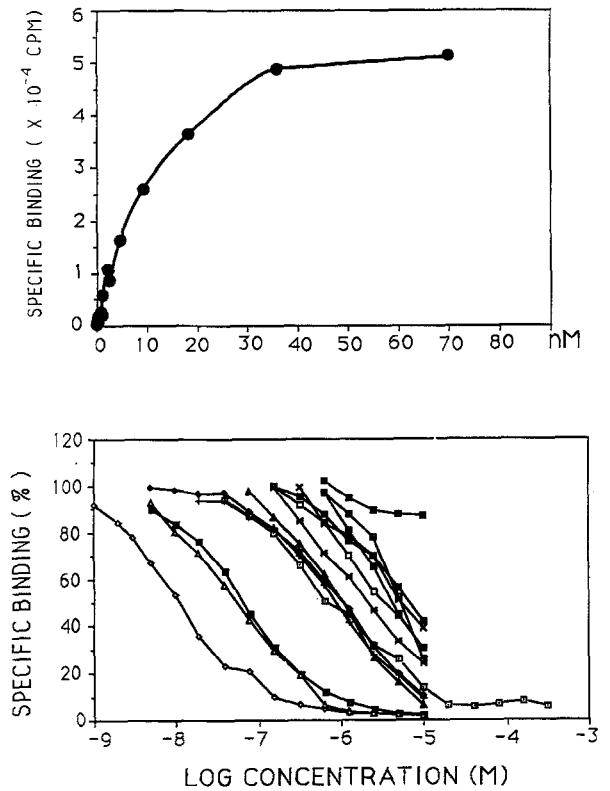

Fig. 2. Radio-ligand receptor binding assay on gpA2R transfected COS-7 cells. (A) Saturation curve of [^{3}H] CGS21680 specific binding. Twenty micromoler of unlabeled CGS21680 are used as total dispacement. (B) Competition of [^{3}H]CGS21680 specific binding by CGS21680 (◇), XAC (△), NECA (■), MECA (+), adenosine (▲), IBMX (⊡), R-PIA (♦), DMPX (*), DPMX (□), CHA (⊠), CPA (X), CCPA (⊞), CPX (⊡), ATP (■).

DISCUSSION

A_2 adenosine receptors, which positively activate adenylyl cyclase through Gs-protein, have been proposed to have A_{2a} and A_{2b} subtypes (1). A_{2a} receptors have high-affinity binding for the specific A_2 agonist CGS21680 and the endogenous ligand adenosine, while A_{2b} bind CGS21680 and adenosine with considerably lower affinity (25,36). The anatomical distributions of the two subtypes of A_2 receptors in the CNS are also very different: A_{2a} receptors are located within the striatum, while A_{2b} receptors are not found in the brain proper but only found in the hypophyseal pars tuberalis of the pituitary (34).

The cloned guinea pig brain cDNA, gpA_2R, exhibits highest homologies to the dog (88%) and human (87%) A_{2a} adenosine receptors; its degrees of homology are lower to the A_{2b} and A_1 adenosine receptors (approximately 60% to A_{2b} and 50% to A_1). The expressed gpA_2R has high affinity binding (5.7 nM) for the A_2 selective agonist CGS21680. Stimulation of cells expressing gpA_2R by CGS21680 increases the cellular cAMP level. The gpA_2R mRNA is located in the striatum of the brain. Therefore, this cloned gpA_2R is likely to encode an A_{2a} subtype adenosine receptor. As of this writing, only the A_{2a} receptors from dog and human have been cloned. Thus the guinea pig A_{2a} receptor represents the first A_{2a} subtype to be cloned from a rodent species.

All the existing adenosine agonists and antagonists were developed, tested and defined with mammalian brain membrane preparations or whole animals prior to the molecular cloning of the adenosine receptors. Obviously, these systems contain a mixture of adenosine receptors (A_1, A_2 and other subtypes). The expression of a pure population of a single receptor subtype from cloned cDNA has proved to be an invaluable system for evaluating pharmacological ligands. With transiently expressed guinea pig A_1 (data not shown) and A_{2a} adenosine receptor, we have tested a dozen adenosine ligands and obtained their ligand selectivity profiles (LSP) and their receptor binding site signatures (BSS) (10). Clearly, CGS21680 displayed highly selective, high-affinity binding to gpA_2R. NECA and MECA bound gpA_2R with moderate affinities and poor selectivities. Antagonists DMPX and DPMX showed some selectivity towards A_2

Table I. GTPase and cAMP Assay on gpA_2R Transfected COS-7 Cells

	Control	CGS21680 (μM) 10	1	0.1	CGS21680 (10 μM) + DPMX (10 μM)	CPA (10 μM)
GTPase activity (%)	100±5	137±6*	128±5*	109±5	113±7	107±4
Cyclic AMP level (%)	100±6	194±7**	172±4**	138±3*	144±5*	128±3*

Data are presented as Mean ± SE of triplicates. In control group, GTPase activity and cAMP level of transfected COS-7 cells were measured without the application of ligands. *$P<0.05$, **$P<0.01$ as compared with control (Student t test).

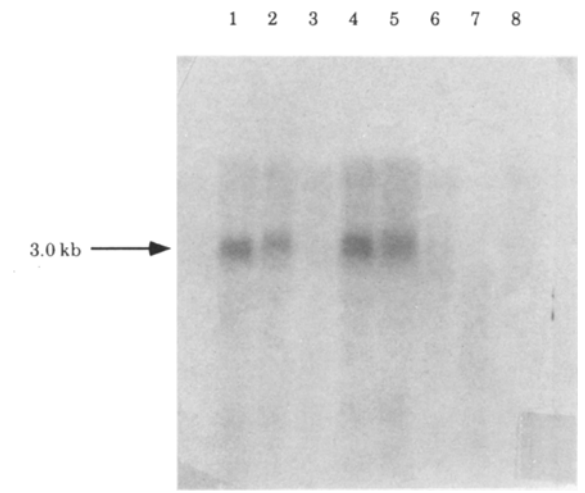

Fig. 3. Northern hybridization of RNAs from different guinea pig tissues. Guinea pig total RNAs from (1) brain, (2) heart, (3) lung, (4) spleen, (5) kidney, (6) liver, (7) stomach, and (8) intestine were probed with gpA_2R riboprobe (9.0×10^6 cpm/ 20 ml) and exposed for 6 days at −80 °C with two intensifying screens. The molecular weight was based on a RNA ladder (BRL) and a Ribosomal RNA marker (Pharmacia).

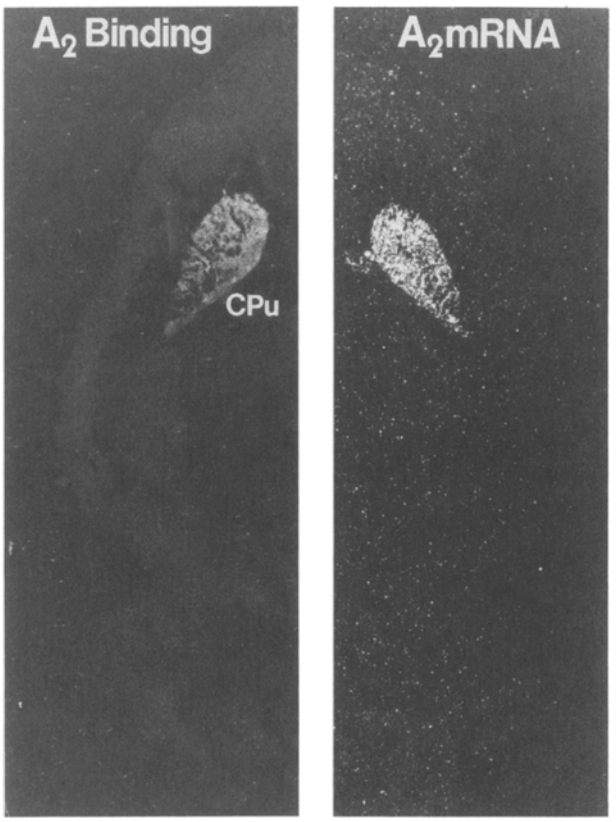

Fig. 4. Comparative autoradiographic localization of A_2 receptor sites (left hemisphere) and gpA_2R mRNA (right hemisphere) in horizontal sections of guinea pig brain. CPu, caudate/putamen.

but their affinities were also moderate. The endogenous ligand adenosine bound gpA_2R as well as A_1 adenosine receptor with an affinity of about 20 nM.

In situ hybridization studies demonstrated that the A_{2a} receptor mRNA is exclusively expressed in the caudate. The complementary distribution of adenosine A_{2a} receptor binding in this region indicates that locally synthesised A_{2a} receptors are present on striatal neurons. Most striatal neurons utilize GABA as a neurotransmitter and can be divided into two sub-populations on the basis of their efferent projections; striatopallidal neurons and striatonigral neurons (9). Striatopallidal neurons have been shown to express enkephalin while striatonigral cells coexpress dynorphin and substance P (9,11). A_{2a} receptor mRNA has been shown to be co-localized with enkephalin in striatal cells but not with substance P (31). Thus, striatal A_{2a} receptors are likely to be more important in relation to striatopallidal circuitry as opposed to striatonigral. Such differentiation has important implications for the functionality of striatal A_{2a} receptors and their involvement in the control of locomotor activity. Indeed, stimulation of these sites may underlie the locomotor depressing effects of adenosine analogs (7). In addition, the A_{2a} receptor appears to be co-localized specifically with dopamine D_2 receptors in the caudate (32,33). Further understanding of the interactions between dopaminergic nigrostriatal inputs and the actions of striatal adenosine are likely to be crucial to our understanding of basal ganglia physiology and pathology.

In addition to its role as a neurotransmitter in the CNS, adenosine acts in an autocrine or paracrine fashion to regulate mammalian cardiovascular, renal, and immune systems (40). The activation of adenosine receptors in the heart increases coronary blood flow via vasodilation of coronary arteries, decreases heart rate and contractility. The increase in coronary blood flow is thought to be mediated by A_2 adenosine receptors (39). In the kidney, adenosine can decrease glomerular filtration rate, stimulate renin release, and increase sodium excretion. The latter function is thought to be mediated by A_2 adenosine receptors (8,13). Evidence also indicates that adenosine is involved in the immune system. While adenosine inhibits the production and stimulation

of lymphocytes (29), the activation of A_2 receptors protects endothelial cells from injury by inhibiting neutrophil adherence (3).

By Northern blot analysis, we have determined that gpA_2R mRNA is found in the brain, kidney, and spleen. Given the reported functions of adenosine, the peripheral distribution of gpA_2R mRNA is consistent with the idea that A_2 type adenosine receptors may mediate some of the effects of adenosine in the cardiovascular, renal, and immune systems, as well as the CNS.

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