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ACCELERATED COMMUNICATION

Cloning and Expression of an A_1 Adenosine Receptor from Rat Brain

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SUMMARY

We have used the polymerase chain reaction technique to selectively amplify guanine nucleotide-binding regulatory protein (G protein)-coupled receptor cDNA sequences from rat striatal mRNA, using sets of highly degenerate primers derived from transmembrane sequences of previously cloned G protein-coupled receptors. A novel cDNA fragment was identified, which exhibits considerable homology to various members of the G protein-coupled receptor family. This fragment was used to isolate a full-length cDNA from a rat striatal library. A 2.2-kilobase clone was obtained that encodes a protein of 326 amino acids with seven transmembrane domains, as predicted by hydropathy analysis. Stably transfected mouse A9-L cells and Chinese hamster ovary cells that expressed mRNA for this clone were screened with putative receptor ligands. Saturable and specific binding sites for the A_1 adenosine antagonist [^{3}H]-1,3-dipropyl-8-cyclopentylxanthine were identified on membranes from transfected cells. The rank order of potency and affinities of various adenosine agonist and antagonist ligands confirmed the identity of this cDNA clone as an A_1 adenosine receptor. The high affinity binding of A_1 adenosine agonists was shown to be sensitive to the nonhydrolyzable GTP analog guanylyl-5′-imidodiphosphate. In adenylyl cyclase assays, adenosine agonists inhibited forskolin-stimulated cAMP production by >50%, in a pharmacologically specific fashion. Northern blot and *in situ* hybridization analyses of receptor mRNA in brain tissues revealed two transcripts of 5.6 and 3.1 kilobases, both of which were abundant in cortex, cerebellum, hippocampus, and thalamus, with lower levels in olfactory bulb, striatum, mesencephalon, and retina. These regional distribution data are in good agreement with previous receptor autoradiographic studies involving the A_1 adenosine receptor. We conclude that we have cloned a cDNA encoding an A_1 adenosine receptor linked to the inhibition of adenylyl cyclase activity.

Adenosine is a ubiquitous modulator of numerous physiological activities, particularly within the cardiovascular and nervous systems. The effects of adenosine appear to be mediated by specific membrane-bound receptor proteins. Biochemical and pharmacological criteria have been used to divide adenosine receptors into two major subtypes, referred to as A_1 and A_2, which either inhibit or stimulate, respectively, the enzyme adenylyl cyclase (1, 2). These receptors have been directly labeled using radioligand binding methods in various tissues, and much information has been generated concerning their regulation and ligand-binding characteristics (1–3).

Recently, substantial progress has also been made in delineating the biochemical properties of adenosine receptors, particularly of the A_1 subtype. The ligand-binding subunit of this receptor has been directly visualized utilizing photoaffinity labeling techniques, and its glycoprotein characteristics have been investigated (4–7). The A_1 receptor has also recently been purified to homogeneity from rat (8) and bovine (9) brains, as well as from rat testes (10). These studies have indicated that the A_1 receptor protein from various tissues is a single subunit glycoprotein with a molecular mass of approximately 36,000 Da. Interestingly, using some purification procedures, the A_1 receptor appears to co-purify with a G protein (11, 12), which may couple this receptor to its signal transduction pathway.

As part of an ongoing effort in our laboratories to isolate and clone G protein-linked receptors, we have recently isolated a cDNA, from a rat striatal library, that encodes a homolog of

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ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; CCPA, 2-chloro-N^6-cyclopentyladenosine; XAC, xanthine amine congener; PIA, N^6-phenyl-2-propyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; CV1808, 2-phenylaminoadenosine; CGS21680, (2-*p*-carboxyethyl)phenylamino-5′-*N*-carboxamidoadenosine; CHA, N^6-cyclohexyladenosine; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; Gpp(NH)p, guanylyl-5′-imidodiphosphate; kb, kilobases.

the RDC7 cDNA clone (13, 14). RDC7 is a cDNA that was isolated from a dog thyroid library and apparently encodes a G protein-linked receptor of unknown function (13, 14). In the present communication, we now demonstrate that the rat cDNA homolog of RDC7 encodes a pharmacologically specific and functional A_1 adenosine receptor protein.

Experimental Procedures

PCR amplification. Poly(A)$^+$ RNA was prepared from rat striatum and fractionated according to size by sucrose gradient centrifugation, as previously described (15). RNA of ~3 kb was used as a template for first-strand synthesis of cDNA. RNA (1–2 μg) was denatured at 65° for 5 min, and reverse transcription was performed at 39–40° in PCR buffer (GeneAmp; Perkin Elmer-Cetus) with avian myeloblastosis virus reverse transcriptase (Promega) and a 1.2 μM concentration of a 64-fold degenerate consensus primer to the sixth transmembrane region of G protein-coupled receptors that was identical to that described previously (13). Second-strand synthesis and subsequent amplification were performed by the addition of a 1.2 μM concentration of a 256-fold degenerate consensus primer to the third transmembrane region that was identical to that previously described (13), plus *Thermoactinomyces aquaticus* polymerase (Perkin Elmer-Cetus). Primers contained *Sal*I and *Hind*III linker sites to facilitate subcloning. Thirty cycles of 93° for 1.5 min (denaturation), 55° for 2 min (annealing), and 72° for 4 min (extension) were carried out, with a final extension for 15 min at 72°, in a 100-μl final volume. Products were analyzed and purified by electrophoresis in 1% LMP-agarose (FMC Bioproducts). Individual bands were excised, extracted with phenol, precipitated with ethanol, digested with *Sal*I and *Hind*III (New England Biolabs), and subcloned into pGEM 11Zf(+) (Promega), followed by transformation into *Escherichia coli* strain JM109. Plasmid preparations were prepared for double-stranded DNA sequencing of individual inserts using SP6 and T7 primers, as described below.

cDNA library screening and DNA sequencing. Recombinants (1×10^6) from a rat striatal cDNA library, constructed in the λ ZAP II vector (Stratagene), were screened with a PCR fragment that was ^{32}P-labeled via nick translation. Duplicate nitrocellulose filters were hybridized in 50% formamide, 0.75 M NaCl/0.075 M sodium citrate (5× standard saline citrate), 5× Denhardt's solution (1 mg/ml Ficoll (type 400), 1 mg/ml poly vinylpyrrlidone, 1 mg/ml bovine serum albumin in H_2O), 0.02 M Na_2HPO_4, 0.25% sodium dodecyl sulfate, 0.15 mg/ml salmon sperm DNA, with 4×10^6 dpm/ml ^{32}P-labeled probe, for 24 hr at 37°. High stringency washing of the filters was performed with 1× standard saline citrate, 0.1% sodium dodecyl sulfate, at 65° before autoradiography. λ Phages found to hybridize to the probe were subsequently plaque purified. *In vivo* excision and rescue of the nested pBluescript plasmids from the λ ZAP II clones were performed using helper phage, according to the Stratagene protocol. Nucleotide sequence analysis was performed, using the Sanger dideoxy nucleotide chain termination method, with Sequenase (US Biochemical Corp.), on denatured doubled-stranded plasmid templates. Primers were synthetic oligonucleotides that were either vector specific or derived from prior sequence information. Sequence analysis and comparisons were performed with GCG sequence analysis software (University of Wisconsin) and Genbank.

mRNA analysis. Northern blot and *in situ* hybridization histochemical analyses were performed as previously described (16). A 48-base oligodeoxynucleotide probe, 5′-CCCGTAGTACTTCTGGGGGT-CACCGGAGGAGGCTGACACCTTTTTTGTT-3′, was synthesized from sequence specific to the putative third intracellular loop. This probe was radiolabeled using terminal deoxynucleotidylransferase (Boehringer Mannheim), with either [α-^{32}P]ATP (Northern blots) or α-^{35}S-ATP (*in situ* hybridization). Hybridization reactions were carried out on 2-μg samples of poly(A)$^+$ RNA or serial 12-μm sagittal and coronal sections of adult rat brain. RNA markers (0.24–9.5 kb) were purchased from Bethesda Research Laboratories.

Expression and transfection. A full-length cDNA insert was subcloned into the pCD-SRα expression vector (17) containing a modified polylinker. Competent DH5α cells were transformed, and clones containing the appropriate cDNA insert were used for large-scale plasmid preparations via the $CsCl_2$ gradient purification method. DNA from the resulting plasmid construct (30 μg), along with 3 μg of pMAMneo (Clontech) for a selectable marker, was used to transfect CHO and A9-L cells by the $CaPO_4$ precipitation technique (18). Cells were cultured in Dulbecco's modified Eagle's medium containing high glucose (4500 mg/liter); 1 mM sodium pyruvate, and 10% fetal bovine serum, in a humidified atmosphere of 5% CO_2 in air at 37°. Selection with the neomycin analog G-418 (500 μg/ml) was started 72 hr after transfection and continued for up to 2 weeks. Cell colonies exhibiting stable transfection of the pCD-SRα-cDNA construct were identified via dot blot hybridization of cellular RNA, as described above for Northern blot analysis. Cell culture media, reagents, and fetal bovine serum were obtained from GIBCO Laboratories (Grand Island, NY).

Membrane preparation. Cells were detached from 150-cm^2 flasks with 1 mM EDTA in Ca^{2+}/Mg^{2+}-free Earle's balanced salt solution and were washed by centrifugation at 300 × *g* and resuspension in cold Earle's balanced salt solution (complete). The cells were then suspended in ice-cold lysis buffer (5 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$), transferred to a Dounce homogenizer on ice, and homogenized using 10 strokes with an A pestle. The homogenate was suspended in 50 mM Tris·HCl, pH 7.4, 1 mM EDTA, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 120 mM NaCl, and centrifuged for 10 min at 43,500 × *g*. The crude membrane pellet was resuspended in 50 mM Tris·HCl, pH 7.2, 1 mM EDTA, 10 mM $MgCl_2$, containing 2 units/ml adenosine deaminase (Sigma, St. Louis, MO), and incubated for 30 min at 30°. The membrane homogenate was subsequently centrifuged for 10 min at 43,500 × *g*, and the resulting membrane pellet was resuspended in the appropriate assay buffer. Protein concentrations were determined using the bicinchoninic acid protein reagent (Pierce, Rockford, IL), as described (19).

Radioligand binding assays. A_1 receptor binding assays using [^{3}H]DPCPX (96 Ci/mmol; Amersham Corp.) were performed as previously described (8, 10). Briefly, the membrane preparation was suspended with the appropriate ligands in 40 mM Tris-acetate, pH 7.5, 0.8 mM EDTA, 4 mM $MgCl_2$, for a final assay concentration of 150 μg of protein/ml. Nonspecific binding was determined in the presence of 1 μM DPCPX. Incubations were carried out at 30° for 1 hr and then terminated by rapid filtration, under vacuum, through Whatman GF/B filters that had been pretreated with 0.3% polyethyleneimine. The filters were washed with 5 × 4 ml of ice-cold 50 mM Tris·HCl (pH 7.4), and the retained radioactivity was quantitated by liquid scintillation counting in 5 ml of Aquasol (National Diagnostics, Palmetto, FL), at a counting efficiency of 47%. All adenosine agonists and antagonists were purchased from Research Biochemicals (Natick, MA). The data were analyzed with the program 'LIGAND' (20), which performs weighted, nonlinear, least squares curve-fitting to the general model of Feldman (21), involving the interaction of several ligands with several independent classes of sites according to the law of mass action. Deviations of the observed points from the predicted values were weighted according to the reciprocal of the predicted variance. Competition curves were analyzed using models for competition of radioligand and competitor for one or two independent sites. Results from fits using a two-site model were retained only when the two-site model fit the data significantly better than a one-site model, as determined by the partial *F* test at a significance level of $p < 0.05$.

Determination of cAMP production. Fifty microliters of cell membranes (100 μg of protein), suspended in AC buffer (75 mM Tris·HCl, pH 7.4, 250 mM sucrose, 12.5 mM $MgCl_2$, 1.5 mM EDTA) containing 1 mM dithiothreitol and 200 μM sodium metabisulfite and supplemented with 2.75 mM phosphoenolpyruvate, 53 μM GTP, 0.12 mM ATP, 1.0 unit of myokinase, 0.2 unit of pyruvate kinase, and 100 μM RO-20-1724 (a phosphodiesterase inhibitor; Biomol, Plymouth Meeting, PA), were added to tubes, on ice, containing 10 μl of H_2O or 10 μl of appropriate test compounds. The membranes were incubated for 5

min at 37°, to generate cAMP, and the reaction was stopped by a 3-min incubation in boiling H_2O. The cAMP generated was assayed by the method of Brown *et al.* (22), by incubation with cAMP-binding protein (prepared from bovine adrenal gland) in the presence of [^{3}H]cAMP (45 Ci/mmol; Amersham Corp., Arlington Heights, IL) at 4° for 2–16 hr, as previously described (23). After incubation with the cAMP-binding protein, free [^{3}H]cAMP was removed by treatment with charcoal-bovine serum albumin solution, and the bound [^{3}H]cAMP remaining in the supernatant was quantitated by liquid scintillation counting. The cAMP concentrations produced in the assay were determined by comparison with a standard curve, which was linear in the range of 1–30 pmol of cAMP/assay tube.

Results and Discussion

As part of an ongoing effort to isolate and clone G protein-linked receptors from rat brain, we have used the PCR technique to selectively amplify cDNA sequences from mRNA purified from rat striatum. Poly(A)$^+$ RNA was used to synthesize cDNA by reverse transcription followed by PCR amplification with a pair of highly degenerate primers, the sequences of which were derived from the third and sixth transmembrane regions of previously cloned G protein-linked receptors. This process resulted in the amplification of several cDNA fragments, which were preliminarily characterized by DNA sequence analysis (data not shown). One of these fragments was found to exhibit considerable (>90%) sequence homology to the RDC7 cDNA clone, which was recently isolated from a dog thyroid library and which apparently encodes a G protein-linked receptor of unknown function (13, 14). This PCR-generated cDNA fragment was subsequently used to screen a rat striatal cDNA library, in order to isolate a full-length clone. One positive clone containing a cDNA insert of ~2.2 kb was isolated, and the complete nucleotide sequence was determined.[2] The longest open reading frame in this cDNA codes for a 326-residue protein with a theoretical molecular mass of 36,692 Da.

The proposed membrane topography of the putative receptor protein is illustrated in Fig. 1. Hydrophobicity analysis (data not shown) of the translated protein revealed seven clusters of ~24 hydrophobic residues, predicted to represent transmembrane-spanning domains, connected by three extracellular and three intracellular loops. This pattern is similar to that observed for other cloned G protein-coupled receptors, where the NH_2 terminus is proposed to be extracellular and the COOH terminus projects into the cytoplasm (25). One potential *N*-linked glycosylation site was observed within the second extracellular loop. The solid circles in Fig. 1 indicate amino acids that differ between the translated rat protein and the dog RDC7 clone (13). Given this high level of amino acid sequence homology (92% overall), it seems likely that the rat cDNA represents a species homolog of RDC7.

Recently, it has been reported that RDC8, a cDNA that was observed to be highly homologous to the RDC7 clone (13), encodes an A_2 adenosine receptor protein (26). This observation might suggest that the RDC7 receptor is also a member of the adenosine or purine receptor family. In order to investigate this possibility, we constructed a series of cell lines that have been stably transfected with the rat RDC7 cDNA. Membranes prepared from these cloned cells were initially examined for the binding of the A_1 adenosine receptor antagonist [^{3}H]DPCPX (Fig. 2). Interestingly, membranes prepared from transfected A9-L cells bound [^{3}H]DPCPX with high (~90%) specificity and saturability (Fig. 2A). No specific binding was observed in membranes from untransfected A9-L cells (data not shown).

[2]The nucleotide sequence reported in this paper has been submitted to the Genbank/EMBL Data Bank, with accession number M64299.

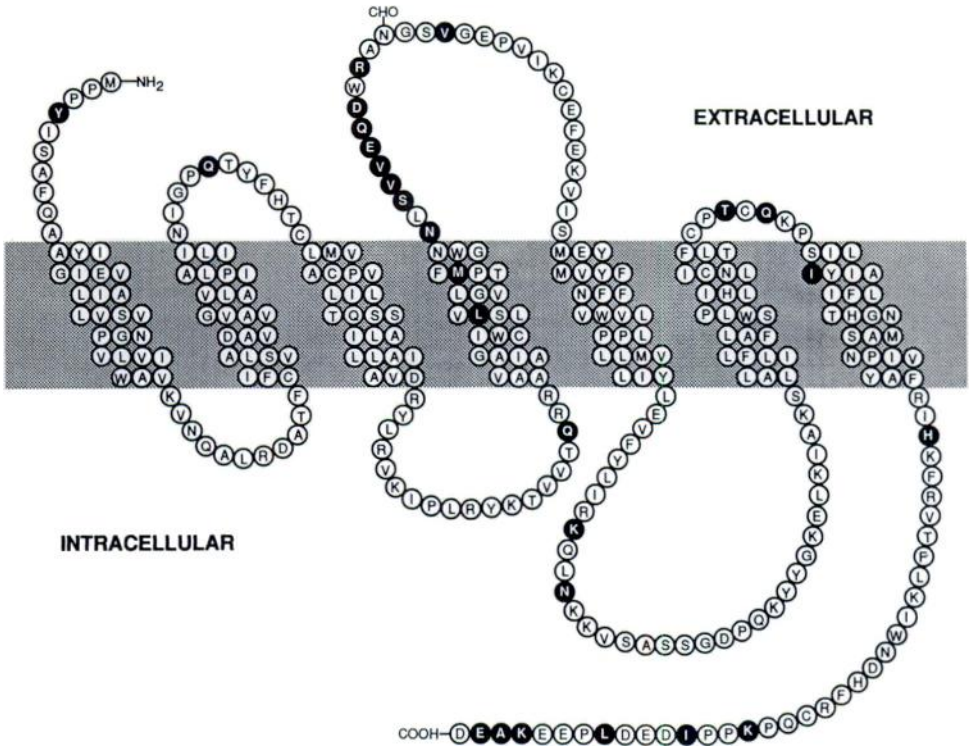

Fig. 1. Proposed membrane topography of the rat homolog of the RDC7 receptor protein. Transmembrane-spanning domains were defined on the basis of hydropathy analysis (24). *Solid circles*, amino acids that differ between the rat and dog (13) clones. *CHO*, potential *N*-linked glycosylation site.

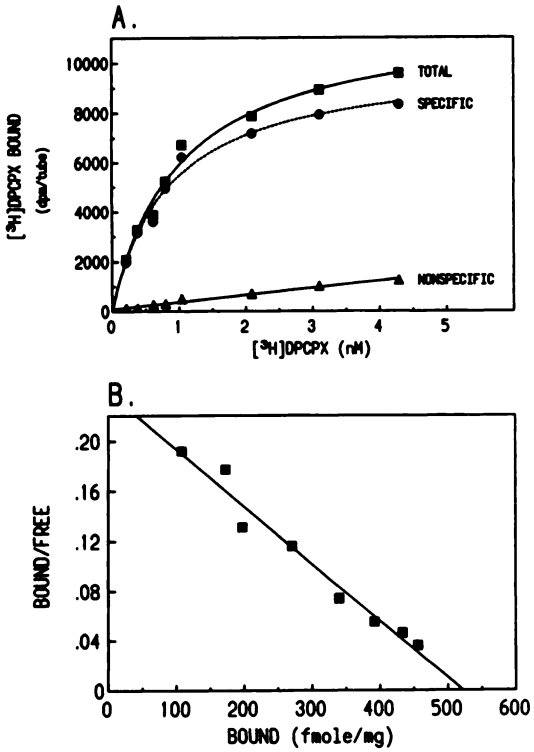

Fig. 2. [³H]DPCPX saturation binding experiment in transfected A9-L cell membranes. A, Transfected A9-L cell membranes were prepared and incubated with the indicated concentrations of [³H]DPCPX, as described in Experimental Procedures. Nonspecific binding was determined by including 1 μM unlabeled DPCPX in the assays and was subtracted from the total binding values to yield the specific binding data. B, Scatchard transformation of the specific binding data from A. Average binding parameters from three separate experiments are given in the text.

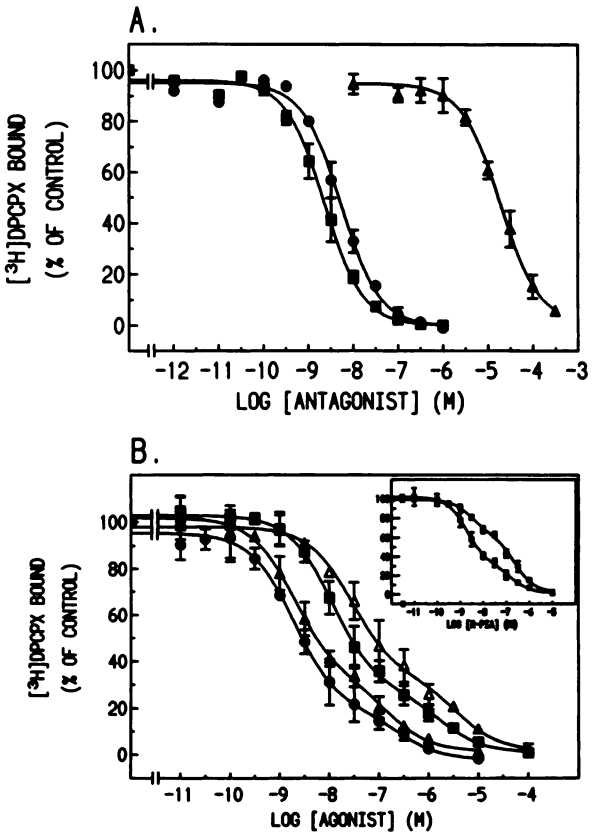

Fig. 3. [³H]DPCPX competition binding assays in transfected A9-L cell membranes. Membranes were prepared and incubated with 0.5 nM [³H] DPCPX and the indicated concentrations of drugs, as described in Experimental Procedures. The *data points* represent the mean ± standard deviation from two independent experiments. The computer-derived affinity constants are given in the text. A, Antagonist competition curves. ■, DPCPX; ●, XAC; ▲, theophylline. B, Agonist competition curves. ●, CCPA; ▲, (*R*)-PIA; ■, NECA; △, (*S*)-PIA. *Inset*, (*R*)-PIA was incubated in the absence (■) or presence (□) of 100 μM Gpp(NH)p.

Scatchard analysis of the binding data (Fig. 2B) revealed a single class of [³H]DPCPX binding sites (Hill coefficient = 1.03 ± 0.01; three experiments), with a dissociation constant (K_D) of 0.53 ± 0.14 nM (three experiments). The maximum binding capacity (B_{max}) of [³H]DPCPX to these membranes was approximately 500 fmol/mg of protein (Fig. 2B). The affinity (K_D) of [³H]DPCPX binding to membranes from transfected A9-L cells was similar to that observed for [³H]DPCPX binding to A_1 adenosine receptors in rat brain membranes (27) or to purified A_1 adenosine receptors from either rat brain or testis (8, 10).

Further identification and characterization of the receptor expressed in the transfected A9-L cells was carried out by radioligand binding competition analysis using adenosine antagonist (Fig. 3A) and agonist (Fig. 3B) ligands. Unlabeled DPCPX was the most potent (K_i = 0.85 nM) antagonist of [³H] DPCPX binding, followed by XAC (K_i = 2.0 nM), a potent A_1-selective antagonist. In contrast, the nonselective adenosine antagonist theophylline was more than 3 orders of magnitude less potent (K_i = 7.3 μM). Such high affinity displacement observed with both DPCPX and XAC is suggestive of an A_1 receptor subtype. The Hill coefficients for all antagonist/[³H] DPCPX competition curves did not differ significantly from unity.

In contrast, all agonist competition curves exhibited Hill coefficients less than unity (ranging from 0.48 to 0.69) and were best described by the existence of one high (K_H) and one low (K_L) affinity agonist-receptor binding state (Fig. 3B). The high affinity agonist state (R_H) constituted the majority of receptor sites (67–78%) in all cases (Fig. 3B). The two A_1-selective agonists CCPA (K_H = 0.71 nM, K_L = 97.5 nM, R_H = 78%) and (*R*)-PIA (K_H = 0.77 nM, K_L = 61.2 nM, R_H = 69%), exhibited the highest affinity (Fig. 3B). The [³H]DPCPX binding sites expressed in A9-L cells demonstrated approximately 15–20-fold stereoselectivity for the (*R*)-PIA enantiomer versus the (*S*)-PIA enantiomer (K_H = 10.7 nM, K_L = 1.4 μM, R_H = 67%). The adenosine agonist NECA was intermediate in potency (K_H = 4.3 nM, K_L = 0.66 μM, R_H = 74%), relative to the PIA enantiomers (Fig. 3B). This rank order of agonist potency, CCPA = (*R*)-PIA > NECA > (*S*)-PIA, is characteristic of the A_1 adenosine receptor subtype in rat brain (8) and other tissues (1). Taken together, the radioligand binding data in Figs. 2 and 3 indicate that this rat striatal cDNA encodes an A_1 adenosine receptor protein.

Linkage of the cloned A_1 adenosine receptor to endogenous G proteins in the A9-L cells was initially investigated using the nonhydrolyzable GTP analog Gpp(NH)p. Addition of 100 μM Gpp(NH)p to the (*R*)-PIA/[³H]DPCPX competition assay (Fig. 3B, *inset*) caused a marked reduction in the number of high affinity agonist binding sites, from 69% to 36%, with an approximate 10-fold shift in the IC_{50} of the competition curve, from ~3 nM to ~50 nM (two experiments). Furthermore, the

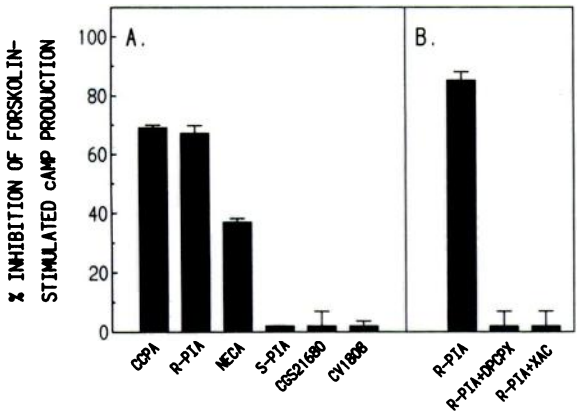

Fig. 4. Adenylyl cyclase assays in transfected CHO cell membranes. Membranes were prepared and adenylyl cyclase assays were performed as described in Experimental Procedures. A, Each of the indicated agonists (10^{-8} M concentration) was examined for its ability to inhibit forskolin-stimulated adenylyl cyclase activity. B, (*R*)-PIA (10^{-6} M) was tested alone or in combination with 10^{-5} M concentrations of either DPCPX or XAC. The data represent the mean ± standard error of replicate values (four determinations) from a single experiment, which was repeated twice with similar results. Typical basal and forskolin-stimulated adenylyl cyclase activities were 5.5 and 30 pmol of cAMP/min/mg, respectively. None of the adenosine agonists affected the basal enzyme activity (data not shown).

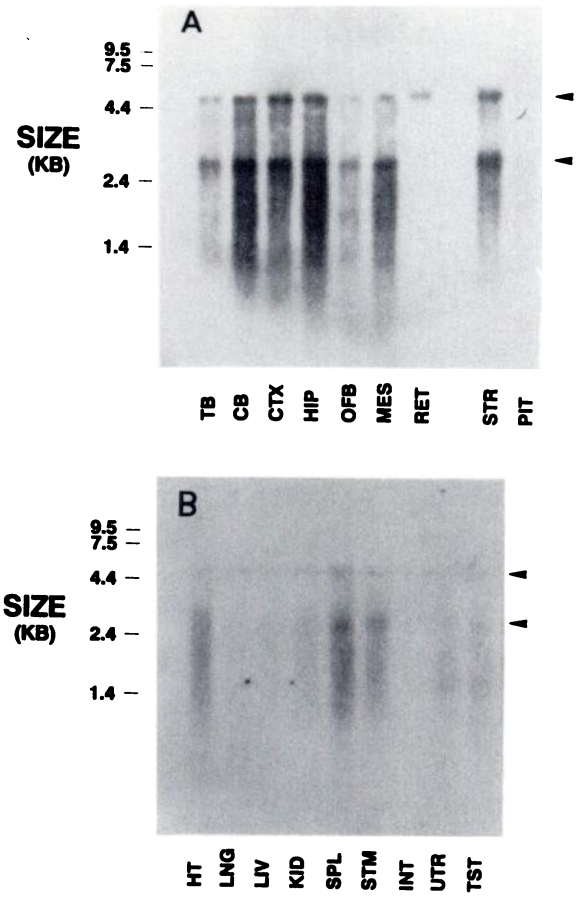

Fig. 5. Northern blot analyses of the A$_1$ adenosine receptor mRNA in various tissues. Poly(A)$^+$ RNA samples (2 μg) were run on denaturing 1% formaldehyde-agarose gels, electrophoretically transferred to GeneScreen (NEN) nitrocellulose, hybridized with a cDNA-specific, ^{32}P-labeled oligodeoxynucleotide probe, washed under high stringency, and exposed to Kodak XAR film, as described in Experimental Procedures. RNA molecular size markers are indicated on the *left*. A, Central nervous system tissues. mRNAs of approximately 3.1 and 5.6 kb (*arrowheads*) in length were detected and located predominantly in the cortex (*CTX*), cerebellum (*CB*), and hippocampus (*HIP*). Other tissues shown are total brain (*TB*), olfactory bulb (*OFB*), mesencephalon (*MES*), retina (*RET*), striatum (*STR*), and pituitary (*PIT*). B, Peripheral tissues. Tissues shown heart (*HT*), lung (*LNG*), liver (*LIV*), kidney (*KID*), spleen (*SPL*), stomach (*STM*), intestine (*INT*), uterus (*UT*), and testis (*TST*).

presence of Gpp(NH)p increased the specific binding of [^{3}H] DPCPX by about 2-fold in these experiments (data not shown), similar to that reported for rat brain membranes (28) and solubilized A$_1$ receptors from rat testis (10). The cloned A$_1$ receptor expressed in A9-L cells thus appears to be functionally coupled in the membranes to an endogenous G protein.

Because A$_1$ receptors have been reported to be functionally linked to the inhibition of adenylyl cyclase activity (1, 2), we tested for this response in the transfected A9-L cell membranes. Preliminary experiments did indeed demonstrate some adenosine agonist inhibition of adenylyl cyclase activity; however, this was variable and confounded by the unexpected presence of an endogenous A$_2$ adenosine receptor linked to stimulation of cAMP production (data not shown). We thus turned to the CHO cells that we had transfected with the A$_1$ receptor cDNA and preliminarily characterized as expressing >1 pmol/mg of protein specific [^{3}H]DPCPX binding sites (data not shown). Fig. 4A shows the effect of various adenosine agonists on inhibition of adenylyl cyclase activity. When tested at 10 nM concentrations, the potent A$_1$-selective agonists CCPA and (*R*)-PIA both significantly inhibited forskolin-stimulated cAMP production. NECA exhibited less of a response, whereas (*S*)-PIA was relatively ineffective at 10 nM (Fig. 4A). In contrast, 10 nM concentrations of the A$_2$-selective agonists CGS21680 and CV1808 did not affect adenylyl cyclase activity (Fig. 4A). No adenosine agonist inhibition of adenylyl cyclase activity (or the presence of specific [^{3}H]DPCPX binding) was observed in untransfected CHO cells (data not shown). Fig. 4B shows that the (*R*)-PIA inhibitory response could be completely blocked by the A$_1$-selective antagonists DPCPX and XAC. This functional agonist and antagonist pharmacology (Fig. 4) agrees well with that observed for the radioligand binding analyses in Figs. 2 and 3. The cloned A$_1$ receptor thus appears to be capable of functional coupling to the inhibition of adenylyl cyclase activity.

As further characterization of the A$_1$ receptor cDNA, we investigated the tissue distribution of its corresponding mRNA. Northern blot analysis of a variety of rat brain tissues revealed two species of mRNA for this A$_1$ adenosine receptor, one of ~3.1 kb and a less prominent, higher molecular weight species of ~5.6 kb (Fig. 5A). Highest expression was observed in the cortex, cerebellum, and hippocampus. Olfactory bulb, mesencephalon, and striatum also exhibited moderate levels of both messages, whereas the retina appeared to predominantly express the 5.6-kb species. No expression was apparent in the pituitary. The 3.1-kb species of mRNA appeared to be the predominant form in all peripheral rat tissues examined (Fig. 5B). These transcripts were detectable in the spleen and stomach, with some expression also being observed in the heart and testis. These data contrast somewhat with Northern blots performed on RDC7 mRNA, where a single transcript of 2.5 kb was observed in dog tissues (13, 28). The origin of the two mRNAs in rat and the relationship of these to the 2.5-kb dog transcript will require further investigation.

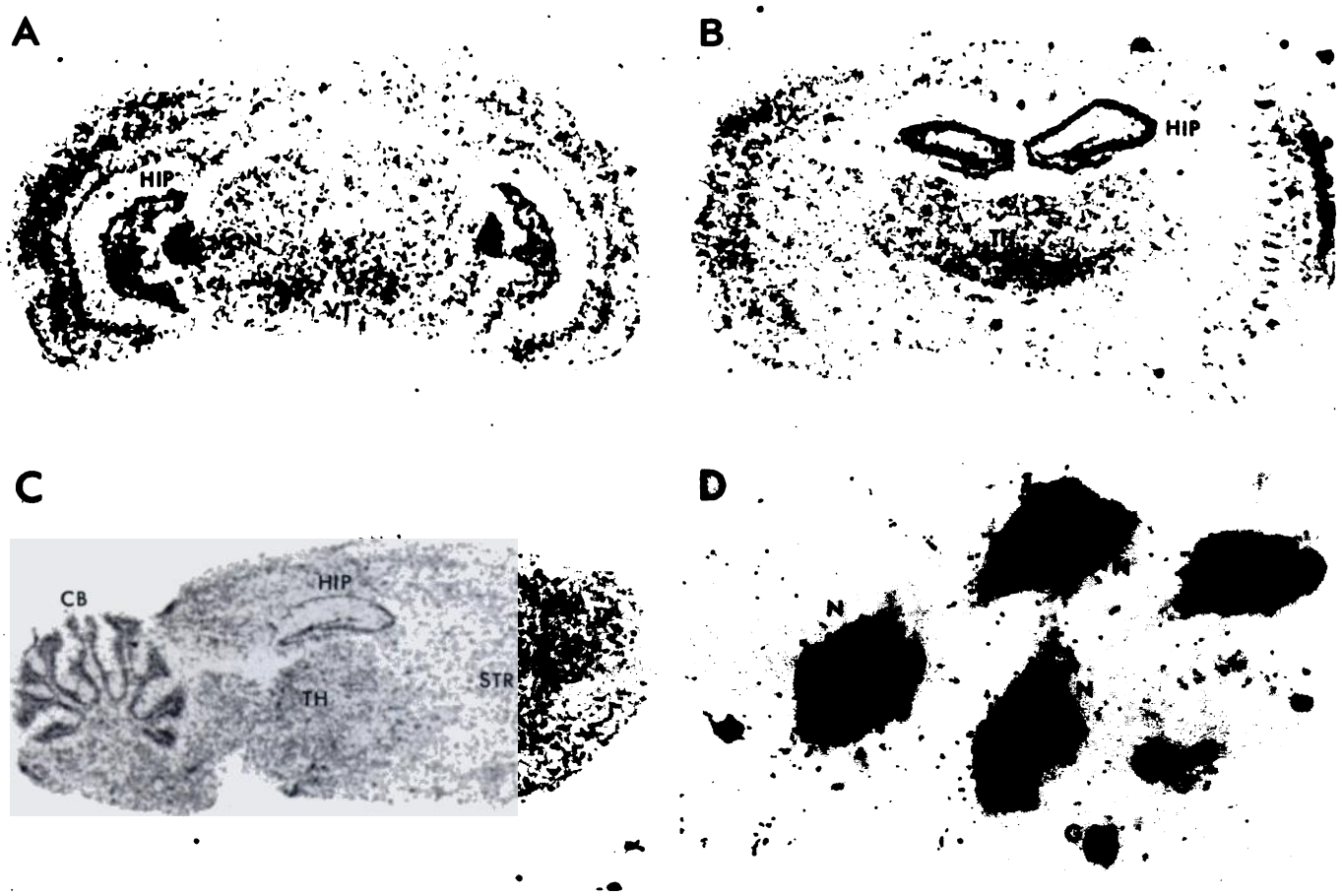

Fig. 6. *In situ* hybridization histochemical analysis of the distribution of mRNA for the A_1 adenosine receptor. A–C, Low magnification autoradiographic analysis of the distribution of mRNA for this receptor in coronal (A and B) and sagittal (C) sections of the adult rat brain. Areas with specific mRNA expression are indicated. *CB*, cerebellum; *CTX*, cortex; *HIP*, hippocampus; *MGN*, medial geniculate nuclei; *STR*, striatum; *TH*, thalamus; *VT*, ventral tegmental area. D, High magnification of the CA4 region of the hippocampus, indicating specific localization to neuronal (*N*) nuclei as opposed to glial (*G*) nuclei.

In situ hybridization histochemical studies (Fig. 6) confirmed the tissue distribution of mRNA expression seen with the Northern blot analyses. In addition, these studies revealed marked expression of A_1 receptor mRNA in thalamic nuclei, the medial geniculate nucleus, and the ventral tegmental area (Fig. 6, A and B). High levels of expression observed in the cerebellum were confined predominantly to the granule cell layer (Fig. 6C). In most cases, autoradiographic labeling was restricted to neuronal, not glial, nuclei (Fig. 6D). The expression of A_1 receptor mRNA and A_1 receptor protein colocalized in a number of brain tissues. Highest levels of A_1 adenosine receptor binding sites (~200–600 fmol/mg of protein) have been observed in the cortex, cerebellum, hippocampus, striatum, and thalamus, utilizing [^{3}H]CHA, an A_1 adenosine agonist, or [^{3}H] DPCPX for binding to membrane preparations or in autoradiographic analyses (27, 29, 30). In addition, these anatomical findings are in agreement with preliminary information on RDC7 mRNA distribution (13, 31).

In summary, we have cloned and expressed a rat cDNA that encodes an A_1 adenosine receptor protein. This conclusion is supported by the observation that, when transfected into mammalian cells, this cDNA directs the expression of a pharmacologically specific and functional A_1 receptor, as determined with radioligand binding and adenylyl cyclase analyses. Moreover, the mRNA corresponding to this cDNA is localized in tissues where the A_1 receptor is known to be expressed. Finally, NH_2-terminal sequence analysis of the A_1 receptor protein purified from rat brain (8) resulted in the identification of 20 amino acid residues that are identical to the NH_2 terminus shown in Fig. 1.[3] It is also interesting to note that the predicted molecular mass of the cloned A_1 receptor (36,692 Da) closely matches that determined using photoaffinity labeling and purification techniques (4–10). Overall, the size of the A_1 receptor is small, relative to other G protein-coupled receptors, particularly when compared with receptors that are linked to adenylyl cyclase inhibition (16, 25).

Although the size of the A_1 receptor appears fairly constant among tissues (4–10), an A_1 receptor with an unusual agonist pharmacology has recently been purified from bovine cerebral cortex (9), suggesting potential heterogeneity in this receptor subtype. Further evidence of receptor heterogeneity comes from studies in which adenosine acting at A_1 receptors has been shown to potently inhibit the release of excitatory neurotransmitters from striatal and hippocampal neurons, both *in vitro* and *in vivo* (32–34). This presynaptic modulation by A_1 receptors appears to be independent of the ability of adenosine to

[3] H. Nakata and M. Haniu, unpublished observations.

effect alterations in intracellular cAMP levels but, rather, may involve inhibition of Ca^{2+} influx or stimulation of K^+ efflux from the cells. Whether these additional properties of the A_1 adenosine receptor reside in one or additional members of this gene family remains to be determined. The cloning of the A_1 as well as A_2 (26) receptors may now allow the testing of these hypotheses as well as the identification of additional gene members within the broader purine receptor family.

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