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Molecular cloning and characterization of an adenosine receptor: The A3 adenosine receptor

QUN-YONG ZHOU*†, CHUANYU LI*, MARK E. OLAH‡, ROBERT A. JOHNSON*, GARY L. STILES‡, AND OLIVIER CIVELLI*§¶

*Vollum Institute for Advanced Biomedical Research, and Departments of †Biochemistry and of §Cell Biology and Anatomy, Oregon Health Sciences University, Portland, OR 97201; and ‡Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, NC 27710

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ABSTRACT **We have previously reported the selective amplification of several rat striatal cDNA sequences that encode guanine nucleotide-binding regulatory protein (G protein)-coupled receptors. One of these sequences (R226) exhibited high sequence identity (58%) with the two previously cloned adenosine receptors. A full-length cDNA clone for R226 has been isolated from a rat brain cDNA library. The cDNA clone encodes a protein of 320 amino acids that can be organized into seven transmembrane stretches. R226 has been expressed in COS-7 and CHO cells and membranes from the transfected cells were screened with adenosine receptor radioligands. R226 could bind the nonselective adenosine agonist tritiated *N*-ethyladenosine 5′-uronic acid ([^{3}H]NECA) and A1-selective agonist radioiodinated N^6-2-(4-amino-3-iodophenyl)-ethyladenosine ([^{125}I]APNEA) but not A1-selective antagonists tritiated 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) and 8-{4-[({[(2-aminoethyl)amino]carbonyl}methyl)oxy]-phenyl}-1,3-dipropylxanthine ([^{3}H]XAC) or the A2-selective agonist ligands tritiated 2-[4-(2-carboxyethyl)phenyl]ethylamino 5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS21680) and radioiodinated 2-[4-({2-[(4-aminophenyl)methylcarbonylamino]ethylaminocarbonyl}ethyl)phenyl]ethylamino 5′-*N*-ethylcarboxamidoadenosine. Extensive characterization with [^{125}I]APNEA showed that R226 binds [^{125}I]APNEA with high affinity (K_d = 15.5 ± 2.4 nM) and the specific [^{125}I]APNEA binding could be inhibited by adenosine ligands with a potency order of (*R*)-N^6-phenyl-2-propyladenosine (*R*-PIA) = NECA > *S*-PIA > adenosine > ATP = ADP but not by antagonists XAC, isobutylmethylxanthine, and DPCPX. In R226 stably transfected CHO cells, adenosine agonists *R*-PIA, NECA, and CGS21680 inhibited by 40–50% the forskolin-stimulated cAMP accumulation through a pertussis toxin-sensitive G protein with an EC_{50} of 18 ± 5.6 nM, 23 ± 3.5 nM, and 144 ± 34 nM, respectively. Based on these observations we conclude that R226 encodes an adenosine receptor with non-A1 and non-A2 specificity, and we thus name it the A3 adenosine receptor. mRNA analyses revealed that the highest expression of R226 was in the testis and low-level mRNAs were also found in the lung, kidneys, heart, and some parts of the central nervous system such as cortex, striatum, and olfactory bulb. The high-expression level of the A3 receptor in the testis suggests a possible role for adenosine in reproduction.**

Adenosine modulates diverse physiological functions including induction of sedation, vasodilatation, suppression of cardiac rate and contractility, inhibition of platelet aggregability, stimulation of gluconeogenesis, and inhibition of lipolysis (1–3). Based on biochemical and pharmacological criteria, two subtypes of adenosine receptors, A1 and A2, have been differentiated, which inhibit and stimulate adenylate cyclase, respectively (1, 3). Adenosine also can stimulate K^+ flux, inhibit Ca^{2+} flux, and modulate inositol phospholipid turnover through receptor-mediated mechanisms (4–7).

Recently, the cDNAs that encode A1 and A2 adenosine receptors have been cloned by a PCR-based strategy (8–12). Molecular cloning of A1 and A2 receptors revealed that they both belong to the superfamily of guanine nucleotide-binding regulatory protein (G protein)-coupled receptors. Physiological and pharmacological studies, however, have suggested subtype heterogeneity of adenosine receptor in addition to the A1 and A2 classification (13–16). We report here the cloning, expression, and functional characterization of a newly discovered adenosine receptor.‖ We present its nucleotide and deduced amino acid sequence, its tissue distribution, its pharmacological characterization, and its ability to inhibit forskolin-stimulated cAMP accumulation, and we conclude that R226 is distinct enough from the A1 and A2 receptors to be called the A3 adenosine receptor.

MATERIALS AND METHODS

PCR Amplification, Library Screening, and Sequencing. Rat striatal cDNA mixture was subjected to 30 cycles of PCR amplification with a pair of degenerate oligonucleotide primers (17). The PCR products sizing from 400 to 750 base pairs (bp) were subcloned and sequenced (17). One fragment, named PCR226, sequentially homologous to A1 and A2 adenosine receptors was identified (8–12). PCR226 was used to screen a rat brain cDNA library in λgt11 and a rat genomic library in λDASH by plaque hybridization (18). One cDNA clone was identified, subcloned into pGemblue (resulting in pGem226), and sequenced. Two identical positive genomic phages were purified, characterized by Southern hybridization, and partially sequenced.

DNA Transfection, Membrane Preparation, and Receptor Binding Assays. The full coding region of R226 was subcloned into expression vectors pBC12BI and Rc-RSV (resulting in pBC226 and Rc-RSV226). For transient expression, 45 μg of pBC226 DNA was transfected into each 150-mm dish of COS-7 cells according to the method of Chen and Okayama (19) and cells were harvested 48 hr later. For stable expression, 1 μg of Rc-RSV226 plasmid DNA was further purified by a Prep-A-Gene kit (Bio-Rad) and transfected into CHO cells by the $CaPO_4$ method (18). CHO cells were selected with neomycin (G418, 700 μg/ml) and screened for expression of R226 by Northern hybridization (18). Membrane preparation and radioligand binding assays were performed as described

Abbreviations: APNEA, N^6-2-(4-amino-3-iodophenyl)ethyladenosine; CGS21680, 2-[4-(2-carboxyethyl)phenyl]ethylamino 5′-*N*-ethylcarboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; NECA, *N*-ethyladenosine 5′-uronic acid; (*R,S*)-PIA, (*R,S*)-N^6-phenyl-2-propyladenosine; XAC, 8-{4-[({[(2-aminoethyl)-amino]carbonyl}methyl)oxy]phenyl}-1,3-dipropylxanthine; G protein, guanine nucleotide-binding regulatory protein.

¶To whom reprint requests should be addressed.

‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M94152).

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(20) except that adenosine deaminase was not included when adenosine competition experiments were performed.

cAMP Measurement. Cells were grown to ≈80% confluence in 150-mm dishes and washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and detached from plates with PBS containing 0.02% EDTA. Cells were spun down at 800 rpm for 10 min at 4°C and resuspended in an appropriate volume of KRH buffer (140 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM KH_2PO_4/6 mM glucose/25 mM Hepes·NaOH, pH 7.4). The cells were washed once with KRH buffer and resuspended in KRH buffer at a concentration of 10^7 cells per ml. Cell suspension (100 μl) was added to each glass tube containing 100 μl of KRH with 200 μM Ro 20-1724 and incubated at 37°C for 10 min. Prewarmed KRH (200 μl) containing 200 μM Ro 20-1724 and test drugs was then added to cells and mixed. After incubation at 37°C for 20 min, 400 μl of 0.5 mM NaOAc (pH 6.2) was added and the glass tubes were transferred to a boiling water bath. Boiling was for 20 min, and then the tubes were cooled to room temperature and centrifuged at 1000 × *g* for 15 min. Supernatants (50 μl) were assayed for cAMP levels (21). For pertussis toxin pretreatment, aseptic pertussis toxin (Sigma) was added to the medium to a final concentration of 100 ng/ml and maintained for ≈18 hr.

Reverse Transcription PCR. Total RNAs (2 μg) isolated from different rat tissues were reverse transcribed (18). The single-stranded cDNA products were denatured and subjected to limited PCR amplification (27 cycles) with two primers (TTCCAGCTGAAGCTTCTC as 5′ primer and GGTGGAGCTGTTTTGAGA as 3′ primer). Each PCR cycle consisted of denaturing at 95°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 90 sec. PCR products were then run on the 1.2% agarose gel and examined by ethidium bromide staining. A standard curve was also developed to test the linearity of the PCR amplification. pGem226 (see above) was linearized by *Xba* I, and RNA was synthesized with T7 RNA polymerase. The *in vitro* synthesized template RNA (640, 320, 160, 80, 40, 20, and 10 fg) was reverse transcribed and PCR amplified as described above. Ethidium bromide staining of the PCR products indicated that the amount of amplified products was approximately proportional to the amount of template added.

RESULTS

A fragment (R226) was identified when rat striatal cDNA was used as template in a PCR amplification with a pair of degenerate oligonucleotides corresponding to the putative third and sixth transmembrane regions of G-protein-coupled receptors (17). The R226 PCR fragment was subsequently used to screen a rat brain cDNA library. One positive clone with an insert of 1.9 kilobases (kb) was isolated and its complete nucleotide sequence was determined (Fig. 1*A*). The longest open reading frame of this cDNA encodes a protein of 320 amino acids (M_r, 36,644). Hydrophobicity analysis of the deduced amino acid sequence showed that the protein contains seven hydrophobic domains of 21–26 amino acids (data not shown). A computer analysis revealed that R226 is most closely related to the adenosine receptors (Fig. 1*B*). In putative transmembrane domains, 58% and 57% of the amino acids of R226 are identical to those of A1 and A2 receptor, respectively (8–12). By comparison to other G-protein-coupled receptor families, such as adrenergic and dopaminergic families, the high sequence similarity among A1, A2, and R226 suggested that they might belong to one family also (22, 23). Furthermore, all these proteins possess a putative N-glycosylation site in the second extracellular loop, while R226 has two extra potential N-glycosylation sites in its N terminus (Fig. 1*A*). R226 also possesses three potential phosphorylation sites (Ser/Thr-Xaa-Arg/Lys) for protein kinase C and one potential phosphorylation site (Ser/Thr-Xaa-Xaa-Glu/Asp) for casein kinase II (17, 24).

Partial characterization of rat genomic clones of R226 revealed the existence of at least one intron (>2 kb) with a donor/acceptor site sequence of TTTTCCTCCCCCCAT-TCAAACCA(G/A)T. This splice site is located just outside the third transmembrane domain of R226, and all the rest of the mRNA sequence of R226 is contained in a single exon.

Initial binding assays of transiently transfected COS-7 cell membrane preparations with the nonselective adenosine radioligand tritiated *N*-ethyladenosine 5′-uronic acid ([^{3}H]N-ECA) showed a saturable (B_{max} = 550 fmol per mg of protein) and high-affinity (K_d = 50 nM) binding. This result suggested that R226 might encode an adenosine receptor. To further characterize this receptor, and also to avoid the low level of endogenous A2 receptor in COS-7 cells, we stably expressed R226 in CHO cells, which do not express endogenous adenosine receptors as judged by [^{3}H]NECA binding (data not shown). We assessed the ability of a variety of radioligands including the A1 selective agonist radioiodinated N^6-2-(4-amino-3-iodophenyl)ethyladenosine ([^{125}I]APNEA), the A1 selective antagonists tritiated 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) and 8-{4-[({[(2-aminoethyl)amino]carbonyl}methyl)oxy]phenyl}-1,3-dipropylxanthine ([^{3}H]XAC), and the A2 selective agonists tritiated 2-[4-(2-carboxyethyl)phenyl]ethylamino 5′-*N*-ethylcarboxamidoadenosine ([^{3}H]-CGS21680) and radioiodinated 2-[4-({2-[(4-aminophenyl)-methylcarbonylamino]ethylaminocarbonyl}ethyl)phenyl]-ethylamino 5′-*N*-ethylcarboxamidoadenosine to specifically bind to the R226 receptor. Only [^{125}I]APNEA gave specific binding as defined by (*R*)-N^6-phenyl-2-propyladenosine (*R*-PIA) (10 μM) or NECA (10 μM). Using direct saturation curves or dilution saturation curves, [^{125}I]APNEA bound with high affinity (15.5 ± 2.4 nM) and in a saturable manner (225 fmol/mg). This affinity is ≈10-fold lower than that found for typical A1 receptors (25). A representative Scatchard plot is shown in Fig. 2. R226 was further characterized in competition assays with a large series of receptor ligands and [^{125}I]APNEA as the labeled ligand. Shown in Fig. 3*A* is the competition of the prototypic adenosine analogs used to define A1 and A2 receptor subtypes. *R*-PIA and NECA were equally potent in competing for binding, with an IC_{50} of 63 ± 19 nM and 74 ± 23 nM, respectively, while *S*-PIA had an IC_{50} of 1140 ± 490 nM. The presumed endogenous hormone adenosine competes with an IC_{50} of 30 ± 4 μM. A constant feature of all previously known adenosine receptors is that alkylxanthines are receptor antagonists. In contrast, none of the alkylxanthines tested including isobutylmethylxanthine, DPCPX, and XAC competes even at 100 μM. Since the pharmacology and ligand affinities described above are not consistent with the expected A1 or A2 receptor pharmacology, we next tested whether R226 might be a purinergic P2 receptor or other nucleoside or nucleotide binding protein (26). ATP at 100 μM inhibits only 50% of specific [^{125}I]-APNEA binding, while ADP at the same concentration inhibits only 25% of specific binding (Fig. 3*B*). p[NH]ppA, adenyl-(β,γ-methylene)diphosphonate, 2-CH_3-S-ATP, UTP, cAMP, and 2-deoxyadenosine all failed to inhibit binding by >20% at 1 mM. The following ligands all failed to compete with [^{125}I]APNEA binding at 100 μM: isoproterenol, carbachol, phentolamine, serotonin, and dopamine. However, p[NH]ppG, a nonhydrolyzable analog of GTP, effectively competed for 60–70% of [^{125}I]APNEA specific binding with an IC_{50} of ≈1 μM (Fig. 3*B*). This is very reminiscent of what is observed for the effects of guanine nucleotides on A1 receptor-agonist binding (25) but is totally distinct from that observed in the A2 receptor system (27).

To analyze the biological activity of the cloned adenosine receptor, we studied its ability to couple to second messenger systems. R226 stably transfected CHO cells were used to

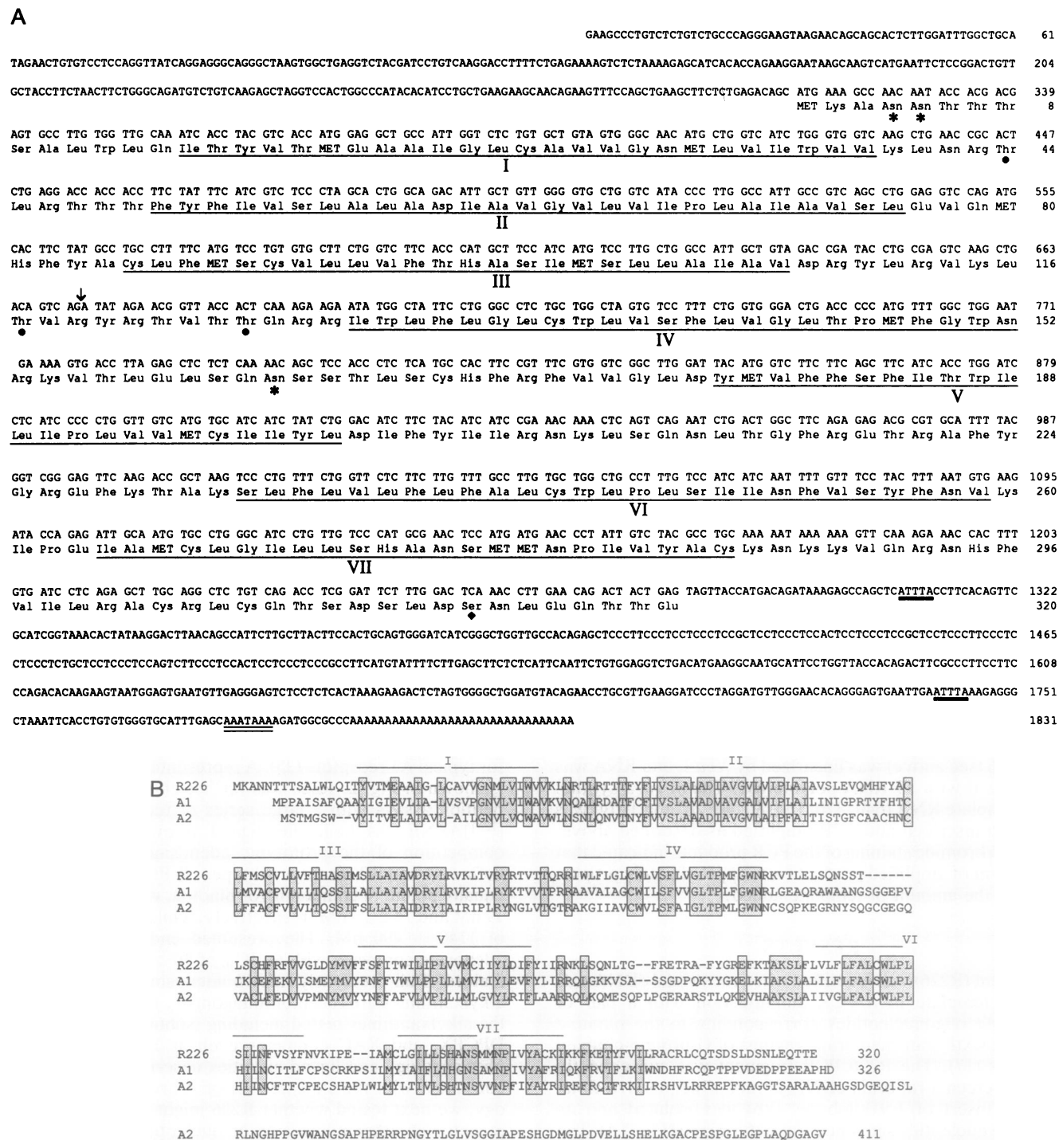

FIG. 1. (*A*) Nucleotide and deduced amino acid sequence of R226 cDNA. Numbering starts with the first nucleotide of the cDNA. Putative transmembrane domains are underlined. A putative polyadenylylation site has two lines below it. The potential N-glycosylation sites are indicated by asterisks. Arrow indicates a splice site. The RNA destability consensus sequences AUUUA have boldface lines below them. The potential protein kinase C phosphorylation sites and casein kinase II phosphorylation site are indicated by solid dots and a diamond, respectively. (*B*) Amino acid sequence alignment of R226, A1, and A2 adenosine receptors. The putative transmembrane domains are boxed and bracketed and labeled with Roman numerals. Shaded amino acid residues indicate conservation in all three adenosine receptors.

study the effects of adenosine agonists on cAMP accumulation. Incubation of wild-type and R226 stably transfected CHO cells with 1 μM forskolin resulted in a 15-fold increase in cellular cAMP levels. In wild-type CHO cells, adenosine agonists had no effect on forskolin-stimulated cAMP production (data not shown). Addition of adenosine receptor agonists *R*-PIA (100 nM), NECA (100 nM), CGS21680 (100 nM), and adenosine (100 μM) on R226 stably transfected CHO cells produced 20–50% inhibition of forskolin-stimulated cAMP accumulation (Fig. 4*A*). ATP and ADP had no effects at 100 μM. Further experiments showed that inhibition of forskolin-stimulated cAMP production by adenosine agonists was dose-dependent with an EC_{50} of 18 ± 5.6 nM for *R*-PIA, 23 ± 3.5 nM for NECA, 144 ± 34 nM for CGS21680, and 6.5 ± 2.1 μM for adenosine (Fig. 5). The maximal inhibition for *R*-PIA, NECA, and CGS21680 was 40–50%, although adenosine itself had not reached its maximal effect (Fig. 5). The inhibitory effect of NECA on forskolin-stimulated cAMP production could not be reversed by incubation with 10 μM DPCPX, a selective A1 adenosine receptor antagonist (Fig.

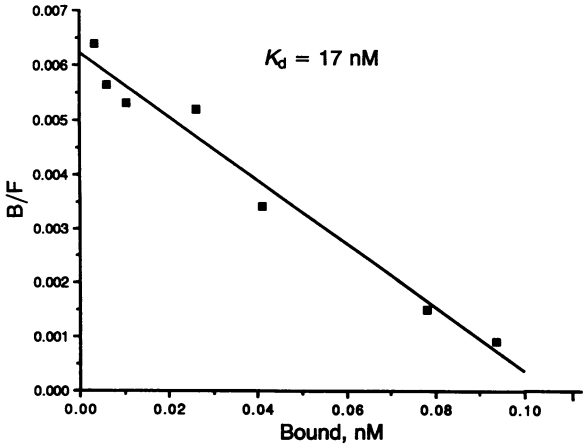

FIG. 2. Scatchard analysis of [^{125}I]APNEA binding to membranes prepared from R226 stably transfected CHO cells (five independent experiments). [^{125}I]APNEA was directly added up to 2 nM and then diluted with APNEA up to 100 nM. Nonspecific binding was defined by 10 μM *R*-PIA. B, bound; F, free.

4*B*). This result agrees with our finding that R226 did not bind any alkylxanthine-type antagonist. Fig. 4*B* also shows that pretreatment with pertussis toxin almost completely abolished the inhibitory effect of *R*-PIA and NECA on forskolin-stimulated cAMP production. These data indicated that this

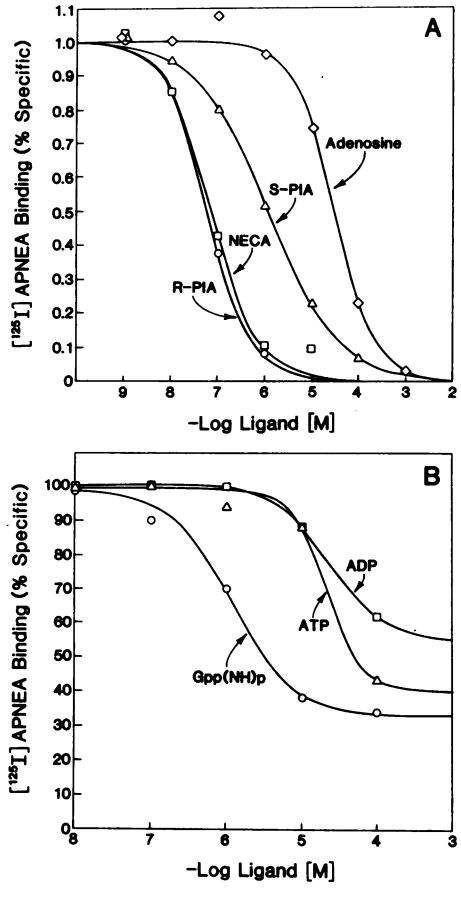

FIG. 3. (*A*) Competition curves of adenosine receptor agonists vs. [^{125}I]APNEA. (*B*) Competition for [^{125}I]APNEA by p[NH]ppG and the P2 receptor agonists ATP and ADP. [^{125}I]APNEA was present at 0.5 nM and competitors were present at the indicated concentration. Competition curves were replicated three to five times depending on the ligands used. The Hill coefficients were near unity.

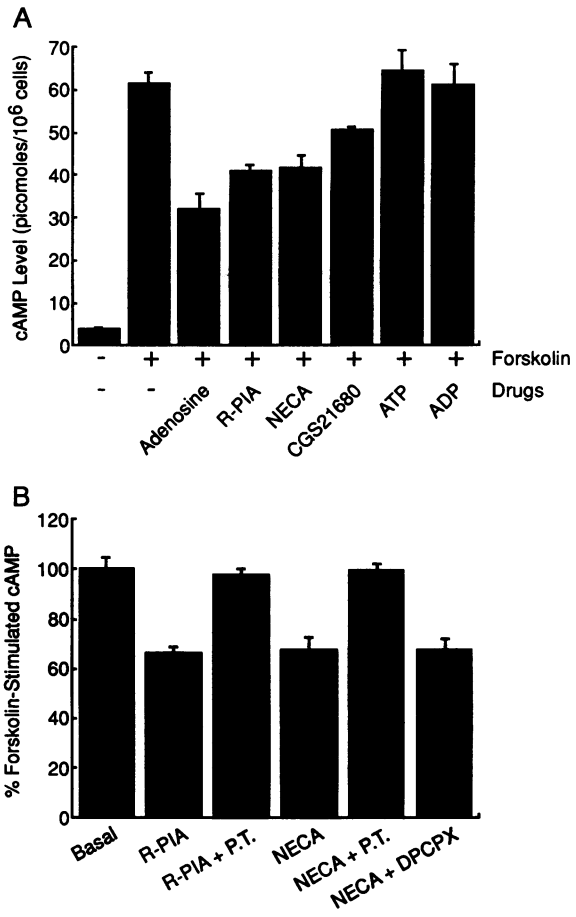

FIG. 4. (*A*) Effects of adenosine agonists on forskolin-stimulated cAMP accumulation in CHO cells stably transfected by R226. Concentrations of adenosine agonists used are 100 nM (*R*-PIA, NECA, and CGS21680) and 100 μM (adenosine, ATP, and ADP), and the concentration of forskolin is 1 μM. Values are obtained from triplicate plates and shown as means ± SE. (*B*) Effects of pertussis toxin (P.T., 100 ng/ml) pretreatment on forskolin-stimulated cAMP accumulation in R226 stably transfected CHO cells. Concentrations of forskolin and adenosine agonists are as in *A*.

adenosine receptor inhibits adenylate cyclase through interaction with a pertussis toxin-sensitive G protein. This conclusion was also supported by the observation that [^{125}I]APNEA binding could be inhibited by p[NH]ppG (Fig. 3*B*).

To gain further insight into the possible responses associated with this adenosine receptor, we have examined the tissue distribution of its corresponding mRNA by reverse transcribed PCR (18). Two primer sequences located in the N terminus and the second extracellular loop were chosen. These primers were chosen so that (*i*) they cross the intronic sequence, which is at least 2 kb long, to avoid detection of possible genomic DNA contamination; (*ii*) they can discriminate R226 from A2 and A1 adenosine receptors. As shown in Fig. 6, highest expression was observed in the testis. The kidneys, the lungs, and the heart exhibited moderate levels of expression. In the central nervous system, low-level expression was detected in cortex, striatum, and olfactory bulb. It is interesting to compare the tissue distribution of R226 with that of A1 and A2 receptors. A1 and A2 adenosine receptors are highly expressed in the brain regions such as cerebral cortex, hippocampus, cerebellum, and thalamus (8–12). In contrast, R226 has relatively low expression in the central nervous system and is mainly expressed in peripheral tissues. The high expression level of R226 found in the testis suggests that adenosine might play a role in reproduction.

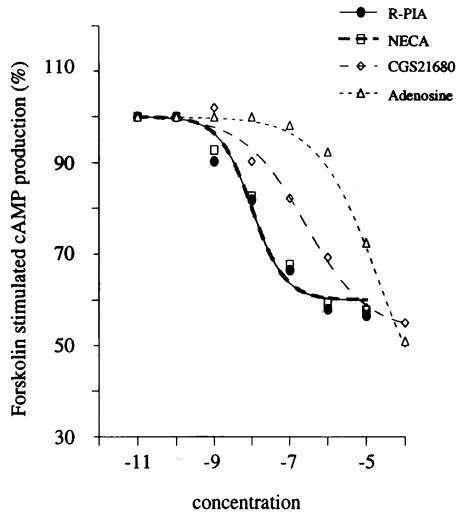

FIG. 5. Dose-dependent inhibition by adenosine agonists of forskolin-stimulated cAMP accumulation in CHO cells stably transfected with R226. Results are shown as percentage maximal stimulation of cAMP accumulation observed with 1 μM forskolin alone. The basal levels of cAMP in the presence of 200 μM Ro 20-1724 were 4.09 ± 0.20 pmol per 10^6 cells. Forskolin increased the cAMP levels to 61.44 ± 2.78 pmol per 10^6 cells. Shown are the results of one of three independent experiments.

DISCUSSION

On the basis of its sequence, the R226 protein belongs to the superfamily of the G-protein-coupled receptors and to the family of the adenosine receptors. Within their putative transmembrane domains, A1 and A2 share 63% amino acid identity. If R226 belonged to a subtype of either A1 or A2, we would expect it to share >63% sequence identity with either A1 or A2 (22, 23). But in its transmembrane domains, R226 is only 58% and 57% identical to A1 and A2, respectively (Fig. 1*B*). Therefore, these comparisons suggest that R226 is probably not a subtype of either A1 or A2 adenosine receptors. The sequence of R226 is the same as the recently reported tgpcr1 cDNA (28). However, the tgpcr1 mRNA was found only in the testis, and the pharmacological profile and biological activity of its protein were not studied.

We found that the R226 receptor binds adenosine receptor agonists with reasonable affinity but without a pharmacological profile consistent with either an A1 or an A2 adenosine receptor (Figs. 2 and 3). In addition, R226 did not bind traditional adenosine receptor antagonists, differentiating it totally from both the A1 and A2 adenosine receptors (2, 6). The R226 receptor was able to inhibit adenylyl cyclase activity through a pertussis toxin-sensitive G protein, but this activity could not be blocked by A1-specific antagonists (Figs. 4 and 5). This suggests that, although R226 is functionally closer to the A1 than to the A2 adenosine receptor, its pharmacological properties are not those of a subtype of A1 adenosine receptor. Therefore, R226 should be considered distinct from both A1 and A2 adenosine receptors. The affinities of adenine nucleotides and nucleosides and UTP for the receptor also suggest that this receptor is not a known subtype of P2 receptors (26). We conclude that R226 is a newly discovered adenosine receptor, and we propose to classify it as the A3 adenosine receptor. We must immediately point out that the relationship between our receptor and the A3 adenosine receptor postulated by Ribeiro and Sebastiao (14) is unknown. Those authors have suggested that there is a distinct adenosine receptor in the brain that is coupled to Ca^{2+} metabolism but no definitive characterization was provided. In addition, this putative receptor is inhibited by alkylxanthines, making it distinct from the A3 adenosine receptor reported here. We propose that newly discovered adenosine receptors, defined by their sequences and functional characterization not to be subtypes of known adenosine receptors, be simply assigned increasing numerical values and, hence, this receptor is the A3 adenosine receptor.

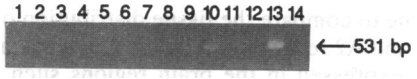

FIG. 6. Tissue distribution of R226 transcripts examined by reverse transcribed PCR. Lanes: 1, striatum; 2, hippocampus; 3, hypothalamus; 4, pituitary; 5, cortex; 6, olfactory bulb; 7, cerebellum; 8, heart; 9, lung; 10, kidney; 11, liver; 12, adipose tissue; 13, testis; 14, negative control.

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1. Stiles, G. L. (1986) *Trends Pharmacol. Sci.* **7**, 486–490.
2. Ramkumar, V., Pierson, G. & Stiles, G. L. (1988) *Prog. Drug Res.* **32**, 195–247.
3. Williams, M. (1987) *Annu. Rev. Pharmacol. Toxicol.* **27**, 315–345.
4. Fredholm, B. & Dunwiddie, T. V. (1988) *Trends Pharmacol. Sci.* **9**, 130–134.
5. Sebastiao, A. M. & Riberio, J. A. (1990) *Br. J. Pharmacol.* **100**, 55–62.
6. Stiles, G. L. (1990) *Clin. Res.* **38**, 10–18.
7. Nakahata, N., Abe, M. T., Matsuoka, I., Uno, T. & Nakanishi, H. (1991) *J. Neurochem.* **57**, 963–969.
8. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M., Dumont, J. E. & Vassart, G. (1989) *Science* **244**, 569–572.
9. Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaegen, J. J., Dumont, J. E., Vassart, G. & Schiffmann, S. (1990) *Biochem. Biophys. Res. Commun.* **173**, 1169–1178.
10. Reppert, S. M., Weaver, D. R., Stehle, J. H. & Rivkees, S. A. (1991) *Mol. Endocrinol.* **5**, 1037–1048.
11. Libert, F., Schiffmann, S. N., Lefort, A., Parmentier, M., Gerard, C., Dumont, J. E., Vanderhaeghen, J. J. & Vassart, G. (1991) *EMBO J.* **10**, 1677–1682.
12. Mahan, L. C., McVittie, L. D., Smyk-Randall, E. M., Nakata, H., Monsma, F. J., Jr., Gerfen, C. R. & Sibley, D. R. (1991) *Mol. Pharmacol.* **40**, 1–7.
13. Lin, W., Marcucci, K. A., Rabin, R. A. & Roth, J. A. (1991) *J. Biol. Chem.* **266**, 14457–14463.
14. Ribeiro, J. A. & Sebastiao, A. M. (1986) *Prog. Neurobiol.* **26**, 179–209.
15. Oliveira, J. C., Sebastiao, A. M. & Riberio, J. A. (1991) *J. Neurochem.* **57**, 1165–1171.
16. Ali, H., Cunha-Melo, J. R., Saul, W. F. & Beaven, M. A. (1990) *J. Biol. Chem.* **265**, 745–753.
17. Zhou, Q.-Y., Grandy, D. K., Thambi, L., Kushner, J. A., Van Tol, H. H. M., Cone, R., Pribnow, D., Salon, J., Bunzow, J. R. & Civelli, O. (1990) *Nature (London)* **347**, 76–80.
18. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
19. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752.
20. Ramkumar, V., Olah, M. E., Jacobson, K. A. & Stiles, G. L. (1991) *Mol. Pharmacol.* **40**, 639–647.
21. Brown, B. L., Albano, J. D., Ekins, R. P. & Sgherzi, A. M. (1971) *Biochem. J.* **171**, 561–562.
22. O'Dowd, B. F., Lefkowitz, R. J. & Caron, M. G. (1989) *Annu. Rev. Neurosci.* **12**, 67–83.
23. Civelli, O., Bunzow, J. R., Grandy, D. K., Zhou, Q.-Y. & Van Tol, H. H. M. M. (1991) *Eur. J. Pharmacol.* **207**, 277–286.
24. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56**, 567–613.
25. Stiles, G. L., Daly, D. T. & Olsson, R. A. (1985) *J. Biol. Chem.* **260**, 10806–10811.
26. Boeynaems, J. M. & Pearson, J. D. (1990) *Trends Pharmacol. Sci.* **11**, 34–37.
27. Nanoff, C., Jacobson, K. A. & Stiles, G. L. (1991) *Mol. Pharmacol.* **39**, 130–135.
28. Meyerhof, W., Muller-Brechlin, R. & Richter, D. (1991) *FEBS Lett.* **284**, 155–160.