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Molecular Cloning and Functional Expression of a Sheep A_3 Adenosine Receptor with Widespread Tissue Distribution

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SUMMARY

Using the polymerase chain reaction, an A_3 adenosine receptor has been cloned from the hypophysial par tuberalis of sheep. The clone encodes a 317-amino acid protein that is 72% identical to the rat A_3 adenosine receptor. In contrast to rat, where abundant A_3 mRNA transcript is found primarily in testis, the sheep transcript is most abundant in lung, spleen, and pineal gland and is present in moderate levels in brain, kidney, and testis. The agonist N^6-amino[^{125}I]iodobenzyladenosine binds with high affinity ($K_d \simeq 6$ nM) and specificity to recombinant A_3 adenosine receptors expressed transiently in COS-1 cells or stably in CHO K1 cells. The potency order of agonists is N^6-aminoiodobenzyladenosine > *N*-ethylcarboxamidoadenosine ≥ (*R*)-phenylisopropyladenosine ≫ cyclopentyladenosine. Little or no binding of purine nucleotides was detected. The potency order of antagonists is 3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenyl-1-propylxanthine (I-ABOPX) ($K_i = 3$ nM) > 1,3-dipropyl-8-(4-acrylate)phenylxanthine (BW-A1433) > 1,3-dipropyl-8-sulfophenylxanthine = xanthine amine cogener ≫ 8-cyclopentyl-1,3-dipropylxanthine. Enprofylline does not bind. These data indicate that, in contrast to A_1 adenosine receptors, A_3 adenosine receptors preferentially bind ligands with aryl rings in the N^6-position of adenine and in the C^8-position of xanthine. Among antagonists, the A_3 adenosine receptor preferentially binds 8-phenylxanthines with acidic versus basic *para*-substituents (I-ABOPX > BW-A1433 > 1,3-dipropyl-8-sulfophenylxanthine = xanthine amine cogener). Agonists reduce forskolin-stimulated cAMP accumulation in Chinese hamster ovary cells stably transfected with recombinant sheep A_3 adenosine receptors; the reduction is blocked by BW-A1433 but not by 8-cyclopentyl-1,3-dipropylxanthine. These data suggest that (i) A_3 adenosine receptors display unusual structural diversity for species homologs, (ii) in contrast to rat, sheep A_3 adenosine receptors have a broad tissue distribution, and (iii) some xanthines with acidic side chains bind with high affinity to A_3 adenosine receptors.

Adenosine acts through specific G protein-coupled membrane-bound receptors, four of which (A_1, A_{2a}, A_{2b}, and A_3) have recently been cloned (reviewed in Ref. 1). Based on radioligand binding and the physiological effects of adenosine analogs on various tissues, A_1, A_{2a}, and A_{2b} adenosine receptors were postulated to exist before they were cloned. A fourth, unanticipated, adenosine receptor subtype with >40% amino acid identity to A_1 and A_2 adenosine receptors, initially called TGPCR1, was cloned from a rat testis cDNA library (2). A subsequently isolated identical clone was designated the A_3 adenosine receptor when binding of the radioligand [^{125}I]APNEA ($K_d = 15.5$ nM) and inhibition of forskolin-stimulated cAMP accumulation with a potency order of NECA = (*R*)-PIA > CGS 21680 were detected in stably transfected CHO cells (3). Of 11 rat tissues examined, Northern blot analysis revealed that rat A_3 mRNA is detectable primarily in testis, particularly in spermatocytes and spermatids but not in spermatogonia (2). Thus, although

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ABBREVIATIONS: [^{125}I]APNEA, N^6-2-(4-amino-3-[^{125}I]iodophenyl)ethyladenosine; PCR, polymerase chain reaction; [^{125}I]IABA, N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine; ABA, N^6-(4-amino-3-benzyl)adenosine; XAC, xanthine amine cogener; CPX, 8-cyclopentyl-1,3-dipropylxanthine; I-ABOPX, 3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine; BW-A1433, 1,3-dipropyl-8-(4-acrylate)phenylxanthine; BW-A533, 1,3-dimethyl-8-(4-acrylate)phenylxanthine; BW-A844, 3-aminophenethyl-8-cyclopentyl-1-propylxanthine; N-0861, (2*S*)-N^6-*endo*-norbornyl-9-methyladenine; SPX, 1,3-dipropyl-8-sulfophenylxanthine; CPA, N^6-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; (*S*)-ENBA, (2*S*)-N^6-*endo*-norbornyladenosine; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; App(NH)p, adenylylimidodiphosphate; SDS, sodium dodecyl sulfate; 1X SSC, standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0); NEM, *N*-ethylmaleimide; NECA, *N*-ethylcarboxamidoadenosine; PIA, phenylisopropyladenosine; SPX, 8-*p*-sulfophenyl-1,3-dipropylxanthine; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s).

it is possible to detect low levels of A_3 adenosine receptor mRNA in rat brain with reverse transcription PCR, speculation on the function of the A_3 adenosine receptor has centered on a possible role in reproduction, based on the primarily testicular distribution of the transcript (2, 3).

We now report the cloning of an adenosine receptor cDNA, designated S17, from sheep that appears to encode a species homolog of the rat A_3 adenosine receptor. The tissue distribution of A_3 receptor mRNA is markedly different in rat and sheep, suggesting other roles for the A_3 adenosine receptors in non-rodents. Furthermore, we report that the agonist [^{125}I] IABA is an improved radioligand for A_3 adenosine receptors and the acidic 8-phenylxanthines I-ABOPX and BW-A1433 are potent antagonists.

Materials and Methods

Drugs were obtained from Sigma Chemical Co. (St. Louis, MO) with the following exceptions: (*S*)-ENBA, CPT, NECA, and CPA were from Research Biochemicals, Inc. (Natick, MA); I-ABOPX (BW-A522), BW-A1433, BW-A533, BW-A844, SPX, IABA, CPX, and enprofylline were gifts of the Burroughs Wellcome Co. (Research Triangle Park, NC); N-0861 was a gift from Whitby Research Inc. (Richmond, VA); APNEA was from Dr. Ray Olsson of the University of South Florida (Tampa, FL); Ro-20–1724 was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA); adenosine deaminase was from Bohringer Mannheim Biochemicals (Indianapolis, IN); and [^{3}H]CPX was purchased from NEN. Ligands were radioiodinated and purified by high performance liquid chromatography essentially as described (4).

PCR. Poly(A)$^+$ RNA was prepared, primed with oligo(dT), and reverse transcribed as described previously (5). The first-strand cDNA was subjected to two rounds of 30 cycles of amplification with 1 μg each of two oligonucleotide primers, A [TCAGAATTCAT(A/C/T)(A/G/T)(C/G)(A/C/G/T)(A/C/G)T(C/G/T)GA(C/T)(A/C)G(A/C/G)TA] and B [TTCAAGCTT(C/G)(A/C/T)(A/G)TA(A/C/G/T)A(C/T)(A/C/G)A(C/G/T)(A/C/G/T)GG(A/G)TT]. Each reaction cycle consisted of incubations at 94° for 1.5 min, 42° for 2 min, and 72° for 2 min, with AmpiTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). The amplified DNA was digested with *Hind*III and *Eco*RI and separated on an agarose gel. DNA bands of approximately 500–900 bp were subcloned into M13 (GIBCO/BRL, Baltimore, MD), and recombinant clones were sequenced (see below).

cDNA library screening. A sheep pars tuberalis library constructed in the λ ZAP II vector (Stratagene, La Jolla, CA) was transferred to Colony Plaque Screen filters (New England Nuclear, Boston, MA). The filters were screened under stringent conditions (50% formamide, 1 M sodium chloride, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm, at 42°). Filters were soaked in 2× SSC/1% SDS at 65° for 1 hr. λ phage that hybridized to the probe were plaque-purified. *In vivo* excision and rescue of pBluescript plasmids from the hybridizing λ ZAP II were performed according to the manufacturer's (Stratagene) instructions.

DNA sequencing. Nucleotide sequences were analyzed by the dideoxynucleotide chain termination method of Sanger *et al.* (6), using Sequenase (United States Biochemical, Cleveland, OH). Sequencing template was either double-stranded plasmid or single-stranded phage. Primers were synthetic oligonucleotides that were either vector specific or derived from sequence information.

Expression studies. For acute transfection studies, S17 was subcloned into pcDNA1 (InVitrogen, San Diego, CA) and introduced into COS-1 cells using the DEAE-dextran method (7). For stable transfection studies, S17 was subcloned into pcDNAI/NEO and introduced into CHO-K1 cells using Lipofectin (GIBCO/BRL) (8). For some studies we used CHO-K1 cells stably transfected with rat A_{2a} and rat A_{2b} (9) receptors.

Membrane preparation and radioligand binding. COS-1 cells were grown in DMEM with 7.5% fetal calf serum for 60 hr after transfection. CHO cells were grown in Ham's F12 medium with 10% fetal calf serum and 0.5 mg/ml G418. For radioligand binding assays cells were washed in phosphate-buffered saline, homogenized in 10 mM EDTA, 10 mM Na-HEPES, pH 7.4, 0.1 mM benzamidine, and centrifuged at 20,000 × *g* for 20 min. Pellets were resuspended and washed twice in 1 mM EDTA, 10 mM Na-HEPES, pH 7.4, 0.1 mM benzamidine, and were resuspended in the same buffer with 10% (w/v) sucrose, at a membrane protein concentration of 1 mg/ml. Membranes were frozen in aliquots at −20°. For radioligand binding to A_3 receptors, cell membranes were incubated in 0.1 ml for 3 hr at 21° with 5 mM $MgCl_2$, 1 unit/ml adenosine deaminase, and 0.5–1 nM [^{125}I]IABA (4). In preliminary studies, two additional radioligands, N^6-(4-hydroxyl-3-[^{125}I]iodophenyl)isopropyladenosine and [^{125}I]APNEA, were evaluated. Both had somewhat lower affinities for the A_3 receptor and lower ratios of specific/nonspecific binding. For equilibrium binding assays the specific activity of [^{125}I]IABA was reduced 10–20-fold with the nonradioactive compound. Nonspecific binding was measured in the presence of 1 or 5 μM IABA, 100 μM NECA, or 100 μM BW-A1433, with equivalent results. The IC_{50} of competitive inhibitors was derived from a three-parameter logistic equation. K_i values were derived from IC_{50} values as described (10). All data points represent the means of triplicate determinations.

cAMP studies. Untransfected CHO cells or cells stably transfected with rat A_{2a}, rat A_{2b}, or sheep A_3 adenosine receptors were grown to confluency on 24-well cluster plates. The medium was changed to serum-free HEPES (10 mM, pH 7.4)-buffered DMEM containing 1 unit/ml adenosine deaminase, and the cells were preincubated at 37° for 1 hr in a shallow water bath. Fresh medium containing drugs was added for 90 sec, removed, and replaced with 550 μl of 0.1 N HCl. cAMP in the acid extract (500 μl) was acetylated by the addition of 22.5 μl of triethylamine/acetic anhydride (3.5:1) and was quantified by automated radioimmunoassay (11). In some experiments cAMP determinations were made on cells in suspension, resulting in improved reproducibility. Cells were removed from plates by treatment with 5 mM EDTA in PBS for 5–10 min, washed twice with and resuspended in serum-free HEPES/DMEM containing 1 unit/ml adenosine deaminase and 20 μM Ro-20–1724, and pipetted into test tubes (50,000–60,0000 cells/0.2 ml, 21°). Drugs were added in 50-μl aliquots and the tubes were transferred to a 37° shaker bath for 15 min. Assays were terminated by the addition of 500 μl of 0.15 M HCl.

Northern analysis. Poly(A)$^+$ RNA was subjected to electrophoresis through a 1% agarose-formaldehyde gel, blotted onto GeneScreen (New England Nuclear), and hybridized with ^{32}P-labeled S17; DNA probes were labeled with [α-^{32}P]dCTP (2000 Ci/mmol) to a specific activity of >10^9 cpm/μg by the method or random priming (Promega, Madison, WI). Hybridizing conditions were 50% formamide, 1 M sodium chloride, 1% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm, at 42° overnight. The final washing of blots was in 0.2× SSC/0.1% SDS at 65° for 40 min. Blots were exposed at −80° to X-ray film with an intensifying screen.

Results

Cloning studies. Using PCR amplification with a pair of degenerate oligonucleotide primers based on regions of the third and seventh transmembrane domains that are highly conserved among several monoamine and peptide receptors, cDNA fragments of several novel G protein-coupled receptors were cloned from the hypophysial pars tuberalis of sheep. Pars tuberalis is a thin sheath of pituitary tissue that covers the ventral surface of the median eminence, surrounds the pituitary stalk, and extends onto the ventral surface of the anterior pituitary gland. This tissue contains high concentrations of receptors for the pineal hormone melatonin (12).

One of the novel cDNA fragments cloned from sheep pars

tuberalis (520 bp) showed greatest identity with members of the adenosine receptor subfamily of G protein-coupled receptors (see below). This fragment was random prime-labeled and used to screen a sheep pars tuberalis library. From 1×10^6 recombinants, several positively hybridizing clones were identified. The restriction map and nucleotide sequence of a clone containing the largest insert, designated S17, are shown in Fig. 1. The clone contains an open reading frame encoding a protein of 317 amino acids. The flanking sequence of the first methionine in this reading frame displays a Kozak consensus sequence for the initiation of translation (13). The initiating methionine is preceded by an upstream, in-frame stop codon. The open reading frame is followed by approximately 400 bp of 3′ untranslated sequence. Hydropathy analysis of the predicted amino acid sequence revealed the presence of seven hydrophobic segments (data not shown), a characteristic feature of G protein-coupled receptors.

Computer searching revealed that the highest homology score of the protein encoded by S17 was with the rat A_3 adenosine receptor (72% overall identity) (Fig. 2). Within the putative transmembrane segments the amino acid identity is 83%. The most dissimilar regions between S17 and the rat A_3 receptor include the amino and carboxyl termini and the second and third extracellular loops. Comparison of S17 with A_1, A_{2a}, and A_{2b} adenosine receptors revealed identity scores of 51%, 47%, and 42%, respectively (Fig. 3).

Expression of S17. To confirm the identity of the receptor encoded by S17, the cDNA was transiently expressed in COS-1 cells and stably expressed in CHO-K1 cells. These cell lines express very low levels of endogenous adenosine receptors. In preliminary experiments it was found that binding of [^{125}I] IABA to CHO cells stably transfected with S17 was inhibited equally well by a large molar excess of nonradioactive IABA (1

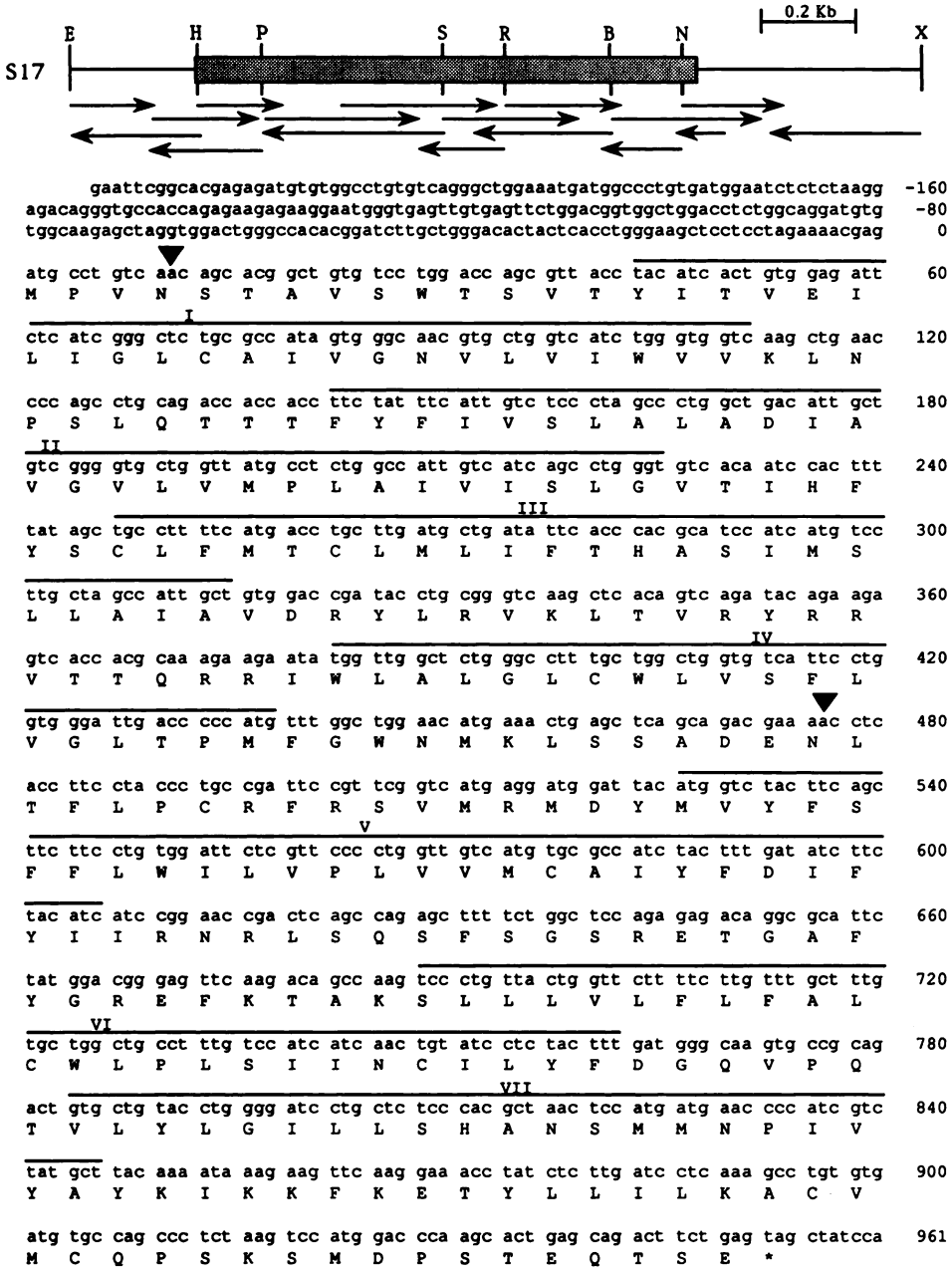

Fig. 1. *Top*, restriction map of S17. *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*cII; *N*, *Nco*I; *P*, *Pst*I; *R*, *Eco*RV; *S*, *Sty*I; *X*, *Xho*I. The sequencing protocol (*arrows*) is depicted below the restriction map. *Bottom*, nucleotide sequence of S17 and deduced amino acid sequence. Nucleotide sequence is numbered from the initiator methionine, as indicated to the *right of each line*. The putative transmembrane regions are *underlined and numbered* (*I-VII*). ▼, Consensus site for *N*-linked glycosylation.

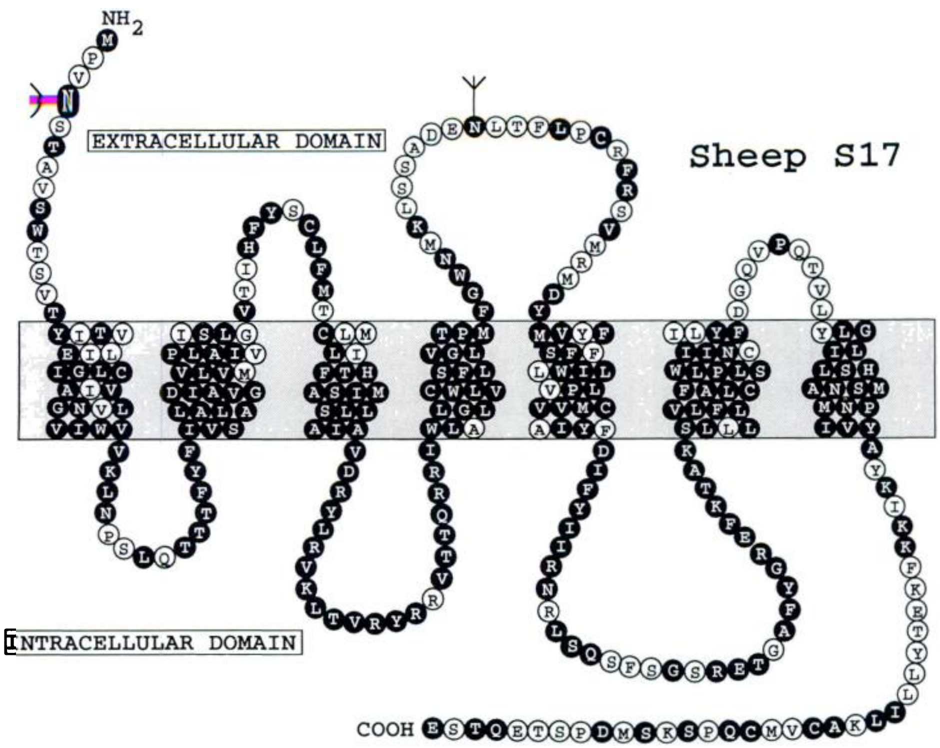

Fig. 2. Structural comparison of the protein encoded by S17 and the rat A_3 adenosine receptor. Depicted is the predicted membrane topology of the S17 protein. *Y*, Potential *N*-linked glycosylation sites. Amino acids that are identical between the two receptors are *shaded*.

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                                  I                                     II                                III
Sheep S17  MPVNSTAVSWTSVTYITVEILIGLCAIVGNVLVIWVVKLNPSLQTTTFYFIVSLALADIAVGVLVMPLAIVISLGVTIHFYSCLFMTCLMLIFTHASIMS  100
Rat A1        MPPYI-AFQAA--GI-V--A-VSVP-------A--V-QA-RDA--C------V--V---A--I----L-NI-PQTY-HT--MVA-PV--L-QS--LA   97
Rat A2a          MG-S..-.-----LA-AVL--L-----C-A-WI-SN--NV-NF-V----A--------AI-F--T--T-FCAACHG---FA-FV-VL-QS--F-   94
Rat A2b        MQLETQDAL-VAL-LV-AA--VA-----CAA-GASSA---P-N--L----T--V---LFAI-F--T----FCTD-H----LA-FV--L-QS--F-   95

                                      IV
Sheep S17  LLAIAVDRYLRVKLTVRYRRVTTQRRIWLALGLCWLVSFLVGLTPMFGWNMKL......SSADENL........TF.....LPCRFRSVMRMDYMVYFSF  181
Rat A1     ------------IPL--KT-V----AAV-IAG--IL-LV----------.N-......-VVEQDWRANGSVGEPV.....IK-E-EK-IS-E-----N-  185
Rat A2a    -----I---IAIRIPL--NGLV-GV-AKGIIAI---L--AI-----L---.NC......-QK-G-S......TK-CGEGR.VT-L-ED-VP-N----YN-  182
Rat A2b    ---V-----LAIRVPL--KGLV-GT-ARGIIAVL-VLA-GI----FL---S-DRATSNCTEPGDGI........-NKSCCPVK-L-EN-VP-S-----N-  187

                V                                      VI
Sheep S17  FLWILVPLVVMCAIYFDIFYIIRNRLSQ..S..FSGSR.ETGAFYGREFKTAKSLLLVLFLFALCWLPLSIINCILYF..DGQV....PQT...VLYLGI  267
Rat A1     -V-V-P--LL-VL--LEV--L--KQ-..NKK..V-A-SGDPQKY--K-L-I----A-I------S----H-L---TL-CPTC-K....-SI...LI-IA-  274
Rat A2a    -AFV-L--LL-L---LR--LAA-RQ-K-ME-QPLP-E-..-RSTLQK-VHA----AIIVG---------H----FTF-..CSTCRHAP-....WLM--A-  274
Rat A2b    -GCV-P--LI-MV--IK--MVACKQ-.-.HMELMEH--.T-...LQ--IHA---LAMI-GI-------VHA----TL-..HPALAKDK-KW...-MNVA-  276

             VII
Sheep S17  LLSHANSMMNPIVYAYKIKKFKETYLLILKACVMCQPSKSMDPSTEQTSE  317
Rat A1     F-T-G--A-------FR-H--RV-F-KWNDHFR.---KPPI-EDLPEEKAED  326
Rat A2a    I---S--VV--FI---R-RE-RQ-FRK-IRTH-L.RRQEPFQAGGSSAWALAAHSTEGEQVSLRLNGHPLGVWANGSATHSGRRPNGYTLGLGGGGSAQG  373
Rat A2b    -------VV-------RNRD-RYSFHR-ISRY-L--.TDTKGG-GQAGGQSTFSLSL 332

Rat A2a    SPRDVELPTQERQEGQEHPGLRGHLVQARVGASSWSSEFAPS  410
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Fig. 3. Comparison of S17 with the rat A_1, A_{2a}, and A_{2b} adenosine receptors. In the rat sequences, *hyphens* indicate identity with the S17 receptor. To maximize homologies, gaps (*dots*) have been introduced in the four sequences. The seven presumed transmembrane domains (*I-VII*) are highlighted by *solid lines*.

μM), by 100 μM NECA, or by 100 μM levels of the acidic 8-phenylxanthine BW-A1433 (Fig. 4A). We concluded that the residual binding was nonspecific. Specific binding was not inhibited by 1 μM levels of the potent A_1 receptor antagonist CPX. The K_d of [^{3}H]CPX binding to sheep pineal gland was found to be 1.09 nM (B_{max} = 0.7 pmol/mg of protein) (data not shown). Thus, CPX in the range of 0.1–1 μM should be effective in blocking A_1 but not A_3 adenosine receptors. No specific [^{125}I]IABA binding was found in nontransfected CHO cells (data not shown).

To determine whether [^{125}I]IABA is an agonist at the S17 receptor, we evaluated the effects of NEM and GTPγS on binding to CHO cell membranes (Fig. 4B). NEM has been shown to selectively uncouple A_1 adenosine receptors from the pertussis toxin-sensitive subpopulation of G proteins (14, 15). NEM and GTPγS reduced specific binding of [^{125}I]IABA by 70–75%, indicating that the radioligand is an agonist and suggesting that most and possibly all of the G proteins coupled to S17 receptors of transfected CHO cells belong to the G_o/G_i family. Residual specific binding may represent receptors that are uncoupled from G proteins.

Fig. 5 shows equilibrium binding of [^{125}I]IABA to recombinant S17 receptors expressed in CHO cells. Specific radioligand binding also was detected in membranes prepared from tran-

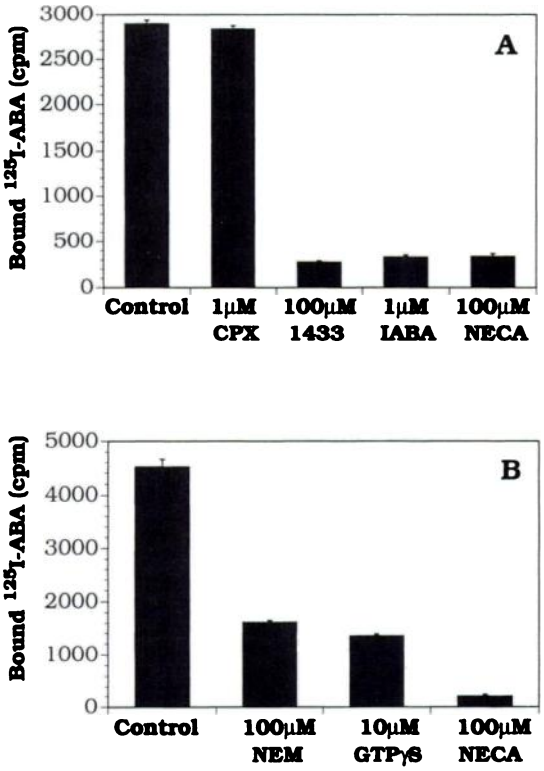


Fig. 4. Inhibition of ^{125}I-ABA binding to S17 receptors of transfected CHO cells by competitive ligands, GTPγS, and NEM. A, Competition by saturating concentrations of competitive ligands. Protein, 30 μg/tube; ^{125}I-ABA, 150,000 cpm/tube (0.40 nM). B, Inhibition of ^{125}I-ABA binding by GTPγS, NEM, or NECA. Protein, 25 μg/tube; ^{125}I-ABA, 133,000 cpm (0.36 nM). Similar results were obtained in a replicate experiment.

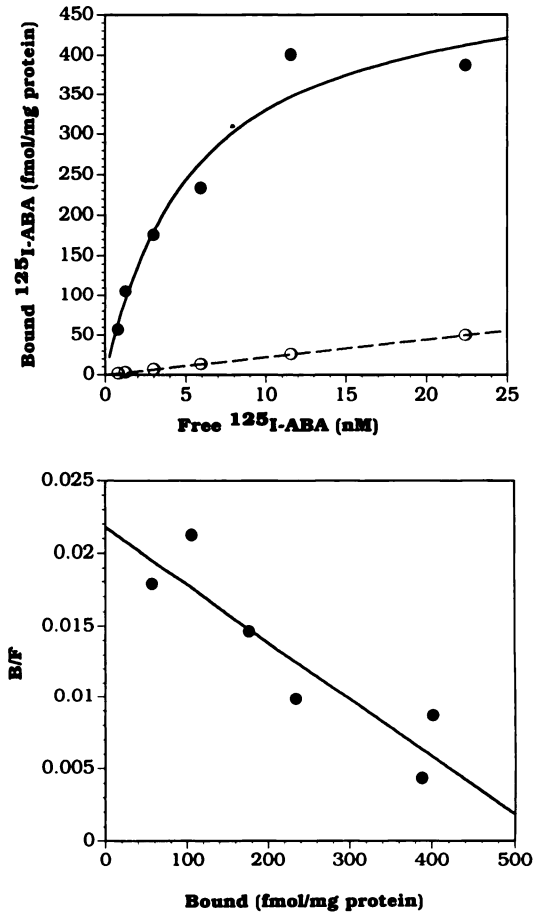


Fig. 5. Equilibrium binding of ^{125}I-ABA to membranes of CHO cells transfected with S17. *Top*, specific and nonspecific (5 μM IABA) binding. *Bottom*, Scatchard transformation; B_{max} = 549 fmol/mg of protein, K_d = 7.2 nM (25 μg of protein/tube). Similar binding was observed in a replicate experiment.

siently transfected COS cells, but the B_{max} was substantially lower (40 fmol/mg of protein). Nearly identical dissociation constants were found in COS and CHO cell membranes (4–8 nM, four experiments). The low B_{max} in COS cell membranes may reflect poor coupling of receptors to G proteins in transiently transfected COS cells and relatively better coupling after stable transfection in CHO cells. As an agonist, [^{125}I] IABA is likely to bind with high affinity only to receptor-G protein complexes.

Fig. 6 shows competition by several adenosine agonists and antagonists for binding to S17 receptors. Dissociation constants and structures of these and additional ligands are summarized in Fig. 7. The potency order for agonists was IABA > NECA ≥ (*R*)-PIA > (*S*)-PIA ≫ CPA = (*S*)-ENBA. The nucleotides AMP, ADP, and App(NH)p (100 μM) did not compete for [^{125}I] IABA binding; 100 μM ATP reudced binding by 28 ± 5%. Among antagonists the potency order was BW-A1433 > 8-SPT = XAC > CPX = N-0861 > 8-CPT. Caffeine and enprofylline (100 μM) produced little inhibition of radioligand binding. These potency rank orders are clearly distinct from those of any of the other adenosine receptors evaluated to date. Because the acidic 8-phenylxanthine BW-A1433 is a potent antatonist, we screened several other structurally similar compounds. I-ABOPX was found to be even more potent than BW-A1433, with a K_i of 3 nM. Although I-ABOPX and BW-A1433 bound with high affinity to sheep A_3 adenosine receptors, they also were potent antagonists of A_1 adenosine receptors (K_i = 20–25 nM, based on competition for [^{3}H]CPX binding to sheep pineal gland membranes) (data not shown). Thus, BW-A1433 is a potent but nonselective antagonist of A_1 and A_3 adenosine receptors and I-ABOPX is somewhat A_3 selective.

cAMP studies. We next examined whether S17 is coupled to the cAMP regulatory system. The nonselective agonist NECA enhanced forskolin-stimulated cAMP accumulation in CHO cells stably transfected with rat A_{2a} or A_{2b} adenosine receptors but significantly reduced forskolin-stimulated cAMP accumulation in the same cells transfected with the S17 receptor (Fig. 8A). The cAMP responses were inhibited by antagonists with a pharmacological profile corresponding to the radioligand binding data, i.e., 1 μM CPX, a concentration sufficient to block A_1 but not A_3 receptors, was ineffective as an inhibitor of the NECA response but 100 μM BW-A1433, sufficient to block A_1 and A_3 receptors, reversed the effect of NECA (Fig. 8B). IABA was much more potent as an agonist than was the A_1-selective agonist CPA (Fig. 8C).

Northern blot analysis. Northern blot analysis of several ovine tissues probed with S17 revealed a major hybridizing transcript of 2.0 kilobases (Fig. 9). In brain, the transcript is modestly expressed in cerebral cortex, striatum, hypothalamus, and cerebellum. Outside the brain, the transcript is prominently located in lung, spleen, pars tuberalis, and pineal gland. Moderate hybridization signals were detected in kidney and testis.

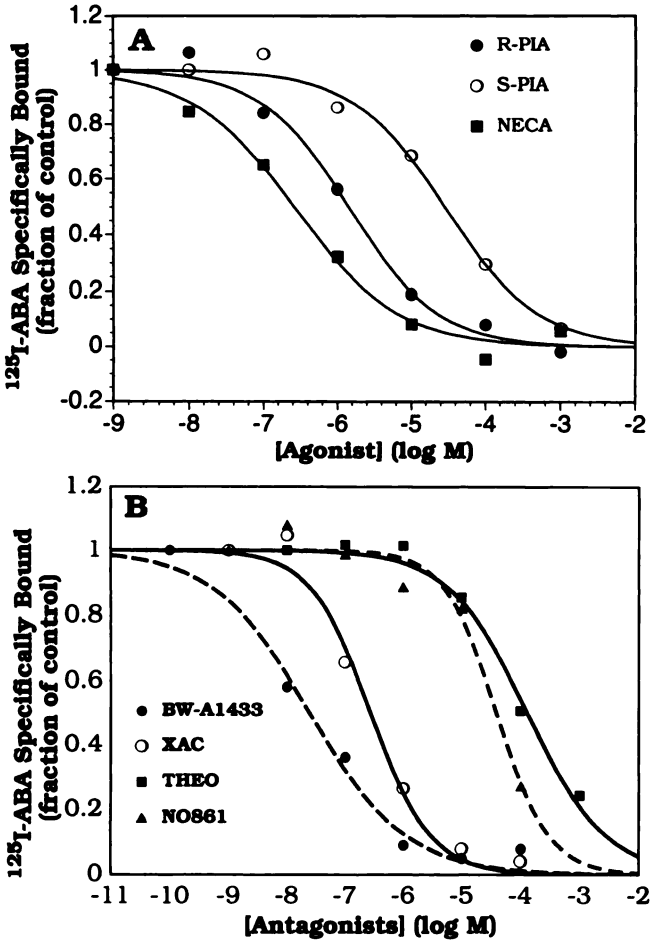


Fig. 6. Competition by various ligands for ^{125}I-ABA binding to membranes of CHO cells transected with S17. Binding is plotted as a fraction of control specific binding. A, Agonists; B, antagonists. Protein, 25 μg/tube; ^{125}I-ABA, 200,000–300,000 cpm/tube (0.54–0.81 nM). K_i determinations for these and other ligands are summarized in Fig. 7. Hill coefficients ranged from 0.66 to 1.01 (mean = 0.83). THEO, theophylline

Discussion

We report the cloning of a novel adenosine receptor cDNA designated S17. The cDNA is similar to a rat clone initially designated TGPCR1 (2) and subsequently found to encode the A_3 adenosine receptor (3). This similarity suggests that S17 is the sheep species homolog (ortholog) of the rat A_3 receptor; hence, we refer to it as the sheep A_3 adenosine receptor. However, the rat and sheep clones differ in some noteworthy respects. The rat A_3 adenosine receptor transcript is confined primarily to testis, and it has been suggested that the physiological role of this receptor is probably limited to this tissue. In striking contrast, the sheep A_3 adenosine receptor transcript has a much broader tissue distribution, with high levels being found in lung, spleen, pars tuberalis, and pineal gland; moderate levels are found in testis, kidney, and most regions of the brain.

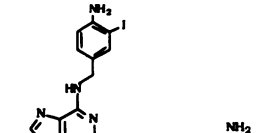
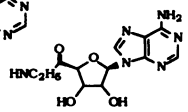
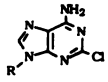
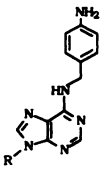
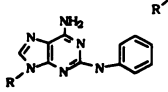
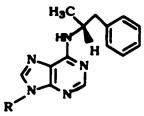
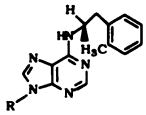
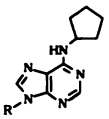
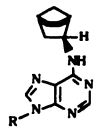
It is worth noting that the cloned rat and sheep A_3 adenosine receptors appear to be distinct from the "A_3 adenosine receptor" proposed on the basis of pharmacological criteria (16). Additional subtypes also have been proposed on the basis of subtle differences in structure-activity profiles in different tissues (reviewed in Ref. 1). Because the existence of uncloned putative subtypes is uncertain, we prefer the use of an adenosine receptor nomenclature based on cloned receptors (1).

The rat and sheep A_3 adenosine receptors are structurally and functionally similar. The receptors are identical in size (317 amino acids) and are coupled to inhibition of cAMP accumulation in stably transfected CHO cells. The amino terminus of the sheep A_3 adenosine receptor is short but contains one potential *N*-linked glycosylation site, similarly to the rat A_3 adenosine receptor. None of the other adenosine receptor subtypes (A_1, A_{2a}, and A_{2b}) has glycosylation sites in the amino-terminal region. The second extracellular loop of S17 also contains a potential *N*-linked glycosylation site, which is similar to that found in the proteins of all cloned adenosine receptors. In the carboxyl-tail region, Cys^{302}, which is part of a cysteine-glutamine pair of amino acids that is conserved between rat and sheep, is a potential palmitoylation site that may be involved in the formation of a fourth intracellular loop (17). This cysteine-glutamine pair also is found in the same position (21 amino acids beyond the seventh transmembrane domain) in the carboxyl-terminal regions of all species of A_1, A_{2b}, and A_3 adenosine receptors cloned to date (dog, rat, and human). All A_{2a} adenosine receptors lack such a potential palmitoylation site and also differ from the other adenosine receptors in having much longer carboxyl tails.

Despite the similarity between the rat and sheep A_3 adenosine receptors, they display unusual structural diversity for orthologs; they are only 72% identical in amino acid sequence. In contrast, orthologs of rat and canine A_1 and A_{2a} adenosine receptors are 92% and 85% identical, respectively. This suggests that the A_3 adenosine receptor may have undergone evolutionarily recent mutational changes. It is also possible that the rat and sheep receptors represent different A_3 subtypes (paralogs). However, we have not yet been able to confirm this speculation by isolating distinct A_3 adenosine receptor cDNAs from a single species. Also, it is not clear whether the rat and sheep A_3 adenosine receptor genes are orthologs or paralogs based on genetic criteria reviewed by Hartig *et al.* (18).

Zhou *et al.* (3) reported that agonists bind to the rat A_3 adenosine receptor with a potency order of NECA = (*R*)-PIA > (*S*)-PIA > ATP > ADP and that xanthine antagonists (CPX and XAC) do not bind. In this study, we have undertaken a more extensive characterization, which reveals some interesting new information about differences between A_3 adenosine receptors and their closest pharmacological relatives, A_1 adenosine receptors. Of note among the agonists is the very low affinity of A_3 adenosine receptors for N^6-substituted compounds with saturated rings, i.e., CPA and (*S*)-ENBA. Thus, for A_1 adenosine receptors the potency order is (*S*)-ENBA = CPA ≥ (*R*)-PIA, whereas for A_3 adenosine receptors the potency order is (*R*)-PIA ≫ *S*-ENBA = CPA. The highest affinity agonist is IABA, which binds more tightly than the noniodinated compound ABA. Iodination of ABA also increases its affinity for A_1 adenosine receptors (4).

Our data suggest that, although the binding profiles of the sheep and rat A_3 adenosine receptors are similar, there appear to be some differences in the binding of adenine nucleotides and XAC. ATP and ADP were reported to bind weakly (IC_{50} = 10–100 μM) to rat A_3 receptors. We found no inhibition of binding by 100 μM AMP or ADP. A small inhibition (28%) by 100 μM ATP might be attributable to contamination or enzymatic formation of GTP from ATP, rather than to direct binding to A_3 adenosine receptors. Two lines of evidence support this idea; (i) ATP failed to reduce cAMP accumulation in

Agonist	K_I (μM) Mean ± SE (n=2-3)
 IABA*	0.006 ± 0.001
 NECA	0.300 ± 0.049
 2-Cl-ADO	0.51 ± 0.17
 ABA	0.913 ± 0.25
 CV1808	1.19 ± 0.046
 *R*-PIA	1.21 ± 0.028
 *S*-PIA	13.72 ± 5.83
 CPA	>100 •
 *S*-ENBA	>100 •

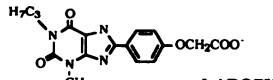
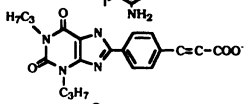
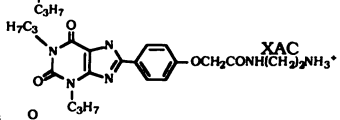
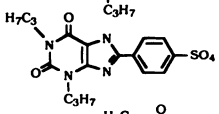
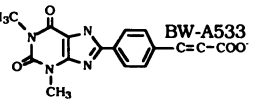
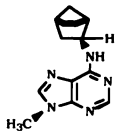
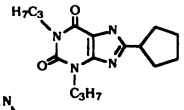
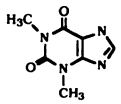
Antagonist	K_I (μM) (Mean ± SE, n=2-3)
 I-ABOPX	0.003 ± 0.001
 BW-A1433	0.021 ± 0.001
 XAC	0.18 ± 0.042
 SPX	0.183 ± 0.061
 BW-A533	7.33 ± 0.194
 N0861	41.4 ± 2.90
 CPX	49.3 ± 14.28
 Theophylline	424.0 ± 166

Fig. 7. Chemical structures and dissociation constants of competitive inhibitors of the S17 receptor. Competition curves such as those shown in Fig. 6 were used to calculate IC_{50} values for competing agonists and antagonists. *R*, ribose. The compounds are listed in descending order of affinity. *, < 20% inhibition at 100 mM, two experiments. The K_d of ^{125}I-ABA was calculated from radioligand binding to CHO cell membranes. *2-Cl-ADO*, 2-chloroadenosine.

stably transfected CHO cells (3), suggesting that it does not activate the rat A_3 adenosine receptor, and (ii) we found no detectable binding of the nonhydrolyzable ATP analog App(NH)p (100 μM) to the sheep A_3 adenosine receptor.

We concur with Zhou *et al.* (3) that XAC and CPX have much lower affinities for A_3 than for A_1 adenosine receptors, but we detected a much larger decrease in the affinity of CPX, compared with that of XAC. Moreover, we have discovered that certain xanthine antagonists bind with high affinity to sheep A_3 adenosine receptors. In this regard, it is interesting that CPX, with a saturated ring in the C^8-position, is far less potent than compounds with aryl rings (I-ABOPX, BW-A1433, and XAC). Van Galen *et al.* (19) have postulated that the N^6-position of adenines and the C^8-position of xanthines occupy the same position in the binding pocket of the A_1 adenosine receptor. Because replacing phenyl with cyclopentyl rings at the N^6/C^8-positions of adenines/xanthines produces the same effect on binding to sheep A_3 adenosine receptors, i.e., markedly reducing affinity, our data are consistent with the idea that this N^6-C^8 orientation also holds for A_3 adenosine receptors. Furthermore, the data suggest that receptor amino acids involved in binding to these ligand moieties differ between the two receptor subtypes, such that the A_1 adenosine receptor can interact more avidly with saturated rings.

Another notable difference between the antagonist binding profiles of A_1 and A_3 adenosine receptors is in the effect of acidic and basic substituents added to the *para*-phenyl position of 8-phenylxanthines. For A_1 adenosine receptors the potency order was XAC (weak base) > BW-A1433 = I-ABOPX (weak acids) > SPX (strong acid). For the sheep A_3 adenosine receptor the potency order was I-ABOPX > BW-A1433 > XAC = SPX. These data suggest a difference in the A_1 versus A_3 adenosine receptor binding pockets, such that the A_1 adenosine receptor interacts more strongly with a basic side chain and the A_3 adenosine receptor interacts more strongly with an acidic side chain.

The radioligands that have been used to characterize A_3 adenosine receptors (IABA and [^{125}I]APNEA) both also bind to A_1 receptors. To detect binding of these radioligands to A_3 adenosine receptors in tissues that also contain A_1 adenosine receptors, e.g., brain and testis, it will be necessary to selectively block binding to A_1 adenosine receptors. Our preliminary unpublished data suggest that addition of 100 nM CPX to binding assays is suitable for this purpose in sheep.

Both the rat and sheep A_3 adenosine receptors produce a modest 40–50% inhibition of forskolin-stimulated cAMP levels in transfected cells. The rat response was shown to be prevented by pertussis toxin (3). In this study we found that the binding

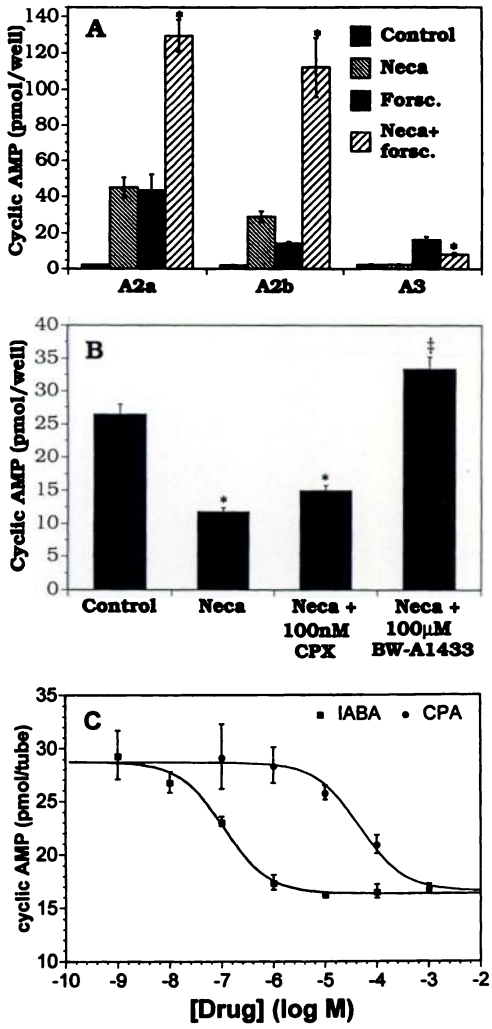


Fig. 8. S17 receptor-mediated inhibition of cAMP accumulation in CHO cells. All incubations contained 1 unit/ml adenosine deaminase. A, CHO cells stably transfected with rat A_{2a} and A_{2b} adenosine receptors and sheep A_3 adenosine receptors in cluster wells were incubated for 1 min with 10 μM NECA or 10 μM forskolin (*Forsc.*) as indicated. *, Different from forskolin alone ($p < 0.01$, four experiments). B, CHO cells transfected with S17 receptors were incubated for 1 min with 50 μM forskolin (all cells), 10 μM NECA, and other drugs as indicated. *, Less than control ($p < 0.05$); ‡, greater than NECA or NECA plus CPX ($p < 0.01$, four experiments). C, Supended CHO cells transfected with S17 adenosine receptors were incubated for 15 min with 5 μM forskolin, 20 μM levels of the phosphodiesterase inhibitor Ro-20–1724, and various concentrations of drugs as indicated (three experiments). Similar findings were obtained in two or three replicate experiments.

of [^{125}I]IABA to the sheep A_3 adenosine receptor was reduced by NEM, which alkylates the same G protein cysteine residue that is ADP-ribosylated by pertussis toxin (16). The fact that the inhibition of cAMP accumulation by A_3 adenosine receptor activation is modest, coupled with the fact that pertussis toxin-sensitive G proteins (G_i and G_o forms) have been found to couple to numerous effectors (21), suggests that additional effectors for A_3 adenosine receptors may exist.

Acknowledgments

We thank Rick Conron and John Lee for expert assistance, John Hadcock of the American Cyanamid Co. (Princeton, NJ) for suggesting a protocol for measuring cAMP in suspensions of CHO cells, and Cheryl Murphy of the University of Virginia Diabetes Center from providing COS cells used for transfection studies.

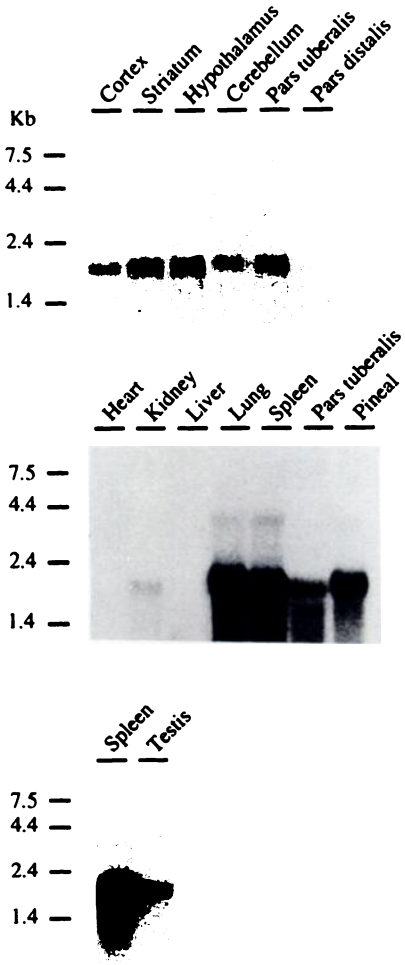


Fig. 9. RNA analysis of S17 receptor transcripts in sheep tissues. Each *lane* contained 5 μg of poly(A)$^+$ RNA. Locations of RNA size markers (BRL) are indicated (*left*). The three blots shown were hybridized separately. The pars tuberalis RNA of the *top two blots* was from the same batch. Similar results were obtained with two other batches of spleen and testis RNA.

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