Characterization of the A_{2B} Adenosine Receptor from Mouse, Rabbit, and Dog

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ABSTRACT

We have cloned and pharmacologically characterized the A_{2B} adenosine receptor (AR) from the dog, rabbit, and mouse. The full coding regions of the dog and mouse A_{2B}AR were obtained by reverse transcriptase-polymerase chain reaction, and the rabbit A_{2B}AR cDNA was obtained by screening a rabbit brain cDNA library. It is noteworthy that an additional clone was isolated by library screening that was identical in sequence to the full-length rabbit A_{2B}AR, with the exception of a 27-base pair deletion in the region encoding amino acids 103 to 111 (A_{2B}AR₁₀₃₋₁₁₁). This 9 amino acid deletion is located in the second intracellular loop at the only known splice junction of the A_{2B}AR and seems to result from the use of an additional 5' donor site found in the rabbit and dog but not in the human, rat, or mouse sequences. [³H]3-isobutyl-8-pyrrolidinoxanthine and 8-[4-[(4-cyano-[2,6-³H]-phenyl)carbamoylmethyl]oxy]phenyl]-

1,3-di(*n*-propyl)xanthine ([³H]MRS 1754) bound with high affinity to membranes prepared from human embryonic kidney (HEK) 293 cells expressing mouse, rabbit, and dog A_{2B}ARs. Competition binding studies performed with a panel of agonist (adenosine and 2-amino-3,5-dicyano-4-phenylpyridine analogs) and antagonist ligands identified similar potency orders for the A_{2B}AR orthologs, although most xanthine antagonists displayed lower binding affinity for the dog A_{2B}AR compared with A_{2B}ARs from rabbit and mouse. No specific binding could be detected with membranes prepared from HEK 293 cells expressing the rabbit A_{2B}AR₁₀₃₋₁₁₁ variant. Furthermore, the variant failed to stimulate adenylyl cyclase or calcium mobilization. We conclude that significant differences in antagonist pharmacology of the A_{2B}AR exist between species and that some species express nonfunctional variants of the A_{2B}AR due to "leaky" splicing.

Four G protein-coupled receptors mediate the biological actions of adenosine designated A₁, A_{2A}, A_{2B}, and A₃ adenosine receptors (AR) (Fredholm et al., 2001). A_{2B}ARs have the unique property of coupling in cells to both G_s and G_q proteins, leading to a rise in intracellular cAMP and calcium levels (Auchampach et al., 1997; Feoktistov and Biaggioni, 1997; Gao et al., 1999; Fredholm et al., 2001). Like the other three AR subtypes, the gene encoding the A_{2B}AR consists of two coding exons interrupted by a single intron, with splicing occurring at the region encoding the second intracellular loop in close proximity to the conserved aspartic acid-arginine-tyrosine (DRY) motif known to be important in regulating the conformational state and G protein coupling of G protein-

coupled receptors (Fredholm et al., 2000; Rovati et al., 2007). A_{2B}ARs are involved in control of vascular tone (Morrison et al., 2002), cell growth (Dubey et al., 2000), proinflammatory cytokine expression (Feoktistov et al., 2002; Zhong et al., 2004), mast cell degranulation (Auchampach et al., 1997), and epithelial water secretion (Strohmeier et al., 1995). Currently, there is great interest in the development of selective A_{2B}AR antagonists that may be useful for treating a wide variety of diseases, including fibrotic lung disease, asthma, inflammatory bowel disease, and diabetes (Feoktistov and Biaggioni, 1997; Ji et al., 2001; Kim et al., 2002). It is interesting that theophylline and enprofylline, drugs that have been used to treat asthma by mechanisms that remain undefined, inhibit A_{2B}ARs at therapeutically relevant concentrations (Auchampach et al., 1997; Kim et al., 2000, 2002; Ji et al., 2001).

To date the A_{2B}AR has been cloned from three different mammalian species: human, rat, and mouse. The structural

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ABBREVIATIONS: AR, adenosine receptor; HEK, human embryonic kidney; CPX, 1,3-dipropyl-8-cyclopentylxanthine; DPSPX, 1,3-dipropyl-8-*p*-sulfophenylxanthine; DRY, aspartic acid-arginine-tyrosine; GFP, green fluorescent protein; RT-PCR, reverse transcriptase-polymerase chain reaction; IB-MECA, *N*⁶-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide; NECA, adenosine-5'-*N*-ethylcarboxamide; 8-PT, 8-phenyltheophylline; *R*-PIA, (–)-*N*⁶-(2-phenylisopropyl)adenosine; 8-SPT, 8-*p*-sulfophenyltheophylline; XAC, xanthine amine congener; Ro 20,1724, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone.

homology of the A_{2B}AR among the three species is relatively high, sharing from 87 (human versus rat or mouse) to 95% (mouse versus rat) identity at the amino acid level. Despite a high degree of structural similarity, however, it has been suggested that the pharmacology of the A_{2B}AR may differ among species similar to that previously reported for A₁ and A₃ARs. In a search for more selective antagonists for the human A_{2B}AR, Kim et al. (2000, 2002) noticed that certain xanthine antagonists bound heterologously expressed recombinant rat A_{2B}ARs with up to 10-fold lower affinity compared with human A_{2B}ARs. Fozard et al. (2003) also suggested that antagonist pharmacology of A_{2B}ARs differs significantly among human, dog, rat, and guinea pig.

The purpose of the present investigation was to further explore the possibility that the pharmacological properties of A_{2B}ARs differ between species. Accordingly, we have compared the binding affinity of a panel of 10 different 1-, 3-, and 8-substituted xanthine derivatives as well as representative compounds from five additional classes of nonxanthine AR antagonists at recombinant mouse, rabbit, and dog A_{2B}ARs expressed in HEK 293 cells. We have also compared the binding affinity of seven different AR agonists composed of traditional adenosine analogs and substituted 6-amino-3,5-dicyano-4-phenylpyridine derivatives (Beukers et al., 2004; Eckle et al., 2007; Baraldi et al., 2008). This work initially involved cloning the A_{2B}AR cDNA from the three different species. In the course of these studies, we identified an intriguing variant of the A_{2B}AR from the rabbit that we describe in detail that seems to result from "leaky" splicing.

Materials and Methods

Materials. All chemicals and ligands were purchased from Sigma-Aldrich (St. Louis, MO) and reconstituted in dimethyl sulfoxide unless stated otherwise. BAY 60-6583 was obtained from Dr. Thomas Krahn at Bayer Healthcare AG (Wuppertal, Germany), and BG 9719 was provided by Dr. Barry Ticho at Biogen IDEC (Cambridge, MA). Adenosine deaminase was from Roche (Indianapolis, IN), and pEGFP-N1 was from Clontech (Mountain View, CA). Cell culture media, such as Lipofectamine, Geneticin (G418 sulfate), fetal bovine serum, ThermoScript RT-PCR assay kits, Fura-2/AM and pcDNA3.1, were purchased from Invitrogen (Carlsbad, CA). Whatman GF/C glass fiber filters were from Brandel (Gaithersburg, MD). Bradford reagent was obtained from Bio-Rad (Hercules, CA). Primers for RT-PCR were from Operon Biotechnologies, Inc. (Huntsville, AL). Restriction endonuclease and cloning enzymes were purchased from New England Biolabs (Beverly, MA), Promega (Madison, WI), and Invitrogen. cAMP radioimmunoassay kits were purchased from Invitrogen and GE Healthcare (Chalfont St. Giles, UK), respectively.

A_{2B}AR cDNA Isolation. The full-length mouse and dog A_{2B}AR cDNAs were amplified by RT-PCR from brain (mouse) and large intestine (dog) total RNA using primers based on conserved regions within the 5'- and 3'-untranslated regions of the human, mouse, and rat A_{2B}ARs. A full-length rabbit A_{2B}AR cDNA clone was isolated from a brain cDNA library by plaque filter hybridization, as described previously (Auchampach et al., 1997), using a fragment of the dog A_{2B}AR as a probe.

Creation of Stable HEK 293 Cell Lines Expressing A_{2B}ARs. Full-length A_{2B}AR cDNAs were subcloned into the mammalian expression vector pcDNA3.1. The plasmids were transfected into HEK 293 cells using Lipofectamine and selected with G418 (2 mg/ml). Cell lines derived from individual clones were maintained in cell culture media (Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics) containing 0.6 mg/ml G418. The full-length rabbit A_{2B}AR and the rabbit A_{2B}AR₁₀₃₋₁₁₁ variant were also sub-

cloned into pEGFP-N1 between the KpnI and NheI sites to allow for expression of the A_{2B}AR variants fused at the C-terminal end with enhanced green fluorescent protein (GFP). Stable HEK 293 cell lines expressing these constructs were used to examine the expression and subcellular localization of the two receptor variants by laser scanning confocal microscopy (Nikon C1 confocal laser-scanning microscope system).

Analysis of A_{2B}AR₁₀₃₋₁₁₁ mRNA Expression. RT-PCR was used to detect mRNA expression of the A_{2B}AR₁₀₃₋₁₁₁ variant in rabbit and mouse tissues. One microgram of total RNA was reverse-transcribed using poly-T primers. The resulting cDNA was amplified by PCR (up to 40 cycles) with primers spanning the splicing region and electrophoresed through 3% agarose gels. The primers for amplification in rabbit studies were forward primer = 5'-CTT CCA CAG CTG CCT CTT TC-3' and reverse primer = 5'-ACC CAG AGG ACA GCA ATG AC-3'. Primers for amplification in mouse studies were forward primer = 5'-TGC TCA CAC AGA GCT CCA TC-3' and reverse primer = 5'-AGT CAA TCC AAT GCC AAA GG-3'.

Synthesis of 6-Amino-3,5-dicyano-4-phenylpyridine AR Agonists. We synthesized a series of 6-amino-3,5-dicyano-4-phenylpyridine derivatives (compounds 1-3) to test in binding assays according to the scheme shown in Fig. 1. Compound 4 (BAY 60-6583) was obtained from Bayer.

Radioligand Binding Assays. Radioligand binding assays were conducted with crude membrane fractions. HEK 293 cells expressing A_{2B}ARs were washed in phosphate-buffered saline followed by homogenization in cold (4°C) HEPES buffer (10 mM Na⁺-HEPES, 10 mM EDTA, and 0.1 mM benzamidine, pH 7.4) using a glass Dounce homogenizer and centrifuged at 20,000g for 30 min. Cell pellets were washed twice with HE buffer (10 mM Na⁺-HEPES, 1 mM EDTA, and 0.1 mM benzamidine, pH 7.4). After the washes, pellets were resuspended in HE buffer with 10% (w/v) sucrose. Samples were aliquoted and stored at -20°C until further use in binding assays after the protein concentration was determined by the Bradford assay.

For equilibrium binding assays, crude membranes were incubated with radioligands and competitors in 100 µl of HE buffer in siliconized glass tubes containing 5 mM MgCl₂ and 5 U/ml adenosine deaminase unless indicated otherwise. Nonspecific binding was determined in most assays by including 100 µM adenosine-5'-N-ethylcarboxamide (NECA). After incubating at 21°C for 3 h, the bound and free radioligands were separated by filtration over glass fiber filters presoaked in 0.5% polyethylene amine to reduce nonspecific binding.

For saturation experiments with [³H]MRS 1754, specific binding data fit optimally to a single-site binding model using Marquardt's nonlinear least-squares interpolation, from which K_d and B_{max} values were obtained. For competition studies, IC₅₀ values were fit to eq. 1:

$$SB_i = B_i - (B_i - NS) [I]/(IC_{50i} + [I]) \quad (1)$$

where *i* is the number of binding sites, SB is specific binding, and NS is nonspecific binding. The K_i value for each competitor was calculated from the IC₅₀, B_{max}, concentration of [³H]MRS 1754, and its K_d value.

cAMP Accumulation Assays. HEK 293 cells were detached from cell culture plates, resuspended in serum-free DMEM containing 25 mM HEPES, pH 7.4, 1 U/ml adenosine deaminase, and 20 µM Ro 20,1724 (phosphodiesterase inhibitor), and then transferred to polypropylene tubes (50,000 cells/tube). The cells were incubated with ligands for 15 min at 37°C with shaking, after which the assays were terminated by adding 500 µl of 0.15 N HCl. cAMP in the acid extract was determined by radioimmunoassay.

Intracellular Ca²⁺ Assays. HEK 293 cells were detached from cell culture plates and loaded with 1 µM Fura-2/AM in buffer containing 100 mM NaCl₂, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄, 25 mM NaHCO₃, 0.5 mM CaCl₂, 2.7 g/liter D-glucose, 20 mM Na-HEPES, pH 7.4, and 0.25% bovine serum albumin for 45 min. Cells

Fig. 1. Synthetic routes to 6-amino-3,5-dicyano-4-phenylpyridine derivatives. Benzaldehyde or 4-hydroxybenzaldehyde was reacted with cyanothioacetamide and a catalytic amount of triethylamine (Et_3N) in ethanol to generate compounds 1 and 2. Compound 2 was further reacted with 2-chloroacetamide and sodium hydrogen carbonate in dimethyl formamide (DMF) to generate compound 3. The compounds were decolorized with activated carbon and recrystallized from methanol or ethanol. Identity of the compounds was performed by melting point analysis, infrared spectroscopy, and NMR. Compound 4 (BAY 60-6583) was provided by Bayer Healthcare.

were washed and resuspended in the same buffer ($1 \times 10^6/\text{ml}$) without bovine serum albumin plus the addition of 1 U/ml adenosine deaminase. Fluorescence at baseline and after the addition of agonists was continuously measured with a spectrofluorimeter (BD Biosciences, Franklin Lakes, NJ) in a stirred thermostable cuvette (30°C) using an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm.

Results

Molecular Cloning of Mouse, Rabbit, and Dog $\text{A}_{2\text{B}}$ ARs.

We chose to characterize mouse, rabbit, and dog $\text{A}_{2\text{B}}$ ARs, given that these species are commonly used in experimental animal models of disease. We obtained the mouse $\text{A}_{2\text{B}}$ AR cDNA for expression studies by RT-PCR because the sequence for this receptor has already been reported previously (GenBank accession number NM_007413). We were also able to amplify the full-length dog $\text{A}_{2\text{B}}$ AR cDNA using primers (forward 5'-GGC CAT GCA/T GCT A/GGA GAC-3'; reverse 5'-AGG CC/GA GAG CCT AGA/G TCA-3') corresponding to untranslated 5' and 3' ends that were highly conserved between sequences for the rat (GenBank accession number NM_017161), human (GenBank accession number AY136748), and mouse. However, all efforts to obtain the rabbit $\text{A}_{2\text{B}}$ AR cDNA sequence by RT-PCR failed, requiring use of a traditional library screening approach. We screened a total of 1×10^6 plaque-forming units from a rabbit

brain cDNA library using a fragment of the dog $\text{A}_{2\text{B}}$ AR as a probe. One positively hybridizing clone containing a 1.5-kilobase insert was sequenced in full (103 and 785 bp of 5'- and 3'-untranslated sequence and 332 bp of coding sequence) and was determined to correspond to the rabbit $\text{A}_{2\text{B}}$ AR based on sequence homology and pharmacological characteristics. Sequences for the rabbit and dog were submitted to GenBank (accession numbers AY630339 and AY313204, respectively).

Alignment of the amino acid sequences of the $\text{A}_{2\text{B}}$ ARs from the mouse, rabbit, and dog, with sequences previously reported for the human and rat, is shown in Fig. 2. All five cloned receptors encode a protein consisting of 332 amino acids. Between species, the greatest degree of homology lies within the transmembrane regions, whereas the least degree of homology lies within the second extracellular loop and the distal half of the carboxyl tail. Unlike the $\text{A}_{2\text{A}}$ AR (which also couples to G_s proteins) but similar to A_1 and A_3 ARs, sequence of the carboxyl tail of the cloned $\text{A}_{2\text{B}}$ AR from the various species is relatively short (~ 40 amino acids versus ~ 70 – 80 amino acids for $\text{A}_{2\text{A}}$ ARs) and contains a conserved putative palmitoylation site at cysteine 311 of the consensus sequence. Of the five mammalian species of $\text{A}_{2\text{B}}$ AR cloned to date, the rat and mouse $\text{A}_{2\text{B}}$ ARs display the greatest (95% identical) degree of sequence similarity, whereas the rat and the human sequences vary the most (87%; Fig. 2). Overall,

	TM I	TM II
human_A2B	MLLETQDALYVALELVIAALSVAGNVILVCAAVGTANTLQTPNTNYFLVS	LAAADVAVGLFA
rabbit_A2B	MQLETQDALYVALELAIAALSVAGNVILVCAAVGTSSALQTPNTNYFLVS	LAAADVAVGLFA
dog_A2B	MQLETQDALYVALELAIAALSVAGNVILVCAAVGTSSALQTPNTNYFLVS	LAAADVAVGLFA
rat_A2B	MQLETQDALYVALELVIAALAVAGNVILVCAAVGASSALQTPNTNYFLVS	LATADVAVGLFA
mouse_A2B	MQLETQDALYVALELVIAALAVAGNVILVCAAVGASSALQTPNTNYFLVS	LATADVAVGLFA
	* *****,*****:*****:..:***** **;***** 60	
	TM III	
human_A2B	IPFAITISLGFCDFYGCFLACFVLVLTQSSIFSILLAVAVDRYLAIC	VPLRYKSLVTGT
rabbit_A2B	IPFAITISLGFCDFHSCFLACFVLVLTQSSIFSILLAVAVDRYLAIL	VPLRYKSLVTGT
dog_A2B	IPFAITISLGFCDFHSCFLACFVLVLTQSSIFSILLAVAVDRYLAIK	VPLRYKSLVTGT
rat_A2B	IPFAITISLGFCDFHSCFLACFVLVLTQSSIFSILLAVAVDRYLAIK	VPLRYKSLVTGT
mouse_A2B	IPFAITISLGFCDFHSCFLACFVLVLTQSSIFSILLAVAVDRYLAIK	VPLRYKSLVTGT
	*****:*****:***** *****.***** 120	
	TM IV	
human_A2B	RARGVIAVLWVLAFFIGLTPFLGWNSKDSATNCTEPWDGTNESCCL	VKCLFENVVPM
rabbit_A2B	RARGVIAVLWVLAFFIGLTPFLGWNSKDSATNCTEPWDGTNESCCL	VKCLFENVVPM
dog_A2B	RARGVIAVLWVLAFFIGLTPFLGWNSKDSAT -NCTEPWDGTNESCCL	VKCLFENVVPM
rat_A2B	RARGVIAVLWVLAFFIGLTPFLGWNSKDSATNCTEPWDGTNESCCL	VKCLFENVVPM
mouse_A2B	RARGVIAVLWVLAFFIGLTPFLGWNSKDSATNCTEPWDGTNESCCL	VKCLFENVVPM
	*****:*****:***** ***** ** :***** *.***** 180	
	TM V	
human_A2B	YMVYFNFFGCVLPPELLIMLVIIKIFLVACRQLQRTLMDSRTTLQR	EIHAAKSLAMIV
rabbit_A2B	YMVYFNFFGCVLPPELLIMLVIIKIFLVACRQLQRTLMDSRTTLQR	EIHAAKSLAMIV
dog_A2B	YMVYFNFFGCVLPPELLIMLVIIKIFLVACRQLQRTLMDSRTTLQR	EIHAAKSLAMIV
rat_A2B	YMVYFNFFGCVLPPELLIMLVIIKIFLVACRQLQRTLMDSRTTLQR	EIHAAKSLAMIV
mouse_A2B	YMVYFNFFGCVLPPELLIMLVIIKIFLVACRQLQRTLMDSRTTLQR	EIHAAKSLAMIV
	*****:*****:***** *****:*****:***** ***** 220	
	TM VI	TM VII
human_A2B	GIFALCWLVPVHAINCVTLFQPAQAKDKPKWAMNTAILLSHANSVNP	VIYAYRNDRFRT
rabbit_A2B	GIFALCWLVPVHAINCVTLFQPAQAKDKPKWAMNTAILLSHANSVNP	VIYAYRNDRFRT
dog_A2B	GIFALCWLVPVHAINCVTLFQPAQAKDKPKWAMNTAILLSHANSVNP	VIYAYRNDRFRT
rat_A2B	GIFALCWLVPVHAINCVTLFQPAQAKDKPKWAMNTAILLSHANSVNP	VIYAYRNDRFRT
mouse_A2B	GIFALCWLVPVHAINCVTLFQPAQAKDKPKWAMNTAILLSHANSVNP	VIYAYRNDRFRT
	*****:*****:***** *****:*****:***** ***** ***** : 280	
human_A2B	FHKIISRYLLCQADVK -SGNGQAGVQPALGVGL	332
rabbit_A2B	FHKIISRYVLCQTDVK -SGNGQSGAQVFLSMGL	332
dog_A2B	FHKIISRYVLCQTDVK -SGNGQSGAQVFLSMGL	332
rat_A2B	FHKIISRYVLCQTDVK -GGSGQAGGQSTFSLSL	332
mouse_A2B	FHKIISRYVLCQAEK -GGSGQAGGQSTFSLSL	332
	**:*	

* - single, fully conserved residue
 : - conservation of strong groups
 . - conservation of weak groups
 - no consensus

Fig. 2. Amino acid alignment of the human, rabbit, dog, rat, and mouse A_{2B}AR sequences. Solids lines indicate putative transmembrane domains (TM) with numbered designations (I–VII). This figure was prepared using Biology Workbench 3.2 (San Diego Supercomputing Center).

sequence variability of the A_{2B}AR among the same five species is similar to that of A₁ and A_{2A}ARs (92–98 and 81–95% similarity, respectively) but less compared with the A₃AR, whereby sequence similarity ranges from 69 (rat versus rabbit) to 88% (mouse versus rat).

During the course of cloning the rabbit A_{2B}AR cDNA by library screening, we identified an additional A_{2B}AR clone (1.3 kilobases) encoding a 323 amino acid protein. The predicted amino acid sequence of this clone, designated A_{2B}AR_{103–111}, is identical to that of the full-length rabbit A_{2B}AR clone described above, with the exception that amino acids 103 to 111 are absent (Fig. 3). This nine amino acid deletion is located at the only known splice junction within the coding region of the A_{2B}AR in the second intracellular loop, which led us to speculate that this clone encodes a splice variant. Indeed, analysis of the A_{2B}AR sequence suggests that the A_{2B}AR_{103–111} variant results from the use of an alternative 5' donor site (see Fig. 3) that is also found in the dog A_{2B}AR sequence but not in the mouse, rat, or human sequences. Splicing normally occurs within the highly conserved DRY motif implicated in conferring constitutive activity, G protein coupling, and receptor stabiliza-

tion in other G protein-coupled receptors (Fredholm et al., 2000; Rovati et al., 2007). It is interesting that this motif is conserved in the A_{2B}AR_{103–111} variant (Fig. 3). Trace mRNA expression of the variant was identified in rabbit brain by RT-PCR (Fig. 4), but no detectable expression was consistently identified in other tissues where the A_{2B}AR is abundantly expressed, including the spleen, small intestine, and large intestine (data not shown). Expression of a similar variant of the A_{2B}AR was not identified in mouse tissues, including the brain (Fig. 4).

We subsequently generated a stable HEK 293 cell line expressing the A_{2B}AR_{103–111} clone to determine whether this variant is capable of activating similar signal transduction pathways as the full-length A_{2B}AR, i.e., G_s protein-mediated activation of adenylyl cyclase by cAMP accumulation assays and G_q protein-mediated Ca²⁺ mobilization using Fura-2 fluorescence. Comparisons were made with stable HEK 293 cell lines transfected with the full-length rabbit A_{2B}AR clone or the empty pcDNA3.1 vector. We used HEK 293 cells for these assays because they endogenously express the A_{2B}AR coupled to adenylyl cyclase and Ca²⁺ mobilization at low levels (Cooper et al., 1997; Gao et al., 1999), ensuring that we

Fig. 3. A, schematic illustration of the genomic organization of the $A_{2B}AR$ depicting the scheme for formation of the $A_{2B}AR_{103-111}$ variant during splicing by use of an alternative 5' donor site. B, nucleotide sequence alignment of the second intracellular loop of the mouse, rat, human, dog, and rabbit $A_{2B}AR$ sequences as well as the $A_{2B}AR_{103-111}$ variant. Proximal sequences of the 5' and 3' ends of the introns (lowercase letters) for the mouse (GenBank accession number NC_000077), rat (NC_005109), and human $A_{2B}AR$ s (NC_000017) are shown. The traditional 5' and 3' donor sites are shown with the exonic and intronic portions depicted in blue and red, respectively. The alternative 5' donor site found in the rabbit and dog sequences is also shown. C, amino acid alignment of the second intracellular loop of the various species showing the 9 amino acid deletion in the rabbit $A_{2B}AR_{103-111}$ variant. The conserved DRY sequence is highlighted in red.

Fig. 4. Trace detection of the $A_{2B}AR_{103-111}$ variant in rabbit brain by RT-PCR. Total RNA obtained from rabbit (top gel) or mouse (bottom gel) brain was reverse-transcribed, subjected to 40 cycles of PCR amplification using species-specific primers spanning the splicing region, and subjected to gel electrophoresis, as described under *Materials and Methods*. The lane labeled Control contained reactions that included full-length (mouse) or a mixture of full-length and $A_{2B}AR_{103-111}$ variant cDNAs (rabbit), and the lane labeled H_2O contained reactions using water in place of cDNA. For studies using rabbit cDNA, a 171-bp fragment corresponds to the full-length receptor, and a 144-bp fragment corresponds to the splice variant. Only a single 158-bp fragment was detected in studies using mouse cDNA, which corresponds to the full-length mouse $A_{2B}AR$ receptor.

would be using a cell system capable of supporting $A_{2B}AR$ signaling. As shown in Fig. 5, the potency and efficacy of the nonselective AR agonist NECA to increase intracellular con-

centrations of cAMP and Ca^{2+} were increased significantly in HEK 293 cells transfected with the full-length rabbit $A_{2B}AR$ compared with cells transfected with the empty vector. However, responses to NECA using cells transfected with the $A_{2B}AR_{103-111}$ clone were essentially identical to those observed in vector control cells. Similar results were obtained in experiments using Chinese hamster ovary cells or COS-7 cells to express the receptors (data not shown). These findings indicate that the $A_{2B}AR_{103-111}$ variant does not couple to the same signaling pathways as the full-length A_{2B} receptor. Because responses elicited by $A_{2B}AR$ s endogenously expressed in HEK 293 cells were not altered, these results also indicate that the variant does not have dominant-negative activity.

We also conducted radioligand binding assays to determine whether we could detect binding to the $A_{2B}AR_{103-111}$ variant in crude membrane fractions obtained from transfected HEK 293 cells. As shown in Fig. 6, we did not detect specific binding with [^3H]MRS 1754 or [^3H]1,3-dipropyl-8-cyclopentylxanthine (CPX) in assays using membranes prepared from $A_{2B}AR_{103-111}$ cells based on inclusion of 100 μM NECA, 5 μM CPX, 2 mM enprofylline, or 10 μM xanthine amine congener (XAC) to define nonspecific binding, whereas substantial specific binding was detected with both radioligands using membranes from HEK 293 cells transfected with the full-length $A_{2B}AR$ clone. Similar negative results were obtained in binding assays using higher concentrations of the radioligands (up to 50 nM) as well as [^3H]ZM 241385 and [^{125}I]ZM 241385 (data not shown).

Because we were not able to detect functional coupling or radioligand binding, stable cell lines expressing $A_{2B}AR$ -

Fig. 5. Changes in intracellular levels of cAMP (A) and Ca²⁺ (B) in HEK 293 cells transfected with the empty vector (pcDNA3.1), the full-length rabbit A_{2B}AR, or the rabbit A_{2B}AR₁₀₃₋₁₁₁ variant in response to increasing concentrations of NECA. For cAMP assays, EC₅₀ (nM; *n* = 6) and *E*_{max} (pmol/50,000 cells/15 min) values were: vector = 2050 ± 1020 and 2145 ± 364; A_{2B}AR = 108 ± 41 and 6932 ± 508; and A_{2B}AR₁₀₃₋₁₁₁ = 2931 ± 940 and 1814 ± 218. For Ca²⁺ assays, EC₅₀ (nM; mean ± S.E.M.; *n* = 6) and *E*_{max} (peak increase in fluorescence ratio from baseline) values were: vector = 1024 ± 440 and 0.69 ± 0.09; A_{2B}AR = 125 ± 38 and 1.00 ± 0.05; and A_{2B}AR₁₀₃₋₁₁₁ = 779 ± 292 and 0.73 ± 0.08. B, inset, the effect of 1 μM NECA on intracellular [Ca²⁺]_i calculated from the fluorescence ratio in the three lines of transfected HEK 293 cells. Note that baseline intracellular Ca²⁺ levels are increased in HEK 293 cells transfected with the full-length A_{2B}AR compared with cells transfected with either the empty vector or the A_{2B}AR₁₀₃₋₁₁₁ variant.

GFP fusion proteins were generated to confirm that the A_{2B}AR₁₀₃₋₁₁₁ variant is expressed in HEK 293 cells and to compare its subcellular localization to the full-length A_{2B}AR using confocal microscopy. We confirmed that the A_{2B}AR₁₀₃₋₁₁₁ variant is indeed expressed in HEK 293 cells (Fig. 7). Unlike the full-length A_{2B}AR, however, distinct expression of the A_{2B}AR₁₀₃₋₁₁₁ variant at the plasma membrane was not evident.

Pharmacological Characterization of Recombinant A_{2B}ARs from Mouse, Rabbit, and Dog. After molecular cloning, we conducted radioligand binding assays using [³H]-

Fig. 6. One-point radioligand binding assays using cell membranes obtained from HEK 293 cells transfected with the empty vector (pcDNA3.1), the full-length rabbit A_{2B}AR, or the rabbit A_{2B}AR₁₀₃₋₁₁₁ variant. Significant specific binding with [³H]MRS 1754 (A) and [³H]CPX (B) was observed in assays using cell membranes obtained from cells transfected with the full-length rabbit A_{2B}AR, but not with the A_{2B}AR₁₀₃₋₁₁₁ variant, using 100 μM NECA, 5 μM CPX, 2 mM enprofylline, or 10 μM XAC to define nonspecific binding. The reactions (3-h incubations with 100-μg membrane protein/tube) included 6 nM [³H]MRS 1754 or 30 nM [³H]CPX. Data are means ± S.E.M. of triplicate determinations. The results are representative of three experiments.

MRS 1754 to compare the binding profile of various AR ligands to the A_{2B}AR orthologs obtained from the various species. We initially conducted saturation experiments to determine the affinity and binding capacity of [³H]MRS 1754 to the three cloned receptors using 200 μM NECA to define nonspecific binding. We found that specific binding of [³H]MRS 1754 to HEK 293 cell membranes expressing either the dog, rabbit, or human A_{2B}AR was saturable fitting optimally to a one-site binding model. A representative saturation isotherm for each species of A_{2B}AR with corresponding Scatchard transformation of the same data is shown in Fig. 8. The *K*_d values obtained from these saturation experiments with [³H]MRS 1754 for the mouse, rabbit, and dog A_{2B}ARs were 3.39 ± 0.18, 1.79 ± 0.24, and 12.8 ± 1.7 nM, respectively. The *B*_{max} values were 1750 ± 249, 1959 ± 244, and 8573 ± 784 fmol/mg, respectively.

We next conducted competition binding assays to compare the binding profiles of a series of xanthine and nonxanthine antagonists. The xanthine compounds included the 1-, 3-, and/or 8-substituted xanthines, including enprofylline, theophylline, 8-PT, 8-SPT, 1,3-dipropyl-8-*p*-sulfophenylxanthine (DP-SPX), XAC, CPX, and BG 9719. The nonxanthine compounds included the adenine antagonist N-0861, the triazolotriazine derivative ZM 241385, the pyridine derivative MRS 1523, and

Fig. 7. Confocal images of HEK 293 cell lines expressing the full-length rabbit A_{2B}AR or the rabbit A_{2B}AR₁₀₃₋₁₁₁ variant fused at the C terminus with GFP.

the quinazoline derivative CGS 15943. N-0861 and ZM 241385 were developed as potent and selective A₁ and A_{2A}AR antagonists, respectively (Shryock et al., 1992; Poucher et al., 1995). MRS 1523 is a relatively potent and moderately selective human/rodent A₃AR antagonist (Li et al., 1998). *K_i* values of these compounds for the dog, rabbit, and mouse A_{2B}AR are shown in Table 1, and, if available, previously reported *K_i* values for the human and rat A_{2B}ARs are also included. For all species, a close correlation between relative affinities of xanthine and nonxanthine antagonists for the A_{2B}AR was found with no striking differences in potency orders (Table 1 and Fig. 9). However, affinities for the xanthine compounds were generally lower for the dog and rat A_{2B}AR versus the other species by as much as 7-fold (Table 1 and Fig. 9). The largest differences occurred with compounds that bound with high affinity, including MRS 1754 (7.2-fold comparing dog versus rabbit) and XAC (6.7-fold comparing dog versus rabbit).

Finally, we conducted competition assays with [³H]MRS 1754 to compare the binding profile of adenosine and non-adenosine agonist ligands. All agonist competition binding data fit best to a one-site model and were not shifted in assays containing 100 μM guanosine 5'-O-(γ-thio)triphosphate (Fig. 10) or in assays where Mg²⁺ was not included in the binding buffer (data not shown). Thus, values obtained in agonist competition binding assays reflect binding to the low affinity, G protein-uncoupled state of the receptor.

Table 2 reports *K_i* values for agonist ligand binding to mouse, rabbit, and dog A_{2B}ARs. The adenosine analogs included the nonselective agonist NECA, the A₁AR-selective agonist (−)-N⁶-(2-phenylisopropyl)adenosine (*R*-PIA), and the potent A₃AR agonist IB-MECA. The 6-amino-3,5-dicyano-4-phenylpyridine analogs included compounds 1 to 3 depicted in Fig. 1 and the newly discovered selective A_{2B}AR agonist, BAY 60-6583. Unlike antagonists, no differences in agonist binding affinities were identified among the three species (Table 2 and Fig. 8). In terms of adenosine analogs, NECA bound with highest affinity to the A_{2B}AR from all three species, with *K_i* values averaging between 1 and 3 μM,

Fig. 8. Saturation isotherm of [³H]MRS 1754 binding to dog, rabbit, and mouse A_{2B}ARs expressed in HEK 293 cells. Insets depict Scatchard transformation of the data. [³H]MRS 1754 (0.5–20 nM) was incubated with 50-μg membrane protein for 3 h at room temperature. Specific binding data, obtained by subtracting nonspecific binding defined by inclusion of 100 μM NECA, is shown. Values are means ± S.E.M. of triplicate determinations. The results are representative of three experiments.

whereas IB-MECA and *R*-PIA bound with ~10 to 20-fold lower affinity. The 2-carbamylmethylthio-substituted 4-phenylpyridine analogs (compounds 3 and 4) bound with relatively high affinity to A_{2B}ARs displaying affinities ranging between 100 and 750 nM among the three different species.

Discussion

We have cloned and characterized by competition radioligand binding analysis agonist and antagonist pharmacology of the A_{2B}AR from the mouse, rabbit, and dog. Our results demonstrate that the primary structure of the A_{2B}AR is highly conserved among mammalian species, and that this

TABLE 1

Dissociation constants for recombinant A_{2B}ARs determined in radioligand binding assaysK_i values were obtained from competition binding experiments using [³H]MRS 1754. All values are the mean ± S.E. of three to four assays.

	R ₁	R ₂	R ₃	Rat*	Human*	Dog	Rabbit	Mouse	Ratio**
<i>nM</i>									
Xanthines									
Enprofylline	H	Propyl	H		4730 ± 270	6960 ± 2730	5840 ± 810	5630 ± 730	1.5 (D/H)
Theophylline	Methyl	Methyl	H	15,100 ± 2700	9070 ± 1490	38,700 ± 6500	17,700 ± 5000	6410 ± 1300	6.0 (D/M)
8-PT	Methyl	Methyl			415 ± 219	371 ± 99	249 ± 48	436 ± 48	1.8 (M/R)
8-SPT	Methyl	Methyl			1330 ± 220	7240 ± 2060	2370 ± 230	4990 ± 1090	5.4 (D/H)
DPSPX	Propyl	Propyl				721 ± 173	200 ± 11	568 ± 15	3.6 (D/R)
XAC	Propyl	Propyl			16.0 ± 0.7	29.8 ± 9.6	4.47 ± 0.40	4.51 ± 0.44	6.7 (D/R)
MRS 1754	Propyl	Propyl		12.8 ± 1.1	1.97 ± 0.31	12.8 ± 1.7	1.79 ± 0.24	3.39 ± 0.18	7.2 (D,Rt/R)
CPX	Propyl	Propyl		186 ± 5	63.8 ± 8.3	147 ± 30	96.0 ± 13.0	86.2 ± 16.0	2.9 (Rt/H)
BG 9719 (CVT-124)	Propyl	Propyl				742 ± 76	470 ± 40	1010 ± 180	2.1 (M/R)
Non-Xanthines									
N-0861 (A ₁ -selective) (adenine derivative)									
ZM 241385 (A _{2A} -selective) (triazolotriazine derivative)					145 ± 15	20,070 ± 2840	17,300 ± 1760	27,500 ± 7350	1.6 (M/R)
Alloxazine (A _{2B} -selective) (benzopteridine derivative)					2040 ± 570	76.2 ± 21.1	109 ± 28	31.3 ± 11.1	4.7 (H/M)
MRS 1523 (A ₃ -selective) (pyridine derivative)					0%***	2840 ± 850	648 ± 82	485 ± 35	5.9 (D/M)
CGS-15943 (quinazoline derivative)					32.4 ± 1.0	0%***	22 ± 3%***	0%***	
						19.1 ± 4.1	24.4 ± 8.0	9.07 ± 1.31	3.6 (H/M)

* Data from Kim et al. (2000, 2002) and Ji et al. (2001).

** Ratio of species with highest K_i/species with lowest K_i (Rt, H, D, R, and M denote rat, human, dog, rabbit, and mouse, respectively).

Fig. 9. Plot of affinities of xanthine antagonists (A) and AR agonists (B) for the mouse A_{2B}AR versus the rabbit and dog A_{2B}ARs. The results of regression analysis are reported within the graphs.

Fig. 10. Competition for [³H]MRS 1754 binding to membranes from HEK 293 cells expressing the mouse A_{2B}AR by NECA (A) or compound 3 (B) in the presence of vehicle or 100 μM guanosine 5'-O-(γ-thio)triphosphate (GTPγS). The reactions (3-h incubations with 100-μg membrane protein/tube) included 6 nM [³H]MRS 1754. Specific binding was defined by use of 100 μM NECA. Data ($n = 5$) are means $\pm$ S.E.M.

translates into similar binding characteristics of synthetic AR ligands. We noticed only slight differences in antagonist pharmacology of the A_{2B}AR from the various species. In particular, we observed that the canine A_{2B}AR tended to bind xanthine antagonists with lower affinity than rabbit and mouse A_{2B}ARs.

Our findings are similar to those of Kim et al. (2002), who

also detected small species differences in antagonist binding to A_{2B}ARs. These investigators noticed in competition binding assays using recombinant receptors that rat A_{2B}ARs tended to bind certain xanthine antagonists with lower affinity compared with human A_{2B}ARs by up to 10-fold. Similar to our work, the differences were more prominent with potent antagonists such as MRS 1754. Combining the results of this previous study by Kim et al. (2002) with our data, we propose that human, rabbit, and mouse A_{2B}ARs have similar binding characteristics, whereas dog and rat A_{2B}ARs can be categorized as having lower binding potency for xanthine antagonists. Our results also agree with those of Fozard et al. (2003), who suggested that antagonist pharmacology of the dog A_{2B}AR differs from that of A_{2B}ARs from human, rat, and guinea pig based on pK_b values calculated from bioassays of various smooth muscle preparations.

In contrast, we observed no species differences in agonist binding to A_{2B}ARs. Our analysis included adenosine analogs as well as members of a new class of substituted 6-amino-3,5-dicyano-4-phenylpyridine agonist ligands recently reported to potently activate the human A_{2B}AR (Beukers et al., 2004; Eckle et al., 2007; Baraldi et al., 2008). It is notable that agonist binding to the A_{2B}AR in our assays was GTP-insensitive (Fig. 10). Thus, agonist affinity values provided in this report reflect binding to the low affinity, G protein-uncoupled form of the A_{2B}AR. It is presumed that expression of specific G protein subunits required for high-affinity agonist binding is low in HEK 293 cells, such that heterologous overexpression of the A_{2B}AR predominantly results in a large pool of uncoupled receptors. It has been reported that other G_s protein-coupled receptors, including the A_{2A}AR, also couple poorly when expressed in HEK 293 cells (Murphree et al., 2002).

Both A₁ and A₃ARs exhibit species differences in ligand binding that are much more pronounced than those observed in the present investigation with A_{2B}ARs. In regard to the A₁AR, affinities of certain N⁶-substituted adenine ligands (i.e., agonists such as R-PIA and antagonists including N-0861) and 8-substituted xanthine antagonists, including 8-PT and CPX vary as much as 200-fold among the different species that have been examined extensively (Murphy and Snyder, 1982; Ukena et al., 1986; Tucker et al., 1994). In regard to the A₃AR, xanthine and nonxanthine antagonists are well known to bind rodent A₃ARs, with nearly 500-fold lower potency than A₃ARs from nonrodent species, including human (Linden, 1994; Auchampach et al., 1997; Li et al., 1998). Until recently, MRS 1523 has been the only readily available antagonist with a binding potency for rodent A₃ARs that is lower than 1 μM (Li et al., 1998; Kreckler et al., 2006). Significant differences in agonist ligand binding also exist between human and rodent A₃ARs (Melman et al., 2008). The complex pharmacology of the A₃AR has hindered progress toward understanding the biological role of this AR subtype and explains why it remained undiscovered until it was cloned in 1991 (Meyerhof et al., 1991; Zhou et al., 1992). Unlike the A₃AR, the pharmacology of A₁ARs differs among species, even though its structure is highly conserved (>90% identical at the amino acid level).

Substantial species differences in pharmacology have been observed for many other families of G protein-coupled receptors, including α-adrenergic, serotonin, and histamine receptors. In many cases, single amino acid mutations were found

TABLE 2
Agonist dissociation constants for recombinant A_{2B}ARs determined in radioligand binding assays
K_i values obtained from competition binding experiments using [³H]MRS 1754. All values are the mean ± S.E. of three to five assays.

	R ₁	R ₂		Dog	Rabbit	Mouse	K _i Ratio*
					μM		
Adenosine derivatives							
NECA				3.01 ± 0.46	2.60 ± 0.65	1.01 ± 0.19	3.0(D/M)
R-PIA				34.9 ± 4.7	46.6 ± 12.5	28.3 ± 6.9	1.6(R/M)
IB-MECA				26.6 ± 2.3	25.5 ± 3.8	12.4 ± 1.5	2.1(D/M)
6-Amino-3,5-dicyano-4-phenylpyridine derivatives							
Compound 1				72.1 ± 8.6	51.3 ± 3.7	45.4 ± 5.6	1.6(D/M)
Compound 2				67.2 ± 8.2	43.6 ± 10.7	46.4 ± 3.9	1.5(D/R)
Compound 3				0.20 ± 0.08	0.27 ± 0.11	0.10 ± 0.02	2.7(R/M)
Compound 4 (BAY 60-6583)				0.75 ± 0.11	0.34 ± 0.07	0.33 ± 0.08	2.3(D/M)

* Ratio of species with highest K_i/species with lowest K_i (D, R, and M denote dog, rabbit, and mouse, respectively).

to be responsible for these differences, usually in transmembrane regions V to VII that predominantly form the ligand binding pocket (Cherezov et al., 2007; Jaakola et al., 2008). In-depth analysis of species differences in pharmacology coupled more recently with molecular modeling and direct structural information has been proven to be a useful strategy in identifying crucial amino acids for ligand-receptor interactions. Upon analysis of the A_{2B} AR sequences (Fig. 2), no remarkable amino acid substitutions were found within any of the transmembrane regions. Substantial sequence variability was found, however, in the second extracellular loop region between transmembrane segments IV and V, and most notably, the canine A_{2B} AR uniquely lacked the variable amino acid at position 152. A recent crystallographic model of the A_{2A} AR bound to the antagonist ligand ZM 241385 suggests that antagonist ligands interact with both the second and third extracellular loops of this receptor in addition to multiple transmembrane regions (Jaakola et al., 2008). Therefore, we speculate that differences in the second extracellular loop region could explain the lower binding affinity observed for the canine homolog of the A_{2B} AR. Through the analysis of interspecies chimeric receptors, Straer et al. (2008) have determined that the second extracellular loop contributes to interspecies differences in pharmacology of the histamine H_1 receptor.

Our antagonist binding data reported in Table 1 highlight very useful information. Note that CPX and ZM 241385, compounds that are commonly used in biological studies as selective A_1 and A_{2A} AR antagonists, respectively, display relatively high-binding affinity for A_{2B} ARs from the various species. Thus, these compounds are not particularly useful for discriminating A_1 or A_{2A} versus A_{2B} AR-mediated responses. Two other compounds that are preferable for selectively inhibiting A_1 ARs include the 8-bicyclo xanthine antagonist BG 9719 and, in particular, the adenine antagonist N-0861; both are potent A_1 AR antagonists ($K_i \sim 1$ nM) (Shryock et al., 1992; Pfister et al., 1997) that are 700 to 1000-fold and >15,000-fold selective versus A_{2B} ARs, respectively. The results of our binding data also confirm that MRS 1523 displays low binding potency for A_{2B} ARs, ensuring that this compound is unlikely to nonspecifically inhibit A_{2B} ARs, even at high concentrations required to inhibit A_3 ARs.

Our receptor binding assays were conducted with the relatively new radioligand [^3H]MRS 1754 (Ji et al., 2001). This ligand displays higher affinity compared with other radiolabels that have been used previously to characterize A_{2B} ARs, including [^3H]ZM 241385 (Ji and Jacobson, 1999), [^{125}I]3-(4-amino-3-iodobenzyl)-8-(phenyl-4-oxyacetate)-1-propyl xanthine (Kim et al., 2000), and [^3H]CPX (Robeva et al., 1996), which bind to A_{2B} ARs with K_d values ranging from 30 to 60 nM. Although [^3H]MRS 1754 is highly lipophilic resulting in considerable nonspecific binding, this problem can be minimized by presoaking filters with 0.5% polyethyleneimine (Ji et al., 2001). We also found that binding assays were improved by using siliconized glass tubes and by adding membrane proteins to binding assays before adding [^3H]MRS 1754 to prevent nonspecific binding to the assay tubes. In general, we found that nonspecific binding comprised 50% of total binding included in assays at concentrations equivalent to the K_d . Note that while MRS 1754 was identified as a potent and moderately selective human A_{2B} AR antagonist (400-, 245-, and 123-fold selective versus A_1 , A_{2A} , and

A_3 ARs), it is not selective versus the A_1 AR in the rat, in part due to its lower binding affinity for rat A_{2B} ARs (Kim et al., 2000, 2002). Considering the results of the present investigation, it is possible that MRS 1754 also possesses limited selectivity for A_{2B} ARs from other species as well.

During the process of cloning the rabbit A_{2B} AR, we isolated a clone shown to be expressed in rabbit brain at very low levels that encodes an A_{2B} AR variant lacking 9 amino acids in the second intracellular loop, which we have designated $A_{2B}AR_{103-111}$. This variant probably resulted from leaky splicing due to the presence of an additional 5' donor site in the rabbit sequence (Fig. 2). Although the $A_{2B}AR_{103-111}$ variant seems to encode a G protein-coupled receptor protein that remains in-frame and retains the highly conserved DRY motif, it did not bind antagonist AR ligands or couple to G protein signaling pathways when expressed in HEK 293 cells. Shortening the second intracellular loop presumably constrains proper orientation of the α -helical transmembrane regions of the receptor, thereby disrupting the ligand binding pocket (Cherezov et al., 2007; Jaakola et al., 2008). Although we cannot exclude the possibility that this variant couples to other unique signaling pathways, serves nonsignaling functions (i.e., participates in receptor dimerization or facilitates trafficking of other receptors) (Milligan and Smith, 2007), or binds other ligands (i.e., netrin-1) (Corset et al., 2000), this variant seems to be a nonfunctional form of the A_{2B} AR found in rabbits and possibly other species. A similar splice variant with questionable physiological significance containing a 17 amino acid insertion within the second intracellular loop of the A_3 AR has also been identified previously in the rat (Sajjadi et al., 1996).

In summary, we have systematically compared agonist and antagonist pharmacology of A_{2B} ARs from three species commonly used in preclinical animal models of human disease. Our results suggest that small but significant differences in antagonist ligand binding exist between species. Our cloning results also demonstrate that some species may express variants of the A_{2B} AR due to alternative or leaky splicing. The results of this work should aid with further elucidation of the pathophysiological roles of the A_{2B} AR.

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