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The human A1 adenosine receptor: ligand binding properties, sites of somatic expression and chromosomal localization

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The A1 adenosine receptor (A1AR) exerts important biological effects in the mammalian biology. To provide insights into the role A1AR action in human physiology, we characterized the pharmacologic properties of the human A1AR, examined somatic sites of A1AR gene expression, and identified the chromosomal location of the human A1AR gene. Using stably transfected CHO cells, the ligand binding properties of human and rat A1ARs were directly compared. Saturation studies showed that the human and rat A1ARs had similar high affinity for the A1 agonist [^{3}H]CCPA (human, $K_d = 517 \pm 64$ pM; B_{max} 438 ± 29 fmol/mg of protein; rat, $K_d = 429 \pm 69$ pM; B_{max} 358 ± 76 fmol/mg of protein). Competition studies performed using seven adenosine agonists and four adenosine antagonists also did not detect differences in the ligand binding properties among the rat and human A1ARs. Northern analysis of 16 human tissues revealed the presence of a single hybridizing transcript of 2.5 kb. Human A1AR receptor mRNA expression was greatest in brain and testis; lower levels of A1AR mRNA were present in heart, pancreas, kidney and spleen. Southern blotting and PCR analysis of human-rodent somatic cell hybrids showed that the A1AR gene is on human chromosome 1. Using fluorescence in situ hybridization, the human A1AR gene was further localized to the 1q32.1 region. These observations show that the human A1AR is a high affinity receptor that has ligand binding properties similar to the rat A1AR, human A1AR mRNA is heavily expressed in brain and testis, and the gene encoding the human A1AR is present on the long arm of chromosome 1.

Keywords: adenosine; adenosine receptors; chromosome; human

Introduction

Adenosine is produced and released by all cells and is among the most ubiquitous chemical signals in mammalian physiology (Jarvis & Willimans 1990). Under basal conditions, extracellular adenosine levels are about 50 nM (Jarvis & Willimans 1990; During & Spencer, 1992). Yet, under conditions that favor the breakdown of ATP, external adenosine levels can reach 1 uM (During & Spencer, 1992). Because adenosine is widely distributed in the local cellular environment (Stiles, 1992), the specificity of its action is largely determined by the distribution of the cell surface receptors through which adenosine acts (Stiles, 1992). To date, four subtypes of adenosine receptors have been cloned. These include the A1, A2a, A2b and A3 adenosine receptors (Londos *et al.*, 1980; Collis & Hourani, 1993; Tucker & Linden, 1993). Studies in rodents and other species show that the different receptor subtypes have distinct ligand binding properties, couple to different effector systems, and have unique patterns of tissue expression (Stiles, 1992; Tucker & Linden, 1993).

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Studies of A1 adenosine receptors (A1ARs) have been of particular interest. The A1AR was initially cloned from the canine thyroid (Libert *et al.*, 1989, 1991b), and later isolated from rats (Mahan *et al.*, 1991; Reppert *et al.*, 1991), bovines (Olah *et al.*, 1992; Tucker *et al.*, 1992), rabbits (Bhattacharya *et al.*, 1993), mice (Marquardt *et al.*, 1994), guinea pigs (Meng, unpublished) and humans (Libert *et al.*, 1992; Townsend-Nicholson & Shine, 1992). In each species, the A1AR cDNA encodes a protein of 326 animo acids containing seven hydrophobic domains. The rat and human A1ARs are 86% identical at the nucleic acid level and are 96% identical at the amino acid level (Libert *et al.*, 1992; Townsend-Nicholson & Shine, 1992).

Pharmacology studies show that A1ARs have unique ligand binding properties, with high affinity for both adenosine agonists and antagonists (Williams & Jacobson, 1990). Differences in A1AR ligand binding properties among different species have also been observed (Olah *et al.*, 1992; Tucker *et al.*, 1992, 1994). Currently, it is not known if the human A1AR has unique ligand binding properties.

The somatic distribution of A1AR receptors has been most extensively studied in rats (Williams & Jacobson, 1990; Reppert *et al.*, 1991). Radioligand binding studies and analyses of patterns of mRNA expression show high levels of A1AR expression in the rat brain, spinal cord, fat, and testis (Reppert *et al.*, 1991). Lower levels of A1ARs are found in several other tissues, including the kidney and heart (Reppert *et al.*, 1991). In humans, A1AR expression has been examined in only a few tissues (Salvatore *et al.*, 1993). Thus, it is not known if human A1ARs are discretely expressed, or are broadly distributed.

The human gene encoding the A1AR was initially localized to the proximal region of the long arm of chromosome 22, while the A2aAR gene was believed to be on chromosome 11 (Libert *et al.*, 1991a). Recently, the localization of the A2aAR gene has been challenged (MacCollin *et al.*, 1994). Thus, it is possible that the human A1AR gene is not located on chromosome 22.

Increasing evidence suggests an important role for A1ARs in the pathogenesis and treatment of human illnesses (Jarvis & Willimans 1990). A1ARs are heavily expressed in brain (Reppert *et al.*, 1991) and A1ARs may play a role in the pathogenesis of seizure disorders (During & Spencer, 1992). A1ARs also modulate the action of neurotransmitters and may protect the central nervous system against excitotoxic injury in animals (Rudolphi *et al.*, 1992). Cardiac A1ARs are sites of action where adenosine acts to terminate arrhythmias (Tucker & Linden, 1993). A1ARs are also heavily expressed in the testes and may play a role in reproduction (Rivkees, 1994).

As the role of A1ARs in human physiology becomes further defined, A1AR activation and blockade may have expanded clinical utility. To provide additional insights about the human A1AR, we have therefore characterized the ligand binding properties of the human A1AR and examined the somatic sites of human A1AR gene expression. To provide

important background information for future genetic analyses of disease states that may be associated with A1ARs, we also have reexamined the chromosomal location of the A1AR gene.

Results/Discussion

Human A1AR ligand binding properties

To determine if human A1ARs have unique ligand binding properties, the ligand binding properties of the rat and human A1ARs were directly compared using CHO cells stably transfected with either the rat or human A1AR cDNAs. Saturation studies were first performed by incubating cells with increasing concentrations of [^{3}H]CCPA (10 to 4000 pM). Scatchard analysis from three separate side-by-side experiments revealed a high affinity binding site for the human A1AR (K_d = 517 ± 64 pM; B_{max} 438 ± 29 fmol/mg of protein) and the rat A1AR (K_d = 429 ± 69 pM; B_{max} 358 ± 76 fmol/mg of protein) (Figure 1). These differences were not statistically significant ($P>0.05$; paired t-test).

Next, competition studies were performed using seven agonists and four antagonists selected for their ability to distinguish different adenosine receptor subtypes (Trivedi *et al.*, 1990). Specific binding was assessed after cells were incubated with a fixed amount of [^{3}H]CCPA (200 pM) and differing concentrations of drugs. K_i values were determined by computer analysis (McPherson, 1985) and are presented in Table 1. These studies revealed the expected rank order of potency for A1ARs (Fastbom *et al.*, 1986; Work *et al.*, 1989; Trivedi *et al.*, 1990; Libert *et al.*, 1992; Nakata, 1992; Townsend-Nicholson & Shine, 1992; Deckert *et al.*, 1993; Townsend-Nicholson & Schofield, 1994). No statistical differences were detected between the rat and human A1AR for any of the drugs compared ($P>0.05$; paired t-test). The high affinity values for agonist compounds suggested that the receptors were in a G-protein coupled state, which is consistent with previous studies of adenosine receptors stably expressed in CHO cells (Rivkees & Reppert, 1992).

Comparison of primary sequence information reveals that the human and rat A1ARs differ by only one animo acid residue within transmembrane domains, by eight amino acids in extracellular domains and by seven amino acids in intracellular domains. In the second extracellular loop, which may play a role in ligand binding (Olah *et al.*, 1994), differences in the amino acids 154–158 of the rat and human A1ARs will result in the rat A1AR having more charged residues in this region than the human A1AR. However, our findings suggest that these differences do not appreciably change the affinity for the drugs examined if these amino acids are involved in ligand binding. It is also important to note that there are no differences among rat and human A1AR at amino acid positions 270 and 277 in the seventh transmembrane domain, which contributes to differences in ligand binding properties among the canine and bovine A1ARs (Tucker *et al.*, 1994).

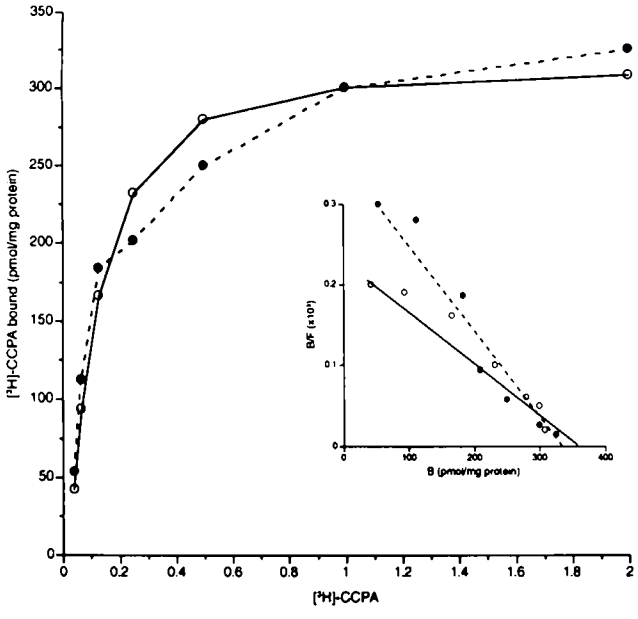

Figure 1 A. Saturation studies of stable CHO cells lines expressing rat(●) or human (○) A1ARs. Cells were incubated with [^{3}H]CCPA. Non-specific binding was assessed using 1 uM CPA. Specific binding is shown from one study that is representative of two other studies. Inset: Scatchard plot

Localization of A1 receptor mRNA in humans

Next, we examined A1AR gene expression by Northern analysis in 16 tissues. We found that a transcript of about 2.5 kb was apparent in tissues yielding a hybridization signal. In contrast to rat A1AR Northern blot studies, in which the hybridization signal appears as a doublet in tissues expressing A1ARs mRNA (Reppert *et al.*, 1991), only a single hybridization signal was apparent for human A1AR mRNA (Figure 2). Salvatore and co-workers previously observed a second hybridizing band of 4.3 kb in human brain (Salvatore *et al.*, 1993). However, similar to observations of Ren and Stiles (1994a), we did not observe this second transcript.

When hybridization signals were expressed relative to the beta-actin hybridization signals to yield relative radioactivity (RR) values, A1AR gene expression was highest in testis (RR = 100) and brain (RR = 96). Lower levels of A1AR mRNA were present in kidney, pancreas, spleen and heart, where RR values were 47, 55, 57 and 41, respectively. In the other tissues examined, A1AR receptor mRNA was either not present, or was expressed in amounts below our level of detection. Because actin expression may vary among different tissues, it is important to note that RR values may not completely reflect differences in A1AR mRNA levels among different sites.

The patterns of A1AR mRNA expression that we observed in human tissues were similar to that previously observed in rats. In rats, high levels of A1AR mRNA are found in spinal cord, brain, testis, epididymis and fat (Reppert *et al.*, 1991), and lower levels of mRNA are expressed in heart, spleen, kidney and stomach (Reppert *et al.*, 1991). The most notable

Table 1 Comparison of affinity of adenosine analogues for human and rat A1ARs

	K_i Values [M]	
	Human	*Rat*
Agonists		
CGS-21680	4.0±3.0 E-5	1.6±1.6 E-5
S-PIA	6.7±1.5 E-7	8.7±2.5 E-7
NECA	2.6±0.5 E-8	5.6±1.2 E-8
CADO	1.7±0.9 E-8	5.1±1.7 E-8
IABA	6.7±2.0 E-9	1.4±0.3 E-8
R-PIA	9.3±2.0 E-10	2.6±5.3 E-9
CPA	8.1±4.3 E-10	2.1±0.7 E-9
Antagonists		
AMN	2.9±1.3 E-6	9.7±2.9 E-7
BWA-1433	1.0±1.4 E-8	8.5±5.0 E-9
DPCPX	3.5±1.3 E-9	1.5±4.7 E-9

Values are mean±S.E.M. of three or four separate studies per drug. No statistical differences were observed between human and rat A1ARs for any of the drugs compared (paired t-test; $P>0.05$). CCPA, 2-chloro-N^{6}cyclopentyl adenosine; S-PIA, N^6-S-phenylisopropyladenosine; R-PIA, N^6-R-phenylisopropyladenosine; CADO, 2-chloroadenosine; NECA, 5'-N-ethylcarboxamidoadenosine; IABA, N^6-(4-amino-3-iodobenzyl) adenosine; BWA-1433, 1,3-dipropyl-8-(4 acrylate)phenylxanthine; AMN; aminophylline; DPCPX, 8-phenyl-1,3-dipropylxanthine. CPA, N^6-cyclopentyladenosine

difference observed among rat and human A1AR mRNA expression was relatively higher testicular A1AR mRNA expression in humans than in rats (Reppert *et al.*, 1991). Our findings thus identify the brain and testis as sites of high level A1AR gene expression in humans.

Chromosomal localization of the human A1AR gene

Previous studies suggested that the A1AR gene was present on human chromosome 22 and that the A2aAR gene was on chromosome 11 (Libert *et al.*, 1991a). Recently, re-analysis of the location of the human A2aAR gene showed that this gene was not located on chromosome 11, but rather was present on chromosome 22 (MacCollin *et al.*, 1994). These conflicting observations led us to reexamine the chromosomal location of the human A1AR gene.

We first assessed the number of human A1AR genes. Human genomic DNA (5 μg) was incubated with PstI, HindIII, BamHI, EcoRI and BglII. Southern blotting revealed single hybridization signal of 2.2, 5.0, 4.6 and 5.1 kb from genomic DNA digested with HindIII, BamHI, EcoRI and BglII, respectively (not shown). Two hybridizing signals of 4.0 and 2.5 kb were seen with PstI digests. Restriction digests of the 1.2 kb human A1AR cDNA clone used to generate our probes and the 10 kb human A1AR genomic clone revealed the presence of an internal PstI site. Thus, the two hybridizing bands of genomic DNA PstI digests represent two fragments of a single gene.

Next we examined a panel of human-hamster or human-murine somatic cell hybrids containing individual human chromosomes (Oncor; Gaithersburg, MD). DNA prepared from these cells had been digested with PstI before Southern blotting. The patterns of hybridization showed that the human A1AR probe labeled hamster and murine genomic DNA as well as human DNA (Figure 3). The patterns of murine, hamster, and human DNA labeling, however, could clearly be differentiated. PstI digests of murine genomic DNA revealed hybridizing fragments of about 6.0 and 3.3 kb. PstI digests of hamster genomic DNA revealed hybridizing fragments of about 5.8 and 3.9 kb. PstI digests of human genomic DNA revealed hybridizing fragments of about 4.0 kb and 2.5 kb. The 2.5 kb human hybridization signal was thus readily distinguishable from the murine and hamster hybridizing signals.

Inspection of the film autoradiographs showed that the hybridization signal (4.0 and 2.5 kb) for the human A1AR was present only over the lane containing DNA from human chromosome 1 (Figure 3). This lane also contained small elements of human chromosomes 13 and 14. However, a signal was not present over lanes containing the entire chromosomes 13 or 14. We also did not detect a hybridization signal over the lane containing human chromosome 22, challenging the assertion that the A1AR gene is present on chromosome 22.

To confirm the above observations, somatic cell hybrids obtained from another source were also examined. For these studies, we examined DNA extracted from cells containing human chromosomes 1, 11 or 22 (Coriell Inst.; Camden, NJ). PCR analysis was performed using primers complementary to nucleotides in transmembrane regions 1 and 2 of the human A1AR. PCR reactions yielded a band of the expected size (about 100 bp) from human genomic DNA and from the somatic cell hybrids containing human chromosome 1 (not shown). In contrast, a PCR product was not obtained from hamster genomic DNA, nor from somatic cell hybrids containing human chromosomes 11 or 22. These observations reinforce the findings of chromosome blot hybridization studies showing that the human A1AR is on chromosome 1.

Next, the position of the A1AR gene was determined on normal human metaphase spreads by two different fluorescent in situ hybridization (FISH) detection methods (Figure 4). First, using FITC (fluorescine isothiocyanate) detection of the human A1AR probe (method A), the A1AR gene was localized to chromosome one at band q32.1 (Figure 4b). Second, using a Cy3 (indocarbocyanine) detection system (method B), labeling also was determined to be at 1q32.1 (Figure 4d and e). Using both methods, labeling was not detected over other chromosomes nor over other regions of chromosome 1.

Collectively, these data show that the human A1AR gene is located on the long arm of human chromosome 1. Currently, genetic abnormalities associated with impaired A1AR gene expression are unknown. We are also unaware of genetic disorders that have been mapped to the chromosome region where we have localized the human A1AR gene (Genom Data Base; Johns Hopkins Univ.). Thus, we are hopeful that knowledge of the correct location of the human A1AR gene will facilitate future studies aimed at identifying illnesses associated with A1ARs by linkage analysis.

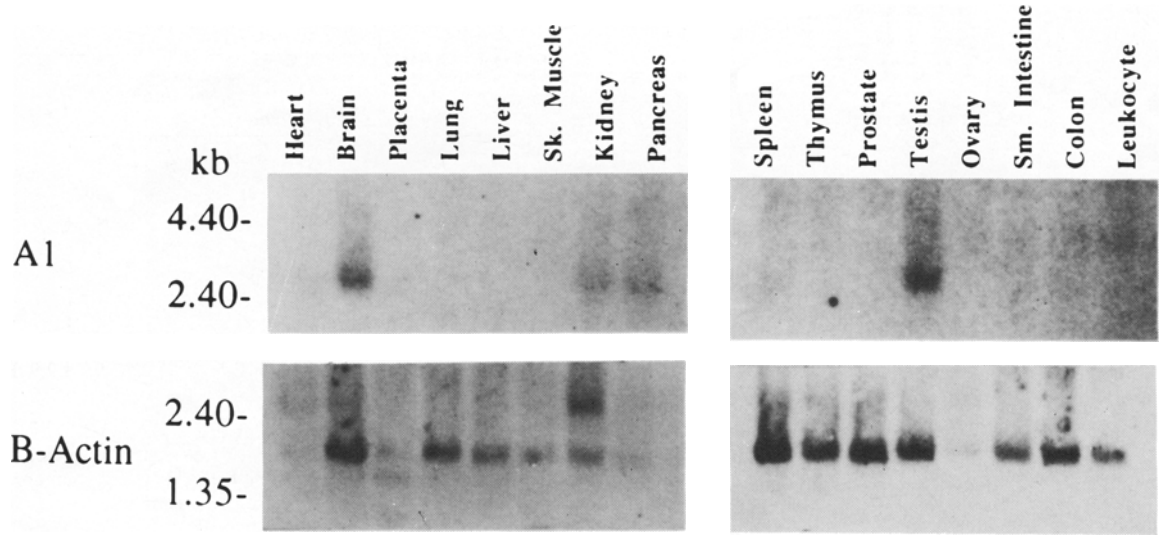

Figure 2 Northern blot analysis of A1AR transcripts in human tissues. 10 ug of poly A$^+$ RNA are present in each lane. Locations of RNA size markers are shown on the left. Blots were hybridized with a ^{32}P-labeled full-length human A1AR probe (top). Blots were then stripped and reprobed with a ^{32}P-labeled beta-actin probe (bottom). The blot shown was exposed for 7 days. The data shown are representative of two such studies

Summary

Our observations show that the human A1AR has high affinity for adenosine agonists and has similar affinities for adenosine analogues as compared to the rat A1AR. Human A1AR mRNA is expressed in several human tissues with highest levels of A1AR mRNA present in testis and brain. The human A1AR is encoded for by a gene that is present on chromosome 1 in the q32.1 region. These observations provide additional insights into the basic biology of this important receptor in human physiology.

Materials and methods

cDNAs

1.2 kb cDNAs encoding the full-length human A1AR and the full-length rat A1AR were graciously provided by Dr S.M. Reppert (Boston, MA). The human A1AR cDNA was identical in sequence to that previously reported for the human A1AR cDNA (Libert *et al.*, 1992). The rat A1AR cDNA has been previously described in full (Reppert *et al.*, 1991). For Northern and Southern blotting the 1.2 kb human A1AR cDNA containing the coding sequence of the human A1AR was used. For receptor expression studies, the 1.2 kb cDNAs containing the coding sequences of the human and rat A1ARs were used. For chromosome localization studies, a 10 kb genomic clone encoding the human A1AR was used. This clone has been previously described in detail (Ren & Stiles, 1994a, b).

Generation of stable cell lines

Stable cell lines were generated similar to as previously described (Reppert *et al.*, 1991). cDNAs encoding the full-length rat or human A1ARs subcloned into the mammalian expression vector pcDNA1-NEO (InVitrogen: La Jolla, CA), were used to transfect Chinese Hamster Ovarian (CHO) cells. Transfections were performed using Lipofectin (Gibco/BRL; Gaithersburg, MD). Transfected cells were selected in the presence of Geneticin (Gibco/BRL; 800 μg/ml). Cell lines (20 distinct clones per cDNA) were then screened for receptor expression by radioreceptor assay using [^{3}H]2-chloro-N^6-cyclopentyladenosine (CCPA; New England Nuclear, Boston, MA; specific activity 33 Ci mmol). The cell line CHO-H6 showed the highest level of human A1AR expression and was selected for further analysis in radioligand binding studies. The cell line CHO-1.7 showed the highest level of rat A1AR expression and was selected for further study in radioligand binding assays. Treatment of both cell lines with N^6-cyclopentyladenosine (CPA; 10 nM) inhibited forskolin-stimulated (100 nM) cAMP accumulation by about 50% in studies executed similar to as previously reported (Reppert *et al.*, 1991). These effects were blocked by 1 uM 8-phenyl-1,3-dipropylxanthine (DPCPX).

Radioreceptor assays

Radioligand binding studies were performed similar to as previously described (Reppert *et al.*, 1991). CHO cells were grown as monolayers in Hams F12 medium (DMEM; Gibco/BRL) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), in 5% CO_2 at 37°C. Binding studies were performed using intact cells. Medium was removed and the culture plates were washed with phosphate buffered saline (PBS). PBS was added to each dish and the cells were mechanically harvested. Cells were pelleted (4000 rpm; 10 min, 4°C) and resuspended in binding buffer consisting of PBS with 10 mM $MgCl_2$ and 2 U/ml of adenosine deaminase (Boehringer Mannheim; Indianapolis,

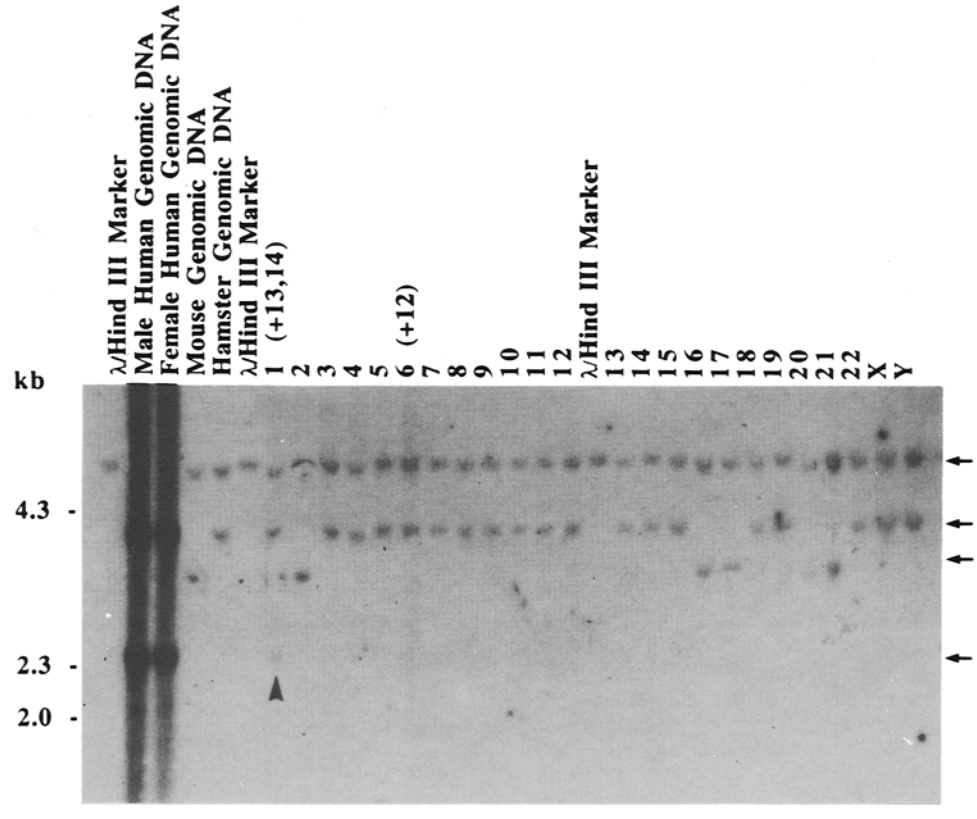

Figure 3 Southern blot analysis of genomic DNA or human-rodent somatic cell hybrids containing individual human chromosomes. 5 ug of DNA digested with PstI are present in each lane. Locations of size markers are shown on the left. Blots were probed with a ^{32}P-labeled 1.2 kb human A1AR probe. The hybridization pattern unique for human genomic DNA was present only over the lane containing human chromosome 1 (arrow). Small arrows on the right side of the figure identify the locations of the 6.0, 3.9, 3.3 and 2.5 kb hybridization signals, respectively. The data shown are representative of two such studies

IN) 37°C for 45 min. Cells were then pelleted and resuspended to a final concentration of 50 μg protein/ml with fresh binding buffer. For binding reactions, 50 μl of cells were added to [^{3}H]CCPA and drugs in a final volume of 150 μl in Millipore (Bedford, MA) multiscreen plates with GF/B 1.2 μm glass fiber filters. Reactions were incubated at 21°C for 1 h with shaking. Bound radioactivity was separated from free by vacuum filtration, and bound radioactivity was determined by liquid scintillation counting. All determinations were performed in quadruplicate. Protein concentration

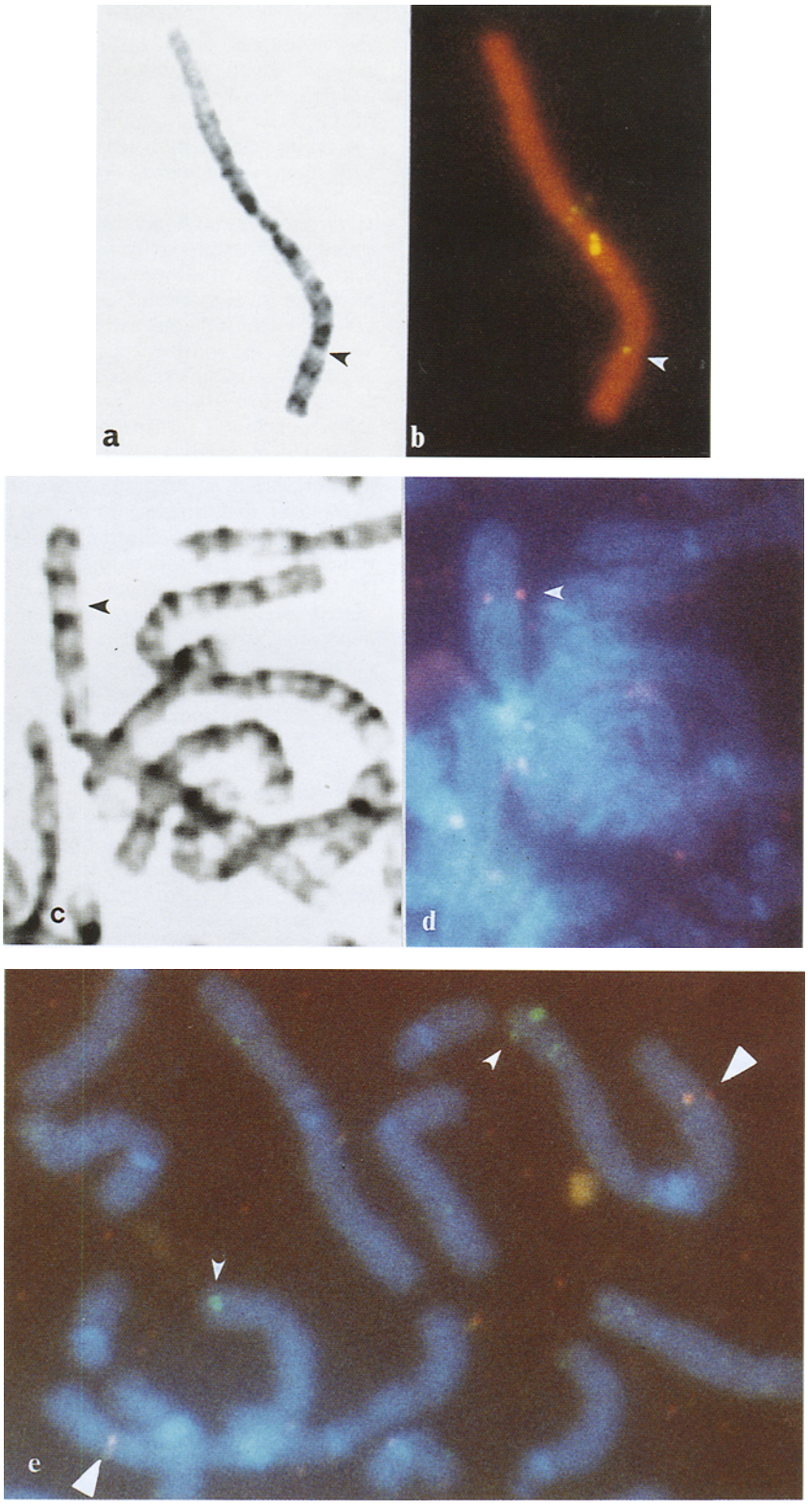

Figure 4 (**a**) G-banded chromosome 1 with arrow designating q32.1. (**b**) FITC detection of A1AR probe on the same chromosome stained with propidium idodide (method A). Arrow points to labeling of the chromosome at 1q32.1 (**c**) Partial G banded metaphase. The arrow points to the corresponding location of the probe on the long arm of chromosome 1 (1q32.1) shown in the adjacent panel. (**d**) FISH results of the same metaphase as in (**c**) using the A1AR probe detected with Cy3 (method B). DAPI counterstain shows an R banding pattern. (**e**) FISH results demonstrating the A1AR probe detected with Cy3 (method B) and a DNP-labeled cosmid control probe localized to 1P36. Two separate chromosomes 1 are shown. The A1AR probe produces a red signal (large arrow) and the DNP-labeled control cosmid produces a green signal (small arrow)

was measured using bicinconinic acid (Pierce; Rockford, IL) with bovine serum albumin standards.

All adenosine analogues were obtained from Research Biochemical Inc. (Natick, MA) except for N^6-(4-amino-3-iodobenzyl)adenosine (IABA) and BWA-1433, which were provided by Dr Joel Linden (Charlottesville, VA). Binding data were analysed by computer using an iterative nonlinear regression program (McPherson, 1985). Comparisons were made by the paired *t*-test. The InStat statistics program (GraphPad: San Diego, CA) was used for all computations.

Northern blotting

Human multiple tissue Northern blots (Clontech; Palo Alto, CA) were hybridized with ^{32}P-labeled, cDNA probes generated by the method of random priming (Prime-It II; Stratagene; La Jolla, CA). The sizes of the probes were assessed by gel electrophoresis and autoradiography of dried gels. Blots were hybridized overnight at 42°C in 50% formamide and washed in 0.2 × SSC and 0.1% SDS at 60°C for 2 × 30 min. Blots were apposed to Kodak (Rochester, NY) X-OMAT AR film for 2–14 days with one intensifying screen at – 80°C. Blots were stripped and reprobed with a ^{32}P-labeled, rat beta-actin probe.

Autoradiographs of blots were scanned using a Microtec (San Diego, CA) scanning system and hybridization signals were quantitated using Sigma Scan/Image software (Jandel; San Rafael, CA), similar to as previously described (Rivkees, 1994). Optical density signals, which were determined in triplicate for each image, were converted to radioactivity levels using Amersham (Arlington, IL) microscale autoradiography standards. Films with short exposure periods were scanned so the intensities of the hybridization signals fell within the mid-range of the autoradiographic images generated from the radioactivity standards. To control for the amount of RNA loaded, A1AR hybridization signals were expressed relative to the beta-actin hybridization signals to yield relative radioactivity (RR) values.

Southern blotting

Human genomic DNA (Clontech) was incubated with restriction endonucleases overnight, separated in a 0.8% agarose gel, and then transferred to nitrocellulose (Stratagene; La Jolla, CA). Southern blots of human-rodent somatic cell hybrids digested with PstI (Oncor; Gaithersburg, MD) were also used. Blots were hybridized with a ^{32}P-labeled human A1AR cDNA probe at 45°C for 16 h. The next day, blots were washed in 0.1 × SSC and 0.1% SDS at 52°C for 60 min. Blots were then exposed to X-ray film for 10 days.

PCR analysis of DNA

Human genomic DNA was obtained from Clontech. Hamster genomic DNA, as well as DNA from human-hamster somatic cell hybrids containing human chromosomes 1 (cell line no. NA-07299), 11 (NA-10972A) or 22 (NA-10888) were obtained from the Coriell Institute (Camden, NJ). Polymerase chain reaction (PCR) primers were designed to amplify a 98 bp fragment of the human A1AR gene. The PCR primer sequences were 5'AACGTGCTGGTGATCTGGGC-3' (transmembrane 1 region) and 5'ACCCACGGCCAC-ATCAGCCA-3' (transmembrane 2 region). PCR reactions were performed using the Gene Amp Kit (Perkin Elmer; Branchburg, NJ) reagents using 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. PCR products were then separated on a 2% agarose gel.

Chromosome localization studies

Chromosome preparation and GTG (G) banding Metaphase chromosome spreads were obtained from phytohemagglutin-stimulated normal human peripheral blood cultures, according to standard laboratory procedures. Slides were either baked for 16 20 h in a 65°C oven and then stored in ethanol at – 20°C or unbaked slides were stored in a desiccator. Slides were banded with a GTG banding method (Seabright, 1971) or Wright's stain (Yunis, 1981). Dividing cells were localized and captured on a hard drive using a Leica microscope and a digital imaging system (Genetiscan A/B Workstation, version 4.0-Persceptive Scientific Instruments).

Probe labeling For FISH localization of the human A1AR gene, a 10 kb genomic clone was used. 400 ng–1 ug of isolated DNA was nick-translated using digoxigenin-11-dUTP (Boehringer Mannheim) following a standard protocol (Ausubul, 1993). Labeled DNA was purified through a Sephadex G-50 (Sigma) column and stored at – 20°C.

Hybridization and detection Method A: Wright-stained slides were destained by immersion in 3:1 methanol: acetic acid, for 5 s, followed by a 3 min immersion in 100% methanol at room temperature, and then air-dried. Cotl DNA (400 ng/ul) and salmon sperm DNA (80 ng/ul) were added to the purified nick-translated DNA. Cold 100% ethanol (2.5 volumes) was added to the solution and stored at – 20°C for 30 min in order to precipitate the DNA. The DNA pellet was resuspended in Hybrisol VII (Oncor) and incubated overnight at 37°C. For each slide analysed, a 10 ul aliquot was denatured for 2 min in a 70°C waterbath and allowed to reanneal for 1.5 h in a 37°C waterbath. 0.5 ul of digoxigenin-labeled chromosome 1 alpha-satellite DNA (Oncor P5001) was denatured separately and added to the A1AR DNA. The A1AR and alpha satellite DNA were placed on a previously G banded slide that had been denatured at 70°C for two minutes and dehydrated in an ethanol series, protected with a coverslip and incubated at 37°C overnight. Post hybridization washes consisted of agitation in 50% formamide and 2 × SSC for 15 min at 47°C and in 2 × SSC for 8 min at 37°C.

Detection was accomplished using a series of antibodies including mouse anti-digoxigenin antibody (Sigma) diluted 1:500 in 3% BSA (Sigma)/4 × SSC, followed by incubation with FITC (fluorescine isothiocyanate)-labeled rabbit anti-mouse antibody (Sigma) and FITC-labeled goat anti-rabbit antibody (Sigma), both in 4 × SSC at dilutions of 1:300 and 1:100 respectively. Following each incubation, slides were washed 3 × 3 min in 1 × PBD buffer (Oncor) at 47°C. Slides were mounted in propidium idodide/antifade (Oncor) which was further diluted 1:4 with additional antifade (Oncor). Slides were examined utilizing a Leica Aristoplan fluorescent microscope.

Method B The GTG banded slides were prepared for FISH by washing in 100% ethanol for 5 min. Slides were air-dried and then rehydrated for 30–60 min in distilled water at 50°C. Pretreatment included exposure to a 0.005% pepsin (Sigma)/0.0N HCl solution for 10 min at 37°C, a rinse in PBS (pH 7.0) and dehydration in a 70%, 90%, 100% ethanol series. The slides were air-dried.

0.5–1 ul labeled probe DNA (5–10 ng) and 4 ul Cot-1 DNA (1 ug/ul) (Gibco/BRL) were mixed with 6 ul hybridization buffer (4 × SSC, 80% formamide, 20% dextran sulfate) then placed on the slide, covered with a 22 × 22 mm coverslip and sealed with rubber cement. A control cosmid probe which localizes to 1p36 was added to the hybridization mixture. This cosmid was labeled with DNP-11-dUTP (Molecular Probes). The chromosomes and probes were denatured simultaneously for 2 min at 75°C and incubated at 37°C overnight. Following hybridization, slides were washed 3 × 4 min in 50% formamide/2 × SSC at 45°C and 3 × 5 min in 0.5 × SSC at 65°C.

For detection of the digoxigenin-labeled A1AR probe, a series of antibodies including mouse anti-digoxigenin (diluted 1:500 in 4 × SSC; Sigma), sheep anti-mouse Cy3 (indocarbocyanine; 1:400 in 4 × SSC; Accurate) and donkey anti-

sheep Cy3 (1:400 in 4 × SSC; Accurate) were used. Detection of the cosmid marker probe was accomplished using an antibody series of rabbit anti-DNP, goat anti-rabbit FITC and donkey anti-goat DTAF. Slides were exposed to each antibody solution for 6–8 min at 37°C, followed by a 3 × 4 min wash in 4 × SSC/0.1% Tween 20 solution at 45°C. Slides were mounted in antifade solution (Oncor) to which DAPI (4,6-diamidino-2-phenylindole) was added to a final concentration of 0.2 ng/ul. This DAPI concentration stained chromosomes with an R banding pattern.

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References

Ausubul, F. (1993). *Current Protocols in Molecular Biology*, p. 18.
Bhattacharya, S., Dewitt, D.L., Burnatowska-Hledin, M., Smith, W.L. & Spielman, W.S. (1993). *Gene*, **128**, 285–288.
Collis, M.G. & Hourani, S.M. (1993). *Trends Pharmacol. Sci.*, **14**, 360–366.
Deckert, J., Berger, W., Kleopa, K., Heckers, S., Ransmayr, G., Heinsen, H., Beckmann, H. & Riederer, P. (1993). *Neurosci. Lett.* **150**, 191–194.
During, M.J. & Spencer, D.D. (1992). *Ann. Neurol.*, **32**, 618–624.
Fastbom, J., Pazos, A., Probst, A. & Palacios, J.M. (1986). *Neurosci. Lett.*, **65**, 127–132.
Jarvis, M.F. & Willimans, M. (1990). *Adenosine and Adenosine Receptors*, (ed) Williams, M. Clifton: Humana, pp. 423–474.
Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande Maenhaut, C., Dumont, J.E. & Vassart, G. (1989). *Science*, **244**, 569–572.
Libert, F., Passage, E., Parmentier, M., Simons, M.J. & VassartMattei, M.G. (1991a). *Genom.*, **11**, 225–227.
Libert, F., Schiffmann, S.N., Lefort, A. & Parmentier, M. (1991b). *EMBO J.*, **10**, 1677–1682.
Libert, F., Van Sande, J., Lefort, A., Czernilofsky, A., Dumont, J.E., Vassart, G., Ensinger, H.A. & Mendla, K.D. (1992). *Biochem. Biophy. Res. Comm.*, **187**, 919 926.
Londos, C., Cooper, D.M. & Wolff, J. (1980). *Proc. Natl. Acad. Sci.*, **77**, 2551–2554.
MacCollin, M., Peterfreund, R., MacDonald, M., Fink, J.S. & Gusella, J. (1994). *Genom.*, **20**, 332–333.
Mahan, L.C., McVittie, L.D., Smyk-Randall, E.M., Nakata, H., Monsma, F.J., Jr., Gerfen, C.R. & Sibley, D.R. (1991). *Mol. Pharm.*, **40**, 1 7.
Marquardt, D.L., Walker, L.L. & Heinemann, S. (1994). *J. Immunol.*, **152**, 4508–4515.
McPherson, G.A. (1985). *J. Pharmacolog. Meth.*, **14**, 213–228.
Nakate, H. (1992). *Eur. J. Biochem.* **206**, 171–177.
Olah, M.E., Jacobson, K.A. & Stiles, G.L. (1994). *J. Biol. Chem.*, **269**, 24692–24698.
Olah, M.E., Ren, H., Ostrowski, J., Jacobson, K.A. & Stiles, G.L. (1992). *J. Biol. Chem.*, **267**, 10764–10770.
Ren, H. & Stiles, G.L. (1994a). *J. Biol. Chem.*, **269**, 3104–3110.
Ren, H. & Stiles, G.L. (1994b). *Proc. Natl. Acad. Sci.*, **91**, 4864–4866.
Reppert, S.M., Weaver, D.R., Stehle, J.H. & Rivkees, S.A. (1991). *Molec. Endocrinol.*, **5**, 1037–1048.
Rivkees, S.A. (1994). *Endocrinology*, **136**, 2307–2313.
Rivkees, S.A. & Reppert, S.M. (1992). *Molec. Endocrinol.* **6**, 1598–1604.
Rudolphi, K.A., Schubert, P., Parkinson, F.E. & Fredholm, B.B. (1992). *Cerebrovasc. Brain Metab. Rev.*, **4**, 346–369.
Salvatore, C.A., Jacobson, M.A., Taylor, H.E., Linden, J. & Johnson, R.G. (1993). *Proc. Natl. Acad. Sci.*, **90**, 10365–10369.
Seabright, M. (1971). *Lancet*, **2**, 971–972.
Stiles, G.L. (1992). *J. Biol. Chem.*, **267**, 6451–6454.
Townsend-Nicholson, A. & Schofield, P R. (1994). *J. Biol. Chem.* **269**, 2373–2376.
Townsend-Nicholson, A. & Shine, J. (1992). *Brain Res. Molec. Brain Res.*, **16**, 365–370.
Trivedi, B.K., Bridges, A.J. & Bruns, R.F. (1990). *Adenosine and Adenosine Receptors*, (ed) Williams, M. Clifton: Humana Press, pp. 57–105.
Tucker, A.L., Linden, J., Robeva, A.S., D'Angelo, D.D. & Lynch, K.R. (1992). *Febs Lett.*, **297**, 107–111.
Tucker, A.L. & Linden, J. (1993). *Cardiovasc. Res.*, **27**, 62–67.
Tucker, A.L., Robeva, A.S., Taylor, H.E., Holeton, D., Bocker, M., Lynch, K.R. & Linden, J. (1994). *J. Biol. Chem.* **269**, 27900–27906.
Williams, M. & Jacobson, K.A. (1990). *Adenosine and Adenosine Receptors*, (ed) Williams, M. Clifton: Humana, pp. 17–56.
Work, C., Hutchinson, K., Prasad, M., Bruns, R.F. & Fox, J.H. (1989). *Arch. Biochem. Biophys.* **268**, 191–202.
Yunis, J.J. (1981). *Hum. Path.*, **12**, 540 549.