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Cloning and Functional Expression of a Novel G_i Protein-Coupled Receptor for Adenine from Mouse Brain

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Running title: Novel Adenine Receptor

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Abbreviations:

AC, adenylate cyclase; ANOVA, analysis of variance; CHO cells, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EDTA, ethylendiaminetetraacetic acid; FBS, foetal bovine serum; GPCR, G protein-coupled receptor; HEK cells, human embryonic kidney cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, Hank´s balanced salt solution; 2-methylthio-ADP, 2-methylthioadenosine 5´-diphosphate; NECA, 5'-(N-ethylcarboxamido)adenosine; NG108-15 cells, mouse neuroblastoma x rat glioma hybrid cell line; PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin; RT-PCR, reverse transcriptase-polymerase chain reaction; Sf21 cells, *Spodoptera frugiperda* 21 cells.

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ABSTRACT

An orphan G protein-coupled receptor from the rat has recently been demonstrated to act as a transmembrane receptor for the nucleobase adenine. The receptor is possibly involved in nociception. Here we report the cloning and functional expression of an additional G_i-coupled receptor for adenine (Genbank accession code DQ386867). mRNA for this receptor was obtained from mouse brain and the mouse neuroblastoma x rat glioma hybrid cell line NG108-15. The new mouse protein sequence shares only 76 % identity with that of the rat adenine receptor suggesting that the receptors are not species homologs but distinct receptor subtypes. In human 1321N1 astrocytoma cells stably expressing the new mouse receptor, adenine and 2-fluoroadenine inhibited the isoproterenol-induced cAMP formation with IC_{50} concentrations of 8 and 15 nM, respectively. The adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 µM) as well as the P2-receptor antagonist suramin (300 µM) failed to change the responses to adenine. In contrast, pretreatment of cells with pertussis toxin abolished the effect of adenine. When the novel adenine receptor was expressed in Sf21 insect cells a specific binding site for [^{3}H]adenine was detected. In competition assays the rank order of potency of selected ligands was identical to that obtained in membranes from NG108-15 cells and rat brain cortex (adenine > 2-fluoroadenine > 7-methyladenine > 1-methyladenine >> N^6-dimethyladenine). In summary, our data show that a second mammalian DNA sequence encodes for a G_i-coupled GPCR activated by low, nanomolar concentrations of adenine.

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INTRODUCTION

Adenine was recently identified as the endogenous ligand of an orphan rat G protein-coupled receptor (GPCR) (Bender et al., 2002). The expression of mRNA for this receptor in dorsal root ganglia neurons (Bender et al., 2002) as well as reports on neurotrophic effects of adenine (Yoshimi et al., 2003; Watanabe et al., 2003) point to a role of adenine as a neuronal signalling molecule. Spinally administered adenine has been shown to facilitate electrically evoked neuronal responses in the rat dorsal horn indicating an enhancement in nociception by the action of adenine (Matthews et al., 2004). Recent studies demonstrating the specific binding of [^{3}H]adenine to membrane preparations provided further evidence for the expression of the adenine receptor in rat neuronal tissues as well as a number of cell lines of neuronal origin (Gorzalka et al., 2005; Watanabe et al., 2005). The study by Gorzalka et al. also showed that an adenine receptor endogenously expressed in the mouse neuroblastoma x rat glioma hybrid cell line NG108-15 (Hamprecht, 1977) mediated an inhibition of adenylate cyclase activity. RT-PCR experiments indicated that NG108-15 cells do not express a rat, but a mouse mRNA sequence encoding for an adenine receptor (Gorzalka et al., 2005). Interestingly, there is evidence for the occurrence of an adenine receptor in human cells (Gorzalka et al., 2005). In healthy human beings plasma concentrations of adenine amount to values of about 70 nM; in patients with chronic renal failure the plasma concentration of adenine even increases to values above 1 µM (see Slominska et al., 2002). Therefore, adenine may play important roles in the pathophysiology of chronic renal failure (for a recent report on a GPCR-mediated effect of adenine on the Na^+-ATPase activity in the proximal tubule see Wengert et al., 2007). The human genome, however, appears to encode no direct ortholog of the rat adenine receptor (see also Bender et al., 2002) indicating that one has to search for a distinct sequence in order to identify human

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adenine receptors. Here, we report the cloning of a novel sequence encoding a GPCR for adenine. The sequence, which was found in mouse brain, mouse spleen and NG108-15 cells, is clearly distinct from the mouse ortholog (mMrgA10) of the rat adenine receptor. Whether the mouse ortholog mMrgA10 or other members of the Mas related gene family found in the mouse genome (see Choi and Lahn, 2003; Zylka et al., 2003) operate as receptors for adenine is not known. We also demonstrate the functional expression of this second member of the family of GPCRs for adenine, which couples via a pertussis toxin-sensitive G protein to inhibition of adenylate cyclase, and characterize the pharmacological properties of the receptor. The presented results may facilitate the identification of a human GPCR for adenine. Some of the results have been presented at a meeting of the German Society of Pharmacology and Toxicology (von Kügelgen et al., 2007).

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MATERIAL AND METHODS.

Molecular biological experiments. Poly A+ mRNA was isolated from homogenized brain tissue, the liver, the kidney and the spleen of adult male MNRI mice (Charles River WIGA, Sulzfeld, Germany) as well as from NG108-15 cells cultured as described previously (Kaulich et al., 2003) using the Oligotex Direct mRNA Mini Kit (Qiagen, Hilden, Germany). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using either the primer pair (sense: CACCATGGGGGAAAGCAGCACCGGTGCAG; antisense: TGGCTCTGCTTTGC-TTTTTGACATCTCC) or for expression analysis primers for a gene-specific sequence of the new mouse gene (240 bp of Genbank accession code DQ386867; sense: CTCCTTTGCCTTTACACCTTCAGG; antisense: ACACCCATAGTCATTTACATATTTGG) and Superscript One Step RT-PCR mix (Invitrogen, Karlsruhe, Germany). Annealing temperature for the PCR reaction was 56.0 °C (35 to 40 cycles). For control experiments the enzyme reverse transcriptase was omitted (instead of the RT-PCR enzyme mix Taq Platinum Polymerase was used; Invitrogen). PCR products were analyzed by ethidium bromide staining after agarose (0.9 %) gel electrophoresis. After amplifying the product of one RT-PCR reaction using Platinum Pfx polymerase (Invitrogen) the total coding sequence was cloned into the expression vector pcDNA3.1D/V5-His-TOPO (Invitrogen). The sequence was then identified by cycle sequencing (SequiTherm Exel II DNA sequencing kit; Epicentre Technologies, Madison WI, USA) using a LICOR Gene READIR 4200 sequencer (MWG-Biotech, Ebersberg, Germany). The sequencing was repeated at GATC (Konstanz, Germany) with identical results.

Expression of recombinant adenine receptors in a mammalian cell line. Human 1321N1 astrocytoma cells (European cell culture collection, Salisbury, UK) were

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cultured at 5 % CO_2 and 36.5 °C in Dulbecco`s modified Eagle medium (DMEM, Invitrogen) containing Glutamax I (Invitrogen) and 10 % fetal bovine serum (FBS; Invitrogen). The cells were split once a week by treating with trypsine-EDTA (0.5 g/l; Invitrogen). 1321N1 astrocytoma cells were transfected using the pcDNA3.1 expression vector and lipofectamine 2000 (Invitrogen) as described by the manufacturer. Cells stably expressing the receptor constructs were selected two days after transfection by culturing in the presence of 800 µg/ml Geneticin (G418; Invitrogen). Cells from passages 4 to 20 of the transfected 1321N1 astrocytoma cells were used for further experiments. For some experiments, 1321N1 astrocytoma cells expressing the mouse adenine receptor were pretreated with pertussis toxin 200 ng/ml for about 20 h (for control experiments see below, Chemicals).

Generation of recombinant baculoviruses. The sequence encoding for the mouse adenine receptor (without vector encoded epitopes) was cloned into the pVL1393 baculovirus transfer vector (PharMingen) between the BamHI and BglII restriction sites using standard techniques. Recombinant baculoviruses were generated using the Baculovirus expression vector system from PharMingen, which is based on the method of Guarino and Summers (Guarino and Summers, 1986). Briefly, 2 x 10^6 cells were co-transfected with 0.5 µg BaculoGold™ DNA (PharMingen, San Diego, USA) and 2 µg transfer vector DNA containing the mouse adenine receptor gene using the calcium phosphate transfection method. Six days after transfection the supernatant was harvested. The transfection supernatant was amplified to produce high titer virus stocks. The titer was determined using a modified end point dilution method (Reed and Muench, 1938). Briefly, in a 24-well plate 2 x 10^5 cells/well were infected with virus dilutions from 10^{-2}-10^{-11}. After three to five days, cells were

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inspected for signs of infection, the highest effective virus dilution was used for further transfection experiments.

Expression of recombinant adenine receptors in insect cells. *Spodoptera frugiperda* (Sf21) cells (gift from J. Höhfeld, Cell Biology, Bonn) were grown in Insect express protein free medium (Bio Whittaker, Verviers, Belgium). Cells were grown at 27°C and subcultured twice a week. Sf21 cells (1.8×10^7) were infected with recombinant baculoviruses at a multiplicity of infection (moi) of 1. They were harvested 72 h after infection. Cells were homogenized with 20 strokes in a tight fitting Dounce homogenizer in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM PMSF. The cell suspension was centrifuged for 10 min at 1,000 g, 4°C. The supernatant was then centrifuged at 48,000 g, 60 min, 4°C and the resulting pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4. The protein concentration was determined by the method of Bradford (Bradford, 1976). Membranes were kept frozen at -80°C until use.

Expression analysis. 1321N1 astrocytoma cells stably expressing the mouse adenine receptor were cultured on coverslips in DMEM supplemented with 800 µg/ml G-418 for 2 days. Expression of the recombinant adenine receptor was assessed by direct immunofluorescence staining using a FITC-coupled monoclonal antibody against the V5-receptor epitope encoded by the expression vector (anti V5-FITC, Invitrogen; 1:500). Expression was then verified by fluorescence microscopy on a Zeiss Axiovert 100 microscope equipped with an oil immersion 100 x objective (Zeiss, Jena, Germany), a CCD camera and a Polychrome II monochromator (TILL Photonics, Planeg, Germany; excitation wavelength 475 nm; exposure time 1 sec).

Analysis of cellular cyclic AMP accumulation. Receptor function was assessed by analysing the activity of cellular adenylate cyclase activity. For this purpose, non-

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transfected 1321N1 astrocytoma cells and 1321N1 astrocytoma cells stably expressing the recombinant mouse adenine receptor or the human $P2Y_{12}$-receptor were cultured on 24-well plates for two days (culture media, see above). After removal of the culture medium, cells were washed with HBSS buffer (containing 20 mM HEPES; pH 7.3) and then incubated with HBSS buffer for 2 h at 36.5 °C. Cellular cAMP production was then stimulated by the addition of 3 nM isoproterenol at 36.5 °C. Solvent (control), adenine, 2-fluoroadenine or 2-methylthio-ADP was added together with isoproterenol. The reaction was stopped after 10 min by removal of the reaction buffer followed by the addition of a hot lysis solution (500 µl; 90 °C; Na_2EDTA 4 mM; Triton X 100 0.1 ‰, Sigma, Munich, Germany). In some experiments the receptor antagonists DPCPX and suramin were given 10 min before adenine. The multi-well plates were shaken on ice for 1 h. cAMP levels in the supernatant were then quantified by incubation of an aliquot with cAMP binding protein and [^{3}H]cAMP (cAMP assay, TRK 432, GE Amersham Biosciences, Freiburg, Germany) and liquid scintillation counting after removal of the unbound [^{3}H]cAMP by charcoal. cAMP levels per well were calculated by linear regression from a standard curve determined for each experiment. The isoproterenol-induced cAMP production in the presence of agonists was expressed as percentage of the isoproterenol-induced cAMP production in the absence of agonists (% of control).

Radioligand binding studies. Saturation and competition assays with [8-^{3}H]adenine (27 Ci/mmol, GE Amersham Biosciences) were carried out essentially as described previously (Gorzalka et al., 2005). In brief, 50 µg of protein (NG108-15 membrane preparations), or 100 µg (Sf21 membrane preparations), respectively, were incubated with 10 nM [^{3}H]adenine (competition assay) in 50 mM Tris-HCl, pH 7.4 in a total volume of 200 µl. Inhibition curves were determined using six to nine different concentrations of adenine or adenine derivative spanning three orders of magnitude. For saturation assays increasing amounts (0.75-150 nM) of [^{3}H]adenine were incubated with the Sf21 membrane preparations (100 µg) in 50 mM Tris-HCl buffer,

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pH 7.4 in a total volume of 200 µl. Three separate experiments were performed each in duplicate or triplicate as noted. Nonspecific binding was determined in the presence of 100 µM adenine. Incubations were carried out for 1 h at room temperature and terminated by rapid filtration through GF/B glass fiber filters (Whatman, Dassel, Germany). Filters were washed three times with ice-cold 50 mM Tris-HCl buffer, pH 7.4, 2 ml each. Filter-bound radioactivity was measured by liquid scintillation counting.

Data analysis and statistics. Results are presented as means ± S.E. from n observations. Data were analysed using Prism 4.03 (Graph Pad, San Diego, USA). Differences between means were tested for significance by the t-test or (for multiple comparisons with the same control) by an analysis of variance followed by the Bonferroni post test (Prism). $p < 0.05$ or lower was the significance criterion. IC_{50} values (concentrations causing half-maximal inhibition) were determined by fitting data to sigmoidal curves.

Chemicals. The following drugs were used: 7-methyladenine (Acros Organics, Geel, Belgium); adenine, ADP sodium salt, ATP sodium salt, N^6-benzyladenine, caffeine, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N^6-dimethyladenine, 2-fluoroadenine, 1-methyladenine, 2-methylthioadenosine 5´-diphosphate sodium salt (2-methylthio-ADP), 5'-(N-ethylcarboxamido)adenosine (NECA), and pertussis toxin (Sigma); suramin hexasodium salt (Bayer, Leverkusen, Germany). Stock solutions of drugs were prepared either with distilled water or DMSO (N^6-benzyladenine, N^6-dimethyladenine, DPCPX, 2-fluoroadenine, 7-methyladenine, 1-methyladenine, NECA). The solvents were added to the buffer used for control experiments. For pertussis toxin control experiments the solvents present in the purchased solution were added to the medium; final concentration: 0.05 % glycerol (Serva, Heidelberg,

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Germany), 0.5 mM NaCl, 0.05 mM Trizma-Base (Sigma) and 0.38 mM glycine (Merck, Darmstadt, Germany).

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RESULTS

Cloning of a novel receptor for adenine. Poly A+ mRNA was isolated from mouse brain homogenates as well as from the mouse neuroblastoma x rat glioma hybrid cell line NG108-15. RT-PCR with both mRNA preparations and with primers for the coding sequence of the putative mouse ortholog MrgA10 of the rat adenine receptor revealed products of the expected length of about 1000 base pairs (for NG108-15 cells see also Gorzalka et al., 2005). Control reactions without the enzyme reverse transcriptase showed no products confirming that the mRNA preparations contained no genomic DNA. The RT-PCR products were subcloned in the pcDNA3.1 TOPO expression vector. Surprisingly, sequencing of the subcloned DNA sequence did not reveal the expected sequence of the mouse ortholog of MrgA10 but a distinct sequence encoding for a protein with 332 amino acids with only 81.6 % identity when compared with the mouse ortholog MrgA10 and only 76.3 % identity when compared with the rat adenine receptor (see Figure 1A). A high degree of variability was found in the predicted transmembrane region 3 and the predicted third extracellular loop (bold residues in Figure 1A). The new mouse nucleotide sequence was submitted to the Genbank under the accession number DQ386867. This novel gene with the accession code DQ386867 is located on mouse chromosome 7B3, whereas MrgA10 is localized in the region 7B4 to 7B5 (NCBI database). Additional RT-PCR experiments using specific primers for the new mouse gene (discriminating it from the mouse MrgA10 sequence) revealed products of the expected length of 240 base pairs from poly A+ mRNA isolated from mouse brain (B+; marked signal) and mouse spleen (S+, weak signal), but not from mRNA isolated from mouse liver (L) or mouse kidney (K; Figure 1B). This indicates the expression of mRNA for the mouse adenine receptor in neuronal tissues as well as in the spleen. Again, there were no products

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in reactions without the enzyme reverse transcriptase ("-" in Figure 1B) in agreement with the absence of genomic DNA in our mRNA preparations.

Expression in astrocytoma cells and functional analysis. Next, experiments were performed to analyze the pharmacological properties of the novel construct when expressed in mammalian cells. In non-transfected human 1321N1 astrocytoma cells, adenine (0.1 and 1 µM) caused no inhibition in cellular cAMP production in the absence and presence of isoproterenol used to stimulate the cellular cAMP accumulation (data not shown). Moreover, in 1321N1 astrocytoma cells stably expressing the recombinant human $P2Y_{12}$-receptor, the $P2Y_{12}$-receptor agonist 2-methylthio-ADP (1 µM) caused the expected inhibition of the isoproterenol (10 nM)-induced increases in cAMP levels (cAMP levels in the presence of 2-methylthio-ADP 1 µM amounted to 52.0 $\pm$ 1.7 % of those without 2-methylthio-ADP, control; n = 3; $p < 0.05$ vs. control, t-test), whereas adenine (10 µM) again failed to cause a change (98.9 $\pm$ 6.0 % of control; n = 3). The lack of any effect of adenine confirms the absence of an endogenous inhibitory receptor for adenine in 1321N1 astrocytoma cells.

1321N1 astrocytoma cells were then transfected with a pcDNA3 vector containing the sequence for the novel adenine receptor and the V5-epitope. Positive clones were selected in the presence of G-418 (see Methods). The expression of the receptor was verified by immunofluorescence staining against the receptor epitope (see Methods). In contrast to non-transfected cells, in 1321N1 astrocytoma cells stably expressing the mouse receptor, adenine (1 nM to 1 µM) inhibited the isoproterenol-induced cAMP formation in a concentration-dependent manner with a halfmaximal concentration (IC_{50}) of 8 nM (95 % confidence interval: 3-23 nM) and a

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maximal inhibition by about 40 % (Figure 2A). Similarly, the analogue 2-fluoroadenine caused a concentration-dependent inhibition of cAMP production with an IC_{50} concentration of 15 nM (95 % confidence interval: 7-30 nM) and a maximal inhibition by about 60 % (Figure 2B). The adenosine A_1-receptor antagonist DPCPX used at the concentration of 1 µM and the P2-receptor antagonist suramin (300 µM) failed to alter the inhibitory effects of adenine in cells expressing the recombinant mouse adenine receptor (Figure 3). DPCPX and suramin also caused no change in the increases in cellular cAMP levels induced by isoproterenol in the absence of adenine (not shown). Finally, the effect of pre-treatment with pertussis toxin was studied in astrocytoma cells expressing the recombinant mouse adenine receptor. Pre-treatment with pertussis toxin (200 ng/ml) for 20 h did not alter the isoproterenol-induced cAMP formation (see legend to Figure 4), but abolished the inhibitory effect of adenine observed in cells pre-treated with the solvent used for pertussis toxin (Figure 4).

Expression of the novel receptor in Sf21 cells and analysis of its binding properties. The novel mouse adenine receptor was subsequently expressed in Sf21 insect cells using the Baculovirus expression system. The adenine receptor sequence was inserted into the Baculovirus transfer vector pVl1393. By cotransfecting the vector and linearized Baculovirus DNA into Sf21 cells recombinant Baculoviruses were generated (see methods). After amplifying the viruses, Sf21 cells were infected for protein expression. Membrane preparations of non-infected Sf21 cells did not show any specific binding for [^{3}H]adenine (10 nM) (Figure 5). In contrast, a specific binding site for [^{3}H]adenine could be detected on membranes of Sf21 cells expressing the mouse adenine receptor (Figure 5). Saturation experiments with [^{3}H]adenine revealed a single high affinity binding site with a K_D value of 113 ± 17

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nM, and a B_{max} value of 1.98 ± 0.39 pmol/mg protein was determined (n=3) (Figure 6).

Selected adenine derivatives, as well as compounds known to activate (NECA) or block (caffeine) adenosine receptors or to activate P2 receptor subtypes (ADP, ATP), were investigated in competition assays at the novel mouse adenine receptor expressed in Sf21 insect cell membranes. Affinity data were compared with those for the adenine receptor expressed in NG108-15 mouse neuroblastoma x rat glioma cell membranes and those expressed in rat brain cortical membrane preparations (Figure 7 and Table 1). Unsubstituted adenine showed the highest affinity in all three test systems exhibiting K_i values in the nanomolar range (IC_{50} = 68.5 nM for the mouse receptor in Sf21 cells, 54.9 nM in NG108-15 cells, K_i = 29.9 nM in rat brain cortex). The rank order of potency for the mouse receptor expressed in Sf21 cells was: adenine > 2-fluoroadenine > 7-methyladenine > 1-methyladenine >> N^6-benzyladenine ≥ N^6-dimethyladenine. The same rank orders of potency were observed for NG108-15 cell membranes (Figure 7B) and rat brain cortical membranes (Table 1). All of the investigated P1 (adenosine) and P2 receptor ligands showed no or only moderate affinity for adenine binding sites in all three membrane preparations (Table 1).

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DISCUSSION

An orphan rat GPCR has previously been shown to operate as a receptor for the nucleobase adenine (Bender et al., 2002) indicating that in addition to P1-receptors for adenosine (Fredholm et al., 2001; Yan et al., 2003) and P2Y-receptors for extracellular nucleotides (Brunschweiger and Müller, 2006; von Kügelgen, 2006) a third group of GPCRs exists for these chemically related compounds (tentatively termed P0-receptors; Brunschweiger and Müller, 2006). Human cells also appear to express receptors for adenine (Gorzalka et al., 2005). The human sequence encoding an adenine receptor, however, has yet to be identified. The present study now demonstrates that a novel sequence encodes for an additional GPCR for adenine. This receptor shares only 76 % identical amino acid residues with the rat receptor suggesting that these proteins are not species homologs, but distinct members of a family of GPCRs for adenine. This view is underlined by the fact that the predicted transmembrane regions 3 of the novel receptor and the rat adenine receptor differ markedly (see TM3 in Figure 1), while species homologs are known to contain conserved transmembrane regions. Moreover, the tissue distribution of the novel receptor and the rat adenine receptor differ; the latter is predominantly expressed in dorsal root ganglia (Bender et al., 2001), while we detected mRNA for the novel sequence in the brain and the spleen suggesting a broader expression. As discussed below there are also differences in pharmacological properties. A related mouse DNA sequence encodes for the mMrgA10 orphan receptor (Bender et al., 2002; see Choi and Lahn, 2003; Zylka et al., 2003). It is not known yet whether mMrgA10 also operates as a receptor for adenine. Neither for the rat adenine receptor (Bender et al., 2002), nor for the new sequence, a closely related human

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ortholog could be identified in the genome database. A search for conserved regions of both sequences, however, may facilitate the identification of the human sequence.

For functional experiments human 1321N1 astrocytoma cells were used. Non-transfected 1321N1 astrocytoma cells do not respond to adenine with a decrease in cAMP formation (present study). Therefore, they are suitable for a pharmacological characterization of a recombinant inhibitory receptor for adenine (despite the fact that the cells possess binding sites for adenine; Gorzalka et al., 2005). In a previous study by Bender et al. (2002) a clone of Chinese hamster ovary (CHO) cells without endogenous adenine receptors had been used for recombinant expression of the rat adenine receptor. However, the clones of CHO cells studied in our laboratory (CHO Flp-In cells and CHO K1 cells) have a high density of adenine binding sites (S. Gorzalka and C.E. Müller, unpublished observations) and possess endogenous receptors for adenine modulating intracellular cAMP levels (K. Hoffmann and I. von Kügelgen, unpublished results).

The novel recombinant mouse adenine receptor shows similar, but not identical pharmacological properties as the adenine receptor endogenously expressed in the mouse neuroblastoma x rat glioma hybrid cell line NG108-15 (which expresses the same mouse sequence; Gorzalka et al., 2005, and, possibly, an additional subtype). Both the native and the recombinant receptor couple to inhibition of adenylate cyclase activity via pertussis toxin sensitive G proteins. Adenine acted as an agonist at the native (Gorzalka et al., 2005) and the recombinant receptor (present study) with half-maximal concentrations of 21 nM (NG108-15 cell membranes) and 8 nM (recombinant receptors), respectively. Adenine has been shown to activate the rat adenine receptor with a similar potency (3 nM, Bender et al., 2002). In addition to

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adenine, the adenine derivative 2-fluoroadenine was active at the native receptor in NG108-15 cells (Gorzalka et al., 2005) and at the recombinant receptor (Figure 2B).

In contrast to the results obtained at the recombinant receptor expressed in astrocytoma cells (present study) and at NG108-15 membranes (Gorzalka et al., 2005), a higher IC_{50} value for the adenine-induced inhibition of forskolin-stimulated cAMP production had been determined in intact NG108-15 cells (IC_{50} 2.54 μM, Gorzalka et al., 2005). The difference is likely to be due to an uptake mechanism for adenine in intact neuroblastoma NG108-15 cells, by which adenine is removed from the extracellular surface. In fact, in a recent study only rat cortical neurons but not astrocytes were found to take up [^{3}H]adenine indicating that astrocytes do not express specific transport systems for adenine (Nagai et al., 2006).

An action of adenine via adenosine receptors was excluded by the lack of any interaction with the adenosine A_1 receptor antagonist DPCPX, which completely blocks adenosine A_1 and A_{2B} receptors and at least partly blocks A_{2A} and A_3 receptors at the concentration used (1 μM; Lohse et al., 1987; Müller, 1996). Moreover, adenine does not act on P2-receptors as shown by the lack of interaction with suramin (acting as an antagonist at $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$-receptors; Table 3 of von Kügelgen, 2006). Hence, these results confirm the idea of a group of GPCRs for the nucleobase adenine distinct from the known adenosine and P2 receptors.

In addition to functional studies, we were interested in obtaining a suitable system for radioligand binding assays. NG108-15 cell membranes natively expressing the mouse adenine receptor had previously been shown to exhibit specific binding of

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[^{3}H]adenine (Gorzalka et al., 2005). However, this cell line may express more than one adenine receptor subtype or other specific binding sites for [^{3}H]adenine. Although we tested a series of cell lines, including CHO, HEK and 1321N1 astrocytoma cells, typically used for heterologous recombinant expression of GPCRs, we could not identify any mammalian cell line that did not natively express a specific binding site for [^{3}H]adenine. Therefore, we turned our attention to non-mammalian cells and found that Sf21 insect cell membranes did not show any specific binding of [^{3}H]adenine. After infection with recombinant baculoviruses containing the sequence for the novel adenine receptor, specific binding of [^{3}H]adenine was detected in these Sf21 insect cell membranes (Figure 5). In homologous competition assays of adenine versus [^{3}H]adenine, concentration-dependent inhibition of [^{3}H]adenine binding was observed (Figure 7). Since Sf21 cell membranes do not express any high-affinity adenine binding proteins, they represent an ideal expression system for adenine receptor proteins for binding assays. It has previously been suggested that [^{3}H]adenine may bind to glass fiber filters in a specific manner (Ye et al., 2006; but see Schiedel et al., 2007, for bacterial proteins binding adenine). However, we did not observe any specific binding of [^{3}H]adenine to glass fiber filters, but observed only nonspecific binding in the absence of Sf21 membranes, which was somewhat lower than the nonspecific binding observed in the additional presence of Sf21 membranes (Figure 5). The specific binding of [^{3}H]adenine to Sf21 insect cell membranes expressing the novel adenine receptor was clearly protein-dependent, and increased with higher protein concentrations (not shown).

In saturation experiments at Sf21 cells expressing the novel adenine receptor, a K_D value of 113 nM was determined for [^{3}H]adenine. Thus, the K_D value was 4-fold higher than that determined for the rat adenine receptor in brain cortical membranes

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(27.2 nM; Gorzalka et al., 2005), but still in the same concentration range. A comparison of affinities of adenine and selected derivatives for the novel receptor showed that affinities were similar to, but not identical with those determined at the native receptors in NG108-15 cells and the rat brain cortex (Table 1). The rank order of potency was: adenine > 2-fluoroadenine > 7-methyladenine > 1-methyladenine >> N^6-benzyladenine, N^6-dimethyladenine. Slight differences could be observed. For example, 2-fluoroadenine and 7-methyladenine showed a four to five-fold lower affinity for the recombinant receptor than for the native receptor (Figure 7 and Table 1). These differences are in agreement with the expression of different receptor subtypes in NG108-15 cells. In addition, different membrane environments may contribute to the differences by changing receptor conformation (Chachisvilis et al., 2006). In radioligand binding studies, the adenosine receptor agonist NECA and the antagonist caffeine did not show any affinity for the mouse adenine receptor (Table 1). The same was observed for the P2 receptor agonists ATP and ADP. IC_{50} values determined in radioligand binding studies were generally several-fold higher than IC_{50} values obtained in functional cAMP assays due to the amplification of the signal mediated by a GPCR.

In conclusion, we have identified, cloned and pharmacologically characterized a novel receptor for the purine nucleobase adenine from mouse brain. The low sequence identity with the previously described rat adenine receptor, particularly in the predicted transmembrane region 3, as well as differences in pharmacology suggest that the new receptor is not a species homolog but rather a distinct receptor subtype. Since human cells also posses specific binding sites for adenine, the existence of a receptor for adenine in human tissues seems to be very likely. However, a human sequence encoding for an adenine receptor has yet to be

identified. The rat adenine receptor discovered by Bender et al. (2002) and the novel adenine receptor described in the present study appear to be members of a new, possibly larger family of receptors for purine nucleobases. The comparison of both sequences may facilitate the identification of the human sequence.

Acknowledgement

We thank Petra Spitzlei for skilful technical assistance and Prof. Dr. Jörg Höhfeld for Sf21 insect cells.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. (A) Alignment of the predicted amino acid sequences of the novel adenine receptor (Novel seq, mouse adenine receptor, present study, Genbank nucleotide sequence accession No. DQ386867; see also XM_001003331), the mouse ortholog of MrgA10 (mMrgA10, Genbank nucleotide sequence accession No. XM_195647, please note that the total sequence of XM_195647 encodes for a protein with an extended N-terminus) and the rat adenine receptor (rAdenine-r, Genbank nucleotide sequence accession No. AJ311952). Residues which differ in the mouse and the rat adenine receptor are shown in bold. The seven predicted transmembrane regions (TM) are indicated by lines (IL, intracellular loop; EL, extracellular loop). (B) RT-PCR experiment indicating the expression of mRNA for the mouse receptor for adenine in tissues using primers specific for amplification of the sequence DQ386867 and poly A^+ mRNA isolated from total brain (B), liver (L), kidney (K) and spleen (S). The figure shows RT-PCR products (+) stained by ethidium bromide after agarose gel electrophoresis. Control experiments without reverse transcriptase (-) revealed no products. One of three independent experiments.

Figure 2. Inhibition of isoproterenol-stimulated cAMP accumulation by adenine (A, IC_{50} = 8 nM, 95 % confidence interval: 3-23 nM) and 2-fluoroadenine (B, IC_{50} = 15 nM, 95 % confidence interval: 7-30 nM) in 1321N1 astrocytoma cells stably expressing the novel (mouse) adenine receptor. Cellular cAMP production was increased by addition of isoproterenol 3 nM for 10 min at 36.5 °C; data are given as percentages of the mean increases in cellular cAMP levels in the presence of isoproterenol alone (% of control; average increase in cAMP levels by 15.3 $\pm$ 1.3 pmol cAMP per well; n = 17). Means $\pm$ S.E. from five to 21 experiments. ** indicates

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significant differences from corresponding control ($p < 0.01$; ANOVA followed by the Bonferroni post test).

Figure 3. Inhibition of isoproterenol-stimulated cAMP accumulation by adenine and interaction with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) 1 µM and suramin 300 µM in 1321N1 astrocytoma cells stably expressing the novel (mouse) adenine receptor. Antagonists or solvent were added 10 min before adenine 10 nM. Data are given as percentages of the mean increases in cellular cAMP levels in the presence of isoproterenol alone (% of control). Means ± S.E. from five to 21 experiments. * indicates significant differences from corresponding control ($p < 0.05$; ANOVA followed by the Bonferroni post test). For further details see legend to Figure 2.

Figure 4. Effect of the pre-treatment with pertussis toxin (PTX) on the adenine-induced inhibition of isoproterenol-stimulated cAMP accumulation in 1321N1 astrocytoma cells stably expressing the novel (mouse) adenine receptor. Cells were pretreated with PTX 200 ng/ml or its solvent for 20 h. Data are given as percentages of the mean increases in cellular cAMP levels in the presence of isoproterenol alone (% of control; pre-treated with solvent: average increase in cAMP levels by 10.9 ± 0.4 pmol cAMP per well; n = 5; pretreated with PTX: 9.8 ± 0.7 pmol cAMP per well; n = 5). Means ± S.E. from five to seven experiments. ** indicates significant difference from corresponding control (no adenine; $p < 0.01$;); ## indicates significant difference from corresponding value obtained with cells pre-treated with solvent ($p < 0.01$; ANOVA followed by the Bonferroni post test). For further details see legend to Figure 2.

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Figure 5. [^{3}H]Adenine binding (10 nM) to membrane preparations (100 µg of protein) of non-infected (Sf21) and infected (Sf21 pVI-mAde; novel mouse receptor for adenine) Sf21 cells as well as to glass fiber filters without protein (samples w/o protein). Nonspecific binding was determined in the presence of 100 µM unlabeled adenine. Data are means ± S.E. from three (Sf21 pVI-mAde), four (samples w/o protein) or five (Sf21) independent experiments performed in triplicate; ns = not significant, *** indicates significant difference ($p < 0.001$; t-test).

Figure 6. Saturation curve of [^{3}H]adenine binding to membranes prepared from Sf21 insect cells expressing the novel (mouse) adenine receptor. A K_D value of 113 ± 17 nM, and a B_{max} value of 1.98 ± 0.39 pmol/mg protein was determined (n=3). Experiments were performed as described under Material and Methods. The result shown represents the mean ± S.E. of three independent experiments, each performed in duplicate.

Figure 7. Competition curves for adenine, 2-fluoroadenine and 7-methyladenine versus 10 nM [^{3}H]adenine obtained with a membrane preparation of Sf21 insect cells recombinantly expressing the novel (mouse) adenine receptor (A) or membranes from NG108-15 cells, natively expressing the novel (mouse) adenine receptor (B). The IC_{50} values were calculated by non-linear regression function for one-site competition (Sf21: IC_{50} adenine = 68.5 ± 9.8 nM (n=5), IC_{50} 2-fluoroadenine = 1.47 ± 0.14 nM (n=2), IC_{50} 7-methyladenine = 5.76 ± 1.02 nM (n=2), NG: IC_{50} adenine = 0.154 ± 0.056 nM (n=2), IC_{50} 2-fluoroadenine = 0.304 ± 0.097 nM (n=3), IC_{50} 7-methyladenine = 1.45 ± 0.44 nM (n=2)). Data are means ± S.E. from two to five independent experiments performed in duplicate as indicated above.

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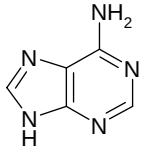
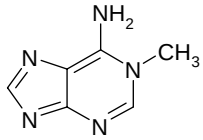
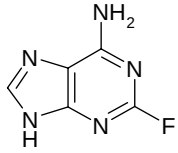
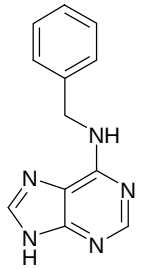
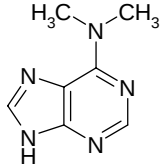
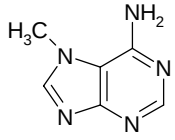
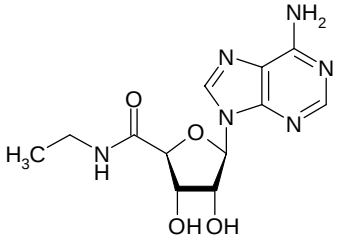
Table 1. Comparison of affinities of adenine and selected compounds for the novel (mouse) adenine receptor and the native rat adenine receptor determined in radioligand binding studies at membrane preparations (NECA, 5'-(N-ethylcarboxamido)adenosine).

Compound	Structure	IC_{50} or K_i value ± SEM [μM] (or % inhibition at 100 μM)[a]		
		Mouse recombinant receptor expressed in Sf21 cells[b]	NG108-15 cells[b]	Rat brain cortex[c]
Adenine		0.0685 ± 0.0098[f]	0.0549 ± 0.0422[d] 0.154 ± 0.056[d,e]	0.0299 ± 0.0034
1-Methyladenine		(62 ± 7)	(60 ± 4)	29.3 ± 9.3
2-Fluoroadenine		1.47 ± 0.14[d]	0.304 ± 0.097	0.622 ± 0.144
N^6-Benzyladenine		(12 ± 12)[d]	(8 ± 12)[d]	(30 ± 3)
N^6-Dimethyladenine		(0 ± 8)[d]	(0 ± 6)[d]	(11 ± 2)
7-Methyladenine		5.76 ± 1.02[d]	1.45 ± 0.44[d]	4.13 ± 1.08
NECA		(28 ± 1)[d]	(7± 1)[d]	(10 ± 2)

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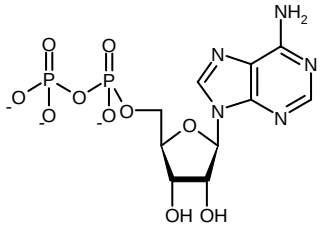
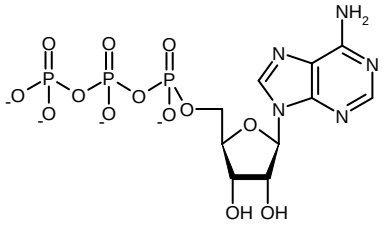
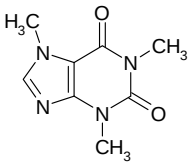
Compound	Structure	IC_{50} or K_i value ± SEM [μM] (or % inhibition at 100 μM)[a]		
		Mouse recombinant receptor expressed in Sf21 cells[b]	NG108-15 cells[b]	Rat brain cortex[c]
ADP		(28 ± 3)[d]	(50 ± 5)[d]	(51 ± 3)
ATP		(14 ± 1)[d]	(61 ± 4)[d]	(39 ± 7)
Caffeine		(1 ± 1)[d]	(0.4 ± 1)[d]	(2 ± 1)

[a] Results are from three independent experiments performed in triplicate, unless otherwise noted
[b] IC_{50} values vs. 10 nM [^{3}H]adenine
[c] K_i values from Gorzalka et al., 2005
[d] Results from two independent experiments performed in duplicate
[e] IC_{50} value from Gorzalka et al., 2005
[f] Result from five independent experiments performed in duplicate

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A

```
Novel seq (001)  MGESSTGAGF LALNISASTM ALTTTNPMDE TIPGSTSIKI
mMrgA10   (001)  MGESSTGAGF LALNTSASSM APSTTNLMDE TIPGSIDIST
rAdenin-r (001)  MGESFTGTGF INLNTSASTI AVTTTNPMDK TIPGSFNGRT
                              N-terminus
Novel seq (041)  LIPNLMIIIF GLVGLTGNAI VFWLLGFHLR RNAFSVYILN
mMrgA10   (041)  LIPHLMIIIF GLVGMTGNAI VFWLLGFRLR KNAFSVYILN
rAdenin-r (041)  LIPNLLIIIS GLVGLIGNAM VFWLLGFRLA RNAFSVYILN
                              TM1           IL1       TM2
Novel seq (081)  LALADFLFLL CRIIASTQKL LTFSSPNITF LLCLYTFRVI
mMrgA10   (081)  LALADFLFLL CHIIASTLFL LKVSYPNIIF RKCFFSVMLV
rAdenin-r (081)  LALADFLFLL CHIIDSTLLL LKFSYPNIIF LPCFNTVMMV
                 TM2                  EL1                TM3
Novel seq (121)  LYIAGLSMLT AISIERCLSV LCPIWYRCHR PEHTSTVMCA
mMrgA10   (121)  LYIAGLSILS AIGTERCLSV LCPIWYRCHC PEHTSTVTCA
rAdenin-r (121)  PYIAGLSMLS AISTERCLSV VCPIWYRCRR PKHTSTVMCS
                 TM3                  IL2                TM4
Novel seq (161)  AIWVLSLLIC ILNRYFSGFL DTKYVNDYGC MASNFFNAAY
mMrgA10   (161)  MIWVLSLLIS ILNKYFCVFL DTKYVNDYGC MASNFFTAAY
rAdenin-r (161)  AIWVLSLLIC ILNRYFCGFL DTKYEKDNRC LASNFFTAAC
                 TM4                  EL2                TM5
Novel seq (201)  LMFLFVVLCV SSLALLARLF CGTGRMKLTR LYVTIMLTIL
mMrgA10   (201)  LMFLFVVLCL SSLALLARLF CGAGRMKLTR LYVTIMLTIL
rAdenin-r (201)  LIFLFVVLCL SSLALLVRSF CGAGRMKLTR LYATIMLTVL
                 TM5                  IL3                TM6
Novel seq (241)  VFLLCGLPCG LYWFLLFWIK NGFAVFDFNF YLASTVLSAI
mMrgA10   (241)  VFLLCGLPCG IYWFLLSKIK NVFIVFDFSL FMASSVLTAL
rAdenin-r (241)  VFLLCGLPFG IHWFLLIWIK IDYGKFAYGL YLAALVLTAV
                 TM6                     EL3             TM7
Novel seq (281)  NSSANPIIYF FVGSFRHRLK HQTLKMVLQS ALQDTPETAE
mMrgA10   (281)  NSCANPIIYF FVGSFRHRLQ HQTLKMVIQS ALQDIPETPE
rAdenin-r (281)  NSCANPIIYF FVGSFRH-QK HQTLKMVLQR ALQDTPETAE
                 TM7                              C-terminus
Novel seq (321)  NMVEMSKSKA EP
mMrgA10   (321)  NIVEMSKSKA EP
rAdenin-r (320)  NTVEMSSSKV EP
                   C-terminus
```

B

B+ B- L+ L- K+ K- S+ S-

Figure 2

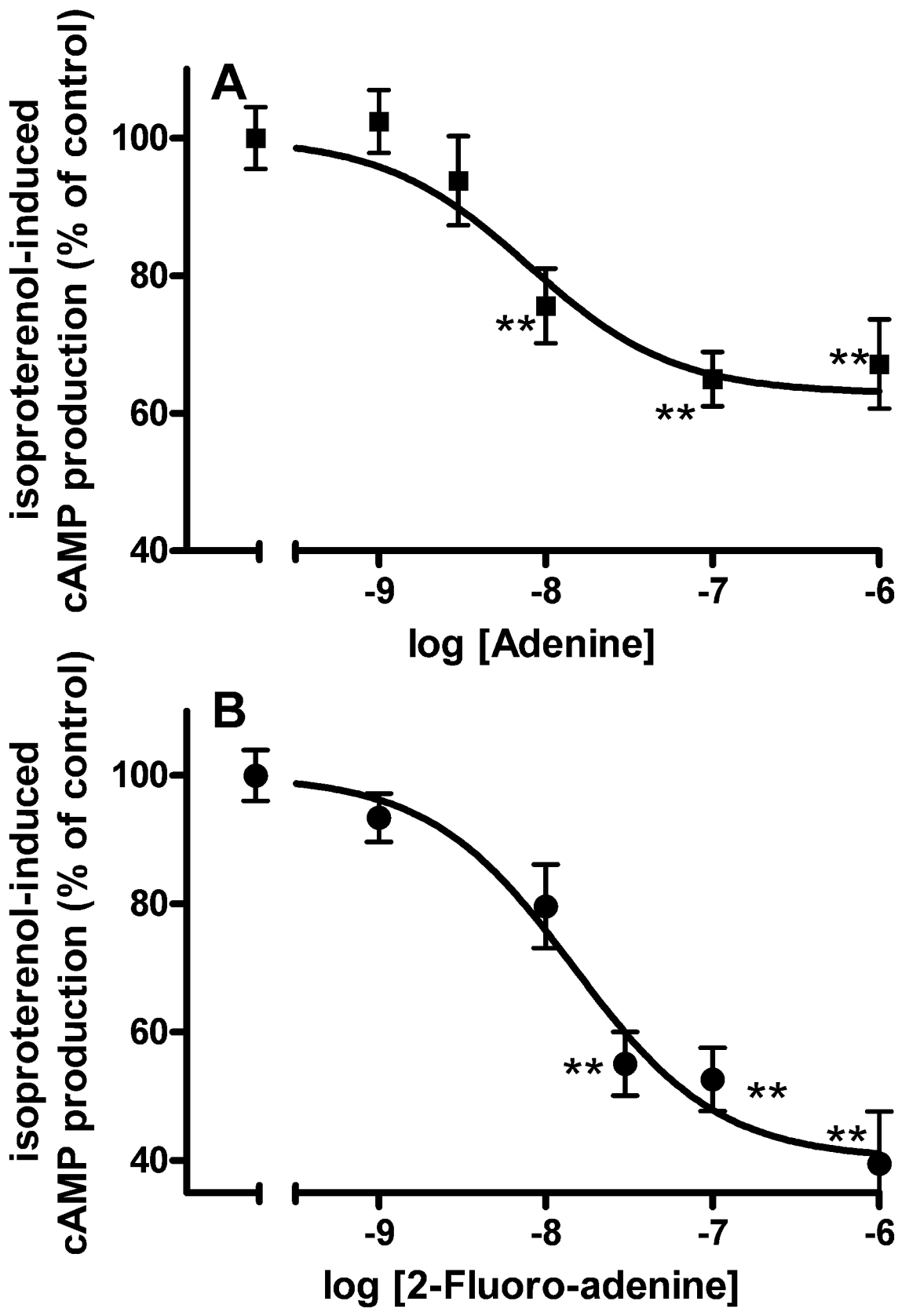

A
isoproterenol-induced cAMP production (% of control)
100
80
60
40
**
**
**
-9
-8
-7
-6
log [Adenine]
B
isoproterenol-induced cAMP production (% of control)
100
80
60
40
**
**
**
-9
-8
-7
-6
log [2-Fluoro-adenine]

Figure 3

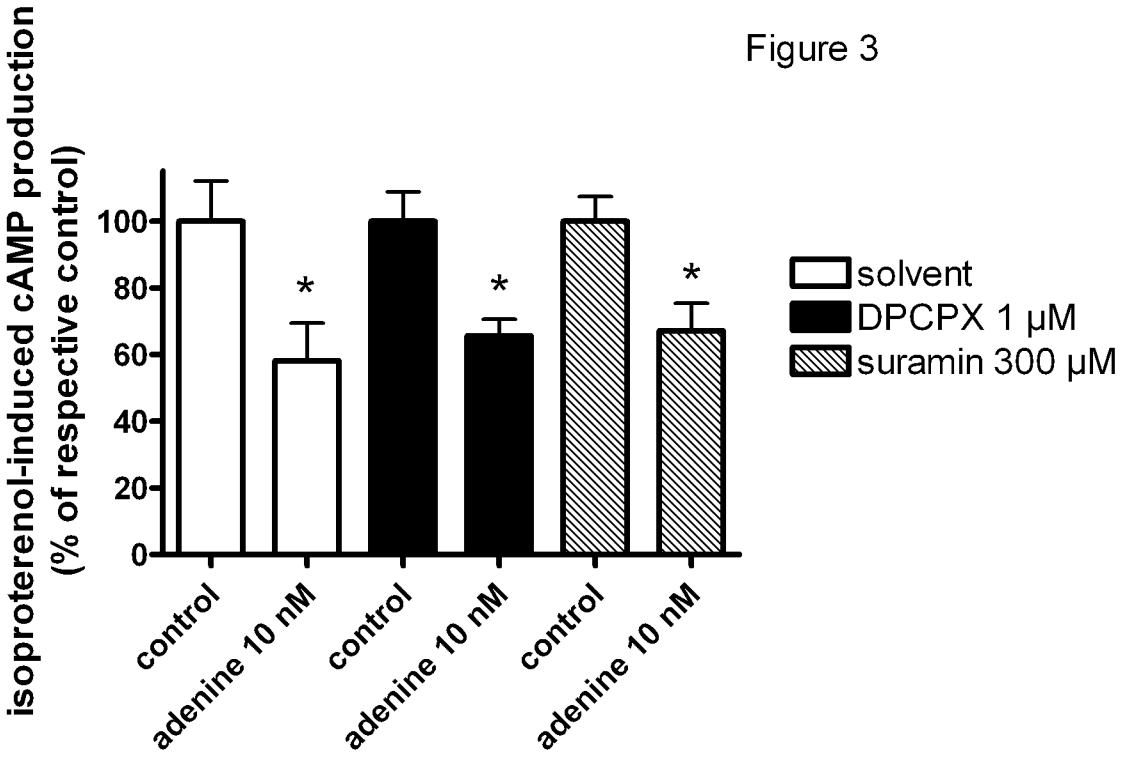

isoproterenol-induced cAMP production
(% of respective control)
100
80
60
40
20
0
*
*
*
control
adenine 10 nM
control
adenine 10 nM
control
adenine 10 nM
solvent
DPCPX 1 µM
suramin 300 µM

Figure 4

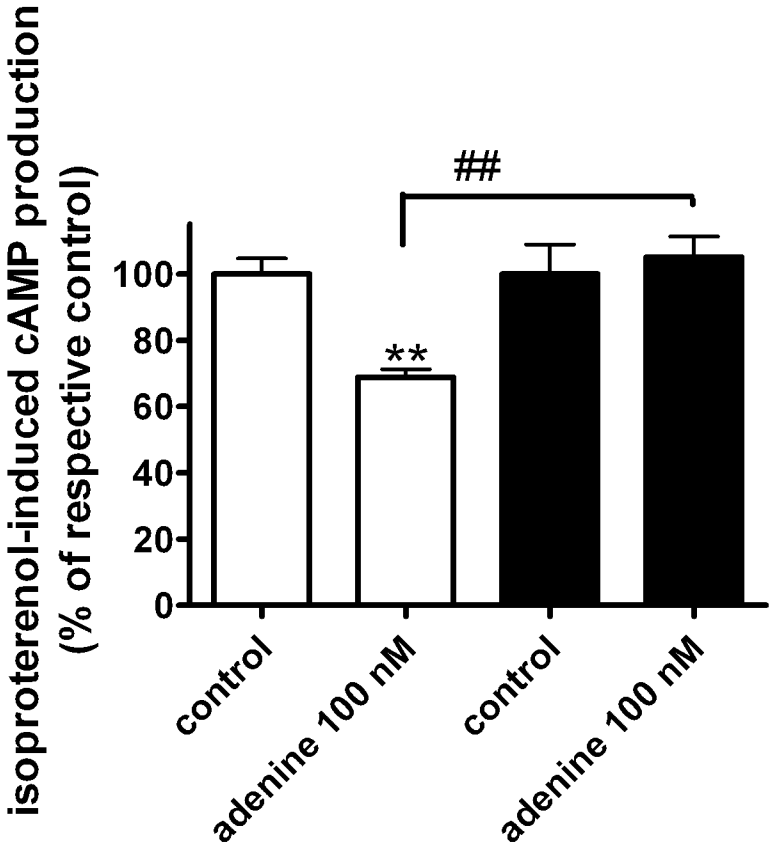

isoproterenol-induced cAMP production
(% of respective control)
##
**
100
80
60
40
20
0
control
adenine 100 nM
control
adenine 100 nM
pretreated with solvent
pretreated with PTX
200 ng/ml for 20 h

Figure 5

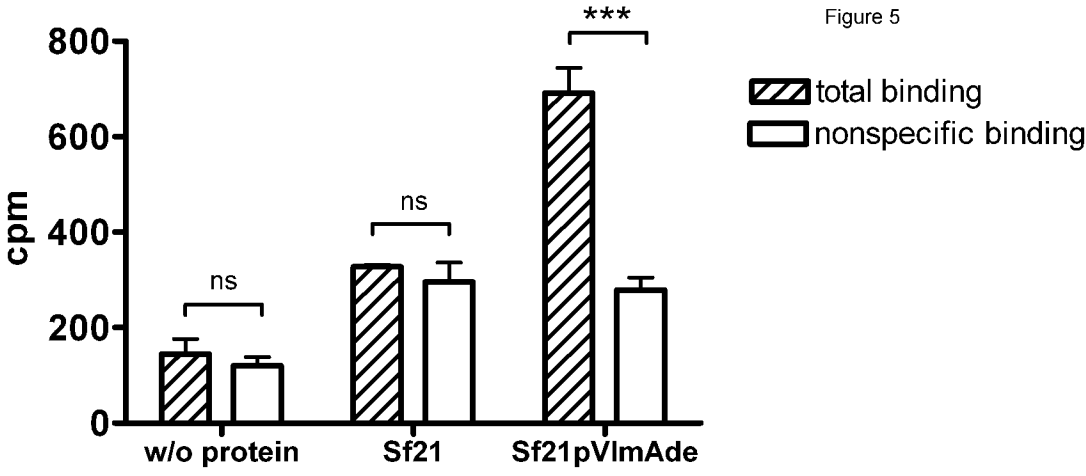

***
800
600
400
200
0
cpm
ns
ns
w/o protein
Sf21
Sf21pVlmAde
total binding
nonspecific binding

Figure 6

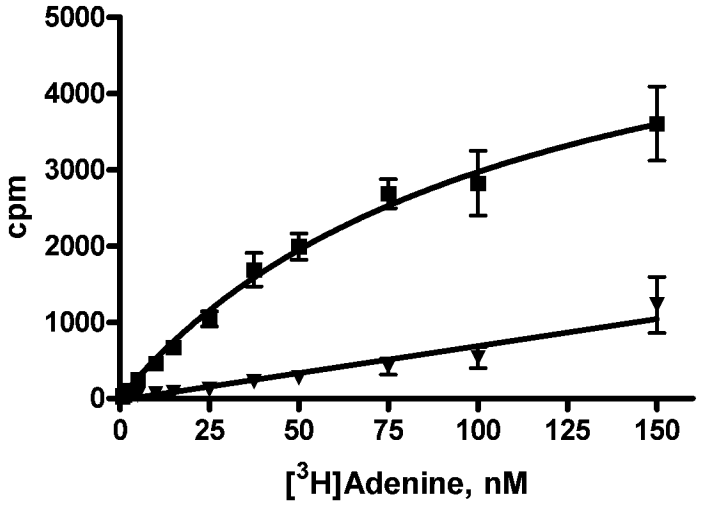

5000
4000
3000
2000
1000
0
cpm
0
25
50
75
100
125
150
[3H]Adenine, nM
specific binding
nonspecific binding

Figure 7A

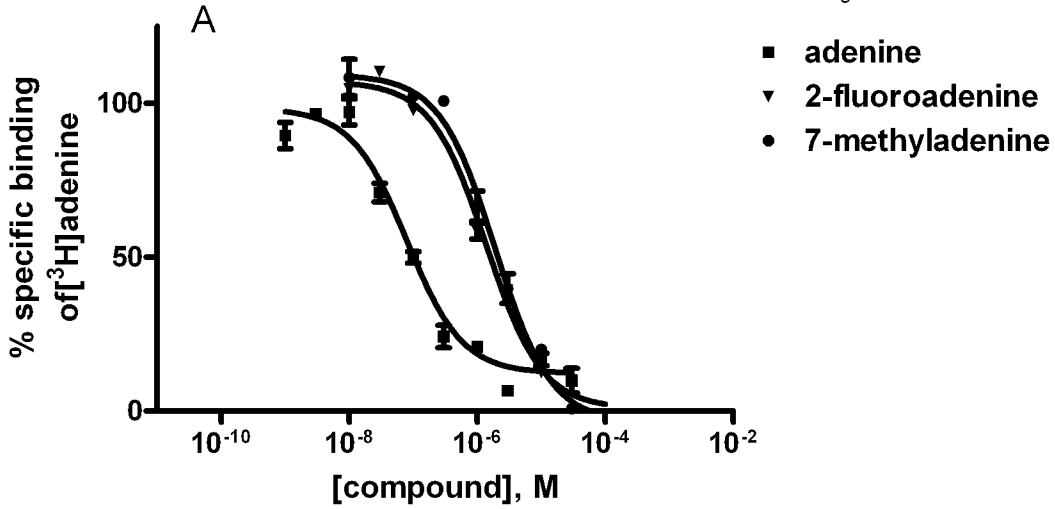
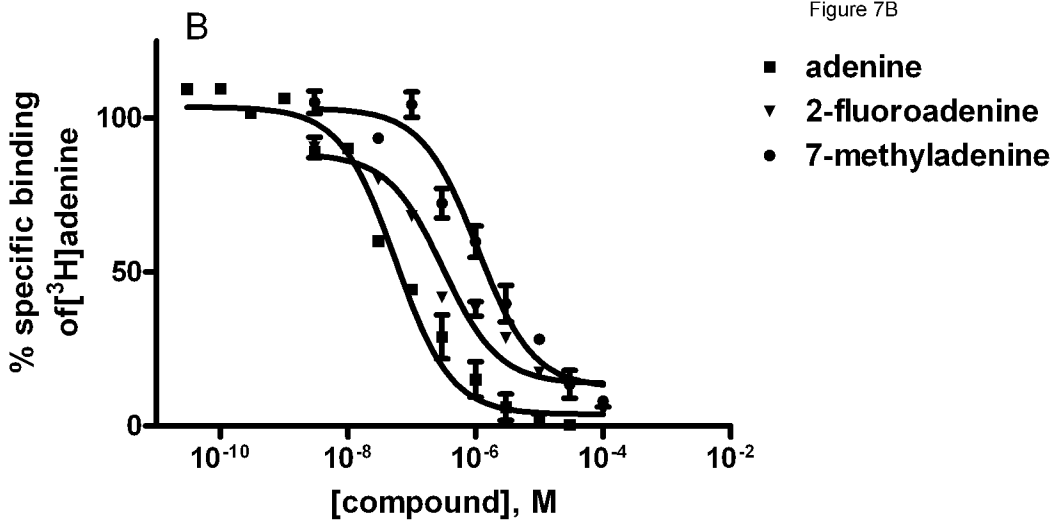

A
% specific binding of[^{3}H]adenine
100
50
0
10^{-10}
10^{-8}
10^{-6}
10^{-4}
10^{-2}
[compound], M
adenine
2-fluoroadenine
7-methyladenine

Figure 7B


B
adenine
2-fluoroadenine
7-methyladenine
% specific binding of[3H]adenine
100
50
0
10-10
10-8
10-6
10-4
10-2
[compound], M