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Evidence for the Functional Expression and Pharmacological Characterization of Adenine Receptors in Native Cells and Tissues

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Running title: Native Adenine Receptors

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Abbreviations:

AC, adenylate cyclase; ADA, adenosine deaminase; BSA, bovine serum albumin; CHO cells, Chinese hamster ovary cells; CPT, 8-cyclopentyltheophylline; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EDTA, ethylendiamine-tetraacetic acid; EGTA, ethyleneglycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; GPCR, G protein-coupled receptor; GppNHp, 5'-guanylylimidodiphosphate; GTPγS, guanosine 5′-[γ-thio]triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxantine; IUPHAR, International Union of Pharmacology; KRH buffer, Krebs-Ringer-HEPES buffer; NECA, 5'-(N-ethylcarboxamido)adenosine; NG108-15 cells, mouse neuroblastoma x rat glioma hybrid cell line; PTX, pertussis toxin; Ro20-1724, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone; RPMI medium, Roswell Park Memorial Institute

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medium; RT-PCR, reverse transcriptase-polymerase chain reaction; SCH58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazole-[4,3-*e*]-1,2,4 triazolo[1,5-*c*]pyrimidine

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ABSTRACT

An orphan G protein-coupled receptor from rat has recently been discovered to be activated by the nucleobase adenine (Bender et al. *Proc. Natl. Acad. Sci. USA* 2002, 99:8573-8578). In the present study we show for the first time that the adenine receptor is expressed in membrane preparations of native tissues and cell lines in high density, including rat brain cortex, rat brain striatum, and the mouse neuroblastoma x rat glioma hybrid cell line NG108-15. Saturation analysis with [^{3}H]adenine at rat brain cortical membranes exhibited a single high affinity binding site with a K_D value of 27.2 nM, and a binding capacity of 2.28 pmol/mg protein. Kinetic studies revealed unusual binding kinetics of [^{3}H]adenine with rapid association and slow dissociation. A series of compounds was investigated in [^{3}H]adenine competition experiments at rat brain cortex. Only minor substitution of the adenine structure was tolerated, the most potent compounds of the present series being 2-fluoroadenine (K_i 620 nM), 8-thioadenine (K_i 2.77 μM), N^6-methyladenine (K_i 3.64 μM) and 7-methyladenine (4.13 μM), all of which were partial agonists (40-60% intrinsic activity). Adenine dose-dependently inhibited forskolin-stimulated adenylate cyclase in membrane preparations of NG108-15 cells as well as in intact cells showing that the receptor is functional in NG108-15 cells. RT-PCR experiments followed by sequencing indicate that the NG108-15 cells express the murine ortholog of the adenine receptor. Moreover, preliminary radioligand binding studies with [^{3}H]adenine at membranes of human astrocytoma 1321N1 cells suggest that a human ortholog of the rat adenine receptor exists.

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Adenine was recently identified as the endogenous ligand of an orphan rat G protein-coupled receptor (GPCR) by a "reverse pharmacology" approach. mRNA localization studies revealed the highest expression in the small neurons of the dorsal root ganglia, suggesting a role in nociception (Bender et al., 2002). Recently, potent neurotrophic effects of adenine have been described: in primary cultures of rat cerebellar Purkinje cells the number of cells was greatly increased by the addition of adenine although relatively high concentrations (1-2 mM) were required (Yoshimi et al., 2003). Adenine was the most potent compound in a series of purine and pyrimidine derivatives that showed protective effects on Purkinje cells and prevented them from cell death, whereas other adenine-based purines, including adenosine, AMP, ADP, and ATP did not promote Purkinje cell survival (Watanabe et al., 2003).

Radioligand binding studies had previously been performed at membrane preparations of CHO cells stably transfected with the rat adenine receptor using [^{3}H]adenine as a radioligand (Bender et al., 2002). The recombinant rat adenine receptor expressed in CHO cells was found to be coupled to inhibition of adenylate cyclase (AC) via G_i protein (Bender et al., 2002). However, the postulated adenine receptor has not been demonstrated to be present in native tissues on the protein level and therefore it has not yet been officially accepted as a novel receptor by the IUPHAR committee on receptor nomenclature. A mouse ortholog (mMrgA10), but no human ortholog of the rat adenine receptor could be identified by sequence analysis and comparison (Bender et al., 2002).

The present study proves the existence of the adenine receptor in brain tissues and native cell lines and its functional coupling to inhibition of adenylate cyclase in NG108-15 cells. Furthermore, we have explored the structure-activity relationships of a large series of compounds structurally related to adenine.

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MATERIALS AND METHODS

Radioligand binding studies.

[^{3}H]Adenine saturation experiments. Saturation binding experiments were carried out at rat brain cortical and rat brain striatal membranes using [8-^{3}H]adenine (27 Ci/mmol, Amersham, Germany) as a radioligand. Frozen rat brain membranes were obtained by Pel Freez®, Rogers, Arkansas, USA, and thawed at 4°C. Cortex and striatum were dissected and membrane fractions were prepared as described (Müller et al., 2002). Protein concentrations of ca. 100 µg/mL were used in the saturation binding experiments, determined by the method of Lowry (Lowry et al., 1951) using a Sigma Aldrich protein assay kit. Incubations were carried out in a total volume of 200 µL Tris-HCl buffer 50 mM, pH 7.4 supplemented with 10 mM $MgCl_2$ and 1 mM EGTA, at room temperature (23°C) for 60 min. Nonspecific binding was determined with 10 µM adenine. In some experiments the stable GTP analog, 5'-guanylylimidodiphosphate (GppNHp, 100 µM) was present.

Incubation was terminated by rapid filtration through GF/B glass fiber filters, presoaked in 0.5% aqueous polyethylenimine solution for 60 min using a Brandel 48-channel cell harvester. Filters were rinsed three times with 2 mL each of ice-cold Tris-HCl buffer, 50 mM, pH 7.4. The wet filters were transferred to mini-vials and scintillation cocktail was added. Drying of the GF/B glass fiber filters prior to the addition of scintillation cocktail did not influence the results. Radioactivity on the punched-out filters was measured after 9 h of preincubation with 2.5 mL of Ultima Gold® scintillation cocktail (Canberra Packard, Dreieich, Germany).

[^{3}H]Adenine competition assays and kinetic studies. The adenine binding assays were carried out at rat brain cortical membranes using 10 nM [^{3}H]adenine as a radioligand. Protein amounts of ca. 100 µg/tube were used in the assays. Inhibition curves were determined using seven to nine different concentrations of the test compound, spanning 3 orders of magnitude.

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Three separate experiments were performed each in triplicate, unless otherwise noted. Nonspecific binding was determined with 10 µM adenine and amounted to 10-20% of total binding. Incubations were carried out in a total volume of 0.5 mL of Tris-HCl buffer 50 mM, pH 7.4 supplemented with 10 mM $MgCl_2$ and 1 mM EGTA, at room temperature (23°C) for 60 min. Further experimental procedures were as described above for saturation studies. Kinetic studies were performed using 15 nM [^{3}H]adenine. Dissociation was initiated by the addition of 1 µM adenine after 60 min of preincubation. Three separate experiments, each in triplicate, yielded similar results. Competition binding assays at membrane preparations of NG108-15 cells and astrocytoma 1321N1 cells were performed as described above for rat brain cortical membranes using ca. 100 µg of protein (NG108-15), or 150 µg of protein (astrocytoma), respectively, per tube.

Cell culture. NG108-15 cells were provided by Dr. Brüss, Pharmacological Institute, University of Bonn, and grown as described previously (Kaulich et al., 2003). For radioligand binding studies, the culture medium was removed and the cells were kept frozen at -80°C until the preparation of the membranes. Cells were thawed and detached in ice-cold Tris-HCl buffer 50 mM, pH 7.4 with a cell scraper. The cell suspension was homogenized on ice and spun down for 10 min at 1,000 g, 4°C. The supernatant was centrifuged at 20,000 g, 30 min, 4°C and the resulting pellet was resuspended in Tris-HCl buffer 50 mM, pH 7.4. The last centrifugation step was repeated once. The protein concentration was determined by the method of Lowry (Lowry et al., 1951). Membranes were kept frozen at -80°C until use. For cAMP assays, membranes were used immediately. Astrocytoma 1321N1 cells were grown adherently and maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum, penicilline G (100 U/mL), streptomycin (100 µg/mL) and 1% glutaMAX™ at 37°C, 5% CO_2. Cells were grown to confluence. For binding assays, culture

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medium was removed and cells were kept frozen at -80°C until membrane preparation following the procedure described by Griessmeier and Müller (2004, in press).

Molecular biological experiments. poly A+ mRNA was isolated from NG108-15 cells cultured as described above using the Oligotex Direct mRNA Mini Kit (Qiagen, Hilden, Germany). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using gene specific primers for the rat adenine receptor (Genbank accession code NM_145787; sense primer: CACCATGGGGGAAAGCTTCACCGGTACAG; anti-sense primer: CGGCTCCACCTTGCTGCTTGACATC) as well as the putative murine ortholog MrgA10 (Genbank accession code XM_195647; sense: CACCATGGGGGAAAGCAGCACCGGTGCAG; antisense: TGGCTCTGCTTTGCTTTTTGACATCTCC) and the Superscript One Step RT-PCR kit (Invitrogen, Karlsruhe, Germany). Annealing temperature for the PCR reaction was 56.0 °C (35 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 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).

[^{35}S]GTPγS Binding Assay. The [^{35}S]GTPγS binding assay was carried out as described (Bender et al., 2002) with minor modifications. The incubation buffer consisted of 20 mM HEPES, pH 7.4 containing 100 mM NaCl, 10 µM GDP, 10 mM $MgCl_2$, and 0.5% BSA. After a preincubation period of 30 min of rat cortical membranes (10 µg/tube) with agonist (adenine

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or N^6-cyclopentyladenosine) in the presence of adenosine deaminase (2 I.U./mL), [^{35}S]GTPγS was added in a final concentration of 0.5 nM (final volume: 200 µL). After a further incubation period of 60 min, bound and free [^{35}S]GTPγS were separated by filtration through a GF/B glass fiber filter, presoaked in Tris-HCl buffer 50 mM, pH 7.4 containing 10 mM $MgCl_2$.

Functional studies.

Cyclic AMP accumulation. (A) Using membrane preparations: Culture flasks with almost confluently grown NG108-15 cells were taken and cells were detached with a cell scraper. A membrane preparation of the cells was obtained according to the method described above. The membrane fraction was resuspended in Tris-HCl buffer 50 mM, pH 7.4 supplemented with 4 mM EDTA, 10 mM $MgCl_2$ and 5 µM GTP. Stock solutions of compounds in DMSO were added to Tris-HCl buffer 50 mM, pH 7.4, containing EDTA (4 mM), $MgCl_2$ (10 mM), GTP (5 µM), forskolin (10 µM) and ATP (1 mM), and the mixture was incubated for 20 min at 37°C with 90 µL of the membrane suspension. Incubation was terminated by rapidly heating to 98°C for 2 min. After cooling down to room temperature, the tubes were centrifuged at 2,000 g for 15 min at 4°C, using an Allegra 21 R centrifuge (Beckman Coulter, Germany). The supernatant (50 µL) was assayed in a charcoal adsorption-assay using a commercially available kit (Amersham, Germany) to determine the cAMP concentration. Each experiment was carried out in duplicates at least three times.

(B) Using whole cells: Cyclic AMP accumulation was determined in intact NG108-15 cells plated on a 48-well culture plate. After cells were almost grown to confluence the cell culture medium was removed, cells were washed twice with Krebs-Ringer-HEPES (KRH) buffer, pH 7.4 containing 130 mM NaCl, 4.7 mM KCl, 4.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$ 2 H_2O, 11.5 mM Glucose, 10 mM HEPES, 0.1% BSA, and were then preincubated with adenosine deaminase (1 I.U./mL), and the phosphodiesterase inhibitors

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Ro20-1724 (500 µM) and 3-isobutyl-1-methylxanthine (IBMX, 500 µM) in KRH buffer, pH 7.4, for 10 min at 37°C. Forskolin (final concentration: 10 µM) and a range of different concentrations of adenine was added. The incubation at 37°C was stopped after 10 min by removal of the reaction buffer. Cells were lysed with 0.1 M HCl for 30 min at room temperature. After neutralization with 1 M NaOH solution and further dilution the amount of cAMP was determined as described above. Each experiment was carried out in duplicates at least three times. In some experiments, cells were preincubated overnight in medium in the presence of pertussis toxin (PTX, 10, 100, or 1000 ng/mL, respectively). All three concentrations gave virtually identical results.

Data analysis. Data were analysed using Prism 3.0 (Graph Pad, San Diego, USA). IC_{50} values were determined by fitting data to a sigmoidal curve with variable slope. The Cheng-Prusoff equation and the K_D value of 28.6 nM for [^{3}H]adenine was used to calculate K_i values from the obtained IC_{50} values.

Chemicals. 1-Methyladenine, 7-methyladenine and 9-methyladenine were purchased from Acros Organics, Belgium. 2-Chloroadenine, 7-ethyladenine, 9-dihydroxypropyladenine, 9-ethyladenine, 8-bromoadenine, 1-deazaadenosine, 3-deazaadenosine, 2',3'-dideoxyadenosine, 2-[3-(1-phenylprop-2-yn-1-ol)-9-ethyladenine, 2-phenylethynyl-9-ethyladenine, 2-(hex-1-yn-1-yl)-9-ethyladenine, 8-phenyl-9-ethyladenine and 8-bromo-9-propyladenine were synthesized according to or in analogy to described procedures (Cristalli et al., 1987; Camaioni et al., 1998; Klotz et al., 2003). All other reagents were obtained from Sigma Aldrich, Germany.

RESULTS

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Saturation binding experiments at rat brain cortical membranes performed with [^{3}H]adenine revealed a single high affinity binding site with a K_D value of 27.2 ± 0.9 nM (n=5, Fig. 1A). A binding capacity (B_{max}) of 2.28 ± 0.01 pmol/mg protein was determined. The presence of a stable analog of GTP, GppNHp (100 μM), led to a small increase in the K_D-value (from 27.2 ± 0.9 (n=5) to 32.6 ± 2.7 nM (n=3)), which appeared to be reproducible, but did not quite reach statistical significance (P=0.058, two-tailed t-test). The B_{max} value was virtually unaltered (2.28 ± 0.01 (n=5, without GppNHp) versus 2.58 ± 0.37 pmol/mg protein (n=3, with GppNHp), P=0.47). Adenine (0.01 nM - 500 μM) did not have any effect on [^{35}S]GTPγS binding to rat brain cortical membranes. In membranes prepared from rat brain striatum [^{3}H]adenine saturation experiments exhibited a single binding site with a K_D value of 72.4 ± 5.0 nM and a B_{max} value of 8.23 + 0.8 pmol/mg protein (n=3, Fig. 1B). For the saturation experiments a two-site model did not give an improved fit over a one-site model. Kinetic studies (Fig. 2) showed that [^{3}H]adenine bound very rapidly to membranes from rat brain cortex. Steady state appeared to be reached as early as the first time point examined (30 sec) and was sustained for at least 200 min (not shown). Binding was slowly reversed by the addition of adenine in a final concentration of 1 μM, with ca. 60% of dissociation being evident after 60 min. A kinetic K_D value could not be determined. The law of mass action did not apply to the system.

A concentration of 10 nM of [^{3}H]adenine was suitable for competition binding assays at rat brain cortical membranes. At this concentration specific binding amounted to 80-90% of total binding. The addition of DMSO in concentrations up to 10% had no effect on [^{3}H]adenine binding (data not shown). NaCl (100 mM) did not alter [^{3}H]adenine binding significantly (data not shown).

Binding affinities for a series of compounds were determined in competition experiments at rat brain cortical membranes. Adenine itself showed a K_i value of 29.9 + 3.4 nM (Fig. 3). The investigated compounds included adenine derivates with substitution in the 1-, 2-, 3-, N^6-, 7-,

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8- or 9-position (Table 1, 2, and 3, Fig. 3), xanthine derivatives (Table 2), a selection of drugs with analgesic properties (salicylic acid, acetylsalicylic acid, acetaminophen, indomethacin, propyphenazone) and with effects against neuropathic pain (the antidepressants amitryptiline, imipramine, doxepine). Only adenine derivatives with small structural modifications showed appreciable affinity for the rat brain adenine receptor. All compounds investigated were considerably (at least 20-fold) less potent than adenine. The most potent derivatives were 2-fluoroadenine (**3**, K_i 620 nM), 8-thioadenine (**14**, K_i 2.77 μM), N^6-methyladenine (**6**, K_i 3.64 μM), and 7-methyladenine (**11**, K_i 4.13 μM) (Fig. 4). The following rank order of potency could be established: adenine >> 2-fluoroadenine > N^6-methyladenine = 7-methyladenine > 9-methyladenine ≥ 1-methyladenine > 3-methyladenine/N^6-dimethyladenine (Table 1). Adenosine (**18**) exhibited binding affinity in the micromolar range (K_i 19.4 ± 6.3 μM) and its 2′,3′-dideoxy analog (**19**) was equally potent (K_i 18.2 ± 3.4 μM) (Table 2). 1-Deazaadenosine (**20**), 3-deazaadenosine (**21**) and the adenosine derivative NECA (**22**) (Table 2) showed no significant affinity for the adenine receptor. The addition of adenosine deaminase, which is used in radioligand binding studies at adenosine receptors in order to remove endogenous adenosine by converting it to inosine, did not have any effect on the [^{3}H]adenine binding studies (data not shown). A selection of adenine derivatives with combinations of substituents in the 2-, 8-, and 9-position, that had previously been shown to be potent adenosine A_{2A} receptor antagonists, were found to be inactive at the rat brain adenine receptor (Table 3).

[^{3}H]adenine binding studies at membrane preparations of two permanent cell lines, the mouse neuroblastoma x rat glioma cells NG108-15, and the human astrocytoma 1321N1 cell line, revealed that both contained specific binding sites for adenine (Fig. 4). Competition assays at NG108-15 cell membranes using 10 nM of [^{3}H]adenine yielded an IC_{50} value of 154 ± 56 nM for unlabeled adenine and similar affinities and the same rank order of potency for a selection of adenine derivatives as seen in binding studies at rat brain cortex (Table 4).

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Freshly prepared membranes from NG108-15 cells and intact NG108-15 cells were used for functional studies (Fig. 5). At membranes adenine dose-dependently inhibited forskolin-stimulated adenylate cyclase with an IC_{50} value of 21 nM (95% confidence interval: 7-67 nM). Forskolin stimulated the basal cAMP production of ca. 6 pmol/mg protein/min about four-fold to ca. 25 pmol/mg protein/min. Maximal inhibition of ca. 50% was observed at 3 μM of adenine. The addition of adenosine deaminase (ADA) had no effect on cAMP accumulation (data not shown). Selected adenine derivatives were investigated in functional assays determining the inhibition of forskolin-stimulated cAMP accumulation at a single, high concentration. All of the compounds were partial agonists at the rat adenine receptor with somewhat weaker maximal inhibition as the full agonist adenine (see Table 5). Functional studies were additionally performed using whole NG108-15 cells, where adenine dose-dependently inhibited forskolin-stimulated adenylate cyclase activity with an IC_{50} value of 2.54 ± 0.99 μM. Maximal inhibition of ca. 70% was observed at 30 μM of adenine. Preincubation of the cells with pertussis toxin (10, 100, or 1000 ng/mL, respectively) led to a decrease of the maximal stimulation by forskolin of the cAMP production by ca. 50% (not shown). Pertussis toxin completely prevented adenine-induced inhibition of the forskolin-stimulated cAMP production (Fig. 6).

RT-PCR with primers specific for the coding sequence of the putative mouse ortholog but not with primers for the rat adenine receptor revealed products of the expected length from mRNA preparations prepared from cultured NG108-15 cells (Fig. 7; one of three independent runs). Control reactions without the enzyme reverse transcriptase showed no products confirming that mRNA but not genomic DNA acted as template for the PCR reaction. Cycle sequencing of one RT-PCR reaction product confirmed that it was a murine and not a rat sequence.

DISCUSSION

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Purinergic membrane receptors for the nucleoside adenosine (P1 receptors) and the adenine nucleotides ATP and ADP (P2 receptors) have been known to exist for more than 20 years. Recently, the aglycon adenine was found to activate an orphan rat G protein-coupled receptor belonging to the MAS-related gene (Mrg) receptor family with high affinity (K_i = 18 nM) (Bender et al., 2002). mRNA expression of the rat adenine receptor was detected in many tissues with highest levels found in the small neurons of the dorsal root ganglia (Bender et al., 2002). We have now for the first time investigated *native* adenine receptors on the protein level with radioligand binding and functional studies.

In saturation studies at rat brain cortical membranes [^{3}H]adenine exhibited a K_D value of 27.2 nM. This is well in accordance with literature data for the recombinant rat adenine receptor expressed in CHO cells (K_D = 24 nM) obtained at virtually identical conditions (Bender et al., 2002), demonstrating that [^{3}H]adenine actually labels the adenine receptor in rat brain cortex. At rat brain striatal membranes the determined K_D value was somewhat lower (72.4 nM) but still in the same concentration range. In both preparations, [^{3}H]adenine labeled only a single binding site with high affinity indicating that only a single adenine receptor appears to be present.

The adenine receptor density in rat cortical membranes was determined to be quite high with a B_{max} value of 2.28 pmol/mg protein; it was even higher in striatal membranes (8.23 pmol/mg protein). In comparison, the density of the highly expressed adenosine A_1 adenosine receptor in the rat brain is several-fold lower (Patel et al., 1982, Lloyd and Stone, 1985). The mRNA profile determined by Bender et al. (2002) had indicated low expression in striatum and somewhat higher expression in brain cortex. Our results show that mRNA levels do not necessarily correlate with protein expression levels of adenine receptors. The detected high densities of adenine receptors in rat brain cortex and striatum point to an important physiological role of these receptors.

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Guanine nucleotides have been shown to decrease the affinity of inhibitory agonists for their receptor (Yeung and Green, 1983, Stiles, 1988). This has been explained by a shift from a high-affinity to a low-affinity receptor state the presence of GTP. Such an effect is sometimes not very pronounced or even undetectable (Kelly et al., 1998). Saturation studies in the presence of the stable GTP analog GppNHp (100 μM) at rat brain cortical membranes revealed that the binding of [^{3}H]adenine was somewhat influenced by the guanine nucleotide, which led to a moderate increase in the K_D value. While GTP and its analogs can lead to decreased binding of an agonist to a G_i-coupled receptor, the reverse allosteric effect has also been observed, namely a stimulation of [^{35}S]GTPγS binding to the G_i protein by a receptor agonist. [^{35}S]GTPγS binding studies work well in artificial systems with high receptor expression levels. In fact, Bender et al. (2002) had shown that adenine increased [^{35}S]GTPγS binding in membranes prepared from CHO cells recombinantly expressing the rat adenine receptor. In native rat cortical membranes we could not show adenine-induced enhancement of [^{35}S]GTPγS binding. Showing such an effect in native tissues is sometimes difficult or even impossible. N^6-Cyclopentyladenosine, an agonist at the G_i-coupled adenosine A_1 receptor, also did not show any measurable increase in [^{35}S]GTPγS binding at native membranes in our hands, while it strongly increased [^{35}S]GTPγS in recombinant CHO cells expressing the human adenosine A_1 receptor in high density (data not shown).

Kinetic studies demonstrated that the binding of [^{3}H]adenine to rat brain cortical membranes was reversible. However, after a very fast association, dissociation initiated by the addition of a high concentration of unlabeled adenine was slow in comparison (Fig. 3). A kinetic K_D value could not be determined. An explanation for the observed unusual kinetics could be that the adenine receptor undergoes a conformational change after binding of [^{3}H]adenine; an initial low-affinity conformation may be converted to a high-affinity conformation for adenine upon binding.

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The binding site of the adenine receptor in rat brain cortex was further characterized by competition assays, and structure-activity relationships were determined by investigating a series of differently substituted adenine derivatives. Bender et al. had evaluated only a very limited selection of compounds at the recombinant rat adenine receptor, namely 1-methyladenine, 6-benzyladenine, adenosine, AMP, ADP, ATP, hypoxanthine and guanine, none of which was remarkably potent. Our results for this collection of compounds at the rat brain cortex receptor was mainly consonant with the results obtained by Bender et al. (2002) at the recombinant adenine receptors. The only major discrepancy was that adenosine appeared to exhibit measurable affinity for the adenine receptor in rat brain cortical membranes (K_i = 19.4 μM), while it was found inactive at the recombinantly expressed receptor in CHO cell membranes. However, the determined K_i value at rat brain cortex was almost 3 orders of magnitude higher than that for adenine. It cannot be excluded at present that adenosine is degraded by a purine nucleoside phosphorylase during the 60 min incubation with rat brain cortical membranes and the detected binding is due to formed adenine. The same may also be true for the adenine nucleotides AMP (**23**), ADP (**24**), and ATP (**25**) which show very weak affinity (39-51% inhibition of radioligand binding at 100 μM): the observed inhibition may be due to adenine formed through enzymatic degradation, although EGTA was present in the assays to inhibit nucleotidases.

In the competition studies adenine itself exhibited a K_i value of 29.9 nM, which is in good agreement with literature data for the recombinant receptor (18 nM; Bender et al., 2002) and with the determined K_D value (27.2 nM). Even only small modifications at the adenine core structure led to a major reduction or loss of affinity. In a series of mono-substituted adenine derivatives (Table 1) substitution with a fluorine atom in the 2-position was best tolerated resulting in 2-fluoroadenine (**3**), which was, however, 20-fold less potent than the unsubstituted adenine (620 nM vs. 30 nM). A fluorine atom is sterically similar to a hydrogen atom but has strong electronic effects (negative inductive effect) which must be responsible

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for the reduced affinity of **3** in comparison with adenine. Replacement of the fluorine atom by the larger halogen atom chlorine (compound **4**) further reduced affinity by 60-fold (K_i = 37.9 μM) showing that the receptor does not have any bulk tolerance in that region. Substitution of the exocyclic amino group was investigated. A methyl group was best tolerated (**6**, K_i = 3.64 μM), while the larger benzyl (**7**) and benzoyl (**8**) residues resulted in very weak binding. Interestingly, the more polar benzoyl substitution was better tolerated (K_i 46.6 μM) than a benzyl residue (K_i > 100 μM). The N^6-hydroxypentenyl-substituted adenine derivative *trans*-zeatin, a cytokinin (phytohormone), was somewhat more potent than **8** (K_i = 22.6 μM); it seems that polar substituents are better accepted by the receptor than unpolar residues. Dimethylation of the amino group abolished affinity indicating that a hydrogen bond donor (N-H) in that position was probably required for binding to the receptor, similarly as in adenosine receptors (Müller, 2000). Small polar substituents in the 8-position, such as thiol (**14**, K_i = 2.77 μM) and bromine (**13**, K_i = 17.3 μM) were tolerated by the receptor leading to moderately potent compounds. Methylation at the ring nitrogen atoms of adenine was explored. Methylated adenine derivatives exhibited quite different binding affinities depending on the position of the methyl group. The rank order of potency was 7-methyladenine (**11**, K_i = 4.13 μM) > 9-methyladenine (**15**, K_i = 17.5 μM) ≥ 1-methyladenine (**2**, K_i = 29.3 μM) >> 3-methyladenine (**5**, K_i >> 100 μM). Replacement of the methyl group in 7- or 9-methyladenine by an ethyl residue resulted in a large drop in affinity (compare **11**/**12** and **15**/**16**) indicating that the receptor did not have bulk tolerance in that positions. However, a more hydrophilic substituent in the 9-position, a dihydroxypropyl residue (compound **17**) was somewhat better tolerated than an ethyl residue.

A series of 9-alkyl-substituted adenine derivatives that bear an additional substituent either in the 2- or 8-position were recently described as relatively potent adenosine A_{2A} receptor

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antagonists (Klotz et al., 2003). A_{2A} antagonists acting at receptors in the striatum (caudate-putamen) are currently developed as novel therapeutics for Parkinson's disease (Chen, 2003). Some of the disubstituted adenine derivatives, including compound **31**, had been evaluated in animal models of Parkinson's disease. The compounds had induced contralateral turning in 6-hydroxydopamine-lesioned rats after systemic application in a dose of 5 mg/kg. In contrast, the standard A_{2A} antagonist SCH58261 had required priming by dopamine to induce the same effect (M. Morelli, oral presentation at the Satellite Symposium on Purinergic Receptors, Camerino, Italy, 2003). Since we have found that adenine receptors are highly expressed in the rat striatum, we investigated whether the adenine derivatives described as adenosine A_{2A} receptor antagonists were selective for the adenosine receptors or whether they showed additional binding to the adenine receptor. In fact, the disubstituted adenine derivatives did not exhibit any affinity for adenine receptors in concentrations up to 100 μM (Table 3).

Adenosine (**18**) exhibited a more than 600-fold lower affinity in comparison with the natural ligand adenine. The potent, non-selective adenosine receptor agonist NECA (**22**), which has been found to also bind with high affinity to non-adenosine receptor binding sites (Lorenzen et al., 1993, Lorenzen et al., 1996, Yoshioka et al., 2001) showed no significant binding affinity for the adenine receptor. As pointed out above, adenosine might be partially degraded to adenine during the assay, while NECA is enzymatically more stable. 2′,3′-Dideoxyadenosine (**19**), which lacks the 2′- and 3′-hydroxyl groups at the ribose moiety, exhibited a similar K_i value as adenosine. 1-Deazaadenosine (**20**) and 3-deazaadenosine (**21**) showed only low affinity for the adenine receptor, but it seems that the N-atom in position 1 is more important for binding than the N-atom in position 3. The adenine nucleotides AMP (**23**), ADP (**24**) and ATP (**25**) appeared to exhibit some affinity to the adenine receptor in similar, high concentrations (39-51 % inhibition of radioligand binding at 100 μM), but this might be due to degradation of the nucleotides to adenosine or adenine by enzymes present in the membrane preparations. The obained structure-activity relationships are not consistent with

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those obtained for the ATP binding site of adenylate cyclases, the so-called P-site (Dessauer et al., 1999; Levy et al., 2003).

All other compounds that we screened and that were not derived from adenine, including the xanthine derivatives caffeine (**26**), theophylline (**27**), CPT (**28**) and DPCPX (**29**), all of which are adenosine receptor antagonists, exhibited no significant binding affinity for the adenine receptor at a high concentration of 100 μM. A set of analgesic and antidepressant compounds was investigated due to the finding that the adenine receptor is highly expressed in the small neurons of the dorsal root ganglia (Bender et al., 2002), suggesting a role in nociception. Neither the analgesics nor the tricyclic antidepressants, which have been shown to be effective in neuropathic pain (Mattia and Coluzzi, 2003), exhibited significant binding affinity for the adenine receptor.

Specific binding sites for [^{3}H]adenine with similar properties as in brain cortex were also detected in the permanent mouse neuroblastoma x rat glioma cell line NG108-15. This cell line can therefore be used as a model system for studying natively expressed adenine receptors. RT-PCR experiments indicated that the cells express the murine ortholog of the adenine receptor. We found that in NG108-15 cells the adenine receptor was coupled to inhibition of adenylate cyclase, as it had previously been shown for CHO cells recombinantly expressing the rat adenine receptor (Bender et al., 2002). The determined IC_{50} value (21 nM) for adenine in inhibiting forskolin-stimulated adenylate cyclase in membrane preparations of NG108-15 cells was roughly in the same concentration range as the previously determined IC_{50} and K_i values obtained in radioligand binding studies. An about 100-fold higher IC_{50} value for adenine of 2.5 μM was determined in adenylate cyclase assays when intact NG108-15 cells were used. The reason for this discrepancy may be the fact that the intact cells have a machinery for quickly and efficiently removing physiological receptor agonists - by degradation or cellular uptake - in order to limit their actions. However, little is known so far about the extracellular metabolism of adenine. Transporter proteins for adenine have been

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described, which may contribute to the removal of adenine from the extracellular compartment (De Koning and Diallinas, 2000; Redzic et al., 2001). Pertussis toxin (PTX) is a modifier of G_i/G_o proteins, which ADP-ribosylates a cysteine residue of the α subunit of G_i/G_o proteins (Yamane and Fung, 1993). This results in the irreversible inactivation of the G protein and in a loss of its ability to transduce signals from activated receptors to adenylate cyclase (Kurose et al., 1986). Preincubation of the cells with PTX resulted in a decrease in the maximal stimulation of cAMP production induced by forskolin. PTX effects on control cAMP levels have previously been described, e.g. Hughes et al. observed an enhancement of the cAMP production by PTX in NG108-15 cells stimulated with prostaglandin E_1 (Hughes et al., 1984). We could show that preincubation of the cells with pertussis toxin at concentrations as low as 10 ng/mL completely prevented the inhibitory effects of adenine on forskolin-stimulated cAMP production indicating that adenine exhibited its effect via a G_i protein. A direct effect of adenine on adenylate cyclase therefore appears to be very unlikely. In addition, adenine does not fulfill the structural requirements for high-affinity binding to the P-site of adenylate cyclase (Dessauer et al., 1999).

Selected adenine derivatives were all found to act as partial agonists at the adenine receptor (Table 5). Bender et al. had tried to identify a potential human ortholog of the rat adenine receptor by sequence comparison but were unsuccessful and concluded that adenine may not be a signaling molecule in humans (Bender et al., 2002). In radioligand binding studies we have detected high specific binding of 10 nM [^{3}H]adenine to membrane preparations of a human cell line, astrocytoma 1321N1 cells (Fig. 5). Therefore we conclude that a human ortholog of the rat adenine receptor is very likely to exist.

In conclusion, we have shown for the first time that adenine receptors are expressed in high density in the rat brain. Functional coupling to inhibition of adenylate cyclase has been found in a native cell line (NG108-15) which most likely express the murine ortholog of the

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receptor. Structure-activity relationships of adenine derivatives have been investigated. Based on radioligand binding studies we postulate that a human ortholog of the rodent adenine receptor exists. Thus, the adenine receptor adds a third family (P3) besides the P1 (adenosine) and the P2 (nucleotide) receptors to the class of purinergic receptors. Like P1 and P2 receptors adenine receptors may be of interest as novel drug targets.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Saturation curves of [^{3}H]adenine binding to (A) rat brain cortical membranes and (B) rat brain striatal membranes and corresponding Scatchard-Rosenthal plots. K_D and B_{max} values were 27.2 ± 0.9 nM and 2.28 ± 0.01 pmol/mg protein for cortex in the absence of GppNHp (n=5), 32.6 ± 2.7 nM and 2.58 ± 0.4 pmol/mg protein in the presence of 100 µM GppNHp (n=3), and 72.4 ± 5.0 nM and 8.23 ± 0.8 pmol/mg protein for striatum (n=3), respectively. Experiments were performed as described under *Materials and Methods*.

Figure 2. Kinetics of [^{3}H]adenine binding (15 nM) to rat brain cortical membranes at 23°C. (A) representative association curve for specific [^{3}H]adenine (15 nM) binding to rat brain cortical membranes (n = 3); (B) representative dissociation curve obtained by the addition of 1 µM unlabeled adenine to equilibrated specific binding of [^{3}H]adenine (15 nM) at rat brain cortical membranes (n = 3).

Figure 3. Competition curves versus 10 nM [^{3}H]adenine at rat brain cortical membranes of adenine and selected adenine derivatives. Data points are means of three independent experiments performed in triplicate.

Figure 4. [^{3}H]Adenine binding (10 nM) to membrane preparations of the mouse neuroblastoma x rat glioma hybrid cell line NG108-15 (100 µg of protein) and the human astrocytoma cell line 1321N1 (150 µg of protein).

Figure 5. Inhibition of forskolin-stimulated cAMP accumulation by adenine determined at (A) membrane preparations of NG108-15 cells (IC_{50} = 21 nM, 95% confidence interval: 7-67 nM) and in (B) intact NG108-15 cells (IC_{50} = 2.54 ± 0.99 nM). Data points are from (A) three and (B) six independent experiments performed in duplicate.

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Figure 6. Forskolin (10 mM)-induced cAMP production in intact NG108-15 cells after pertussis-toxin pretreatment in the absence of adenine (control, set at 100%) and in the presence of 30 μM adenine. Data bars represent means from two independent experiments performed in duplicate.

Figure 7. RT-PCR experiment indicating the expression of the murine ortholog of the adenine receptor in NG108-15 cells. Poly A^+ mRNA was amplified by RT-PCR with specific primers for the rat adenine receptor (R+) or the putative murine ortholog MrgA10 (M+). 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. bp, 100 base pair ladder.

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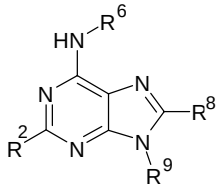
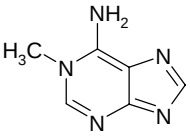
Table 1. Affinities of mono- and disubstituted adenine derivatives for adenine receptors in rat brain cortical membranes



1, 3-4, 6-9, 13-17



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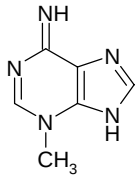

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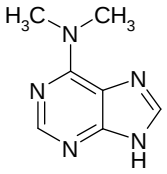

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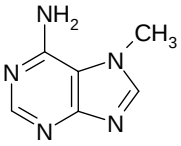

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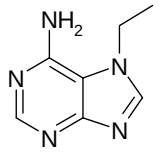

12

	Compound	R^2	R^6	R^8	R^9	K_i ± SEM [μM] (or % inhibition of radioligand binding at 100 μM)[a]
1	adenine	H	H	H	H	0.0299 ± 0.0034
2	1-methyladenine	-	-	-	-	29.3 ± 9.3
3	2-fluoroadenine	F	H	H	H	0.62 ± 0.14
4	2-chloroadenine	Cl	H	H	H	37.9 ± 4.4
5	3-methyladenine	-	-	-	-	(5% ± 2)
6	N^6-methyladenine	H	methyl	H	H	3.64 ± 0.36
7	N^6-benzyladenine	H	benzyl	H	H	(30% ± 3)
8	N^6-benzoyladenine	H	benzoyl	H	H	46.6 ± 15.3
9	*trans*-zeatin	H	(*E*)-$CH_2CH=C(CH_3)CH_2OH$	H	H	22.6 ± 2.1
10	N^6-dimethyladenine	-	-	-	-	(11% ± 2)
11	7-methyladenine	-	-	-	-	4.13 ± 1.08
12	7-ethyladenine	-	-	-	-	47.3 ± 4.1
13	8-bromoadenine	H	H	Br	H	17.3 ± 2.7
14	8-thioadenine	H	H	SH	H	2.77 ± 0.12
15	9-methyladenine	H	H	H	methyl	17.5 + 3.0
16	9-ethyladenine	H	H	H	ethyl	(27% ± 2)
17	9-dihydroxy-propyladenine	H	H	H	$-CH_2CHOHCH_2OH$	78.6 ± 14.6

[a]results from 3 independent experiments performed in triplicate

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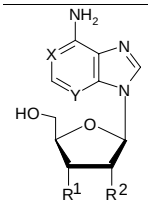
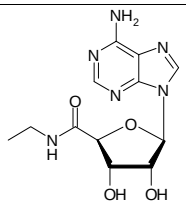
Table 2. Affinities of adenosine, adenosine derivatives and xanthine derivatives to adenine receptors in rat brain cortical membranes



18-21



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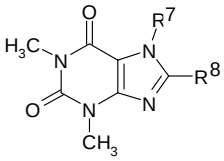
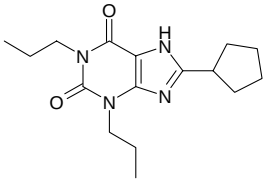


26-28



29

	Compound	R^1	R^2	X	Y	R^7	R^8	% inhibition of radioligand binding at 100 μM[a] (or K_i ± SEM)[a]
18	adenosine	OH	OH	N	N	-	-	46% ± 2 (K_i 19.4 ± 6.3 μM)
19	2′,3′-dideoxyadenosine	H	H	N	N	-	-	78% ± 1 (K_i 18.2 ± 3.4 μM)
20	1-deazaadenosine	OH	OH	CH	N	-	-	9% ± 2
21	3-deazaadenosine	OH	OH	N	CH	-	-	26% ± 5
22	NECA	-	-	-	-	-	-	10% ± 2
23	AMP	-	-	-	-	-	-	47% ± 1
24	ADP	-	-	-	-	-	-	51% ± 3
25	ATP	-	-	-	-	-	-	39% ± 7
26	caffeine	-	-	-	-	CH_3	H	2% ± 1
27	theophylline	-	-	-	-	H	H	3% ± 2
28	CPT	-	-	-	-	H	cyclopentyl	3% ± 2
29	DPCPX	-	-	-	-	-	-	4% ± 2

[a]results from 3 independent experiments performed in triplicate

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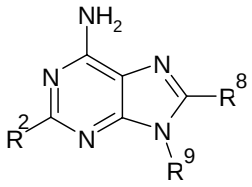
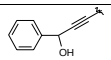
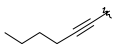
Table 3. Adenine receptor affinities of selected adenine derivates with high affinity for A_{2A} adenosine receptors



				K_i (nM)	
	R^2	R^8	R^9	A_{2A} adenosine receptor vs. [^{3}H]NECA[a]	adenine receptor vs. [^{3}H]adenine
30		H	ethyl	35	> 100,000[b]
31	H	ethoxy	ethyl	46	> 100,000[b]
32	H	Br	propyl	300[c]	> 100,000[b]
33	H	phenyl	ethyl	360	> 100,000[b]
34		H	ethyl	390	> 100,000[b]
35		H	ethyl	400	> 100,000[b]

[a] Data from Klotz et al., 2003

[b] Inhibition of radioligand binding (10 nM [^{3}H]adenine) was less than 10% at a concentration of 100 μM of test compound

[c] Data from Volpini et al., 2003

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Table 4. Comparison of the inhibition of radioligand binding at the adenine receptor in rat brain cortical membranes and in membrane preparations of NG108-15 cells

Compound	% inhibition of [^{3}H]adenine binding at drug concentration of 100 μM (or IC_{50}) ± SEM	
	rat brain cortex vs. [^{3}H]adenine[a]	NG108-15 cells vs. [^{3}H]adenine[b]
adenine	(IC_{50} = 41.1 ± 4.7 nM)	(IC_{50} = 154 ± 56 nM)
1-methyladenine	39 ± 3	5 ± 7
2-fluoroadenine	93 ± 2	94 ± 2
7-methyladenine	95 ± 1	89 ± 1
N^6-benzyladenine	30 ± 3	8 ± 12
N^6-dimethyladenine	11 ± 2	0 ± 6
adenosine	46 ± 2	57 ± 4

[a] results from 3 independent experiment performed in triplicate
[b] results from 2 independent experiments performed in duplicate

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Table 5. Inhibition of forskolin-stimulated adenylate cyclase by adenine and adenine derivatives

Compound	Concentration [μM] (fold K_i value)	% Inhibition with respect to adenine (= 100 %)
adenine	3.00 (100-fold)	100
2-fluoroadenine	60.0 (100-fold)	56 ± 2
7-methyladenine	250 (60-fold)	41 ± 3
9-methyladenine	250 (15-fold)	60 ± 7
N^6-methyladenine	250 (70-fold)	52 ± 13
8-thioadenine	250 (90-fold)	60 ± 3
trans-zeatin	250 (10-fold)	60 ± 8

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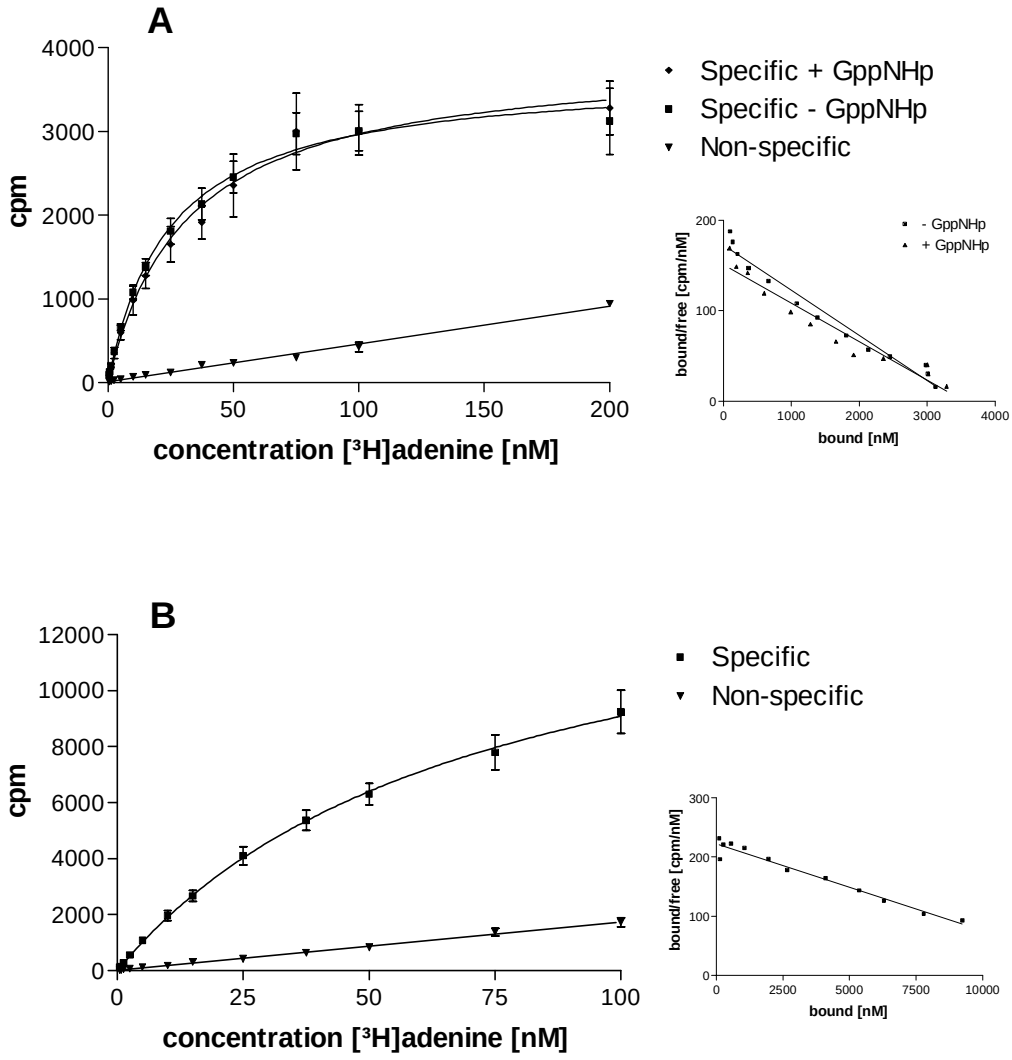

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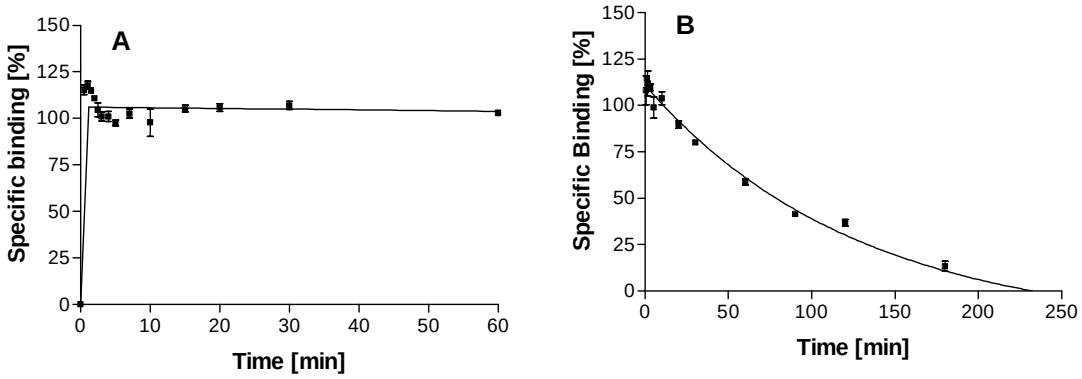

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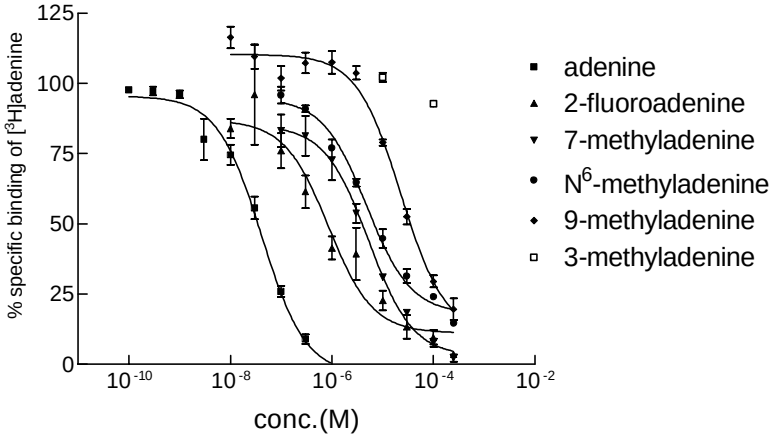

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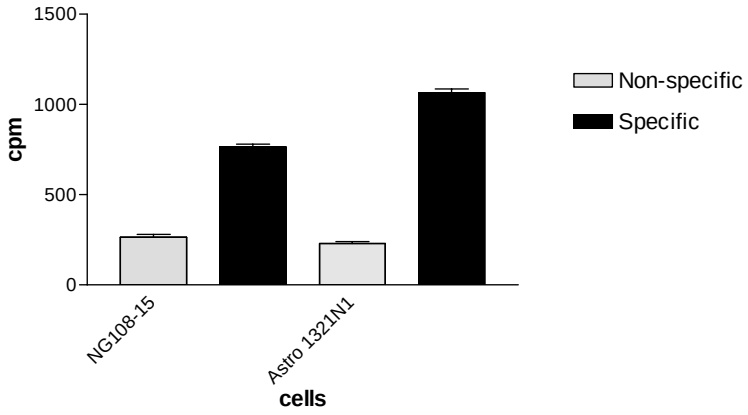

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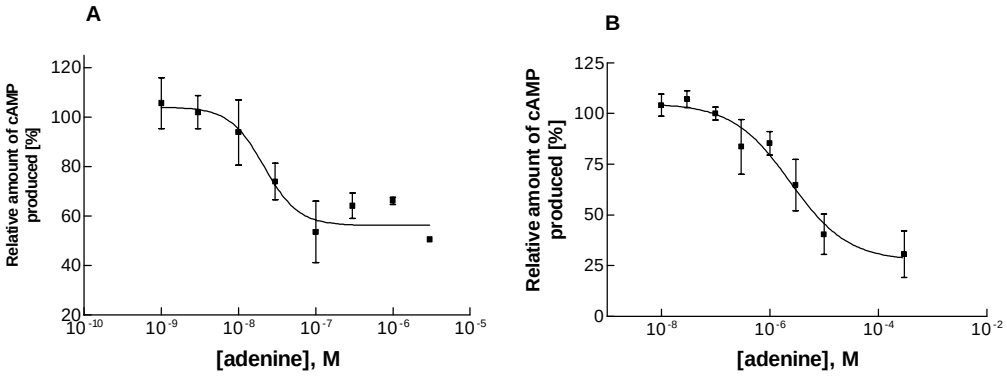

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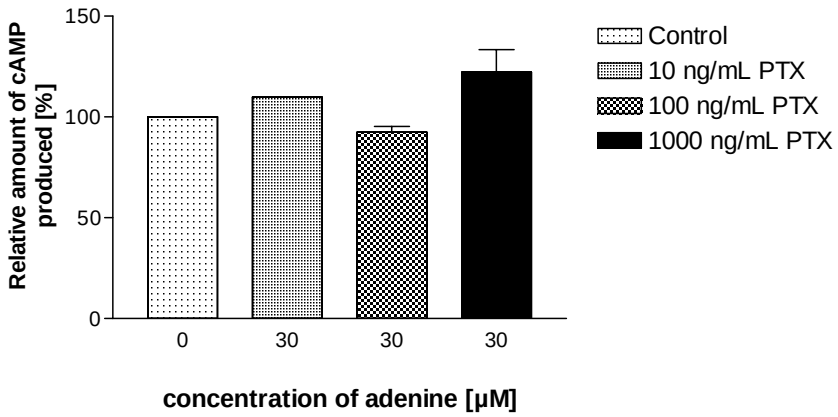

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