Adenosine-induced neuroprotection
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Chapter 4

The mouse brain adenosine A₁ receptor: functional expression and pharmacology

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Summary

The adenosinergic system is involved in many important physiological functions. Adenosine exerts its extracellular effects through four types of G-protein coupled receptors: A1, A2A, A2B and A3. Adenosine acts as an important regulator of metabolic processes. In the brain adenosine mediates prominent neuroprotective functions via the adenosine A1 receptor. Whereas the pharmacological characteristics of the rat and human adenosine A1 receptor have been intensively studied, the mouse adenosine A1 receptor has not yet been characterized. Accordingly, we have cloned the mouse brain adenosine A1 receptor and present here a pharmacological characterization of the mouse adenosine A1 receptor using functional studies and radioligand binding assays. The results show that the binding affinities of several ligands for the mouse adenosine A1 receptor are similar to the affinities for the rat and human adenosine A1 receptor with some exceptions.

Introduction

The four distinct adenosine receptor subtypes, A1, A2A, A2B and A3, belong to the family of G-protein coupled receptors [11]. Originally adenosine A1 and A3 receptors were described to interact mainly with Gi-proteins and induce inhibition of adenylyl cyclase whereas adenosine A2A and A2B receptors are coupled mainly to Gs-proteins, consequently stimulate adenylyl cyclase and increase cAMP levels [12, 32]. However, since adenosine receptors have also been reported to interact with different G-proteins and signal through various other pathways, independent of adenylyl cyclase, as reviewed recently [37] the above mentioned classification should be regarded as historical and may not solely reflect the situation in vivo.

Since adenosine was first described to be involved cardiovascular regulation [9], extensive research has been performed and a large number of physiological functions of adenosine has been described (for reviews see: [10, 17, 22, 28, 35, 38, 42]). After the cloning of the first adenosine receptor [20], different adenosine receptor subtypes have been characterized. The adenosine A1 receptor has been cloned from several species: [1, 21, 25, 27, 29, 31, 33, 43, 45]. Fewer reports described the cloning of adenosine A3 [29, 43, 45], adenosine A2A [26] and adenosine A2B [33] receptor subtypes.

Mouse strains with targeted deletions of adenosine A1, A2A and A3 receptor subtypes have been generated [16, 19, 44]. These transgenic mice prove to be a useful tool to unravel the function of these adenosine receptor subtypes [30]. Despite the large interest in mouse adenosine A1 receptors, its pharmacological properties are largely unknown [12]. This is in contrast to the human and rat adenosine receptors, which have been well characterized in pharmacological studies. In order to elucidate the pharmacological properties of the mouse
adenosine A\textsubscript{1} receptor, we have cloned the adenosine A\textsubscript{1} receptor from mouse brain and expressed it in Chinese Hamster Ovary (CHO) cells for pharmacological characterization and functional studies. Additionally, further binding studies were performed on rat and human adenosine A\textsubscript{1} receptor in order to compare the binding affinities for all three species.

Materials and methods

Reagents

Phosphate-buffered saline, Dulbecco’s Modified Eagle medium (DMEM), DMEM-F12, fetal calf serum and penicillin/streptomycin were obtained from Gibco. Reverse transcriptase enzyme and buffer were purchased from Promega. Taq-polymerase and buffer for polymerase chain reaction (PCR) were obtained from Eppendorf (Boulder, CO, U.S.A). PCR-primers were ordered from Genset oligo’s. N\textsuperscript{6}-cyclopentyladenosine (CPA), R-N\textsuperscript{6}-phenylisopropyladenosine (R-PIA), cyclopentyltheophylline (CPT), N\textsuperscript{6}-cyclopentyl-9-methyladenine (N-0840) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from RBI (Natick, MA, U.S.A.). N-ethylcarboxamidoadenosine (NECA), 2-chloro-N\textsuperscript{6}-cyclopentyladenosine (CCPA) and forskolin were purchased from Sigma (St.Louis, MO, U.S.A.), while adenosine deaminase was from Roche Biochemicals (Mannheim, Germany). 2-[(p-(2-carboxyethyl)phenyl-ethylamino]-5’-N-ethylcarboxamidoadenosine (CGS21680) was from Ciba Geigy. 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241358) was from Zeneca Pharmaceuticals. [\textsuperscript{3}H]DPCPX was purchased from Amersham, whereas [\textsuperscript{3}H]cAMP was from NEN (Du Pont Nemours, ‘s-Hertogenbosch, NL). GTP was obtained from Aldrich. 2-chloro- N\textsuperscript{6}-(3-iodobenzyl)-N-methyl-5’-carbamoyladenosine (Cl-IB-MECA) was a kind gift from K.A. Jacobson, NIH, USA.

Glial cultures

Mixed astrocyte cell cultures were established as described previously [3]. In brief, mouse cortex was dissected from new-born mouse pups (<1 day). Brain tissue was gently dissociated by trituration in phosphate-buffered saline and filtered through a cell strainer (70 mm Ø, Falcon) into Dulbecco’s Modified Eagle medium (DMEM). After two washing steps (200 x g for 10 min), cells were seeded in culture flasks (75 cm\textsuperscript{2}, Greiner; 10 x 10\textsuperscript{6} cells/flask). Cultures were maintained up to 4 weeks in DMEM containing 10% fetal calf serum with 0.01% penicillin and 0.01% streptomycin in a humidified atmosphere (5% CO\textsubscript{2}) at 37\textdegree C. Culture medium was changed the second day after preparation and every 6 days thereafter.
Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were lysed in guanidinium isothiocyanate/mercaptoethanol buffer and total RNA was extracted with one phenol/chloroform step, precipitated according to Chomczynski and Sacchi [5]. Subsequently RNA was treated with RNase free DNase (Sigma-Aldrich, Bornhem, Belgium) for 2 hours and precipitated. Reverse transcription (RT): 1 µg of total RNA was transcribed into cDNA in a total volume of 25 µl containing 0.5 µl of M-MLV reverse transcriptase, 1 µl of RNase inhibitor, 1 µl of random hexamers (0.2 mM), 5 µl of 5x buffer, 5 µl of deoxynucleosidetriphosphates (dNTPs) (2.5 mM) and H2O adjusted to 25 µl. After 60 min at 42°C, the reaction was stopped by heating at 95°C for 5 min. The quality of the cDNA was controlled using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers [4] and potential contamination by genomic DNA was checked for by running the reactions without reverse transcriptase and using GAPDH primers in subsequent polymerase chain reaction (PCR) amplifications. PCR: 2 µl of the RT reaction was used in subsequent PCR amplification as described previously [3]. In brief, the following reagents were added: 5 µl 10x PCR-buffer, 2.5 µl MgCl2 (50 mM), 0.5 µl dNTPs (10 mM), 1 µl of each primer, 38 µl H2O and 0.1 µl Taq-polymerase. Primer sequences for mouse full-length adenosine A1 receptor:

- forward primer: 5’-CATGCCGCGCATCCTTC-3’;
- backward primer: 5’-TCTAGCTCAGCTTCTC-3’.

Cycle numbers were 35 and annealing temperature was 58°C. The sequence of the PCR product was verified by TA cloning into pCRII (Invitrogen). In brief PCR products were stored on ice after the amplification. 20 µl of the resulting PCR product was checked by gel electrophoresis and 2 µl was used for ligation into linearized pCRII (Invitrogen) according to the instructions of the manufacturer. The ligation product was used to transform competent bacteria (TOP-10F, Invitrogen) and resulting bacterial colonies were grown overnight in 5 ml LB medium. Plasmid preparation was done by standard methods and positive plasmids were sequenced by ALF (sequencing facility; University of Groningen). A XhoI /BamHI fragment, containing the full-length adenosine A1 receptor coding region, was excised from the pCRII vector clone and ligated into a XhoI /BamHI digested pcDNA3.1(-) expression vector (Invitrogen), incorporating a neomycin resistance gene. Sequence analysis was performed to check the orientation of the adenosine A1 receptor insert.

Stable expression in Chinese hamster ovary (CHO) cells

CHO cells were cultured at 37°C in cell culture flasks (25 cm², Corning), containing 5 ml DMEM-F12 medium, supplemented with 10% fetal calf serum and 2 mM L-glutamine in a humidified atmosphere (5% CO2). Cells were seeded into a six-well dish and transfected with 1 µg of the pcDNA3.1(-)-mouse
adenosine A1 receptor expression vector, using 3 µl of fuGENE 6 transfection reagent (Roche). After transfection, cells were seeded in cell culture flasks. After 2 weeks of selection with 50 µg/ml G 418, monoclonal CHO cell lines, stably expressing the mouse adenosine A1 receptor (CHO-mA1), were generated and adenosine A1 receptor mRNA expression levels of 16 clones were determined by RT-PCR. The CHO-mA1 cell line with highest expression levels was used for binding studies and cAMP measurements.

Radioligand binding assay
For membrane preparation CHO-mA1 cells were cultured in cell culture flasks (75 cm², Greiner), grown to confluency and harvested by trypsinization for 30 sec with 0.25% w/v porcine trypsin at 37°C. The cell suspension was spun for 10 min at 500 g. After removing the supernatant, the pellet was resuspended in 50 mM Tris/HCl pH 7.4 (at 25°C, approx. 10 x 10⁶ cells/ml) and spun for 40 min (4°C) at 18,000 g. After discarding the supernatant, the pellet was resuspended in 50 mM Tris/HCl pH 7.4 (at 4°C, approx. 20 x 10⁶ cells/ml) and stored at –80°C until analysis.

Rat cortical membranes were prepared according to the method of Lohse and colleagues [23] and incubated with 2 IU/ml adenosine deaminase for 30 min at 37°C before storage [34]. CHO-hA1 cell membranes were prepared as described previously [7]. For displacement studies, membranes (75 µg) were incubated for 1 hour at 25°C in 50 mM Tris/HCl (pH 7.4) in the presence of 1.6 nM [3H]DPCPX and different concentrations of ligand to determine the Kᵢ. To determine non-specific binding 100 µM CPA was used. Total volume during incubation was 200 µl.

Incubations were stopped by rapid dilution with 1 ml ice-cold buffer and bound radioligand was subsequently recovered by filtration through Whatman GF/B filters using either Millipore system or Brandel Harvester under reduced pressure. Filters were then washed three times with 2 ml buffer. The retained radioactivity was measured by liquid scintillation counting (LKB Wallac, 1219 Rackbeta).

Saturation experiments were carried out under the similar conditions. Increasing concentrations of [3H]DPCPX (0 – 4 nM) were used. Filters were washed five times with 2.5 ml buffer to remove excess of radioligand.

cAMP-accumulation assay
CHO-mA1 cells were seeded in 24 wells plate at a density of 2 x 10⁵ cells/well. The next day, growth medium was aspirated and washed twice with DMEM/HEPES (pH 7.4). Cells were then incubated for 30 min at 37°C with DMEM/HEPES supplemented with adenosine deaminase (2 IU/ml), cilostamide (50 µM) and rolipram (50 µM). After incubation different concentrations of CPA, ranging from 1 nM till 100 µM, were added for another 10 min. Subsequently,
forskolin (10 µM) was added. After another 15 min, the cAMP generation was stopped by aspirating incubation medium and adding 200 µl ice-cold 0.1 N HCl to the cells.

The amount of cAMP was determined by competition with [3H]cAMP for protein kinase A binding protein (PKA). Briefly, sample or cAMP standard (0 – 16 pmol), 1.8 nM [3H]cAMP and PKA-solution were incubated on ice for at least 2.5 hours. The incubation was stopped by dilution with ice-cold TrisHCl buffer (pH 7.4). Bound radioactivity was recovered by filtration through Whatman GF/C filters using a Brandel harvester.

Data analysis
All radioligand binding data were analyzed using the Software program PRISM 3.0 (GraphPad, San Diego, CA), based on non-linear curve fitting procedures, yielding K_D and B_max values in case of saturation binding experiments and K_i values in case of displacement studies. Data derived from cAMP determinations were also analyzed using PRISM (GraphPad, San Diego, CA), providing EC_{50} values.

Results
Sequence
The sequence that was obtained for the present adenosine A_1 receptor gene cloned from mouse brain (Figure 4.1, accession number (AC): AJ555877) showed 99% similarity with the sequence suggested to be the mouse adenosine A_1 receptor previously cloned by Marquardt and colleagues ([26], AC: U05671), 95% similarity with the rat adenosine A_1 receptor (AC: M69045) and 89% similarity with the human adenosine A_1 receptor (AC: S56143). See Figure 4.1 for an alignment of the protein sequences of the mouse, rat and human adenosine A_1 receptor. The previously cloned mouse adenosine A_1 receptor [26] differs from the present receptor sequence at position 10. We describe here an alanine, which is also found in the rat and human sequence, whereas the previously described sequence showed a glycine at this position.

Radioligand binding
Saturation binding experiments were performed at CHO-mA_1 membranes, using the ligand [3H]DPCPX. A typical experiment is shown in Figure 4.2A. Analysis of three experiments resulted in a K_D of 0.53 nM with a 95% confidence limit of 0.44-0.63 nM and a B_max of 97 ± 60 (standard deviation) fmol/mg. Competition binding experiments were performed with the following ligands: the selective adenosine A_1 receptor agonists CCPA, CPA and R-PIA, the non-selective adenosine
receptor agonist NECA and the selective adenosine A1 receptor antagonists CPT, DPCPX and N0840. Furthermore binding affinities towards the mouse adenosine A1 receptor were determined for ligands selective for other adenosine receptors: the adenosine A2A receptor agonist CGS21680, the adenosine A2A receptor antagonist ZM241385 and the selective adenosine A3 receptor agonist Cl-IB-MECA. Figure 4.2B shows a representative competition experiment of increasing concentrations of selective agonists CPA and R-PIA with the radioligand [\(^{3}\text{H}\)]DPCPX. Adding GTP (1 mM) shifted the displacement curve of CPA to the right, resulting in a Ki value of 4.2 ± 2.7 nM in the control situation in contrast to a Ki of 17 ± 8 nM in the presence of GTP. This finding suggested agonistic activity of CPA at CHO-mA1 membranes, which was further corroborated by functional studies (see below). An overview of the affinity values of the binding assays is provided in Table 4.1.
Figure 4.2. A) Saturation analysis of $[^3\text{H}]$DPCPX binding to membranes of CHO cells expressing the mouse adenosine $A_1$ receptor. A representative experiment is shown. B) Displacement studies of specific $[^3\text{H}]$DPCPX binding (1.6 nM) from membranes of CHO cells expressing the mouse adenosine $A_1$ receptor by CPA, CPA + GTP and by R-PIA. A representative experiment is shown.

Table 4.1. Binding affinities ($K_i$ values ± S.E.M. in nM or % displacement) of reference ligands for adenosine $A_1$ receptors from mouse, rat and human.

<table>
<thead>
<tr>
<th></th>
<th>mouse$^a$</th>
<th>rat$^b$</th>
<th>human$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCPA</td>
<td>21 ± 2</td>
<td>8.1 ± 0.4</td>
<td>6.4 ± 1.8$^d$</td>
</tr>
<tr>
<td>CPA</td>
<td>4.2 ± 2.7</td>
<td>6.1 ± 0.8</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>R-PIA</td>
<td>14 ± 3</td>
<td>11 ± 5$^e$</td>
<td>n.d.</td>
</tr>
<tr>
<td>NECA</td>
<td>238 ± 55</td>
<td>98 ± 22</td>
<td>12 (9.6-15)$^d$</td>
</tr>
<tr>
<td>DPCPX</td>
<td>1.4 ± 0.1</td>
<td>0.73 ± 0.08</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>8-CPT</td>
<td>12 ± 2</td>
<td>11 ± 3</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>N0840</td>
<td>303 ± 70</td>
<td>182 ± 53</td>
<td>1081 ± 69</td>
</tr>
<tr>
<td>ZM 241385</td>
<td>116 ± 18</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CGS 21680</td>
<td>3 - 5 % (10$^{-6}$)$^f$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>18 - 20 % (10$^{-5}$)$^f$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cl-IB-MECA</td>
<td>10 - 12 % (10$^{-6}$)$^f$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>45 - 49 % (10$^{-5}$)$^f$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^a$ membranes of CHO cells expressing the mouse adenosine $A_1$ receptor
$^b$ rat brain cortical membranes
$^c$ membranes of CHO cells expressing the human adenosine $A_1$ receptor
$^d$ $K_i$ value with 95% confidence interval; value taken from [2]
$^e$ value taken from [41]
$^f$ % displacement of specific binding at the designated molar concentration (n=2)

n.d. = not determined
cAMP measurements

In recombinant CHO cells stably expressing the mouse adenosine A₁ receptor baseline cAMP levels were too low to allow analysis of A₁ receptor-induced inhibition of cAMP production. Therefore, cAMP levels were raised by adding forskolin at a concentration of 10 µM. Co-administration of 1 nM up to 100 µM of the selective adenosine A₁ receptor agonist CPA concentration-dependently inhibited the forskolin-induced cAMP accumulation in CHO-mA₁ cells (Figure 4.3). An EC₅₀ value of 16.7 ± 2.2 (S.E.M.) nM was calculated from 3 independent experiments.

Discussion

The adenosinergic system has been studied intensively for several decades. A large number of physiological and metabolic functions of adenosine have been described. Thus adenosine is essentially involved in the coupling of metabolic requirement to energy supply. In order to control metabolic rate, adenosine for example, retains neuronal firing, increases heart rate and increases blood flow by inducing vasodilatation of smooth muscle cells [6, 8, 15, 28, 39]. Furthermore, adenosine is also involved in other functions such as inducing sleep and causing analgesic effects and anti-inflammatory effects [10, 13, 22]. Especially in the brain adenosine shows its importance as a metabolic control factor. Adenosine inhibits the release of excitatory neurotransmitters and causes a hyperpolarization of the membrane potential in neurons. Adenosine exerts these neuroprotective effects mainly by stimulation of adenosine A₁ receptors.
present throughout the brain [10, 36]. Although adenosine is obviously involved in essential physiological functions, studies show that adenosine A₁ receptor deficient mice function relatively normal compared to wild type mice [14, 16]. However, exposed to pathophysiological conditions like hypoxia, mice lacking adenosine A₁ receptors show more neuronal damage and have a lower survival rate. It is therefore concluded that adenosine A₁ receptors are primarily important in mediating effects of adenosine during pathophysiological conditions [14, 16].

Despite the fact that human and rat adenosine receptors have been extensively characterized, the pharmacological properties of the mouse adenosine receptors have not yet been investigated in detail compared to other species [12]. The two previous studies that investigated binding affinities of adenosine A₁ receptor ligands in membrane fractions from fresh material, used only a limited set of adenosine receptor ligands [24, 40]. Therefore, we have cloned the adenosine A₁ receptor from mouse brain, expressed it in CHO cells, a cell line that has been successfully used in a detailed study on rat and human adenosine receptors [18] and studied its characteristics in radioligand binding assays and second messenger studies.

The sequence of the present adenosine A₁ receptor gene cloned from mouse brain was almost identical to the mouse adenosine A₁ receptor gene that was previously described by Marquardt and colleagues [26] and the sequence was similar to the rat and human adenosine A₁ receptor sequence. The radioligand binding assays revealed that the binding characteristics of the mouse receptor in this study are similar to the characteristics of the rat and human adenosine A₁ receptor (Table 4.1), although the non-selective ligand NECA showed a slightly lower affinity for the mouse receptor than for the rat receptor. Moreover, inhibition of cAMP production was observed after receptor stimulation with CPA with an EC₅₀ value of 16.7 ± 2.2 nM. The results of the radioligand binding and cAMP assays show that ligands commonly used to study the rat and human adenosine A₁ receptor have similar binding affinities to the mouse adenosine A₁ receptor. It is thus concluded that these ligands can be used to study pharmacological and physiological effects of the mouse adenosine A₁ receptor.

In summary, we have cloned the mouse adenosine A₁ receptor, which showed to be functional after expression in CHO cells. Our results provide a specification of mouse adenosine A₁ receptor pharmacology. This information will be of use for the characterization of physiological effects of the adenosine A₁ receptor in mouse models.
References
