Adenosine A1 receptors are crucial in keeping an epileptic focus localized

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Abstract

Adenosine is an endogenous neuromodulator with anticonvulsant and neuroprotective properties presumably mediated by activation of adenosine A1 receptors (A1Rs). To study the involvement of A1Rs in neuroprotection during epileptogenesis, we induced status epilepticus by a unilateral intrahippocampal kainic acid (KA) injection (1 nmol) in wild-type C57BL/6 and homozygous adenosine A1R knock out (A1R-KO) mice of the same genetic background. Whereas the KA injection caused non-convulsive status epilepticus in wild-type mice, in A1R-KO mice KA induced status epilepticus with severe convulsions and subsequent death of the animals within 5 days. 24 h after KA injection, brains from wild-type C57BL/6 mice were characterized by slight neuronal cell loss confined to the immediate location of the KA injection. In contrast, KA-injected A1R-KO mice displayed massive neuronal cell loss in the ipsilateral hippocampus, and, importantly, the contralateral hippocampus was also affected with significant cell loss in the hilus and in the CA1 region of the pyramidal cell layer. We conclude that activation of A1 receptors by ambient adenosine is crucial in keeping epileptic foci localized. These results open up a new dimension of the A1 receptor’s role in controlling excitotoxic cell death and further demonstrate its importance in preventing the progression of status epilepticus to lethal consequences. © 2006 Elsevier Inc. All rights reserved.

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Introduction

Adenosine is an endogenous neuromodulator that is released during seizures, ischemia, and hypoxia (Berman et al., 2000; Fredholm et al., 1984; Olsson et al., 2004). It exerts antiepileptic and neuroprotective effects (Fredholm, 1996; Ribeiro et al., 2003) mediated by adenosine receptors, of which the A1 receptor (A1R) is the most abundant in regions critical for epileptogenesis, such as the hippocampus (Fredholm et al., 2001, 2005b). Studies in animal models of ischemia and excitotoxicity have shown that A1R stimulation is neuroprotective (Fredholm, 1996; Fredholm et al., 2005a), while A1R blockade either fails to provide neuroprotection (Lee et al., 2004) or aggravates neuronal damage (Vianna et al., 2005). Similarly, adenosine released during seizures is thought to be responsible for terminating seizures (Dunwiddie et al., 1981; During and Spencer, 1992; Ribeiro et al., 2003) primarily by reducing neuronal excitability via activation of A1Rs. Thus, blockade of A1Rs prolongs seizure activity and can cause a transition from seizure activity to status epilepticus (Avsar and Empson, 2004; Fredholm et al., 2005a), and A1R agonists or increased extracellular adenosine result in the suppression of seizures (Anschel et al., 2004; Boison, 2005; Huber et al., 2001). Since certain types of seizures and, in particular, status epilepticus, are capable of causing neuronal cell loss (Henshall and Simon, 2005), the neuroprotective effects of A1R activation may be of additional therapeutic benefit. Status epilepticus is a condition of persistent seizures, ranging from focal to generalized convulsive seizures with a mortality in humans of approximately 20% (Walker et al., 2002). The extensive neuronal damage that is a hallmark of status epilepticus results from peripheral physiological changes, the excitotoxic effects of excessive neuronal activity, and finally from the induction of apoptosis (Henshall and Simon, 2005).

To elucidate the function of the A1R in vivo, A1R knockout mice (A1R-KO) have recently been generated (Johansson et al., 2001; Kochanek et al., 2005; Sun et al., 2001). Phenotypically, A1R-KO mice were characterized by hyperalgesia, anxiety, aggressiveness, and a heightened neuronal sensitivity to hypoxia (Gimenez-Llort et al., 2002; Johansson et al., 2001). However, in
adult A1R-KO mice, the severity of ischemic damage was not increased as demonstrated both in vivo and in organotypic hippocampal slices (Olsson et al., 2004), but the incidence of death after traumatic brain injury was increased (Kochanek et al., 2005). The A1R-KO model has not yet been used to study a potential role of the A1R in controlling the spread of status epilepticus-induced cell death. To further understand the combined anticonvulsive and neuroprotective effects of A1R activation, an intrahippocampal injection of kainic acid (KA) was administered to A1R-KO and C57BL/6 control mice. Previously reported effects of unilateral intrahippocampal KA administration include the immediate onset of status epilepticus with concurrent excitotoxic damage of ipsilateral hilar interneurons, and CA1 and CA3 pyramidal neurons, thus mimicking closely the damage observed in human tissue after acute status epilepticus (Bouilleret et al., 1999; Meldrum, 1991). Here, we demonstrate in a knockout model that activation of the A1R is crucial for preventing the spread of the KA-induced lesion to the contralateral brain hemisphere and for preventing the death of the animals during kainate-induced status epilepticus.

Materials and methods

Animals

A1R-KO mice were generated as described previously (Johansson et al., 2001) and back-bred onto C57BL/6 until deemed congenic by 140 genomic markers. Male and female A1R-KO mice, aged 8 to 10 weeks, were used for all in vivo analyses, with age- and sex-matched wild type C57BL/6 mice as controls. The animals were caged individually with food and water ad libitum under standard 12-h light/dark cycle conditions (light on at 7:00). All animal procedures were conducted in accordance with the regulations of the local animal welfare authority, which conform to EU and NIH guidelines (license number 138/2004 from the veterinary office of the Canton of Zurich). All efforts were made to minimize animal suffering and to limit the number of animals used for experimentation.

Intrahippocampal kainic acid injections

Under general anesthesia (equithesin, 4 ml/kg, i.p.) 15 homozygous A1R-KO mice and 14 control mice were stereotactically injected into the right dorsal hippocampus (coordinates with bregma as reference: anteroposterior (AP) = −1.6, mediolateral (ML) = −1.8, dorsoventral (DV) = −1.9) with 50 nl of 20 mM of the same batch of kainic acid (KA, Tocris Cookson Ltd., UK) dissolved in 0.9% saline (Gouder et al., 2004). All mice were then implanted with a bipolar electrode into the right dorsal hippocampus and a monopolar reference electrode over the cerebellum. The electrodes were fixed in place with dental acrylic cement as described previously (Gouder et al., 2003).

Electroencephalography

Hippocampal activity was recorded from KA treated mice using bipolar electrodes implanted into the dorsal hippocampus as described above. The EEG activities were recorded from freely moving animals in a Faraday cage using a computer-based digital acquisition system (MP100WSW System; Biopac Systems, Inc., Santa Barbara, CA, USA, sampling rate 200 Hz). EEG recordings were made from immediately after the KA injection for up to 24 h. Status epilepticus recorded immediately after the KA injection was characterized by continuous spiking activity at a minimum frequency of 1 Hz.

Histological analysis

24 h after KA administration, A1R-KO and control mice (n = 7, each) were transcardially perfused with 4% paraformaldehyde and 15% saturated picric acid solution in phosphate buffer (0.15 M, pH 7.4). Seven more control mice were perfused at 4–6 weeks after KA injection. As additional controls, 3 non-treated A1R-KO and 3 non-treated control mice were perfused as well. The brains were then post-fixed in the same fixative at 4°C for 6 h and cryoprotected in 10% DMSO in PBS (v/v) before being cut.
into 40-μm coronal sections using a sliding microtome. Sections were then mounted onto gelatin-coated slides and stained with Cresyl violet to determine the extent of cell loss within the hippocampus as a result of the KA injection.

Analysis of KA-induced cell death

Sections adjacent to the Cresyl violet stained sections were mounted in medium containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories Inc., Burlingame, CA, USA) to assess nuclear morphology. Analysis of cells exhibiting DNA fragmentation was performed using fluorescein-linked TUNEL (Roche Molecular Biochemicals) to label double-stranded DNA breaks as previously described (Henshall et al., 2002). DAPI labeling and TUNEL were analyzed using a Leica microscope (Leica Microsystems Inc., Bannockburn, IL, USA) equipped for epifluorescent illumination under excitation/emission wavelengths of 340/425 nm (blue) and 500/550 nm (green). Counts of TUNEL-positive cells in CA1 or in the hilus were performed at 40× magnification and averaged (n = 7 counts). Data are presented as mean ± SD. Data were analyzed using a one-sided t test with a power of 0.9 using Welch’s approximation for the degrees of freedom.

Results

Kainic acid-induced status epilepticus is lethal in A1R-KO mice

To investigate the role of the A1R in epileptogenesis and specifically in the persistence of status epilepticus, the KA mouse model of mesial temporal lobe epilepsy was used. For our purposes, this model was considered to be ideal since in KA-susceptible wild-type mice (i) the intrahippocampal injection of KA causes immediate status epilepticus which lasts for up to 10 h after the injection, and (ii) approximately 3 weeks thereafter, the animals develop chronic, recurrent, non-convulsive partial seizures, which are observable only by EEG recordings from the injected hippocampus (Bouilleret et al., 1999; Meldrum, 1991; Riban et al., 2002). Eight homozygous A1R-KO mice and 7 wild-type mice were injected with KA on the same day in alternating order to rule out any effects from the time of injection. As expected, all KA-injected wild-type mice survived for at least 4 weeks when they were perfused for histological analysis. Immediately after the KA injection, EEG recordings from wild-type mice showed persistent spiking with variable frequencies and amplitudes (Fig. 1A). Out of the 7 wild-type mice injected with KA, only one developed a single...
convulsion of short duration (less than 1 min), while recovering from anesthesia. In contrast, A1R-KO mice responded to the KA injections with continuous violent convulsive seizures, which were fatal within 4 h after KA injection in 4 of the 8 mice. The remaining mice also exhibited convulsive seizures for approximately 4 h, which subsided to a lethargic state characterized by little movement and prostration. In these surviving mice, persistent spike activities of variable frequencies and amplitudes were recorded (Fig. 1B), which did not differ considerably from the recordings taken from wild-type controls. In the mutants, the lethargic state persisted, eventually resulting in the death of the animals within 5 days. Thus, kainate-induced status epilepticus, which is normally non-convulsive in wild-type animals, is highly exacerbated in A1R-KO mice causing convulsive seizures and subsequently the death of the animals within 5 days.

A1 receptor knockout mice display contralateral cell loss after unilateral kainate injection

In order to study the extent of status epilepticus-induced cell death, another group of 7 homozygous A1R-KO and 7 control mice were injected with KA, recorded and perfused 24 h after the injection. Of these 7 A1R-KO mice, 1 died during this 24-h period. The reduced fatality in this group of knockout mice was due to more careful observation and the placement of water-soaked food pellets within the recording chamber to ease water and food intake. Coronal brain sections containing the dorsal hippocampus were analyzed for cell loss using the Cresyl violet stain. Whereas the 7 control mice exhibited only slight neuronal cell loss and only within the immediate vicinity of the KA injection (asterisk, Fig. 2A) and in the ipsilateral hilus, the six surviving A1R-KO mice exhibited extensive cell loss in the KA injected hippocampus including hilar neurons, and CA1 and CA3 pyramidal cells (Fig. 2B). The neuronal KA-induced cell loss seen in A1R-KO mice was reminiscent of the neuronal cell loss observed in wild-type mice 4 weeks after KA injection (Gouder et al., 2004). Thus, the absence of the A1 receptor leads to an acceleration of neuronal cell loss after KA injection. While contralateral cell loss was not prominent in KA injected wild-type mice (Figs. 2C, E) (Gouder et al., 2004), profound neuronal cell loss was observed as early as 24 h after KA injection both in the hilus and in CA1 pyramidal cells contralateral to the injection site in 4 of the 6 A1R-KO mice (Figs. 2D, F). This indicates that the A1R prevents the spread of the KA-induced lesion to the contralateral brain hemisphere. We conclude that the adenosine acting at the A1R has a significant neuroprotective effect in the early phase after KA-induced status epilepticus.

Quantification of contralateral KA-induced cell loss in A1 receptor knockout mice

To quantify contralateral cell loss after unilateral KA injection, we performed a double immunofluorescence analysis with DAPI

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Fig. 3. Neuronal cell death observed 24 h after KA injection. Coronal sections from C57BL/6 (A, C) and A1R-KO (B, D) mice sacrificed 24 h after the intra-hippocampal KA injection were double stained with DAPI (blue) and TUNEL (green) and analyzed for apoptotic cell death. Representative sections are shown, which are adjacent to those shown in Fig. 2. Cell nuclei stained with DAPI (A–D1, 3) give an idea of the location of the TUNEL-positive cells shown in (A–D2, 4). (A1, 2) Cell death in wild-type mice in the CA1 region ipsilateral to the injection of KA. (A3, 4) Contralateral hippocampus in wild-type mice. (B1, 2) Ipsilateral hippocampus in A1R knockout mice. Note the increase of TUNEL-positive cells in B2 compared to A2. (B3, 4) Contralateral hippocampus in A1R knockout mice. Note the increase of TUNEL-positive cells in the CA1 region and in the hilus of B4 in comparison with A4 and B2. (C1, 2) Contralateral hilus in wild-type mice. (D1, 2) Contralateral hilus in A1R knockout mice. Note the increase of TUNEL-positive cells in D2 compared to C2. Panels A, B were taken at 10× magnification, panels C, D at 40× magnification. Scale bars: 250 μm.
loss in the contralateral hilus of mutant animals in Cresyl violet
hippocampus is more pronounced than in the ipsilateral hippo-
thus, in the mutant animals, apoptotic cell death in the contralateral
stained sections (Fig. 2D), we quantified this as well with the DAPI
positive cells per section; Fig. 4). By contrast, cell death in the
and TUNEL stain. In wild-type animals evidence of cell death in
contralateral hilus of the A1R KO mice was increased more than 7-
sections from wild type and A1R knockout mice taken 24 h after
jection. Data shown represent the averaged (n = 7) total number of TUNEL-positive
mice (Johansson et al., 2001; Sun et al., 2001) constitute an ideal
to study the role of this receptor in hippocampal function and
disease. To study the involvement of A1Rs in neuroprotection
during epileptogenesis, we used a mouse model of intrahippo-
campal kainic acid-induced status epilepticus. To be able to detect a
wider range of potential cell loss after kainic acid-induced status
epilepticus in the A1R knockout mice, we chose a mouse strain
(C57BL/6), which is known to be resistant to KA-induced cell loss
in some models (Schauecker and Steward, 1997) but susceptible to
modest KA-induced cell loss and seizure generation after intra-
amygdala KA injection (Araki et al., 2002). Here, we demonstrate
for the first time that the A1R is essential in preventing the spread of
unilateral epilepsy associated cell death to the contralateral brain
hemisphere. These findings are all the more striking for being
observed in a mouse strain that is not very susceptible to KA-
induced cell loss.

Adenosine is known to suppress repetitive neuronal firing, thus suggesting a role as an endogenous modifier of seizures (Dragunow et al., 1985; Dunwiddie et al., 1981; Lee et al., 1984). Indeed, intracerebral adenosine concentrations rise acutely during seizure activity and are thought to be responsible for terminating seizures and establishing a period of post-ictal refractoriness (During and Spencer, 1992). In line with these findings, seizure-inducing lesions, which involve astrogliosis, lead to an increase in endogenous astrocytic adenosine kinase activity, thus reducing the levels of protective adenosine (Gouder et al., 2004). Indeed, overexpression of adenosine kinase in the hippocampus and concomitant reduction of adenosine can directly result in seizures (Fedele et al., 2005). Since the A1R is the major receptor mediating adenosinergic neuromodulation in the hippocampus (Johansson et al., 2001), one would expect lower seizure thresholds and aggravated seizures in A1R-KO animals unless major compensatory mechanisms come into effect. In our study, we were able to demonstrate that in the absence of the A1R a normally non-
convulsive status epilepticus progresses into a convulsive status
epilepticus with lethal consequences. These findings are in
agreement with a recent study in which A1R-KO mice develop
lethal status epilepticus after traumatic brain injury (TBI)
(Kochanek et al., 2005). Thus, it is clear that the hippocampal
A1R limits the progression of seizure activity induced in many
different ways. The findings also imply that the loss of A1
receptors does not lead to a major compensation. Since
convulsions reduce the number of A1 receptors in the cerebral
cortex (Rebola et al., 2003, 2005) and reduced levels of protec-
tive adenosine may result as a consequence of increased
adenosine kinase activity in chronic epilepsy (Fedele et al.,
2005; Gouder et al., 2004), defective adenosinergic neuromodu-
lation may indeed contribute significantly to the progressive
course of many epilepsies. Our finding that A1R knockout mice
display a highly aggravated seizure response after KA injection
further supports this notion.

Apart from seizure control, adenosine is involved in the
protection of cells from damage resulting from excessive energy/
oxygen consumption, insufficient energy/oxygen supply, or a
combination of both. These phenomena have been studied
extensively in the ischemic heart (Mubagwa and Flameng,
2001). Studies using adenosine receptor agonists and antago-
nists, as well as animals with a transgenic overexpression of the

![Fig. 4. Quantification of TUNEL-positive cells in coronal brain sections from C57BL/6 and A1R-KO mice sacrificed 24 h after the intra-hippocampal KA injection. Data shown represent the averaged (n = 7) total number of TUNEL-positive cells in the ipsilateral CA region (CAi), the contralateral CA region (CAc), or the contralateral hilus (Hic) of wild-type (WT) or A1R knockout mice. The increase of TUNEL-positive cells in the mutants was statistically significant in each brain region. *P < 0.05; **P < 0.01.](image-url)
A1R, indicate that adenosine exerts an anti-ischemic action. Adenosine is also involved in the phenomenon of preconditioning, in which short periods of ischemia induce cardioprotection to a subsequent sustained ischemia. This protective action is mediated by the A1 and A3 receptor subtypes (Mubagwa and Flameng, 2001). Just as it is involved in cardioprotection, adenosine is also involved in neuroprotection of the brain (Johansson et al., 2001; Ribeiro et al., 2003), and adenosine is likely to play a role in the acute responses leading to ischemic tolerance and neuroprotection (Dirmagl et al., 2003). Adenosine-mediated neuroprotection has been studied most extensively in models of hypoxia and ischemia. In contrast, less is known about the mechanisms linking the adenosine system to the prevention of seizure-induced cell death (Ribeiro, 2005; Ribeiro et al., 2003). Similar to findings in the heart, activation of the adenosine system is also involved in the phenomenon of epileptic preconditioning (Blondeau et al., 2000), and a transient downregulation of adenosine kinase has been observed during status epilepticus (Gouder et al., 2004), which may increase amounts of adenosine available for preconditioning.

Surprisingly, in adult A1R-KO mice, the severity of ischemic damage was not increased as demonstrated both in vivo and in vitro (Olsson et al., 2004), but the incidence of cell death after traumatic brain injury (Kochanek et al., 2005) or kainic acid-induced status epilepticus (this study) was increased. There are different potential explanations for this apparent discrepancy. The results may imply that compensatory mechanisms in A1R-KO mice may become effective in ischemia, but not after TBI or status epilepticus. From an evolutionary perspective, it seems feasible that protective mechanisms against hypoxia have evolved earlier and may involve redundant compensatory constituents, than protective mechanisms against less common TBI or status epilepticus, which consequently would require more complex components. The nature of these adaptive changes remains unknown, although changes in the expression of other adenosine receptors or changes in GABAB receptor-mediated responses could be excluded (Johansson et al., 2001). Elucidation of adaptive responses after different types of brain injury will be important for the development of effective therapies after stroke, TBI, or status epilepticus. Another, not necessarily mutually exclusive, hypothesis, is that excitotoxicity influenced by A1R is very important in status epilepticus, but not so important in ischemic cell death. A recent study demonstrated that bone-marrow-derived cells are critically important in mediating effects of A2A receptor activation on ischemic cell death (Yu et al., 2004).

Here, we demonstrate that the A1 receptor is critically involved in limiting the spread of a seizure-induced lesion, which was apparently not previously known. While there was moderate acute cell death after focal kainic acid-induced status epilepticus that was confined to the hippocampus ipsilateral to the injection site in wild-type mice, cell loss in the ipsilateral hippocampus of A1R-KO mice was highly aggravated and also associated with the spread of the lesion to CA1 and hilar neurons of the contralateral hippocampus (Fig. 2). Cell loss in the contralateral hippocampus of mutant animals was verified and quantified by TUNEL staining (Fig. 3) and found to be highly significant (P < 0.002). This is a remarkable finding as it points to a potential novel role of the A1 receptor in preventing the spread of status epilepticus-induced cell death to the contralateral brain hemisphere with lethal consequences. These are novel findings and go far beyond the previously known neuroprotective function of the A1 receptor. Mechanistically, our results are supported by a recent study, which demonstrated increased levels of adenosine in the contralateral brain hemisphere after the unilateral application of strongly depolarizing stimuli (Shepel et al., 2005). We thus conclude that widespread adenosine release following kainic acid-induced status epilepticus in our model, by activation of A1 receptors, is crucial in keeping an epileptic focus localized. Thus, therapeutic intervention aimed at augmentation of the A1 receptor-mediated reduction of the spread of a lesion, e.g., by locally increasing the extracellular levels of adenosine by cellular implants (Göttinger et al., 2005a,b; Huber et al., 2001), may not only prevent seizures, but may actually be anti-epileptogenic by limiting the spread of seizure-induced cell death.

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