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Adenosine A_1 receptor agonism in the immature rat brain and heart

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Abstract

We examined if the adenosine A_1 receptor agonist adenosine amine congener (ADAC, 100 μg/kg i.p.) is neuroprotective in 7-day-old rats subjected to hypoxic ischemia. Brain damage, evaluated as weight deficit and gross morphology, was not affected by ADAC treatment. Nonetheless, ADAC (100 μg/kg i.p.) reduced heart rate by 44% ($p < 0.0001$), indicating that the dose given was pharmacologically active. Adenosine A_1 receptors were determined by [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine (DPCPX)-binding and levels were 23% of the adult levels. GTP did not affect [^{3}H] DPCPX-binding in the cerebral cortex at postnatal day 7 whereas there was strong enhancement of [^{3}H] DPCPX-binding in the heart. This suggested a poor G-protein coupling at postnatal day 7 in the brain, which also was confirmed using GTP [γ-^{35}S]-binding in the presence of an adenosine A_1 receptor agonist. Thus, the lack of a neuroprotective effect of ADAC may be explained by the fact that adenosine A_1 receptors are not part of a functional unit in the 7-day-old rat brain.

Keywords: Ontogeny; Newborn; Heart; A_1 receptor

1. Introduction

Although the newborn brain tolerates a much longer period of oxygen deprivation and ischemia than does the adult brain, perinatal hypoxic ischemia is still an important cause of neurological dysfunction, cerebral palsy and epilepsy later in life (Volpe, 2001). Hence, it is important to investigate the mechanisms that modulate the extent of perinatal ischemic brain damage.

There is good evidence that endogenous adenosine acts as a neuroprotective agent in models of ischemia in the mature brain (Rudolphi et al., 1992; De Mendonca et al., 2000). Adenosine activates receptors of four subtypes—A_1, A_{2A}, A_{2B} and A_3—each having a distinct pattern of distribution in the brain (Fredholm et al., 1994). Low levels of adenosine are present in the extracellular fluid in the brain under physiological conditions, but levels increase dramatically during ischemic conditions (Hagberg et al., 1987). Enhancement of extracellular adenosine leads to reduced brain damage (Andine et al., 1990a; Dux et al., 1990; Miller et al., 1996; Jiang et al., 1997; Tatlisumak et al., 1998). Adenosine A_1 receptors, which are widely distributed in the mammalian brain (Fastbom et al., 1987), are believed to mediate neuroprotective effects of adenosine in the mature brain. Thus, adenosine A_1 receptor specific agonists attenuate (Von Lubitz et al., 1996a,b, 1999) and A_1 receptor specific antagonists aggravate brain damage in models of both focal and global ischemia (reviewed by Rudolphi et al., 1992; De Mendonca et al., 2000), partly via pre-synaptic effects on glutamate release (Lupica et al., 1992) and stabilization of post-synaptic membrane potential (Siggins and Schubert, 1981).

Less is known about the effects of adenosine in the immature brain. There is good evidence that cerebral adenosine production increases also in newborn animals in response to hypoxia ischemia (Park et al., 1987; Kjellmer et al., 1989; Aranda et al., 1989). Elevation of extracellular adenosine levels reduces ischemic brain injury also in immature rats (Hagberg et al., 1990; Gidday et al., 1995). However, it is still unclear which of the adenosine receptor subtypes could mediate such an effect. Numerous studies have shown that the response to hypoxia and ischemia of the immature brain differs from that of the mature brain (for review see Hagberg et al., 1997). Although adenosine A_1 receptors can be detected at embryonic day 18 in rat

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brain, the levels are very low (Rivkees, 1995; Weaver, 1996; Aden et al., 2000) and blockade of the endogenous adenosine with the adenosine A_1 antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) does not affect brain damage in 7-day-old rats subjected to hypoxic ischemia (Bona et al., 1997). Whereas these results indicate that endogenous adenosine acting at A_1 receptors is not of major consequence in determining the outcome of hypoxic ischemia in perinatal rats, they do not tell us if this is due to a lack of receptors or to a limited amount of adenosine. Therefore, we have investigated the post-ischemic administration of a selective adenosine A_1 receptor agonist. We used adenosine amine congener, ADAC (Jacobson et al., 1985), which has been reported not to have any acute cardiovascular side effects and not to produce hypothermia at the neuroprotective dose (100 μg/kg i.p.,Von Lubitz et al., 1996b).

This is important because adenosine A_1 receptor agonists can have cardiovascular side effects, such as bradycardia and hypotension (Williams, 1989) and effects on body temperature (Vapaatalo et al., 1975) that could affect the outcome of ischemic brain damage. Our results show a lack of effect of the compound in the brain but a clearcut effect in the heart. These results can be explained by a delayed maturation of the adenosine A_1 receptor signaling in brain compared to periphery.

2. Materials and methods

2.1. Chemicals

Adenosine amine congener N^6-[4-[[(2-aminoethyl)-amino] carbonylmethyl]phenyl]adenosine (ADAC) and N^6-cyclohexyladenosine (CHA) were purchased from RBI (Natick, MA, USA). The drug was dissolved to a concentration of 10 μg/ml in a 20/80 (v/v) mixture of phosphate buffered saline and Alkamuls EL-620 (Rhone-Poulenc, Cranbury, NJ, USA). [^{3}H] DPCPX and GTP [γ-^{35}S] were purchased from NEN-Dupont (Brussels, Belgium). *R*-N^6-phenylisopropyladenosine (R-PIA), N^6-cyclopentyladenosine (CPA), GTP and GDP were from Sigma (Stockholm, Sweden). Adenosine deaminase and guanylyl imidodiphosphate (Gpp(NH)p) were from Boeringer-Mannheim (Bromma, Sweden). All other chemicals were from Merck (Stockholm, Sweden).

2.2. Hypoxic ischemia in immature rats

To induce damage, we used the well-established model of combined unilateral common carotid ligation and hypoxia (Rice et al., 1981) in 7-day-old rats. Inbred Wistar Furth rat pups of both sexes were used. At postnatal day 7, the pups were exposed to hypoxic ischemia as follows: the pups were anesthetized with halothane (3.5% for induction and 1% for maintenance) in a mixture of nitrous oxide and oxygen (1:1). The left common carotid artery was dissected out and cut between double ligatures of prolene sutures (6-0). The duration of anesthesia was < 10 min. After the surgical procedure, a local anesthetic was applied to the wounds before skin sutures were made. The pups were left to recover for 1 h. The litters were then placed in a chamber perfused with humidified air and were preheated for 15–30 min and then perfused with a gas mixture (7.7 ± 0.01% oxygen in nitrogen, 3 l/min) for 70 min. The temperature in the gas chamber was kept at 36.0 °C. After hypoxic exposure, the pups were returned to their dams. They were then reared at 20 °C environmental temperature with a light–dark cycle of 12:12 h of food and water ad libitum. The model of hypoxic ischemia is well established and is believed to share important features with brain injury seen in term newborn infants exposed to an acute episode of severe birth asphyxia (Rice et al., 1981; Volpe, 2001).

Adenosine amine congener (100 μg/kg) was administered i.p. (10 ml/kg) 15 min after exposure to hypoxia ($n = 31$). Littermates that were also subjected to hypoxic ischemia as above served as controls and received vehicle i.p. (10 ml/kg, $n = 33$). Drug dose and timing of administration of ADAC were selected based on the effects of ADAC after bilateral common carotid ligation in adult gerbils (Von Lubitz et al., 1996b).

One rat pup died during hypoxia, i.e. before drug administration. Three pups treated with ADAC and six vehicle-treated pups died before evaluation at postnatal day 21. ADAC and vehicle-treated rats displayed similar weights at postnatal day 21 (31.3 ± 0.7 g vs. 32.4 ± 0.5 g).

Animal experiments were approved by the ethical committee of Göteborg (N289-97) and that of Stockholm (N166/98) and were performed according to the principles of European Community guidelines for the use of experimental animals.

2.3. Brain damage evaluation

At postnatal day 21, the pups were decapitated and the brains were dissected out. The brains were evaluated from the dorsal view by a five-step gross morphology score originally described by Yager et al. (1992) and modified by Bona et al. (1997). Brains were given the score 0 if the two hemispheres were equal in size, 1 for hypotrophy of the lateral-posterior part of the left hemisphere, 2 for atrophy of the anterior and posterior parts of the left hemisphere, 3 for large cysts in the left hemisphere or 4 for parasagittal viable tissue left in the midline.

Then the brainstem and the cerebellum were removed from the forebrain. The two cerebral hemispheres were separated at the midline and weighed on a high precision balance. The brain damage was expressed as ipsilateral hemisphere weight deficit in percent of the weight of the contralateral hemisphere.

Brain weight is a valuable measure of brain injury 14 days after unilateral hypoxic ischemia in this immature rat model but is not a good measure of brain damage in adult models of focal ischemia. The reason is likely the limited growth of injured brain tissue in the immature brain. The 14-day recovery period is important, as hypotrophy of the damaged hemisphere compared to the undamaged hemisphere develops over time. There is now good evidence that the weight deficit 14 days after hypoxic ischemia the 7-day-old rat correlates well to the loss of brain tissue examined with histopathology (Andine et al., 1990b; Gilland et al., 1994; Hagberg et al., 1994) and to the loss of evoked response activity (Andine et al., 1990b).

2.4. Temperature measurements

Temperature recordings were made in all the rats that underwent hypoxic ischemia and a group of nine adult male rats that were not subjected to hypoxic ischemia. Thin temperature probes (0.4 mm outer diameter microprobe type IT-21, Physitemp, Clifton, NJ, USA) were used and measurements were made using BAT-10 thermometer (Physitemp). The temperature probe was inserted rectally 0.5 cm in 7-day-old rats and 2 cm in adult rats.

2.5. Electrocardiography (ECG)

The effect of ADAC on heart rate was investigated in a separate group of 14 rats aged 7–10 days. Prior to measurements, animals were anesthetized with enflurane 2% for induction and $1.0 \pm 0.2\%$ for maintenance and body temperature was kept at 37°C with a heating pad. Two bipolar limb leads were placed subcutaneously and instant ECG was acquired and analyzed digitally using the computer program PC-LAB v5.0 (Axenborg, 1993). The heart rate was calculated using the interval between the QRS-complexes.

2.6. Autoradiography

For the autoradiographic experiments, a separate group of animals at different ages ($n = 4$–6 per timepoint) was used. These animals were anesthetized with carbon dioxide before decapitation and brains were dissected out and immediately frozen on dry ice and stored at −80 °C. Ten-micrometer sagittal sections were cut on a Leitz cryostat and collected from the lateral part of the olfactory bulb of the left hemisphere.

2.6.1. DPCPX autoradiography

Receptor density was determined using receptor autoradiography with the adenosine A_1 receptor antagonist [^{3}H] DPCPX (0.5 nM) (Fastbom and Fredholm, 1990a). Non-specific binding was determined using R-PIA (100 μM). Ten-micrometer-thick sections were preincubated in 170 mM Tris–HCl buffer containing 1 mM EDTA and 2 U/ml adenosine deaminase at 37 °C for 30 min. Sections were then washed twice for 10 min at 23 °C in 170 mM Tris–HCl buffer. Incubations were performed for 2 h at 23 °C in 170 mM Tris–HCl buffer containing [^{3}H] DPCPX (120 Ci/mmol, 0.5 nM) and 2 U/ml adenosine deaminase. $MgCl_2$ (1 mM) was added to preincubation and to incubation buffer. The incubation with DPCPX was done in the presence or absence of 100 μM GTP. Sections were then washed twice for 5 min each in ice-cold Tris–HCl, dipped three times in ice-cold distilled water and dried at 4 °C over a strong fan. Slides were exposed to [^{3}H] film with [^{3}H] microscales for 8 weeks (brain) or 1 year (heart).

2.6.2. Adenosine A_1 receptor agonist-stimulated GTP [γ-^{35}S] autoradiography

G-protein coupling of adenosine A_1 receptors during development was determined using GTP [γ-^{35}S] autoradiography (Sim et al., 1995) with the adenosine A_1 receptor agonist CPA (30 nM). When GTP [γ-^{35}S] binding is assessed with an agonist, only the G-proteins that are coupled to the specific receptor are detected. Ten-micrometer-thick sections mounted on gelatin-coated slides were incubated in 50 mM Tris–HCl buffer (pH 7.7) containing 3 mM $MgCl_2$, 0.2 mM EGTA and 100 mM NaCl at 25 °C for 10 min. Slides were then incubated in Tris–HCl buffer containing GDP (1 mM) at 25 °C for 25 min. Incubations were performed for 2 h at 25 °C in 50 mM Tris–HCl buffer containing GTP [γ-^{35}S] (1250 Ci/mmol, 0.04 nM), GDP (30 mM) and CPA (30 mM). Parallel sections were incubated with a buffer lacking CPA. The basal activity was assessed with GDP in the absence of adenosine A_1 receptor agonist and non-specific binding was assessed in the presence of the GTP analog Gpp(NH)p (100 μM). Sections were then washed twice for 10 min each in ice-cold Tris–HCl, dipped three times in ice-cold distilled water and dried at 4 °C over a strong fan. Slides were exposed to Hyperfilm β-max for 24 h.

2.7. Statistics

Statistical procedures in the software package Graph Pad Prism (Graph Pad Software, San Diego, USA) were used. Temperature measurements were analyzed with repeated measures analysis of variance (ANOVA) and DPCPX-binding data were analyzed with Student's *t*-test. Data are given as mean ± S.E.M.

3. Results

3.1. Brain damage

In agreement with previous results (Bona et al., 1997), the hypoxic ischemia resulted in a major unilateral brain damage as determined by both a weight loss in the affected hemisphere and by gross morphology (Fig. 1). However,

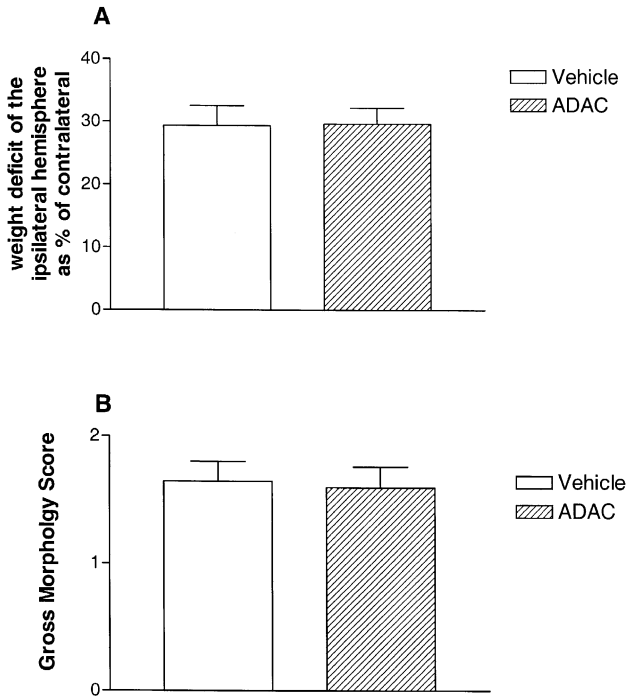

Fig. 1. The effect of ADAC (100 μg/kg, $n = 31$) given i.p. 15 min after HI vs. vehicle injection ($n = 33$) on the degree of brain damage. Brain injury was evaluated by (A) weighing the brains (brain damage expressed as ipsilateral hemisphere weight deficit in percent of contralateral hemisphere weight) and (B) blinded scoring of the gross morphology 14 days after hypoxic ischemia.

i.p. injection of ADAC at 100 μg/kg 15 min after hypoxic ischemia in 7-day-old rats did not result in any change in brain damage (Fig. 1).

3.2. Heart rate

The failure of ADAC to affect brain damage was not due to the fact that the drug was inactive, for example, owing to poor absorption. We found that 100 μg/kg of ADAC clearly reduced the heart rate in 7-day-old rats and the maximal effect was reached 50 min after injection, when the heart rate was reduced by 44% compared to vehicle-treated rats (Fig. 2, $p < 0.0001$). This effect persisted at least 300 min. Also, 50 μg/kg of ADAC produced a reduction in heart rate, although not as pronounced as 100 μg/kg, whereas 1000 μg/kg led to the death of one rat. Thus, it was not possible to use a higher dose due to systemic effects.

3.3. Rectal temperature

A possible read-out of the functionality of brain adenosine A_1 receptors is body temperature, as adenosine A_1 receptor agonists are known to produce hypothermia (Jonzon et al., 1986; Williams, 1989). Rectal temperature was measured in all 7-day-old rats that were subjected to hypoxic ischemia and was not affected by ADAC (100 μg/kg) at 30 min, 4 h, 30 h, 48 h and 56 h after hypoxic ischemia (not shown). By contrast, in adult rats 100 μg/kg ADAC caused a small transient reduction in rectal temperature (ADAC-treated group 34.9 ± 0.1 °C vs. vehicle-treated group 36.0 ± 0.3 °C, $p < 0.05$) at 30 min after hypoxic ischemia. The adenosine A_1 receptor agonist CHA (200 μg/kg) was used as a positive control and also produced a reduction in rectal temperature 30 min after injection (CHA-treated group 32.3 ± 0.4 °C vs. vehicle-treated group 36.0 ± 0.3 °C, $p < 0.05$) in adult rats. The effect of ADAC was maintained for at least 4 h, whereas the effect of CHA was more transient.

3.4. [^{3}H] DPCPX-binding in brain and heart

To determine why ADAC did not appear to have any effects in the brain despite its prominent effects in the heart, we first determined adenosine A_1 receptors by [^{3}H] DPCPX-binding. These results are shown in Fig. 3. The incubation with DPCPX was done in the presence or absence of 100 μM GTP. The presence of GTP converts all the receptors to the low-affinity state for agonists and thereby removes all endogenous adenosine (Fastbom and Fredholm, 1990a) that is otherwise cryptically bound to the receptor and decreases apparent receptor number (Parkinson and Fredholm, 1992). Thus, we compared the binding of [^{3}H] DPCPX in the presence of GTP in adult and 7-day-old rats. Using this measure, we found that at postnatal day 7 the binding of 0.05 nM [^{3}H] DPCPX in the

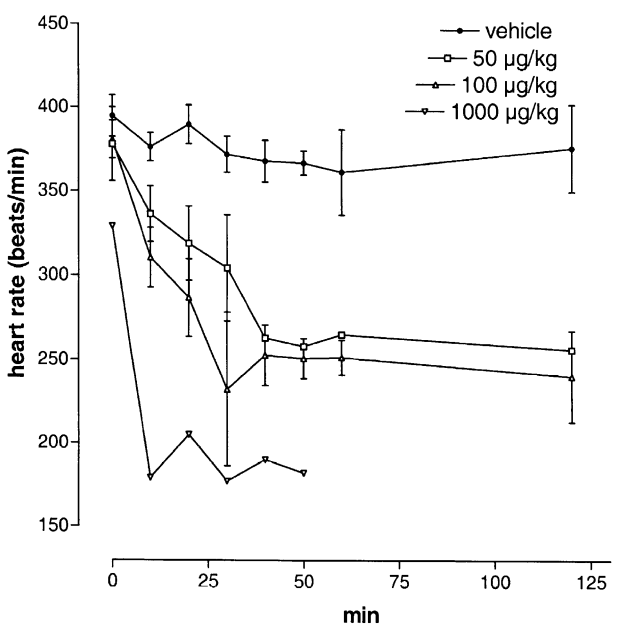

Fig. 2. Dose-dependent reduction in heart rate in 7-day-old rats after injection of ADAC. 100 μg/kg ADAC given i.p. ($n = 6$) reduced heart rate compared to vehicle-treated rats ($p < 0.0001$, repeated measures ANOVA). There was no change in heart rate in vehicle-treated rats ($n = 5$). Two rats that were injected with 50 μg/kg were followed for 120 min. One rat died after injection of 1000 μg/kg ADAC.

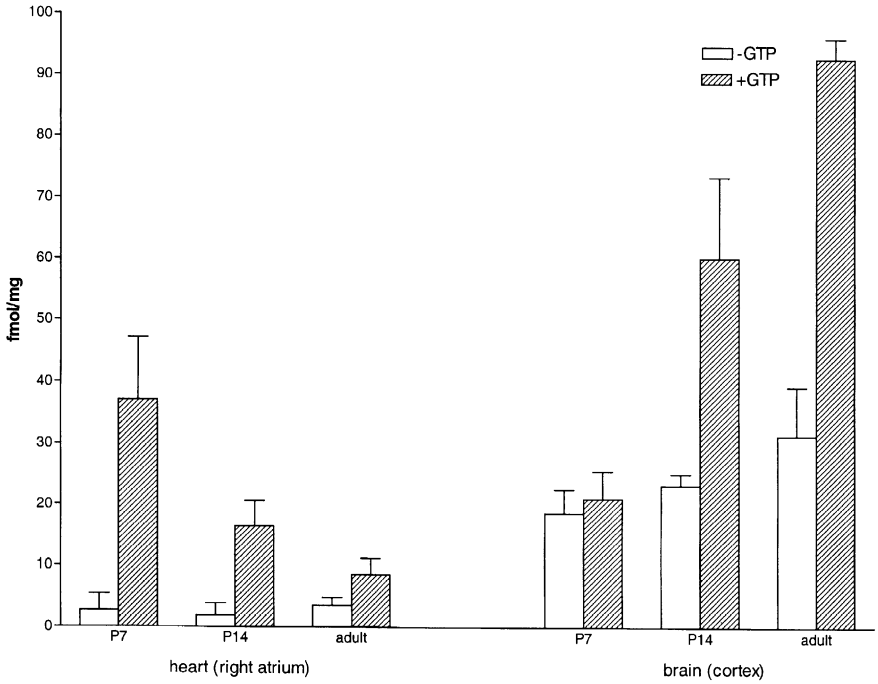

Fig. 3. [^{3}H] DPCPX-binding in the presence or absence of GTP at different ages.

cortex was only 23% of that found in adults. The difference was much smaller when [^{3}H] DPCPX-binding was studied in the absence of GTP.

For reasons mentioned above, the difference between [^{3}H] DPCPX-binding in the presence and absence of GTP indicates the amount of adenosine bound to the G-protein coupled conformation of the receptor. In the cerebral cortex, [^{3}H] DPCPX-binding was influenced by adding GTP to the incubation and by age and the interaction between those two (Fig. 3, $p < 0.05$, $F = 5.1$). In 7-day-old rats, GTP did not increase [^{3}H] DPCPX-binding significantly in the cerebral cortex (Fig. 3), thus indicating a defective G-protein coupling. However, at postnatal day 14, GTP increased [^{3}H] DPCPX-binding by 159% ($p < 0.05$) and in adult rats by 196% ($p < 0.001$, Fig. 3). Similar results were obtained in the hippocampus (not shown). In the hearts of these rats, the opposite pattern was seen: GTP significantly increased [^{3}H] DPCPX-binding (Fig. 3, $p < 0.001$, $F = 17.0$) at all ages studied, but this influence was weaker with increasing age (interaction between GTP and age: $p < 0.05$, $F = 4.0$). At postnatal day 7, GTP increased [^{3}H] DPCPX-binding by 1275% ($p < 0.01$), at postnatal day 14 by 763% ($p < 0.01$), but in adult hearts GTP produced only a small increase in [^{3}H] DPCPX-binding (139%, $p < 0.05$). These results suggested that the adenosine A_1 receptors in the atria were well coupled to G-proteins at postnatal day 7, whereas those in the brain were not.

3.5. GTP [γ-^{35}S]-binding

To examine this further, we used adenosine A_1 receptor agonist (CPA) stimulated GTP [γ-^{35}S]-binding to detect G-proteins that are coupled to adenosine A_1 receptors. At prenatal stages (embryonic days 14, 18, and 21, not shown) and early postnatal stages (postnatal days 3 and 7, Fig. 4),

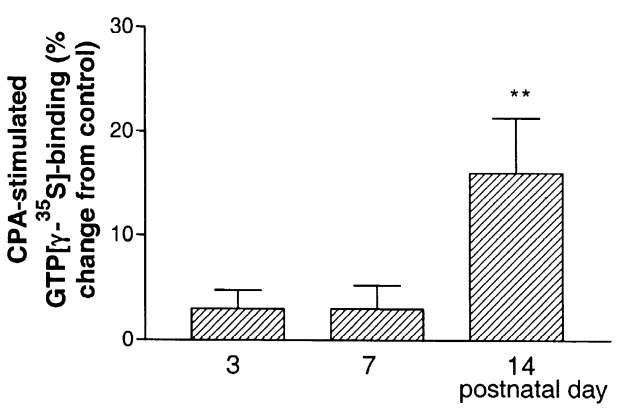

Fig. 4. CPA (adenosine A_1 receptor agonist) stimulated GTP [γ-^{35}S] binding in rat brain at different ages. The basal activity was assessed with GDP in the absence of adenosine A_1 receptor agonist and non-specific binding was assessed in the presence of the GTP analog Gpp(NH)p (100 μM). The adenosine A_1 receptor agonist CPA or buffer (control) was added to consecutive sections and the difference between optical density in non-stimulated and stimulated sections represents the adenosine A_1 receptor coupled G-proteins. Measurements of optical density were made in the cerebral cortex and are given as percent change from control. At prenatal stages (embryonic days 14, 18 and 21 were studied, not shown) and early postnatal stages (0–7 days after birth), there was no significant CPA stimulated GTP [γ-^{35}S]-binding. Optical density was 0.55 ± 0.008 in CPA stimulated vs. 0.54 ± 0.011 in control sections at postnatal day 3, 0.57 ± 0.009 in CPA stimulated vs. 0.59 ± 0.014 in control sections at postnatal day 7 and 0.61 ± 0.027 in CPA stimulated sections vs. 0.70 ± 0.040 in control sections at postnatal day. At postnatal day 14, there was a significant increase in GTPγS binding following CPA stimulation of adenosine A_1 receptors ($p < 0.05$), indicating G-protein coupling at this stage.

there was no significant CPA stimulated GTP [γ-^{35}S]-binding in cerebral cortex. At postnatal day 14, there was a significant increase in GTP [γ-^{35}S]-binding in cerebral cortex following CPA stimulation of adenosine A_1 receptors ($p < 0.05$), indicating a functional G-protein coupling at this stage. Similar results were found in the hippocampus (not shown).

4. Discussion

Given the strong evidence that adenosine A_1 receptor agonists are neuroprotective in mature animals (Von Lubitz et al., 1996b, 1999), it was surprising that the adenosine A_1 receptor agonist ADAC did not prevent brain damage after hypoxic ischemia in 7-day-old rats. There are two major possibilities to explain this lack of cerebroprotective effect: (1) cardiovascular effects are more pronounced in immature animals and they diminish the cerebroprotective effect, and (2) A_1 receptors are poorly developed in the CNS of immature rats. The second possibility was supported by the finding that rectal temperature was not affected by 100 μg/kg ADAC in 7-day-old rats, but was decreased in adult Wistar rats (present study). We therefore further examined the possibility that in the immature rat brain, cerebral adenosine A_1 receptors that regulate body temperature and modulate outcome of cerebral ischemia do not respond to an adenosine A_1 receptor agonist, whereas they do in adult rats.

A possible explanation is that adenosine A_1 receptors in the brain are too few for a functional response to be observed. However, adenosine A_1 receptors are present in the brains of 7-day-old rats, albeit in lower levels than in adults, and their distribution is similar to that in the adult brain (Aden et al., 2000). On the other hand, the present results suggest that the adenosine A_1 receptors may be less well coupled to G-proteins. Earlier studies (Morgan and Marangos, 1987; Daval et al., 1991) have shown that the binding of the radioactive agonist [^{3}H] CHA was decreased by Gpp(NH)p already at postnatal day 5. This was interpreted as evidence for G-protein coupling at that age, but these studies were not performed in the presence of Mg^{2+} known to promote such coupling and they were not controlled for effects of Gpp(NH)p independent of G-proteins. In the present study, we found, as expected, a major enhancement of [^{3}H] DPCPX binding by GTP in slices from mature rat brain incubated in Mg^{2+}-containing buffer (Parkinson and Fredholm, 1992). By contrast, no significant enhancement by GTP of antagonist binding was observed in immature 7-day-old brain. Since the enhancement by GTP is due to displacement of endogenous adenosine via a G-protein-dependent mechanism (Fastbom and Fredholm, 1990b), this indicated that the coupling was less pronounced in brains from very young animals. This conclusion agrees with the results on agonist stimulated GTP [γ-^{35}S] binding (Laitinen, 1999). Thus, in the present study in the brains from 7-day-old rats, an adenosine A_1 receptor agonist did not significantly stimulate GTP [γ-^{35}S] binding, whereas in brain sections from 14-day-old rats a significant stimulation was observed. Hence, our data suggest that at postnatal day 7 there are few adenosine A_1 receptors in the brain and those that exist are not yet fully coupled to G-proteins. This could adequately explain why the adenosine A_1 receptor agonist ADAC does not prevent brain damage in the 7-day-old rat brain. Furthermore, other studies have shown that if G-protein binding to adenosine A_1 receptors is enhanced by PD 81,273 (2-amino-4,5-dimethyl-3-thienyl-[3(triflouromethyl)-phenyl]methanone, a drug that modifies the receptor via allosteric binding), brain injury is reduced after hypoxic ischemia in the same model (Halle et al., 1997).

The present study and the study by Von Lubitz et al. (1996a,b) have found disparate results concerning heart rate after injection of ADAC. Whereas in the study by Von Lubitz, heart rate was unaltered in adult gerbils, we found a striking bradycardia in 7-day-old rats that were injected with ADAC. Also, this difference might be partly attributed to the developmental changes in G-protein coupling to adenosine A_1 receptors. Previous studies have shown that adenosine A_1 receptors indeed are present at birth in the heart and that they are more abundant in the atria compared to the ventricle (Rivkees, 1995; Matherne et al., 1996). Furthermore, the receptor density actually decreases with age (Cothran et al., 1995). When ventricular cardiomyocytes from 3-day-old Wistar rats were exposed to a β-adrenoceptor agonist and cAMP formation was measured, the adenosine A_1 receptor agonist R-PIA was clearly able to reduce the cAMP formation (Cothran et al., 1995), indicating that adenosine A_1 receptors are well coupled to second-messenger systems at this stage.

We confirm that [^{3}H] DPCPX binding in the heart decreases with age. Furthermore, GTP had a larger effect on the heart of young than of older animals: 10-fold increase in the right atrium at postnatal day 7, but only twofold in the adult atrium. This suggests that the number of adenosine A_1 receptors that are G-protein coupled is actually higher at postnatal day 7 than at later stages. On the basis of these results and previous data (Cothran et al., 1995) showing that receptor levels and coupling to second-messenger systems are even higher at birth, one might speculate that adenosine A_1 receptors provide a protective mechanism against the surge of catecholamines at birth.

Accordingly, another possible contributory cause for the lack of ameliorating effect on ischemic brain damage in the present study is that the cardiovascular side-effects of ADAC (bradycardia/hypotension) seen in young animals might have counteracted any cerebroprotective effect. Thus, the decreased cerebral effects of adenosine A_1 receptor stimulation coupled to increased cardiovascular responsiveness indicates that therapies designed to increase activ-

ity via A_1 receptors are not likely to be very useful to prevent cerebral damage in young animals.

In summary, the present results indicate that an adenosine A_1 receptor agonist given after hypoxic ischemia to 7-day-old rats does not counteract the development of brain damage. Although this is a negative finding it is important because both enhancing the extracellular levels of adenosine via uptake inhibitors and adenosine A_1 receptor stimulation have been suggested to ameliorate brain damage in the mature and in the immature brain. We also suggest that a major reason for the lack of effect is that the adenosine A_1 receptors are poorly developed in the brains of young rats, but that the situation is the opposite in the heart. The present results therefore suggest that other subtypes of adenosine receptors than the adenosine A_1 receptor are responsible for the protective effect of increased endogenous adenosine after hypoxic ischemia in the immature brain. This is currently under investigation.

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