ELSEVIER

European Journal of Pharmacology 316 (1996) 171–179

ejp

Postischemic administration of adenosine amine congener (ADAC): analysis of recovery in gerbils

Dag K.J.E. Von Lubitz [a,*], Rick C.-S. Lin [b], Ian A. Paul [c], Mark Beenhakker [a], Mellonie Boyd [a], Norbert Bischofberger [d], Kenneth A. Jacobson [a]

[a] *Molecular Recognition Section, NIH / NIDDK, Building 8, Room 1A15, Bethesda, MD 20892, USA*
[b] *Department of Anatomy and Neurobiology, Pennsylvania Medical College and Hahnemann University, Philadelphia, PA 19102, USA*
[c] *Department of Psychiatry and Human Behavior, University of Mississippi, Jackson, MS 39216, USA*
[d] *Gilead Sciences, Inc., Foster City, CA 94404, USA*

Received 25 April 1996; revised 2 July 1996; accepted 16 August 1996

Abstract

Although adenosine receptor-based treatment of cerebral ischemia and other neurodegenerative disorders has been frequently advocated, cardiovascular side effects and an uncertain therapeutic time window of such treatment have constituted major obstacles to clinical implementation. Therefore, we have investigated the neuroprotective effects of the adenosine A_1 receptor agonist adenosine amine congener (ADAC) injected after either 5 or 10 min ischemia at 100 μg/kg. When the drug was administered at either 6 or 12 h following 5 min forebrain ischemia, all animals were still alive on the 14th day after the occlusion. In both ADAC treated groups neuronal survival was approximately 85% vs. 50% in controls. Administration of a single dose of ADAC at times 15 min to 12 h after 10 min ischemia resulted in a significant improvement of survival in animals injected either at 15 or 30 min, or at 1, 2, or 3 h after the insult. In all 10 min ischemia groups, administration of ADAC resulted in a significant protection of neuronal morphology and preservation of microtubule associated protein 2 (MAP-2). However, postischemic Morris' water maze tests revealed full preservation of spatial memory and learning ability in animals injected at 6 h. On the other hand, the performance of gerbils treated at 12 h postischemia was indistinguishable from that of the controls. Administration of ADAC at 100 μg/kg in non-ischemic animals did not result in bradycardia, hypotension, or hypothermia. The data indicate that when ADAC is used postischemically, the most optimal level of protection is obtained when drugs are given at 30 min to 6 h after the insult. Although the mechanisms involved in neuroprotective effects of adenosine A_1 receptor agonists require further studies, the present results demonstrate the feasibility of their clinical applications.

Keywords: Ischemia, treatment; Adenosine; Memory; MAP2 (microtubule-associated protein 2); (Gerbil)

1. Introduction

Adenosine-based treatment of cardiovascular disease reached its clinical reality, and drugs such as Adenocard or Persantine are now widely available to the worldwide community of cardiologists. Yet, apart from carbamazepine, whose antagonistic properties at adenosine A_1 receptors are marginal, and which is used in prophylaxis of affective disorders (Van Calker and Berger, 1993), the treatment of neurodegenerative diseases with adenosine A_1 receptor active drugs is still limited to experimental practice. While both the neuroprotective and anticonvulsant impact of acute administration of adenosine A_1 receptor agonists became fully recognized (reviews by Deckert and Gleiter, 1994; Rudolphi and Schubert, 1996; Von Lubitz et al., 1995; Von Lubitz and Jacobson, 1995), the presence of disturbing side effects (e.g., bradycardia and hypotension (Williams, 1989, 1993)) severely mitigated introduction of these drugs as clinically viable alternatives in treatment of such disorders as stroke or seizures.

Very recently, a new series of adenosine A_1 receptor agonists characterized by the absence of noxious cardiac and vascular side effects has been developed (Knutsen et al., 1995) opening new possibilities for their practical introduction. We have also shown that preischemic administration of 100 μg/kg adenosine amine congener (ADAC), a highly potent and selective adenosine A_1 receptor agonist (Jacobson et al., 1985; Maillard et al., 1993) results in an extensive prevention of neuronal damage and

* Corresponding author. Tel.: (1-301) 496-3938; Fax: (1-301) 402-0008.

PII S0014-2999(96)00667-X

postischemic memory deficits following global ischemia in gerbils (Von Lubitz et al., 1996). At that dose ADAC does not induce either bradycardia or hypotension (unpublished and present paper).

While the reports of anticonvulsant and neuroprotective (Knutsen et al., 1995; Von Lubitz et al., 1996) effects of adenosine A_1 receptor agonists that are uncontaminated by side effects are highly encouraging, several aspects of the therapeutic effects of these drugs need further clarification. Thus, Rudolphi et al. (1992) indicated that postischemic treatment with an unspecified adenosine A_1 agonist appears to preserve, at least in part, its efficacy when administered as late as 14 h after reperfusion of brain with blood (Rudolphi et al., 1992). However, the therapeutic 'time window' of such therapy has not been systematically investigated. Furthermore, although several studies described neuronal preservation consequent to both pre- and postischemic treatment with adenosine A_1 receptor agonists (reviewed by Rudolphi et al., 1992; Miller and Hsu, 1992; Von Lubitz et al., 1995), none of these reports indicate the impact of these drugs upon individual structural components of neurons known to be particularly prone to ischemic damage, e.g., cytoskeleton (Matesic and Lin, 1994). Such data are of an unquestionable importance both in understanding therapeutic effects of acutely administered adenosine A_1 receptor agonists, and in providing further information necessary for the putative clinical use of these drugs. We have, therefore, attempted to assess the impact of ADAC administered at different times following global brain ischemia in gerbils by means of several independent measures of recovery, i.e., survival, neuronal damage and memory deficits.

2. Materials and methods

2.1. Animals

Female gerbils (70 g, Tumblebrook Farms, MA, USA) were used. All animals were tested for susceptibility to spontaneous seizures (Lee et al., 1984) and convulsion-prone animals were rejected from further studies. Each experimental group consisted of 15 animals.

2.2. Drug and its administration

Adenosine amine congener N^6-[4-[[[(2-aminoethyl) amino]carbonyl]methyl]phenyl]adenosine (ADAC) was purchased from RBI (Natick, MA, USA). ADAC was dissolved in a 20:80 (v/v) mixture of Alkamuls EL-620 (Rhône-Poulenc, Cranbury, NJ, USA) and phosphate-buffered saline. The drug was administered as a single 0.15 cc i.p. injection of 100 μg/kg ADAC (Von Lubitz et al., 1996) at 15 or 30 min, or at 1, 2, 3, 6, 12 or 18 h postischemia. Since pilot studies showed that the time point of postischemic vehicle injection had no influence on the subsequent course of recovery in control animals, control groups were given a 0.15 cc injection of the vehicle at 15 min postischemia.

2.3. Blood pressure and cardiac rate measurements

The effect of ADAC on blood pressure and cardiac rate was investigated in 6 gerbils. Prior to the measurements, the animals were lightly anesthetized with halothane (induction at 2% and maintenance at 0.5% in 30% O_2/70% N_2O) and placed on a heating pad (Harvard Apparatus, South Natick, MA, USA). Simultaneous measurements of both parameters were made prior to injection of 100 μg/kg ADAC and were repeated at 15, 30, and 60 min after the injection. Blood pressure was monitored using Harvard Apparatus (South Natick, MA, USA) rat tail blood pressure monitor (Von Lubitz et al., 1994) while Silogic EC-60 cardiac and respiratory monitor (Stewartstown, PA, USA) was used to measure cardiac rate. EEG clip electrodes were attached to the unshaven lose skin of the armpits and to both thighs saturated with EEG gel (Siemens Burdick, Milton, WI, USA). Lead II position proved to provide the maximum signal strength. All measurements were performed with the animals under anesthesia and their body temperature was maintained as described above.

2.4. Ischemia

The details of all procedures involved in the induction and monitoring of forebrain ischemia have been described previously (Von Lubitz et al., 1992, 1993). In the present experiments, bilateral carotid occlusion was maintained for either 5 or 10 min. Preischemic body/brain temperature was maintained within ±0.5°C during the entire course of the occlusion by means of a heating blanket (Harvard Apparatus) and a switch-controlled infrared lamp illuminating the head of the animal. Body temperature was measured at 10, 20, and 30 min postischemia. Since all gerbils maintained it spontaneously at the normal (i.e., preischemic) level, postischemic heating of recovery cages was not employed.

Following release of the occlusion, animals were injected at the indicated time points and kept at the laboratory until the next day. During this period, their survival was monitored every hour. One day after ischemia, gerbils were moved to the animal facility, and their subsequent survival was assessed daily for 14 days.

2.5. Neuronal survival and analysis of MAP-2 preservation

At the end of the survival monitoring period (14 days), all controls and drug treated survivors in both 5 and 10 min ischemia groups were heavily anesthetized with Nembutal (60 mg/kg, i.p.) and perfused with phosphate

buffered (pH 7.4) 3.5% formaldehyde as described previously (Lin et al., 1990). Cryostat sections were stained with the method of Nissl, and the extent of the neuronal loss in the hippocampal region was investigated according to a published protocol (Von Lubitz et al., 1992, 1994).

The fate of microtubule associated protein 2 (MAP-2) was determined in additional groups of animals (n = 10/group) injected with 100 μg/kg ADAC at 2, 6 and 12 h following 10 min occlusion of the carotid arteries. Animals were allowed to survive either 2 or 7 days. They were then perfused with a freshly made 3.5% solution of paraformaldehyde in phosphate-buffered saline (pH 7.4). After removal, brains were cut at 40 μm, and the sections were stained immunohistochemically with a monoclonal MAP-2 antibody (Sigma) diluted 1:500–1:1000. Standard peroxidase anti-peroxidase procedure of ABC complex with Vector kit intensified with heavy metal was used (Hsu et al., 1981). For studies of immunofluorescence, the sections were incubated at 4°C with the primary antibody overnight. Following three 5–10 min washes the sections were exposed to the secondary antibodies, i.e., fluorescein isothiocyanate, rhodamine isothiocyanate, and Texas Red conjugated goat anti-mouse IgG. The secondary antibodies were diluted at 1:20–1:100 in 0.1 M phosphate buffer. Qualitative morphological and immunostaining characteristics of the sections were studied using an epifluorescent microscope (Nikon Labophot) equipped with filters (Nikon B2 and Nikon G1B) appropriate for the detection of the secondary antibodies. Quantitative analysis of MAP-2 presence in the dendritic fields of CA1 was made using a computer image analysis system (Biographics, Winston-Salem, NC, USA).

2.6. Water maze training

Physical details of the water maze have been described previously (Von Lubitz et al., 1993, 1996).

Due to the possibility that the preocclusive water maze stress might have an influence on postischemic recovery, all animals received water maze training prior to the occlusion. However, since the study of postischemic damage in the hippocampus indicated substantial neuronal and MAP-2 preservation in groups treated with ADAC as late as 6 and 12 h postischemia, only these two treatment groups and a corresponding control group injected with the vehicle at 6 h postischemia (n = 15/group) were used in the subsequent experiments investigating the effects of the drug on postischemic memory and learning.

The preischemic water maze training consisted of the invisible target acquisition phase (a single 120 s trial/day). Invisible target acquisition trials were interrupted before the learning curve reached its plateau (average target latency 40 s; for further details see Von Lubitz et al., 1996) and were followed by a single probe trial (60 s) administered the next day. Immediately after the probe trial, animals were randomly divided into treatment and control groups (n = 15/group) and subjected to cerebral ischemia (10 min) one day later.

Since postischemic hyperactivity (Gao and Phillis, 1994) may result in a spurious improvement of water maze performance, locomotor activity (horizontal displacement) of all animals has been tested prior to postischemic maze training. Measurements of locomotor activity (expressed as the total distance traveled) were performed 13 days after ischemia with each surviving animal tested in 2 min epochs for 10 min using a Digiscan locomotor activity monitor (Omnitech Electronics, Columbus, OH, USA).

Postischemic target acquisition trials were initiated 14 days after ischemia and were conducted once daily for the subsequent 10 days. A single probe trial followed the acquisition phase. The final water maze test consisted of the visible target trial performed in order to eliminate the possibility of visual impairment as a factor affecting performance during the preceding stages of the experiment.

2.7. Statistics

Dunnett's test was used to analyze histological data, while Fisher's exact test was used to determine the statistical significance of the end-point survival data. The analyses were performed using the Genstat 3.0 program (Infallible Software, Research Triangle Park, NC, USA) with $P < 0.05$ considered significant.

Single factor repeated measures analysis of variance (ANOVA) was used to determine the significance of locomotor and water maze data. Individual group differences were assessed by means of contrast analyses using univariate F-tests with $P < 0.01$ indicating significance. Statistical parameters of the behavioral data were computed using the Systat 5.03 program (Evanston, IL, USA).

3. Results

3.1. Body temperature and cardiovascular effects of ADAC

Administration of the drug at 100 μg/kg did not result in any significant changes of body temperature, cardiac rate or mean arterial blood pressure (Table 1).

Table 1
Cardiovascular effects of 100 μg/kg ADAC in nonischemic animals

Time	Cardiac rate (beats/min ± S.E.M.)	Blood pressure (mmHg ± S.E.M.)
Preinjection	384 ± 10	81 ± 2
5 min post	385 ± 6	83 ± 1
15 min post	382 ± 7	81 ± 1
30 min post	383 ± 8	82 ± 2
60 min post	383 ± 6	80 ± 2

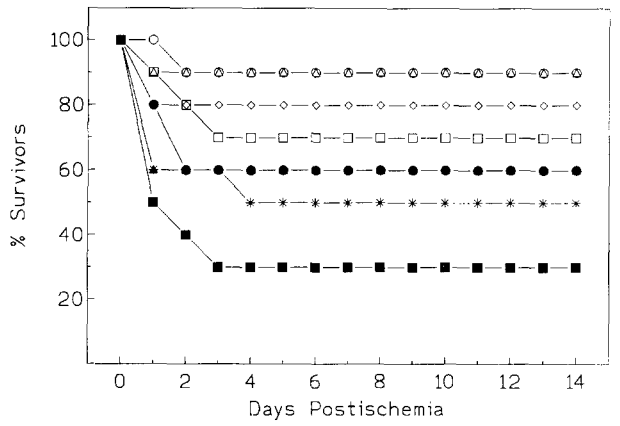

Fig. 1. Survival of 10 min ischemia. Symbols: black squares – controls; open squares – ADAC 15 min post; open triangles – ADAC 30 min post; open circles – ADAC 1 h post; open diamonds – ADAC 2 h post; black circles – ADAC 3 h post; black triangles – ADAC 6 h post; asterisks – ADAC 12 h post. Only groups given ADAC at 15, 30, 60 and 120 min are statistically different from the controls ($P < 0.05$, Bonferroni corrected Fisher's test).

3.2. Survival

No deaths occurred in any of the groups exposed to 5 min ischemia. Following 10 min ischemia, the overall mortality pattern (Fig. 1) was similar to that described in our previous studies (Von Lubitz et al., 1994, 1996). Between 40 and 50% of all deaths took place within the initial 5–6 h postischemia. Therefore, a large number of animals in the 6 and 12 h ADAC groups died prior to drug administration.

Only 30% controls exposed to 10 min ischemia survived until the end of the monitoring period (i.e. 14 days, Fig. 1). At that time, statistically significant ($P < 0.05$) improvement of survival was evident in groups injected with ADAC at 15 min (70%), 30 min (90%), 1 h (90%), and 2 h (80%) postischemia. In the three remaining treatment groups (i.e., 3, 6 and 12 h postischemia), the numerical improvement of postischemic survival was quite evident although its statistical significance was lost.

3.3. Neuronal preservation

3.3.1. Five minutes ischemia

Fourteen days after 5 min ischemia, the number of intact neurons in the CA1 sector of control animals was reduced to $49 \pm 10\%$. In animals treated with ADAC, neuronal survival was significantly ($P < 0.05$) elevated with $86 \pm 2\%$ and $83 \pm 4\%$ present in the 6 and 12 h groups, respectively (Fig. 2).

3.3.2. Ten minutes ischemia

Neuronal survival 14 days after 10 min ischemia is shown in Fig. 3. Increased duration of the occlusion resulted in a corresponding expansion of neuronal CA1 damage in the control group (only $25 \pm 3\%$ neurons still

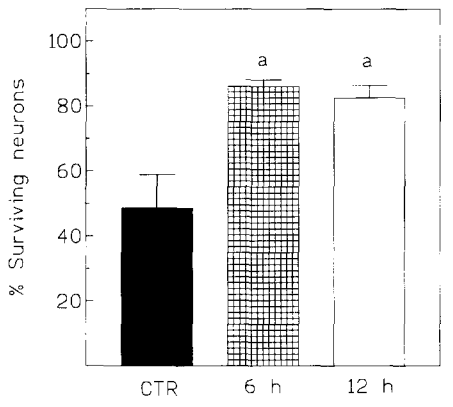

Fig. 2. Neuronal survival following 5 min ischemia. [a] $P < 0.05$. Bars: S.E.M.

present). Postischemic treatment with ADAC resulted in a statistically significant ($P < 0.05$) improvement of neuronal population in the CA1 region in all treatment groups. Administration of ADAC at 1 h postischemia produced best results ($88 \pm 4\%$ neurons still intact, Fig. 3). Treatment at 0.5, 2 or 3 h resulted in a similar degree of neuronal preservation among these groups ($\sim 75\%$, no statistically significant differences among groups). Although injection of ADAC at either 15 min, or at 6 or 12 h postischemia significantly improved neuronal survival when compared to control animals ($P < 0.05$), the neuron sparing effect was significantly poorer (approximately 60% neurons surviving) than when the drug was given at any other time (i.e., 0.5, 1, 2 or 3 h postocclusion, Fig. 3).

3.4. MAP-2 preservation

While in the CA1 sector of control animals there was a 50% loss of MAP-2 density already 2 days after 10 min ischemia (Fig. 4), there was no loss in animals treated with ADAC at 2, 6 or 12 h postischemia. Five days later (i.e., 7 days after the occlusion), MAP-2 degeneration in control

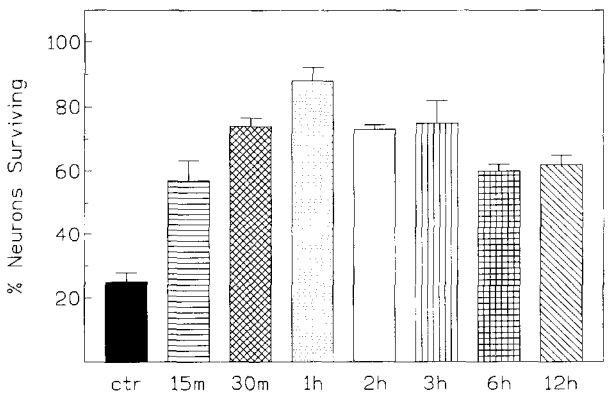

Fig. 3. Neuronal survival following 10 min ischemia. Groups injected at 30 min or at 1, 2 or 3 h postischemia are statistically different from all other treatment groups ($P < 0.05$, Student-Newman-Keuls test). Bars: S.E.M.

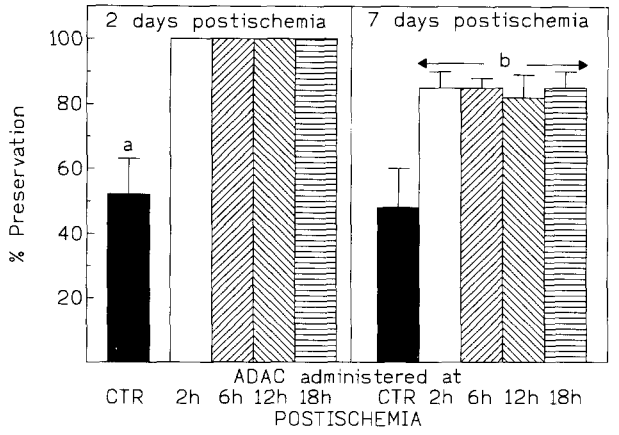

Fig. 4. MAP 2 preservation at 2 or 7 days postischemia. [a] $P < 0.05$ vs. all treatment groups; [b] $P < 0.05$ treatment groups at 2 days vs. 7 days postischemia (Student-Newman-Keuls test). Bars: S.E.M.

animals did not progress any further. In the treated groups, however, the density of MAP-2 decreased by approximately 15% ($P < 0.05$, Figs. 4 and 5).

3.5. Postischemic memory

3.5.1. Locomotor activity

Compared to preischemic measurements, locomotor activity of both controls and gerbils injected with ADAC at 12 h postischemia was significantly elevated on the 13th day after the occlusion (Table 2). No hyperactivity was observed in the group injected with ADAC at 6 h postischemia.

3.5.2. Postischemic target latency

Control gerbils showed a statistically significant increase in postischemic target latency during the initial six trials. Thereafter, target latencies were fully comparable to those observed during the last preischemic trial (Fig. 6). In animals injected with ADAC at 12 h postischemia, postischemic latencies were significantly longer during the first two postocclusive trials. Subsequent values remained within the preischemic range (Fig. 6). Statistically significant differences between target latencies of 12 h ADAC and control animals were present only during the initial

Table 2
Locomotor activity measured prior and after ischemia (mean distance traveled ± S.E.M.)

Epoch	Preischemia	Postischemia (13 days)		
		Nontreated	ADAC 6 h	ADAC 12 h
2 min	671 ± 63	1249 ± 112	773 ± 51	1146 ± 127
4 min	478 ± 58	1052 ± 95	613 ± 65	900 ± 77
6 min	462 ± 40	895 ± 34	493 ± 33	836 ± 65
8 min	396 ± 40	815 ± 50	433 ± 50	733 ± 77
10 min	375 ± 46	759 ± 51	403 ± 31	751 ± 96

Locomotor activity of animals treated with ADAC at 6 h postischemia does not differ statistically from the preischemic values. At all times, postischemic locomotor activity of both nontreated gerbils and animals treated with ADAC at 12 h is significantly higher ($P < 0.05$) than prior to ischemia.

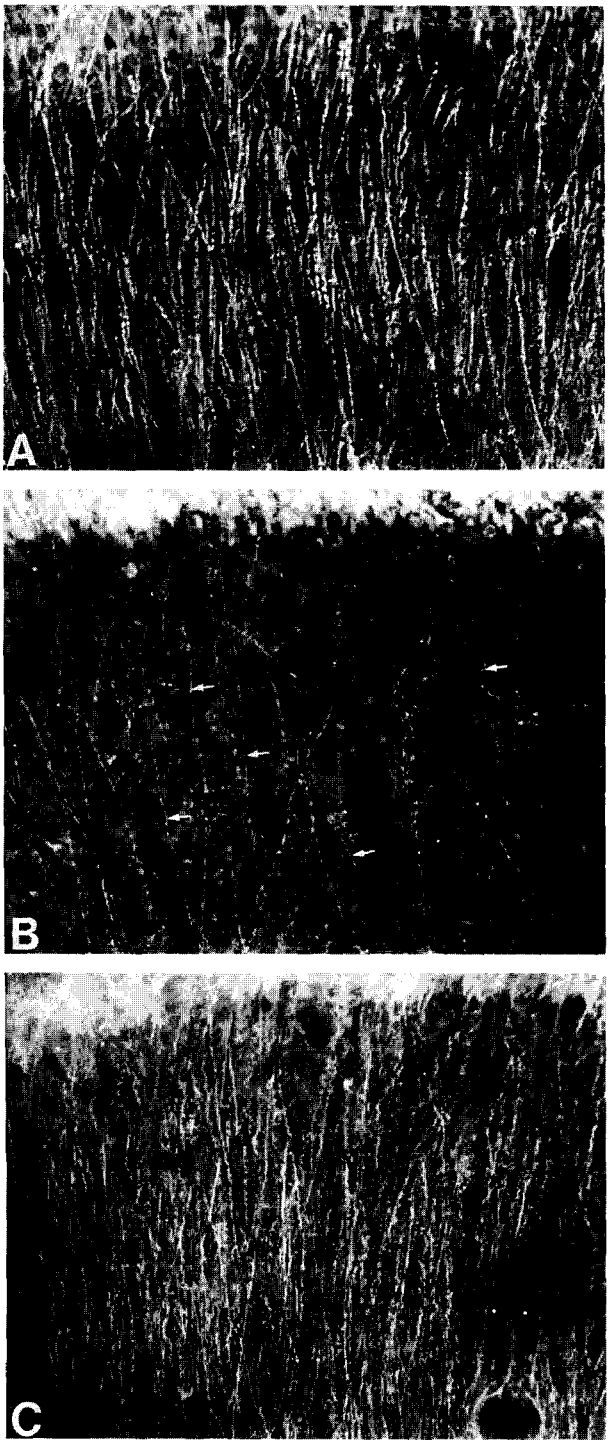

Fig. 5. Immunofluorescent staining pattern of MAP 2 (monoclonal antibody linked to Texas Red) in the hippocampal CA1 sector of a normal gerbil (A). Seven days after 10 min ischemia, the destruction of MAP 2 is evident (B). Seven days following postischemic treatment with ADAC (100 μg/kg, 2 h after 10 min ischemia) results in extensive preservation of this cytoskeletal protein (C). Arrows: dendrites with normal appearance of MAP-2.

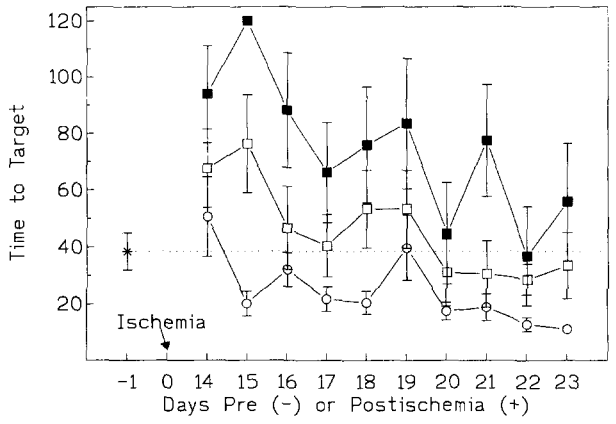

Fig. 6. Target latencies prior to and 14 days after ischemia. Single factor repeated measures ANOVA revealed significant main effects of treatment group ($F(2,21) = 13.261$, $P < 0.001$) and trial days ($F(9,189) = 5.599$, $P < 0.001$). No significant interaction between treatment group and trial days was observed ($F(1,189) = 0.805$, $P = 0.693$). Symbols: asterisk – the last preischemic trial; black squares – controls; open circles – ADAC at 6 h postischemia; open squares – ADAC at 12 h postischemia.

three postischemic trials. Injection of ADAC at 6 h postischemia resulted, on the other hand, in a statistically insignificant increase of postischemic target latencies during the first postocclusive trial. Thereafter, with the exception of trials 3 and 6, the latencies were significantly shorter than those observed during the last preischemic trial. Moreover, during the entire course of postocclusive training, target latencies of the 6 h ADAC group were very significantly shorter than in the control group (Fig. 6).

3.5.3. Probe trials

Since the preischemic training sequence was interrupted prior to the plateau of the learning curve, preischemic quadrant preference was, predictably, not fully established. Nonetheless, all animals showed a statistically significant reduction in time spent in the starting quadrant (Fig. 7). Compared to the preischemic values, the time spent in the starting quadrant increased significantly during the postocclusive trials in both controls and in gerbils injected with ADAC at 12 h postischemia. Despite this increase, neither of these two groups showed a significant quadrant preference (Fig. 7). Significant preference for the target quadrant was present in animals injected with ADAC at 6 h (Fig. 7). Moreover, while the preference for the SW quadrant was similar in the latter group to that seen in both control and 12 h ADAC animals, time spent in the two other quadrants (i.e., NE and SE) decreased significantly (Fig. 7).

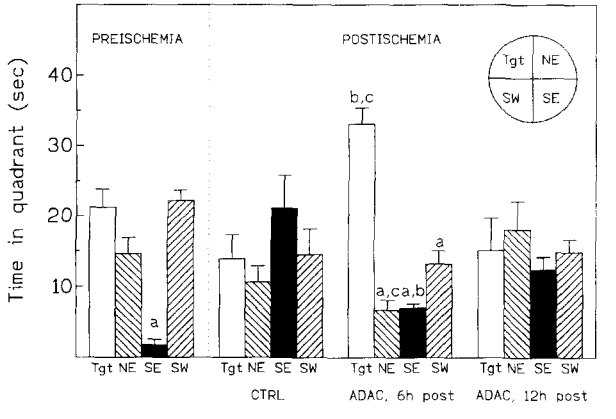

Fig. 7. Probe trials (quadrant preference) pre- and after ischemia. Single factor repeated measures ANOVA revealed no significant main effect of treatment group ($F(2,21) = 0.189$, $P = 0.83$) or quadrant latency ($F(3,63) = 2.376$, $P < 0.078$). However, a significant interaction between treatment group and quadrant latency was observed ($F(6,63) = 4.272$, $P < 0.001$). Abbreviations: [a] $P < 0.05$ within the group; [b] $P < 0.05$ between pre- and postischemic groups; [c] $P < 0.05$ between postischemic controls and treatment groups. Quadrant designations: Tgt – target; NE – north/east; SE – south/east; SW – south/west quadrants. Inset: plan of the maze.

4. Discussion

The therapeutic potential of adenosine A_1 receptor agonists in treatment of neurodegenerative pathologies such as cerebral ischemia or convulsant disorders has been suggested over a decade ago (Phillis and Wu, 1981). A large number of subsequent studies proved the validity of this concept (reviewed by Rudolphi et al., 1992; Deckert and Gleiter, 1994; Von Lubitz et al., 1995). While the observed neuroprotection was very convincing, the deleterious cardiovascular side effects accompanying therapeutically relevant doses of adenosine A_1 receptor agonists were equally apparent (Williams, 1993). In an attempt to eliminate hypotension and bradycardia induced by the stimulation of peripheral adenosine A_1 receptors, a blood-brain barrier impermeable adenosine A_1 antagonist, 8-sulphophenyltheophylline, was co-administered with the agonist N^6-cyclohexyladenosine prior to forebrain ischemia in gerbils (Von Lubitz and Marangos, 1990). Although the resultant neuroprotection was fully comparable to that obtained with N^6-cyclohexyladenosine alone, the usefulness of such approach was clearly limited to the experimental environment. As demonstrated in the present paper, administration of ADAC at the therapeutic dose of 100 μg/kg obviates concerns of undesirable cardiovascular side effects. However, in similarity to other adenosine A_1 receptor agonists, treatment with ADAC at doses of 300 μg/kg and above results in both bradycardiac and hypotensive side effects as well. Both their intensity and duration depend on the amount of the injected drug (unpublished data).

Enhancement of Ca^{2+} influx, increased liberation of excitatory neurotransmitters, and hyperactivation of their postsynaptic receptors are among the earliest processes leading to the subsequent cascade of destructive events that follow cerebral ischemia (Siesjö, 1981). Stimulation of adenosine A_1 receptors results in a marked diminution of all these processes (reviewed by Von Lubitz et al., 1995). However, it has been also shown that systemic administra-

tion of N^6-cyclohexyladenosine significantly reduces body/brain temperature (Miller and Hsu, 1992). Due to the neuroprotective effects of hypothermia alone (Busto et al., 1989), it has been postulated that lowered brain temperature evoked either by a systemic or intracerebrovascular administration of adenosine A_1 receptor agonists might be the chief mechanism involved in prevention of postischemic neuronal damage (see the review by Miller and Hsu, 1992). However, neuron-sparing actions of these drugs have been reported both in situations where body and brain temperatures were subject to strict normothermic control (reviews by Rudolphi et al., 1992; Von Lubitz and Jacobson, 1995) and in in-vitro preparations (Goldberg et al., 1988). It is highly unlikely that hypothermic mechanisms are involved in protection of cerebral neurons demonstrated in the present study since neither 15 min of halothane anesthesia, required to perform surgery and ischemia, nor 100 μg/kg ADAC result in a significant depression of body/brain temperature either alone or in combination (Von Lubitz et al., 1994, present study, and unpublished data).

In similarity to other adenosine A_1 agonists (review by Fredholm and Dunwiddie, 1988), ADAC has been shown to depress the release of glutamate from cortical slice preparations in a dose dependent manner (Boyd et al., 1996). However, in addition to decreased neurotransmitter liberation, postsynaptic mechanisms involving Ca^{2+} homeostasis might be also involved. The latter possibility is indicated by a significant preservation of microtubule associated protein 2 (MAP-2). Breakdown of cytoskeletal proteins such as MAP-2 has been described as one of the earliest stages of postischemic neurodegeneration (Matesic and Lin, 1994). One of the main enzymes involved in this process is calpain 1 whose activation depends, in turn, on stimulation of NMDA receptors and the concomitant elevation of cytosolic Ca^{2+} (Regehr and Tank, 1990; Seubert et al., 1988; Siman and Noszek, 1988). Since excitability of NMDA receptors has been shown to be closely modulated by adenosine A_1 receptors (Schubert and Kreutzberg, 1990; Schubert et al., 1995), it is therefore quite likely that stimulation of the latter by ADAC may result in a significant impairment of NMDA receptor-mediated Ca^{2+} influx with consequential depression of calpain 1 activity. However, the presence of MAP-2 in the control animals seen in the present study appears to be substantially higher than that described by previous authors in normothermic, nonmedicated rats or gerbils. Thus, Miyazawa et al. (1993) have shown that 7 days following 30 min global ischemia, there was a complete loss of MAP-2 immunostaining in the CA1 sector of both normothermic and spontaneously hypothermic rats, while in the animals with induced hypothermia the loss was variable. Also Matesic and Lin (1994) demonstrated that 5 min bilateral carotid artery occlusion in gerbils results in a virtually complete preservation of MAP-2 on the 1st day postischemia, followed by initial signs of breakdown visible 48 h after the insult, and a complete disappearance of immunostaining on the 4th day after ischemia. In the present study, control animals showed approximately 50% reduction of MAP-2 immunostaining on the 2nd day of the reflow and no further progression of MAP-2 damage on day 7. Sugaya and Kitani (1993) showed that halothane exposure reduces MAP-2 degeneration to the extent similar to that reported in the present paper. Thus, the effect of halothane anesthesia to which our control animals have been exposed both during the surgery and ischemia may explain the comparatively high level of MAP-2 observed in these animals. Nonetheless, although halothane may also enhance the level of intact MAP-2 seen in the drug treated groups, there is no doubt that ADAC alone is capable of a significant reduction of the postischemic breakdown of this protein. Such protection is particularly evident in animals injected with the drug as late as 6 and 12 h postischemia, i.e., at the time when there is no further exposure to volatile anesthetics for several hours. Yet, in both cases of the delayed postischemic treatment with ADAC, preservation of MAP 2 seen at 7 days after the occlusion is still much better than in the control animals.

Immediately postischemic disturbances of cerebral blood flow (Kagström et al., 1983) might result in an inadequate delivery of the drug administered at 15 min postischemia and explain slightly diminished neuronal protection in these animals. However, the sustained beneficial outcome of very late postischemic administration of ADAC (e.g., 6, 12 h) is surprising in view of the rapid decrease in adenosine A_1 receptor agonist binding following cerebral ischemia. Lee et al. (1983) have shown in normal rats the regional differences in adenosine A_1 receptor density are the source of very substantial differences in the depression of electrophysiological responses caused by administration of N^6-cyclohexyladenosine. Thus, an approximately 50% higher B_{max} in the dorsal aspect of the rat CA1 sector results in an almost 60% greater reduction of EPSPs when compared to the ventral sector of the same region (Lee et al., 1983). Onodera and Kogure (1990), on the other hand, have demonstrated that in the rat CA1 sector the binding of [^{3}H]N^6-cyclohexyladenosine decreases by 12–31% at 6–12 h following 20 min global cerebral ischemia. It is, therefore, conceivable that postischemic depletion of adenosine A_1 receptor population may have an adverse effect on the intensity of the neuroprotective effect of ADAC administered at late stages postischemia (6 or 12 h post, 60% neurons surviving, present study) compared to either earlier time points (e.g., 1 h post) or even to the effect of an identical dose given 15 min prior to the insult (81%; Von Lubitz et al., 1996). Yet, the involvement of other factors cannot be excluded since neuroprotective effects of 100 μg/kg ADAC injected within 1 h postischemia (present study) are almost identical to those seen following preischemic treatment with N^6-cyclopentyladenosine (CPA) given, however, at a 10 times higher dose (Von Lubitz et al., 1994). The receptor affinities but not

chemical structures of ADAC and CPA are very similar (Jacobson et al., 1985; Maillard et al., 1993). These differences may indicate that not only receptor density but possibly even minute differences in the properties of adenosine A_1 receptor agonists (such as pharmacokinetics) may play a very significant role in the extent of the ensuing neuroprotection. Similar concerns have been recently discussed in the context of acute vs. chronic administration of other agents acting at adenosine receptors (Jacobson et al., 1996).

The present study indicates that in situations of very brief ischemic episodes (5 min), ADAC can be given as late as 12 h after the insult without any marked deterioration in the number of morphologically intact neurons. These data are in agreement with the findings of Rudolphi et al. (1992). However, when the duration of ischemia is doubled, the optimal time window for ADAC administration narrows to between 30 min and 3 h postischemia. Either very early (i.e., 15 min post) or late (i.e., 6 or 12 h) injections result in a significant improvement of neuronal morphology compared to controls, but its extent is markedly poorer when compared to administration within the optimal treatment window.

Although neuronal preservation may serve as a general indicator of the protective efficacy of a drug, the results of the study of postischemic memory retention and learning indicate that the neuroprotective effects of ADAC alone do not accurately depict the overall effect of treatment. We have previously shown that preischemic treatment with ADAC results in a significant retention of spatial memory in gerbils (Von Lubitz et al., 1996). Since learning impairment, at least in the gerbil model of chronic hypotension, is associated with the degradation of cytoskeletal proteins rather than neuronal loss per se (Kudo et al., 1990), the memory sparing effects of either pre- or postischemic treatment with ADAC could be ascribed to the preservation of MAP-2 (present study). However, while both survival and morphological parameters of gerbils injected with ADAC at either 6 or 12 h postischemia were statistically indistinguishable, only the group treated at 6 h postischemia showed target latencies and that were indistinguishable from those observed preischemia. Postischemic target quadrant preference was also much better in this group as demonstrated during the probe test. On the other hand, the performance of animals treated with the drug at 12 h postischemia did not differ from that observed in the vehicle injected controls. These data indicate that the high degree of postischemic morphologic integrity at the light microscope level does not preclude the possibility for more discrete aberrations of either structure or function. This conclusion is also supported by the locomotor data tests which showed unchanged activity in gerbils injected with ADAC at 6 h postischemia. On the other hand, both postischemic controls and gerbils in which ADAC was given 12 h after the occlusion were significantly hyperactive, although the degree of neuronal integrity in the latter group was fully comparable to that seen following treatment with ADAC at 6 h postischemia.

Substantial changes of pre- and postsynaptic ultrastructure involving modification of synaptic densities, reduction in the area of synaptic contact, and redistribution of synaptic vesicles have been detected very shortly after global cerebral ischemia in rats (Von Lubitz and Diemer, 1983). Furthermore, diminished dendritic spine density and loss of synaptic vesicle protein have been associated with the loss of learning ability in congenitally hydrocephalic rats (Suda et al., 1994), while the density of synaptic connections between granule and CA3 pyramidal cells appeared to be one of the important factors involved in water maze performance of CFY rats (Schwegler et al., 1993). Functional recovery rather than dendrite sparing per se was also found to be the determining factor in water maze tests of recovery in rats subjected to neonatal frontal lesions. Adenosine A_1 receptors modulate both synaptically and NMDA receptor-evoked Ca^{2+} influx (Schubert and Kreutzberg, 1990), and Andiné (1993) has shown that at 6 h postischemia, the ability of the adenosine A_1 receptor antagonist theophylline to antagonize this influx is lost. The latter finding may indicate a severe impairment of the receptor function. It is thus conceivable that administration of ADAC at 6 h after the insult represents the last possible stage at which the drug is capable of exerting the full range of its protective actions. Later treatment, while still resulting in the preservation of some aspects of neuronal morphology, may be without any additional benefits.

The present study indicates that several aspects of the neuroprotective actions of both ADAC and other adenosine A_1 receptor agonists require further exploration. Nonetheless, the advent of drugs whose administration is free of cardiovascular side effects yet offers a broad-spectrum protection against morphological and functional consequences of cerebral ischemia makes adenosine receptor-based treatment of neurodegenerative disorders into a feasible practical concept.

References

Andiné, P., 1993, Involvement of adenosine in ischemic and postischemic calcium regulation, Mol. Chem. Neuropathol. 18, 35.

Boyd, M., Y. Meshulam, K.A. Jacobson and D.K.J.E. Von Lubitz, 1996, Effect of A_1 adenosine receptor agonist and antagonist on [^{3}H]glutamic acid release from cortical tissue in vitro, FASEB J. Abstracts 10, A159, Abstract 920, Washington, DC, April 14–17.

Busto, R., W.D. Dietrich, M.Y.T. Globus and M.D. Ginsberg, 1989, The importance of brain temperature in cerebral ischemic injury, Stroke 20, 1113.

Deckert, J. and C.H. Gleiter, 1994, Adenosine – an endogenous neuroprotective metabolite and neuromodulator, J. Neural Transm. (Suppl.) 43, 23.

Fredholm, B.B. and T.V. Dunwiddie, 1988, How does adenosine inhibit transmitter release?, Trends Pharmacol. Sci. 9, 130.

Gao, Y. and J.W. Phillis, 1994, CGS 15943, an adenosine A_2 receptor antagonist, reduces cerebral ischemic injury in the Mongolian gerbil, Life Sci. 3, PL 61.

Goldberg, M.P., H. Monyer, J.H. Weiss and D.W. Choi, 1988, Adenosine reduces cortical neuronal injury induced by oxygen and glucose deprivation in vitro, Neurosci. Lett. 89, 323.

Hsu, S., L. Raine and H. Fanger, 1981, Use of avidin-biotin-peroxidase (ABC) immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedure, J. Histochem. Cytochem. 29, 577.

Jacobson, K.A., K.L. Kirk, W.L. Padgett and J.W. Daly, 1985, Functionalized congeners of adenosine: preparation of analogues with high affinity for A_1 adenosine receptors, J. Med. Chem. 28, 1341.

Jacobson, K.A., D.K.J.E. Von Lubitz, J.W. Daly and B.B. Fredholm, 1996, Adenosine receptor ligands: differences with acute versus chronic treatment, Trends Pharmacol. Sci. 17, 108.

Kagström, E., M.-L. Smith and B.K. Siesjö, 1983, Local cerebral blood flow in the recovery period following complete cerebral ischemia in the rat, J. Cereb. Blood Flow Metab. 3, 170.

Knutsen, L.S.J., J. Lau, M.J. Sheardown, K. Eskesen, C. Thomsen, J.U. Weis, M.E. Judge and H. Klitgaard, 1995, Anticonvulsant actions of novel and reference adenosine agonists, in: Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology, eds. L. Belardinelli and A. Pelleg (Kluwer Academic Publishers, Boston, MA) p. 479.

Kudo, T., K. Tada, M. Takeda and T. Nishimura, 1990, Learning impairment and microtubule-associated protein 2 decrease in gerbils under chronic cerebral hypoperfusion, Stroke 21, 1205.

Lee, K.S., P. Schubert, M. Reddington and G.W. Kreutzberg, 1983, Regulation of strength of adenosine modulation in the hippocampus by a differential distribution of the density of A_1 receptors, Brain Res. 260, 156.

Lee, R.J., J.G. Bajorek and P. Lomax, 1984, Similar anticonvulsive, but unique behavioral effects of opioid agonists in the seizure-sensitive Mongolian gerbil, Neuropharmacology 5, 517.

Lin, C.-S., K. Polsky, J.V. Nadler and B. Crain, 1990, Selective neocortical and thalamic cell death in the gerbil after transient ischemia, Neuroscience 2, 289.

Maillard, M.C., O. Nikodijević, K.F. LaNoue, D. Berkich, X.-D. Ji, R. Bartus and K.A. Jacobson, 1993, Adenosine receptor prodrugs: synthesis and biological activity of derivatives of potent A_1 selective agonists, J. Pharm. Sci. 1, 46.

Matesic, D.F. and R.C.-S. Lin, 1994, Microtubule-associated protein 2 as an early indicator of ischemia-induced neurodegeneration in the gerbil forebrain, J. Neurochem. 63, 1012.

Miller, L.P. and Ch. Hsu, 1992, Therapeutic potential for adenosine receptor activation in ischemic brain injury, J. Neurotrauma Suppl. 2, S563.

Miyazawa, T., P. Bonnekoh and K.-A. Hossmann, 1993, Temperature effect on immunostaining of microtubule-associated protein 2 and synaptophysin after 30 min of forebrain ischemia in rat, Acta Neuropathol. 85, 526.

Onodera, H. and K. Kogure, 1980, Differential localization of adenosine A_1 receptors in the rat hippocampus: quantitative autoradiographic study, Brain Res. 458, 212.

Phillis, J.W. and P.H. Wu, 1983, The role of adenosine and its nucleotides in central synaptic transmission, Prog. Neurobiol. 15, 187.

Regehr, W.G. and D.W. Tank, 1990, Postsynaptic NMDA receptor-mediated calcium accumulation in hippocampal CA1 pyramidal cell dendrites, Nature 345, 807.

Rudolphi, K.A. and P. Schubert, 1996, Purinergic interventions in traumatic and ischemic injury, in: Novel Therapies for CNS Injuries, Rationales and Results, eds. P.L. Peterson and J.W. Phillis (CRC Press, Boca Raton, FL) p. 327.

Rudolphi, K.A., P. Schubert, F.E. Parkinson and B.B. Fredholm, 1992, Adenosine and brain ischemia, Cerebrovasc. Brain Metab. Rev. 4, 346.

Schubert, P. and G.W. Kreutzberg, 1990, Neuroprotective mechanisms of endogenous adenosine action and pharmacological implications, in: Pharmacology of Cerebral Ischemia, eds. J. Krieglstein and H. Oberpichler (Wissenschaftliche Verlagsgesellschaft, Stuttgart) p. 417.

Schubert, P., J. Pintor and M.T. Miras-Portugal, 1995, Inhibitory action of adenosine and adenine dinucleotides on synaptic transmission in the central nervous system, in: Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology, eds. L. Belardinelli and A. Pelleg (Kluwer Academic Publishers, Boston, MA) p. 281.

Schwegler, H., G.G. Müller, W.E. Crusio, L. Szemes and L. Seras, 1993, Hippocampal morphology and spatially related behavior in Long-Evans and CFY rats, Hippocampus 3, 1.

Seubert, P., G. Ivy, J. Larson, J. Lee, K. Shahi, M. Baudry and G. Lynch, 1988, Lesions of entorhinal cortex produce a calpain-mediated degradation of brain spectrin in dentate gyrus, I. Biochemical studies, Brain Res. 459, 226.

Siesjö, B.K., 1981, Cell damage in the brain: a speculative synthesis, J. Cereb. Blood Flow Metab. 1, 155.

Siman, R. and J.C. Noszek, 1988, Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo, Neuron 1, 279.

Suda, K., K. Sato, N. Takeda, T. Miyazawa and H. Arai, 1994, Early ventriculoperitoneal shunt – effect on learning ability and synaptogenesis of the brain in congenitally hydrocephalic HTX rats, Child. Nerv. Syst. 10, 19.

Sugaya, T. and Y. Kitani, 1993, Isoflurane reduces microtubule-associated protein 2 degradation compared with halothane during forebrain ischemia in the rat, Br. J. Anaesth. 71, 247.

Van Calker, D. and M. Berger, 1993, Possible role of adenosine receptors in psychiatric disorders, Drug Dev. Res. 3, 354.

Von Lubitz, D.K.J.E. and N.H. Diemer, 1983, Cerebral ischemia in the rat: ultrastructural and morphometric analysis of synapses in stratum radiatum of the hippocampal CA-1 region, Acta Neuropathol. 1, 52.

Von Lubitz, D.K.J.E. and K.A. Jacobson, 1995, Behavioral effects of adenosine receptor stimulation, in: Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology, eds. L. Belardinelli and A. Pelleg (Kluwer Academic Publ., Boston, MA) p. 489.

Von Lubitz, D.K.J.E. and P.J. Marangos, 1990, Cerebral ischemia in gerbils: postischemic administration of cyclohexyladenosine and 8-sulphophenyl-theophylline, J. Mol. Neurosci. 2, 53.

Von Lubitz, D.K.J.E., R.C.-S. Lin, R.J. McKenzie, T.M. Devlin, P. Skolnick and R.T. McCabe, 1992, A novel treatment of global cerebral ischemia with a glycine partial agonist, Eur. J. Pharmacol. 219, 153.

Von Lubitz, D.K.J.E., I.A. Paul, R.T. Bartus and K.A. Jacobson, 1993, Effects of chronic administration of adenosine A_1 receptor agonist and antagonist on spatial learning and memory, Eur. J. Pharmacol. 249, 271.

Von Lubitz, D.K.J.E., R.C.-S. Lin, P. Popik, M.F. Carter and K.A. Jacobson, 1994, Adenosine A_3 receptor stimulation and cerebral ischemia, Eur. J. Pharmacol. 263, 59.

Von Lubitz, D.K.J.E., M.F. Carter, M. Beenhakker, R.C.-S. Lin and K.A. Jacobson, 1995, Adenosine: a prototherapeutic concept in neurodegeneration, Ann. NY Acad. Sci. 765, 163.

Von Lubitz, D.K.J.E., M. Beenhakker, R.C.-S. Lin, M.F. Carter, I.A. Paul, N. Bischofberger and K.A. Jacobson, 1996, Reduction of postischemic brain damage and memory deficits following treatment with the selective adenosine A_1 receptor agonist, Eur. J. Pharmacol. 302, 43.

Williams, M., 1989, Adenosine: the prototypic neuromodulator, Neurochem. Int. 14, 249.

Williams, M., 1993, Purinergic drugs: opportunities in the 1990s, Drug Dev. Res. 28, 438.