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Brain Research 762 (1997) 79–88

BRAIN RESEARCH

Research report

Neurotoxicity in organotypic hippocampal slices mediated by adenosine analogues and nitric oxide

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Accepted 11 March 1997

Abstract

Adenosine (ADO) and nitric oxide (NO) have been implicated in a variety of neurophysiological actions, including induction of long-term potentiation, regulation of cerebral blood flow, and neurotoxicity/neuroprotection. ADO has been shown to promote NO release from astrocytes by a direct effect on A_1 and A_2 receptors, thus providing a link between actions of NO and adenosine in the brain. However, while adenosine acts as an endogenous neuroprotectant, NO is believed to be the effector of glutamate neurotoxicity. To resolve this apparent paradox, we have further investigated the effects of adenosine and NO on neuronal viability in cultured organotypic hippocampal slices exposed to sub-lethal (20′) in vitro ischemia. Up to a concentration of 500 μM ADO did not cause toxicity while exposures to 100 μM of the stable ADO analogue chloroadenosine (CADO) caused widespread neuronal damage when paired to anoxia/hypoglycemia. CADO effects were significantly prevented by the ADO receptor antagonist theophylline and blockade of NO production by L-NA (100 μM). Moreover, CADO effects were mimicked by the NO donor SIN-1 (100 μM). Application of 100 μM ADO following blockade of adenosine deaminase (with 10 μM EHNA) replicated the effects of CADO. CADO, ADO + EHNA but not ADO alone caused a prolonged and sustained release of nitric oxide as measured by direct amperometric detection. We conclude that at high concentrations and/or following blockade of its enzymatic catabolism, ADO may cause neurotoxicity by triggering NO release from astrocytes. These results demonstrate for the first time that activation of pathways other than those involving neuronal glutamate receptors can trigger NO-mediated neuronal cell death in the hippocampus. © 1997 Elsevier Science B.V.

Keywords: Organotypic hippocampal culture; Excitotoxicity; Neuron/glia interaction; Adenosine deaminase; Neuroprotection; Ischemia; NOS inhibitor

1. Introduction

In the central nervous system, the physiologic actions of adenosine (ADO) are achieved by a variety of actions spanning from a direct effect on neuronal ion channels and neurotransmitters [8,10,11,35] to its well-characterized function as an endogenous modulator of cerebral blood flow (CBF) [20,43]. Nitric oxide (NO) has been similarly implicated in neuroregulation and in the control of CBF [14,27]. Owing to this mosaic of actions shared by ADO and NO, it is not surprising that both have been proposed as modulators of hippocampal excitability as well as synaptic plasticity [7,9,22,39,40,42].

In addition to its physiological role, adenosine, which acts through a variety of receptors, has been proposed as an endogenous neuroprotectant [16,17,36]. The mechanism of adenosinergic protection is largely mediated by its hyperpolarizing actions via neuronal ion channels and presynaptic depression of excitatory neurotransmission, inasmuch as the same mechanisms that enhance or maintain inhibition of neuronal networks are also capable of decreasing excitotoxic damage triggered by a variety of noxious stimuli (e.g., ischemia). In this respect, the actions of nitric oxide and adenosine seem to diverge: adenosine, acting via A_1 receptors, has been shown to limit post-ischemic neuronal death [45], while NO is believed to be directly toxic for neurons [5,6,21]. Surprisingly, some of the actions mediated by ADO appear to favor toxicity more than protection; thus, activation of adenosine receptors results in Ca-dependent astrocytic swelling [2,13] and A_2 receptors promote excitatory neurotransmitter release from cortical neurons [4,38]. Therefore, under different conditions, and depending on the cellular target for its

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PII S0006-8993(97)00348-X

actions, adenosine may act as a neuroprotectant or neurotoxicant.

In a recent study, we have shown that adenosine causes receptor-mediated nitric oxide release from cortical astrocytes [19]. NO production was presumably mediated by the well-known ADO action on glial phospholipase C [13] and subsequent release of calcium from intracellular stores [33]. In contrast to adenosine-mediated neuromodulation and neuroprotection, NO release was observed only after application of high concentrations of adenosine, or following exposure of the cells to metabolically stable ADO analogues. It thus appears that abnormally high concentrations of adenosine and/or prolonged exposures may promote, rather than prevent, neuronal cell death by triggering NO production.

We have investigated in an in vitro model of brain ischemia [28,29] the effects of prolonged exposures to stable adenosine analogues on neuronal viability. In addition, we performed experiments aimed at the understanding of the effects of adenosine-induced NO release on hippocampal neurons. We have chosen the in vitro hippocampal cell culture model to isolate direct neuronal/glial mechanisms from the effects of cerebral blood flow in a cortical structure sensitive to ischemic damage. Furthermore, this approach allowed us to directly measure NO production and adenosine degradation in the hippocampus. Specifically, we wanted to investigate the role of NO in neurotoxicity; the effects of the stable adenosine analogue chloroadenosine (CADO) on neuronal survival following transient, sublethal ischemia; and the effect of enzymatic blockade of adenosine metabolism as possible triggers for NO-mediated toxicity.

2. Materials and methods

Details of the methods used to grow organotypic hippocampal slices have been described elsewhere [17,28,29]. Briefly, hippocampal slice cultures were prepared from 5- to 7-day old neonatal rats (Sprague–Dawley, Bantin and Kingman Inc., Fremont, CA) according to the method of Stoppini et al. [41] and grown on sterile transparent Anocel membranes (Whatman Inc., Clifton, NJ) for 12–14 days before experimentation. The growth medium consisted of 50% MEM (Gibco Laboratories, Grand Island, NY) supplemented with HEPES and sodium bicarbonate, 25% Hanks balanced salt solution, 25% horse serum, and glucose to a final concentration of 6.5 mg/ml. The cultures were grown at 36.5°C, 90–100% humidity and 5% CO_2. Propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO), which rapidly enters cells with damaged membranes and becomes fluorescent after binding to nucleic acids [24], was added (0.5 μg/ml) to the culture medium as an indicator of cell death during the experiments.

Adenosine and 3-morpholinosydnonimine (SIN-1) were obtained from Sigma Chemical Co. (St. Louis, MO). 2-Chloroadenosine (CADO), theophylline, N^{ω}-nitro-L-arginine (L-NA), and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) were obtained from RBI (Natik, MA). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; a selective A_1 receptor antagonist), 3,7-dimethyl-L-propragylxanthine (DMPX; a selective A_2 antagonist; 1000 × stock solution prepared in ethanol), 2-chloro-N^6-cyclopentyladenosine (CCPA; a highly selective A_1 agonist), 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680; an A_2 selective agonist) and 5′-*N*-ethylcarboxamidoadenosine (NECA) were also purchased from RBI. Drugs were added to the medium as small aliquots in glucose-free HBSS and left for 18 h before wash out. After addition of drugs, the cultures were either directly put back into the incubator or briefly exposed to anoxia/hypoglycemia (Ax/Hg). Cultures were then grown for further 48 h before cell death assessment.

For exposure to Ax/Hg, cultures in glucose-free HBSS were transferred into an anaerobic chamber (Forma Scientific) which was pre-equilibrated at 37°C and had an atmosphere of 0% oxygen, 10% hydrogen, 5% CO_2, and 85% nitrogen. The hydrogen was present for interaction with a palladium catalyst which maintained the oxygen concentration at 0%. Exposure times of either 20 or 35 min were used, depending on the experimental protocol. Upon removal from the anaerobic chamber, cultures were transferred back into the incubator in pre-warmed growth medium containing drug or vehicle in the same concentrations used for pretreatment.

For assessment of cell death, cultures were examined using a Nikon Diaphot inverted fluorescent microscope. Fluorescent images were obtained using a Dage 72 CCD camera (Michigan City, IN) and were digitized using Optimas image analysis software (Bio-Scan Inc., Edmonds, WA). The intensity of PI fluorescence in the CA1 area was used as an index of cell death. The first measurement of fluorescent intensity was performed 40 to 48 h after initial exposure. The remaining neurons were killed by exposing the cultures to 3 h of anoxia. The fluorescent intensity obtained 24 h after 3 h of anoxia was set equal to 100% damage and was then compared to the fluorescent intensity following the initial insult. The integrated gray values (fluorescent intensity) from CA1 following the initial insult were then expressed as a percentage of the value obtained following 3 h of anoxia. The values were averaged for each group and expressed as mean S.E.M. Statistical analysis of the results was performed using the Mann–Whitney test with Bonferroni correction for multiple comparisons. Significant differences were considered at P value < 0.05.

For measurement of ADO concentration and ADO metabolites in the growth medium, culture wells containing three cultures each were placed into new culture trays and 1 ml of fresh growth medium was pipetted on the upper surface of the Anocell membrane, thus in direct contact with the hippocampal cultures. A fixed concentra-

tion of ADO (100 μM) was added to the growth medium and four samples were taken and immediately frozen at $t = 0$, 5, 10 and $t = 30$ min. ADO and its breakdown products were quantified by high performance liquid chromatography as previously described [16].

NO was measured directly using an isolated NO meter and sensor (Iso-NO, WPI) [18]. NO release was measured in a saline buffer containing (mM/l) 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 dextrose. The probe was positioned 4 mm below the surface of the solution bathing the cultures. The NO released from the cultures diffuses through a polymeric membrane at the tip of the probe casing and is oxidized at the working platinum electrode, resulting in an electrical current that is proportional to the concentration of NO in the sample. The current was read on an oscilloscope and stored on a PC after AD conversion (at 48 kHz). Signals were filtered by a Bessel low-pass filter at 500 Hz. The probe was calibrated by titrating a solution of 0.1 M H_2SO_4 + 0.1 M KI with 50 μM KNO_2, a method based on the following equation: $2\,KNO_2 + 2KI + 2\,H_2SO_4 \rightarrow 2NO + I_2 + 2K_2SO_4$. Current responses can then be linearly correlated with nanomolar concentrations of NO.

3. Results

Potential neurotoxic effects on hippocampal cultures were evaluated by adding increasing doses of ADO or the non-hydrolizable ADO analogue CADO to the growth

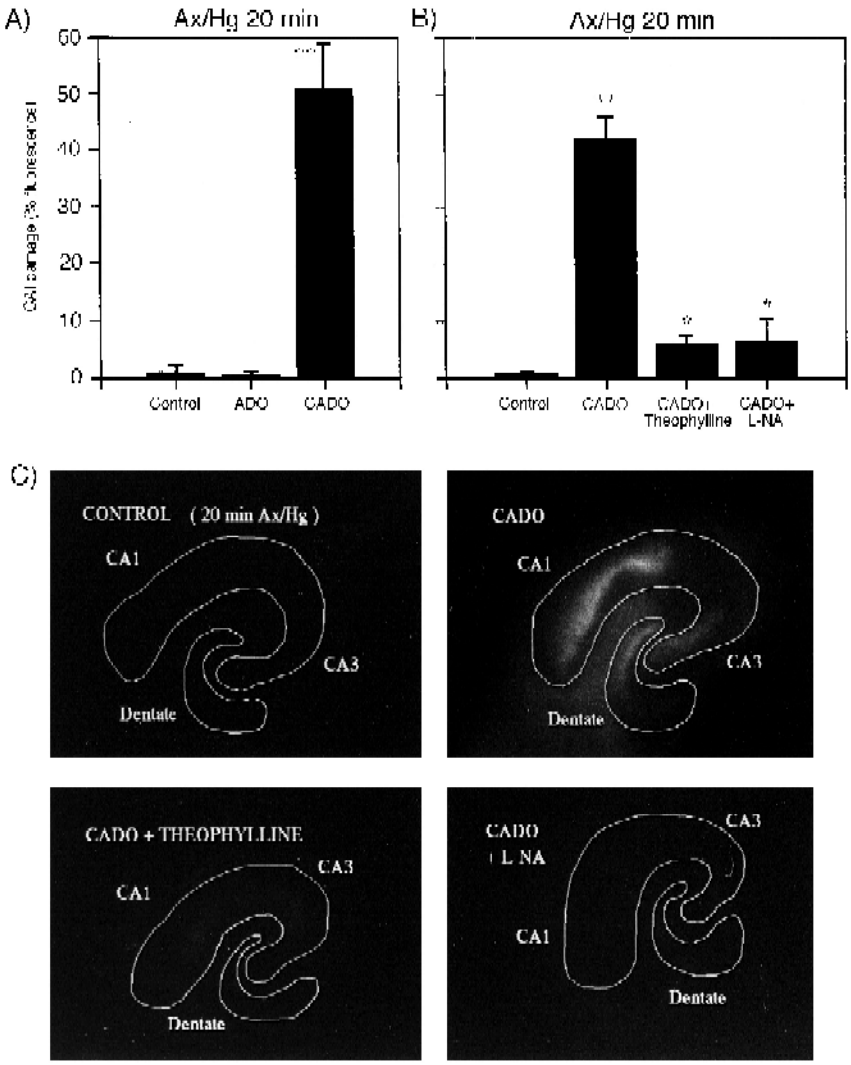

Fig. 1. Chloroadenosine-mediated neurotoxicity in cultured organotypic hippocampal slices. A: different effects of ADO and CADO (100 μM) on organotypic hippocampal slices. Drugs were added immediately before exposure to sublethal Ax/Hg (20 min). Only the non-hydrolizable ADO analogue CADO produced significant neurotoxicity. B: the ADO-receptor blocker theophylline (100 μM) and the NO-synthase inhibitor L-NA (100 μM) both significantly reduced CADO-induced neurotoxicity. $n = 9$ for each group. * $P < 0.004$ (compared to CADO alone), ** $P < 0.0001$ (compared to controls), using Mann–Whitney test with Bonferroni correction for multiple comparisons. C: dark-field photographs at low magnification showing the appearance of the propidium iodide fluorescence. Note the extensive damage in the CA1 subfield and in the upper blade of the dentate gyrus in slices treated with CADO.

medium. Up to a concentration of 400 μM, no adverse effect on culture survival was observed with either drug. At 500 μM, hippocampal cultures exposed to ADO for 18 h still did not show any development of propidium iodide fluorescence, indicating absence of neuronal cell death ($0.4 \pm 0.4\%$ cell death with ADO vs. $0.7 \pm 0.07\%$ in controls). In contrast, some discrete, yet significant neurotoxicity was observed in cultures similarly treated with 500 μM CADO ($14.1 \pm 5.1\%$ cell death with CADO vs. $0.7 \pm 0.07\%$ in controls; $P < 0.009$).

This difference between ADO and CADO effects on culture survival was greatly enhanced when exposure to the drugs was paired to 20 min of Ax/Hg, a sublethal ischemic insult which by itself did not produce any visible damage in our preparation (Fig. 1A,C, upper left panel). Hippocampal cultures treated with 100 μM ADO under these conditions did not significantly differ from controls ($0.5 \pm 0.5\%$ cell death with ADO vs. $1.0 \pm 1.0\%$ in controls). In contrast, marked propidium iodide fluorescence developed in cultures exposed to 100 μM CADO and sublethal Ax/Hg (Fig. 1A,C, upper right panel). This fluorescence was predominantly restricted to the CA1 region and corresponded to $50.85 \pm 7.9\%$ cell death (vs. $1.0 \pm 1.0\%$ in controls; $P < 0.0001$).

Neuronal damage induced by CADO and sublethal Ax/Hg was significantly reduced by the ADO receptor

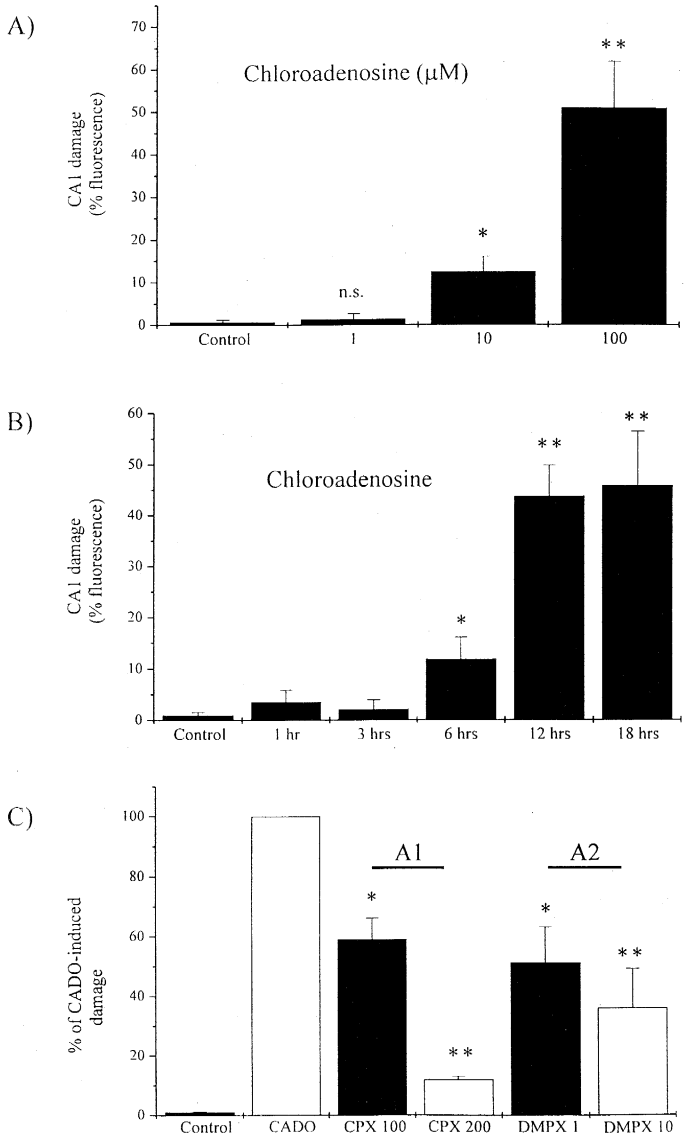

Fig. 2. Characterization of hippocampal neurotoxicity induced by the stable adenosine analogue CADO. A: dose–response of the CADO-induced toxicity. Maximal toxicity was obtained at 100 μM CADO (** $P < 0.01$), but 10 μM CADO caused significant ($P < 0.05$) damage. B: time course of CADO-mediated toxicity. Slices were incubated with 100 μM CADO for variable time intervals following exposure to sublethal anoxia/hypoglycemia (e.g., Fig. 1). Maximal damage was observed following > 12 h incubations, while significant ($P < 0.05$) damage was observed as early as 6 h after CADO. C: pharmacology of the CADO-mediated toxicity. Slices were exposed to two concentrations of the A_1 and A_2 specific antagonists DPCPX (CPX in Fig. C, concentration expressed in nM) and DMPX (concentrations in μM). Both manipulations successfully reduced the toxic response.

blocker theophylline (100 μM) (42.1 ± 3.9% cell death with CADO vs. 6.2 ± 1.2% with CADO + theophylline; $P < 0.004$), indicating that the neurotoxic effects of CADO were mediated by activation of ADO receptors (Fig. 1B,C, lower left panel). The NO-synthase inhibitor L-NA (100 μM) provided significant protection against neurotoxicity caused by CADO and sublethal Ax/Hg (42.1 ± 3.9% cell death with CADO vs. 6.4 ± 3.8% with CADO + L-NA; $P < 0.004$). This result suggest that neuronal cell death induced by CADO and sublethal ischemia was mediated by NO production (Fig. 1B,C, lower right panel).

In a separate set of experiments, the dose/response for CADO actions was investigated by exposing hippocampal slices to increasing concentrations of CADO (Fig. 2A). A significant damage (12.59 ± 3.34%; $P < 0.05$, $n = 10$) was observed following exposure to 10 μM CADO, while maximal damage was obtained at a CADO concentration of 100 μM (50.88 ± 10.99%; $P < 0.001$); 1 μM CADO was ineffective (0.75 ± 0.44%). In a similar set of experiments we investigated the time course of CADO toxicity (Fig. 2B). Slices were exposed to the anoxic/hypoglycemic insult and 100 μM CADO was added to the medium for variable durations prior to imaging for propidium iodide fluorescence. Brief (< 6 h) CADO exposures were not sufficient to cause any significant damage (3.54 ± 2.3% and 2.09 ± 1.81% at 1 and 3 h, respectively); significant damage (11.86 ± 4.2%, $P < 0.05$) was first observed following exposures of 6 h whereas maximal damage was observed following 12 and 18 h of incubation (43.01 ± 6.0% and 45.85 ± 10.55%, respectively; $P < 0.001$).

Since CADO actions may be due to the activation of both A_1 and A_2 receptors, we performed a number of experiments aimed to dissect out the relative contribution of A_1- vs. A_2-related mechanisms. To this end, we attempted to prevent hippocampal damage induced by CADO by exposing the slices to specific ADO receptor antagonists ((Jacobson, 1992; Fig. 2C). Both DPCPX (an A_1-selective antagonist, see Jacobson, 1992); 100–200 nM) and DMPX (1–10 μM) significantly reduced CADO-mediated damage ($P < 0.01$ at DPCPX 200 nM and DMPX 10 μM; $P < 0.05$ at DPCPX 100 nM and DMPX 10 μM). Since the stable adenosine analogue used, chloroadenosine, is also a substrate for uptake, hippocampal slices were exposed to NECA, a stable adenosine receptor agonist not recognized by the uptake mechanism [37]. Similar to what was observed after CADO, 100 μM NECA caused significant hippocampal damage (53 ± 11.51%, $P < 0.001$ vs. control, $n = 11$) limited to the CA1 region.

To further investigate the relationships between NO production and CADO/ischemia-induced neurotoxicity, we examined whether NO could directly promote neuronal cell death following artificial ischemia in our preparation. For that purpose, a significant ischemic injury corresponding to 75.4 ± 8.6% cell death was produced in control cultures by increasing the exposure time of Ax/Hg from

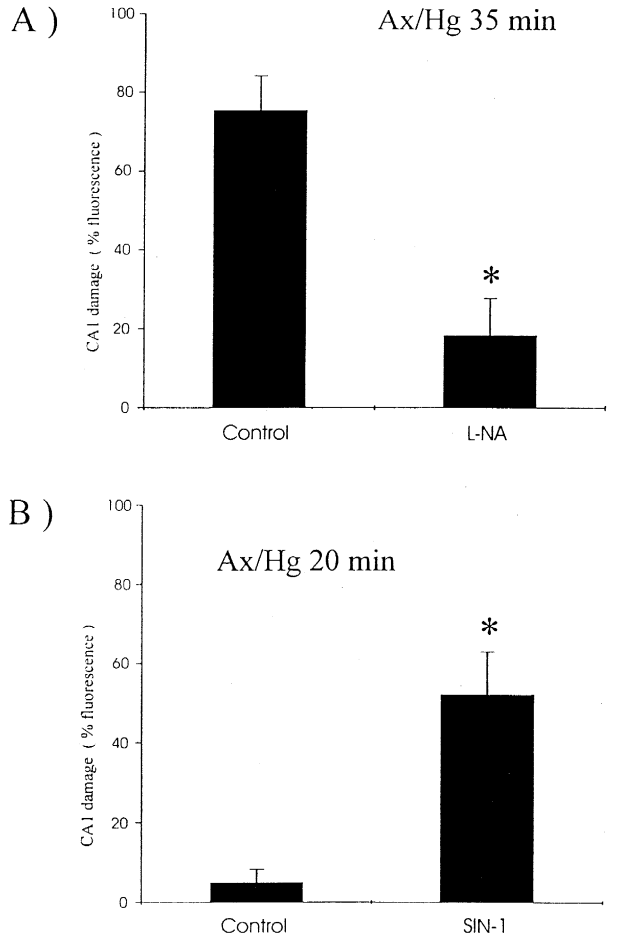

Fig. 3. Anoxia/hypoglycemia-induced CA1 neurotoxicity is mediated by nitric oxide; NO directly causes neuronal cell death. Hippocampal cultures pre-treated with the NO-synthase inhibitor L-NA (100 μM) were significantly protected against neuronal injury caused by 35 min of Ax/Hg. B: conversely, marked neuronal damage was produced in hippocampal cultures exposed to the NO-donor SIN-1 (100 μM) coupled with sublethal Ax/Hg of 20 min (* $P < 0.001$ using Mann–Whitney test). The distribution and extent of cellular damage as determined by propidium iodide fluorescence imaging following exposure to 35 min of ischemia was similar to that observed after CADO (e.g., Fig. 1).

20 min to 35 min (Fig. 3). Pre-treatment with 100 μM L-NA significantly reduced this ischemia-induced neuronal damage to 18.3 ± 9.3% cell death ($P < 0.001$). Conversely, the addition of 1 mM 3-morpholinosydnonimine (SIN-1, Fig. 3B) to the culture medium for 18 h under normoxic/normoglycemic conditions caused a moderate, yet significant neuronal injury corresponding to 19.2 ± 5.8% cell death (vs. 0.7 ± 0.07% in controls; $P < 0.005$). Similar to the effects of CADO, neuronal cell death following exposure to SIN-1 (100 μM) was greatly enhanced when paired with sublethal Ax/Hg (20 min) (52.1 ± 10.8% cell death with SIN-1 vs. 5.2 ± 3.3% in controls; $P < 0.001$). These results strongly support the involvement of NO in mediating ischemic neuronal injury in our preparation.

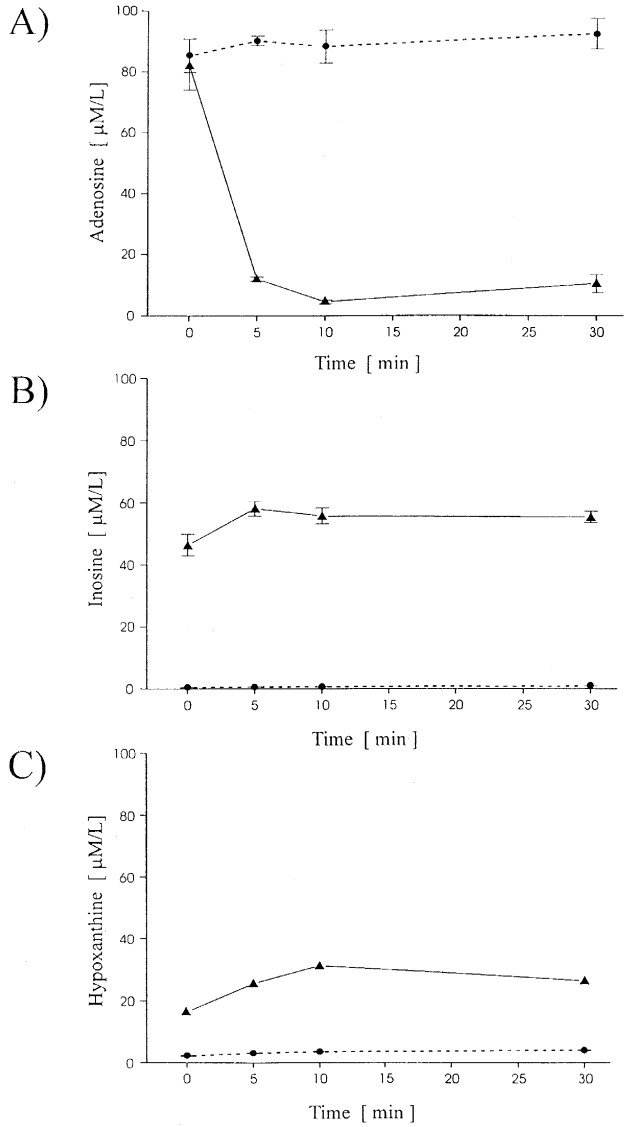

Fig. 4. Rapid metabolic degradation of exogenous adenosine in organotypic slices. A: time-course of ADO metabolism in organotypic hippocampal cultures in the absence (solid line) or presence (dashed line) of the ADO deaminase inhibitor EHNA. ADO and EHNA (both 100 μM) were added to 1 ml growth medium bathing three hippocampal cultures per well. The degradation of ADO observed under control conditions was completely blocked by inhibition of ADO-deaminase by EHNA. B,C: correspondingly, the sharp rise of ADO metabolites (inosine and hypoxanthine) observed under control conditions was completely blocked in the presence of EHNA; $n = 3$ wells for each data point.

Since the effects of CADO were mediated by ADO receptors but could not be mimicked by ADO itself, we hypothesized that blockade of enzymatic breakdown of ADO may unmask a potential neurotoxic effect of this endogenous neurotransmitter. To address this hypothesis, we first tested the ability of hippocampal cultures to metabolize ADO by measuring the variations of a fixed ADO concentration (100 μM) added to the growth medium bathing the cultures over a period of 30 min. Under these conditions, ADO concentration rapidly dropped to 12.0 μM ± 0.6 after 5 min and 4.5 μM ± 0.6 after 10 min (Fig. 4A). However, after blockade of ADO deaminase with 100 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), ADO levels remained virtually unchanged over 30 min. To further confirm the biochemical substrates of this manipulation, we measured the levels of ADO metabolites in the same experiments. As expected, inosine and hypoxanthine levels increased immediately following application of exogenous ADO; this was prevented by addition of EHNA to the growth medium (Fig. 4B,C).

Since enzymatic blockade of ADO degradation maintained high levels of the neurotransmitter in our preparation, we then examined whether application of ADO + EHNA would cause some neurotoxic damage comparable to the non-hydrolizable ADO analogues, CADO or NECA. Under sublethal ischemic conditions (20 min Ax/Hg), hippocampal cultures exposed to ADO alone (100 μM) did not significantly differ from controls (1.5 ± 0.9% cell death with ADO vs. 0.4 ± 0.4% in controls) (Fig. 5). In contrast, addition of 100 μM EHNA to the medium resulted in marked ADO-induced neurotoxicity equivalent to the damage caused by CADO (42.9 ± 8.1% cell death with ADO + EHNA; $P < 0.0001$, compared to controls). This neurotoxic effect induced by the association of ADO and EHNA was significantly reduced by L-NA to 3.6 ± 1.6% cell death ($P < 0.001$, compared to ADO + EHNA).

These results, combined with the finding that CADO, but not ADO, exacerbates neuronal damage, suggested that

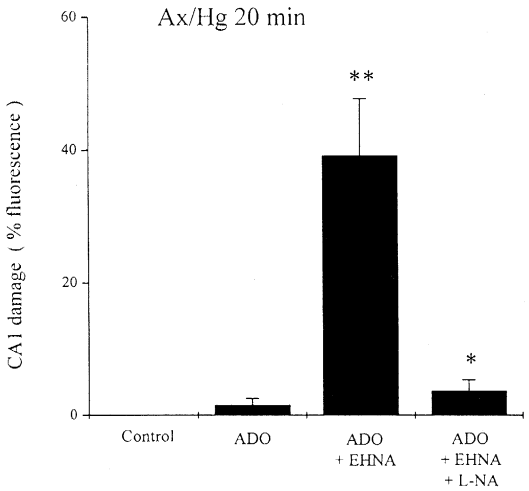

Fig. 5. Pharmacological enhancements of ADO levels in the slice reveal adenosine-mediated neurotoxicity. Blockade of ADO degradation by the ADO-deaminase inhibitor EHNA (+sublethal Ax/Hg of 20 min) resulted in marked development of propidium iodide fluorescence in hippocampal cultures, indicating a neurotoxic effect of ADO under conditions of decreased ADO metabolism. This neurotoxic effect of ADO was significantly reduced by addition of 100 μM L-NA to the medium ($n = 10$ for each group; * $P < 0.001$, compared to ADO + EHNA; ** $P < 0.0001$, compared to controls, using Mann–Whitney test with Bonferroni correction for multiple comparisons). As for the neurotoxic effects of prolonged ischemia or exposure to CADO, slices exposed to ADO + EHNA displayed pronounced yet CA1 region-specific neuronal damage (not shown).

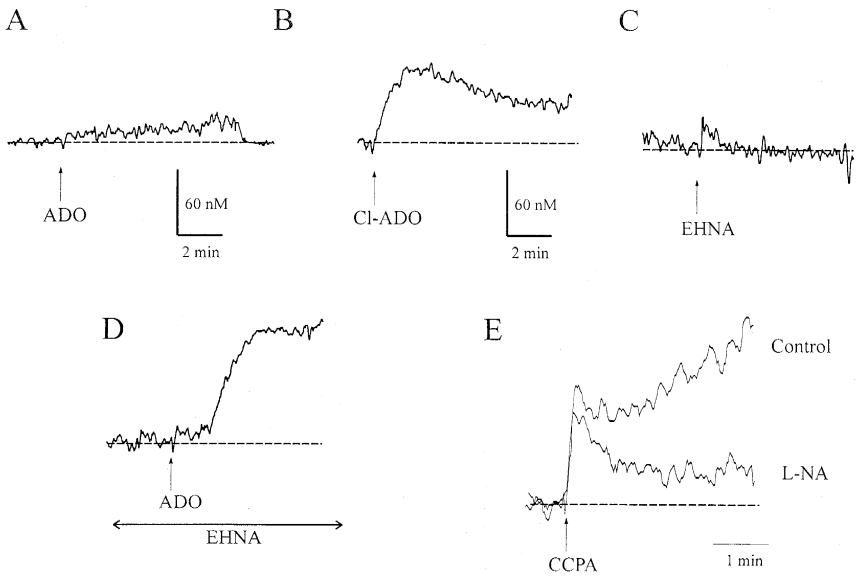

Fig. 6. Amperometric detection of NO release from organotypic hippocampal slice cultures. A: response to 100 μM ADO. In this experiment, 100 μM adenosine released 17.88 nM/min of NO. B: response to 100 μM CADO under identical conditions; same slice as in A. Note the prolonged, large increase in NO following CADO application (47.09 nM/min). C: the ADO-deaminase inhibitor EHNA (10 μM) alone failed to cause appreciable release of NO, however (D) pretreatment with EHNA greatly enhanced the response to ADO (to 72.01 nM/min). E: the effects of the stable, non-specific adenosine analogue CADO were mimicked by application of the A_1-specific adenosine agonist 2-chloro-N^6-cyclopentyladenosine (CCPA, 100 nM). The effect of this stable analogue was inhibited by pre-exposure of the hippocampal slice to the NOS inhibitor L-NA (100 μM, for 30 min).

CADO-mediated neurotoxicity was due to a prolonged activation of ADO receptors and possibly release of NO. To confirm this hypothesis, we directly measured the production of NO following application of ADO or CADO in our preparation (Fig. 6). ADO (or EHNA) caused a modest release of NO when applied alone, while at the same concentration (100 μM) CADO promoted a sustained ($> 15'$) and pronounced release of NO (70 ± 12 nM peak response, $n = 3$). ADO + EHNA similarly triggered a massive and prolonged production of measurable NO (60 ± 18 nM).

Since the neurotoxic effects of CADO were partially abolished by both A_1 and A_2 antagonists (Fig. 2), and since the adenosine-induced NO release from cultured glia could be mimicked by both A_1 and A_2 agonists (Janigro et al., 1996b), we exposed the organotypic hippocampal slices to specific A_1 and A_2 agonists and measured NO release. Activation of A_1 receptors was achieved by application of CCPA (100 nM, Fig. 6E, $n = 3$), while A_2-mediated effects were obtained following exposure to CGS 21680 (100 nM, $n = 3$, data not shown). Both procedures caused measurable NO release that was sensitive to blockade of NOS activity by 100 μM L-NA (after 30 min incubations; Fig. 6E).

4. Discussion

The main finding of this study is that hippocampal slices are sensitive to neurotoxic damage induced by exposure to stable adenosine analogues or, following blockade of adenosine catabolism, to ADO itself. Thus, we here characterize a novel NO- and NOS-mediated form of neurotoxicity in the hippocampus. When paired to sublethal Ax/Hg, CADO caused widespread neuronal injury resembling the post-ischemic damage observed in the same culture preparation after withdrawing oxygen and glucose for > 20 min. CADO-induced toxicity was mediated by adenosine receptors and nitric oxide production since it could be prevented by theophylline, the specific adenosine receptor antagonists DPCPX and DMPX, and L-NA.

In order to study this nitric oxide-mediated effects of adenosinergic receptor activation, we first investigated the role of NO in hippocampal ischemic damage and observed a direct neurotoxic effect of NO donors. A role for NO in ischemic neurotoxicity was further confirmed by the finding that L-NA protected against prolonged Ax/Hg. Similar data demonstrating neuroprotection by NOS inhibition against ischemic damage were obtained by other investigators [5,6]. In addition, we developed a system that enables nitric oxide detection from hippocampal slice cultures. This approach, combined with biochemical detection of extracellular ADO following enzymatic blockade of purine catabolism, led us to the discovery of a novel, NO-mediated action of adenosine in cultured rat hippocampal slices.

4.1. Mechanism of ischemic damage in the hippocampus: role of NO

Ischemia causes selective neuronal death in the vulnerable CA1 region of the hippocampus. This damage is mediated by widespread depolarization of neuronal termi-

nals, release of glutamate, and subsequent activation of NMDA receptors [26,28,34]. The NMDA receptor/channel complex allows massive entry of cations including calcium, and under ischemic conditions, this additional depolarization remains unchallenged by failing inhibitory mechanisms and thus overrides normal brain electrical and ionic homeostasis. In our in vitro experiments, short exposure of hippocampal slices to anoxia/hypoglycemia did not cause any significant neuronal damage. This is in agreement with previous findings by others showing that brief exposure to ischemia does not result in a significant change in brain 'excitotoxic index' [15]. However, sublethal ischemia appeared to predispose neuronal cells for extensive damage induced by further noxious challenge with Ax/Hg itself, nitric oxide donors, or promoters of nitric oxide release.

Based on results obtained from NOS 'knock-outs', ischemic and NMDA-induced neurotoxicity can be attributed primarily to release of NO from glia [6]. In agreement with these findings, prolonged ischemia in organotypic slices produced extensive delayed neuronal cell death mediated by excitotoxic glutamate release [28,29]. Furthermore, these effects were largely prevented by inhibition of nitric oxide production via incubation of the tissue with the nitric oxide synthase (NOS) inhibitor L-NA. This putative NOS inhibitor was used in our experiments at a concentration known to specifically block NOS without affecting baseline synaptic transmission [30]. In addition to non-specific *neuronal* effects, arginine analogues may also affect vascular responses that may, in turn, be neuroprotective. By using the organotypic hippocampal slice preparation, we were able to dissect out any unwarranted vascular effects. As one would expect based on the neuroprotection observed after treatment with L-NA, the NO donor molecule SIN-1 was neurotoxic. These results confirm that NO produced in response to ischemia and mediated by glutamate release can cause neuronal cell death. These effects were prevented by NOS inhibition, suggesting that enzymatic production of NO from L-Citrulline was involved.

4.2. Adenosine-mediated NO production

We have previously demonstrated that the qualitative results of electrochemical NO detection from cultured cells are identical to those obtained by [^{3}H]citrulline assay [18]. By using the same electrochemical methods, we provided direct evidence demonstrating that adenosine can cause release of nitric oxide from cultured astrocytes. Adenosine-induced NO release from astrocytes was prevented by inhibition of NOS by L-NA and depended on $[Ca^{2+}]_i$ [19]. In the present study, we have further expanded these findings by measuring nitric oxide production from cultured organotypic hippocampal slices exposed to adenosine and adenosine analogues. These experiments were performed by amperometric detection of NO and thus allowed us to determine the temporal profiles of nitric oxide production with a time resolution < 5 s.

Adenosine caused a modest and transient increase in baseline NO, comparable to what we observed when recording NO signals from purified cortical astrocytes (peak response around 10–20 nM NO, see [19]). This provides indirect evidence that in hippocampal slices ADO-induced nitric oxide release occurs via activation of glial, and perhaps not by neuronal, adenosine receptors. The hypothesis that adenosinergic NO release is, in the hippocampus, segregated to actions on non-neuronal cells is further supported by the fact that the *neuronal* actions of adenosine are entirely inconsistent with the known mechanisms underlying NO production (e.g., [32]). For example, while neuronal NO production occurs following glutamate release and activation of neuronal NMDA receptors, adenosine has been shown to inhibit excitatory amino acid release [22] and post-synaptic NMDA activation [8,10,11,23,25]. In addition, adenosine has been shown to decrease neuronally mediated NO production [1]. Furthermore, ADO causes neuronal hyperpolarization by increasing a potassium conductance [12], thus decreasing the probability of calcium influx through low- and high-threshold Ca^{2+} channels. These combined effects are likely to result in a 'concentration clamp' of neuronal $[Ca^{2+}]_i$, hence preventing NO synthesis by the calcium-dependent NO synthase. In contrast, adenosine actions on astrocytes are consistent with ADO-induced NO release since adenosine increases $[Ca^{2+}]_i$ concentrations in glia by promoting Ca^{2+} release from internal stores [33]. A similar mechanism (increased $[Ca^{2+}]_i$) in axonal terminals may also be responsible for the excitatory actions that follow activation of neuronal A_2 receptors.

Under our experimental conditions, ADO-mediated NO release was not sufficient to cause neuronal damage unless exposure to this neurotransmitter was paired to blockade of adenosine metabolism *and* to a sublethal ischemic episode. We thus hypothesized that the unequivocal cytotoxic effects of the stable adenosine analogue CADO were due to prolonged ADO receptor-triggered NO release. To test this hypothesis, we measured the time course of adenosine degradation in hippocampal slices and compared the results to the development of the NO increase (Fig. 4 and Fig. 6). ADO-induced responses were, as expected, transient unless adenosine metabolism was blocked by EHNA: the latter manipulation essentially promoted an adenosine-induced NO release indistinguishable from that observed following application of CADO.

Taken together, these results suggest that ADO and its stable analogues can trigger NO production from hippocampal astrocytes: these levels of NO, however, were not sufficient to cause widespread neuronal cell death, since pairing of adenosine (or CADO) applications to Ax/Hg were required to produce neurotoxicity. Therefore, our findings suggest that in addition to NO, other mechanisms, possibly including adenosine-enhanced, ischemia-

evoked glutamate release, are also operative in producing post-ischemic CA1 hippocampal damage.

4.3. Mechanisms of adenosine-induced neuronal damage and its relationship to adenosine neuroprotection

Several reports have shown that adenosine acting at A_1 receptors may function as an endogenous neuroprotectant following cerebral ischemia [16,34,36,45]. A neuroprotective role for endogenous adenosine is further supported by the finding that blockade of ADO receptors enhances post-ischemic damage [45]. While in vivo these effects may be due to the association of direct neuronal effects combined with actions on CBF, in isolated cultured slices ADO inhibition of excitotoxic transmission is likely to account for most of its beneficial effects. While on the one hand it may appear difficult to conciliate these beneficial effects with the adenosine receptor-mediated neurotoxicity reported herein, one must keep in mind that a similar apparent contradiction characterizes the neuronal effects of nitric oxide; both neurotoxicity and protection can be ascribed to nitric oxide, depending on its redox status and the cellular and molecular targets of its actions [21].

Chloroadenosine is highly cytotoxic for neoplastic cells [3], but this toxicity does not depend upon activation of selective adenosine receptors. It is thus unlikely that the effects observed in our study depended on the direct toxicity of CADO on cell division. Furthermore, CADO actions on neuronal cells are indistinguishable from those of adenosine (membrane hyperpolarization and decreased glutamate release): hence, direct effects on neuronal receptors do not appear as likely candidates for CADO neurotoxicity. In contrast, both ADO and CADO cause release of NO from glia by actions on A_1 and A_2 receptors [19]. Furthermore, astrocytic A_1 receptor activation has been shown to inhibit glutamate reuptake [13], a powerful, yet indirect action potentially linked to toxicity. A_2 receptors mediate activation of glutamate release from ischemic brain [31], and adenosine induces swelling of glia [2]. All these potentially neurotoxic actions were obtained with either high ($> 10\ \mu M$) ADO concentrations or by use of metabolically stable agonists (such as CADO or NECA), suggesting that these effects are not obtainable under physiological conditions but require an interstitial environment where significant (and pathologic) accumulation of adenosine may occur. It is worth noting that under pathological conditions, ADO concentrations equal or greater than 100 μM can be measured [44].

We attempted to characterize the adenosine receptor subtypes involved in the neurotoxic response. In agreement with our previous findings with cultured astrocytes, both A_1 and A_2 receptors seemed to be involved since both the A_1-specific receptor antagonist DPCPX and the A_2 antagonist DMPX were capable of significantly decreasing the CADO-induced CA1 toxicity. Furthermore, NO release from hippocampal slices was obtained following exposure to both A_1- and A_2-specific agonists (Fig. 6E). We also ruled out the possible involvement of an adenosine transporter in CADO neurotoxicity, since results identical to those obtained with chloroadenosine were obtained with NECA, a poor substrate for the adenosine transporter [37].

Taken together, our results suggest that in addition to its well known effects as an endogenous vasodilator and neuroprotectant, glial adenosine receptor activation and/or activation of neuronal A_2 receptors may lead to neurotoxicity. There are important inferences that result from our findings: exogenous drugs aimed at increasing adenosine concentrations in the brain as a therapeutic tool to counteract ischemic damage may exert limited effects due to overlapping neurotoxicity and neuroprotection. A foreseeable approach aimed at enhancing the latter while reducing the former may consist in developing selective pharmacological agonists targeting neuronal but not glial receptors.

Acknowledgements

We would like to thank Vera Papermaster for preparing the hippocampal cultures and Raimondo D'Ambrosio for helping in part of the experiments. This work was supported through grants of the Swiss Foundation for Biomedical Grants and the Ciba-Geigy Jubilee-Foundation (A.B.), NIH Grant 1 P50 NS 30305-01, National Stroke Association Award, and Clinician Investigator Development Award 1 K08 NS 01596901 (D.W.N.), and NIH Grants NIH 51614, 21076 and NIEHS ES 07033 (D.J.).

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