The Journal of Experimental Biology **198**, 1621–1628 (1995)
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ROLE OF ADENOSINE IN NMDA RECEPTOR MODULATION IN THE CEREBRAL CORTEX OF AN ANOXIA-TOLERANT TURTLE (*CHRYSEMYS PICTA BELLI*)

L. T. BUCK AND P. E. BICKLER
Department of Anesthesia, University of California, San Francisco, CA 94143-0542, USA

Accepted 14 March 1995

Summary

Accumulation of the neuromodulator adenosine in the anoxia-tolerant turtle brain may play a key role in a protective decrease in excitatory neurotransmission during anoxia. Since excitatory neurotransmission is mediated largely by Ca^{2+} entry through *N*-methyl-D-aspartate (NMDA) receptors, we measured the effect of adenosine on NMDA-mediated Ca^{2+} transients in normoxic and anoxic turtle cerebrocortical sheets. Intracellular [Ca^{2+}] was measured fluorometrically with the Ca^{2+}-sensitive dye Fura-2. Baseline intracellular [Ca^{2+}] and [ATP] were also measured to assess cortical sheet viability and potential toxic effects of NMDA. Baseline [Ca^{2+}] did not change significantly under any condition, ranging from 109±22 to 187±26 nmol l^{-1}. Throughout normoxic and 2 h anoxic protocols, and after single and multiple NMDA exposures, [ATP] did not change significantly, ranging from 16.0±1.9 to 25.3±4.9 nmol ATP mg^{-1} protein. Adenosine caused a reduction in the normoxic NMDA-mediated increase in [Ca^{2+}] from a control level of 287±35 to 103±22 nmol l^{-1} (64 %). This effect is mediated by the A_1 receptor since 8-phenyltheophylline (a specific A_1 antagonist) effectively blocked the adenosine effect and N^6-cyclopentyladenosine (a specific A_1 agonist) elicited a similar decrease in the NMDA-mediated response. Cortical sheets exposed to anoxia alone exhibited a 52 % decrease in the NMDA-mediated [Ca^{2+}] rise, from 232±30 to 111±9 nmol l^{-1}. The addition of adenosine had no further effect and 8-phenyltheophylline did not antagonize the observed decrease. Therefore, the observed down-regulation of NMDA receptor activity during anoxia must involve additional, as yet unknown, mechanisms.

Key words: cerebral cortex, A_1 receptor, Fura-2, calcium, fluorometry, turtle, *Chrysemys picta belli*.

Introduction

Freshwater turtles possess the most anoxia-tolerant central nervous system of any vertebrate species, surviving hours of complete anoxia at high temperatures (25 °C) and weeks to months at low temperatures (15–3 °C) (Jackson *et al.* 1984; Ultsch and Jackson, 1982). There is a great deal of interest in understanding the mechanisms underlying anoxia-tolerance in this group. One key to prolonged anoxic survival is the matching of ATP production and utilization to maintain essential transmembrane ion gradients (Hochachka, 1986). However, the biochemical signaling mechanisms that achieve a coordinated reduction in metabolism and ion fluxes remain unclear. A candidate for a metabolic signal is adenosine, since adenosine acting through its specific receptors (A_1 and A_2) has been shown to have numerous physiological and metabolic effects that could be beneficial to anoxic survival. These effects include: (1) vasodilation, to increase blood flow and therefore substrate delivery; (2) stimulation of glycogenolysis, providing substrate for anaerobic glycolysis; (3) stimulation of glycolysis, increasing ATP production to meet utilization; and (4) decreasing neuronal excitability (postsynaptic inhibition) as well as neurotransmitter release (presynaptic inhibition), effectively reducing neuronal energy requirements (for a review of adenosine effects, see Collis and Hourani, 1993). Three lines of evidence suggest that adenosine plays such a regulatory role in the transition from normoxia to anoxia in turtle brain: (1) extracellular adenosine in the intracerebral space accumulates during 2 h of anoxia to levels 10-fold greater than during normoxia (from approximately 2 to 20 μmol l^{-1}; Nilsson and Lutz, 1992); (2) antagonizing A_1 receptors during anoxia in isolated turtle cerebellum leads to a release of intracellular K^+ (Perez-Pinzon *et al.* 1993), retention of K^+ being characteristic of anoxia-tolerance in the turtle brain; and (3) blood flow increases and decreases in the cerebral vasculature in concert with the appearance and disappearance of adenosine during anoxia, and this could be inhibited by A_1 receptor antagonists (Hylland *et al.* 1994).

A major pathophysiological lesion leading to irreversible anoxic injury in mammalian central nervous systems is a large increase in intracellular calcium concentration ($[Ca^{2+}]_i$) (Siesjo, 1990). The excessive influx of Ca^{2+} is thought to be caused by an increase in the level of the excitatory amino acid glutamate during ischemic or anoxic periods (Siesjo, 1990).

Of the glutamate receptor subtypes, the *N*-methyl-D-aspartate (NMDA) receptor is a high-flux ligand-gated cation channel that is highly permeable to Ca^{2+} and is therefore a major source of Ca^{2+} entry during anoxia. This conclusion is supported by evidence showing that antagonism of this channel reduces the rate of Ca^{2+} entry and the degree of tissue damage (for reviews, see Choi, 1992; Lobner and Lipton, 1993). Recently, adenosine application to rat hippocampal slices has been shown to reduce NMDA-receptor-mediated excitatory potentials (de Mendonca and Ribeiro, 1993). Furthermore, the apparent reduction in NMDA receptor sensitivity was shown to be Mg^{2+}-dependent. Since adenosine is known to activate K^+ currents in hippocampal neurons (Krnjevic, 1993), this is probably the result of membrane hyperpolarization and strengthening of the voltage-dependent Mg^{2+} block of the NMDA receptor (Ascher and Nowak, 1987). We reasoned that in an anoxia-tolerant species such as the western painted turtle, where adenosine levels increase (Nilsson and Lutz, 1992) and electrical activity decreases (Sick *et al.* 1982) with the onset of anoxia, adenosine would modulate the activity of NMDA receptors in cortical slices. In fact, glutamate receptors in turtle brain slices prepared from turtles breathing a N_2 atmosphere for 18 h have been shown to become unresponsive to glutamate (Bickler and Gallego, 1993). Even though levels of glutamate in the anoxic turtle brain decrease (Nilsson *et al.* 1990), these data indicate that at some level there is direct or indirect regulation of postsynaptic glutamate receptors. We are studying the effect of adenosine on the NMDA-mediated increase in $[Ca^{2+}]_i$ in cortical slices during the initial 2 h of anoxia because [glutamate] remains within 20 % of normoxic levels and [adenosine] peaks during this time (Nilsson *et al.* 1990). Therefore, our main goal was to determine the effect of adenosine on the NMDA-mediated increase in $[Ca^{2+}]_i$ in normoxic and anoxic turtle cerebrocortical sheets. Since NMDA and glutamate are neurotoxic to rat brain slices, the toxicity of these compounds to turtle brain sheets in single and multiple exposures was also determined.

Materials and methods

Animal care and cortical slice preparation

These studies were approved by the University of California at San Francisco committee on Animal Research and conform to relevant guidelines for the care of experimental animals. Spring and summer animals (*Chrysemys picta belli* Sneider) of either sex weighing between 250 and 500 g were obtained from Lemberger, Oshkosh, WI, USA. Animals were housed in a large aquarium equipped with a flow-through dechlorinated freshwater system operating at 15–20 °C and a basking platform and lamp. All animals were maintained on the same natural photoperiod and given continuous access to food and water.

Cortical brain sheets were prepared after decapitation and rapid removal of the cranium. The entire cerebral cortex was dissected free and placed in artificial cerebrospinal fluid at 3–5 °C (aCSF, in mmol l^{-1}: 86.5 NaCl, 26.5 $NaHCO_3$, 2.0 Na_2HPO_4, 2.6 KCl, 2.5 $CaCl_2$, 0.2 $MgCl_2$, 20 glucose and Hepes, pH 7.4 at 25 °C). Individual cortical sheets (6–8 in total) were cut from larger cortical sheets with fine scissors as described by Blanton *et al.* (1989).

Normoxic and anoxic measurement of $[Ca^{2+}]_i$

Cortical sheets were loaded with the Ca^{2+}-sensitive dye Fura-2/AM (Molecular Probes, Eugene, OR) to facilitate measurement of $[Ca^{2+}]_i$. Cortical sheets were placed in a 20 ml capped vial containing 5 ml of aCSF and 4–6 μmol l^{-1} Fura-2/AM (from a 1 mmol l^{-1} stock solution in dimethyl sulfoxide and 0.05 % pluronic acid) for 2 h at room temperature and continually gassed with 95 % O_2/5 % CO_2. Cortical sheets were then subdivided into three treatment groups containing 2 μmol l^{-1} Fura-2/AM (to prevent dye loss); these were: control; 100 μmol l^{-1} adenosine; and 100 μmol l^{-1} adenosine plus the A_1 receptor antagonist 8-phenyltheophylline (8-PT, 10 μmol l^{-1}). Anoxic experiments were conducted in a similar fashion. All solutions were prebubbled with 95 % N_2/5 % CO_2, and 0.5 mmol l^{-1} NaCN was added to prevent reoxygenation artifacts during transfer of cortical sheets to fluorometry cuvettes. Normoxic preincubation was carried out as above, cortical sheets were subdivided and placed into three treatment groups as above. Anoxic conditions were maintained by continually gassing capped vials with 95 % N_2/5 % CO_2. Anoxic solutions were routinely assayed for residual oxygen using a CIBA-Corning 178pH/blood gas analzyer (Medfield, MA) calibrated with Radiometer (Copenhagen) P_{O_2}-zero solution. Residual P_{O_2} values of anoxic solutions did not differ from the P_{O_2}-zero calibration buffer. All experiments were conducted at 25 °C.

Single cortical sheets loaded with Fura-2 were mounted on a plastic mesh support that was suspended from a Teflon coverslip holder and placed in a stirred 3 ml quartz cuvette. The cuvette contained 2 ml of normoxic or anoxic aCSF and the respective treatment compounds adenosine, 8-PT or N^6-cyclopentyladenosine. The cuvette was then placed in the thermostatically regulated cuvette chamber of a Hitachi F-2000 fluorometer (Tokyo), carefully positioning the excitation beam on the cortical sheet. Cortical sheets loaded with Fura-2 were alternately excited at 340 and 380 nm, and fluorescence intensity at 510 nm was recorded at 0.5 s intervals with the fluorometer. Fluorescence at 510 nm was collected from the Fura-2-loaded cortical sheet for 200 s before addition of the glutamate receptor agonist NMDA. Calculation of $[Ca^{2+}]_i$ was performed using the following equation (Jensen and Chiu, 1991):

$$[Ca^{2+}]_i = K_D[(R - R_{min})/(R_{max} - R)](F_0/F_1),$$

where K_D is 263 nmol l^{-1} (temperature-corrected K_D; Shuttleworth and Thompson, 1991) and R is the ratio of fluorescence intensity at 510 nm elicited by excitation at 340 and 380 nm, after first correcting for background (see below). R_{max} and R_{min} are the respective 340 nm/380 nm ratios at saturating and zero $[Ca^{2+}]$. F_0/F_1 is the 380 nm ratio at zero and saturating $[Ca^{2+}]$, respectively.

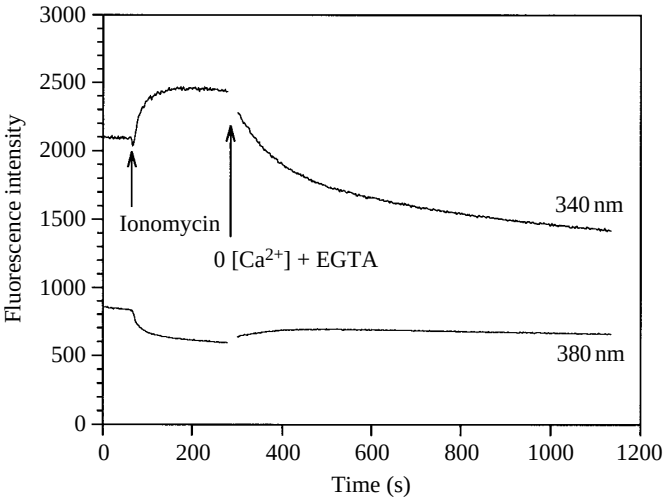

Fig. 1. A representative intracellular Fura-2 calibration showing Fura-2 fluorescence at 340 nm (upper trace) and 380 nm (lower trace). Arrows indicate points of 2 μmol l^{-1} ionomycin addition in aCSF containing 2.5 mmol l^{-1} Ca^{2+} (to obtain R_{max}) and medium exchange to 0 mmol l^{-1} Ca^{2+} and 10 mmol l^{-1} EGTA (to obtain R_{min}).

Calibration of Fura-2

To calibrate the response of intracellular Fura-2 to changes in $[Ca^{2+}]_i$ an *in vivo* calibration was performed, essentially as described by Jensen and Chiu (1991). A flat baseline fluorescence signal was recorded over 200 s, after which ouabain (1 mmol l^{-1}, from a 40 mmol l^{-1} stock in ethanol) was introduced. Ouabain incubation was maintained for 10 min to inhibit Na^+/K^+-ATPase and to dissipate the Na^+ gradient, therefore preventing Ca^{2+} extrusion *via* the Na^+/Ca^{2+} exchanger. Ionomycin (2 μmol l^{-1}, from a 5 mmol l^{-1} stock in ethanol) was added and a Ca^{2+}-saturated Fura-2 signal obtained, corresponding to an R_{max} ranging from 4.2 to 10 (Fig. 1). To obtain an estimate of R_{min}, the cuvette solution was removed and replaced with aCSF containing 0 μmol l^{-1} Ca^{2+} and 10 mmol l^{-1} EGTA. The 340 and 380 nm fluorescent signals then converge (Fig. 1), indicating Ca^{2+} removal from the intracellular space and therefore an increasing unbound Fura-2 level. At least 930 min was required for the slopes of these lines to reach zero and obtain an R_{min} ranging from 0.44 to 1.1. The R_{min} and R_{max} values were determined routinely as were autofluorescence (background) values from cortical slices not loaded with Fura-2. This procedure was performed with both normoxic and anoxic cortical sheets.

Repeat NMDA exposure and CPA incubation

To determine whether cortical sheets could be washed free of NMDA and re-used in a 'repeat measures' design, Fura-2-loaded slices were exposed to NMDA for 2 min, then removed and washed for 90 min, this cycle being repeated three times. NMDA washout consisted of placing cortical sheets in 5 ml of aCSF containing 2 μmol l^{-1} Fura-2 and gassed with 95 %O_2/5 %CO_2. Following the first exposure, cortical sheets were placed in aCSF with 60 μmol l^{-1} N^6-cyclopentyladenosine (CPA, Research Biochemicals Int., Natick, MA, USA) for 90 min. Subsequently an NMDA-mediated increase in $[Ca^{2+}]_i$ was determined and the cortical sheets were washed and assayed a third time. Finally, cortical sheets were sampled for ATP measurement (see below). Cortical sheets were handled and agonist response measured as described above.

ATP and tissue protein measurement

To measure cortical sheet [ATP] and protein content, cortical sheets were rapidly placed into 1 ml of boiling 50 mmol l^{-1} Hepes buffer at pH 7.8 and sonicated for 20 s. They were incubated at high temperature for an additional 2 min before being removed and subdivided into two equal samples, one for ATP and one for protein determination. An Analytical Luminescence Laboratory model 2001 luminometer (San Diego, CA, USA) was used to measure ATP. Stock luciferase–luciferin was obtained from Analytical Luminescence Laboratory (San Diego, CA, USA) and reconstituted into firefly diluent (Sigma). Total protein was determined using a Pierce protein assay kit (Rockford, IL, USA).

Statistical methods

Analysis of variance followed by a *post-hoc* multiple-comparison test (Student–Neuman–Keuls or Dunnett's) was used to compare NMDA-mediated Ca^{2+} influx under the different experiment conditions. Values are reported as means ± S.E.M., and $P \leqslant 0.05$ was considered statistically significant.

Results

Effect of adenosine on the NMDA-mediated increase in $[Ca^{2+}]_i$

After 120 min of incubation in the presence of 100 μmol l^{-1} adenosine, the NMDA-mediated increase in $[Ca^{2+}]_i$ was reduced by 75 % (Fig. 2). This was blocked by the inclusion of 10 μmol l^{-1} 8-PT in the incubation mixture (Fig. 2). The data are summarized in Fig. 3, where the time course of the adenosine effect is shown. Since adenosine levels in turtle brain have been shown to peak around 100 min after the onset of breathing N_2 (Nilsson and Lutz, 1992), we measured the effect of adenosine incubation on the NMDA-mediated increase in $[Ca^{2+}]_i$ at 60, 90 and 120 min. The NMDA-mediated Ca^{2+} increase (control 287±35 nmol l^{-1}) is reduced by 33, 51 and 64 %, at 60, 90 and 120 min (103±22 nmol l^{-1}) of adenosine incubation, respectively (Fig. 3). The increases in $[Ca^{2+}]$ measured at 90 and 120 min were significantly different from the control and 180 min control responses ($P \leqslant 0.05$). Cortical sheets incubated with adenosine and 8-PT showed NMDA responses that were not significantly different from the control and 180 min control responses (Fig. 3). The effect of anoxic incubation on the NMDA-mediated increase in $[Ca^{2+}]_i$ is shown in Fig. 4. 120 min of anoxic incubation resulted in a statistically significant 52 % decrease in the increase in $[Ca^{2+}]_i$ elicited by NMDA; from a normoxic control value of 232±30 to 111±9 nmol l^{-1} Ca^{2+} ($P \leqslant 0.05$). The addition of adenosine,

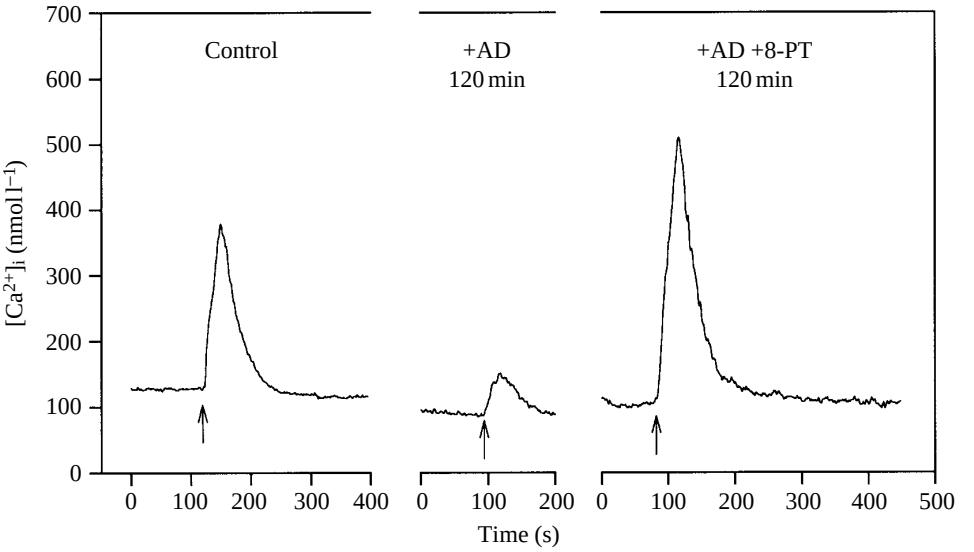

Fig. 2. A typical experiment showing the effect of incubation with adenosine (AD) or adenosine + 8-phenyltheophylline (8-PT) on the NMDA-mediated increase in cytosolic $[Ca^{2+}]$. Data shown are from three separate cortical slices: a control slice, a slice incubated for 120 min with 100 μmol l^{-1} adenosine and a slice incubated with 100 μmol l^{-1} adenosine and 10 μmol l^{-1} 8-PT. Arrows mark the point of NMDA addition.

or adenosine and 8-PT, did not markedly alter this result: corresponding values were 108±26 and 116±29 nmol l^{-1} Ca^{2+}, respectively (Fig. 4). The $[Ca^{2+}]_i$ values at 90 min of anoxia were not significantly different from the control values. Following 120 min of normoxic recovery, $[Ca^{2+}]_i$ returned to levels not significantly different from control levels.

To assess the specificity of adenosine action further, the effect of the A_1 receptor agonist CPA on the NMDA-mediated increase of $[Ca^{2+}]_i$ was examined (Fig. 5). The initial NMDA exposure resulted in a 630±72 nmol l^{-1} increase in $[Ca^{2+}]_i$. Following a 90 min wash period, a second NMDA exposure resulted in an approximately 24 % greater increase. This effect was maintained during a second wash period and a third NMDA exposure. The inclusion of 60 μmol l^{-1} CPA in the first wash incubation resulted in a 44 % decrease in the NMDA-mediated $[Ca^{2+}]_i$ increase, similar to the decrease measured following incubation in adenosine (Fig. 3). The decrease in the NMDA-elicited response was temporary; a second 90 min wash period removed the effect of CPA. Since CPA was dissolved in ethanol (a potential inhibitor of NMDA channel activity), an equal amount of ethanol was added to control incubations. There was no significant decrease in the control response due to ethanol (Fig. 5).

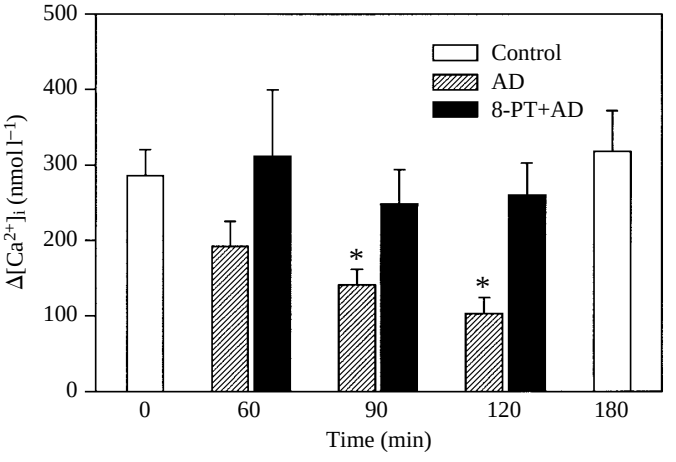

Fig. 3. The effect of the A_1 receptor antagonist 8-PT on the NMDA-mediated increase in cytosolic $[Ca^{2+}]$ in the presence and absence of adenosine (AD). Slices were incubated for the indicated time in the presence of 100 μmol l^{-1} adenosine or 100 μmol l^{-1} adenosine + 10 μmol l^{-1} 8-PT. Each bar represents the mean + S.E.M. from 5–23 separate animals. Asterisks indicate values significantly different from control values.

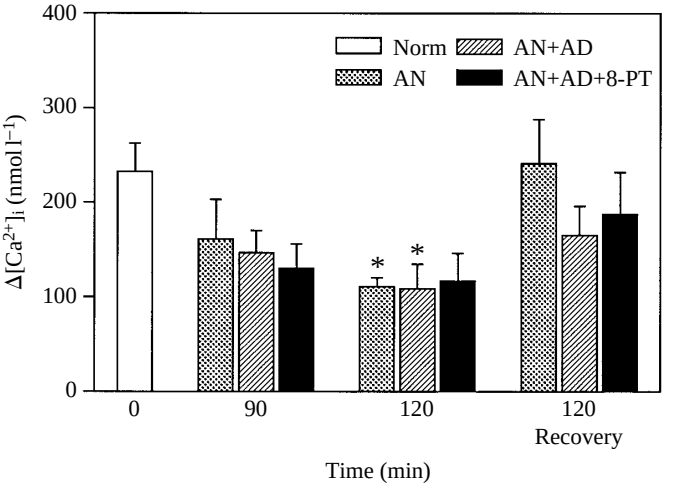

Fig. 4. The effect of anoxic incubation (AN) on the NMDA-mediated increase in cytosolic $[Ca^{2+}]$. Cortical sheets were incubated in anoxic aCSF containing 0.5 mmol l^{-1} NaCN for the indicated times. Recovery sheets were maintained under anoxia for 120 min, exposed to NMDA and placed in control normoxic aCSF. Subsequently, these slices were re-exposed to NMDA. Each bar represents the mean + S.E.M. from 5–10 separate animals. Asterisks indicate values significantly different from control values. Norm, normoxic control; AD, adenosine; 8-PT, 8-phenyltheophylline.

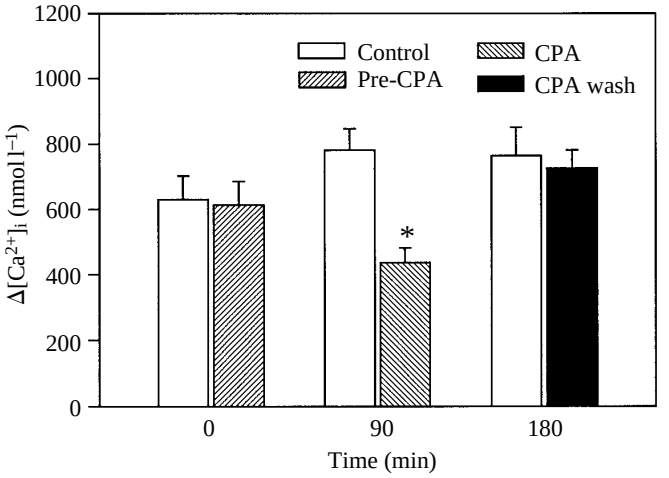

Fig. 5. The effect of the A_1 agonist CPA on the NMDA-mediated increase in $[Ca^{2+}]_i$. Each bar at time 0 represents the mean + S.E.M. of 15 separate slices: six slices obtained from each of five animals and subdivided into control and CPA treatment groups. Slices were washed free of NMDA and one group was incubated with 60 μmol l^{-1} CPA, then re-exposed to NMDA at 90 min. Slices were again washed free of NMDA and CPA, and again tested for an NMDA response at 180 min. The asterisk indicates a value significantly different from control.

Table 1. *Comparison of intracellular [ATP] in cortical slices before and after exposure to glutamate receptor agonists and incubation with adenosine receptor agonists and antagonists*

	[ATP] (nmol mg^{-1} protein)		
Exposure	Normoxic	Anoxic, 2 h	Normoxic recovery, 1 h
Control (t=0)	22.7±2.4 (8)	20.1±1.2 (5)	
NMDA 1×	25.3±4.9 (4)	15.0±2.4 (4)	16.0±1.9 (6)
NMDA + adenosine	18.9±1.4 (9)	18.3±2.1 (6)	17.7±2.1 (5)
NMDA + 8-PT	19.4±1.1 (6)	16.3±1.9 (6)	17.9±3.3 (3)
Control (180 min)	24.9±3.9 (9)		
NMDA 3× + CPA	20.5±1.2 (3)		
Glutamate 1×	19.8±2.5 (4)		
CN + IAA	1.02 (2)*		

For multiple exposure experiments, slices were washed in aCSF for 90 min between agonist exposures; 1× and 3× refer to single and triple agonist exposures, respectively.

Agonist, antagonist and inhibitor concentrations were as follows: NMDA, 100 μmol l^{-1}; adenosine, 100 μmol l^{-1}; 8-PT, 10 μmol l^{-1}; CPA, 60 μmol l^{-1}; glutamate, 1 mmol l^{-1}; CN, 100 μmol l^{-1}; IAA, 5 mmol l^{-1}.

Values are means ± S.E.M. with the number of slices studied in parentheses.

*Significantly different from all others (ANOVA, followed by Student–Neuman–Keuls *post-hoc* multiple comparison, $P \leqslant 0.05$.

NMDA, *N*-methyl-D-aspartate; 8-PT, 8-phenyltheophylline; CPA, cyclopentyladenosine; CN, cyanide; IAA, iodoacetate.

Baseline $[Ca^{2+}]_i$

To rule out the possibility that adenosine was altering baseline $[Ca^{2+}]_i$ in a way that could affect the NMDA-mediated $[Ca^{2+}]_i$ increase, baseline $[Ca^{2+}]_i$ was compared between treatment groups. Normoxic baseline $[Ca^{2+}]_i$ ranged from 157±19 (control) to 187±26 nmol l^{-1} (180 min control) and there were no significant differences between the treatment groups, control, adenosine and adenosine plus 8-PT. This was reflected in the anoxic baseline values, which ranged from 109±22 to 170±42 nmol l^{-1} Ca^{2+} without statistically significant differences between groups.

Cortical slice viability

Slice [ATP] was not altered by adenosine, nor by A_1 receptor agonists or antagonists, nor by single or multiple NMDA exposure (Table 1). Similarly glutamate (1.0 mmol l^{-1}) had no significant effect. Anoxic incubation of cortical sheets for 2 h in the presence and absence of adenosine and 8-PT did not result in any statistically significant changes in [ATP]. Following a 1 h normoxic recovery period, cortical sheet [ATP] did not differ from [ATP] in normoxic or anoxic cortical sheets. Complete metabolic blockade with cyanide and iodoacetate resulted in a statistically significant 95 % loss of [ATP] in less than 10 min.

Discussion

Our data show that adenosine inhibits NMDA receptor activity, significantly reducing the increase in $[Ca^{2+}]_i$ observed following NMDA receptor stimulation in normoxic turtle cerebral cortex. This effect is mediated by the A_1 receptor since 8-PT (a specific A_1 antagonist) effectively blocked the effect of adenosine and CPA (a specific A_1 agonist) elicited a similar decrease in the NMDA-mediated $[Ca^{2+}]$ increase. These results are consistent with what is known of the pharmacology of the A_1 receptor in mammalian brain (Rudolphi *et al.* 1992) and are consistent with a major protective role of adenosine during anoxia in turtle brain (Nilsson and Lutz, 1992; Perez-Pinzon *et al.* 1993). However, anoxia in the absence of exogenous adenosine resulted in a similar decrease in the NMDA-mediated response. The anoxic result suggests that there are other mechanisms leading to the observed decrease, such as increased concentrations of γ-aminobutyric acid (GABA) or glycine. Levels of both of these compounds have been shown to increase significantly in turtle brain following 2 h of anoxic exposure (Nilsson *et al.* 1990).

Adenosine is well established as a potent pre- and postsynaptic neuromodulator. Mechanisms underlying these effects have recently been characterized. Presynaptically, adenosine reduces neurotransmitter release (Burke and Nadler, 1988), probably as a result of reduced Ca^{2+} entry through N-type channels (rat hippocampal pyramidal neurons, Scholz and Miller, 1991; rat hippocampal CA3 region, Mogul *et al.* 1993; chick ciliary ganglia, Yawo and Chuhma, 1993; mouse motoneurons, Mynlieff and Beam, 1994; rat hippocampal CA1

region, Wu and Saggau, 1994). Postsynaptically, adenosine activates K^+ and Cl^- currents in hippocampal neurons, causing membrane hyperpolarization (Dunwiddie, 1985; Gerber *et al.* 1989; for a review, see Rudolphi *et al.* 1992). Hyperpolarization decreases the likelihood of NMDA receptor activation *via* a voltage-dependent Mg^{2+} block (Ascher and Nowak, 1987). Recently, an adenosine-evoked decrease in electrical activity in the CA1 region of rat hippocampus was shown to be Mg^{2+}-dependent and to be modulated by both A_1 receptor and NMDA receptor ligands (Stone *et al.* 1990; Bartrup and Stone, 1990; de Mendonca and Ribeiro, 1993). These data suggest that membrane hyperpolarization is one link between A_1 receptors and reduced NMDA receptor activity.

A presynaptic effect of adenosine (reduction of neurotransmitter release) is unlikely to be the mechanism behind the reduced NMDA receptor activity reported here, because in our experiment a change in $[Ca^{2+}]_i$ was elicited by the exogenous application of agonist (NMDA). Furthermore, in preliminary experiments the Na^+ channel blocker tetrodotoxin, which blocks action potential propagation, reduced the NMDA-mediated $[Ca^{2+}]$ increase by only 16 % (L. T. Buck and P. E. Bickler, unpublished observation). This decrease cannot account for the adenosine-evoked 64 % reduction in the NMDA-mediated $[Ca^{2+}]_i$ increase (Fig. 3) and indicates that a major component of the $[Ca^{2+}]$ increase is not depolarization-dependent (*via* N-type Ca^{2+} channels). These observations support the conclusion that the effect of adenosine is likely to be postsynaptic. In addition, turtle brain NMDA receptors have previously been shown to be Mg^{2+}-sensitive (Blanton and Kriegstein, 1992), a result that we have also observed ($2\,mmol\,l^{-1}$ Mg^{2+} in the bathing medium reduced NMDA-mediated Ca^{2+} flux by approximately 80 %; L. T. Buck and P. E. Bickler, unpublished observation). Taken together, the known Mg^{2+}-dependent adenosine effects on brain electrical activity and the data presented here suggest a mechanism involving an adenosine-induced membrane hyperpolarization and reduction in NMDA receptor activity by a strengthening of the voltage-dependent Mg^{2+} block. Alternatively, K^+ and Cl^- may be distributed close to their equilibrium potentials in resting cells, so that activation of their respective ion channels (by adenosine, GABA or glycine) does not necessarily result in hyperpolarization. Rather, opening of K^+ and Cl^- channels may serve to counter membrane depolarization, preventing the depolarization-dependent removal of Mg^{2+} and inhibiting NMDA-mediated Ca^{2+} currents.

Although adenosine accumulation might decrease NMDA activity during anoxia, Sakurai *et al.* (1993) reported that anoxia did not induce functional changes in the NMDA receptor from turtle forebrain. They employed a [^{3}H]MK-801 binding assay to detect functional changes in the NMDA receptor. However, their method assumes that any functional change in receptor activity is maintained throughout the tissue preparation protocol. The binding assay protocol employed would not detect a Mg^{2+}-dependent block of the NMDA receptor, since this is dependent on membrane potential and an extracellular source of Mg^{2+} (Ascher and Nowak, 1987), both of which were absent throughout the protocol.

The decrease in NMDA receptor activity observed here may be the result not only of membrane hyperpolarization but also of phosphorylation or dephosphorylation of the receptor. Phosphorylation of the NMDA receptor effectively increases Ca^{2+} currents, and dephosphorylation decreases Ca^{2+} currents (Chen and Huang, 1992; Wang and Salter, 1994; Wang *et al.* 1994; Lieberman and Mody, 1994). The role that receptor phosphorylation status plays in the transition to anoxia is unknown. Furthermore, both A_1 and A_2 receptors have been shown to have a depressant effect on neural activity (Lin and Phillis, 1991). Both of these receptors regulate the activity of G-proteins that switch on and off the protein kinases and phosphatases necessary to modify NMDA receptors allosterically. Additionally, adenosine has been shown to have a tonic regulatory role in the normoxic functioning of neurons, because uptake pathway inhibitors and adenosine deaminase inhibitors sharply increase its concentration in microdialysis perfusates (Pazzagli *et al.* 1993). Moreover, [adenosine] has been shown to increase in response to NMDA application in rat cerebrocortex (Chen *et al.* 1992; Craig and White, 1993), and this induced increase can be blocked with tetrodotoxin, pointing to an activity-dependent component of adenosine release (Pazzagli *et al.* 1993). Therefore, the NMDA receptor could become less active by becoming less phosphorylated in the presence of adenosine. This mechanism would also lead to energy savings, in terms of ATP, during an anoxic transition, since protein kinase activity requires ATP.

The role that phosphorylation/dephosphorylation cycles play in the transition from normoxia to anoxia in the turtle brain remains uncertain; but one scenario is as follows. (1) Blood and tissue O_2 stores fall below a critical level and there is a net breakdown of ATP to adenosine, resulting from an imbalance between ATP utilization and production. (2) Adenosine reduces Ca^{2+} influx at the presynaptic membrane, decreasing vesicular glutamate release into the synaptic cleft and hyperpolarizing the postsynaptic membranes, which effectively desensitizes the NMDA receptors to glutamate and reduces detrimental Ca^{2+} loading and/or reduces the steady-state phosphorylation level to a more (or completely) dephosphorylated state.

As a measure of cortical slice viability, [ATP] was measured under control, normoxic and anoxic experimental conditions. There were no significant decreases in [ATP] under any condition except when slices were incubated with metabolic inhibitors. Remarkably, there was no significant loss of ATP following three exposures to NMDA, and $[Ca^{2+}]_i$ returned to pre-NMDA exposure levels each time, indicating that the preparation was healthy. Furthermore, cortical slice [ATP] and baseline $[Ca^{2+}]_i$ were similar to those previously reported (Bickler, 1992; Bickler and Gallego, 1993). The tolerance of turtle cortex to glutamate ($1\,mmol\,l^{-1}$) exposure agrees with previous results using histochemical techniques (Wilson and Kriegstein, 1991). The tolerance of turtle brain to repeat exposures of NMDA at relatively high concentrations is even

more remarkable than its tolerance to glutamate, since there is no known uptake pathway for NMDA. At the concentrations employed here, excitatory neurotransmitters such as glutamate and agonists such as NMDA are toxic to mammalian neurons (Choi, 1992). The mechanisms behind this tolerance to excitotoxic cell death are unclear but are likely to be related to the innate anoxia-tolerance of freshwater turtles.

In conclusion, we have demonstrated a functional change in the activity of the NMDA receptor from turtle cerebral cortex in response to adenosine application during normoxia. Futhermore, anoxia alone resulted in a significant decrease in NMDA receptor activity, independent of adenosine or 8-PT. The lack of an adenosine effect and 8-PT antagonism during anoxia is unclear and we must conclude that there are other mechanisms involved, perhaps involving GABA or glycine. We speculate from our normoxic data, and the known effects of adenosine on neuronal function, that adenosine application results in membrane hyperpolarization and decreased NMDA receptor activity by strengthening of the Mg^{2+}-dependent block. Additionally, turtle cortical slices are tolerant of normoxic and anoxic exposures of NMDA that are toxic to mammalian cortical slices.

This research was supported by a Medical Reseach Council of Canada Fellowship to L.T.B. and a National Institute of Health (USA) Grant (no. 52212) to P.E.B. We thank Dr A. Gray for commenting on the manuscript.

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