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ADENOSINE AND ANOXIA REDUCE *N*-METHYL-D-ASPARTATE RECEPTOR OPEN PROBABILITY IN TURTLE CEREBROCORTEX

LESLIE T. BUCK[1,*] AND PHILIP E. BICKLER[2]

[1]*University of Toronto, Department of Zoology, Toronto, Canada ON M5S 3G5* and [2]*University of California, Department of Anesthesia, San Francisco, CA 94143-0542, USA*

*e-mail buckl@zoo.utoronto.ca

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Summary

During normoxia, glutamate and the glutamate family of ion channels play a key role in mediating rapid excitatory synaptic transmission in the central nervous system. However, during hypoxia, intracellular [Ca^{2+}] increases to neurotoxic levels, mediated largely by the *N*-methyl-D-aspartate (NMDA) subfamily of glutamate receptors. Adenosine has been shown to decrease the magnitude of the hypoxia-induced increase in $[Ca^{2+}]_i$ in mammalian brain slices, delaying tissue injury. Turtle brain is remarkably tolerant of anoxia, maintaining a pre-anoxic $[Ca^{2+}]_i$ while cerebral adenosine levels increase 12-fold. Employing cell-attached single-channel patch-clamp techniques, we studied the effect of adenosine (200 μmol l^{-1}) and anoxia on NMDA receptor open probability (P_{open}) and current amplitude. After 60 min of anoxic perfusion, channel P_{open} decreased by 65 % (from 6.8±1.6 to 2.4±0.8 %) an effect that could also be achieved with a normoxic perfusion of 200 μmol l^{-1} adenosine (P_{open} decreased from 5.8±1.1 to 2.3±1.2 %). The inclusion of 10 μmol l^{-1} 8-phenyltheophylline, an A_1 receptor blocker, prevented the adenosine- and anoxia-induced decrease in P_{open}. Mean single-channel current amplitude remained at approximately 2.7±0.23 pA under all experimental conditions. To determine whether a change in the membrane potential could be part of the mechanism by which P_{open} decreases, membrane and threshold potential were measured following each experiment. Membrane potential did not change significantly under any condition, ranging from −76.8 to −80.6 mV. Therefore, during anoxia, NMDA receptors cannot be regulated by Mg^{2+} in a manner dependent on membrane potential. Threshold potentials did decrease significantly following 60 min of anoxic or adenosine perfusion (control −33.3±1.9 mV, anoxia −28.4±1.5 mV, adenosine −23.4±2.8 mV). We conclude that anoxia modulates NMDA receptor activity and that adenosine plays a key role in mediating this change. This is the first direct measurement of ion channel activity in anoxic turtle brain and demonstrates that ion channel regulation is part of the naturally evolved anoxic defence mechanism of this species.

Key words: turtle, *Chrysemys picta*, anoxia, adenosine, *N*-methyl-D-aspartate, NMDA receptor.

Introduction

A large increase in intracellular calcium concentration ($[Ca^{2+}]_i$) is a major pathophysiological lesion leading to irreversible anoxic injury in the mammalian central nervous system. The excessive influx of Ca^{2+} is thought to be caused by an increase in the concentration of the excitatory amino acid glutamate during ischaemic or anoxic periods (Choi, 1992). 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 *in vivo* and in cell culture (for reviews, see Choi, 1992; Lobner and Lipton, 1993). The NMDA receptor subtype is also involved in long-term potentiation and long-term depression and may be involved in the mechanism underlying memory and learning (Bliss and Collingridge, 1993). The physiological significance of this receptor may be linked to its ability to transduce a presynaptic excitatory input to a second messenger response *via* Ca^{2+} entry. A receptor with such an important role in signal transduction is likely to be closely regulated *in vivo*. There is evidence that second messenger systems regulate neuronal excitability by phosphorylation of voltage- and ligand-gated ion channels, such as the NMDA receptor. The NMDA receptor has been shown to undergo phosphorylation by protein kinase A (Cerne *et al.* 1993), protein kinase C (Chen and Huang, 1992; Tingley *et al.* 1993) and tyrosine kinase (Wang and Salter, 1994). More recently, it has become necessary to consider the regulatory role of a 95 kDa postsynaptic density protein (PSD 95) that has been shown to bind to the NMDA receptor (Kornau *et al.* 1995). The NMDA receptor has been shown

to be dephosphorylated by the corresponding phosphatases: tyrosine phosphatase (Wang and Salter, 1994), protein phosphatases 1 and 2A (Wang *et al.* 1994; Tong *et al.* 1995) and Ca^{2+}-dependent phosphatase (Lieberman and Mody, 1994). It is becoming clear that a complex interplay of second messenger phosphorylation/dephosphorylation cycles regulates neuronal excitability and that these cycles could be involved in decreasing excitability in some hypoxia-tolerant neurones such as those from western painted turtle (*Chrysemys picta*).

In the brain of the western painted turtle, decreased neuronal excitability (termed 'spike arrest' by Sick *et al.* 1993) and the associated energy savings may be the key to its impressive anoxia-tolerance. Adenosine is an important modulator during the transition to anoxia in the turtle brain, and levels increase 12-fold during 2 h of anoxia (from approximately 2 to 20 μmol l^{-1}, Nilsson and Lutz, 1992). Antagonizing A_1 receptors during anoxia in isolated turtle cerebellum leads to a release of intracellular K^+ (Perez-Pinzon *et al.* 1993), the absence of cellular K^+ release being characteristic of anoxia-tolerance in the turtle brain. It has been shown that blood flow increases and decreases in the cerebral vasculature in concert with the appearance and disappearance of adenosine during anoxia, and these changes can be inhibited by A_1 receptor antagonists (Hylland *et al.* 1994). Recently, we demonstrated that adenosine reduced the NMDA-mediated rise in intracellular [Ca^{2+}] by 62 % in turtle cortex and that this effect was blocked by the A_1 receptor inhibitor 8-phenyltheophylline (Buck and Bickler, 1995). Furthermore, the decrease in intracellular Ca^{2+} accumulation resulting from adenosine application was similar in magnitude to the 58 % decrease in accumulation induced by anoxia. We concluded that during the transition to anoxia adenosine interacts with the A_1 receptor, decreasing NMDA receptor activity and neuronal excitability. Whether the decreased NMDA receptor activity was due to a direct second-messenger-mediated effect on the receptor or to membrane hyperpolarization and strengthening of the voltage-dependent Mg^{2+} block was unclear. The effect of adenosine in reducing neuronal excitability has previously been shown to be Mg^{2+}-dependent (Stone *et al.* 1990; de Mendonca and Ribeiro, 1993). However, using whole-cell attached-patch recordings of NMDA-elicited currents in rat hippocampal slices, de Mendonca *et al.* (1995) demonstrated that the NMDA current could be inhibited by adenosine application irrespective of the presence of Mg^{2+}.

In the present study, we characterized the NMDA receptor in turtle cortex, using cell-attached single-channel patch-clamp methods, and directly measured changes in receptor open probability in response to anoxia and adenosine application. Since a potential mechanism regulating the NMDA receptor is membrane hyperpolarization and strengthening of the Mg^{2+} block, the channel properties and the membrane potential were also measured in the presence and absence of Mg^{2+} in the recording electrode and during anoxia and adenosine application.

Materials and methods

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, summer and autumn 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 at 28 °C and a basking platform and lamp. Turtles were maintained on a 12 h:12 h light:dark 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 turtle cerebrospinal fluid at 3–5 °C (aCSF, in mmol l^{-1}: 97 NaCl, 26.5 $NaHCO_3$, 2.0 NaH_2PO_4, 2.6 KCl, 2.5 $CaCl_2$, 2.0 $MgCl_2$, 20 glucose and 10 Hepes, pH 7.4 at 20 °C). Individual cortical sheets (6–8 in total) were cut from larger cortical sheets as described by Blanton *et al.* (1989). All bathing and electrode filling solution osmolarities were measured with a vapour pressure osmometer (Wescor, model 5500, Logan, UT, USA) and adjusted to 290–300 mosmol l^{-1}.

Cortical sheets were supported by nylon mesh and held in place by a coil of platinum wire in a tissue-slice recording chamber (Fine Science Tools Inc., N. Vancouver, British Columbia). Cell-attached patch-clamp recordings were obtained from submerged and perfused cortical sheets (aCSF at 2–3 ml min^{-1}, 20 °C). The tissue-slice chamber was gravity-perfused from two glass 1 l intravenous bottles with glass intravenous drippers and thick-walled tubing of low gas permeability. Three-way stopcocks and an intravenous flow controller were used to switch between intravenous bottles and the control flow. The line volume from the three-way valve to the chamber was 1 ml, and the chamber volume was 1.3 ml.

For anoxic experiments, the treatment reservoir was gassed with 95 % N_2/5 % CO_2 and contained 0.5 mmol l^{-1} sodium cyanide to prevent oxygenation artefacts. Excised patch experiments were performed to determine the effects of cyanide on the NMDA receptor. Cyanide had no significant effect on the current amplitude or slope conductance of the channel (data not shown). The recording chamber was fitted with a cap and a gas line allowing the chamber head space to be gassed with 95 % N_2/5 % CO_2. A small hole was made in the cap for the recording electrode to pass through and to vent positive gas pressure. In sham experiments, the patch-clamp recording electrode was replaced with an oxygen electrode. When the perfusate and head space gas was switched from 95 % O_2/5 % CO_2 to 95 % N_2/5 % CO_2, the time required for aCSF P_{O_2} to drop below 1 mmHg (0.133 kPa) was 8 min.

Single-channel recordings were performed with fire-polished 6–10 MΩ electrodes containing (in mmol l^{-1}): NaCl, 115; CsCl, 5; $CaCl_2$, 2.5; EGTA, 10; Hepes acid, 10; glycine 0.001; NMDA, 0.01; pH 7.4. Cell-attached 5–20 GΩ seals were obtained using a blind technique (Blanton *et al.* 1989). Briefly, an electrode was placed close to the ventricular surface of the

cortical sheet, advanced in 0.5 μm steps with slight positive pressure, and a 2 mV pulse was applied. A slow sustained increase in resistance indicated that the electrode tip was pressing against the glial cell layer that forms the outer cell layer of the cortex (Ulinski, 1990). The electrode was then advanced further (approximately 2 μm) to break through the outer cell layer, re-establishing the original electrode resistance. A sharp decrease in the square-wave pulse signalled that the tip was close to a neurone. At this time, pressure was removed and a seal formed spontaneously or slight suction was applied to initiate sealing. NMDA channels were identified from their current/voltage relationship.

Single-channel data were collected using an Axopatch-1D patch-clamp amplifier, CV-4 headstage and TL-1 DMA interface (Axon Instruments, Burlingame, CA, USA). Data were low-pass-filtered at 1 kHz (four-pole Bessel, −3 dB) and stored on tape using a Vetter Instruments data recorder (model 420; A. R. Vetter Co. Rebersburg, PA, USA). Data were digitized off-line at 20 kHz and analyzed *via* computer using PClamp software (version 6, Axon Instruments, Burlingame, CA, USA). When Mg^{2+} was included in the recording electrode solution, resulting in characteristic NMDA open-channel flickering behaviour, the PClamp analysis software was adjusted to detect the short-duration open events.

To determine whether NMDA receptors are modulated by adenosine receptor stimulation, recordings were obtained from tissue perfused with 200 μmol l^{-1} adenosine. In a separate experiment, brain sheets were perfused for 60 min with 200 μmol l^{-1} adenosine and 10 μmol l^{-1} 8-phenyltheophylline (8-PT, an adenosine receptor blocker that is 10 times more specific for A_1 than for A_2 receptors, Rudolphi *et al.* 1992) to block any adenosine-mediated effects and to test for a possible second messenger pathway. To determine whether the anoxic response is regulated by A_1 receptor stimulation, anoxic perfusions including 10 μmol l^{-1} 8-PT were also performed. Tissue was preincubated for 20 min with 10 μmol l^{-1} 8-PT before adenosine or anoxic perfusion experiments. In all experiments, data were collected at 10 min intervals throughout the 60 min treatment perfusion. Data were acquired for approximately 60 s at each 10 min time point.

Intracellular recordings were made using the whole-cell patch-clamp method immediately following each of the single-channel recording experiments described above. Therefore, the electrode solution contained the same solution as in the single-channel experiments. To break into a cell, the recording electrode potential was set at −70 mV and a sharp pulse of suction was applied. Once a whole-cell attached-patch configuration had been established, capacitance transients were removed using the capacitance compensation circuit of the Axo-patch 1D, and access resistance was measured (ranging from 100 to 200 MΩ). From the voltage-clamp configuration, the Axo-patch 1D amplifier was switched to the zero-current position and the membrane potential was read off the main meter. The patch was allowed to stabilize for 2 min before resting membrane potential was determined. To measure threshold potential, the cell was voltage-clamped at −70 mV and the potential was made positive until an action potential was observed on the oscilloscope. The entire process from obtaining a whole-cell patch-clamp configuration to measurement of threshold potential required less than 8 min.

Analysis of variance (ANOVA) followed by a *post-hoc* multiple-comparison test (Dunnett's) was used to compare control *versus* treatment open probabilities and current amplitudes. An ANOVA followed by a *post-hoc* multiple comparison test (Student–Neuman–Keuls) was used to compare resting membrane potential control *versus* treatment, and a similar test was used for the control threshold *versus* treatment threshold. Values are reported as means ± S.E.M., and $P<0.05$ was considered statistically significant.

Results

Four diagnostic features of the NMDA receptor were used to verify NMDA receptor currents. (1) 200 μmol l^{-1} Mg^{2+} was added to the recording pipette solution, resulting in characteristic channel-flickering open events typical of NMDA receptors in the presence of Mg^{2+} (Fig. 1A). (2) A cell-attached current–voltage (*I–V*) relationship (relative to cellular membrane potential) was obtained (Fig. 1B) both in the presence and in the absence of Mg^{2+} in the recording electrode (Fig. 1C). The presence of Mg^{2+} effectively blocked inward currents at more positive electrode holding potentials, demonstrating an inward rectification that is characteristic of the receptor (Fig. 1C). (3) The slope conductance of the receptor in the absence of Mg^{2+} was 46 pS (Fig. 1C), a value similar to the mammalian value of 50 pS (Nowak *et al.* 1984) and identical to that of hatchling turtle NMDA receptors (46 pS; Blanton and Kriegstein, 1992). (4) Finally, the transmembrane potential was close to zero. Since the mean resting membrane potential is −80.6 mV (see Fig. 4), a current reversal should be observed at an electrode holding potential of approximately −80 mV. This value is in agreement with the data in Fig. 1B,C, showing a reversal potential of approximately −80 mV.

Typical open-channel amplitude and P_{open} histograms from the five different experimental conditions are shown in Fig. 2: control (95 % O_2/5 % CO_2), 200 μmol l^{-1} adenosine, 200 μmol l^{-1} adenosine plus 10 μmol l^{-1} 8-PT, anoxia (0.5 mmol l^{-1} cyanide and 95 % N_2/5 % CO_2) and anoxia plus 10 μmol l^{-1} 8-PT. Fig. 2A,B shows an open-channel amplitude histogram and a P_{open} histogram compiled from data acquired following 60 min of normoxic perfusion. A Marquardt least-squares analysis (Marquardt LSQ), used to fit a curve to the data, determined the open-channel amplitude to be 2.48 pA, with an *r* value of 0.89 and a goodness-to-fit value of 2. The control normoxic P_{open} plot shows a random distribution of open probabilities throughout the recording period with a mean P_{open} of 0.039 (or 3.9 %). Fig. 2C,D shows the results of a 60 min normoxic perfusion with 200 μmol l^{-1} adenosine. The channel amplitude of 2.31 pA, with an *r* value of 0.81 and a goodness-to-fit value of 2.1 (Fig. 2C), did not differ from the control value; however, the open probability decreased to

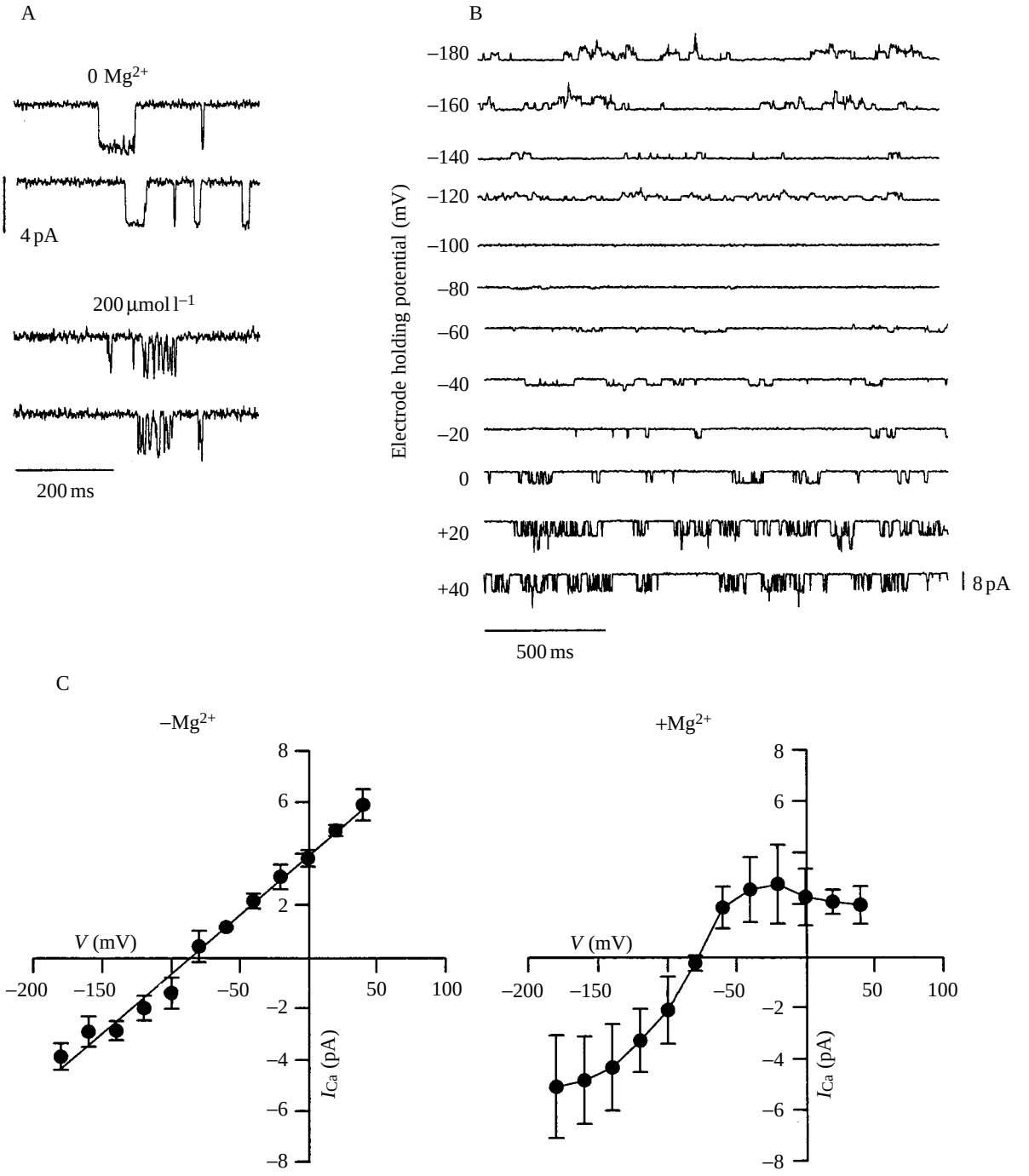

Fig. 1. Representative raw data recordings and *I–V* relationships characterizing the NMDA receptor. Single-channel recordings of an NMDA receptor in the absence (A, top traces) and in the presence (A, bottom traces) of 200 µmol l^{-1} Mg^{2+} in the recording pipette. In the presence of Mg^{2+}, the channel openings show typical flickering open-channel behaviour. (B) A typical series of raw data recordings used to generate an NMDA receptor *I–V* relationship. At least two channels are present in this patch as indicated by the double-amplitude events. When the recording electrode holding potential is driven more positive (–60 to +40 mV), typical inward currents are observed; when it is driven more negative (–100 to –180 mV), outward currents are observed. A reversal potential typical of the NMDA receptor is observed at approximately –80 mV (cellular membrane potential being approximately –80 mV, giving a transmembrane potential of 0 mV). (C) A summary of eight separate NMDA receptor *I–V* relationships in the absence (left) and eight experiments in the presence (right) of 200 µmol l^{-1} Mg^{2+}. All data are filtered at 1 kHz with a headstage gain of 200 mV pA^{-1} and at 20 °C. Data in A were obtained with an electrode holding potential of –40 mV. Values are means ± S.E.M. The slope conductance in the absence of Mg^{2+} was 46 pS.

0.011 (or 1.1 %, Fig. 2D). In Fig. 2E,F, data from a 60 min normoxic perfusion experiment with 200 µmol l^{-1} adenosine and 8-PT are shown. The current amplitude did not change over this time course, resulting in a current amplitude of

2.53 pA at 60 min (*r* value of 0.88 and a goodness-to-fit value of 2.1). P_{open} is also unchanged over the 60 min time course by this treatment (0.04 or 4 %). Fig. 2G,H shows data from a 60 min anoxic experiment. The open-amplitude data are fitted with a Marquardt LSQ analysis as above, and the open amplitude is 2.71 pA (*r* value of 0.76 and goodness-to-fit value of 2.1). P_{open} decreased significantly to 0.023 following 60 min of anoxia (2.3 %, Fig. 2H). Fig. 2I,J shows data from a 60 min anoxic experiment including 10 μmol l^{-1} 8-PT. The amplitude data are fitted as above, resulting in an open-channel amplitude of 1.8 pA (*r* value of 0.93 and goodness-to-fit value of 2.1), a value no different from the normoxic, adenosine, adenosine plus 8-PT and anoxia plus 8-PT values. Following 60 min of anoxic perfusion with 8-PT, P_{open} did not decrease significantly (0.03 or 3.0 %, Fig. 2J). In the amplitude histograms shown in Fig. 2C,E,G, there is an indication of a possible lower-amplitude open-channel event following 60 min of perfusion with adenosine or with adenosine plus 8-PT or during anoxia. However, analysis of the Marquardt LSQ curve fit using the compare function in Pstat indicated that these events were not significant.

Fig. 3 gives summary data of the above time course experiments. Over the control normoxic 60 min time course, there was no significant change in open probability, with values of 5.4±1.3 to 5.6±1.0 % at 60 min. However, adenosine application caused P_{open} to decrease from the control value of 5.8±1.1 % to a value of 2.3±1.2 % at 60 min. This represents a significant (P<0.05) 57 % decrease in P_{open} due to adenosine application over the 60 min time course (Fig. 3A). When a lower concentration of adenosine (100 μmol l^{-1}) was used, the onset of the observed decrease in P_{open} was delayed or occasionally was absent. The decrease in P_{open} due to the application of 200 μmol l^{-1} adenosine is rapid, with a significant decrease of 51 % following 10 min of adenosine application. Anoxic perfusion also resulted in a significant decrease in P_{open} (Fig. 3B); however, this was not as rapid as the decrease caused by adenosine exposure. Anoxic exposure resulted in a 65 % decrease in P_{open} from a control value of 6.8±1.6 to a value of 2.4±0.8 % after 60 min (Fig. 3B). The addition of 200 μmol l^{-1} Mg^{2+} to the recording electrode had no effect on the anoxia-induced decrease in P_{open} (Fig. 3B). The decrease in P_{open} due to adenosine was blocked by the presence of 10 μmol l^{-1} 8-PT (Fig. 3C). The initial P_{open} value of 3.2±0.6 % was not significantly different from the value of 3.6±0.8 % (Fig. 3B) measured following a 40 min adenosine plus 8-PT perfusion. In these experiments, late summer animals were used, and control P_{open} values were significantly lower than values from spring and early summer animals. The reason for this is not known, but experiments with these animals showed a similar significant reduction of P_{open} in the presence of adenosine to that found in the spring and summer animals (data not shown). During these experiments, patches routinely became unstable after 40 min and broke down. From the recordings that were obtained following this time point, P_{open} did not decrease. The decrease in P_{open} due to anoxia was also blocked by the presence of 10 μmol l^{-1} 8-PT (Fig. 3C). The control value of 3.6±0.69 % is not significantly different from the 60 min value of 3.8±0.77 % (Fig. 3C, late summer animals again resulted in lower starting values). In all cases, changes in P_{open} reflect a real change in mean open time of the NMDA receptor which, under control conditions, is 5.9±0.39 ms.

Under control conditions, mean current amplitude at time zero is 2.7±0.13 pA, which is not different from the value of 2.57±0.18 pA at 60 min (P>0.05, Fig. 3D). Perfusion with adenosine, adenosine plus 8-PT or exposure to anoxia ($NaCN/N_2$) failed to produce a statistically significant difference from control values over the 60 min time course (Fig. 3D). Additionally, the NMDA receptor slope conductance of 46 pS was not affected by adenosine or anoxic perfusion (data not shown).

Following the control, adenosine and anoxic experiments, resting membrane potential and action potential thresholds were measured (Fig. 4). There were no significant differences in resting membrane potential between the three 60 min treatments: control −80.6±2.47 mV, anoxia −76.8±1.23 mV, adenosine −78.0±1.36 mV. However, threshold potentials were significantly affected. The control threshold potential decreased from −33.3±1.85 mV to −28.4±1.52 mV with anoxic perfusion (a 15 % reduction) and to −23.4±2.79 mV with adenosine perfusion (a 30 % reduction). In addition, the resting membrane potential remained unchanged from the moment of first measurement, approximately 10 s after achieving the whole-cell patch configuration, to the measurement made 60 min after first recording in this configuration. This indicates that the recording solution used for this measurement did not significantly alter membrane properties.

Discussion

Our data show that, in turtle cerebral cortex, NMDA receptors undergo a large reduction in open probability with the onset of anoxia. This may contribute to preventing intracellular [Ca^{2+}] from rising to neurotoxic levels and leading to cell death from hypoxic exposure. This response could be blocked with the A_1 receptor antagonist 8-PT and could be reproduced by adenosine perfusion during normoxia, an effect that was also sensitive to blockade by 8-PT. The absence of Mg^{2+} from the recording electrode did not alter the decrease in open probability observed during anoxia. Furthermore, resting membrane potential did not change significantly during anoxic exposure or adenosine application, but there was a significant positive shift in action potential threshold during these exposures. The lack of a change in membrane potential and the insensitivity to Mg^{2+} rule out the possibility of a strengthened Mg^{2+} blockade of the NMDA receptor as a mechanism for regulating neuronal excitability during hypoxia. Taken together, these results point to a mechanism for the control of neuronal excitability and protection from hypoxic/anoxic damage that includes the following: accumulation of extracellular adenosine, A_1 receptor

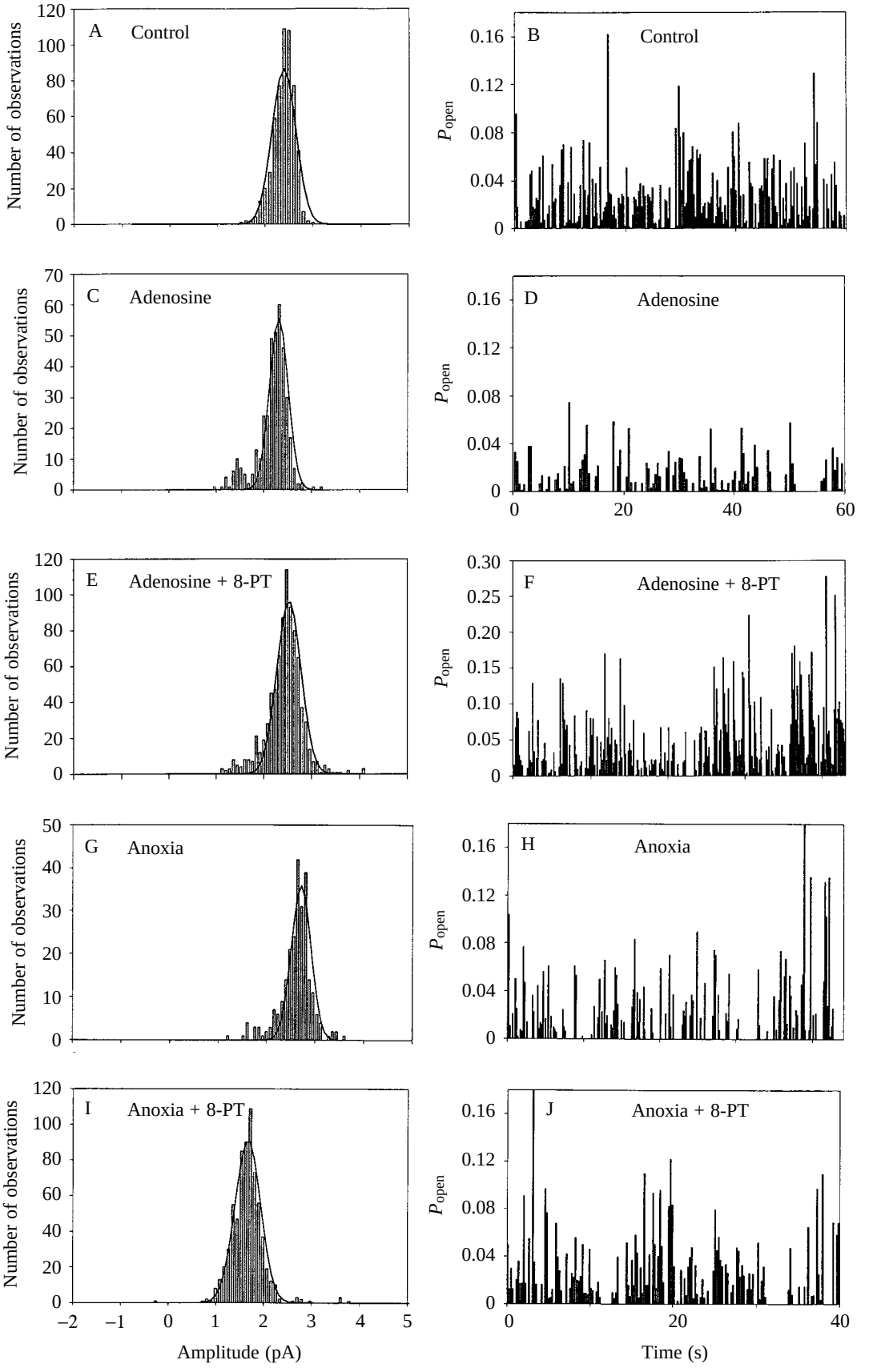
A Control
B Control
C Adenosine
D Adenosine
E Adenosine + 8-PT
F Adenosine + 8-PT
G Anoxia
H Anoxia
I Anoxia + 8-PT
J Anoxia + 8-PT
Number of observations
P_{open}
Amplitude (pA)
Time (s)

Fig. 2

Fig. 2. Representative NMDA receptor open-channel amplitude (left) and open probability (P_{open}, right) histograms obtained from attached-patch recordings following 60 min perfusion experiments. (A,B) Control normoxic data (95 % O_2/5 % CO_2); (C,D) data from a patch that had been perfused with 200 μmol l^{-1} adenosine; (E,F) data from a normoxic perfusion experiment with adenosine (200 μmol l^{-1}) and 10 μmol l^{-1} 8-phenyltheophylline (8-PT); (G,H) data from a patch perfused with anoxic artificial cerebrospinal fluid (95 % N_2/5 % CO_2 + 0.5 mmol l^{-1} NaCN); (I,J) data from an anoxic experiment including 10 μmol l^{-1} 8-PT. All open-channel amplitude data were fitted using a Marquardt least-squares analysis with a goodness-to-fit value of approximately 2.0 and r values of 0.89 (A), 0.81 (C), 0.88 (E), 0.76 (G) and 0.93 (I). The open-probability histograms show a random distribution of open probabilities throughout the recording period. Throughout the 60 min period of normoxic perfusion, open-channel amplitude and P_{open} did not change. However, adenosine and anoxic perfusion significantly reduced P_{open} but not receptor amplitude (see Results).

stimulation, and activation of a second messenger pathway regulating NMDA receptor open probability.

The blind patch-clamp method of Blanton *et al.* (1989) employed here resulted in a very high attached-patch recording success rate. Patches with active NMDA receptors were obtained approximately 90 % of the time, and half were high-quality gigaohm seals suitable for patch-clamp recording. A critical factor in this success was the use of the ventricular side of the cerebral cortex. This surface has a densely staining region of neurones (Ulinski, 1990) just below the ependymal layer that is easily accessible to patch-clamp electrodes. The NMDA receptor recordings obtained are very similar to those obtained from hatchling turtle neurones by Blanton and Kriegstein (1992). In both cases, a slope conductance of 46 pS was obtained, which is similar to the value of 50 pS reported for NMDA receptors from rat cortex (Nowak *et al.* 1984). It is important to point out that the *I–V* relationships shown in

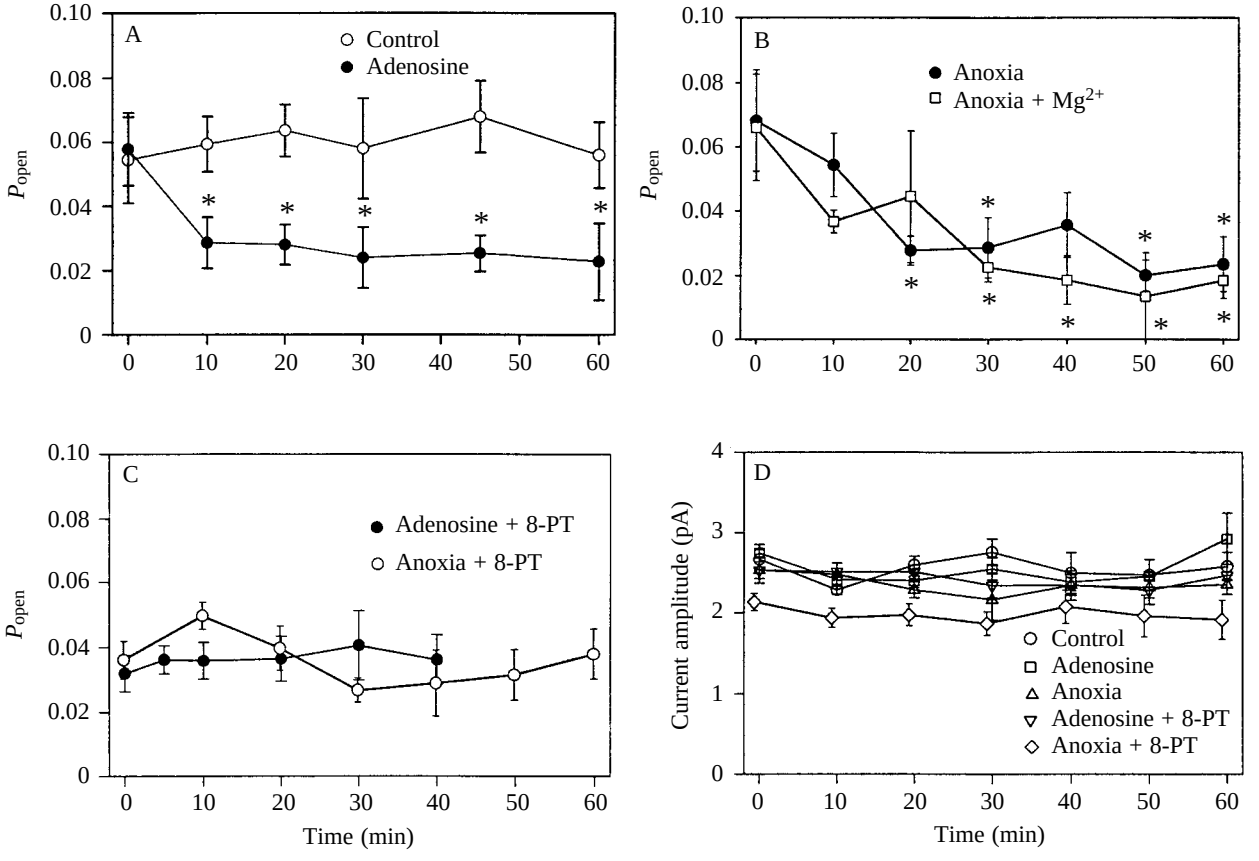

Fig. 3. The effect of adenosine, adenosine plus 8-phenyltheophylline (8-PT), anoxia (95 % N_2/5 % CO_2 + 0.5 mmol l^{-1} NaCN) and anoxia plus 8-PT on NMDA receptor open probability (P_{open}). Control normoxic (95 % O_2/5 % CO_2) perfusion does not result in a significant decrease in P_{open} over the 60 min time course of the experiment (A). Inclusion of 200 μmol l^{-1} adenosine in the normoxic perfusion significantly reduces P_{open} within 10 min, a reduction that is maintained over the 60 min time course (A). A significant decrease in P_{open} is observed during a 60 min anoxic perfusion both in the absence and in the presence of 200 μmol l^{-1} Mg^{2+} (B). Pre-incubation of the tissue or inclusion of 10 μmol l^{-1} 8-PT during anoxic or normoxic adenosine perfusion prevents both the anoxic and the adenosine-induced decrease in P_{open} (C). (D) Channel open amplitudes under all the conditions described above. There are no significant changes in channel open amplitude under any of these conditions. Asterisks indicate points that are significantly different from control values when the data are analyzed using analysis of variance followed by the Student–Neuman–Keuls *post-hoc* test, P<0.05. Open-probability data are the mean ± S.E.M. of 5–9 separate experiments. Receptor open-amplitude data are the mean ± S.E.M. of 4–6 individuals experiments.

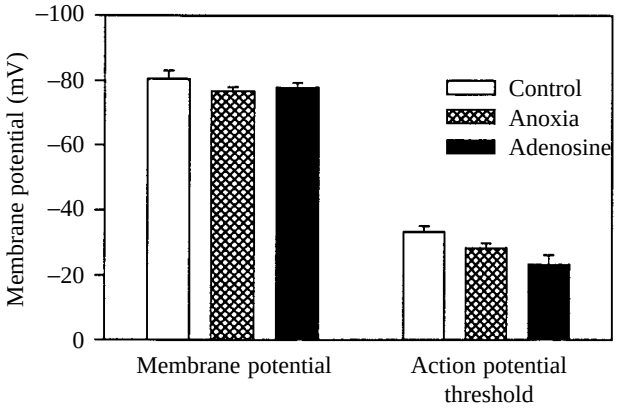

Fig. 4. The effect of anoxia and adenosine on resting membrane potential and threshold potential. Following 60 min of normoxic perfusion, resting membrane potential and threshold values did not change (not shown). Neither anoxic perfusion nor the inclusion of 200 μmol l^{-1} adenosine in the normoxic perfusion affected resting membrane potential significantly. Threshold potential was significantly reduced from the normoxic control value by anoxia (15 %) and by adenosine perfusion (30 %). Asterisks indicate points that are significantly different from control values. Data were analyzed using analysis of variance followed by the Student–Neuman–Keuls *post-hoc* test, $P<0.05$ considered statistically significant. Points represent the mean + S.E.M. of 15–17 individual experiments.

Fig. 1C are whole-cell attached recordings and are plotted with reference to the resting membrane potential of the neurone of −80 mV. Interestingly, knowing that the NMDA receptor reversal potential is close to zero, one can estimate the cell membrane potential from the intersection of the regression line and the *x*-axis (Fig. 1C). In this case, it is estimated to be −85.0 mV, which agrees well with the empirical measurement of −80.6 mV shown in Fig. 4. Further evidence that we are recording from NMDA receptors comes from the effect of Mg^{2+} on receptor gating properties and the inward rectification by Mg^{2+} at more positive holding potentials, as observed for the hatchling turtle and mammalian receptor (Blanton and Kriegstein, 1992; Nowak *et al.* 1984). When NMDA was excluded from the electrode filling solution, NMDA-like channel activity was never observed.

The use of high concentrations of adenosine in these experiments is reasonable. As mentioned above, lower concentrations resulted in variable results and required long exposure times to elicit a response. Turtle brain cortical sheets were used in this study rather than tissue slices; sheets maintain an intact ependymal layer, which has been suggested to be a barrier to adenosine diffusion (Rudolphi *et al.* 1992). A barrier to adenosine diffusion from tissue to cerebrospinal fluid, together with a large Na^+-driven adenosine uptake into glial and vascular endothelia cells and erythrocytes, suggests that microdialysis methods underestimate true interstitial adenosine concentrations. Additionally, mammalian cerebrocortex and hippocampus have been reported to have a higher density of A_1 than of A_2 receptors (Rudolphi *et al.* 1992; Ramkumar *et al.* 1988). Taken together with the 10-fold greater specificity of 8-PT for A_1 over A_2 receptors, we feel justified in the use of high pharmacological adenosine concentrations and selective inhibition of A_1 receptors.

Previously, we demonstrated that anoxia and adenosine decrease the degree to which Ca^{2+} accumulates in Fura-2-loaded brain sheets stimulated by NMDA application (Buck and Bickler, 1995). Using fluorometric methods to measure NMDA-mediated changes in intracellular [Ca^{2+}] during anoxia, we were unable to distinguish unequivocally between direct NMDA receptor regulation or other mechanisms regulating intracellular Ca^{2+} pools. On the basis of the present evidence, we can now conclude that adenosine and anoxia produce an effect on NMDA receptor modulation since single-channel properties were measured directly. The precise mechanism by which a decrease in open probability is achieved during anoxia or adenosine application is uncertain. However, it is known that the adenosine A_1 receptor is a G-protein effector and, in turn, can modulate the activity of phospholipase C (PLC) and the inositol trisphosphate (InsP_3) cascade (for a review, see Rudolphi *et al.* 1992). Adenylate cyclase activation *via* the G-protein pathway would result in increased intracellular cyclic AMP levels. Alternatively, the production of InsP_3 *via* PLC would result in increased intracellular Ca^{2+} levels. Both of these pathways are capable of modulating protein kinases and phosphatases, which are known effectors of NMDA receptors (as discussed above). How the intracellular second messenger traffic is regulated in a way that selectively activates the appropriate protein kinases and phosphatases is uncertain.

Recently, de Mendonca *et al.* (1995), using whole-cell patch-clamp methods, demonstrated that the adenosine analogue 2-chloroadenosine reversibly inhibited NMDA- mediated currents and that this effect was independent of Mg^{2+} in the bathing solution. These results are consistent with our findings regarding both the effect on the NMDA receptor and the lack of effect of Mg^{2+}. It is interesting that we did identify a significant change in threshold potential during anoxia or adenosine application. This is consistent with the observed shift to more depolarized threshold potentials in anoxic turtle cerebellar Purkinje cells (Perez-Pinzon *et al.* 1990) and the down-regulation of Na^+ channel density in turtle cerebellum during anoxia (Perez-Pinzon *et al.* 1992). The positive shift in threshold potential, 5 mV more positive during anoxia and 10 mV more positive with adenosine, that we observe is intermediate between the 14 mV shift due to anoxia observed in turtle cerebellar Purkinje cells (Perez *et al.* 1990) and the 3–5 mV positive shift observed in neurones of rat hippocampal slices (Fujiwara *et al.* 1987). The resting membrane potentials (RMPs) and firing threshold potentials reported here fall within the typical range reported for turtle neurones (Doll *et al.* 1991, RMP −74.0 mV, threshold −36.0 mV; Perez *et al.* 1992, RMP −55 to −85 mV).

In summary, we have demonstrated a functional decrease in NMDA receptor open probability with the onset of anoxia or with adenosine application. This response was insensitive to the presence of Mg^{2+}, and the resting membrane potential remained constant throughout all experiments. This evidence rules out mechanisms involving strengthening of the voltage-dependent

Mg^{2+} block of the receptor. The adenosine- and anoxia-evoked response was sensitive to the A_1 receptor antagonist 8-PT, suggesting that an endogenous second messenger pathway regulates the NMDA receptor. In addition, the action potential threshold was decreased both by anoxia and by adenosine application, suggesting regulation of voltage-gated Na^+ channels with the onset of anoxia, possibly by phosphorylation/dephosphorylation of the channel (Cukierman, 1996). We conclude that the anoxia-induced decrease in P_{open} of the NMDA receptor is mediated *via* a second messenger pathway, probably involving adenosine receptor stimulation. This mechanism is part of a larger underlying mechanism that regulates neuronal excitability and forms the basis of the natural anoxia tolerance of the western painted turtle. This is the first direct measurement of ion channel activity in anoxic turtle brain and demonstrates that ion channel regulation is part of the naturally evolved anoxic defence mechanism of this species.

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