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Calcium and protein phosphatase 1/2A attenuate *N*-methyl-D-aspartate receptor activity in the anoxic turtle cortex

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Abstract

Excitotoxic cell death (ECD) is characteristic of mammalian brain following min of anoxia, but is not observed in the western painted turtle following days to months without oxygen. A key event in ECD is a massive increase in intracellular Ca^{2+} by over-stimulation of *N*-methyl-D-aspartate receptors (NMDARs). The turtle's anoxia tolerance may involve the prevention of ECD by attenuating NMDAR-induced Ca^{2+} influx. The goal of this study was to determine if protein phosphatases (PPs) and intracellular calcium mediate reductions in turtle cortical neuron whole-cell NMDAR currents during anoxia, thereby preventing ECD. Whole-cell NMDAR currents did not change during 80 min of normoxia, but decreased 56% during 40 min of anoxia. Okadaic acid and calyculin A, inhibitors of serine/threonine PP1 and PP2A, potentiated NMDAR currents during normoxia and prevented anoxia-mediated attenuation of NMDAR currents. Decreases in NMDAR activity during anoxia were also abolished by inclusion of the Ca^{2+} chelator — BAPTA and the calmodulin inhibitor — calmidazolium. However, cypermethrin, an inhibitor of the Ca^{2+}/calmodulin-dependent PP2B (calcineurin), abolished the anoxic decrease in NMDAR activity at 20, but not 40 min suggesting that this phosphatase might play an early role in attenuating NMDAR activity during anoxia. Our results show that PPs, Ca^{2+} and calmodulin play an important role in decreasing NMDAR activity during anoxia in the turtle cortex. We offer a novel mechanism describing this attenuation in which PP1 and 2A dephosphorylate the NMDAR (NR1 subunit) followed by calmodulin binding, a subsequent dissociation of α-actinin-2 from the NR1 subunit, and a decrease in NMDAR activity.

Keywords: Protein phosphatase; Calcium; NMDA receptors; Turtle; Cortex; Anoxia

1. Introduction

While most vertebrates suffer brain damage within min of anoxia, the Western painted turtle, *Chrysemys picta bellii*, can survive anoxia for days to months without any apparent brain injury (Ultsch and Jackson, 1982). The key to this anoxia tolerance is their ability to maintain ATP supply by reducing ATP demand in the absence of oxygen (Lutz, 1992; Buck et al., 1993; Hochachka et al., 1996; Hochachka and Lutz, 2001; Jackson, 2000). One way this is achieved is by down-regulating energy demanding ion movement across the cell membrane via the process of "channel arrest" (Hochachka, 1986).

Of all mammalian tissues, the brain is the most energy demanding, utilizing 20% of the total body oxygen consumption. Furthermore, 50–60% of the total ATP utilized by normoxic brain is used by the Na^+K^+ATPase to maintain ionic gradients (Hansen, 1985; Erecinska and Silver, 1989). The "Channel arrest" hypothesis predicts that membranes become less permeable to ions, thereby lowering Na^+/K^+ATPase activity and decreasing cellular ATP demand. A plausible mechanism to reduce ATP demand is to attenuate synaptic activity by down-regulating the activity of the *N*-methyl-D-aspartate receptor (NMDAR) (Buck and Bickler, 1995, 1998; Bickler et al., 2000); an ionotropic glutamate receptor. This high-flux ligand and voltage-gated receptor is highly permeable to Ca^{2+} and underlies fast synaptic transmission in the vertebrate brain. It also plays an important role in learning and memory, vision and cognitive

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function (Collingridge et al., 1983; Miller et al., 1992; Tingley et al., 1993; Wang and Salter, 1994; de Mendonca et al., 1995; Zhang et al., 1998; Gingrich et al., 2000). The uncontrolled activation of this receptor and subsequent influx of Ca^{2+} leads to an ionic imbalance that is a hallmark of excitotoxic cell death (ECD) in mammals (Choi, 1995). In contrast, over-stimulation of NMDAR activity and subsequent increase in intracellular Ca^{2+} is not seen in the anoxic turtle brain. Therefore, it is possible that turtle neurons prevent ECD by reducing NMDAR activity as a means for anoxic survival, which could decrease neuronal excitability and energy requirements.

The mammalian NMDAR is modulated by histamine (Bekkers, 1993), arachidonic acid (Miller et al., 1992), calmodulin (Hisatsune et al., 1997), ethanol (Wirkner et al., 1999), pH (Tang et al., 1990), adenosine (de Mendonca et al., 1995) and cytoskeletal elements (Rosenmund and Westbrook, 1993). Modulation of NMDARs by kinases and protein phosphatases (PPs) appear to be particularly important. NMDAR activity is attenuated by PP1 and 2A (Wang et al., 1994) and the Ca^{2+}/calmodulin-dependent phosphatase PP2B (calcineurin) (Mulkey et al., 1994; Wang and Kelly, 1996, 1997). In the turtle, Bickler et al., (2000) found evidence of PP1 and 2A-mediated decreases in NMDAR open probability during anoxia using single-channel recordings and calcium fluorometry. In the presence of okadaic acid and calyculin A, inhibitors of PP1 and 2A, they reported that anoxia led to a marked elevation in the NMDA-induced change in $[Ca^{2+}]_i$. We have also monitored NMDAR currents directly (Shin and Buck, 2003) demonstrating that NMDAR currents decrease by 65% during 40 min of anoxia; however, the mechanism underlying this attenuation was not examined.

The aim of this study was to determine whether serine/threonine protein phosphatases modulate NMDAR whole-cell currents during anoxia in turtle cortical neurons. The protein phosphatase inhibitors, calyculin A and okadaic acid, were used to examine the role of PP1 and 2A. The role of Ca^{2+}, as an intracellular signaling molecule during anoxia, was also investigated by employing the Ca^{2+} chelator — BAPTA, and cypermethrin, an inhibitor of the Ca^{2+}/calmodulin-dependent PP2B (calcinuerin). Lastly, the inhibitor calmidazolium was used to determine whether calmodulin is involved in the anoxia-mediated attenuation in NMDAR activity in turtle brain since Ca^{2+} and calmodulin are co-involved in many signaling cascades.

2. Materials and methods

2.1. Animals

This study conforms to relevant guidelines for the care of experimental animals and was approved by the University of Toronto Animal Care committee. Spring, summer, and autumn adult female turtles (*Chrysemys picta bellii*, Schneider) weighing 600–750 g were obtained from Lemberger Inc. (Oshkosh, WI, USA). Animals were housed in a large aquarium equipped with a flow-through dechlorinated freshwater system at 17°C, a basking platform, and a lamp. Turtles were maintained on a 12L:12D photoperiod and given continuous access to food and water.

2.2. Dissection and whole-cell patch-clamp protocol

Basic methods for dissection and whole-cell patch-clamp are described in Shin and Buck (2003). Briefly, cortical 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 (aCSF at 3–5 °C; in mM): 107 NaCl, 2.6 KCl, 1.2 $CaCl_2$, 1 $MgCl_2$, 2 NaH_2PO_4, 26.5 $NaHCO_3$, 10 Glucose, 5 Imidazole (pH 7.4 at room temperature). Six cortical sheets 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, UT, USA) and were between 280–290 mOsm.

Cortical sheets were placed on a coverslip in a perfusion chamber system (RC-26 open bath with a P1 chamber platform, Warner Instruments, CT, USA). The tissue slice chamber was gravity perfused from a 1 L glass bottle with an intravenous (IV) dripper attached. During normoxic experiments, aCSF was gassed with 95% O_2/5% CO_2. A second 1 L glass bottle with an attached IV dripper was used to perfuse the chamber with aCSF bubbled with 95% N_2/5% CO_2. A fast-step perfusion system (VC-6 model perfusion valve controller and SF-77B fast-step perfusion system, Warner Instruments, CT, USA) was used to deliver the following: 1 μM tetrodotoxin (TTX), 300 μM *N*-methyl-D-aspartate (NMDA), 500 nM Okadaic acid, 20 nM Calyculin A, 400 pM Cypermethrin or 100 nM Calmidazolium onto the slice, depending upon the experiment.

To achieve anoxia, perfusion tubes from IV bottles were double jacketed, and the outer jacket gassed with 95% N_2/5% CO_2. Anoxic aCSF was constantly bubbled with 95% N_2/5% CO_2. A plastic cover with a hole for the electrode was placed over the perfusion chamber and the space between the fluid surface and cover was gently gassed with 95% N_2/5% CO_2. The aCSF was gassed with N_2/CO_2 for 30 min and then 2 mM Na_2SO_3 (sodium sulfite) was added to reduce any remaining oxygen. An OM2000 oxygen and temperature meter (Cameron Instruments, TX, USA) with an E5046 PO_2 electrode was used to measure PO_2 in the perfusion chamber. The partial pressure of oxygen (PO_2) in the recording chamber decreased from approximately 610 mm Hg PO_2 (hyperoxia) to 0.5 mm Hg PO_2 (anoxia) within 5 min, which is the limit of detection for the PO_2 electrode and not different from that in the N_2/CO_2 bubbled reservoir. PO_2 levels were maintained at this low level for the entire 40 min of anoxia (data not shown).

Whole-cell recordings were performed using the whole-cell voltage-clamp method with 5–8 MΩ electrodes containing the following (in mM): 8 NaCl, 0.0001 $CaCl_2$, 10 NaHepes, 20 KCl, 110 Kgluconate, 1 $MgCl_2$, 0.3 NaGTP, 2 NaATP (pH 7.4, adjusted with methanesulfonic acid). Cell-attached 5–20 GΩ seals were obtained using the blind-patch technique of Blanton et al., (1989). To break into the cell, the recording electrode potential was set at −60 mV, and a sharp pulse of suction was applied to establish the whole-cell configuration. In the voltage-clamp configuration, an Axopatch 1D amplifier was switched to the zero-current position and the resting membrane potential was read from the main meter. The cell-attached patch was allowed to stabilize for 2–5 min before the resting membrane potential was determined (ranging from −55 to −85 mV). Data were collected using an Axopatch-1D amplifier, a CV-4 headstage, and a TL-1 DMA interface (Axon Instruments) and then digitized and stored on computer using Clampex 6 software (Axon Instruments, CA, USA).

2.3. Normoxic and anoxic NMDAR currents

Normoxic experiments consisted of an O_2/CO_2 aCSF perfusion. Prior to each NMDA application, TTX was delivered onto the patched neuron for 5 min to prevent action potentials and vesicle-mediated release of glutamate. This procedure was used throughout all experiments. The initial peak NMDAR current was set to 100% and subsequent NMDA currents normalized to this value. For anoxic experiments, NMDA was initially applied to cortical sheets in normoxic aCSF, and the evoked whole-cell current was set to 100%. Cortical sheets were then exposed to anoxic aCSF for 40 min followed by reperfusion with oxygenated aCSF for another 40 min. NMDA evoked currents were monitored at 0, 20, 40, 60 and 80 min.

2.4. Protein phosphatase inhibitor experiments

Prior to whole-cell recordings for both normoxia and anoxia, cortical slices were pre-incubated with either

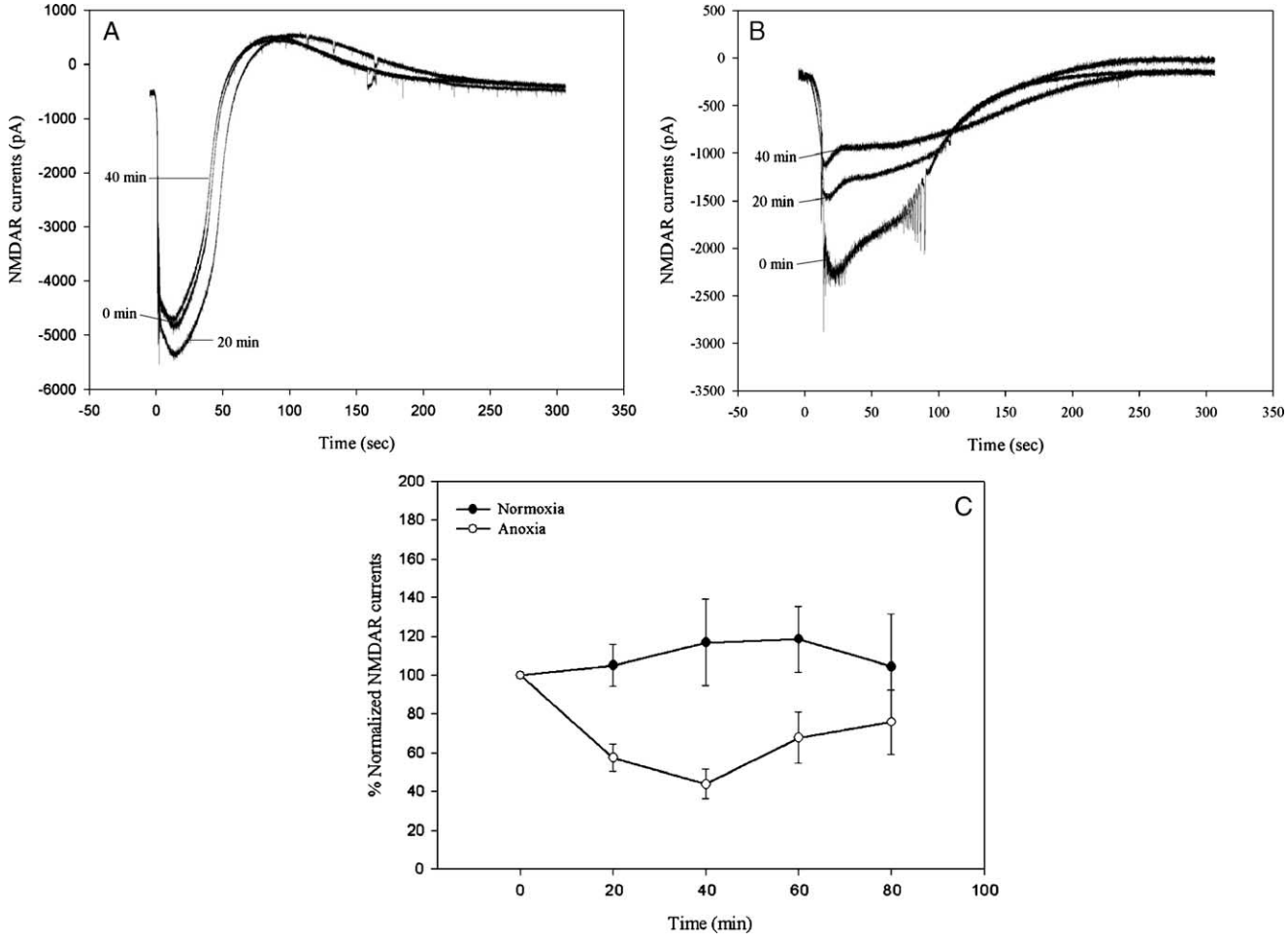

Fig. 1. Raw data traces of NMDA receptor whole-cell currents during (A) normoxic and (B) anoxic perfusion from two different neurons. (C) Group data showing normalized whole-cell NMDA currents in turtle cortical neurons. Solid line represents duration of anoxic exposure (95% N_2/5% CO_2). Daggers represent values significantly different from time 0 ($P<0.05$) while asterisks represent values significantly different from corresponding normoxic values ($P<0.05$). Data are represented as mean and standard error of mean (SEM) from 10 to 6 separate experiments for normoxia and 11 to 5 separate experiments for anoxia.

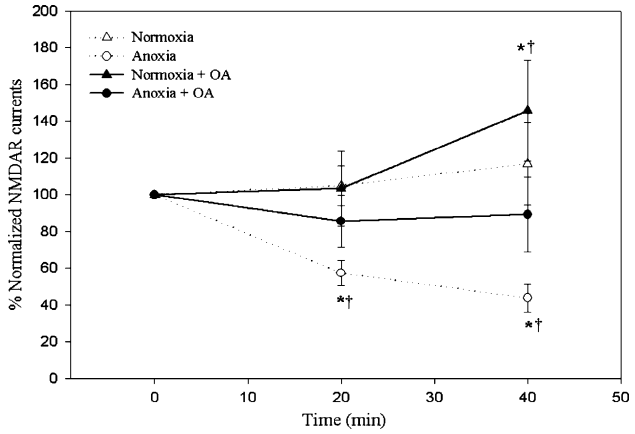

Fig. 2. Summary data showing the effects of okadaic acid (OA) on whole-cell NMDAR currents during 40 min of recording. Cortical sheets were pre-incubated with 6 μM okadaic acid for 15 min and bath perfused to maintain a constant level of okadaic acid (500 nM) during the recording. Data presented as dotted lines represents previously shown data from Fig. 1C. Treatment perfusion began immediately after the first recording ($T=0$ min). Data are represented as mean and SEM from 11 to 8 separate experiments. Daggers represent values significantly different from time 0 ($P<0.05$) while asterisks represent values significantly different from corresponding normoxic values ($P<0.05$).

okadaic acid (6 μM), calyculin A (200 nM) or cypermethrin (4 nM) for a period of 15 min. These inhibitors were first dissolved in DMSO (dimethylsulfoxide) and then diluted with aCSF. Vehicle alone had no effect on NMDAR whole-cell currents (data not shown). NMDA application and measurement of evoked whole-cell currents were performed as described above; however, recovery measurements were not made.

2.5. BAPTA experiments

To determine whether changes in intracellular calcium are required for modulating whole-cell NMDAR currents during normoxia and anoxia, the Ca^{2+} chelator BAPTA (5 mM), was included in the recording electrode solution. Both normoxic and anoxic experiments lasted 80 min with NMDA application every 20 min as described above.

2.6. Calmidazolium experiments

Prior to whole-cell recordings (both normoxic and anoxic) cortical slices were incubated with the calmodulin inhibitor Calmidazolium (1 μM) for 15 min. NMDA application and measurement of evoked whole-cell currents were performed over 40 min as described above.

2.7. Chemicals

All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) except for cypermethrin, which was purchased from Calbiochem (San Diego, CA, USA).

2.8. Statistical analysis

NMDAR whole-cell current data were analyzed using two-way ANOVA with a Tukey post-hoc test to compare within and against treatment and normoxic values. Prior to ANOVA analysis data were arcsine transformed. Significance was determined at $P<0.05$, and all data are expressed as the mean ± SEM (standard error of mean).

3. Results

3.1. Normoxic and anoxic whole-cell NMDAR currents

Raw normoxic and anoxic whole-cell NMDAR current traces and summary data are shown in Fig. 1. Throughout 40 min of normoxia NMDAR currents do not change significantly (Fig. 1A). However, 20 and 40 min of anoxic perfusion resulted in a 40% and 60% reduction in NMDAR current, respectively ($P<0.001$) (Fig. 1C).

3.2. Inhibition of PP1 and 2A experiments

Okadaic acid, a non-selective PP1 and 2A inhibitor, abolished the anoxia-induced decrease in NMDAR currents at 20 and 40 min; however a 46% increase in NMDAR currents was seen after 40 min of normoxic perfusion (Fig. 2).

Compared to okadaic acid, calyculin A is a more potent inhibitor of PP1, with similar inhibitory effects on PP2A (Ishihara et al., 1989). NMDAR currents in cortical sheets treated with calyculin A showed a gradual increase throughout the normoxic period, and by 40 min, currents

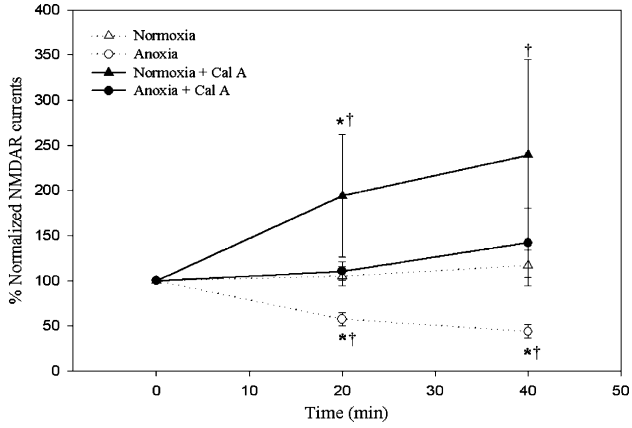

Fig. 3. Summary data showing the effects of calyculin A (Cal A) on whole-cell NMDAR currents during 40 min of recording. Cortical sheets were pre-incubated with 200 nM calyculin A for 15 min and bath perfused to maintain a constant level of calyculin A (20 nM) during the recording. Data presented as dotted lines represents previously shown data from Fig. 1C. Treatment perfusion began immediately after the first recording ($T=0$ min). Data are represented as mean and SEM from 11 to 8 separate experiments. Daggers represent values significantly different from time 0 ($P<0.05$) while asterisks represent values significantly different from corresponding normoxic values ($P<0.05$).

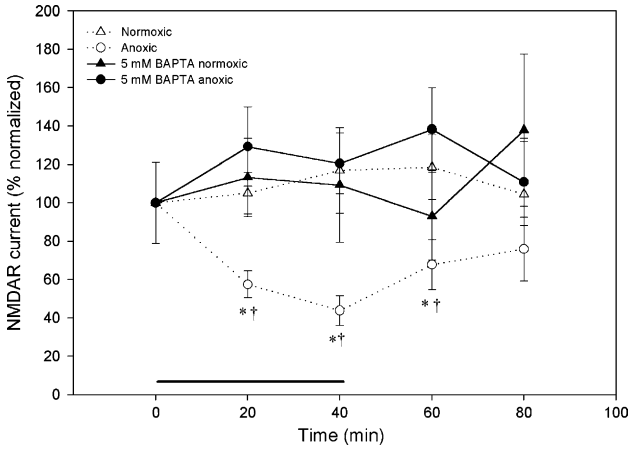

Fig. 4. Summary data showing normalized whole-cell NMDA currents in turtle cortical neurons with 5 mM BAPTA in the recording electrode. Solid line represents duration of anoxic exposure (95% N_2/5% CO_2). Data presented as dotted lines represents previously shown data from Fig. 1C. Asterisks represent values significantly different from time 0 ($P<0.05$). Daggers represent values significantly different from time 0 ($P<0.05$) while asterisks represent values significantly different from corresponding normoxic values ($P<0.05$).

had increased by 140% (Fig. 3). Calyculin A also prevented the anoxia-induced reduction in NMDAR currents in turtle cortical sheets. Indeed, there was a 45% increase in NMDAR currents after 40 min of anoxia in the calyculin A treated sheets (Fig. 3).

3.3. Calcium dependence of the anoxic response

To determine if intracellular Ca^{2+} modulates NMDAR activity during anoxia, the calcium chelator BAPTA (5 mM) was included in the recording electrode solution. In the presence of BAPTA NMDAR currents did not change over a normoxic 80 min time course (Fig. 4). However, the previously observed anoxia-mediated decrease in whole-cell NMDAR currents at 20 and 40 min were completely abolished in the presence of BAPTA (Fig. 4). Since Ca^{2+} was involved in abolishing the anoxia-mediated attenuation in NMDAR activity, NMDAR currents were monitored following application of cypermethrin, a Ca^{2+}/calmodulin-dependent protein phosphatase 2B/calcineurin blocker (PP2B). During anoxia, cypermethrin dampened the anoxic response of turtle cortical neurons at 20 min, but by 40 min NMDAR currents were not significantly different from those observed during anoxia alone (Fig. 5).

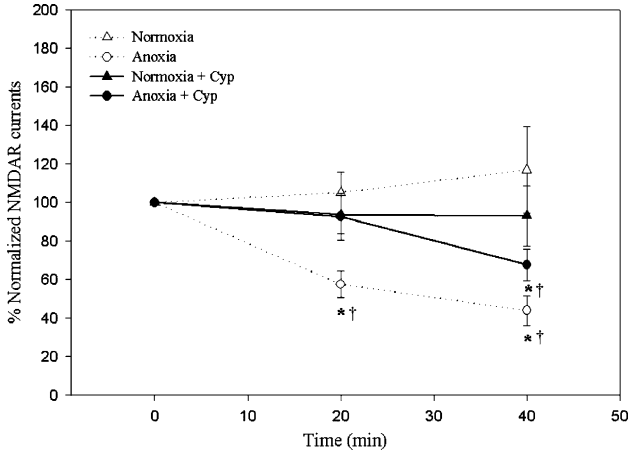

Fig. 5. Summary data showing the effects of cypermethrin (Cyp) on whole-cell NMDAR currents during 40 min of recording. Cortical sheets were pre-incubated with 4 nM cypermethrin for 15 min and bath perfused to maintain a constant level of cypermethrin (400 pM) during the recording. Data presented as dotted lines represents previously shown data from Fig. 1C. Treatment perfusion began immediately after the first recording ($T=0$ min). Data are represented as mean and SEM from 11 to 8 separate experiments. Daggers represent values significantly different from time 0 ($P<0.05$) while asterisks represent values significantly different from corresponding normoxic values ($P<0.05$).

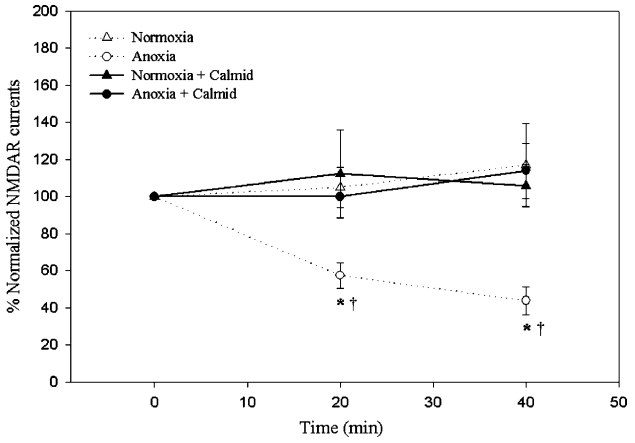

Fig. 6. Summary data showing the effects of calmidazolium (Calmid) on whole-cell NMDAR currents during 40 min of recording. Cortical sheets were pre-incubated with 1 μM calmidazolium for 15 min and bath perfused to maintain a constant level of calmidazolium (100 nM) during the recording. Data are presented as dotted lines represents previously shown data from Fig. 1C. Treatment perfusion began immediately after the first recording ($T=0$ min). Data are represented as mean and SEM from 10 to 7 separate experiments. Daggers represent values significantly different from time 0 ($P<0.05$) while asterisks represent values significantly different from corresponding normoxic values ($P<0.05$).

3.4. Involvement of calmodulin in the anoxic response

Since Ca^{2+} appears to be involved in the anoxia-mediated decrease in NMDAR activity, the calmodulin inhibitor calmidazolium was perfused onto turtle cortical sheets during normoxia and anoxia. During 20 and 40 min of normoxic aCSF perfusion, NMDAR currents remained constant. During anoxia, the application of calmidazolium completely abolished the anoxia-mediated decrease in NMDAR activity at 20 and 40 min (Fig. 6).

4. Discussion

In this study, an anoxia-mediated decrease in NMDAR currents was measured directly using the whole-cell patch-

clamp technique in turtle cortical neurons. The anoxia-mediated decrease was abolished by the inclusion of PP1 and 2A inhibitors. PP1 may have more involvement than PP2A in dephosphorylating the NMDAR during normoxia and anoxia since calyculin A, an inhibitor more specific to PP1, elicited a greater increase in NMDAR currents. In addition, the down-regulation of turtle cortical neuron NMDAR activity appears to be Ca^{2+} and calmodulin dependent since BAPTA and calmidazolium completely abolished the anoxia-mediated attenuation of NMDAR currents. Interestingly, the Ca^{2+}/calmodulin-dependent protein phosphatase PP2B inhibitor abolished the anoxic response at 20 min, but not at 40 min.

In the present study, NMDAR currents decreased during anoxia, but remained constant during normoxia. These results are consistent with previously reported data showing a 65% decrease in whole-cell NMDAR currents in turtle cortical neurons during 40 min of anoxia (Shin and Buck, 2003). This decrease in NMDAR activity is thought to be important for prolonging anoxic survival by preventing or reducing ECD (Choi, 1995). In mammalian models, a hallmark of anoxia induced cell death is the influx of Ca^{2+} via NMDARs (Benveniste et al., 1984; Choi, 1995). The reduction of Ca^{2+} influx via this receptor is paramount for anoxia tolerance since extracellular [Ca^{2+}] can reach as high as 25 mM in the Western painted turtle within months (Ultsch and Jackson, 1982; Cserr et al., 1988). Since over-stimulation of NMDAR, Ca^{2+} influx and ultimately ECD, is not seen in the turtle brain; it is possible that the attenuation in NMDAR activity is key to this process. Yet, the mechanism for modulating NMDARs during anoxia has not been elucidated.

It has been established under normoxic conditions that protein phosphatases and kinases modulate NMDAR activity in a tightly balanced manner. Using calyculin A and okadaic acid to inhibit PP1 and 2A's activity, Wang et al., (1994) demonstrated that these phosphatase inhibitors increased single channel open time duration and frequency of NMDARs by 62%, demonstrating that PP1 and 2A reduce NMDAR activity in cultured rat hippocampal neurons. Similarly, PP1 and 2A also appear to play a role in attenuating turtle cortical neuron NMDAR activity via dephosphorylation since inhibition by okadaic acid and calyculin A elicited a similar potentiation of receptors under normoxic, and to a lesser extent anoxic conditions. The present results suggest that PP1 may play a greater role in reducing NMDAR activity since calyculin A caused a greater increase in NMDAR currents during normoxia and anoxia.

The Ca^{2+}/calmodulin-dependent protein phosphatase 2B may also play an early role in attenuating NMDAR activity. Although inhibition of PP2B reduced the anoxia-induced suppression of NMDAR activity at 20 min, the full NMDAR anoxic response was established at 40 min. It is therefore possible that a transient increase in $[Ca^{2+}]_i$, due to the NMDAR, activates PP2B. Indeed, Bickler et al., (2000) demonstrated using fluorometric Ca^{2+} measurements, a temporary rise in intracellular Ca^{2+} following NMDA application. Thus, at 20 min, $[Ca^{2+}]_i$ may be sufficient to activate PP2B, but not at 40 min. Nevertheless, the initial reduction in whole-cell NMDAR currents during anoxia appears to be due to a calcium-dependent inactivation (CDI) of NMDARs. In mammalian studies, CDI is possible only when Ca^{2+} influx is via the NMDAR and not other calcium channels such as L-type Ca^{2+} channels (Mayer et al., 1987; Rosenmund and Westbrook, 1993; Tymianski et al., 1993; Tong et al., 1995; Medina et al., 1996). Indeed, the slight elevation in intracellular Ca^{2+} in turtle neurons during brief and prolonged anoxia reported by Bickler et al., (2000) may have acted as a second messenger to control neuronal activity. They also showed that calmodulin modulation is partially responsible for NMDAR inactivation and suggested that calmodulin positively modulates NMDAR activity during normoxia and its activity is reduced during anoxia. Our study showed that calmodulin plays a significant role in attenuating NMDAR activity during anoxia. Interestingly, our results demonstrated a considerable role for calmodulin in attenuation since application of calmidazolium completely blocked the reduction in NMDAR activity during anoxia. In contrast, Bickler et al., (2000) concluded that calmodulin is partially responsible for the decrease in NMDAR activity during anoxia since the decrease observed cannot account for the 65% decrease in NMDAR activity.

Some of the data presented in our study is confirmatory of Bickler et al., (2000) except in two respects. First, we used whole-cell recordings to measure NMDAR activity instead of calcium fluorometry and single channel open probability (P_{open}). The whole-cell recording technique allows for a more integrative view of NMDAR activity. Single channel recordings are very useful but only measure channel activity within the diameter of the electrode. Most, if not all single channel recordings are done at the cell soma since seal formation is more likely here due to the greater surface area and visibility under infrared microscopy, thus synaptic contributions are disregarded. Secondly, our study shows clear evidence of Ca^{2+} involvement in attenuating NMDAR activity during anoxia.

It is currently unknown whether or not protein phosphatases and Ca^{2+}/calmodulin independently modulate NMDAR activity in the anoxic turtle brain. Our data suggests that both mechanisms work in concert to modulate NMDAR activity during anoxia. When PP1 and 2A were inhibited during anoxia, the anoxia-mediated decrease in NMDAR activity was completely abolished. If Ca^{2+}/calmodulin modulated NMDAR activity independently of PP1 and 2A, then a partial decrease in NMDAR current would be observed during anoxia; this was not the case. Further, when Ca^{2+} was chelated and calmodulin inhibited, the anoxic response was also completely abolished. These results suggest that PP1, 2A, Ca^{2+} and calmodulin act in concert to decrease NMDAR activity in the anoxic turtle brain. Ehlers et al., (1996) reported two

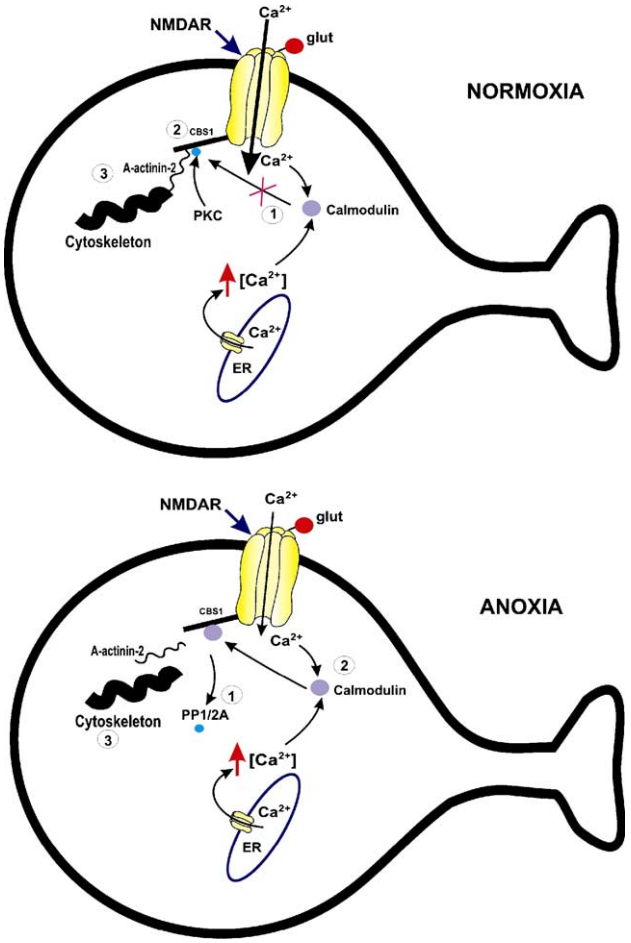

Fig. 7. (Top): diagram outlining the inability (1) of calmodulin (purple circle) to bind to CBS1 (calmodulin binding site) on the C-terminus of the NR1 subunit of the NMDAR during normoxia. Its binding is blocked by (2) a serine residue that is phosphorylated (blue circle) by PKC and the interaction of (3) α-actinin-2 anchoring CBS1 to the cytoskeleton. (Bottom): diagram showing the dephosphorylation of the serine residue on the CBS1 by PP1 and 2A (1) during anoxia. Upon activation by Ca^{2+}, calmodulin (2) outcompetes α-actinin-2 and binds to CBS1 (3) resulting in the dissociation of NMDAR from the cytoskeleton and a decrease in receptor activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

calmodulin binding sites (CBS1 and CBS2) on the C-terminus of the NR1 subunit. Using single-channel recordings, Ehlers et al., (1996) demonstrated that calmodulin-binding to the C-terminus of the NR1 subunit decreased the open probability of the NMDAR. Hisatsune et al., (1997), using a yeast two-hybrid system, reported that calmodulin interacts with the C-terminal of the NR1 subunit and inactivates the NMDA channel in a Ca^{2+} dependent manner. Furthermore, they showed that protein kinase C (PKC)-mediated phosphorylation of NR1 serine residues decreased its affinity for calmodulin. Later, it was found that the CBS1 region of the NR1 subunit also interacts with α-actinin-2, an actin-associated protein (Wyszynski et al., 1997). Zhang et al., (1998) investigated whether calmodulin and α-actinin-2 were involved in CDI of the NMDAR using whole-cell currents mediated by recombinant and native NMDARs. They concluded that disrupting the association of the NMDAR with α-actinin-2 by Ca^{2+}/calmodulin mediated CDI. Furthermore, this disruption involved the CBS1 site, and not the CBS2 NR1 site.

Based on these findings, we propose the following mechanism describing PP1, 2A, Ca^{2+}, and calmodulin-mediated attenuation of NMDAR activity in the turtle cortex (Fig. 7). During anoxia, PP1 and 2A dephosphorylate the serine residue on the C-terminus of the NR1 subunit. This enables a Ca^{2+}/calmodulin-mediated disruption of the NMDAR from the cytoskeleton by out-competing binding with α-actinin-2. Interestingly, it has been shown that PP2B dephosphorylates NR2A and not the NR1 subunit (Krupp et al., 2002). This is consistent with our data showing a limited role of PP2B in the anoxia-mediated decrease in NMDAR activity in the turtle cortex.

In conclusion, our results show that the reduction in NMDAR activity in turtle cortical neurons during anoxia is mediated by its dephosphorylation via PP1/2A. This attenuation is Ca^{2+} and calmodulin-dependent, but does not involve PP2B after 20 min of anoxia. Based on these data we propose a novel mechanism for NMDAR attenuation in the turtle cortex involving a dependent role of PP1, 2A and Ca^{2+}/calmodulin.

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