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OXYGEN-SENSITIVE REDUCTION IN CA^{2+}-ACTIVATED K^+ CHANNEL OPEN PROBABILITY IN TURTLE CEREBROCORTEX☆

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Abstract—In response to low ambient oxygen levels the western painted turtle brain undergoes a large depression in metabolic rate which includes a decrease in neuronal action potential frequency. This involves the arrest of *N*-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) currents and paradoxically an increase in γ-amino-butyric acid receptor (GABAR) currents in turtle cortical neurons. In a search for other oxygen-sensitive channels we discovered a Ca^{2+}-activated K^+ channel (K_{Ca}) that exhibited a decrease in open time in response to anoxia. Single-channel recordings of K_{Ca} activity were obtained in cell-attached and excised inside-out patch configurations from neurons in cortical brain sheets bathed in either normoxic or anoxic artificial cerebrospinal fluid (aCSF). The channel has a slope conductance of 223 pS, is activated in response to membrane depolarization, and is controlled in a reversible manner by free [Ca^{2+}] at the intracellular membrane surface. In the excised patch configuration anoxia had no effect on K_{Ca} channel open probability (P_{open}); however, in cell-attached mode, there was a reversible fivefold reduction in P_{open} (from 0.5 ± 0.05 to 0.1 ± 0.03) in response to 30-min anoxia. The inclusion of the potent protein kinase C (PKC) inhibitor chelerythrine prevented the anoxia-mediated decrease in P_{open} while drip application of a phorbol ester PKC activator decreased P_{open} during normoxia (from normoxic 0.4 ± 0.05 to phorbol-12-myristate-13-acetate (PMA) 0.1 ± 0.02). Anoxia results in a slight depolarization of turtle pyramidal neurons (~8 mV) and an increase in cytosolic [Ca^{2+}]; therefore, K_{Ca} arrest is likely important to prevent Ca^{2+} activation during anoxia and to reduce the energetic cost of maintaining ion gradients. We conclude that turtle pyramidal cell Ca^{2+}-activated K^+ channels are oxygen-sensitive channels regulated by cytosolic factors and are likely the reptilian analog of the mammalian large conductance Ca^{2+}-activated K^+ channels (BK channels).

Key words: anoxia-tolerant, metabolism, channel arrest, neuron, PKC, phosphorylation.

INTRODUCTION

The western painted turtle (*Chrysemys picta bellii*) survives days and even months of anoxia without apparent injury to energetically costly neuronal tissue (Jackson et al., 1984; Jackson, 2002). Mammalian neurons are not anoxia-tolerant and survive only minutes of anoxia due to an increase in neuronal ATP demand that outpaces ATP supply. Failure of ion motive ATPases to maintain transmembrane ion gradients results in neuronal depolarization, a rapid increase in extracellular K^+ concentration ($[K^+]_o$), Ca^{2+} influx through NMDA receptors, hyperexcitability, and excitotoxic cell death (ECD) (Choi, 1992, 1994; Love, 2003). It has become clear that a reduction in both ATP supply and demand is an important anoxia-tolerance strategy in the turtle (Staples and Buck, 2009), and this is accomplished via ion channel arrest (CA) and spike arrest (SA) (Hochachka, 1986; Sick et al., 1993). In turtle cortical neurons, anoxia decreases the activity of glutamatergic NMDA receptors and AMPA receptors (Buck and Bickler, 1998; Shin and Buck, 2003; Pamenter et al., 2008a), thereby decreasing excitation, and increases the activity of inhibitory GABA receptors, thereby decreasing action potential frequency (Pamenter et al., 2011).

Initiation of CA and SA must include O_2 sensors; cells must first sense a change in O_2 tension and then trigger mechanisms to reduce ATP demand. Opening of mitochondrial ATP-sensitive K^+ channels (mK_{ATP}) has been linked to a reduction in both NMDAR and AMPAR activities in the turtle cortex and may be central to an oxygen sensor (Pamenter et al., 2008b; Zivkovic and Buck, 2010). An O_2-sensing mechanism may consist of a decrease in mitochondrial [ATP] due to the lack of oxidative phosphorylation which activates mK_{ATP}, leading to mitochondrial K^+ influx, depolarization, Ca^{2+} efflux to the cytosol, protein kinase/phosphatase activation, and ion channel modulation (Shin et al., 2005; Pamenter et al., 2008b). Mitigating a neurotoxic NMDAR-mediated increase in intracellular [Ca^{2+}] ($[Ca^{2+}]_i$) is key to preventing ECD in the mammalian

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Abbreviations: aCSF, artificial cerebrospinal fluid; APV, (2*R*)-amino-5-phosphonovaleric acid; CA, channel arrest; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; ECD, excitotoxic cell death; EGTA, ethylene glycol tetraacetic acid; *I*/*V*, current/voltage; IbTX, iberiotoxin; K_{Ca}, Ca^{2+}-activated K^+ channel; mK_{ATP}, mitochondrial ATP-sensitive K^+ channels; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; P_{open}, open probability; RM-ANOVA, repeated measures analysis of variance; SA, spike arrest; SD, spreading depression; TEA, tetraethylammonium chloride; TTX, tetrodotoxin.

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central nervous system. The less dramatic increase in $[Ca^{2+}]_i$ associated with mK_{ATP} opening in the turtle cortex appears to be part of the natural signaling mechanism leading to the anoxic reduction in NMDAR activity (Pamenter et al., 2008b).

We used patch-clamp techniques to record oxygen-sensitive spontaneous single-channel currents in turtle cortical neurons. Single-channel currents were identified in cell-attached and excised inside-out patches using a combination of channel blockers and ion substitution in the recording electrode. We identified a large conductance Ca^{2+}-activated K^+ channel (K_{Ca}) similar to BK or maxi K_{Ca} in mammals (for review see Gribkoff et al., 2001; Magleby, 2003) and directly measured changes in channel open probability (P_{open}) in response to anoxia and in the presence of protein kinase and phosphatase modulators.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed using adult male and female western painted turtles (*Chrysemys picta bellii*) weighing approximately 200 g. This study conforms to the relevant guidelines for the care and handling of experimental animals as determined by the Canadian Council on Animal Care and was approved by the University of Toronto Animal Care Committee. Turtles were obtained from Niles Biological (Sacramento, California, USA) and housed in large aquariums equipped with flow-through dechlorinated freshwater systems (20 °C), basking platforms, and lamps. Turtles were maintained under a 12-h:12-h light:dark photoperiod and given continuous access to food and water.

Dissection and cortical brain sheet preparation

Turtles were decapitated and the whole brain was rapidly excised from the cranium within 30 s. Six cortical sheets were isolated (for details see Blanton et al., 1989) and collected in chilled (3–5 °C) turtle artificial cerebrospinal fluid (aCSF) containing (in mM): 107 NaCl, 2.6 KCl, 1 $MgCl_2$, 2 NaH_2PO_4 ($2H_2O$), 26.5 $NaHCO_3$, 10 glucose, 5 imidazole, 1.2 $CaCl_2$ ($2H_2O$) (pH 7.4, adjusted with 12 N HCl; osmolarity 285–290 mOsm). All chemicals used in this study were obtained from Sigma–Aldrich (Oakville, Ontario, Canada).

Individual cortical sheets were placed on a coverslip that formed the bottom of a flow-through perfusion chamber system of about 1 ml in volume (RC-26 chamber with P1 platform; Warner Instruments), and held in place for recording by a slice hold-down anchor (Warner Instruments). The tissue chamber was gravity-perfused with turtle aCSF from a 1 L glass bottle via an intravenous (IV) dripper at a rate of 2–3 mL min^{-1}. A second 1 L glass bottle was used to gravity-perfuse the tissue chamber with aCSF containing varying $[K^+]$ and $[Ca^{2+}]$ or aCSF bubbled with 95% N_2/5% CO_2 (see details below). A fast-step drug perfusion system (VC-6 model perfusion valve controller and SF-77B fast-step perfusion system; Warner Instruments) was used to deliver 1 μM tetrodotoxin (TTX) and aCSF containing 0–50 μM Ca^{2+} directly above the cortical sheet (see details below). All experiments were performed at a room temperature of 22–25 °C.

Single-channel patch-clamp recording protocol

Single-channel recordings were obtained from cell-attached and excised inside-out patches using fire-polished 8–10 MΩ borosilicate glass pipettes. An Ag–AgCl electrode connected to a CV-4 headstage and MultiClamp 700B amplifier (Molecular Devices) was inserted into pipettes. Cell-attached 5–10 GΩ seals were obtained using the blind-patch technique previously described (Blanton et al., 1989). Once sealed, the potential of the membrane patch was clamped to the desired level by polarizing the electrode with respect to ground and single-channel currents of the patch were recorded. All data were collected at 20 kHz, low-pass-filtered at 1 kHz, and digitized using a Digidata 1440A interface (Molecular Devices). Following GΩ seal formation, excised inside-out membrane patches were obtained by reversing the recording electrode away from the cortical sheet using the stepper-motor. The tip of the electrode was positioned above the cortical sheet but remained immersed in the bath.

Single-channel K_{Ca} current/voltage relationships

For cell-attached single-channel recordings, electrodes were filled with turtle aCSF which contained physiological extracellular $[K^+]$ and $[Ca^{2+}]$'s (see above for molar concentrations). Cortical sheets were also bathed with turtle aCSF during all cell-attached patch-clamp experiments. After obtaining a GΩ seal, patches were allowed to stabilize for 5 min before a 5-min perfusion of 1 μM TTX to suppress action potentials. 1 μM TTX was perfused onto the cortical sheet every 20 min. In the voltage-clamp configuration the initial holding potential was set to −80 mV. Assuming a cellular resting membrane potential of approximately −80 mV, this resulted in a patch transmembrane potential of 0 mV. The following formula was used to calculate the transmembrane potential of the patch (MP) from the electrode holding potential (HP) and a cellular resting membrane potential of −80 mV:

$$MP = (-80\ mV) - (HP).$$

To test for spontaneous single-channel activity, voltage was stepped from −120 mV to +40 mV in 20 mV steps in the cell-attached configuration. The patch was held at each potential for 60 s. Single-channel currents were observed and could be characterized as outward rectifying. Depolarization of the patch was required to activate the channel and channel P_{open} was calculated from amplitude histograms generated from 60-s recordings of single-channel activity at transmembrane potentials of −60 mV to +40 mV. To rule out confounding NMDAR activity, 3 mM $MgCl_2$ was added to the electrode solution (4 mM total Mg^{2+}) as well as 30 μM (2*R*)-amino-5-phosphonovaleric acid (APV; NMDAR antagonist). To eliminate AMPAR and non-specific K^+ channel activity, 30 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; AMPAR antagonist), 5 mM CsCl (Cs^{2+}; K^+ channel antagonist), and 10 mM tetraethylammonium chloride (TEA; voltage-gated K^+ channel antagonist) were added to the electrode solution. To eliminate K_{Ca} activity, 150 nM iberiotoxin (IbTX) was added to the electrode solution. Cell-attached patches obtained with the above electrode solutions were interspersed among those obtained with a control aCSF electrode solution that did not contain any channel antagonists. Based on the effects of TEA and IbTX, outward rectification, and current responses at depolarized transmembrane potentials, we hypothesized that our channel of interest was a K_{Ca}.

A full current/voltage (*I*/*V*) relationship could not be obtained in cell-attached mode because of the outward rectification of the channel. To generate a full *I*/*V* relationship, we obtained stable excised inside-out membrane patches and bathed both sides of the membrane patch with a solution containing high $[K^+]$ (mM: 100 KCl; 11 NaCl; 1 $MgCl_2$; 2 NaH_2PO_4 ($2H_2O$); 26.5 $NaHCO_3$; 10 glucose; 5 imidazole; pH 7.4). The solution was identical on either side of the membrane patch, except with respect to $[Ca^{2+}]$. It was previously shown that 0.5 μM free $[Ca^{2+}]$ at the