Acute reduction in whole cell conductance in anoxic turtle brain

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Ghai, H. S., and L. T. Buck. Acute reduction in whole cell conductance in anoxic turtle brain. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R887–R893, 1999.—We tested the effect of anoxia, a “mimic” turtle artificial cerebrospinal fluid (aCSF) consisting of high Ca2+ and Mg2+ concentrations and low pH and adenosine perfusions, on whole cell conductance (Gw) in turtle brain slices using a whole cell voltage-clamp technique. With EGTA in the recording electrode, anoxic or adenosine perfusions did not change Gw significantly (values range between 2.15 ± 0.24 and 3.24 ± 0.56 nS). However, perfusion with normoxic or anoxic mimic aCSF significantly decreased Gw. High [Ca2+] (4.0 or 7.8 mM) perfusions alone could reproduce the changes in Gw found with the mimic perfusions. With the removal of EGTA from the recording electrode, Gw decreased significantly during both anoxic and adenosine perfusions. The A1-receptor agonist N6-cyclopentyladenosine reduced Gw in a dose-dependent manner, whereas the A2-receptor specific antagonist 8-cyclopentyl-1,3-dipropylxanthine blocked both the adenosine- and anoxic-mediated changes in Gw. These data suggest a mechanism involving A1-receptor-mediated changes in intracellular [Ca2+] that result in acute changes in Gw with the onset of anoxia.

calcium; channel arrest; cortical neurons; membrane conductance; western painted turtle

The Western Painted Turtle is the most anoxic-tolerant vertebrate presently known, surviving as much as 6 mo of anoxia at low temperatures (29). The most effective strategy employed by such a species to survive long-term anoxia is probably the coordinated suppression of ATP-utilizing and -producing pathways. Such a strategy has been demonstrated in anoxic turtle brain, with reductions in energy metabolism of 49–80% (10, 21). The anoxic turtle brain still maintains ionic gradients, actively pumps ions (28), and is electrically active, although at a reduced level (11). Similarly, the onset of anoxia reduces hepatocyte metabolism by 90% and ion pumping via the Na+-K+ ATPase by 75% without an apparent change in membrane potential (5). A hypothesis that can account for such alterations is the “channel arrest” hypothesis (16), which predicts that 1) in hypoxia-tolerant species, the plasma membrane should be less permeable to ions than in hypoxia-intolerant species, and 2) that tolerant species should have mechanisms to acutely regulate cellular ion permeability. Indeed, various forms of channel arrest do occur in the anoxic turtle brain. Anoxia induces a 42% decrease in voltage-gated Na+ channel density as determined by a [3H]brevetoxin-binding assay in brain slices (24). There is also an apparent change in K+ channel permeability; the rate of K+ efflux in ouabain (Na+-K+ ATPase inhibitor)-treated brain is reduced by 70% during anoxia (23). Furthermore, with the use of microelectrodes, rates of K+ leakage are 50% lower in anoxic turtle cerebellum than in normoxic turtle cerebellum (6). In a direct measure of channel arrest, N-methyl-D-aspartate (NMDA) receptor open probability has been shown to decrease by 65% in response to anoxia (3). These studies point to a reduction in plasma membrane permeability that would be accompanied by a reduction in the amount of ATP required to maintain cellular ionic homeostasis in anoxic turtle brain.

The mechanisms by which membrane permeability is regulated are largely unknown. However, adenosine is considered to play an important neuroprotective role during the transition to anoxia in the turtle brain and may be part of a mechanism regulating membrane permeability. Evidence supporting this notion is as follows: extracellular adenosine has been shown to increase from 2 to 20 µM during anoxia (22); application of an A1-receptor antagonist elicits the release of intracellular K+ in the isolated turtle cerebrum during anoxia, the absence of such release being characteristic of anoxia tolerance in the turtle brain (25); adenosine reduces NMDA-mediated rise in intracellular Ca2+ concentration ([Ca2+]i) by 62% (4) and reduces NMDA receptor open probability by 65% in the turtle cortex (3). On the basis of this evidence, we have reasoned that adenosine would modulate neuronal whole cell conductance (Gw). In fact, previous whole cell voltage-clamp studies found no significant changes in Gw in response to anoxia in a turtle brain slice preparation (8, 9). However, these studies were conducted with high-resistance electrodes (30–70 MΩ) that may be unable to achieve an effective whole cell clamp configuration and were thus unable to accurately measure a change in Gw. Furthermore, high levels of the calcium ion chelator EGTA were used in the recording electrode. If changes in [Ca2+]i are an important event mediating the cellular response to anoxia, then any change would have been buffered by the presence of EGTA.

In the present study, we have repeated these experiments with lower resistance electrodes (6–8 MΩ) and EGTA-free recording electrode solutions to determine whether a change in Gw occurs in anoxic turtle brain slices. Because turtle blood concentrations of Ca2+, Mg2+, and H+ have been shown to increase greatly in a dived turtle (15), we also examined the effect of an acute perfusion of a “mimic” turtle artificial cerebrospinal fluid (aCSF) consisting of Ca2+ and Mg2+ concentrations and low pH on Gw. Finally, the effect of adenosine

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MATERIALS AND METHODS

These studies were approved by the University of Toronto Animal Care Committee and conform to relevant guidelines for the care of experimental animals. Spring, summer, and autumn female turtles (Chrysemys picta bellii, Sneider), weighing between 250 and 500 g, were obtained from Lemberger (Oshkosh, WI). Animals were housed in a large aquarium equipped with a flow-through dechlorinated freshwater system at 23°C, basking platform, and lamp. Turtles were maintained on a 12-h light-to-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 aCSF (in mM: 97 NaCl, 26.5 NaHCO3, 2.0 Na2HPO4, 2.6 KCl, 2.5 CaCl2, 2.0 MgCl2, 20 glucose, and 20 HEPEs; pH 7.4 at 23°C) at 3–5°C. Individual cortical sheets (6–8 total) were cut from larger cortical sheets as described by Blanton et al. (2). All bathing and electrode filling solution osmolarities were measured with a vapor pressure osmometer (Wescor model 5500) and adjusted to ~280 mosM.

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, Vancouver, BC). Voltage-clamp recordings in the whole cell configuration were obtained from submerged and perfused cortical sheets (aCSF at 2–3 ml/min, 23°C). The tissue-slice chamber was gravity perfused from two 1-l glass bottles with intravenous drippers and thick-walled, low-gas permeability tubing. A three-way stopcock and a pinch clamp were used to switch between intravenous bottles and regulate flow. All plastic tubing and intravenous drippers were double jacketed; N2 gas was blown through the outer jacket to prevent O2 from diffusing into perfusion solutions. The switching valve was as close as possible to the perfusion chamber. The measured line volume between switch and chamber was 1 ml, and chamber volume was 1.3 ml. Whole cell recordings were performed using the whole cell voltage-clamp method with 6- to 8-MΩ electrodes containing (in mM): 8 NaCl, 0.1 CaCl2, 5 EGTA, 10 HEPEs Na, 20 KCl, 110 KOH, 1 MgCl2, 0.3 NaGTP, and 2 MgATP; pH 7.4 adjusted with methanesulfonic acid. For some experiments EGTA was removed from the recording solution and Ca2+ was adjusted to 1 mM. This did not have a significant effect on osmolarity, remaining at ~280 mosM. Cell-attached 5- to 20-GΩ seals were obtained using the blind-patch technique of Blanton et al. (2). To break into a cell, the recording electrode potential was set at −60 mV and a sharp pulse of suction was applied. Once the whole cell configuration had been established, capacitance transients were removed using the capacitance compensation circuit of the Axopatch 1D, and access resistance was measured (ranging from 100 to 200 MΩ). From the voltage-clamp configuration, the Axopatch 1D amplifier was switched to the zero-current position and the membrane potential was read off of the main meter. The whole cell configuration was allowed to stabilize for 2 min before resting membrane potential was determined (ranging from −65 to −85 mV). Data were collected using an Axopatch 1D amplifier, CV-4 headstage, and TL-1 DMA interface (Axon Instruments, Burlingame, CA). Data were digitized offline at 20 kHz and analyzed via computer using pCLAMP software (version 6.0, Axon Instruments; Fig. 1A). To measure whole cell conductance, Clampex software was used to step voltage from −60 to −93 mV in −3-mV increments lasting 250 ms. Current values were measured between 200 and 220 ms to avoid any capacitance effects, and a slope conductance (or $G_w$) was determined from the resultant current-voltage relationship (Fig. 1B).

Control and anoxic experiments. Experiments consisted of 130- or 70-min time courses in which two control points (t = 0 or 10) were obtained before a 60-min treatment perfusion. Control experiments involved brain sheets perfused for 130 min with normoxic aCSF in the presence of EGTA in the recording electrode. Control normoxic experiments were also conducted for 130 min with the exclusion of EGTA from the recording electrode. For anoxic experiments, the treatment reservoir was gassed with 95% N2-5% CO2 and contained 0.5 mM NaCN to prevent oxygenation artifacts. The recording chamber was fitted with a cap and a gas line that allowed the chamber head space to be gassed with 95% N2-5% CO2. A small hole was made in the cap for the recording electrode to pass through and to vent positive gas pressure. Whereas recovery measurements were deemed useful, tissue was reperfused with normoxic aCSF for 60 min. In sham experiments the patch-clamp recording electrode was replaced with an oxygen electrode. When the perfusate and head space gas were switched from 95% O2-5% CO2 to 95% N2-5% CO2 the time required for aCSF PO2 to drop below 1 torr was 8 min.

Mimic aCSF experiments. Mimic aCSF composition designed from values reported by Herbert and Jackson (15) was...

![Fig. 1. Representative raw data recording and current-voltage (I-V) relationship to determine whole cell conductance ($G_w$). A: typical current response to a series of stepped voltages (from −60 to −93 mV in 3-mV increments). B: I-V relationship obtained from whole cell current responses shown in A. A regression analysis of these data gives a slope conductance (or $G_w$) of 2.65 nS.](image)
(in mM): 97 NaCl, 26.5 NaHCO₃, 2.0 NaH₂PO₄, 2.6 KCl, 7.8 CaCl₂, 4.0 MgCl₂, 20 glucose, and 10 HEPES; pH 7.1 at 23°C. Cortical sheets were perfused with mimic aCSF containing 0.5 mM NaCN and gassed with 95% N₂-5% CO₂ to detect whether Gₓ changes under conditions that mimic the composition of anoxic turtle blood. To determine if the mimic solution had effects on Gₓ separate from those of anoxia alone, a normoxic mimic experiment was carried out.

Calcium experiments. To determine if high extracellular Ca²⁺ levels could have an effect on Gₓ, 5 mM EGTA was removed from the recording electrode during both anoxic and adenosine treatments of the tissue. Furthermore, in one set of experiments, cortical sheets were perfused with normoxic aCSF containing 4 mM Ca²⁺ in the absence of EGTA from the recording electrode. Additionally, in two separate sets of experiments, cortical sheets were perfused with aCSF containing 7.8 mM Ca²⁺ both in the presence and absence of EGTA in the recording electrode. These three sets of experiments were performed to demonstrate whether increases in extracellular [Ca²⁺]([Ca²⁺]ₑ) play a role in modulating Gₓ.

Tissue viability and energy charge determination. Tissue viability was assessed by measurements of [ATP], [ADP], and [AMP] and subsequent energy charge calculation in cortical sheets incubated for 0, 40, and 80 min in aCSF containing 1.2, 4, and 7.8 mM Ca²⁺. For this purpose, individual cortical sheets were weighed and rapidly placed in 0.5 ml of boiling 50 mM HEPES buffer at pH 7.8 and sonicated for 25 s. They were incubated at high temperature for an additional 3 min before being removed and chilled in an ice bath for 5 min. The samples were then centrifuged for 10 min at 10,000 g (at 2°C) and a 400-µl aliquot of supernatant was used for adenosine nucleotide determination by HPLC. To separate these nucleotides, a reverse-phase column (150 × 4.6 mm) (Supelco model LC-18-T, Scarborough, Ontario) was used. For the mobile phase, two buffer solutions were used: buffer A [0.1 M monophosphate monobasic ammonium phosphate (Sigma, Ontario) in deionized water, adjusted to pH 6.0 with KOH] and buffer B (70% buffer A and 30% methanol). Buffer solutions were degassed with helium before use. The gradient elution protocol consisted of an initial perfusion with 100% buffer A, gradient perfusion of buffer B at 5 min, and then transition to 100% perfusion with buffer B at 13 min and lasting to 17 min. At this time the perfusion was switched back to buffer A (100% at 18 min) and perfused for an additional 7 min. Adenylate peaks were eluted at 1.5 ml/min, and a variable wavelength ultraviolet-visual spectrophotometer operating at 254 nm was used for detection. The adenylate peak areas were quantified by comparison with known peak areas of ATP, ADP, and AMP standards.

Adenosine experiments. To determine if Gₓ modulation is adenosine-receptor based, recordings were obtained from cortical sheets perfused with 200 µM adenosine. To distinguish between metabolic- and receptor-based effects, tissue was perfused with either 1, 10, or 100 µM N₆-cyclopentyladenosine (CPA), a selective A₁-receptor agonist. To further characterize the receptor-based response, cortical sheets were preincubated for 10 min with adenosine-receptor blocker 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 µM) and then perfused with 200 µM adenosine and 1 µM DPCPX followed by a recovery perfusion. In a similar fashion, anoxic experiments were performed on tissue preincubated and perfused with 1 µM DPCPX.

Statistical analysis. Repeated-measures ANOVA with a Dunnett’s post hoc test was used to compare treatment values with control. The first two control time points [t (in min) = 0 and 10] were pooled together for comparison with the treatment values. The same statistical test using the control points individually does not alter the results. A similar test

![Fig. 2. Effect of anoxic and mimic turtle artificial cerebrospinal fluid (aCSF) perfusion on Gₓ. A: control normoxic and anoxic perfusions with EGTA in recording electrode. B: anoxic and normoxic mimic aCSF perfusions with EGTA. C: control normoxic and anoxic perfusions without EGTA in recording electrode.](http://ajpregu.physiology.org/Downloaded from)
was used to detect differences in energy charge. All data are expressed as means ± SE.

RESULTS

Under control conditions and using a standard electrode recording solution containing EGTA, G_w was found to remain stable throughout a 130-min time course (Fig. 2A). Similarly, anoxic perfusion resulted in no significant changes in G_w (Fig. 2A). To test whether G_w is effected by elevated levels of [Ca^{2+}], [Mg^{2+}], and low pH, such as those measured in blood of dived turtles, tissue was perfused with a mimic aCSF solution under both normoxic and anoxic conditions (Fig. 2B). After the initiation of normoxic or anoxic mimic aCSF perfusion, significant 31 and 26% reductions occurred in G_w [2.69 ± 0.26 to 1.86 ± 0.13 nS at t = 30 (P = 0.00869) and 2.61 ± 0.23 to 1.94 ± 0.19 nS at t = 30 (P = 0.00914), respectively]. In both sets of experiments G_w returned to control values within 20 min of control aCSF reperfusion. Exclusion of EGTA from the recording electrode during anoxic aCSF perfusion did result in a significant decrease in G_w (3.37 ± 0.34 to 2.46 ± 0.30 nS at t = 60, P = 0.0014), subsequently returning to control values on normoxic reperfusion (Fig. 2C). The removal of EGTA from the recording electrode had no effect on the normoxic control values (Fig. 2C).

To further investigate the role of calcium in modulating G_w, perfusion of cortical sheets with control aCSF containing 4 and 7.8 mM Ca^{2+} was carried out (Fig. 3A). Perfusion with aCSF containing 4 mM Ca^{2+}, without EGTA in the recording electrode, resulted in a significant 36% decrease in G_w (3.72 ± 0.22 to 2.40 ± 0.22 nS at t = 50, P = 0.0156). Perfusion with aCSF containing 7.8 mM Ca^{2+}, in the absence of EGTA, resulted in a 52% reduction in G_w (3.65 ± 0.59 to 1.78 ± 0.29 nS at t = 50, P = 0.0269). In the absence of EGTA the whole cell configuration consistently broke down at t = 50 min. However, with inclusion of EGTA in the recording electrode, the whole cell configuration was maintained throughout the treatment and recovery period and a 32% reduction in G_w was measured with the 7.8 mM Ca^{2+} treatment (2.68 ± 0.13 to 1.82 ± 0.16 nS at t = 50, P = 0.0000227).

Tissue [ATP], [ADP], and [AMP] were determined from cortical sheets incubated for 0, 40, or 80 min in saline containing 1.2, 4, or 7.8 mM Ca^{2+}. Energy charge values were calculated to determine if high [Ca^{2+}] was injurious to the cortical sheets, resulting in loss of the whole cell configuration. Tissue [ATP] and energy charge did not change significantly at any [Ca^{2+}] or exposure duration; values ranged from 0.69 ± 0.06 to 0.72 ± 0.03 and from 1.91 ± 0.05 to 2.58 ± 0.24 µmol/g wet weight, respectively (Table 1). Tissue [ADP] and
Table 1. Cortical sheet energy charge and [ATP] on exposure to varying [Ca^{2+}]

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<th>[Ca^{2+}] (mM)</th>
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<tr>
<td>7.8</td>
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Values are means ± SE for 6–8 separate experiments. Energy charge (EC) values were calculated with formula EC = ATP + 0.5 (ADP)/ATP + ADP + AMP, *[ATP] in μmol/g wet wt.

Our data show that G_w decreases in anoxic turtle brain. This finding is in contradiction to those previously reported (9) in which no change in neuronal G_w was detected during anoxic perfusion. It is unlikely that the relatively high-resistance (30–70 MΩ) electrodes used in the previous study compromised measurement of G_w, because their values for G_w (2.73 ± 0.36 nS) (9) are similar to our values (2.80 ± 0.30 nS) and those reported in mammalian brain (5.68 ± 0.60 nS) (27). Rather, it is likely that the inclusion of EGTA (10 mM) in the recording electrode prevented an anoxia-induced decrease in G_w because, in our experiments, inclusion of EGTA in the recording electrode blocked changes in G_w. Additionally, our findings support the second prediction of the channel arrest hypothesis that anoxia-tolerant species should have mechanisms to acutely regulate cellular ion permeability.

Both a normoxic and anoxic mimic perfusion significantly decreased G_w, suggesting that either elevated levels of Ca^{2+}, Mg^{2+}, low pH, or a combination of these constituents is responsible for the observed reduction in G_w. Perfusion of cortical sheets with aCSF containing high Mg^{2+} or low pH (7.1) did not alter G_w significantly (unpublished data). However, increasing [Ca^{2+}], from 1.2 to 4.0 mM in the perfusion did result in significant reductions in G_w. Because [Ca^{2+}] increases in turtle blood throughout an anoxic dive (15) and it has been recently shown that [Ca^{2+}] increases in dived anoxic turtle brain (1), then changes in [Ca^{2+}] may be an important event leading to decreased membrane ion permeability and therefore G_w.

Adenosine perfusion also reduced G_w, suggesting a role for adenosine in channel arrest. This response is likely receptor mediated, because the A_1-receptor specific agonist CPA decreased G_w in a dose-dependent manner, and the potent A_1-receptor antagonist DPCPX blocked the adenosine-mediated decrease in G_w, further strengthening the argument for a receptor-based mechanism. The antagonist also prevented the anoxia-induced reductions in G_w, suggesting that endogenous adenosine released from cortical sheets during anoxia results in the observed decrease in G_w.

We propose three potential mechanisms to explain our results. First, nonselective, voltage-independent K^+ channels, or "leakage K^-channels" (12, 19, 20), are thought to contribute to basal leakage currents and establish a resting neuronal membrane potential. Of these, the rTASK channel type is known to have several regulatory sites, including protein A and C kinases, tyrosine kinase, and postsynaptic density (PSD-95) sites (19). Forskolin (adenylate cyclase stimulator) application to rTASK channels exogenously expressed in Xenopus oocytes results in a 42% reduction in K^+ currents (19). Thus it is possible that, during the transition to anoxia, K^+ leakage-channel permeability is decreased through the action of second-messenger pathways, resulting in decreased G_w. Second, A_1-receptor activation leads to changes in [Ca^{2+}], and...
cAMP. Failure of adenosine perfusion to illicit a reduction in \( G_w \) in the presence of EGTA suggests that adenosine exerts its effects through changes in \( [Ca^{2+}] \). The \( A_1 \) receptor is a G protein-linked receptor able to modulate several intracellular cascades including the phospholipase C, inositol triphosphate, and adenylyl cyclase pathways (26). It is possible that \( A_1 \)-receptor activation leads to increases in \( [Ca^{2+}] \) and cAMP. The anoxia-induced increase in intracellular calcium may function as a molecular switch as described for the neuronal \( Ca^{2+} \)-binding protein calmodulin, which regulates the action of various protein kinases and phosphatases (17). These in turn change the phosphorylation state and thus the permeability of ion channels and receptors, resulting in an altered \( G_w \). Third, there is electrical continuity via gap junctions among various cell types. Gap junctions provide a third mechanism by which anoxia, \( Ca^{2+} \), and adenosine may effect \( G_w \). Gap junctions provide extensive coupling among glia in the turtle cerebral cortex (7), and experimental evidence suggests that gap junctions can be modulated functionally in response to the neurotransmitter application (13) and second messenger action (18). Preliminary work in our lab using Lucifer yellow to observe nonsynaptic connectivity among turtle neurons showed physical alterations in neuronal coupling between pyramidal cells that occur on anoxic exposure (unpublished data). Therefore, it is possible that induction of anoxia triggers a series of second messenger cascades that doses gap junctions and decrease \( G_w \).

In summary, our data outline a cellular response to anoxia that includes increased adenosine levels, stimulation of \( A_1 \) receptors, increases in intracellular \( Ca^{2+} \) and possibly cAMP, activation of protein kinases and phosphatases that target one or more of the three possible candidates capable of changing whole cell permeability, and therefore, conductance. We conclude that adenosine and \( Ca^{2+} \) play a major role in the anoxic regulation of \( G_w \) and are involved in the natural cellular anoxic defense mechanism of this species.

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