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Channel arrest: implications from membrane resistance in turtle neurons

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DOLL, CHRISTOPHER J., PETER W. HOCHACHKA, AND PETER B. REINER. *Channel arrest: implications from membrane resistance in turtle neurons.* Am. J. Physiol. 261 (Regulatory Integrative Comp. Physiol. 30): R1321–R1324, 1991.—A widespread defense strategy used by hypoxia-tolerant animals is metabolic depression. One possible mechanism for metabolic depression is "channel arrest." This hypothesis predicts that ion leakage through plasma membrane leakage channels is reduced during an anoxic episode. The decreased ion flux would result in the conservation of energy through the reduction of ATP-demanding ion pumping. We tested this hypothesis with the anoxia-tolerant turtle (*Chrysemys picta*) as a model system. With intracellular recording used in cortical slices, whole cell input resistance and specific membrane resistivity were monitored under control and anoxic conditions. There were no significant changes in resistance, indicating that the channel arrest defense mechanism was not utilized for energy conservation during short-term anoxia (≤120 min).

intracellular recording; ion leakage; ion pumping; turtle cortical neurons

BRAINS OF HOMEOTHERMS are very sensitive to oxygen deprivation. The evolutionary strategy in this group, even among diving species, has been to develop mechanisms that prolong and conserve oxygen stores for the nervous system (2). In marked contrast to this survival strategy, many ectotherms have developed nervous systems that can tolerate and function during long periods of anoxia. Most notable of these is the turtle (*Chrysemys picta*), documented to survive for >6 mo at 3°C (23) and 48 h at 25°C (16) in anoxic environments. We have documented neurons in vitro to remain intact and to function for up to 18 h of anoxia at 25°C (4).

We suspect that the turtle brain is able to survive anoxia because it has evolved mechanisms that allow adequate production of energy (ATP) throughout the insult. These mechanisms include a low normoxic metabolic rate (22), large glycolytic capacity (22), presence of a transient Pasteur effect (10), and metabolic depression. Depressed metabolism for the turtle brain during anoxia is supported by both in vivo (11) and in vitro (20) studies. Reduced metabolism during times of stress has been documented for several species of invertebrates and vertebrates, but precisely which metabolic processes are being suppressed is unknown (9).

All cell membranes leak ions. Leakage is the result of both intracellular and extracellular ions flowing down their electrochemical gradients. Several cellular processes contribute to this phenomenon in neurons, including activation of voltage- and ligand-gated channels, neurotransmitter release and uptake, co- and countertransport systems, and leakage channels (voltage-independent ion channels) (7). Maintenance of a homeostatic intracellular environment requires the redistribution of these ions through the use of energy-demanding pumping systems such as that of Na^+-K^+-ATPase, which may consume as much as 50% of the cell's resting metabolic rate (15). One mechanism by which the turtle brain could reduce its metabolism would be to reduce ion leakage (12), which could conceivably occur through the reduction of any of the above leakage processes. In this paper we explore one facet of the "channel arrest" hypothesis, which predicts that leakage channels close in response to anoxia (8).

MATERIALS AND METHODS

Turtles (*C. picta*) were cold anesthetized before decapitation. The brain was rapidly dissected free and immersed in precooled oxygenated artificial cerebrospinal fluid (aCSF). The cortical tissue was then dissected free as described by Connors and Kreigstein (3). Slices were stored at room temperature ≈22°C in a holding chamber for at least 60 min until their use in the recording chamber. The recording chamber was a modification of a previous design (6) in which slices were continuously superfused with aCSF at a flow rate of 1.5–2.0 ml/min.

Intracellular recordings were carried out using micropipettes (1.2 mm OD) filled with 2 M KCl with resistances ranging from 30 to 70 MΩ connected to an Axoclamp 2A amplifier. Data were collected using the Pclamp suite of programs and an Axolab 1100 interface, which also served to generate current commands. Data

were also independently digitized at 49 kHz and stored on videotape for off-line analysis.

Pyramidal neurons in the turtle were identified by location and action-potential shape as well as input resistance and time constants as previously discussed (3). Criteria for healthy cells included a stable membrane potential for 15 min maintained without the assistance of current, positive-going action potentials, and whole cell input resistance (R_w) >70 MΩ. Ground shift was corrected by subtracting the reading after withdrawing from the cell from the membrane potentials recorded during the experiment.

R_w and time constant (T_c) were calculated using hyperpolarizing or depolarizing current pulses of 500-ms duration sufficient to elicit a 10 ± 2.5-mV change in membrane potential. R_w was calculated using the equation

$$V = IR_w \quad (1)$$

where V is the change in membrane potential and I is the current (Ohm's law). T_c was calculated by fitting the membrane charging curves to the equation

$$Y = A_0 + A_1 e^{-t/T_c} \quad (2)$$

where Y is the voltage at any given time t, A_0 is the offset, and A_1 is the maximum voltage, using the Clampfit feature of Pclamp. Specific membrane resistance (R_m) was calculated from the equation

$$T_c = R_m \times C_m \quad (3)$$

where C_m is the capacitance of the membrane per unit area. All computer fits showed a >0.9800 least squares residual (R) value or they were rejected.

The aCSF for the turtle was a modification of that used by Connors and Kreigstein (3) and consisted of (in mM) 96.5 NaCl, 2.6 KCl, 2.5 $CaCl_2$, 2.0 $MgCl_2$, 2.0 NaH_2PO_4, 10 glucose, 26.5 $NaHCO_3$, and 0.03 phenol red as a pH indicator. For normoxic solutions, the aCSF was bubbled with 95% O_2-5% CO_2. For anoxia, the aCSF was bubbled with 95% N_2-5% CO_2. Final pH of both solutions was 7.4 when saturated with the gases used. Turtles were obtained from Lemberger, chemicals were purchased from Sigma Chemical and Fisher Scientific, data were analyzed using Pclamp software (Axon Instruments), statistics were carried out using Systat (SYSTAT), and graphics were done using Sigmaplot (Jandel).

RESULTS

We tested whether leakage channels were closing in response to an anoxic episode using intracellular recording. This method allowed the continuous monitoring of R_w, T_c, and R_m. Two separate measures of resistance were performed, R_w and R_m (Fig. 1). R_w gives the resistance of the whole cell. If the area of the cell were known, resistance per unit area could be calculated (R_m). Surface area of the cell is unknown, but R_m can be derived by using *Eq. 3*. The calculation is based on a 1 $\mu F/cm^2$ capacitance of the membrane. This number, although not specific for turtle neurons, is an average for biological membranes (7). The capacitance measure is necessary for comparison purposes only, and its exact value is not important as-

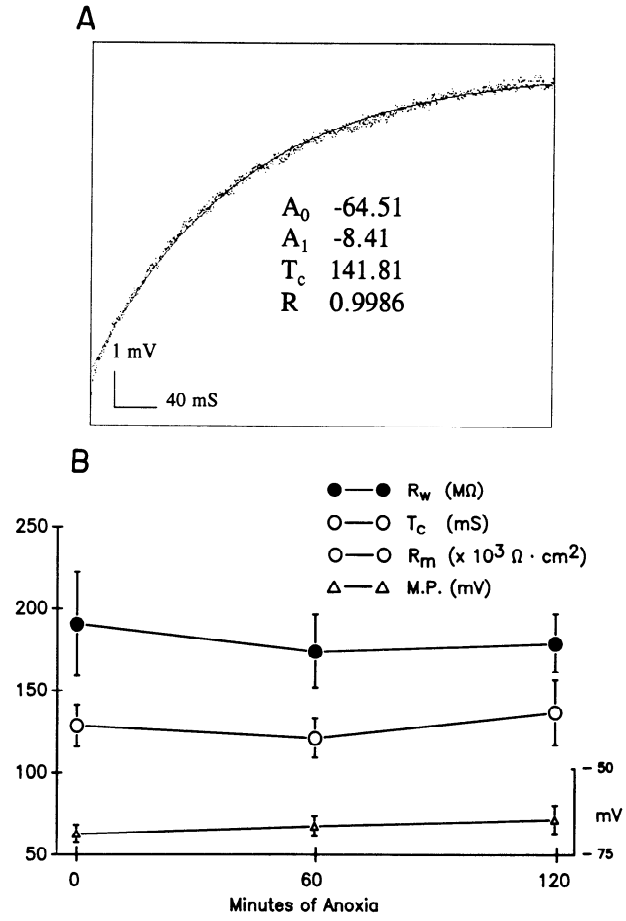

FIG. 1. *A*: an example of a computer-fit membrane charging curve for a single neuron. Time constants (T_c) were computer fit using the Clampfit feature of Pclamp. All fits showed a least squares residual value (R) >0.9800 or they were rejected. Two independent measures of membrane resistivity were calculated: whole cell input resistance (R_w) and specific membrane resistivity (R_m). A_0, offset; A_1, maximum voltage. *B*: changes in R_w, R_m, T_c, and membrane potential (MP) over a period of 120 min of anoxia for 8 repeatedly measured cells from 8 turtles. R_m is calculated from T_c assuming 1 $\mu F/cm^2$ capacitance for the membrane. Data are illustrated with SE bars. All data were analyzed by a repeated measures design over time. There was no significance of fit for either a linear or a quadratic function ($P > 0.05$) for any measured parameter.

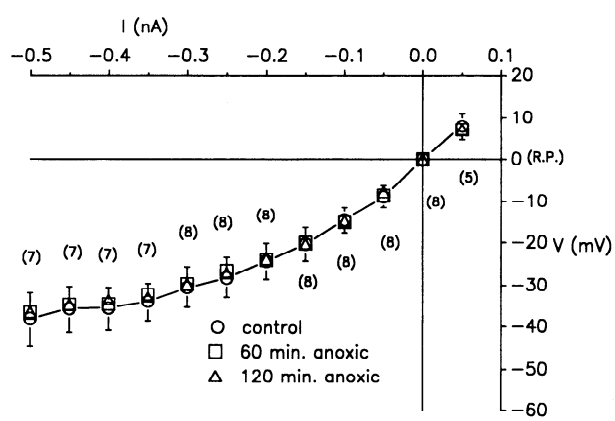

FIG. 2. Current (I)-voltage (V) plot for 8 repeatedly measured cells over a time course of 120 min of anoxia (0.1 to −0.5 by 0.05-nA steps). Numbers in parentheses are no. of cells for that point set. Data are illustrated with 95% confidence bars. Each point set was analyzed across anoxic time course for either a linear or quadratic fit (repeated measures design). There was no significance for increasing resistance over time ($P > 0.05$). RP, resting potential.

suming that it does not change over anoxia, which is an unlikely event because changes in the lipid composition of the membrane would have to occur. Two measures of resistance were performed for two reasons. First, these two measures of resistance are independently derived and thus provide a way of double-checking the values obtained. Although the values for R_m and R_w are different, their changes through anoxia should parallel each other, as supported by Fig. 1. Second, we have found that the use of T_c tends to be more sensitive to small changes in resistance than R_m. However, the calculation of R_w is based on no assumptions and is therefore the standard way of measuring resistance.

A third observation of changes in resistance is obtained through the current-voltage plot (Fig. 2). The slope of the line represents voltage-dependent changes in R_w. As can be seen in Fig. 2, the voltage dependence of R_w does not significantly change over the course of 120 min of anoxia. Thus neither steady-state nor voltage-dependent R_w changes during anoxia.

DISCUSSION

In an earlier paper (4), we documented that turtle cortical neurons were able to withstand long-term exposure to both N_2 and pharmacological anoxia (N_2 + cyanide) without dramatically losing resistance or membrane potential as observed in the rat cortical neuron. In this study, we performed detailed measures of turtle cortical neuronal resistance to assess whether ion channels are closing in response to an anoxic episode.

We chose to use anoxia over pharmacological anoxia for several reasons. First, the anoxic chamber aCSF was tested for oxygen contamination using a radiometer and an O_2 electrode. No detectable oxygen was measured compared with a control (sodium dithionite) solution, indicating that our chamber provides a good anoxic mimic. Second, we have shown that in this same chamber setup, the rat cortical and hippocampal neuron shows degenerative signs within 1 min of exposure to the anoxic solution and complete loss of membrane resistance and potential in ~30 min (4, 19). Third, the use of artificial substances such as cyanide may be affecting synaptic activity and ion channels, causing artificial increases in membrane resistance (1).

Channel arrest predicts an increase in R_m and R_w with anoxia. The results presented here do not support this expectation. This hypothesis was proposed as a mechanism of metabolic depression because of the high amount of energy assumed to be required to maintain ion homeostasis. However, evidence for this is not conclusive. Cell preparations where oxygen consumption is monitored in the presence and absence of ouabain (Na^+-K^+-ATPase inhibitor) create two problems. First, ouabain rapidly depolarizes the cell (4, 5); as a result, the cell becomes increasingly unhealthy, causing an overestimation of energy consumed by ion pumping. Second, plasma membrane ion pumps may be relying on glycolytic ATP production for function rather than on oxidative ATP production (13). Glycolytic energy production in relation to oxidative energy production has never been simultaneously measured during a ouabain experiment. One reason neurons were used in this study was because of their large surface-to-volume ratio, suggesting a comparatively high metabolic maintenance cost for ion homeostasis.

Although only cortical pyramidal neurons were investigated in this study, we hypothesize that the results can be applied to other types of turtle neurons for several reasons. The cells used in this experiment survive anoxia, indicating that channel arrest is not necessary for survival of the neuron. Intracellular measurements of R_w in turtle cerebellum during anoxia do not support acute channel arrest defense mechanisms (18). Both in vivo (11) and in vitro (20) studies indicate a 50% reduction in metabolism by 120 min into anoxia, suggesting that metabolic depression is occurring via other mechanisms.

Two questions need to be more fully addressed before mechanisms of metabolic depression are investigated further. First, what fraction of metabolism does leakage consume in the brain? If it is small, as some evidence suggests (14, 17), then channel arrest is not sufficient to explain metabolic depression in the turtle brain. Second, accurate measures of metabolic depression are needed. Current measures of turtle brain metabolic depression in vivo are based on brain lactate accumulation over the duration of anoxic insult (11). Because lactate is not trapped in the tissue (21), the concentration of lactate that remains may underestimate lactate produced, resulting in overestimates of metabolic depression.

As a final note, the rate of adenosinetriphosphatase activity was not directly measured. Pumping activity may be reduced during anoxia because ion leakage can occur through a variety of processes of which leakage channels are but one, as reviewed in the introduction. Thus reduced pumping activity during anoxia may occur through reduction of other leakage processes, such as suppression of electrical activity. However, until the metabolic costs of leakage processes are known, mechanisms of metabolic depression will not be fully understood.

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REFERENCES

1. AITKEN, P. G., AND D. J. BRAITMAN. The effects of cyanide on neural and synaptic function in hippocampal slices. *Neurotoxicology* 10: 239–248, 1989.
2. BUTLER, P. J., AND D. R. JONES. The comparative physiology of diving in vertebrates. *Adv. Comp. Physiol. Biochem.* 8: 179–364, 1982.
3. CONNORS, B. W., AND A. R. KREIGSTEIN. Cellular physiology of the turtle visual cortex: distinctive properties of pyramidal and stellate neurons. *J. Neurosci.* 6: 164–177, 1986.
4. DOLL, C. J., P. W. HOCHACHKA, AND P. B. REINER. Effects of anoxia and metabolic arrest on turtle and rat cortical neurons. *Am.*

J. Physiol. 260 (*Regulatory Integrative Comp. Physiol.* 29): R747–R755, 1991.
5. FUJIWARA, N., H. HIGASHI, K. SHIMOJI, AND M. YOSHIMURA. Effects of hypoxia on rat hippocampal neurons in vitro. *J. Physiol. Lond.* 384: 131–151, 1987.
6. HAAS, H. L., B. SCHAERER, AND M. VOSMANSKY. A simple perfusion chamber for the study of nervous tissue slices in vitro. *Neurosci. Methods* 1: 323–325, 1979.
7. HILLE, B. *Ionic Channels of Excitable Membranes.* Sunderland, MA: Sinauer, 1984, p. 426.
8. HOCHACHKA, P. W. Defense strategies against hypoxia and hypothermia. *Science Wash. DC* 231: 234–241, 1986.
9. HOCHAHCKA, P. W., AND M. GUPPY. *Metabolic Arrest and the Control of Biological Time.* Cambridge, MA: Harvard Univ. Press, 1987, p. 227.
10. KELLY, D. A., AND K. B. STOREY. Organ-specific control of glycolysis in anoxic turtles. *Am. J. Physiol.* 255 (*Regulatory Integrative Comp. Physiol.* 24): R774–R779, 1988.
11. LUTZ, P. L., P. M. MCMAHON, M. ROSENTHAL, AND T. J. SICK. Relationship between aerobic and anaerobic energy production in turtle brain in situ. *Am. J. Physiol.* 247 (*Regulatory Integrative Comp. Physiol.* 16): R740–R744, 1984.
12. LUTZ, P. L., M. ROSENTHAL, AND T. J. SICK. Living without oxygen: turtle brain as a model of anaerobic metabolism. *Mol. Physiol.* 8: 411–425, 1985.
13. LYNCH, R. M., AND R. S. BALABAN. Coupling of aerobic glycolysis and Na^+-K^+-ATPase in renal cell line MDCK. *Am. J. Physiol.* 253 (*Cell Physiol.* 22): C269–C276, 1987.
14. MATA, M., D. FINK, H. GAINER, C. SMITH, L. DAVIDSEN, H. SAVAKI, W. J. SCHWARTZ, AND L. SOKOLOFF. Activity-dependent energy metabolism in rat posterior pituitary primarily reflects sodium pump activity. *J. Neurochem.* 34: 213–215, 1980.
15. MCBRIDE, B. W., AND L. P. MILLIGAN. Magnitude of ouabain-sensitive respiration of lamb hepatocytes (*Ovis aries*). *Int. J. Biochem.* 17: 43–49, 1985.
16. MUSACCHIA, X. J. The viability of *Chrysmys picta* submerged at various temperatures. *Physiol. Zool.* 32: 47–50, 1959.
17. OKAMOTO, K., AND J. H. QUASTEL. Water uptake and energy metabolism in brain slices from the rat. *Biochem. J.* 120: 25–36, 1970.
18. PEREZ-PINZON, M., C. CHAN, M. ROSENTHAL, AND T. SICK. Changes in membrane properties and survival strategies for turtle brain during anoxia. *Soc. Neurosci. Abstr.* 16: 741, 1990.
19. REINER, P. B., A. G. LAYCOCK, AND C. J. DOLL. A pharmacological model of ischemia in the hippocampal slice. *Neurosci. Lett.* 119: 175–178, 1990.
20. ROBIN, E. D., N. LEWISTON, A. NEWMAN, L. M. SIMON, AND J. THEODORE. Bioenergetic pattern of turtle brain and resistance to profound loss of mitochondrial ATP generation. *Proc. Natl. Acad. Sci. USA* 76: 3922–3926, 1979.
21. SCHURR, A., C. A. WEST, AND B. M. RIGOR. Lactate-supported synaptic function in the rat hippocampal slice preparation. *Science Wash. DC* 240: 1326–1328, 1988.
22. SUAREZ, R. K., C. J. DOLL, A. E. BUIE, T. G. WEST, G. D. FUNK, AND P. W. HOCHACHKA. Turtles and rats: a biochemical comparison of anoxia-tolerant and anoxia-sensitive brains. *Am. J. Physiol.* 257 (*Regulatory Integrative Comp. Physiol.* 26): R1083–R1088, 1989.
23. ULTSCH, G. The viability of nearctic freshwater turtles submerged in anoxia and normoxia at 3 and 10°C. *Comp. Biochem. Physiol. A Comp. Physiol.* 81: 607–611, 1985.