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Protein kinase C in turtle brain: changes in enzyme activity during anoxia

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Abstract. Protein kinase C from the anoxia-tolerant turtle *Pseudemys scripta elegans* was investigated to determine its role in mediating changes in brain metabolism associated with anoxia. Measurements of protein kinase C distribution in cytosol and membrane-associated fractions of cerebrum and hindbrain were performed with warm (18 °C)- and cold (7 °C)-acclimated animals exposed to normoxic or anoxic conditions. In cerebrum, the percentage of bound protein kinase C decreased from 48.5% to 35.1% in warm-acclimated animals and from 45.0% to 25.6% in cold-acclimated animals. In the hindbrain, bound protein kinase C increased from 45.0% to 72.9% in warm-acclimated animals and from 40.3% to 68.8% in cold-acclimated animals. The presence of three distinct protein kinase C isozymes (Types I, II and III) was confirmed by hydroxylapatite chromatography. The distribution of isozymes between cytosolic and membrane-associated fractions in cerebrum was 24% I, 37% II and 39% III (cytosolic) and 32% I, 35% II and 34% III (membrane-associated). In the hindbrain, the protein kinase C isozyme distribution was 34% I, 40% II and 26% III (cytosolic) and 18% I, 47% II and 35% III (membrane-associated). Kinetic characterization of the three isozymes showed that Type I was 27% activated by Ca^{2+}, whereas Types II and III were only 4% and 2% activated by Ca^{2+}, respectively. Full activity for all enzymes was observed only in the presence of phosphatidylserine and diacylglycerol. No differences in the K_m for ATP, the K_a for Ca^{2+} or the K_a for phosphatidylserine were observed.

Key words: Anoxia – Protein kinase C – Phosphorylation – Brain – Turtle, *Pseudemys elegans*

Abbreviations: EDTA, ethylenediaminetetra-acetic acid; EGTA, ethyleneglycol-*bis*-(2-aminoethyl)-tetraacetic acid; H7, 1-(5-isoquinolinyl sulphonyl)-2-methyl-piperazine; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol; PIPES, piperazine-n,n′-bis(2-ethanesulfonic acid); PKC, protein kinase C; PS, phosphatidylserine; TCA, trichloroacetic acid

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Introduction

Turtles show a remarkable ability to endure prolonged periods of apnea during diving and underwater hibernation (Belkin 1966; Ultsch and Jackson 1982). This ability is dependent on adaptive strategies which may include: (1) the existence of large stores of glycogen in all tissues, (2) a high tolerance for metabolic acidosis, as well as the ability to buffer and retard the development of acidosis, and (3) an ability to depress metabolic rates to levels that are 15% of the normoxic values (Jackson 1968; Penny 1974). The combination of these processes permit cells to remain functional for long periods of anoxia (up to 4 months at 3 °C). For example, turtle brain retains excitability and ion balance over 48 h of N_2 inspiration even though oxidative phosphorylation is completely shut down (Lutz et al. 1984, 1985; Chih et al. 1989).

Studies of glycolytic regulation during environmental anoxia, hibernation and estivation in a wide variety of animals have served to illustrate the molecular mechanisms which mediate metabolic depression in these animals (Storey and Storey 1990). In *Trachemys scripta elegans,* these mechanisms include: (1) control of carbohydrate utilization via fructose 2,6-bisphosphate regulation of phosphofructokinase (Brooks and Storey 1988, 1989), (2) changes in the percentages of enzymes associated with subcellular structures to directly alter enzyme activity (Duncan and Storey 1991), and (3) covalent modification of regulatory enzymes via reversible protein phosphorylation (Brooks and Storey 1988, 1989).

Cellular control of enzyme covalent modification, such as phosphorylation, is mediated by second-messenger-dependent protein kinases such as PKC, cAMP-dependent protein kinase and cGMP-dependent protein kinase. The Ca^{2+}- and phospholipid-dependent PKC is involved in regulating various cellular functions including secretion, muscle contration and nerve signal propagation all related to changes in protein phosphorylation (Nishizuka 1989; Lotan et al. 1990; Vaccarino et al. 1991). Relationships between signalling pathways may also be mediated by PKC; recent studies

suggest that PKC may potentiate cAMP- and cGMP-mediated responses (Ho et al. 1988; Aasheim et al. 1989; Houslay 1991). PKC activity is regulated by changes in cellular free Ca^{2+} and free lipid concentrations which cause a translocation of PKC from the cytosol (where it is inactive) to the membrane where it becomes activated through binding to membrane lipids (Ho et al. 1988; Bell and Burns 1991). Thus, activity in vivo may be monitored by following PKC translocation before and after physiological stress (Boneh and Tenenhouse 1988; Cleland et al. 1989).

This study examines the possible role of PKC in mediating the metabolic response of turtle brain to environmental anoxia. Overall activity was monitored by following enzyme translocation and the PKC isozymes were kinetically characterized to assess possible differences which may play a role in enzyme regulation. The potential role of PKC during anoxia was suggested by (1) previously observed changes in enzyme phosphorylation patterns during anoxia (Brooks and Storey 1988, 1989), (2) the involvement of Ca^{2+} ions in suppressing the Pasteur effect during anoxia in mammalian brains (Kauppinen and Nicholls 1986), and (3) the role of protein kinases in modulating ion channels (Lotan et al. 1990) and neuronal excitability (Vaccarino et al. 1991).

Materials and methods

Chemicals and animals. Whatman DE–52 was purchased from Canlab (Montreal, P.Q., Canada) hydroxylapatite from Bio-Rad (Mississauga, Ont., Canada) and gamma-labelled ^{32}P–ATP (6000 Ci · $mmol^{-1}$) from Amersham (Montreal). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), except for staurosporine which was purchased from Boehringer-Mannheim (Montreal). Red-eared slider turtles *(Trachemys scripta elegans)* were obtained from Boreal Canada and held in large tanks with running, dechlorinated water at 18 °C for 3 weeks before use. Animals were fed a diet of egg shells, lettuce and trout food pellets supplied ad libitum.

Normoxic (control) turtles were sampled directly from the tank. Anoxia (at both 18 °C and 7 °C) was imposed by submerging turtles for 5 h in a sealed tank of water that had been previously bubbled with 100% N_2 gas for 16 h. Animals were killed by decapitation and brain tissue was divided into cerebrum and hindbrain (optic lobe, pons, cerebellum and medulla oblongata, brain stem). The tissue was quickly removed and frozen at −80 °C in liquid N_2. In agreement with Crumrine and LaManna (1991) we found that freezing had no effect on the distribution of PKC between cytosol and membrane-bound fractions (data not shown). For low temperature studies, turtles were acclimated at 7 °C for 2 days prior to imposition of anoxia.

Preparation of semi-purified PKC. All steps were performed at 4 °C. Brain was homogenized 1:4 (w:v) in buffer A [containing (mmol · l^{-1}): 20 PIPES (pH 6.6), 10 β-mercaptoethanol, 5 EDTA, 5 EGTA, and 250 sucrose, plus (μg · ml^{-1}) 10 leupeptin and 1 aprotinin] for 20 s in an Ultra-Turrax (Tekmar) tissue homogenizer and centrifuged at 15000 × *g* for 30 min (Sorvall, SS–34 head). The supernatant (cytosolic fraction) was immediately loaded onto a Whatman DE–52 ion-exchange column (0.5 ml of bed volume per 0.2 g tissue) equilibrated in buffer A and washed with 5 volumes of buffer B [containing (mmol · l^{-1}): 20 HEPES (pH 7.4), 20 NaCl, 10 β-mercaptoethanol, 1 EGTA, 1 EDTA]. Enzyme activity was eluted by washing the column with 2 ml 0.5 mol NaCl · l^{-1} in buffer B; this concentration was optimal as determined from the elution position in a NaCl gradient. The pellet was rehomogenized in one-half the original homogenate volume of buffer A plus 0.1% Triton X–100, incubated on ice for 15 min, and centrifuged at 15000 × *g* for 30 min. The resulting supernatant (membrane-associated fraction) was loaded onto a fresh DE–52 column and treated in an identical fashion as the supernatant.

PKC isozymes were identified by hydroxylapatite column chromatography. The 0.5-mol · l^{-1} salt washes from the DE–52 columns were diluted 1:3 (v:v) with buffer C [containing (mmol · l^{-1}): 20 potassium phosphate (pH 7.0), 10 β-mercaptoethanol, 0.1 EDTA, 0.1 EGTA; 20% glycerol] to reduce the ionic strength and loaded onto 1-ml hydroxylapatite columns pre-equilibrated in buffer C. Columns were washed with buffer C until the absorbance at 280 nm was zero. The PKC isozymes were eluted with a 30-ml gradient of 20–200 mmol potassium phosphate · l^{-1} in buffer C. Phosphate concentration was monitored by following changes in conductivity.

PKC activity assay. PKC activity was assayed by following the incorporation of [^{32}P]phosphate into histone III–S (Diaz-Guerra et al. 1991). The final assay contained (mmol · l^{-1}): 20 HEPES (pH 7.0), 1 magnesium acetate, 10 β-mercaptoethanol, 0.5 $CaCl_2$, plus (μg · ml^{-1}) 20 PS, 200 histone III–S, 2 OAG with 10 μmol ^{32}P–ATP · l^{-1} (0.2 μCi per assay) in a final volume of 150 μl. Reactions were incubated for 5 min at 22 °C and were terminated by addition of 1 ml of 5% TCA/10 mmol H_3PO_4 · l^{-1}. Samples were incubated for 10 min on ice and filtered through Whatman GF/c filters. Filters were washed four times with 5% TCA/10 mmol H_3PO_4 · l^{-1} and then counted for Cerenkov radiation. Two control reactions were always included: control reaction 1 was immediately stopped by addition of 5% TCA/10 mmol H_3PO_4 · l^{-1} (zero time control). Control reaction 2 contained 1 mmol EGTA · l^{-1} instead of $CaCl_2$ (no-Ca^{2+} control). Both tubes gave similar results. Kinetic constants were determined by varying the appropriate substrate or activator. Activation energies were determined by incubating PKC at temperatures between 5 and 35 °C. The pH of the reaction was determined at 22 °C and allowed to vary with temperature. Although pH changed by as much as 0.45 units when compared with the assay at 22 °C, this did not appreciably affect activity (see Fig. 2). One unit (IU) is defined as the amount of enzyme required to transfer 1 pmol phosphate · min^{-1}.

Results

The total PKC activity in turtle brain as well as the percentage of PKC associated with the plasma membrane for warm- (18 °C) and cold-acclimated (7 °C) ani-

Table 1. Effect of anoxia and temperature on the total and membrane-associated PKC activity of turtle cerebrum and hindbrain

	Condition	Total activity (IU · g ww^{-1})	% Membrane associated
Cerebrum			
18 °C-acclimated	Normoxic	223.7 ± 28.5	48.5 ± 2.5
	Anoxic	246.8 ± 22.4	35.1 ± 4.7*
7 °C-acclimated	Normoxic	203.0 ± 44.8	46.2 ± 6.3
	Anoxic	225.5 ± 22.8	25.6 ± 4.5*
Hindbrain			
18 °C-acclimated	Normoxic	187.8 ± 22.8	45.0 ± 3.4
	Anoxic	142.5 ± 16.2	72.9 ± 5.2*
7 °C-acclimated	Normoxic	133.5 ± 55.2	42.4 ± 7.4
	Anoxic	128.9 ± 51.3	68.8 ± 8.4*

Values are means ± SEM for $n = 3$ or $n = 4$ animals in each condition. Asterisk denotes values which are significantly different from corresponding normoxic values at the $P < 0.05$ level as determined by the two-tailed Student's *t*-test

mals under normoxic and anoxic conditions is presented in Table 1. These temperatures represent body temperatures for animals on land (18 °C) or submerged in water (7 °C) during the summer months. The total PKC activity in turtle brain was 5- to 10-fold lower than that of mammalian brain when the difference in the assay temperature was taken into account [mammalian brains were assayed at 37 °C, turtle enzymes at 22 °C; Huang et al. (1987)]. Differences between cerebrum and hindbrain were also apparent: PKC activity in hindbrain averaged only 65.9% of that found in cerebrum. Exposing turtles to anoxia by submergence in N_2-bubbled water caused significant changes in the percentage of PKC associated with membranes. In the cerebrum 13.4% (18 °C) and 19.4% (7 °C) less PKC was membrane associated. In the hindbrain the amount of membrane-associated PKC increased by 28% (18 °C) and 28.5% (7 °C).

Nine members of the PKC family have been identified by molecular cloning (Nishizuka 1989) of which seven protein isozymes have now been isolated. The isozymes are expressed differently in different tissues (Huang et al. 1986; Ono et al. 1988). For example, Type I occurs only in neural tissue (Huang et al. 1986, 1987; Marais and Parker 1989), whereas Types II and III are more ubiquitous. Hydroxylapatite chromatography of a crude PKC homogenate revealed the presence of three PKC isozymes (Fig. 1). These were identified by the relative positions of their elution peaks as described in Huang et al. (1987). The isozyme distribution of Fig. 1 was obtained from a crude homogenate which contained soluble plus membrane-associated PKC. Analysis of the isozyme pattern from cytosolic and membrane-associated PKC activities revealed that some isozymes were not equally distributed between these two fractions. Comparison of the isozymic distribution between these two fractions revealed that a larger proportion of the membrane-bound activity was Type I isozyme and smaller proportion was Type III. This pattern was exactly opposite to that found in the hindbrain where a smaller proportion of the membrane-bound activity consisted of Type I isozyme and a larger proportion consisted of the Type III isozyme. The distribution of Type II was similar in both fractions making up 35–40% of the cytosolic or membrane-bound PKC activity (Table 2).

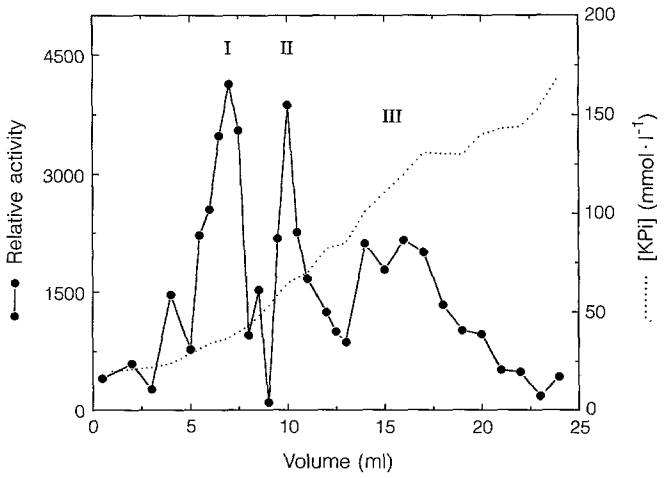

Fig. 1. Hydroxylapatite chromatography of turtle brain PKC types I, II and III. Normoxic turtle brain was homogenized in buffer A + 0.2% Triton X-100, centrifuged, and chromatographed on DE-52. This fraction represents total brain PKC activity. The eluate was loaded onto a 1-ml hydroxylapatite column and developed as described in Materials and methods. Fractions of 0.5 ml were collected and assayed for PKC activity. Relative activity in the presence of $CaCl_2$ was subtracted from that measured in the absence of $CaCl_2$ (+EGTA)

The Ca^{2+} and phospholipid requirements of the three PKC isozymes were investigated to determine how each isozyme is regulated. Table 3 shows that PKC Types I, II and III were all dependent on Ca^{2+} for activity. Addition of Ca^{2+} increased Type I activity to 27% of the fully active isozyme, but Types II and III were not appreciably activated by Ca^{2+} alone. Addition of phospholipid (PS/OAG plus EGTA to chelate free Ca^{2+}) increased the activity of all three isozymes with Type I showing the greatest increase. Table 3 also shows that complete activity was dependent on the presence of both Ca^{2+} and PS + AG. All isozymes were inhibited by iso-H7 and staurosporine which have been shown to inhibit mammalian PKC isozymes, although the test with staurosporine is not definitive since staurosporine is not a specific PKC inhibitor (Kawamaoto and Hidaka 1984; Tamaoki et al. 1986).

Table 2. Turtle brain isozyme distribution in normoxic cerebrum and hindbrain

Fraction	% of cytosolic or membrane-bound activity		
	I	II	III
Cerebrum			
Cytosol	24.0 ± 2.6	37.3 ± 1.6	38.9 ± 1.1
Membrane	31.5 ± 1.1*	34.6 ± 1.6	34.0 ± 0.4*
Hindbrain			
Cytosol	33.9 ± 1.9	39.7 ± 0.3	26.4 ± 2.2
Membrane	18.1 ± 3.2*	46.9 ± 4.1	35.0 ± 3.3*

Values are means ± SEM for $n = 3$ separate determinations. * Values are significantly different from cytosol at the $P < 0.05$ level as measured by the two-tailed Student's t-test

Table 3. Effect of Ca^{2+}, phospholipids and inhibitors on the activity of turtle brain PKC isozymes I, II and III

Addition	Relative activity (%)		
	I	II	III
EGTA	0.3 ± 1	2.0 ± 2	1.2 ± 1
Ca^{2+}	27.1 ± 12	4.2 ± 3	1.6 ± 1
EGTA + PS/OAG	49.5 ± 1	28.6 ± 5	20.4 ± 8
Ca^{2+} + PS/OAG	100.0	100.0	100.0
+ H7	7.4 ± 1	3.3 ± 3	6.8 ± 1
+ Staurosporine	< 0.5	< 0.5	2.3 ± 2

Values are mean ± SEM for $n = 3$ separate determinations except for Ca^{2+} + PS/OAG which was set at 100%. Where indicated, 50 μmol · l^{-1} 1-(5-isoquinolinyl sulphonyl)-2-methyl piperazine (H7) or 0.2 μg · ml^{-1} staurosporine was added. When present, Ca^{2+} was 0.5 mmol · l^{-1}, EDTA was 1 mmol · l^{-1}, PS (phosphatidylserine) was 20 μg · ml^{-1} and OAG (1-oleoyl-2-acetyl-*rac*-glycerol) was 2 μg · ml^{-1}

Table 4. Kinetic constants of turtle brain PKC isozymes

Substrate/activator	Value of constant		
	I	II	III
K_m ATP (μmol · l^{-1})	27.0 ±5.4	33.8 ±13	49.7 ±7.5
K_a $CaCl_2$ (μmol · l^{-1})	4.35±0.56	3.93± 1.1	3.05±1.0
K_a PS (μg · ml^{-1})	0.23±0.09	0.34± 0.19	0.35±0.13

Values are means±SEM for $n=3$ separate determinations

Table 5. Effect of temperature on the maximal activity of three different PKC isozymes from turtle brain

Isozyme	E_a (kcal · mol^{-1})	A	Q_{10}
I	12.8±2.7	$4.32 \cdot 10^{13}$	2.20
II	10.7±1.6	$1.98 \cdot 10^{12}$	1.94
III	5.9±1.6*	$6.97 \cdot 10^{7}$	1.44

Activation energy (E_a) values represent slope±SD for pooled values from two determinations. Values of intercepts (A) and (Q_{10}) are means of two separate determinations. Data was plotted according to the Arrhenius Equation: $\ln(\text{rate}) = \ln A + \frac{-E_a}{(R \cdot T)}$. Temperature was varied by 5-°C increments between 5 and 35 °C. The plots were linear throughout this range. Q_{10} values were calculated from: $\ln(Q_{10}) = \frac{R \cdot T_1 \cdot T_2 \cdot E_a}{13}$ with $T_1 = 5$ °C and $T_2 = 18$ °C (two temperatures naturally encountered by the turtle). * Significantly different from the Type I and Type II isozymes as determined by an F-test for differences between two regression coefficients (Sokal and Rolf 1981)

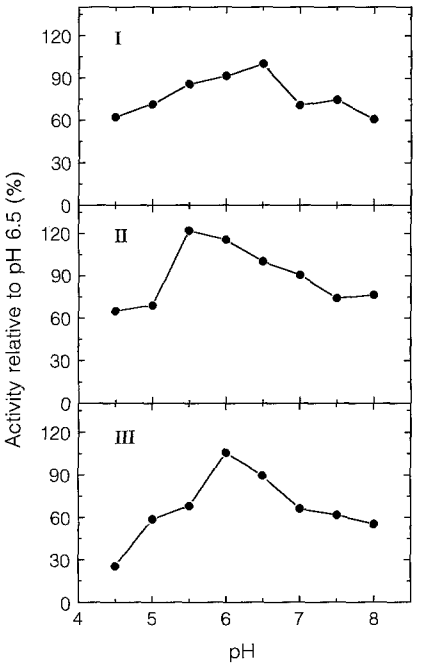

Fig. 2. Activity versus pH profiles for the three PKC isozymes identified from hydroxylapatite chromatography. The pH was varied by addition of acid or base to a mixture containing (mmol · l^{-1}): 20 HEPES, 20 PIPES, 10 β-mercaptoethanol, 1 magnesium acetate, and (μg · ml^{-1}): 200 histone III-S, 20 PS and 2 OAG. PKC and ^{32}P–ATP were then added and the reaction incubated as described in Materials and methods. Points represent means of two separate determinations

The kinetic constants for ATP, Ca^{2+} and phospholipid for the three isozymes are presented in Table 4. All isozymes had a high affinity for ATP and PS with K_m values for ATP between 27 and 50 μmol · l^{-1} and K_a values for PS between 0.23 and 0.35 μg · ml^{-1}. There were no statistically significant differences between the three isozymes in the values for any of the constants. Analysis of the temperature dependence of PKC Types I, II and III is presented in Table 5. The relationship between the logarithm of the reaction velocity and the reciprocal temperature was linear throughout the range tested (5–35 °C). Types I and II showed the greatest temperature dependence with Q_{10} values of approximately 2; Type III was less temperature dependent with a Q_{10} of 1.44. Because of the well-characterized decrease in tissue pH associated with anoxia (Lutz 1989) the pH optima of the three PKC isozymes were also determined. Figure 2 shows that the pH optima of turtle PKC isozymes were 6.5, 5.5 and 6.0 for Types I, II and III, respectively.

Discussion

Analysis of PKC binding in anoxic and normoxic turtle brain shows that changes in PKC activity may be intimately associated with the metabolic reorganization that accompanies the anoxia response in turtle brains. The changes in PKC activity were inferred by changes in the percentage of enzyme that was associated with the membrane fraction during anoxia (Table 1). In several mammalian cell lines (pineal cells, liver cells, skeletal muscle) increased enzyme activity has been associated with a translocation of the enzyme from cytosol to particulate fractions (Boneh and Tenenhouse 1988; Ho et al. 1988, 1989; Cleland et al. 1989). This increase is thought to arise from PKC binding to phospholipids in the plasma membrane (Bell and Burns 1991); membrane phospholipids, and in particular PS and OAG, activate mammalian PKC several fold in vitro. Table 3 shows that this is also true for turtle brain PKC.

The data in Table 1 show that anoxia had a significant effect on PKC activity. However, this effect was opposite in direction when cerebrum and hindbrain were compared; binding decreased in cerebrum and increased in the hindbrain in anoxic animals. These results suggest that either (1) different regions of the turtle brain have different metabolic activities during anoxia, (2) PKC plays different roles in regulating metabolism in each brain region, or (3) the time-courses for depressing metabolic processes in cerebrum and hindbrain are different. The importance of PKC in regulating ion channels, and ultimately brain excitability, has been demonstrated in several studies. For example, increases in PKC activity were associated with a decrease in Na^+ and K^+ channel permeability in *Xenopus* oocytes injected with chick brain PKC (Lotan et al. 1990). Purified PKC also phosphorylated rat brain voltage-gated Na^+ channel α subunits expressed in Chinese hamster ovary cells to inhibit channel function (Numann et al. 1991). These results suggest that changes in PKC activity are directly related to neuronal excitability in vivo. A link between decreased neuronal function and increased PKC activity in mammalian

brains has also been suggested by results from several laboratories [for a review see Kaczmarek (1987)]. By analogy with these systems, translocation of PKC to membranes in the presence of increased intracellular Ca^{2+} concentrations should decrease turtle brain neuronal activity in vivo.

At the present time it is not possible to determine the possible significance of decreased PKC activity in the cerebrum and increased PKC activity in the hindbrain. The data suggest that cerebral neuronal activity does not change (or increases) and hindbrain activity decreases in anoxic turtles. In mammals, the cerebrum is responsible for memory, cognitive thought, processing of sensory signals and initiation of movement. The hindbrain, in our rough separation of the turtle brain, is responsible for many of the lower functions including coordinating motor impulses (cerebellum), and for regulating the autonomic nervous system (Grossman 1976). It is possible that the motor control functions of the cerebrum may remain active during short-term anoxia (5 h) if the animal is to remain mobile and process sensory stimuli during the initial stages of diving. However, decreases in breathing, heart rate and changes in vasoconstriction occur almost immediately during diving (control by selected regions of the hindbrain). These speculations are supported by measurements of phosphofructokinase activity and fructose 2,6-bisphosphate concentrations in white and red muscle which suggest that muscle metabolism is not depressed after 5 h of anoxia (Brooks and Storey 1989). Irrespective of the physiological basis, the differences in PKC activity in different areas of the turtle brain is an intriguing result.

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