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EFFECT OF CHEMICAL ANOXIA ON PROTEIN KINASE C AND Na^+/K^+-ATPase IN HEPATOCYTES OF GOLDFISH (*CARASSIUS AURATUS*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

Protein kinase C (PKC) and Na^+/K^+-ATPase in hepatocytes from the anoxia-tolerant goldfish (*Carassius auratus*) and the anoxia-intolerant rainbow trout (*Oncorhynchus mykiss*) were studied to determine their role in the anoxic response of these cells. PKC and Na^+/K^+-ATPase activities were measured for up to 90 min in the absence (normoxia) and presence (chemical anoxia) of 2 mmol l^{-1} sodium cyanide. PKC activity of normoxic cells from both species remained constant for the entire experimental period. Addition of cyanide had no effect on PKC activity of trout cells, which was maintained at 25 % of maximal PKC activity. In goldfish hepatocytes, PKC activity remained constant at 56 % of maximal PKC activity for 30 min but fell to 27 % after 90 min of anoxic exposure.

ATPase activity was measured in hepatocytes exposed to 100 nmol l^{-1} phorbol-12,13-dibutyrate (PdBu), a treatment which enhanced PKC activity to its maximum level. In trout cells, there was no significant change in Na^+/K^+-ATPase activity whereas in goldfish hepatocytes a significant increase to about 150 % of the respective controls was observed.

On the basis of the experimental evidence that in hepatocytes of goldfish (1) PKC and Na^+/K^+-ATPase activities decreased in parallel during chemical anoxia and (2) a stimulation of PKC activity by PdBu increased Na^+/K^+-ATPase activity, we postulate that PKC activity in goldfish, but not in trout, may be implicated in the Na^+/K^+-ATPase inhibition observed under anoxia.

Key words: ATPase, Rb^+ uptake, K^+ efflux, phorbol ester, chemical anoxia, Na^+/K^+-ATPase, goldfish, *Carassius auratus*, rainbow trout, *Oncorhynchus mykiss*.

Introduction

In cells, whatever mechanisms are used to maintain viability under anoxia, ATP-producing and ATP-utilizing reactions must be curtailed in concert so that transmembrane ionic gradients are maintained (Hochachka, 1986; Storey and Storey, 1990). When this balance is broken, cells necessarily become sensitive to anoxia, a process which eventually leads to inhibition of ion transport with loss of transmembrane ion gradients and anoxic depolarization. However, if cells are to adapt to the new situation, a new steady state must be achieved and this must include a general reduction of metabolic processes and probably a changed allocation of energy utilization (Wieser, 1989).

In previous studies (Krumschnabel *et al.* 1994, 1996), we have described a similar strategy in hepatocytes of goldfish (*Carassius auratus*), a good anaerobic species: cells subjected to chemical anoxia (by cyanide incubation) sustained ionic gradients by a parallel reduction of both K^+ influx and K^+ efflux, while ATP concentration remained constant. In contrast, in hepatocytes of an anoxia-intolerant species, the rainbow trout (*Oncorhynchus mykiss*), chemical anoxia led to a rapid decline of ATP concentration and of active transport but K^+ efflux remained unaffected (Krumschnabel *et al.* 1996).

Although some of the molecular properties and mechanisms involved in metabolic suppression have been elucidated (see Storey, 1993), many of them remain unknown.

The present paper examines the possible role of protein kinase C (PKC) in mediating the suppression response of Na^+/K^+-ATPase activity during chemical anoxia in goldfish hepatocytes. This role is suggested by previously observed changes in enzyme phosphorylation patterns in the anoxic turtle brain (Brooks and Storey, 1988), the turtle being an exceptionally anoxia-tolerant species. Several reports in

mammals have also implicated PKC activation in the regulation of Na^+/K^+-ATPase activity, resulting in either stimulation or inhibition of the pump depending on the tissue examined (Bertorello and Katz, 1993; Nørby and Obel, 1994). In the liver, activators of PKC stimulated Na^+/K^+-ATPase activity (Lynch *et al.* 1986).

PKC activity is regulated by transient changes in the concentration of diacylglycerol, which induce translocation of PKC from the cytosol (where it is inactive) to the plasma membrane where it becomes activated (Ho *et al.* 1988; Epand and Lester, 1990). Thus, its involvement in physiological responses may be monitored by following PKC translocation before and after experimental perturbations (Nishizuka, 1984; Brooks and Storey, 1993).

In consequence, hepatocytes were subjected to chemical anoxia (cyanide incubation) for times covering the range where we had observed a cyanide-dependent decrease of Na^+/K^+-ATPase activity (Krumschnabel *et al.* 1994, 1996), and then PKC activity was estimated.

In order to discriminate which properties are inherent to cells from good anaerobic species, we compared the responses of goldfish hepatocytes with those of the rainbow trout.

Additionally, irrespective of the possible role of PKC in anoxia, we tested the 'potential' role of PKC in modulating Na^+/K^+-ATPase activity. Cells were incubated in the presence and absence of a phorbol ester which has been shown to activate PKC in many systems (Kikkawa *et al.* 1983; Doerner *et al.* 1990). Ouabain-sensitive uptake of Rb^+ (a K^+ analogue) was used to estimate the activity of the Na^+/K^+-ATPase, together with parallel determinations of K^+ efflux to check whether a change in the net flux of K^+ was induced by PKC activation.

Materials and methods

Chemicals

Phosphocellulose filters (PC/B) were obtained from Whatman International Ltd (England) and [γ-^{32}P]ATP (111 GBq mmol^{-1}) was from Amersham (London). Protein kinase C substrate peptide {[ser25]PKC(19–31)} was purchased from Gibco BRL (Life Technologies Inc., NY, USA). All other chemicals were analytical reagent grade from Sigma Chemical Co. (St Louis, MO, USA).

Experimental animals and hepatocyte isolation

Goldfish (40–80 g) and trout (230–430 g) were obtained commercially and were maintained in 200 l aquaria with running water at 15 °C (trout) or at 15 and 20 °C (goldfish). Fish were fed trout pellets (EWOS Aquaculture International) once or twice a day *ad libitum* and were starved for 20 h prior to experimentation.

Goldfish and trout hepatocytes were isolated as described in detail elsewhere (Krumschnabel *et al.* 1994, 1996). After isolation, goldfish hepatocytes were resuspended in Hepes-buffered saline (in mmol l^{-1}): 10 Hepes, 135 NaCl, 3.8 KCl, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 $NaHCO_3$, pH 7.6 at 20 °C, including 2 % bovine serum albumin). Trout hepatocytes were suspended in medium containing (in mmol l^{-1}): 10 Hepes, 136.9 NaCl, 5.4 KCl, 1 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 5 $NaHCO_3$, 1.5 $CaCl_2$, and 5 glucose, pH 7.6 at 20 °C, including 2 % bovine serum albumin. All media used for cell incubation and ion-flux measurements were fully saturated with air.

Cells were preincubated in a shaking water bath at 15 °C for 1 h before use.

In trout and goldfish hepatocytes, viability (assessed by Trypan Blue exclusion) and cell density remained constant during exposure to cyanide.

Conversion factors from cell number to fresh mass were 1.43 mg fresh mass per 10^6 cells for trout and 4.52 mg fresh mass per 10^6 cells for goldfish.

PKC activity

Changes in PKC activity were inferred from changes in the percentage of enzyme that was associated with the membrane fraction. Accordingly, we designed a protocol to obtain a crude microsomal fraction (here called 'membranes') from the hepatocyte suspension and then we extracted the proteins by detergent solubilization. The solubilized proteins were then used for the activity assay.

Preparation of membranes followed the method of Busacker and Chavin (1981) with modifications: the hepatocytes suspension was centrifuged for 4 min at 70 ***g***. The cell pellet was resuspended in 2 ml of ice-cold isolation medium (0.25 mmol l^{-1} sucrose, 5 mmol l^{-1} EDTA, 1 mmol l^{-1} phenylmethylsulphonylfluoride, 1 mg ml^{-1} leupeptine, 30 mmol l^{-1} imidazole–HCl, pH 7.45 at 20 °C) and centrifuged for 4 min at 70 ***g***. 0.5–1 g of the cell pellet and 2 ml of ice-cold isolation medium were homogenized using an Ultraturrax homogenizer for 4×5 s (goldfish) or 2×5 s (trout) at 13 500 revs min^{-1}, cooling on ice between centrifugations. Then 10 ml of cold isolation medium was added and the homogenate was centrifuged at 121 ***g*** for 4 min. The supernatant was centrifuged for 30 min at 12 000 ***g***. The resulting pellet was resuspended in 0.5 ml of 30 mmol l^{-1} imidazole–HCl (pH 7.45 at 20 °C). All centrifugation steps were performed using a Sorval centrifuge with fixed-angle SS-34 rotor (0–4 °C). The presence of 5 mmol l^{-1} EDTA in the isolation medium ensured that only the chelator-stable form of membrane-associated PKC was separated.

Membranes were incubated at room temperature in 10 mmol l^{-1} Tris–HCl (pH 7.45 at 20 °C) containing 0.5 % Triton X-100 (goldfish membranes) or 0.25 % Triton X-100 (trout membranes). After incubation for 15 min, the mixture was centrifuged for 5 min at 10 000 revs min^{-1} in an Eppendorf centrifuge. The supernatant was collected and used for activity measurements as well as for protein determination.

PKC activity assay

PKC was assayed by following the incorporation of [^{32}P]phosphate into a specific substrate peptide {[ser25]PKC(19–31)}. The final assay contained: 20 mmol l^{-1}

Tris–HCl (pH 7.45 at 20 °C), 10 mmol l^{-1} $MgCl_2$, 100 μg ml^{-1} phosphatidylserine (PS), 6 μmol l^{-1} phorbol-12,13-dibutyrate (PdBu), 2 μmol l^{-1} substrate peptide, 1 mmol l^{-1} $CaCl_2$ and 30–60 μg of protein, in a final volume of 100 μl. Tubes containing PdBu and PS were prepared as described by Thomas *et al.* (1987). Briefly, stock solutions of PdBu (10 mg ml^{-1}) and PS (1.2 mmol l^{-1}) were prepared in ethanol; samples of both were added to a tube on ice and ethanol was evaporated under nitrogen gas. The rest of the salts for this medium were added to this lipid mixture and the mixture was sonicated with three 1 min bursts.

The reaction was started by adding [γ-^{32}P]ATP (final concentration 100 μmol l^{-1}; 5×10^6 cts min^{-1} $assay^{-1}$). After incubation for 10 min at 15 °C, the reaction was terminated by adding 5 ml of ice-cold 75 mmol l^{-1} H_3PO_4. Samples were briefly vortexed and filtered through phosphocellulose filters which were then washed three times with 10 ml of 75 mmol l^{-1} H_3PO_4. The filters were allowed to dry and radioactivity was measured by scintillation counting.

Protein kinase C activity was calculated from the difference in ^{32}P incorporated into the substrate peptide in the presence and absence of (PdBu+PS). Phosphorylation was linear with respect to time and protein added under these conditions. Results are expressed as nmol of ^{32}P incorporated per minute per milligram of protein. Maximal PKC activity was obtained from cells which were incubated with PdBu for 30 min. PdBu was dissolved in ethanol, the final concentration of the alcohol being 0.17 %. Final [PdBu] was 100 nmol l^{-1}, a concentration high enough to produce maximal activation but low enough to prevent unspecific effects (TerBush and Holz, 1986; Witters and Blackshear, 1987).

Protein determination was carried out using the method of Lowry *et al.* (1951).

K^+ efflux and Rb^+ influx in isolated hepatocytes

Cation fluxes were determined at 15 °C as described previously (Krumschnabel *et al.* 1996). Briefly, rates of Rb^+ influx and K^+ efflux were measured in a thermostatted Oxygraph chamber (Haller *et al.* 1994) with constant stirring at 500 revs min^{-1}. Hepatocytes were added to the chamber to yield a concentration of 3×10^6 cells ml^{-1}. Samples of the cell suspension were collected by rapid centrifugation (3 s at 10 000 ***g***). The cell pellet was washed twice in 40 volumes of isotonic $MgCl_2$ medium (10 mmol l^{-1} imidazole–HCl, 100 mmol l^{-1} $MgCl_2$, pH 7.6 at 5 °C) and rapidly centrifuged at 60 ***g*** (trout cells) or 50 ***g*** (goldfish cells). The supernatant was removed and the cell pellet was resuspended and incubated in an assay medium in which K^+ had been replaced by Rb^+, so that at time zero the intracellular compartment contained K^+ and no Rb^+ and the opposite was true for the extracellular medium. This allowed the influx of Rb^+ and efflux of K^+ to be measured simultaneously by following the appearance of K^+ in the medium and the accumulation of Rb^+ in the hepatocytes. Concentrations of both cations were measured by flame emission photometry.

The rate of ouabain-sensitive Rb^+ uptake was used to estimate the activity of Na^+/K^+-ATPase.

Statistics

Results are expressed as means ± S.E.M. (*N*=number of independent hepatocyte preparations). Means were compared using Student's *t*-test with $P\leqslant0.05$.

Results

Maximal PKC activity

Addition of 100 nmol l^{-1} PdBu to fish hepatocytes for 30 min led to approximately threefold and 1.7-fold increases in PKC activities of trout and goldfish, respectively. No further increase was seen after this time; therefore, we used these values as estimates of maximal PKC activity under those conditions (Table 1).

Table 1. *Protein kinase C activity of goldfish and trout hepatocytes*

		Protein kinase C activity (nmol ^{32}P mg^{-1} protein min^{-1})			
Species	Treatment	5 min NaCN	30 min NaCN	90 min NaCN	Maximal
Goldfish	Control	0.122±0.019 (*N*=6)	0.136±0.039 (*N*=5)	0.126±0.019 (*N*=5)	0.243±0.035 (*N*=3)
Goldfish	Cyanide	0.148±0.025 (*N*=5)	0.137±0.030 (*N*=4)	0.065±0.011* (*N*=5)	
Trout	Control	0.028±0.004 (*N*=4)	0.023±0.008 (*N*=4)		0.113±0.016 (*N*=3)
Trout	Cyanide	0.026±0.004 (*N*=4)	0.038±0.012 (*N*=4)		

Cells were treated with 0 (control) or 2 mmol l^{-1} NaCN (cyanide) for different periods (0–90 min).
Results are expressed as mean ± S.E.M.
*Significantly different from the respective control, $P<0.05$ (Student's *t*-test).
Maximal activity was measured 30 min after addition of 100 nmol l^{-1} PdBu.

Effect of cyanide on the distribution of membrane-associated PKC

Hepatocytes were incubated with or without 2 mmol l^{-1} NaCN for 5, 30 and 90 min (goldfish) or for 5 and 30 min (trout). Preliminary experiments showed that, in goldfish as well as in trout hepatocytes, addition of 2 mmol l^{-1} NaCN suppressed more than 96 % of total oxygen consumption within 1 min after exposure of cells to the inhibitor.

As shown in Table 1, PKC activity of control cells from both species remained constant for the entire experimental period. Addition of cyanide had no effect on PKC activity of trout cells, which was maintained at about 25 % of maximal PKC activity. After 30 min of exposure to cyanide, PKC activity appeared to have increased, though this change was not significant (P=0.43). In goldfish hepatocytes, in contrast, PKC activity remained constant at 56 % of maximal activity for 30 min but fell to 27 % after 90 min of exposure.

Effect of PdBu on K^+(Rb^+) fluxes

PdBu under normoxia

Table 2 shows the effect of 30 min of exposure of the hepatocytes to PdBu on Rb^+ influx and K^+ efflux. In trout cells, there were small increases in the Rb^+ influx and K^+ efflux, but these were not significant. In goldfish hepatocytes, however, influx significantly increased 1.20-fold and efflux 1.54-fold with respect to controls.

A similar result was obtained with goldfish acclimated to 20 °C: Rb^+ influx and K^+ efflux increased about 1.5-fold with respect to controls. Results are summarized in Fig. 1, where fluxes are expressed as a percentage of the control value for both 15 °C- and 20 °C-acclimated groups. It can be seen that, irrespective of acclimation temperature, goldfish cells showed a significant increase of unidirectional Rb^+(K^+)-fluxes.

PdBu under chemical anoxia

Results for goldfish hepatocytes showed an anoxia-induced decrease in PKC activity (Table 1) and a PdBu stimulation of Rb^+ influx and K^+ efflux (Fig. 1). We then performed an additional control experiment so as to follow the effect of PdBu under chemical anoxia.

Goldfish hepatocytes were incubated for 30 and 90 min with cyanide, and 30 min before the end of the anoxic exposure 0 or 100 nmol l^{-1} PdBu was added to the cells. After 30 min and 90 min of cyanide incubation, we measured a 1.2-fold increase of Rb^+ influx but no significant change in K^+ efflux (Fig. 1). Since the response to chemical anoxia was independent of its duration, the 30 min and 90 min groups were pooled (see Fig. 2).

Table 2. *Rb^+ influx and K^+ efflux rates in goldfish and trout hepatocytes exposed to 100 nmol l^{-1} PdBu for 30 min*

		Flux (nmol 10^6 cells^{-1} min^{-1})	
Species	Treatment	Rb^+ influx	K^+ efflux
Goldfish	Control	0.90±0.10 (N=5)	0.76±0.07 (N=5)
Goldfish	PdBu	1.09±0.15* (N=5)	1.13±0.07* (N=5)
Trout	Control	0.64±0.08 (N=6)	0.65±0.27 (N=6)
Trout	PdBu	0.86±0.41 (N=6)	0.79±0.37 (N=6)

*Significantly different from the respective control, P<0.05 (Student's t-test).

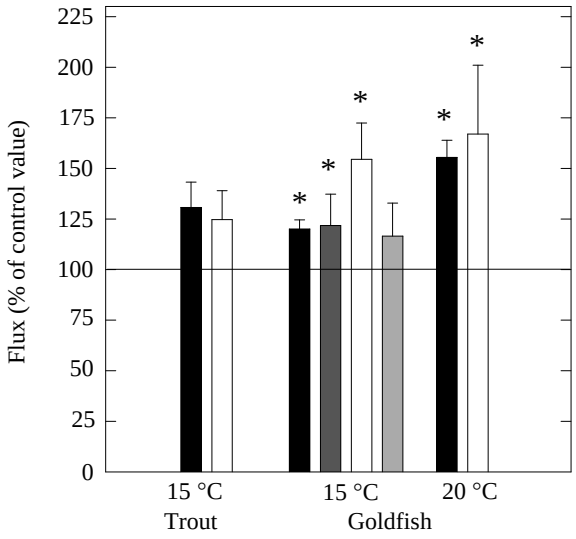

Fig. 1. Rb^+ influx and K^+ efflux rates in hepatocytes. Black columns, Rb^+ influx; dark grey column, Rb^+ influx after 30–90 min of chemical anoxia; white columns, K^+ efflux; light grey column, K^+ efflux after 30–90 min of chemical anoxia. In each case, cells were exposed to 100 nmol l^{-1} PdBu for 30 min. Results are expressed as a percentage of the control value obtained in the absence of PdBu. Values are means + S.E.M. of 5–10 independent preparations. The asterisks identify values significantly different from the respective controls, P<0.05 (Student's t-test). Hepatocytes were obtained from trout acclimated at 15 °C and goldfish acclimated at 15 or 20 °C.

Discussion

We have recently reported two species-specific patterns of Na^+/K^+-ATPase activity after chemical anoxia of fish hepatocytes (Krumschnabel *et al.* 1994, 1996).

In hepatocytes of goldfish acclimated at 15 or at 20 °C, cyanide treatment led to a reduction of Na^+/K^+-ATPase activity, slowing down at a fairly constant rate between 30 and 90 min. ATP concentration dropped to about 50 % during the first 30 min, but remained constant thereafter (Fig. 2). Recent experiments show that ATP is maintained at this lower level for up to 8 h (M. Dorigatti and G. Krumschnabel, unpublished observation). In contrast, in hepatocytes of trout, ATPase activity and ATP concentration dropped very fast, the

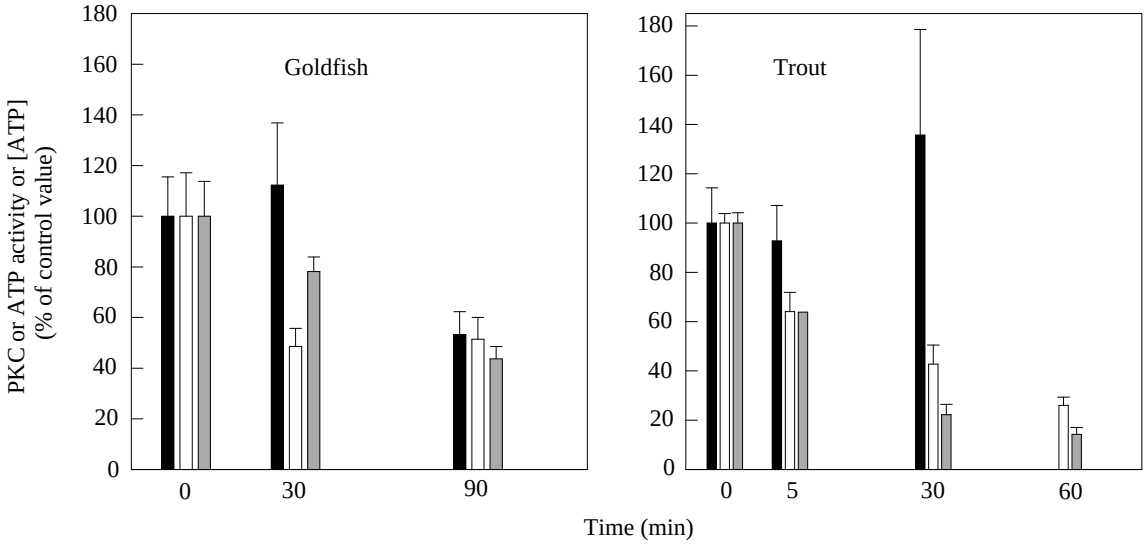

Fig. 2. Kinetics of PKC activity (black columns), Na^+/K^+-ATPase activity (grey columns) and ATP concentration (white columns) after exposure of hepatocytes to 2 mmol l^{-1} NaCN. Values are means ± S.E.M. (N=4–7) expressed as percentage of the respective control value. ATPase activity and ATP concentration in goldfish and trout hepatocytes were taken from Krumschnabel *et al.* (1994, 1996). For trout hepatocytes, Na^+/K^+-ATPase activity at 5 min was calculated by interpolation using the following equation: $ATPase_t=ATPase_0e^{-0.068t}+c$, where $ATPase_t$ is activity at time t, $ATPase_0$ is activity at time zero and c is a constant.

reduction in ATPase activity being 90 % and in ATP concentration 70 % of the initial level after 60 min of exposure to cyanide.

Some authors have discussed the possible role of PKC in mediating such a suppression response under energy-limiting conditions. Anoxia was thought to trigger PKC translocation to the plasma membranes (activation) and inhibit transport processes by phosphorylation (Storey and Storey, 1990; Brooks and Storey, 1993; Buck and Hochachka, 1993). It has recently been demonstrated in a cell-free system that purified PKC phosphorylates the α-subunit of the Na^+/K^+-ATPase (Chibalin *et al.* 1992; Feschenko and Sweadner, 1995).

If this had happened in our system, a negative correlation between Na^+/K^+-ATPase activity and PKC would have been measured.

To our surprise we found that, in goldfish hepatocytes, 90 min of chemical anoxia caused PKC activity to decrease to about 45 % of control values (Table 1), so that the above hypothesis must be discarded. Moreover, the results presented in Table 2 show that PKC can activate Na^+/K^+-ATPase activity and K^+ efflux simultaneously. Therefore, we postulate a direct correlation between PKC and Na^+/K^+-ATPase activity in goldfish cells: under control conditions, PKC remained active at a high level (about 50 % is bound PKC), activating Na^+/K^+-ATPase to a certain extent. Under cyanide treatment, the decline in PKC activity would be responsible at least for part of the observed Na^+/K^+-ATPase inhibition (Fig. 2). This hypothesis is supported by the results of Fig. 1, which show that, even under chemical anoxia, PdBu activation of PKC significantly increased Na^+/K^+-ATPase activity.

In trout cells, in contrast, the situation is different: (1) the relative proportion of active (bound) PKC is about half of that seen in goldfish; (2) chemical anoxia does not cause any decrease in PKC activity, even at 30 min where we had observed a 70–80 % reduction of Na^+/K^+-ATPase activity.

As far as the results in goldfish are concerned, only one mechanism for direct correlation between PKC and Na^+/K^+-ATPase has been postulated. Bertorello and Katz (1993) postulated that stimulation of Na^+/K^+-ATPase may be achieved by a PKC-mediated regulation of cytoskeletal structures adjacent to the pump.

More recently, the combined effect of PKC activity and partial pressure of oxygen on Na^+/K^+-ATPase activity has been reported in rat proximal convoluted tubule (Feraille *et al.* 1995). Under normoxia, PdBu activation of PKC activity stimulated ouabain-sensitive Rb^+ uptake. However, under hypoxic conditions, PdBu inhibited Na^+/K^+-ATPase activity, probably as an adaptation to protect rat proximal convoluted tubule against the deleterious effects of hypoxia.

Compared with goldfish hepatocytes, these results mark a qualitative difference in the way that PKC contributes to the hypoxia/anoxia response of both systems, since PdBu in goldfish hepatocytes increased ouabain-sensitive Rb^+ uptake under normoxia and chemical anoxia. If PKC activation under chemical anoxia had occurred, it would have induced a compensatory increase in Na^+/K^+-ATPase activity, which would delay the metabolic arrest of goldfish cells. However, the results of Table 1 show that PKC activation under anoxia, although pharmacologically possible (Fig. 1), did not take place and it is the decrease in PKC activity which contributes to the anoxic response.

In anoxic goldfish cells, K^+ influx and efflux decreased simultaneously (Krumschnabel *et al.* 1996). Thus, as in the hepatocytes of the anoxia-tolerant freshwater turtle (Buck and

Hochachka, 1993), the anoxia-induced decrease in Na^+/K^+-ATPase activity occurs without alteration of K^+ transmembrane gradients and presumably at constant membrane potential. The present work adds independent confirmation to this strategy and to the means by which a concerted metabolic shut-down may be achieved, since the reduced PKC and Na^+/K^+-ATPase activities after cyanide exposure of goldfish hepatocytes must result in ATP savings and therefore contribute to the observed pseudo steady state of ATP concentration after 30 min of cyanide incubation (Fig. 2). Decreased ATP utilization may, in turn, help to maintain ion homeostasis (Sick *et al.* 1982).

It is worth stressing that cell viability and density were maintained throughout the experiments in both species, so that the responses reported here are not due to anoxic selection of an anoxia-tolerant subpopulation of the cells. Rather, they represent the mean response of the whole population of hepatocytes.

What putative modulators could provide a link to the observed results? In anoxia-sensitive systems, there is an anoxia-induced cascade of events including relaxation of transmembrane ion gradients leading to membrane depolarization, an increase in $[Ca^{2+}]_i$ followed by activation of phospholipases, membrane lysis and eventually cell death. Anoxia-tolerant systems, in contrast, are thought to avoid this sequence by partially shutting down ATP-consuming functions with the benefits of maintaining ATP levels and ion homeostasis.

Even if anoxia-sensitive systems did not survive, the anoxic response shows several aspects which deserve clarification. Taking, for example, Ca^{2+} regulation, we can see the complexity of the problem by making a few assumptions: (1) $[Ca^{2+}]_i$ increases in anoxia-sensitive systems exposed to anoxia; (2) elevated $[Ca^{2+}]_i$ may, in turn (a) increase the relative proportion of active PKC (Thomas *et al.* 1987; Bazzi and Nelsestuen, 1988) and (b) increase the K^+ efflux relative to the K^+ influx as a result of the opening of Ca^{2+}-sensitive K^+ channels. In consequence, a putatively elevated $[Ca^{2+}]_i$ would explain why, in trout hepatocytes, unlike in goldfish hepatocytes (1) PKC may not be downregulated (Table 1; Fig. 2) and (2) an uncoupled K^+ efflux takes place (as seen in Krumschnabel *et al.* 1996). Moreover, the fact that PdBu does not induce a change in $[Ca^{2+}]_i$ (Garrison *et al.* 1984) agrees well with experimental data showing that, under normoxia, in neither species does PdBu change K^+ influx relative to efflux (Table 2; Fig. 1).

Several authors have raised concerns about the extrapolation of results from 'chemical anoxia' to the physiological anoxia situation (see Aw and Jones, 1989; Gores *et al.* 1989), the major concern being the possible formation of toxic oxygen species that may contribute to lethal cell injury. We are aware of these limitations, but the results of this and previous papers on the stoichiometric coupling of metabolism and membrane functions under chemical anoxia support the utility of the model system under the conditions applied in this study.

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