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Arsenic Impairs the Effect of Low Temperature on the Regulation of Na^+-K^+ ATPase Activity in Skeletal Muscle of Fish (*Channa punctata*)

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Abstract

To characterize whether environmental low temperature is involved in metabolic regulation, *Channa punctata* species of fish were exposed to cold (4~8°C) for 30 min, 1 hr and 2 hrs since they are much energetic and survive in the critical environment. The inorganic phosphate (Pi) in skeletal muscle was significantly increased after 1 hr in cold compared to control fish kept in ambient temperature, however, although lower, stimulatory response to cold for 2 hrs was also observed. In separate examinations, Na^+-K^+ ATPase activity in the extract of skeletal muscle was determined and enhanced significantly after 1 hr and 2 hrs. The results appear to indicate that the increased Pi in response to cold might be due to the higher activity of Na^+-K^+ ATPase. To clarify the regulatory mechanism of the enzyme, groups of fish were exposed to cold with 100 mM Na_2HAsO_4 for 1 hr and 2 hrs. The enhanced Na^+-K^+ ATPase activity was reduced significantly when treated with Na_2HAsO_4 compared to the control and also to the respective cold exposed fish. The experimental findings indicate that arsenic is involved in the regulation of cold induced Na^+-K^+ ATPase activity. The enhanced Na^+-K^+ ATPase activity in skeletal muscle of this species in cold and its inhibition by arsenic may play a critical role in environment.

Keywords: Skeletal muscle, arsenic, Na^+-K^+ ATPase, sympathetic innervations, cold exposure.

Arseniğin *Channa Punctata*'nın İskelet Kasında Na^+-K^+ Atpaz Aktivitesi Düzenine Düşük Sıcaklığın Etkilerini Zayıflatması

Özet

Channa punctata'nın metabolizmasının düşük çevre sıcaklığına ihtiyaç duyup duymadığını belirlemek için, balıklar 4~8°C'de 30 dakika, 1 ve 2 saat tutulmuştur. Bir saatin sonunda, normal şartlarda tutulan kontrole kıyasla, soğukta tutulan balıklarda iskelet kasındaki inorganik fosfat (Pi) birikiminin önemli derecede arttığı buna rağmen, iki saatin sonunda soğuğa karşı verilen tepkinin azaldığı gözlemlenmiştir. Ayrı ayrı yapılan denemelerde iskelet kası ekstraktındaki Na^+-K^+ ATPase aktivitelerinin 1. ve 2. saatin sonunda önemli derecede arttığı belirlenmiştir. Sonuçlar, soğuğa karşı artan Pi'nin Na^+-K^+ ATPase'ın yüksek aktivitesine bağlı olduğunu göstermektedir. Balık grupları, enzimin düzenleyici mekanizmasını netleştirmek için 1 saat ve 2 saat boyunca soğuk ile birlikte 100 mM Na_2HAsO_4'e maruz bırakılmıştır. Artan Na^+-K^+ ATPase aktivitesi Na_2HAsO_4 ile muamele edilince kontrole kıyasla önemli derecede azalmıştır. Artan Na^+-K^+ ATPaz aktivitesi, Na_2HAsO_4 ile muamele edildiğinde sırasıyla kontrol grubuna ve soğuğa maruz kalan balıklara kıyasla önemli derecede azalmıştır. Deneysel bulgular, soğuk indüklenmiş Na^+-K^+ ATPaz aktivitesinin düzeninde arsenikin gerekli olduğunu göstermiştir. Bu türün iskelet kasındaki soğukta artan Na^+-K^+ ATPaz aktivitesi ve bunun arsenik ile inhibisyonu çevre için kritik bir rol oynayabilir.

Anahtar Kelimeler: İskelet kası, arsenik, Na^+-K^+ ATPaz, sempatik inervasyon, soğuk muamele.

Introduction

Taki fish (*Channa punctatus)* are generally found in fresh water of haor, bil, river in Bangladesh. They are the major sources of protein in the diet for human and are energetic; survive in critical circumstances, e.g., water deprivation, cold. The mechanism is not known for this species. The proposed concept may be employed to clarify the mechanism involving the regulation of enzyme Na^+-K^+ ATPase. In the biological process, ATP is generated endogenously and is used in various purposes predominantly for mechanical work. It is well known that Na^+-K^+ ATPase is involved in the cleavage of ATP to release Pi (Jørgensen *et al*., 1998). Therefore, it is assumed that the organism uses Pi for

their survival process.

Na^+-K^+ ATPase is dominantly expressed in skeletal muscles (Torben, 2003) and is involved in the transport of Na^+ and K^+ in the membrane. The Na^+-K^+-ATPase is a heterodimer, composed of two protein subunits, a catalytic α-subunit involved in the splitting of ATP (molecular mass ~112 kDa) and a β-subunit (~35 kDa). The α-subunit, which actually pumps Na^+ and K^+, contains binding sites for Na^+, K^+, ATP, and digitalis glycosides. It has 10 transmembrane segments and depends on the β-subunit for transport activity. The β-subunit is a glycoprotein, necessary for the transfer of the entire enzyme molecule from its site of synthesis in the endoplasmic reticulum to its site of insertion in the plasma membrane. A γ-subunit has been described in several tissues and seems to have regulatory function. Like many other proteins, the subunits of the Na^+-K^+-ATPase are expressed in various isoforms, which can be detected using specific antibodies (Sweadner, 1989; Lavoie *et al.* 1997). Four isoforms of the α-subunit (α_1-α_4) and three isoforms of the β-subunit (β_1-β_3) have been identified. In skeletal muscle, α_1, α_2, β_1, and β_2 are expressed, only minor amounts of α_3 have been found, and the γ-subunit has not been detected.

The transport of the Na^+ and K^+ produces the gradient, which drives the reaction for cleavage of ATP. Therefore, it is assumed that the synthesized gradient is also involved for the survival process. Several factors are involved in the regulation of the activity of Na-K ATPase. Cold exposure is a stressful environmental sympathetic stimuli (Rayner *et al*., 2001) that elicits different thermogenic adaptive responses in endotherms and exotherms. The increased sympathetic nerve activity in response to cold might be involved in the regulation of this enzyme because Na^+-K^+-ATPase activity has been shown to be dynamically regulated in a number of tissues by hormones, neurotransmitters, and local factors (Ewart *et al*., 1995). Moreover, skeletal muscle has been recognized to be supplied with noradrenergic sympathetic axons, which are distributed to the muscle spindles and extrafusal muscle fibres (Barker *et al*., 1981). Therefore, in the present study, to clarify the survival process, the following project was considered.

Arsenic is a famous environmental toxic substance to the living organisms. Prolonged exposure of arsenic has detrimental effects in tissues. It may impair the glycolysis as well as the oxidative processes (Tchounwou *et al.*, 2003). It causes different types of pathogenic syndromes in rodents, fish and other organisms. However, the mechanism of arsenic toxicity involving the metabolic impairment is not well characterized. Therefore, in this study, we also clarify the regulatory process of the enzyme Na^+-K^+ ATPase in response to arsenic exposure of the cold induced metabolic process.

Materials and Methods

Fish

Taki fish (*Channa punctatus*) weighing 50 g to 60 g were used and maintained in normal tap water with ambient temperature (25.5°C). In the day of experiment, cold exposure (4~8°C) was given to the different groups of fish in the cold chamber for 30 min, 1 hr and 2 hrs with full aeration and with free access of water. After the cold exposure treatment, the fish were quickly decapitated and the skeletal muscle from the dorsal area was sampled carefully in cold. Control fish were similarly used for sampling of tissues except giving cold exposure.

Arsenic Treatment

Groups of fish were examined to find the role of arsenic on the regulation of changes of metabolic activity in response to cold. The fish were kept in 100 mM of arsenic compound ($Na_2HAsO_4.7H_2O$, BDH Chemical Ltd.) and exposed to cold for 1 hr and 2 hrs in the cold chamber. The fish were quickly decapitated after arsenic treatment and the tissues were sampled similarly.

Assay of Tissue Metabolite

For analysis of inorganic phosphate (Pi) in skeletal muscles of fish exposed to cold the tissues were homogenized with water. The resulting homogenate was centrifuged at 7000 rpm for 10 min and the supernatant was used for the estimation of inorganic phosphate (Pi) by spectrophotometrically (Ramnik, 1999). The protein content in the crude extract of tissues was determined by the method of Lowry *et al*. (1951).

Assay of Na^+-K^+ ATPase Activity

The Na^+-K^+ ATPase activity was determined by the procedure of Katz *et al*. (1967). Briefly, about 2.0 g of skeletal muscle from the dorsal area of fish was homogenized in cold with homogenization buffer (10~15 ml), 0.2 M sucrose, 30 mM L-histidine, 2.4 mM sodium deoxycholate (pH 6.8). The supernatant was collected after centrifugation of the homogenate at 6000 rpm for 15 minutes and used as crude extract. 0.2 ml extract was transferred to 2.9 ml of incubation buffer without KCl (100 mM NaCl, 6 mM $MgCl_2.6H_2O$, 6 mM ATP and 10 mM imidazole, pH 7.8) and with KCl (100 mM NaCl, 20 mM KCl, 6 mM $MgCl_2.6H_2O$, 6 mM ATP and 10 mM imidazole, pH 7.8), incubated at 37°C for 15 minutes. After incubation, 0.4 ml of 0.1 N NaOH was added to each tube quickly and kept in ice to stop the reaction. 2.0 ml from the incubation mixture was used to determine Pi by calorimetric method (Ramnik, 1999) and for protein estimation by Folin-Lowry method (Lowry *et al*.,

1951), 50 µl of crude extract was used. The Na^+-K^+ ATPase activity was expressed as µmole of Pi/mg of protein/hr.

Statistical Analysis

Results of the experiments were expressed as mean and standard error of different groups. The differences between the mean values were evaluated by ANOVA followed by pared t-test using SPSS software.

Results

Effect of Low Temperature on the Regulation of Inorganic Phosphate (Pi)

To examine the effect of low temperature on the regulation of Pi release from the skeletal muscle, groups of fish were exposed to cold. The amount of inorganic phosphate (Pi) for 30 min, 1 hr and 2 hrs in cold were 2.50±0.19 mg, 9.01±0.40 mg and 4.78±0.20 mg/100 g of tissue weight respectively while for the control fish, the amount of Pi was 3.87±0.37 mg/100 g of tissue weight. A significant 132.8% ($P<0.001$) and 23.5% ($P<0.05$) increased Pi in skeletal muscle was found after 1 hr and 2 hrs respectively while 35.4% ($P<0.05$) reduced Pi was observed after 30 min of cold exposure compared to control fish kept in ambient temperature (Figure 1-3). The changes of Pi in response to cold exposure might be involved in the regulation of metabolic functions in skeletal muscle.

Effect of Low Temperature on the Regulation of Na^+-K^+ ATPase Activity

To clarify the mechanism of the enhancement of Pi in response to cold, groups of fish were examined to find the role of Na^+-K^+ ATPase. As shown in Figure 4, the activity of Na^+-K^+ ATPase in skeletal muscles of fish exposed to cold for 1 hr and 2 hrs were 11.71±3.35 µmole and 8.75±4.25 µmole of Pi/mg of protein/hr respectively while for the control fish the value was 5.69±2.21 µmole of Pi/mg of protein/hr. The Na^+-K^+ ATPase activities were increased significantly by 105.8% ($P<0.05$) and 53.8% ($P<0.01$) respectively when compared to the control fish. The results demonstrate that cold exposure induces Pi in skeletal muscles through activation of the enzyme Na^+-K^+ ATPase. The increased Pi and higher activity of the enzyme might play a role to survive in the environment for these species of fish.

Effect of Na_2HAsO_4 on the Regulation of Na^+-K^+ ATPase Activity

Groups of fish were used to examine the role of arsenic on the regulation of cold induced Na^+-K^+ ATPase activity. As shown in Table 1, the Na^+-K^+ ATPase activity of arsenic treated fish (100 mM Na_2HAsO_4) for 1 hr and 2 hrs in cold were 7.25±3.35 µmole and 7.96±1.96 µmole of Pi/mg of protein/hr respectively where as for control fish kept in ambient temperature, the enzyme activity was 5.69±2.21 µmole of Pi/mg of protein/hr. The Na^+-K^+ ATPase in response to 100 mM Na_2HAsO_4 has been significantly influenced. A significant 38.0% ($P<0.05$) and 9.0% reduced enzyme activities in cold was observed after 1 hr and 2 hrs respectively when compared with the respective enzyme activities in tissues in cold only while significant enhanced 105.8% ($P<0.05$) and 53.8% ($P<0.01$) activities were observed after 1 hr and 2 hrs respectively in response to cold compared to control fish (Figure 4 and Table 1). The enzyme activities in these groups of fish were 11.71±3.35 µmole of Pi/mg of protein/hr and 8.75±4.25 µmole of

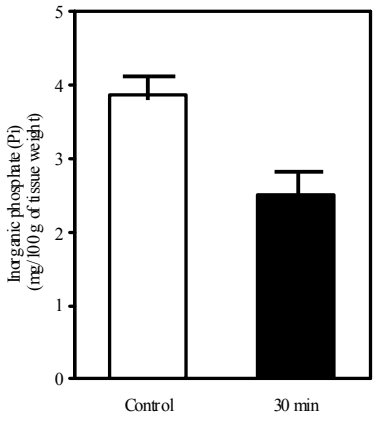

Figure 1. Release of inorganic phosphate (Pi) in skeletal muscle of fish exposed to cold.

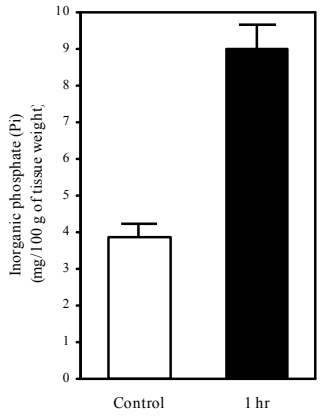

Figure 2. Release of inorganic phosphate (Pi) in skeletal muscle of fish exposed to cold.

The fish were exposed to cold for 30 min and 1 hrs in the cold chamber. After the cold exposure, the fish were immediately decapitated and the skeletal muscle was separated. Control fish were similarly used for sampling of tissue without cold exposure. The Pi was estimated in the cold exposed and the control fish. The data are ± SEM for 4 fish in each group.

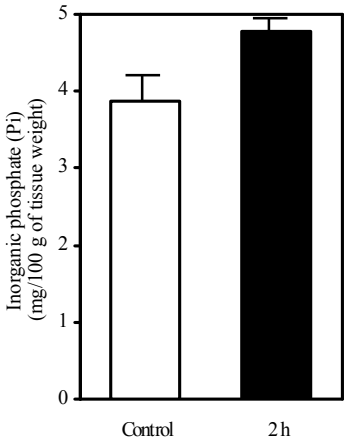

Figure 3. Changes in inorganic phosphate (Pi) in skeletal muscle of fish subjected to acute cold exposure. The fish were exposed to cold for 2 hrs in the cold chamber. After cold exposure, the fish were immediately decapitated and the skeletal muscle was separated. Control fish were similarly used for sampling of tissue without cold exposure. The Pi was estimated in the cold exposed and the control fish. The data are ± SEM for 4 fish in each group.

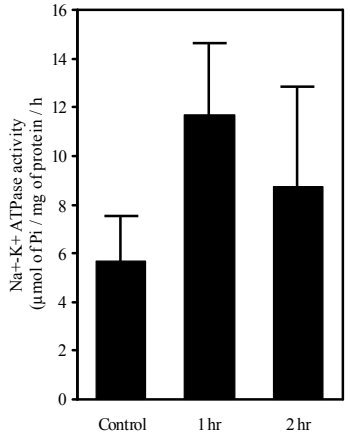

Figure 4. Influence of acute cold exposure on Na^+-K^+ ATPase activity. The fish were exposed to cold for 1 hr and 2 hrs in the cold chamber. After cold exposure, the fish were immediately decapitated and the skeletal muscle was separated. Control fish were similarly used for sampling of tissue without cold exposure. The Na^+-K^+-ATPase activity was measured in the cold exposed and the control fish. The data are ± SEM for 3 fish in each group.

Table 1. Effects of arsenic (100 mM Na_2HAsO_4) on Na^+-K^+ ATPase activity in skeletal muscle of respective groups of fish. The fish were exposed to cold with arsenic solution for 1 hr and 2 hrs in the cold chamber. The respective controls were exposed with cold only. After the treatment, the fish were immediately decapitated and sampling of tissue was performed. Control fish were similarly used except giving cold exposure

	Control (n=3)	Cold (1 hr) (n=3)	Cold (2 hrs) (n=3)	Na_2HAsO_4+ cold (1 hr) (n=3)	Na_2HAsO_4+ cold (2 hrs) (n=3)
Na^+-K^+-ATPase activity (µmol of Pi / mg of protein / hr)	5.69 ± 2.21	11.71 ± 3.35 [A]	8.75 ± 4.25 [B]	7.25 ± 3.35 [C]	7.96 ± 1.96

The data are means ± SE for 3 fish in each group. [A]$P<0.05$ versus control. [B]$P<0.01$ versus control. [C]$P<0.05$ versus cold (1 hr).

Pi/mg of protein/hr respectively for 1 hr and 2 hrs in cold. The results demonstrate that the reduced Na^+-K^+ ATPase activity in response to arsenic may cause the adverse environment for the regulation of the survival process of the fish.

Discussion

The results of the present investigation demonstrated the regulation of metabolic functions in skeletal muscles of *Channa punctata* varieties of fish in response to environmental temperature and adverse effect of sodium arsenate (Na_2HAsO_4) in cold induced fish. This variety of fish generally is energetic and survives in the critical environment, for instance, in water deficiency, or food deprivation. Therefore, it is assumed to be it as a major source for characterization of the regulation of metabolic activities. We found that acute cold exposure triggered inorganic phosphate (Pi) release from skeletal muscles. The released Pi might be due to the increased activity of Na^+-K^+ ATPase because acute cold exposure for 1 hr and 2 hrs effectively stimulates the activity of the enzyme. Cold exposure has dynamic effects on cellular activity. Although variation of temperature causes the alteration of cell activity, effect of acute and prolonged exposure to cold is used to maintain the homeostasis of the species. Among the peripheral tissues of fish, skeletal muscles play the critical role in the regulation of metabolic functions, however, the mechanism involving the survival process in the environment for this species is not known. The increased Pi might be involved in the regulation of this process.

Differential centrifugation of muscle homogenates combined with electron microscopic identification of membrane vesicles as well as by immunofluorescence labeling (Caswell *et al.,* 1976; Williams *et al*., 2001) reveals that the Na^+-K^+ pumps in skeletal muscle are located in the sarcolemma and the t tubules. In skeletal muscle, Na^+ and K^+ are exchanged across the plasma membrane of sarcolemma and t tubules via a number of specific transport systems. The passive movements of Na^+ and K^+ are counterbalanced by one single active transport system, the Na^+-K^+ pump. The major passive fluxes are mediated by the voltage-sensitive Na^+ channels and at least four different categories of K^+ channels.

During excitation, the action potentials are elicited by a rapid and marked influx of Na^+ via the Na^+ channels, immediately followed by an almost equivalent efflux of K^+. These fluxes may exceed the capacity of the Na^+-K^+ for restoring Na^+-K^+ distribution. Studies on frog and snake skeletal muscle indicate that at variance with many other membrane proteins, the Na^+ channels and most of the K^+ channels are immobile, possibly due to an anchoring to the cell membrane (Roberts *et al*., 1986).

Immunofluorescence labeling showed that in the human soleus muscle, the α_1-subunit isoform was mainly located in the sarcolemma, whereas the α_2-subunit iso-form was observed both in the sarcolemma and diffusely distributed in the muscle fibers, possibly located in the t tubules (Hundal *et al*., 1994). The plasma membrane seems to contain the α_3-subunit isoform. In the guinea pig heart, the t tubules and sarcolemma contain only the α_1-subunit isoform of the Na^+-K^+ pump, whereas in the rat heart, t tubules contained both α_1- and α_2-subunit isoforms (McDonough *et al*., 1996). More recent studies *in vivo* showed the expression of both α_1- and α_2- subunit as well as β subunits (Hundal *et al*., 1992.) differentially in the fractions of the tissues of rat skeletal muscle.

Na^+-K^+-ATPase activity has been shown to be dynamically regulated in a number of tissues by hormones, neurotransmitters, and local factors (Ewart *et al*., 1995). Vasodilators acting through the cyclic nucleotide pathways (cAMP and cGMP) increase Na^+ pump activity by activating their specific protein kinases. Therefore, an activation of Na^+-K^+-ATPase has been suggested to contribute, at least partly, to the mechanism of nitric oxide- and β -adrenoceptor agonist-induced vasodilatation (Tamaoki *et al*., 1997; Webb *et al*., 1981). Paradoxically, several vasoconstrictors may also stimulate Na^+-K^+-ATPase. In vascular smooth muscle cells, it has been suggested (Xia *et al*., 1995) that PKC induces a direct stimulation of Na^+-K^+-ATPase, whereas it indirectly inhibits the enzyme by activation of phospholipase A_2 (PLA_2) and the subsequent release of arachidonic acid and its metabolites that are known to inhibit the enzyme (Satoh *et al*., 1993; Schwartzman *et al*., 1985).

Inorganic arsenic is naturally occurring and ubiquitously present in the environment. Contamination of drinking water by arsenic, either by natural means or through industrial pollution, represents the major route of human exposure (Chan *et al*., 1997; Gebel, 2000). It is a serious environmental problem worldwide because of the large number of contaminated sites that have been identified and the hundreds of millions of people at risk, particularly in developing countries, such as West Bengal in India and Bangladesh (Dahr *et al*., 1997; Nickson *et al*., 1998.). Accumulation of arsenic either from water or other sources to fish is a general phenomenon. The toxic arsenic compound is consumed by the peripheral tissues of fish and was assumed to be involved in the interaction with metabolic functions. It is speculated that 100 mM Na_2HAsO_4 deactivates the sympathetic nervous system because it effectively suppresses cold-induced Na^+-K^+-ATPase activity. Degradation of ATP is an essential biological process for survives. The higher the consumption of O_2, higher the degradation of the molecule. The reduced Na^+-K^+-ATPase activity by arsenic might be linked to the lower consumption of O_2 uptake in such adverse environment. Recent investigation reveals that higher arsenic level prevents cellular oxygen uptake (Madsen, 1992), moreover, cellular respiration is also prohibited by arsenic accumulation (Stanton *et al*., 2006).

We found that 100 mM Na_2HAsO_4 effectively but not completely prevents Na^+-K^+-ATPase activity, therefore, further studies are needed to find the optimum concentration of arsenic on the regulation of this enzyme. The increased nerve activity in response to cold has been observed (Rayner *et al*., 2001; Spiegelman *et al*., 2001). Norepinephrine (NE) and other excitatory substances might be involved in the regulation of Na^+-K^+-ATPase activity. Therefore, it is also possible that arsenic could not prevent cold-induced Na^+-K^+ ATPase level or the toxic effects of arsenic could be prohibited by the environmental low temperature. The inhibitory effect of arsenic on Na^+-K^+ ATPase is an index for the regulation of the survival process in the environment for the species of fish. Collectively, we conclude that cold exposure elicits Pi release through activation of the enzyme Na^+-K^+-ATPase in skeletal muscle. Higher activity of Na^+-K^+-ATPase showing its existence in the skeletal muscle and its inhibition by arsenic are the index for characterization of the metabolic status of this species and may play a critical role in augmenting for survive in the environment.

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