J. exp. Biol. ***180****, 15–25 (1993)*
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THE EFFECT OF SEROTONIN (5-HYDROXYTRYPTAMINE) ON GLYCOLYSIS IN THE PERFUSED VENTRICLE OF THE FRESHWATER BIVALVE *ANODONTA CYGNEA*: EVIDENCE FOR PHOSPHORYLATION/DEPHOSPHORYLATION CONTROL OF PHOSPHOFRUCTOKINASE

BASILE MICHAELIDIS, EVAGELIA ROFALIKOU and ISIDOROS BEIS*

Laboratory of Animal Physiology, Department of Zoology, Science School, University of Thessaloniki, GR-54006 Thessaloniki, Greece

Accepted 24 March 1993

Summary

The ventricles of the freshwater mollusc *Anodonta cygnea* were isolated and perfused with serotonin in order to examine its effect on glycolysis. Serotonin induces an increase in the concentration of glycolytic intermediates. Phosphofructokinase (PFK) preparations from ventricles perfused with serotonin exhibited an increased sensitivity to activation by the substrate fructose 6-phosphate (F6P) and to the activators AMP and fructose 2,6-bisphosphate ($F2,6P_2$) and a reduced sensitivity to inhibition by ATP. *In vitro* phosphorylation/dephosphorylation experiments revealed that incubation of PFK preparations from ventricles perfused with normal saline in the presence of ATP, cyclic AMP and $MgCl_2$ alters the degree of activation by F6P. In contrast, when enzyme preparations from ventricles treated with serotonin were incubated in the presence of alkaline phosphatase and $MgCl_2$, PFK showed a reduced sensitivity to activation by F6P. Serotonin had no significant effect on the kinetic properties of PK, while it increased the proportion of the active a form of glycogen phosphorylase. These results indicate that serotonin induces an increase in the concentration of glycolytic intermediates in the ventricle of *A. cygnea* and that this is at least partly due to the activation of PFK, possibly *via* phosphorylation by an endogenous cyclic-AMP-dependent protein kinase.

Introduction

Serotonin (5-hydroxytryptamine), some invertebrate neuropeptides and acetylcholine are involved in the control of the molluscan heart rate (Jones, 1983; Walker, 1986). Serotonin excites the cardio-excitor nerves (Hill and Welsh, 1966; Welsh, 1971; Jones, 1983); however, an inhibitory action in some molluscan species has been reported (Painter and Greenberg, 1982). The excitatory action of serotonin on the molluscan heart may be mediated by cyclic AMP, since it has been shown that serotonin activates

*To whom reprint requests should be addressed.

Key words: *Anodonta cygnea*, ventricle, phosphofructokinase, pyruvate kinase, glycogen phosphorylase, serotonin, covalent modification.

adenylate cyclase and increases the intracellular cyclic AMP concentration in the bivalve heart (Higgins, 1974; Wolleman and S.-Rozsa, 1975).

In vitro studies have shown that the activities of the glycolytic regulatory enzymes, phosphofructokinase (PFK) and pyruvate kinase (PK), from molluscan tissues are partly modulated *via* phosphorylation/dephosphorylation mechanisms. Phosphorylation of PFK from molluscan tissues by endogenous cyclic-AMP-dependent protein kinases turns the enzyme into a more active form compared with the dephosphorylated form. In particular, phosphorylation of PFK increases the affinity of the enzyme for its substrate fructose 6-phosphate (F6P), decreases K_a values for activators and increases I_{50} values for inhibitors (Michaelidis and Storey, 1990, 1991; Biethinger *et al.* 1991). In contrast, phosphorylation of PK, possibly by cyclic-GMP-dependent protein kinase, alters its kinetic properties towards a less active enzyme form (Plaxton and Storey, 1984, 1985; Michaelidis *et al.* 1988; Brooks and Storey, 1990). As far as the control of glycogen phosphorylase is concerned, *in vivo* studies have shown that, in molluscan muscular tissues, an interconversion of a and b forms is observed. For example, hypoxia reduces the amount of the a form while an increase in the contractile activity of molluscan muscles causes the conversion of the b form to the a form (Chih and Ellington, 1986).

The question of whether serotonin modulates *in vivo* the PFK and PK activities *via* phosphorylation and the ratio of glycogen phosphorylase forms and consequently the glycolytic flux in the molluscan hearts, remained unanswered. The present study examines the effect of serotonin on (1) the concentration of glycolytic intermediates, (2) the kinetic properties of PFK and PK, and (3) the ratio of glycogen phosphorylase forms in the perfused ventricle of a freshwater bivalve mollusc, *Anodonta cygnea*. Serotonin stimulates an increase in cyclic AMP level in the heart of *A. cygnea* (Wolleman and S.-Rozsa, 1975). In order to examine whether such an increase in cyclic AMP level can modulate the kinetic properties of the glycolytic regulatory enzymes PFK and PK *via* enzyme phosphorylation, as well as modulating glycolysis, the ventricles of *A. cygnea* hearts were isolated and perfused with serotonin. Concentrations of glycolytic intermediates were measured and enzyme kinetic constants determined on homogenates of ventricles. In addition, *in vitro* phosphorylation/dephosphorylation studies were performed in order to determine whether PFK activity can be modified by phosphorylation and dephosphorylation. The experiments suggest that serotonin increases the concentration of glycolytic intermediates, possibly *via* PFK phosphorylation and glycogen phosphorylase interconversion, whereas serotonin has very little effect on PK regulation.

Materials and methods

Animals and chemicals

Anodonta cygnea were acquired from a local dealer in the vicinity of Lake Ioannina and were kept starved for 5–6 days in running tap water at 15˚C. All biochemicals and coupling enzymes were purchased from Serva (Heidelberg, FRG) and Sigma Chemical (St Louis, USA).

Perfusion of the ventricles of Anodonta cygnea

The heart was exposed by opening the valves and removing most of the soft tissues. The auricle–ventricle junctions were ligatured and the heart was pinned down through one ligature. A cotton thread was hooked through the other ligature and connected to an isotonic transducer (model ST-2, Phipps and Bird, Inc.). A cannula was placed in the anterior aorta and perfusates were delivered through a three-way tap. The saline used in all experiments contained 12mmol l^{-1} $NaHCO_3$, 3.7mmol l^{-1} NaCl, 0.45mmol l^{-1} KCl, 8 mmol l^{-1} $CaCl_2$, 0.19mmol l^{-1} $MgCl_2$ and 1mmol l^{-1} glucose. The above composition of saline was based on the inorganic composition of *Anodonta* blood (Potts, 1954). Serotonin was dissolved in the same saline to give a final concentration of 3pmol l^{-1}, which is the threshold to achieve a 100% increase in the amplitude of heart beats in *Anodonta* species (Painter and Greenberg, 1982). The ventricles were perfused for 10min with saline containing serotonin; prior to this, they were perfused with normal saline (without serotonin) until a stable heart rate had been achieved. The perfusate that had flowed outside the ventricles was removed by a suction pipette. In these studies, ventricles were beating against a constant pressure head of 0.49kPa (Peggs, 1975).

Preparation of homogenates for measuring metabolite concentrations and enzyme kinetic constants

Upon completion of perfusion, ventricles were removed immediately from the perfusion system and frozen in liquid nitrogen. For metabolite measurements, the ventricles were homogenized in a ground-glass homogenizer with 3 volumes of ice-cold $HClO_4$ (10% w/v). The precipitated proteins were removed by centrifugation at 4000 ***g*** for 10min and supernatants were neutralized with 3mol l^{-1} $KHCO_3$. Precipitated potassium perchlorate was removed by centrifugation as above and the supernatants were taken for determination of metabolites. Glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, phosphoenolpyruvate, pyruvate, lactate and adenosine phosphates (ATP, ADP, AMP) were measured as reported elsewere (Michaelidis and Beis, 1990). In control experiments, levels of the above metabolites were measured in hearts that had been freeze-clamped immediately after dissection and were compared with those of control hearts freeze-clamped after perfusion. The results showed no significant differences in the levels of metabolites between the immediately clamped hearts and control perfused hearts (data not given).

For studies of enzyme kinetic constants, ventricles were perfused as described above and then homogenized 1:3 (w/v) in 50mmol l^{-1} imidazole–HCl buffer (pH7.0) containing 100mmol l^{-1} NaF, 5mmol l^{-1} EDTA, 5mmol l^{-1} EGTA, 0.1mmol l^{-1} phenylmethylsulphonyl fluoride, 30mmol l^{-1} 2-mercaptoethanol and 40% (v/v) glycerol (buffer A). After centrifugation at 25000 ***g*** for 20min at 4°C, the supernatant was removed and passed through a 5ml column of Sephadex G-25 equilibrated in 40mmol l^{-1} imidazole–HCl buffer (pH7.0) containing 5mmol l^{-1} EDTA, 15mmol l^{-1} 2-mercaptoethanol and 20% (v/v) glycerol in order to remove low-molecular-weight metabolites (Helmerhost and Stokes, 1980). The column was centrifuged in a desktop centrifuge at top speed for 1min and the filtrate was used as the source of enzymes for

studies of kinetic constants. Enzyme assays and studies of kinetic constants were perfomed as described by Michaelidis *et al.* (1990).

In vitro *phosphorylation and dephosphorylation studies on PFK*

For the phosphorylation experiments, ventricles were perfused with normal saline, as described above, and homogenized 1:2.5 (w/v) in buffer A. After centrifugation, low-molecular-weight metabolites were removed by passing the supernatant through columns of Sephadex G-25 equilibrated in 40mmol l^{-1} imidazole–HCl (pH7.0), 10mmol l^{-1} 2-mercaptoethanol, 0.5mmol l^{-1} EDTA, 20% (v/v) glycerol and 10mmol l^{-1} potassium phosphate. Columns were centrifuged as described previously and the filtrate was collected. Samples of filtrates (100 μl) were added to 100 μl of a solution containing 40mmol l^{-1} imidazole–HCl (pH7.0), 40mmol l^{-1} NaF, 10mmol l^{-1} 2-mercaptoethanol, 20% (v/v) glycerol and one of the following: (1) 3mmol l^{-1} ATP and 5mmol l^{-1} F6P with no $MgCl_2$; (2) 3mmol l^{-1} ATP, 20mmol l^{-1} $MgCl_2$ and 0.5mmol l^{-1} cyclic AMP. The first condition was the control incubation, omitting one of the two metabolites (ATP, Mg^{2+}) required for protein kinase function. Incubations were carried out for 3h at 30˚C; then low-molecular-weight compounds were removed by passage through columns, as described above, and PFK kinetics were assessed.

For experiments involving *in vitro* dephosphorylation, the ventricles were perfused with saline containing serotonin, as described previously, and then the protocol described above for homogenization and centrifugation was followed. After the homogenates had been centrifuged, the filtrates were incubated in the presence of alkaline phosphatase (0.3units ml^{-1}) and 20mmol l^{-1} $MgCl_2$; NaF was omitted from the incubation mixture. Samples without alkaline phosphatase and $MgCl_2$, but containing 40mmol l^{-1} NaF, were used as controls. After incubation at 30˚C for 2h, samples were centrifuged as above and the filtrates were used for the analysis of PFK activity.

Results

Under the experimental conditions of perfusion with normal saline, the ventricles beat at a stable rate of about 12beats min^{-1}. Serotonin applied to the ventricle preparation increased both the frequency and the amplitude of the beat (Fig. 1). Specifically, the heart rate increased by 84±1.87% (N=3) and the amplitude increased by 90±2.35% (N=3). After perfusion of the ventricle, metabolite measurements were made and studies of enzyme kinetic properties were performed as described in the Materials and methods section.

Serotonin evoked a significant increase in the level of glycolytic metabolites (Table 1). These data suggest that the increase in both frequency and amplitude of ventricle beats is accompanied by an increase in the content of glycolytic intermediates.

Studies of saturation curves of PFK for the substrate F6P on both untreated and serotonin-treated ventricles showed that serotonin induced an increase in $S_{0.5}$ for F6P (Fig. 2). Moreover, studies of the kinetic properties of the enzyme from ventricles treated with serotonin revealed that serotonin induced changes in the PFK kinetic properties towards a more active enzyme form. Data presented in Table 2 show that the $S_{0.5}$ value

Fig. 1. Response of an isolated ventricle of *Anodonta cygnea* to perfusion with normal saline and with saline containing serotonin.

for the substrate F6P and the K_a value for the activator AMP decreased by about twofold and the K_a value for the other activator, fructose 2,6-bisphosphate ($F2,6P_2$), decreased by 40% compared with PFK from control ventricles. In addition, I_{50} value for ATP, an inhibitor of PFK, increased by twofold.

Table 3 shows that serotonin does not have any significant effect on the kinetic properties of the other key glycolytic enzyme, PK, studied under the same conditions.

In order to determine whether the changes in PFK kinetic properties induced by serotonin were due to enzyme phosphorylation, enzyme preparations from untreated and serotonin-treated ventricles were subjected to phosphorylation and dephosphorylation experiments as described in Materials and methods. When enzyme preparations, derived from ventricles perfused with normal saline, were incubated with ATP, cyclic AMP and Mg^{2+}, an increased sensitivity to F6P was observed (Fig. 3). In contrast, enzyme

Table 1. *Concentrations of glycolytic metabolites in the ventricle of* Anodonta cygnea *perfused without (−5-HT) or with (+5-HT) serotonin*

	Metabolite concentration (μmol g^{-1} wetmass)	
Metabolite	−5-HT	+5-HT
Glucose 6-phosphate	0.015±0.001	0.043±0.003*
Fructose 6-phosphate	0.011±0.001	0.031±0.001*
Fructose 1,6-bisphosphate	0.042±0.003	0.095±0.009*
Dihydroxyacetone phosphate	0.081±0.004	0.150±0.012*
Phosphoenolpyruvate	0.055±0.005	0.068±0.004
Pyruvate	0.100±0.007	0.220±0.016*
Lactate	0.600±0.040	1.390±0.025*
ATP	0.780±0.031	0.440±0.037*
ADP	0.079±0.004	0.120±0.010
AMP	0.030±0.003	0.067±0.003*

Values are means ± S.E.M., N=5 animals.
*Significantly different from corresponding controls (−5-HT) by Student's t-test, P<0.005.

preparations from ventricles treated with serotonin exhibited a reduced sensitivity to F6P after incubation with alkaline phosphatase and Mg^{2+} (Fig. 4).

The total glycogen phosphorylase (a+b) activity was not affected significantly by serotonin. However, the percentage of the active a form increased by about twofold in the ventricles perfused with saline containing serotonin (Table 4).

Discussion

Serotonin is present in all three major classes of molluscs. It can function as a neurotransmitter, modulator or neurohormone on target cells (Walker, 1986). The effect

Fig. 2. Fructose 6-phosphate saturation curves for phosphofructokinase from the ventricles of *Anodonta cygnea* at pH7.0 after perfusion of the ventricles with normal saline (●) or with saline containing serotonin (○). Values are given as mean ± S.E.M., N=3 determinations on separate preparations of enzyme from different animals.

Table 2. *Kinetic properties of phosphofructokinase from the ventricle of* Anodonta cygnea *after perfusion without (–5-HT) or with (+5-HT) serotonin*

	−5-HT	+5-HT
V_{max} (μmol min^{-1} g^{-1} wet mass)	2.93±0.20	3.91±0.24
$S_{0.5}$ (F6P) (mmol l^{-1})	2.24±0.18	1.19±0.05*
n_H	1.25±0.04	1.30±0.02
K_m (ATP) (μmol l^{-1})	28±2	35±2
K_a (AMP) (μmol l^{-1})	56±5	25±2*
K_a (F2, 6P$_2$) (μmol l^{-1})	0.65±0.025	0.40±0.015*
I_{50} (ATP) (mmol l^{-1})	2.45±0.086	4.41±0.20*
I_{50} (PEP) (mmol l^{-1})	0.50±0.024	0.62±0.018

Values are means ± S.E.M., N=3 determinations on separate preparations of enzyme from different animals. Effector constants were determined at 0.5 mmol l^{-1} F6P for K_a values for both controls and 5-HT-treated enzyme forms, and at 2.0 mmol l^{-1} F6P for I_{50} values.

*Significantly different from the corresponding control form (−5-HT) by Student's t-test, P<0.005.

F6P, fructose 6-phosphate; F2, 6P$_2$, fructose 2,6-bisphosphate; PEP, phosphoenolpyruvate.

of serotonin on different molluscan tissues seems to be mediated by cyclic AMP. An increase in the level of cyclic AMP in molluscan tissues induced by serotonin can stimulate several processes at the cellular level *via* phosphorylation mechanisms. For example, serotonin-induced relaxation of anterior byssus retractor muscle (ABRM) of bivalve molluscs is attributed to the phosphorylation of myosin rods by an endogenous cyclic-AMP-dependent protein kinase *via* cyclic AMP (Castellani and Cohen, 1987). Higgins and Greenberg (1974) have isolated a cyclic-AMP-dependent protein kinase from bivalve myocardium and reported that an increase in cyclic AMP concentration augments the uptake of Ca^{2+} by myocardial microsomes. Na^{+} and water uptake by freshwater mussels is also stimulated by serotonin *via* cyclic AMP (Dietz *et al.* 1982; Scheide and Dietz, 1986).

Table 3. *Kinetic properties of pyruvate kinase from the ventricle of* Anodonta cygnea *after perfusion without (–5-HT) or with (+5-HT) serotonin*

Values are means ± S.E.M., N=3 determinations on separate preparations of enzyme from different animals. Effector constants were determined at 0.05mmol l^{-1} PEP for I_{50} values for both controls (−5-HT) and 5-HT-treated enzyme forms.

NE, no effect on enzyme activity up to 0.2mmol l^{-1} F1, $6P_2$.

PEP, phosphoenolpyruvate; Ala, alanine; F1, $6P_2$, fructose 1,6-bisphosphate.

Fig. 3. Fructose 6-phosphate saturation curves for phosphofructokinase at pH7.0 from the ventricles of *Anodonta cygnea* perfused with normal saline, after incubation of enzyme preparations (Sephadex G-25 filtered) at 30°C for 2h with (□) or without (■) cyclic AMP and $MgCl_2$. Values are given as means ± S.E.M., N=3 determinations on separate preparations of enzyme from different animals.

Fig. 4. Fructose 6-phosphate saturation curves for phosphofructokinase at pH7.0 from the ventricles of *Anodonta cygnea* perfused with saline containing serotonin, after incubation of enzyme preparations (Sephadex G-25 filtered) at 30°C for 2h with (●) or without (○) alkaline phosphatase and $MgCl_2$. Values are given as means ± S.E.M., *N*=3 determinations on separate preparations of enzyme from different animals.

The results presented here show that perfusion of the ventricles of the freshwater mussel *A. cygnea* with serotonin increases the content of glycolytic intermediates (Table 1), suggesting that serotonin stimulates the oxidation of carbohydrates through glycolysis. Serotonin stimulates the adenylate cyclase of the heart of *A. cygnea*, resulting in an increase in cyclic AMP content (Wolleman and S.-Rozsa, 1975). This observation suggests that cyclic AMP participates in the modulation of the rate of glycolysis by serotonin.

Mechanisms involving phosphorylation of glycolytic regulatory enzymes, especially PFK, may be responsible in part for the effect of serotonin on glycolysis in the ventricle of *A. cygnea*. The kinetic behaviour of PFK changed when the ventricles were perfused with serotonin. Specifically, PFK exhibited a saturation curve which showed enhanced activation by F6P compared with enzyme from ventricles untreated with serotonin (Fig. 2). Table 2 presents additional evidence that serotonin modified PFK towards a

Table 4. *The total glycogen phosphorylase (a+b) activity and the percentage of the active a form in the ventricles of* Anodonta cygnea *after perfusion without (–5-HT) or with (+5-HT) serotonin*

more active form. After the ventricles had been perfused with serotonin, PFK exhibited an increased sensitivity to activation by AMP and $F2,6P_2$ and a reduced sensitivity to inhibition by ATP compared with PFK from control ventricles.

These results suggest that a covalent modification of PFK occurs when ventricles of *A. cygnea* are stimulated by serotonin. An endogenous cyclic-AMP-dependent protein kinase appears to phosphorylate PFK molecules. When filtrates from homogenates of control ventricles were incubated in the presence of ATP, cyclic AMP and Mg^{2+}, PFK exhibited kinetic behaviour similar to PFK from ventricles perfused with serotonin (Fig. 3). Dephosphorylation can modify PFK activity towards a less active form. As shown in Fig. 4, PFK exhibited a reduced sensitivity to activation by F6P after filtrate preparations of the ventricles perfused with serotonin had been incubated with alkaline phosphatase compared with the same filtrates not incubated with alkaline phosphatase. In agreement with these findings are the results obtained from phosphorylation experiments on isolated PFK from the molluscs *Helix pomatia* and *Mytilus edulis*. In these experiments, the catalytic subunit of the cyclic-AMP-dependent kinase isolated from the same species incorporated ^{32}P into PFK molecules, resulting in a more active form of PFK (Biethinger *et al.* 1991).

Our present results on the activation of PFK by phosphorylation agree with those we obtained previously. *In vitro* studies of PFK kinetic behaviour in tissues from different molluscs showed that PFK activity is modulated by phosphorylation and dephosphorylation, with phosphorylation increasing enzyme activity (Michaelidis and Storey, 1990, 1991). Results presented in this paper suggest that in the heart of molluscs such a phosphorylation of PFK can be induced by serotonin *via* cyclic AMP. This effect of cyclic AMP on molluscan heart PFK resembles the situation described for the parasitic helminths. In these parasites, serotonin also stimulates an increase in intracellular cyclic AMP level (Mansour *et al.* 1960; Donahue *et al.* 1981), and the administration of either serotonin or cyclic AMP increases the activity of PFK when they are added directly to *Fasciola hepatica* homogenate (Mansour and Mansour, 1962). Recent studies have shown that serotonin stimulates the phosphorylation of PFK in both *F. hepatica* and *Ascaris suum* (Harris *et al.* 1982; Kamemoto *et al.* 1989) and that the phosphorylated form of the enzyme has a lower $S_{0.5}$ value for F6P than the dephosphorylated enzyme (Hofer *et al.* 1982; Kamemoto and Mansour, 1986; Kamemoto *et al.* 1987).

In addition to the activation of PFK by covalent modification, two other mechanisms have been found to influence glycolysis through the PFK step in the tissues of molluscs. These include the synthesis of the potent PFK activator $F2,6P_2$ and the binding of PFK to subcellular particles (Storey, 1985). Whether these mechanisms are stimulated by serotonin in the mollusc heart is at present under investigation.

In contrast to PFK, PK does not show any significant changes in its kinetic properties after application of serotonin to the ventricle of *A. cygnea* (Table 3). Determination, however, of values for the mass action ratio for the PK from untreated (17.95) ventricles and ventricles treated with serotonin (11.86) indicates an inhibition of PK. Studies on PK from molluscan tissues revealed that covalent modification of PK by phosphorylation results in a less active form compared with the dephosphorylated enzyme (Holwerda *et al.* 1983; Plaxton and Storey, 1984, 1985; Michaelidis *et al.* 1988). Exposure of molluscs

to anoxia induced phosphorylation of PK, which was stimulated by a cyclic-GMP-dependent protein kinase rather than by a cyclic-AMP-dependent enzyme (Brooks and Storey, 1990). However, as judged by the results presented in the Table 3, the suggestion that PK is regulated by phosphorylation after treatment of the ventricles with serotonin can be excluded. Thus, it is more logical to suggest that other mechanisms, including allosteric effectors, are involved in the regulation of PK in the heart of *A. cygnea* under the above perfusion conditions.

Whether the increase in the percentage of the active a form of glycogen phosphorylase induced by serotonin is due to the increased level of cyclic AMP and/or to the increased concentration of Ca^{2+} released from the sarcoplasmic reticulum or to some other factor is not certain.

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