J Comp Physiol B (1990) 160:309–316

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

cGMP-Stimulated protein kinase phosphorylates pyruvate kinase in an anoxia-tolerant marine mollusc

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Accepted April 3, 1990

Summary. A cystosolic protein kinase that phosphorylates pyruvate kinase (PK) in vitro has been identified in crude homogenates of heart, radular retractor, and foot muscle from the anoxia-tolerant marine whelk *Busycon canaliculatum*. Protein kinase action was measured by following changes in PK kinetic parameters: phosphorylated PK has a higher K_m value for phosphoenolpyruvate and a lower I_{50} value for L-alanine. The crude protein kinase readily phosphorylated PK in a Mg^{2+}- and ATP-dependent manner in the absence of any added effector. This activity was not affected by the addition of either cAMP (a stimulator of protein kinase A) or Ca^{2+} plus phorbol 12-myristate 13-acetate (stimulators of protein kinase C) to the incubation medium. Addition of cGMP to the homogenate, however, increased the rate of PK phosphorylation giving a 3–4-fold increase in the rate of change in PK kinetic parameters that was readily apparent after 5 h. Complete time-courses of changes in PK kinetic parameters in the presence and absence of cGMP showed that cGMP increased the rate, but not the final extent, of PK phosphorylation. These results indicate that PK inactivation by enzyme phosphorylation in response to anoxia in whelk tissues may be mediated by a cyclic GMP stimulated protein kinase in response to changing levels of cGMP. This conclusion was further supported by data indicating that the total activity of protein kinase was the same in both anoxic and aerobic animals, and that the total PK phosphatase activity was also constant. Changes in PK phosphorylation during anoxia are not, therefore, the result of changes in the total amount of protein kinase or phosphatase.

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Abbreviations: *cAMP* adenosine 3′:5′-monophosphate; *cGMP* guanosine 3′:5′-monophosphate; *PK* pyruvate kinase; *PMA* phorbol 12-myristate 13-acetate; *PEP* phosphoenolpyruvate; K_m Michaelis constant; I_{50} inhibitor concentration that reduces enzyme activity by 50%

Key words: Anaerobiosis – Pyruvate kinase – cGMP-dependent protein kinase – Marine molluscs – Reversible phosphorylation control of enzymes

Introduction

Many marine molluscs have the ability to survive extended periods of time in the absence of environmental oxygen (Hochachka 1980). In the anoxic state, the survival of these facultative anaerobes depends on biochemical adaptations that reorganize metabolic processes so that energy production balances energy demands over the long term with limited accumulation of toxic end products (de Zwaan 1983; Livingstone and de Zwaan 1983; Storey 1985). A central part of this re-organization is the ability to depress metabolic rates to values that are only 5–10% of the normoxic rates (de Zwaan and Wijsman 1976; Shick et al. 1983; Storey 1988a).

The overall process by which facultative anaerobes coordinate metabolic depression is not well understood, although the end results of this process are easily identified in marine invertebrates. Three control mechanisms have been identified in the whelk *Busycon canaliculatum* that act to regulate glycolytic flux in the anoxic state: (1) covalent modification of regulatory enzymes via reversible protein phosphorylation (affecting glycogen phosphorylase, 6-phosphofructo-1-kinase, 6-phosphofructo-2-kinase, and pyruvate kinase; Storey 1984, 1988b; Plaxton and Storey 1984, 1985; Bosca and Storey 1990); (2) changes in the subcellular location of enzymes via reversible binding to macromolecular structures (Plaxton and Storey 1986); and (3) fructose 2,6-bisphosphate regulation of 6-phosphofructo-1-kinase to control the biosynthetic use of carbohydrate reserves (Storey 1988b). Control over glycolytic rate is particularly important in facultative anaerobes since carbohydrate fermentation is the primary means of ATP synthesis during anoxia.

Studies on whelk tissues have clearly demonstrated that for pyruvate kinase (PK) the changes in kinetic parameters observed during anoxia result from changes in the degree of enzyme phosphorylation; both the anoxia-stimulated incorporation of ^{32}P into PK, and the action of exogenous phosphatases in reconverting the anoxic enzyme form to the aerobic form, confirm this (Plaxton and Storey 1984). Changes in glycogen phosphorylase activity and in 6-phosphofructo-1-kinase and 6-phosphofructo-2-kinase kinetic parameters are also known to be caused by changes in the phosphorylation state of the enzyme in mammalian systems (Cohen 1980; Sale and Denton 1983; Kemp and Foe 1983). In mammalian organs, the phosphorylation of phosphofructokinase, PK, and glycogen phosphorylase can be initiated and coordinated through the addition of specific hormones that act by changing the concentration of second messengers (most notably cAMP and Ca^{2+}; see Cohen 1980; Sale and Denton 1985; Kagimoto and Uyeda 1979; Claus et al. 1979; Feliu et al. 1976). Available evidence suggests, therefore, that the coordinated control of regulatory enzymes of glycolysis in marine facultative anaerobes is probably achieved via changes in the activity of either protein kinases or phosphoprotein phosphatases during the transition to the anoxic state. Preliminary tests of this hypothesis used common protein kinase activators such as dibutyryl cAMP (Krebs and Beavo 1979; Pallen et al. 1987) or Ca^{2+} (with the calcium ionophore A23187) plus phorbol 12-myristate 13-acetate (Connelly et al. 1987; Mieskes et al. 1987) in an attempt to mimic anoxia-induced changes in vivo, but failed to detect changes in either PK activity, 6-phosphofructo-1-kinase activity, or fructose 2,6-bisphosphate concentrations in isolated whelk tissues (Brooks and Storey 1989). The study did, however, demonstrate the existence of a stable endogenous protein kinase activity which could phosphorylate PK.

The present study analyzed the nature of the processes regulating PK phosphorylation in whelk tissues. The activities of protein kinases and phosphatases were analyzed in anoxic and aerobic tissues and the second-messenger-dependence of protein kinase activity was identified.

Materials and methods

Animals and chemicals. Whelks (*Busycon canaliculatum*) were obtained from the Marine Biological Laboratory, Woods Hole, Mass., and were held in recirculating (aerated) sea water (1000 mOsm) at 15–18 °C without feeding until use. Control animals were sampled directly from the tank. Anoxia was imposed according to Plaxton and Storey (1986). Briefly, animals were placed in a large pail containing sea water (previously bubbled with N_2 gas for 12 h) for 16 h prior to sampling. Animals were held at 15–18 °C during the exposure to anoxic conditions. All biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Pyruvate kinase assays. The kinetic parameters of PK were determined by coupling the reaction to excess lactate dehydrogenase and monitoring the oxidation of NADH at 340 nm using a Gilford model 240 spectrophotometer. The assay was performed by varying phosphoenolpyruvate (PEP) or L-alanine concentrations in 50 m*M* imidazole HCl-buffer (pH 7.0) containing 50 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* ADP, and 0.15 m*M* NADH at 22 °C. The I_{50} values for L-alanine were determined in the presence of 0.2 m*M* PEP.

Phosphorylation of pyruvate kinase. Whelk tissues (radular retractor, foot, or ventricle) from either aerobic or anoxic animals were homogenized 1:2 (w/v) in ice-cold 50 m*M* imidazole, pH 7.0, 100 m*M* NaF, 25% (v/v) glycerol, 5 m*M* EDTA, 5 m*M* EGTA, 10 m*M* β-mercaptoethanol (Buffer A) containing 0.1 m*M* phenylmethylsulfonyl fluoride using a Polytron PT 10 homogenizer. Homogenates were then centrifuged at $21000 \times g$ for 10 min, and the supernatant was desalted by centrifugation through a Sephadex G25 "spun" column (Helmerhorst and Stokes 1980) pre-equilibrated in 50 m*M* imidazole, pH 7.0, 25% (v/v) glycerol, 10 m*M* β-mercaptoethanol, 0.1 m*M* EDTA, 10 m*M* potassium phosphate (Buffer B). For the analysis of protein kinase activity in extracts from anoxic whelks, enzyme homogenates were first treated to dephosphorylate the endogenous PK; 300 µl supernatant was incubated overnight at 20 °C in the presence of 15 m*M* $MgCl_2$, and then desalted prior to use. A 40-µl aliquot of desalted homogenate (either incubated overnight (anoxic) or freshly prepared (aerobic)) was added to 80 µl Buffer B, and the appropriate effectors where then added to give a final total volume of 150 µl. All tubes for phosphorylation time-course determinations contained 30 m*M* NaF to inhibit protein phosphatases (optimal concentration under our conditions) and 10 m*M* ATP. Experimental tubes also contained other effectors as noted in individual figure and table legends. Reactions were incubated for specified times at 30 °C and stopped by immersion in liquid N_2. Samples were stored at −80 °C (for not longer than 4 d) until assayed for K_m or I_{50} values. Storage at −80 °C did not affect the maximal PK activity, the K_m or I_{50} values. Samples were desalted by centrifugation through "spun columns" immediately prior to assay.

Dephosphorylation of pyruvate kinase. Dephosphorylation time courses for PK were measured in desalted extracts from either control or anoxic animals. This procedure relied on endogenous phosphatases to alter the PK kinetic constants. In order to measure dephosphorylation in aerobic animal homogenates, a phosphorylation experimental tube was set up containing 40 µl desalted extract, 80 µl Buffer B, 15 m*M* $MgCl_2$, and 10 m*M* ATP (total volume = 150 µl). This mixture was incubated for 16 h at 30 °C to phosphorylate PK, and then desalted to remove Mg^{2+} and ATP. Dephosphorylation of PK was then initiated by adding 4.5 µl 0.5 *M* $MgCl_2$ (15 m*M* $MgCl_2$ final concentration) to 150 µl of this eluate. Samples were removed at specified time points and the reaction stopped by freezing at −80 °C. The PK kinetic constants were determined from desalted samples thawed immediately prior to assay.

Dephosphorylation time-courses of PK from anoxic animals were measured by preparing PK-containing homogenates from anoxic animals essentially as described for control animal extracts. Tissues from freshly killed animals were homogenized 1:2 in Buffer A and centrifuged for 10 min at $21000 \times g$. The supernatant was then desalted through a Sephadex G-25 "spun column" previously equilibrated in Buffer B. A 40 µl aliquot of eluant was incubated with 80 µl Buffer B containing 15 m*M* $MgCl_2$ in a total volume of 150 µl for defined periods as described above.

Dephosphorylation time-courses were fitted to a modified Hill equation which served as a general relationship between the initial K_m values (K_m^{P}, the K_m value for the phosphorylated enzyme), the final K_m values (K_m^{deP}, the K_m value for the dephosphorylated enzyme), the half time for enzyme phosphorylation ($t_{0.5}$), the time of incubation (t), the Hill coefficient (h) and the K_m value observed at any time t (K_m^{obs}).

$$K_m^{obs} = K_m^{P} - (K_m^{P} - K_m^{deP}) * t^{h} / (t_{0.5}{}^{h} + t^{h}) \quad (1)$$

Equation 1 is a generalized equation for changes in the kinetic patterns of multi-subunit enzymes and as such is not intended as a specific solution to a detailed phosphorylation mechanism.

The solution to Eq. 1 gives the midpoints of the dephosphorylation time courses ($t_{0.5}$) as well as the K_m values for phosphorylated and non-phosphorylated PK.

Results

Phosphorylation of pyruvate kinase by endogenous protein kinases

The data of Figs. 1 and 2 demonstrate that whelk homogenates contain an endogenous protein kinase activity that can alter PK kinetic constants under appropriate conditions. This is shown by the dependence of changes in the K_m for PEP (Fig. 1) and the I_{50} for L-alanine (Fig. 2) on the presence of both ATP and Mg^{2+} ions in the incubating medium. Thus, extracts of whelk radular retractor, foot, and ventricle showed no changes in kinetic parameters when incubated in the presence of ATP plus EDTA or in the absence of ATP. In the presence of both ATP and Mg^{2+}, however, the K_m values for PEP increased and the I_{50} values for L-alanine decreased with increasing length of incubation until a new, constant, value was obtained. These new final values (observed after 24 h of incubation) agree with previous values from whelk muscle tissues obtained by holding the whole animal under anoxic conditions for 21 h (Plaxton and Storey 1985; Whitwam and Storey 1990) and with changes observed in radular retractor muscle PK during in vitro incubations with exogenous *E. coli* phosphatase (Plaxton and Storey 1984). This suggests that phosphorylation by endogenous protein kinases in vitro is a good model system for studying changes observed during anoxia in vivo.

Although the curves of Figs. 1 and 2 were obtained using tissue homogenates from aerobic animals, identical curves were also obtained when the enzyme system in tissue homogenates of anoxic animals were tested. PK in these preparations was first dephosphorylated by overnight incubation of the homogenate to allow the action of endogenous phosphatases. When phosphorylation was then stimulated by the addition of ATP and

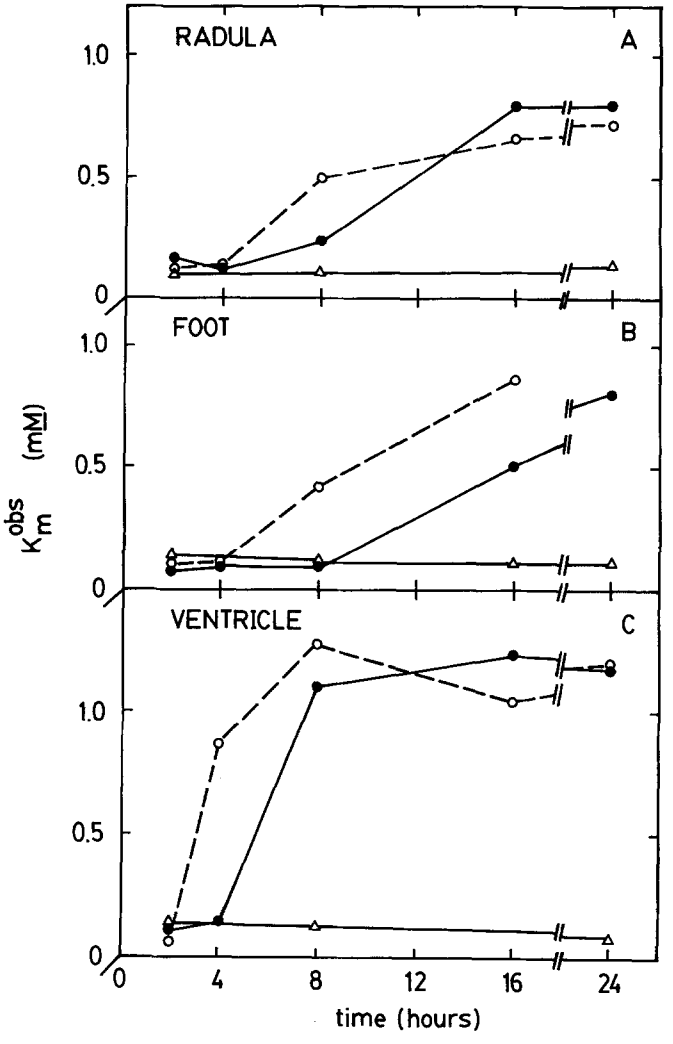

Fig. 1A–C. PK phosphorylation time-course: effect of cGMP on the PEP K_m value for PK from radular retractor muscle **A**, foot muscle **B**, and ventricle **C**. K_m values for PEP (in m*M*) were measured after 2, 4, 8, 16, and 24 h of incubation under one of three conditions: △, plus 20 m*M* EDTA; ●, plus 15 m*M* $MgCl_2$; ○, plus 0.4 m*M* cGMP and 15 m*M* $MgCl_2$. All tubes contained 10 m*M* ATP. All curves were determined in tissue homogenates from aerobic animals

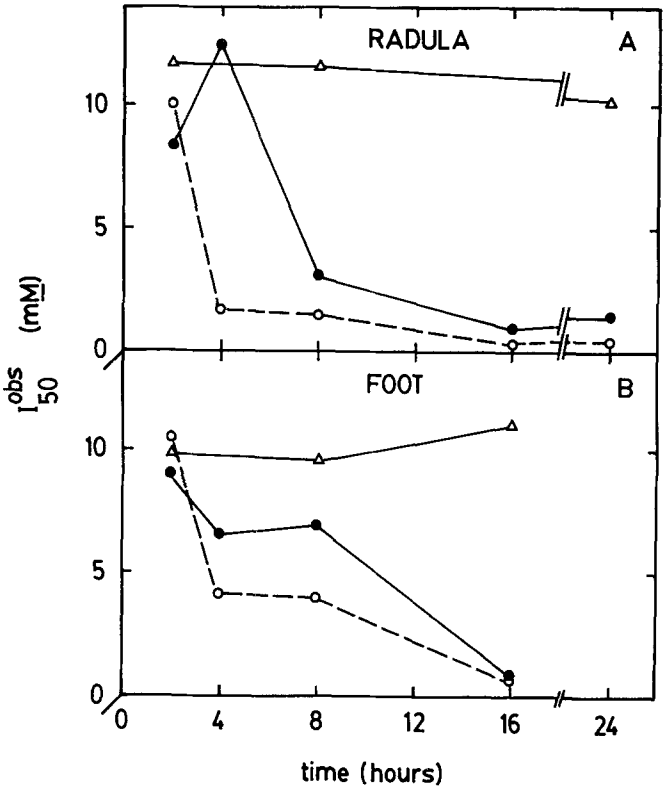

Fig. 2A, B. PK phosphorylation time-course: effect of cGMP on the I_{50} value for L-alanine for PK from radular retractor muscle **A** or foot **B**. I_{50} values (in m*M*) for L-alanine were measured after 2, 4, 8, 16, and 24 h of incubation under one of three conditions: △, plus 20 m*M* EDTA; ●, plus 15 m*M* $MgCl_2$; ○, plus 0.4 m*M* cGMP and 15 m*M* $MgCl_2$. All tubes contained 10 m*M* ATP. All curves were determined in tissue homogenates from aerobic animals

Table 1. Effect of cAMP, cGMP, and Ca^{2+} plus PMA on PK phosphorylation in vitro. Values are mean ± SEM ($n=3$). Samples were incubated at 30 °C for 5 h before assay. All tubes contained 10 m*M* ATP and 15 m*M* $MgCl_2$. Other additions were either: 0.4 m*M* cAMP, 0.4 m*M* cGMP, or 2 m*M* $CaCl_2$ plus 40 $\mu g \cdot ml^{-1}$ phorbol 12-myristate 13-acetate (Ca^{2+} + PMA)

Tissue	Experimental condition	K_m PEP (m*M*)	I_{50} L-alanine (m*M*)
Radular retractor	No second messenger	0.053 ± 0.005	10.1 ± 3.8
	+cAMP	0.050 ± 0.006	18.2 ± 7.6
	+cGMP	0.229 ± 0.033[a]	2.5 ± 1.5[a]
	+Ca^{2+} + PMA	0.052 ± 0.006	15.5 ± 8.9
	+cAMP + Ca^{2+} + PMA	0.071 ± 0.018	13.7 ± 6.9
	+cGMP + Ca^{2+} + PMA	0.084 + 0.015	6.7 ± 4.6[a]
Foot	No second messenger	0.073 ± 0.019	8.1 ± 2.2
	+cAMP	0.045 ± 0.005	10.2 ± 3.6
	+cGMP	0.325 ± 0.079[a]	2.0 ± 1.0[a]
	+Ca^{2+} + PMA	0.080 ± 0.012	8.2 ± 2.9
	+cAMP ± Ca^{2+} + PMA	0.067 ± 0.028	8.9 ± 2.3
	+cGMP + Ca^{2+} + PMA	0.128 ± 0.034	7.9 ± 3.2
Ventricle	No second messenger	2.65 ± 0.86	N.D.
	+cAMP	1.74 ± 0.14	N.D.
	+cGMP	3.78 ± 1.02[a]	N.D.
	+Ca^{2+} + PMA	0.83 ± 0.12[a]	N.D.
	+cAMP + Ca^{2+} + PMA	0.92 ± 0.26[a]	N.D.
	+cGMP + Ca^{2+} + PMA	1.25 ± 0.15	N.D.

[a] Significantly different from condition without added second messenger as determined by the Paired Student's *t*-test at the $P<0.05$ level. N.D. not determined

Mg^{2+} (with or without effectors), the same response of PK was found (data not shown).

The effect of various protein kinase second messengers on the extent of PK phosphorylation is presented in Table 1. These measurements were made after 5 h incubation at 30 °C because the data of Figs. 1 and 2 indicated this time would show a maximal difference between control and experimental values; the kinetic constants were just beginning to change after 5 h of incubation. Three different types of second messengers were used: cAMP (activates protein kinase A; Krebs and Beavo 1979; Pallen et al. 1987); cGMP (activates cGMP activated protein kinase; Lincoln 1983); and Ca^{2+} plus phorbol 12-myristate 13-acetate (activates protein kinase C and calmodulin dependent protein kinase; Connelly et al. 1987; Mieskes et al. 1987). The effect of adding each compound separately, or in combination, to the desalted PK-containing supernatants is shown in Table 1. It is apparent from Table 1 that neither cAMP alone, nor Ca^{2+} plus phorbol 12-myristate 13-acetate (PMA), nor cAMP plus Ca^{2+} plus PMA, had any effect on the extent of PK phosphorylation in radular retractor or foot muscle after 5 h of incubation. In ventricle, however, supernatants incubated with either Ca^{2+} + PMA alone, or cAMP plus Ca^{2+} + PMA, showed significantly lower K_m values for PEP. This latter result is opposite from that expected for an activation of protein kinase.

The effect of cGMP on PK kinetic parameters is also presented in Table 1. In contrast to the actions of the other second messengers, the addition of cGMP to PK-containing supernatants resulted in a significant increase in the K_m value for PEP, and a decrease in the I_{50} value for L-alanine, in all three muscle tissues assayed. This effect was abolished when Ca^{2+} + PMA was added to the cGMP-containing incubations; the cGMP + Ca^{2+} + PMA samples were not significantly different from the control tubes except for the L-alanine I_{50} value for radular retractor muscle PK.

The data of Table 1 show that the addition of cGMP significantly altered the PK kinetic parameters measured after 5 h of incubation. The data of Figs. 1 and 2 show that this effect was due to an enhanced rate of PK phosphorylation and not to an increase in the overall extent of PK phosphorylation. This was demonstrated by the fact that cGMP affected only PK kinetic constants measured before 12 h of incubation. After this time, the presence of cGMP had no effect on the measured PK kinetic constants. The cGMP effect was observed in all three muscle tissues examined; cGMP increased the rate of change of both the K_m for PEP and the I_{50} for L-alanine (open circles, Figs. 1 and 2).

A comparison of the time-courses for K_m (Fig. 1) and I_{50} (Fig. 2) values shows that the changes did not occur over the same time periods. Figures 1 and 2 demonstrate that the I_{50} values changed first, followed changes in the K_m value. This pattern is identical to that observed in vivo (Whitman and Storey 1990) suggesting that this time-course differential is a property of the PK phosphorylation process and not of the in vitro system used.

Measurement of PK activity also showed no significant changes in V_{max} over the time-course of Fig. 1 (data not shown). This shows that increased enzyme phosphorylation affected only the PK, K_m, and I_{50} values and did not effect enzyme turnover rate.

The protein kinase activity observed in these homogenates was completely soluble as demonstrated by measurements with crude homogenates which contained membrane fragments. In these experiments, the centrifugation step was omitted during the purification procedure (thus retaining large membrane vesicles and insoluble cellular material) and samples were dialysed for 4 h versus 2 × 500 ml Buffer B. Both second-messenger-insensitive and cGMP-sensitive endogenous protein kinase activity was identical to that measured in samples centrifuged at 21 000 × *g* for 10 min.

Dephosphorylation of pyruvate kinase by endogenous phosphatases

Initial experiments into PK phosphorylation by endogenous protein kinases showed that the extent of PK phosphorylation was a function of the amount of NaF added to the incubation mixture. In our system, 30 m*M* NaF gave optimal phosphorylation after a 5-h incubation. Since NaF is a well-known phosphatase inhibitor, this suggested that an endogenous phosphatase activity was present in the PK-containing supernatant. The existence of this phosphatase activity is demonstrated in Fig. 3; adding only Mg^{2+} ions to phosphorylated PK led to

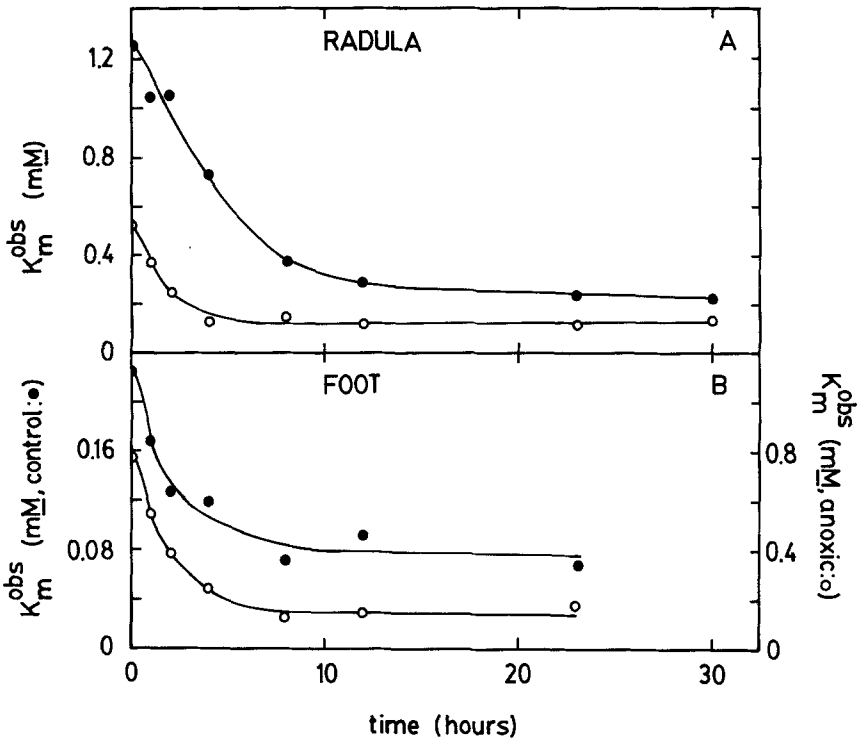

Fig. 3A, B. Time-courses for PK dephosphorylation: comparison of endogenous PK phosphatase activity in tissue extracts from aerobic versus anoxic animals. Figure shows PEP K_m values (in mM) measured after 0, 1, 2, 4, 8, 12, 23, and 30 h of incubation. Reactions were initiated by the addition of 15 mM $MgCl_2$ to desalted tissue extracts and represent endogenous phosphatase activity. Values represented by solid circles were obtained by phosphorylating PK in vitro overnight in homogenates from aerobic animals (PK from aerobic animals is dephosphorylated) prior to the start of the experiment (see Materials and methods). Phosphatase activity in anoxic homogenates (open circles) was obtained by measuring the K_m value for PK obtained directly from tissue homogenates (PK from anoxic tissues is phosphorylated). The solid lines were obtained by a non-linear regression (Gauss-Newton algorithm) fit of the data to Eq. 1. The half-time values for the reaction are presented in Table 2

a decrease in K_m for PEP in both radular retractor and foot muscle. This process was time-dependent; the K_m values decreased with increasing length of incubation until a final value was obtained. Figure 3 also shows the PK dephosphorylation time-courses for homogenates from both anoxic animals and from aerobic animals where the homogenate was previously treated to phosphorylate PK in vitro. These dephosphorylation time-courses were obtained using homogenates diluted by the same factor in order to compare the rates of dephosphorylation in the two different homogenates.

Table 2. Action of endogenous phosphatases in tissue extracts from aerobic versus anoxic animals on a phosphorylated PK substrate. $t_{0.5}$ values are best-fit values (± 1 standard deviation) obtained by a non-linear least squares regression (Gauss-Newton algorithm) fit of the data of Fig. 3 to Eq. 1. $t_{0.5}$ values represent the time required for one half the maximal response and are thus proportional to the total phosphatase activity in the samples. The phosphorylated PK required to measure phosphatase activity in homogenates of aerobic tissues (PK from aerobic tissues is dephosphorylated) was obtained by phosphorylating PK in vitro overnight prior to the start of the experiment (see Materials and methods). Phosphatase activity in anoxic homogenates was obtained by measuring its effect on phosphorylated PK (obtained directly from anoxic tissues). Phosphorylation was carried out by endogenous phosphatases; the reaction was initiated simply by addition of 15 mM $MgCl_2$ (see Materials and methods)

Tissue	Experimental condition	$t_{0.5}$ (h)
Radula	aerobic	3.4 ± 1.1
Radula	anoxic	1.4 ± 0.2
Foot	aerobic	1.7 ± 0.7
Foot	anoxic	1.5 ± 0.2

Because it is difficult to compare the curves using only graphical procedures, the data of Fig. 3 was fitted to Equation 1. The $t_{0.5}$ values (half-time for complete reaction) from the data of Fig. 3 are presented in Table 2. Since the rate of PK dephosphorylation is proportional to the time required to obtain the half maximal change in the K_m value ($t_{0.5}$), a comparison of this value for homogenates from both aerobic and anoxic animals allowed a relative measure of the phosphatase activity present in anoxic and aerobic tissues. Table 2 shows a 2.4-fold lower $t_{0.5}$ for phosphatase action in homogenates from anoxic as compared to aerobic radular retractor muscle, whereas foot muscle showed no difference between phosphatase activity in aerobic and anoxic extracts. These data reveal that the phosphatase activity in anoxic animals is equal to or greater than that of aerobic animals. Note that the curves in Fig. 3 are sigmoidal (the cooperativity factor for these processes was greater than 1.0) suggesting that the observed decrease in the K_m value for PEP required the removal of more than one phosphate per active subunit (data not shown).

Discussion

The data presented in this paper show that pyruvate kinase in whelk muscle tissues can be reversibly phosphorylated in vitro by endogenous protein kinases to give an enzyme with altered kinetic properties. This phosphorylated enzyme was similar to that isolated directly from tissues of anoxic animals in both the direction and the magnitude of the effect on the K_m value for PEP and the I_{50} value for L-alanine. Table 3 compares data for the in vitro phosphorylated and in vitro

Table 3. Comparison of in vitro phosphorylated and in vitro dephosphorylated PK kinetic parameters to values for PK isolated from aerobic and anoxic whelks

Enzyme source	K_m PEP (mM)	I_{50} L-alanine (mM)
Radular retractor		
purified anoxic PK[b]	0.88	0.15
in vitro phosphorylated[a]	0.89	0.90
purified aerobic PK[b]	0.07	72
in vitro dephosphorylated[a]	0.18	N.D.
Foot muscle		
in vivo anoxic PK[c]	1.8	0.28
in vitro phosphorylated[a]	0.9	0.35
in vitro aerobic PK[c]	0.3	2.2
in vitro dephosphorylated[a]	0.11	N.D.
Ventricle		
in vivo anoxic PK[c]	2.4	0.006
in vitro phosphorylated[a]	1.2	N.D.
in vivo aerobic PK[c]	0.38	5.5
in vitro dephosphorylated[a]	N.D.	N.D.

[a] Values determined from this study. Phosphorylated values are from Figs. 1 and 2, dephosphorylated values are from Fig. 3

[b] Values are for PK purified from aerobic and anoxic individuals (Plaxton and Storey 1984)

[c] Values are from Whitwam and Storey (1990) and represent measurements performed in crude homogenates from aerobic and anoxic individuals

dephosphorylated PK kinetic parameters obtained from the present study with values measured directly in tissues from aerobic and anoxic whelks. In all cases, the kinetic parameters of the in vitro phosphorylated PK are similar to those of the enzyme isolated from anoxic animals, and the kinetic parameters of in vitro dephosphorylated PK are similar to those of the aerobic enzyme form. This indicates that the endogenous protein kinase activity identified in these tissue homogenates is responsible for generating the observed changes in PK during the aerobic to anoxic transition in vivo. It also shows that the phosphatase activity measured in foot and radular retractor muscles catalyses the changes in kinetic parameters during the anoxic to aerobic recovery in whelks.

In whole animals, a significant decrease in PK maximal activity is associated with exposure to anoxic conditions (Plaxton and Storey 1985). The present data suggest that this decrease results from changes in PK protein turnover rates, and not from changes in the degree of enzyme phosphorylation since no changes in V_{max} were observed during the course of our experiments. It is apparent, then, that phosphorylation alters only enzyme-substrate and enzyme-inhibitor affinities, and does not affect maximal enzyme activity.

The results of Figs. 1 and 2 demonstrate that a substantial protein kinase activity exists in the absence of any added second messenger. Thus, in the presence of Mg^{2+} and ATP alone, foot and radular retractor PK were phosphorylated with a half-time of approximately 11 h, whereas the half maximal response for ventricle PK occurred at approximately 6 h. These results suggest the existence of a second messenger-insensitive protein kinase activity present in all three muscles. This activity may result either from protease action on a protein kinase C (to give a PK-M protein kinase) or from the dissociation of an active subunit from a larger "regulatory protein-catalytic subunit" protein kinase complex. Second-messenger-independent protein kinase activities have also been identified from systems such as *Ascaris suum* muscle (Thalhofer et al. 1988) and vertebrate muscle (see Edelman et al. 1987). These often represent the active subunit of a cAMP dependent protein kinase. Although this explanation may account for the second messenger-insensitive activity observed in whelk muscle homogenates, it seems more likely that the second messenger-insensitive protein kinase activity comes from dissociation of a non-cAMP dependent protein kinase. This is supported by the complete insensitivity of endogenous protein kinase activity to the addition of cAMP in the whelk homogenates; it appears that cAMP is not involved in the phosphorylation of PK in the three muscle tissues studied.

The second-messenger insensitive protein kinase is also not, apparently, regulated by a membrane-bound cofactor, or by binding to membranes (such as observed for protein kinase C; Moruzzi et al. 1987) since omitting the centrifugation step during the sample preparation did not alter the expression of either the second-messenger-insensitive or cGMP-sensitive protein kinase activities. However, if the second-messenger-insensitive activity represents the dissociation of a larger protein complex, the affinity between catalytic subunit and regulatory protein must not be very high because the two putative subunits were dissociated under the very mild conditions of our tissue homogenization. It is possible, then, that this activity represents a genuine second-messenger-insensitive protein kinase, distinct from the cGMP catalytic subunit. Such protein kinases have been found in several animals including *Ascaris suum* muscle tissue (Thalhofer et al. 1988) and usually form part of a larger phosphorylating cascade such as that described for insulin stimulation of serine kinases (Czech et al. 1988). Thus, the second-messenger-insensitive protein kinase activity may represent the final enzyme in a cascade responsible for mediating the response of PK to anoxia. This cascade may be similar to others (such as the serine kinase pathway) with several "layers" of phosphorylated kinases.

The results from Figs. 1 and 2 also show that the endogenous protein kinase activity responded to increased concentrations of cGMP, and not to cAMP or Ca^{2+} plus PMA. This effect was maximal after approximately 5 h of incubation and was the result of an increase in the rate, but not the overall extent of PK phosphorylation as measured by the changes in enzyme kinetic parameters. Although the existence of cGMP-dependent protein kinases has been well established in invertebrates (Bodnaryk 1983; Kuo et al. 1971; Higgins and Greenberg 1974) and mammalian tissues (Lincoln et al. 1988), endogenous protein substrates have not yet been identified for this enzyme (Takahashi 1985) even though

artificial substrates have included PK, fructose 1,6-bisphosphatase, glycogen synthase, and phosphorylase *b* kinase (Lincoln and Corbin 1977). The present paper, however, demonstrates a physiologically relevant substrate, and function, for a cGMP activated protein kinase.

Although cGMP dependent protein kinases are not ubiquitous (see Krebs and Beavo 1979), the level of cGMP stimulated activity can be higher than the cAMP stimulated activity in some arthropod tissues (Kuo et al. 1971) and in bivalve myocardium (Higgins and Greenberg 1974). Changes in cGMP concentrations have also been linked to developmental changes in insects (Bodnaryk 1983; Takahashi et al. 1975), to anoxia in marine bivalves (Holwerda et al. 1981) and to increased acetylcholine concentrations in bivalves (Kohler and Lindl 1980), showing that cGMP protein kinases regulate important changes in invertebrate cellular processes. The present study suggests that cGMP may play a similar role in the reorganization of metabolism associated with the transition from aerobic to anoxic conditions in the channelled whelk. Changes in protein kinase second-messenger concentrations in response to differing metabolic conditions are well documented in hormone-regulated mammalian systems (Krebs and Beavo 1979; Lincoln 1983) as well as in invertebrate tissues (Bodnaryk 1983; Takahashi 1985; Holwerda et al. 1981). This hypothesis, therefore, proposes that an increase in cellular cGMP levels, stimulating cGMP protein kinase activity, probably mediates the anoxia-induced phosphorylation of PK, and possibly also the coordinated covalent modification of glycogen phosphorylase, 6-phosphofructo-1-kinase, and 6-phosphofructo-2-kinase that is seen in whelk tissues (Storey 1988b; Bosca and Storey 1990). A decrease in the rate of PK dephosphorylation during anoxia, which would also result in increased PK phosphorylation, is ruled out by the data of Fig. 3 and Table 2 which show identical rates of phosphatase activity in food tissue homogenates of aerobic and anoxic animals, and an apparent higher phosphatase activity in anoxic radula. An increase in the concentration of protein kinase activity is also ruled out by measurements of identical total and cGMP-stimulated activity in extracts from both anoxic and aerobic foot and radular retractor muscles.

Finally, the data in Table 1 also suggest that either a Ca^{2+} activated PK phosphatase activity is present in *B. canaliculatum* muscle tissue, or that low concentrations of Ca^{2+} ions inhibit the endogenous protein kinase. This is most clearly illustrated when the K_m values for PEP of ventricle PK are compared for 5-h control versus addition of Ca^{2+} (in the presence or absence of other effectors); ventricle homogenates incubated in the presence of Ca^{2+} ions and cGMP had a PEP K_m value that was identical to the 0-h control. This shows that PK was dephosphorylated with respect to 5-h control PK and suggests either Ca^{2+} activation of an endogenous phosphatase activity and/or Ca^{2+} inhibition of the cGMP-stimulated protein kinase. Although this effect was most evident in ventricle homogenates (because of the greater degree of ventricle PK phosphorylation after 5 h incubation) the effect is also seen in foot and radular retractor tissue (Table 1). Interactions between Ca^{2+} and cGMP second messenger systems are well documented: Ca^{2+} ions indirectly mediate an increase in the rate of cGMP synthesis (increase guanylate cyclase activity) and directly stimulate its degradation (increase Ca^{2+} dependent phosphodiesterase; Tremblay et al. 1988). These results suggest that the control of PK phosphorylation during anoxia in whelks is the result of a complex balance between several cellular signals including cGMP and Ca^{2+} concentrations.

Acknowledgements. Supported by an N.S.E.R.C. Canada operating grant to KBS and a post-doctoral fellowship to SPJB. Thanks to J. Storey for proof reading.

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