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A kinetic re-interpretation of the regulation of rabbit skeletal-muscle phosphorylase kinase activity by Ca^{2+} and phosphorylation

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The regulation of phosphorylase kinase has been proposed to occur physiologically under conditions of zero-order ultrasensitivity [Meinke & Edstrom (1991) J. Biol. Chem. **266**, 2259–2266]. This is also one of the conditions that recent theoretical approaches have indicated to be essential in order for an interconvertible enzyme cascade to generate a sensitive response to an effector [Cardenas & Cornish-Bowden (1989) Biochem. J. **257**, 339–345]. In contrast, all published kinetic data to date have strongly suggested that activation of phosphorylase kinase by Ca^{2+} or phosphorylation is attributable solely to a change in affinity for phosphorylase, with no effect on the $V_{max.}$ of the reaction. In this study an attempt is made to resolve this conflict. Findings suggest that changes in $V_{max.}$ can fully account for the activation of phosphorylase kinase by the physiological mechanisms of cyclic AMP-dependent phosphorylation and increase in Ca^{2+} concentration.

INTRODUCTION

Phosphorylase kinase (EC 2.7.1.38) is a key enzyme in the interconvertible enzyme cascade that regulates glycogenolysis. The physiological benefits of the complexity of this bicyclic cascade for the regulation of the rate of glycogenolysis remain to be fully established. The question of pertinence is what advantage(s) does this multistep regulation provide that a more direct regulation would not. The regulation of glycogenolysis appears to involve the interconversion of at least three enzymes, i.e. phosphorylase, phosphorylase kinase and the one or more protein phosphatases that dephosphorylate these two enzymes. In an extensive series of studies, Stadtman, Chock and co-workers have argued that interconvertible enzyme cascades can allow a far more sensitive response to an effector acting on one or both of the interconverting enzymes than if that effector acts directly on the enzyme catalysing a specific reaction of a metabolic pathway (Stadtman & Chock, 1977, 1978; Chock & Stadtman, 1977; Chock *et al.*, 1980). Cardenas & Cornish-Bowden (1989) have more precisely detailed the specific attributes that an interconvertible enzyme cascade must possess in order to allow this increase in sensitivity to an allosteric regulator. From their theoretical analysis of a monocyclic cascade (involving the interconversion of one enzyme by two interconverting enzymes), they deduced that increased sensitivity will occur only when the following conditions are met: (i) the allosteric effector must alter the catalytic constant ($k_{cat.}$) of one of the interconverting enzymes; (ii) the effector must work on both interconverting enzymes, inhibiting one and activating the other; (iii) both modifier enzymes must act under conditions of near-saturation with substrate. These conditions were assessed for an allosteric regulation of an interconverting enzyme, but a similar rationale can be applied to regulation by covalent modification, i.e. constituting a bicyclic cascade of covalent control (Stadtman & Chock, 1977, 1978). The last required condition as proposed by Cardenas & Cornish-Bowden (1989) has been defined further by Goldbeter & Koshland (1981) and LaPorte & Koshland (1983) as 'zero-order ultrasensitivity'. Interconverting enzymes operating out of the region of first-order kinetics with respect to their target substrate were illustrated to have a markedly enhanced regulatory sensitivity. The control of muscle glycogenolysis has been detailed by Meinke *et al.* (1986) and Meinke & Edstrom (1991) to be highly favoured under zero-order ultrasensitivity conditions, and most likely to occur under such conditions physiologically, with phosphorylase in excess of its K_m value for phosphorylase kinase. Importantly, under conditions of zero-order ultrasensitivity, little response would be obtained were a regulatory event to lead to a change in the K_m of an interconverting enzyme, i.e. for an already saturating substrate, whereas under such conditions the system is exquisitely sensitive to changes in $V_{max.}$.

To date, evaluations of phosphorylase kinase regulation, in particular with respect to the effects of activation on its kinetic parameters, have been quite scant. Where evaluated (Krebs *et al.*, 1964; Heilmeyer *et al.*, 1970; Chan & Graves, 1982; also reviewed in Carlson *et al.*, 1979; Pickett-Gies & Walsh, 1986), activation by either phosphorylation or by Ca^{2+} appeared to lead solely to a change in the K_m for phosphorylase and to have no significant effect on $V_{max.}$ (an indicator of $k_{cat.}$). The kinetic assessments so far undertaken for phosphorylase kinase are thus not consistent with control under the conditions of zero-order ultrasensitivity, which appear to be those that exist for this enzyme physiologically (Meinke & Edstrom, 1991). These previous investigations may not have provided the information that is of relevance for this assessment. Phosphorylation of the enzyme for some of these studies was not restricted solely to that catalysed by cyclic AMP-dependent protein kinase, and/or assays were undertaken at pH 8.2 (near the pH optimum of phosphorylase kinase). The effect on phosphorylase kinase of raising the pH from 7.0 to 8.2 is to cause a massive change in activity (typically 20–40-fold), which may then obscure a conformational change and resultant change in kinetic parameter(s) that would occur in response to phosphorylation at a physiological pH. Many of these kinetic studies were also undertaken before the adoption of precautions to eliminate proteolysis of phosphorylase kinase during its purification, which even at very low level can have an

Abbreviations used: PhK_b, PhK_a and PhK_t refer to non-activated phosphorylase kinase and to phosphorylase kinase activated by the cyclic AMP-dependent protein kinase and trypsin respectively, as described in the Experimental section. 'Phosphorylase kinase' is used for all generic discussion of the enzyme.

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intense effect on enzyme activity and its regulation (Cohen, 1980). Given the apparent disparity between the theoretical considerations for optimum regulation of phosphorylase phosphorylation and the kinetic data so far accumulated, we have undertaken a more in-depth evaluation of the effects on the kinetic parameters of phosphorylase kinase of allosteric regulation by Ca^{2+} and also of cyclic AMP-dependent phosphorylation.

EXPERIMENTAL

Protein preparation

The non-activated form of rabbit skeletal-muscle phosphorylase kinase (PhK_b) was purified to homogeneity as described previously by this laboratory (Pickett-Gies & Walsh, 1985) by using zonal sucrose-gradient ultracentrifugation as the terminal step in the purification and a cocktail of proteolytic inhibitors during the preparation. Purified PhK_b had a pH 6.8/8.2 activity ratio of 0.02–0.04 and exhibited < 1% contaminants as assessed by SDS/PAGE.

Phosphorylase kinase activated by cyclic AMP-dependent phosphorylation (PhK_a) was prepared as described previously with a Hepes buffer system (Ramachandran *et al.*, 1987), except that EDTA was omitted from the phosphorylation reaction mixture, the concentration of phosphorylase kinase was 0.5 mg/ml, the concentration of the catalytic subunit of the cyclic AMP-dependent protein kinase was 2.25 munits/ml, the concentration of ATP was 0.22 mM and the amount of EDTA used to stop the reaction gave a final concentration of 5 mM. The final preparation had a pH 6.8/8.2 activity ratio of 0.38 and was phosphorylated to a stoichiometry of 6–7 mol of phosphate/mol of α_4-subunit and 2.5–3.0 mol of phosphate/mol of β_4-subunit. This level of phosphorylation, especially with the increased level of cyclic AMP-dependent α-subunit phosphorylation, is as we have described previously (Ramachandran *et al.*, 1987). Partial phosphorylation of phosphorylase kinase was achieved by using the same protocol as for PhK_a, but with modifications of the amount of catalytic subunit of the cyclic AMP-dependent protein kinase (0.05–2.25 munits/ml) and incubation time (5–30 min) to give the indicated levels of subunit phosphorylation. Subunit phosphorylation was determined as detailed previously (Pickett-Gies & Walsh, 1985).

Trypsin-activated phosphorylase kinase (PhK_t) was prepared by incubation of PhK_b with 3 units of trypsin covalently attached to agarose beads (Sigma Chemical Co.)/ml, for 30 min at 30 °C, in 50 mM-Hepes/0.2 mM-EDTA/10% (w/v) sucrose, pH 6.8. The limited proteolysis was allowed to proceed to give the maximum possible pH 6.8 activity, at which point the pH 8.2 activity was typically 65% of that of the intact holoenzyme. The reaction was terminated by the addition of 1 μg of Bowman–Birk trypsin/chymotrypsin inhibitor (Sigma Chemical Co.), the trypsin–agarose beads were removed by centrifugation, and the protein was concentrated by centrifugation in a Centricon-10 (Amicon). Glycerol and BSA were added to final concentrations of 50% and 1 mg/ml respectively, and the protein was stored at −20 °C. PhK_t was stable for 2 weeks with a pH 6.8/8.2 activity ratio of 0.63.

Phosphorylase *b* and bovine heart cyclic AMP-dependent protein kinase catalytic subunit were purified to homogeneity, characterized and stored as described previously (Sul *et al.*, 1983; Fletcher *et al.*, 1986). One unit of cyclic AMP-dependent protein kinase is that amount catalysing transfer of 1 μmol of phosphate from [γ-^{32}P]ATP to a histone mixture (Sigma IIA) in 1 min at 30 °C.

Phosphorylase kinase assay

Phosphorylase kinase was diluted immediately before assay in an ice-cold buffer consisting of 50 mM-Hepes, 5 mM-dithiothreitol and 0.1% BSA, pH 6.8. It was then assayed by addition of 10 μl of diluted enzyme to 50 μl of assay reaction mixture to give final concentrations of 50 mM-Hepes/42 mM-Tris, pH 6.8, 1 mM-EGTA, 1.1 mM-$CaCl_2$ (or as indicated), 3 mM-[γ-^{32}P]ATP (specific radioactivity 50 c.p.m./pmol of ATP), 10 mM-magnesium acetate, 2 mM-dithiothreitol, 82 μM-phosphorylase *b* (or for kinetic assays varied from 5 to 150 μM, assuming a molecular mass of 97.4 kDa) and either 1.5 μg of PhK_a or PhK_t/ml or 35 μg of PhK_b/ml. Ca^{2+} concentration, where indicated, was varied by means of a Ca^{2+}/Mg^{2+}/EGTA/H^+ chelation buffer (Cooper *et al.*, 1980). The final Ca^{2+} concentration was determined by means of a Ca^{2+} electrode (Radiometer F2112Ca). The concentration of free Ca^{2+} in the standard assay defined above was 170 μM. Activity was determined for a 10 or 15 min incubation at 30 °C, and ^{32}P incorporation into phosphorylase was determined as previously described (Pickett-Gies & Walsh, 1985). Under all conditions, at least 80% of substrate remained unphosphorylated at the termination of the assay. Activity is expressed as μmol of phosphorylase *a* monomer produced/min per mg of phosphorylase kinase. Reaction rates in the Hepes/Tris buffer system were linear under all conditions for at least 10 min and were examined for each of the levels of phosphorylase kinase phosphorylation evaluated in this study. This is in contrast with previous assays using a β-glycerophosphate-buffered assay, with which a marked lag in phosphorylase kinase activity occurs over the first few minutes of assay (Pickett-Gies & Walsh, 1986). Kinetic constants were determined as described previously (Whitehouse *et al.*, 1983).

RESULTS AND DISCUSSION

Fig. 1 provides a kinetic characterization of PhK_b (panel *a*), PhK_a (panel *b*) and PhK_t (panel *c*) as a function of Ca^{2+} concentration. The kinetic constants derived from these data are summarized in Table 1. The assays presented in this paper were undertaken at a physiological pH for exercising skeletal muscle, 6.8, and at 3 mM-ATP, a value within the physiological range of ATP concentrations (2–5 mM) and in 10-fold excess of the K_m^{ATP} of phosphorylase kinase (Sahlin, 1978; Hermansen, 1981; Pickett-Gies & Walsh, 1986). It is apparent from these data (Table 1), and in contrast with a previous report (Heilmeyer *et al.*, 1970), that activation of both PhK_b and PhK_a by Ca^{2+} occurs by a change in $V_{max.}$, whereas K_m values remain relatively constant. The sensitivity to Ca^{2+} was indistinguishable for the two forms (K_a approx. 3.0 μM), and closely matched previously determined values (Kilimann & Heilmeyer, 1982; Burger *et al.*, 1982).

In addition to Ca^{2+} regulating phosphorylase kinase by a change in $V_{max.}$, at each of the Ca^{2+} concentrations tested cyclic AMP-dependent phosphorylation of phosphorylase kinase also leads to a large change in $V_{max.}$ (40–50-fold), whereas K_m values remain either unchanged or increase slightly (Table 1). These results are apparently contrary to the two previous reports in which phosphorylation was reported to change the K_m for phosphorylase but was without effect on the $V_{max.}$ of the reaction (Krebs *et al.*, 1964; Chan & Graves, 1982). However, the latter report only examined the effects of cyclic AMP-dependent phosphorylation on enzyme already activated by elevated pH (8.2), whereas this current study was undertaken at a pH more closely reflecting physiological conditions, and the study by Krebs *et al.* (1964) did not report effects on $V_{max.}$ and used enzyme that was extensively autophosphorylated in multiple sites. As is illustrated by the data in Table 1, there is a clear

synergy in the effects on $V_{max.}$ of the activation by phosphorylation and Ca^{2+}, with the fold increases in velocity with both effectors much more closely reflecting the product rather than the sum of the velocities with either effector alone.

The effects of activation by limited proteolysis were distinct from those caused by cyclic AMP-dependent phosphorylation (Table 1). Proteolytic activation of phosphorylase kinase leads to a large change in $V_{max.}$ but in addition promotes a large increase in affinity for Ca^{2+}. Further, Ca^{2+} activates PhK_t by decreasing the K_m for phosphorylase *b*, while having no effect on the $V_{max.}$. The increased sensitivity of PhK_t to Ca^{2+} is as reported previously (Cohen, 1980; Kilimann & Heilmeyer, 1982), and proteolysis may also account for the previous report that Ca^{2+} altered the K_m of phosphorylase kinase for phosphorylase in a partially purified preparation (Heilmeyer *et al.*, 1970).

Phosphorylase kinase has a subunit stoichiometry of $\alpha_4\beta_4\gamma_4\delta_4$. The effect of stepwise phosphorylation, first primarily of the β-subunit and secondly of the α-subunit, on the kinetic parameters of phosphorylase kinase was examined. As we have documented previously (Ramachandran *et al.*, 1987), the phosphorylation of the β-subunit is essential for activation by cyclic AMP-dependent phosphorylation, whereas the phosphorylation of the α-subunit does not modulate activity in the absence of β-subunit phosphorylation but amplifies the effects caused by β-subunit phosphorylation. Compatible with these results, both β-subunit phosphorylation and the subsequent phosphorylation of the α-subunit resulted in an increase in $V_{max.}$ (Fig. 2). No

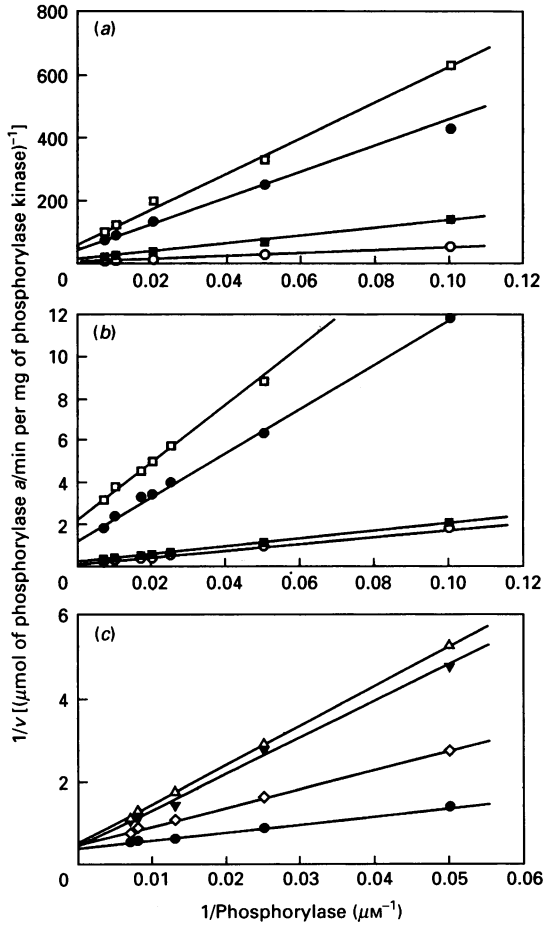

Fig. 1. Double-reciprocal Lineweaver–Burk plots of PhK_b (panel *a*), PhK_a (panel *b*) and PhK_t (panel *c*) at pH 6.8

Reactions were performed as described in the Experimental section with various phosphorylase *b* concentrations, 3 mM-ATP and various free Ca^{2+} concentrations. Free Ca^{2+} concentrations were: panels (*a*) and (*b*), 8.40 μM (○), 3.20 μM (■), 1.10 μM (●) and 0.96 μM (□); panel (*c*), 1.10 μM (●), 0.83 μM (◇), 0.58 μM (▼) and < 0.01 μM (△).

Table 1. Summary of the effects of Ca^{2+}, cyclic AMP-dependent phosphorylation and proteolysis on the kinetic parameters of phosphorylation kinase

Assays were as described in the Experimental section with various concentrations of phosphorylase *b* and 3 mM-ATP. Abbreviation: N.D., not determined.

Calcium concentration (μM)	Phosphorylase K_m (μM)			$V_{max.}$ (μmol of phosphorylase *a*/min per mg)		
	PhK_b	PhK_a	PhK_t	PhK_b	PhK_a	PhK_t
< 0.01	N.D.	N.D.	172	N.D.	N.D.	1.86
0.58	N.D.	N.D.	183	N.D.	N.D.	2.16
0.83	N.D.	N.D.	81	N.D.	N.D.	1.86
0.96	72	72	N.D.	0.013	0.488	N.D.
1.10	68	81	38	0.018	0.77	2.16
3.2	70	70	N.D.	0.062	4.0	N.D.
8.4	70	107	N.D.	0.16	6.7	N.D.
170	67	125	N.D.	0.16	9.5	N.D.

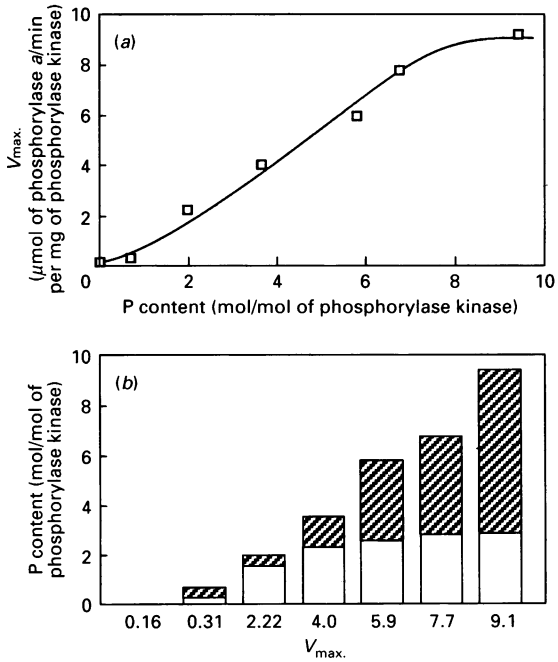

Fig. 2. (*a*) Effect of phosphorylation state on the $V_{max.}$ of phosphorylase kinase at pH 6.8 and (*b*) the contribution of β- and α-subunit phosphorylation to $V_{max.}$ changes

$V_{max.}$ values were determined for each of the species of partially phosphorylated phosphorylase kinase with various phosphorylase *b* concentrations, 3 mM-ATP and 170 μM free Ca^{2+}. Full reaction conditions are given in the Experimental section. The levels of β- and α-subunit phosphorylation, as depicted in panel (*b*), were in mol/mol of subunit$_4$: $\alpha = 0.38$, $\beta = 0.31$; $\alpha = 0.38$, $\beta = 1.58$; $\alpha = 1.23$, $\beta = 2.29$; $\alpha = 3.20$, $\beta = 2.56$; $\alpha = 3.94$, $\beta = 2.77$; $\alpha = 6.52$, $\beta = 2.84$. ▨, mol of P/mol of α_4; □, mol of P/mol of β_4.

significant differences were observed between the K_m values for phosphorylase of the partially phosphorylated species (results not shown), although there appeared to be a trend for a slight increase with increasing phosphorylation. Thus even under the more limited conditions of phosphorylation of this enzyme that occur physiologically (Pickett-Gies *et al.*, 1987; Angelos *et al.*, 1987), the changes in activity are fully attributable to changes in the $V_{max.}$ of the enzyme.

The conclusion from this study is that the activation of phosphorylase kinase, either by an increase in Ca^{2+} concentration or by cyclic AMP-dependent phosphorylation, is solely a consequence of a change in $V_{max.}$. Thus these results fit precisely into the proposed regulation of this enzyme under conditions of zero-order ultrasensitivity (Meinke *et al.*, 1986; Meinke & Edstrom, 1991), and are also as predicted from theoretical considerations (Cardenas & Cornish-Bowden, 1989). The properties of this enzyme are thus compatible with the bicyclic cascade of control of glycogenolysis being a sensitive mode of regulation. The characteristics of this system also allow for the full co-ordination of regulation by the cyclic AMP and Ca^{2+} second-messenger systems. The kinetic properties of phosphorylase kinase are such that incremental changes in both cyclic AMP and Ca^{2+} would lead to synergistic increases in the state of phosphorylase phosphorylation.

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