Skeletal muscle glycogen phosphorylase α kinetics: effects of adenine nucleotides and caffeine

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Rush, James W. E., and Lawrence L. Spriet. Skeletal muscle glycogen phosphorylase α kinetics: effects of adenine nucleotides and caffeine. J Appl Physiol 91: 2071–2078, 2001.—This study aimed to determine physiologically relevant kinetic and allosteric effects of Pi, AMP, ADP, and caffeine on isolated skeletal muscle glycogen phosphorylase α (Phos α). In the absence of effectors, Phos α had Vmax = 221 ± 2 U/mg and Km = 5.6 ± 0.3 mM Pi at 30°C. AMP and ADP each increased Phos α Vmax and decreased Km in a dose-dependent manner. AMP was more effective than ADP (e.g., 1 μM AMP vs. ADP: Vmax = 354 ± 2 vs. 209 ± 8 U/mg, and Km = 2.3 ± 0.1 vs. 4.1 ± 0.3 mM). Both nucleotides were relatively more effective at lower Pi levels. Experiments simulating a range of contraction (exercise) conditions in which Pi, AMP, and ADP were used at appropriate physiological concentrations demonstrated that each agent singly and in combination influences Phos kinase-dependent phosphorylation; glycogen Phos kinase-dependent phosphorylation in activity at 2–10 mM Pi). The present in vitro data support a possible contribution of substrate (Pi) and allosteric effects to Phos α regulation in many physiological states, independent of covalent modulation of the percentage of total Phos in the Phos α form and suggest that caffeine inhibition of Phos α activity may contribute to the glycogen-sparing effect of caffeine.

glycogenolysis; enzyme kinetics; allosteric control; adenosine 5'-phosphate; adenosine 5'-diphosphate; physiological biochemistry; inorganic phosphate

SKELETAL MUSCLE GLYCOGENOLYSIS is determined by the catalytic rate of glycogen phosphorylase (Phos; glycogenn + Pi → glycogenn−1 + glucose 1-phosphate). Control of Phos enzymatic activity involves three main regulatory mechanisms: phosphorylation-dependent activation of latent enzyme (enzyme transformation), substrate effect of Pi availability, and allosteric effects (3, 11). Traditionally, phosphorylation-dependent activation of the enzymatic capacity has been regarded as the most important control mechanism in Phos regulation; glycogen Phos kinase-dependent phosphorylation of the less-active Phos b results in the more-active Phos α. The activity of Phos kinase in turn is sensitive to cellular and hormonal signals associated with muscle contraction and exercise (3, 11–13, 25). However, several recent studies demonstrate that phosphorylation of Phos [percentage of total Phos in the Phos α form (%Phos α)] is not necessarily associated with relative glycogenolytic flux (5, 6, 9, 10, 22–24). These results indicate an important role for phosphorylation-independent or posttransformational control of skeletal muscle Phos activity, including substrate (Pi) availability and allosteric activation (e.g., AMP, ADP).

Reported Km values for Phos α toward glycogen are ~1 mM (4, 20). Because resting muscle glycogen concentration is on the order of ~100 mM, it is generally accepted that Phos is saturated with glycogen under most physiological conditions. It has been recognized that Pi may play a major role in the control of skeletal muscle glycogenolysis in vivo because the concentration of Pi in resting muscle (~1–3 mM; 21) is lower than the reported Km for Phos α in skeletal muscle homogenates (~26 mM; 5). Pi increases stoichiometrically with the decline in phosphocreatine that occurs during energetically demanding contractions, which prompt enhanced rates of glycogenolysis (18, 21, 27), and can reach levels of ~20 mM after intense exercise. In addition, muscle energy turnover is necessary to activate glycogenolysis because %Phos α and Pi can be elevated in resting skeletal muscle by physiological and pharmacological interventions (6, 23), but glycogenolysis does not occur (i.e., Phos enzymatic activity does not increase) unless energy turnover is accelerated. These observations point to a potentially important physiological role for signals associated with ATP turnover in control of Phos α activity in vivo. Indeed, it has been demonstrated that ADP and AMP, the free concentrations of which increase with elevated ATP turnover rates (7, 17, 21, 27), are potent allosteric activators of Phos α in vitro (1, 20). Specifically, AMP and ADP have been reported to activate Phos α by lowering the Km of the enzyme toward Pi (20, 24). On the basis of these results, it has been hypothesized that alterations in intramuscular Pi, AMP, and ADP could account for observed differences in muscle glycogenolysis that occurred with similar %Phos α in different

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physiological states (7, 8, 14, 17). For instance, the reduced rate of net glycogenolysis that occurs during exercise after short-term aerobic exercise training (7, 17) has been proposed to result from decreased allosteric activation of Phos because accumulations of Pi, AMP, and ADP are reduced during the same exercise after compared with before training, whereas %Phos a is not different (7). Similar explanations have been proposed for the glycogen-sparing effects observed during exercise after caffeine ingestion (8) and with increased free fatty acid availability (14).

Although an extensive body of literature has revealed potentially important roles for Pi and purines in the control of Phos a activity, many of these studies are limited either by a lack of control of reaction conditions (i.e., control of effector concentrations and Phos phosphorylation state in studies using muscle homogenates; 5, 24) or by the use of unphysiological effector conditions (i.e., concentrations exceeding what occurs in muscle; 19, 20). Therefore, the purpose of this study was to test the hypothesis that the purines AMP and ADP at free concentrations representative of a dynamic range that occurs in skeletal muscle during contractions can activate purified Phos a by increasing the sensitivity of the enzyme’s kinetics with respect to Pi. Another purine, caffeine, is associated with skeletal muscle glycogen sparing during aerobic exercise in certain individuals (8, 28), and an ancillary report suggests that caffeine at millimolar (supraphysiological) concentrations can inhibit Phos a under certain conditions (19). Therefore, a further purpose of this study was to test the hypothesis that physiological levels of caffeine can inhibit Phos a by decreasing the sensitivity of Phos a kinetics with respect to Pi. These studies were performed in vitro using a purified Phos a preparation.

METHODS

Determinations of Phos a Purity

Commercially prepared, purified, 2× crystallized glycogen Phos a (rabbit skeletal muscle source) was obtained from Sigma Chemical (St. Louis, MO). The purity of the Phos a preparations was determined by SDS-PAGE and by isoelectric focusing (IEF). For SDS-PAGE, Phos a was solubilized in extraction buffer containing 6 M urea, 2% SDS, 100 mM dithiothreitol, and 50 mM Tris•HCl, pH 7.4. This extract was incubated for 30 min at 70°C, and protein concentration was determined (bicinchoninic acid assay, Pierce, Rockford, IL). Aliquots containing 0.25, 0.5, 1, and 2 μg of protein were loaded into separate lanes of a 4–20% gradient polyacrylamide gel and electrophoresed. A prestained molecular mass standard mix was included in a separate lane. The gels were stained with Coomassie brilliant blue, destained, and photographed, and scanning densitometry was performed. For IEF, Phos a was solubilized in water, a 1-μg protein aliquot was loaded on a nondenaturing continuous pH gradient 3–10 IEF minislab gel (Ready-Gel, Bio-Rad, Hercules, CA), and IEF was performed (100 V, 1 h; 250 V, 1 h; 500 V, 30 min). A 1-μg aliquot of a Phos b preparation (Sigma Chemical) was run in an adjacent lane of the same gel to distinguish mobility of Phos a from Phos b. After IEF, the gel was stained with Coomassie R-250 and Crocein scarlet, and scanning densitometry was performed.

Glycogen Phosphorylase Kinetics Experiments

For kinetics studies the lyophilized enzyme was solubilized in 100 mM KCl and 50 mM imidazole, pH 7.0, to yield 0.5 μg Phos a/μl. These preparations were stable for at least 7 days when stored at 4°C (constant specific activity) but were used within 24 h of enzyme solubilization. Phos a reactions in the direction of glycogen degradation were performed with an assay system similar to that previously used to determine Phos activities in muscle homogenates (7, 9, 22, 30). Briefly, reactions were initiated by adding 5 μg of Phos a to 10 μl to a reaction buffer containing 100 mM KCl, 1.25 mM MgCl2, 5 μM glucose 1,6-diphosphate, 0.5 mM dithiothreitol, 3 μg/ml phosphoglucomutase, 0.25% bovine serum albumin, 10 mg/ml AMP-free glycogen, and 50 mM imidazole-HCl, pH 7.0. Orthophosphate was present at the indicated concentrations (1, 2, 5, 10, 15, 20, 25, or 42 mM) in kinetic analyses. Purine nucleotides (stocks prepared in 100 mM KCl and 50 mM imidazole, pH 7.0) were included in the reaction buffer at indicated concentrations. For experiments simulating the conditions in resting and exercising muscle (Fig. 4), Pi, AMP, and ADP were added at the concentrations indicated in the table below Fig. 4. These values were chosen from previously reported data for human muscle (18, 27) under the exercise conditions indicated for each simulation. The Phos a reaction conditions were pH 7.0, at 30°C, and total reaction volume of 600 μl. Reactions were terminated by the addition of 60 μl of 0.5 M HCl. This first stage of the assay degrades glycogen to glucose 1-phosphate, and, because of the presence of the near-equilibrium enzyme phosphoglucomutase, the glucose 1-phosphate is converted to glucose 6-phosphate. A 50-μl aliquot of the acid-stopped primary reaction was then added to 1 ml of a glucose-6-phosphate dehydrogenase reaction buffer containing the following: 1 mM EDTA, 250 μM NADP, 0.5 μg/ml glucose-6-phosphate dehydrogenase, and 50 mM Tris•HCl, pH 8.0. The second part of the assay converts glucose 6-phosphate to 6-phosphoglucono-δ-lactone, coupled to the reduction of NADP to NADPH. This reaction proceeded to completion in <10 min at room temperature. The absorbance at 340 nm of the resultant samples was determined spectrophotometrically against appropriate blanks to determine the degree of conversion of NADP to NADPH and, therefore, the content of glucose 6-phosphate formed in the first reaction. Glycogen phosphorylase a activities are expressed in micromoles per minute per milligram protein (U/mg). Standard conditions were 5 μg of Phos a and 10-min reactions (first stage, Phos a reaction). This assay system was linear with respect to both time and quantity of Phos a used under the described conditions using between 1 and 15 μg of Phos a and allowing reactions to occur for 2–30 min. In total, five separate enzyme preparations of Phos a were used in these studies, and each set of reactions was performed a total of five separate times.

Calculations and Statistics

To determine kinetic constants K0 and Vmax, kinetic data were plotted as double-reciprocal plots [1/enzyme velocity vs. 1/substrate (Pi) concentration] and the intercepts determined by linear regression. Depending on the nature of the data comparison, either one-way analysis of variance followed by Tukey’s procedure or the unpaired t-test was used to determine whether differences were statistically significant (P < 0.05). All illustrated data are expressed as means ± SE. Where error bars cannot be seen, it is because they are small enough to be hidden by the symbol.
RESULTS

Purity of the Phos a Preparation

SDS-PAGE and Coomassie brilliant blue staining of the Phos a preparation revealed a single band at a molecular mass of ~100 kDa, characteristic of Phos a (Fig. 1A). No other bands could be resolved from baseline in scanning densitometry of the gels; thus, for practical purposes, it was assumed that the Phos a protein preparation was essentially free of contamination by other proteins of the same molecular mass. The possibility remained, however, that Phos a could be contaminated by other proteins of different molecular masses. Purity of the Phos a preparation revealed a single band at a molecular mass of 100 kDa, characteristic of Phos a preparation as in scanning densitometry of the gels; thus, for practical purposes, it was assumed that the Phos a protein preparation was essentially free of contamination by other proteins of the same molecular mass. The possibility remained, however, that Phos a could be contaminated by other proteins of different molecular masses.

Phos a Kinetics

The kinetic response of Phos a to P_i at saturating glycogen in the absence of effectors was first evaluated using either orthophosphoric acid or KH_2PO_4 as the phosphate source. The kinetics were Michaelis-Menten type [rectangular hyperbola in enzyme velocity vs. substrate (P_i) concentration plot], and K_m was found not to depend on the phosphate source under these conditions (K_m = 5.58 ± 0.29 vs. 5.94 ± 0.06 mM for orthophosphate and KH_2PO_4, respectively; n = 5 per group; data not shown). Orthophosphoric acid was used as the phosphate source in all subsequent experiments reported herein. The highest concentration of P_i used was 42 mM, and the Phos a activity values at 42 mM P_i were essentially identical to the extrapolated V_max values (Table 1).

Effects of AMP and ADP. AMP decreased the K_m and increased the V_max of Phos a (Table 1), even at the lowest concentration used, 0.1 μM. The magnitude of Phos a activation was AMP concentration dependent, up to the highest AMP used, 100 μM, which reduced K_m to approximately one-third of the control value and increased V_max by greater than twofold (Table 1, Figs. 2 and 3). Phos a was less sensitive to ADP than to AMP at a given nucleotide concentration (Figs. 2 and 3, Table 1). In fact, at low ADP (0.1 and 1 μM), ADP inhibited or had no effect on Phos a activity (at submaximal P_i levels) compared with no-effector values (Fig. 3). Thus there appears to be some quantitative specificity for monophosphorylated over diphosphorylated purine nucleotides in Phos a activation. Catalytic efficiency assessed by the V_max/K_m ratio increased in a dose-dependent manner for both AMP and ADP (Table 1).

Activation of Phos a by purine mono- and diphosphorylated nucleotides was found to be more specific to adenosine- vs. guanosine-based structures because 100 μM GDP and 100 GMP increased Phos a activity by only 0.5 ± 3 and 37 ± 3%, respectively (n = 5 per group, data not illustrated), compared with the 60 ± 4 and

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**Fig. 1. Electrophoretic analysis of phosphorylase (Phos) a preparation.** A: aliquots of the Phos a preparation were resolved by SDS-PAGE and Coomassie brilliant blue staining, the gel was photographed, and scanning densitometry was performed for the 2-μg protein load lane. μg protein, load in each indicated lane (0.25–2); M, prestained molecular mass markers lane; nos. to the left of M lane, molecular mass (in kDa) of each individual marker; position of the Phos a band is ~100 kDa. B: nondenaturing isoelectric focusing experiment with Phos a (lane a) and Phos b (lane b) preparations. 1 μg of protein was loaded per lane and resolved in a pH 3–10, 5%T polyacrylamide nondenaturing slab gel, followed by Coomassie R-250 and Crocein scarlet staining and densitometry. These analyses were performed twice with similar results.
165 ± 5% increases in Phos a $V_{\text{max}}$ elicited by 100 µM ADP and AMP, respectively (Table 1). Although IMP was effective at reducing Phos a $K_m$ at P_i levels, IMP did not increase Phos a $V_{\text{max}}$ even when used at millimolar concentrations (Table 1).

The impact of AMP and ADP on Phos a activity was found to depend on the prevailing P_i concentration, at lower P_i, the relative activating effect of each given concentration of nucleotide on Phos a activity is greater than at higher P_i (Fig. 3). The complexity of this interaction of allosteric and P_i regulation of Phos a is of possible importance in the context of glycogenolytic flux regulation in skeletal muscle because AMP, ADP, and P_i all increase in muscle during contractions, which induces glycogenolysis (5, 7, 11, 17, 18, 21, 27). Therefore, we performed experiments to simulate P_i, AMP, and ADP concentrations existing in human skeletal muscle during different resting and exercising conditions on the basis of previously reported experimental values (18, 27). Using these conditions, we were able to assess the independent and combined influences of these physiological levels of P_i and adenine nucleotides on Phos a flux (Fig. 4). Two different conditions in the context of glycogenolytic flux regulation in skeletal muscle because AMP, ADP, and P_i all increase in muscle during contractions, which induces glycogenolysis (5, 7, 11, 17, 18, 21, 27). Therefore, we performed experiments to simulate P_i, AMP, and ADP concentrations existing in human skeletal muscle during different resting and exercising conditions on the basis of previously reported experimental values (18, 27). Using these conditions, we were able to assess the independent and combined influences of these physiological levels of P_i and adenine nucleotides on Phos a flux (Fig. 4). Two different conditions. 

### Table 1. Phosphorylase a kinetic constants for $P_i$ at different effector conditions

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration, µM</th>
<th>$V_{\text{max}}$, U/mg</th>
<th>$K_m$, mM $P_i$</th>
<th>$V_{\text{max}}/K_m$</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No effectors</td>
<td>221 ± 2</td>
<td>5.6 ± 0.3</td>
<td>40 ± 1</td>
<td>0.998</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>0.1</td>
<td>292 ± 5†</td>
<td>2.9 ± 0.1†</td>
<td>101 ± 8†</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>354 ± 2†</td>
<td>2.3 ± 0.1†</td>
<td>152 ± 8†</td>
<td>0.992</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>393 ± 1†</td>
<td>2.0 ± 0.0†</td>
<td>199 ± 6†</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>497 ± 2‡</td>
<td>1.7 ± 0.0‡</td>
<td>287 ± 14‡</td>
<td>0.990</td>
</tr>
<tr>
<td>ADP</td>
<td>0.1</td>
<td>238 ± 7*</td>
<td>7.5 ± 0.5*</td>
<td>32 ± 2*</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>209 ± 8</td>
<td>4.1 ± 0.3†</td>
<td>51 ± 2†</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>244 ± 9†</td>
<td>2.5 ± 0.1†</td>
<td>97 ± 6†</td>
<td>0.991</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>305 ± 2†</td>
<td>1.5 ± 0.1†</td>
<td>205 ± 10†</td>
<td>0.994</td>
</tr>
<tr>
<td>IMP</td>
<td>100</td>
<td>229 ± 5</td>
<td>2.2 ± 0.1†</td>
<td>106 ± 4†</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>234 ± 7</td>
<td>2.2 ± 0.1†</td>
<td>107 ± 6</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td>189 ± 2†</td>
<td>3.3 ± 0.2†</td>
<td>57 ± 5†</td>
<td>0.999</td>
</tr>
<tr>
<td>Caffeine</td>
<td>10</td>
<td>204 ± 5†</td>
<td>6.8 ± 0.5*</td>
<td>30 ± 3†</td>
<td>0.996</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>197 ± 3†</td>
<td>8.4 ± 0.2†</td>
<td>24 ± 3</td>
<td>0.987</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>175 ± 6†</td>
<td>13.8 ± 0.6†</td>
<td>13 ± 3†</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>153 ± 7†</td>
<td>16.0 ± 1.2†</td>
<td>10 ± 2†</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Values are means ± SE for 5 observations per group. $V_{\text{max}}$ and $K_m$ were each determined from double-reciprocal plots of the kinetic data; $r$ is the coefficient of the regression line from the double-reciprocal plots. $V_{\text{max}}/K_m$ is the simple ratio of paired values from each individual set of kinetics. *P < 0.05, †P < 0.01 vs. no effectors. $V_{\text{max}}$ and $V_{\text{max}}/K_m$ are significantly greater for the AMP group vs. the ADP group at each given effector concentration (P < 0.01).
resting conditions (Rest 1 and Rest 2) were simulated with the only difference being the P_i concentration; this was to accommodate the range of reported resting P_i values (21). Increases in P_i in the physiological range activated Phos a (Fig. 4), as expected from other data in this paper (Fig. 2). At each simulation condition and resultant P_i level, the condition-appropriate AMP and ADP levels resulted in activation of Phos a over the P_i-only values (Fig. 4). The combined effects of AMP and ADP at each simulation condition were greater than the single effects but were less than the additive result of the single effects (Fig. 4). At simulations of more intense contractions with resulting higher P_i levels, there was less relative activation effect of nucleotides (Fig. 4). Thus there was a large activation by nucleotides in the 35% maximal O_2 uptake contraction simulation, which was sustained, but not substantially altered, by the simulations of other more intense contraction conditions (Fig. 4). The ability of small physiological changes in nucleotide levels to influence Phos a activity at fixed P_i was illustrated, however, by comparing the 90 and 250% maximal O_2 uptake conditions (Fig. 4); the increased AMP and ADP at 250% resulted in greater activation of Phos a.

**Effects of caffeine.** In contrast to the effects of ADP and AMP, caffeine inhibits Phos a activity (Table 1, Figs. 5 and 6). Caffeine increased K_m, decreased V_max, and decreased V_max/K_m ratio in a concentration-dependent manner (Table 1). The plasma caffeine concentration that results from moderate caffeine consumption and that can result in a glycogen-sparing effect during exercise is ~50–100 μM (8, 16, 28). At this concentration of caffeine in this study, the K_m for Phos a was approximately twofold higher than control (Table 1). The sensitivity of caffeine inhibition to the prevailing P_i concentration (Figs. 5 and 6) was not as great as that observed for the influence of P_i concentration on sensitivity to AMP or ADP activation of Phos a. For example, the relative percent inhibition effect of 50–100 μM caffeine compared with no caffeine was similar at 2 and 20 mM P_i (~40–50% inhibition; Fig. 6), although this effect was greater than at saturating P_i (i.e., under V_max conditions, in which case ~25% inhibition was observed; Table 1).

**DISCUSSION**

The major findings of this study confirm our hypothesis that AMP and ADP at free concentrations representing a dynamic range that occurs in skeletal muscle during contractions are capable of activating skeletal...
muscle Phos $a$ in vitro and of increasing the sensitivity of Phos $a$ to its substrate, $P_i$. In addition, the results with caffeine support our second hypothesis that caffeine, at levels known to result from moderate caffeine consumption, can inhibit Phos $a$ and decrease the sensitivity of Phos $a$ to $P_i$. This study is the first to demonstrate the potentially physiologically relevant regulation of Phos $a$ by AMP, ADP, and caffeine.

In the absence of AMP or other effectors and at saturating glycogen concentration, the $K_m$ for Phos $a$ toward $P_i$ in a relatively pure preparation has previously been reported to be $\sim 10 \text{ mM}$ (20). In contrast, crude muscle homogenates from human subjects have previously been used for a similar kinetic analysis and have yielded values on the order of $\sim 25–30 \text{ mM}$ (5). Our data using a Phos $a$ preparation of known purity ($\sim 90\%$ Phos $a$, $\sim 10\%$ Phos $b$) from rabbit skeletal muscle support the lower end of $K_m$ values; we found it to be $5.6 \pm 0.3 \text{ mM}$ (Table 1). The quantitative difference in results from different preparations is important here, because muscle free $P_i$ ranges from $\sim 2 \text{ mM}$ at rest to $\sim 15–20 \text{ mM}$ during intense muscle contractions (21, 27). Thus an accurate assessment of the $K_m$ for Phos $a$ is essential for understanding the relative importance of $P_i$ and differences in $P_i$ on the activity enzyme AMP deaminase, which converts added AMP to mimic a given simulated condition produced roughly equal activation of Phos $a$, as did AMP at appropriate concentrations to mimic the same simulated condition (Fig. 4). This occurs despite the fact that the specific activation of Phos $a$ by AMP is much greater than that by ADP (Table 1, Figs. 2 and 3) due to the fact that free ADP in muscle is $50–250$ times greater than free AMP under any given conditions (18, 21, 27; Fig. 4). Thus both AMP and ADP, at the concentrations occurring in skeletal muscle, could make contributions to the regulation of Phos $a$ at rest and during contractions. The combination of AMP and ADP in a given simulation produced a greater activation than AMP or ADP alone, although the effect was not completely additive (Fig. 4). The refinements in understanding the control of Phos $a$ by physiological levels of $P_i$, AMP, and ADP from the present study using purified Phos $a$ and physiological substrate and effector concentrations support the suggestion that these agents are highly effective controllers of Phos $a$ in contracting muscle.

Inhibition of Phos $a$ by caffeine has been reported in an earlier study (19). Kasvinsky and co-workers (19) used a purified Phos $a$ preparation and measured Phos $a$ activity reacting in the nonphysiological direction of glycogen synthesis, thereby precluding direct comparisons to our data, which were generated with Phos $a$ reacting in the physiologically relevant direction, in which $P_i$ is a substrate. In addition, caffeine was used at supraphysiologic concentrations (e.g., $1 \text{ mM}$) and inhibited skeletal muscle Phos $a$ by $\sim 50\%$ in the Kasvinsky et al. study. In our studies, we found Phos $a$ to be more sensitive to caffeine inhibition. For instance, at $P_i$ concentrations in the physiological range, $50–100 \text{ mM}$ caffeine, which is similar to plasma caffeine levels after moderate caffeine consumption (8, 16, 28), inhibited Phos $a$ by $\sim 50\%$ (Table 1, Fig. 6). This suggests that caffeine is an inhibitor of Phos $a$ when the enzyme reacts in the direction of glycogen breakdown and that this action of caffeine could influence Phos $a$ activity under biochemical conditions existing in intact, contracting skeletal muscle of animals after caffeine consumption.

Speculative Physiological Importance of in Vitro Allosteric Effector Results

The relevance of these in vitro, purified enzyme effector studies to the complex control of Phos $a$ activity in intact exercising muscle depends on how the influence of these effectors interact with other factors that determine Phos $a$ flux (such as covalent interconversion of Phos $a$ and Phos $b$) as well as the cellular conditions established as a result of muscle activity. These data establish that the effectors studied are capable of significantly influencing Phos $a$ activity at...
concentrations that result in muscle under physiological circumstances, thus bolstering the argument for their importance in vivo. Furthermore, changes in effector concentrations under different experimental treatments are associated with changes in muscle glycogenolysis in exercising humans that would be predictable from the demonstrated roles of these effectors in the allosteric control of Phos a in vitro (7, 8, 14, 17). Two examples illustrate this point: the effects of short-term aerobic exercise training and of caffeine consumption on muscle glycogenolysis during exercise in human skeletal muscle. Short-term training [STT; ∼2 h/day cycle exercise at ∼65% pretraining peak O₂ uptake (V₀₂ peak)] results in lower rates of muscle glycogenolysis during a subsequent acute submaximal exercise trial (i.e., 15–30 min at 70–80% V₀₂ peak; 7, 17). The extent of the increase in Phos a mole fraction (%Phos a) response to acute exercise is not altered by STT (7), but the exercise-induced decrease in phosphocreatine and the increases in Pᵢ, ADP, and AMP are substantially tempered by STT (7, 17). Thus, under conditions of constant work and constant %Phos a, lower rates of glycogenolysis are coincident with lower levels of Phos a substrate (Pᵢ) and allosteric activators (ADP and AMP) in the muscle of STT subjects. Under these circumstances, it is possible that a reduced substrate and allosteric pressure on Phos a mediated by lower Pᵢ, AMP, and ADP could contribute to the lower rate of glycogenolysis during exercise after STT. It is not known, however, whether lower glycogenolysis is the cause or effect of probable compensatory metabolic changes after STT to provide adequate fuel for the constant work output. For instance, respiratory exchange ratio data suggest an enhancement of fat oxidation after STT (7, 26), and muscle and blood lactate levels are correspondingly lower (7, 17, 26).

Caffeine ingestion provides another context in which to study differences in glycogenolysis during exercise. Caffeine ingestion (9 mg/kg body wt, 1 h before exercise) that results in plasma caffeine levels in the range of ∼50–100 μM at the time of exercise (7, 16) can result in glycogen sparing (reduced net rate of glycogenolysis) during the nonfatiguing phase of moderate-to-intense aerobic exercise. For instance, a group of subjects used ∼30% less glycogen during a 15-min 80% V₀₂ peak cycling trial after caffeine ingestion than during an identical trial without caffeine (8). %Phos a was not significantly different between caffeine and placebo trials (it tended to be higher after caffeine). Thus, at constant work and constant (or higher) %Phos a, lower rates of glycogenolysis were observed in the presence of caffeine, an allosteric inhibitor of Phos a (8). It is possible that inhibition of Phos a by caffeine may have contributed, together with increased fat availability and use and with tempered perturbations to the energy status of exercising muscle (8, 15, 28), to the glycogen-sparing effect of caffeine during the early stages of moderately intense aerobic exercise (8, 28). Thus it is likely that multiple, independent downstream effects of caffeine are involved in the inhibition of glycolysis, but quantitative data now suggest possible involvement of direct allosteric inhibition of Phos a by caffeine in this phenomenon.

The two examples discussed above are the clearest correlates to the physiological importance that can be made with the in vitro data generated in this study. This is so because the other potentially very important factor in determining Phos a flux, the %Phos a, was not different between treatments in the studies of these two examples. It should again be recognized, however, that transformation of Phos to the α form sets the upper limit of Phos a flux and glycogenolysis. The posttransformational regulators, Pᵢ, and allosteric effectors such as AMP, ADP, and caffeine can fine-tune the actual flux through Phos a under given cellular conditions.

Limitations

The impetus to use the isolated enzyme approach in this study comes from a recent plethora of data demonstrating dissociation of %Phos a from glycogenolytic rate in skeletal muscle under a number of conditions (5–10, 22–24). Furthermore, increasing evidence gathered in physiological studies suggests that factors associated with the energy status of the cell that are also Phos a substrate and allosteric effectors may play a significant role in determining Phos a flux in vivo (7, 8, 17, 22–24). These studies were performed to address the question of sensitivity of Phos a to its substrate, Pᵢ, and to allosteric effectors at concentrations within the dynamic range that occurs in muscle during rest and intense activity. In this paper, we have provided novel data using isolated Phos a under precisely controlled conditions that support the possible importance of alterations in Pᵢ, AMP, ADP, and caffeine on the control of Phos a activity in vivo. A potential limitation of this study is that the enzyme preparation was not 100% pure; i.e., it consisted of ∼90% Phos a and ∼10% Phos b. Careful consideration of the situation indicates that this low level of Phos b contamination probably did not taint the results, however. Phos b is inactive in the absence of allosteric activators; thus the Pᵢ-only data and the caffeine inhibition data reflect Phos a activity only and should not be affected by the presence of 10% Phos b. Furthermore, the physiological levels of AMP and ADP used in this study (see Fig. 4 values) are below those necessary to significantly activate Phos b in vitro (2). Even at the highest AMP concentration used (100 μM), Phos b is activated to only approximately one-sixth of its enzymatic capacity. Combined with the fact that only an ∼10% Phos b contamination exists, this would result in a <2% contribution of Phos b to the overall measured Phos activities in this study. Thus it is unlikely that the low level of Phos b contamination of the Phos a preparation used herein significantly affected the interpretation of the data. The possible influences of other regulatory motifs, such as complex formation between Phos, glycogen, and organelles and/or other subcellular structures, on enzyme kinetics were not addressed in this study. In addition, these data do not address the issues of control of the %Phos a in muscle, or the possible muscle fiber
type differences (that characterize many other enzyme systems) in regulation of Phos. If it is recognized that the conversion of Phos b to Phos a at the onset of contractions is incomplete (i.e., not 100% Phos a) and transitory (i.e., decreased %Phos a over time during contractions), the regulatory properties of Phos b may make a significant contribution to the control of glycogenolysis; this issue was not investigated in the present study. All of these issues must be taken into account for a global appreciation of Phos control.

In summary, we have demonstrated that Phos a is sensitive to regulation by P_i, AMP, and ADP within a concentration range characteristic of the dynamic range that occurs between rest and contractile activity during exercise. The interaction of these factors is complex, and differences in the levels of P_i, AMP, and ADP may contribute to altered rates of glycogenolysis at fixed %Phos a under different physiological circumstances such as comparing trained with untrained muscle. The K_m for Phos a is on the order of the resting P_i concentration in skeletal muscle. This limits the dynamic range of activation of Phos a but increases the sensitivity of the enzyme to P_i in the physiological range. In addition, we have established that caffeine inhibits Phos a by altering the kinetics with respect to P_i. These effects are substantial at caffeine concentrations such as comparing trained with untrained muscle. The F_s for Phos a is the role of IMP in the regulation of phosphorylase activity in skeletal muscle. The Enzymes

REFERENCES


