STIMULATION OF RAT LIVER PHOSPHORYLASE KINASE BY MICROMOLAR CONCENTRATIONS OF Ca^{2+}

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Received 24 June 1975

1. Introduction

Phosphorylase kinase from rabbit skeletal muscle [1–3], cardiac muscle [3], insect flight muscle [4], guinea pig brain [5] and human platelets [6] has been shown to require micromolar concentrations of Ca^{2+} for optimal activity. Recently, we have shown that phosphorylase kinase from adipose tissue also has a Ca^{2+} requirement [7,8].

The results presented below show that phosphorylase kinase from rat liver requires Ca^{2+} for optimal activity. It was inhibited 75% by 0.2 mM EGTA [ethyleneglycol-*bis* (β-aminoethyl ether)-*N*, *N'*-tetra-acetic acid]. This inhibition was promptly and fully reversed by Ca^{2+}. The possible involvement of liver phosphorylase phosphatase was ruled out by inclusion of fluoride (F^-) and β-glycerolphosphate (β-GP) in the assay system, which effectively blocked phosphatase activity.

2. Materials and methods

Livers from Sprague–Dawley rats (150–180 g) were homogenized in 2 volumes of a solution containing 0.25-M sucrose and 10 mM Tris–Cl, pH 7.4. The homogenate was centrifuged at 1000 *g* and the fat cake was removed. The infranatant fluid was then centrifuged at 50 000 *g* for 30 min. Any floating fat was suctioned off and the remaining supernatant fluid (S_{50}) was used as the source of enzyme.

Phosphorylase kinase was assayed by a modification [9] of the method of Krebs et al. [10]. Phosphorylase *a* formed was assayed according to the method of Hardman et al. [11]. One unit of phosphorylase activity was defined as one μmole of glucose-1-P produced per min. The phosphorylase kinase reaction mixture consisted of 50 mM β-GP, 10 mM KF, 13 mM β-mercaptoethanol (β-ME), 5 mM $Mg(Ac)_2$, 1 mM ATP, purified rabbit skeletal muscle phosphorylase *b* (240 U/ml), 15 μl (about 0.4 mg protein) of the rat liver S_{50} fraction and an ATP-regenerating system (2 mg/ml creatine kinase and 5 mM phosphocreatine) in a final volume of 50 μl. A 5-min incubation time at 30°C was routinely used (unless otherwise indicated) during which time the increase of phosphorylase *a* was linear. The reaction was terminated by 20-fold dilution with a stopping reagent consisting of 40 mM β-GP, 40 mM KF and 13 mM β-ME, pH 6.8 at -5°C (ice-salt bath). Aliquots of 5 μl were immediately transferred to 50 μl of phosphorylase *a* assay mixture at pH 6.8 which contained 50 mM Pi, 10 mg/ml glycogen and, to eliminate effects of 5'-AMP present in reagents or generated during assay, 5'-nucleotidase (1.6 U/ml)was added. Phosphorylase *a* was assayed for 5 min at 30°C. Under these assay conditions, the contribution of endogenous phosphorylase activity was negligible.

The phosphorylase phosphatase assay mixture consisted of purified rabbit muscle phosphorylase *a* (about 150 U/ml), 15 μl of rat liver S_{50} fraction, 13 mM β-ME and 50 mM PIPES buffer [piperazine-*N*, *N'*-bis(2-ethane sulfonic acid)] at pH 6.8 in a final volume of 50 μl. The initial phosphorylase activity ratio (assayed in the absence and presence of 2 mM 5'-AMP) was about 0.72. The rate of decrease in phosphorylase *a* was taken as a measure of phosphorylase phosphatase activity. The reaction was terminat-

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ed by 20-fold dilution with stopping reagent as described for the phosphorylase kinase assay.

3. Results

As shown in fig.1A, EGTA markedly inhibited the conversion of exogenous phosphorylase *b* to *a* by 75% at 0.2 mM. The inhibition was prevented by addition of $CaCl_2$ along with EGTA as shown in fig.1B The optimal concentration of $CaCl_2$ was 0.3 mM in the presence of 0.5 mM EGTA. Higher concentrations of $CaCl_2$ were inhibitory. The pH of the reaction mixture changed very little with the addition of $CaCl_2$ (less than 0.1 pH unit when 0.5 mM $CaCl_2$ was added to 0.5 mM EGTA). The lower abscissa of fig.1B shows the estimated free Ca^{2+} concentration in equilibrium with EGTA–Ca^{2+} at each point, based on a dissociation constant of 1.3×10^{-6} M as reported by Portzehl et al. [12]. Half-maximal stimulation was obtained at a free Ca^{2+} concentration of 0.6 μM and maximal at 4 μM. Similar results have been observed using endogenous liver phosphorylase (4 U/ml) as substrate. Phosphorylase kinase was inhibited 77% by 0.1 mM EGTA at pH 8.0. We have also found that phosphorylase kinase from rabbit liver was inhibited by EGTA. Again, full stimulation was obtained at concentrations of Ca^{2+} less than those of the added EGTA. Ca^{2+} and EGTA at equimolar ratios were inhibitory as in the case of the rat liver enzyme.

The Ca^{2+} reversal of EGTA inhibition was rapid as shown in fig.2. The phosphorylase *b* to *a* conversion was measured in the absence and presence of 0.5 mM EGTA at intervals over a 20-min incubation period. The initial of phosphorylase *a* formation was inhibited by 60%. At 6 min, a 50 μl aliquot of the reaction mixture containing EGTA was transferred to a tube containing enough $CaCl_2$ to yield a final concentration of 0.3 mM. The inhibition due to EGTA was promptly released and the rate of phosphorylase *a* production increased immediately.

Crude liver extracts contain high levels of phosphorylase phophatase activity. To rule out the possible involvement of phosphorylase phosphatase in the observed reciprocal effects of EGTA and Ca^{2+}, 10 mM F^- and 50 mM β-GP were included in the phosphory-

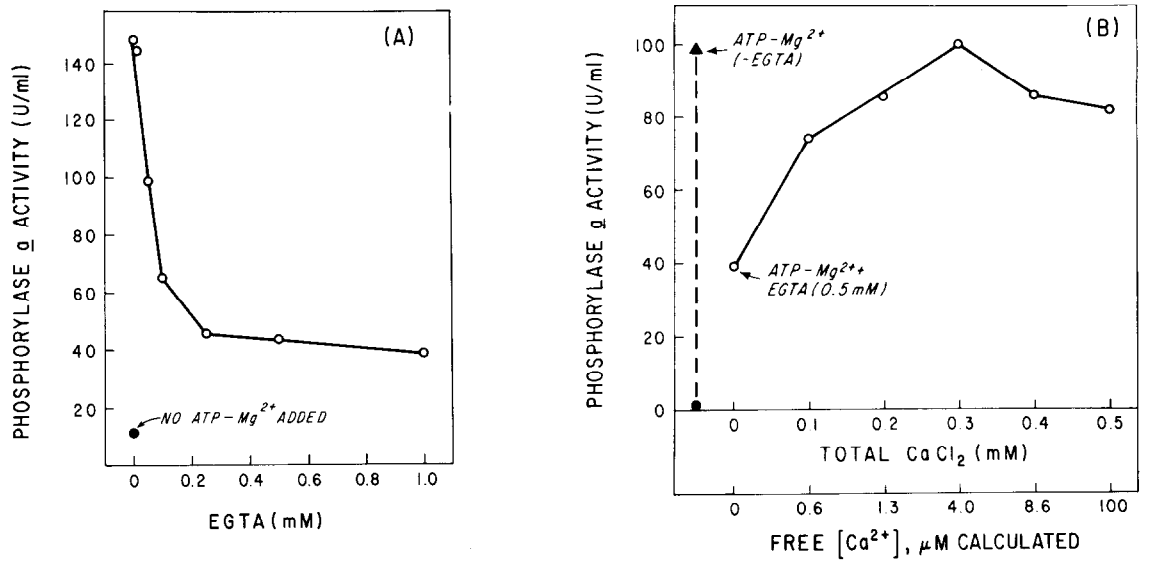

Fig.1. Effect of EGTA and Ca^{2+} on phosphorylase kinase activity. Phosphorylase kinase, assayed for 5 min at 30°C, is expressed in terms of units phosphorylase *a* formed. (•), phosphorylase *a* activity in control incubation (without addition of ATP–Mg^{2+}). Panel A: inhibition as a function of EGTA concentration. Panel B: prevention of EGTA inhibition by addition of $CaCl_2$ in the presence of 0.5 mM EGTA. The lower abscissa shows the calculated concentration of free Ca^{2+} at each point based on a dissociation constant of 1.3×10^{-6} M [12].

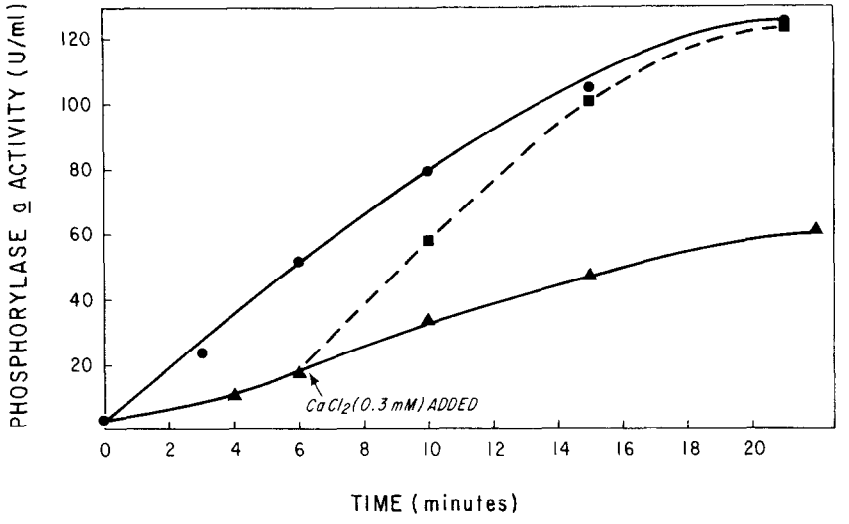

Fig.2. Rapid reversibility of EGTA inhibition of phosphorylase kinase activity by Ca^{2+}. (•), control time course of phosphorylase kinase assay; (▲), time course in presence of 0.5 mM EGTA; (■), effect of adding $CaCl_2$ at 6 min to give a final Ca^{2+} concentration of 0.3 mM.

lase kinase reaction mixture. This combination effectively inhibited the phosphatase activity as shown in fig.3. At 5 min, the time used for phosphorylase kinase assays, there was no measurable change in phosphorylase *a* activity unless F^- and β-GP were omitted. There

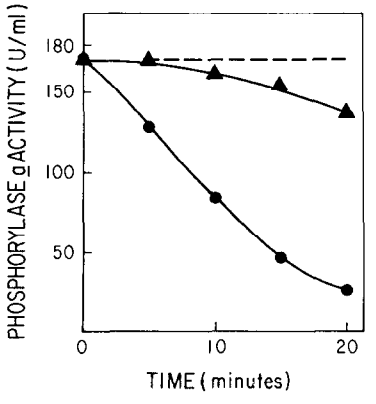

Fig.3. Inhibition of phosphorylase phosphatase by F^- and β-GP. Purified phosphorylase *a* (170 U/ml) from rabbit skeletal muscle was used as substrate. (•), phosphorylase phosphatase assayed with 15 μl of rat liver S_{50} in 13 mM β-ME and 50 mM PIPES, pH 6.8, in a final volume of 50 μl; (▲), as above with additions of 50 mM β-GP and 10 mM F^-.

remained the possibility that EGTA or EGTA–Ca^{2+} might affect phosphorylase phosphatase activity. However, as shown in table 1, neither EGTA nor EGTA–Ca^{2+} affected phosphorylase phosphatase activity either in the presence or absence of β-GP and F^-.

Gratecos and Fischer [13] have reported that rabbit muscle phosphorylase activated using ATP γS [adenosine 5′-*O*(3-thiotriphosphate)] in place of ATP is resistant to the action of the muscle phosphorylase phosphatase. Assuming that thiophosphorylase *a* is also resistant to liver phosphorylase phosphatase, one would not expect to see an EGTA inhibition of phosphorylase *a* formation using this ATP analog if the EGTA effect were exclusively on activation of the phosphatase. In fact, the inhibition by 0.5 mM EGTA was fully as great (70%) when ATPγS was substituted for ATP (data not shown).

4. Discussion

These results clearly show that phosphorylase kinase activity in crude rat liver fractions requires Ca^{2+} for optimal activity using either exogenous rabbit skeletal muscle phosphorylase *b* or endogenous liver phosphorylase as substrate. The possibility that the

Table 1
Effects of EGTA and Ca^{2+}–EGTA on phosphorylase phosphatase activity in rat liver S_{50} fraction

Exp. nol	Assay conditions	Time (min)	Additions		
			None	EGTA (0.5 mM)	$CaCl_2$ (0.3 mM) + EGTA (0.5 mM)
			Phosphorylase *a* activity (U/ml)*		
1.	Assayed in the presence of F^- (10 mM) and β-GP (50 mM), pH 6.8	5	124	110	118
		10	104	105	102
		15	93	94	86
		20	80	82	87
2.	Assayed in the absence of F^- and β-GP. PIPES buffer (50 mM), pH 6.8	5	130	128	131
		10	92	93	99
		15	58	59	60
		20	36	38	38

* Zero time phosphorylase *a* activity, 130 U/ml in exp. 1; 170 U/ml in exp. 2.

observed results might be due to changes in phosphorylase phosphatase activity was ruled out: (1) by the inclusion of β-GP and F^- at pH 6.8 which was shown to inhibit phosphorylase phosphatase during the phosphorylase kinase assays; (2) by the demonstration that neither EGTA nor EGTA–Ca^{2+} showed any direct effect on apparent phosphorylase phosphatase activity; and (3) by the comparable EGTA inhibition when ATPγS was substituted for ATP.

Thus, it is possible that phosphorylase kinases generally require Ca^{2+} for optimal activity. However, there appears to be variability in the level of activity in the absence of Ca^{2+}, i.e. in the degree of inhibition by EGTA. The enzyme from rabbit skeletal muscle was completely inhibited by EGTA [1–3] whereas in the present studies, the liver enzyme was inhibited at most by 60–75%. In this connection , it is interesting to note that phosphorylase kinase from insect flight muscle was inhibited at most by 70% by EGTA [14]. This may explain the previously reported absence of EGTA inhibition of phosphorylase kinase from rabbit liver [15] and also that from rat adipose tissue when endogenous phosphorylase *b* served as substrate [16]. It is possible that Ca^{2+} dependency of the enzyme may be altered during preparation and handling. Suggestive evidence for such a change during prolonged dialysis against EGTA has been noted [8]. While this manuscript was in preparation, Shimazu and Amakawa [17] reported that phosphorylase kinase purified 80-fold from rabbit liver was inhibited 70% by 0.1 mM EGTA using purified liver phosphorylase as substrate. However, addition of Ca^{2+} did not fully restore kinase activity in contrast to the results reported here (fig.1B).

Acknowledgements

The authors wish to express their appreciation to Drs D. L. Severson, J. T. Stull and S. E. Mayer for valuable discussions and to Mrs. M. Silvestre and Miss A. Aquino for their excellent technical assistance. This work was supported by USPHS Grant HL-12373 of the National Heart and Lung Institute.

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