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STUDIES ON GLYCOGEN PHOSPHORYLASE KINASE: CATALYTIC PROPERTIES AND ISOZYMES*

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INDEXING WORDS

calcium; calmodulin; cyclic AMP; protein kinase; protein phosphorylation; glycogen metabolism; antiserum

SYNOPSIS

The present study is concerned with the multiple functions and isozymes of glycogen phosphorylase kinase. Skeletal muscle glycogen phosphorylase kinase [EC 2.7.1.38] is able to phosphorylate in vitro multiple species of unidentified proteins from various subcellular fractions of rat liver. The phosphorylation is dependent on the presence of Ca^{2+}. Phosphate acceptor proteins of subcellular fractions are most abundant in the soluble and microsomal fractions. The spectrum of phosphate acceptor proteins entirely differs from that for cyclic AMP-dependent protein kinase (protein kinase A). The enzyme also phosphorylates calf thymus histone fractions and H1 histone is the most preferable substrate. The phospho-

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Abbreviations: protein kinase A, cyclic AMP-dependent protein kinase; protein kinase C, Ca^{2+}-activated, phospholipid dependent protein kinase; protein kinase G, cyclic GMP-dependent protein kinase; SDS, sodium dodecyl sulfate, EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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rylation sites in H1 histone for this enzyme are different from those for protein kinase A by N-bromosuccinimide bisection and fingerprint analysis. It is suggested that the enzyme is potentially multifunctional and plays roles in controlling some of the Ca^{2+} dependent processes.

Properties of glycogen phosphorylase kinase, present in 100,000 x g supernatant from various rat tissues, were examined. The enzymes appear to be divided into at least three types; muscle, brain and liver types. The muscle and liver types are distinguished from each other in the pH 6.8/8.5 activity ratio, Ca^{2+} dependency, activation by protein kinase A and inhibition by the antiserum. The enzymes from heart and skeletal muscle belong to the muscle type, and those from liver, kidney, spleen, lung and testis to the liver type. The enzyme from brain apparently differs from these types and seems to be an intermediate type or mixture of the two.

INTRODUCTION

Glycogen phosphorylase kinase [EC 2.7.1.38] is known to play a key role in the hormonal regulation of glycogen breakdown. The enzyme has been purified to apparent homogeneity in the skeletal muscle and its molecular weight is about 1,300,000,[4] consisting of four kinds of subunits in the stoichiometry $\alpha_4\beta_4\gamma_4\delta_4$.[24] The δ-subunit appears to be identical with calmodulin.[24] Both cyclic AMP-dependent protein kinase [EC 2.7.1.37] (protein kinase A) and Ca^{2+} are important for the activation mechanism of this enzyme.[20, 32] The enzyme catalyzes not only the conversion of phosphorylase b to phosphorylase a, but also the phosphorylation of muscle glycogen synthase I, casein, troponin I and T, sarcoplasmic reticulum, cardiac sarcolemma, calf thymus whole histone and a synaptic membrane protein of 40,000 daltons.[1, 3, 14, 25] These observations may suggest that phosphorylase kinase is one of the multifunctional protein kinases, although the physiological function of this enzyme other than glycogen breakdown has not been substantiated.

Heart enzyme, in which the α'-subunit replaces the α-subunit of skeletal muscle, has been purified to near homogeneity and its molecular weight, enzyme properties and Ca^{2+} sensitivity are similar to those of skeletal muscle enzyme.[5] However, the liver enzyme, which has been partially purified in this laboratory, has quite different enzyme properties and Ca^{2+} sensitivity from those of the skeletal muscle and the heart enzymes.[22] In other tissues the enzymes have not been well studied.

We report in this paper that: 1) the skeletal muscle glycogen phos-

phorylase kinase has the ability to phosphorylate various unidentified endogenous proteins and the spectrum of phosphate acceptor proteins differs from that for protein kinase A, 2) the enzyme has the potential to phosphorylate seryl residues of Hl histone and the sites of phosphorylation differ from those for protein kinase A and 3) the enzymes in various rat tissues are classified to at least three types; muscle, brain and liver types.

MATERIALS AND METHODS

Chemicals and materials

[γ-^{32}P]ATP was prepared as described by Glynn and Chappell.[9] [U-^{14}C]Glucose-1-phosphate was purchased from Radiochemical Centre, Amersham. Phosphocreatine and creatine phosphokinase were purchased from Sigma. Complete Freund adjuvant was purchased from Nakarai Chemicals, Kyoto. Trypsin (Code TRTPCK) was obtained from Worthington. Calmodulin was a generous gift of Dr. S. Kakiuchi, Osaka University School of Medicine. Other chemicals were obtained from commercial sources.

Subcellular fractions of male Sprague-Dawley rat liver were prepared as described earlier.[13] Histone fractions were prepared from calf thymus and purified as described previously.[10] Crystalline muscle phosphorylase b was prepared from rabbit skeletal muscle as described by Fischer and Krebs.[8] Protein kinase inhibitor of protein kinase A was prepared from rabbit skeletal muscle as described by Walsh et al.[31]

Enzyme preparations

Rabbit skeletal muscle phosphorylase kinase was purified by the method of Cohen.[4] This preparation was essentially homogenous, and four bands corresponding α, α', β, and γ-subunits were detected upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Catalytic and regulatory subunits of protein kinase A were purified from rabbit skeletal muscle by the method described by Yamamura et al.[34] and the catalytic subunit was further purified by using a Sephadex G-100 column. In order to examine the properties of each phosphorylase kinase in various rat tissues, the supernatant for 60 min at 100,000 x g was employed as described earlier.[27]

Enzyme assay

Protein kinase was assayed by measuring the amount of ^{32}P incorpo-

rated into various substrate proteins. For purified muscle phosphorylase kinase the reaction mixture (0.1 ml) contained 5 μmol of Tris-HCl at pH 8.5, 10 nmol of [γ-^{32}P]ATP (1-10 x 10^4 cpm/nmol), 1 μmol of magnesium acetate, 0.5 μmol of 2-mercaptoethanol, and 100 μg of substrate protein, and an enzyme preparation in various concentration of Ca^{2+}. For protein kinase A the reaction mixture (0.1 ml) contained 5 μmol of Tris-HCl at pH 7.5, 0.5 μmol of magnesium acetate, 1 nmol of [γ-^{32}P]ATP (1-10 x 10^5 cpm/nmol), 5 μg of catalytic units of protein kinase A, and 100 μg of substrate protein in each subcellular fraction or 250 μg of histone fraction. $CaCl_2$ showed no effect on protein kinase A, and cyclic AMP was not added since the catalytic subunit was employed. The reaction was started by the addition of enzyme, and was terminated with 10% trichloroacetic acid except that 25% was used in histone phosphorylation.

For phosphorylase kinase in 100,000 x g supernatant solution, the assay was measured the conversion of phosphorylase b to phosphorylase a as described previously.[27] One unit of phosphorylase kinase in 100,000 x g supernatant was defined as the amount of enzyme which produced one unit of phosphorylase a per min. One unit of phosphorylase a was the amount that transferred one μmol of glucose into glycogen per min under the conditions mentioned above.

Determinations

The radioactivity of ^{32}P- and ^{14}C-samples was determined using a Nuclear-Chicago Geiger Muller gas flow counter, Model 4338 and a Packard Tri-Carb liquid scintillation spectrometer, Model 3320, respectively. Protein was determined by the method of Lowry et al.[16] with bovine serum albumin as a standard.

Gel electrophoresis and other procedures

The reaction samples of SDS-polyacrylamide gel electrophoresis was terminated by the method of Ueda and Greengard.[29] After boiling 3 min, an aliquot of this mixture was subjected to this gel electrophoresis as described by Kii et al.[13] Gel electrophoresis of H1 histone has been done by the method of Panyim and Chalkley.[21] Densitometric tracing was performed at 660 nm using an ISCO gel scanner, Model 659, and the radioactivity in each protein band was determined by transverse sectioning of the gel into 2 mm width followed by counting individual slices directly in 10 ml of distilled water with a liquid scintillation spectrometer. Acid hydrolysis of radioactive histone preparations and determinations of phos-

phoserine and phosphothreonine were studied as described by Criss et al. [6] Autoradiograms (fingerprints) were made as described earlier.[10] The anti-skeletal muscle phosphorylase kinase antiserum was prepared as described previously.[27]

RESULTS

Phosphorylation of subcellular fractions of rat liver

Fig. 1 showed the phosphorylation of native and heat-treated subcellular fractions of rat liver in the presence of exogenously added muscle phosphorylase kinase. Using native fractions as substrate, the amount of phosphate incorporated into each fraction reached maximum at 5 min and then declined quickly, probably due to the action of phosphoprotein phosphatases (Fig. 1A). In order to avoid the actions of phosphoprotein phosphatases, endogenous protein kinases and other interfering enzymes, each fraction was heated for 2 min at 80°C before being phosphorylated.

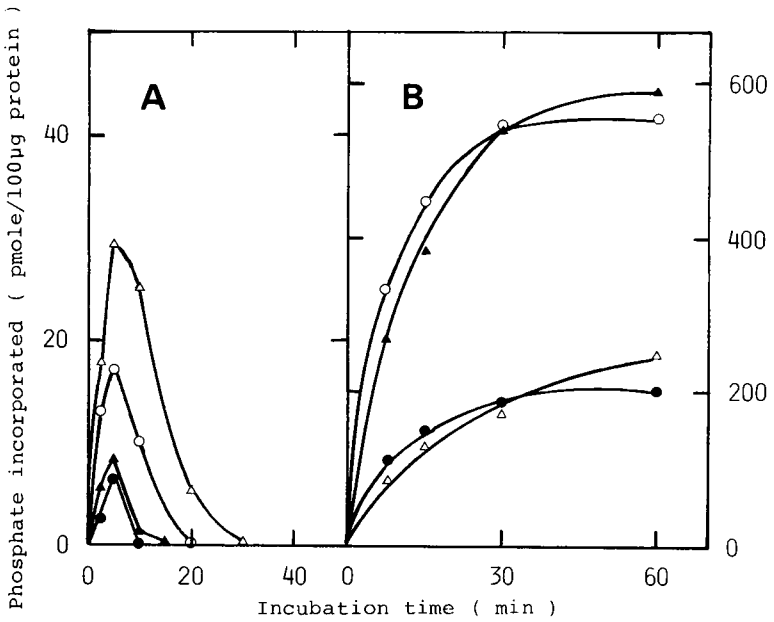

Fig. 1 Phosphorylation of native and heat-treated subcellular fractions of rat liver in the presence of skeletal muscle phosphorylase kinase. Assay conditions are described in detail under "MATERIALS AND METHODS". A, native fractions; B, heat-treated fractions; (△, nuclear fractions; ●, mitochondrial fractions; ○, microsomal fractions; ▲, supernatant fractions). Values are subtracted by phosphate incorporation of enzyme alone.

Fig. 1B showed the time course of phosphorylation of heat-treated subcellular fractions. The soluble and microsomal fractions were rich sources of phosphate acceptor proteins. In the absence of phosphorylase kinase a slight degree of endogenous incorporation of phosphate was detected using native fractions as substrate, however no incorporation of radioactive phosphate was detected using heat-treated fractions as substrate (data not shown).

In the next series of experiments, each heat-treated subcellular fraction was fully phosphorylated with [γ-^{32}P]ATP in the presence of muscle

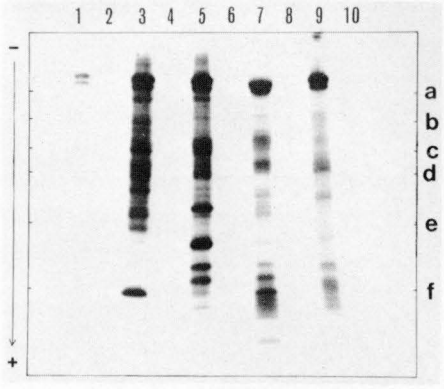

Fig. 2
Electrophoretogram of heat-treated subcellular fractions with and without phosphorylase kinase. Experimental conditions are described in detail under "MATERIALS AND METHODS". Lane 1 and 2, no substrate; Lane 3 and 4, supernatant fractions; Lane 5 and 6, microsomal fractions; Lane 7 and 8, mitochondrial fractions; Lane 9 and 10, nuclear fractions. Odd and even numbered lanes represent the experiments with and without phosphorylase kinase, respectively. Letters represent the marker proteins; a, skeletal muscle phosphorylase b; b, bovine serum albumin; c, ovalbumin; d, glyceraldehyde 3 phosphate dehydrogenase; e, trypsin; f, horse heart cytochrome c.

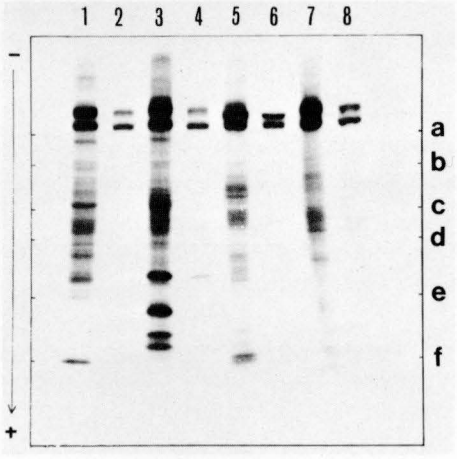

Fig. 3
Electrophoretogram of heat-treated subcellular fractions labeled with phosphorylase kinase with absence and presence of EGTA. Experimental conditions are described in detail under "MATERIALS AND METHODS". Lane 1 and 2, supernatant fractions; Lane 3 and 4, microsomal fractions; Lane 5 and 6, mitochondrial fractions; Lane 7 and 8, nuclear fractions. Odd and even numbered lanes represent the experiments without and with EGTA, respectively. Letters represent the same marker proteins shown in the legend to Fig. 2.

phosphorylase kinase, and the radioactive protein bands were revealed by SDS-gel electrophoresis. The results are shown in Fig. 2 and 3. In Fig. 2, autophosphorylation showed two radioactive bands which corresponded to α-and β-subunits of the enzyme (Lane 1). In addition to these bands, multiple radioactive protein bands were distinguished for all subcellular fractions and were abundant in the soluble and microsomal fractions (Lane 3, 5, 7 and 9). No radioactive protein bands appeared in any subcellular fraction without exogenously added phosphorylase kinase (Lane 2, 4, 6, 8 and 10). It should be noted that phosphorylase kinase phosphorylates in vitro numerous proteins of subcellular fractions of rat liver in addition to autophosphorylation. Fig. 3 showed that phosphorylation of various proteins by phosphorylase kinase absolutely required Ca^{2+}. However, the autophosphorylation of phosphorylase kinase was not absolutely dependent on Ca^{2+} (Lane 2, 4, 6 and 8).[33] The result excluded the possibility that the phosphorylation of each subcellular fraction was catalyzed by protein kinase A, which might be contaminated in the preparation of phosphorylase kinase.

Phosphorylation of calf thymus H1 histone

Phosphorylase kinase catalyzed the phosphorylation of five fractions of calf thymus histone and this reaction was stimulated by calmodulin.[26] H1 histone was the most preferable substrate and H2A histone was also phosphorylated by this enzyme about 50% as active as H1 histone. The other histones including H2B histone were about 10% as active as H1 histone. However, protein kinase A greatly phosphorylated H2B histone more than other histones under similar conditions. In order to clarify that H1 histone itself was phosphorylated, next experiments were performed. H1 histone phosphorylated by phosphorylase kinase was subjected to gel electrophoresis,[21] and densitometric tracing and determination of radioactivity incorporated into H1 histone were performed. As illustrated in Fig. 4, radioactivity corresponded directly to protein bands. In the next experiments, it was explored that the phosphorylation of calf thymus H1 histone was truely catalyzed by phosphorylase kinase. As shown in Fig. 5, the phosphorylation of H1 histone by phosphorylase kinase was not affected at all by protein kinase inhibitor. The reaction was absolutely dependent on Ca^{2+} and it was not affected by the addition of cyclic AMP, cyclic GMP or the regulatory subunit. Myosin light chain was not phosphorylated by this enzyme.[26] These results indicate that phosphorylase kinase employed in this experiment itself has catalyzed the phosphorylation of H1 histone.

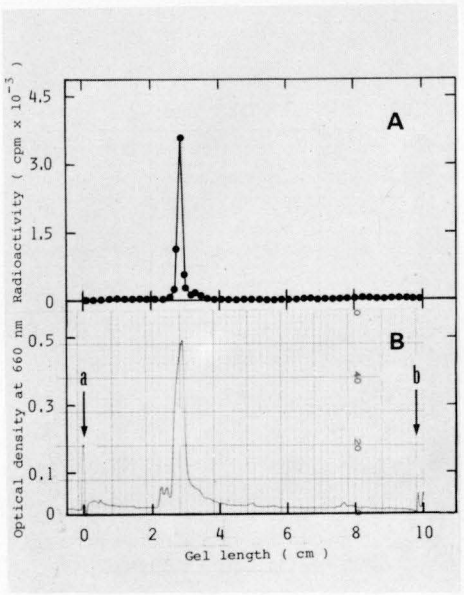

Fig. 4
Phosphorylation of calf thymus H1 histone by phosphorylase kinase. Fully phosphorylated radioactive H1 histone was subjected to gel electrophoresis as described under "MATERIALS AND METHODS". After staining with Coomassie brilliant blue, densitometric tracing and determination of radioactivity were performed. A, radioactivity of phosphorylated H1 histone. B, densitometric tracing of phosphorylated H1 histone. The arrows a and b represent the origin and dye front of the gel, respectively.

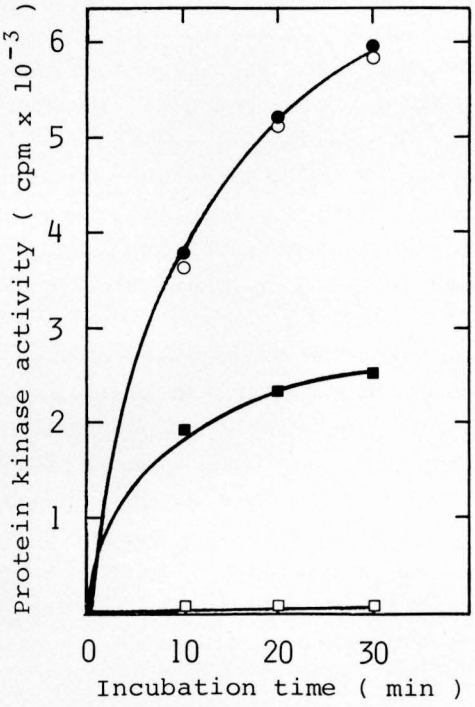

Fig. 5
Phosphorylation of calf thymus H1 histone by phosphorylase kinase and protein kinase A with and without protein kinase inhibitor. Each protein kinase was assayed, as described under "MATERIALS AND METHODS", with 20 nM $CaCl_2$. At the indicated times the reaction was terminated for determination of ^{32}P incorporation. ●, by phosphorylase kinase in the absence of inhibitor protein; ○, by phosphorylase kinase in the presence of inhibitor protein; ■, by protein kinase A in the absence of inhibitor protein; □, by protein kinase A in the presence of inhibitor protein.

In order to determine phosphorylation sites of H1 histone, next sets of experiments were performed. Phosphorylase kinase and protein kinase A phosphorylated different portions of this histone molecule as shown in Table 1. Each fully phosphorylated H1 histone was bisected chemically at Tyr-74 with N-bromosuccinimide.[2] Then, these fragments were separated to two polypeptide portions by a Sephadex G-100 column. The result indicates that phosphorylase kinase phosphorylated both NH_2-and COOH-terminal portions of H1 histone, although NH_2-terminal portion was phosphorylated about half as much as COOH-terminal portion. However, protein kinase A almost catalyzed the phosphorylation of NH_2-terminal portion. By acid hydrolysis of the radioactive peptide portions, it was revealed that phosphorylase kinase phosphorylated almost exclusively (more than 95%) seryl residues in both NH_2-and COOH-terminal portions. Protein kinase A also reacted mainly with seryl residues, and Ca^{2+}-activated, phospholipid dependent protein kinase (protein kinase C) reacted equally with both seryl and threonyl residues.[11] By fingerprint analysis radioactive H1 histone molecule gave apparently different patterns for these enzymes as shown in Fig. 6. The major radioactive spot obtained for protein kinase A has been previously identified as a phosphopeptide containing Ser-38.[10] The results are also different from that for protein kinase C.[11] As it has been already reported that phosphorylation site of H1 histone by cyclic GMP-dependent protein kinase (protein kinase G) is the same as that by protein kinase A,[10] the sites by phosphorylase kinase are also different from that by protein kinase G.

Table 1 N-bromosuccinimide-bisected fragments of H1 histone phosphorylated by phosphorylase kinase and protein kinase A.

H1 histone	Phosphorylation	
	Phosphorylase kinase	Protein kinase A
	(percentage)	
COOH-terminal portion	67.8 ± 1.2	3.3 ± 1.7
NH_2-terminal portion	32.2 ± 1.2	96.7 ± 1.7

Fully phosphorylated radioactive H1 histone was treated with N-bromosuccinimide. Detailed experimental conditions were described previously.[26] Percentage of non-bisected histone was less than 5% in each case. Values are means of five separate determinations with standard errors.

Properties of phosphorylase kinase and its isozymes

In 100,000 x g supernatant fractions of various tissues, phosphorylase kinase activity was examined under the condition described in "MATERIALS AND METHODS". The activity was found in various tissues thus far examined, as shown in Fig. 7. Specific activity in skeletal muscle was about 7.3 unit/mg protein in average. The activities in other tissues were less than 7% of that of skeletal muscle. Table 2 summarizes the properties of phosphorylase kinase in various tissues. The values of activity ratio (pH 6.8/8.5) obtained from skeletal muscle and heart were about 0.15 and 0.24, respectively. Whereas, the values from liver, kidney, spleen, lung and testis were more than 0.7. The value from brain showed an intermediate range at about 0.4.

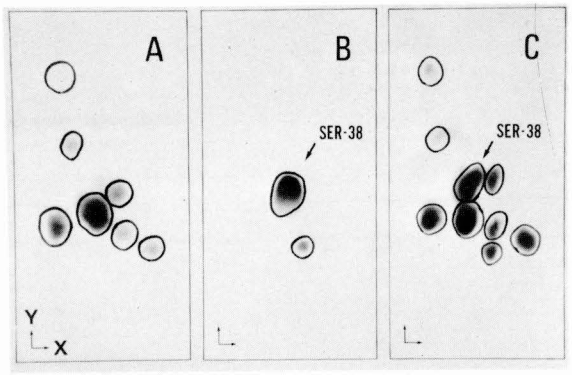

Fig. 6
Fingerprints of tryptic phosphopeptides of radioactive Hl histone phosphorylated by phosphorylase kinase and protein kinase A. Each radioactive Hl histone preparation was digested with trypsin and subjected to paper chromatography (direction X) followed by paper electrophoresis (direction Y) under the conditions as described previously.[10] A, with phosphorylase kinase; B, with protein kinase A; C, with A + B.

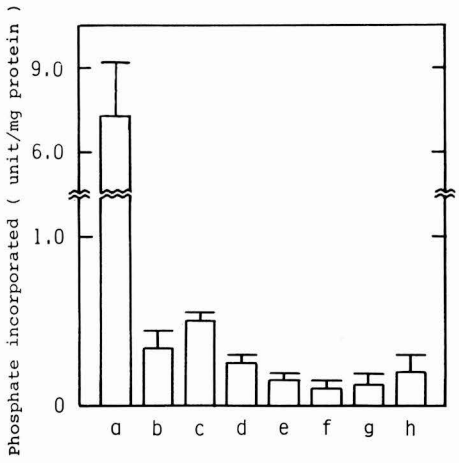

Fig. 7
Phosphorylase kinase activity in various rat tissues. Phosphorylase kinase activity of 100,000 x g supernatants in various tissue was examined. The enzyme activity was assayed as described under "MATERIALS AND METHODS". Values are means of experimental values obtained for 5 rats with standard errors. Small letters represent the tissues: a, skeletal muscle; b, heart; c, brain; d, liver; e, kidney; f, spleen; g, lung; h, testis.

Table 2 Properties of phosphorylase kinase reactions in various tissues.

Tissue	Activity ratio of phosphorylase kinase (pH 6.8/8.5)[a/]	Ca^{2+} requirement for phosphorylase kinase[b/]	Activation by protein kinase A[c/]	Inhibition by anti-muscle phosphorylase kinase antiserum[d/]
		(percentage)	(percent increase)	(percentage)
Skeletal muscle	0.15 ± 0.07	94.0 ± 2.0	180.2 ± 65.2	92.1 ± 4.0
Heart	0.24 ± 0.05	87.8 ± 5.8	171.1 ± 63.1	91.8 ± 6.1
Brain	0.40 ± 0.04	84.3 ± 5.2	39.3 ± 28.2	76.2 ± 7.2
Liver	0.85 ± 0.06	60.5 ± 4.4	20.1 ± 18.2	56.5 ± 8.4
Kidney	0.78 ± 0.10	59.3 ± 13.4	18.4 ± 14.3	52.4 ± 11.0
Spleen	0.85 ± 0.07	52.3 ± 6.4	20.5 ± 18.7	46.2 ± 14.3
Lung	0.70 ± 0.07	63.4 ± 9.9	30.8 ± 27.3	46.3 ± 12.2
Testis	0.85 ± 0.07	57.6 ± 11.0	9.1 ± 8.3	55.1 ± 14.1

Values are means of experimental values obtained for 5 rats with standard errors.

a/ Phosphorylase kinase activity was measured under the conditions described in the text except that 25 mM Tris-25 mM glycerol 1-phosphate buffer at pH 6.8 and 8.5 was employed.

b/ Phosphorylase kinase activity was measured under the conditions described in the text except that 10 nmol of EGTA was added instead of $CaCl_2$. Percentage of inhibition by EGTA is expressed as Ca^{2+} requirement.

c/ Enzymatic activation of phosphorylase kinase by protein kinase A was assayed under the conditions described previously,[22] except for measuring the phosphorylase kinase activity as described in "MATERIALS AND METHODS".

d/ In this experiment, one unit of phosphorylase kinase was incubated with 100 µl of the antiserum.

It has been already reported that the activity of phosphorylase kinase from skeletal muscle is almost absolutely dependent on the presence of Ca^{2+},[20] whereas the liver enzyme retains about 40% of its activity in the absence of Ca^{2+}.[22] Percentage of Ca^{2+} requirement for phosphorylase kinase was tested either in the presence or absence of EGTA. Phosphorylase kinases in skeletal muscle and heart were almost absolutely dependent on the presence of Ca^{2+} for its activity. However, enzymes from other tissues appeared to be of the liver type. Brain enzyme was relatively close to the muscle type in this respect.

It has been well established that skeletal muscle protein kinase A can phosphorylate and activate the skeletal muscle phosphorylase kinase.[32] Phosphorylase kinases in skeletal muscle and heart were stimulated by protein kinase A about 170-180%. However, the activities from other tissues were slightly stimulated by protein kinase A. It was noted that brain enzyme was stimulated about 40% by protein kinase A.

In order to clarify the properties of phosphorylase kinase from various tissues more precisely, anti-skeletal muscle phosphorylase kinase antiserum was employed. Twentyfive μl of antiserum almost completely inhibited one unit of the enzyme activity from skeletal muscle, but one unit from liver was not inhibited more than 50% even in the presence of 100 μl antiserum.[27] In the presence of 100 μl antiserum, the activities of phosphorylase kinase in skeletal muscle and heart were inhibited more than 90% and in brain about 76%, respectively. The activities from other tissues were inhibited only about 50%. The results indicate that skeletal muscle and heart enzymes were immunologically crosswise reactive, but were distinguishable from enzymes in other tissues such as liver, kidney, spleen, lung and testis which belonged to the liver type. Brain enzyme again behaved differently and it seems to be an intermediate type or mixture of the two.

DISCUSSION

In mammalian systems, there appear to be three species of multifunctional protein kinase; namely protein kinase A, protein kinase G and protein kinase C.[28] These enzymes are activated selectively by different second messengers and each enzyme may play specific roles in protein phosphorylation. The intracellular substrate proteins have not yet been fully clarified, but it is suggested that these enzymes recognize apparently similar spectra of phosphate acceptor protein as far as tested in vitro, although the relative reaction velocities towards various proteins are

markedly different.[19] Glycogen phosphorylase kinase is activated by protein kinase A in the presence of Ca^{2+} and exhibits potentially broad catalytic activity as described in this paper. The present results seem to indicate that catalytic function of phosphorylase kinase differs from those of the three species of enzymes as mentioned above. Presumably, phosphorylase kinase plays roles in controlling some of the Ca^{2+}-dependent processes in addition to glycogen breakdown. In sharp contrast to phosphorylase kinase, myosin light chain kinase, which is also Ca^{2+}-calmodulin dependent enzyme, is very specific for the substrate.[13]

The isozymes of phosphorylase kinase have been first suggested by the finding in several laboratories[7, 18] that enzyme deficiency in liver is not accompanied with that in skeletal muscle,[15, 18] and vice versa.[17] In rabbit skeletal muscle, white and red muscles contain two different types of enzymes with subunit structures of $\alpha_4\beta_4\gamma_4$ and $\alpha_4'\beta_4\gamma_4$, respectively.[12] Although these types show very similar kinetics and catalytic properties, only white muscle isozyme may be activated by exogenously added calmodulin.[23] It is uncertain whether the red muscle and heart phosphorylase kinases are identical. Liver phosphorylase kinases were partially purified in recent years in rabbit and rat[22, 30] and their molecular weights are the same as that of skeletal muscle enzyme. Although the subunit structure of liver enzyme has not been examined, the enzyme is clearly distinguished from muscle enzyme in their enzyme properties.[22] Results in this paper indicate that the enzymes found in various tissues may apparently be divided into three types; muscle, brain and liver types. Although subunits of skeletal muscle and heart enzymes are distinguished from each other as mentioned above, both enzymes appear to belong to the muscle type, which well responds to Ca^{2+} and the antiserum as well as to phosphorylation by protein kinase A. In contrast, the enzymes from other tissues such as liver, kidney, spleen, lung and testis seem to belong to the liver type. This enzyme type has not been fully substantiated but clearly differs from the muscle type. Liver enzyme partially reacts with the anti-skeletal muscle phosphorylase kinase antiserum, and these results are consistent with the data reported by Daegelen-Proux et al.[7] Brain enzyme differs from both entities and presumably belongs to an additional entity; an intermediate type or mixture of the two. It seems that further purification of phosphorylase kinase in various tissues may explain the classification of isozymes more precisely.

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