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PHOSPHORYLATION/ACTIVATION OF PHOSPHORYLASE b KINASE BY cAMP/Ca^{2+}-INDEPENDENT, AUTOPHOSPHORYLATION-DEPENDENT PROTEIN KINASE

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Phosphorylase b kinase from rabbit skeletal muscle can be phosphorylated and activated by a cyclic nucleotide- and Ca^{2+}-independent protein kinase previously identified as an autophosphorylation-dependent multifunctional protein kinase (auto-kinase) from brain and liver (Yang et al., J. Biol. Chem. 262, 7034-7040 (1987) and Yang et al. J. Biol. Chem. 262, 9421-9427 (1987)). This independent kinase phosphorylates both α and β subunits of phosphorylase b kinase and results in a ~5-fold activation of the kinase when 0.55 and 0.5 mol of phosphate are incorporated into the α and β subunits, respectively. Activation of phosphorylase b kinase catalyzed by auto-kinase is about 70% of that observed with cAMP-dependent protein kinase. Analysis of phosphopeptide maps of α and β subunits further reveals that both kinases phosphorylate almost the same sites on both α and β subunits, suggesting that activation of phosphorylase b kinase by the two kinases may be through a common molecular action mechanism. Taken together with the prevous result that auto-kinase can inactivate glycogen synthase, the present study provides initial evidence that a coordinate control mechanism for simultaneous regulation of glycogenolysis and glycogenesis can be modulated by autophosphorylation-dependent protein kinase in a cAMP- and Ca^{2+}-independent pathway, representing a new mode of control mechanism for the regulation of glycogen metabolism in cells.

Phosphorylase b kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38) from skeletal muscle is a key regulatory enzyme involved in the regulation of glycogenolysis in response to neural stimulation of muscle contraction (1, 2). It exists in nonactivated and activated froms and the two forms are interconverted by protein phosphorylation and dephosphorylation (3-7). Phosphorylase b kinase from rabbit skeletal muscle is a hexade-

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Abbreviations: Auto-kinase, autophosphorylation-dependent protein kinase; PKA, cAMP-dependent protein kinase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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camer made up of four types of subunits in the stochiometry α(or α')$_4\beta_4\gamma_4\delta_4$ (3, 8). The γ subunit is the catalytic subunit (9), and the δ subunit, which has the same amino acid sequence as calmodulin (10), is a Ca^{2+}-binding protein and is responsible for the Ca^{2+}-mediated activation of phosphorylase b kinase. The function of the α and β subunits is thought to be regulatory (3, 4) and direct inhibition of the isolated catalytic subunit (γ) of phosphorylase b kinase by its α/β subunits has been reported (11). The α and β subunits can be phosphorylated by various protein kinases and the phosphorylation concomitantly results in the activation of the enzyme. These include cAMP-dependent protein kinase (3, 4), cGMP-dependent protein kinase (12, 13), Ca^{2+}/calmodulin-dependent protein kinase (14), Ca^{2+}/phospholipid-dependent protein kinase (15, 16) and casein kinase I (17, 18).

In this report, we provide further evidence that phosphorylase b kinase from rabbit skeletal muscle can be phosphorylated and activated by a cyclic nucleotide- and Ca^{2+}-independent protein kinase, which was previously identified as an autophosphorylation-dependent protein kinase from brain (19) and as a potent glycogen synthase kinase capable of phosphorylating and inactivating glycogen synthase to terminate glycogen synthesis in liver (20). Taken together, the results provide a new mode of coordinate control mechanism for simultaneous regulation of glycogen metabolism in cells via a cyclic nucleotide- and Ca^{2+}-independent pathway.

MATERIALS AND METHODS

Materials---[γ-^{32}P]ATP was purchased from Amersham. Tris base, β-glycerophosphate, NaF, [ethylenebis(oxyethylenenitrilo)]tetraacetic-acid (EGTA), 1,4-dithiothreitol, cellulose-coated thin layer plates were from Merck. NH_4HCO_3, Ponceau S dye, polyvinylpyrolidone (PVP) and TPCK-treated trypsin were from Sigma. Polyvinylidenedifluoride (PVDF) membrane (Immobilon-P) was from Millipore. Sepharose 4B was from Pharmacia. Inhibitory peptide of cAMP-dependent protein kinase (PKIPT) was synthesized by a peptide synthesizer (Milligen).

Purification of Enzymes and Proteins---Phosphorylase b (21) and phosphorylase b kinase (3) were purified from rabbit skeletal muscle. Catalytic subunit of the cAMP-dependent protein kinase (PKA) was purified from pig heart (22). Myelin basic protein (MBP) was purified from pig brain (23). Autophosphorylation-dependent protein kinase (auto-kinase) was purified from pig liver as described by Yang et al. (19, 20).

Enzyme Assays---Activities of PKA and auto-kinase were determined as described by Kinzel and Kubler (22) and by Yang et al. (19, 20), respectively, using MBP (1 mg/ml) as substrate. A unit of PKA or auto-kinase is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of phosphate/min into 1 mg/ml of MBP at 30°C. Phosphorylation and activation of phosphorylase b kinase by PKA or by auto-kinase was performed under the following conditions: reaction mixture (25 µl) containing phosphorylase b kinase (0.18 mg/ml), 0.5 mM dithiothreitol, 0.2 mM EGTA, 10 mM NaF, 0.1 mM [γ-^{32}P]ATP, 3 mM $MgCl_2$, 25 mM Tris-HCl, 25 mM β-glycerophosphate at pH 6.8, and 0.4 µg of PKA or 0.1 µg of auto-kinase. The reaction was initiated by the addition of Mg.ATP-γ-^{32}P. Aliquots were removed from the reaction mixtures at the indicated time intervals for the determination of the phosphate incorporation into the kinase or for the activity assay of the kinase basically as described by Cohen (3) and Hayakawa et al. (4). The activity of phosphorylase b kinase was assayed in a 25-µl reaction mixture containing phosphorylase b kinase (20-40 µg/ml), phosphorylase b (1 mg/ml), 0.5 mM dithiothreitol, 0.2 mM EGTA, 10 mM NaF, 0.2 mM [γ-^{32}P]ATP, 20 mM $MgCl_2$, 25 mM Tris-HCl and 25 mM β-glycerophosphate at pH 6.8. The reaction time was 2 min. A unit of phosphorylase b

kinase is defined as the amount of enzyme catalyzing the incorporation of 1 μmol of phosphate/min into phosphorylase b at 30°C.

Gel Electrophoresis, Autoradiography and Determination of Phosphorylation Stoichiometry of α and β subunits---Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ^{32}P-labelled phosphorylase b kinase was performed according to Laemmli (24) using 8% gels. The gels were subjected to autoradiography with a Fuji RX x-ray film using Kodak X-Omatic cassette with intensifying screen at -70°C. The protein-staining bands of α and β subunits were localized in the autoradiogram and cut out from the dried gel. The ^{32}P-incorporation of each subunit was determined by direct counting the bands in 5 ml liquid scintillator in a liquid scintillation counter (model 1600TR, Packard).

Determination of Protein Concentration---Protein concentration was determined by the method of Lowry et al. (25) using bovine serum albumin as standard.

Preparation of Tryptic Peptides from α and β Subunits and Two-Dimensional Phosphopeptide Mapping---To obtain the tryptic peptides from α and β subunits of phosphorylase b kinase separately, the method of in situ protease-digestion of membrane-bound protein (26) was used. Briefly, ^{32}P-labelled phosphorylase kinase was subjected to 8% SDS-PAGE followed by transferring to PVDF membrane. The bands of α and β subunits visualized by staining the membrane with Ponceau S dye were cut out and washed several times with deionized water. After treating the membrane with 0.5% polyvinylpyrolidone in 100 mM acetic acid for 1 h to block the free protein-binding sites, membrane-bound proteins were subjected to trypsin digestion in 50 mM NH_4HCO_3 buffer at pH 8.0 for 24 h at 37°C. The enzyme to substrate ratio (w/w) is 1/20. During digestion, fixed amount of trypsin was added to the reaction mixture every 6 h to ensure complete digestion. After digestion, the reaction mixture was concentrated to dry by Speed-Vac concentrator (Savant) and analyzed by two dimensional peptide mapping (27). High-voltage electrophoresis on cellulose-coated TLC plates in the first dimension was performed on HTLE-7000 electrophoresis set (C.B.S. Scientific Co.) in solution A (formic acid/acetic acid/water = 50/156/1794, pH 1.9) at 1,000 V for 40 min. Assending chromatography in the second dimension was carried out in solution B (n-butanol/pyridine/acetic acid/water = 15/10/3/12) for 6 h. After being air-dired, plates were exposed to x-ray films and autoradiographed.

Phosphoamino Acid Analysis---Phosphoamino acid analysis was performed by the method as described by Kamps and Sefton (28). Briefly, ^{32}P-labelled protein transferred to PVDF membrane was directly hydrolyzed in 5.7 N HCl under N_2 at 110°C for 1 h. The hydrolysate was dried by Speed-Vac concentrator and subjected to high-voltage electrophoresis on cellulose-coated TLC plates in solution C (acetic acid/pyridine/water = 10/1/189, pH 3.5) at 1,000 V for 1 h. The positions of phosphoamino acids in plates were localized by ninhydrin-staining of standards run in parallel. The dried plates were exposed to x-ray films and autoradiographed.

RESULTS AND DISCUSSION

Incubation of phosphorylase b kinase with the catalytic sububit of cAMP-dependent protein kinase (PKA) or autophosphorylation-dependent protein kinase (auto-kinase) in the presence of 0.1 mM [γ-^{32}P]ATP and 3 mM $MgCl_2$ results in phosphorylation and activation of phosphorylase b kinase (Fig. 1A and 1B). In agreement with the previous reports (3, 4), autophosphorylation of phosphorylase b kinase also results in a time-dependent activation and ~0.8 mol of phosphate was incorporated into one mol of monomer. However, PKA could incorporate 3 additional mol of phosphate and stimulate the activity of phosphorylase b kinase up to ~7-fold (Fig. 1A and 1B). In similarity, approximately one additional mol of phosphate and ~5-fold activation of phosphorylase b kinase catalyzed by auto-kinase could also be observed (Fig. 1A), demonstrating that auto-

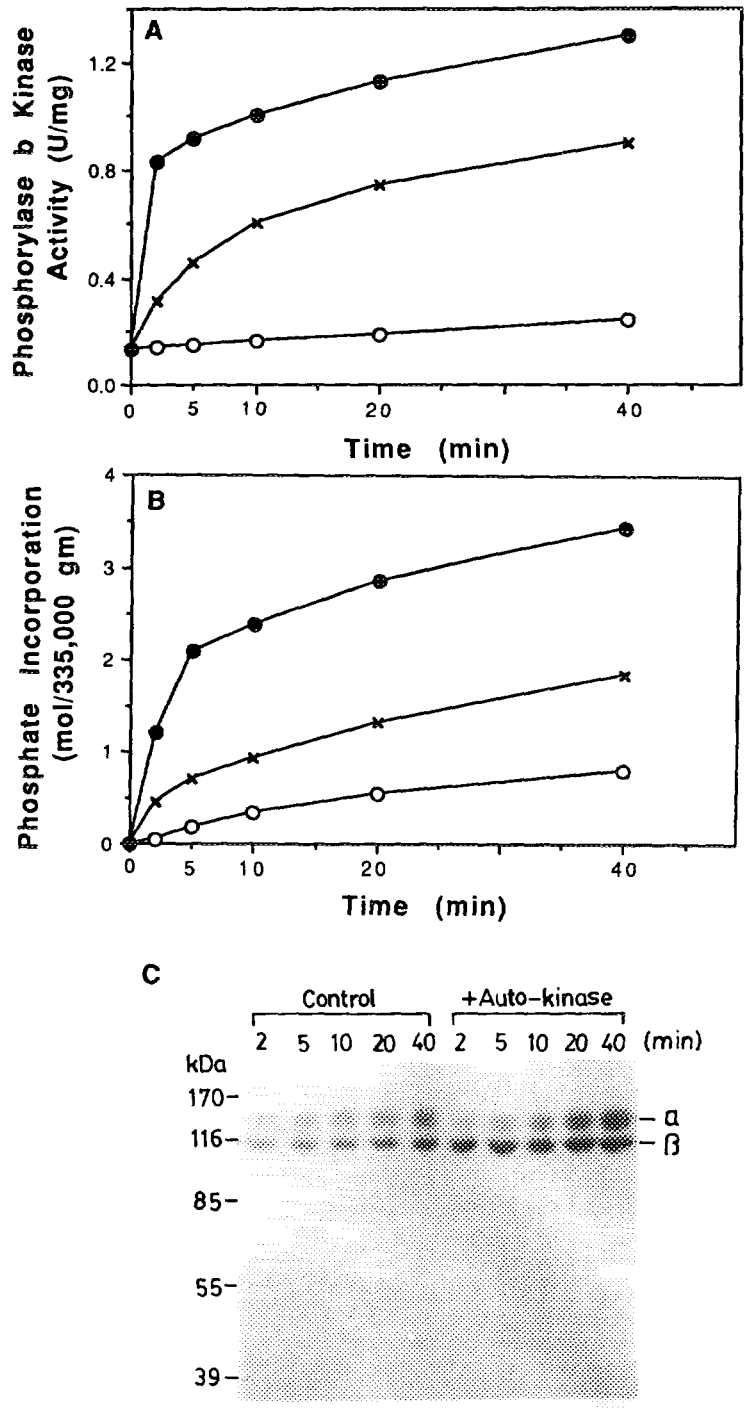

Fig. 1. Time course activation and phosphorylation of phosphorylase b kinase by PKA and auto-kinase.
Phosphorylase b kinase (0.18 mg/ml) was self-phosphorylated (o) or phosphorylated by PKA (•) or by auto-kinase (x) under the condition as described in "MATERIALS AND METHODS". Aliquots were removed from the reaction mixture at indicated times for the measurement of enzyme activity (A) or of the ^{32}P incorporation into the kinase (B) or quenched by adding Laemmli sample buffer and subjected to 8% SDS-PAGE followed by autoradiography (C).

kinase is a potent phosphorylase b kinase kinase. During the auto-kinase-mediated activation process, both α and β subunits of phosphorylase b kinase were phosphorylated but no phosphate was incorporated into γ and δ subunits (Fig. 1C). Phosphorylation of β subunits reached maximal level (~0.5 mol of phosphate/monomer) within 20 min whereas phosphorylation of α subunit continuously increased up to about 0.55 mol of phosphate/monomer after a 40-min reaction with auto-kinase (Fig. 1C). Phosphoamino acid analysis further revealed that both α and β subunits were phosphorylated by auto-kinase on the serine residue (data not shown).

To eliminate the possibility that the activation of phosphorylase b kinase catalyzed by auto-kinase might be due to a contamination of PKA, the inhibitory peptide of PKA (PKIPT) was included in the assay mixture. As shown in Fig. 2, the activity of PKA was completely inhibited by 10 μM PKIPT whereas the activity of the same amount of auto-kinase was totally unaffected by PKIPT even at concentrations up to 80 μM, demonstrating that phosphorylation/activation of phosphorylase b kinase by auto-kinase is not due to a contaminant of PKA in auto-kinase preparation. The phosphorylation and activation of phosphorylase b kinase by auto-kinase was also unaffected by 10 μM cAMP and 0.1 mM Ca^{2+}, further indicating that auto-kinase is a newly-described phosphorylase b kinase kinase independent of cAMP and Ca^{2+}.

To further elucidate the activation mechanism of phosphorylase b kinase by auto-kinase, the tryptic phosphopeptides derived from α and β subunits phosphorylated by PKA or by auto-kinase were subjected to two dimensional peptide separation followed by autoradiography. At least 7 major (spots 1-7) and several minor ^{32}P-spots were detected in the map derived from the α subunit phosphorylated by PKA (Fig. 3A) and by auto-kinase (Fig. 3B). The only difference between these two maps appeared to be spot 7, which could be phosphorylated by PKA but not by auto-kinase. The results indicated that most of the phosphorylation sites of the α subunit catalyzed by PKA are also recognized by auto-

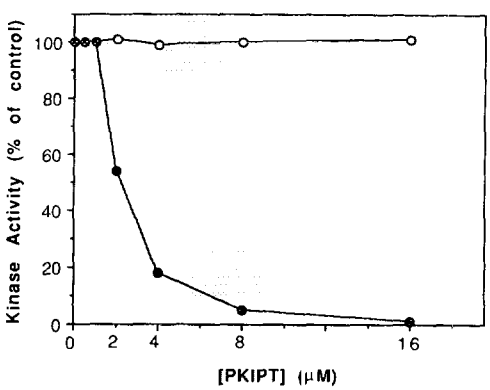

Fig. 2. Dose effect of PKIPT on the activities of PKA and auto-kinase.
0.15 unit of PKA (●) or auto-kinase (o) was incubated with various concentrations of PKIPT as indicated at 30°C for 5 min and the kinase activities were assayed as described in "MATERIALS AND METHODS" using myelin basic protein (1 mg/ml) as substrate.

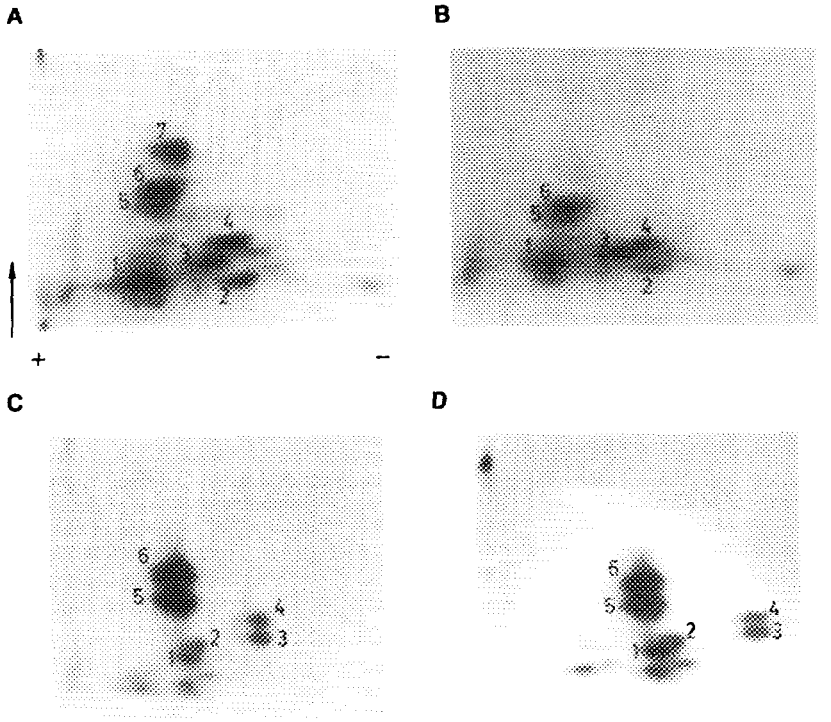

Fig. 3. Two-dimemsional phosphopeptide mapping of α and β subunits phosphorylated by PKA and auto-kinase.
Phosphorylase b kinase was ^{32}P-phosphorylated by PKA (A and C) or by auto-kinase (B and D) and the tryptic peptides derived from ^{32}P-labelled α (A and B) and β (C and D) subunits were analyzed by two-dimemsional phosphopeptide mapping as described in "MATERIALS AND METHODS". Direction of electrophoresis (+,-) and ascending chromatography (arrow) are shown in A.

kinase. On the other hand, the patterns of the phosphopeptide maps derived from β subunit phosphorylated by both kinases were found to be identical (Figs. 3C and 3D), indicating that auto-kinase and PKA phosphorylate the same sites on the β subunit of phosphorylase b kinase. All the results taken together indicate that activation of phosphorylase b kinase by the two kinases appeared to be through a common molecular action mechanism.

It has been reported that Ca^{2+}-stimulated phosphorylase b kinase can be further activated by phosphorylation catalyzed by PKA or by itself and the Ca^{2+} sensitivity of phosphorylase b kinase can be increased after phosphorylation by PKA (29, 30). In similarity, we found that after phosphorylation by auto-kinase, phosphorylase b kinase could also be further activated by Ca^{2+} ion to higher activity levels. As shown in Fig. 4, the three forms of phosphorylase b kinase (i.e. nonactivated, autophosphorylation-activated and auto-kinase-activated froms) have similar bell-shape Ca^{2+} responsive curves. The Ca^{2+} ion concentration required for half-maximal activation ($C_{0.5}$) of both nonactivated and

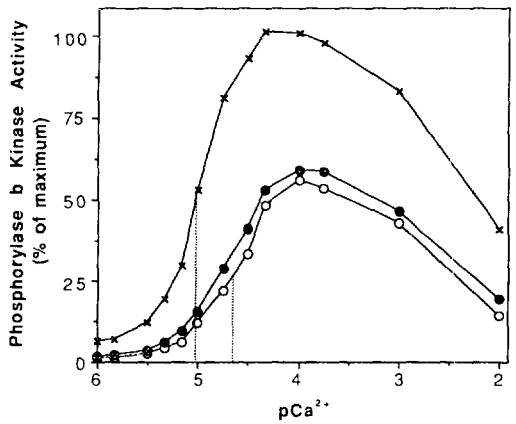

Fig. 4. Effect of phosphorylation on the Ca^{2+}-sensitivity of phosphorylase b kinase by auto-kinase.

Phosphorylase b kinase without any modification (o) or with prior phosphorylation by itself (•) or by auto-kinase (x) in the presence of 0.1 mM ATP and 3 mM $MgCl_2$ at 30°C for 30 min was assayed for enzyme activity in the presence of 1 mM EGTA and various concentrations of Ca^{2+} ion. Detailed phosphorylation and assay conditions were as described in "MATERIALS AND METHODS". Under this phosphorylation condition, the total phosphate incorporation into phosphorylase b kinase was 0.71 mol/mol of monomer by autocatalysis and 1.56 mol/mol of monomer by auto-kinase. The apparent free Ca^{2+} ion concentration in reaction mixtures containing Ca^{2+} chelators was calculated as deacribed by Storer and Cornish-Bowden (31). The Ka values for various complexes involving Ca^{2+}, Mg^{2+}, EGTA, and ATP were taken from Fabiato and Fabiato (32).

autophosphorylated phosphorylase b kinase were about 20 μM, which is close to the value of 23 μM reported by Cohen (30). In contrast to nonactivated and autophosphorylation-activated froms, the $C_{0.5}$ value of auto-kinase-activated phosphorylase b kinase toward Ca^{2+} ion was significantly reduced to ~9 μM (Fig. 4), indicating that the Ca^{2+}-sensitivity of phosphorylase b kinase was enhanced after phosphorylation by auto-kinase. All the results taken together provide initial evidence that autophosphorylation-dependent protein kinase may function as a potent and unique phosphorylase b kinase kinase capable of phosphorylating and concurrently activating phosphorylase b kinase in a cAMP- and Ca^{2+}-independent pathway. This, taken together with the previous report (20) that autophosphorylation-dependent protein kinase may also function as a potent glycogen synthase kinase capable of phosphorylating sites 2 and 3 of glycogen synthase to cause inactivation of glycogen synthase to terminate glycogen synthesis in liver, provides a coordinate control mechanism for simultaneous regulation of glycogenolysis and glycogenesis via modulation of this autophosphorylation-dependent protein kinase in a cAMP and Ca^{2+}-independent pathway, representing a new mode of control mechanism for the regulation of glycogen metabolism in cells.

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