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Studies on the Phosphorylation of Protein Kinase B by Ca^{2+}/Calmodulin-dependent Protein Kinases[1]

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Protein kinase B (PKB) was recently reported to be activated on the phosphorylation of Thr^{308} by Ca^{2+}/calmodulin-dependent protein kinase kinase α (CaM-kinase kinase α), suggesting that PKB was regulated through not only the phosphoinositide 3-kinase pathway but also the Ca^{2+}/calmodulin protein kinase pathway. The activation of PKB by CaM-kinase kinase α was as high as 300-fold after incubation for 30 min under the phosphorylation conditions, and still increased thereafter, suggesting that the maximal activation of PKB on phosphorylation of the Thr^{308} residue is several hundred fold. On the other hand, the V_{max} value of CaM-kinase kinase α for the phosphorylation of PKB was more than two orders of magnitude lower than that for CaM-kinase IV, although the K_m values for PKB and CaM-kinase IV were not significantly different, raising the question of whether or not PKB is a physiological substrate of CaM-kinase kinase α. Besides CaM-kinase kinase α, CaM-kinase II also remarkably activated PKB. However, the specific activities of CaM-kinase kinase α and CaM-kinase II as to the activation of PKB were more than three orders of magnitude lower than that of 3-phosphoinositide-dependent protein kinase 1 (PDK1).

Key words: Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-kinase kinase), Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II), protein kinase B (PKB), signal transduction, upstream protein kinase.

Protein kinase B (PKB), also called Akt, is involved in the regulation of cell survival in response to a variety of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, thrombin, and nerve growth factor (NGF) (*1*). PKB is activated on the phosphorylation of Thr^{308} located in the "activation-loop" by 3-phosphoinositide-dependent protein kinase 1 (PDK1), which is activated on the binding of phosphatidylinositol-3,4,5-triphosphate to the pleckstrin homology (PH) domain of the enzyme (*1*, *2*). It was recently demonstrated that PKB is also activated on the phosphorylation of Thr^{308} by Ca^{2+}/calmodulin-dependent protein kinase kinase α (CaM-kinase kinase α) (*3*), which has been reported to activate CaM-kinases IV and I, selectively, through the phosphorylation of Thr^{196} and Thr^{177}, respectively, located in their "activation-loop" (*4*). To ascertain the biological significance of CaM-kinase kinase α in the regulation of the activity of PKB, detailed analysis of the phosphorylation and activation of PKB by the CaM-kinase kinase is carried out in the present study.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (5,000 Ci/mmol), glutathione-Sepharose 4B, and Sephadex G-50 (superfine) were from Amersham Pharmacia Biotech. Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). DEAE-cellulose (DE52), phosphocellulose paper (P81), and 3MM paper were from Whatman. Akt substrate (RPRAATF) was from Santa Cruz Biotechnology. PKA inhibitor peptide (TTYADFIASGRTGRRNAIHD) was from Sigma. AIP [CaM-kinase II inhibitor (*5*), KKALRRQEAVDAL] was synthesized by Takara Biomedicals. Syntide-2 (PLARTLSVAGLPGKK) (*6*) was synthesized by the American Peptide Company. The IMPACT™ T7 system (Intein Mediated Purification with an Affinity Chitin-binding Tag) was from New England Biolabs. All other reagents were of the highest grade commercially available.

Protein Preparations—The GST-PKB [α isoform (*7*)] and GST-PKB (K179M) (in which Lys^{179}, the ATP-binding site, was replaced with Met) fusion proteins were expressed in Sf9 cells using the pAcGHLT-A vector (Pharmingen), and then purified by affinity chromatography on glutathione-Sepharose 4B (Matsuzaki, H., Konishi, H., and Kikkawa,

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Abbreviations: GST, glutathione S-transferase; CaMKI, calmodulin-dependent protein kinase I; CaMKII, calmodulin-dependent protein kinase II; CaMKIV, calmodulin-dependent protein kinase IV; CaM-kinase kinase, calmodulin-dependent protein kinase kinase; PAGE, polyacrylamide gel electrophoresis; PDK1, 3-phosphoinositide-dependent protein kinase 1; PH, pleckstrin homology; PKA, catalytic subunit of cyclic AMP–dependent protein kinase; PKB, protein kinase B.

U., unpublished work). The purities of the purified GST-PKB and GST-PKB (K179M) were estimated to be 74.3 and 32.2%, respectively, on the basis of the results of analysis of the intensities of the protein bands separated on SDS-PAGE with a MasterScan image analyzer and a RFLP scan program (Scanalytics). The major contaminant was endogenous 25-kDa GST (in Sf9 cells): the content of free GST in the purified GST-PKB (K179M) preparation was estimated to be 55.2% on MasterScan image analysis, and accordingly the molar ratio of GST-PKB (K179M) to the contaminating GST was roughly calculated to be about 0.17 from the molecular weights of 85,489 for GST-PKB (K179M) and 25,000 for GST, respectively. CaM-kinase kinase α expressed in *E. coli* (*8*) was purified as described previously (*9*). CaM-kinase IV (*10*) expressed in Sf9 cells was purified as described previously (*11*). CaM-kinase IV (K71R), in which Lys^{71} (ATP-binding site) was replaced with arginine, expressed in Sf9 cells was purified as described previously (*9*). Since the incubation of CaM-kinase IV with calmodulin in the presence of Ca^{2+} results in a decrease in the enzyme activity and the resulting less active form is converted to the original active form on incubation with Mg^{2+} in the presence of EGTA (*11*, *12*), the purified less active CaM-kinase IV and CaM-kinase IV (K71R) eluted from the calmodulin-Sepharose column were incubated in 40 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA,1 mM dithiothreitol, 0.05% Tween 40, 10% ethylene glycol, and 5 mM $Mg(CH_3COO)_2$ at 30°C for 5min for conversion to their active forms, and then frozen in aliquots at –85°C. CaM-kinase I expressed in Sf9 cells was purified as described previously (*13*). A cDNA fragment containing the entire coding sequence of CaM-kinase IIα (*14*) was introduced into a baculovirus, AcNPV, using a Bac-To-Bac Baculovirus Expression System (Life Technologies). Sf9 cells infected with the recombinant baculovirus were grown at 27°C for 2 days in Grace's medium containing 10% fetal bovine serum, 0.35 g/liter $NaHCO_3$, 100 μg/ml kanamycin, and 2.6 g/liter Bacto tryptose phosphate broth (Difco) (pH 6.1). The harvested cells (1.07 g) were washed once with phosphate-buffered saline, suspended in approximately 10 volumes of 50 mM Tris-HCl buffer (pH 7.5 at 4°C) containing 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Tween 40, and 5 μg/ml of each of the microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin), and then disrupted by sonic oscillation. The supernatant obtained on centrifugation was applied to a column of DE52 (10 ml) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, and 0.05% Tween 40. The column was washed extensively with the equilibration buffer containing 25 mM NaCl and then the enzyme was eluted with the buffer containing 0.2 M NaCl. To the eluate was added solid ammonium sulfate to a final saturation level of 60%, and the resulting precipitate, collected by centrifugation, was dissolved in 10 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 0.05% Tween 40, 0.4 mM $CaCl_2$, 0.2 M NaCl, and 10 μg/ml of each of the microbial protease inhibitors, and then applied to a column of calmodulin-Sepharose (2 ml), prepared as described previously (*15*), equilibrated with the same buffer. The column was washed extensively with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 0.05% Tween 40, 0.2 mM $CaCl_2$, and 2 M NaCl, and then with the same buffer with NaCl omitted, and then the enzyme was eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 0.05% Tween 40, 5 mM EGTA, and 0.2 M NaCl, and dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 0.05% Tween 40, and 0.1 mM EDTA. Ethylene glycol was added to the dialysate to 10% (v/v), and then the dialysate was stored at –80°C in aliquots. Approximately 0.77 mg of the purified CaM-kinase IIα, which gave a single protein band on SDS-PAGE, was obtained. The specific activity of the final preparation was approximately 14,000 nmol/min/mg protein, when assayed with 50 μM syntide-2 as a substrate at 30°C. A cDNA fragment containing the entire coding sequence of CaM-kinase phosphatase (*16*) was introduced into an expression vector, pTYB 1, to create an in-frame fusion between the C-terminus of the enzyme and the N-terminus of the gene encoding the intein, to the C-terminus of which a chitin binding domain had been added, for affinity purification of the enzyme (IMPACT™ T7 system; New England Biolabs). This construct was transformed into *E. coli* strain BL21 (DE3). The bacteria were grown to an A_{600} of 0.6 to 1.0 at 30°C in M9ZB medium containing 200 μg/ml ampicillin, and then IPTG was added to a final concentration of 1 mM. After 2.5 h, the harvested bacteria (2.6 g) were suspended in 10 volumes of 50 mM Tris-HCl buffer (pH 7.5 at 4°C) containing 10 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml of each of the microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin), and then disrupted by sonic oscillation. The supernatant obtained on centrifugation was applied to a column of chitin beads (2 ml), and the column was washed with 70 ml of 40 mM Tris-HCl buffer (pH 7.5 at 4°C) containing 0.5 mM EDTA, 0.5 mM EGTA, 0.1% Tween 40, and 0.5 M NaCl, and then with 6 ml of 40 mM Tris-HCl buffer (pH 8.0 at 4°C) containing 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Tween 40, 5% ethylene glycol, and 50 mM NaCl. The column was quickly flushed with 6 ml of the buffer containing 50 mM dithiothreitol, left at 4°C overnight, and then eluted with 6 ml of the buffer containing 0.5 mM dithiothreitol. The elution procedure was repeated every day for 18 days. The active eluate was dialyzed against 40 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Tween 40, 5% ethylene glycol, and 0.5 mM dithiothreitol, and frozen in aliquots at –85°C. Approximately 2.4 mg of the purified CaM-kinase phosphatase, which gave an almost single protein band at the position of CaM-kinase phosphatase purified from rat brain (*17*) on SDS-PAGE, was obtained after elution 18-times. PKA (catalytic subunit of cyclic AMP-dependent protein kinase) was purified from bovine heart as described previously (*18*). Calmodulin was purified from *E. coli* cells transformed with expression vector pET11d carrying a cDNA encoding chicken calmodulin as described previously (*11*).

Analytical Procedures—SDS-PAGE was performed by the method of Laemmli (*19*). The concentration of calmodulin was determined spectrophotometrically using an absorption coefficient, A_{280} (1 mg/ml), of 0.21 (*20*) and a molecular weight of 16,700 (*21*, *22*). Other proteins were determined by the method of Lowry *et al.* (*23*), as modified by Peterson (*24*), with bovine serum albumin as a standard. The molar concentrations of GST-PKB and GST-PKB (K179M) were calculated using molecular weight values of

85,486 and 85,489, respectively, which were estimated from their amino acid sequences (Matsuzaki, H., Konishi, H., and Kikkawa, U., unpublished work). The amount of CaM-kinase IV was corrected for overestimation by a factor of 1.15 by means of Lowry's method (*25*). The molar concentrations of CaM-kinase kinase α, CaM-kinase I, CaM-kinase IIα, CaM-kinase IV, CaM-kinase IV (K71R), PKA, and CaM-kinase phosphatase were calculated using molecular weights values of 55,907 (*8*), 41,643 (*26*), 54,111 (*27*), 53,133 (*28, 29*), 53,161, 40,862 (*30*), and 49,165 (*16*), respectively.

Protein phosphorylation was carried out at 30°C in the standard phosphorylation mixture comprising 50 mM Mops-NaOH (pH 7.0 at 30°C), 5 mM $Mg(CH_3COO)_2$, 0.4 mM ATP, 2 mM dithiothreitol, 0.1 mM $CaCl_2$, 3 μM calmodulin, and the indicated amounts of protein substrate and kinase.

The activity of PKB was determined by measuring the phosphorylation of Akt substrate at 30°C for 1 min in the standard assay mixture comprising 50 mM Hepes-NaOH (pH 7.5 at 30°C), 10 mM $Mg(CH_3COO)_2$, 0.1 mM [γ-^{32}P]ATP (200 cpm/pmol), 1 mM dithiothreitol, 0.2 mM EGTA, 10 μM Akt substrate, and the indicated amounts of PKB. The incorporation of [^{32}P]phosphate into the peptide substrate was determined by the phosphocellulose paper method (*31*).

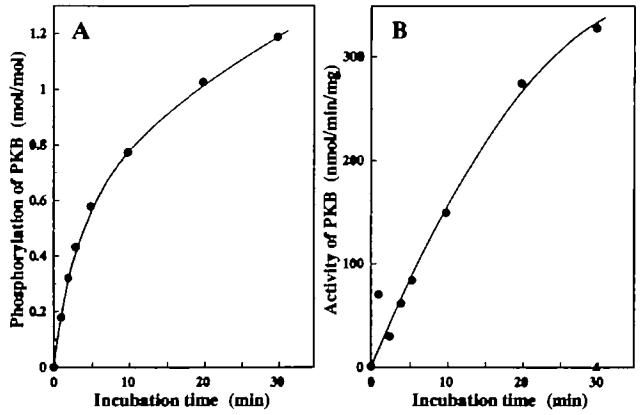

Fig. 1. **Time courses of phosphorylation (A) and activation (B) of PKB by CaM-kinase kinase α.** (A) GST-PKB (K179M) (about 0.95 μM, 81 μg/ml) was incubated at 30°C with CaM-kinase kinase α (about 1 μM, 56 μg/ml) in the standard phosphorylation mixture containing [γ-^{32}P]ATP (1,000 cpm/pmol). At the indicated times, aliquots were subjected to SDS-PAGE on a 7.5% acrylamide gel, followed by autoradiography. The radioactive region of the gel corresponding to GST-PKB was excised, and the radioactivity was determined with a liquid scintillation counter. The efficiency of the counting was estimated to be approximately 82%, on the basis of a comparison of the ^{32}P-radioactivity of autophosphorylated CaM-kinase IIα determined as described above to that determined by the 3MM paper method of Corbin and Reimann (*32*), except that the filter papers were washed with ice-cold 10% trichloroacetic acid containing 2 mM ATP. The results are expressed as moles of phosphate incorporated into 1 mole of GST-PKB. (B) GST-PKB (0.95 μM) was incubated at 30°C with (●) or without (▲) CaM-kinase kinase α (1 μM) in the standard phosphorylation mixture. At the indicated times, aliquots were assayed for PKB activity as described under "EXPERIMENTAL PROCEDURES." The results are presented as activities/PKB protein in the GST-PKB.

RESULTS AND DISCUSSION

Phosphorylation and Activation of PKB by CaM-Kinase Kinase α—It was recently reported that not only PDK1 but also CaM-kinase kinase α, which had been reported to selectively activate CaM-kinases IV and I through the phosphorylation of threonine residues within their "activation-loop" (*4*), can activate PKB on the phosphorylation of Thr^{308} located in the activation-loop of PKB (*3*). Indeed, PKB was phosphorylated and activated by CaM-kinase kinase α, as shown in Fig. 1. When 81 μg/ml (about 1 μM) GST-PKB was incubated at 30°C with 56 μg/ml (1 μM) CaM-kinase kinase α under the Ca^{2+}/calmodulin-depen-

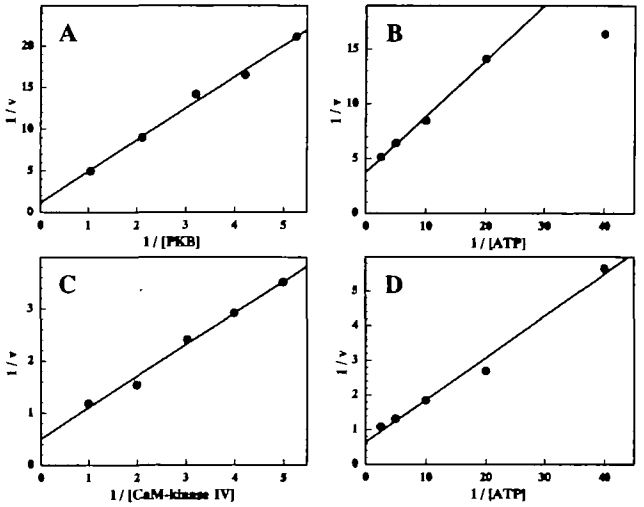

Fig. 2. **Effects of the concentrations of substrates on the phosphorylation of PKB and CaM-kinase IV by CaM-kinase kinase α.** (A and B) CaM-kinase kinase α (0.1 μM) was incubated in the standard phosphorylation mixture containing 0.4 mM [γ-^{32}P]ATP (1,000 cpm/pmol) and various concentrations of GST-PKB (K179M) (A), or various concentrations of [γ-^{32}P]ATP and 0.76 μM GST-PKB (K179M) (B) at 30°C for 5 min. After reaction, aliquots were subjected to SDS-PAGE on a 7.5% acrylamide gel, followed by autoradiography. The radioactive region of the gel corresponding to GST-PKB was excised, and the radioactivity was determined with a liquid scintillation counter. (C and D) CaM-kinase kinase α (1 nM) was incubated in the standard phosphorylation mixture containing 0.4 mM [γ-^{32}P]ATP (1,000 cpm/pmol) and various concentrations of CaM-kinase IV (K71R) (C), or various concentrations of [γ-^{32}P]ATP and 1 μM CaM-kinase IV (K71R) (D) at 30°C for 5 min. After reaction, aliquots were analyzed by SDS-PAGE on a 7.5% acrylamide gel, followed by autoradiography. The radioactive region of the gel corresponding to CaM-kinase IV was excised, and the radioactivity was determined with a liquid scintillation counter. The results are corrected for the counting efficiency (82%) and plotted as double-reciprocal plots.

TABLE I. **Kinetic parameters of phosphorylation of PKB by CaM-kinase kinase α.** Kinetic parameters were obtained from the plots presented in Fig. 2. The V_{max} values were calculated from apparent V_{max} values on the basis of the Michaelis equation $v = V/(1 + K_m/[S])$.

Substrate	K_m for substrate (μM)	K_m for ATP (μM)	V_{max} (nmol/min/mg)
PKB	3.1	140	23 (0.6%)
CaM-kinase IV	1.0	120	4,000 (100%)

dent protein phosphorylation conditions, the PKB activity increased progressively as the incorporation of phosphate into the enzyme increased. After incubation for 30 min, about 1.2 mol of [^{32}P]phosphate was incorporated into 1 mol of the enzyme and the enzyme was concomitantly activated about 300-fold. The extent of the activation of PKB by CaM-kinase kinase α was thus very great, but the rate of the activation was very slow compared with that of CaM-kinase IV: CaM-kinase IV (8.7 μg/ml, 0.16 μM) is fully activated by CaM-kinase kinase α at as little as 2 μg/ml (0.036 μM) instantaneouly (*33*), but PKB (about 1 μM) was only gradually activated by CaM-kinase kinase α at as much as 1 μM. In order to address the mechanism by which the activation of PKB by CaM-kinase kinase α occurred very slowly, the availability of PKB as a substrate for the CaM-kinase kinase was examined by kinetic analysis of the rate of phosphorylation of PKB by the CaM-kinase kinase, compared with that of CaM-kinase IV, as shown in Fig. 2. To avoid autophosphorylation of the substrates, PKB (K179M) and CaM-kinase IV (K71R), in which Lys^{179} and Lys^{71} (ATP-binding sites) were replaced with methionine and arginine, respectively, were used for the experiment. CaM-kinase kinase α showed normal kinetic behavior with respect to both PKB and CaM-kinase IV. The kinetic parameters obtained from the double-reciprocal plots shown in Fig. 2 are summarized in Table I. The K_m value for PKB, approximately 3 μM, was not significantly different from that for CaM-kinase IV, 1 μM, but the estimated V_{max} value toward PKB was more than two orders of magnitude lower than that toward CaM-kinase IV, inconsistent with the finding that the activation of PKB requires an about 10-fold higher concentration of CaM-kinase kinase than the activation of CaM-kinase IV does (*3*, *34*). Thus, the kinetic analysis revealed that the activity of CaM-kinase kinase α towards PKB is much lower than reported, raising doubts concerning the physiological significance of CaM-kinase kinase α in the activation of PKB. The K_m values for ATP with PKB were almost the same as that with CaM-kinase IV.

Activation of PKB by Other Protein Kinases—The fact that CaM-kinase kinase α, besides PDK1, can activate PKB led us to examine the possibility that there may be protein kinases, other than PDK1 and CaM-kinase kinase α, capable of activating PKB. Our preliminary experiments suggested that PKA, and multifunctional CaM-kinases such as CaM-kinases I, II, and IV phosphorylated GST-PKB (K179M) significantly (data not shown). Among them, PKA, and CaM-kinases I and IV did not significantly activate PKB, but CaM-kinase II activated PKB, as shown in

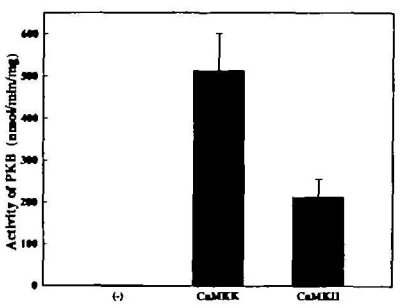

Fig. 3. **Activation of PKB by various protein kinases.** GST-PKB (0.95 μM) was incubated at 30°C for 30 min in the standard phosphorylation mixture with CaM-kinase kinase α (1 μM), PKA (1 μM), CaM-kinase IIα (1 μM), CaM-kinase I (1 μM) activated by preincubation with 0.01 μM CaM-kinase kinase α at 30°C for 5 min, or CaM-kinase IV (1 μM) activated by 0.01 μM CaM-kinase kinase α, and aliquots were assayed for PKB activity as described under "EXPERIMENTAL PROCEDURES," except that the assays for the enzymes incubated with PKA and CaM-kinase II were carried out in the presence of 2 μM PKA inhibitor peptide and 10 μM AIP, respectively. Among the five protein kinases, only CaM-kinase kinase α and CaM-kinase IIα significantly activated PKB. The results are expressed as the means and sample standard deviation for three independent experiments. The phosphorylation of Akt substrate in the absence of GST-PKB is subtracted from the experimental data.

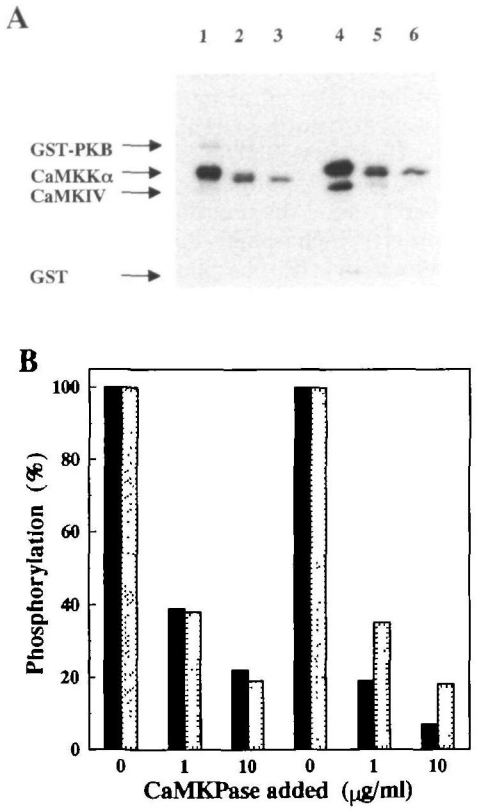

Fig. 4. **Dephosphorylated PKB by CaM-kinase phosphatase.** (A) GST-PKB (K179M) (0.95 μM) (lanes 1–3) and CaM-kinase IV (K71R) (1 μM) (lanes 4–6) were phosphorylated at 30°C for 30 min with 1 μM CaM-kinase kinase α in the standard phosphorylation mixture containing [γ-^{32}P]ATP (1,000 cpm/pmol), and then the mixture was applied to a small column of Sephadex G-50 (superfine) (0.9 ml) equilibrated with 50 mM Hepes-NaOH buffer (pH 7.0) containing 0.1 mM dithiothreitol, 0.05% Tween 40, 10% ethylene glycol, and 2 mM $MnCl_2$. The column was eluted with the same buffer by the spin column method (*37*), and the radioactive fractions eluted near the void volume were pooled. Aliquots were incubated at 30°C for 10 min with 0 μg/ml (lanes 1 and 4), 1 μg/ml (0.02 μM) (lanes 2 and 5), or 10 μg/ml (0.2 μM) (lanes 3 and 6) CaM-kinase phosphatase in a reaction mixture comprising 50 mM Hepes-NaOH buffer (pH 7.0) containing 0.1 mM dithiothreitol, 0.05% Tween 40, 10% ethylene glycol, and 2 mM $MnCl_2$. After reaction, samples were subjected to SDS-PAGE on a 7.5% acrylamide gel, followed by autoradiography. (B) The radioactive regions of the gel corresponding to GST-PKB (■), CaM-kinase kinase α (▨), and CaM-kinase IV (▩) were excised, and the radioactivities were determined with a liquid scintillation counter. The results are expressed as percentages of the radioactivities without CaM-kinase phosphatase added.

Fig. 3. When about 1 μM GST-PKB was incubated at 30°C for 30 min with 1 μM CaM-kinase II under the Ca^{2+}/calmodulin-dependent phosphorylation conditions, PKB was activated about 140-fold. The extent of the activation by CaM-kinase II was almost half of that by CaM-kinase kinase α under the experimental conditions used.

Alessi *et al.* (*35*) have reported that the specific activity of PDK1 purified from rabbit skeletal muscle toward GST-PKBα is about 100,000 units/mg, when one unit of activity is defined as the amount required to increase the basal activity of GST-PKB by 1 unit of activity in 1 min; 1 unit of GST-PKB activity is the amount of enzyme required to catalyze the phosphorylation of 1 nmol of the peptide RPRAATF in 1 min in an assay mixture containing 0.1 mM RPRAATF. CaM-kinase kinase α and CaM-kinase II showed specific activities of 35.1 and 20.6 units/mg, respectively, toward GST-PKB under similar assay conditions. Thus, the activation activities of CaM-kinase kinase α and CaM-kinase II toward PKB were more than three orders of magnitude lower than that of PDK1.

Dephosphorylation of Phosphorylated PKB by CaM-Kinase Phosphatase—CaM-kinase kinase α phosphorylated Thr^{308} located in the activation-loop of PKB (*3*), although the activity was very low compared with that toward Thr^{196} in the activation-loop of CaM-kinase IV, resulting in an increase in the activity of PKB, and this raised the question of whether or not CaM-kinase phosphatase can dephosphorylate Thr^{308} of PKB phosphorylated by CaM-kinase kinase, because CaM-kinase kinase is known to particularly efficiently phosphorylate the threonine residues located in the activation-loops of CaM-kinases I and IV, and both the phosphorylated residues are efficiently dephosphorylated by CaM-kinase phosphatase (*17*, *36*). As shown in Fig. 4, 1 μg/ml CaM-kinase phosphatase dephosphorylated about 80% of CaM-kinase IV phosphorylated by CaM-kinase kinase, but as much as 10 μg/ml of the phosphatase was required to produce the same extent of dephosphorylation of PKB under the experimental conditions used. Thus, the threonine residue in the activation-loop of PKB did not appear to be a good substrate for not only CaM-kinase kinase but also CaM-kinase phosphatase, compared with the threonine residue in the activation-loop of CaM-kinases IV and I.

The present study revealed that the rate of phosphorylation of Thr^{308} of PKB by CaM-kinase kinase α was more than two orders of magnitude lower than that of Thr^{196} of CaM-kinase IV (Table I), and that the specific activity of CaM-kinase kinase α as to activation of PKB was as low as 0.035% of that of PDK1, raising the question of whether or not CaM-kinase kinase α is involved in the activation of PKB. CaM-kinase II, besides CaM-kinase kinase, was found to remarkably activate PKB, but the specific activity was also as low as 0.02% of that of PDK1, raising doubts concerning the involvement of CaM-kinase II in the regulation of PKB. However, in contrast to the low rate of phosphorylation, the magnitude of the activation of PKB upon phosphorylation was as great as several hundred fold (Figs. 1 and 3). CaM-kinase kinase α (*15*) and CaM-kinase II (*38*) have been purified to apparent homogeneity from brain extracts by about 2,000- and 700-fold, respectively, while PDK1 has been purified from skeletal muscle extracts by about 500,000-fold (*39*). Thus, the possibility cannot be ruled out that the low rate of phosphorylation of PKB by CaM-kinases such as CaM-kinase II and CaM-kinase kinase α is compensated for by the great magnitude of the activation of PKB and the abundant occurrence of the CaM-kinases.

REFERENCES

1. Marte, B.M. and Downward, J. (1997) PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *TIBS* **22**, 355–358
2. Hemmings, B.A. (1997) Akt signaling: linking membrane events to life and death decisions. *Science* **275**, 628–630
3. Yano, S., Tokumitsu, H., and Soderling, T.R. (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature* **396**, 584–587
4. Okuno, S., Kitani, T., and Fujisawa, H. (1997) Studies on the substrate specificity of Ca^{2+}/calmodulin-dependent protein kinase kinase. *J. Biochem.* **122**, 337–343
5. Ishida, A., Kameshita, I., Okuno, S., Kitani, T., and Fujisawa, H. (1995) A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. *Biochem. Biophys. Res. Commun.* **212**, 806–812
6. Hashimoto, Y. and Soderling, T.R. (1987) Calcium·calmodulin-dependent protein kinase II and calcium·phospholipid-dependent protein kinase activities in rat tissues assayed with a synthetic peptide. *Arch. Biochem. Biophys.* **252**, 418–425
7. Konishi, H., Shinomura, T., Kuroda, S., Ono, Y., and Kikkawa, U. (1994) Molecular cloning of rat RAC protein kinase α and β and their association with protein kinase C ζ. *Biochem. Biophys. Res. Commun.* **205**, 817–825
8. Okuno, S., Kitani, T., and Fujisawa, H. (1996) Evidence for the existence of Ca^{2+}/calmodulin-dependent protein kinase IV kinase isoforms in rat brain. *J. Biochem.* **119**, 1176–1181
9. Kitani, T., Okuno, S., and Fujisawa, H. (1997) Studies on the site of phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase) IV by CaM-kinase kinase. *J. Biochem.* **121**, 804–810
10. Okuno, S. and Fujisawa, H. (1993) Requirement of brain extract for the activity of brain calmodulin-dependent protein kinase IV expressed in *Escherichia coli*. *J. Biochem.* **114**, 167–170
11. Kitani, T., Okuno, S., and Fujisawa, H. (1995) Inactivation of Ca^{2+}/calmodulin-dependent protein kinase IV by Ca^{2+}/calmodulin and restoration of the activity by Mg^{2+}/EGTA. *J. Biochem.* **117**, 1070–1075
12. Okuno, S., Kitani, T., and Fujisawa, H. (1997) Regulation of Ca^{2+}/calmodulin-dependent protein kinase IV (CaM-kinase IV) by changing its susceptibility to phosphorylation by CaM-kinase kinases. *J. Biochem.* **122**, 897–900
13. Okuno, S., Kitani, T., and Fujisawa, H. (1997) Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase kinase β from rat cerebellum. *J. Biochem.* **121**, 155–160
14. Tobimatsu, T. and Fujisawa, H. (1989) Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. *J. Biol. Chem.* **264**, 17907–17912
15. Okuno, S., Kitani, T., and Fujisawa, H. (1994) Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase IV kinase from rat brain. *J. Biochem.* **116**, 923–930
16. Kitani, T., Ishida, A., Okuno, S., Takeuchi, M., Kameshita, I., and Fujisawa, H. (1999) Molecular cloning of Ca^{2+}/calmodulin-dependent protein kinase phosphatase. *J. Biochem.* **125**, 1022–1028
17. Ishida, A., Kameshita, I., and Fujisawa, H. (1998) A novel protein phosphatase that dephosphorylates and regulates Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **273**, 1904–1910
18. Okuno, S. and Fujisawa, H. (1990) Stabilization, purification and crystallization of catalytic subunit of cAMP-dependent protein kinase from bovine heart. *Biochim. Biophys. Acta* **1038**, 204–208
19. Laemmli, U.K. (1970) Cleavage of structural proteins during

the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
20. Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D., and Means, A.R. (1977) Physicochemical properties of rat testis Ca^{2+}-dependent regulator protein of cyclic nucleotide phosphodiesterase. Relationship of Ca^{2+}-binding, conformational changes, and phosphodiesterase activity. *J. Biol. Chem.* **252**, 8415–8422
21. Dedman, J.R., Jackson, R.L., Schreiber, W.E., and Means, A.R. (1978) Sequence homology of the Ca^{2+}-dependent regulator of cyclic nucleotide phosphodiesterase from rat testis with other Ca^{2+}-binding proteins. *J. Biol. Chem.* **253**, 343–346
22. Watterson, D.M., Sharief, F., and Vanaman, T.C. (1980) The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain. *J. Biol. Chem.* **255**, 962–975
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
24. Peterson, G.L. (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**, 346–356
25. Kameshita, I. and Fujisawa, H. (1995) Preparation and characterization of calmodulin-dependent protein kinase IV (CaM-kinase IV) free of CaM-kinase IV kinase from rat cerebral cortex. *J. Biochem.* **117**, 85–90
26. Cho, F.S., Phillips, K.S., Bogucki, B., and Weaver, T.E. (1994) Characterization of a rat cDNA clone encoding calcium/calmodulin-dependent protein kinase I. *Biochim. Biophys. Acta* **1224**, 156–160
27. Lin, C.R., Kapiloff, M.S., Durgerian, S., Tatemoto, K., Russo, A.F., Hanson, P., Schulman, H., and Rosenfeld, M.G. (1987) Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **84**, 5962–5966
28. Means, A.R., Cruzalegui, F., LeMagueresse, B., Needleman, D.S., Slaughter, G.R., and Ono, T. (1991) A novel Ca^{2+}/calmodulin-dependent protein kinase and a male germ cell-specific calmodulin-binding protein are derived from the same gene. *Mol. Cell. Biol.* **11**, 3960–3971
29. Ohmstede, C.-A., Bland, M.M., Merrill, B.M., and Sahyoun, N. (1991) Relationship of genes encoding Ca^{2+}/calmodulin-dependent protein kinase Gr and calspermin: A gene within a gene. *Proc. Natl. Acad. Sci. USA* **88**, 5784–5788
30. Shoji, S., Ericsson, L.H., Walsh, K.A., Fischer, E.H., and Titani, K. (1983) Amino acid sequence of the catalytic subunit of bovine type II adenosine cyclic 3′,5′-phosphate dependent protein kinase. *Biochemistry* **22**, 3702–3709
31. Roskoski, R., Jr. (1983) Assays of protein kinase. *Methods Enzymol.* **99**, 3–6
32. Corbin, J.D. and Reimann, E.M. (1974) Assay of cyclic AMP-dependent protein kinases. *Methods Enzymol.* **38**, 287–290
33. Okuno, S., Kitani, T., and Fujisawa, H. (1995) Full activation of brain calmodulin-dependent protein kinase IV requires phosphorylation of the amino-terminal serine-rich region by calmodulin-dependent protein kinase IV kinase. *J. Biochem.* **117**, 686–690
34. Tokumitsu, H., Takahashi, N., Eto, K., Yano, S., Soderling, T.R., and Muramatsu, M. (1999) Substrate recognition by Ca^{2+}/calmodulin-dependent protein kinase kinase. Role of the Arg-Pro-rich insert domain. *J. Biol. Chem.* **274**, 15803–15810
35. Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., MacDougall, C.N., Harbison, D., Ashworth, A., and Bownes, M. (1997) 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase. *Curr. Biol.* **7**, 776–789
36. Ishida, A., Okuno, S., Kitani, T., Kameshita, I., and Fujisawa, H. (1998) Regulation of multifunctional Ca^{2+}/calmodulin-dependent protein kinases by Ca^{2+}/calmodulin-dependent protein kinase phosphatase. *Biochem. Biophys. Res. Commun.* **253**, 159–163
37. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed., pp. E.37–E.38, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
38. Yamauchi, T. and Fujisawa, H. (1983) Purification and characterization of the brain calmodulin-dependent protein kinase (Kinase II), which is involved in the activation of tryptophan 5-monooxygenase. *Eur. J. Biochem.* **132**, 15–21
39. Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R.J., Reese, C.B., and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Curr. Biol.* **7**, 261–269