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Regulation of Multifunctional Ca^{2+}/Calmodulin-Dependent Protein Kinases by Ca^{2+}/Calmodulin-Dependent Protein Kinase Phosphatase

Atsuhiko Ishida, Sachiko Okuno, Takako Kitani, Isamu Kameshita, and Hitoshi Fujisawa[1]
Department of Biochemistry, Asahikawa Medical College, Asahikawa 078-8510, Japan

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We have recently reported a novel protein phosphatase which dephosphorylates and thereby deactivates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activated through autophosphorylation (Ishida, A., Kameshita, I., and Fujisawa, H. (1998) *J. Biol. Chem.* 273, 1904–1910). In the present study, we show that this protein phosphatase also catalyzed dephosphorylation of Ca^{2+}/calmodulin-dependent protein kinases I (CaMKI) and IV (CaMKIV) which had been phosphorylated and activated by Ca^{2+}/calmodulin-dependent protein kinase kinase α, resulting in reversible deactivation of the enzymes. The fairly high degree of the substrate specificity of this protein phosphatase suggests that the physiological significance of the phosphatase may be the regulation of the three multifunctional Ca^{2+}/calmodulin-dependent protein kinases, CaMKI, CaMKII, and CaMKIV, which are the key enzymes in a Ca^{2+}-signaling system in the cell.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a Ca^{2+}-responsive multifunctional protein kinase occurring abundantly in the brain (1), is thought to be involved in a variety of neuronal functions mediated by Ca^{2+} (reviewed in Refs. 2–4), and the regulation of its activity is therefore very important. The fact that CaMKII activity increases upon autophosphorylation of Thr^{286} suggested that to define the mechanism for dephosphorylation of the autophosphorylated CaMKII was necessary to understanding the regulatory mechanism of CaMKII. With a novel in-gel protein phosphatase assay, we detected three distinct protein phosphatases capable of dephosphorylating the residue corresponding to Thr^{286} of CaMKII in the rat brain extract (5), and purified one of the three phosphatases (6). The purified enzyme is a calyculin A-insensitive, Mn^{2+}-dependent, and poly-L-lysine (poly(Lys))-stimulated protein phosphatase with an apparent molecular weight of 54,000 (6). This phosphatase dephosphorylates autophosphorylated CaMKII with the concomitant deactivation of the enzyme, but cannot dephosphorylate phosphorylase kinase, mixed histones, myelin basic protein, α-casein (phosphorylated by PKA), and phosphorylase (phosphorylated by phosphorylase kinase), suggesting a strict substrate specificity of the enzyme (6).

Ca^{2+}/calmodulin-dependent protein kinases I (CaMKI) and IV (CaMKIV) are also multifunctional protein kinases (2, 7, 8), and therefore thought to play various roles in a Ca^{2+}-signaling system, along with CaMKII. CaMKI (9, 10) and CaMKIV (11–13) are activated by Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) through phosphorylation at the threonine residues located within their activation loop (Thr^{177} of CaMKI and Thr^{196} of CaMKIV) (14–17), suggesting the importance of dephosphorylation of the phosphorylated threonine residues in the regulation of the activities of CaMKI and CaMKIV. A Mg^{2+}-dependent protein phosphatase was reported to deactivate the activated CaMKIV (18), and thereafter protein phosphatase 2A (PP2A) was reported to deactivate the activated CaMKIV (19) and CaMKI (20). Very recently, a complex of CaMKIV with PP2A, in which PP2A dephosphorylates CaMKIV, has been identified (21), suggesting the regulation of CaMKIV by PP2A. The present study reports that CaMKI and CaMKIV which had been activated through phosphorylation by CaMKK-α were dephosphorylated and concomitantly deactivated by the novel protein phosphatase de-

[1] To whom correspondence should be addressed. Fax: 81-166-68-2349. E-mail: fujisawa@asahikawa-med.ac.jp.

Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKI, Ca^{2+}/calmodulin-dependent protein kinase I; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase IV; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; CaMKPase, Ca^{2+}/calmodulin-dependent protein kinase phosphatase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PKA, catalytic subunit of cAMP-dependent protein kinase; PKC, protein kinase C; poly(Lys), poly-L-lysine; PP2A, protein phosphatase 2A.

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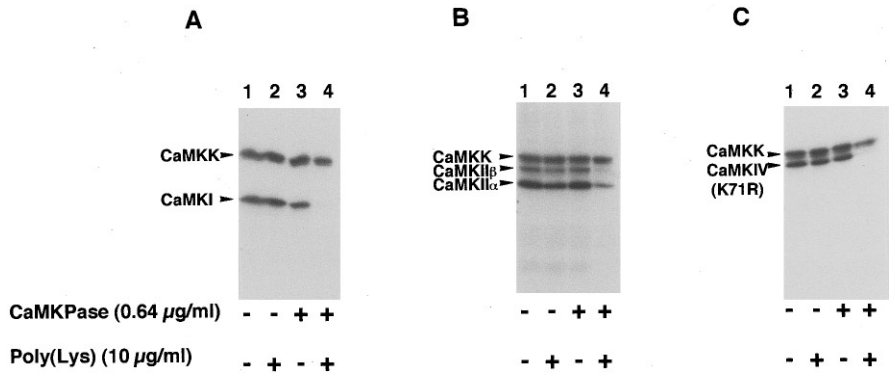

FIG. 1. Dephosphorylation of CaMKs by CaMKPase. (A) CaMKI (17 nM), which had been phosphorylated by CaMKK-α, as described under Materials and Methods, and autophosphorylated CaMKK-α (17 nM) were incubated at 30°C in a reaction mixture consisting of 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 100 mM KCl, 0.1 mM EGTA, and 0.01% Tween 20 with the indicated additions and omissions. After incubation for 1 min, aliquots were analyzed by SDS-PAGE followed by autoradiography. (B) autophosphorylated CaMKII (61 nM) and autophosphorylated CaMKK-α (17 nM) were incubated as described above. After incubation at 30°C for 1 min, aliquots were analyzed by SDS-PAGE followed by autoradiography. (C) CaMKIV(K71R) (17 nM) expressed in Sf9 cells phosphorylated by CaMKK-α and autophosphorylated CaMKK-α (17 nM) were incubated as described above. After incubation at 30°C for 1 min, aliquots were analyzed by SDS-PAGE followed by autoradiography. The positions corresponding to CaMKI, CaMKII, CaMKIV(K71R), and CaMKK-α are indicated with the arrow heads.

scribed above, indicating that this phosphatase plays important roles as a Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKPase) in regulating the activities of the three multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), CaMKI, CaMKII, and CaMKIV.

MATERIALS AND METHODS

Materials. ATP and poly(Lys) (average molecular weight 87,000) were purchased from Sigma. [γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham International.

Protein preparations. CaMKPase was purified from rat brain stem as described previously (6). Recombinant chicken brain calmodulin expressed in *E. coli* was prepared as described previously (22). Catalytic subunit of cAMP-dependent protein kinase (PKA) was purified form bovine heart as described previously (23). Protein kinase C (PKC) was purified from rat cerebral cortex essentially according to the method of Woodgett and Hunter (24). Recombinant rat CaMKI expressed in Sf9 cells was purified as described previously (25). Rat brain CaMKII was purified as described (26, 27). Recombinant rat CaMKIV expressed in Sf9 cells was purified as described previously (22). Recombinant rat CaMKIV(K71R), in which Lys^{71} (ATP-binding site) was replaced with Arg, expressed in Sf9 cells was purified as described (17). Recombinant rat CaMKK-α expressed in *E. coli* (28) was purified as described previously (17).

Preparation of protein phosphatase substrates. Purified recombinant rat CaMKI (87 μg/ml) and CaMKIV(K71R) (117 μg/ml) expressed in Sf9 cells were phosphorylated by recombinant rat CaMKK-α (3 μg/ml) at 30°C for 5 min in a reaction mixture comprising 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-NaOH (pH 7.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 5 μM calmodulin, 0.3 mM $CaCl_2$, 2 mM DTT, and 50 μM [γ-^{32}P]ATP. Autophosphorylation of recombinant rat CaMKK-α (87 μg/ml) was carried out at 30°C for 30 min in the same reaction mixture. Rat brain CaMKII was autophosphorylated at 5°C for 1 min as described previously (6). The specific activity of [γ-^{32}P]ATP used for phosphorylation of these proteins were 17,000-19,000 cpm/pmol. The phosphorylation reactions were terminated by adding excess EDTA (12.3 mM) and bovine serum albumin (1 mg/ml), and the phosphorylated proteins were separated from undesirable small molecules by gel filtration upon Sephadex G-50 and stored as described previously (6). The substrate concentrations presented in the text represent the concentrations of ^{32}P bound to the substrate proteins. The purified recombinant rat CaMKI (226 μg/ml) and CaMKIV (94 μg/ml) were activated by incubation with CaMKK-α (3 μg/ml) at 30°C for 5 min in the reaction mixture as described above, except that nonradioactive ATP and 0.6-0.7 mM $CaCl_2$ were used. The activated enzymes were stored after gel filtration as described above.

Other analytical procedures. Protein phosphatase assay using SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described previously (6). The activities of PKA and PKC were measured using syntide-2 as a substrate as described previously (29). CaMKI and CaMKIV activities were determined using syntide-2 as a substrate as described previously (25), except that the $CaCl_2$ concentration was raised to 0.5 mM and the incubation times were 3 min for CaMKI and 5 min for CaMKIV. SDS-PAGE was carried out according to the method of Laemmli (30). Protein concentrations were determined by the method of Lowry et al. (31) as modified by Peterson (32).

RESULTS AND DISCUSSION

CaMKI and CaMKIV are good substrates for CaMKPase. The previous finding that a novel protein phosphatase (CaMKPase) purified from rat brain dephosphorylated autophosphorylated CaMKII with a concomitant decrease in the enzyme activity but did not significantly dephosphorylate any other proteins tested (6) raised the question of whether the role of this phosphatase is limited to the regulation of CaMKII. To solve this question, we examined whether other CaMKs, such as CaMKI, CaMKIV, and CaMKK-α, can serve as substrates for CaMKPase, as shown in Fig. 1. Since CaMKIV undergoes intensive autophosphorylation at many sites (33, 34) after being activated on the phosphorylation of Thr^{196} by CaMKK (16, 17), CaMKIV(K71R) whose activity is lost was used in place of CaMKIV in the experiment. CaMKI (Fig. 1A) and

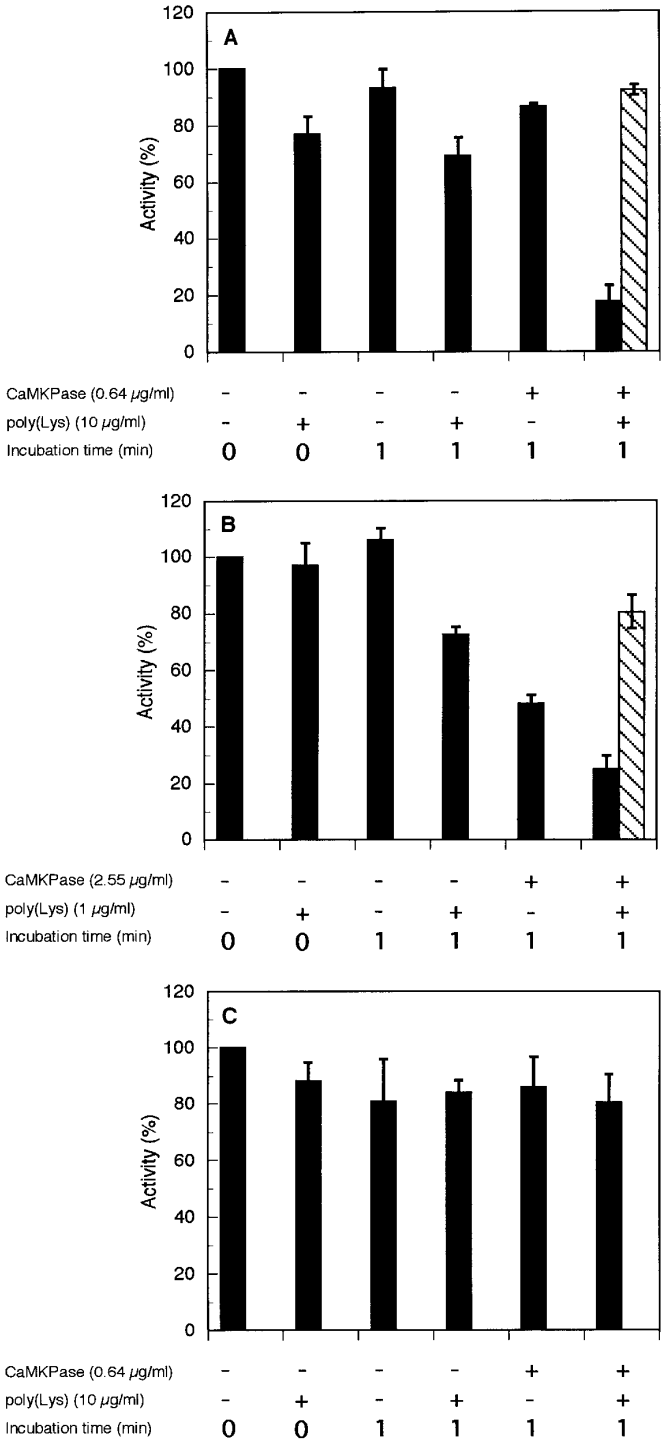

FIG. 2. Effect of CaMKPase on the activities of CaMKI, CaMKIV, and PKA. (A) CaMKI, which had been activated through phosphorylation with nonradioactive ATP by CaMKK-α as described under Materials and Methods, was incubated at 30°C with or without CaMKPase (0.64 μg/ml) in the presence or absence of poly(Lys) (10 μg/ml) as indicated in the figure, as described in the legend to Fig. 1A. After incubation for 1 min, aliquots were mixed with 4 volumes of an ice-cold stop solution consisting of 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.05% Tween 40, and 1 mM DTT and, immediately thereafter, the CaMKI activity was determined as described under Materials and Methods (filled bars). In the control experiment of 0-min incubation, the stop solution was added to the mixture on ice before addition of CaMKI. To confirm the reversibility of the deactivation of CaMKI by CaMKPase, CaMKI which had been deactivated by incubation with CaMKPase and poly(Lys) was mixed with the stop solution as described above, then incubated with CaMKK-α (3 μg/ml) at 30°C for 1 min in the assay mixture for CaMKI without syntide-2, and then incubated for 3 min with syntide-2 added for determination of CaMKI activity (hatched bar). Under the assay conditions, phosphorylation of syntide-2 by CaMKK-α was negligible. (B) CaMKIV, which had been activated by CaMKK-α, was incubated at 30°C for 1 min with or without CaMKPase (2.55 μg/ml) in the presence or absence of poly(Lys) (1 μg/ml), as described above. After incubation, CaMKIV activity was determined with syntide-2 as a substrate at 30°C for 5 min (filled bars). Reactivation of the CaMKPase-deactivated CaMKIV by CaMKK-α was carried out and assayed as in A (hatched bar). (C) bovine heart PKA was incubated as described in *A*, and PKA activity was determined as described under Materials and Methods. The results are expressed as a percentage of the initial activity, and each value represents the average of three independent experiments ± SD.

CaMKIV(K71R) (Fig. 1C), which had been phosphorylated by CaMKK-α, and CaMKII (Fig. 1B), which had been autophosphorylated at 5°C for 1 min, were efficiently dephosphorylated by CaMKPase in the presence of poly(Lys), while autophosphorylated CaMKK-α was not significantly dephosphorylated. Judging from silver staining, the amounts of proteins corresponding to CaMKI, CaMKII, and CaMKIV(K71R) virtually unaltered during the incubation under these conditions (data not shown), indicating that disappearance of the radioactive bands was not due to decrease in the amounts of the proteins. It has been reported that when CaMKI or CaMKIV(K71R) is incubated with CaMKK under the phosphorylation conditions, phosphorylation occurs almost exclusively on Thr^{177} of CaMKI (14) and Thr^{196} of CaMKIV(K71R) (17), respectively. When CaMKII is incubated at 5°C for a short time under the phosphorylation conditions, autophosphorylation occurs predominantly on Thr^{286} (35–37). These phosphorylation sites are known to be involved in the regulation of the respective CaMKs. The results of Fig. 1, together with these earlier findings, suggest that CaMKPase plays crucial roles in controlling the activities of the three multifunctional CaMKs.

CaMKI and CaMKIV activities are reversibly regulated by CaMKPase. To confirm the important roles of CaMKPase in the regulation of the multifunctional CaMKs, effect of this phosphatase on the activities of the CaMKs was examined, as shown in Fig. 2. The activities of CaMKI (Fig. 2A) and CaMKIV (Fig. 2B), which had been activated by CaMKK-α, were both decreased by incubation with CaMKPase in the presence of poly(Lys), and the decreased activities were almost completely reversed by subsequent incubation with CaMKK-α under the phosphorylation conditions (Fig. 2, *hatched bars*). Since the activity of the activated CaMKIV was strongly inhibited by 10 μg/ml poly(Lys),

the concentration of poly(Lys) was lowered to 1 μg/ml and that of CaMKPase was raised to 2.55 μg/ml (Fig. 2B). Incubation of the activated CaMKIV with CaMKPase even in the absence of poly(Lys) caused a significant decrease in the activity, probably owing to the basal activity of CaMKPase in the absence of poly(Lys) (6). These results, taken together, indicate that CaMKPase reversibly dephosphorylates the CaMKK-phosphorylated threonine residues located within the activation loops of CaMKI and CaMKIV, thereby deactivating the CaMKs. This raises the question of whether CaMKPase can dephosphorylate phosphorylated threonine or serine residues located in the activation loops of other protein kinases. It has been reported that PKA is spontaneously activated upon autophosphorylation of Thr^{197} (38), which is located within the activation loop, and deactivated upon dephosphorylation by PP2A or PP2A-like phosphatases (39). Since the phosphorylation of the threonine residue located in the activation loop of PKA is thus necessary for its activity, effect of CaMKPase on the PKA activity was examined. However, no significant decrease in the activity was observed after incubation with CaMKPase under the standard experimental conditions (Fig. 2C) and even with a large amount of CaMKPase (2.55 μg/ml) (data not shown). PKC, another second-messenger-responsive multifunctional protein kinase, is activated upon phosphorylation of the residue located within the activation loop (Thr^{500} of PKC-βII) by an unidentified protein kinase, followed by subsequent autophosphorylation of the residues located at the carboxyl-terminal region (Thr^{641} and Ser^{660} of PKC-βII) , and deactivated by protein phosphatase 1 through dephosphorylation of these residues (40–42). When PKC was incubated with CaMKPase and poly(Lys), no significant decrease in the PKC activity was observed (data not shown). These results, taken together with our earlier finding that CaMKPase catalyzes the dephosphorylation of autophosphorylated CaMKII with a concomitant decrease in the activity (6), suggest that the physiological function of CaMKPase is the regulation of the activities of multifunctional CaMKs, such as CaMKI, CaMKII, and CaMKIV. The activity of CaMKII may be reversibly regulated by the activation upon autophosphorylation of Thr^{286} located in the autoinhibitory domain and by the deactivation upon its dephosphorylation through CaMKPase. The activities of CaMKI and CaMKIV may be also reversibly regulated by the activation upon phosphorylation by CaMKK of Thr^{177} and Thr^{196}, respectively, located in the activation loops, and by the deactivation upon their dephosphorylation through CaMKPase. Thus, CaMKPase may participate in regulation of the activities of multifunctional CaMKs which mediate a variety of intracellular Ca^{2+}-signaling pathways.

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