A novel protein phosphatase that dephosphorylates and regulates Ca\(^{2+}\)/calmodulin-dependent protein kinase II

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A synthetic peptide corresponding to the autophosphorylation site of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) (residues 281–289) was conjugated to paramagnetic particles, and phosphorylated by a constitutively active CaMKII fragment. Using this phosphopeptide conjugate as a substrate, a calycin A-insensitive, Mn\(^{2+}\)-dependent, and poly-L-lysine-stimulated protein phosphatase activity was detected in the crude extract of rat brain. The protein phosphatase (designated as CaMKII phosphatase) (CaMKII(Pase)) was purified to near homogeneity from rat brain. CaMKII(Pase) showed apparent molecular weights of 54,000 and 65,000, on SDS-polyacrylamide gel electrophoresis and gel-filtration analysis, respectively. It was not inhibited by 100 mM calycin A or 10 \(\mu\)M okadaic acid. Mn\(^{2+}\), but not Mg\(^{2+}\), was absolutely required for activity. CaMKII(Pase) was potently activated by polycations. Autophosphorylated CaMKII was dephosphorylated by CaMKII(Pase), whereas phosphorylase kinase, mixed histones, myelin basic protein, and \(\alpha\)-casein (which had been phosphorylated by cAMP-dependent protein kinase and phosphorylase \(a\) (phosphorylated by phosphorylase kinase) were not significantly dephosphorylated. No other proteins than CaMKII in rat brain extract which had been phosphorylated by CaMKII were dephosphorylated. The stimulated Ca\(^{2+}\)-independent activity of autophosphorylated CaMKII was reversed by the action of CaMKII(Pase). Thus, CaMKII(Pase) appears to be a specialized protein phosphatase for the regulation of CaMKII.

Calmodulin-dependent protein kinase II (CaMKII)\(^1\) occurs abundantly in the brain, and has a broad substrate specificity (1). It plays a number of roles in the functioning of the central nervous system in response to intracellular Ca\(^{2+}\) (reviewed in Refs. 2–4). The possible involvement of CaMKII in the regulation of neuronal functions such as neurotransmitter synthesis (5, 6), neurotransmitter release (7, 8), long term potentiation (9–11), and the formation of spatial learning (12) has so far been suggested. CaMKII is known to be regulated by autophosphorylation at multiple sites. Among them, autophosphorylation at Thr\(^{286}\) results in generation of Ca\(^{2+}\)/calmodulin-independent activity (13–17), full activation of the total activity (18–20), and trapping of Ca\(^{2+}\)/calmodulin (21, 22). Therefore, the protein phosphatases that dephosphorylate the residue may be important for regulation of CaMKII activity. It has so far been reported that protein phosphatase 1 (PP1) (23), protein phosphatase 2A (PP2A) (14, 16, 24, 25), and protein phosphatase 2C (PP2C) (26) dephosphorylate Thr\(^{286}\) in vitro and that the dephosphorylation is catalyzed by distinct phosphatases in distinct subcellular compartments (27). Recently, we developed a novel in-gel protein phosphatase assay, using polyacrylamide gels containing phosphorylated peptide conjugates, and found that at least three distinct phosphatases existed in the rat brain extract which catalyzed dephosphorylation of the residue corresponding to Thr\(^{286}\) of CaMKII in the gels (28).

In the present study, we achieved purification and characterization of the protein phosphatase with an apparent molecular weight of 54,000, which had been previously detected by the in-gel assay (28). The purified phosphatase, which was designated as CaMKII phosphatase (CaMKII(Pase)), was highly specific for autophosphorylated CaMKII, and actually reversed the activated Ca\(^{2+}\)/calmodulin-independent activity of autophosphorylated CaMKII.

EXPERIMENTAL PROCEDURES

Materials

ATP, poly-L-lysine (poly(Lys)), average molecular weight 87,000 and 9,600, poly-L-arginine (poly(Arg)), average molecular weight 40,000, poly-L-(glutamic acid,lysine,tyrosine) 6:3:1 (poly(Glu,Lys,Tyr) 6:3:1, average molecular weight 23,000), and heparin were purchased from Sigma. W-7 was from Toronto Research Chemicals. Okadaic acid and calyculin A were from Wako Pure Chemical Industries. \(\beta\)-Glycerophosphate (disodium salt) was from nacalai tesque. A hetero-bifunctional reagent, N-(6-maleimidocaproyloxy)succinimide (EMCS), was obtained from Dojindo Laboratories. Amino paramagnetic particles (1–2 \(\mu\)m) was from Polysciences, Inc. \([\gamma^{-32}\text{P}]\text{ATP} (5,000 \text{Ci/mmol}) was from Amersham International. CaMKII(281–289) (MHRQETVDC) (29) was synthesized by a Shimadzu FSSFM automated peptide synthesizer and purified by reversed-phase HPLC.

Protein Preparations

CaMKII and its constitutively active 30-kDa fragment were prepared as described previously (30, 31). Catalytic subunit of cAMP-dependent protein kinase (PKA) was purified as described (32). Protein phosphatase inhibitor 2 was obtained from BIOMOL Research Labs., Inc. Protamine sulfate was from nacalai tesque. Calmodulin was prepared as described previously (33). Mixed histones (type II-A), myelin basic protein, myoglobin, transferrin, \(\gamma\)-globulin, bovine serum albumin (BSA), ovalbumin, and catalase were purchased from Sigma. Phosphorylase \(b\), cytochrome \(c\), and ferritin were from Boehringer Mannheim.

Purification of CaMKII(Pase) from Rat Brain Stem

All procedures were carried out at 4 °C. Approximately 20 g of rat brain stems, which had been frozen in liquid nitrogen immediately after freezing, were homogenized in 30 ml of 50 mM Tris-HCl (pH 7.4) containing 0.5 mM EGTA, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.25 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM sodium 3-iodotyrosine, 0.5 mM \(\alpha\)-thiogalactoside, 0.1 mM leupeptin, 1 mM pepstatin, 1 mM diisopropylfluorophosphate, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol.

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1 The abbreviations used are: CaMKII, calmodulin-dependent protein kinase II; BSA, bovine serum albumin; CaMKII(Pase), CaMKII phosphatase; DTT, dithiothreitol; EMCS, N-(6-maleimidocaproyloxy)succinimide; HPLC, high performance liquid chromatography; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PKA, catalytic subunit of cAMP-dependent protein kinase; PMI, peptide-magnetic particle conjugate; PMSE, phenylmethanesulfonfluoride; poly(Lys), poly-L-lysine; poly(Arg), poly-L-arginine; poly(Glu,Lys,Tyr); poly-L-(glutamic acid,lysine,tyrosine); PP, protein phosphatase.
CaMK II-specific Protein Phosphatase

Preparations of Protein Phosphatase Substrates

Detailed procedures for preparation of [32P]PMC-KII(281–289) (peptide-magnetic conjugate of CaMKII(281–289)), a phosphopeptide corresponding to amino acid residues 281–289 of autophosphorylated CaMKII conjugated to paramagnetic particles, are described elsewhere (34). Briefly, poly(Lys) (M, 87,000) was conjugated to amino paramagnetic particles by glutaraldehyde according to the manufacturer's instruction. To the particles, CaMKII(281–289) was conjugated using EMCS, a hetero-bifunctional reagent. The resulting peptide conjugate PMC-KII(281–289) was phosphorylated by a constitutively active 30-kDa proteolytic fragment of CaMKII to yield [32P]PMC-KII(281–289). After phosphorylation, the conjugate was exhaustively washed with 10 mM sodium phosphate buffer (pH 7.2) containing 0.85% NaCl, 50 g/ml each of pepstatin, leupeptin, antipain, and chymostatin with a Teflon/glass homogenizer. The homogenate was centrifuged at 100,000 × g for 60 min and the supernatant was withdrawn. A crude extract of rat cerebral cortex (407 mg/ml) was determined to the bottom of the tube by a neodymium iron magnet (Polysciences, Inc.) when the supernatant was withdrawn. One unit of enzyme was defined as the amount that catalyzed the release of 1 pmol of phosphate/min at 30 °C.

Immobilized Phosphopeptide Assay—For analysis with the purified enzyme, dephosphorylation of [32P]PMC-KII(281–289) was carried out at 30 °C for 5 min in a reaction mixture (30 l) containing an appropriate amount of CaMKII-Pase, 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM MgCl2, 2 mM MnCl2, 0.1 mM EDTA, 0.4 mM EGTA, 0.01% Tween 20, 10 pmol [32P]ATP (28,000 cpm/pmol). Termination of the reaction and dephosphorylation of the phosphoproteins were carried out as described for autophosphorylated CaMKII except that 100 mM calcium A was included in the equilibration/elution buffer for the spin column. The phosphorylated sample was stored in aliquots at −80 °C.

Protein Phosphatase Assay

CaMKII-Pase activity was determined by the following four assay methods. Unless otherwise stated, poly(Lys) means M, 87,000 species of poly(Lys). Basic proteins and polycations including poly(Lys) were initially dissolved in phosphate-buffered saline to yield 10 mg/ml solutions and they were diluted with H2O to appropriate concentrations prior to use.

Immobilized Phosphopeptide Assay—For analysis with the purified enzyme, dephosphorylation of [32P]PMC-KII(281–289) was carried out at 30 °C for 5 min in a reaction mixture containing 40 mM Hepes-NaOH (pH 8.0), 5 mM Mg(CH3CO2)2, 0.1 mM EGTA, 0.4 mM EDTA, 1 μM calmodulin, 1.2 mM CaCl2, 0.01% Tween 20, 0.96 μg/ml CaM Kinase II-specific Protein Phosphatase 1905, 50 μM [γ-32P]ATP (28,000 cpm/pmol). The reaction and dephosphorylation of the phosphoproteins were carried out as described for autophosphorylated CaMKII except that 100 mM calcium A was included in the equilibration/elution buffer for the spin column. The phosphorylated sample was stored in aliquots at −80 °C.

SDS-PAGE Assay—After preincubation for 30 min at 30 °C, the reaction was started by adding the phosphoprotein substrate. After incubation for 30 s or 1 min, a 10-μl aliquot was withdrawn and transferred to a tube containing 100 μl of 20% trichloroacetic acid, and then 100 μl of 6 mg/ml BSA was added to the tube, followed by vortexing. The mixtures were allowed to stand for 10 min on ice, and then 30 μl of ice-cold 5% trichloroacetic acid was added. After centrifugation for 5 min at 4 °C in a microcentrifuge at maximum speed, 450 μl of the supernatant was withdrawn and added to the tube containing 20 μl of 10% SDS in SDS-PAGE loading buffer. The supernatant was loaded to the bottom of the tube by a neodymium iron magnet (Polysciences, Inc.) when the supernatant was withdrawn. One unit of enzyme was defined as the amount that catalyzed the release of 1 μmol of phosphate/min at 30 °C.

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SDS-PAGE Assay—After preincubation for 30 min at 30 °C, the reaction was started by adding the phosphoprotein substrate. After incubation for 30 s or 1 min, a 10-μl aliquot was withdrawn and transferred to a tube containing 100 μl of 20% trichloroacetic acid, and then 100 μl of 6 mg/ml BSA was added to the tube, followed by vortexing. The mixtures were allowed to stand for 10 min on ice, and then 30 μl of ice-cold 5% trichloroacetic acid was added. After centrifugation for 5 min at 4 °C in a microcentrifuge at maximum speed, 450 μl of the supernatant was withdrawn and counted for 32P radioactivity.
**TABLE I**  
**Purification of CaMKII-Pase**

| Method       | Total activity | Total protein | Specific activity | Purification Yield |
|--------------|----------------|---------------|------------------|--------------------|
| Crude extract| 56.7 mg        | 664 milliunits| 0.0854 mg/milliunits | 1 100 |
| Phosphocellulose | 58.6 mg      | 288 milliunits| 0.204 mg/milliunits | 2.4 103 |
| DEAE-cellulose | 36.7 mg      | 78.2 milliunits| 0.47 mg/milliunits | 5.5 65 |
| Phenyl-5PW    | 5.4 mg         | 1.35 milliunits| 4.0 mg/milliunits | 47 9.5 |
| Superdex 200  | 3.8 mg         | 0.29 milliunits| 13.1 mg/milliunits | 153 6.7 |
| DEAE-NPR      | 1.34 mg        | 0.0215 milliunits| 62.3 mg/milliunits | 730 2.4 |

In-gel Assay—In-gel phosphatase assay of CaMKII-Pase was carried out as described previously (28).

Other Analytical Procedures

SDS-PAGE was carried out according to the method of Laemmli (36). Protein concentrations were determined by the method of Lowry et al. (37), as modified by Peterson (38).

**RESULTS**

Purification of CaMKII-Pase—Since our previous in-gel assay for protein phosphatase with a synthetic peptide corresponding to the autophosphorylation site of CaMKII (residues 281–289) as a substrate suggested the existence of two protein phosphatases specific for the autophosphorylation site in rat brain (28), we attempted to purify and characterize them. Our preliminary experiments using immobilized phosphopeptide assay showed that a calyculin A-insensitive, Mn2+-dependent, and poly(Lys)-stimulated phosphatase activity toward [32P]PMC-KII(281–289) was present in a rat brain extract (data not shown). The possibility that the activity detected under such conditions was due to a protease could be eliminated, since almost all the radioactivities released into the supernatant of the assay mixture during the reaction were extracted with ammonium the assay mixture during the reaction were extracted with ammonium ions tested, such as Mg2+, Zn2+, Co2+, Ca2+, Ni2+, and Ba2+. EDTA completely inhibited the enzyme activity, presumably by removing Mn2+ essential for the activity. Inhibitor 2, a protein inhibitor specific for PP1, and okadaic acid and calyculin A, both of which are known to be potent inhibitors for PP1 and PP2A, but not PP2C, inhibited the activity of CaMKII-Pase. Another common phosphatase inhibitor, β-glycerophosphate also inhibited the activity. Orthovanadate, a potent inhibitor of tyrosine phosphatase that also inhibits PP1, PP2A, and PP2B, did not inhibit the activity of CaMKII-Pase. Heparin, which is known to inhibit PP1, inhibited CaMKII-Pase. EDTA completely inhibited the enzyme activity, presumably by removing Mn2+ essential for the activity. Inhibitor 2, a protein inhibitor specific for PP1, and okadaic acid and calyculin A, both of which are known to be potent inhibitors for PP1 and PP2A, did not significantly affect the activity of CaMKII-Pase. W-7, a calmodulin antagonist, and Ca2+/calmodulin had no significant effect. ZnCl2, CoCl2, or NiCl2 completely inhibited CaMKII-Pase at a concentration of 2 mM, whereas 2 mM BaCl2 had no effect. CaCl2 poorly inhibited the activity at

**FIG. 1.** Molecular weights of CaMKII-Pase. A, approximately 0.4 μg of CaMKII-Pase was subjected to SDS-PAGE on a 10% acrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. B, approximately 15 ng of CaMKII-Pase was analyzed by in-gel assay as described under “Experimental Procedures.” C, approximately 14 μg of CaMKII-Pase was subjected to gel filtration on a Superdex 200HR 10/30 column (Pharmacia) attached to a non-metal HPLC system (Tosoh) equilibrated and eluted with 25 mM Tris-HCl (pH 7.5) containing 1 mM EGTA, 100 mM NaCl, 0.05% Tween 40, and 0.5 mM DTT at a flow rate of 0.4 ml/min. The column was calibrated with the following molecular weight standards: ferritin, 450,000; catalase, 240,000; γ-globulin, 160,000; transferrin, 75,000; BSA, 67,000; ovalbumin, 45,000; myoglobin, 18,000; cytochrome c, 13,000. The elution of the proteins were monitored by their absorbance at 280 nm. The logarithm of molecular weights of proteins was plotted against their elution volume. The arrow shows the position of the elution of CaMKII-Pase.
Dephosphorylation of Autophosphorylated CaMKII by CaMKIIPase—In our experiments described so far, we had used \[^{32}P\]PMC-KII(281–289), a synthetic phosphopeptide conjugate corresponding to residues 281–289 of autophosphorylated CaMKII, as a substrate for assay of CaMKIIPase. The important question of whether the purified enzyme can actually catalyze dephosphorylation of autophosphorylated CaMKII was examined as shown in Fig. 4. CaMKIIPase dephosphorylated autophosphorylated CaMKII in the presence of poly(Lys), and the dephosphorylation was almost completely blocked by the omission of poly(Lys). The dephosphorylation was not inhibited by calyculin A, but inhibited by NaF or EDTA, in good agreement with the results obtained with \[^{32}P\]PMC-KII(281–289) as a substrate (Table II). The dephosphorylation of autophosphorylated CaMKII by CaMKIIPase was also confirmed by trichloroacetic acid precipitation assay. When \[^{32}P\]autophosphorylated CaMKII (50 nM) was incubated with CaMKIIPase (64 ng/ml) in the presence of poly(Lys) (10 μg/ml) and then precipitated with trichloroacetic acid, a rapid release of \[^{32}P\]radioactivity into the supernatant was observed, while no significant radioactivity was recovered in the supernatant when poly(Lys) was omitted from the incubation mixture (data not shown). When much higher concentrations of CaMKIIPase was used in this assay, however, a slow but significant dephosphorylation was observed in the absence of poly(Lys) (data not shown), indicating that CaMKIIPase possessed a low dephosphorylating activity toward autophosphorylated CaMKII even in the absence of poly(Lys). The activation by 10 μg/ml

![Graph](image1)

**FIG. 2. Effect of varying the concentration of poly(Lys) on CaMKIIPase activity.** The activity of CaMKIIPase was measured by immobilized phosphopeptide assay using \[^{32}P\]PMC-KII(281–289) as a substrate in the presence of the indicated concentrations of poly(Lys) as described under “Experimental Procedures.” The results are expressed as the ratio of activity in the presence of poly(Lys) to that in its absence. Each value represents the average of three independent experiments ± S.D.

![Graph](image2)

**FIG. 3. Activation of CaMKIIPase by polycations and basic proteins.** The activity of CaMKIIPase was measured by immobilized phosphopeptide assay using \[^{32}P\]PMC-KII(281–289) as a substrate as described under “Experimental Procedures,” except that 10 μg/ml poly(Lys) was replaced by the indicated polycations or basic proteins of 10 μg/ml. The results are expressed as the ratio of activity in the presence of the indicated additions to that in their absence. Each value represents the average of three independent experiments ± S.D.

![Graph](image3)

**FIG. 4. Dephosphorylation of autophosphorylated CaMKII by CaMKIIPase.** Approximately 73 nM CaMKII, which had been autophosphorylated with \[^{γ-32}P\]ATP as described under “Experimental Procedures,” was incubated at 30 °C for 1 min as described under “SDS-PAGE Assay” under “Experimental Procedures,” with the indicated additions and omissions, and aliquots were analyzed by SDS-PAGE, followed by autoradiography. The positions corresponding to α and β isoforms of CaMKII are indicated.

CaMKII was examined as shown in Fig. 4. CaMKIIPase dephosphorylated autophosphorylated CaMKII in the presence of poly(Lys), and the dephosphorylation was almost completely blocked by the omission of poly(Lys). The dephosphorylation was not inhibited by calyculin A, but inhibited by NaF or EDTA, in good agreement with the results obtained with \[^{32}P\]PMC-KII(281–289) as a substrate (Table II). The dephosphorylation of autophosphorylated CaMKII by CaMKIIPase was also confirmed by trichloroacetic acid precipitation assay. When \[^{32}P\]autophosphorylated CaMKII (50 nM) was incubated with CaMKIIPase (64 ng/ml) in the presence of poly(Lys) (10 μg/ml) and then precipitated with trichloroacetic acid, a rapid release of \[^{32}P\]radioactivity into the supernatant was observed, while no significant radioactivity was recovered in the supernatant when poly(Lys) was omitted from the incubation mixture (data not shown). When much higher concentrations of CaMKIIPase was used in this assay, however, a slow but significant dephosphorylation was observed in the absence of poly(Lys) (data not shown), indicating that CaMKIIPase possessed a low dephosphorylating activity toward autophosphorylated CaMKII even in the absence of poly(Lys). The activation by 10 μg/ml

**TABLE II Effects of various phosphatase inhibitors and divalent cations on CaMKIIPase activity**

| Addition       | Concentration | Activity (%) |
|----------------|---------------|--------------|
| None           | 100           | 100          |
| Orthovanadate  | 1 mM          | 203 ± 7.9    |
| Calyculin A    | 100 nM        | 106 ± 8.1    |
| Okadaic acid   | 10 μM         | 111 ± 8.1    |
| Inhibitor 2    | 20 nM         | 96.2 ± 1.0   |
| NaF            | 100 mM        | 28.5 ± 5.1   |
| β-Glycerophosphate | 50 mM  | 8.3 ± 4.1    |
| Heparin        | 100 μg/ml     | 10.8 ± 4.1   |
| EDTA           | 25 mM         | 0            |
| W-7            | 100 μM        | 101 ± 2.1    |
| CaCl\(_2\)     | 0.3 mM/1 μM   | 136 ± 8.9    |
| CdCl\(_2\)     | 0.3 mM        | 97.6 ± 1.2   |
| CaCl\(_2\)     | 2 mM          | 73.4 ± 0.9   |
| ZnCl\(_2\)     | 2 mM          | 0            |
| CoCl\(_2\)     | 2 mM          | 0            |
| NiCl\(_2\)     | 2 mM          | 0            |
| BaCl\(_2\)     | 2 mM          | 93.5 ± 3.1   |

2 mM, but did not inhibit at 0.3 mM.

CaMKII was assayed by immobilized phosphopeptide method with \[^{32}P\]PMC-KII(281–289) as a substrate as described under “Experimental Procedures,” with the indicated additions. The results are expressed as a percentage of activity determined with no additions. The data represent the average of three independent experiments ± S.D.
CaM Kinase II-specific Protein Phosphatase

**FIG. 5.** Substrate specificity of CaMKII-Pase. A, approximately 16.7 nm phosphorylase kinase (lanes 1 and 2), mixed histones (lanes 3 and 4), phosphorylase α (lanes 5 and 6), MBP, MBP, and α-casein (lanes 9 and 10), which had been phosphorylated with [γ-32P]ATP as described under “Experimental Procedures,” were incubated with (lanes 2, 4, 6, 8, and 10) or without (lanes 1, 3, 5, 7, and 9) 1.6 μg/ml CaMKII-Pase and 10 μg/ml poly(Lys) in the presence of autophosphorylated CaMKII for 1 min at 30 °C, and aliquots were analyzed by SDS-PAGE, followed by autoradiography. B, a crude extract of rat cerebral cortex was phosphorylated with [γ-32P]ATP by CaMKII as described under “Experimental Procedures,” and aliquots amounting to 13.5 nm [32P]phosphate incorporated were incubated with CaMKII-Pase and poly(Lys), as indicated. Prior to addition of the autophosphorylated CaMKII, the mixture was preincubated for 30 s, and the reaction was started by adding the CaMKII. After incubation for 60 min, a 5-μl aliquot was withdrawn and mixed with 20 μl of an ice-cold stop buffer consisting of 50 mM Tris–HCl (pH 7.5), 2 mM MgCl2, 2 mM MnCl2, 0.1 mM EDTA, and 0.01% Tween 20, in the presence or absence of 12.8 μg/ml CaMKII-Pase and/or 1 μg/ml poly(Lys), as indicated. Prior to addition of the autophosphorylated CaMKII, the mixture was preincubated for 30 s, and the reaction was started by adding the CaMKII. After incubation for 60 min, a 5-μl aliquot was withdrawn and mixed with 20 μl of an ice-cold stop buffer consisting of 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.05% Tween 40, and 40 mM DTT, and immediately thereafter the activity of CaMKII-Pase was determined in the presence and absence of CaCl2 as described previously. For 0-min incubation, the stop buffer was added before addition of CaMKII. The results are expressed as autonomy, i.e. the ratio (%) of the activity in the absence of Ca2+ to that in its presence. The total activities of the kinase expressed as a percentage of the initial value after 60 min of incubation were as follows: −CaMKII-Pase and −poly(Lys), 91.9 ± 7.0%; −CaMKII-Pase and +poly(Lys), 32.1 ± 1.7%; +CaMKII-Pase and −poly(Lys), 88.0 ± 1.1%; +CaMKII-Pase and +poly(Lys), 64.6 ± 6.6%. Each value represents the average of three independent experiments ± S.D.

**FIG. 6.** The decrease in the increased autonomy of autophosphorylated CaMKII by CaMKII-Pase. CaMKII, which had been autophosphorylated with nonradioactive ATP, was incubated at 5 °C for 0 or 60 min in a reaction mixture containing 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 2 mM MgCl2, 2 mM MnCl2, 0.1 mM EDTA, and 0.01% Tween 20, in the presence or absence of 12.8 μg/ml CaMKII-Pase and/or 1 μg/ml poly(Lys), as indicated. Prior to addition of the autophosphorylated CaMKII, the mixture was preincubated for 30 s, and the reaction was started by adding the CaMKII. After incubation for 60 min, a 5-μl aliquot was withdrawn and mixed with 20 μl of an ice-cold stop buffer consisting of 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.05% Tween 40, and 40 mM DTT, and immediately thereafter the activity of CaMKII was determined in the presence and absence of CaCl2 as described previously. For 0-min incubation, the stop buffer was added before addition of CaMKII. The results are expressed as autonomy, i.e. the ratio (%) of the activity in the absence of Ca2+ to that in its presence. The total activities of the kinase expressed as a percentage of the initial value after 60 min of incubation were as follows: −CaMKII-Pase and −poly(Lys), 91.9 ± 7.0%; −CaMKII-Pase and +poly(Lys), 32.1 ± 1.7%; +CaMKII-Pase and −poly(Lys), 88.0 ± 1.1%; +CaMKII-Pase and +poly(Lys), 64.6 ± 6.6%. Each value represents the average of three independent experiments ± S.D.

**A.** Ishida, I. Kameshita, and H. Fujisawa, unpublished observations.
CaMKII in the presence of poly(Lys) caused a marked decrease in the autonomy. Thus, the Ca\(^{2+}/\)calmodulin-independent activity of CaMKII generated by autophosphorylation at Thr\(^{286}\) was reversed by incubation with CaMKII in the presence of poly(Lys). When the dephosphorylated kinase was assayed after brief incubation (30 °C, 10 s) under the autophosphorylating conditions, generation of Ca\(^{2+}/\)calmodulin-independent activity was observed again (44% of the total activity), suggesting that the dephosphorylation process is reversible.

**DISCUSSION**

Since CaMKII, which is involved in controlling a variety of neuronal functions, is markedly activated through autophosphorylation at Thr\(^{286}\) (2-4, 13-22), the dephosphorylation of the Thr\(^{286}\) is thought to be very important for the regulation of the activity of CaMKII. Recently, the existence of protein phosphatases catalyzing the dephosphorylation of the Thr\(^{286}\) in the rat brain was suggested by in-gel protein phosphatase assay using a synthetic peptide CaMKII(281-289) (MHRQETVDC) corresponding to the autophosphorylation site of CaMKII (residues 281-289) as a substrate (28). In the present study, we purified and characterized a novel protein phosphatase, designated CaMKII-Pase, catalyzing the dephosphorylation of [\(^{32P}\)]PMC-KII(281-289), a poly(Lys) and paramagnetic particles. Table III compares some catalytic properties of CaMKII-Pase with those of other well known protein Ser/Thr phosphatases, such as PP1, PP2A, PP2B, and PP2C shown in this table are from a review by Shenolikar and Nairn (39). It has been reported that some protein phosphatases having a decrease in the autonomy. Thus, the Ca\(^{2+}/\)calmodulin-dependent activity of CaMKII, in good agreement with our previous finding (28) that the band corresponding to CaMKII-Pase detected by the in-gel protein phosphatase assay was observed when CaMKII(281-289) was used as a substrate but not observed when other phosphopeptides such as C-syntide-2 and CAMKAKS peptide were used as substrates. An apparent K_m value of CaMKII-Pase for autophosphorylated CaMKII estimated from double-reciprocal plots of the rates of the dephosphorylation determined by trichloroacetic acid precipitation assay as functions of the concentrations of CaMKII was in the range of 30–80 nM (data not shown). This value is much lower than the K_m values of PP2A ranging from 1 to 100 μM obtained for various substrates (44) or the K_m value of PP2C from turkey gizzard smooth muscle of 7.9 μM estimated for myosin light chain (45), suggesting that autophosphorylated CaMKII is a good specific substrate for CaMKII-Pase.

Since the autophosphorylation of CaMKII at Thr\(^{286}\) is known to generate the Ca\(^{2+}/\)calmodulin-dependent activity, the dephosphorylation of the phosphorylated Thr\(^{286}\) should result in a decrease in the activity. However, autophosphorylated CaMKII was very labile to poly(Lys) added for the reaction of CaMKII-Pase, and therefore the reverse of the activity due to the dephosphorylation of Thr\(^{286}\) of CaMKII was monitored by a decrease in the autonomy of the activity of CaMKII (Fig. 6). A maximum decrease in the autonomy was observed after incubation with CaMKII-Pase in the presence of poly(Lys), suggesting that CaMKII-Pase dephosphorylated the autophosphorylated Thr\(^{286}\) of CaMKII, thereby resulting in a decrease in the autonomy. The results, taken together, suggest the possibility that CaMKII-Pase purified in the present study is a specialized protein phosphatase for the regulation of CaMKII.

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