Phosphorylation and activation of Ca^{2+}/calmodulin-dependent protein kinase phosphatase by Ca^{2+}/calmodulin-dependent protein kinase II

Isamu Kameshita, Atsuhiko Ishida, Hitoshi Fujisawa*

Department of Biochemistry, Asahikawa Medical College, Asahikawa 078-8510, Japan

Received 21 June 1999

Abstract Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKPase) is a protein phosphatase which dephosphorylates autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and deactivates the enzyme (Ishida, A., Kameshita, I. and Fujisawa, H. (1998) J. Biol. Chem. 273, 1904–1910). In this study, a phosphorylation-dephosphorylation relationship between CaMKII and CaMKPase was examined. CaMKPase was not significantly phosphorylated by CaMKII under the standard phosphorylation conditions but was phosphorylated in the presence of poly-L-lysine, which is a potent activator of CaMKPase. The maximal extent of the phosphorylation was about 1 mol of phosphate per mol of the enzyme and the phosphorylation resulted in an about 2-fold increase in the enzyme activity. Thus, the activity of CaMKPase appears to be regulated through phosphorylation by its target enzyme, CaMKII.

Key words: Calmodulin-dependent protein kinase II; Enzyme activation; Protein phosphatase; Protein phosphorylation; Polylysine

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a Ca^{2+}-responsive multifunctional protein kinase, is known to occur abundantly in the brain [1] and is thought to play important roles in a variety of neuronal functions mediated by Ca^{2+} [2,3]. CaMKII is markedly activated upon autophosphorylation at Thr^{286} and therefore, protein phosphatases which dephosphorylate the phosphorylated threonine residue may be expected to deactivate CaMKII. It has been reported that protein phosphatases 1 [4], 2A [5–8] and 2C [9] dephosphorylate Thr^{286} in vitro. Recently, using an in-gel phosphatase assay, we detected three protein phosphatase activities with apparent molecular weights of 54 000, 58 000 and 74 000, which dephosphorylate CaMKII at Thr^{286} in a crude brain rat extract [10], and the protein phosphatase with a molecular weight of 54 000 was purified and characterized [11]. This enzyme, designated as Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKPase), exhibited a much higher substrate specificity than do the well-known multifunctional protein phosphatases such as protein phosphatases 1, 2A and 2C and required Mn^{2+} and polycations such as poly-L-lysine (poly(Lys)), protamine and histones for its activity [11].

In the present paper, we reported that CaMKPase was phosphorylated by CaMKII, which is its target, only in the presence of polycations such as poly(Lys), which is its activator, and was thereby activated about 2-fold. These results suggest the possibility that CaMKPase regulates CaMKII and vice versa.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (5000 Ci/mmol) was obtained from Amersham. Poly-$(Lys)_{9.6K}$, poly$(Lys)_{23K}$, poly$(Lys)_{87K}$, histone type IIIS from calf thymus, protamine from salmon testis and bovine serum albumin were purchased from Sigma Chemicals. Lysine oligomers such as $(Lys)_5$, $(Lys)_{10}$ and $(Lys)_{20}$ were synthesized using a Shimadzu PSSM-8 automated peptide synthesizer and purified by reverse-phase HPLC.

Calmodulin was purified from rat testis by the method of Dedman et al. [12]. CaMKPase was purified from rat brain stem as described previously [11]. CaMKII and its constitutively active 30 kDa fragment (30 kDa CaMKII) were prepared as described [13,14]. Myosin light chain was prepared from chicken gizzard [15,16].

2.2. Phosphorylation of CaMKPase

Phosphorylation of CaMKPase by CaMKII was carried out at 30°C for 10 min in a final volume of 10 μl of a reaction mixture containing 40 mM HEPES-NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 2 mM dithiothreitol, 50 μM [γ-^{32}P]ATP, 40–100 ng CaMKPase and an appropriate amount of poly(Lys) and 8.35 ng of 30 kDa CaMKII (or 10 ng CaMKII). The reaction was stopped by the addition of 10 μl SDS sample buffer and the mixture was analyzed by SDS-PAGE, followed by autoradiography.

2.3. Protein phosphatase assay

CaMKPase activity was determined with [^{32}P]CaMKII as a substrate essentially as described previously [11]. The reaction mixture contained, in a final volume of 30 μl, 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 10 μg/ml poly(Lys) and 40 nM [^{32}P]CaMKII. The reaction was started by the addition of CaMKPase after preincubation for 1 min at 30°C to warm the reaction mixture. After incubation for 1 min, the reaction was stopped by the addition of 100 μl of 20% trichloroacetic acid and then, 100 μl of 6 mg/ml bovine serum albumin was added. The mixture was allowed to stand for 10 min on ice and then, 300 μl of ice-cold 5% trichloroacetic acid was added. After centrifugation, 450 μl of the supernatant was withdrawn for measurement of ^{32}P radioactivity.

2.4. Other analytical procedures

Incorporation of [^{32}P]phosphate into CaMKPase was determined by the Whatmann 3MM chromatography paper method of Corbin and Reimann [17]. PAGE in the presence of SDS was performed by

*Corresponding author. Fax: (81) (166) 68-2349.
E-mail: fujisawa@asahikawa-med.ac.jp

Abbreviations: CaMK, Ca^{2+}/calmodulin-dependent protein kinase; 30 kDa CaMKII, 30 kDa fragment of Ca^{2+}/calmodulin-dependent protein kinase II; CaMKPase, Ca^{2+}/calmodulin-dependent protein kinase phosphatase; poly(Lys), poly-L-lysine; poly$(Lys)_{9.6K}$, poly-$(Lys)_{23K}$ and poly$(Lys)_{87K}$, poly-L-lysine with average molecular weights of 9600, 23 000 and 87 000, respectively; $(Lys)_5$, $(Lys)_{10}$ and $(Lys)_{20}$, oligopeptides composed of 5, 10 and 20 lysines, respectively

0014-5793/99/$20.00
PII: S0014-5793(99)00958-8

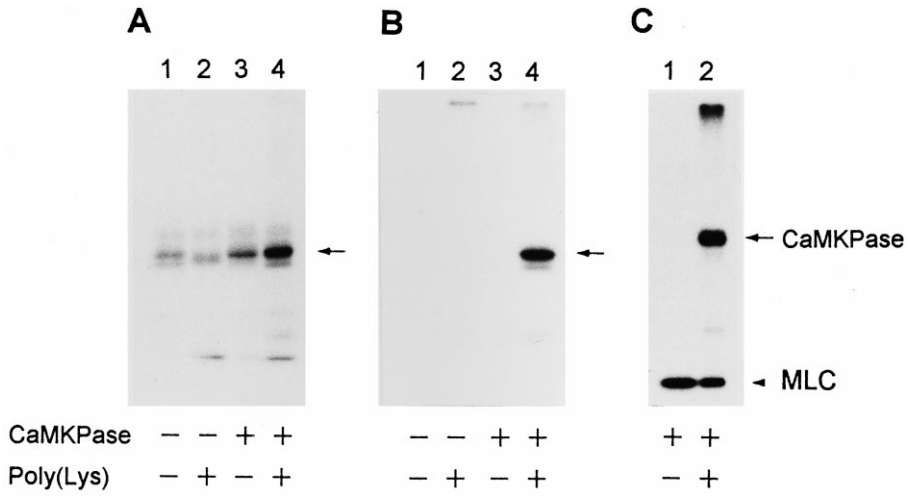

Fig. 1. Phosphorylation of CaMKPase by CaMKII. Purified CaMKPase (100 ng) was incubated in the standard phosphorylation mixture containing 50 μM [γ-^{32}P]ATP (9000 cpm/pmol) and 10 μg/ml poly(Lys)$_{87K}$ and 10 ng of CaMKII (A) or 8.35 ng of 30 kDa CaMKII (B), with the indicated omissions, and was analyzed by SDS-PAGE followed by autoradiography, as described in Section 2. (C) CaMKPase (40 ng), together with myosin light chain (MLC) (100 ng), was phosphorylated by 30 kDa CaMKII as described above. The arrows and arrowhead indicate the positions of CaMKPase and MLC, respectively.

the method of Laemmli [18] with slab gels consisting of an 8 or 10% acrylamide separation gel and a 3% stacking gel. Proteins were determined by the method of Bensadoun and Weinstein [19] using bovine serum albumin as a standard.

3. Results

3.1. Phosphorylation of CaMKPase by CaMKII

When CaMKPase was incubated with CaMKII under the phosphorylation conditions in the presence of Ca^{2+}/calmodulin, CaMKPase was not significantly phosphorylated, but when poly(Lys)$_{87K}$, which is a potent activator of CaMKPase, was added to the reaction mixture, it became phosphorylated (Fig. 1A). Since the migration position of autophosphorylated CaMKII, which migrates more slowly than does the non-phosphorylated enzyme, was close to that of phosphorylated CaMKPase upon SDS-PAGE as shown in Fig. 1A, the active 30 kDa fragment of CaMKII [14] was used in place of CaMKII in the experiment as shown in Fig. 1B. The results indicate a clear-cut dependence of the phosphorylation of CaMKPase by CaMKII on poly(Lys)$_{87K}$. When myosin light chain, a well-known substrate of CaMKII [20], and CaMKPase were incubated with 30 kDa CaMKII in the presence or absence of poly(Lys) under the phosphorylation conditions, CaMKPase was phosphorylated only in the presence of poly(Lys) but myosin light chain was phosphorylated similarly in the pres-

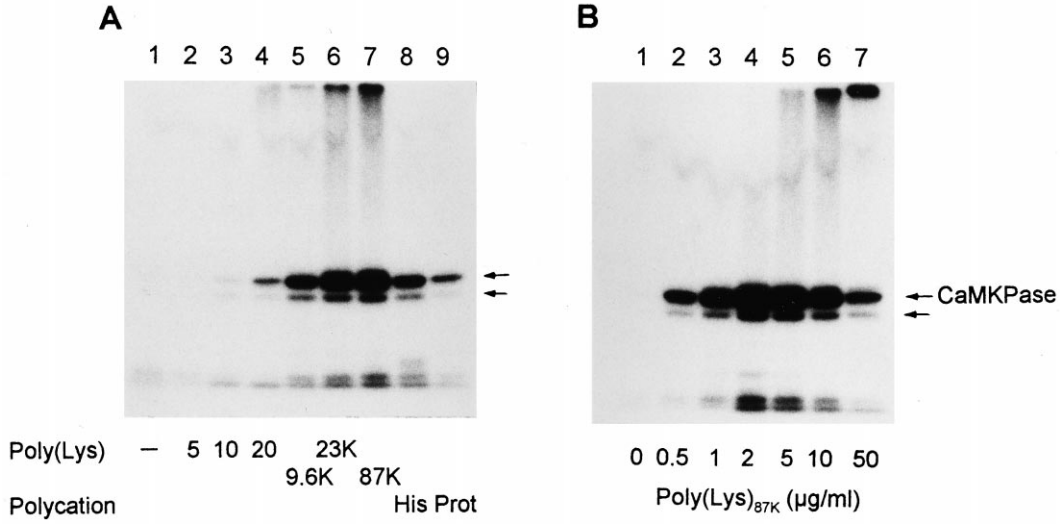

Fig. 2. Effects of polycations on phosphorylation of CaMKPase by CaMKII. (A) Purified CaMKPase (100 ng) was incubated in the standard phosphorylation mixture containing 50 μM [γ-^{32}P]ATP (9000 cpm/pmol) and 30 kDa CaMKII (8.35 ng) and 10 μg/ml (Lys)$_5$ (lane 2), (Lys)$_{10}$ (lane 3), (Lys)$_{20}$ (lane 4), poly(Lys)$_{9.6K}$ (lane 5) poly(Lys)$_{23K}$ (lane 6), poly(Lys)$_{87K}$ (lane 7), histone (lane 8), protamine (lane 9) or no polycation (lane 1) and was analyzed by SDS-PAGE followed by autoradiography, as described in Section 2. (B) CaMKPase (100 ng) was phosphorylated in the reaction mixture containing 0 μg/ml (lane 1), 0.5 μg/ml (lane 2), 1 μg/ml (lane 3), 2 μg/ml (lane 4), 5 μg/ml (lane 5), 10 μg/ml (lane 6) or 50 μg/ml (lane 7) poly(Lys)$_{87K}$, as described above. The arrows indicate the positions of CaMKPase. The bands shown by the arrowheads may be proteolytic products of CaMKPase.

ence and absence of poly(Lys) (Fig. 1C), indicating that the stimulation of the CaMKPase phosphorylation by poly(Lys) was not due to the activation of CaMKII.

Fig. 2A shows the effects of various polycations such as poly(Lys)s of various lengths, histone and protamine on the phosphorylation of CaMKPase by CaMKII. The magnitude of the stimulation of the phosphorylation varied depending on the size of poly(Lys) and the stimulation increased with increasing the length of poly(Lys) under the experimental conditions. Basic proteins such as histone and protamine also stimulated the phosphorylation of CaMKPase. When the effects of various concentrations of poly(Lys)$_{87K}$, which was the most effective in stimulating the CaMKPase phosphorylation, on the stimulation was examined upon SDS-PAGE followed by autoradiography (Fig. 2B), the most intense radioactive band corresponding to the position of CaMKPase was observed at concentrations of 2–5 μg/ml. With increasing concentrations of poly(Lys)$_{87K}$ above 5 μg/ml, the intensity of the radioactive band corresponding to the position of CaMKPase decreased and the intensity of the band at the top of the gel increased inversely (Fig. 2B, lanes 5–7), suggesting that poly(Lys)$_{87K}$ at a high concentration tends to form high-molecular weight complexes with CaMKPase.

When CaMKPase was incubated with CaMKII in the presence of 2 μg/ml poly(Lys)$_{87K}$ under the experimental conditions, the phosphorylation of CaMKPase reached a maximum level within 10 min, the maximal extent being about 0.95 mol of phosphate/mol of the enzyme (Fig. 3). CaMKPase was also phosphorylated only in the presence of poly(Lys) by the catalytic subunit of cAMP-dependent protein kinase, but the rate of the phosphorylation was much slower than that by CaMKII (data not shown).

3.2. Activation of CaMKPase by CaMKII

The finding that CaMKPase was phosphorylated by CaMKII in the presence of polycations raised the question whether the phosphorylation of CaMKPase caused changes in the enzyme activity. As shown in Fig. 4, the activity of CaMKPase which had been phosphorylated with 30 kDa CaMKII was approximately twice that of the non-phosphorylated enzyme, when assayed with autophosphorylated CaMKII as a substrate. The phosphorylated enzyme required poly(Lys)$_{87K}$ for full activity, as the non-phosphorylated enzyme did, and the activities of both enzymes showed a similar dependence on the poly(Lys)$_{87K}$ concentration.

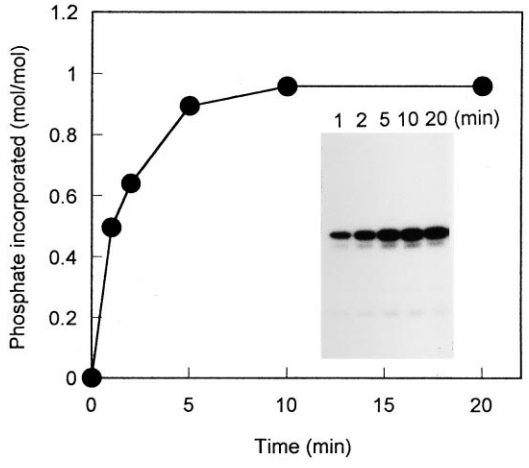

Fig. 3. Time course of phosphorylation of CaMKPase by CaMKII. Purified CaMKPase (500 ng) was incubated at 30°C in the standard phosphorylation mixture (50 μl) containing 50 μM [γ-^{32}P]ATP, 30 kDa CaMKII (31.3 ng) and 2 μg/ml poly(Lys)$_{87K}$. At the indicated times, two 4 μl aliquots were withdrawn. One aliquot was spotted onto Whatmann 3MM paper and the incorporation of [^{32}P]phosphate into protein was determined according to the method of Corbin and Reimann [17] and the other aliquot was subjected to analysis by SDS-PAGE followed by autoradiography (inset). The stoichiometry of phosphorylation was calculated using a molecular weight of 54 000 for CaMKPase.

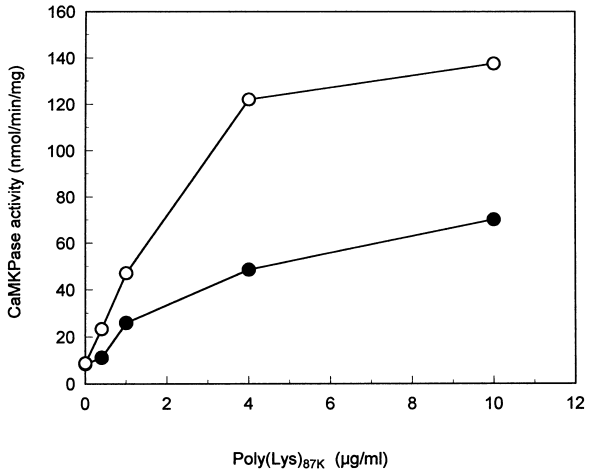

Fig. 4. Effect of phosphorylation of CaMKPase on its activity. Purified CaMKPase (80 ng) was incubated with (○) or without (●) 30 kDa CaMKII (16.7 ng) in the standard phosphorylation mixture (10 μl) containing 2 μg/ml poly(Lys)$_{87K}$. After incubation at 30°C for 10 min, the reaction was terminated by the addition of 190 μl of 50 mM Tris-HCl (pH 7.4) containing 0.05% Tween 40 and 1 mM EDTA and the activity of CaMKPase (1.2 ng) was determined with [^{32}P]CaMKII as a substrate in the presence of the indicated concentrations of poly(Lys)$_{87K}$, as described in Section 2.

4. Discussion

In the present paper, we demonstrate that CaMKPase was phosphorylated and activated by CaMKII only in the presence of a polycation such as poly(Lys). Since a polycation is not required for the phosphorylation of protein substrates other than CaMKPase by CaMKII and it is required for the activity of CaMKPase [11], a conformational change in CaMKPase induced by the binding of a polycation may be required for not only its enzyme activity but also its phosphorylation by CaMKII. The preliminary finding that CaMKII and CaMKPase co-precipitated with poly(Lys)$_{87K}$ (data not shown) may indicate that the binding of a polycation helps access of CaMKII to the phosphorylation site of CaMKPase.

The phosphorylation of CaMKPase by CaMKII resulted in an incorporation of approximately 1 mol of phosphate into 1 mol of the enzyme (Fig. 3) and a more than 2-fold increase in the activity of CaMKPase (Fig. 4). CaMKPase specifically dephosphorylates the phosphorylated threonine residues of multifunctional CaMKs involved in the marked activation of the enzymes (Thr^{286}, Thr^{196} and Thr^{177} of CaMKII, CaMKIV and CaMKI, respectively) [11,21]. Among the CaMKs, CaMKIV and CaMKI are phosphorylated by upstream protein kinases [22,23] but CaMKII is phosphorylated by itself.

Thus, CaMKII autophosphorylates the Thr^{286}, resulting in the marked activation of itself on the one hand, and phosphorylates CaMKPase, which dephosphorylates the autophosphorylated Thr^{286} of CaMKII and thereby deactivates the enzyme, possibly resulting in an efficient deactivation of itself on the other hand. The phosphorylation and concomitant activation of CaMKPase by CaMKII reported in the present paper may be physiologically important for a rapid and efficient deactivation of CaMKII, activated in response to an increase in intracellular Ca^{2+}.

Acknowledgements: This work was supported by Grants-in-Aid number 10102002 for Specially Promoted Research, the Ministry of Education, Science, Sports and Culture of Japan and by grants from the Byotai Taisha Research Foundation and the Akiyama Foundation.

References

[1] Yamauchi, T. and Fujisawa, H. (1980) FEBS Lett. 116, 141–144.
[2] Fujisawa, H. (1990) BioEssays 12, 27–29.
[3] Soderling, T.R. (1996) Biochim. Biophys. Acta 1297, 131–138.
[4] Schworer, C.M., Colbran, R.J. and Soderling, T.R. (1986) J. Biol. Chem. 261, 8581–8584.
[5] Lai, Y., Nairn, A.C. and Greengard, P. (1986) Proc. Natl. Acid. Sci. USA 83, 4253–4257.
[6] Saitoh, Y., Yamamoto, H., Fukunaga, K., Matsukado, Y. and Miyamoto, E. (1987) J. Neurochem. 49, 1286–1292.
[7] Miller, S.G., Patton, B.L. and Kennedy, M.B. (1988) Neuron 1, 593–604.
[8] Lou, L.L. and Schulman, H. (1989) J. Neurosci 9, 2020–2032.
[9] Fukunaga, K., Kobayashi, T., Tamura, S. and Miyamoto, E. (1993) J. Biol. Chem. 268, 133–137.
[10] Kameshita, I., Ishida, A., Okuno, S. and Fujisawa, H. (1997) Anal. Biochem. 245, 149–153.
[11] Ishida, A., Kameshita, I. and Fujisawa, H. (1998) J. Biol. Chem. 273, 1904–1910.
[12] Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D. and Means, A.R. (1977) J. Biol. Chem. 252, 8415–8422.
[13] Ishida, A., Kitani, T., Okuno, S. and Fujisawa, H. (1994) J. Biochem. 115, 1075–1082.
[14] Ishida, A. and Fujisawa, H. (1995) J. Biol. Chem. 270, 2163–2170.
[15] Perrie, W.T. and Perry, S.V. (1970) Biochem. J. 119, 31–38.
[16] Ebashi, S. (1976) J. Biochem. 79, 229–231.
[17] Corbin, J.D. and Reimann, E.M. (1974) Methods Enzymol. 38, 287–290.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[20] Yamauchi, T. and Fujisawa, H. (1983) Eur. J. Biochem. 132, 15–21.
[21] Ishida, A., Okuno, S., Kitani, T., Kameshita, I. and Fujisawa, H. (1998) Biochem. Biophys. Res. Commun. 253, 159–163.
[22] Okuno, S. and Fujisawa, H. (1993) J. Biochem. 114, 167–170.
[23] Okuno, S., Kitani, T. and Fujisawa, H. (1997) J. Biochem. 121, 155–160.