Vol. 180, No. 1, 1991
October 15, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 191-196

# PHOSPHORYLATION AND FUNCTIONAL MODIFICATION OF CALMODULIN-DEPENDENT PROTEIN KINASE IV BY cAMP-DEPENDENT PROTEIN KINASE

Isamu Kameshita and Hitoshi Fujisawa

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

Received August 24, 1991

**SUMMARY:** Calmodulin-dependent protein kinase IV (CaM-kinase IV), a neuronal calmodulin-dependent multifunctional protein kinase, undergoes autophosphorylation in response to $Ca^{2+}$ and calmodulin, resulting in activation of the enzyme (Frangakis et al. (1991) J. Biol. Chem. 266, 11309-11316). In contrast, the enzyme was phosphorylated by cAMP-dependent protein kinase, leading to a decrease in the enzyme activity. Thus, the results suggest differential regulation of CaM-kinase IV by two representative second messengers, $Ca^{2+}$ and cAMP. 

cAMP, diacylglycerol, and $Ca^{2+}$ are known to play important roles as representative second messengers in controlling diverse cellular functions by activating multifunctional protein kinases, cAMP-dependent protein kinase, protein kinase C, and CaM-kinase II, respectively (1,2). Among several protein kinases responsible for $Ca^{2+}$/calmodulin, only CaM-kinase II was thought to be a multifunctional enzyme (3,4) until CaM-kinase IV (5,6), also called calmodulin-dependent protein kinase Gr (7), was recognized as a multifunctional enzyme capable of phosphorylating a number of substrates, including myosin light chain, microtubule-associated protein 2, tau protein, myelin basic protein, synapsin I, histone H1, and tyrosine hydroxylase (8).

The abbreviations used are: CaM-kinase II, calmodulin-dependent protein kinase II; CaM-kinase IV, calmodulin-dependent protein kinase IV; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate.

0006-291X/91 $1.50

Frangakis et al. (9) recently reported that autophosphorylation of CaM-kinase IV leads to the formation of a $Ca^{2+}$-independent activity state, as well as potentiation of the $Ca^{2+}$/calmodulin-dependent activity. The present study reports the phosphorylation of CaM-kinase IV by cAMP-dependent protein kinase, with a concomitant decrease in the enzyme activity. Thus, CaM-kinase IV, a calmodulin-dependent multifunctional protein kinase presumably participating in the regulation of diverse neuronal functions, appears to be under the control of two important intracellular signaling systems, the cAMP system and the $Ca^{2+}$ system.

## MATERIALS AND METHODS

Materials: [$\gamma$-$^{32}$P]ATP (5,000 Ci/mmol) was purchased from the Radiochemical Center, Amersham. Syntide-2 (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys) (10), a synthetic peptide substrate of CaM-kinases II and IV, was synthesized by American Peptide Company. Protein kinase inhibitor (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp) (11), a peptide inhibitor of cAMP-dependent protein kinase, was obtained from Sigma Chemical Co. Calmodulin was prepared from rat testis by the method of Dedman et al. (12). Catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as desribed previously (13). CaM-kinase IV was purified from rat cerebral cortex essentially according to the method used for purification of cerebellar CaM-kinase IV (8).

Enzyme Assay: The activity of CaM-kinase IV was determined from the slope of the linear portion of the time course of the phosphate incorporated into syntide-2. The reaction was carried out at 30°C in an assay mixture, in a final volume of 50 µl, containing 40 mM Hepes-NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 50 µM [$\gamma$-$^{32}$P]ATP (200-1,000 cpm/pmol), 0.1 mM EGTA, 2 mM dithiothreitol, 20 µM syntide-2 and a suitable amount of CaM-kinase IV, in the presence and absence of 0.15 mM $CaCl_2$ and 0.84 µM calmodulin. An aliquot was withdrawn at an appropriate interval and the incorporation of [$^{32}$P]phosphate into syntide-2 was determined by the phosphocellulose paper method of Roskoski (14). CaM-kinase IV activity in a mixture containing both CaM-kinase IV and cAMP-dependent protein kinase was measured in the assay mixture containing 1.4 µM protein kinase inhibitor which selectively inhibited cAMP-dependent protein kinase but did not affect the activity of CaM-kinase IV at this concentration.

Phosphorylation of CaM-kinase IV was measured in a reaction mixture, in a final volume of 50 µl or 100 µl, containing 40 mM Hepes-NaOH (pH 7.0), 5 mM $Mg(CH_3COO)_2$, 50 µM [$\gamma$-$^{32}$P]ATP (4,000-20,000 cpm/pmol), 2 mM dithiothreitol, 0.1 mM EGTA and suitable amounts of CaM-kinase IV and the catalytic subunit of cAMP-

dependent protein kinase. Incorporation of [$^{32}$P]phosphate to CaM-kinase IV was determined by whatman 3MM chromatography paper method of Corbin and Reimann (15).

Other Procedures: Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmli (16) with slab gels (7.8 x 8.4 x 0.1 cm) consisting of a 10% acrylamide separation gel and a 3% stacking gel.

Protein was determined by the method of Lowry et al. (17) using bovine serum albumin as a standard after samples were made free of interfering substances (18).

## RESULTS AND DISCUSSION

When CaM-kinase IV was incubated with [$\gamma$-$^{32}$P]ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase under phosphorylating conditions and analyzed by SDS-polyacrylamide gel electrophoresis, a major band of $^{32}$P radioactivity was observed at the position corresponding to that of CaM-kinase IV with a molecular weight of 63,000 and a faint band was at the position corresponding to that of the catalytic subunit of cAMP-dependent protein kinase with a molecular weight of 41,000 (lane 3 in Fig. 1B). The phosphorylation of CaM-kinase IV was completely blocked by the omission of the catalytic subunit of cAMP-dependent protein kinase from the reaction mixture (lane 2 in Fig. 1B), indicating that CaM-kinase IV was phosphorylated by cAMP-dependent portein kinase. Phosphorylation of CaM-kinase IV was observed with $Ca^{2+}$/calmodulin added even in the absence of cAMP-dependent protein kinase (lane 1 in Fig. 1B), in agreement with the recent results of Frangakis et al. (9) that CaM-kinase IV undergoes autophosphorylation in response to $Ca^{2+}$/calmodulin. Incubation of CaM-kinase IV with protein kinase C under phosphorylating conditions caused no significant phosphorylation of CaM-kinase IV (data not shown).

In order to know whether phosphorylation of CaM-kinase IV by cAMP-dependent protein kinase was involved in the regulation of CaM-kinase IV activity, time course of phosphorylation of the

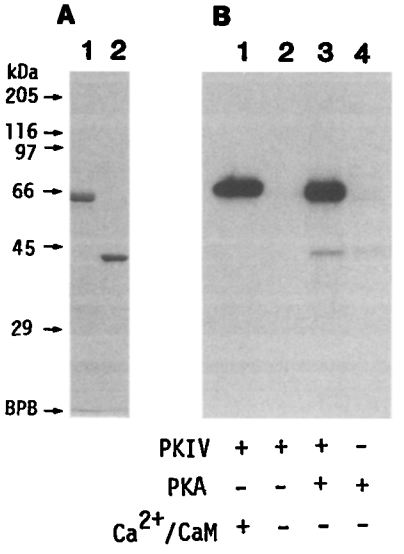


Figure 1. Phosphorylation of CaM-kinase IV by cAMP-dependent protein kinase. A, Approximately 1 µg of purified CaM-kinase IV (lane 1) and cAMP-dependent protein kinase (lane 2) were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue R-250. B, 1.4 µg of purified CaM-kinase IV was incubated with 50 µM [$\gamma$-$^{32}$P]ATP in the reaction mixture (final volume of 50 µl) containing 2.8 µg of the catalytic subunit of cAMP-dependent protein kinase as described under "Materials and Methods," with the indicated omissions. After incubation for 30 min at 30°C, a 10-µl aliquot was subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The migration positions of molecular weight markers are indicated. The abbreviations used are: PKIV, CaM-kinase IV; PKA, cAMP-dependent protein kinase; and CaM, calmodulin.

enzyme and alterations in the enzyme activity was examined as shown in Fig. 2. Incubation of CaM-kinase IV with cAMP-dependent portein kinase under phosphorylating conditions resulted in a progressive increase of the phosphorylation of the enzyme and a progressive decrease in the enzyme activity during the reaction for 60 min under the experimental conditions, and both the phosphorylation and deactivation of the enzyme were almost completely blocked by the addition of protein kinase inhibitor, indicating that phosphorylation of CaM-kinase IV by cAMP-dependent protein kinase caused deactivation of CaM-kinase IV. The amount of phosphate incorporated into CaM-kinase IV after incubation for 60 min was 1.17 nmol of phosphate/100 µg of the

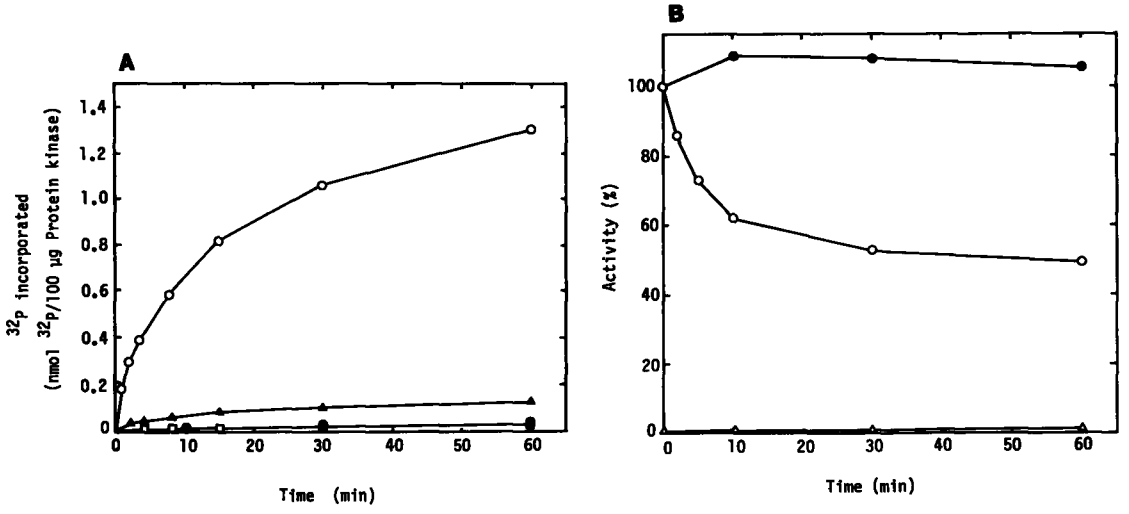


Figure 2. Time course of phosphorylation (A) and deactivation (B) of CaM-kinase IV by cAMP-dependent protein kinase. A, 2.1 µg of CaM-kinase IV was incubated with 50 µM [$\gamma$-$^{32}$P]ATP in the reaction mixture (final volume of 100 µl) containing 2.1 µg of the catalytic subunit of cAMP-dependent protein kinase as described under "Materials and Methods" (○), without either CaM-kinase IV (▲) or cAMP-dependent protein kinase (□), or with 7 µM protein kinase inhibitor (●). After incubation for the indicated periods at 30°C, 10-µl aliquots were withdrawn and the incorporation of [$^{32}$P]phosphate into acid-insoluble materials was determined. B, 2.1 µg of CaM-kinase IV was incubated with 50 µM ATP in the reaction mixture (final volume of 100 µl) containing 2.1 µg of the catalytic subunit of cAMP-dependent protein kinase, as described under "Materials and Methods" (○,△), with 7 µM protein kinase inhibitor (●). After incubation for the indicated periods at 30°C, 10-µl aliquots were withdrawn and the kinase activity toward syntide-2 in the presence (○,●) or absence (△) of $Ca^{2+}$/calmodulin was measured.

enzyme, which represents 0.74 mol of phosphate/mol of the enzyme on the basis of the assumption that the molecular weight of the enzyme is 63,000 and that Lowry's method of protein determination (17) is adequately valid for the determination of CaM-kinase IV. Thus, the activity of CaM-kinase IV appears to be inversely regulated by autophosphorylation in response to $Ca^{2+}$ and phosphorylation by cAMP-dependent protein kinase in response to cAMP. Studies on the phosphorylation sites of CaM-kinase IV for autophosphorylation and cAMP-dependent protein kinase and the functional relationship between the phosphorylations are now in progress.

**ACKNOWLEDGMENTS:** This work was supported, in part, by grants-in-aid for Scientific Research from Ministry of Education, Science and Culture of Japan, grants from the Ministry of Health and Welfare of Japan, and the Akiyama Research Foundation.

## REFERENCES

1. Nestler, E. J. and Greengard, P. (1983) Nature 305, 583-588
2. Schulman H. and Lou, L. L. (1989) Trends Biochem. Sci. 14, 62-66
3. Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y., Rich D. P., Smith, M. K., and Soderling, T. R. (1989) Biochem. J. 258, 313-325
4. Fujisawa, H. (1990) BioEssays 12, 27-29
5. Sikela, J. M. and Hahn, W. E. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 3038-3042
6. Sikela, J. M., Law, M. L., Kao, F., Hartz, J. A., Wei, Q., and Hahn, W. E. (1989) Genomics 4, 21-27
7. Ohmstede, C-A., Jensen, K. F., and Sahyoun, N. (1989) J. Biol. Chem. 264, 5844-5875
8. Miyano, O., Kameshita, I., and Fujisawa, H. (1991) submitted for publication
9. Frangakis, M. V., Ohmstede, C-A., and Sahyoun, N. (1991) J. Biol. Chem. 266, 11309-11316
10. Hashimoto. Y., and Soderling, T. R. (1987) Arch. Biochem. Biophys. 252, 418-425
11. Cheng, H-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M., and Walsh, D. A. (1986) J. Biol. Chem. 261, 989-992
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. Okuno, S., and Fujisawa, H. (1990) Biochim. Biophys. Acta 1038, 204-208
14. Roskoski, R. Jr. (1983) Methods Enzymol. 99, 3-6
15. Corbin, J. D., and Reimann, E. M. (1974) Methods Enzymol. 38, 287-290
16. Laemmli, U. K. (1970) Nature 227, 680-685
17. Lowry, O.H., Rosebrough, N-J., Farr, A.L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
18. Bensadoun, A., and Weinstein, D. (1976) Anal. Biochem. 70, 241-250