Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 213-219

SELF-REGULATION OF CALMODULIN-DEPENDENT PROTEIN KINASE II AND GLYCOGEN SYNTHASE KINASE BY AUTOPHOSPHORYLATION

Takashi Yamauchi and Hitoshi Fujisawa

Department of Biochemistry, Asahikawa Medical College, Asahikawa 078-11, Japan

Received April 18, 1985

SUMMARY: Calmodulin-dependent protein kinase II from rat brain underwent autophosphorylation and the autophosphorylation caused a marked decrease in the enzyme activity. Calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle was also inactivated by incubation under autophosphorylating conditions. The inactivation of the protein kinases by the autophosphorylation may be an important self-regulatory mechanism in controlling the enzyme activities.

In 1980, we demonstrated that three distinct calmodulin-dependent protein kinases, designated calmodulin-dependent protein kinase I, II, and III in the order of decreasing molecular weight, were present in rat brain (1) and that calmodulin-dependent protein kinase II (CaM kinase II) showed a broad substrate specificity, suggesting that it might play a number of roles in the functioning of the nervous system (1). Recently, we, in collaboration with Woodgett and Cohen, demonstrated that calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle showed the same substrate specificity and phosphorylation site specificity as CaM kinase II (2).

CaM kinase II was reported to be autophosphorylated (3) and the autophosphorylation was thereafter demonstrated to lead to the activation of this kinase (4). On the other hand, autophosphor-

Abbreviations: CaM kinase II, calmodulin-dependent protein kinase II; MAP 2, microtubule-associated protein 2; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.

0006-291X/85 $1.50

ylation of calmodulin-dependent glycogen synthase kinase was also reported but in this case the autophosphorylation did not alter the activity of the kinase (5).

Contrary to the previous findings, the present study reports that the autophosphorylation of CaM kinase II and calmodulin-dependent glycogen synthase kinase led to a large decrease in their enzyme activities, suggesting that the activities of the two kinases may be controlled by the mechanism of self-regulation.

EXPERIMENTAL PROCEDURES

CaM kinase II was purified from rat cerebral cortex as described previously (6). Calmodulin-dependent glycogen synthase kinase was purified from rabbit skeletal muscle by the method of Woodgett et al. (5). Calmodulin was purified from rat testis by the method of Dedman et al. (7). MAP 2 was purified from heat stable microtubule-associated proteins from rat brain by chromatography on Bio-Gel A-1.5m (8).

CaM kinase II was assayed as described previously (6). The standard incubation mixture contained 50 mM Hepes buffer, pH 6.5, 0.05 mM [γ-^{32}P]ATP (5-10 X 10^4 cpm/nmol), 5 mM $Mg(CH_3COO)_2$, 0.12 mM $CaCl_2$, 0.1 mM EGTA, 1000 nM calmodulin, 1 mg/ml of α-casein and a suitable amount of the enzyme in a total volume of 0.07 ml. When MAP 2 was used as the substrate of CaM kinase II, 50 mM Hepes buffer, pH 6.5, and 1 mg/ml of α-casein were replaced by 50 mM Hepes buffer, pH 8.0, and 0.3 mg/ml of MAP 2. Reaction was carried out for 2 min at 30 °C. Incorporation of [^{32}P]-phosphate into protein was estimated by the method of Corbin and Reimann (9).

Autophosphorylation of CaM kinase II or glycogen synthase kinase was carried out in the mixture contained 50 mM Hepes buffer, pH 8.0, 0.05 mM ATP or [γ-^{32}P]ATP, 5 mM $Mg(CH_3COO)_2$, 0.1 mM $CaCl_2$, 800 nM calmodulin, 1 mg/ml of α-casein and a suitable amount of the enzyme in a total volume of 0.05 ml. After incubation at 30 °C, an aliquot was withdrawn and the kinase activity was determined under the standard assay conditions described above. When incorporation of [^{32}P]phosphate into CaM kinase II was measured, the phosphorylation mixture was subjected to SDS disc gel electrophoresis, and the gels were sliced, and the radioactivity of the slice containing the protein band of CaM kinase II was measured by a liquid scintilation counter.

SDS polyacrylamide gel electrophoresis was carried out according to the electrophoretic system of Laemmli (10).

Phosphoamino acid analysis of CaM kinase II was carried out by cellulose thin layer electrophoresis as described previously (11). [^{32}P]Radioactivity was detected by autoradiography.

Protein was determined by the method of Lowry et al. (12).

RESULTS AND DISCUSSION

Upon SDS polyacrylamide gel electrophoresis in a system developed by Laemmli (10), CaM kinase II showed a major 50-kDa

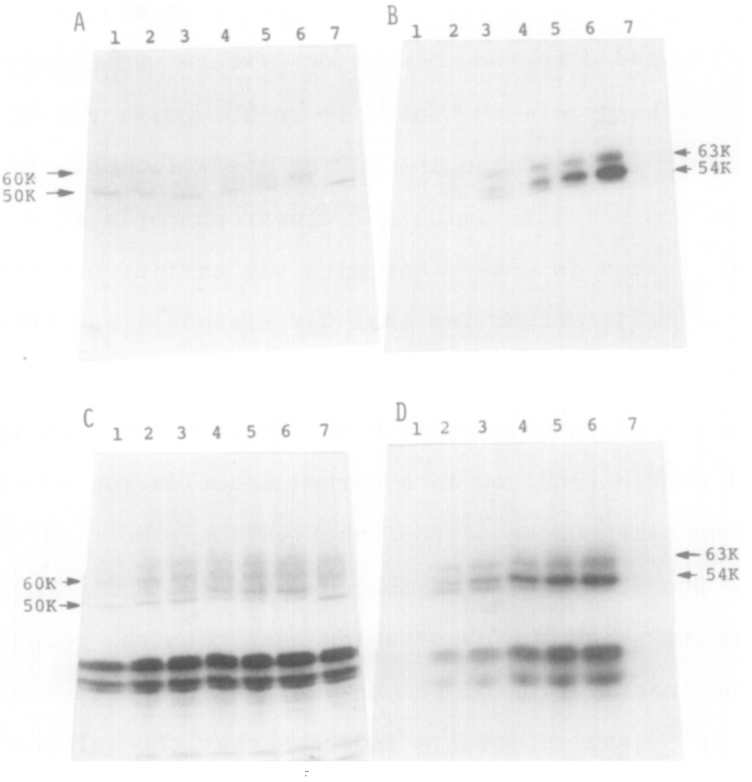


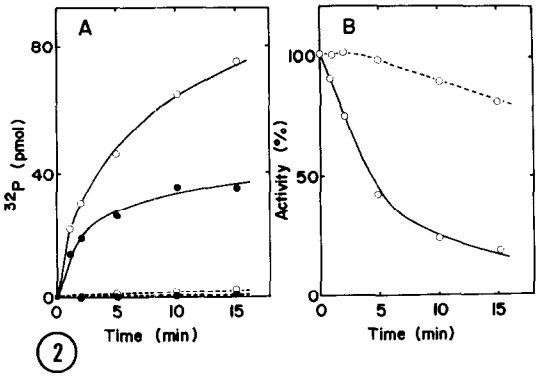
Fig. 1. Autophosphorylation of CaM kinase II analyzed by SDS polyacrylamide slab gel electrophoresis. CaM kinase II (2 μg) was incubated with [γ-^{32}P]ATP in the absence (A and B) and presence (C and D) of α-casein under the standard conditions. After the incubation at 30 °C, the reaction mixture was subjected to SDS polyacrylamide slab gel electrophoresis and autoradiography. A and C, protein stained by Coomassie brilliant blue; B and D, autoradiograph; lanes 1 to 6, incubated for 0, 1, 2, 5, 10 and 15 min, respectively; lane 7, incubated for 15 min in the absence of Ca^{2+}. 50 K and 60 K indicate the molecular weights of the major and minor bands of CaM kinase II, and 54 K and 63 K indicate the molecular weights corresponding to the mobilities of the major and minor bands of phosphorylated CaM kinase II, respectively.

and a minor 60-kDa component (Fig. 1A, lanes 1 and 2), in agreement with our earlier observation (2). When CaM kinase II was incubated with [γ-^{32}P]ATP in the presence of calmodulin and analyzed by SDS polyacrylamide gel electrophoresis, both protein staining (Fig. 1A) and autoradiography (Fig. 1B) revealed a major 54-kDa and a minor 63-kDa band. These results indicate that the autophosphorylation of CaM kinase II may affect its electrophoretic mobility, presumably converting the 60-kDa band to the 63-kDa band and the 50-kDa band to the 54-kDa band. The

presence of casein, which was very effective in stabilizing CaM kinase II, added to the incubation mixture was effective in sharpening the bands of CaM kinase II on SDS polyacrylamide gel electrophoresis (Fig. 1C and 1D). The shift in mobility of CaM kinase II on SDS polyacrylamide gel electrophoresis induced by autophosphorylation is consistent with the earlier observation by Bennett et al. (3), suggesting that CaM kinase II and their enzyme may be identical.

Incubation of CaM kinase II with ATP at 30 °C even in the presence of casein resulted in a marked inactivation of the enzyme. The time course of the inactivation almost paralleled that of the phosphorylation of the enzyme (Fig. 2). These results together with the observation that both the phosphorylation and the inactivation of the enzyme required the presence of Ca^{2+}, ATP, Mg^{2+}, and calmodulin indicate that the autophosphorylation of CaM kinase II may cause a decrease in the enzyme activity. About 80 pmol and 40 pmol of [^{32}P]phosphate were incorporated into 50-kDa and 60-kDa components from 2 μg of CaM kinase II, respectively, indicating that about 3.4 mol of phosphate was incorporated per mol of subunit of the enzyme, taking the average molecular mass of subunit to be 55 kDa. When the [^{32}P]labeled CaM kinase II was hydrolyzed in 6 N HCl at 110 °C for 2.5 hours and subjected to cellulose thin layer electrophoresis, serine and threonine residues were identified as the phosphate acceptors. The ratio of the activities incorporated into serine and threonine was approximately 6:4.

Shields et al. reported that CaM kinase II was activated by autophosphorylation and the activation was more pronounced at low calmodulin concentrations (4). Fig. 3 shows the effect of varying the concentration of calmodulin on the activities of phosphorylated and unphosphorylated CaM kinase II. The rate of



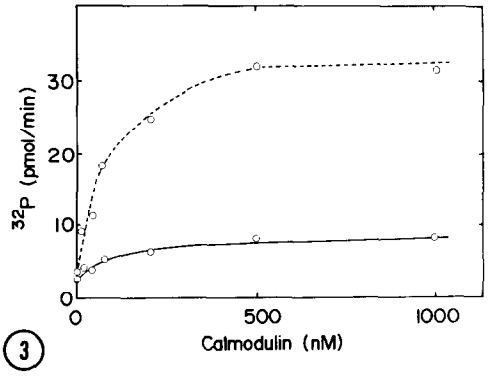


Fig. 2. Time course of autophosphorylation and inactivation of CaM kinase II. (A) CaM kinase II (2 μg) was incubated with [γ-^{32}P]ATP under the standard conditions (o——o, ●——●). Control experiments were carried out without Ca^{2+} (o---o, ●---●). After the incubation at 30 °C for the indicated times, the reaction mixture was subjected to SDS polyacrylamide disc gel electrophoresis. After electrophoresis, the major (o——o, o---o) and minor (●——●, ●---●) protein bands of CaM kinase II were located by staining with Coomassie brilliant blue and the gel slices containing the protein bands of CaM kinase II were placed into liquid scintilation vials and the radioactivities were determined. (B) CaM kinase II (1 μg) was preincubated with 0.05 mM ATP under the standard conditions (o——o). Control experiments were carried out without Ca^{2+} (o---o). After the incubation at 30 °C for the indicated times, an aliquot was withdrawn and the activity of CaM kinase II was determined by measuring the phosphorylation of α-casein.

Fig. 3. Effect of varying the concentration of calmodulin on the activity of phosphorylated CaM kinase II. CaM kinase II (0.3 μg) was incubated with 0.05 mM ATP under the standard conditions (o——o). Control experiments were carried out without Ca^{2+} (o---o). After incubation for 15 min, the reaction mixture was diluted to 5 volumes, and a 5-μl aliquot was withdrawn and CaM kinase II activity was determined by measureing the phosphorylation of MAP 2 at various concentrations of calmodulin.

the phosphorylation of MAP 2, a very good substrate for CaM kinase II (13), by phosphorylated CaM kinase II was much lower than that by unphosphorylated CaM kinase II over the range of calmodulin concentrations from 10 to 1000 nM and the activation of CaM kinase II by autophosphorylation was not observed at any concentration of calmodulin.

Calmodulin-dependent glycogen synthase kinase shares many catalytic properties with CaM kinase II (2,14) and it has been reported to undergo autophosphorylation without effect on its enzymatic activity (5). Fig. 4 shows the time course of the

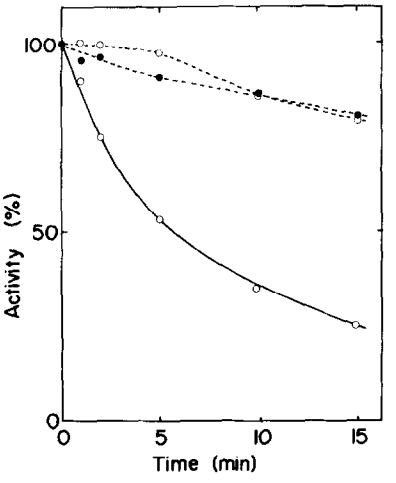


Fig. 4. Inactivation of glycogen synthase kinase by exposure to phosphorylating conditions. Calmodulin-dependent glycogen synthase kinase (1 μg) was incubated with 0.05 mM ATP under the standard conditions (o——o). Control experiments were carried out without Ca^{2+} (o----o) or ATP (●----●). After the incubation for the indicated times, an aliquot was withdrawn and the activity of glycogen synthase kinase was determined by measuring the phosphorylation of α-casein.

changes in the activity of glycogen synthase kinase incubated under phosphorylating conditions. The incubation elicited a marked decrease in the enzyme activity and the inactivation required the presence of Ca^{2+} and ATP, suggesting that this inactivation might occur as a result of the autophosphorylation.

The present study reports that CaM kinase II from rat brain and calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle underwent autophosphorylation and that the autophosphorylation of these enzymes led to the inactivation of the respective enzymes. Both protein kinases require Ca^{2+} and calmodulin for their catalytic action and therefore an influx of Ca^{2+} into the cytosol is thought to activate the kinases. The kinases would be maintained maximally active in the presence of Ca^{2+} without specific regulatory mechanisms. It is conceivable that the inactivation of the kinases by the autophosphorylation may play an important role in controlling their enzymatic activities in the presence of Ca^{2+}. Thus, in the presence of

Ca^{2+}, CaM kinase II and calmodulin-dependent glycogen synthase kinase may be regulated by this self-ragulatory mechanism.

ACKNOWLEDGEMENTS

This work has been supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, by Grant No.84-14105 from NCNMMD of the Ministry of Health and Welfare of Japan, and by a grant from the Naito Foundation.

REFERENCES

1. Yamauchi, T. and Fujisawa, H. (1980) FEBS Lett. 116, 141-144
2. Woodgett, J. R., Cohen, P., Yamauchi, T. and Fujisawa, H. (1984) FEBS Lett. 170, 49-54
3. Bennett, M. K., Erondu, N. E. and Kennedy, M. B. (1983) J. Biol. Chem. 258, 12735-12744
4. Shields, S. M., Vernon, P. J. and Kelly, P. T. (1984) J. Neurochem. 43, 1599-1609
5. Woodgett, J. R., Davison, M. T. and Cohen, P. (1983) Eur. J. Biochem. 136, 481-487
6. Yamauchi, T. and Fujisawa, H. (1983) Eur. J. Biochem. 132, 15-21
7. Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. and Means, A. R. (1977) J. Biol. Chem. 252, 8415-8422
8. Fellous, A., Francon, J., Lennon, A. -M. and Nunez, J. (1977) Eur. J. Biochem. 78, 167-174
9. Corbin, J. D. and Reimann, E. M. (1974) Methods Enzymol. 38, 287-290
10. Laemmli, U. K. (1970) Nature 227, 680-685
11. Yamauchi, T. and Fujisawa, H. (1984) Arch. Biochem. Biophys. 234, 89-96
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
13. Yamauchi, T. and Fujisawa, H. (1982) Biochem. Biophys. Res. Commun. 109, 975-981
14. McGuinness, T. L., Lai, Y., Greengard, P., Woodgett, J. R. and Cohen, P. (1983) FEBS Lett. 163, 329-334