Autophosphorylation of Ca^{2+} /Calmodulin-Dependent Protein Kinase II: Effects on Interaction between Enzyme and Substrate

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ABSTRACT—Characteristics of the autophosphorylation of Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) from the cytosol and in the postsynaptic densities (PSD) of rat brain were investigated. Several proteins were surveyed for their abilities to serve as a substrate for non-autophosphorylated and autophosphorylated CaM kinase IIs from the cytosol and PSD. The tested substrates were separated into two groups. Autophosphorylation of the kinase slightly decreased or did not change its activities towards substrates of the first group: myosin light chain of chicken gizzard, synapsin I, tau factor and microtubule-associated protein 2. In contrast, autophosphorylation of the enzyme increased its activities towards substrates of the second group: syntide-2, histone H1, calcineurin and myelin basic protein. The Ca^{2+} /calmodulin-independent kinase activity increased by autophosphorylation with any of substrates tested. Similar results were obtained with the cytosolic and PSD CaM kinase II. Tri-fluoperazine and mastoparan, calmodulin binding antagonists, inhibited the activity of the non-autophosphorylated CaM kinase II, but had no effect or only a slight inhibitory effect on the activity of the autophosphorylated CaM kinase II, indicating that the autophosphorylated kinase has no requirement for calmodulin for Ca^{2+} -dependent activity and/or a higher affinity for calmodulin. The results suggest that the autophosphorylation of CaM kinase II is a subtle mechanism for regulating the interaction between the enzyme and substrate.

CaM kinase II which belongs to a class of calmodulin-dependent protein kinases is highly concentrated in the brain as the soluble and particulate forms (1–3). The enzyme has a multiple substrate specificity and can be involved in many neuronal functions such as the

biosynthesis of neurotransmitters, the release of neurotransmitters and hormones, the assembly-disassembly of microtubules and the activation and inactivation of several enzymes (1–3). It was reported that CaM kinase II may be related to the generation of long-term

Abbreviations used: CaM kinase II, Ca^{2+} /calmodulin-dependent protein kinase II; PSD, postsynaptic densities; EGTA, [ethylenbis(oxyethylenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; MAP 2, microtubule-associated protein 2; BSA, bovine serum albumin; MLC, myosin light chain; MBP, myelin basic protein.

potentiation in CA1 cells of the hippocampus (4, 5).

CaM kinase II undergoes autophosphorylation in a Ca^{2+} /calmodulin-dependent manner (6). It was recently reported that autophosphorylation of CaM kinase II occurs in rat fibroblast 3Y1 cells (7) and cerebellar granule cells (8) in response to the addition of growth factors and KCl, respectively. Because the autophosphorylation reaction is reversed by protein phosphatases (9–15), it may be an important mechanism for the regulation of CaM kinase II.

The role of autophosphorylation in the living system is assumed to prolong the effects triggered by the transient increase in cytosolic Ca^{2+} concentration by the generation of Ca^{2+} /calmodulin-independent activity. We have previously reported that the autophosphorylation of CaM kinase II increased this enzyme's activities towards specified substrates (16). In the present communication, we describe a further study on the effect of autophosphorylation of the cytosolic and PSD CaM kinase II on its activities towards several substrates. The autophosphorylation of the enzyme brought about a change in the interaction between the enzyme and its substrates.

MATERIALS AND METHODS

Materials

[γ - ^{32}P]ATP was purchased from New England Nuclear; mastoparan, from Peptide Institute Inc.; trifluoperazine, from Yoshitomi Co.; EGTA, from Dojin Chemicals; histone H1, from Sigma; casein of milk, from Wako Co. Syntide-2 was a gift of Dr. T.R. Soderling (Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School). All other chemicals used were of analytical grade.

Preparation of proteins

Calmodulin was purified from bovine brain (17). CaM kinase II was purified to homogeneity from rat brain cytosol (6). The specific activity of the enzyme was 320–760 nmole of

Pi/mg/min with the chicken gizzard MLC as substrate, under standard conditions. Calcineurin was purified from rat brain (18). The specific activity of the purified calcineurin was 0.2 $\mu\text{mol/mg/min}$ using chicken gizzard MLC, phosphorylated by MLC kinase, as substrate. PSD were prepared from rat forebrain according to the method of Cohen et al. (19). The content of CaM kinase II in PSD proteins was about 20%, as determined by densitometric analysis and immunoblotting. PSD themselves were used as the source of CaM kinase II. Using published methods, we prepared calmodulin-deficient MLC from chicken gizzard (20), MBP from bovine brain (21), synapsin I from rat brain (22), and tau factor and MAP 2 from porcine brain (23).

Autophosphorylation of CaM kinase II

Cytosol CaM kinase II (3.0 μg) or PSD CaM kinase II (10 μg) was autophosphorylated at 0°C for 0–70 min in a final volume of 0.1 ml containing 25 mM Tris-HCl buffer (pH 7.5), 10 mM Mg^{2+} , 0.1 mM Ca^{2+} , 0.3 μM calmodulin, 1 mM DTT and 20 μM [γ - ^{32}P]ATP containing 10–80 $\times 10^5$ cpm. Incubation was terminated by the addition of 0.02 ml of the SDS stop solution (24). Aliquots of the samples were subjected to SDS-PAGE with 10% acrylamide. After staining and destaining, radiolabeled subunit bands were cut from the gel and the incorporated phosphate was directly quantified by liquid scintillation spectrometry.

Effects of autophosphorylation on kinase activity

CaM kinase II from the cytosol (1.0–1.5 μg) or that in PSD (5 μg) was autophosphorylated at 0°C for 10 min in a final volume of 0.05 ml containing 25 mM Tris-HCl buffer (pH 7.5), 10 mM Mg^{2+} , 0.1 mM Ca^{2+} , 0.3 μM calmodulin, 1 mM DTT and 20 μM nonradioactive ATP. The effects of the autophosphorylation on enzyme activity were examined as follows: Fifty microliters of each sample containing the autophosphorylated CaM kinase II were added to the incubation mixture for protein phosphorylation. The mixture contained

25 mM Tris-HCl buffer (pH 7.5), 10 mM Mg^{2+} , 1 mM DTT, 30 μM [γ - ^{32}P]ATP containing $10-80 \times 10^5$ cpm and the indicated amount of substrate with either 0.1 mM Ca^{2+} plus 0.3 μM calmodulin (Ca^{2+}/CaM) or 2 mM EGTA, 0.05 mM Ca^{2+} plus 0.15 μM calmodulin (EGTA) in a final volume of 0.1 ml. Incubation was performed at 30°C for 1 min. The phosphorylation reaction was linear for at least the first 4 min, under the standard conditions. Incubation was terminated by the addition of 0.02 ml of the SDS-stop solution. Aliquots of the samples were subjected to SDS-PAGE with 10 or 13.5% acrylamide. After staining and destaining, radiolabeled protein bands were cut from the gel, and the radioactivities of the proteins were directly quantified by liquid scintillation spectrometry. The assay for the phosphorylation of syntide-2 was carried out using Whatman P81 ion exchange paper, according to the method of Roskoski (25). The papers which adsorbed the radiolabeled syntide-2 were directly counted by liquid scintillation spectrometry.

Assay for phosphorylated calcineurin

Cytosol CaM kinase II (4.5 μg) which was previously autophosphorylated at 0°C for 10 min under the standard conditions was added to the incubation mixture for calcineurin phosphorylation. The mixture contained 25 mM Tris-HCl buffer (pH 7.5), 10 mM Mg^{2+} , 1 mM DTT, 30 μM nonradioactive ATP, 0.1 mM Ca^{2+} , 0.3 μM calmodulin and 60 μg of calcineurin in a final volume of 0.3 ml, and incubation was performed at 30°C for 5 min. An aliquot containing 10 μg of calcineurin was used for the assay of phosphatase activity. Calcineurin was assayed at 30°C for 20 min with *p*-nitrophenylphosphate as substrate, according to the method of Pallen and Wang (26).

Other methods

SDS-PAGE was performed by the method of Laemmli (24). Protein was determined by the method of Bradford (27) with BSA as the standard. The phosphoamino acid composition

of the excised protein bands from the SDS-PAGE gel was analyzed essentially as described (28) with slight modifications (7). Other procedures used in the present study were the same as described previously (14, 16).

RESULTS

Autophosphorylation of CaM kinase II

The autophosphorylation of CaM kinase II from either the cytosol or the PSD was observed in a Ca^{2+} /calmodulin-dependent manner. Figure 1 shows the time course of autophosphorylation of cytosolic CaM kinase II. The reaction of the incorporation of phosphate into the α and β subunits of the enzyme reached plateaus in about 30 min, at which 0.3 and 1.8 mol of phosphate per mol of the α and β subunits were incorporated, respectively. Assuming that the ratio of the α subunit to the β subunit is 4 and that the molecular weight of the holoenzyme is 550 K (1, 12), the amount of phosphate incorporated into the holoenzyme was calculated to be about 6.7 mol/mol.

Phosphoamino acid analysis revealed that no tyrosine residue was phosphorylated; and at 1-min incubation time, the ratio of phos-

Fig. 1. Time course of autophosphorylation of cytosolic CaM kinase II. The amount of phosphate incorporated into the α subunit (●) and the β subunit (○) of cytosol CaM kinase II was determined during the time course of autophosphorylation. The experiments were carried out twice, and data were comparable to each other. One of the results is shown.

Fig. 2. Phosphoamino acid analysis of the autophosphorylated cytosol CaM kinase II. Autophosphorylation of cytosol CaM kinase II ($3 \mu\text{g}$) was performed at 0°C for 1–30 min with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, under standard conditions. Samples were subjected to SDS-PAGE. After staining and destaining, the bands of the α and β subunits were cut out and treated by the procedures described in Materials and Methods, using trypsin ($100 \mu\text{g}/\text{ml}$) and chymotrypsin ($50 \mu\text{g}/\text{ml}$) at 30°C for 24 hr. Phosphoamino acids were cut out from the chromatogram after high voltage paper electrophoresis, and the amount of phosphate incorporated into each phosphoamino acid was quantified by liquid scintillation spectrometry. Phosphoserine ($\blacksquare$) and phosphothreonine ($\blacktriangle$) in the α subunit and phosphoserine ($\square$) and phosphothreonine ($\triangle$) in the β subunit are shown. The experiments were carried out four times, and data were comparable to each other. One of the results is shown.

phosphoserine to phosphothreonine was about 1 in both the α and β subunits of the enzyme and thereafter increased to greater than 2 (Fig. 2).

Figure 3 shows the time course of autophosphorylation of PSD CaM kinase II. The incorporation of phosphate into the α and β subunits of PSD CaM kinase II reached the maximum at a 30-min incubation. Because it was assumed that the content of CaM kinase II in PSD proteins is about 20%, as described above, the amounts of phosphate incorporated into the α and β subunits were 0.2 and 1.4 mol/mol, respectively. If the composition and the ratio of the α and β subunits in the holoenzyme is the same as those of the cytosolic enzyme, the amount of phosphate incorporated into the holoenzyme in PSD was calculated to be 5.0 mol/mol. As described for the cytosolic

Fig. 3. Time course of autophosphorylation of PSD CaM kinase II. The amount of phosphate incorporated into the α subunit ($\bullet$) and the β subunit ($\circ$) of PSD CaM kinase II was determined during the time course of autophosphorylation. The experiments were carried out 5 times, and data were comparable to each other. One of the results is shown.

CaM kinase II, the ratio of phosphoserine to phosphothreonine was about 1 at 1-min incubation, and thereafter, serine residue was more phosphorylated.

Effects of autophosphorylation on activity of CaM kinase II

We previously showed that the autophosphorylation of CaM kinase II resulted in no change or an increase in activity with specified substrates (16). Figure 4 shows the changes in activity of cytosolic CaM kinase II with calcineurin or histone H1 as a substrate during the time course of autophosphorylation. With both substrates, the autophosphorylation of cytosol CaM kinase II had stimulatory effects on the enzyme's activity. The kinase activity attained a plateau with either substrate by a 10-min autophosphorylation. The time course of the increase in activity by autophosphorylation differed from that of the autophosphorylation of CaM kinase II (compare Figs. 1 and 2 to Fig. 4), indicating that the site related to the increase in kinase activity by autophosphorylation is phosphorylated at the earlier stage of autophosphorylation. The Ca^{2+} /calmodulin-independent activity also increased by autophosphorylation, although the ratio of

Fig. 4. Time course of effects of autophosphorylation on cytosolic CaM kinase II activity. After autophosphorylation of cytosolic CaM kinase II (1.5 μg) for the indicated times, the enzyme activity was assayed with 20 μg of calcineurin (A) or 20 μg of histone H1 (B) as substrate in the presence of EGTA (○) or $\text{Ca}^{2+}/\text{calmodulin}$ ($\text{Ca}^{2+}/\text{CaM}$) (●), under standard conditions. The experiments were carried out twice, and data were comparable to each other. One of the results is shown.

the $\text{Ca}^{2+}/\text{calmodulin}$ -dependent activity to the $\text{Ca}^{2+}/\text{calmodulin}$ -independent activity differed between the substrates used.

We then examined which proteins can serve as the substrates for autophosphorylated CaM kinase II. As shown in Table 1, substrates for CaM kinase II can be separated into two groups. Towards the substrates of group-1: casein, MLC, synapsin I, tau factor and MAP2, the total activity of the autophosphorylated CaM kinase II determined with $\text{Ca}^{2+}/\text{calmodulin}$ was similar to that of the unphosphorylated enzyme, and only the $\text{Ca}^{2+}/\text{calmodulin}$ -independent activity increased with any substrate. In contrast, towards the sub-

strates of group-2, which contained histone H1, calcineurin and MBP, the total activity determined with $\text{Ca}^{2+}/\text{calmodulin}$ increased with an increase in the time of autophosphorylation. The $\text{Ca}^{2+}/\text{calmodulin}$ -independent activity determined with EGTA also increased by autophosphorylation of the enzyme.

We next examined the effects of autophosphorylation on the activity of PSD CaM kinase II with several substrates. Figure 5 shows the time course of the kinase activity affected by autophosphorylation. When synapsin I was used as the substrate, the total activity determined with $\text{Ca}^{2+}/\text{calmodulin}$ did not change or slightly decreased during the course of autophosphorylation (Fig. 5A). In contrast, when calcineurin (Fig. 5B) or MBP (Fig. 5C) was used as the substrate, the total activity increased with an increase in the time of autophosphorylation and reached the maximum at a 10-min autophosphorylation. The $\text{Ca}^{2+}/\text{calmodulin}$ -independent activity of the kinase determined with EGTA increased with any of substrates used during the time course of autophosphorylation. On the basis of these results, several proteins were surveyed for their effectiveness as a substrate for PSD CaM kinase II (Table 2). Similar results to those for cytosolic CaM kinase II were obtained. Substrates examined were separated into two groups. Under the conditions of the substrates tested, the generation of the total activity (with $\text{Ca}^{2+}/\text{calmodulin}$) and the $\text{Ca}^{2+}/\text{calmodulin}$ -independent activity (with EGTA) of the PSD enzyme showed the same tendency as the cytosol enzyme. There was qualitatively no prominent difference between the cytosolic and PSD CaM kinase II (Tables 1 and 2).

Effects of mastoparan and trifluoperazine on kinase activity

Trifluoperazine (29) and mastoparan (30, 31) are known to bind calmodulin and thereby inhibit the activity of calmodulin-activatable enzymes. We examined whether or not the autophosphorylated CaM kinase II requires $\text{Ca}^{2+}/\text{calmodulin}$ for activity.

When CaM kinase II is not autophosphoryl-

Table 1. Effects of autophosphorylation on activity of cytosolic CaM kinase II

Substrate	Autophosphorylation (min)			
	0		10	
	EGTA	Ca ²⁺ /CaM	EGTA	Ca ²⁺ /CaM
	pmole			
Group-1				
Casein	0.2	4.5	0.8	4.2
MLC	2.4	72.4	19.7	70.2
Synapsin I	1.2	108.0	56.2	118.2
Tau factor	1.3	111.2	83.8	118.5
MAP 2	0.1	141.6	38.0	148.7
Group-2				
Histone H1	1.0	1.5	6.0	6.0
Calcineurin	0.6	2.6	3.4	40.3
MBP	3.9	16.4	47.4	74.4

Cytosolic CaM kinase II was autophosphorylated at 0°C for 0 or 10 min. Then 50-μl of aliquots containing 1.5 μg of CaM kinase II were assayed for the kinase activity with each substrate indicated. The amounts of substrates used were 20 μg of casein, 20 μg of myosin light chain of chicken gizzard (MLC), 5 μg of synapsin I, 20 μg of tau factor, 5 μg of microtubule-associated protein 2 (MAP 2), 20 μg of Histone H1, 20 μg of calcineurin and 20 μg of myelin basic protein (MBP). The experiments were carried out 3 times, and data were comparable to each other. One of the results is shown.

Table 2. Effects of autophosphorylation on activity of PSD CaM kinase II

Substrate	Autophosphorylation (min)			
	0		10	
	EGTA	Ca ²⁺ /CaM	EGTA	Ca ²⁺ /CaM
	pmole			
Group-1				
Casein	1.0	3.1	2.7	3.4
Tau factor	0.0	11.7	1.8	9.8
MLC	0.9	8.8	6.4	10.6
MAP 2	0.4	17.1	1.0	14.4
Synapsin I	0.1	23.6	6.7	17.1
Group-2				
Histone H1	0.1	0.1	0.4	0.6
Calcineurin	0.6	2.4	1.2	14.7
MBP	1.9	4.1	10.3	16.0

Postsynaptic densities (PSD) CaM kinase II was autophosphorylated at 0°C for 0 or 10 min. Then 50-μl of aliquots containing 5 μg of PSD CaM kinase II were assayed for the kinase activity with each substrate indicated. The amounts of substrates used were as described in Table 1. The experiments were carried out twice, and data were comparable to each other. One of the results is shown.

Fig. 5. Time course of effects of autophosphorylation on PSD CaM kinase II activity. After autophosphorylation of PSD CaM kinase II ($5 \mu\text{g}$) for the indicated times, the enzyme activity was assayed with $5 \mu\text{g}$ of synapsin I (A), $20 \mu\text{g}$ of calcineurin (B) or $20 \mu\text{g}$ of myelin basic protein (MBP) (C) as substrate in the presence of EGTA ($\circ$) or Ca^{2+} /calmodulin ($\bullet$), under standard conditions. The experiments were carried out 5 times, and data were comparable to each other. One of the results is shown.

ated, trifluoperazine or mastoparan inhibited the Ca^{2+} /calmodulin-dependent activity with MLC, MBP or syntide-2 as substrate (Table 3). As the activity of the non-autophosphorylated kinase was undetectable with calcineurin as substrate even in the presence of Ca^{2+} /calmodulin, the inhibitors apparently had no effect. On the other hand, regarding the autophosphorylated enzyme, the results obtained were rather unexpected. The removal of calmodulin with trifluoperazine or mastoparan had little or only a slight effect on the activity of the autophosphorylated enzyme with any of the substrates used, determined in

the presence of Ca^{2+} /calmodulin. The dependence of the kinase activity on calmodulin slightly differed among the substrates used. That is, the activity was inhibited with trifluoperazine or mastoparan using MBP or syntide-2 as substrate, but this inhibition was not obviously observed with MLC or calcineurin as substrate. The Ca^{2+} /calmodulin-independent activity of the autophosphorylated enzyme was not affected by the removal of calmodulin. Because the activities with Ca^{2+} /calmodulin were apparently higher than those without Ca^{2+} /calmodulin ($+2 \text{ mM}$ EGTA) with any of substrates used, the addition of Ca^{2+} was at least required.

Effects of autophosphorylation of CaM kinase II on kinetic parameters

The kinetic parameters were compared using the autophosphorylated or non-autophosphorylated CaM kinase II.

Table 4 shows the ED_{50} value and the maximal activity of the cytosol CaM kinase II with MBP as substrate before or after autophosphorylation. The concentration of MBP to give a half maximal activity was not changed by autophosphorylation, although a slight difference was observed with EGTA by autophosphorylation. In contrast, the maximal activity greatly increased by autophosphorylation with or without Ca^{2+} /calmodulin.

We next examined the effects of phosphorylation of calcineurin on the K_m and V_{max} values of the protein phosphatase activity of calcineurin using *p*-nitrophenylphosphate as substrate (Table 5). The kinetic parameters determined showed no difference between the non-phosphorylated and phosphorylated calcineurin. Under the conditions employed, the amount of phosphate incorporated into calcineurin was 0.23 mol/mol . The relatively small amount of phosphate incorporated may have little effect on calcineurin activity before or after phosphorylation. Phosphoamino acid analysis revealed that only the serine residue was phosphorylated in calcineurin.

Table 3. Effects of trifluoperazine and mastoparan on activity of non-autophosphorylated and autophosphorylated CaM kinase II

Substrate	Calmodulin antagonist	Autophosphorylation (min)			
		0		10	
		EGTA	Ca ²⁺ /CaM	EGTA	Ca ²⁺ /CaM
pmole					
MLC	None	0.0	32.1	8.2	31.0
	TFP	0.0	1.0	8.2	24.3
	Mastoparan	0.1	4.2	9.4	31.5
MBP	None	2.2	16.6	28.5	49.7
	TFP	1.6	3.3	16.1	26.5
	Mastoparan	1.8	2.4	22.7	25.0
Calcineurin	None	0.2	0	4.2	21.3
	TFP	0.1	0.1	3.1	19.5
	Mastoparan	0.2	0.2	1.5	18.7
Syntide-2	None	3.1	136.1	53.9	156.5
	TFP	3.1	7.9	53.9	96.5
	Mastoparan	3.2	9.1	57.4	95.5

Cytosolic CaM kinase II (1.0 μ g) was autophosphorylated at 0°C for 0 or 10 min. Then, the kinase activity was assayed with 20 μ g of myosin light chain (MLC), 20 μ g of myelin basic protein (MBP), 20 μ g of calcineurin and 0.6 μ g of syntide-2 as substrate, with or without each antagonist as indicated, in the presence of 2 mM EGTA, 0.1 mM Ca²⁺ and 0.3 μ M calmodulin (EGTA) or 0.1 mM Ca²⁺ and 0.3 μ M calmodulin (Ca²⁺/CaM) in the incubation mixture of 0.1 ml, under standard conditions. The concentrations of trifluoperazine (TFP) and mastoparan used were 50 μ M and 5 μ M, respectively. The experiments were carried out 4 times in duplicate determinations, and data were comparable to each other. One of the results is shown.

Table 4. Effects of autophosphorylation on kinetic parameters of cytosolic CaM kinase II with myelin basic protein as substrate

Addition	Autophosphorylation (min)			
	0		10	
	ED ₅₀ (μ M)	Maximal activity (nmol/min/mg)	ED ₅₀ (μ M)	Maximal activity (nmol/min/mg)
EGTA	1.83	0.91	0.81	35.71
Ca ²⁺ /calmodulin	0.71	20.41	0.76	64.34

Cytosolic CaM kinase II was autophosphorylated at 0°C for 0 or 10 min. Then, 50- μ l of aliquots containing 0.76 μ g of the enzyme were assayed at 30°C for 1 min for the activity with 5-160 μ g of myelin basic protein as substrate and 50 μ M [γ -³²P]ATP in the presence of 2 mM EGTA, 0.05 mM Ca²⁺ plus 0.15 μ M calmodulin (EGTA) or 0.1 mM Ca²⁺ plus 0.3 μ M calmodulin (Ca²⁺/CaM), under standard conditions.

Table 5. Effects of phosphorylation of calcineurin on protein phosphatase activity

	Nonphosphorylated		Phosphorylated	
	K_m (mM)	V_{max} (μ mol/min/mg)	K_m (mM)	V_{max} (μ mol/min/mg)
Calcineurin	28.6	0.59	28.6	0.59

Calcineurin was phosphorylated at 30°C for 5 min by the autophosphorylated cytosolic CaM kinase II. Under these conditions, the amount of phosphate incorporated into calcineurin was 0.23 mol/mol. Calcineurin was assayed for activity with 2.5–40 mM *p*-nitrophenylphosphate as substrate. The experiments were carried out 4 times, and data were comparable to each other. One of the results is shown.

DISCUSSION

The effects of autophosphorylation on kinase activity reported previously can be summarized as follows: a) The Ca^{2+} /calmodulin-dependent activity becomes partially Ca^{2+} /calmodulin-independent by the autophosphorylation of Thr 286/287 (32–35). b) The enzyme is translocated from the membrane fraction to the cytosol fraction in the neurons of *Aplysia* (36). c) The effect of the inhibitory domain is relieved by the autophosphorylation of Thr 286/287 (37). d) The calmodulin-binding ability of the enzyme is lost by the autophosphorylation of Thr 305/306; and therefore, the activity becomes fully Ca^{2+} /calmodulin-independent (38, 39). In addition, the present study represents another aspect of the effects of autophosphorylation on kinase activity, which has not been reported, although preliminary data were reported previously (16).

It was reported that a decrease (12, 14, 40–42) or increase (43, 44) in kinase activity is brought about by autophosphorylation. Because autophosphorylation is usually examined using a single substrate for each study, the above conflicting findings may be at least in part due to the substrates used. The effects of autophosphorylation on kinase activity should be examined with various substrates. Kwiatkowski et al. (44) explained that the autophosphorylation was an obligatory step for the full activation of CaM kinase II using syntide-2 as

substrate. In fact, we could demonstrate in the present study that the kinase activity increased with syntide-2 by autophosphorylation. Thus, the findings obtained were concluded to account for one of the effects of autophosphorylation on kinase activity.

It was reported that the generation of Ca^{2+} /calmodulin-independent activity by autophosphorylation is due to the phosphorylation of Thr 286/287 in the α and β subunits, respectively (32–35). Although we could not identify the phosphorylation site on which an increase in kinase activity is caused by autophosphorylation, it may be based on the phosphorylation of Thr 286/287. a) An increase in kinase activity was observed as early as 15 sec after autophosphorylation. This is consistent with the finding that Thr 286 is phosphorylated as early as 15 sec (33, 45) and faster than the serine residue. b) The patterns of the increase in kinase activity and generation of Ca^{2+} /calmodulin-independent activity were almost identical during the time course (Figs. 4 and 5). c) The increase in kinase activity reached the maximum at 10 min (Figs. 4 and 5), while the autophosphorylation of the enzyme attained the maximum at 30 min (Figs. 1 and 3). d) Phosphoamino acid analysis revealed that threonine residue was phosphorylated earlier than serine residue and that thereafter, the phosphorylation of serine residue increased (Fig. 2).

Trifluoperazine and mastoparan are known as calmodulin inhibitors which act by binding

to calmodulin and inhibiting calmodulin-activatable enzymes. We examined the requirement of the autophosphorylated CaM kinase II for calmodulin and compared it to that of the non-autophosphorylated enzyme (Table 3). In the present study, the autophosphorylation of the enzyme was performed in the presence of Ca^{2+} /calmodulin. Therefore, the enzyme was expected to maintain the calmodulin-binding ability, since the extensive autophosphorylation (Thr 305) was attained in the absence of Ca^{2+} /calmodulin (38, 39). The removal of calmodulin from the assay mixture for the autophosphorylated enzyme did not have an inhibitory effect on the kinase activity. This finding is consistent with that by Hashimoto et al. (46) who showed that the phosphorylation of calcineurin requires Ca^{2+} but not calmodulin. Because this was also observed when the MLC, MBP and syntide-2 were used as substrates, it was not based on the Ca^{2+} -binding site of calcineurin B. These results may be explained as follows: a) The autophosphorylated CaM kinase II may require only Ca^{2+} but not calmodulin, and/or b) the autophosphorylated enzyme may have a higher affinity for calmodulin. Even in the presence of the inhibitors, calmodulin might not be removed from the enzyme. In the cases for MBP and syntide-2, the Ca^{2+} /calmodulin-dependent activity of the autophosphorylated kinase was slightly inhibited with trifluoperazine and mastoparan. The interactions among the enzyme, calmodulin and substrate may be changed by autophosphorylation, although the precise mechanism remains to be elucidated.

The K_m and V_{max} values of calcineurin phosphorylated by the autophosphorylated CaM kinase II were the same as those of the non-phosphorylated calcineurin. Hashimoto et al. (46) reported that the phosphorylated calcineurin had a higher K_m using MLC as substrate and a lower V_{max} using *p*-nitrophenyl-phosphate as substrate. The difference between both results may be due to the amount of phosphate incorporated into calcineurin. In the present study, only 0.2 mol of phosphate was incorporated into 1 mol of calcineurin,

while in their study, 0.8 and 0.9–1.0 mol/mol by CaM kinase II (46) and protein kinase C (47), respectively, were incorporated into calcineurin.

Several proteins may serve as substrates only for the autophosphorylated CaM kinase II. This kind of endogenous substrate should be further investigated. Furthermore, calmodulin is no longer required (and/or calmodulin is tightly bound to the autophosphorylated kinase) for phosphorylation of substrates by the autophosphorylated kinase, and the substrate specificity obviously differs between the autophosphorylated and non-autophosphorylated CaM kinase IIs. The results suggest that, in addition to the generation of Ca^{2+} /calmodulin-independent activity, the autophosphorylation has a physiological role in the regulation of CaM kinase II.

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