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Purification and Characterization of Active Fragment of Ca^{2+}/Calmodulin-Dependent Protein Kinase II from the Post-Synaptic Density in the Rat Forebrain[1]

Yoshiyuki Yoshimura, Tomohiko Nomura, and Takashi Yamauchi[2]

Department of Biochemistry, Faculty of Pharmaceutical Sciences, The University of Tokushima, Tokushima 770

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Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) of the post-synaptic density (PSD) was solubilized and activated 4- to 5-fold by limited α-chymotrypsin digestion with prior autophosphorylation of the kinase. The enzyme was also activated by trypsin and μ-calpain, a Ca^{2+}-dependent protease. The active catalytic fragment was purified to homogeneity using gel filtration and ion exchange chromatography. The purified active fragment was completely Ca^{2+}/calmodulin-independent and exists as a monomer. Kinetic studies with the purified fragment revealed similar K_m values for ATP and synthetic peptide substrate, and an about 8-fold increment in V_{max}, compared with native PSD CaM kinase II.

Key words: active catalytic fragment, Ca^{2+}/calmodulin-dependent protein kinase II, post-synaptic density, protein phosphorylation, rat forebrain.

The post-synaptic density (PSD) is a specialization of the submembranous cytoskeleton visible in the electron microscope that appears to represent a tight complex of post-synaptic junctional proteins (*1*). The PSD fraction is believed to be involved in the regulation of synaptic events such as receptor function, structural modification and synaptic plasticity. Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is associated with the PSD fraction and it is one of the major components of PSD (*2-4*). CaM kinase II is one of the most abundant protein kinases in the mammalian brain (*5-9*). The PSD fraction also contains some receptors, and proteins that may participate in receptor regulation and in regulation of other forms of synaptic efficacy. In the post-synaptic cells, CaM kinase II activation is required to generate long-term potentiation (LTP) (*10*). Relatively few studies have focused on the PSD CaM kinase II.

Neuronal CaM kinase II regulates a broad array of nerve functions, including the synthesis and secretion of neurotransmitters, receptor function, structural modification of the cytoskeleton, axonal transport, LTP, and perhaps gene expression (for reviews, see Refs. *11-13*). The soluble CaM kinase II displays a native molecular mass of about 500-600 kDa, and is composed of two distinct, but closely related polypeptides of 50 (α) and 60 (β) kDa. The native form of CaM kinase II consists of a distinct homooligomer of either the α or the β polypeptide (*14*). Autophosphorylation of CaM kinase II has profound effects on its activity (*15, 16*). CaM kinase II exhibits an absolute requirement for Ca^{2+} and calmodulin for its initial enzyme activity and phosphorylates itself as well as its substrate proteins. Upon phosphorylation in a Ca^{2+}/calmodulin-dependent manner, CaM kinase II is converted to a partially Ca^{2+}-independent form. Thr-286 and -287 of the α and β isoforms, respectively, are autophosphorylation sites that generate Ca^{2+}-independent activity (*17-20*).

In contrast to soluble CaM kinase II, much of the PSD CaM kinase II appears to be inactive (*21, 22*). Although PSD CaM kinase II is solubilized with 8 M urea and has physical properties very similar to those of the cytosolic kinase, PSD CaM kinase II is not solubilized by non-ionic detergents in the active form (*23*). It is possible that PSD CaM kinase has a low specific activity, the majority of the kinase molecule is inactive, or the active site of the kinase is masked in the PSD structure. Rich *et al.* have provided evidence suggesting that, in analogy to the soluble kinase, Thr-286 on PSD CaM kinase II is phosphorylated in the presence of Ca^{2+} (*24*). This suggests that CaM kinase II is very similar to soluble CaM kinase II in terms of regulation by autophosphorylation. We confirmed that proposal and in addition, we suggest that additional distinct sites are phosphorylated and that PSD CaM kinase II is different from the soluble kinase in terms of regulatory consequences (*25*).

In this study, we purified the active catalytic fragment of PSD CaM kinase II by means of a simple procedure. CaM kinase II of the PSD is solubilized and activated 4- to 5-fold by limited proteolytic digestion.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP was purchased from Amersham International. TLCK-treated α-chymotrypsin and TPCK-treated trypsin were from Sigma. μ-Calpain was from Nakarai Chemicals. Calpain inhibitor I was from Boehrin-

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[2] To whom correspondence should be addressed. Phone: +81-886-31-3111, Fax: +81-886-33-0510

ger Mannheim. Horseradish peroxidase-coupled anti-mouse goat IgG was from Bio-Rad Laboratories. The Western blot chemiluminescence reagent was from DuPont NEN Research Products. DEAE cellulose (DE-52) was from Whatman and Sephadex G-75 was from Pharmacia. The CaM kinase II substrate syntide 2 (PLARTLSVAGLPGKK) was synthesized and purified as described (*26*). Soluble CaM kinase II was purified from the rat forebrain (*9*). A monoclonal antibody specific to CaM kinase II α isoform (3E11) was as described (*27*). The antibody recognizes the catalytic domain of CaM kinase II (*14*).

Purification of PSD—PSD was purified from the rat forebrain according to the procedures of Cohen *et al.* (*28*), as modified by Wu *et al.* (*29*). Briefly, a crude synaptosomal fraction was exposed to 1% Triton X-100, then separated by discontinuous sucrose density gradient centrifugation. The yield of PSD fraction was about 3.9 mg of total PSD protein from 15 g wet weight of forebrain. PSD CaM kinase II α isoform in the PSD fraction accounted for about 10% of the total PSD protein, which is consistent with published results (*25*).

Assay of CaM Kinase II—CaM kinase II activity was assayed in terms of the Ca^{2+}-dependent phosphorylation of syntide 2 as described (*25*). The standard reaction mixture contained 50 μM [γ-^{32}P]ATP ($3-5\times10^5$ cpm), 8 mM $Mg(CH_3COO)_2$, 20 μM syntide 2, 2 μM calmodulin, 0.25 mM $CaCl_2$, 0.1 mM EGTA, 50 mM Hepes buffer, pH 8.0, and a suitable amount of enzyme in a total volume of 50 μl. Ca^{2+}-independent activity was assayed under the same conditions except for the presence of 1 mM EGTA instead of $CaCl_2$. The reaction was carried out at 30°C. One unit of CaM kinase II activity was defined as 1 nmol of phosphate transferred/min under the standard conditions.

Generation of Active Catalytic Fragment of PSD CaM Kinase II—The active fragment of PSD CaM kinase II was generated by means of a modification of the described procedure (*30*). PSD CaM kinase II was first autophosphorylated for 10 min at 0°C in a reaction mixture containing about 0.5-1.0 mg/ml PSD, 50 mM Hepes, pH 8.0, 8 mM $Mg(CH_3COO)_2$, 0.25 mM $CaCl_2$, 0.1 mM EGTA, 2 μM calmodulin, and 50 μM ATP. Control experiments proceeded under the same conditions except for the presence of EGTA instead of $CaCl_2$ or in the absence of ATP (non-phosphorylating conditions). Thereafter, α-chymotrypsin (5-10 μg/ml) was added to the incubation mixture. After 60 min at 0°C, the reaction was terminated with 1 mM phenylmethylsulfonyl fluoride (PMSF), then 1 mM dithiothreitol (DTT) and 10% ethyleneglycol were added. Trypsin or μ-calpain digestion proceeded under the same conditions, and the reactions were terminated with PMSF and 4 μg of calpain inhibitor I, respectively.

Purification of Active Catalytic Fragment of PSD CaM Kinase II—PSD fraction (3.92 mg protein) was incubated with ATP in 5 ml of the standard autophosphorylation mixture for 10 min at 0°C, then α-chymotrypsin (19.6 μg) was added. A protease (α-chymotrypsin): substrate (PSD fraction) ratio of 1:200 was used to generate a relatively high specific activity of the active fragment. The mixture was centrifuged for 30 min at $105,000\times g$. The supernatant was concentrated to 0.2 ml by ultrafiltration using a Centricon 10. The solution was applied to a Sephadex G-75 (1×40 cm) column equilibrated with 40 mM Tris buffer, pH 7.6, containing 1 mM DTT and 10% ethyleneglycol, then eluted with the same buffer at a flow rate of 1.4 ml/h. The active fractions were pooled and applied to a DEAE cellulose column (bed volume = 40 μl) equilibrated with 40 mM Tris buffer, pH 7.6, containing 1 mM DTT and 10% ethyleneglycol. The column was washed with 5 volumes of the same buffer containing 20 mM NaCl, then eluted with the equilibration buffer containing 70 mM NaCl. The active fractions were pooled. The purified active fragment was divided into 40-μl aliquots and stored at −80°C.

Protein concentration was determined as described (*31*) using bovine serum albumin as the standard.

SDS-PAGE and Immunoblotting—SDS-PAGE proceeded on 10% polyacrylamide gels in the presence of 0.1% SDS as described by Laemmli (*32*). Immunoblotting was performed as described (*27*) with slight modifications. Briefly, proteins were separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated with 5% non-fat skim milk in Tris-buffered saline, pH 7.4, for 1 h, then with 3 μg/ml of a monoclonal antibody specific to CaM kinase II α isoform at 4°C overnight. After washing, the transblots were incubated with peroxidase-coupled anti-mouse IgG (1:3,000 dilution) for 30 min at room temperature. CaM kinase II was detected by using an enhanced chemiluminescence procedure as recommended by the manufacturer.

RESULTS

Effect of Limited Proteolysis on the Activation and Solubilization of PSD CaM Kinase II—Since autophosphorylation of CaM kinase II and proteolytic modifications have been proposed as a regulatory mechanism in LTP (*33*), it was important to determine whether the proteolysis had any effect on the activity of PSD CaM kinase II after autophosphorylation of the kinase. As shown in Fig. 1, CaM kinase II activity was solubilized, increased 4- to 5-fold for

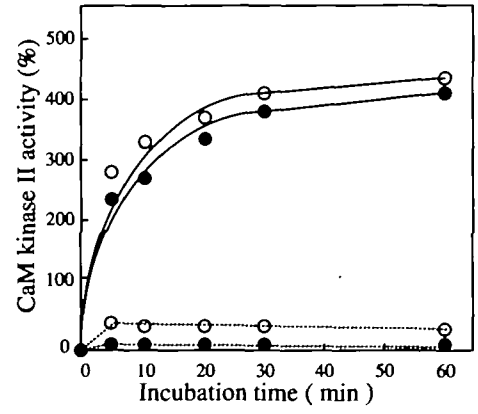

Fig. 1. **Activation and solubilization of PSD CaM kinase II by limited proteolytic digestion.** The PSD fraction (100 μg) was autophosphorylated under the standard conditions in 100 μl for 10 min at 0°C, then 1 μg of α-chymotrypsin was added. After incubation for the indicated periods at 0°C, 1 mM PMSF, 1 mM DTT, and 10% ethylene glycol were added and the mixture was centrifuged at $105,000\times g$ for 30 min. Control experiments proceeded without autophosphorylation. An aliquot of the supernatant was assayed for CaM kinase II activity in the presence (○) or absence (●) of Ca^{2+} under the standard conditions. 100% activity shows the CaM kinase II activity of PSD fraction (192 units/mg). Solid line, autophosphorylated; dashed line, non-autophosphorylated.

30 min and remained at these levels for more than 60 min after addition of α-chymotrypsin. The CaM kinase II activity was completely Ca^{2+}/calmodulin-independent. The increase in activity by α-chymotrypsin was dependent on the autophosphorylation prior to the proteolytic digestion (Fig. 1). When PSD was digested by α-chymotrypsin without prior autophosphorylation, the CaM kinase II activity was slightly solubilized, and partially Ca^{2+}/calmodulin-dependent. Under the same conditions, proteolysis of soluble CaM kinase II by α-chymotrypsin without prior autophosphorylation inactivated the kinase (data not shown), consistent with the published report (*30*).

Similar results were obtained after μ-calpain and trypsin digestion (Table I). Proteolytic digestion of PSD CaM kinase II by μ-calpain and trypsin resulted in about a 5-fold activation of CaM kinase II. Although the activity was Ca^{2+}/calmodulin-independent after tryptic digestion, about 30% of the Ca^{2+}/calmodulin-dependent activity remained after μ-calpain digestion. When the PSD fraction was digested by μ-calpain without prior autophosphorylation, PSD CaM kinase II was partially activated and Ca^{2+}/calmodulin-independent activity was solubilized to some extent.

These results indicated that the inactive forms of PSD CaM kinase II can be activated or unmasked from PSD structures by limited proteolytic digestion.

Characterization of Solubilized Protein by Limited Proteolytic Digestion—Proteins solubilized by α-chymotrypsin were resolved by SDS-PAGE. Figure 2 shows 4 major (140, 72, 45, and 30 kDa) and some minor bands. Proteolysis by α-chymotrypsin with prior autophosphorylation generated a fragment of about 30 kDa (lane 2), but proteolysis without prior autophosphorylation resulted in the generation of a fragment of about 32 kDa instead (lane 3). The 30 kDa fragment may correspond to the active fragment of CaM kinase II generated from purified soluble CaM kinase II (lane 8), suggesting that the one of the major

TABLE I. **Effect of autophosphorylation on the activation of PSD CaM kinase II by limited proteolysis.** The PSD fraction (100 μg protein) was autophosphorylated in a 100 μl reaction mixture under the standard conditions for 10 min at 0°C. Non-autophosphorylated reaction proceeded under the same conditions but without ATP. After autophosphorylation, 1 μg of α-chymotrypsin, 5 μg of μ-calpain, or 1 μg of trypsin was added. After incubation for 60 min at 0°C, 1 mM PMSF or 4 μg of calpain inhibitor I, 1 mM DTT, and 10% ethyleneglycol were added to the mixture. The mixture was centrifuged at 105,000 × *g* for 30 min, and Ca^{2+}-dependent and -independent CaM kinase II activity of the supernatant was determined under the standard conditions. When PSD was not digested with protease, the CaM kinase II activity of the reactions was determined without centrifugation, since PSD CaM kinase II was insoluble.

Protease	Autophosphorylated		Non-autophosphorylated	
	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
	(units)			
α-Chymotrypsin	72.7	70 2	5.5	1.7
μ-Calpain	77 5	59.2	27 0	19 7
Trypsin	87.6	84.5	4.2	3.9
None	14.0	7 7	16.4	0 4

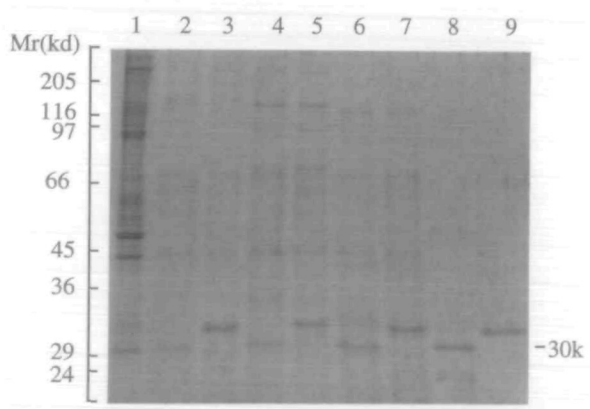

Fig. 2. **SDS-PAGE of proteins solubilized from PSD fraction by limited proteolytic digestion.** The PSD fraction (100 μg protein) was incubated under the autophosphorylated or non-autophosphorylated conditions, then digested with 1 μg of α-chymotrypsin, 5 μg of μ-calpain, or 1 μg of trypsin as described in Table I. After incubation for 60 min at 0°C, 1 mM PMSF or 4 μg of calpain inhibitor I, 1 mM DTT, and 10% ethyleneglycol were added to the mixture. The mixture was centrifuged at 105,000 × *g* for 30 min, and a 15-μl aliquot of the supernatant was analyzed by SDS-PAGE. Thereafter, the gel was stained with Coomassie Blue. Lane 1, PSD fraction; lanes 2, 4, 6, and 8, autophosphorylated; lanes 3, 5, 7, and 9, non-autophosphorylated; lanes 2 and 3, α-chymotrypsin-digested, lanes 4 and 5, μ-calpain-digested; lanes 6 and 7, trypsin-digested; lanes 8 and 9, purified soluble CaM kinase II (2 μg) was digested with α-chymotrypsin (0 2 μg) under the standard conditions. Molecular markers are shown on the left, and the position of active 30 kDa fragment is shown on the right.

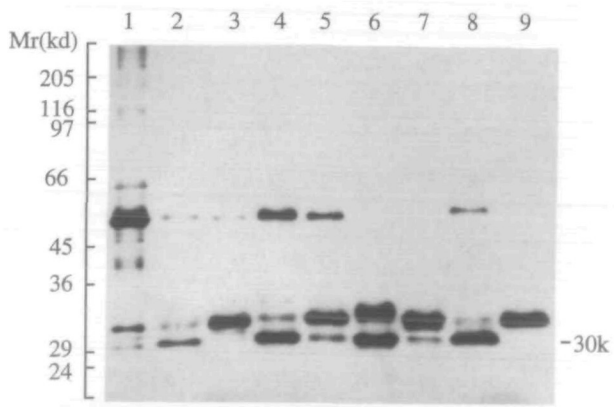

Fig 3. **Immunoblots of the proteolytic fragment of PSD CaM kinase II.** The PSD fraction was autophosphorylated and solubilized as described in the legend to Fig. 1 An 8-μl aliquot of the supernatant was separated by SDS-PAGE and immunoblotted Lane 1, PSD fraction; lanes 2, 4, 6, and 8, autophosphorylated; lanes 3, 5, 7, and 9, non-autophosphorylated, lanes 2 and 3, α-chymotrypsin-digested; lanes 4 and 5, μ-calpain-digested; lanes 6 and 7, trypsin-digested; lanes 8 and 9, purified soluble CaM kinase II (2 μg) was digested with α-chymotrypsin (0.2 μg) under the standard conditions

TABLE II. **Summary of purification of active catalytic fragment of PSD CaM kinase II.** The active catalytic fragment was purified from PSD fraction (3 9 mg) after autophosphorylation followed by α-chymotrypsin. CaM kinase II activity was assayed under the standard conditions.

Fraction	Protein (mg)	Activity (unit)	Specific activity (unit/mg)	Yield (%)
PSD	3.93	774	197	100
Chymotrypsin-treated	1.12	2,408	2,140	311
Sephadex G-75	0.34	2,160	6,430	279
DEAE-cellulose	0.11	1,308	12,222	169

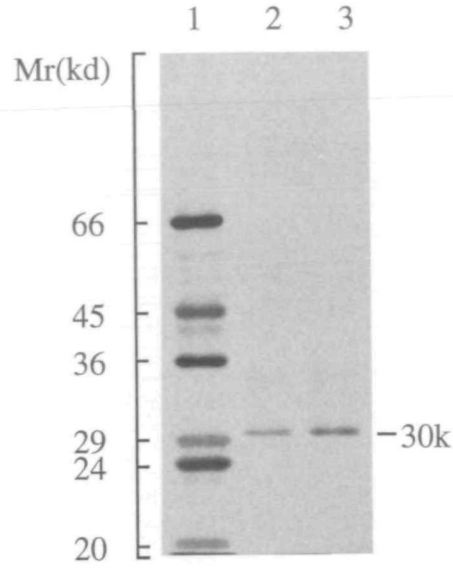

Fig. 4 **SDS-PAGE of purified active catalytic fragment of CaM kinase II.** Lane 1, molecular markers, lanes 2 and 3, 1 1 and 3 8 μg of purified active fragment, respectively After electrophoresis, the gel was stained with Coomassie Blue.

proteins solubilized by α-chymotrypsin was an active fragment of CaM kinase II from PSD fraction. The 30 kDa protein was the active fragment of CaM kinase II α isoform, as confirmed by immunoblotting (Fig. 3, lanes 2 and 8). The 32 kDa fragment had low activity and was partially Ca^{2+}/calmodulin-dependent, although its molecular mass was similar to that of the inactive fragment of soluble CaM kinase II (Figs. 2 and 3, lane 9).

Similar results were obtained after digestion with μ-calpain and trypsin. Although the three proteases generated active fragments with similar molecular mass, the sites of cleavage may be somewhat different because the substrate specificity of the proteases differs. The less active 32 kDa fragment was also generated by μ-calpain and trypsin, when proteolytic digestion proceeded without prior autophosphorylation of PSD CaM kinase II. The results of this study did not indicate the sequences of the active 30 kDa and the less active 32 kDa fragments.

Purification of Active Fragment of PSD CaM Kinase II—Since the ratio of active 30 kDa fragment of PSD CaM kinase II to total solubilized protein was the highest after α-chymotrypsin digestion, the active fragment of PSD CaM kinase II was purified after α-chymotrypsin treatment as described under "MATERIALS AND METHODS." A typical purification procedure is summarized in Table II. From 3.9 mg of PSD, 0.11 mg of the purified active fragment was obtained. The active fragment was purified 5.7-fold from the soluble fraction digested by α-chymotrypsin with 54% yield. The protein thus obtained was almost homogeneous, with a molecular mass of 30 kDa upon SDS-PAGE (Fig. 4). The elution profile from Sephadex G-75 indicated that the active fragment is monomeric.

The inactive 32 kDa fragment of PSD was purified after α-chymotrypsin treatment without prior autophosphorylation by the same method, except that the inactive fragment was recovered in the flow-through fraction of the DEAE cellulose column. The inactive fragment was neither phosphorylated nor activated by native CaM kinase II (data not shown), suggesting that autophosphorylation induces a conformational change of the kinase that favors limited proteolysis.

TABLE III **Catalytic properties of purified active catalytic fragment of PSD CaM kinase II.** The apparent K_m values for syntide 2 and ATP and the apparent V_{max} values were obtained from double-reciprocal plots of the data. To determine the K_m for syntide 2 and ATP, 50 μM ATP and 20 μM syntide 2 were used, respectively. To determine the apparent V_{max}, 50 μM ATP was used and the concentration of syntide 2 was changed. [a] V_{max} is expressed on the assumption that the content of PSD CaM kinase II is 10%

Parameter	Active fragment of PSD CaM kinase II	CaM kinase II of PSD fraction
K_m, syntide 2 (μM)	19 1	21.7
K_m, ATP (μM)	26 9	21.8
V_{max} (unit/mg)	22,322	2,830[a]

The active 30 kDa fragment was relatively stable at low temperature, and the CaM kinase II activity was almost completely retained for 24 h and about 70% retained after 8 days at 4°C in 40 mM Tris buffer, pH 7.4, containing 1 mM DTT, 50 mM NaCl, 10% ethyleneglycol, and 0.05% Tween 40 (data not shown). However, thermal inactivation of the active 30 kDa fragment was observed. Incubation of the active fragment resulted in a progressive decrease in the activity with increasing incubation temperature at above 20°C, and more than 90% of the original activity was lost at 40°C for 3 min, while the activity of native CaM kinase II was not significantly changed under the conditions (data not shown). A similar thermal inactivation of the active 30 kDa fragment of soluble CaM kinase II was reported by Ishida and Fujisawa (*34*).

Catalytic Properties of the Purified Active Fragment—Kinetic parameters of the purified active fragment were compared with those of native PSD CaM kinase II using the synthetic peptide substrate syntide 2 (Table III). The enzyme activity was completely independent of Ca^{2+} and calmodulin. The active fragment exhibited almost the same K_m values for syntide 2 and ATP as those of the native PSD CaM kinase II. The V_{max} of the active fragment increased about 7.9-fold, compared with the native enzyme, assuming that the content of PSD CaM kinase II is 10%. These results suggest that the active site of the inactive PSD CaM kinase II may be masked in PSD structures rather than be disrupted in PSD fraction.

The active fragment phosphorylated more than 10 proteins (220, 190, 165, 125, 77, 74, 65, 56, 27, 24 kDa, and others) of the PSD fraction on SDS-PAGE (data not shown).

DISCUSSION

Malenka *et al.* (*35*) and Malinow *et al.* (*36*) have demonstrated, using specific peptide inhibitors, that CaM kinase II activation in post-synaptic cells is required to generate LTP. Studies of mice homozygous for a targeted disruption of CaM kinase II α isoform gene have further demonstrated that this kinase is required for LTP in the hippocampus and acquisition of spatial learning (*37, 38*). The PSD CaM kinase II may play a critical role in LTP generation, since the autophosphorylation of CaM kinase II in the presence of Ca^{2+} and calmodulin induces the Ca^{2+}-independent activity. The Ca^{2+}-independent activity may prolong the effects

triggered by the transient Ca^{2+} signal after intracellular Ca^{2+} has been restored to the original level (*13*). On the other hand, much of the PSD CaM kinase II appears to be inactive or has a low specific activity, in contrast to the soluble CaM kinase II (*21, 22*). We reported that PSD CaM kinase II differs from the soluble CaM kinase II in terms of regulatory consequences and sites of autophosphorylation, and that there are three species of the PSD CaM kinase II α isoform (*25*). If PSD CaM kinase II plays a critical role in LTP, an inactive or less active form of PSD CaM kinase II may be activated, and then Ca^{2+} signals may be enhanced by the action of the kinase.

In this study, we examined the activation of PSD CaM kinase II and purification of the activated enzyme in order to confirm that PSD CaM kinase II is likely to play a critical role in LTP. Inactive or less active PSD CaM kinase II is not known to be activated under physiological conditions at present. CaM kinase II was reported to be activated by limited proteolytic digestion, but this is controversial (*39, 40*). Levine and Sahyoun have reported that the generation of Ca^{2+}/calmodulin-independent activity by limited proteolysis is not required for prior autophosphorylation of the PSD CaM kinase II (*39*). Rich *et al.* have reported that incubation of PSD fraction with α-chymotrypsin or μ-calpain results in a three- to fivefold enhancement of total activity and generation of Ca^{2+}/calmodulin-independent activity (*24*). Yamagata *et al.* have demonstrated that the proteolytic generation of the active fragment requires prior autophosphorylation of purified soluble CaM kinase II (*30*). The disparity among these findings may be due to a difference in the autoregulatory mechanism of the soluble and PSD CaM kinase II. In this report, however, we demonstrated that PSD CaM kinase II is much more activated by proteolytic digestion with prior autophosphorylation than without, and that the catalytic active fragment is generated by limited proteolytic digestion with prior autophosphorylation of the kinase.

We demonstrated that the active 30 kDa fragment purified from PSD was relatively stable at 4°C. However, thermal inactivation of the active fragment was observed. A similar thermal instability was reported for the active 30 kDa fragment of soluble CaM kinase II (*34*). Therefore, the removal of the autoinhibitory domain of both soluble and PSD CaM kinase II may cause a marked decrease in thermal stability.

The 30 kDa active fragment of PSD CaM kinase II can be purified by the relatively simple procedure described in this study. The active fragment of soluble CaM kinase II has been purified from purified CaM kinase II (*30*). The purification of PSD is much easier than that of CaM kinase II. The purified active fragment of PSD CaM kinase II should provide a useful tool for the study of the functional role of CaM kinase II in various biological systems. The studies here support the notion that the irreversible activation of CaM kinase II by proteolysis also functions as a persistent mechanism for the regulation of synaptic events. The activation of inactive or less active PSD CaM kinase II may be required for the induction and maintenance of LTP. The physiological role of PSD CaM kinase II activation is under investigation.

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