

# Protein phosphatase 1 is involved in the dissociation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II from postsynaptic densities

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**Abstract** Autophosphorylation-dependent translocation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaM kinase II) to postsynaptic densities (PSDs) from cytosol may be a physiologically important process during synaptic activation. We investigated a protein phosphatase responsible for dephosphorylation of the kinase. CaM kinase II was shown to be targeted to two sites using the gel overlay method in two-dimensional gel electrophoresis. Protein phosphatase 1 (PP1) was identified to dephosphorylate CaM kinase II from its complex with PSDs using phosphatase inhibitors and activators, and purified phosphatases. The kinase was released from PSDs after its dephosphorylation by PP1.

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**Key words:** Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; Postsynaptic density; Translocation; Protein phosphatase 1; Autophosphorylation; Dissociation

## 1. Introduction

Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaM kinase II) is one of the most abundant protein kinases in the mammalian brain [1–5]. It is activated by a variety of signal transduction pathways that increase the intracellular free Ca<sup>2+</sup> concentration. It regulates a broad array of neuronal functions, including the induction of long-term potentiation (LTP) [6–8] and acquisition of spatial memory [9]. A distinct property is that autophosphorylation of the kinase induces Ca<sup>2+</sup>-independent activity. This reaction has been proposed as a molecular switch, translating transient calcium elevation into prolonged kinase activity [10]. Presumably, protein phosphatases (PPs) contribute to the regulation of CaM kinase II autophosphorylation in vivo by dephosphorylating the site.

CaM kinase II is concentrated at postsynaptic densities (PSDs), which are considered to be the major site of neurotransmission of excitatory synapses. The  $\alpha$  isoform of CaM kinase II is a major component of PSDs [11–13]. The concentration of a calcium-regulated kinase in PSDs might modulate synaptic activity in response to a rise in postsynaptic calcium levels.

In contrast to the soluble CaM kinase II, most of the PSD-

associated CaM kinase II appears to be inactive [14,15]. However, PSD-associated kinase is very similar to soluble CaM kinase II in terms of regulation by autophosphorylation [15–17]. Similar to soluble kinase, Thr-286 on PSD-associated kinase was demonstrated to be phosphorylated in the presence of Ca<sup>2+</sup> and calmodulin by high performance liquid chromatography (HPLC) analysis and peptide mapping of phosphopeptides [15,17]. A recent study in this laboratory suggested that many of the active sites of PSD-associated kinase are masked in the PSD structure [18]. We also demonstrated that CaM kinase II reversibly translocates to PSDs in a phosphorylation-dependent manner [19]. Strack et al. recently reported a similar translocation of autophosphorylated CaM kinase II to the PSDs [20]. In order to understand the physiological significance of the translocation, it is important to identify the protein phosphatase responsible for dissociation of CaM kinase II from its complex with PSDs.

## 2. Materials and methods

### 2.1. Materials

Human recombinant PP1 was purchased from Boehringer Mannheim, PP2A from human red cells was from Seikagaku Co., Tokyo, protein phosphatase inhibitor 2 from rabbit skeletal muscle was from New England Biolabs, a monoclonal antibody specific to the NR2B subunit of NMDA receptor was from Santa Cruz Biotechnology Inc. Partially purified CaM kinase II was prepared as described previously [19].

### 2.2. Assay of CaM kinase II

CaM kinase II activity was assayed as described [19]. The standard reaction mixture contained 50  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP ( $3\text{--}5 \times 10^5$  cpm), 8 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 20  $\mu$ M syntide 2, 2  $\mu$ M calmodulin, 0.25 mM CaCl<sub>2</sub>, 0.1 mM EGTA, 50 mM HEPES buffer, pH 8.0, and a suitable amount of enzyme in a total volume of 50  $\mu$ l. Ca<sup>2+</sup>-independent activity was assayed under the same conditions except for the presence of 1 mM EGTA instead of CaCl<sub>2</sub>.

### 2.3. Autophosphorylation of CaM kinase II

Autophosphorylation of CaM kinase II was carried out at 0°C for 10 min as described previously [19]. The reaction mixture contained 50 mM HEPES buffer, pH 8.0, 50  $\mu$ M non-radioactive ATP or [ $\gamma$ -<sup>32</sup>P]ATP, 1 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 0.2 mM CaCl<sub>2</sub>, 0.1 mM EGTA, 2  $\mu$ M calmodulin, 1 mM DTT, 2  $\mu$ M okadaic acid, 10 mM NaF, 1 mM orthovanadate, protease inhibitors (10  $\mu$ g/ml each of antipain, leupeptin, and pepstatin A), and a suitable amount of CaM kinase II in a total volume of 50  $\mu$ l. Under these conditions, Thr-286 and Thr-287 in  $\alpha$  and  $\beta$  isoforms, respectively, are almost specifically autophosphorylated, although there are some different autophosphorylation sites [21].

### 2.4. Preparation of PSD-CaM kinase II complex

The PSD fraction was prepared from rat forebrain by the methods of Wu et al. [22] as modified by Suzuki et al. [23].

The PSD fraction (about 30  $\mu$ g protein) was added to an autophosphorylated reaction mixture in a total volume of 60  $\mu$ l. After incubation for 10 min at 0°C, the reaction mixtures were centrifuged at 18 000  $\times$  g for 30 min at 0°C. The precipitate was washed once with

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**Abbreviations:** CaM kinase II, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; PSD, postsynaptic density; LTP, long-term potentiation; DTT, dithiothreitol; EGTA, ethylene glycol bis( $\beta$ -aminoethylether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PP1, PP2A, PP2B, PP2C, protein phosphatase 1, 2A, 2B and 2C, respectively; NMDA, *N*-methyl-D-aspartic acid; AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionate

200  $\mu$ l of 40 mM Tris-HCl pH 7.6, containing 1 mM DTT, 50 mM NaCl, 10% ethyleneglycol, and 0.05% Tween 40, and centrifuged at 18 000  $\times g$  for 30 min. The precipitate was then suspended in the same buffer and used as PSD-CaM kinase II complex.

### 2.5. Dephosphorylation of CaM kinase II from its PSD complex

CaM kinase II was autophosphorylated with [ $\gamma$ - $^{32}$ P]ATP to prepare PSD- $^{32}$ P-labeled CaM kinase II complex as described above. The  $^{32}$ P-labeled complex was incubated at 30°C in solution A (40 mM Tris buffer, pH 7.6, containing 1 mM DTT, 50 mM NaCl, 10% ethyleneglycol, 0.05% Tween 40 and protease inhibitors (10  $\mu$ g/ml each of antipain, leupeptin, and pepstatin A)). After the incubation, an aliquot of the reaction mixture was withdrawn and radioactivity of  $^{32}$ P-labeled CaM kinase II was measured by P-cellulose filter method [24]. For analysis of dephosphorylation by SDS-PAGE, an aliquot of the reaction mixture was subjected to SDS-PAGE, and radioactive bands were detected by autoradiography. The level of radioactivity was measured by an imaging analyzer BAS 1500 Mac.

### 2.6. Release of CaM kinase II from its complex with PSDs

PSD-CaM kinase II complex was incubated at 30°C in solution A. After an appropriate incubation, reactions were terminated by adding okadaic acid in a final concentration of 1  $\mu$ M, and then centrifuged at 36 000  $\times g$  for 30 min at 4°C. To avoid the incomplete precipitation of PSDs, the force of centrifugation was 2-fold stronger than that for isolation of PSD-CaM kinase II complex. CaM kinase II activity in the supernatant was measured.

### 2.7. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described previously [25,26] with a slight modification. The first dimensional gel was made in a glass tube (2.6  $\times$  180 mm), the second dimensional gel in a standard slab-gel apparatus (20  $\times$  13 cm).

### 2.8. Gel overlay method

The PSD protein was separated by two-dimensional gel electrophoresis, electrophoretically transferred to a PVDF membrane at 22 V in 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS) buffer, pH 11.0, containing 10% methanol, at 4°C overnight, and then the membrane was blocked with 5% non-fat skim milk in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.05% Tween 20. The transblots were incubated with  $^{32}$ P-labeled CaM kinase II for 3 h at room temperature with constant rocking in rinse buffer. The membrane was extensively washed with at least six changes of Tris-HCl buffer, pH 7.4, containing 200 mM NaCl and 0.2% Triton X-100. Radioactive spots were detected by autoradiography and quantified by an imaging analyzer.

## 3. Results and discussion

Since we demonstrated that PSDs bind to autophosphorylated CaM kinase II [19], a gel overlay assay in two-dimensional gel electrophoresis was developed to detect proteins capable of binding to the kinase. CaM kinase II was autophosphorylated with [ $\gamma$ - $^{32}$ P]ATP and incubated with PSD proteins separated by two-dimensional gel electrophoresis, and then transferred to a PVDF membrane. CaM kinase II was targeted to two sites using gel overlay method as shown in Fig. 1A. The major spot had a molecular mass of about 170 kDa and an isoelectric point of about pH 6. This protein contained 60–65% radioactivity, and corresponded to the protein staining spot of about 170 kDa shown by the arrowhead in Fig. 1B. The minor binding protein had a molecular mass of about 140 kDa and an isoelectric point of about pH 5.8. This protein contained about 35–40% radioactivity, but did not correspond to the protein staining spot in Fig. 1B.

Protein phosphatases responsible for dephosphorylation of CaM kinase II associated with PSDs were investigated using phosphatase inhibitors and activators (Fig. 2). PSD- $^{32}$ P-labeled CaM kinase II complex was incubated at 30°C for

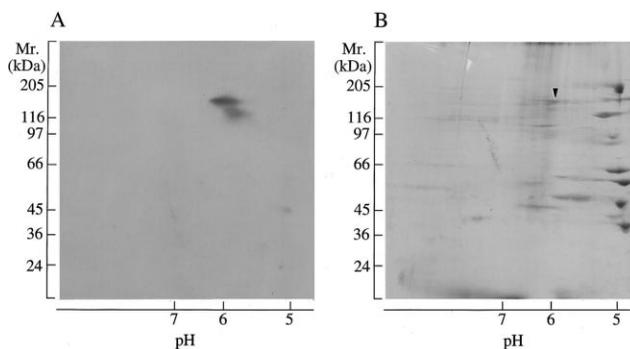


Fig. 1. Detection of proteins capable of binding to autophosphorylated CaM kinase II by gel overlay assay in two-dimensional gel electrophoresis. A: Gel overlay assay. PSD protein (200  $\mu$ g) was separated by two-dimensional gel electrophoresis, and electrophoretically transferred to a PVDF membrane. The membrane was incubated with  $^{32}$ P-labeled CaM kinase II ( $5 \times 10^4$  cpm) and detected by autoradiography as described in Section 2. B: Protein staining. PSD protein (200  $\mu$ g) was separated by two-dimensional gel electrophoresis, and stained by Coomassie brilliant blue. Arrowhead: protein corresponding to the major radioactive spot in A. The pH gradient in the isoelectric focusing gels was measured after serial gel sections were incubated in distilled water. Myosin heavy chain (205 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), and trypsinogen A (24 kDa) were used as references. Data are representative of at least three experiments.

30 min, and then dephosphorylation was determined. An endogenous protein phosphatase in PSDs dephosphorylated CaM kinase II to about 50%. Dephosphorylation was stimulated by the addition of PP1, but not PP2A. Dephosphorylation was completely inhibited in the presence of protein phosphatase inhibitor 2, a specific inhibitor of PP1, and microcystin-LR, an inhibitor of PP1 and PP2A. Okadaic acid, an inhibitor of PP1 and PP2A, also inhibited dephosphorylation at 0.1  $\mu$ M, but to a lesser extent than microcystin-LR. These results indicate that PP1, not PP2A is involved in dephosphorylation of CaM kinase II, consistent with the view that the inhibition by okadaic acid of PP1 is weaker than that of PP2A [27]. The dephosphorylation was not stimulated in the presence of  $\text{Ca}^{2+}$  and calmodulin, activators of PP2B, and  $\text{Mg}^{2+}$ , an activator of PP2C (data not shown).  $\text{Mn}^{2+}$  and poly-L-lysine, activators of recently identified CaM kinase II phosphatase [28], did not stimulate the dephosphorylation, indicating that PP2B, PP2C and CaM kinase II phosphatase are not involved in the dephosphorylation of the kinase.

To confirm dephosphorylation of CaM kinase II, the reaction mixtures were analyzed by SDS-PAGE. When PSD- $^{32}$ P-labeled CaM kinase II complex was incubated, dephosphorylation of the  $\alpha$  and  $\beta$  isoforms of the kinase (50 and 60 kDa bands, respectively) was observed depending on the incubation time as shown in Fig. 3A. Dephosphorylation of CaM kinase II was further stimulated by adding purified PP1, and completely inhibited in the presence of microcystin-LR and protein phosphatase inhibitor 2. Radioactivity of the  $\alpha$  and  $\beta$  isoforms was reduced about 40% and 50% at 15 min of incubation, respectively (Fig. 3B). The phosphorylation level of the  $\beta$  isoform was significantly higher compared to the ratio of the  $\alpha$  and  $\beta$  isoform proteins in the forebrain, suggesting that binding affinity of the  $\beta$  isoform was higher than that of the  $\alpha$  isoform. These results indicated that autophos-

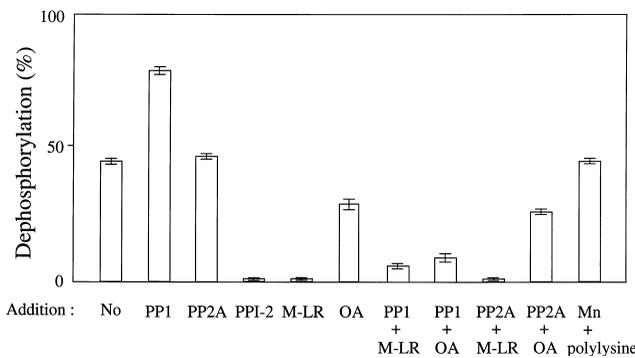


Fig. 2. Effects of protein phosphatases and their inhibitors or activators on dephosphorylation of CaM kinase II from its PSD complex. PSD-<sup>32</sup>P-labeled CaM kinase II complex (25 µg of protein, 5 × 10<sup>4</sup> cpm) was incubated in the presence or absence of PP1 (1 mU/ml), PP2A (5 mU/ml), protein phosphatase inhibitor 2 (2 µg) (PPI-2), microcystin-LR (M-LR) (0.1 µM), okadaic acid (OA) (0.1 µM), MnCl<sub>2</sub> (Mn) (2 mM), and/or poly-L-lysine (polylysine) (50 µg/ml) at 30°C. After incubation for 30 min, an aliquot of the reaction mixture was withdrawn and the radioactivity released was measured as described in Section 2.

phorylated CaM kinase II translocated to PSDs was dephosphorylated by PP1.

Since Ca<sup>2+</sup>-independent activity induced by autophosphorylation of the kinase is retained in the PSD-CaM kinase II complex, effects of protein phosphatases and their activators or inhibitors on the reduction of Ca<sup>2+</sup>-independent activity of the PSD complex were examined. When the complex was incubated at 30°C, Ca<sup>2+</sup>-independent activity was reduced by incubation as shown in Fig. 4A. The activity was further reduced by the addition of PP1, but not PP2A. The activity was retained in the presence of protein phosphatase inhibitor 2 and microcystin-LR. Okadaic acid was also effective in maintaining the activity to some extent. The remaining Ca<sup>2+</sup>-independent activity was less than 100%, because of the thermal instability of the autophosphorylated kinase [21]. These results indicated that the Ca<sup>2+</sup>-independent activity of the kinase was reduced by dephosphorylation. When the reaction mixture was subjected to autophosphorylating conditions after the reduction of the activity, the activity was restored (data not shown), also indicating that the decrease in Ca<sup>2+</sup>-independent activity was due to dephosphorylation.

The release of the kinase from the complex by PP1 was examined. PSD-CaM kinase II complex was incubated at 30°C in the presence or absence of PP1 and microcystin-LR, and then centrifuged. The kinase activity of the supernatant increased depending on the incubation time, and reached almost a maximum at 15 min (Fig. 4B), indicating that the release from the complex was slow as compared to its formation [19]. CaM kinase II activity further increased on the addition of PP1 as shown in Fig. 4B. The activity was not released in the presence of microcystin-LR. CaM kinase II was not released from control PSDs at all. The extent of release was significantly low as compared to the extent of dephosphorylation. These results indicated that CaM kinase II was released from the complex after its dephosphorylation by PP1 and that the dissociation of kinase from the complex took place slowly as compared to the formation of complex.

Autophosphorylation of CaM kinase II induces the translocation of the cytosolic kinase to PSDs, and dephosphorylation of the kinase induces its dissociation from PSDs [19,20].

In the present study, we investigated a protein phosphatase responsible for dephosphorylation of autophosphorylated CaM kinase II using protein phosphatase inhibitors and activators. It has been reported that purified PP1, PP2A, and PP2C dephosphorylate Thr-286 in vitro, and that PP1 activity is enriched in the P2 fraction (cytoskeletal fraction) while PP2A and PP2C are enriched in the soluble fraction [29]. Here, we demonstrated that PP1 is involved in dephosphorylation of autophosphorylated CaM kinase II associated with PSDs, and the kinase was gradually released from PSDs.

We demonstrated that CaM kinase II was targeted to two sites using the gel overlay method. The major spot had a molecular mass of about 170 kDa and an isoelectric point of about pH 6. McNeill and Colbran have reported that <sup>32</sup>P-labeled CaM kinase II binds to p190 and p140, but the extent of binding of CaM kinase II to PSDs was not shown [30]. Strack and Colbran reported recently that the NR2B subunit of the NMDA receptor directly and specifically interacted with autophosphorylated CaM kinase II α isoform [31]. We confirmed that the 170 kDa protein reacted with an antibody against the NR2B subunit of the NMDA receptor by immunoblotting using one-dimensional SDS-PAGE (data not shown). Taken together, the major binding protein is thought to correspond to the NR2B subunit of the NMDA receptor.

In neurons, autophosphorylation of Thr-286 of the CaM kinase II α isoform in response to Ca<sup>2+</sup> or to increased synaptic activity may be important in synaptic plasticity. It renders the kinase persistently active and causes a translocation

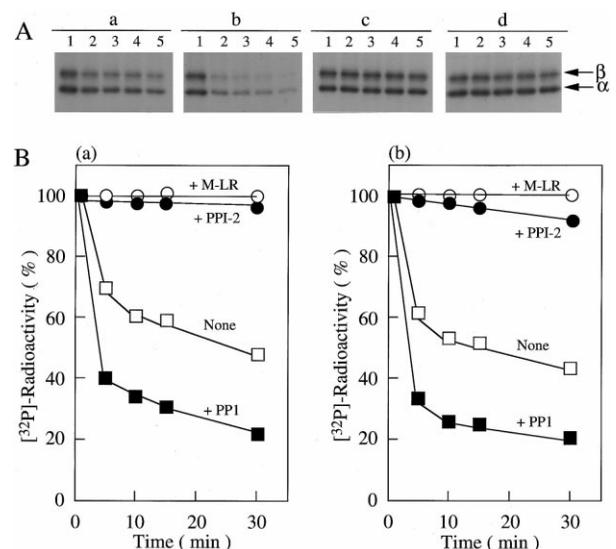


Fig. 3. Dephosphorylation of CaM kinase II from its PSD complex. PSD-<sup>32</sup>P-labeled CaM kinase II complex (25 µg of protein, 5 × 10<sup>4</sup> cpm) was incubated in the absence or presence of PP1 (1 mU/ml), microcystin-LR (0.1 µM), or protein phosphatase inhibitor 2 (2 µg) at 30°C. After incubation, an aliquot of the reaction mixture was withdrawn and subjected to SDS-PAGE. The radioactive bands were detected by autoradiography or an imaging analyzer. A: Autoradiography: panel a, no addition; panel b, in the presence of PP1; panel c, in the presence of microcystin-LR; panel d, in the presence of protein phosphatase inhibitor 2. Lanes 1–5; 0, 5, 10, 15, 30 min incubation, respectively. Upper and lower arrows show the positions of β and α isoforms, respectively. B: Changes in the level of phosphorylation of α and β isoforms measured by an imaging analyzer: panel a, α isoform; panel b, β isoform, in the absence (none) or presence of microcystin-LR (M-LR), PP1, and protein phosphatase inhibitor 2 (PPI-2), as indicated.

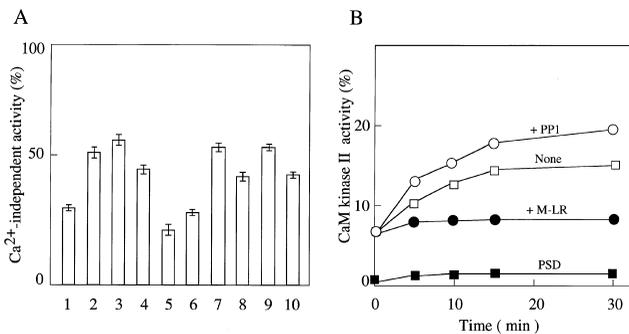


Fig. 4. Effect of protein phosphatases and their inhibitors on Ca<sup>2+</sup>-independent activity and on the release of the CaM kinase II activity. PSD-CaM kinase II complex was incubated in the absence or presence of protein phosphatase inhibitor 2 (2 µg), PP1 (1 mU/ml), PP2A (5 mU/ml), microcystin-LR (0.1 µM), and/or okadaic acid (0.1 µM) at 30°C. A: After incubation for 15 min, an aliquot of the reaction mixture was withdrawn and Ca<sup>2+</sup>-independent activity was measured as described in Section 2. 1, no addition; 2, in the presence of protein phosphatase inhibitor 2; 3, in the presence of microcystin-LR; 4, in the presence of okadaic acid; 5, in the presence of PP1; 6, in the presence of PP2A; 7, in the presence of PP1+microcystin-LR; 8, in the presence of PP1+okadaic acid; 9, in the presence of PP2A+microcystin-LR; 10, in the presence of PP1+okadaic acid. Data were expressed as a percentage compared to that of PSD-CaM kinase II complex without incubation (100%). B: At the indicated time, release of CaM kinase II activity was measured as described in Section 2. Data were expressed as a percentage compared to the original activity of PSD-CaM kinase II complex (100%).

of cytosolic CaM kinase II to the PSDs. Autophosphorylation of postsynaptic CaM kinase II is known to be necessary for NMDA receptor-dependent LTP [9,32]. PSD-CaM kinase II complex phosphorylates more than 15 PSD proteins (unpublished data) including glutamate receptors. Phosphorylation of PSD proteins may provide a mechanism for increased synaptic strength during LTP. Activated CaM kinase II may be dephosphorylated by PSD-associated PP1. However, the dephosphorylated kinase may be retained for a short time at PSDs, perhaps the NR2B subunit of the NMDA receptor, since the dissociation of the kinase from PSDs occurs slowly as compared to its association. The kinase may be re-autophosphorylated and further activated by the next NMDA receptor activation. Thus, the prolonged activation of CaM kinase II may be involved in LTP. When NMDA receptor activation does not continue for some while, the activity of PP1 in PSDs may regulate the translocation of CaM kinase II from cytosol to PSDs and synaptic plasticity.

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