# Post-synaptic density-95 promotes calcium/calmodulin-dependent protein kinase II-mediated Ser$^{847}$ phosphorylation of neuronal nitric oxide synthase

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Post-synaptic density-95 (PSD-95) is a neuronal scaffolding protein that associates with *N*-methyl-D-aspartate (NMDA) receptors and links them to intracellular signalling molecules. In neurons, neuronal nitric oxide synthase (nNOS) binds selectively to the second PDZ domain (PDZ2) of PSD-95, thereby exhibiting physiological activation triggered via NMDA receptors. We have demonstrated previously that $Ca^{2+}$/calmodulin-dependent protein kinase II$\alpha$ (CaM-K II$\alpha$) directly phosphorylates nNOS at residue Ser$^{847}$, and can attenuate the catalytic activity of the enzyme in neuronal cells [Komeima, Hayashi, Naito and Watanabe (2000) J. Biol. Chem. **275**, 28139–28143]. In the present study, we examined how CaM-K II participates in the phosphorylation by analysing the functional interaction between nNOS and PSD-95 in cells. The results showed that PSD-95 directly promotes the nNOS phosphorylation at Ser$^{847}$ induced by endogenous CaM-K II. In transfected cells, this effect of PSD-95 required its dual palmitoylation and the PDZ2 domain, but did not rely on its guanylate kinase domain. CaM-K I$\alpha$ and CaM-K IV failed to phosphorylate nNOS at Ser$^{847}$ in transfected cells. Thus PSD-95 mediates cellular trafficking of nNOS, and may be required for the efficient phosphorylation of nNOS at Ser$^{847}$ by CaM-K II in neuronal cells.

Key words: calmodulin-dependent protein kinase II, cellular trafficking, KN-93, neuronal nitric oxide synthase, PDZ domain, PSD-95.

## INTRODUCTION

Many of the intracellular actions of $Ca^{2+}$ in eukaryotic cells are mediated by the family of $Ca^{2+}$/calmodulin (CaM)-dependent protein kinases [1,2]. $Ca^{2+}$/CaM-dependent protein kinase II (CaM-K II) is a broad-specificity enzyme with central roles in synaptic plasticity, learning and memory [3]. Although an abundant soluble protein in brain, it is concentrated in post-synaptic densities (PSDs) [4], which are cytoskeletal 'scaffolds' for the *N*-methyl-D-aspartate (NMDA) receptors and their regulators [5,6], and translocates to PSDs following synaptic stimulation [7,8]. The NMDA receptor and Densin-180, a transmembrane protein tightly associated with PSD, have been identified as target proteins for CaM-K II [9–13].

Neuronal nitric-oxide synthase (nNOS) is also a $Ca^{2+}$/CaM-dependent enzyme, which catalyses the oxidation of L-arginine to generate nitric oxide and L-citrulline [14]. NO, formed by nNOS, has major signalling functions in the central and peripheral nervous systems [15,16]. In neurons, nNOS is associated with cell membranes in association with PSD-95 [17], a major protein constituent of PSD that has an activity in clustering *Shaker*-type $K^+$ channels [18] and NMDA receptor subunits 2A and 2B [19]. PSD-95 mediates the interaction of the tyrosine kinase Fyn with NMDA receptor subunit 2A, this resulting in a promotion of Fyn-mediated tyrosine phosphorylation of the subunit [20], and is required for the efficient coupling of NMDA receptor activity to NO toxicity [21]. A ternary complex containing nNOS, PSD-95 and NMDA receptor subunit 2B has been identified in the brain [22]. It has been established that nNOS is phosphorylated by CaM-K II [23,24], and is directly phosphorylated at Ser$^{847}$ by CaM-K II$\alpha$, leading to a reduction in its enzyme activity [25,26]. It was recently shown that the Ser$^{847}$ phosphorylation of nNOS, blocked by the CaM-kinase inhibitor KN-93, occurs in rat hippocampus after transient forebrain ischaemia [27]. Co-localization of CaM-K II, nNOS and PSD-95 in PSDs could be important in regulating CaM-K II phosphorylation of nNOS. We therefore examined whether or not the phosphorylation of nNOS at Ser$^{847}$ is regulated by PSD-95.

In the present paper, we document evidence that PSD-95 is important for Ser$^{847}$ phosphorylation of nNOS and that CaM-K II, but not CaM-K I or CaM-K IV, is involved. These findings imply that a dynamic molecular composition mediated by PSD-95 is important for the phosphorylation of nNOS at Ser$^{847}$ by CaM-K II in cells.

## EXPERIMENTAL

### Materials

The cDNA for rat brain nNOS was generously given by Dr Solomon H. Snyder (Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.) [14]. Restriction enzymes and DNA-modifying enzymes were obtained from Takara Shuzo (Osaka, Japan). Electrophoresis reagents were products of Bio-Rad. All other materials and reagents were of the highest quality available from commercial suppliers.

Abbreviations used: CaM, calmodulin; CaM-K I$\alpha$/II$\alpha$/IV, $Ca^{2+}$/CaM-dependent protein kinases I$\alpha$, II$\alpha$ and IV respectively; CaM-KK, $Ca^{2+}$/CaM-dependent protein kinase kinase; CREB, cAMP-response-element-binding protein; GST, glutathione S-transferase; HA, haemagglutinin; mAb, monoclonal antibody; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; pAb, polyclonal antibody; PSD-95, post-synaptic density-95; PDZ, PSD-95/Dlg/ZO-1 homology; T286A, etc., a mutation bearing a replacement of Thr$^{286}$ with alanine, etc.

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### Antibodies

The rabbit polyclonal antibody (pAb) NP847, recognizing phosphorylation at $Ser^{847}$ on nNOS, was prepared as described previously [25]. The anti-(PSD-95) pAb was generated by immunization of rabbit with recombinant glutathione S-transferase (GST)–PSD-95-(256–432). The rabbit anti-CREB (cAMP-response-element-binding protein) and phospho-CREB (phosphorylated at $Ser^{133}$) pAbs were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). The mouse anti-(CaM-K II$\alpha$) monoclonal antibody (mAb) used in the present study was obtained from Oncogene (Cambridge, MA, U.S.A.), whereas a mouse anti-(CaM-K IV) mAb was from Transduction Laboratories, Inc. (San Diego, CA, U.S.A.) The rabbit anti-(CaM-K) I pAb was kindly provided by Dr Angus C. Nairn (Yale University School of Medicine, New Haven, CT, U.S.A.) [28].

### Plasmid construction

The plasmids pME18s-PSD-95 and pME18s-nNOS were generated as described previously [20,26]. The pME18s-FLAG-tagged nNOS was constructed as follows. The initial 500 nt section of nNOS (from the ATG start codon to the *Nco*I restriction site, containing an *Apa*I site) was generated by digestion of the nNOS cDNA with *Nco*I and filling- in. The fragment was ligated to the pME18s-FLAG vector [29], digested with *Eco*RI and filled in. The *Apa*I/*Not*I fragment of nNOS, containing the stop codon, was ligated into the plasmid obtained as described above. Plasmids encoding PSD-95 mutants with C-terminal or internal deletions were generated as described previously [20]. These cDNAs were then cloned into pME18s. For GST fusion protein production, the PSD-95 cDNA fragments encoding the proteins PSD-95/Dlg/ZO-1 homology 1 (PDZ1; $Asn^{55}$–$Lys^{157}$), PDZ2 ($Val^{158}$–$Tyr^{255}$) and PDZ1 + 2 ($Asn^{55}$–$Tyr^{255}$) were cloned in-frame into pGEX-3X vector (Amersham Biosciences, Piscataway, NJ, U.S.A.). The mutants CaM-K II$\alpha$ $Thr^{286} \rightarrow$ Ala (T286A) and K42A, and PSD-95 C3,5S (i.e. a mutant bearing replacements of both $Cys^3$ and $Cys^5$ with Ser) were subcloned into pME18s. CaM-K I$\alpha$, CaM-K II$\alpha$, CaM-K IV and $Ca^{2+}$/CaM-dependent protein kinase kinase (CaM-KK) were cloned from a rat brain cDNA library, and the cDNAs were then cloned into pME18s. The constitutively active construct, pME18s-CaM-KK-(1–434), was generated as described previously [30]. Haemagglutinin (HA)–CaM-K IV was subcloned into pME18s. The nucleotide sequence of each mutant was confirmed.

### Cell culture, transfection and stimulation

HEK-293 cells were maintained in Dulbecco's modified Eagle medium containing 10 % (v/v) fetal-calf serum and subcultured in 6 cm dishes for 24 h. They were then transfected with plasmid pME18s containing FLAG-tagged nNOS (0.3 $\mu$g) alone, together with either wild-type (3 $\mu$g) or a series of PSD-95 constructs (3 $\mu$g) using LIPOFECTAMINE™ Plus method (Life Technologies, Inc.). After 36–48 h incubation, the cells were incubated for 30 min in serum-free medium, before an additional 30 min incubation in the same medium alone or with 5 $\mu$M KN-93 (Seikagaku, Tokyo, Japan). The cells were then stimulated with 10 $\mu$M A23187 (Sigma) for 3.5 min. For the determination of exogenous CaM kinases that phosphorylate $Ser^{847}$ of nNOS, the cells were transfected with pME18s plasmid containing nNOS (0.3 $\mu$g) and wild-type PSD-95 (3 $\mu$g) or nNOS (0.3 $\mu$g) and $\Delta$2 PSD-95 (3 $\mu$g), with each combination containing CaM-KK-(1–434) (0.075 $\mu$g), CaM-K I$\alpha$ (0.15 $\mu$g)/CaM-KK-(1–434) (0.075 $\mu$g), CaM-K II$\alpha$ (0.15 $\mu$g), HA–CaM-K IV (0.15 $\mu$g)/CaM-KK-(1–434) (0.075 $\mu$g), CaM-K II$\alpha$ T286D (0.15 $\mu$g) or CaM-K II$\alpha$ K42A (0.15 $\mu$g). The amount of DNA transfected was adjusted in each experiment by using a control expression vector, pME18s.

### Preparation of lysates, purification of nNOS and immunoprecipitation

For preparation of lysates from HEK-293 cells, cells were sonicated with 0.3 ml of TNE buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM PMSF/10 $\mu$g/ml leupeptin/1 $\mu$g/ml pepstatin A/1 $\mu$g/ml aprotinin/1 mM sodium orthovanadate/50 mM sodium fluoride/2 mM sodium pyrophosphate/1 % Nonidet P40]. After centrifugation at 15 000 ***g*** for 15 min, a 10 $\mu$l ADP agarose gel (50 % slurry; Sigma) was added to the supernatant, and the mixture was incubated for 1 h at 4 °C. After precipitation of the resin by centrifugation and removal of the supernatant, the resin was washed three times with 300 $\mu$l of TNE buffer, and nNOS was eluted with 40 $\mu$l of elution buffer (TNE buffer containing 10 mM NADPH). For immunoprecipitation of FLAG-tagged nNOS, 15 $\mu$l of an anti-FLAG M2-agarose affinity gel (50 % slurry; Sigma) was added to the supernatant, and the mixture was incubated for 1 h at 4 °C. After precipitation by centrifugation and removal of the supernatant, the resin was washed three times with 300 $\mu$l of TNE buffer and boiled with 50 $\mu$l of SDS/PAGE sample buffer. For immunoprecipitation using a PSD-95 pAb, lysates were pre-cleared by centrifugation with an excess amount of Protein G–Sepharose (Amersham Biosciences), and then incubated with 2 $\mu$g of the antibody on ice for 1 h. Immune complexes were collected on Protein G Sepharose (15 $\mu$l) and washed three times with TNE buffer.

### Immunocytochemistry and immunoblotting

Cells were fixed with 4 % (w/v) formaldehyde in PBS for 10 min, followed by treatment with cooled methanol at –20 °C for 10 min. For double immunostaining, cells were incubated with a mixture of a mouse anti-nNOS mAb and NP847 pAb, each diluted 1:50 in PBS at 4 °C overnight. The immunoreactivity was visualized by incubation with Alexa 488-conjugated anti-rabbit IgG diluted 1:200 and Alexa 594-conjugated anti-mouse IgG diluted 1:500 (Molecular Probes, Inc.) and the samples were examined under an epifluorescence microscope (Nikon TE300). Images were acquired through a charged-couple device camera using Metamorph software (Universal Imaging). Immunoprecipitates or lysates were resolved by SDS/PAGE and transferred to PVDF membranes (Bio-Rad), which were then blocked and probed with appropriate antibodies. Finally, the membranes were developed with the ECL® system, and band intensities were quantified by densitometric scanning (LumiVision PRO; Aisin Seiki, Aichi, Japan) using the LumiVision Imager program (Aisin Seiki). Calibration was performed using various amounts of recombinant nNOS as the control. For the calibration of densitometric quantification, 19–75 ng of stoichiometrically phosphorylated nNOS at $Ser^{847}$ and 75–600 ng of nNOS were immunoblotted with NP847 and anti-nNOS antibodies respectively. Band intensities were then analysed by densitometric scanning to show that these were in the linear range when densitometric ratio was determined.

### GST fusion protein chromatography

Purified GST fusion proteins (10 $\mu$g) bound to glutathione–Sepharose (Amersham Biosciences) were incubated in 50 $\mu$l of TNE buffer with 1.5 $\mu$g of nNOS purified from *Escherichia coli* for 1 h at 4 °C. Bound proteins were separated by centrifugation and washed with TNE buffer.

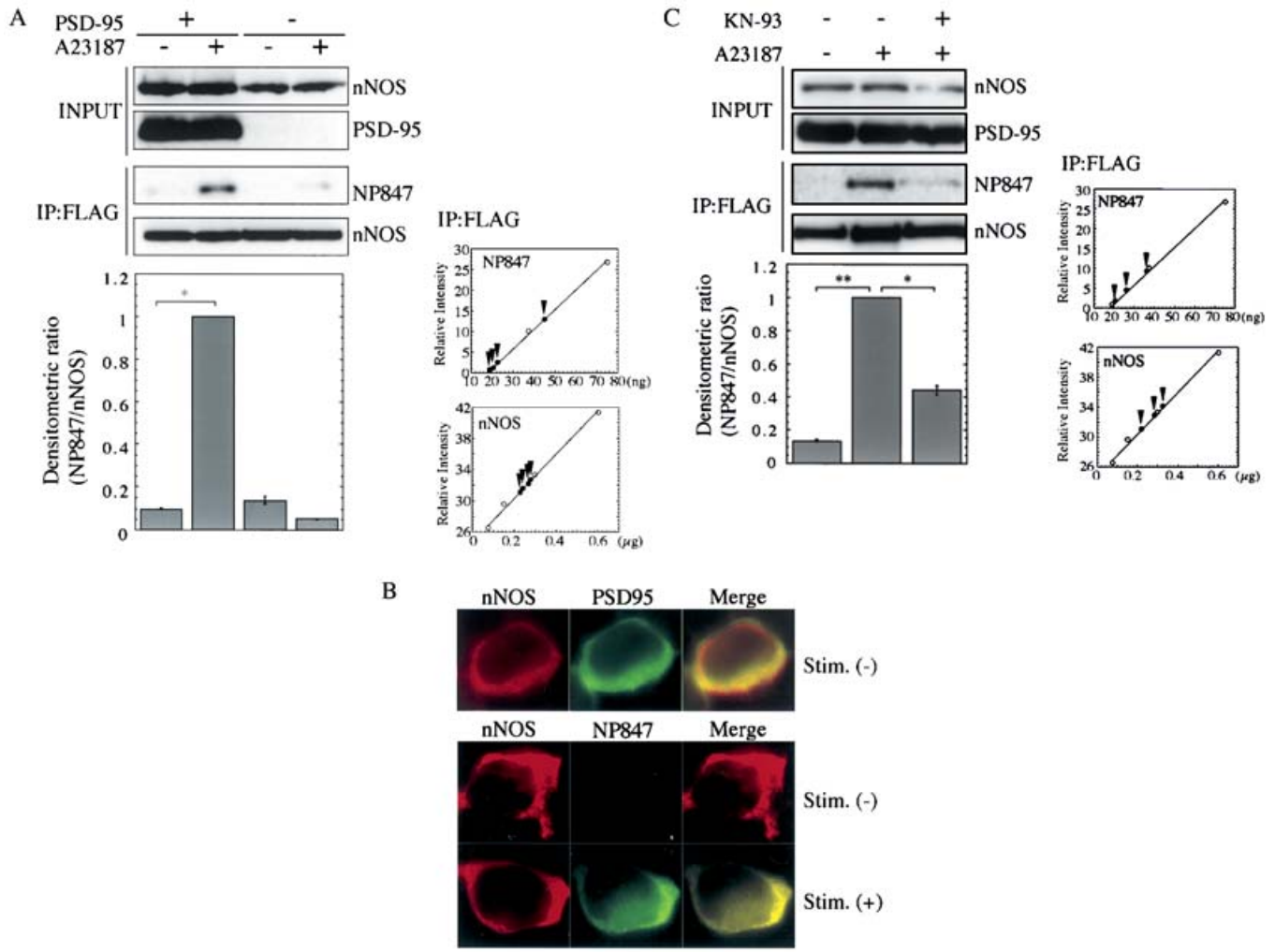


**Figure 1 Promotion of endogenous CaM-K-mediated phosphorylation of nNOS at Ser$^{847}$ by PSD-95 in transfected cells**

(**A**) Immunoblot analysis of $Ca^{2+}$-induced nNOS phosphorylation at Ser$^{847}$ in HEK-293 cells co-expressing PSD-95. HEK-293 cells expressing the FLAG-tagged nNOS alone or together with PSD-95 were stimulated with buffer alone ( $-$ ) or 10 $\mu$M A23187 (+) for 3.5 min. Anti-FLAG immunoprecipitates from lysates of the cells were immunoblotted with anti-nNOS antibody (nNOS) or NP847. The histogram shows the amounts of immunoreactive NP847 relative to that of nNOS in the membranes. The means $\pm$ S.E.M. for three experiments using three independent transfections are shown; asterisks represent significant differences ($^*P < 0.01$). Input, 30 $\mu$g of lysates in each reaction. The calibration of densitometric intensities was performed as described in the Experimental section. The right panels show that the intensities of NP847- and nNOS-immunoreactive bands (indicated by the arrowheads) in the anti-FLAG immunoprecipitates were in the linear range. (**B**) Immunostaining analysis showing $Ca^{2+}$-induced nNOS phosphorylation at Ser$^{847}$ in HEK-293 cells co-expressing PSD-95. HEK-293 cells transfected with FLAG-tagged nNOS and PSD-95 were stimulated with buffer alone ( $-$ ) or 10 $\mu$M A23187 (+) for 3.5 min. The cells were then double-immunostained with anti-nNOS (shown by the red fluorescence) and NP847 (shown by the green fluorescence) antibodies, or anti-(PSD-95) (green fluorescence). The panels in the third column (labelled 'Merge') show the overlaid image. Note that the nNOS-positive neuron contained PSD-95. (**C**) Immunoblot analysis showing the effects of KN93 on the $Ca^{2+}$-induced phosphorylation of nNOS at Ser$^{847}$. HEK-293 cells expressing FLAG-tagged nNOS and PSD-95 were pre-incubated for 30 min with buffer alone or 5 $\mu$M KN93, and stimulated with buffer alone ( $-$ ) or 10 $\mu$M A23187 (+) for 3.5 min. Anti-FLAG immunoprecipitates from lysates of the cells were immunoblotted with anti-nNOS antibody or NP847. The histogram shows the amounts of immunoreactive NP847 relative to that of nNOS in the membranes. The means $\pm$ S.E.M. for three experiments using three independent transfections are shown; asterisks represent significant differences ($^*P < 0.05$; $^{**}P < 0.005$). Input, 30 $\mu$g of lysates in each reaction. The calibration of densitometric intensities was performed as described in the Experimental section. The right panels show that the intensities of NP847- and nNOS-immunoreactive bands (shown by the arrowheads) in the anti-FLAG immunoprecipitates (IP) were in the linear range.

## RESULTS

### Promotion of endogenous CaM-K-mediated phosphorylation of nNOS at Ser$^{847}$ by PSD-95 in transfected cells

We examined the effect of PSD-95 on the phosphorylation of nNOS at Ser$^{847}$ in HEK-293 cells possessing CaM-K IV and undetectable levels of CaM-K I and CaM-K II$\alpha$, as determined by immunoblotting (see Figure 2). It has been reported that these cells contain $\delta$, but neither $\alpha$ nor $\beta$, isoforms of CaM-K II [31]. We employed the endogenous CaM kinases, CaM-K II$\delta$ and/or CaM-K IV, in HEK-293 cells for a study of *in situ* phosphorylation of nNOS at Ser$^{847}$ using a pAb, NP847, which reacts with nNOS phosphorylated at Ser$^{847}$ [25]. For this purpose, we transfected HEK-293 cells with the FLAG-tagged nNOS either alone or together with PSD-95. The anti-FLAG immunoprecipitates from lysates of the cells were immunoblotted with NP847 either with or without $Ca^{2+}$ ionophore A23187 stimulation (10 $\mu$M) for 3.5 min. In the absence of stimulation, phosphorylation of nNOS at Ser$^{847}$ occurred only faintly, or was undetectable. A23187 treatment led to an increase in nNOS phosphorylation when PSD-95 was present (Figure 1A). Immunostaining analysis also detected $Ca^{2+}$-ionophore-induced phosphorylation at Ser$^{847}$ of nNOS in the

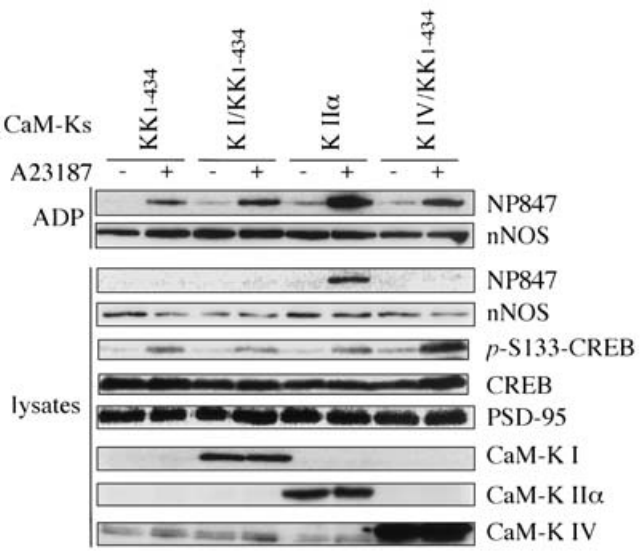


**Figure 2 Determination of CaM kinases that phosphorylate Ser$^{847}$ of nNOS in transfected cells by immunoblot analysis**

HEK-293 cells expressing nNOS and PSD-95 with CaM-KK-(1–434), CaM-K Iα/CaM-KK-(1–434), CaM-K IIα, or HA–CaM-K IV/CaM-KK-(1–434) were stimulated with buffer alone (−) or 10 μM A23187 for 3.5 min (+). Cell lysates were immunoblotted with anti-nNOS (nNOS), NP847, anti-CREB (CREB), anti-phospho-CREB (phosphorylated at Ser$^{133}$) (*p*-S133-CREB), anti-PSD-95 (PSD-95), anti-CaM-K I (CaM-K I), anti-CaM-K IIα (CaM-K IIα) or anti-CaM-K IV (CaM-K IV) antibodies. Notice the $Ca^{2+}$-induced phosphorylation of nNOS at Ser$^{847}$ observed with CaM-K IIα, but not with either CaM-K Iα or HA–CaM-K IV transfection, in cell lysates. $Ca^{2+}$-induced phosphorylation of CREB at Ser$^{133}$ by endogenous CaM-K IIδ and/or CaM-K IV, enhanced by overexpression of HA–CaM-K IV, is also evident. Expressed nNOS was also affinity-purified from transfected cells using the ADP-agarose chromatography technique (ADP). and subjected to immunoblotting with anti-nNOS (nNOS) and NP847. Note that A23187-induced Ser$^{847}$ phosphorylation was remarkably enhanced when CaM-K IIα was transfected. The data are representative of at least two independent experiments.

cytoplasm of the cells with co-expression of PSD-95 (Figure 1B). The presence of PSD-95 in nNOS-expressing cells was also confirmed by double immunostaining (Figure 1B). This was inhibited significantly by the CaM-kinase inhibitor, KN-93 (Figure 1C), at a concentration of 10 μM, indicating the phosphorylation at Ser$^{847}$ to be mediated by endogenous CaM-K IIδ and/or CaM-K IV.

### CaM-Ks that phosphorylate Ser$^{847}$ of nNOS *in situ*

Since Ser$^{847}$ of nNOS was phosphorylated by endogenous CaM-Ks in transfected cells (Figure 1), we examined further which enzymes were involved *in situ*. HEK-293 cells were transfected with CaM-K Iα/CaM-KK-(1–434), CaM-K IIα or HA–CaM-K IV/CaM-KK-(1–434), and lysates were immunoblotted with NP847 either with or without 10 μM A23187 stimulation for 3.5 min. In the absence of stimulation, no phosphorylation was detectable. Stimulation of the cells expressing nNOS, PSD-95 and CaM-K IIα with A23187 led to a remarkable increase in nNOS phosphorylation (Figure 2). However, nNOS was not phosphorylated at Ser$^{847}$ upon A23187 stimulation in cells expressing CaM-K Iα/CaM-KK-(1–434) or HA–CaM-K IV/CaM-KK-(1–434) or CaM-KK-(1–434) alone. Although the $Ca^{2+}$-stimulated endogenous CaM-kinase activity appears to be significant (Figures 1A and 1C), this was not detectable in cell lysates. Therefore we partially purified nNOS by ADP–agarose affinity chromatography from lysates to concentrate the enzyme, quantified the phosphorylation state at Ser$^{847}$ and expressed protein levels. In the absence of stimulation, phosphorylation of nNOS at Ser$^{847}$ was faint or undetectable. A23187 treatment led to an increase in the nNOS phosphorylation. A23187-induced nNOS phosphorylation at Ser$^{847}$ was enhanced remarkably when the cells were transfected with CaM-K IIα (Figure 2). To confirm that expressed CaM kinases were functionally active, we measured enzyme activity using syntide-2 as a substrate. Although an A23187-induced increase in CREB phosphorylation on Ser$^{133}$ was observed (Figure 2), CaM-kinase activities in cell lysates were too low to be analysed in non-transfected cells. Expression of CaM-Ks resulted in substantial $Ca^{2+}$/CaM-dependent activity (results not shown). A23187-induced CREB phosphorylation at Ser$^{133}$ was enhanced when the cells were transfected with HA–CaM-K IV/CaM-KK-(1–434) (Figure 2), indicating that expressed HA–CaM-K IV was functionally active in transfected cells. These data indicate that an endogenous CaM-K IIδ, but not CaM-K IV, is able to phosphorylate nNOS in HEK-293 cells expressing PSD-95.

### The domain of PSD-95 that is crucial for the promotion of Ser$^{847}$ phosphorylation of nNOS in transfected cells

To determine how PSD-95 promotes CaM-K II-mediated Ser$^{847}$ phosphorylation of nNOS, we constructed a series of C-terminal and internal-deletion mutants, and also a palmitoylation-deficient mutant, C3,5S [32], of PSD-95 (see Figure 6). It is known that nNOS interacts with the second PDZ motif (i.e. PDZ2) of PSD-95 [17]. We initially constructed fusion proteins containing PDZ1, PDZ2 or PDZ1 + PDZ2 of PSD-95 (see Figure 6), and evaluated the binding of each of these constructs to recombinant nNOS (Figure 3A). We found PDZ2 to be competent for binding, and this association was not enhanced when both PDZ1 and PDZ2 were included. We then examined whether PSD-95 PDZ2 binds to nNOS in HEK-293 cells transfected with FLAG-tagged nNOS and wild-type PSD-95 or PSD-95 mutants lacking the PDZ1 (Δ1) or PDZ2 (Δ2) domains. When probed with anti-(PSD-95) antibody, PSD-95 was co-precipitated with FLAG-tagged nNOS after transfection of wild-type or the Δ1 mutant, but not Δ2 mutant, of PSD-95 (Figure 3B). Conversely, FLAG-tagged nNOS was present in PSD-95 immunoprecipitates in the cells expressing wild-type or the Δ1 mutant, but not the Δ2 mutant, of PSD-95. These results indicate that PSD-95 PDZ2 interacts with nNOS in transfected cells, even though nNOS binding to the Δ1 mutant is weaker than to the wild-type PSD-95.

Finally, we examined the effects of a series of PSD-95 constructs on CaM-K II-mediated Ser$^{847}$ phosphorylation of nNOS. The Δ2 and C3,5S mutants failed to promote Ser$^{847}$ phosphorylation of nNOS (Figure 4), indicating that the PDZ2 domain and dual palmitoylation of PSD-95 are required for this phosphorylation process. In transfected cells, targeting of PSD-95 to an intracellular membrane compartment required its dual palmitoylation [32]. These observations suggest that PSD-95 directly promotes CaM-K II-mediated Ser$^{847}$ phosphorylation of nNOS through direct binding, and thereby the intracellular trafficking, of nNOS in cells.

### PSD-95 promotes exogenous CaM-K II-mediated phosphorylation of nNOS at Ser$^{847}$ in transfected cells

The above results demonstrate that PSD-95 promotes endogenous CaM-K II-mediated Ser$^{847}$ phosphorylation of nNOS. To test whether this phosphorylation is also promoted by PSD-95 in cells expressing exogenous CaM-K II, we transfected

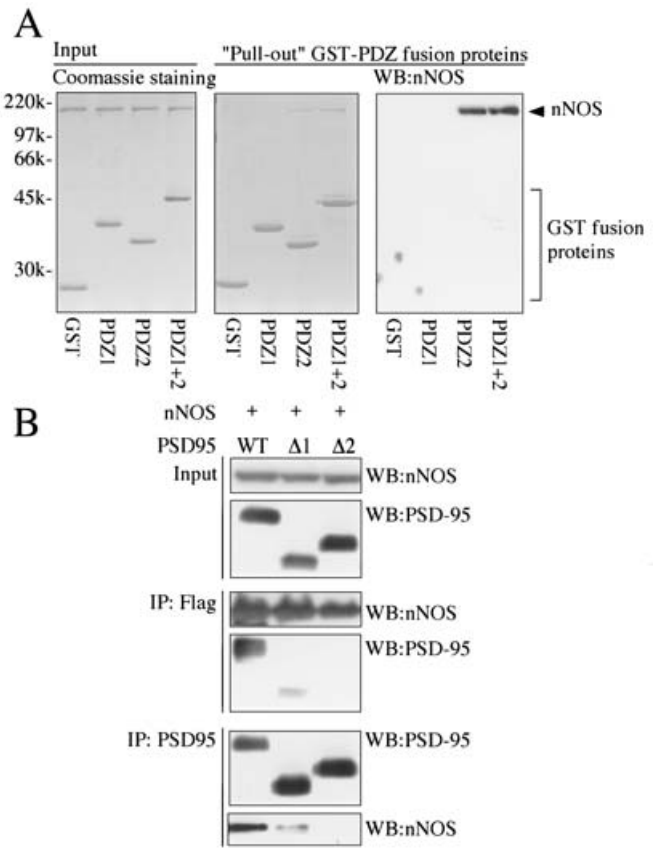


**Figure 3 Interaction of PSD-95 PDZ2 with nNOS *in vitro* and *in situ***

(**A**) GST-PDZ1, PDZ2 and PDZ1 + 2 fusion proteins bound to glutathione–Sepharose (10 $\mu$g of each) were incubated with 1.5 $\mu$g of nNOS purified from *E. coli*. After extensive washing, bound nNOS was eluted with SDS sample buffer, separated by SDS/PAGE and analysed either by Coomassie Brilliant Blue staining (middle panel) or by Western blotting (WB) with an antibody to nNOS (nNOS; right panel). Input, 0.15 $\mu$g of nNOS and 1 $\mu$g of each GST construct in each binding reaction. (**B**) HEK-293 cells were transfected with FLAG-tagged nNOS and either wild-type PSD-95 or PSD-95 lacking the PDZ1 ($\Delta$1) or the PDZ2 ($\Delta$2) domain. FLAG immunoprecipitates from the lysates were probed with anti-(PSD-95) antibody (PSD-95) (IP:Flag). Conversely, PSD-95 immunoprecipitates from the lysates were probed with anti-nNOS antibody (nNOS) (IP:PSD-95). Input, 30 $\mu$g of lysates in each binding reaction. The data are representative of at least three independent experiments.

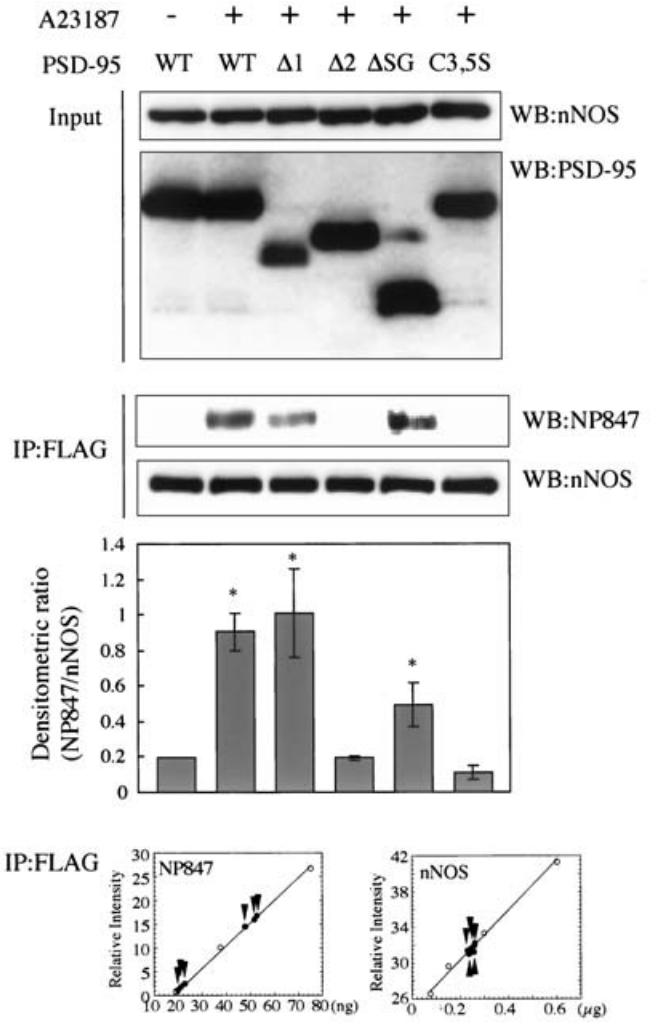


**Figure 4 Analysis of the domain of PSD-95 that is crucial for the promotion of Ser$^{847}$ phosphorylation of nNOS in transfected cells**

HEK-293 cells transfected with the FLAG-tagged nNOS with a series of PSD-95 constructs (see Figure 6) were stimulated with buffer alone (–) or 10 $\mu$M A23187 for 3.5 min (+), and anti-FLAG immunoprecipitates from lysates of the cells were immunoblotted with anti-nNOS antibody (nNOS) or NP847. Promotion of Ser$^{847}$ phosphorylation requires not only the PDZ2 domain, but also the palmitoylated N-terminus. The histogram shows the amounts of immunoreactive NP847 relative to that of nNOS in the membranes. The means $\pm$ S.E.M. for three experiments using three independent transfections are shown; asterisks represent significant differences ($^*P < 0.05$ compared with the buffer control). Input, 30 $\mu$g of lysates in each reaction. The calibration of densitometric intensities was performed as described in the Experimental section. The bottom panels show that the intensities of NP847- and nNOS-immunoreactive bands (shown by arrowheads) in the anti-FLAG immunoprecipitates were in the linear range.

HEK-293 cells with a constitutively active mutant of CaM-K II$\alpha$, CaM-K II$\alpha$ T286D [33], and wild-type PSD-95 or the $\Delta$2 mutant. The anti-FLAG immunoprecipitates from lysates of the cells were immunoblotted with NP847 without A23187 stimulation. The precipitates expressing the $\Delta$2 mutant showed a faint or undetectable phosphorylation of nNOS at Ser$^{847}$. Expression of wild-type PSD-95 led to a remarkable increase in nNOS phosphorylation (Figure 5). Cells expressing an inactive mutant of CaM-K II$\alpha$, CaM-K II$\alpha$ K42A (Lys$^{42}$ being an essential residue in the ATP-binding motif [34]), failed to phosphorylate Ser$^{847}$ of nNOS in the presence of wild-type PSD-95. These results are summarized in Figure 6, and strongly support the idea that PSD-95 promotes endogenous CaM-K II-mediated Ser$^{847}$ phosphorylation of nNOS in transfected cells.

## DISCUSSION

We have previously shown that down-regulation of nNOS activity by CaM-K II may represent an important component of the 'cross-talk' between $Ca^{2+}$- and NO-regulated signal-transduction pathways [25–27,35]. In transfected cells and in neurons, polarized targeting and vesiculotubular sorting of PSD-95 require its dual palmitoylation [32]. The results of the present study provide strong evidence that PSD-95 directly promotes CaM-K II-mediated Ser$^{847}$ phosphorylation of nNOS, in association with intracellular trafficking of nNOS, in cells. Although it was observed that all the expressed mutants of PSD-95 except for the $\Delta$2 mutant could bind nNOS in cells, the results of the present study do not exclude the potential involvement of major conformational changes induced by the mutations. Although the predicted molecular masses of GST fusion proteins containing PDZ1 and PDZ2 were similar, the former migrated more slowly

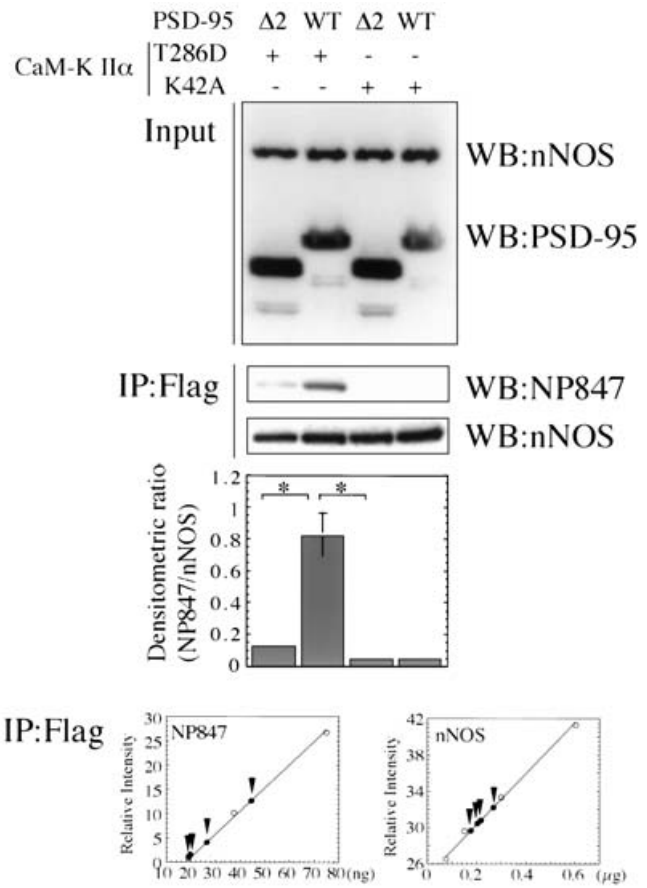


**Figure 5 Promotion of exogenous CaM-K IIα-mediated phosphorylation of nNOS at Ser[847] in transfected cells by PSD-95**

Active (T286D) or inactive (K42A) CaM-K IIα was co-transfected with the FLAG-tagged nNOS and wild-type PSD-95 or the Δ2 mutant (Figure 6) and anti-FLAG immunoprecipitates (IP) from lysates of the cells were immunoblotted with anti-nNOS antibody (nNOS) or NP847 without A23187 stimulation. The histogram shows the amounts of immunoreactive NP847 relative to that of α-nNOS in the membranes. The means ± S.E.M. for three experiments using three independent transfections are shown; asterisks represent significant differences (* $P < 0.05$). Input, 30 μg of lysates in each reaction. The calibration of densitometric intensities was performed as described in the Experimental section. The bottom panels shows that the intensities of NP847- and nNOS-immunoreactive bands (shown by the arrowheads) in the anti-FLAG immunoprecipitates were in the linear range.

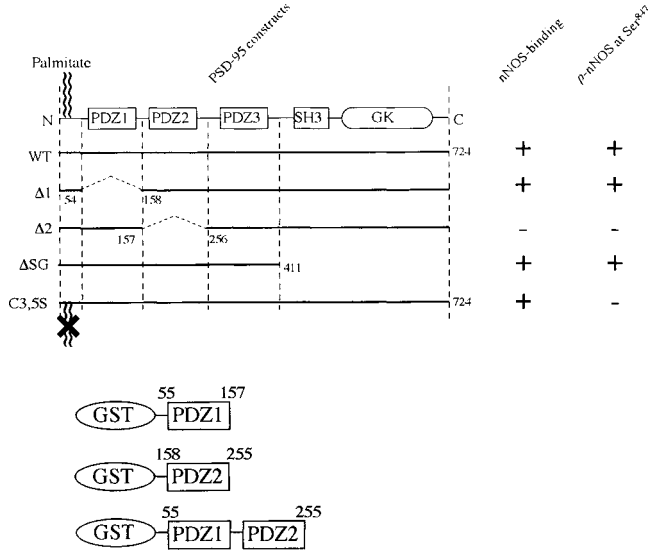


**Figure 6 Schematic illustration of PSD-95 mutants and summary of their effects on nNOS binding and phosphorylation of nNOS at Ser[847]**

Residue numbers correspond to those in the amino acid sequence of human PSD-95. WT, wild-type; C3,5S, the palmitoylation-deficient mutant of PSD-95. The Δ1 mutant carried a deletion of 103 amino acids in the PDZ1 domain. The Δ2 mutant lacked 98 amino acids in the PDZ2 domain. The mutant ΔSG carried a deletion of 313 C-terminal amino acids, which included both the SH3 and guanylate kinase domains. Note that the mutations in the palmitoylation motif of Cys[3] and Cys[5] to serine (C3,5S) failed to promote Ser[847] phosphorylation of nNOS.

than the latter (Figure 3A). The calculated isoelectric points (pI values) of PDZ1 and PDZ2 were 4.68 and 6.34 respectively. Different migration patterns of these GST constructs might be due to internal charged residues. We also show that CaM-K Iα and CaM-K IV fail to phosphorylate nNOS at Ser[847] in transfected cells, even though they do phosphorylate it *in vitro* [25]. CaM-K II would be a good candidate for phosphorylation of nNOS in cells, consistent with the kinetics of CaM kinases utilizing the synthetic peptide nNOS-(836–859) [26]. As we reported previously [26], nNOS is phosphorylated stoichiometrically, and thereby its enzyme activity is attenuated by CaM-K II in cells transfected with a 10:1 molar ratio of CaM-K IIα cDNA to nNOS cDNA. When PSD-95 is present in cells, small amounts of CaM-K II would be sufficient to promote phosphorylation of nNOS. Indeed, endogenous CaM-K II and the same kinase transfected with half the amount of cDNA of CaM-K II relative to that of nNOS were able to phosphorylate nNOS in HEK-293 cells expressing PSD-95 (Figures 1, 2 and 5). However, attenuation of nNOS activity through the increased phosphorylation of nNOS by PSD-95 was not observed, due to a low stoichiometry of nNOS phosphorylation in transfected cells (results not shown). There might be another molecule that promotes the stoichiometric phosphorylation of nNOS in cells when small amounts of CaM-K II are also present.

A dynamic molecular state mediated by PSD-95 and CaM-K II might have a functional role in the regulation of nNOS activity through the phosphorylation of Ser[847]. PSD-95 appears to be important in coupling the NMDA receptor with biochemical intracellular pathways controlling bidirectional synaptic plasticity [36,37]. The results of the present study shed new light on mechanisms important for CaM-K II phosphorylation of nNOS in neuronal cells. Further studies are required to clarify the other processes involved in the promotion of CaM-K II-dependent phosphorylation of NOS in cells, and the role of PSD-95 in modulating phosphorylation in neuronal cells.

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