Correspondence

Dephosphorylation of nNOS at Ser^{847} by protein phosphatase 2A

Keiichi Komeima[a,b], Yasuo Watanabe[a,*]

First published online 13 April 2001

Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaM-K II) is a broad-specificity enzyme with central roles in synaptic plasticity, learning, and memory. It is present at high concentrations in postsynaptic densities (PSDs), cytoskeletal scaffolds for the *N*-methyl-D-aspartate (NMDA) receptors and their regulators. Neuronal nitric oxide synthase (nNOS) is a Ca^{2+}/CaM-dependent enzyme, which catalyzes the oxidation of L-arginine to generate nitric oxide (NO) and L-citrulline. NO, formed by nNOS, has major signaling functions in the central and peripheral nervous system. In neurons, nNOS is associated with cell membranes in association with PSD-95, a major protein constituent of PSD that activates clustering shaker-type K^+ channels and the NMDA receptor subunit 2B. It has been established that nNOS is phosphorylated by CaM-K II linking to the decreased catalytic activity [1]. It has been reported recently that constitutively active CaM-K IIα can phosphorylate nNOS at Ser^{847}, and when transfected into NG108-15 neuronal cells, can attenuate the catalytic activity of nNOS [2]. Thus, co-localization of CaM-K II and nNOS in PSDs could be important in regulating CaM-K II phosphorylation of nNOS. While nNOS phosphorylation has been investigated in several studies, the reverse reaction is not well documented.

Our previous observations indicated that okadaic acid-sensitive protein phosphatases are involved in dephosphorylation of nNOS at Ser^{847} and reversible activation of the enzyme in NG108-15 neuronal cells [2]. Okadaic acid and caliculin A are highly specific inhibitors of the protein phosphatase family, targeting protein phosphatases 1 (PP1) and 2A (PP2A) in a highly selective manner [3]. We used these and other inhibitors of protein phosphatases (cyclosporin A and protein phosphatase inhibitor-2 (I-2)) to assess the potential involvement of protein phosphatase in regulating nNOS. We performed immunoblot analysis with NP847, which reacts with nNOS phosphorylated at Ser^{847} [1]. To characterize protein phosphatase involved in the dephosphorylation of nNOS at Ser^{847}, rat brain extracts were used as the source (Fig. 1A). Recombinant nNOS phosphorylated with CaM-K IIα at Ser^{847} was dephosphorylated with the extracts in a time-dependent manner. Immunoreactivity of NP847 with the heat-inactivated extracts was not changed during the incubation at 30°C. Inclusion of 5 μM okadaic acid completely inhibited the dephosphorylation. In contrast, addition of 5 μM cyclosporin A or 300 nM I-2 was without significant effect. Since 5 μM okadaic acid should completely block PP1 and 2A and partially block CaM [4], the above data indicate that the major endogenous protein phosphatase in rat brain responsible for the dephosphorylation is PP2A. Inclusion of a lower concentration of okadaic acid (1 μM) in the mixture resulted in the same effects on the dephosphorylation by rat brain extracts (data not shown). We then used caliculin A, another potent inhibitor of PP1 and 2A, to assess the potential involvement of protein phosphatase for such dephosphorylation. The immunoreactivity of NP847 demonstrated similar dose–response effects of okadaic acid and caliculin A with IC_{50} values of 4.3–4.6 and 6.3–6.8, respectively (Fig. 1B). Since both are reported to inhibit PP2A with a similar potency [5], these results suggest that the dominant protein phosphatase for the dephosphorylation of nNOS at Ser^{847} in rat brain extracts is PP2A, consistent with the result of Fig. 1A.

PP1 is regulated by its interaction with a variety of protein subunits that act in a manner distinct from the inhibitor proteins and that appear to target the catalytic subunit to specific subcellular compartments in vivo. PP2A is regulated by the differential expression of regulatory proteins that control en-

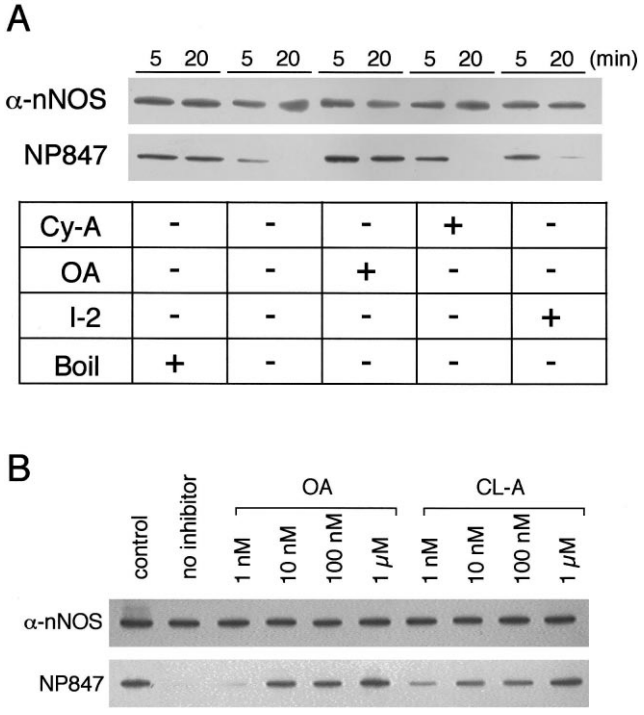

Fig. 1. Effects of protein phosphatase inhibitors on the time course of dephosphorylation of nNOS at Ser^{847} by endogenous protein phosphatase. A: Recombinant nNOS expressed in *Escherichia coli* was phosphorylated at Ser^{847} by CaM-K IIα with 1 mM ATP. Dephosphorylation with endogenous protein phosphatase was initiated by addition of rat brain extract (150 μg/ml of protein), pre-incubated with (I-2) or without 300 nM protein inhibitor-2 at 30°C for 15 min, and further incubated at 30°C in the presence or absence of 5 μM okadaic acid (OA) or cyclosporin A (Cy-A). Aliquots (1 μg of nNOS) of the samples were removed at the indicated times, subjected to 7.5% SDS–PAGE and transferred to polyvinylidene difluoride membrane and immunoblotted with nNOS (α-nNOS) or NP847 antibody (NP847). The data are representative of experiments repeated twice. The phosphorylated nNOS at Ser^{847} was also incubated with the extract which had been heat-inactivated at 60°C for 10 min (Boil). B: Dephosphorylation of nNOS with or without (control) rat brain extract (3 μg of protein) was analyzed as in A in the presence or absence (no inhibitor) of the indicated concentration of OA or caliculin A (CL-A). These data are representative of an experiment repeated twice.

zyme specificity and activity by formation of oligomeric complexes in vivo. Thus, establishing the identity of protein phosphatases responsible for specific dephosphorylation by comparison of different protein phosphatase preparations is very difficult. Here we could identify PP2A as a major protein phosphatase involved in the dephosphorylation of nNOS at Ser^{847} using rat brain extracts as a source of protein phosphatase.

Increased phosphorylation of nNOS at Ser^{847} by CaM-K IIα in NG108-15 cells is associated with inhibition of nNOS enzyme activity [2]. From these data, taken together, we conclude that PP2A is a major contributor to dephosphorylation of Ser^{847} of nNOS, responsible for reversible activation of the enzyme in neuronal cells.

Acknowledgements: We wish to express our appreciation to Drs. Akira Takai (Nagoya University, Japan) and Hitoshi Nakagama (National Cancer Research Institute, Japan) for valuable discussion and suggestions during the course of these studies and the Japanese Ministry of Education, Science, Sports and Culture for financial support. We are also grateful to Dr. Malcolm Moore for critical reading of the manuscript and Masami Iwahori for expert technical support.

References

[1] Hayashi, Y., Nishio, M., Naito, Y., Yokokura, H., Nimura, Y., Hidaka, H. and Watanabe, Y. (1999) J. Biol. Chem. 274, 20597–20602.
[2] Komeima, K., Hayashi, Y., Naito, Y. and Watanabe, Y. (2000) J. Biol. Chem. 275, 28139–28143.
[3] Favre, B., Turowski, P. and Hemmings, B.A. (1997) J. Biol. Chem. 272, 13856–13863.
[4] Bialojan, C. and Takai, A. (1988) Biochem. J. 256, 283–290.
[5] Takai, A., Sasaki, K., Nagai, H., Mieskes, G., Isobe, M., Isono, K. and Yasumoto, T. (1995) Biochem. J., 657–665.

*Corresponding author. Present address: First Department of Physiology, Faculty of Medicine, Kagawa Medical University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan.
Fax: (81)-87-891 2096. E-mail: yasuwata@kms.ac.jp

[a] *Department of Molecular Pharmacology, Nagoya University, Graduate School of Medicine, Showa-ku, Nagoya 466-8550, Japan*
[b] *Department of Ophthalmology, Nagoya University, Graduate School of Medicine, Showa-ku, Nagoya 466-8550, Japan*

PII: S0014-5793(01)02389-4