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## **Inhibitors of the Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatase family**

### **(CaMKP and CaMKP-N)**

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### **Footnotes to the title page**

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Abbreviations : CaMK, Ca<sup>2+</sup>/calmodulin-dependent protein kinase; CaMKK,  
Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase; CaMKP, Ca<sup>2+</sup>/calmodulin-dependent  
protein kinase phosphatase; CaN, calcineurin ; DMEM, Dulbecco's modified Eagle's  
medium; PP, protein phosphatase.

## Abstract

Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatase (CaMKP) and its nuclear isoform CaMKP-N are unique Ser/Thr protein phosphatases that negatively regulate the Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaMK) cascade by dephosphorylating multifunctional CaMKI, II, and IV. However, the lack of specific inhibitors of these phosphatases has hampered studies on these enzymes *in vivo*. In an attempt to obtain specific inhibitors, we searched inhibitory compounds and found that Evans Blue and Chicago Sky Blue 6B served as effective inhibitors for CaMKP. These compounds also inhibited CaMKP-N, but inhibited neither protein phosphatase 2C, another member of PPM family phosphatase, nor calcineurin, a typical PPP family phosphatase. The minimum structure required for the inhibition was 1-amino-8-naphthol-4-sulfonic acid. When Neuro2a cells cotransfected with CaMKIV and CaMKP-N were treated with these compounds, the dephosphorylation of CaMKIV was strongly suppressed, suggesting that these compounds could be used as potent inhibitors of CaMKP and CaMKP-N *in vivo* as well as *in vitro*.

*Keywords:* CaMKP; CaMKP-N; CaMK; PP2C; inhibitor; permeability; calcineurin; PPM family; chemical screening

## Introduction

Multifunctional  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases (CaMKs) are activated in response to  $\text{Ca}^{2+}$  stimulus, and play pivotal roles in the regulation of neuronal functions such as learning, memory, and neuronal cell death [1-4].  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase phosphatase (CaMKP) and its nuclear homolog CaMKP-N are unique protein phosphatases that specifically dephosphorylate and regulate multifunctional CaMKs such as CaMKI, II, and IV [5-9]. CaMKP and CaMKP-N are  $\text{Mn}^{2+}$ -dependent, calyculin A/okadaic acid-insensitive Ser/Thr protein phosphatases that belong to the PPM family and share 28% homology with the catalytic domain of protein phosphatase 2C (PP2C)  $\alpha$  [5-9].

There are several reports regarding the physiological functions of CaMKP and CaMKP-N. Tan et al. identified CaMKP as a human homologue of FEM-2, a product of a gene that participates in sex-determination in *C. elegans* [10]. Transient expression of nematode FEM-2, human CaMKP, or rat CaMKP in HeLa cells resulted in apoptosis, whereas the expression of PP2C, another member of the PPM family of protein phosphatases, did not induce apoptosis, suggesting that CaMKP is involved in apoptotic signaling. Harvey et al. showed that the overexpression of CaMKP in fibroblasts causes a significant decrease in CaMKII-dependent phosphorylation of vimentin, suggesting that CaMKP functions as a negative regulator of CaMKII *in vivo* [11]. On the other hand, we reported that antisense knockdown of CaMKP-N during zebrafish embryogenesis induces enhanced apoptosis in the central nervous system, where CaMKP-N exclusively expresses. We also revealed that cotransfection of

CaMKP-N in CaMKIV-transfected Neuro2a cells significantly decreases phospho-CaMKIV in ionomycin-stimulated cells, suggesting that CaMKP-N dephosphorylates phosphorylated CaMKIV *in vivo* [12]. Further studies will be necessary to clarify the physiological significance of CaMKP/CaMKP-N, but such studies have been much constrained by the lack of specific and effective inhibitors of CaMKP and/or CaMKP-N.

In an attempt to obtain useful inhibitors of CaMKP and/or CaMKP-N, we carried out screening of a commercially available compounds library. Out of over 800 compounds screened, 4 known as dyes such as Evans Blue were found to be potent inhibitors of CaMKP and CaMKP-N and had no significant inhibition toward PP2C, which belongs to the PPM family as do CaMKP/CaMKP-N, and calcineurin, which belongs to the PPP family, the other gene family of Ser/Thr phosphatases.

## **Materials and methods**

*Materials.* A compound library (containing receptor ligands, enzyme inhibitors, and enzyme substrates) was obtained from TOCRIS Bioscience. Other compounds used for screening were from Tokyo Chemical Industry Co., Wako Pure Chemical Industries, and NACALAI TESQUE. Inc. Ionomycin calcium salt was from Calbiochem. ATP, bovine serum albumin, and poly L-Lys (average molecular weight of 84,000) were purchased from Sigma Chemicals. Anti-CaMKIV antibody was from BD Biosciences. Goat anti-mouse IgG+A+M and goat anti-rabbit IgG, conjugated with horseradish peroxidase, were from ICN Pharmaceuticals and PIERCE, respectively. Monoclonal

antibody to detect phospho-CaMKIV at Thr-196 was prepared as described [13].

Recombinant rat calmodulin [14], rat CaMK kinase  $\alpha$  (CaMKK) [15], rat CaMKI [16], rat CaMKP [16], rat PP2C  $\alpha$  [17], and zebrafish CaMKP-N [12] were expressed in *E. coli*, and purified as described. Rat calcineurin A  $\alpha$  (CaNA  $\alpha$ , Acc. No. X57115) and rat calcineurin B (CaNB, Acc. No. L03554) were expressed as His<sub>6</sub>-tagged protein at the C-terminus, and purified by HiTrap Chelating HP (GE Healthcare).

*Production of antibodies.* Antibodies against CaMKI and phospho-CaMKI at Thr-177 were produced by immunizing mice with purified CaMKI (for anti-CaMKI) or antigenic phosphopeptide (MSDHGVMSpTACGTPYC, for anti-phospho-CaMKI). An antibody against CaMKP-N was produced as described [12]. Immunization was carried out essentially according to the procedure described previously [18].

*Protein phosphatase assay.* The activities of CaMKP, CaMKP-N, and PP2C were determined using phosphopeptide pp10 (YGGMHRQET(p)VDC) as a substrate [19, 20]. The activity of CaN was measured by using phosphopeptide RII (DLDVPIPGRFDRRVS(p)VAAE) [21]. In this assay, heterodimeric CaN consisting of CaNA  $\alpha$  (4 pmol) and CaNB (4 pmol) were activated by incubating at 30°C for 30 min in 50 mM Tris-HCl (pH 8.0, 50  $\mu$ l) containing 0.1 mM EGTA, 0.01% Tween 20, 1 mM dithiothreitol, 1.2 mM MnCl<sub>2</sub>, 6 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.35  $\mu$ M calmodulin, and 1 mM NaCl, followed by adding 160  $\mu$ M phosphopeptide RII. After 10 min of incubation at 30°C, the inorganic phosphate released in the mixture was determined by

malachite green assay [20]. The compound to be tested was added in the reaction systems mentioned above at the indicated concentrations. Protein phosphatase assay using CaMKI as a phosphoprotein substrate was carried out essentially as described previously [5, 16]. The reaction was started by the addition of CaMKP and terminated by the addition of an equal volume of SDS sample buffer. The sample was then subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting analysis using phospho-CaMKI antibody.

*Cell culture and transfection.* Mouse neuroblastoma, Neuro2a cells, were cultured, transfected, stimulated, and analyzed essentially as described previously [12] with minor modifications. The transfected cells were cultured in serum free DMEM containing compounds 1-6 for 6 h to starve the cells, and then stimulated by 1  $\mu$ M ionomycin in DMEM containing the same compound at room temperature for 10 min. To stop the reaction, 70  $\mu$ l of SDS sample buffer was added.

*SDS-polyacrylamide gel electrophoresis and Western blotting.* SDS- polyacrylamide gel electrophoresis was performed essentially according to the method of Laemmli [22] on a slab gel consisting of a 10% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Protran BA85, Schleicher and Schuell) and immuno-reactive protein bands were detected essentially according to a previously described method [23].



## Results

### *Chemical screening of CaMKP/CaMKP-N-specific inhibitors*

To search for CaMKP-specific inhibitors, we screened more than 800 compounds obtained from TOCRIS and other chemical companies. When we examined the effects of 40  $\mu\text{M}$  each of the compounds on the phosphatase activities of CaMKP and PP2C, we found that Evans Blue (Fig. 1. compound 1, Fig. 2A) and Chicago Sky Blue 6B (Fig. 1. compound 2) significantly inhibited CaMKP. Evans Blue and Chicago Sky Blue inhibited CaMKP activity with concentrations for half-maximal inhibition ( $\text{IC}_{50}$ ) of  $6.2 \pm 0.2 \mu\text{M}$  and  $4.1 \pm 0.3 \mu\text{M}$ , respectively. By contrast, under the same conditions, these compounds showed no significant effects on the activity of PP2C, another PPM family phosphatase, or of CaN, which is a member of the PPP family of phosphatases (Fig. 2A, Table 1). Since CaMKP shares 64% of sequence homology with the catalytic domain of CaMKP-N, these CaMKP-inhibiting compounds were anticipated to potentially inhibit CaMKP-N. As expected, Evans Blue and Chicago Sky Blue effectively inhibited CaMKP-N with  $\text{IC}_{50}$  of  $0.9 \pm 0.1 \mu\text{M}$  and  $1.0 \pm 0.1 \mu\text{M}$ , respectively, indicating that these compounds could inhibit CaMKP/CaMKP-N without affecting other PPM and PPP family phosphatases.

### *Identification of a structural unit indispensable for potent inhibition of CaMKP*

Next, we examined the inhibition of CaMKP by Oxamine Blue B (Fig. 1. compound 3) and Azo Blue (Fig. 1. compound 4), respectively, both of which have

structures similar to that of Evans Blue. Oxamine Blue and Azo Blue also inhibited CaMKP activity with an  $IC_{50}$  of  $7.9 \pm 0.3 \mu\text{M}$  and  $16.1 \pm 1.2 \mu\text{M}$ , respectively, indicating that they are significantly less potent inhibitors than Evans Blue. As shown in Table 1, however, they still showed specificity for CaMKP-inhibition because they had essentially no significant effect on PP2C activity.

The chemical structure of Evans Blue, Chicago Sky Blue, Oxamine Blue and Azo Blue can be divided into three structural components: A central unit comprised of a biphenyl ring, and two lateral units comprised of a naphthalene ring (Fig. 1). In an attempt to identify the structural unit essential for CaMKP-specific inhibition, we examined the inhibition of CaMKP by compounds 5-9, which were derived from the partial structure of the lateral unit, and by compounds 10-12, which were derived from the partial structure of the central unit (Fig. 1). We then compared the inhibitory activities of these compounds; only compound 5 (1-amino-8-naphthol-2,4-disulfonic acid) and compound 6 (1-amino-8-naphthol-4-sulfonic acid) showed potent inhibition toward CaMKP (Fig. 2B, Table 1). They also strongly inhibited CaMKP-N, but did not significantly inhibit either PP2C or CaN (Table 1).

#### *Characterization of the types of inhibition of CaMKP by Evans Blue and*

##### *1-amino-8-naphthol-2,4-disulfonic acid*

To characterize the inhibition of CaMKP by Evans Blue and 1-amino-8-naphthol-2,4-disulfonic acid, we carried out kinetic analyses of the inhibition by these compounds, as shown in Fig. 2C and Fig. 2D. The patterns of the

Lineweaver-Burk plot suggested that these compounds were competitive with respect to phosphopeptide substrates ( $K_m = 21.3 \mu\text{M}$ ,  $V_{\text{max}} = 3.72 \mu\text{mol}/\text{min}/\text{mg}$ ). The apparent  $K_i$  values for Evans Blue and 1-amino-8-naphthol-2,4-disulfonic acid were estimated from the plot to be  $4.1 \mu\text{M}$  and  $3.6 \mu\text{M}$ , respectively.

#### *Effects of the inhibitors on the dephosphorylation of a phosphoprotein substrate*

We also examined whether these compounds could effectively inhibit not only the dephosphorylation of phosphopeptide substrates but also that of phosphoprotein substrates. For this purpose, we used phosphorylated CaMKI as a substrate, and examined the effects of the compounds on dephosphorylation (Fig. 3). Recombinant CaMKI, which had been previously phosphorylated by CaMKK, was incubated with CaMKP in the presence or absence of the compound, and the dephosphorylation was estimated by Western blotting using anti-phospho-CaMKI. Although phosphorylated CaMKI was completely dephosphorylated by CaMKP in the absence of the inhibitors (Fig. 3, lanes 2 and 8), phosphorylated CaMKI was clearly detected even after incubation with CaMKP in the presence of Evans Blue ( $10 \mu\text{M}$ , Fig. 3, lane 3), Chicago Sky Blue ( $10 \mu\text{M}$ , Fig. 3, lane 4), Oxamine Blue ( $20 \mu\text{M}$ , Fig. 3, lane 5), 1-amino-8-naphthol-2,4-disulfonic acid ( $10 \mu\text{M}$ , Fig. 3, lane 6), Azo Blue ( $40 \mu\text{M}$ , Fig. 3, lane 9), and 1-amino-8-naphthol-4-sulfonic acid ( $10 \mu\text{M}$ , Fig. 3, lane 10), respectively, indicating that these compounds could inhibit dephosphorylation of phosphoprotein substrates by CaMKP, as in the case of phosphopeptide substrates.

*Effects of the inhibitors on the phosphatase activity of CaMKP-N in Neuro2a cells*

Ionomycin treatment of CaMKIV-transfected Neuro2a cells resulted in a marked increase in the phosphorylated form of CaMKIV due to cytosolic  $\text{Ca}^{2+}$ -rise caused by ionomycin and the subsequent activation of endogenous CaMKK. However, cotransfection with zebrafish CaMKP-N significantly decreased phospho-CaMKIV in ionomycin-stimulated cells, suggesting that CaMKP-N could dephosphorylate phospho-CaMKIV in cells [12]. Using this experimental system, we examined whether these compounds could effectively act on CaMKP-N expressed in the cells. As shown in Fig. 4, cotransfection of CaMKP-N with CaMKIV resulted in a significant decrease in the phosphorylation level of CaMKIV in the presence of ionomycin, as compared to that of CaMKIV in cells transfected with just CaMKIV (Fig. 4, lanes 2 and 7). When cells cotransfected with CaMKIV and CaMKP-N were incubated with Evans Blue (100  $\mu\text{M}$ , Fig. 4, lane 8), Chicago Sky Blue (100  $\mu\text{M}$ , Fig. 4, lane 9), and Oxamine Blue (100  $\mu\text{M}$ , Fig. 4, lane 10), respectively, followed by ionomycin treatment, the phosphorylation level of CaMKIV significantly increased compared to that of CaMKIV without the compounds (Fig. 4, lane 7). These compounds did not affect the phosphorylation level of CaMKIV after ionomycin treatment of the cells without CaMKP-N transfection (Fig. 4, lanes 3-5). These data suggested that Evans Blue, Chicago Sky Blue, and Oxamine Blue can be effectively incorporated into cells to inhibit CaMKP-N expressed in those cells.

## Discussion

Ser/Thr protein phosphatases can be classified into two superfamilies on the basis of similarity in the primary amino acid sequence. One is the PPP family and the other the PPM family. The former consists of the most abundant Ser/Thr protein phosphatases of the eukaryotes, PP1, PP2A, PP2B (CaN), and some other novel phosphatases. The latter consists of the PP2C family and other structurally related phosphatases such as CaMKP and CaMKP-N, which requires  $Mg^{2+}$  or  $Mn^{2+}$  for activities. For PPP family phosphatases, potent and specific inhibitors such as calyculin A, okadaic acid, microcystin LR, and cyclosporin, which are derived from natural products, are already available. They have greatly contributed to clarifying the physiological functions of PPP family phosphatases [24, 25]. For PPM phosphatases, however, only two compounds have been so far reported as specific inhibitors. One is a compound obtained by a virtual screening, reported by Rogers et al. [26], while the other is a peptide inhibitor developed by Yamaguchi et al. [27]. Unfortunately, the former inhibitor showed only modest specificity to PP2C with substantial inhibitory activity toward PPP family phosphatases [26]. The latter inhibitor is potent and highly specific to a PPM phosphatase, Wip1, but it is thought to have poor cell permeability because it is a peptide-based inhibitor with multiple negative charges, which may prevent permeation of the cell membrane. Besides these, some chelators of metal ions essential for PP2C activity and vanadate, which widely inhibits various protein phosphatases, are known to inhibit PP2C. Heparin and NaF are also reported to inhibit

CaMKP, but they are non-specific inhibitors that affect the activities of a variety of protein phosphatases and other enzymes. Thus, no any specific and potent inhibitors for PPM phosphatases with sufficient cell permeability, for example like okadaic acid for PPP phosphatases, have thus far been reported. This has hampered studies on the physiological significance of PPM phosphatases, whereas those of PPP phosphatases have been greatly facilitated by the specific inhibitors.

The compounds found in this study possessed satisfactory cell permeability, and showed specificity among PPM family phosphatases, inhibiting CaMKP and CaMKP-N but not PP2C. Since Evans Blue and Chicago Sky Blue 6B, however, are reported to be potent inhibitors of the vesicular uptake of glutamate [28], it is likely that these compounds also inhibit functions of other proteins or enzymes. Therefore, improvements to the inhibitors are necessary so that they might be specific enough to CaMKP and/or CaMKP-N for pharmacological studies on them. It is interesting that some of the partial structures derived from the CaMKP-inhibiting dyes, 1-amino-8-naphthol-2,4-disulfonic acid (compound 5) and 1-amino-8-naphthol-4-sulfonic acid (compound 6) are potent CaMKP/CaMKP-N inhibitors without any significant inhibition toward PP2C and CaN, but that the other partial structures tested were very poor inhibitors toward CaMKP and CaMKP-N (Table 1). These data strongly suggested that the simultaneous presence of an amino group and a hydroxyl group at positions 1 and 8, respectively, on the naphthalene ring is important for specificity and potency as CaMKP/CaMKP-N inhibitors. Based on the structure-function relationship obtained in this study, we can rationalize and design the

structure of inhibitors to have more specificity to CaMKP/CaMKP-N. Once this has been achieved, the inhibitors created can be expected to be useful tools for exploring the physiological functions of CaMKP/CaMKP-N.

The three-dimensional structure of PP2C was reported by Das et al [29]. In their study, they identified amino acid residues essential for catalysis and those involved in binding to a metal ion at the active site and a phosphate group of a substrate. These residues are conserved between PP2C and CaMKP [16]. Since site-directed mutagenesis of these residues results in complete loss of CaMKP activity, it has been thought that the structure around the active center of CaMKP is very similar to that of PP2C, on the basis of their primary structures [16]. In this study, however, it was revealed that a series of compounds found as potent CaMKP-inhibitors did not inhibit PP2C at all. This means that they can discriminate subtle differences between the three-dimensional structures of the active sites of CaMKP/CaMKP-N and of PP2C. Therefore, the findings obtained in this study should greatly contribute to the design of more specific and effective inhibitors of CaMKP/CaMKP-N, as well as to clarifying the tertiary structures of the catalytic center of PPM phosphatases, in combination with X-ray crystallography.

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## References

- [1] S.S. Hook, A.R. Means,  $\text{Ca}^{2+}$ /CaM-dependent kinases: from activation to function, *Annu. Rev. Pharmacol. Toxicol.* 41 (2001) 471-505.
- [2] H. Fujisawa, Regulation of the activities of multifunctional  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases, *J. Biochem.* 129 (2001) 193-199.
- [3] T.R. Soderling, J.T. Stull, Structure and regulation of calcium/calmodulin-dependent protein kinases, *Chem. Rev.* 101 (2001) 2341-2352.
- [4] A. Hudmon, H. Schulman, Neuronal  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function, *Annu. Rev. Biochem.* 71 (2002) 473-510.
- [5] A. Ishida, I. Kameshita, H. Fujisawa, A novel protein phosphatase that dephosphorylates and regulates  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II, *J. Biol. Chem.* 273 (1998) 1904-1910.
- [6] A. Ishida, S. Okuno, T. Kitani, I. Kameshita, H. Fujisawa, Regulation of multifunctional  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase phosphatase, *Biochem. Biophys. Res.*



Commun. 253 (1998) 159-163.

[7] T. Kitani, A. Ishida, S. Okuno, M. Takeuchi, I. Kameshita, H. Fujisawa, Molecular cloning of Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatase, J. Biochem. 125 (1999) 1022-1028.

[8] M. Takeuchi, A. Ishida, I. Kameshita, T. Kitani, S. Okuno, H. Fujisawa, Identification and characterization of CaMKP-N, nuclear calmodulin-dependent protein kinase phosphatase, J. Biochem. 130 (2001) 833-840.

[9] A. Ishida, Y. Shigeri, T. Taniguchi, I. Kameshita, Protein phosphatases that regulate multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinases: from biochemistry to pharmacology. Pharmacol. Ther. 100 (2003) 291-305.

[10] K.M. Tan, S.L. Chan, K.O. Tan, V.C. Yu, The *Caenorhabditis elegans* sex-determining protein FEM-2 and its human homologue, hFEM-2, are Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatases that promote apoptosis, J. Biol. Chem. 276 (2001) 44193-44202.

[11] B.P. Harvey, S.S. Banga, H.L. Ozer, Regulation of the multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinase II by the PP2C phosphatase PPM1F in fibroblasts, J. Biol. Chem. 279 (2004) 24889-24898.

[12] T. Nimura, N. Sueyoshi, A. Ishida, Y. Yoshimura, M. Ito, H. Tokumitsu, Y. Shigeri, N. Nozaki, I. Kameshita, Knockdown of nuclear Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatase causes developmental abnormalities in zebrafish, Arch. Biochem. Biophys. 457 (2007) 205-216.

[13] H. Tokumitsu, N. Hatano, H. Inuzuka, S. Yokokura, N. Nozaki, R. Kobayashi,

- Mechanism of the generation of autonomous activity of Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV, *J. Biol. Chem.* 279 (2004) 40296-40302.
- [14] N. Hayashi, M. Matsubara, A. Takasaki, K. Titani, H. Taniguchi, An expression system of rat calmodulin using T7 phage promoter in *Escherichia coli*, *Protein Express. Purif.* 12 (1998) 25-28.
- [15] S. Kinoshita, N. Sueyoshi, H. Shoji, I. Suetake, M. Nakamura, S. Tajima, I. Kameshita, Cloning and characterization of a novel Ca<sup>2+</sup>/calmodulin-dependent protein kinase I homologue in *Xenopus laevis*, *J. Biochem.* 135 (2004) 619-630.
- [16] Y. Tada, T. Nimura, N. Sueyoshi, A. Ishida, Y. Shigeri, I. Kameshita, Mutational analysis of Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatase (CaMKP), *Arch. Biochem. Biophys.* 452 (2006) 174-185.
- [17] Y. Sugiyama, N. Sueyoshi, Y. Shigeri, Y. Tatsu, N. Yumoto, A. Ishida, T. Taniguchi, I. Kameshita, Generation and application of a monoclonal antibody that detects a wide variety of protein tyrosine kinases, *Anal. Biochem.* 347 (2005) 112-120.
- [18] I. Kameshita, S. Kinoshita, Y. Shigeri, Y. Tatsu, N. Yumoto, A. Ishida, Generation of a polyclonal antibody that simultaneously detects multiple Ser/Thr protein kinases, *J. Biochem. Biophys. Methods* 60 (2004) 13-22.
- [19] A.A. Baykov, O.A. Evtushenko, S.M. Avaeva, A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay, *Anal. Biochem.* 171 (1988) 266-270.
- [20] A. Ishida, Y. Shigeri, Y. Tatsu, Y. Endo, I. Kameshita, S. Okuno, T. Kitani, M. Takeuchi, N. Yumoto, H. Fujisawa, Substrate specificity of Ca<sup>2+</sup>/calmodulin-dependent

protein kinase phosphatase: kinetic studies using synthetic phosphopeptides as model substrates, *J. Biochem.* 129 (2001) 745-753.

[21] D.K. Blumenthal, K. Takio, R.S. Hansen, E.G. Krebs, Dephosphorylation of cAMP-dependent protein kinase regulatory subunit (type II) by calmodulin-dependent protein phosphatase. Determinants of substrate specificity, *J. Biol. Chem.* 261 (1986) 8140-8145.

[22] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680-685.

[23] I. Kameshita, T. Tsuge, T. Kinashi, S. Kinoshita, N. Sueyoshi, A. Ishida, S. Taketani, Y. Shigeri, Y. Tatsu, N. Yumoto, K. Okazaki, A new approach for the detection of multiple protein kinases using monoclonal antibodies directed to the highly conserved region of protein kinases, *Anal. Biochem.* 322 (2003) 215-224.

[24] J.J. Fernandez, M.L. Candenas, M.L. Souto, M.M. Trujillo, M. Norte, Okadaic acid, useful tool for studying cellular processes, *Curr. Med. Chem.* 9 (2002) 229-262.

[25] S. Martinez-Martinez, J.M. Redondo, Inhibitors of the calcineurin/NFAT pathway, *Curr. Med. Chem.* 11 (2004) 997-1007.

[26] J.P. Rogers, A.E. Beuscher IV, M. Flajolet, T. McAvoy, A.C. Nairn, A.J. Olson, P. Greengard, Discovery of protein phosphatase 2C inhibitors by virtual screening, *J. Med. Chem.* 49 (2006) 1658-1667.

[27] H. Yamaguchi, S.R. Durell, H. Feng, Y. Bai, C.W. Anderson, E. Appella, Development of a substrate-based cyclic phosphopeptide inhibitor of protein phosphatase 2C $\delta$ , Wip1, *Biochemistry* 45 (2006) 13193-13202.

- [28] S. Roseth, E. M. Fykse, F. Fonnum, Uptake of L-glutamate into synaptic vesicles: Competitive inhibition by dyes with biphenyl and amino- and sulphonic acid-substituted naphthyl groups, *Biochem. Pharmacol.* 56 (1998) 1243-1249.
- [29] A.K. Das, N.R. Helps, P.T.W. Cohen, D.Barford, Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 Å resolution, *EMBO J.* 15 (1996) 6798-6809.

### Figure legends

Fig. 1. Chemical structures of the compounds used in this study. 1, Evans Blue; 2, Chicago Sky Blue 6B; 3, Oxamine Blue B; 4, Azo Blue; 5, 1-amino-8-naphthol-2,4-disulfonic acid; 6, 1-amino-8-naphthol-4-sulfonic acid; 7, 1-naphthol-5-sulfonic acid; 8, 4-amino-1-naphthalenesulfonic acid; 9, 5-amino-1-naphthol; 10, 3,3'-dihydroxybenzidine; 11, 3,3'-dimethylbenzidine; 12, 3,3'-dimethoxybenzidine. Compounds 1, 2, 3, 5, 7 were dissolved in H<sub>2</sub>O and others were in dimethylsulfoxide.

Fig. 2. Effects of Evans Blue (compound 1) (A) and 1-amino-8-naphthol-2,4-disulfonic acid (compound 5) (B) on CaMKP and PP2C activities. The activities of CaMKP (50 ng, ■) and PP2C (50 ng, ○) were measured using pp10 (20 μM) as a substrate in the absence or presence of the compound. (C, D) Kinetics of CaMKP inhibition by compound 1 (C) and compound 5 (D). The Lineweaver-Burk plot of pp10 hydrolysis catalyzed by CaMKP in the absence and

presence of compound 1 (2.5, 5  $\mu$ M) or compound 5 (2.5, 5  $\mu$ M) are shown.

Fig. 3. Effects of compounds on phospho-CaMKI hydrolysis by CaMKP. CaMKI (10 ng), phosphorylated by CaMKK, was incubated with CaMKP (50 pg) in the absence or presence of compounds for 4 min at 30°C. Then, samples were electrophoresed on SDS-polyacrylamide gel and analyzed by Western blotting with anti-phospho-CaMKI antibody (upper panel) and anti-CaMKI antibody (lower panel). Compound 1 (10  $\mu$ M), compound 2 (10  $\mu$ M), compound 3 (20  $\mu$ M), compound 5 (10  $\mu$ M) were dissolved in H<sub>2</sub>O and compound 4 (40  $\mu$ M) and compound 6 (10  $\mu$ M) in dimethylsulfoxide.

Fig. 4. Effects of compounds 1, 2, 3, 5 on CaMKP-N expressed in Neuro2a cells. A plasmid for CaMKIV (0.83  $\mu$ g) was cotransfected with or without that for CaMKP-N (0.33  $\mu$ g) into Neuro2a cells and the total amount of DNA was adjusted to 1.16  $\mu$ g with the empty vector. Transfected cells were cultured in serum free DMEM with or without 100  $\mu$ M compound 1, 2, 3, or 10  $\mu$ M compound 5 for 6 h, and then stimulated by 1  $\mu$ M ionomycin in DMEM at room temperature. After stimulation for 10 min, cells were lysed with 70  $\mu$ l of SDS sample buffer and the cell lysates (2.5  $\mu$ g protein) analyzed by Western blotting using anti-phospho-CaMKIV (upper panel), anti-CaMKIV (middle panel) and anti-CaMKP-N (lower panel) antibodies.









