

**Negative regulation of multifunctional Ca²⁺/calmodulin-dependent protein kinases:
physiological and pharmacological significance of protein phosphatases**

Running title: CaMK-regulating protein phosphatases

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Summary

Multifunctional Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) play pivotal roles in intracellular Ca^{2+} signaling pathways. There is growing evidence that CaMKs are involved in the pathogenic mechanisms underlying various human diseases. In this review, we begin by briefly summarizing our knowledge of the involvement of CaMKs in the pathogenesis of various diseases suggested to be caused by the dysfunction/dysregulation or aberrant expression of CaMKs. It is widely known that the activities of CaMKs are strictly regulated by protein phosphorylation /dephosphorylation of specific phosphorylation sites. Since phosphorylation status is balanced by protein kinases and protein phosphatases, the mechanism of dephosphorylation/deactivation of CaMKs, corresponding to their “switching off”, is extremely important, as is the mechanism of phosphorylation/activation corresponding to their “switching on”. Therefore, we focus on the regulation of multifunctional CaMKs by protein phosphatases. We summarize the current understanding of negative regulation of CaMKs by protein phosphatases. We also discuss the biochemical properties and physiological significance of a protein phosphatase that we designated as Ca^{2+} /calmodulin-dependent protein kinase phosphatase (CaMKP), and those of its homologue CaMKP-N. Pharmacological applications of CaMKP inhibitors are also discussed. These compounds may be useful not only for exploring the physiological functions of CaMKP/CaMKP-N, but also as novel chemotherapies for various diseases.

Keywords: Ca^{2+} /calmodulin-dependent protein kinase; Protein phosphatase; Phosphorylation; Dephosphorylation; Deactivation; Disease; Pathogenesis; Inhibitor; Chemical screening

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+} /calmodulin-dependent protein kinase; CaMKK, Ca^{2+} /calmodulin-dependent protein kinase kinase; CaMKP, Ca^{2+} /calmodulin-dependent protein kinase phosphatase; LTP, long-term potentiation; PKA, cAMP-dependent protein kinase; PP, protein phosphatase; PSD, postsynaptic density; zCaMKP, zebrafish homolog of CaMKP; zCaMKP-N, zebrafish homolog of CaMKP-N

Introduction

All of the biological responses observed in cells are elegantly regulated by intracellular signaling systems. Of these, regulatory pathways mediated through protein phosphorylation catalyzed by protein kinases are of particular importance. Protein kinases not only phosphorylate their substrate proteins, but they are also phosphorylated by themselves or by other protein kinases. In many cases, the phosphorylation reactions on protein kinases are important steps in the activation of these kinases (Johnson et al., 1996) considered to represent “switch on” mechanisms. On the other hand, protein kinases that are activated by phosphorylation are subsequently deactivated by dephosphorylation mediated by protein phosphatases (PP); this dephosphorylation reaction is usually regarded to represent a “switch off” mechanism. Consequently, protein phosphatases that dephosphorylate protein kinases are also responsible for the regulation of these kinases. Thus, intracellular signal transduction is constructed on the basis of the subtle balance between phosphorylation and dephosphorylation. Heretofore, little attention has been given to the roles of protein phosphatases regarding the “switch off” mechanisms of protein kinases. In this review, we focus on the protein phosphatases that dephosphorylate and regulate multifunctional Ca^{2+} /calmodulin-dependent protein kinases (CaMKs), which are widely known to play critical roles in intracellular Ca^{2+} -signaling, and have been recently shown to be involved in the pathogenesis of various diseases. The possibility that these regulating phosphatases could represent potential drug targets is also discussed.

Multifunctional CaMKs

Calcium ions are known to play important roles in the regulation of a variety of neuronal functions, and most of the diverse actions of Ca^{2+} are mediated by protein phosphorylation by three multifunctional calmodulin-dependent protein kinases (CaMKs), CaMKI, CaMKII and CaMKIV (Fujisawa, 2001; Hook and Means, 2001; Soderling and Stull, 2001; Hudmon and Schulman, 2002; Yamauchi, 2005). These kinases have broad substrate specificities and are involved in a variety of physiological responses in response to intracellular Ca^{2+} rises through the phosphorylation of various substrate proteins. The biochemical properties and physiological significance of multifunctional CaMKs are briefly summarized in Table 1. CaMKII exhibits extremely broad substrate specificity, and a variety of proteins have been reported to serve as substrates for CaMKII. The possible involvement of CaMKII in the regulation of neuronal functions, such as neurotransmitter synthesis, neurotransmitter release, long-term potentiation (LTP) and the formation of spatial learning, has been suggested (Hudmon and Schulman, 2002; Yamauchi, 2005).

CaMKIV occurs abundantly in the brain and thymus. CaMKII is known to be activated following autophosphorylation of Thr286, whereas CaMKIV is strongly activated upon phosphorylation by another CaMK, CaMK kinase (CaMKK) (Fujisawa, 2001; Hook and Means, 2001; Soderling and Stull, 2001), and phosphorylates a number of proteins including synapsin I, microtubule associated protein 2 (MAP2), tau, myosin light chain, tyrosine hydroxylase and CREB (Miyano et al., 1992; Hook and Means, 2001). CaMKIV is thought to play a role in mediating Ca²⁺-regulated transcription through phosphorylation of transcription factors such as CREB, ATF and SRF (Hook and Means, 2001), and was shown to be involved in neuronal functions such as learning and memory (Kang et al., 2001; Wei et al., 2002; Mizuno and Giese, 2005; Colomer and Means, 2007). CaMKIV is also expressed in testis, and plays an important role in spermatogenesis (Wu et al., 2000). The participation of CaMKIV in cardiac hypertrophy (Passier et al., 2000) and in mitochondrial biogenesis in skeletal muscle (Wu et al., 2002) has been suggested. CaMKI, which is distributed in various tissues including brain, shows a substrate specificity similar to that of CaMKIV (Hook and Means, 2001). Although its physiological significance mostly remains to be clarified, a possible involvement of CaMKI in cell cycle regulation has been reported, as discussed below (Rodriguez-Mora et al., 2005; Colomer and Means, 2007).

Multifunctional CaMKs and diseases

There is growing evidence that CaMKs are involved in the pathogenic mechanisms underlying various human diseases. Since CaMKs play pivotal roles not only in the central nervous system, but also in other tissues including heart, pancreas and bone, aberrant expression or misregulation of CaMKs may be responsible for various diseases, such as neurological disorders, heart failure, diabetes and osteoporosis. Thus, it has been pointed out that specific inhibitors or activators of CaMKs, which artificially regulate CaMK activities in a specific manner, might have potential as therapeutics for these diseases. In this chapter, we will briefly summarize recent progress regarding the involvement of CaMKs in various diseases.

The central nervous system

It is widely accepted that CaMKII plays important roles in the regulation of higher-order neuronal functions such as memory. Transgenic mice with genes encoding mutant CaMKII in which the autophosphorylation site Thr286 had been replaced with a non-phosphorylatable residue Ala exhibited reduced LTP and impaired spatial memory, suggesting that autophosphorylation of Thr286 of CaMKII plays an important role in the formation of spatial memory (Giese et al., 1998).

However, transgenic mice expressing mutant CaMKII with a Thr286→Asp mutation, which mimicked the autophosphorylation of Thr286, also exhibited impaired learning and memory (Mayford et al., 1995). On the other hand, autophosphorylation of Thr305/306 caused inactivation of CaMKII due to reduced CaM-binding (Hudmon and Schulman, 2002; Yamauchi, 2005). Mutant CaMKII in which Thr305/306 had been replaced with Val or Ala did not show such inactivation. Transgenic mice with these mutations exhibited normal LTP, but reversal learning and contextual discrimination were impaired (Elgersma et al., 2002; Elgersma et al., 2004). Replacement of the Thr residue with Asp, which mimicked phosphorylation, resulted in impaired activation of CaMKII due to inhibition of CaM-binding. Transgenic mice carrying this mutation also exhibited reduced LTP and reduced learning ability (Elgersma et al., 2002; Elgersma et al., 2004).

Taken together, these data suggest that the autophosphorylation status of CaMKII is closely related to learning and memory ability, and that CaMKII activity must be finely regulated by autophosphorylation at an appropriate level and in a timely manner for the development of normal learning and memory. The autophosphorylation level of CaMKII is also regulated by protein phosphatases that dephosphorylate it. Genoux et al. (2002) examined the effects of inhibition of PP1, which is believed to be a major protein phosphatase responsible for the dephosphorylation of CaMKII in the postsynaptic density (PSD), on learning and memory. For this purpose, they generated transgenic mice that inducibly expressed the activated form of I-1 (I-1*), a specific inhibitory protein for PP1, in a brain-specific manner. These transgenic mice showed significant improvements in learning and memory in an I-1*-dependent manner, with the improvements being especially remarkable in aged mice.

CaMKII is likely to be involved in the pathogenesis of various diseases of the central nervous system. It is reported that autophosphorylation of Thr286 and Thr305 of CaMKII are significantly enhanced in Ube3a ubiquitin ligase gene knockout mice, a pathological model for Angelman's syndrome, which is characterized by severe cognitive impairment and convulsive seizure (Weeber et al., 2003). These neurological deficits were rescued by introducing a Thr305→Val/Thr306→Ala double mutation, which prevented self-inhibition of CaMKII by abrogating autophosphorylation of these residues (van Woerden et al., 2007). These findings strongly suggest that aberrant autophosphorylation of CaMKII is closely related to some central nervous system diseases. Moreover, abnormal autophosphorylation of CaMKII may be involved in the pathophysiology of Parkinson's disease. Using CaMKII inhibitors, Picconi et al. (2004) showed that hyperphosphorylated CaMKII plays a causal role in the alteration of striatal plasticity and motor behavior that follow dopamine denervation in a rat model of parkinsonism. Brown et al. (2005) also reported that dopamine depletion in a rat model of parkinsonism increased

autophosphorylation of CaMKII, which was reversed by treating the rats with L-DOPA, a well-known therapeutic agent for the disease. Aberrant autophosphorylation of CaMKII associated with Parkinson's disease and other diseases of the central nervous system might be due to misregulation of the protein phosphatases responsible for the dephosphorylation/regulation of CaMKII (see below), although this possibility remains to be explored. Yoshimura et al. (2003) reported that one fourth of the phosphorylation sites in anomalously phosphorylated tau protein, a major component of the neurofibrillary tangles (NFATs) characteristic of Alzheimer's disease, were phosphorylated by CaMKII, suggesting that CaMKII is involved in the pathogenic mechanism underlying Alzheimer's disease.

Transient cerebral ischemia leads to the delayed and selective degeneration of certain populations of neurons. This phenomenon, called “delayed neuronal death”, often causes serious clinical problems such as sequelae after cerebral infarction. It is suggested that CaMKII is involved in the process of delayed neuronal death (Laabich and Cooper, 2000; Takano et al., 2003). On the other hand, Yano et al. (2005) provided evidence for the possible involvement of activation of protein kinase B (Akt) and CaMKIV in the induction of neuroprotective action against delayed neuronal death.

Heart

Recently, attention has been given to the roles of CaMKII in heart (Zhang and Brown, 2004; Anderson, 2005; Hund and Rudy, 2006). CaMKII δ_B , which has a nuclear localization signal, executes a hypertrophic gene program by transcriptional regulation via the phosphorylation of histone deacetylase leading to the development of cardiac hypertrophy. On the other hand, CaMKII δ_C in the cytosol facilitates Ca²⁺ leakage from the sarcoplasmic reticulum by inducing phosphorylation of ryanodine receptors to cause systolic/diastolic heart failure (Zhang and Brown, 2004). CaMKII δ_C associates with myocardial Na⁺ channels, and is involved in the pathogenesis of arrhythmia by phosphorylating these channels (Wagner et al., 2006). In addition, CaMKII δ_C also associates with L-type Ca²⁺ channels, which play an important role in the myocardial systolic/diastolic cycle by regulating Ca²⁺ influx across the plasma membrane. It is reported that the activity of these channels is augmented by phosphorylation by CaMKII (Grueter et al., 2006). Although the detailed mechanism is not yet clarified, CaMKII is likely to participate in cardiomyocyte apoptosis (Zhang and Brown, 2004; Anderson, 2005; Hund and Rudy, 2006; Zhu et al., 2007). Thus, CaMKII plays important roles in various aspects of the myocardial systolic/diastolic cycle, and dysfunction of CaMKII could cause heart failure. Based on these lines of evidence, the possibility that CaMKII-specific inhibitors provide a promising therapeutic

approach for heart failure has been pointed out. Indeed, transgenic mice expressing a peptide inhibitor of CaMKII exhibited a resistant phenotype against myocardial infarction (Zhang et al., 2005). However, transgenic expression of a similar CaMKII peptide inhibitor, AIP, specifically in the myocardial longitudinal sarcoplasmic reticulum of mice, was reported to cause stimulated cardiac hypertrophy (Ji et al., 2003). Therefore, temporal and spatial regulation of CaMKII expression should be taken into consideration for the therapeutic use of CaMKII-specific inhibitors or activators. Meanwhile, CaMKIV is reported to be involved in cardiac hypertrophy by inducing MEF2-mediated transcriptional activation (Passier et al., 2000).

Insulin secretion from the pancreas

Since insulin secretion from pancreatic β -cells is a Ca^{2+} -dependent process, it has been suggested that a Ca^{2+} -dependent protein kinase is involved in this process. To assess the relevance of CaMKs to insulin secretion, KN-62, an inhibitor of CaMKs, was used. Since KN-62 had been shown to inhibit L-type Ca^{2+} channels as well as CaMKs, there has been some controversy over whether CaMKs were involved in insulin secretion. Thereafter, using KN-93, another structurally different inhibitor of CaMKs, and AIP, a highly specific CaMKII inhibitor, careful experiments were performed and the data obtained strongly suggested that CaMKII plays important roles in insulin secretion (Easom, 1999). A recent study using a more specific CaMKII inhibitor also supports this contention (Vest et al., 2007). Thus, CaMKII might be involved in the pathogenesis of diabetes. Although it has not yet been clarified at which stage of the insulin secretion process CaMKII is involved, CaMKII has been shown to participate in cyclic ADP-ribose-mediated intracellular Ca^{2+} mobilization via ryanodine receptors leading to insulin secretion (Takasawa et al., 1995).

Bone formation

There are far fewer reports of an involvement of CaMKs other than CaMKII, such as CaMKI and CaMKIV, in the pathogenesis of diseases. Recently, Sato et al. (2006) reported that CaMKIV plays important roles in the differentiation and function of osteoclasts, which are crucial for bone metabolism, via the CaMKIV-CREB pathway; bone volume and bone mineral density were significantly increased in CaMKIV knockout mice. Administration of KN-93, a specific inhibitor of CaMKs including CaMKIV, in model mice with postmenopausal osteoporosis induced by ovariectomy, showed significant therapeutic effects on the bone loss associated with ovariectomy. These results suggest that CaMKIV is a promising drug target for the treatment of osteoporosis.

Cancer

CaMKs may be involved in the pathogenesis of cancer. Previous studies suggest that CaMKs including CaMKII participate in cell cycle regulation. Recently, CaMKI and CaMKK have been shown to be essential for cell cycle progression through G1 phase into S phase using RNA interference technology (Rodriguez-Mora et al., 2005). CaMKI is likely to act on the cyclin D1/CDK4 complex to regulate its function (Colomer and Means, 2007). It was also reported that CaMKII plays an important role in the duplication of the centrosome (Matsumoto and Maller, 2002), and that CaMKII phosphorylates Cdc25, an important regulator of the G2/M transition, to activate its phosphatase activity (Patel et al., 1999). There are many reports suggesting an involvement of CaMKII and CaMKIV in the apoptosis of various cells (See et al., 2001; Fladmark et al., 2002; Yang et al., 2003). Xiao et al. (2002) pointed out the possibility that CaMKII inhibitors show anti-tumor activity against gliomas through stimulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. The cytotoxicity of ionizing irradiation and anti-tumor drugs often causes serious side effects in cancer therapy. Rodriguez-Mora et al. (2005) proposed an interesting hypothesis that CaMK inhibitors sensitize cancer cells to ionizing irradiation and anti-tumor drugs that generate reactive oxygen species through the suppression of cellular stress response systems. If that is the case, combined use of CaMK inhibitors with radiotherapy or chemotherapy might be expected to relieve the harmful side effects associated with these therapies by enabling a decrease in the dose of radiation or the anti-tumor drug.

Mechanisms of activation of multifunctional CaMKs

As described above, CaMKs play crucial roles *in vivo*, and so their dysfunction causes various diseases. Therefore, it is extremely important to explore the mechanisms underlying the activation of CaMKs in view of not only basic biological science, but also clinical pharmacology. Phosphorylation of multifunctional CaMKs, as well as binding of Ca²⁺/calmodulin (CaM), plays an important role in the regulation of their kinase activities. The mechanisms by which multifunctional CaMKs are activated by phosphorylation have been extensively studied as “switch on” mechanisms.

CaMKII activity is complicatedly regulated by autophosphorylation, and multiple autophosphorylation sites of CaMKII have been identified. Among them, Thr286, which is located within the autoinhibitory domain, is the most important for regulation. Following activation, rapid autophosphorylation at Thr286 is observed, resulting in dramatic changes in enzymatic properties such as the generation of Ca²⁺/CaM-independent activity, and a thousand-fold elevation in its affinity for Ca²⁺/CaM. These changes in enzymatic properties are thought to be

essential for CaMKII to induce LTP at synapses. There are many excellent reviews detailing the regulation of CaMKII activity by autophosphorylation (Fujisawa, 2001; Hook and Means, 2001; Soderling and Stull, 2001; Hudmon and Schulman, 2002; Yamauchi, 2005).

In the cases of CaMKI and CaMKIV, phosphorylation at a Thr residue (Thr177 for CaMKI, and Thr196 for CaMKIV) located within the region called the “activation loop” is a key event in their activation. This phosphorylation reaction is catalyzed by a distinct protein kinase designated CaMKK (Fujisawa, 2001; Hook and Means, 2001; Soderling and Stull, 2001). Interestingly, CaMKK is also a member of the CaMK family. Although CaMKK is highly specific for CaMKI and CaMKIV (Okuno et al., 1997), constituting a so-called “CaMK cascade”, AMP-activated protein kinase (Hawley et al., 1995; Colomer and Means, 2007) and protein kinase B (Akt) (Yano et al., 1998; Okuno et al., 2000) are also reported to serve as substrates of CaMKK. In view of structural biology, the regulatory mechanisms of CaMKI were clarified in detail, with the three-dimensional structure being determined by X-ray crystallography (Goldberg et al., 1996). Recently, the three-dimensional structure of CaMKII has also been solved (Hoelz et al., 2003; Rosenberg et al., 2005).

Negative regulation of multifunctional CaMKs by commonly known protein phosphatases

As discussed above, volumes of data on the activation of multifunctional CaMKs via phosphorylation, in so-called “switch on” mechanisms, have been accumulated. By contrast, the deactivation, “switch off” mechanisms of dephosphorylation have remained uncertain until recently. However, several groups including ours have recently identified protein phosphatases that dephosphorylate multifunctional CaMKs, prompting a better understanding (Ishida et al., 2003; Colbran, 2004). In the following sections, we summarize the protein phosphatases involved in the negative regulation of multifunctional CaMKs and discuss their physiological significance. The biochemical properties of these phosphatases are summarized in Table 2. Ser/Thr protein phosphatases can be classified into two superfamilies on the basis of similarities in primary amino acid sequence. One is the PPP family and the other the PPM family (Barford et al., 1998). The former consists of the most abundant Ser/Thr protein phosphatases in eukaryotes, PP1, PP2A, PP2B (calcineurin) and some other novel phosphatases. The latter group consists of PP2C and other structurally related phosphatases, which require Mg^{2+} or Mn^{2+} for their activity, and which exist as monomers devoid of regulatory subunits.

PP1 and CaMKs

PP1 is a Ser/Thr protein phosphatase composed of catalytic and regulatory subunits (Shenolikar and Nairn, 1991; Cohen, 2002; Ceulemans and Bollen, 2004). Several isoforms of the catalytic subunit and various regulatory subunit molecules of PP1 have been identified. The involvement of the regulatory subunits in a variety of functions, such as regulation of catalytic activity, subcellular localization and substrate specificity of PP1, has been reported. The dephosphorylation of autophosphorylated CaMKII in the PSD seems to be mainly catalyzed by PP1 anchored to the PSD through its scaffolding proteins (Strack et al., 1997; Yoshimura et al., 1999; Colbran, 2004). A hypothesis that PP1 in the PSD plays a pivotal role in the expression of LTP through the dephosphorylation of CaMKII has been presented (Lisman and Zhabotinsky, 2001). PP1 was shown to enhance the apparent cooperativity for autophosphorylation of CaMKII, making it an ultra sensitive molecular switch toward Ca^{2+} (Bradshaw et al., 2003). Very recently, however, Mullasseril et al. (2007) reported that PP1 in the PSD dephosphorylates many sites on CaMKII, but not Thr286, which is responsible for key regulatory mechanisms including generation of Ca^{2+} /CaM-independent activity. These authors suggested a novel mechanism that maintains the “on-state” of CaMKII in the PSD by structural constraints. CaMKII also plays important physiological roles in various tissues other than brain, as discussed above. PP1 and Mg^{2+} -dependent protein phosphatases (Easom et al., 1998) and PP1 (Hwang et al., 1996) have been shown to play major roles in the dephosphorylation of autophosphorylated CaMKII in pancreatic β cells and pancreatic acinar cells, respectively. On the other hand, Kasahara et al. (1999) reported that CaMKIV that had been phosphorylated and activated *in vitro* was markedly dephosphorylated and deactivated by PP1.

PP2A and CaMKs

PP2A ubiquitously occurs in various cells and tissues, and exists as dimer (AC) or trimer (ABC) composed of a catalytic subunit (C) and regulatory subunits (A and/or B) (Shenolikar and Nairn, 1991; Mumby, 2007). In particular, a marked molecular diversity of the regulatory subunit B (B'/B''/PR72), also called the third subunit, has been reported. Such a diversity of PP2A subunits, in conjunction with the covalent modification of each subunit and regulation by specific activators/inhibitors, produces elegant but intricate regulation of catalytic activity, substrate specificity and intracellular localization of PP2A. In contrast to PSD-associated CaMKII, cytosolic CaMKII seems to be dephosphorylated *in vivo* mainly by protein phosphatases other than PP1 (Fukunaga et al., 2000). In a pharmacological study using rat brain slices and protein phosphatase inhibitors, it was deduced that negative regulation of cytosolic CaMKII activity is mainly carried out by PP2A in the mammalian forebrain (Bennechib et al., 2001). Interestingly, induction and maintenance of LTP in the rat CA1 hippocampal region are associated with a

significant decrease in PP2A activity, which appears to be due to direct phosphorylation of the regulatory subunit B' of PP2A by CaMKII (Fukunaga et al., 2000). On the other hand, it was reported that the δ isoform of CaMKII, which is expressed in cardiac muscle and can induce cardiac gene expression and hypertrophy as discussed above, forms a complex with PP2A (Zhang et al., 2002). PP2A is also known to form a complex with the catalytic domain of CaMKIV. PP2A is suggested to play an important role in the rapid deactivation of CaMKIV after cellular stimulation through a complex formation with CaMKIV (Westphal et al., 1998). In the case of CaMKI, it was reported that PP2A deactivated *in vitro* CaMKI that had been purified from rat brain (DeRemer et al., 1992).

PP2B and CaMKs

PP2B (calcineurin), a Ca^{2+} /CaM-dependent protein phosphatase that consists of a catalytic subunit (A) and a regulatory subunit (B) (Shenolikar and Nairn, 1991; Aramburu et al., 2000), is believed to be unable to directly dephosphorylate autophosphorylated CaMKII (Table 2). However, PP2B dephosphorylates I-1, which is a specific endogenous protein inhibitor of PP1. I-1 inhibits PP1 when it is phosphorylated by cAMP-dependent protein kinase (PKA), and the inhibition of PP1 is cancelled through the dephosphorylation of I-1 by PP2B. Based on these observations, an indirect role of PP2B in the regulation of CaMKII via I-1/PP1 by PKA/PP2B is suggested. The indirect regulatory mechanism of CaMKII activity by PKA/PP2B in the modulation of synaptic transmission in response to the frequency of the neuronal stimulation is now widely accepted (Makhinson et al., 1999; Winder and Sweatt, 2001; Colbran, 2004). PP2B may also participate in the negative regulation of CaMKIV by inducing its direct dephosphorylation *in vivo* (Kasahara et al., 1999).

PPM phosphatases and CaMKs

At least 12 distinct PPM phosphatases with a marked molecular diversity, 2C α , 2C β , 2C γ /FIN13, 2C δ /ILKAP, 2C ϵ , 2C ζ , 2C η , Wip1, CaMKP, CaMKP-N, NERPP-2C, and SCOP/PHLPP, have been identified in mammalian cells (Tamura et al., 2006). PP2C is the prototype of a PPM family phosphatase (see Table 2). It has been suggested that both okadaic acid-insensitive and -sensitive protein phosphatases are involved in the dephosphorylation of CaMKII in rat cerebellar granule cells (Fukunaga et al., 1989). These authors also showed that PP2C dephosphorylates and regulates CaMKII *in vitro* (Fukunaga et al., 1993); however, a lack of specific inhibitors for PP2C hampers full elucidation of how CaMKII activity is regulated by PP2C *in vivo*.

A protein phosphatase that specifically dephosphorylates and regulates multifunctional CaMKs: CaMKP and CaMKP-N

All of the protein phosphatases mentioned above are well-known Ser/Thr protein phosphatases with broad substrate specificity. We assumed that there could be another protein phosphatase that might specifically act on CaMKII to negatively regulate its activity. Making use of newly developed assay techniques, we purified a novel protein phosphatase that dephosphorylated Thr286 of CaMKII, from rat brain (Ishida et al., 1998a; 2003). This phosphatase is highly specific for multifunctional CaMKs, and the activated CaMKs were reversibly deactivated by the phosphatase. Thus, we called the phosphatase CaMK phosphatase (CaMKP) (Ishida et al., 1998b; 2001; 2003). Although CaMKP is a protein phosphatase belonging to the PPM family, its sequence identity to PP2C α is only 28%, even in the phosphatase domain (Figure 1a) (Kitani et al., 1999; Ishida et al., 2003). At the N-terminus, it has a large domain that is not shared by PP2C, with a characteristic cluster of glutamic acid residues. It seems that the N-terminal domain of CaMKP functions as an association domain to bind/recognize other proteins including substrates and modulators (Ishida et al., 2005), and that the N-terminal region of CaMKP is crucial for its unique substrate specificity (Tada et al., 2006). Western blotting analysis with a specific antibody to CaMKP revealed that CaMKP is expressed ubiquitously in all of the tissues examined, including lung, thymus, brain, spleen, uterus and pancreas (Kitani et al., 1999). Immunocytochemical analysis of PC12 cells (Kitani et al., 1999) and rat brain tissue (Nakamura et al., 2000) showed that CaMKP is localized only in the cytoplasm, and was never observed in the nucleus. The distribution of CaMKP and CaMKs overlapped in various regions in the brain and spinal cord.

A cDNA clone showing 52% identity to human CaMKP was found in human cDNA databases using the sequence of rat CaMKP as a query. This homologue is highly homologous to human CaMKP, but has large regions without homology to CaMKP in both N- and C-termini (Figure 1b) (Takeuchi et al., 2001). Unlike CaMKP, the mRNA of this homologue was specifically expressed in brain. When the cDNA was expressed in COS cells, the expressed protein was localized to the nucleus, in contrast to CaMKP. Biochemical analysis of a partially purified preparation of the protein, obtained from Sf9 cells expressing the cDNA, revealed that the enzymatic properties are similar to those of CaMKP. Thus, we named the enzyme CaMKP-N after its localization in the nucleus. The differences in tissue and subcellular distribution of CaMKP and CaMKP-N raise the possibility that CaMKP and CaMKP-N play some complementary roles in cells (Kitani et al., 2003; Ishida et al., 2003).

Functional analysis of CaMKP and CaMKP-N *in vivo*

There are several papers regarding the biological functions of CaMKP and CaMKP-N. Tan et al. (2001) identified CaMKP as a human homologue of FEM-2, a product of a gene that participates in sex-determination in *C. elegans*. Transient expression of nematode FEM-2, human CaMKP or rat CaMKP in HeLa cells resulted in apoptosis; by contrast, the expression of PP2C α , another PPM family protein phosphatase, did not induce apoptosis. These data suggest that CaMKP is involved in apoptotic signaling, although it is unclear how the promotion of apoptosis relates to the intracellular dynamics of CaMKs. Since there are many reports that CaMKII and/or CaMKIV is involved in apoptosis (See et al., 2001; Fladmark et al., 2002; Yang et al., 2003), CaMKP and CaMKP-N might regulate cellular apoptosis by modulating CaMK activities. Harvey et al. (2004) reported that overexpression of human CaMKP in fibroblasts resulted in a marked attenuation of the CaMKII-dependent phosphorylation of vimentin. This means that CaMKP could actually function as a negative regulator of CaMKII in cells. However, Koh et al. (2002) reported that CaMKP/CaMKP-N participated in the regulation of PAK, which is a Ser/Thr protein kinase that interacts with the activated GTP-bound forms of Cdc42.

We examined what roles CaMKP and CaMKP-N play in the early development of vertebrates using zebrafish as a model animal. We found a CaMKP homolog of zebrafish (zCaMKP) in the GenBank zebrafish whole genome shotgun database, and cloned its full-length cDNA by PCR. Microinjection of zebrafish embryos with antisense morpholino oligonucleotides against zCaMKP, to eliminate CaMKP, resulted in severe morphological abnormality of the zebrafish with apoptotic cells throughout the whole body. These observations strongly suggest that zCaMKP plays an essential role in the early development of the zebrafish embryo (Sueyoshi et al., *in preparation*). Likewise, the full-length cDNA of zebrafish homolog of CaMKP-N (zCaMKP-N) was obtained by a BLAST search of the whole genome shotgun database and subsequent PCR cloning. When Neuro2a cells expressing rat CaMKIV with or without zCaMKP-N were stimulated by the Ca²⁺ ionophore ionomycin, the phosphorylation level of CaMKIV was greatly reduced in cells co-expressing CaMKIV together with zCaMKP-N, suggesting that zCaMKP-N functions as a negative regulator of CaMKIV *in vivo* as well as *in vitro*. Gene knockdown of zCaMKP-N using morpholino-based antisense oligonucleotides induced significant morphological abnormalities of the head and spinal cord in zebrafish embryos. Acridine orange staining indicated that numerous cells of the brain and spinal cord exhibited typical apoptosis (Figure 2). Thus, it was revealed that zCaMKP-N is essential for early development of the brain and spinal cord in zebrafish (Nimura et al., 2007).

Inhibitors of PPM family phosphatases: pharmacological applications

As discussed earlier, CaMKs play important roles not only in the central nervous system, but also in other tissues such as heart, pancreas and bone. Moreover, since CaMKs are intimately involved in cell cycle control and in the regulation of apoptosis, they are suggested to be closely related to the mechanisms of carcinogenesis and mode of action of anticancer drugs. Therefore, dysfunction of their regulatory mechanisms and/or their aberrant expression would cause various diseases. As we saw above, the activities of CaMKs are strictly regulated through (auto)phosphorylation, and their phosphorylation levels are also under the control of protein phosphatases that dephosphorylate the phosphorylation sites responsible for activation. Therefore, the protein phosphatases that regulate CaMKs represent alternative targets to artificially control CaMK activities, instead of directly inhibiting or stimulating CaMK activities themselves. Indeed, as mentioned, transgenic mice that inducibly expressed the activated form of I-1, a specific inhibitor of PP1, in a brain-specific manner, showed significant improvements in learning and memory (Genoux et al., 2002). This means that the alternative approach of regulating CaMK activities by inhibiting the responsible protein phosphatase is promising. Since CaMKP is highly specific for multifunctional CaMKs, unlike PP1, a specific inhibitor of CaMKP is expected to have relatively fewer systemic side effects than those of PP1 or PP2A. Furthermore, such an inhibitor would be extremely useful for exploring the physiological significance of CaMKP. Unfortunately, however, specific inhibitors for PPM family phosphatases, including CaMKP and PP2C, are not yet available.

In an attempt to obtain useful inhibitors or activators 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 and Chicago Sky Blue 6B, were found to be potent inhibitors of CaMKP and CaMKP-N with satisfactory cell permeability, but showed no significant inhibition toward PP2C and PP2B (Figure 3) (Sueyoshi et al., 2007). This observation suggests that these compounds can discriminate subtle differences in the structures of the active sites of CaMKP/CaMKP-N and PP2C, although it has so far been thought that the three-dimensional structure around the active center of CaMKP is very similar to that of PP2C (Tada et al., 2006). We also identified 1-amino-8-naphthol-4-sulfonic acid as the minimum structure required for the inhibition of CaMKP/CaMKP-N (Figure 3, compound 5). However, since Evans Blue and Chicago Sky Blue 6B are reported to be potent inhibitors of the vesicular uptake of glutamate (Roseth et al., 1998), improvements to the inhibitors on the basis of the structure-function relationship are necessary so that they might be specific enough to CaMKP and/or CaMKP-N for

their pharmacological use.

To date, no specific and potent inhibitors of PPM phosphatases, including CaMKP/CaMKP-N, with sufficient cell permeability, have been reported. This has hampered studies on the physiological significance of PPM phosphatases, whereas those of PPP phosphatases have been greatly facilitated by the existence of specific inhibitors, such as okadaic acid. There is increasing evidence that many PPM family phosphatases modulate a variety of stress response systems. For instance, PP2C ϵ participates in the negative regulation of an apoptotic pathway mediated by reactive oxygen species via Ask1 (Tamura et al., 2006). Therefore, an effective inhibitor specific to PP2C ϵ might be expected to reduce the side effects associated with cancer therapy by sensitizing cancer cells to irradiation or anticancer drugs, like the CaMKII inhibitors discussed above (Rodriguez-Mora, 2005). Wip1 has been shown to be intimately involved in the oncogenic transformation of cells by suppression of the activation of p53. Wip1-specific inhibitors are expected to be a novel type of anticancer drug, and efforts to develop such drugs are now underway (Belova et al., 2005; Yamaguchi et al., 2006). Exploiting specific inhibitors of PPM family phosphatases, including CaMKP/CaMKP-N, is an important subject not only for the elucidation of the physiological functions of these enzymes, but also for clinical application aimed at the development of novel chemotherapies.

Concluding Remarks

CaMKs, which are positioned in the center of intracellular Ca²⁺-signaling pathways, are regulated by phosphorylation like many other protein kinases. Since phosphorylation status is balanced by protein kinases and protein phosphatases, the mechanism of dephosphorylation /deactivation of CaMKs corresponding to the “switch off” mechanism is as important as the mechanism of phosphorylation/activation corresponding to the “switch on” mechanism. Thus, phosphorylation reactions are to dephosphorylation reactions what heads of a coin are to tails of a coin; neither reaction can be discussed separately. However, research on protein kinases, including CaMKs, have so far been largely biased towards the “switch on” mechanism, and so it seems that little attention has been paid to the “switch off” mechanism catalyzed by protein phosphatases. For example, in the case of CaMKII, there are various protein phosphatases that are likely to be involved in the “switching off” of CaMKII (Ishida et al., 2003; Colbran, 2004), but the details of “who does what” in cells are not yet fully understood. It is very important to address this issue as well as to elucidate the temporal and spatial interactions *in vivo* among these phosphatases and CaMKII, in detail. We believe that unraveling the detailed molecular mechanisms underlying the

negative regulation of protein kinases by protein phosphatases can provide an overview of the complicated network of intracellular signal transduction mediated by protein phosphorylation. Efforts to uncover these mechanisms are now underway.

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Statement of conflict of interest

The authors declare no conflict of interest.

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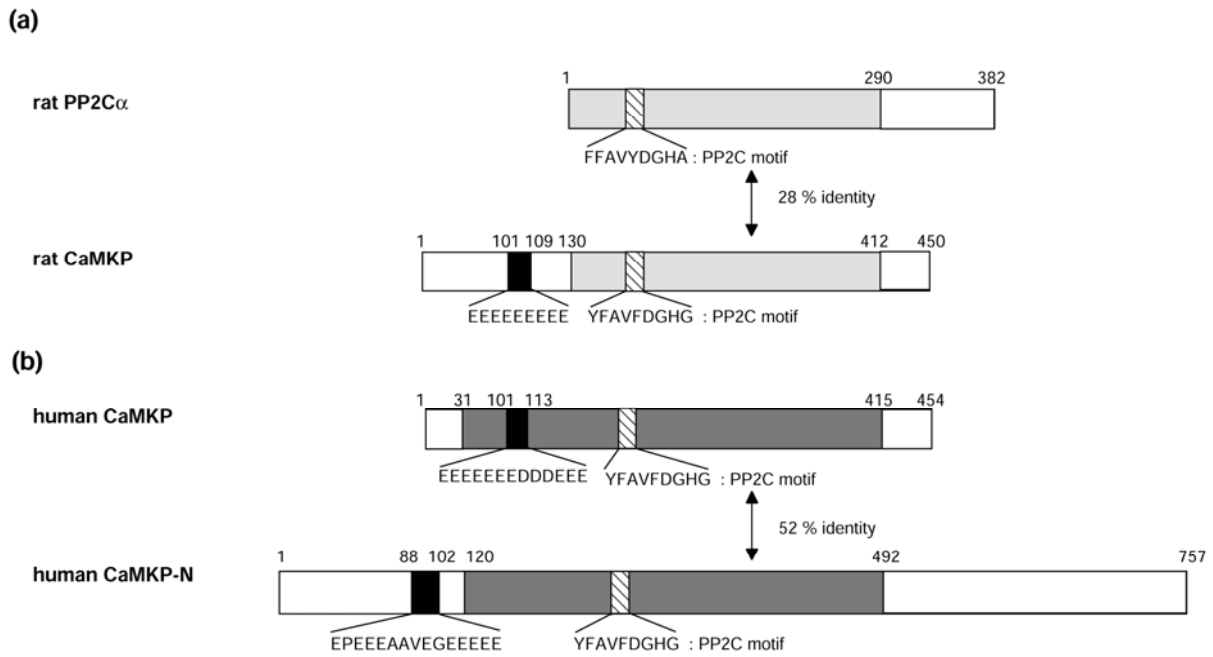
Zhu W, Woo AY, Yang D, Cheng H, Crow MT, Xiao RP (2007). Activation of CaMKII δ_C is a common intermediate of diverse death stimuli-induced heart muscle cell apoptosis. *J Biol Chem* **282**: 10833-10839.

Figure legends

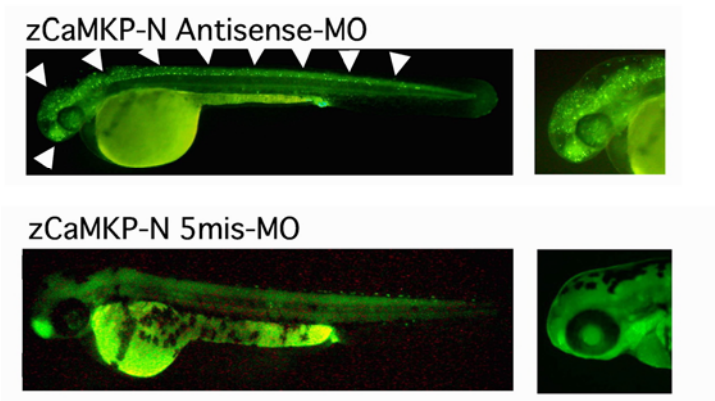
Figure 1 Domain structures of CaMKP and CaMKP-N. (a) The domain structures of rat PP2C α and CaMKP are shown. Phosphatase domains of PP2C α and CaMKP, which show modest sequence homology (light gray, 28% identity), were aligned with the PP2C motifs located within these regions being indicated (hatched bar). A Glu cluster located within the N-terminal domain of CaMKP (101-109, filled bar) is also shown. (b) The domain structures of human CaMKP and human CaMKP-N are shown. Regions of human CaMKP and CaMKP-N, which show significant sequence homology (dark gray, 52% identity), were aligned with the PP2C motifs located within the indicated regions (hatched bar). Cluster sequences with acidic amino acids located within the N-terminal regions of human CaMKP and CaMKP-N are also shown (filled bar).

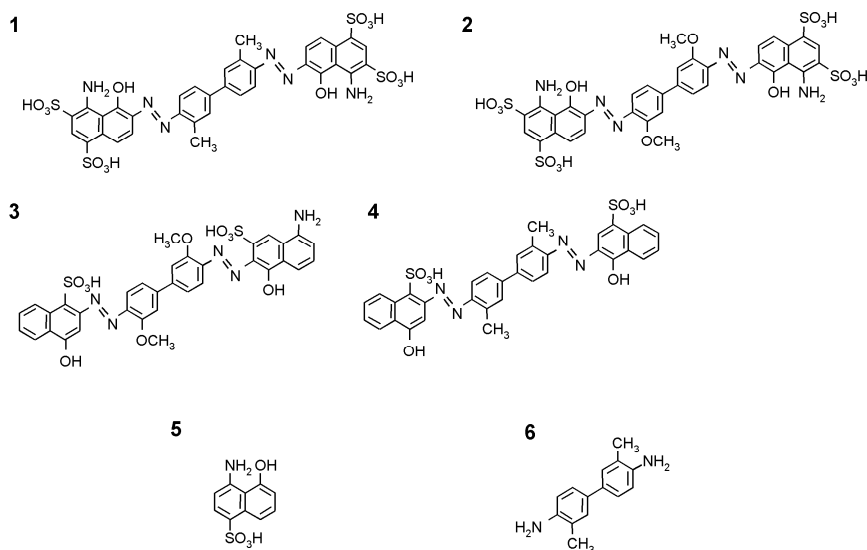
Figure 2 Gene knockdown experiments using an antisense morpholino oligonucleotide designed on the basis of the sequence at the 5'-untranslated region of zCaMKP-N mRNA. Zebrafish embryos at the 1-4 cell stage were injected with an antisense morpholino oligo (Antisense-MO) or a 5-base mismatch morpholino oligonucleotide (5mis-MO). At 48 hpf, embryos were stained with acridine orange and observed by stereoscopic microscopy. Injection of the antisense-MO resulted in abnormal apoptotic cell death during embryogenesis. Arrowheads indicate apoptotic cells stained with acridine orange (white spots).

Figure 3. Compounds that inhibit CaMKP and CaMKP-N with no significant inhibition toward PP2C and PP2B. 1, Evans Blue; 2, Chicago Sky Blue 6B; 3, Oxamine Blue B; 4, Azo Blue; 5, 1-amino-8-naphthol-4-sulfonic acid; 6, 3,3'-dimethylbenzidine. The IC₅₀ values for these compounds are also shown in the table.



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Name	Compound No.	IC ₅₀ (μM)			
		CaMKP	CaMKP-N	PP2C	PP2B
Evans Blue	1	6.2 ± 0.2	0.9 ± 0.1	*	**
Chicago Sky Blue 6B	2	4.1 ± 0.3	1.0 ± 0.1	*	**
Oxamine Blue B	3	7.9 ± 0.3	2.2 ± 0.3	>40	ND
Azo Blue	4	16.1 ± 1.2	3.0 ± 0.3	>40	ND
1-amino-8-naphthol-4-sulfonic acid	5	3.3 ± 0.2	3.8 ± 0.8	*	*
3,3'-dimethylbenzidine	6	>40	ND	>40	ND

*, Less than 10% inhibition at 10 μM. **, Less than 20% inhibition at 10 μM. ND, not determined.