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Protein phosphatases that regulate multifunctional Ca^{2+}/calmodulin-dependent protein kinases: from biochemistry to pharmacology

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Abstract

Multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) play pivotal roles in Ca^{2+} signaling pathways, such as the regulation of the neuronal functions of learning, memory and neuronal cell death. The activities of the kinases are strictly regulated by protein phosphorylation/dephosphorylation. Although the activation mechanisms for multifunctional CaMKs through phosphorylation, which correspond to “switch on”, have been extensively studied, the negative regulatory mechanisms through dephosphorylation, which correspond to “switch off”, have not. In this review, we focused on the regulation of multifunctional CaMKs by the protein phosphatases responsible. We first summarized the current understanding of negative regulation of CaMKs by known protein phosphatases and their physiological significance. We then discussed newly developed methods for detection of protein phosphatases involved in the regulation of CaMKs. We also summarized the biochemical properties of a novel protein phosphatase, which we isolated with the new methods and designated as Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP), and its homologue. Pharmacological implications for neuronal functions including memory and neuronal cell death are discussed from the viewpoint that regulation of protein kinase activity can be elucidated by focusing on protein phosphatases involved in its “switch off” mechanism.

Keywords: Ca^{2+}/calmodulin-dependent protein kinase, Protein phosphatase, Phosphorylation, Dephosphorylation, Negative regulation, Central nervous system

Contents

Abbreviations: AIP, autocamtide-2-related inhibitory peptide; 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; MAPK, mitogen-activated protein kinase; PAK, p21-activated protein kinase; PKA, cAMP-dependent protein kinase; poly(Lys), poly-L-lysine; PSD, postsynaptic density.

1. Introduction

About one-third of the intracellular proteins in mammalian cells are considered to be phosphorylated. The phosphorylation and the subsequent dephosphorylation reactions are catalyzed by protein kinases and protein phosphatases, respectively. They play important roles in signal transduction mediating, for example, cell proliferation and death; so that dysregulation of protein kinases and phosphatases is closely related to various diseases. 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 for regulation of the kinases (Johnson et al., 1996). Consequently, protein phosphatases that dephosphorylate phosphorylated protein kinases are also responsible for the regulation of the protein kinases. Thus, intracellular signal transduction is constructed on the basis of the subtle balance between phosphorylation and dephosphorylation. In this review, we focus on the protein phosphatases that dephosphorylate and regulate multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs).

An increase in intracellular Ca^{2+} upon cellular stimulation leads calmodulin (CaM) to bind Ca^{2+}; resulting in the conformational change of CaM. The resulting Ca^{2+}/CaM complex activates a number of Ca^{2+}/calmodulin-dependent enzymes, and elicits a variety of Ca^{2+}-dependent cellular responses. Among these enzymes, a group of Ser/Thr protein kinases activated by Ca^{2+}/CaM, called CaMKs, plays pivotal roles in the Ca^{2+} signaling pathways (Nairn & Picciotto, 1994; Soderling, 1999; Hook & Means, 2001; Fujisawa, 2001). CaMKs are classified into two categories: one is dedicated CaMKs, which have strict substrate specificities, and the other is made up of multifunctional CaMKs that can phosphorylate multiple protein substrates. Myosin light chain kinase, phosphorylase kinase, and CaMKIII belong to the former, and each kinase is responsible for the phosphorylation of a specific substrate in response to Ca^{2+} (myosin light chain for myosin light chain kinase, glycogen phosphorylase for phosphorylase kinase, and EF-2 for CaMKIII). In contrast, multifunctional CaMKs have broad substrate specificities and are involved in a much greater variety of physiological

responses through phosphorylation of various substrate proteins.

2. Multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs)

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, which occurs abundantly in brain, and is known to play important roles in the central nervous system through the regulation of the synthesis, release, and signaling of neurotransmitters (Braun & Schulman, 1995; Soderling et al., 2001; Hudmon & Schulman, 2002a; Hudmon & Schulman, 2002b). Recently, the functions of CaMKII in brain have been revealed by behavioral studies using gene knockout and transgenic mice (Matynia et al., 2002). The long-lasting enhancement of the efficacy of synaptic transmission following brief, repetitive stimulation of afferent pathways is called long-term potentiation (LTP); the molecular mechanism of its expression has been extensively studied as an elementary process in neuronal memory. It is widely accepted that CaMKII plays a crucial role in the expression of LTP, and also occurs in various tissues other than brain; and has been shown to be involved in a variety of physiological pathways including the regulation of carbohydrate metabolism, functions of various ion channels, transcription, cytoskeletal organization, and intracellular Ca^{2+} homeostasis.

CaMKIV as well as CaMKII also phosphorylates various proteins, and is enriched in brain and thymus. It has been shown that CaMKIV is involved in the regulation of Ca^{2+}-dependent gene expression through phosphorylation of various transcription factors including CREB and ATF-1 (Hook & Means, 2001). Especially, transcriptional regulation by CaMKIV through phosphorylation of CREB plays important roles in the regulation of memory and neuronal plasticity (Kang et al., 2001; Wei et al., 2002). CaMKIV is also expressed in testis, and plays an important role in spermatogenesis (Wu et al., 2000). Participation of CaMKIV in cardiac hypertrophy (Passier et al., 2000) and in mitochondrial biogenesis in skeletal muscle (Wu et al., 2002) have been suggested by experiments using transgenic mice that express constitutively

active CaMKIV.

CaMKI, distributed in various tissues including brain, shows a substrate specificity similar to that of CaMKIV; however, its physiological significance remains to be clarified (Hook & Means, 2001; Picciotto et al., 1996).

Not only Ca^{2+}/CaM, but also the phosphorylation of multifunctional CaMKs themselves plays an important role in the regulation of kinase activities. Mechanisms by which multifunctional CaMKs are activated by phosphorylation have been extensively studied as "switch on" mechanisms of these kinases.

CaMKII activity is complicatedly regulated by autophosphorylation, and multiple autophosphorylation sites of CaMKII have been identified. Among them, Thr^{286}, which is located within the autoinhibitory domain, is the most important for regulation. Following activation, the rapid autophosphorylation at Thr^{286} is observed, resulting in dramatic changes in enzymatic properties such as generation of Ca^{2+}/CaM-independent activity, and a thousand times elevation of affinity for Ca^{2+}/CaM. These changes in the enzymatic properties are thought to be essential for CaMKII to induce LTP at synapse. Many excellent reviews detailing the regulation of CaMKII activity by autophosphorylation are available. (Soderling et al., 2001; Fujisawa, 2001; Hudmon & Schulman, 2002a; Hudmon & Schulman, 2002b).

In the cases of CaMKI and CaMKIV, phosphorylation at a Thr residue (Thr^{177} for CaMKI, and Thr^{196} for CaMKIV) located within the region called the "activation loop" is a key event in their activation. This phosphorylation is catalyzed by a distinct protein kinase designated as CaMK kinase (CaMKK) (Soderling, 1999; Hook & Means, 2001; Fujisawa, 2001). Interestingly, CaMKK is also a member of the Ca^{2+}/calmodulin-dependent protein kinase family, and is highly specific for CaMKI and CaMKIV(Okuno et al., 1997), constituting a so-called "CaMK cascade."

The regulatory mechanisms of CaMKI from a structural point of view have been clarified in detail, with the three-dimensional structure being determined by X-ray crystallography (Goldberg et al., 1996).

3. Negative regulation of CaMKs by protein phosphatases

As discussed above, the activation mechanisms, called the "switch on" mechanisms, for multifunctional CaMKs through phosphorylation have been extensively studied. Whereas the deactivation, "switch off" mechanisms of dephosphorylation have been uncertain until recently. However, several groups including ours have recently identified protein phosphatases that dephosphorylate multifunctional CaMKs, prompting the better understanding. In the following sections, we summarize the protein phosphatases involved in the negative regulation of multifunctional CaMKs and discuss their physiological significance.

3.1. Negative regulation of CaMKII by protein phosphatases

As described in the previous chapter, CaMKII activity is regulated mainly by autophosphorylation at several sites including Thr^{286}. Protein phosphatases so far reported to dephosphorylate and regulate CaMKII *in vitro* are of four species: PP1, PP2A, PP2C, and Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP). The biochemical properties of these protein phosphatases are briefly summarized in Table 2 (see also reviews by Shenolikar & Nairn, 1991; Barford et al., 1998; Price & Mumby, 1999; Winder & Sweatt, 2001). Protein phosphatases that dephosphorylate Ser and Thr residues are classified into PPP and PPM families, which are defined by distinct amino acid sequences and three-dimensional structures. PP1, PP2A, and PP2B belong to the PPP family, whereas PP2C and CaMKP belong to the PPM family.

PP1 is a Ser/Thr protein phosphatase composed of a catalytic subunit and regulatory subunits. Several isoforms of the catalytic subunit and various regulatory subunit molecules of PP1 have been identified. 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.

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). In particular,

a marked molecular diversity of the regulatory subunit B (B'/B''/PR72), also called the third subunit, is reported. Such 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 the catalytic activity, substrate specificity, and intracellular localization of PP2A.

PP2C is a Ser/Thr protein phosphatase that belongs to the PPM family. PP2C shows okadaic acid-insensitive phosphatase activity, requires Mg^{2+} or Mn^{2+} for activity, and exists as a monomer devoid of a regulatory subunit (see Table 2). Recently, various PP2C homologs with a marked molecular diversity have been identified (Li et al., 2003; Kashiwaba et al., 2003).

PP1 and PP2A have long been known to regulate CaMKII negatively by dephosphorylation *in vitro* (Shields et al., 1985; Lai et al., 1986; Schworer et al., 1986). For example, in rat forebrain PP1 and PP2A are reported to be responsible for dephosphorylation of insoluble CaMKII associated with postsynaptic density (PSD) and soluble CaMKII, respectively (Strack et al., 1997a). Recently, a hypothesis that PP1 plays a pivotal role in the expression of LTP through dephosphorylation of CaMKII has been presented (Lisman & Zhabotinsky, 2001). It is reported that CaMKII translocates to PSD when it is autophosphorylated (Yoshimura & Yamauchi, 1997; Strack et al., 1997b), and the PSD-associated CaMKII is then dissociated from PSD when it is dephosphorylated (Yoshimura & Yamauchi, 1997). The concentration of CaMKII in PSD is fairly high; estimated to be 100–200 μM. The dephosphorylation of autophosphorylated CaMKII in PSD seems to be mainly catalyzed by PP1 anchored to PSD through its scaffolding proteins (Strack et al., 1997a; Yoshimura et al., 1999), and CaMKII/PP1 undergo autophosphorylation/dephosphorylation under such circumstances. Lisman and Zhabotinski proposed that CaMKII/PP1 would be an ideal molecular switch to regulate the efficacy of synaptic transmission through phosphorylation of AMPA type glutamate receptor, which is responsible for synaptic transmission, due to the saturation of PP1 by high concentrations of CaMKII (Lisman & Zhabotinsky, 2001).

In contrast to PSD-associated CaMKII, cytosolic CaMKII seems to be dephosphorylated *in vivo* mainly by protein phosphatases other than PP1. It is reported that PP2A and PP2C

contribute to 90% of the dephosphorylation activity in cell extracts from the CA1 hippocampal region, with PP1 activity being only 10% of the total activity, when the phosphatase activities are assessed using ^{32}P-autophosphorylated CaMKII as substrate (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 mammalian forebrain (Bennecib 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. This reduction of PP2A activity might be important for maintaining a highly autophosphorylated and activated state of CaMKII, maintaining the stable phosphorylation of synaptic proteins that might underlie the expression of LTP (Fukunaga et al., 2000).

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). Fukunaga et al. 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*.

CaMKII also plays important physiological roles in various tissues other than brain. For example, in pancreatic β cells, CaMKII is essential for secretion of insulin in response to glucose stimulation (Takasawa et al., 1995). PP1 and Mg^{2+}-dependent protein phosphatases (Easom et al., 1998) and PP1 (Hwang et al., 1996) are shown to play major roles in the dephosphorylation of autophosphorylated CaMKII in pancreatic β cells and in pancreatic acinar cells, respectively, based on biochemical and pharmacological analysis using phosphatase inhibitors, okadaic acid and calyculin A. Further, it has been reported recently that the δ isoform of CaMKII, which is expressed in cardiac muscle and can induce cardiac gene expression and hypertrophy, forms a complex with PP2A (Zhang et al., 2002). Thus, protein phosphatases responsible for the dephosphorylation of autophosphorylated CaMKII vary depending on the localization of CaMKII in tissues or cells; probably because differences in the environments in which the phosphatases and CaMKII interact with each other largely influence

the dephosphorylation reaction of CaMKII.

PP2B (calcineurin), a Ca^{2+}/calmodulin-dependent protein phosphatase, is believed to be unable to dephosphorylate autophosphorylated CaMKII directly (Table 2). However, PP2B dephosphorylates I-1, which is a specific endogenous protein inhibitor for 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. In fact, the possible involvement of indirect regulation of CaMKII activity by PKA/PP2B in the modulation of synaptic transmission in response to the frequency of the stimulation is indicated (Makhinson et al., 1999; Winder & Sweatt, 2001).

On the other hand, it is reported that autophosphorylated Thr^{286} of CaMKII is dephosphorylated in the presence of excess ADP by the reversed kinase reaction, not by protein phosphatases, leading to the deactivation of CaMKII (Kim et al., 2001).

3.2. Negative regulation of CaMKI/CaMKIV by protein phosphatases

As mentioned above, CaMKI/CaMKIV are activated through phosphorylation of the Thr residues in their activation loops by CaMKK. Originally, CaMKIV was called CaMK-Gr, and the phosphorylation of CaMKIV/Gr incorrectly recognized as “autophosphorylation, ” which was later found to be phosphorylation by contaminated CaMKK (Kameshita & Fujisawa, 1995). Frangakis et al. found that an unidentified Mg^{2+}-dependent protein phosphatase prepared from rat cerebellar extract could dephosphorylate and deactivate the “autophosphorylated” CaMKIV/CaMK-Gr (Frangakis et al., 1991). CaMKK was found thereafter (Okuno & Fujisawa, 1993), and a marked deactivation of the activated CaMKIV by PP2A treatment (Tokumitsu et al., 1994; Park & Soderling, 1995) but not by PP1 treatment (Park & Soderling, 1995) was shown using CaMKIV activated *in vitro* and *in vivo* by CaMKK. However, Kasahara et al. reported that CaMKIV phosphorylated and activated *in vitro* was markedly dephosphorylated and deactivated by PP1 (Kasahara et al., 1999). The reason for the discrepancy between the two reports is unclear, though a difference in the isoform of PP1 used is

pointed out. They also reported that activated CaMKIV was dephosphorylated and deactivated not only by PP1 but also by PP2A, PP2B, and PP2C. Interestingly, enhancement of the phosphorylation of CaMKIV, caused by glutamate stimulation of hippocampal neurons, was further facilitated by the addition of cyclosporin A, a PP2B specific inhibitor (Kasahara et al., 1999). PP2B may also participate in the negative regulation of CaMKIV by dephosphorylation *in vivo*.

PP2A is known to form a complex with CaMKIV through the catalytic domain of CaMKIV. In addition, it is reported that the expression of SV40 small T antigen, an inhibitory protein for PP2A, activates the CREB-dependent transcription caused by CaMKIV. Based on these observations, 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). The "signaling complex" with PP2A as a component, is also observed in other protein kinases such as S6 kinase, casein kinase, p21-activated protein kinase (PAK) 1, RAF-1, JAK2, and CaMKII (Price & Mumby, 1999; Millward et al., 1999; Zhang et al., 2002).

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). However, extensive study remains to be carried out.

4. A protein phosphatase that dephosphorylates and regulates multifunctional CaMKs

All of the protein phosphatases mentioned above are well-known Ser/Thr protein phosphatases with broad substrate specificity. Recently, using novel methods to detect protein phosphatase activity, we isolated a new family of protein phosphatases that shows strict substrate specificity to CaMKs. Since these methods are widely applicable to identification and analysis of other protein phosphatases, we introduce them here.

4.1. A methodology for identification of novel protein phosphatases

Previously, we developed an in-gel protein kinase assay that could detect protein kinase

activities in a SDS-polyacrylamide gel as bands in autoradiography (Kameshita & Fujisawa, 1989; Kameshita & Fujisawa, 1996). This method has the advantage that activities and molecular weights of protein kinases in crude samples can be simultaneously analyzed without purification of the samples when appropriate substrate peptides or proteins are available. In an attempt to explore novel protein phosphatases, we modified it to an in-gel protein phosphatase assay. To analyze protein phosphatases that dephosphorylate the autophosphorylation site Thr^{286} of CaMKII, a synthetic peptide derived from the sequence surrounding the autophosphorylation site was conjugated to poly-L-lysine (poly(Lys)), followed by phosphorylation using a CaMKII active fragment to prepare a protein phosphatase substrate. Samples were electrophoresed in a SDS-polyacrylamide gel containing the phosphorylated peptide conjugate and SDS removed from the gel. The denatured sample proteins were then renatured in the gel to be visualized as radio-negative bands at the positions of the phosphatase proteins, as the dephosphorylation reactions proceeded in the gel (Fig. 1A). When crude extracts of the rat cerebellums were analyzed by the in-gel protein phosphatase assay, three transparent bands which represented the protein phosphatases were detected by autoradiography as shown in Fig. 1B (Kameshita et al., 1997). This method has several disadvantages; enzymes that are difficult to renature cannot be detected, it is only applicable to enzymes that show protein phosphatase activities with a single subunit, and it is inadequate for rapid processing of a large number of samples such as fraction assay for protein purification. However, it does provide an extremely useful means for examining activities and molecular weights of protein phosphatases in a crude sample without purification.

Next, to purify protein phosphatases detected by an in-gel protein phosphatase assay, we developed another assay that was more rapid and convenient, and thus was suitable for checking the activity of each fraction during purification. In general, most of the protein phosphatases have so far been assayed with phosphorylated proteins as substrate, which are available in large amounts such as histone and casein with phosphorylation by appropriate protein kinases and [γ-^{32}P]ATP (MacKintosh, 1993). However, this method has disadvantages that the preparation procedure is tedious, and that protein phosphatases unable to dephosphorylate these

substrates cannot be detected. Although an assay method using *p*-nitrophenylphosphate is convenient and is often employed (MacKintosh, 1993), it is only applicable to some of the protein phosphatases that can dephosphorylate *p*-nitrophenylphosphate. Therefore, we developed a novel assay method for rapid detection of protein phosphatase activities that could not be detected by conventional protein phosphatase assays. Instead of phosphoproteins, a peptide conjugate, which had been prepared by conjugation of a synthetic peptide to magnetic particles, was used as a substrate in this method called the "immobilized phosphopeptide assay" (Ishida et al., 1997) (Fig. 1C). We prepared the peptide conjugate using a synthetic peptide derived from the sequence surrounding Thr^{286} of CaMKII, the same peptide used for the in-gel assay, followed by phosphorylation using the CaMKII active fragment to obtain the phosphatase substrate. When the phosphatase activities in the fractions prepared from rat brain extract with DEAE-5PW column chromatography were examined, a peak of phosphatase activity was detectable in the presence of Mn^{2+} and poly(Lys). This peak was not seen in the conventional method with *p*-nitrophenylphosphate as a substrate (Ishida et al., 1997). This assay is a useful method with the following advantages. (1) Due to magnetic particles, separation of excess ATP and a protein kinase from the conjugate, an indispensable step for the substrate preparation, is easy. (2) Operability of the assay is excellent, and (3) A variety of protein phosphatase substrates can readily be prepared, employing different combination of various synthetic peptides and protein kinases. Since it is possible to detect much more diverse protein phosphatase activities by changing not only the combination of synthetic peptides and protein kinases used for preparation of the phosphopeptide conjugate, but also the conditions in the phosphatase reactions such as the addition of activators/inhibitors, and chelators/metals; the assay seems to be widely applicable to the studies of various protein phosphatases.

4.2. Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP)

Using the unique techniques mentioned above, we purified a novel protein phosphatase, which dephosphorylated Thr^{286} of CaMKII, from rat brain. The purified protein phosphatase has a molecular weight of 54000, which corresponded to one of the three bands detected by the

in-gel protein phosphatase assay (Fig. 1B)(Ishida et al., 1998a). This enzyme is a Mn^{2+}-dependent, okadaic acid/calyculin A-insensitive, Ser/Thr protein phosphatase, and exists as a monomer, unlike PP1 and PP2A. However, like PP2A, it is markedly activated by polycations such as poly(Lys) and protamine. Phosphorylated forms of casein, histone, myelin basic protein, phosphorylase, and phosphorylase kinase were hardly dephosphorylated by this phosphatase under conditions where autophosphorylated CaMKII was completely dephosphorylated. It is also shown that the phosphatase is highly specific for multifunctional CaMKs such as phosphorylated CaMKI/CaMKIV and autophosphorylated CaMKII, as the protein phosphatase hardly dephosphorylated other phosphorylated protein kinases such as CaMKK and mitogen-activated protein kinase (MAPK)(Fig. 2). These CaMKs were reversibly deactivated by CaMKP, whereas PKA and protein kinase C, which were reported to be deactivated by PP2A (Liauw & Steinberg, 1996) and PP1 (Pears et al., 1992; Keranen et al., 1995; Newton, 1997), respectively, were not deactivated by CaMKP. Thus, we called the enzyme Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP)(Ishida et al., 1998b).

Since the biochemical properties of CaMKP are quite different from the protein phosphatases so far reported, this strongly suggested that CaMKP was a novel enzyme (Table 2); as demonstrated by its primary structure deduced from the nucleotide sequence of the corresponding cDNA (Kitani et al., 1999). Although CaMKP is a protein phosphatase which belongs to the PPM family, homology to PP2Cα is only 28% even in the phosphatase domain indicated by a hatched bar in Fig. 3A. At the N-terminus, it has a large domain, which is not shared by PP2C, with a characteristic cluster of glutamic acid. Based on mutational analysis, the glutamic acid cluster was found to be important for binding to polycations and the subsequent activation (Ishida et al., 2002).

As CaMKP is highly specific for multifunctional CaMKs, we examined its substrate specificity in detail using a variety of chemically synthesized phosphopeptides. CaMKP showed unexpectedly broad substrate specificity towards phosphopeptide substrates; thus no consensus sequences of dephosphorylation were identified. However, it was shown that CaMKP is fairly specific to phospho-Thr for dephosphorylation, and that the minimal length of

the substrate peptide is only 2 to 3 amino acid residues including the dephosphorylation site. Thus, it is likely that the substrate specificity of CaMKP is determined by the higher-order structure of the substrate protein, rather than by the primary structure around its dephosphorylation site (Ishida et al., 2001).

CaMKP is phosphorylated and activated by CaMKII, which is a substrate for CaMKP, in the presence of poly(Lys). The physiological significance of the activation reaction is currently unclear. However, since CaMKP is activated by a substrate, CaMKII, leading to enhancement of dephosphorylation of CaMKII, it is possible that this phenomenon is a kind of a mechanism for feedback regulation (Kameshita et al., 1999).

Using a specific antibody for CaMKP, we examined tissue distribution and subcellular localization of the enzyme. When examined by Western blotting, CaMKP was found to be expressed ubiquitously in all the tissues examined. It was highly expressed in 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) revealed that CaMKP localized only in the cytoplasm, and was never observed in the nucleus. Moreover, the distribution of CaMKP and CaMKs overlapped in various regions in the brain and spinal cord (Nakamura et al., 2000). Although the content of CaMKP in PSD fraction is usually very low, it is occasionally observed that PSD is clearly stained with anti-CaMKP antibody in some cells (Nakamura et al., 2000). Since the existence of CaMKP-binding proteins in PSD fraction are suggested (Kameshita et al., unpublished data), it seems to be possible that CaMKP participates in the dephosphorylation of CaMKII in PSD under some conditions.

4.3. CaMKP-N

A cDNA clone showing 78% homology in its amino acid sequence to rat CaMKP was found in human cDNA databases with the sequence of rat CaMKP as a query. When the cDNA was expressed in *E. coli*, it showed enzymatic properties similar to those of rat CaMKP. RT-PCR analysis for expression of mRNA in various human tissues indicated that the expression profiles were in good agreement with those in rat tissues. Based on these data, this homologue

was concluded to be human CaMKP (Shigeri et al., 2001).

Another homologue that was clearly distinct from human CaMKP was found in the human databases (Takeuchi et al., 2001). This homologue possesses a region which shows 64% homology to human CaMKP in its phosphatase domain, but large regions without homology to CaMKP in the both N- and C-terminus (Fig. 3B). Unlike CaMKP, 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: it dephosphorylated CaMKIV in a poly(Lys)- and Mn^{2+}-dependent manner. Thus, we named the enzyme CaMKP-N after its localization in the nucleus. A nuclear localization signal of the enzyme turned out to be in the C-terminal region after the amino acid residue 574, which was lacking in both CaMKP and PP2C (Takeuchi et al., 2001). 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 (Fig. 4) (Kitani et al., 2003).

4.4. Physiological roles of CaMKP

It is important to investigate whether CaMKP and CaMKP-N participate in the regulation of CaMKs *in vivo*. Recently, 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* (Tan et al., 2001). Transient expression of either nematode FEM-2, human CaMKP, or rat CaMKP in Hela cells resulted in apoptosis; in contrast the expression of PP2Cα, another member of PPM family protein phosphatases, 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 or CaMKIV is involved in apoptosis, it seems to be possible that, amongst other activities, CaMKP promotes cell death by modulating CaMK activity.

Koh et al. reported that CaMKP participated in the regulation of PAK, which is a Ser/Thr

protein kinase that interacts with the activated GTP-bound forms of Cdc42 or Rac1 (Koh et al., 2002). POPX1 and POPX2 were identified as binding partners for PIX, the PAK interacting guanine nucleotide exchange factor. Interestingly, POPX1 and POPX2 are identical to human CaMKP-N and human CaMKP, respectively. CaMKP-N and CaMKP could dephosphorylate the phosphorylated form of PAK *in vitro* to deactivate it. They also inhibited the breakdown of actin stress fiber and morphological changes driven by active $Cdc42^{V12}$. Based on these data, it was concluded that CaMKP-N (POPX1) and CaMKP (POPX2) are involved in the signaling pathways for negative regulation of PAK.

5. Pharmacological implications

It is well-known that CaMKIIα knock out mice show profound spatial learning deficits (Silva et al., 1992b). Since transgenic mice expressing mutant CaMKII, in which autophosphorylation site Thr^{286} was replaced with Ala, had no LTP and no spatial learning, the autophosphorylation of CaMKII at Thr^{286} appears to be critical for LTP and spatial learning (Giese et al., 1998). Consequently, it may be possible that enhancement of the autophosphorylation at Thr^{286} improves learning efficacy and memory. One of the means to enhance the autophosphorylation level is to inhibit protein phosphatases that are responsible for the dephosphorylation of the autophosphorylation site. CaMKII in PSD is believed to play essential roles for LTP as the molecular basis of memory (Lisman & Zhabotinsky, 2001). It has been shown that PP1 mainly participates in dephosphorylation of CaMKII in PSD (Strack et al., 1997a; Yoshimura et al., 1999), so that PP1 specific inhibitor may be effective in improving learning efficacy and memory. Indeed, transgenic mice that inducibly expressed the activated form of I-1, a specific inhibitory protein for PP1, in a brain specific manner showed significant improvements of learning and memory (Genoux et al., 2002), with the improvements being especially remarkable in aged mice. Since I-1 and I-2, both of which are endogenous inhibitory proteins, had no membrane permeability, their pharmacological use seems to be limited. Although potent and highly specific inhibitors for PP1 and PP2A, such as okadaic

acid and calyculin A, are widely used as tools for molecular pharmacology, their clinical use appears to be difficult because of their toxicity and carcinogenecity. Since PP1 and PP2A are widely distributed in various tissues and involved in regulation of the phosphorylation status of not only CaMKII but also various other proteins, it is conceivable that these inhibitors, which uniformly inhibit them, largely affect overall signal transduction systems with cumbersome systemic side effects. It seems to be necessary to develop PP1 inhibitors that specifically act on the central nervous system, especially on PSD for the improvement of learning and memory.

Recently, based on the experiments using transgenic mice or knockout mice, it has been shown that not only CaMKII but also CaMKIV are involved in memory formation (Kang et al., 2001; Wei et al., 2002). Since CaMKIV activity is strictly regulated by phosphorylation and dephosphorylation, it may be possible that drugs that elevate the phosphorylation level of CaMKIV will be effective in improving memory. As mentioned above, since phosphorylation of Thr^{196} of CaMKIV caused by glutamate stimulation of hippocampal neuron is reported to be enhanced by cyclosporin A, a PP2B inhibitor (Kasahara et al., 1999), such a drug might be effective in improving memory; though the results of pharmaco-behavioral studies so far argue against this contention (Bennett et al., 1996; Bennett et al., 2002).

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. Unfortunately, specific inhibitors for protein phosphatases of PPM family including CaMKP and PP2C have not yet been found. The absence of a specific inhibitor is one of the causes for the delay in the elucidation of the physiological functions of PPM protein phosphatases. Recently, Wakamiya et al. established a convenient method for the synthesis of phosphoseryl and phosphothreonyl peptides, which had previously been difficult to chemically prepare (Wakamiya et al., 1994; Wakamiya et al., 1997). For the purpose of exploring substrate specificity of CaMKP, we obtained a variety of chemically synthesized phosphopeptides by this protocol, and established a convenient non-RI assay method for CaMKP using these peptides as substrates (Ishida et al., 2001). This method is based on a sensitive assay for inorganic phosphate using Malachite green as a coloring reagent. Although the

sensitivity is considerably lower than that of the assay described in section 4.1, it is available when a sufficient quantity of the purified enzyme can be obtained. We hope to establish this as a high-throughput screening method for inhibitors/activators of CaMKP.

CaMKs have been shown to play key roles in the pathogenesis of some diseases. In the central nervous system, transient cerebral ischemia leads to the delayed and selective degeneration of certain populations of neurons. This phenomenon called “delayed neuronal death” often caused serious clinical problems such as sequelae after cerebral infarction. CaMKII is involved in the process of delayed neuronal death. Autocamtide-2-related inhibitory peptide (AIP) is widely used as a CaMKII-specific inhibitory peptide (Ishida & Fujisawa, 1995; Ishida et al., 1995; Takasawa et al., 1995; Ishida et al., 1998c), and its myristylated form, which is cell-permeable, has protective effects from neuronal death induced by administration of NMDA (Laabich & Cooper, 2000) or a Na^+ channel activator (Takano et al., 2003). This suggests that the aberrant enhancement of autophosphorylation/activation of CaMKII caused by Ca^{2+} influx, which is induced by these drugs, is one of the causes for delayed neuronal death. If this is the case, similar protective effects on the neuronal death may be expected by administration of not only CaMKII inhibitors but also activators for protein phosphatases responsible for the negative regulation of CaMKII. However, specific activators for particular protein phosphatases are not yet established.

In cardiomyocytes, hypertrophic growth is an adaptive response of the heart to a variety of pathological stimuli. However, chronic hypertrophy often leads to dilated cardiomyopathy and eventually to heart failure. Recently, it has been suggested that overexpression of CaMKIV (Passier et al., 2000) and δ_B isoform of CaMKII (Zhang et al., 2002) in cardiomyocytes induce hypertrophy. Since KN62, a CaMKII/CaMKIV-specific inhibitor, can block cardiomyocyte hypertrophy in response to endothelin-1 (Zhu et al., 2000), inhibition of CaMKII/CaMKIV would seem effective for prevention of cardiac hypertrophy. Therefore, activators of protein phosphatases responsible for negative regulation of CaMKII/CaMKIV might have similar effects on cardiac hypertrophy. Development of specific activators of protein phosphatases responsible for the negative regulation of CaMKII/CaMKIV, especially those of CaMKP and/or

CaMKP-N, could provide a new therapeutic approach for cardiac hypertrophy.

6. Conclusions and perspectives

Multifunctional CaMKs, which play crucial roles in Ca^{2+} signaling, are regulated by protein phosphorylation; thus, the mechanism of dephosphorylation/deactivation by protein phosphatases, which corresponds to the "switch off" mechanism, along with the mechanism of activation by phosphorylation, is extremely important. In this article, we summarized the current status of studies regarding protein phosphatases involved in the regulation of multifunctional CaMKs. At present, the protein phosphatases revealed to regulate directly CaMKs *in vitro* are of five species: PP1, PP2A, PP2B, PP2C, and CaMKP. Each CaMK is regulated by some of these protein phosphatases. However, this review is far from the elucidation of an overview of the regulation of protein phosphatases themselves *in vivo*. How is the activity of each protein phosphatase regulated in a complex cellular system? How do the protein phosphatases share the work required? These are important issues remaining for future study. In addition, the spatial or temporal dynamics of the interactions of these protein phosphatases with their substrate CaMKs appear to be important determinants for the regulation of dephosphorylation reactions *in vivo*. Interaction of CaMKII with PP1 in PSD and a signaling complex composed of CaMKIV and PP2A, described in sections 3.1 and 3.2, respectively, are good examples of such interactions. Interestingly, it seems likely that the interaction of CaMKII with PP1 in PSD is essential for the expression of LTP, as discussed in detail by Lisman and Zhabotinski (see section 3.1). Analysis that takes into consideration such subcellular distribution/compartmentation will be central to future discussions. Development/exploration of specific inhibitors of CaMKP, a novel protein phosphatase, as well as the elucidation of its regulatory mechanism and its roles in the regulation of CaMK *in vivo*, are important issues to be resolved. An approach that targets the "switch off" mechanism of CaMKs by protein phosphatases may also lead to new drug developments, and provide clues to understand the molecular mechanisms of neuronal functions in which CaMKs are involved, such

as memory.

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Figure legends

Fig. 1 Newly developed methods for detection of protein phosphatase activity. A: in-gel protein phosphatase assay. A schematic representation of in-gel protein phosphatase assay using a synthetic peptide derived from the sequence surrounding Thr^{286} of CaMKII (CaMKII(281-289)) as a substrate. B: developmental changes of CaMKII(281-289)-dephosphorylating protein phosphatases. Crude extracts of the cerebellums obtained from the indicated ages of rats were analyzed by the in-gel protein phosphatase assay. C: a schematic representation of immobilized phosphopeptide assay using phospholylated CaMKII(281-289) as a substrate.

Fig. 2 Dephosphorylation of CaMKs by CaMKP. A: autophosphorylated CaMKKα and CaMKI, which had been phosphorylated by CaMKKα, were incubated at 30 ˚C with the indicated additions and omissions. After incubation for 1 min, aliquots were analyzed by SDS-PAGE followed by autoradiography. B: autophosphorylated CaMKKα and autophosphorylated CaMKII were incubated and analyzed as described above. C: autophosphorylated CaMKKα and CaMKIV(K71R) expressed in Sf9 cells, which had been phosphorylated by CaMKKα, were incubated and analyzed as described above. D: GST-MAPK (Erk2) phosphorylated by MEK1 and CaMKIV(K71R) expressed in *E. coli*, which had been phosphorylated by CaMKKα, were incubated and analyzed as described above. The bands shown by the arrow were proteolytic fragments generated from CaMKIV protein expressed in *E. coli.* during the preparation. The positions corresponding to CaMKI, CaMKII, CaMKIV(K71R), GST-MAPK, and CaMKKα are indicated with the arrow heads (Ishida et al., 1998b; Ishida et al., 2001).

Fig. 3 Domain structures of CaMKP and CaMKP-N. A: domain structures of rat PP2Cα and CaMKP are shown. Phosphatase domains of PP2Cα and CaMKP, which show significant sequence homology (28% identity), were aligned (hatched box) with the PP2C

motifs located within these regions being indicated. A Glu cluster located within the N-terminal domain of CaMKP (101-109, closed box) is also shown. B: domain structures of human CaMKP and human CaMKP-N are shown. Regions of human CaMKP and CaMKP-N, which show significant sequence homology (64% identity), were aligned (netted box) with the PP2C motifs located within these regions being indicated. Cluster sequences with acidic amino acids located within N-terminal regions of human CaMKP and CaMKP-N are also shown.

Fig. 4 Regulation of CaMK cascade by CaMKP and CaMKP-N. Asterisk shows the phosphorylated form of CaMKs.

Table 1. Some features of multifunctional CaMKs.

	CaMKII	CaMKIV	CaMKI
Structure	oligomeric (~550 kDa) (subunit = 50-60 kDa)	monomeric (~60 kDa)	monomeric (~40 kDa)
Distribution			
tissue	ubiquitous, abundant in brain	limited (abundant in brain and thymus)	ubiquitous
subcellular	PSD and cytosol	nucleus	cytosol
Substrates	tyrosine hydroxylase, tryptophan hydroxylase, synapsin I, GluR1, glycogen synthase, MAP2, phospholamban, etc.	synapsin I, MAP2, myelin basic protein, Rap-1b, CREB, SRF, MFE2D, etc.	synapsin I, CFTR, CREB, etc.
Physiological roles	carbohydrate metabolism, transcription, cytoskeletal organization, LTP, neuronal memory, cardiac functions, etc.	transcription, spermatogenesis, LTP, neuronal memory, mitochondrial biogenesis, etc.	transcription ?
Knockout/ trangenic mice	Silva et al. 1992a, Silva et al. 1992b, Mayford et al. 1995, Bach et al. 1995, Giese et al. 1998, Cho et al. 1998, Elgersma et al. 2002, Zhan et al. 2002, etc.	Anderson et al. 1997, Passier, et al. 2000, Wu et al. 2000, Ho et al. 2000, Kang et al. 2001, Wei et al. 2002, Wu et al. 2002, etc.	
Specific inhibitor	CaMKII(281-309)[1], CaMKII(273-302)[2] , KN-62[3], KN-93[4], AIP[5], CaM-KIIN[6], PEP-19[7]	KN-62[8]	KN-62[9], CaMKI (294-321)[10]
Activation mechanism	activated upon autophosphorylation on Thr-286 located in autoinhibitory domain	activated upon phosphorylation by CaMK kinase on Thr-196 located in activation loop	activated upon phosphorylation by CaMK kinase on Thr-177 located in activation loop
Dephosphorylating protein phosphatase	PP1, PP2A, PP2C, CaMKP	PP1, PP2A, PP2B, PP2C, unidentified Mg^{2+}-dependent protein phosphatase, CaMKP	PP2A, CaMKP

[1] Malenka et al. 1989, [2] Malinow et al. 1989, [3]Tokumitsu et al. 1990, [4] Sumi et al. 1991, [5] Ishida et al. 1995, Ishida et al. 1998c, [6]Chang et al. 1998, [7] Johanson et al. 2000, [8] Enslen et. al. 1994, [9] Mochizuki et al. 1993, [10] Yokokura et al. 1995. See text for other references.

Table 2. Biochemical properties of protein phosphatases that dephosphorylate multifunctional CaMKs.

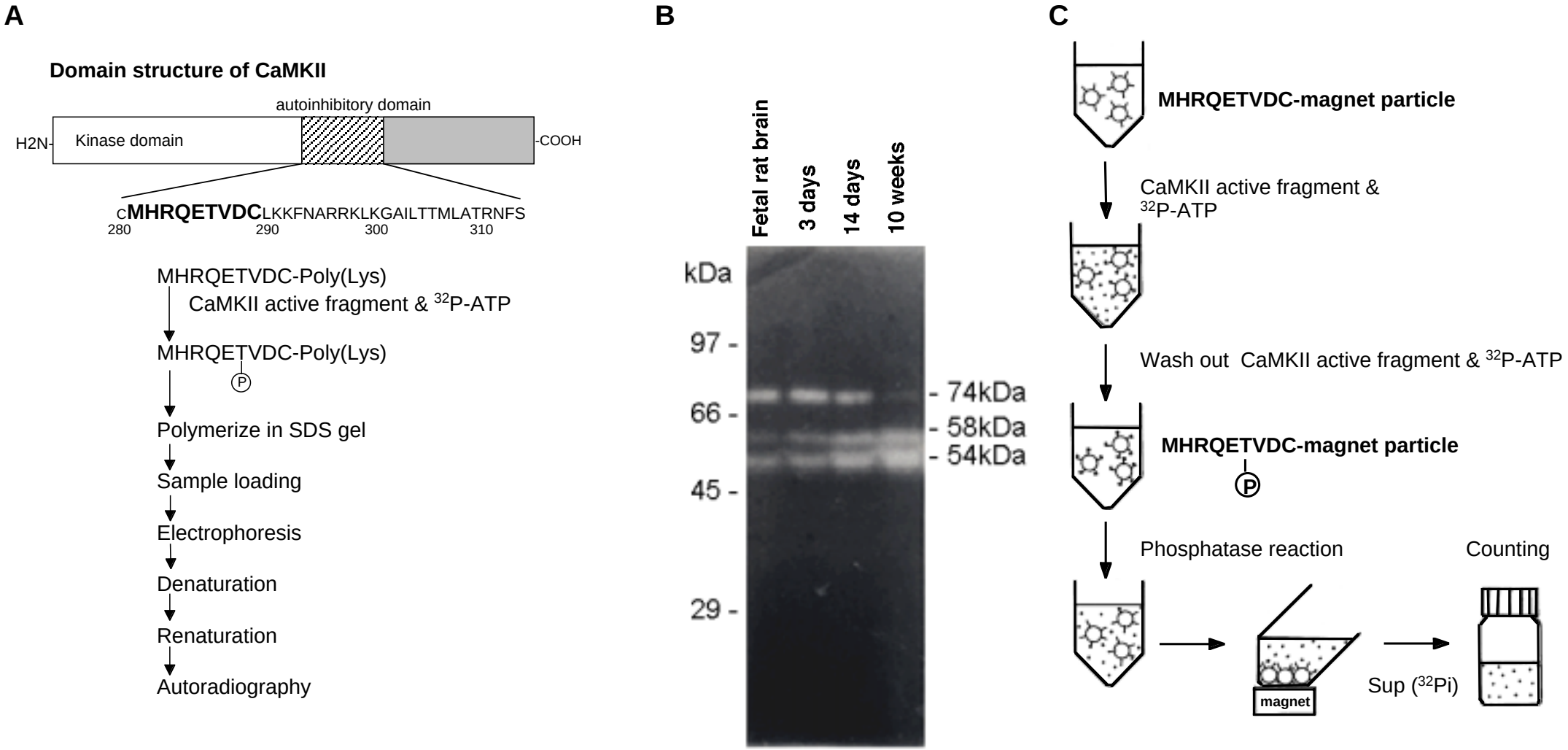
	PP1	PP2A	PP2B/calcineurin	PP2C(α)	CaMKP

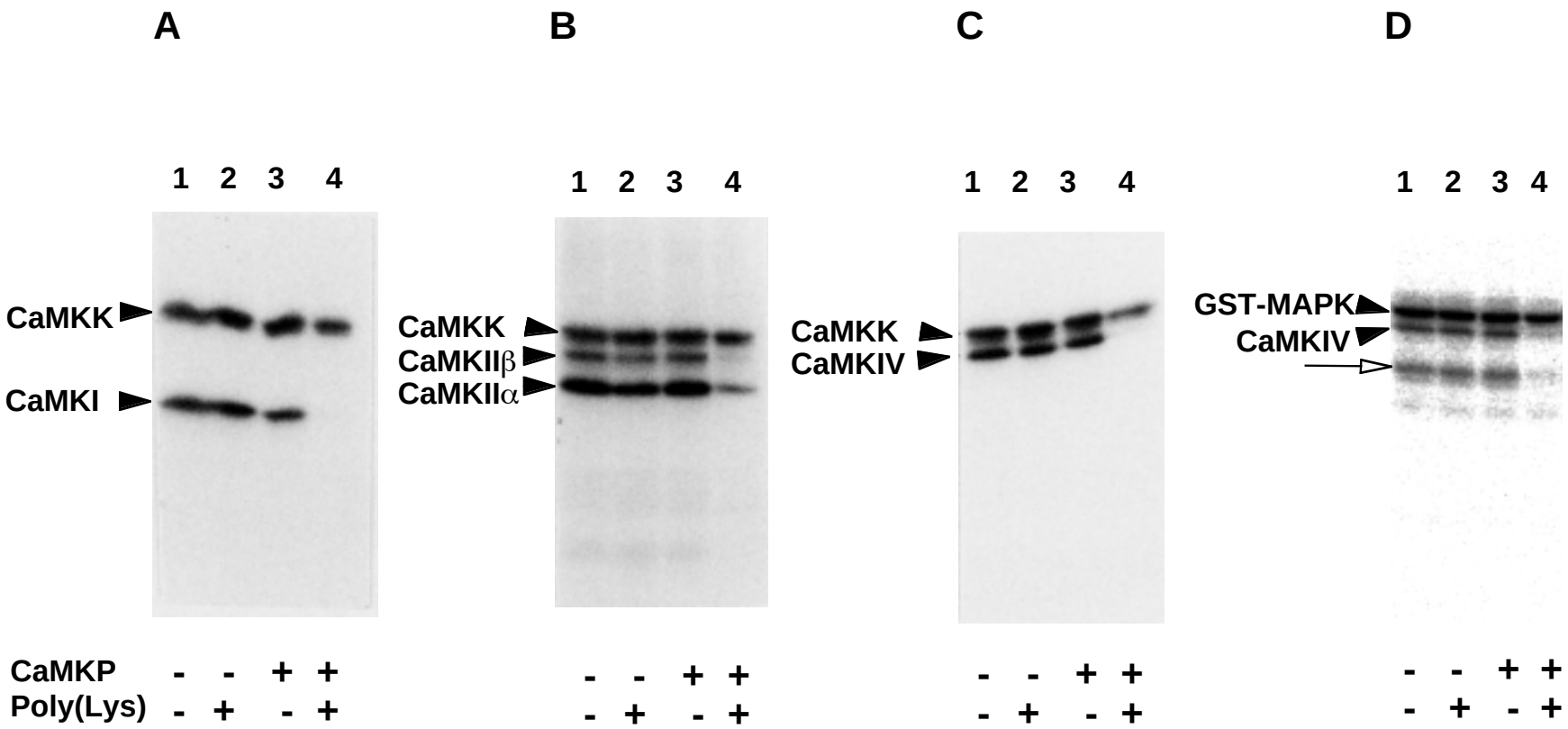
Subunit structure	Oligomeric	Oligomeric	Oligomeric	Monomeric	Monomeric
catalytic subunit	C (~37 kDa)	C (36 kDa)	A (58~64 kDa)	(42 kDa)	(54 kDa)
regulatory/targeting proteins	DARPP-32 family (23-32 kDa), Spinophilin family (90-120 kDa), Yotiao (200 kDa), etc.	A (65 kDa) B/PR55 (55 kDa) B'/B" (52-74 kDa) PR72 (59-130 kDa), etc.	B (19 kDa) AKAP79 (79 kDa) FKBP12 (12 kDa) CAIN (240 kDa), etc.		
Activation by polycations	-	+	-	-	+
Metal requirement	-	-	Ca^{2+}	Mg^{2+}	Mn^{2+}
Inhibition by					
Heparin	+	-	-	-	+
Inhibitor 2	+	-	-	-	-
Okadaic acid	+	+	-	-	-
Calyculin A	+	+	-	-	-
NaF	+	+	+	-	+
Orthovanadate	+	+	-	+	-
Other pharmacological inhibitors	Microcystin[1], Tautomycin[1], Nodularin[2], Cantharidin[3], Fostriecin[4], Inhibitor 1	Microcystin, Tautomycin, Nodularin, Cantharidin, Fostriecin	Cyclosporin[5], FK506[5], Cypermethrin[6], Deltamethrin[6]		
Dephosphorylation of					
CaMKII	+	+	-	+	+
Phosphorylase *a*	+	+	-	-	-

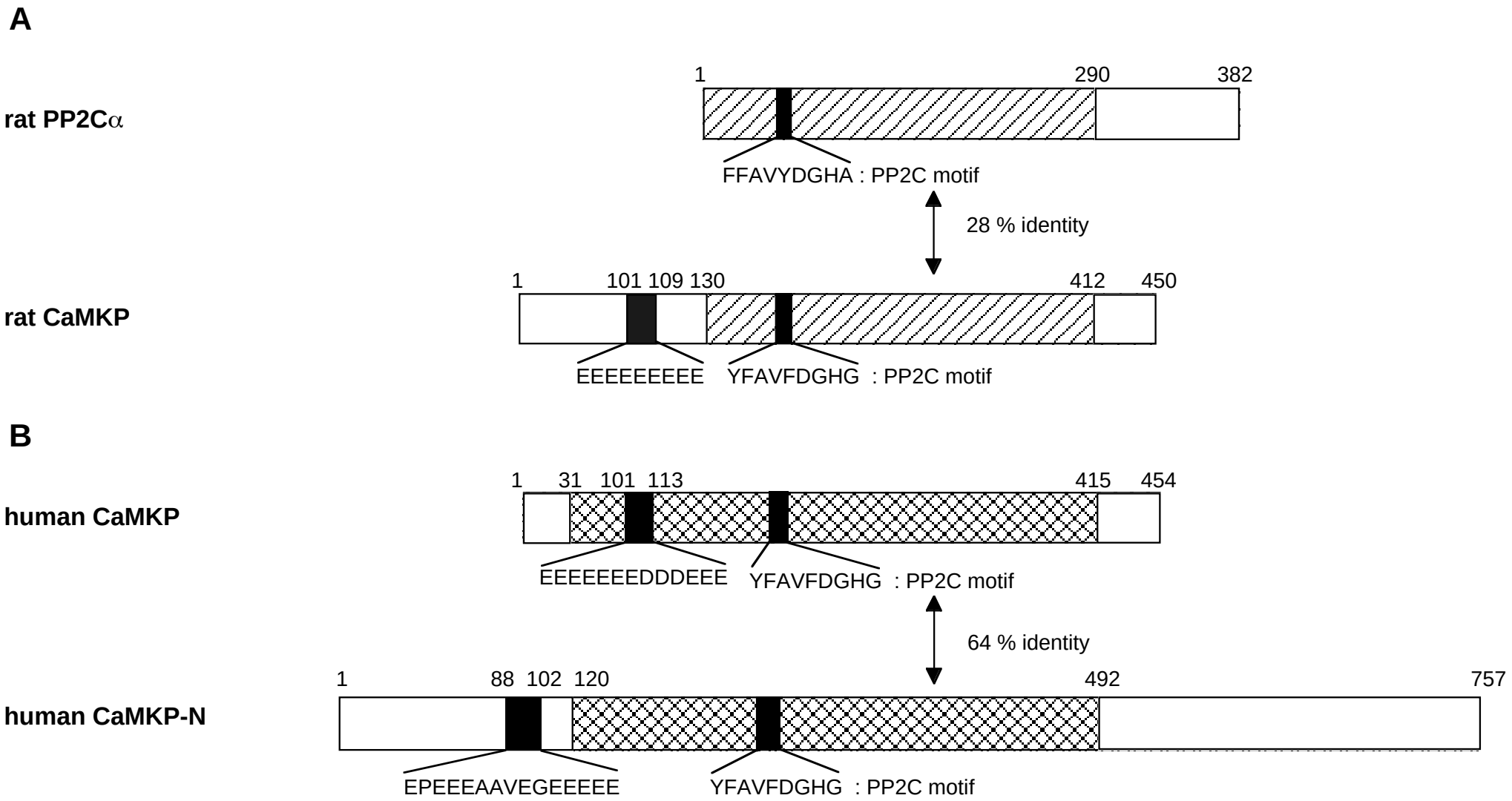
[1]Takai et al. 1995, [2]Honkanen et al. 1991, Craig et al. 1996, [3]Li et al. 1992, Li et al. 1993, [4]Walsh et al. 1997. [5]Bram et al. 1993, Price & Mumby 1999, [6]Enan and Matsumura 1992.

Some of the characteristics of protein phosphatases shown in this table are from reviews by Shenolikar & Nairn 1991, Price & Mumby 1999, and Winder & Sweatt 2001.

A. Ishida et al. Fig.1




A
B
C
D
1 2 3 4
CaMKK
CaMKI
CaMKK
CaMKIIβ
CaMKIIα
CaMKK
CaMKIV
GST-MAPK
CaMKIV
CaMKP - - + +
Poly(Lys) - + - +


A
rat PP2Cα
1
290
382
FFAVYDGHA : PP2C motif
28 % identity
rat CaMKP
1
101 109 130
412
450
EEEEEEEEE
YFAVFDGHG : PP2C motif
B
human CaMKP
1
31
101
113
415
454
EEEEEEEDDDEEE
YFAVFDGHG : PP2C motif
64 % identity
human CaMKP-N
1
88
102
120
492
757
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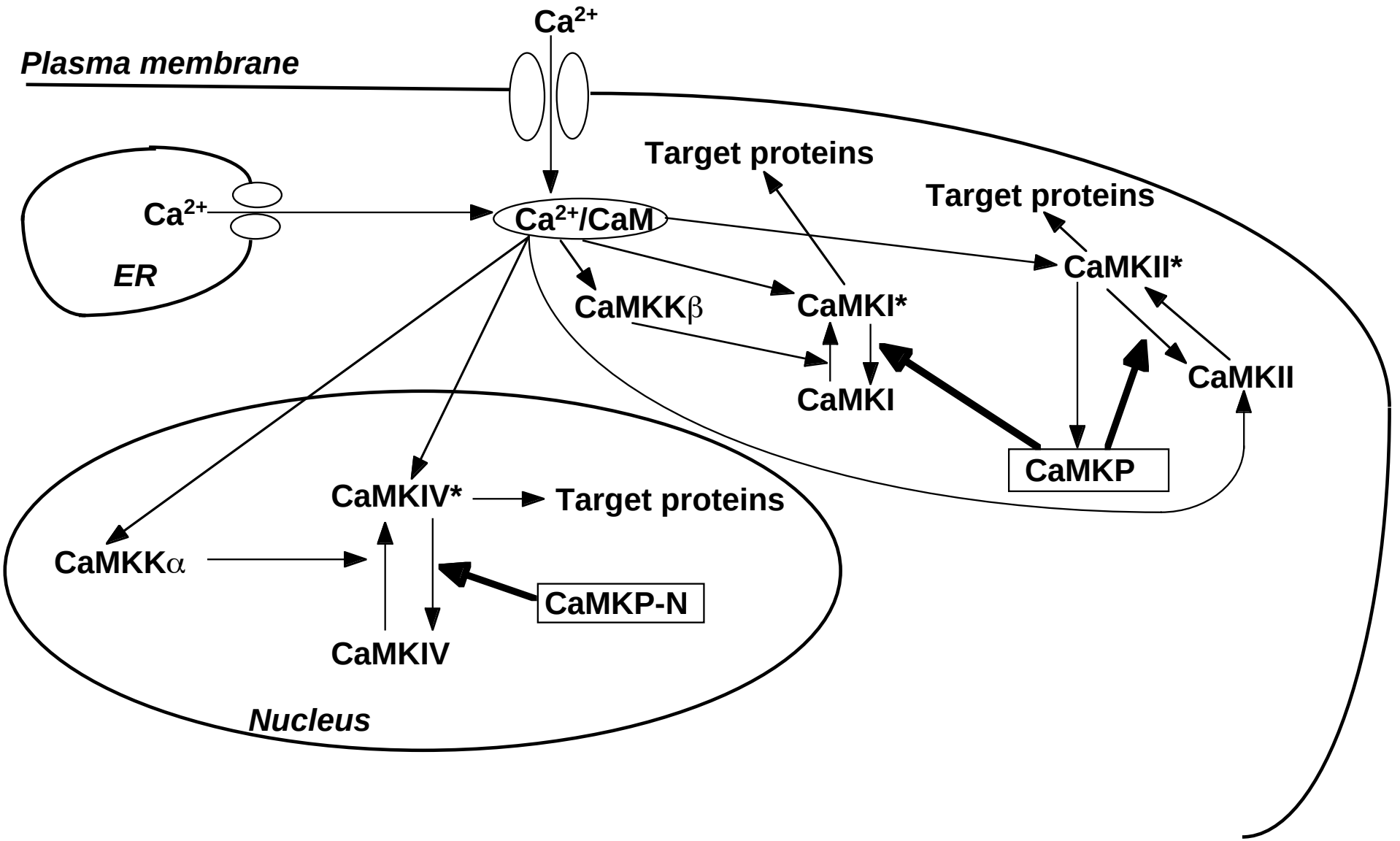
Ca2+
Plasma membrane
Target proteins
Target proteins
Ca2+
Ca2+/CaM
ER
CaMKIIδ*
CaMKKβ
CaMKIδ*
CaMKIIδ
CaMKIδ
CaMKP
CaMKIV*
Target proteins
CaMKKα
CaMKP-N
CaMKIV
Nucleus