Regulation of Ca^{2+}/calmodulin-dependent protein kinase kinase β by cAMP signaling

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Running title: *Regulatory phosphorylation of CaMKKβ by PKA*

The abbreviations used are: CaMKKβ, Ca^{2+}/CaM-dependent protein kinase kinase β; AID, autoinhibitory domain; CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; AMPK, 5'AMP-activated protein kinase; PKA, cAMP-dependent protein kinase; CDK5, cyclin-dependent kinase 5; GSK3, glycogen synthase kinase 3; DAPK, death-associated kinase

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ABSTRACT

BACKGROUND: Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) is a pivotal activator of CaMKI, CaMKIV and 5'-AMP-activated protein kinase (AMPK), controlling Ca^{2+}-dependent intracellular signaling including various neuronal, metabolic and pathophysiological responses. Recently, we demonstrated that CaMKKβ is feedback phosphorylated at Thr144 by the downstream AMPK, resulting in the conversion of CaMKKβ into Ca^{2+}/CaM-dependent enzyme. However, the regulatory phosphorylation of CaMKKβ at Thr144 in intact cells and *in vivo* remains unclear.

METHODS: Anti-phosphoThr144 antibody was used to characterize the site-specific phosphorylation of CaMKKβ in immunoprecipitated samples from mouse cerebellum and in transfected mammalian cells that were treated with various agonists and protein kinase inhibitors. CaMKK activity assay and LC-MS/MS analysis were used for biochemical characterization of phosphorylated CaMKKβ.

RESULTS: Our data suggest that the phosphorylation of Thr144 in CaMKKβ is rapidly induced by cAMP/cAMP-dependent protein kinase (PKA) signaling in CaMKKβ-transfected HeLa cells, that is physiologically relevant in mouse cerebellum. We confirmed that the catalytic subunit of PKA was capable of directly phosphorylating CaMKKβ at Thr144 *in vitro* and in transfected cells. In addition, the basal phosphorylation of CaMKKβ at Thr144 in transfected HeLa cells was suppressed by AMPK inhibitor (compound C). PKA-catalyzed phosphorylation reduced the autonomous activity of CaMKKβ *in vitro* without significant effect on the Ca^{2+}/CaM-dependent activity, resulting in the conversion of CaMKKβ into Ca^{2+}/CaM-dependent enzyme.

CONCLUSION: cAMP/PKA signaling may confer Ca^{2+}-dependency to the CaMKKβ-mediated signaling pathway through direct phosphorylation of Thr144 in intact cells.

GENERAL SIGNIFICANCE: Our results suggest a novel cross-talk between cAMP/PKA and Ca^{2+}/CaM/ CaMKKβ signaling through regulatory phosphorylation.

Keywords: CaMKK, PKA, phosphorylation, intracellular Ca^{2+}, Calmodulin, signal transduction

INTRODUCTION

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) was originally identified as an activator of CaMKIα and CaMKIV by phosphorylating their activation loop Thr residue (Thr177 in CaMKIα and Thr196 in CaMKIV) [1, 2]. In mammals, CaMKK is composed of two isoforms (α and β) and is expressed in lower eukaryotes, including *Caenorhabditis elegans* and *Aspergillus nidulans* [3-7]. Recently, accumulated evidence indicated that CaMKKβ activates 5'AMP-activated protein kinase (AMPK) through phosphorylation of Thr172 in AMPKα, resulting in various metabolic and pathophysiological responses including hepatic steatosis and cancer cell growth [8-15]. CaMKK is a member of the CaMK family, which is regulated by intrasteric autoinhibition and activated by Ca^{2+}/CaM-binding to the regulatory domain [16, 17], however, does not belong to CAMK group according to the classification by Manning *et al*. [18]. In addition to Ca^{2+}/CaM-binding, CaMKK is regulated by phosphorylation, including autophosphorylation [19] and trans-phosphorylation by multiple protein kinases. It has been demonstrated that CaMKKα is negatively regulated by phosphorylation with cAMP-dependent protein kinase (PKA), resulting in the recruitment of 14-3-3 proteins [20-22]. Unlike CaMKKα, which is strictly regulated by autoinhibitory mechanism [17], CaMKKβ contains an N-terminal regulatory domain (residues 129-151) that suppresses the autoinhibitory mechanism and, consequently, generates autonomous activity [23]. Despite the significant Ca^{2+}/CaM-independent activity of CaMKKβ, the activation of CaMKKβ-mediated signaling, including the CaMKKβ/AMPK cascade, requires an increasing concentration of intracellular Ca^{2+} [8-10]. Recent studies demonstrated that the phosphorylation of Ser129, Ser133, and Ser137 in the N-terminal regulatory domain of human CaMKKβ by CDK5 and GSK3 reduced autonomous activity [24]. Moreover, feedback phosphorylation of Thr144 in the same region by activated AMPK converts CaMKKβ into a Ca^{2+}/CaM-dependent kinase [25], indicating that phosphorylation of the N-terminal regulatory domain suppresses the inhibitory effect of the region in the autoinhibitory mechanism. This post-translational modification of the N-terminal regulatory domain of CaMKKβ may be essential for Ca^{2+}-dependent activation of CaMK cascade although the regulatory phosphorylation of CaMKKβ at Thr144 in intact cells remains unclear.

In this study, we investigated the intracellular signaling system controlling Thr144 phosphorylation of

CaMKKβ in cultured cells. In addition, we identified the cAMP/PKA signaling, which contributes to the regulatory phosphorylation of CaMKKβ, maintaining CaMKKβ as a Ca^{2+}/CaM-dependent enzyme that might be occurred *in vivo*.

RESULTS

cAMP/PKA signal enhances phosphorylation of CaMKKβ at Thr144 in HeLa cells

CaMKKβ is phosphorylated at Thr144 by activated AMPK, resulting in the conversion of the enzyme into a Ca^{2+}/CaM-dependent kinase *in vitro* and in transfected cells [25]. To further examine the Thr144 phosphorylation in cultured cells, we stimulated HeLa cells expressing CaMKKβ [8-10, 26] with various agonists and performed immunoblot analysis to detect the phosphorylation of CaMKKβ at Thr144. Since the immunoreactivity of the antibody against phosphoThr144 of CaMKKβ is not sensitive enough to detect the phosphorylation of endogenous CaMKKβ in HeLa cells and cultured mouse hippocampal neurons (data not shown), we transfected rat CaMKKβ expression plasmid to examine Thr144 phosphorylation. We found that treatment with 10 μM isoproterenol, a selective β-adrenoceptor agonist, rapidly enhanced CaMKKβ phosphorylation at Thr144 (peaking at 1-5 min, Fig. 1A). Subsequently, the Thr144 phosphorylation gradually decreased. In addition, treatment with 50 μM H-89 (PKA inhibitor) completely abolished the isoproterenol-induced Thr144 phosphorylation (Fig. 1B), indicating that this phosphorylation was catalyzed by PKA and dynamically regulated by protein phosphatase(s). We also observed isoproterenol-induced phosphorylation of cAMP response element binding protein (CREB) at Ser133 as well as phosphorylation of CaMKKβ in transfected HeLa cells (supplemental Fig. S1), indicating the activation of cAMP/PKA signaling in the cells. To confirm these results, we treated transfected HeLa cells with 10 μM forskolin (an activator of adenylate cyclase) and subsequently analyzed Thr144 phosphorylation in exogenously expressed CaMKKβ. Figure 1C shows the enhanced phosphorylation of CaMKKβ at Thr144 in HeLa cells following treatment with forskolin. This effect was also completely abolished by pretreatment with H-89 (Fig. 1D) in a similar manner to treatment with isoproterenol (Fig. 1B). These results suggest that the phosphorylation of CaMKKβ at Thr144 is induced by activation of cAMP-PKA signaling in these cells.

PKA directly phosphorylates CaMKKβ at Thr144 in cultured cells and in vitro

To confirm the direct involvement of PKA in the phosphorylation of CaMKKβ at Thr144 in living cells, we co-transfected an expression plasmid of the catalytic subunit of PKA (PKAc) with that of CaMKKβ in COS-7 cells (Fig. 2A). The Thr144 phosphorylation was examined through immunoblot analysis using an anti-phosphoThr144 antibody. Although the phosphorylation of CaMKKβ at Thr144 was very weak or undetectable without co-expression of PKAc, the exogenous expression of PKAc significantly enhanced phosphorylation of CaMKKβ at Thr144 in transfected COS-7 cells. Next, we performed *in vitro* phosphorylation of recombinant CaMKKβ by the purified catalytic subunit of PKA (PKAc), demonstrating that PKAc directly phosphorylates CaMKKβ at Thr144 (Fig. 2B). Since PKA-mediated phosphorylation of CaMKKβ was monitored only at Thr144 using a phosphoThr144 specific antibody, we attempted to identify other PKA-phosphorylation sites through LC-MS/MS analysis (Fig. 2D). Comparison of the phosphorylation sites between two CaMKKβ samples, which were phosphorylated with (autophosphorylated + PKA-phosphorylated CaMKKβ) and without (autophosphorylated CaMKKβ) PKAc for 10 min in the presence of Mg-ATP, detected three PKA-phosphorylation sites including Thr144, Ser494, and Ser510 (Fig. 2 C, D), in addition to the autophosphorylation sites previously identified (data not shown) [25]. These three PKA-phosphorylation sites were recently identified as sites phosphorylated by activated AMPK [25]. Moreover, Ser510 was shown to be phosphorylated by death-associated kinase (DAPK) *in vitro* and in SH-SY5Y cells [27]. These results clearly indicate that CaMKKβ was directly phosphorylated at Thr144 by PKA *in vitro* and in cultured cells.

Basal phosphorylation of CaMKKβ at Thr144 in HeLa cells is mediated by AMPK

Our pharmacological and biochemical data indicate that cAMP/PKA signaling mediates isoproterenol-induced phosphorylation of CaMKKβ at Thr144 (Fig. 1 and 2), recently identified as an AMPK phosphorylation site [25]. In CaMKKβ-transfected HeLa cells, endogenous AMPK is expected to be activated constitutively by phosphorylation with overexpressed CaMKKβ, which exhibits a significant autonomous kinase activity [25]. Therefore, we next investigated whether AMPK was

involved in the basal phosphorylation of CaMKKβ at Thr144 in transfected HeLa cells. As shown in Fig. 1A, Thr144 in CaMKKβ was weakly phosphorylated in unstimulated cells. When we treated CaMKKβ-transfected HeLa cells without or with compound C (AMPK inhibitor) for 20 min, 10 μM compound C almost completely inhibited basal phosphorylation of CaMKKβ (at Thr144) (Fig. 3A). We observed that the endogenous AMPK was phosphorylated at an activation Thr172 residue in CaMKKβ transfected HeLa cells, whose phosphorylation level was not affected by compound C treatment (supplemental Fig. S2). These results indicate that the basal phosphorylation of CaMKKβ at Thr144 is possibly mediated by AMPK that was much weaker than isoproterenol-induced phosphorylation of CaMKKβ (Fig. 1A). Then we compared the catalytic efficiency of PKA with that of AMPK for CaMKKβ (at Thr144) as a substrate (Fig. 3B). We prepared an activated AMPK (hexahistidine-tagged), which had been phosphorylated by CaMKKβ, followed by purification with Ni-NTA column chromatography to remove Mg-ATP and CaMKKβ as described in the MATERIALS and METHODS section. When we measured Thr144-phosphorylation in CaMKKβ by PKA and an activated AMPK with various enzyme concentrations, PKA exhibited significantly higher Thr144-phosphorylation activity than activated AMPK. This is consistent with the results of basal and isoproterenol-induced phosphorylation of CaMKKβ in transfected HeLa cells (Fig. 1A, C and Fig.3 A).

Phosphorylation at Thr144 by PKA converts CaMKKβ into Ca^{2+}/CaM-dependent enzyme

Subsequently, we examined the effect of PKA-mediated phosphorylation on CaMKKβ activity. CaMKKβ activity was measured by dot blot assay using 100 μM ATP and GST-CaMKI 1-293, K49E (kinase dead mutant) as substrates [25]. The phosphate incorporation into Thr177 of the substrate was detected and quantitated using an anti-phosphoThr177 antibody instead of the conventional CaMKK activity assay using [γ-^{32}P]-ATP [17] to exclude the PKA-catalyzed phosphorylation of GST-CaMKI 1-293, K49E. According to our recent results regarding the effect of AMPK phosphorylation on CaMKKβ activity [25], we suspected that Thr144 phosphorylation by PKA reduced the autonomous activity of CaMKKβ, resulting in Ca^{2+}/CaM-dependency. In accordance with previous results [4, 5, 23], CaMKKβ expressed in *E. coli* exhibited significantly enhanced autonomous activity in the absence of Ca^{2+}/CaM. Subsequently,

we incubated the recombinant CaMKKβ with the purified catalytic subunit of PKA in the presence of Mg-ATP for various time points and under the conditions described in Fig. 2B. The autonomous activity of CaMKKβ was gradually decreased by PKA treatment (Fig. 4A) in a similar manner to the increasing level of Thr144 phosphorylation (Fig. 2B). However, the activity was not affected by autophosphorylation (without PKAc). We confirmed that the autonomous activity of Thr144Ala mutant was no longer suppressed by PKA phosphoryaltion unlike wild-type CaMKKβ (Fig. 4B). Then we prepared the CaMKKβ phosphorylated without or with PKAc for 60 min and measured the autonomous and total (in the presence of Ca^{2+}/CaM) activities of both CaMKKβ samples. In accordance with Fig. 4A, the autonomous activity of CaMKKβ was significantly decreased by PKA-mediated phosphorylation without significant effect on the total activity (Fig. 4C). This finding indicates that PKA-mediated phosphorylation converts CaMKKβ into a Ca^{2+}/CaM-dependent enzyme in a similar manner to AMPK-mediated phosphorylation [25].

CaMKKβ is phosphorylated at Thr144 in vivo

To evaluate the physiological significance of Thr144 phosphorylation of CaMKKβ, we attempted to detect the Thr144-phosphorylated form of CaMKKβ in mouse brain tissue, especially the cerebellum, in which CaMKKβ is highly expressed [28]. Since the immunoblot analysis of mouse brain extracts using the anti-phosphoThr144 antibody is unable to detect the phosphorylation of CaMKKβ, probably due to the lower abundance of the phosphorylated form (data not shown), we attempted to immunoprecipitate phospho-CaMKKβ using the anti-phosphoThr144 antibody to concentrate the phosphorylated enzyme. First, we prepared recombinant CaMKKβ either phosphorylated by PKA or untreated (unphosphorylated) to assess the ability of the anti-phosphoThr144 antibody to specifically immunoprecipitate the phosphorylated form of CaMKKβ. Subsequently, we performed the immunoprecipitation using samples with two different concentrations of CaMKKβ (438 ng/mL and 219 ng/mL) and the anti-phosphoThr144 antibody (Fig. 5A). Immunoblot analysis showed that phosphorylated CaMKKβ (by PKA) and unphosphorylated CaMKKβ were equally detected by immunoblotting with the anti-CaMKKβ antibody. Although immunoprecipitated CaMKKβ from the PKA-phosphorylated recombinant enzyme was readily

detected by immunoblotting using both anti-CaMKKβ and anti-phosphoThr144 antibodies, the immunoprecipitated CaMKKβ from the unphosphorylated enzyme was not detected by either antibody. This finding indicates that phosphorylated CaMKKβ at Thr144 is specifically immunoprecipitated by the phospho-specific antibody, unlike the unphosphorylated form of CaMKKβ. When we used this anti-phosphoThr144 antibody for the immunoprecipitation of samples from mouse cerebellum extracts, the immunoprecipitated CaMKKβ using the anti-phosphoThr144 antibody (but not with control mouse IgG), was readily detected by both anti-phosphoThr144 (Fig. 5B *left panel*) and anti-CaMKKβ antibodies (Fig. 5B *right panel*). We detected two immunologically reacted bands on both blots (Fig. 5B *asterisks*), probably due to alternative splicing isoforms of CaMKKβ [26, 29]. These results document that the phosphorylation of CaMKKβ at Thr144 occurs *in vivo*.

DISCUSSION

Accumulated evidence indicates that the CaMKK-mediated phosphorylation cascade plays an important role in a wide variety of physiological responses, including neuronal and metabolic signaling as well as pathophysiological pathways, including cancer cell growth [30, 31]. Conventional CaMK cascades (i.e., CaMKK/CaMKI and CaMKK/CaMKIV) are strictly regulated by the dual mode of Ca^{2+}-signaling, including Ca^{2+}/CaM-binding to downstream CaMKs (CaMKI and CaMKIV) and upstream CaMKKs for activation [1]. CaMKKα is simply activated by Ca^{2+}/CaM-binding in a similar manner to other CaMKs [17, 32, 33]. In contrast, CaMKKβ is elaborately regulated by multiple mechanisms including Ca^{2+}/CaM-binding, autophosphorylation, and trans-phosphorylation by multiple kinases [5, 19, 24, 25]. Unlike CaMKKα, CaMKKβ exhibits significant autonomous activity in the absence of Ca^{2+}/CaM due to the N-terminal regulatory domain (residues 129-151) disrupting the autoinhibitory mechanism [23]. Recent studies demonstrated that phosphorylation of multiple residues (Ser129, Ser133, and Ser137 in human CaMKKβ) by CDK5 and GSK3, and phosphorylation of Thr144 by activated AMPK in the N-terminal regulatory region, impair the function of this region [24, 25]. This phosphorylation results in a decrease in autonomous activity of CaMKKβ and the conversion of the enzyme into a Ca^{2+}/CaM-dependent enzyme [24, 25]. In this study, we observed that Thr144-phosphorylation of

CaMKKβ was significantly induced by cAMP/PKA signaling stimulated by treatment with isoproterenol and forskolin in mammalian cells as well as co-transfection of the catalytic subunit of PKA. It is noteworthy that Thr144 phosphorylation in CaMKKβ is very weak without stimulation, which was almost completely inhibited by treatment with an AMPK inhibitor (compound C), suggesting the involvement of AMPK in the basal phosphorylation of Thr144. A result showing that the phosphorylation level at Thr144 was gradually decreased after 5 min of isoproterenol-stimulation (Fig. 1A), indicates that Thr144 is dynamically modified by reversible phosphorylation. We also confirmed that Thr144 in CaMKKβ was phosphorylated by PKA *in vitro*, that is associated with a decrease in autonomous activity without any observed effects on the total activity. This finding is consistent with recent results indicating that the phosphomimetic Thr144Glu mutant of CaMKKβ exhibited complete Ca^{2+}/CaM-dependent kinase activity [25]. Contrary to our results, Psenakova *et al*. found that the phosphorylation by PKA significantly suppressed the activity of phosphorylated human CaMKKβ (93-517) relative to the non-phosphorylated enzyme by ~50% and ~30% for human CaMK1D (kinase-dead mutant) and human AMPKα2 (kinase-dead mutant) as substrates, respectively [34]. This discrepancy can perhaps be explained on the ground that the reaction time for PKA phosphorylation used in this study was apparently short (Fig. 4B and C, at 30 ºC for 60 min) as compared to that in a recent report (at 30 °C for 3 h and then overnight at 4 °C [34]) and/or the kinase activity of truncated form of CaMKKβ (93-517) used in a recent report [34] might be more sensitive to PKA-mediated phosphorylation than that of wild-type enzyme. It is noteworthy that CaMKKα has demonstrated to be phosphorylated at Thr108 by PKA that is equivalent to Thr144 in CaMKKβ, resulting in suppression of Ca^{2+}/CaM-dependent kinase activity [20, 21] unlike CaMKKβ. Furthermore, it has been reported that the catalytic activity of phosphorylated CaMKK2 (CaMKKβ) by PKA was not inhibited by 14-3-3γ binding [34] whereas the 14-3-3 protein binding suppressed the activity of phosphorylated CaMKKα [22]. Therefore, cAMP/PKA signaling may differentially regulate CaMKK isoforms in intact cells. In addition to Thr144, we detected through LC-MS/MS analysis that PKA phosphorylates Ser494 and Ser510. However, we could not detect the phosphorylation of Ser99, whose equivalent site in human CaMKKβ (Ser100) was shown to be stoichiometrically phosphorylated by PKA [34]. This is probably due to an amino acid difference

between two species at the -3 position where position 0 is the primed phosphorylation site (GKMS[99]LQ in rat CaMKKβ; RKLS[100]LQ in human CaMKKβ). It has been well established that an arginine residue on the P-3 position is an important determinant for PKA recognition [35]. Moreover, we recently observed that activated AMPK phosphorylates Ser494; however, the autonomous activity of the Ser494Ala mutant was reduced by activated AMPK-mediated phosphorylation in a similar manner to wild-type CaMKKβ [25]. Previous study showed that the phosphorylation of rat CaMKKα by PKA reduced the Ca^{2+}/CaM-binding possibly due to the Ser458 (equivalent to Ser494 in rat CaMKKβ) phosphorylation in the CaM-binding region according to the result demonstrating that the Ca^{2+}/CaM-binding of Ser458Ala mutant was no longer suppressed by PKA phosphorylation [20]. In this study, Ca^{2+}/CaM-dependent activity of CaMKKβ was not apparently affected by PKA-mediated Ser494 phosphorylation (Fig. 4C), probably due to the fact that we used relatively high concentration (6 μM) of CaM in the kinase assay as compared to CaM-binding assay (~30 nM biotinylated CaM was used for CaM-overlay method [36]). CaM concentration (6 μM) used in our CaMKK assay is comparable to that in the soluble fraction of various mammalian tissues including brain, testis, lung, adrenal grand and prostate [37]. It has been shown that DAPK-mediated phosphorylation at Ser511 in human CaMKKβ (Ser510 in rat CaMKKβ) results in attenuation of Ca^{2+}/CaM-stimulated CaMKK autophosphorylation (~40% reduction) [27]. However, we were unable to detect any significant reduction of the total activity of CaMKK in the presence of Ca^{2+}/CaM during PKA phosphorylation, suggesting that the phosphorylation of Ser510 affects the autophosphorylation of CaMKKβ, but not the phosphorylation of the substrate. These results indicate that PKA-catalyzed Ser494 and Ser510 phosphorylation may not be involved in the reduction of autonomous activity. This is supported by Green *et al.* showing that human CaMKKβ expressed in mammalian cells exhibits relatively low autonomous activity although it contains phosphoSer495 and phosphoSer511. However, CaMKKβ Ser100Ala, Ser495Ala, and Ser511Ala triple mutant had activity similar to that of the wild-type enzyme [24]. Collectively, Thr144 phosphorylation by PKA may be involved in the conversion of CaMKKβ into a Ca^{2+}/CaM-dependent enzyme in a similar manner to AMPK-mediated phosphorylation. We have succeeded in detecting Thr144 phosphorylation of immunoprecipitated CaMKKβ from the mouse cerebellum. These analyses indicated that phosphorylation

at Thr144 is physiologically relevant. However, we cannot exclude the possibility that other kinases except AMPK and PKA may be involved in this phosphorylation reaction in the brain. The findings of this study demonstrated that cAMP/PKA signaling may be a reasonable candidate for the regulation of CaMKKβ through phosphorylation of Thr144 in cultured HeLa cells and cerebellum. However, further studies are warranted to identify the kinase(s) responsible for the phosphorylation of CaMKKβ at Thr144 *in vivo*. Taken together with this and previous reports, phosphorylation of the N-terminal regulatory domain (residues 129-151) by multiple kinases including CDK5/GSK3 [24], activated AMPK [25], and PKA confers Ca^{2+}/CaM-dependency to CaMKKβ, that may be required for CaMKKβ-mediated signaling cascades triggered by increasing concentration of intracellular Ca^{2+} (Fig. 6).

MATERIALS AND METHODS

Materials

Recombinant rat CaMKKβ was expressed in *E. coli* BL21 Star (DE3) cells and purified by CaM-sepharose and Q-sepharose chromatography [23]. GST-rat CaMKIα 1–293, Lys49Glu (GST-CaMKI 1–293, KE) was expressed in *E. coli* JM109 and purified as previously described [17]. Recombinant wild-type AMPK and Thr144Ala mutant were expressed in *E. coli* BL21-CodonPlus (DE3) (Stratagene, La Jolla, CA, USA) using a tricistronic pγ1β1His-α1 plasmid (kindly provided by Dr. Dietbert Neumann, Swiss Federal Institute of Technology, Zurich, Switzerland), and purified as previously described [25, 38]. Recombinant rat CaM was expressed in *E. coli* BL21 (DE3) using the plasmid pET-CaM (kindly provided by Dr. Nobuhiro Hayashi, Tokyo Institute of Technology, Yokohama, Japan) [39]. The anti-phosphoCaMKI at Thr177 (clone 9H8) monoclonal antibody was generated as previously described [40]. The anti-phosphoCaMKKβ at Thr144 (clone A04) monoclonal antibody was generated as previously described [25]. The anti-CaMKKβ monoclonal antibody was generated as previously described [28]. The anti-phosphoAMPKα subunit at Thr172 (2535) was purchased from Cell Signaling Technology (Danvers, MA). Purified bovine cardiac PKA was kindly provided by Dr. Yasuo Watanabe (Showa Pharmaceutical Univ.). Isoproterenol and compound C (Dorsomorphin) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Forskolin was purchased from Wako Pure Chemical (Osaka, Japan). H-89 was

obtained from Cayman Chemical (Michigan, USA). All other reagents were obtained from standard commercial sources.

PKA phosphorylation of CaMKKβ in vitro

Recombinant CaMKKβ (1 μg/20 μL) was incubated without or with purified bovine cardiac PKA (3 ng – 0.33 μg) or an activated AMPK (10 ng – 0.33 μg) prepared as described below at 30°C for indicated time periods in a solution containing 50 mM HEPES pH7.5, 10 mM Mg $(CH_3COO)_2$, 1 mM DTT, and 1 mM ATP in the presence of 2 mM EGTA. The reactions were terminated by adding an equivolume of 1 x SDS-PAGE sample buffer or using a 20-fold dilution in ice-cold dilution buffer (50 mM HEPES pH 7.5, 2 mg/mL bovine serum albumin, 10% ethylene glycol, 2 mM EDTA), followed by immunoblot analysis or measurement of CaMKK activity.

Preparation of activated AMPK

Recombinant AMPK (100 μg) was incubated with CaMKKβ (10 μg) in a solution containing 50 mM HEPES, pH 7.5, 10 mM Mg $(CH_3COO)_2$, 1 mM DTT, 1 mM ATP, 2 mM $CaCl_2$ and 6 μM CaM at 30 ºC for 60 min and then subjected to Ni-NTA column chromatography (200 μL gel volume, Qiagen, Hilden, Germany) to remove CaMKKβ and Mg-ATP. Activated AMPK was eluted from the Ni-NTA column with 300 mM imidazole containing buffer, followed by dialysis against 50 mM NaCl, 50 mM HEPES, pH 7.5, 1 mM DTT and stored at -30 ºC until use for kinase assay.

Identification of phosphorylation sites by LC-MS/MS

Recombinant CaMKKβ (1 μg) was phosphorylated using purified bovine cardiac PKA (0.1 μg/20 μL) at 30°C for 10 min, as described earlier in this section. SDS-PAGE was followed by protease treatment and LC-MS/MS analysis to identify the phosphorylation sites using an LCMS-IT-TOF instrument (Shimadzu, Kyoto, Japan) interfaced with a nano reverse-phase LC system (Shimadzu), as previously described [25]. The MS/MS data were acquired in the datum-dependent mode using the LC-MS solution software (Shimadzu) and converted into a single text file (containing the observed precursor peptide *m/z*, fragment

ion *m/z*, and intensity values) using the Mascot Distiller (Matrix Science, London, UK). The MS/MS data were obtained independently and merged for the Mascot analysis. The following search parameters were used: database, rat CaMKKβ (578 amino acid residues); enzyme, all; variable modifications, carbamidomethyl (Cys), oxidation (Met), propionamide (Cys), and phospho (Ser/Thr).

In vitro CaMKK activity assay

CaMKK activity was measured at 30°C for 5 min in a solution (20 μL) containing 2.5 ng CaMKKβ, 50 mM HEPES pH 7.5, 10 mM Mg $(CH_3COO)_2$, 1 mM DTT, 100 μM ATP, and 10 μg GST-CaMKI 1–293 K49E in the presence of 2 mM EGTA (autonomous activity) or 2 mM $CaCl_2$/6 μM CaM (total activity). Each reaction was initiated by the addition of ATP. Reactions were terminated by spotting the samples (4 μL) onto a nitrocellulose membrane. Thr177-phosphorylated GST-CaMKIα 1–293 K49E was detected using an anti-phospho-CaMKI antibody [25]. Antibody immunoreactivity was detected using a chemiluminescent reagent (PerkinElmer Life Sciences, Waltham, MA, USA) and ChemiDoc XRS (Bio-Rad Laboratories, Inc., Hercules, CA, USA), followed by quantification of Thr177-phosphorylation using the Quantity One® software (ver. 4.6.5, Bio-Rad Laboratories, Inc.).

Cell culture and transfection

HeLa cells and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37ºC in 5% CO_2. HeLa cells placed in 6-well dishes were transfected with 2 μg of CaMKKβ expression plasmid (pME-CaMKKβ) using polyethylenimine "Max" (Polysciences, Inc. Warrington, PA, USA) according to the manufacturer's protocol. After a 44-h culture, the cells were either treated or not treated with 50 μM H-89 for 1 h or 10 μM compound C for 20 min, followed by treatment with 10 μM isoproterenol or forskolin for indicated time periods. Similarly, COS-7 cells placed in 6-well dishes were transfected with or without 1 μg of CaMKKβ expression plasmid (pME-CaMKKβ) together with 1 μg of the catalytic subunit of PKA expression plasmid (pME-PKAc) or empty vector (pME18s) using polyethylenimine "Max" and cultured for 44 h. Subsequently, cells were extracted using 1×

SDS-PAGE sample buffer (100 µL), followed by immunoblot analyses (15 µL).

Immunoprecipitation

Recombinant CaMKKβ (2 µg) phosphorylated with or without purified bovine cardiac PKA (0.1 µg/40 µL) at 30°C for 60 min, as described earlier in this section, was diluted using an immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl pH.7.5, 1 mM EDTA, 0.1 µM okadaic acid) at two concentrations of CaMKKβ (438 ng/mL and 219 ng/mL), followed by immunoprecipitation as follows. The mouse cerebellum was homogenized using 3 mL of immunoprecipitation buffer and centrifuged at 15,000 rpm for 15 min at 4ºC. One mL of the supernatant (cerebellum extracts) or recombinant CaMKKβ with or without PKA phosphorylation was pre-cleared using 25 µL of protein G-sepharose (GE-Healthcare), followed by incubation with either normal mouse IgG (1 µg) or anti-phosphoThr144 antibody (1 µg) for 12 h at 4ºC. Subsequently, 25 µL of protein G-sepharose were added to the reaction mixture and incubated overnight. After washing (x 5) the immunocomplex with immunoprecipitation buffer, 1 X SDS-PAGE sample buffer was added to the sample and boiled at 100ºC for 10 min, followed by immunoblot analysis (10 µL sample) using the anti-CaMKKβ or anti-phosphoThr144 antibody.

Other methods

Immunoblot and dot blot analyses were performed using the indicated primary antibodies and horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare UK, Ltd.) as the secondary antibody. A chemiluminescent reagent (PerkinElmer Life Sciences, Waltham, MA, USA) was used for signal detection, followed by quantification of the immunoreactivity using the ImageJ software [41]. Protein concentrations in the samples were estimated using Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc.) and bovine serum albumin as a standard. The Student's *t* test was used to evaluate the statistical significance of comparisons between two groups. A p value < 0.05 denoted statistical significance.

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FIGURE LEGENDS

Figure 1. cAMP-signaling mediates CaMKKβ phosphorylation at Thr144 in transfected HeLa cells. HeLa cells transfected with rat CaMKKβ expression plasmid were stimulated without or with either 10 μM isoproterenol (A) or 10 μM forskolin (C) for indicated time periods (1-60 min). Subsequently, cell lysates were subjected to immunoblot analysis using either an anti-phosphoThr144 antibody (*inset upper panel*) or anti-CaMKKβ antibody (*inset lower panel*). The phosphorylation level of CaMKKβ at Thr144 was quantitated, expressed as a percentage of the average value of stimulated cells with 10 μM isoproterenol for 5 min (A) or 10 μM forskolin for 60 min (C) and plotted as duplicate experiments. HeLa cells transfected with rat CaMKKβ expression plasmid were untreated (B and D, -) or treated with 50 μM H-89 for 1 h (B and D, +H-89), followed by treatment with 10 μM isoproterenol (B) and forskolin (D) for 5 min and 60 min, respectively. The cell lysates (triplicates) were subjected to immunoblot analysis using either an anti-phosphoThr144 antibody (B and D, *inset upper panel*) or anti-CaMKKβ antibody (B and D, *inset lower panel*). The phosphorylation level of CaMKKβ at Thr144 was quantitated and expressed as a percentage of the average value of stimulated cells not treated with H-89. Results in B and D are expressed as the mean ± S.D. from triplicate experiments. *Error bars* represent S.D. Statistical differences are marked: *, $p < 0.005$; **, $p < 0.001$. Arrows and asterisks in *inset panels* indicate phosphoCaMKKβ (at Thr144) and CaMKKβ, respectively. The molecular mass in kilodaltons is indicated on the left in each blot. D, Similar results were obtained in at least three independent experiments.

Figure 2. Phosphorylation of CaMKKβ at Thr144 by PKA in transfected COS-7 cells and *in vitro.* A, COS-7 cells were transfected with either mock (-) or rat CaMKKβ expression plasmid (+) in the absence (-) or presence of PKAc expression plasmid (+), followed by immunoblot analysis (10 μL cell lysate) using either an anti-phosphoThr144 antibody (*upper panel*) or anti-CaMKKβ antibody (*lower panel*). The data represent triplicate experiments. B, recombinant rat CaMKKβ (1 μg) was incubated with purified PKAc (0.1 μg) in the presence of Mg-ATP for indicated time periods, followed by immunoblot analysis (62.5 ng protein) using either an anti-phosphoThr144 antibody (*inset upper panel*) or anti-CaMKKβ antibody (*inset lower panel*). The phosphorylation level of CaMKKβ at Thr144 was

quantitated and expressed as a percentage of the average value of the 60-min time point and plotted as duplicate experiments. Arrows and asterisks in *panels A* and *B* indicate phosphoCaMKKβ (at Thr144) and CaMKKβ, respectively. The molecular mass in kilodaltons is indicated on the left in each blot. Similar results were obtained in at least three independent experiments. C, schematic representation of rat CaMKKβ with PKA phosphorylation sites (Thr144, Ser494, and Ser510) identified through LC-MS/MS analysis as shown in *panel D*. P in a black circle indicates phosphorylation. N, N-terminal regulatory domain (residues 129–151) [23]; RP, Arg-Pro rich insert domain [42]; Catalytic, catalytic domain (residues 162–470); AID/CBD, autoinhibitory domain containing the Ca^{2+}/CaM binding region (residues 474–499) [17]. D, recombinant CaMKKβ phosphorylated by PKAc for 10 min as shown in *panel B* was separated by SDS-PAGE and digested using a protease cocktail, followed by LC-MS/MS analysis to identify phosphorylation sites. The phosphorylation of Thr144 (*upper panel*), Ser494 (*middle panel*), and Ser510 (*lower panel*) was analyzed through LC-MS/MS of the singly charged ion for peptides containing residues 142-148, residues 494-503, and residues 508-522, respectively. The observed b- and y-ion fragment series generated by collision-induced dissociation are indicated above and below the peptide sequences. PhosphoThr517 was observed in autophosphorylated CaMKKβ [25].

Figure 3. Phosphorylation of CaMKKβ at Thr144 by AMPK in transfected HeLa cells and *in vitro.*

A, HeLa cells transfected with rat CaMKKβ expression plasmid were untreated (-) or treated with 10 μM compound C for 20 min (+). The cell lysates (triplicates) were subjected to immunoblot analysis using either an anti-phosphoThr144 antibody (*inset upper panel*) or anti-CaMKKβ antibody (*insert lower panel*). The phosphorylation level of CaMKKβ at Thr144 was quantitated and expressed as a percentage of the average value of the cells not treated with compound C. Results are expressed as the mean ± S.D. from triplicate experiments. *Error bars* represent S.D. Statistical differences are marked: *, $p < 0.05$. Similar results were obtained in three independent experiments. B, recombinant rat CaMKKβ (1 μg) was incubated with indicated concentrations of either purified PKAc (closed circles) or activated AMPK (open circles) in the presence of Mg-ATP for 60 min at 30 ºC, followed by immunoblot analysis (375 ng protein) using an anti-phosphoThr144 antibody to quantitate the phosphorylation of CaMKKβ (at Thr144).

The phosphorylation level of CaMKKβ at Thr144 was expressed as a percentage of the average value of the phosphorylation level with 409 nM PKA and plotted as duplicate experiments.

Figure 4. PKA-catalyzed phosphorylation reduces autonomous activity, resulting in the conversion of CaMKKβ into a Ca^{2+}/CaM-dependent enzyme.

A, recombinant rat CaMKKβ was incubated without (open circle) or with purified PKAc (closed circle) in the presence of Mg-ATP for indicated time periods as described in Figure 2B, followed by measurement of the autonomous activity of CaMKKβ (2.5 ng) in the presence of EGTA as described in the MATERIALS and METHODS section. CaMKKβ autonomous activity (phosphorylation of GST-CaMKI 1–293 K49E at Thr177) was quantitated and expressed as a percentage of the average value of the 60-min time point and plotted as duplicate experiments. B, rat CaMKKβ wild-type (WT) or Thr144Ala mutant (Thr144Ala) was incubated without (-, *open column*) or with purified PKAc (+, closed column) in the presence of Mg-ATP for 60 min as shown in in *panel A*. After termination of the reaction, the CaMKKβ activity was measured in the absence (autonomous activity) of Ca^{2+}/CaM as described in *panel A*. The CaMKKβ activity was quantitated and expressed as a percentage of the average value of the autonomous activity without PKA phosphorylation. Results are represented as an average ± S.D. from triplicate experiments. *Error bars* represent S.D. Statistical differences are marked: **, $p < 0.001$; n.s., not significant *versus* the activity without PKA phosphorylation. C, recombinant rat CaMKKβ was incubated without (-PKA) or with purified PKAc (+PKA) in the presence of Mg-ATP for 60 min as shown in *panel A*. After termination of the reaction, the CaMKKβ activity was measured in the presence (total activity, *open column*, +) or absence (autonomous activity, *closed column*, -) of Ca^{2+}/CaM as described in the MATERIALS and METHODS section. The CaMKKβ activity was quantitated and expressed as a percentage of the average value of the total activity without PKA phosphorylation. Results are represented as an average ± S.D. from triplicate experiments. *Error bars* represent S.D. Statistical differences are marked: **, $p < 0.001$; n.s., not significant *versus* total activity without PKA phosphorylation. Immunoblot analysis of CaMKKβ (25 ng) with (+PKA) or without (-PKA) PKA phosphorylation using an anti-phosphoThr144 antibody is shown in *inset.* An arrow in *inset panel* indicates phosphoCaMKKβ

(at Thr144). The molecular mass in kilodaltons is indicated on the left. Similar results were obtained in at least three independent experiments.

Figure 5. Phosphorylation of CaMKKβ at Thr144 in the mouse cerebellum.

A, immunoprecipitation of phosphorylated CaMKKβ at Thr144 using an anti-phosphoThr144 antibody. Recombinant rat CaMKKβ (2 μg) was incubated without (-) or with purified PKAc (100 ng, +) in the presence of Mg-ATP for 60 min, as described in the MATERIALS and METHODS section. Diluted samples using immunoprecipitation buffer (438 and 219 ng/mL CaMKKβ) were incubated with 1 μg of anti-phosphoThr144 antibody, followed by incubation with protein G sepharose. After thorough washing of the resin, the immunoprecipitated samples (IP) together with the input samples (Input) (20 ng CaMKKβ in left panels and 10 ng CaMKKβ in right panels, respectively) were subjected to immunoblotting using either an anti-phosphoThr144 (*upper panels*) or anti-CaMKKβ antibody (*lower panels*). An arrow and an asterisk indicate phosphoCaMKKβ (at Thr144) and CaMKKβ, respectively. B, phosphorylation of CaMKKβ at Thr144 in the mouse cerebellum. Mouse cerebellum extracts were immunoprecipitated using 1 μg of either an anti-phosphoThr144 antibody (α-pThr144 Ab) or normal mouse IgG (Normal IgG), as described in the MATERIALS and METHODS section. Immunoprecipitated samples were subjected to immunoblotting using either an anti-phosphoThr144 antibody (*left panel*) or an anti-CaMKKβ antibody (*right panel*). Asterisks indicates phosphoCaMKKβ (at Thr144) (*left panel*) and CaMKKβ (*right panel*), respectively. Arrows indicate IgG. The molecular mass in kilodaltons is indicated on the left in each blot. Similar results were obtained in three independent experiments.

Figure 6. Schematic representation of cross-talk between CaMKKβ and cAMP/PKA signaling.

The molecular structure of CaM used in this model was obtained from the Protein Data Bank (PDB) entry 1UP5 [43] visualized using the UCSF Chimera [44]. GPCR, G-protein coupled receptor; α/β/γ, G-protein α/β/γ subunit; CaMKI, Ca^{2+}/CaM-dependent protein kinase I; CaMKIV, Ca^{2+}/CaM-dependent protein kinase IV; AMPK, 5'AMP-activated protein kinase; CREB, cAMP response element binding protein; ACC, acetyl-CoA carboxylase. T, Thr residue. P in a black circle indicates phosphorylation.

Figure 1

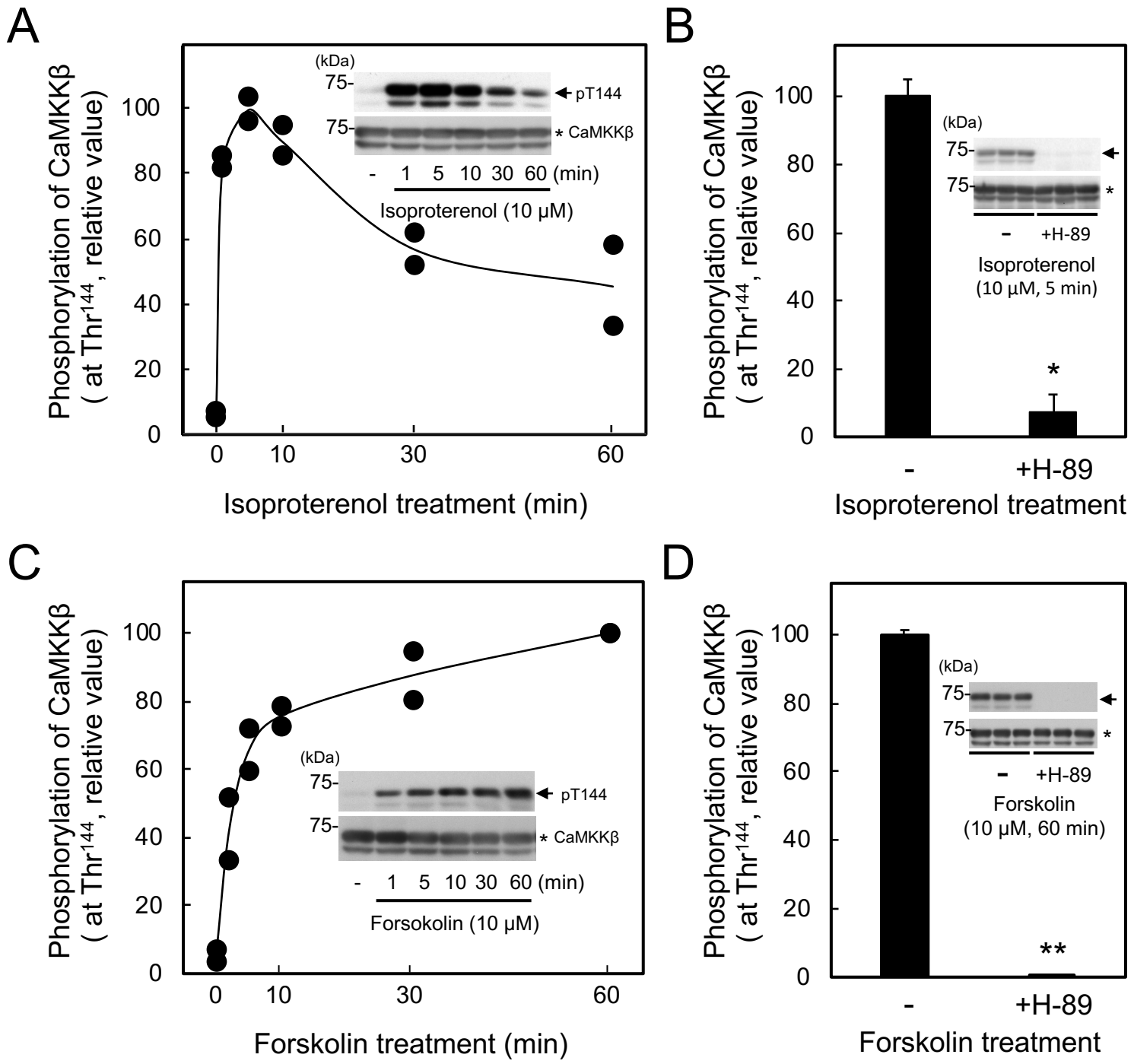
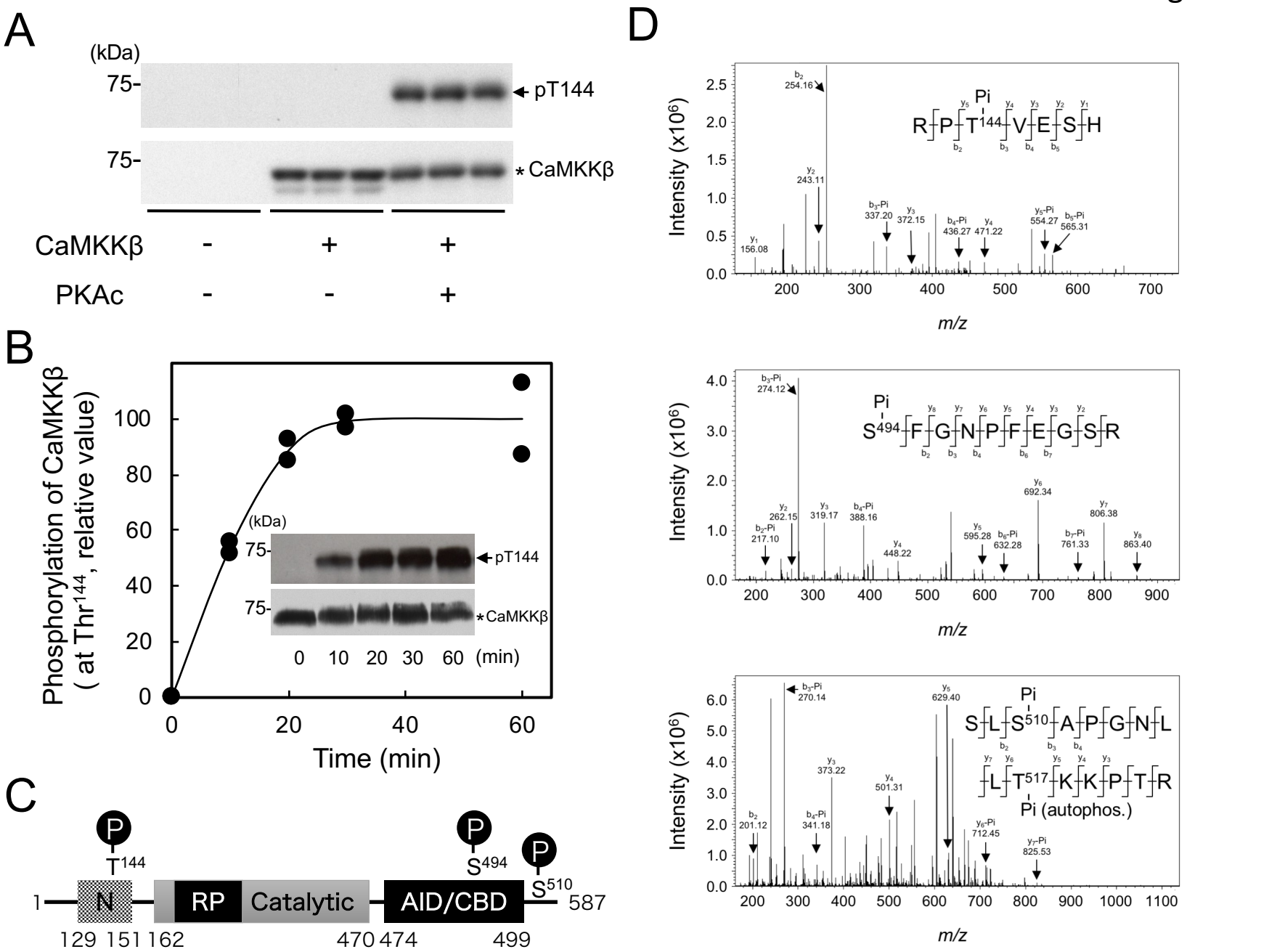

A
Phosphorylation of CaMKKβ
(at Thr144, relative value)
(kDa)
75-
pT144
75-
* CaMKKβ
- 1 5 10 30 60 (min)
Isoproterenol (10 μM)
Isoproterenol treatment (min)
B
Phosphorylation of CaMKKβ
(at Thr144, relative value)
(kDa)
75-
75-
*
- +H-89
Isoproterenol
(10 μM, 5 min)
*
- +H-89
Isoproterenol treatment
C
Phosphorylation of CaMKKβ
(at Thr144, relative value)
(kDa)
75-
pT144
75-
* CaMKKβ
- 1 5 10 30 60 (min)
Forsokolin (10 μM)
Forskolin treatment (min)
D
Phosphorylation of CaMKKβ
(at Thr144, relative value)
(kDa)
75-
75-
*
- +H-89
Forskolin
(10 μM, 60 min)
**
- +H-89
Forskolin treatment

Figure 2


A
(kDa)
75-
pT144
75-
*CaMKKβ
CaMKKβ - + +
PKAc - - +
B
Phosphorylation of CaMKKβ
(at Thr144, relative value)
100
80
60
40
20
0
0 20 40 60
Time (min)
(kDa)
75-
pT144
75-
*CaMKKβ
0 10 20 30 60 (min)
C
P
T144
P
S494
P
S510
1
N
RP
Catalytic
AID/CBD
587
129 151 162
470 474
499
D
Intensity (x10^6)
m/z
b2 254.16
y2 243.11
y1 156.08
b3-Pi 337.20
y3 372.15
b4-Pi 436.27
y4 471.22
y5-Pi 554.27
b5-Pi 565.31
Pi
R P T144 V E S H
b3-Pi 274.12
y2 262.15
b2-Pi 217.10
y3 319.17
b4-Pi 388.16
y4 448.22
y6 692.34
y5 595.28
b6-Pi 632.28
y7 806.38
b7-Pi 761.33
y8 863.40
Pi
S494 F G N P F E G S R
b3-Pi 270.14
y5 629.40
y3 373.22
y4 501.31
b2 201.12
b4-Pi 341.18
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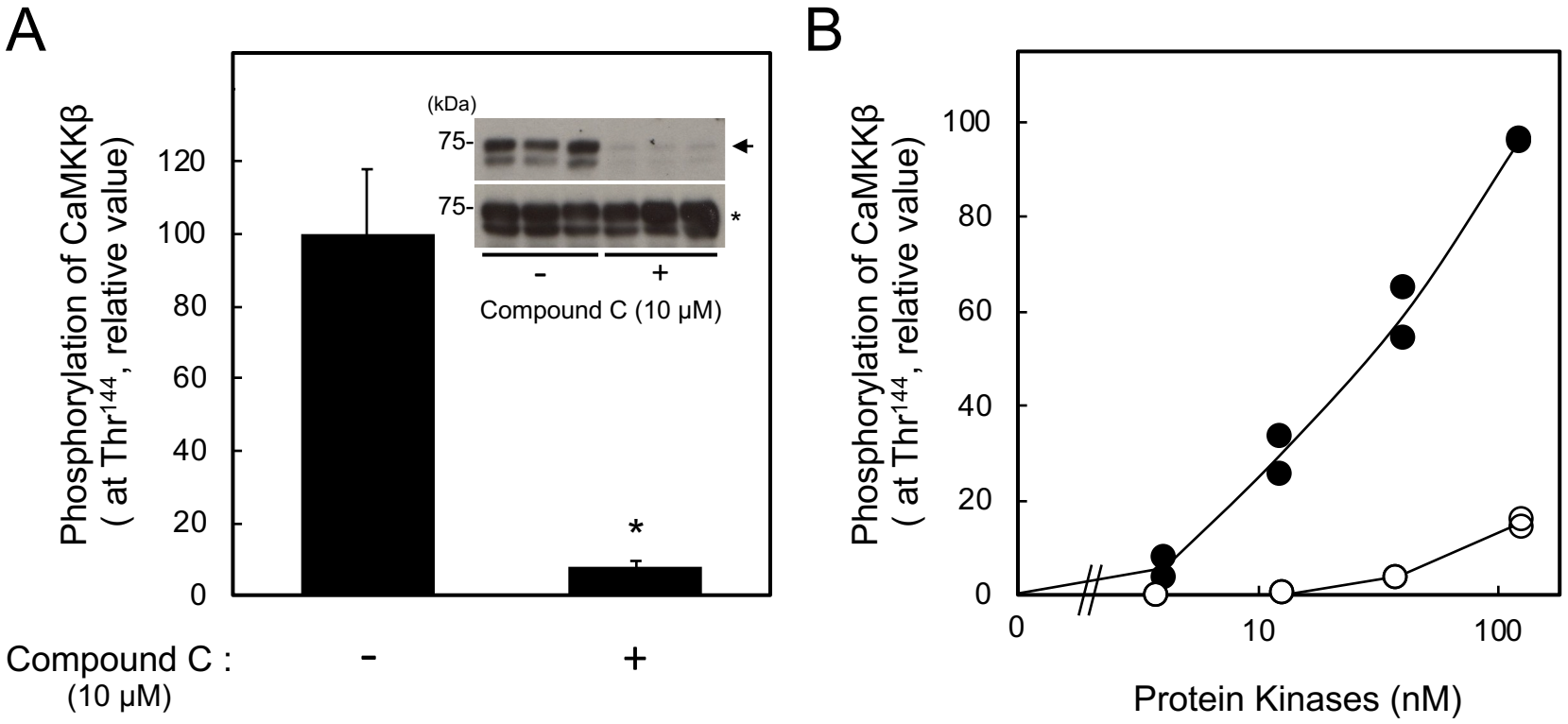
y7-Pi 825.53
Pi
S L S510 A P G N L
L T517 K K P T R
Pi (autophos.)


A
Phosphorylation of CaMKKβ
(at Thr144, relative value)
120
100
80
60
40
20
0
(kDa)
75-
75-
*
-
+
Compound C (10 μM)
*
Compound C :
(10 μM)
-
+
B
Phosphorylation of CaMKKβ
(at Thr144, relative value)
100
80
60
40
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0
0
10
100
Protein Kinases (nM)

Figure 4

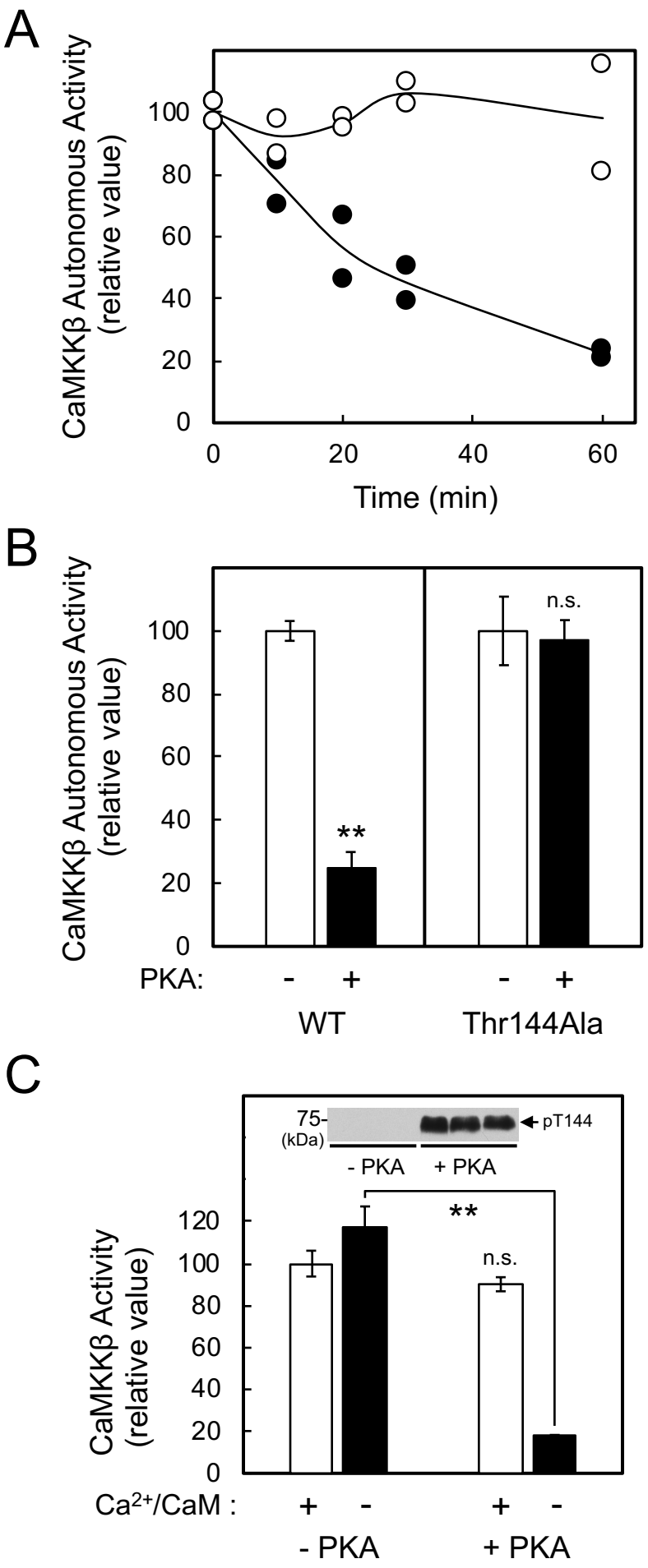
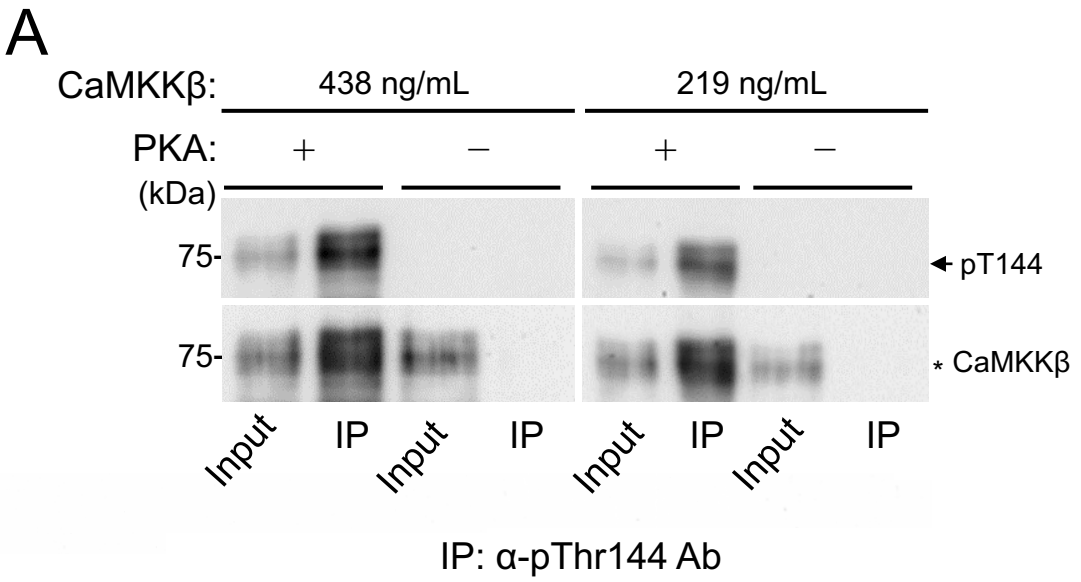

A
CaMKKβ Autonomous Activity
(relative value)
100
80
60
40
20
0
0
20
40
60
Time (min)
B
CaMKKβ Autonomous Activity
(relative value)
100
80
60
40
20
0
**
n.s.
PKA: - + - +
WT
Thr144Ala
C
75-
(kDa)
pT144
- PKA + PKA
**
n.s.
CaMKKβ Activity
(relative value)
120
100
80
60
40
20
0
Ca2+/CaM : + - + -
- PKA
+ PKA

Figure 5


A
CaMKKβ:
438 ng/mL
219 ng/mL
PKA:
+
−
+
−
(kDa)
75-
75-
pT144
* CaMKKβ
Input
IP
Input
IP
Input
IP
Input
IP
IP: α-pThr144 Ab

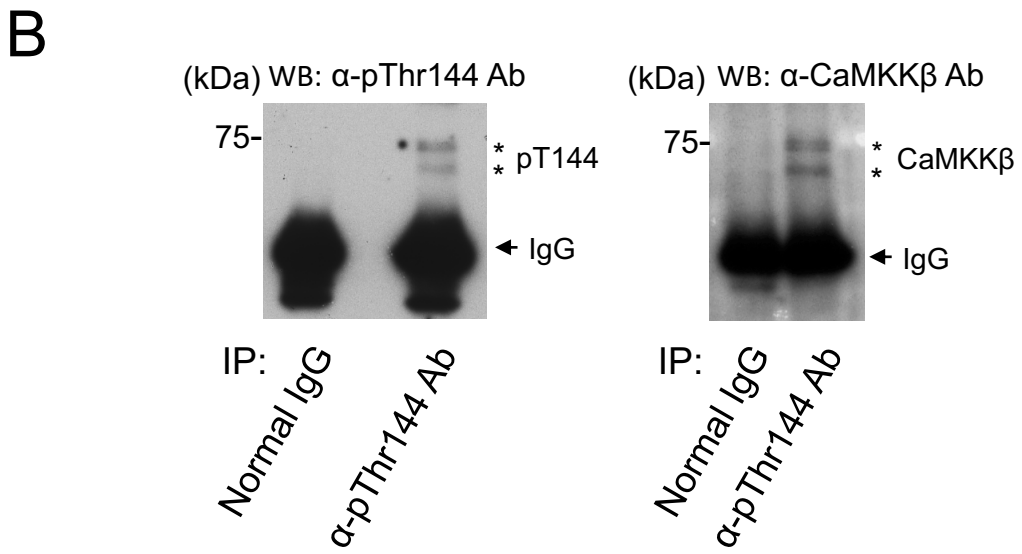
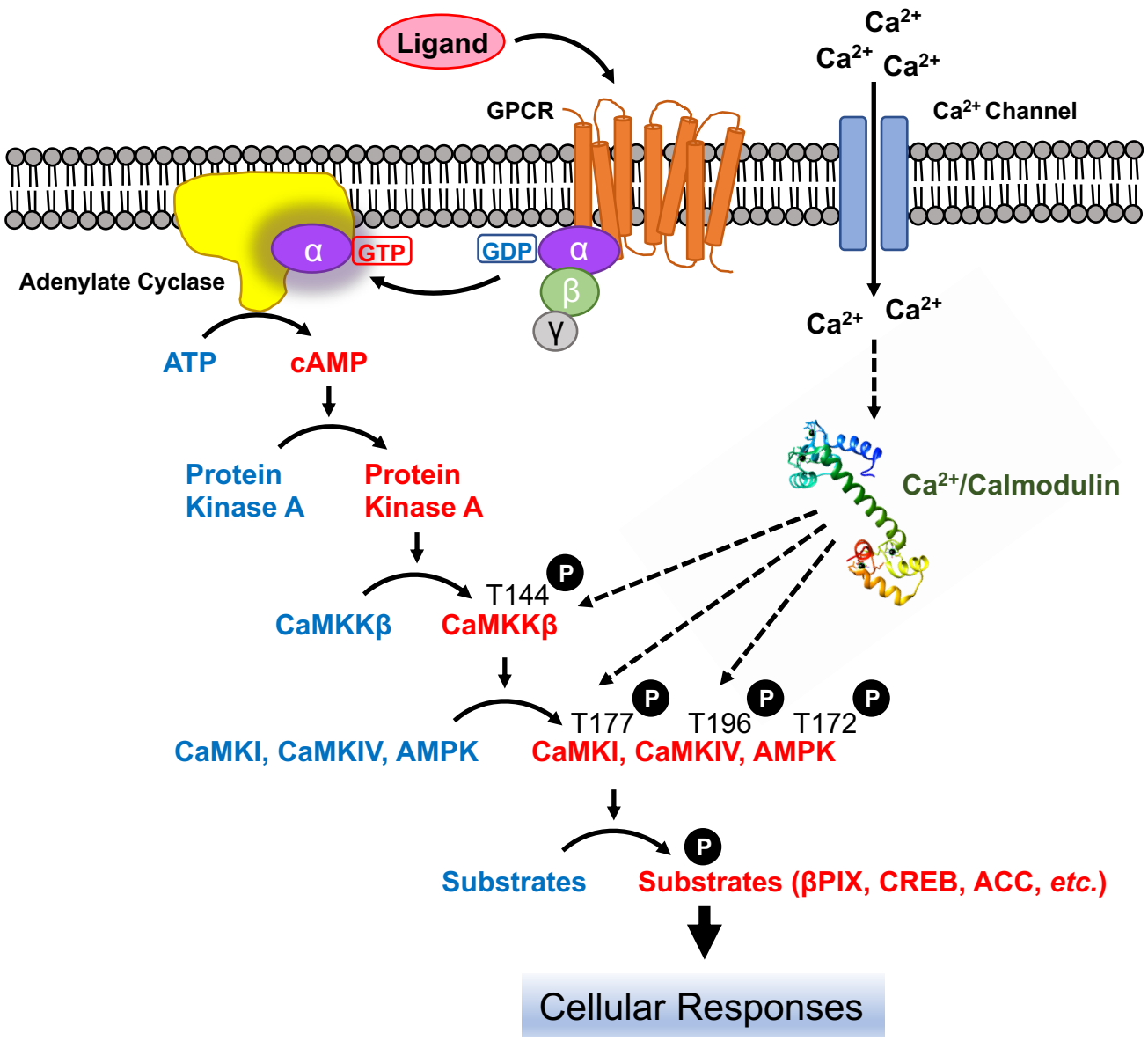

B
(kDa) WB: α-pThr144 Ab
75-
* pT144
IgG
IP:
Normal IgG
α-pThr144 Ab
(kDa) WB: α-CaMKKβ Ab
75-
* CaMKKβ
IgG
IP:
Normal IgG
α-pThr144 Ab

Figure 6


Ligand
GPCR
Ca2+
Ca2+ Ca2+
Ca2+ Channel
α
GTP
GDP
α
β
γ
Adenylate Cyclase
Ca2+ Ca2+
ATP
cAMP
Protein Kinase A
Protein Kinase A
Ca2+/Calmodulin
T144
P
CaMKKβ
CaMKKβ
T177
P
T196
P
T172
P
CaMKI, CaMKIV, AMPK
CaMKI, CaMKIV, AMPK
P
Substrates
Substrates (βPIX, CREB, ACC, etc.)
Cellular Responses