

Characterization of Autonomous and Ca²⁺/Calmodulin-Dependent Activities of CaMKK Isoforms In Vitro and in Mouse Tissues

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ABSTRACT

Ca^{2+} /CaM-dependent protein kinase kinase (CaMKK) phosphorylates and activates downstream kinases including CaMKI, CaMKIV, PKB and AMPK, regulating in various cellular functions such as neuronal morphogenesis, metabolic control and pathophysiological pathways, such as cancer progression. CaMKK α /1 is tightly regulated by autoinhibitory mechanism. CaMKK β /2 activity is highly Ca^{2+} /CaM-independent (autonomous activity) in vitro and Ca^{2+} /CaM-dependent in cultured cells. Whether these two activity states of CaMKK β /2 exist in vivo and the detailed regulatory mechanisms for transition of both activity states remain unclear due to the difficulty in distinguishing the two activity states. In this study, we detected Ca^{2+} -dependent and autonomous CaMKK activity in HeLa cells and successfully separated both activity states of CaMKK β /2 in mouse brain and testis extracts using a recently developed CaMKK inhibitor (TIM-063)-coupled Sepharose, which binds to the catalytic domain in the active state but not in the autoinhibited state. Furthermore, lambda protein phosphatase treatment converted Ca^{2+} /CaM-dependent form to the autonomous form of CaMKK β /2, which was not affected by Ala mutation of Ser129, Ser133, and Ser137. The two activity forms of CaMKK β /2 had equivalent Ca^{2+} /CaM-binding ability. The findings demonstrate the presence of autonomous and Ca^{2+} /CaM-dependent forms of CaMKK β /2 independently in mouse tissues and cultured cells. The transition of these states of CaMKK β /2 may be dynamically regulated by phosphorylation/dephosphorylation of serine residues in the N-terminal regulatory domain.

INTRODUCTION

Ca^{2+} /CaM-dependent protein kinase kinase (CaMKK) is a serine/threonine (Ser/Thr) kinase originally identified as an activator of CaM kinase I (CaMKI) and IV (CaMKIV) that phosphorylates Thr residues (Thr177 in CaMKI and Thr196 in CaMKIV) in the activation loop of each.¹⁻⁶ CaMKK-mediated activation of CaMKs regulates various cellular functions, such as neuronal morphogenesis, synaptic plasticity, and transcriptional activation.^{7,8} In addition to CaMKI and CaMKIV, 5'AMP-kinase (AMPK), which is a key regulator of cellular and whole-body metabolism, is also activated with phosphorylation of a Thr residue in the activation loop in the catalytic subunit (α subunit) by CaMKK β /2, however, not CaMKK α /1.⁹⁻¹¹ Similarly, protein kinase B (PKB/Akt) is activated by CaMKK-catalyzed phosphorylation, which protects NG108 neuroblastoma cells from apoptosis¹² and participates in the growth of OVCAR-3, SKOV-3, and Caov-3 ovarian cancer cells.¹³ The CaMKK β /2-mediated kinase cascade plays important roles in pathophysiological pathways, such as cancer progression, metabolic syndrome, and mental disorders.¹⁴⁻¹⁷

CaMKK consists of a catalytic domain followed by a regulatory domain containing an autoinhibitory segment and a Ca^{2+} /CaM-binding sequence and is activated by Ca^{2+} /CaM-binding to the regulatory domain.³ Unlike CaMKK α /1, which is tightly regulated by an autoinhibitory mechanism, CaMKK β /2 contains an N-terminal regulatory domain (residues 129-151) that suppresses the autoinhibitory mechanism, resulting in autonomous activity.^{18,19} Therefore, CaMKK β /2 has significant Ca^{2+} /CaM-independent activity.^{18,20} However, the activation of CaMKK-mediated kinase cascade including CaMKK β /2-AMPK pathway, requires an increase in intracellular Ca^{2+} concentration.⁹⁻¹¹ A recent study demonstrated that

the phosphorylation of Ser129, Ser133, and Ser137 in the N-terminal regulatory domain of human CaMKK β /2 by cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3) reduces the autonomous activity.²¹ Moreover, phosphorylation of Thr144 also reduces autonomous activity of CaMKK β /2 and is dynamically regulated by the AMPK and PKA upstream kinases and protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) phosphatases.^{22–24} Importantly, the mutation of Thr85Ser, which is an autophosphorylation site mutant of CaMKK β /2, fails to generate autonomous activity and has been implicated in human anxiety, bipolar, and schizophrenia behavioral disorders.²⁵ These findings suggest that two forms of CaMKK β /2 with distinct Ca²⁺/CaM dependencies may exist in vivo and that post-translational modifications that regulate the autonomous activity may be essential for precise regulation of the CaMK cascade. However, the existence of an autonomous activity state of CaMKK β /2 in vivo, its physiological significance, and the regulatory mechanisms underlying the transition between the Ca²⁺/CaM-dependent and autonomous activation states remain to be elucidated. This is because no antibody is currently available to distinguish between the two activation states of CaMKK β /2 in vivo, and conventional CaM-sepharose chromatography cannot separate them.

Recently, we developed a novel pan-CaMKK inhibitor, TIM-063 (2-hydroxy-3-nitro-7*H*-benzo[de]benzo[4,5]-imidazo[2,1-*a*]isoquinolin-7-one) and demonstrated that TIM-063 interacts with CaMKK in a Ca²⁺/CaM-dependent manner.^{26,27} The finding suggests that the inhibitor binds to the catalytic domain of CaMKK in the active, however, not the autoinhibited state. In this study, we succeeded in separating the two active forms (Ca²⁺/CaM-dependent and autonomous states) of CaMKK β /2 using TIM-063-coupled

Sepharose (TIM-127; 1-amino-N-(2-((2-hydroxy-3-nitro-7-oxo-7H-benzo[de]benzo[4,5]imidazo[2,1-a]isoquinolin-11-yl)amino)-2-oxoethyl)-3,6,9,12,15,18-hexaoxahenicosan-21-amide (major)-Sepharose) from mouse tissues. The transition of two CaMKK β /2 activity states by dephosphorylation has also been demonstrated.

MATERIALS AND METHODS

Materials

Glutathione S-transferase (GST)-fused rat CaMKK α /1 and β /2 were expressed in *E. coli* BL21 Star (DE3) and purified using glutathione-Sepharose chromatography.¹⁸ GST-rat CaMKI α 1–293, Lys49Glu (GST-CaMKI 1–293, K49E) was produced in *E. coli* JM109 and purified.¹⁹ Recombinant rat CaM was expressed in *E. coli* BL21 (DE3) using pET-CaM (kindly provided by Dr. Nobuhiro Hayashi, Tokyo Institute of Technology, Yokohama, Japan).²⁸ GST-tagged rat CaMKK α /1 (pME-GST-rat CaMKK α /1)²⁹ and GST-rat CaMKK β /2 (pME-GST-rat CaMKK β /2)²³ expression plasmids were constructed previously. Hemagglutinin (HA)-tagged rat CaMKK β /2 (pME-HA-rat CaMKK β /2) was constructed by PCR and subcloned into EcoRV site of pME-18s-HA vector. Anti-phospho-CaMKI α (at Thr177, clone 9H8) monoclonal antibody³⁰ and anti-CaMKK β /2-3/3x³¹ antibodies were previously generated. Anti-CaMKK α /1, anti-CaMKK β /2-1 and anti-CaMKI α antibodies were produced as previously described.^{32,33} Anti-HA (12CA5) and anti-GST (27457701V) antibodies were purchased from Roche Applied Sciences (Indianapolis, IN, USA) and GE Healthcare (Buckinghamshire, UK), respectively. Lambda protein phosphatase (λ PP) was

purchased from New England Biolabs (Ipswich, MA, USA; P0753S). TIM-063 (2-hydroxy-3-nitro-7*H*-benzo[de]benzo[4,5]-imidazo[2,1-*a*]isoquinolin-7-one)²⁶ and TIM-127 (1-amino-*N*-(2-((2-hydroxy-3-nitro-7-oxo-7*H*-benzo[de]benzo[4,5]imidazo[2,1-*a*]isoquinolin-11-yl)amino)-2-oxoethyl)-3,6,9,12,15,18-hexaoxahenicosan-21-amide (major))²⁷ were synthesized as described previously. TIM-127-coupled Sepharose was prepared using NHS-activated Sepharose (GE Healthcare).²⁷

Construction of expression plasmids for CaMKK β /2 mutants

Expression plasmid of rat CaMKK β /2 Ser128/132/136Ala mutant was constructed by ligation of a DNA fragment prepared by annealing of sense (5'-GCTCCAGCCAGCGCTCCACAGTCCGCACCCC-3') and antisense (5'-GGGGTGCGGACTGTGGAGCGCTGGCTGGAGC-3') oligonucleotides into an inversed PCR fragment of either pME-HA-rat CaMKK β /2 or pME-GST-rat CaMKK β /2 using forward (5'-CGGATGCCCCGGCGGCCAC-3') and reverse (5'-GTAGGACAGGGATGGGCAGA-3') PCR primers.

Transfection of HA-CaMKI α in HeLa cells

Human cell line derived from cervical cancer (HeLa, RRID: CVCL_0030) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5 % CO₂ in mycoplasma-free conditions. HeLa cells in 6-well dishes were transfected with 0.25 μ g of HA-rat CaMKI α expression plasmids (pME-HA-CaMKI α)

including wild type and truncation mutant (1–293, Lys49Glu) using polyethylenimine “Max” (Polysciences, Inc., Warrington, PA, USA) according to the manufacturer’s protocol. After 36 h of culture, the cells were further cultured in serum-free medium for 6 h and then treated with or without 10 μ M ATP for 5 min. Cell lysates were prepared by addition of 1x SDS-PAGE buffer (200 μ L), followed by immunoblot analysis.

Transfection and purification of rat CaMKK β /2 in COS-7 cells

SV40-transformed African green monkey kidney fibroblast-like cell line (COS-7, RRID: CVCL_0224) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in 5 % CO₂ in mycoplasma-free conditions. For the purification of rat CaMKK β /2, cells in two 10-cm plates were transfected with 10 μ g of either GST-rat CaMKK β /2 (pME-GST-CaMKK β /2) or GST-rat CaMKK β /2 Ser128/132/136Ala mutant (pME-GST-CaMKK β /2 Ser128/132/136Ala) expression plasmid using polyethylenimine “MAX” according to the manufacturer’s protocol (Polysciences, Inc.). After 48 h of culture, the cells were lysed with 500 μ L of buffer A (150 mM NaCl, 50 mM Tris-HCl pH 8.0, and 0.05% Tween 20) containing a 1:100 dilution of protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The cell lysate was mixed with 100 μ L of glutathione Sepharose for 30 min at 4°C. The resin was washed three times with 1 mL of buffer A at 4°C. Subsequently, CaMKK β /2 was eluted by digestion overnight with 2 U/mL PreScission™ Protease (Cytiva, Uppsala, Sweden) in 200 μ L PreScission Protease buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and

0.05% Tween 20). For the purification of His₆-rat CaMKK α /1, cells in two 10-cm plates were transfected with 10 μ g of His₆-rat CaMKK α /1 (pME-His₆-CaMKK α /1) expression plasmid using polyethylenimine “MAX” according to the manufacturer’s protocol. After 48 h of culture, the cells were lysed with 500 μ L of buffer A containing 10 mM imidazole and a 1:100 dilution of protease inhibitor cocktail (Nacalai Tesque). The cell lysate was mixed with 100 μ L of nickel Sepharose for 30 min at 4°C. The resin was then washed three times with 1 mL of buffer A containing 20 mM imidazole at 4°C. Subsequently, His₆-CaMKK α /1 was eluted by 300 mM imidazole in 600 μ L of buffer A and the imidazole was removed using spin desalting column (Zeba™ Spin Desalting Column 40K; Thermo Fisher Scientific, Waltham, MA, USA). The purified His₆-CaMKK α /1 and CaMKK β /2 were then subjected to λ PP treatment followed by separation assay as described below.

λ PP treatment

Purified CaMKK β /2 (400 nM) or His₆-CaMKK α /1 (1.2 μ M) in 300 μ L of a solution containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM DTT, 2 mM MnCl₂ and 0.01% Brij 35 was incubated with λ PP (400 units) or without at 30°C for 30 min. These incubated samples then received 2 mM CaCl₂ and 4 μ M CaM, and separation assay was performed as described below.

Separation of Ca²⁺/CaM-dependent and autonomous activity of CaMKK

Three mouse brains or testes were homogenized with buffer B (150 mM NaCl, 50 mM Tris-HCl pH 7.5, and 0.05% Tween 20) using 3 mL for the brain samples and 5 mL for the testis,

as well as a 1:50 dilution of protease inhibitor cocktail. To separate Ca^{2+} /CaM-dependent and autonomous activity states from recombinant CaMKK isoforms or mouse tissues, purified GST-rat CaMKK isoforms (0.7 μM) in 400 μL of buffer B or 3 to 5 mL mouse tissue lysates were added to TIM-127-Sepharose (for recombinant GST-CaMKKs: 100 μL , for tissue lysates: 200 μL) in the presence of 2 mM CaCl_2 and 6 μM CaM at 4°C. Subsequently, the Sepharose was washed three times with 5 mL of buffer B in the presence of 2 mM CaCl_2 . After the Sepharose resin was extensively washed, the Ca^{2+} /CaM-dependent form of CaMKK was eluted with 2 mM EGTA in buffer B using 1/2 volume of Sepharose resin for fraction 1 and an equal volume of Sepharose resin for fractions 2 to 10. Subsequently, 400 μL of 100 μM TIM-063 in buffer B was added to dissociate the autonomous activity state of CaMKK remaining on the TIM-127-Sepharose. After concentrating each eluate (EGTA-eluted fractions 2–4 and TIM-063-eluted fractions 1–3) to 100 μL with Amicon Ultra-0.5 mL (Ultracel 30 K; Merck Millipore Ltd. Burlington, MA, USA), EGTA or TIM-063 in the eluates was removed using a Zeba™ Spin Desalting Column 40K (Thermo Fisher Scientific). These CaMKK samples were subjected to CaMKK activity assay as described below. For separation of the two activity states of CaMKK from cultured cell lysates, 25 μL of TIM-127-Sepharose was added to 100 μL of 400 nM purified CaMKK β /2 or 1.2 μM His₆-CaMKK α /1 incubated with or without λPP or COS-7 cell lysates as described above in the presence of 2 mM CaCl_2 and 1 μM CaM. This was followed by incubation for 30 min at 4°C. Subsequently, the Sepharose was extensively washed three times with 400 μL aliquots of buffer B in the presence of 2 mM CaCl_2 . After the Sepharose resin was extensively washed, 150 μL of 2 mM EGTA in buffer B was added to the resin to elute the Ca^{2+} /CaM-dependent

activity form of CaMKK. After washing the Sepharose with 400 μ L of 2 mM EGTA in buffer B, 100 μ L of 100 μ M TIM-063 in buffer B was added to dissociate the autonomous activity state of CaMKK remaining on the TIM-127-Sepharose. Each eluate (11.25 μ L of EGTA eluate and 7.5 μ L of TIM-063 eluate) was subjected to immunoblot analyses using either anti-CaMKK β /2-1, anti-CaMKK α /1, or anti-HA antibody.

In vitro CaMKK activity assay

CaMKK activity was measured at 30°C for 10 min in a solution (20 μ L) containing 20 ng CaMKK α /1 or β /2, 50 mM HEPES pH 7.5, 10 mM Mg(CH₃COO)₂, 1 mM DTT, 100 μ M ATP, and 10 μ g GST-CaMKI α 1–293, K49E in the presence of 2 mM CaCl₂/6 μ M CaM. Each reaction was initiated by the addition of ATP. The reactions were terminated by spotting 3 μ L of samples onto nitrocellulose paper (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, the phosphorylation of CaMKI α at Thr177 on the blotted membrane was detected using anti-phospho-CaMKI α (at Thr177) antibody and quantified using ImageJ software (NIH, Bethesda, MD, USA) as previously described.³⁴

Other methods

Dot blot and immunoblot analyses were performed with the indicated primary antibodies and secondary horseradish peroxidase-conjugated anti-mouse IgG (NA931V, GE Healthcare), anti-rabbit IgG (NA934V, GE Healthcare) or anti-goat IgG (6420-05, SouthernBiotech, Birmingham, AL, USA). CaM-overlay was performed using biotinylated CaM in the presence of 2 mM CaCl₂ as previously described.³⁵ A chemiluminescent reagent

(PerkinElmer Life Sciences, Waltham, MA, USA) was used for signal detection of dot blotting, immunoblotting, and CaM-overlay. The protein concentration of the samples was estimated using Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc.) with bovine serum albumin as the standard.

RESULTS

Autonomous and Ca^{2+} -dependent CaMKK activities in HeLa cells

A previous in vitro kinase assay demonstrated that rat brain extracts contained CaMKI α -phosphorylating activity (at Thr177) in both Ca^{2+} /CaM-independent (autonomous activity) and -dependent manners.¹⁸ Based on these findings, we first attempted to detect both CaMKK activities in HeLa cells using exogenously expressed HA-tagged rat CaMKI α as a CaMKK substrate (Figure 1). Phosphorylation of HA-CaMKI α wild type was not detected in resting HeLa cells. However, stimulation by 10 μM ATP markedly induced phosphorylation of HA-CaMKI α at Thr177, due to catalyzed by endogenous CaMKK activated with ATP-sensitive P2 receptor-mediated Ca^{2+} -mobilization (Figure 1).^{36–38} Notably, depletion of extracellular Ca^{2+} completely blocked the ATP-stimulated CaMKI α phosphorylation (data not shown). The use of HA-CaMKI α 1–293, K49E (a kinase dead mutant lacking C-terminal autoinhibitory and CaM-binding domains) as a CaMKK substrate resulted in significant Thr177 phosphorylation of the CaMKI α mutant in untreated cells, which was further induced (at 2.4-fold) by ATP-stimulation (Figure 1). These results indicate that endogenous CaMKK in HeLa cells exhibits both autonomous and Ca^{2+} -dependent activities, consistent with a

previous result.¹⁸ We could not detect the Thr177 phosphorylation of HA-CaMKI α wild type in unstimulated HeLa cells because CaMKI α maintains a closed conformation in the absence of Ca²⁺/CaM, which prevents Thr177 phosphorylation even by the Ca²⁺/CaM-independent form of CaMKK, in good agreement with a previous result.³⁹

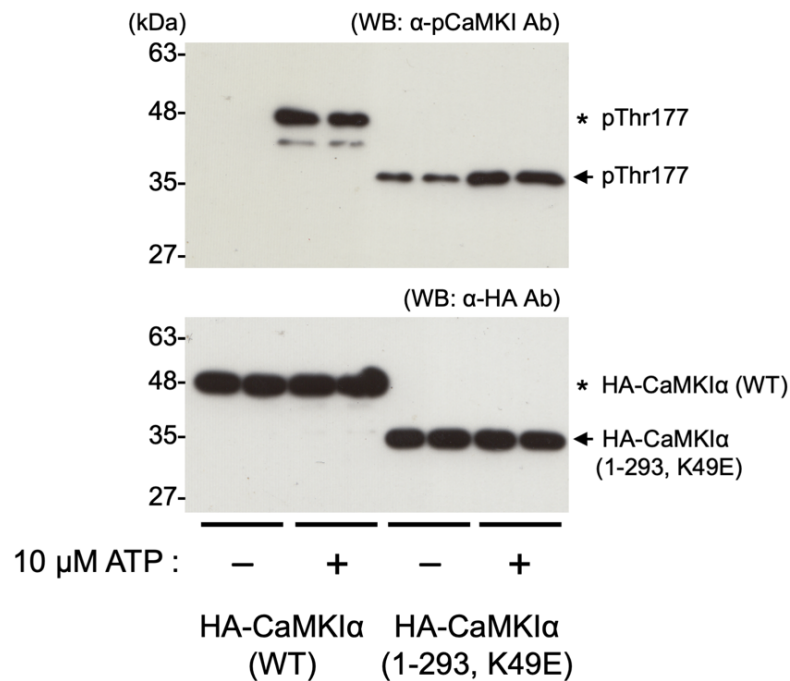


Figure 1. Autonomous and Ca²⁺-dependent CaMKK activities in HeLa cells.

HeLa cells were transfected with an expression vector for either HA-rat CaMKI α wild type (WT) or residues 1–293, Lys49Glu (1–293, K49E) and stimulated with 10 μ M ATP for 5 min after 6 h serum depletion as described in “**MATERIALS and METHODS**”. The cells were then lysed with 1x SDS-PAGE sample buffer, followed by western blot (WB) analysis with either anti-pCaMKI at Thr177 (*upper panel*) or anti-HA tag (*lower panel*) antibody. The results are from duplicate experiments. The left lanes in both panels show the molecular mass markers. Asterisks and arrows indicate HA-rat CaMKI α WT and HA-rat CaMKI α 1–293, K49E, respectively. Similar results were obtained in at least three independent experiments.

Separation of autonomous and Ca²⁺/CaM-dependent activities of CaMKK by TIM-063 coupled Sepharose chromatography

Unlike CaMKK α /1¹⁹, recombinant CaMKK β /2 expressed in *Escherichia coli* or mammalian cells exhibits Ca²⁺/CaM-independent (autonomous) activity in vitro.^{18,20} CaMKK β /2 requires Ca²⁺-mobilization for phosphorylation and activation of downstream protein kinases, including AMPK in cells.⁹⁻¹¹ These observations suggest that CaMKK β /2 possesses both autonomous and Ca²⁺/CaM-dependent activities that might be transitioned in cells. However, the existence of both CaMKK β /2 activity states has not been proven directly in vivo because of the difficulty in separating both CaMKK β /2 activity states from organ or tissue samples. Therefore, we attempted to separate autonomous and Ca²⁺/CaM-dependent forms of CaMKK β /2 using the recently developed pan-CaMKK inhibitor, TIM-063-coupled Sepharose with a spacer arm (TIM-127-Sepharose). Based on the previous demonstrations that the catalytic domain of CaMKK α binds to TIM-127-Sepharose in a Ca²⁺/CaM-dependent manner and dissociates with the addition of either TIM-063 in a competitive manner or the Ca²⁺ chelating reagent EGTA, which converts to the enzyme an autoinhibited form,²⁷ we attempted the inhibitor-based chromatographic separation of the autonomous and Ca²⁺/CaM-dependent states of CaMKK. Specifically, recombinant CaMKKs bound to TIM-127-Sepharose in the presence of Ca²⁺/CaM were eluted by EGTA to obtain the Ca²⁺/CaM-dependent form, followed by elution with TIM-063 to obtain the remaining CaMKK bound to TIM-127 that could be the Ca²⁺/CaM-independent form (Figure 2A).

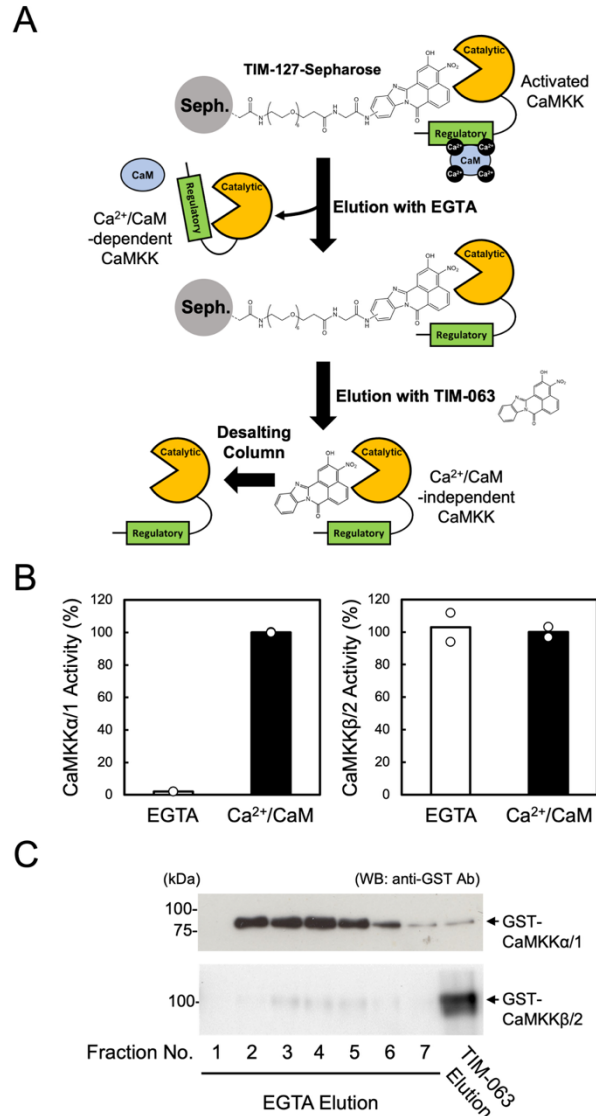


Figure 2. Separation of autonomous and Ca²⁺/CaM-dependent forms of CaMKK by TIM-127-Sepharose chromatography.

(A) Protocol for separation of autonomous and Ca²⁺/CaM-dependent forms of CaMKK using TIM-127-Sepharose chromatography. CaMKK was applied to TIM-127-Sepharose, and after extensive washing of resin, Ca²⁺/CaM-dependent form of CaMKK was eluted by the addition of 2 mM EGTA containing buffer. Subsequently, the remaining Ca²⁺/CaM-independent form of CaMKK bound to TIM-127 was eluted with 100 μM TIM-063. Catalytic, catalytic domain; Regulatory, regulatory domain; CaM, calmodulin; Seph., Sepharose. (B) Protein kinase activities of recombinant CaMKKα/1 (*left panel*) and CaMKKβ/2 (*right panel*) were measured at 30°C for 5 min in a solution containing 50 mM HEPES pH 7.5, 10 mM Mg(CH₃COO)₂, 1 mM dithiothreitol, 10 μg GST-CaMKIα 1–293 K49E, 100 μM ATP containing 2 mM

CaCl₂, and 6 μ M CaM (*black columns*) or 2 mM EGTA (*white columns*). CaMKK activities were quantified and expressed as a percentage of the average value in the presence of Ca²⁺/CaM. The results represent duplicate experiments. (C) Recombinant GST-CaMKK α /1 or GST-CaMKK β /2 (0.7 μ M) was subjected to TIM-127-Sepharose chromatography as described in panel A to separate into Ca²⁺/CaM-dependent (EGTA Elution) and autonomous (TIM-063 Elution) forms of CaMKKs as described in “**MATERIALS and METHODS**”. Eluted fractions of GST-CaMKK α /1 (*upper panel*) and GST-CaMKK β /2 (*lower panel*) from TIM-127-Sepharose were subjected to western blot (WB) analysis with the anti-GST antibody. Molecular mass markers (kDa) in each panel are indicated in the left lanes. The data are representative of at least two independent experiments.

First, we measured the activity of GST-fused rat CaMKK isoforms expressed in *E. coli* in the absence or presence of Ca²⁺/CaM. Contrary to the CaMKK α /1 activity that was completely dependent on Ca²⁺/CaM (Figure 2B, *left panel*), CaMKK β /2 was highly constitutively active (Figure 2B, *right panel*), consistent with previous reports.^{18,20} In good agreement with the CaMKK activities as shown in Figure 2B, CaMKK α /1 was eluted by EGTA from TIM-127-Sepharose without remaining enzyme bound to TIM-127 (Figure 2C, *upper panel*). Conversely, CaMKK β /2 was eluted by TIM-063 containing buffer with a trace amount of the enzyme eluted with EGTA (Figure 2C, *lower panel*). Collectively, these results clearly indicate that the TIM-127-based sequential chromatographic method can efficiently separate the two CaMKK activities.

Separation of autonomous and Ca²⁺/CaM-dependent activities of CaMKK from mouse tissues

To prove that autonomous and Ca²⁺/CaM-dependent activities of CaMKK exist in vivo, we performed TIM-127-based chromatography to separate both activities from mouse brain and

testis (Figure 3). Mouse brain extracts were applied to TIM-127-Sepharose in the presence of 2 mM CaCl_2 and 1 μM recombinant CaM. After extensive washing of the TIM-127-Sepharose with 2 mM CaCl_2 -containing buffer, elution of CaMKK was achieved using EGTA containing buffer followed by TIM-063. Each of eluted fractions was analyzed by immunoblotting using either anti-CaMKK β /2-1 (Figure 3A, *upper panel*), anti-CaMKK β /2-3/3x (Figure 3A, *middle panel*) or anti-CaMKK α /1 (Figure 3A, *bottom panel*) antibody, since CaMKK α /1 and CaMKK β /2 including two active splice variants (CaMKK β /2-1 and CaMKK β /2-3) and a low activity variant (CaMKK β /2-3x) are expressed in mouse tissues.³¹ *CaMKK β /2-1* transcript is an originally identified longest isoform of CaMKK β /2 which consists of 17 exons, and C-terminal splice variants *CaMKK β /2-3* (lacking exon 16) and *CaMKK β /2-3x* (lacking exons 14/16) are transcribed in mice.³¹ Both CaMKK β /2-1 and CaMKK β /2-3/3x were readily detected in both EGTA-eluted and TIM-063-eluted fractions with comparable amounts. CaMKK α /1 was detected only in EGTA-eluted fractions. These results suggest that CaMKK β /2-1 and CaMKK β /2-3/3x have two activity states: Ca^{2+} /CaM-dependent and Ca^{2+} /CaM-independent, and CaMKK α /1 is a Ca^{2+} /CaM-dependent form. To confirm the Ca^{2+} /CaM-dependency of CaMKK activities in both fractions, after removing EGTA and TIM-063 in the fractions (EGTA-eluted fractions 2–4 and TIM-063-eluted fractions 1–3) using spin desalting column, we measured the CaMKK activity using GST-CaMKI α 1–293, K49E as a substrate and phospho-CaMKI α (at Thr177) antibody for detection of substrate phosphorylation.

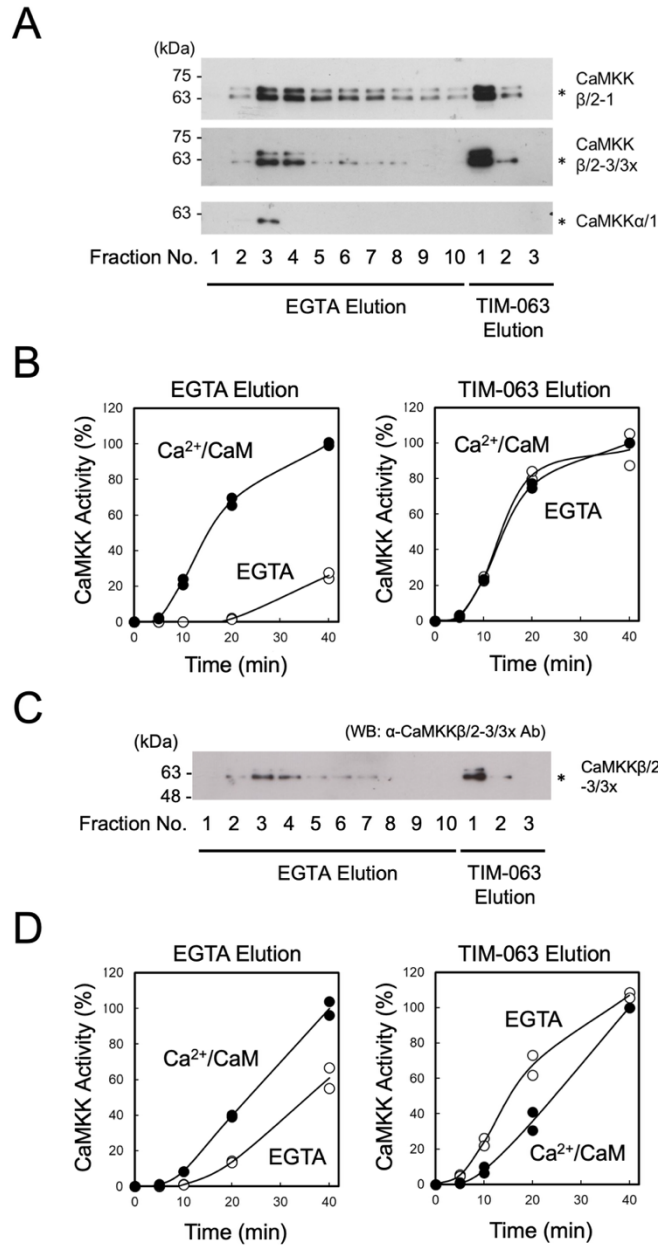


Figure 3. Separation of autonomous and Ca²⁺/CaM-dependent forms of CaMKK from mouse tissues.

Tissue extracts from mouse brain (A, B) or testis (C, D) were subjected to TIM-127-Sepharose chromatography as shown in Figure 2A and detailed in “**MATERIALS and METHODS**” to partially purify the Ca²⁺/CaM-dependent and autonomous activity states of CaMKK. Subsequently, EGTA-eluted and TIM-063-eluted fractions were subjected to immunoblot analysis using anti-CaMKKβ/2-1 (A, *upper panel*), anti-CaMKKβ/2-3/3x (A, *middle panel* and C) or CaMKKα/1 antibody (A, *lower panel*).

Molecular mass markers (kDa) in each panel are indicated in the left lanes. The data are representative of at least two independent experiments. After removing EGTA and TIM-063 in the fractions by using spin desalting column, CaMKK activities in the EGTA-eluted fraction (Fractions 2–4, B and D, *left panels*) or subsequent TIM-063-eluted fraction (Fractions 1–3, B and D, *right panels*) from mouse brain (B) or testis (D) were measured at 30°C for 5–40 min in a solution containing 50 mM HEPES pH 7.5, 10 mM $\text{Mg}(\text{CH}_3\text{COO})_2$, 1 mM dithiothreitol, 10 μg GST-CaMKI 1–293, K49E, 100 μM ATP containing 2 mM CaCl_2 , and 6 μM CaM (*closed circles*) or 2 mM EGTA (*open circles*). CaMKK activities were quantified and expressed as a percentage of the average value in the presence of Ca^{2+} /CaM at 40 min. The results represent duplicate experiments.

As shown in Figure 3B, CaMKK activity in the fractions eluted by EGTA exhibited highly Ca^{2+} /CaM-dependent activity and the TIM-063 eluted fraction featured CaMKK activity that was completely Ca^{2+} /CaM-independent. Notably, CaMKK activity in the TIM-063 eluted fraction was completely inhibited by TIM-063 (Supporting Information; Figure S1). These results clearly indicate that mouse brain contains both autonomous and Ca^{2+} /CaM-dependent activity states of CaMKK, which can be separated. The autonomous CaMKK activity is probably due to CaMKK β /2 isoforms. We also tested the chromatographic separation of autonomous and Ca^{2+} /CaM-dependent activities of CaMKK from mouse testis extracts. Similar to the results using mouse brain extracts (Figure 3A and B), CaMKK β /2-3/3x highly abundant in testis,³¹ was detected in both EGTA-eluted and TIM-063-eluted fractions (Figure 3C). The CaMKK activities of the EGTA-eluted and TIM-063-eluted fractions were Ca^{2+} /CaM-dependent and Ca^{2+} /CaM-independent, respectively (Figure 3D). Taken together, the findings provide the first direct evidence of autonomous and Ca^{2+} /CaM-dependent activity states of CaMKK in mouse brain and testis, which could be sequentially separated by CaMKK inhibitor-coupled Sepharose (TIM-127-Sepharose) chromatography. These

results also indicate that the Ca^{2+} /CaM-independent (autonomous) CaMKK activity was due to CaMKK β /2 isoforms, but not CaMKK α /1.

Dephosphorylation of Ser residues on the N-terminal regulatory domain converts CaMKK β /2 activity from CaM-dependent to CaM-independent without altering its CaM-binding ability

Phosphorylation of Ser129, Ser133, and Ser137 in the N-terminal regulatory domain of human CaMKK β /2 (rat CaMKK β /2 Ser128, Ser132, Ser136) by CDK5 and GSK3 reduces autonomous activity.²¹ However, the extent to which phosphorylation contributes to regulating autonomous activity of CaMKK β /2 is unclear. Therefore, we investigated the contribution of phosphorylation to the regulation of autonomous activity by lambda protein phosphatase (λ PP) catalyzed dephosphorylation. Purified CaMKK β /2 from transfected COS-7 cells were treated with λ PP and subsequently separated by TIM-127-based chromatography. As shown in Figure 4A, incubation with λ PP resulted in a reduction of Ca^{2+} /CaM-dependent activity of CaMKK β /2. Importantly, the triple alanine (Ala) mutant of Ser128, Ser132, and Ser136, which mimics the dephosphorylated state of CaMKK β /2, reduced Ca^{2+} /CaM-dependent activity, and was unaffected by λ PP treatment (Figure 4B). Contrary to CaMKK β /2, CaMKK α /1 lacking an N-terminal regulatory domain was detected in only the Ca^{2+} /CaM-dependent fraction (EGTA-eluted fraction), regardless of λ PP treatment (Figure 4C).

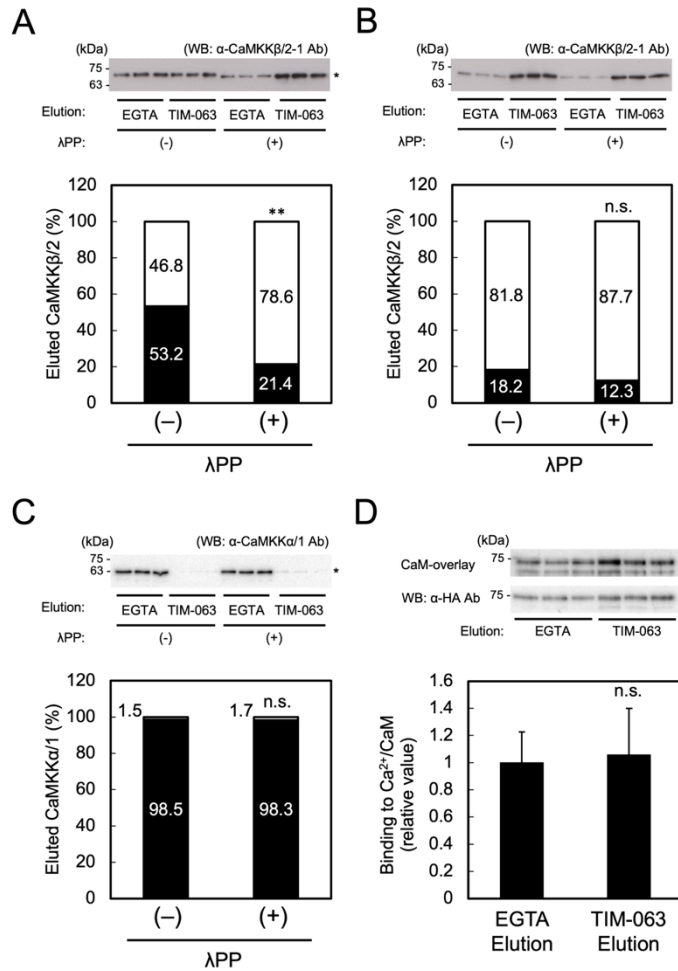


Figure 4. Lambda protein phosphatase (λ PP) treatment converts Ca^{2+} /CaM-dependent form to the autonomous form of CaMKK $\beta/2$.

Purified CaMKK $\beta/2$ WT (A), CaMKK $\beta/2$ Ser128/132/136Ala (B), or His₆-CaMKK $\alpha/1$ (C) from transfected COS-7 lysate were treated with 400 units of λ PP at 30°C for 30 min (+) or were not treated (-), followed by subjecting to TIM-127-Sepharose chromatography to separate two Ca^{2+} /CaM-dependent (EGTA Elution) and autonomous (TIM-063 Elution) states of CaMKKs as described in “**MATERIALS and METHODS**”. EGTA and TIM-063 eluted fractions were subjected to western blot (WB) analysis with either anti-CaMKK $\beta/2$ -1 (A and B, *upper panels*) or anti-CaMKK $\alpha/1$ (C, *upper panel*) antibody. Molecular mass markers (kDa) in each panel are indicated in the left lanes. Ca^{2+} /CaM-dependent (EGTA-elution, *black column*) and autonomous state (TIM-063-elution, *white column*) of CaMKK were quantified from immunoblot analyses (A, B and C, *upper panels*) and are expressed as a percentage relative to the total amount of CaMKK. The numbers inserted in the columns show the percentages. The results are from

triplicate experiments. Statistical differences are marked: **, $p < 0.005$, n.s., not significant. (D) HA-CaMKK β /2 in transfected COS-7 lysate was separated using TIM-127-Sepharose chromatography, followed by CaM-overlay analysis in the presence of 2 mM CaCl_2 (*upper panel*) and immunoblot analysis using anti-HA-tag antibody (*lower panel*). CaM-binding of EGTA-eluted (EGTA Elution) and TIM-063 eluted (TIM-063 Elution) was quantified by CaM-binding signal on CaM-overlay plot/WB signal for anti-HA antibody as a relative value. The molecular mass markers (kDa) in each panel are shown in the left lane. The data are representative of at least two independent experiments.

These results indicate that phosphorylation of these Ser residues on N-terminal regulatory domain of CaMKK β /2 is required for acquisition of Ca^{2+} /CaM-dependent activity, in good agreement with a previous report.²¹ To test whether phosphorylation of these Ser residues (Ser128, 132, 136) affects CaM-binding ability, we performed CaM-overlay analysis of two forms of CaMKK β /2 separated by TIM-127-based chromatography. As shown in Figure 4D, autonomous and Ca^{2+} /CaM-dependent forms of CaMKK β /2 exhibited comparable CaM-binding. This result shows that dephosphorylation of Ser residues on the N-terminal regulatory domain converts CaMKK β activity from CaM-dependent to CaM-independent, which is not due to altered CaM-binding ability.

DISCUSSION

In this study, we successfully separated autonomous and Ca^{2+} /CaM-dependent activities of CaMKK isoforms from mouse brain and testis, especially CaMKK β /2, using inhibitor-coupled Sepharose chromatography. A previous study revealed significant CaMKK activity (CaMKI-phosphorylating activity) in the absence of Ca^{2+} /CaM and Ca^{2+} /CaM-induced CaMKK activity in rat brain extracts. Presently, we detected both CaMKK activities in HeLa

cells (Figure 1). The findings resolve the long-standing inability to biochemically separate the activities. Based on our chromatographic separation method, CaMKK α /1 likely exhibits Ca²⁺/CaM-dependent form, in good agreement with a previous result.¹⁹ CaMKs, such as myosin light chain kinase,⁴⁰ calmodulin-dependent protein kinase I (CaMKI),⁴¹ and death-associated protein kinase,⁴² exhibit only Ca²⁺/CaM-dependent activity, with no autonomous activity. However, CaMKII converts itself to an autonomous state from the Ca²⁺/CaM-dependent state by Ca²⁺/CaM-dependent autophosphorylation of Thr286 in the autoinhibitory domain.^{43–47} CaMKIV also exhibits significant autonomous activity by phosphorylation of activation loop Thr residue (Thr196) by CaMKK.^{6,30} The transition of CaMKII and CaMKIV from the Ca²⁺/CaM-dependent state to an autonomous state is mediated by phosphorylation and is due to suppression of the autoinhibitory mechanism. Conversely, according to our results showing that λ PP treatment generates autonomous activity of CaMKK β /2, the autonomous state of CaMKK β /2 is likely converted to Ca²⁺/CaM-dependent state by phosphorylation at the N-terminal regulatory domain. This is in a good agreement with a previous report indicating that phosphorylation of N-terminal Ser residues (Ser129, Ser133, and Ser137 in human CaMKK β /2) by CDK5/GSK3 is decreased in the autonomous activity of CaMKK β /2.²¹ As an autonomous and Ca²⁺/CaM-dependent forms of CaMKK β /2 exist independently in vivo, both activities are likely dynamically regulated by phosphorylation/dephosphorylation (Figure 5). An artificial CaMKK substrate (GST-CaMKI α 1–293, K49E) is basally phosphorylated in resting transfected HeLa cells and further phosphorylated by increasing concentration of intracellular Ca²⁺ (Figure 1), probably due to autonomous CaMKK β /2 and Ca²⁺/CaM-dependent CaMKK isoforms, respectively.

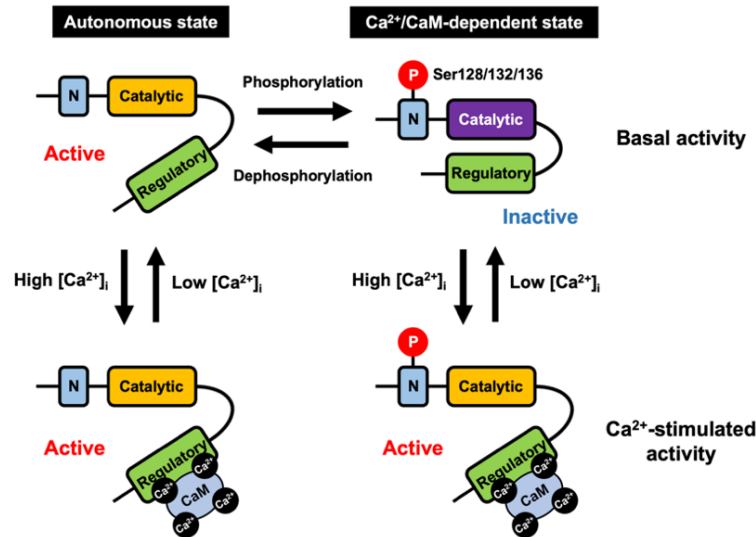


Figure 5. Schematic of transition between autonomous and Ca^{2+} /CaM-dependent activities of CaMKK β /2.

The autonomous state of CaMKK β /2 exists in mouse tissues and may be involved in basal phosphorylation of target kinases (Basal activity). Phosphorylation at Ser128/132/136 in the N-terminal regulatory domain converts CaMKK β /2 activity from Ca^{2+} /CaM-independent (autonomous) to Ca^{2+} /CaM-dependent without altering its Ca^{2+} /CaM-binding ability. Increasing concentration of intracellular Ca^{2+} further stimulates CaMKK-mediated signaling through Ca^{2+} /CaM-dependent CaMKK β /2 activity (Ca^{2+} -stimulated activity). N, N-terminal regulatory domain; Catalytic, catalytic domain; Regulatory, regulatory domain containing the autoinhibitory domain and the Ca^{2+} /CaM-binding region. P in a red circle indicates phosphorylation.

However, whether the activity transition of CaMKK β /2 is reversible in vivo and, if so, which protein phosphatase is involved, remains unclear. When we stimulated the CaMKK β /2-transfected cells with 10 μM ATP for 5 min to induce an intracellular Ca^{2+} concentration, we did not observe dynamic changes in the two activity states (autonomous and Ca^{2+} /CaM-dependent) using our developed chromatographic separation method (data not shown). However, our method of separating the autonomous and Ca^{2+} /CaM-dependent forms of

CaMKK may be useful for detecting the dynamic transition between the two activity states of CaMKK β /2 and for quantifying the CaMKK isoforms in those states in cells.

CONCLUSIONS

Our studies also raise a possibility that the autonomous activity of CaMKK β /2 may be required for target effector kinases, such as AMPK and PKB, to keep them basally phosphorylated and activated to some extent in resting cells. This could be relevant to previous reports showing that AMPK is weakly phosphorylated in unstimulated HeLa cells, which lack the liver kinase B1 (LKB1) AMPK activating kinase.^{9,11} Furthermore, accumulated evidence has demonstrated that CaMKK β /2 is highly expressed in certain cancer cells including prostate¹⁴ and hepatic⁴⁸ cancers, and is involved in cancer cell growth. In these cases, the autonomous activity of CaMKK β /2 is likely significantly elevated. Thus, the autonomous activity of CaMKK β /2 may play a role in the fine-tuning of the CaMKK-mediated kinase cascade in resting cells, as well as in pathophysiological processes. Further investigations are required to test these hypotheses and to elucidate how the activity states of CaMKK β /2 are modulated under both physiological and pathophysiological conditions.

The abbreviations: CaMKK β /2, calcium/calmodulin-dependent protein kinase kinase β /2; CaM, calmodulin; CaMK, calcium/calmodulin-dependent protein kinase; AMPK, 5'AMP-activated protein kinase; PKB, protein kinase B; λ PP, lambda protein phosphatase; HA, hemagglutinin; GST, glutathione-S-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; cDNA, complementary DNA; PMSF,

phenylmethylsulfonyl fluoride; EGTA, Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol; IgG, immunoglobulin G; TIM-063, 2-hydroxy-3-nitro-7*H*-benzo[de]benzo[4,5]-imidazo[2,1-*a*]isoquinolin-7-one; TIM-127, 1-amino-N-(2-((2-hydroxy-3-nitro-7-oxo-7*H*-benzo[de]benzo[4,5]imidazo[2,1-*a*]isoquinolin-11-yl)amino)-2-oxoethyl)-3,6,9,12,15,18-hexaoxahenicosan-21-amide (major)

Accession codes

Rattus norvegicus CaMKK α : UniProtKB - P97756 (KKCC1_RAT)

Rattus norvegicus CaMKK β : UniProtKB - O88831 (KKCC2_RAT)

Rattus norvegicus calmodulin : UniProtKB - P0DP29 (CALM1_RAT)

Rattus norvegicus CaMKI α : UniProtKB - Q63450 (KCC1A_RAT)

Author contributions: **Satomi Ohtsuka:** Investigation, Data curation, Funding acquisition, and Writing-Original Draft, **Yerun Chen:** Visualization, Investigation, Data curation, **Masaki Magari:** Supervision and Writing-Reviewing and Editing, **Teruhiko Ishikawa:** Synthesis of TIM-063 and TIM-127, **Hiroyuki Sakagami:** Resources, Writing-Reviewing and Editing. **Futoshi Suizu:** Supervision, Funding Acquisition, Writing-Reviewing and Editing, **Hiroshi Tokumitsu:** Conceptualization, Data curation, Writing-Original Draft, Funding Acquisition, Writing-Reviewing and Editing.

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Conflicts of Interest: The authors declare that they have no conflict of interest with the contents of this article.

Data Availability Statement: The datasets analyzed in this study are available from the corresponding authors upon reasonable request.

Supporting Information: Figure S1 (Inhibition of CaMKK activity in the TIM-063-eluted fraction of TIM-127 Sepharose chromatography from mouse brain extracts by TIM-063).

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