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Analysis of Distinct Roles of CaMKK Isoforms Using STO-609-Resistant Mutants in Living Cells

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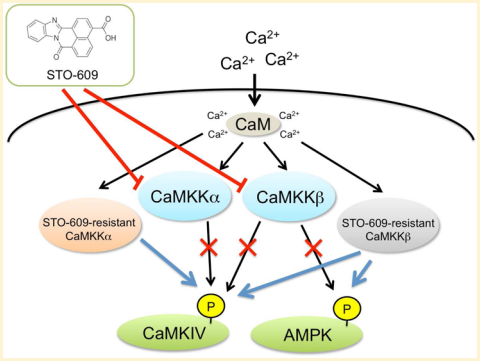
Supporting Information

ABSTRACT: To assess the isoform specificity of the Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK)-mediated signaling pathway using a CaMKK inhibitor (STO-609) in living cells, we have established A549 cell lines expressing STO-609-resistant mutants of CaMKK isoforms. Following serial mutagenesis studies, we have succeeded in obtaining an STO-609-resistant CaMKKα mutant (Ala292Thr/Leu233Phe) and a CaMKKβ mutant (Ala328Thr/Val269Phe), which showed sensitivity to STO-609 that was 2−3 orders of magnitude lower without an appreciable effect on kinase activity or CaM requirement. These results are consistent with the results obtained for CaMKK activities in the extracts of A549 cells stably expressing the mutants of CaMKK isoforms. Ionomycin-induced 5′-AMP-activated protein kinase (AMPK) phosphorylation at Thr172 in A549 cells expressing either the wild-type or the STO-609-resistant mutant of CaMKKα was completely suppressed by STO-609 treatment but resistant to the inhibitor in the presence of the CaMKKβ mutant (Ala328Thr/Val269Phe). This result strongly suggested that CaMKKβ is responsible for ionomycin-induced AMPK activation, which supported previous reports. In contrast, ionomycin-induced CaMKIV phosphorylation at Thr196 was resistant to STO-609 treatment in A549 cells expressing STO-609-resistant mutants of both CaMKK isoforms, indicating that both CaMKK isoforms are capable of phosphorylating and activating CaMKIV in living cells. Considering these results together, STO-609-resistant CaMKK mutants developed in this study may be useful for distinguishing CaMKK isoform-mediated signaling pathways in combination with the use of an inhibitor compound.



Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) is a member of the CaMK family that is composed of α (505 amino acid residues) and β (587 amino acid residues) isoforms.[1,2] CaMKKα was originally identified and cloned from rat brain as an activating protein kinase for CaMKIV, also proving to be a CaMKI activator,[3−5] and another β isoform was later cloned.[6,7] These protein kinases were accordingly named CaMKKs. CaMKK has been found in lower eukaryotes such as *Caenorhabditis elegans*[8,9] and *Aspergillus nidulans*[10] as well as in higher eukaryotes.

Unlike multifunctional CaMKs, including CaMKI, -II, and -IV, the α and β isoforms of CaMKK have relatively narrow substrate specificity. The kinase can phosphorylate only the activation loop Thr residue of particularly downstream protein kinases, including CaMKI and -IV, protein kinase B/Akt (PKB), 5′-AMP-activated protein kinase (AMPK), and SAD kinase, resulting in induction of their kinase activities.[11−14] This strict substrate specificity is probably due to a unique Arg-Pro-rich insertion domain (RP domain) between subdomains II and III in the catalytic region of CaMKK, because a CaMKKα mutant lacking the RP domain has been shown to impair their ability to phosphorylate and activate CaMKI and -IV without a marked loss of kinase activity toward CaMKIV peptide substrate and autophosphorylation.[8] Multiple target protein kinases, including CaMKI, CaMKIV, PKB, and members of the AMPK family, have been shown to be regulated by CaMKK phosphorylation, suggesting that CaMKK is involved in the regulation of a wide variety of Ca^{2+}-dependent physiological functions through the activation of these downstream protein kinases. Thus, manipulating CaMKK activity in living cells by either gene silencing or pharmacological treatment could yield important information about the signal transduction system mediated by these protein kinase cascades.[15−17]

In 2002, we developed a selective inhibitor (STO-609) of CaMKK that was an ATP-competitive and cell-permeable compound.[18] Since then, STO-609 has frequently been used for investigating the physiological significance of the protein kinase cascade mediated by CaMKK. The application of STO-609 to cultured neurons revealed the regulation of axonal extension and growth cone motility by the CaMKK-mediated CaMKI

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activation pathway,[19] leptin-induced spine formation mediated by a CaMKK/CaMKIγ cascade,[20] the involvement of Ca^{2+}-dependent CaMKK in activity-enhanced dendritic development,[21] and CaMKK-mediated cortical axonal growth.[22] A recent study using STO-609 treatment of hippocampal neurons indicated the requirement of CaMKK for NMDA receptor-dependent structural plasticity of dendritic spines.[23] The treatment of bone marrow-derived osteoblast precursor cells with STO-609 suppressed osteoblast differentiation, suggesting the involvement of a CaMKK-mediated pathway in this differentiation process.[24] In addition to the analysis of CaMK cascades, including CaMKK/CaMKI and CaMKK/CaMKIV, STO-609 has been used for analysis of the physiological functions of the CaMKK/AMPK pathway. In HeLa and A549 cells, mannitol, 2-deoxyglucose, ionomycin, and A23187 induced phosphorylation at Thr172 and activation of AMPK were inhibited by STO-609 treatment.[15−17] Thrombin-induced AMPK activation in human endothelial cells was suppressed by STO-609 treatment,[25] and i.c.v. infusion of STO-609 in adult mice resulted in the acute suppression of neuropeptide Y expression and food intake, possibly via suppression of AMPK activation.[26]

The cumulative evidence suggests that STO-609 could be a useful tool for investigating the CaMKK-mediated signal transduction pathway. However, Bain et al. reported that STO-609 did not show complete specificity toward CaMKK like other protein kinase inhibitors but could inhibit other protein kinases with a potency similar to that of CaMKKα, suggesting that results obtained by the use of this compound should be interpreted with caution.[27] More than 500 protein kinase genes are encoded in the human genome, indicating the practical difficulty of precisely determining the specificity of a kinase inhibitor *in vivo* as well as *in vitro*. Furthermore, STO-609 may interfere with the function of cellular proteins other than protein kinases. STO-609 itself is incapable of distinguishing the signaling pathways mediated by each CaMKK isoform. Of particular interest is the question of whether each CaMKK isoform activates different downstream protein kinase(s) or redundant activators. It is necessary to develop an experimental method to confirm the isoform-dependent effect of the inhibitor, for example, by testing whether the effects of STO-609 are no longer observed in cells that express an STO-609-resistant mutant of a CaMKK isoform. Here we describe the development of STO-609-resistant CaMKK isoforms that can be used to analyze the distinct roles of CaMKK isoforms in living cells as well as to confirm the specificity of the inhibitor.

EXPERIMENTAL PROCEDURES

Materials. CaMKKα cDNA (GenBank accession number L42810) was obtained from a rat brain cDNA library.[5] Rat CaMKKβ was cloned by reverse transcriptase polymerase chain reaction (PCR), as described previously.[28] Recombinant rat CaM was expressed in *Escherichia coli* strain BL-21(DE3) using pET-CaM (kindly provided by N. Hayashi, Tokyo Institute of Technology, Yokohama, Japan) and was purified by phenyl-sepharose column chromatography.[29] The expression vector for HA (hemagglutinin-tagged)-CaMKIV (pME-HA-CaMKIV) was constructed as previously described.[18] Rat CaMKIα 1−293, K49E (GST-CaMKI 1−293 KE) was expressed in *E. coli* JM-109 as a GST fusion protein and purified by glutathione-sepharose column chromatography.[11] STO-609 (7*H*-benzimidazo[2,1-*a*]benz[de]isoquinoline-7-one-3-carboxylic acid, MW of 314.29) was synthesized as previously described.[18] Anti-phosphoCaMKIα (at Thr177) and anti-phosphoCaMKIV (at Thr196) were produced as described previously.[30] An anti-β actin antibody (sc-47778) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-HA (12CA5) and anti-FLAG (clone M2) were obtained from Roche Applied Sciences (Indianapolis, IN) and Sigma-Aldrich (St. Louis, MO), respectively. Antibodies against the AMPK α subunit (2532) and AMPK α subunit phosphorylated at Thr172 (2535) were purchased from Cell Signaling (Danvers, MA). All other chemicals were obtained from standard commercial sources.

Construction and Expression of CaMKK Catalytic Domain Mutants by Mutagenesis. GST fused with rat CaMKKα (126−434) was constructed as described previously.[31] Random mutagenesis was performed with a Diversify PCR Random Mutagenesis Kit (Clontech Laboratories, Inc., Mountain View, CA) using GST-CaMKKα (126−434) cDNA as a template with a sense primer (5′-TCTGTTCCAGGGG-CCCATTCTAGAG-3′) and an antisense primer (5′-ATTAA-GCTTGAGCTCGAGTCGACTA-3′), followed by ligation into an *Xba*I/*Sal*I site in a pGEX-PreS vector (see the figure in the Supporting Information). The ligated plasmids were then introduced into *E. coli* JM-109. *E. coli* harboring pGEX-PreS-CaMKKα (126−434) mutants, including the wild-type, were cultured at 37 °C until the OD_{600} reached 0.6. The cells were then cultured for a further 5 h, after 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) had been added, to induce the expression of recombinant enzymes. Site-directed mutagenesis of pGEX-PreS-CaMKKα-(126−434), pET-CaMKKα, pcDNA3-FLAG-CaMKKα, pGEX-PreS-CaMKKβ, and pcDNA3-FLAG-CaMKKβ was performed by inverse PCR using the following primer pairs: CaMKKα K205E, 5′-CGTGGTCCAGTTCCTTTAAAATGGC-3′ and 5′-TGAAT-GTAGTCAAGTTGATCGAGGT-3′; CaMKKα A292T, 5′-CACCAAAGTCGGTGATCTTCACGTG-3′ and 5′-TCAGC-AACCAGTTTGAGGGGAATGA-3′; CaMKKα T325S, 5′-AGCTCTGGCCGGAGTCAGAAATGGC-3′ and 5′-TCAGT-GGGAAGGCCTTGGATGTATG-3′; CaMKKα D384G, 5′-TTCAGGATCAGGCCTTTGAGTTCCT-3′ and 5′-GATGC-TAGACAAGAATCCTGAAACA-3′; CaMKKα L405S, 5′-ACCCAAGGGTGTGACTTGATATCAG-3′ and 5′-GACCA-AGCATGGAGAGGAGCCCCTC-3′; CaMKKβ A328T, 5′-CACATCAAGATAACCGACTTCGGCG-3′ and 5′-CCCGT-CCTCCCCCACTAGGAGGTTG-3′.

Mutations for CaMKKα L233F and CaMKKβ V269F were created by PCR as described previously.[31] Recombinant CaMKKα and GST-CaMKKβ (wild-type and mutants) were bacterially expressed and purified.[33]

The nucleotide sequences of all constructs were confirmed by sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA)

Screening of an STO-609-Resistant Mutant of CaMKKα. After induction of expression of GST-CaMKKα (126−434) wild-type and mutants with IPTG for 5 h, *E. coli* (2 mL culture) was collected by centrifugation at 21880*g* for 5 min, followed by sonication with 500 μL of ice-cold PBS containing 0.2 mM PMSF. After centrifugation at 21880*g* for 5 min, 2.5 μL of the supernatant was incubated with 1 μg of GST-CaMKI 1−293 KE at 30 °C for 10 min in a solution (20 or 25 μL) containing 50 mM HEPES (pH 7.5), 10 mM $Mg(Ac)_2$, 1 mM dithiothreitol, 2 mM EGTA, and 50 μM ATP with indicated concentrations of STO-609 (0−10 μg/mL in Me_2SO at a final concentration of 4%). The reaction was initiated by the addition of ATP and terminated by spotting

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aliquots (2 μL) onto a nitrocellulose membrane (Hybond C, GE Healthcare UK, Ltd., Buckinghamshire, U.K.), followed by detection of phosphorylated CaMKIα at Thr177 by anti-phosphoCaMKI antibody, or terminated by the addition of 5 μL of sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer and the mixture subjected to SDS−7.5% PAGE, followed by immunoblotting using anti-phosphoCaMKI antibody.

Cell Culture and Transfection. Human lung adenocarcinoma epithelial cell line A549 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 units/mL streptomycin at 37 °C in 5% CO_2. A549 cells in six-well dishes were transfected with or without 2 μg of HA-CaMKIV expression plasmid (pME-HA-CaMKIV) using polyethleneimine "MAX" (Polysciences, Inc., Warrington, PA) according to the manufacturer's protocol. After being cultured for 20 h, the cells were cultured in the absence of FBS for 6 h and then treated with 1 μM ionomycin for 5 min. The cells were extracted with 1× SDS−PAGE sample buffer (100 μL), followed by immunoblot analysis. To measure CaMKK activity in the cell extracts, A549 cells in a 10 cm dish were lysed with 300 μL of ice-cold lysis buffer [150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1% NP-40, 10% glycerol, and 1:1000 protease inhibitor cocktail], and then the cell lysate was obtained by centrifugation at 17970*g* for 10 min.

Generation of Stable A549 Cell Lines Expressing FLAG-CaMKK Isoforms. To establish cell lines stably expressing wild-type FLAG-CaMKKβ or STO-609-resistant FLAG-CaMKKβ (A328T, V269F), each CaMKKβ gene was retrovirally transduced in A549 cells. Briefly, *Spe*I fragments containing FLAG-tagged CaMKKβ and CaMKKβ mutant genes were elicited from pcDNA3-FLAG CaMKKβ and pcDNA3-FLAG-CaMKKβ (A328T, V269F) vectors, respectively. These fragments were blunted using a Blunting Kit (Takara Bio Inc., Ohtsu, Japan) and then inserted into blunted *Eco*RI/*Bgl*II sites in a pMSCV-based retroviral vector (pMSCV-MCS-IRES-EGFP) that contains enhanced GFP cDNA as an expression marker downstream of the internal ribosomal entry site. For CaMKKα, a FLAG-CaMKKα (wild-type) fragment was digested and ligated into a pMSCV-MCS-IRES-EGFP. The FLAG-CaMKKα mutant (A292T, L233F) was amplified by PCR using primers 5′-TCCATGTCGACGCCACCATGGAC-TACAAGGACGACG-3′ and 5′-ATAGCGGCCGCTCAGGA-TGCAGCCTCATCTT-3′ with pcDNA3-FLAG-CaMKKα (A292T, L233F) as a template. The PCR fragment was digested with *Sal*I/*Not*I and then subcloned into a pMSCV-MCS-IRES-EGFP. The retroviral transfer vectors were transformed into PT-67 packaging cells (Clontech Laboratories, Inc.) to produce recombinant retrovirus according to the manufacturer's protocol. Cells were infected with the recombinant retroviruses in the presence of 16 μg/mL Polybrene (Sigma-Aldrich). After being cultured for 1 week, GFP^+ cells were isolated by single-cell sorting with a FACS Aria cell sorter (BD Biosciences, San Jose, CA). Expression levels of FLAG-CaMKK proteins in isolated clones were determined by immunoblotting using anti-FLAG antibody.

In Vitro CaMKK Activity Assay. CaMKK activity was measured for recombinant CaMKKs (10 ng) and cell extracts (2.5−5 μg of protein) in a solution containing 50 mM HEPES (pH 7.5), 10 mM $Mg(Ac)_2$, 1 mM dithiothreitol, 4 mM $CaCl_2$, the indicated concentrations of CaM, 10 μg of GST-CaMKI 1−293 KE, and 50−100 μM ATP or [γ-^{32}P]ATP in the presence of 0.5 μM okadaic acid (for the cell extracts) with various concentrations of STO-609 (0−10 μg/mL in Me_2SO at a final concentration of 4−5%). CaMKK activity was measured by a P-81 phosphocellulose paper assay for recombinant enzymes[11] or immunoblot analysis using anti-phosphoCaMKI antibody for cell extracts, followed by quantification of substrate (GST-CaMKI 1−293 KE) phosphorylation with ImageJ.[38]

Other Methods. Immunoblot analysis was performed with the indicated primary antibodies and with horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare UK, Ltd.) as the secondary antibody. A chemiluminescence reagent (PerkinElmer, Inc., Waltham, MA) was used for signal detection. CaM overlay was performed using biotinylated CaM as previously described.[32] The protein concentration was estimated by staining with Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc., Hercules, CA) using bovine serum albumin as a standard. Student's *t* tests were used to calculate significance when two groups were compared. Probability (*p*) values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Screening of STO-609-Resistant CaMKKα Mutants. To develop STO-609-resistant mutants of CaMKKs, we introduced random mutations by PCR-based mutagenesis (~8 mutations/1 kb of DNA) into the CaMKKα catalytic domain (residues 126−434), which was then inserted into an *E. coli* expression GST fusion vector. The reason for our choice of the α isoform instead of the β isoform of CaMKK for the mutagenesis template was that CaMKKα has been shown to be less sensitive to STO-609 inhibition than the β isoform.[18,31] After construction of a mutagenized library, each CaMKKα mutant was expressed in the JM109 strain of *E. coli* (2 mL culture) using 0.5 mM IPTG induction for 5 h. The bacterial lysate was then prepared by sonication and used for measurement of CaMKK activity in the absence or presence of 10 μg/mL STO-609. CaMKK activity of the mutant in the bacterial lysate was measured by phosphorylation of GST-rat CaMKIα 1−293, K49E (GST-CaMKI 1−293 KE) at Thr177, as detected with the anti-phosphoCaMKI monoclonal antibody using a dot blot assay (Figure 1A). Figure 1A shows the result of STO-609 inhibition of CaMKK activity of 8 of 27 enzymatically active mutants (data not shown), including the wild-type enzyme. CaMKK activity was easily detected for each mutant in the absence of the inhibitor under our experimental conditions but varied depending on the mutant, and the activities of the GST-CaMKKα catalytic domain mutants, except for clone 4, as well as of the wild-type enzyme were completely inhibited in the presence of 10 μg/mL STO-609. The CaMKK activity of clone 4 was no longer sensitive to the inhibitor. We also confirmed the resistance of clone 4 to STO-609 by a CaMKK activity assay in the presence of various concentrations (0−10 μg/mL) of STO-609 (Figure 1B). In contrast to wild-type GST-CaMKKα 126−434, whose activity was inhibited by STO-609 in a dose-dependent manner, clone 4 was markedly resistant to STO-609. The nucleotide sequence of the clone 4 cDNA contained five mutations that resulted in changing amino acid residues (K205E, A292T, T325S, D384G, and L405S) in the catalytic domain of CaMKKα (Figure 1C and Figure 1 of the Supporting Information). Next, to identify the residue(s) involved in the sensitivity of the enzyme to STO-609, we produced GST-CaMKKα catalytic domain mutants carrying each individual mutation, expressed the mutant enzymes, and measured the CaMKK activity of the mutants in the absence or

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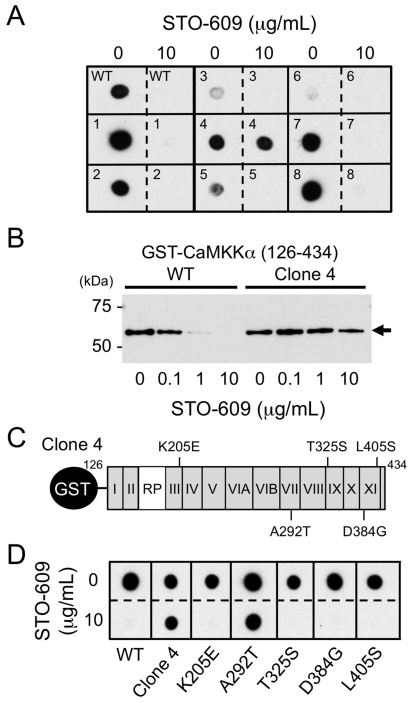

Figure 1. STO-609-resistant CaMKKα catalytic mutants generated by random mutagenesis. (A) The GST-CaMKKα catalytic domain (residues 126−434) was mutated by PCR-based random mutagenesis, and the CaMKK activity of each mutant (clones 1−8), including the wild-type (WT), was assayed in a solution containing 50 μM ATP in the absence or presence of 10 μg/mL STO-609 by phosphorylation of GST-CaMKI 1−293 K49E, followed by a dot blot assay using anti-phosphoCaMKI antibody. (B) CaMKK activities of both the wild-type (WT) and clone 4 (Clone 4) were measured in the absence or presence of various concentrations of STO-609 (0.1, 1, and 10 μg/mL) by immunoblotting using anti-phosphoCaMKI antibody. The arrow indicates phosphoGST-CaMKI 1−293 K49E. (C) Schematic representation of GST-CaMKKα 126−434 (clone 4) with five amino acid mutations (K205E, A292T, T325S, D384G, and L405S) according to the nucleotide sequence of clone 4 cDNA (see Figure 1 of the Supporting Information). The kinase subdomains (I−XI) are indicated. RP, Arg/Pro-rich insert domain. (D) The STO-609 sensitivity of GST-CaMKKα catalytic domain (residues 126−434) point mutants (K205E, A292T, T325S, D384G, and L405S), including the wild-type (WT) and clone 4 (Clone 4), was measured by a dot blot kinase assay as shown in panel A.

presence of 10 μg/mL STO-609 using the dot blot kinase assay (Figure 1D). Figure 1 D clearly shows that the A292T mutant was markedly resistant to STO-609 in a manner similar to that of clone 4 without any apparent loss of CaMKK activity and that the activities of the other four mutants, including the wild-type enzyme, were completely inhibited by 10 μg/mL STO-609. These results indicated that replacement of Ala292 in subdomain VII with Thr markedly reduced the inhibitor sensitivity without strong impairment of catalytic activity. By comparison of the amino acid sequences of rat CaMKK α and β isoforms, it was determined that Ala292 in the α isoform is conserved in the β isoform (Ala328) and is located in subdomain VII in the CaMKK catalytic domain (Figure 1C). It is noteworthy that STO-609 hydrogen bonds with the backbone of Asp330 in human CaMKKβ (Asp329 in rat CaMKKβ and Asp293 in rat CaMKKα).[39,40] This may suggest that the decreased sensitivity to STO-609 by a mutation of rat CaMKKα at Ala292 (Ala328 in rat CaMKKβ) to Thr as shown in Figure 1D is due to steric inhibition of STO-609 binding.

Characterization of STO-609-Resistant CaMKK Mutants. A previous study revealed that the replacement of Val269 in CaMKKβ with hydrophobic residues with larger side chains such as Leu, Met, His, and Phe efficiently blocked drug−enzyme interaction, resulting in reduction of the STO-609 sensitivity of CaMKKβ.[31] This report is consistent with the observation that CaMKKα carrying Leu233, which is equivalent to Val269 in CaMKKβ, is ∼10-fold less sensitive to STO-609 than CaMKKβ is. We accordingly introduced the Thr mutation at the equivalent residue (Ala328) into a GST-fused full length CaMKKβ mutant carrying another STO-609-resistant mutation (V269F) as described previously.[31] We then expressed the wild-type, the A328T mutant, and a double mutant (A328T, V269F; AT/VF) in *E. coli*, followed by purification with glutathione-sepharose and CaM-sepharose chromatographies. We also introduced equivalent mutations (A292T; AT and A292T, L233F; AT/LF) into full length CaMKKα and purified both wild-type and mutant enzymes. We then investigated the effect of STO-609 on the activities of the wild-type and the mutant CaMKK isoforms (Figure 2A). The activities of the single mutants of CaMKK isoforms (A292T in the α isoform and A328T in the β isoform) were slightly suppressed by 1 μg/mL STO-609 (12 and 34% inhibition, respectively), but the CaMKK activities remained in the presence of 10 μg/mL STO-609. The activity of the double mutant (A328T, V269F) of CaMKKβ was not inhibited by 1 μg/mL STO-609, which almost completely inhibited the activity of wild-type CaMKKβ. Even in the presence of 10 μg/mL inhibitor, more than 60% of CaMKKβ activity of the mutant remained (Figure 2A, right panel). We observed similar results with CaMKKα, indicating that the inhibitory effect of STO-609 on CaMKKα activity was ∼100-fold decreased by the double mutations [A292T, L233F (Figure 2A, left panel)]. These results indicated that these two mutations in the catalytic domain of CaMKK isoforms reduced STO-609 sensitivity synergistically. According to the 2.4 Å crystal structure of the catalytic domain of the human CaMKKβ complexed with STO-609, STO-609 hydrogen bonds with the backbones of Val270 (Val269 in rat CaMKKβ) and Asp330 (Asp329 in rat CaMKKβ) adjacent to Ala328 in rat CaMKKβ (equivalent to Ala292 in rat CaMKKα),[39] which suggests that the mutations of Val269 (Leu233 in rat CaMKKα) to Phe and Ala328 (Ala292 in rat CaMKKα) to Thr in rat CaMKKβ may directly impact the CaMKK−STO-609 interaction by steric hindrance. We next confirmed the requirement of Ca^{2+}/CaM for the activities of CaMKK mutants as compared with those of wild-type CaMKK isoforms (Figure 2B). The maximal activities of wild-type CaMKKα and its double mutant were 1.7 and 1.2 μmol min^{-1} mg^{-1}, respectively, and those of wild-type GST-CaMKKβ and its double mutant were 0.6 and 0.4 μmol min^{-1} mg^{-1}, respectively. The concentrations of CaM giving half-maximal CaMKK activity for wild-type CaMKKα and its double mutant were indistinguishable [∼2 nM (Figure 2B, left panel)], which were similar to those for wild-type CaMKKβ and its double mutant (Figure 2B, right panel), although the autonomous activity (in the absence of Ca^{2+}/CaM) of CaMKKβ enzymes is relatively high (40−50% of total activity), consistent with previous reports.[7,28,33] These results indicate that double mutations leading to STO-609 insensitivity did not markedly affect either catalytic activity or CaM dependency of either CaMKK isoform.

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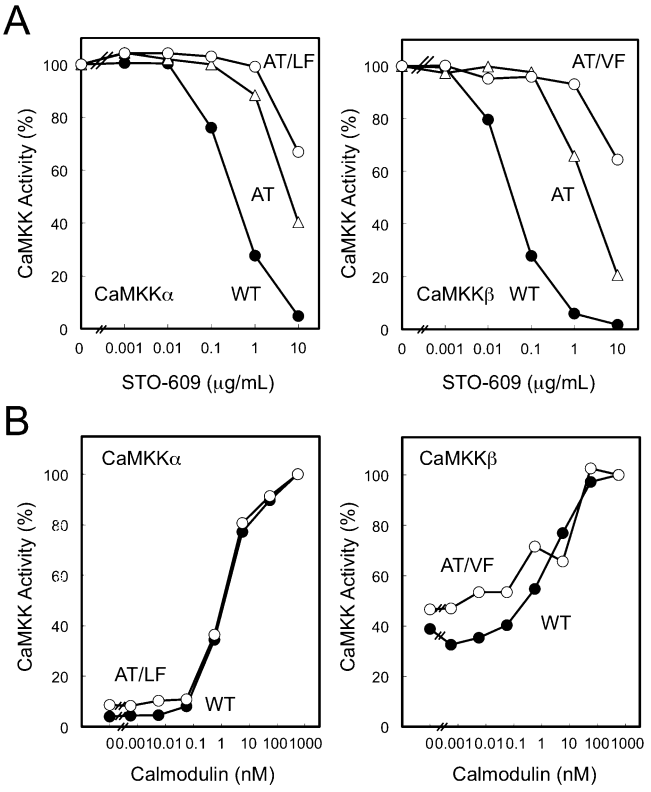

Figure 2. Characterization of STO-609-resistant mutants of CaMKKα and CaMKKβ. (A) CaMKK activities of recombinant CaMKKα mutants [A292T, AT (△); A292T, L233F, AT/LF (○, left panel)], CaMKKβ mutants [A328T, AT (△); A328T, V269F, AT/VF (○, right panel)], and each wild-type (WT, ●) enzyme were measured in the absence or presence of various concentrations of STO-609 (0.001−10 μg/mL) at 30 °C for 10 min with 50 μM [γ-^{32}P]ATP, 2 mM $CaCl_2$, and 14 μM CaM. The results are expressed as a percentage of the value in the absence of STO-609. (B) CaM requirement for the activities of the recombinant CaMKKα mutant [A292T, L233F, AT/LF (○, left panel)], CaMKKβ mutant [A328T, V269F, AT/VF (○, right panel)], and each wild-type (WT, ●) enzyme were measured in the absence or presence of various concentrations (0.56 pM to 560 nM) of CaM at 30 °C for 10 min with 2 mM $CaCl_2$. The results are expressed as a percentage of the value in the presence of 560 nM CaM. The data are representative of at least two independent experiments.

A549 Cells Expressing STO-609-Resistant CaMKK Isoforms. We next attempted to establish A549 cells (a human lung adenocarcinoma epithelial cell line) expressing the STO-609-resistant mutant of FLAG-tagged CaMKKα (A292T, L233F, AT/LF) and FLAG-tagged CaMKKβ (A328T, V269F, AT/VF) as well as the wild-type (WT) using a retroviral expression system. Expression of FLAG-CaMKK mutants in A549 cells, including wild-type CaMKK isoforms, was confirmed by immunoblot analysis using anti-FLAG antibody (Figure 3A, top panel). The CaM binding ability of FLAG-CaMKK mutants in A549 cells, including wild-type CaMKK isoforms, was confirmed by CaM overlay analysis in the presence of 1 mM $CaCl_2$ (Figure 3A, middle panel). CaMKK activity in the cell lysate of CaMKK-expressing A549 cells, including the wild-type and the double mutant of CaMKK isoforms, was measured as phosphorylating activity toward GST-CaMKI 1−293 KE as a substrate. Phosphorylation of GST-CaMKI 1−293 KE at Thr177 was monitored at various time points by immunoblot analysis using anti-phosphoThr177 antibody. Whereas endogenous CaMKK activity was very weak, relatively high CaMKK activities were observed in the cell lysate of all A549 cells stably expressing CaMKK isoforms, including the wild-type and mutants (Figure 3B). We next confirmed the sensitivity of CaMKK activity to STO-609 in the cell lysate of stable A549 cell lines (Figure 3C). CaMKK activity in the cell lysate of stable A549 cell lines expressing wild-type CaMKK isoforms was inhibited by STO-609 in a dose-dependent manner. In contrast, CaMKK activities of the A549 cell lysate of the mutant CaMKKs were resistant to STO-609 up to 10 μg/mL, whereas 10 μg/mL STO-609 almost completely inhibited the activity in the cell lysate of A549 cells expressing wild-type CaMKKs (Figure 3C). These results are consistent with the results from *E. coli*-expressed CaMKK mutants as described above (Figure 2A).

Phosphorylation of AMPK in A549 Cells Expressing STO-609-Resistant CaMKK Isoforms. To evaluate CaMKK-mediated phosphorylation of AMPK, we next treated A549 cells with ionomycin to induce the concentration of intracellular Ca^{2+} and detected the phosphorylation of the AMPK α subunit at Thr172 by immunoblot analysis using anti-phosphoAMPK antibody. Treatment of A549 cells with 1 μM ionomycin strongly induced AMPK phosphorylation, which was suppressed by STO-609 (Figure 4A). This observation is consistent with previous findings that CaMKK phosphorylates the activation loop Thr of AMPK in living cells.[15−17] Recent evidence using siRNA revealed that the CaMKKβ isoform is responsible for phosphorylation of AMPK but not the α isoform.[15,16] However, it has been shown that purified CaMKKα can phosphorylate AMPKα *in vitro*, although less effectively than CaMKKβ,[15,34] indicating that the role for CaMKKα as an AMPK kinase has yet to be conclusively established in mammalian cells. We accordingly used A549 cells expressing STO-609-resistant CaMKK isoforms to address this question. Ionomycin-induced AMPK phosphorylation was suppressed by STO-609 treatment in A549 cells expressing STO-609-resistant FLAG-CaMKKα (A292T, L233F, AT/LF) in a manner similar to those of both A549 mother cells and cells expressing wild-type CaMKK isoforms (Figure 4A,B). As compared with exogenously expressed wild-type CaMKKα, FLAG-CaMKKα (A292T, L233F, AT/LF) seems to slightly prevent STO-609 from reducing the level of AMPK phosphorylation (Figure 4A). This may be due to a slight impact of the mutations (A292T, L233F) on the affiliation between CaMKKα and AMPK because it has been shown that CaMKKβ was able to form a complex with AMPK and CaMKKα was not.[42] Alternatively, the mutations may modestly affect the substrate specificity of CaMKKα without forming a stable complex with AMPK.[43] In contrast, ionomycin-induced AMPK phosphorylation was not affected by inhibitor treatment in the cells expressing STO-609-resistant FLAG-CaMKKβ (A328T, V269F, AT/VF) (Figure 4B). A previous report showed that extracellular nucleotides such as ATP, UTP, and ADP induced phosphorylation of AMPK at Thr172 in human umbilical vein EC (HUVEC) and HeLa cells by CaMKK in response to an increasing concentration of intracellular Ca^{2+} that was blocked by STO-609 treatment.[41] Therefore, we treated A549 cells expressing STO-609-resisitant CaMKK isoforms with 100 μM ATP for 1 min in presence or absence of STO-609 (Figure 2 of the Supporting Information). An ATP treatment rapidly induced AMPK phosphorylation, which was blocked by STO-609 treatment in A549 mother cells and in A549 cells expressing STO-609-resistant FLAG-CaMKKα (A292T, L233F, AT/LF) but resistant to STO-609 treatment

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Biochemistry 2015, 54, 3969−3977

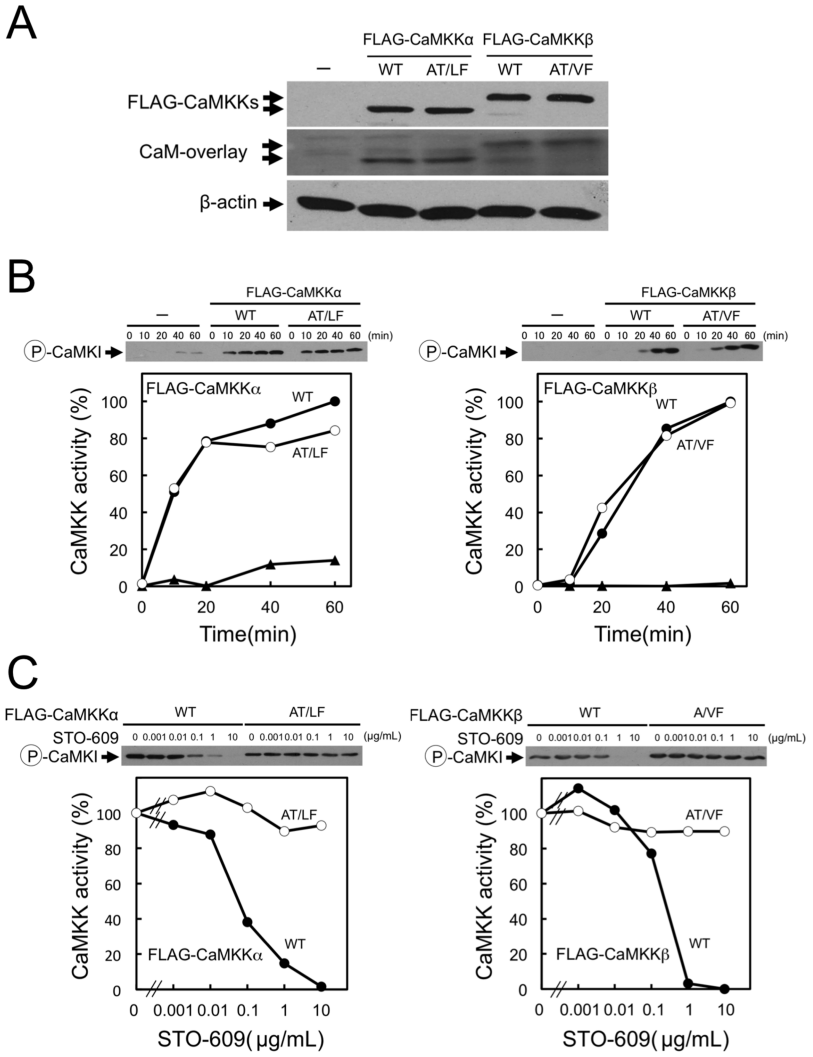

Figure 3. Characterization of STO-609-resistant mutants of CaMKK isoforms expressed in A549 cells. (A) Expression and CaM binding ability of FLAG-CaMKK*α* mutant (A292T, L233F, AT/LF), FLAG-CaMKK*β* mutant (A328T, V269F, AT/VF), and wild-type (WT) enzymes stably expressed in A549 cells were detected in the cell lysate by immunoblot analysis using anti-FLAG antibody (top) and CaM overlay (middle), respectively. Control A549 cells (−) were also analyzed. Expression of *β*-actin was confirmed as a loading control (bottom). (B) CaMKK activities in the extracts of A549 cells stably expressing FLAG-CaMKK*α* mutant [A292T, L233F, AT/LF (○, left panel)], FLAG-CaMKK*β* mutant [A328T, V269F, AT/VF (○, right panel)], and FLAG-tagged wild-type [WT (●)] enzymes, including control A549 cells (−, ▲) were measured at 30 °C at the indicated time points with 4 mM $CaCl_2$, 12 *μ*M CaM, and 100 *μ*M ATP. Phosphorylation of GST-CaMKI 1−293 K49E at Thr177 by each cell extract was detected by immunoblot analysis using anti-phosphoCaMKI antibody (inset), followed by quantification of the phosphorylation signal. (C) CaMKK activities in the extracts of A549 cells stably expressing FLAG-CaMKK*α* mutant [A292T, L233F, AT/LF (○, left panel)], FLAG-CaMKK*β* mutant [A328T, V269F, AT/VF (○, right panel)], and FLAG-tagged wild-type [WT (●)] enzymes were measured in the absence or presence of various concentrations of STO-609 (0.001−10 *μ*g/mL) at 30 °C for 10 min with 4 mM $CaCl_2$, 12 *μ*M CaM, and 100 *μ*M ATP. Phosphorylation of GST-CaMKI 1−293 K49E at Thr177 by each cell extract was detected by immunoblot analysis using anti-phosphoCaMKI antibody (inset), followed by quantification of the phosphorylation signal. The results are expressed as a percentage of the value in the absence of STO-609. The data are representative of at least three independent experiments.

in cells expressing STO-609-resistant FLAG-CaMKK*β* (A328T, V269F, AT/VF). These results are similar to those of the experiments with ionomycin treatments as shown in panels A and B of Figure 4. These results strongly support previous findings that CaMKK*β* rather than CaMKK*α* is an activator for AMPK.[15−17] This inference is also strengthened by the observation that basal AMPK phosphorylation without ionomycin or ATP treatment was significantly enhanced in CaMKK*β*-expressing A549 cells (Figure 4B and Figure 2 of the Supporting Information), in good agreement with the high level of autonomous activity of CaMKK*β* (Figure 2B, right panel).

Phosphorylation of HA-CaMKIV in A549 Cells Expressing STO-609-Resistant CaMKK Isoforms. It has been shown that CaMKK is capable of phosphorylating and activating other downstream targets, including CaMKIV, CaMKI, and PKB/Akt,[12,35] but the isoform specificity for activation of those kinases remains unknown. We attempted to identify the CaMKK isoform responsible for phosphorylation of CaMKIV at Thr196 in living cells using A549 cells expressing STO-609-resistant CaMKK isoforms and STO-609 treatment. Given that CaMKIV is not expressed in A549 cells or its expression is undetectable (data not shown), we exogenously expressed HA-CaMKIV in A549 cells to monitor the

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Biochemistry 2015, 54, 3969−3977

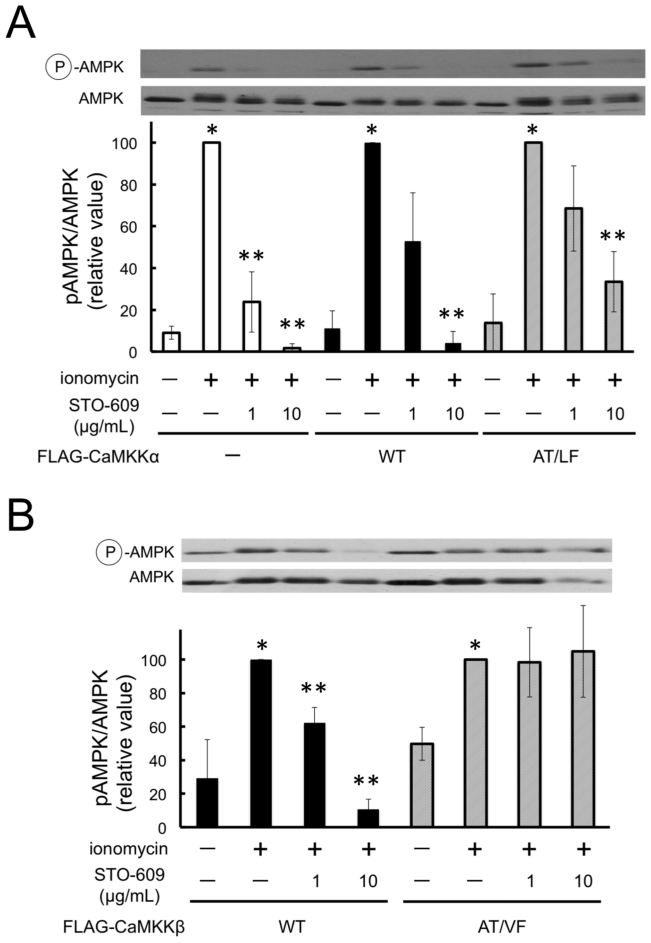

Figure 4. Ionomycin-induced AMPK phosphorylation in A549 cells stably expressing STO-609-resistant mutants of CaMKK isoforms. (A) Either A549 cells (−) or A549 cells stably expressing either wild-type FLAG-CaMKKα (WT) or FLAG-CaMKKα mutant (A292T, L233F, AT/LF) were stimulated with 1 μM ionomycin for 5 min without or with STO-609 treatment (1 and 10 μg/mL). (B) A549 cells stably expressing either wild-type FLAG-CaMKKβ (WT) or FLAG-CaMKKβ mutant (A328T, V269F, AT/VF) were stimulated with 1 μM ionomycin for 5 min without or with STO-609 treatment (1 and 10 μg/mL). Stimulation was terminated, and then AMPK phosphorylation at Thr172 was analyzed by immunoblotting with either anti-phosphoAMPK antibody (top inset) or anti-AMPKα antibody (bottom inset), followed by quantification of the phosphorylation signal. The results are expressed as a percentage of the value in the absence of STO-609 with ionomycin treatment. Results represent means and standard errors of three experiments. Statistical differences are marked: $^{**}p < 0.05$ vs ionomycin-treated cells in the absence of STO-609, and $^{*}p < 0.05$ vs control cells.

ionomycin-induced phosphorylation of CaMKIV at Thr196 by anti-phosphoCaMKIV antibody (Figure 5). Ionomycin-induced phosphorylation of HA-CaMKIV in A549 cells was easily detected and suppressed by STO-609 treatment. Unlike AMPK phosphorylation, HA-CaMKIV phosphorylation was not inhibited in cells expressing both STO-609-resistant CaMKK isoforms. This result suggested that both CaMKK α and β isoforms are capable of phosphorylating CaMKIV at Thr196 in living cells.

In summary, STO-609 has been widely used to analyze the CaMKK-mediated signaling pathway, including the CaMKK/CaMKI, CaMKK/CaMKIV, and CaMKK/AMPK signaling cascades. However, because the specificity of the compound

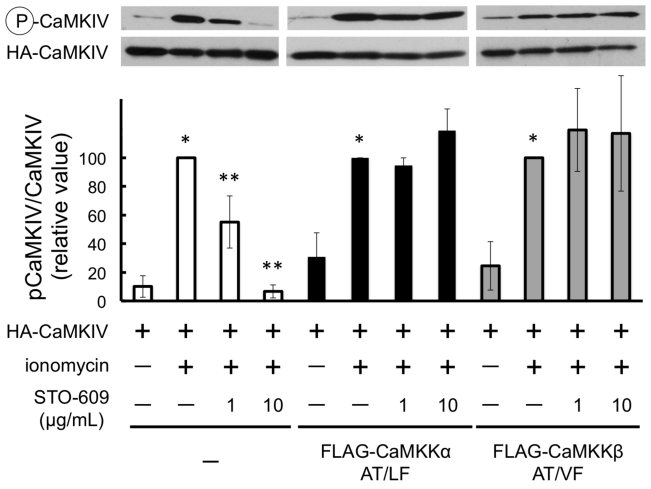

Figure 5. CaMKIV phosphorylation in A549 cells stably expressing STO-609-resistant mutants of CaMKK isoforms. The expression plasmid for HA-CaMKIV was transfected into either A549 cells (−) or A549 cells stably expressing either FLAG-CaMKKα mutant (A292T, L233F, AT/LF) or FLAG-CaMKKβ mutant (A328T, V269F, AT/VF), and then transfected cells were stimulated with 1 μM ionomycin for 5 min without or with STO-609 treatment (1 and 10 μg/mL). Stimulation was terminated, and then CaMKIV phosphorylation at Thr196 was analyzed by immunoblotting with either anti-phospho-CaMKIV antibody (top inset) or anti-HA antibody (bottom inset), followed by quantification of the phosphorylation signal. The results are expressed as a percentage of the value in the absence of STO-609 with ionomycin treatment. Results represent means and standard errors of three experiments. Statistical differences are marked: $^{**}p < 0.05$ vs ionomycin-treated cells in the absence of STO-609, and $^{*}p < 0.05$ vs control cells.

is not perfect like that of other kinase inhibitors, the results obtained using this compound should be interpreted with caution.[27] In addition, STO-609 inhibits both CaMKK isoforms (α and β), indicating the difficulty of dissecting CaMKK isoform-dependent signaling pathways.[18] To overcome this problem, we have succeeded in obtaining STO-609-resistant CaMKK isoforms as well as stable cell lines expressing mutant CaMKKs to distinguish the CaMKK isoform-dependent inhibitory effects of STO-609. In combination with using STO-609 treatment, we have shown that increasing the concentration of intracellular Ca^{2+} stimulated AMPK activation through the phosphorylation of the Thr residue (Thr172) in the activation loop that was catalyzed by CaMKKβ in living cells but not likely by the α isoform, an observation in good agreement with previous reports.[15−17] We confirmed that an ~50% reduction of CaMKKα expression by siRNA treatment did not affect the ionomycin-induced AMPK phosphorylation in A549 cells, which was completely suppressed by STO-609 treatment (data not shown). In addition, we have shown that both CaMKK isoforms are capable of phosphorylating another target kinase, CaMKIV, in living cells using this strategy. Although it has been reported that CaMKK α and β isoforms exhibited different expression patterns in mature rat brain as well as during neural development,[36,37] the apparent redundancy of CaMKK isoforms with respect to phosphorylation of CaMKIV at Thr196 suggests that phosphorylation-dependent CaMKIV activation may occur in various regions and developmental stages in the brain. The CaMKK isoform specificity of CaMKK/CaMKI and the CaMKK/PKB activation pathway remain to be analyzed. According to our study, a

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Biochemistry 2015, 54, 3969−3977

combination of the use of STO-609 and the inhibitor-resistant CaMKK mutants developed in this study may be useful for distinguishing CaMKK isoform-mediated signaling pathways in living cells as well as for confirming the specificity of STO-609.

■ ASSOCIATED CONTENT

Supporting Information

Two figures and their associated legends. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00149.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; CaMKK, CaMK kinase; AMPK, 5′-AMP-activated protein kinase; PKB, protein kinase B; GST, glutathione *S*-transferase; DTT, dithiothreitol; HA, hemagglutinin; WT, wild-type.

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