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The active-site cysteine residue of Ca^{2+}/calmodulin-dependent protein kinase I is protected from irreversible modification via generation of polysulfidation

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ABSTRACT

Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaMK) I is activated by the phosphorylation of a crucial activation loop Thr^{177} by upstream kinases, CaMK kinase (CaMKK), and regulates axonal or dendritic extension and branching. Reactive sulfur species (RSS) modulate protein functions via polysulfidation of the reactive Cys residues. Here, we report that the activity of CaMKI was reversibly inhibited via its polysulfidation at Cys^{179} by RSS. *In vitro* incubation of CaMKI with the exogenous RSS donor Na_2S_3 resulted in a dose-dependent inhibition of the phosphorylation at Thr^{177} by CaMKK and inactivation of the enzymatic activity. Dithiothreitol (DTT), a small molecule reducing reagent, rescued these inhibitions. Conversely, mutated CaMKI (C179V) was resistant to the Na_2S_3-induced inactivation. In transfected cells expressing CaMKI, ionomycin-induced CaMKI activity was decreased upon treatment with Na_2S_4, whereas cells expressing mutant CaMKI (C179V) proved resistant to this treatment. A biotin-polyethylene glycol-conjugated maleimide capture assay revealed that CaMKI was a target for polysulfidation in cells. Furthermore, the polysulfidation of CaMKI protected Cys^{179} from its irreversible modification, known as protein succination. Thus, we propose that CaMKI was reversibly inhibited via polysulfidation of Cys^{179} by RSS, thereby protecting it from irreversible modification.

1. Introduction

Ca^{2+}/CaM (calmodulin)-dependent protein kinases (CaMKs) play important roles in processing cellular signal transduction mediated by an increase of intracellular Ca^{2+} levels [1,2]. CaMKI is a member of the CaMKs that is widely distributed in most mammalian cell types but is highly abundant in the brain, where it phosphorylates and regulates numerous protein substrates [3]. Activated CaMKI plays pivotal roles in various cellular processes such as outgrowth of both axons and dendrites [4,5]. Two features are critical for activation of CaMKI: the binding of Ca^{2+}/CaM for release of auto-inhibition, and the phosphorylation of a Thr^{177} residue in the activation loop by the upstream CaMK kinase (CaMKK) [6]. Thus, the regulation of CaMKI requires binding of Ca^{2+}/CaM to and phosphorylation of the enzyme that initiate the process.

Reactive sulfur species (RSS) comprise a group of sulfur-containing molecules that play regulatory roles in biological systems. Among various RSS, cysteine hydropersulfide (CysSSH), glutathione persulfide (GSSH), and longer chain sulfur compounds (polysulfides, including CysS/GS–$(S)_n$–H) are present in remarkable amounts in cultured cells and in the tissues of mice and humans [7–10]. These RSS act as potent antioxidants and cellular protectants, and show a strong redox signaling regulatory function via electrophile thiolation [7–12]. Notably, the chemical reactivity of RSS has been thought to be involved in the catalytic activity of particular proteins. In particular, a wide variety of proteins are regulated by polysulfidation, a RSS-induced modification [13–17]. Thus, the identification of novel polysulfidated proteins is of interest in studies of RSS.

We reported previously other regulatory mechanisms of CaMKs, such as *S*-glutathionylation or *S*-nitrosylation of CaMKI at Cys^{179} [18,19], *S*-nitrosylation of CaMKII at $Cys^{6/30}$ [20] and *S*-oxidation of CaMKIV at Cys^{198} [21]. We have recently reported that CaMKII and

Abbreviations: 2SC, *S*-(2-succino)cysteine; A23187, Ca^{2+} ionophore; biotin-PEG-MAL, biotin-polyethylene glycol-conjugated maleimide; CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKK, CaMK kinase; CSE, cystathionine-γ-lyase; CysSSH, cysteine hydropersulfide; DMF, dimethyl fumarate; DTT, dithiothreitol; MS, mass spectrometry; RSS, reactive sulfur species

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CaMKIV were inactivated by RSS via polysulfidation of the specific Cys residue [22,23]. In the present study, we investigated the molecular mechanisms of RSS-dependent regulation of CaMKI.

2. Materials and methods

2.1. Materials

Recombinant glutathione *S*-transferase (GST)-fused rat CaMKIα [24], rat CaMKKα [25], and rat CSE [26] were expressed in *Escherichia coli* DH5α and purified as described previously. Purification of GST-cleaved CaMKI was performed using Turbo3C protease as described in the manufacturer's protocol. Recombinant rat CaM was expressed in *E. coli* BL21 (DE3) using pET-CM, which was provided by Dr. Nobuhiro Hayashi (Tokyo Institute of Technology, Yokohama, Japan) [27]. The monoclonal antibody specific to phosphorylated CaMKI (*p*-CaMKI, Thr^{177}) was provided by Dr. Naohito Nozaki (MAB Institute, Inc., Nagano, Japan). The rabbit *anti*-CaMKI polyclonal antibody was provided by Dr. Angus C. Nairn (Yale University School of Medicine, New Haven, CT, USA). Na_2S_3 and Na_2S_4 were obtained from Dojindo Laboratories (Kumamoto, Japan). ECL prime (enhanced chemiluminescence) immunoblotting detection reagents were obtained from GE Healthcare (Piscataway, NJ, USA). All other materials and reagents were of the highest quality available from commercial suppliers.

2.2. Inactivation of CaMKI by RSS

To generate CysSSH, recombinant CSE (100 μg/mL) was incubated in 30 mM HEPES buffer (pH 7.5) containing 50 μM pyridoxal phosphate with buffer alone or 1 mM cystine for 60 min at 37 °C. Recombinant CaMKI (20 μg/mL) was also incubated with one-tenth of the products for 30 min or increasing amounts of polysulfides (Na_2S_3) (1–300 μM) for 10 min at 30 °C in 30 mM HEPES (pH 7.5) and 400 μg/mL bovine serum albumin. After this, pretreated CaMKI (5 μg/mL) was activated with CaMKK (1 μg/mL) for 15 min at 30 °C in 40 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 μM CaM, and 50 μM ATP. An aliquot of the reaction mixture was then removed and analyzed for activity. Kinase activity was measured at 30 °C for 3 min in 40 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 μM CaM, 10 μM [γ-^{32}P] ATP, 50 μM synthetic peptide Syntide-2 (PLARTLSVAGLPCKK), and CaMKI (1 μg/mL) in a final volume of 25 μL. ^{32}P incorporation was evaluated by spotting 20-μL aliquots onto Whatman P-81 phosphocellulose paper, followed by washing in 75 mM phosphoric acid [28].

2.3. Construction of plasmids

The CaMKI C179V mutant was generated using pGEX6P and pME18s-FLAG CaMKI plasmid DNA using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The nucleotide sequences of all constructs were confirmed by sequencing analysis.

2.4. Cell culture, transfection, and stimulation

HEK293 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum and subcultured for 24 h in 6-cm dishes in a humidified atmosphere at 37 °C. Next, the cells were transfected with the pME18s-FLAG CaMKI construct (1 μg) using Lipofectamine LTX (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After a 24–36-h incubation, the cells were serum-starved for 18 h and pre-incubated with or without Na_2S_4 for 10 min, followed by stimulation with or without 10 μM A23187 for 3.5 min.

2.5. Detection of polysulfidated CaMKI using a biotin-polyethylene glycol-conjugated maleimide capture method

Polysulfidated proteins were detected using a biotin-polyethylene glycol-conjugated maleimide (biotin-PEG-MAL) capture method, performed as described previously [23,29]. Briefly, cells were lysed with ice-cold RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM phenylmethane sulfonyl fluoride, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Nonidet P40] containing 1 mM biotin-PEG-MAL. In the initial step, both protein Cys thiol (-SH) and polysulfide (-$(S)_n$-H) groups are alkylated by using biotin-PEG-MAL. After a 3-h incubation at 37 °C, whole biotinylated proteins in the lysates were captured and enriched with streptavidin agarose (Thermo Fisher Scientific, Inc., Waltham, MA, USA), polysulfidated proteins were collected by using reducing agent, 2-mercaptoethanol and subjected to western blotting for CaMKI. Some biotin-PEG-MAL capture methods were performed using recombinant enzymes aside from cell lysates.

2.6. Western blot analysis

Supernatants (20 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene fluoride membranes. The membranes were incubated for 1 h at room temperature with *anti*-phospho-CaMKI (1:40), *anti*-CaMKI (1:1000), or *anti*-CaMKK (BD Biosciences, USA, 610544; 1:1000), and then with anti-horseradish peroxidase-linked secondary antibodies. Blots were developed using ECL prime detection reagent. Signals were detected and analyzed using a Luminescent Image Analyzer LAS-4000 with image analysis software MultiGauge (FujiFilm, Tokyo, Japan).

2.7. Statistical analysis

All results are represented as the mean ± standard error (SE) of at least three determinations. Statistical evaluation was performed using a one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. CaMKI was inactivated by treatment with RSS

To attain full activity, CaMKI require phosphorylation of a Thr^{177} residue present in its crucial activation loop by CaMKK, an upstream kinase [6]. In initial experiments, we determined the effects of RSS on CaMKI activity and phosphorylation of Thr^{177}. Recombinant CaMKI (20 ng/μL) was incubated with Na_2S_3 (1–300 μM) for 10 min and then activated by CaMKK. The activity of CaMKI was measured using the synthetic peptide Syntide-2 as a substrate. As shown in Fig. 1A, both the activity and Thr^{177} phosphorylation of CaMKI in the absence of CaMKK were negligible. Both the activity and Thr^{177} phosphorylation of CaMKI by CaMKK inhibited with Na_2S_3 in a concentration-dependent manner. We next examined whether dithiothreitol (DTT), a small molecule reducing agent, would reverse the inhibition by removal of polysulfidation from CaMKI. Fig. 1B shows that the addition of DTT completely reversed the inhibition of enzymatic activity and Thr^{177} phosphorylation by 100 μM Na_2S_3. We carried out comparative studies the effects of Na_2S_3 and Na_2S_4, resulting that these polysulfides both inhibited to CaMKI activity *in vitro* (Supplementary Fig. 1). Next, we tested whether CaMKK-induced phosphorylation at Thr^{177} of CaMKI affected Na_2S_3-induced inactivation of the enzyme. We pretreated CaMKI either with or without a CaMKK-containing reaction mixture and analyzed the Na_2S_3-induced CaMKI inhibition. As shown in Fig. 1C, prior phosphorylation of CaMKI with CaMKK did not prevent subsequent Na_2S_3-induced inactivation. We tested the effects of RSS on CaMKK activity using GST-CaMKI-(1–293)-K49E as a substrate [30]. We did not observe a significant decrease in CaMKK (α and β subunits) activity upon treatment with Na_2S_4 below the concentration of 1 mM (data not shown). Thus, it is not most likely that CaMKK α activity is not

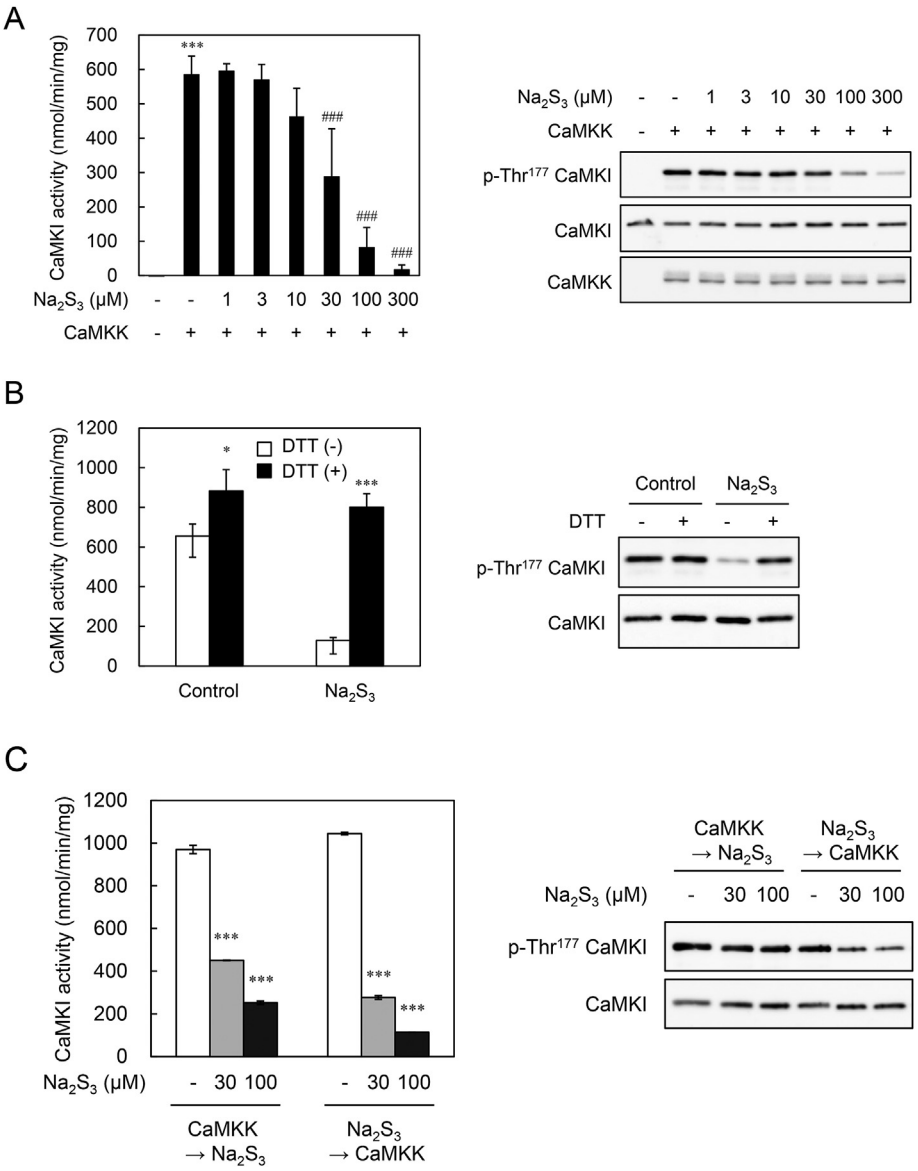

Fig. 1. CaMKI is reversibly inactivated by Na_2S_3. (**A**) Purified CaMKI was treated with 1–300 μM Na_2S_3 at 30 °C for 10 min. Samples were incubated with CaMKK at 30 °C for 15 min and then assayed for 3 min using 50 μM Syntide-2, 1.5 mM $CaCl_2$, 1 μM CaM, and 10 μM [γ-^{32}P] ATP (left panel). The means ± SE of three independent experiments are shown. $^{***}P < 0.001$ as compared with absence of incubation with CaMKK, $^{###}P < 0.001$ as compared with CaMKI activity incubated with CaMKK in the absence of Na_2S_3. Each sample was subjected to western blotting with phospho-Thr^{177} CaMKI, and *anti*-CaMKI, *anti*-CaMKK antibodies (right panel). (**B**) Purified CaMKI was initially treated with or without 100 μM Na_2S_3 at 30 °C for 10 min and then additionally incubated with or without 20 mM DTT at 25 °C for 10 min. Samples were incubated with CaMKK at 30 °C for 15 min and subjected to the kinase assay (left panel) or western blotting (right panel) as described in (A). The means ± SE of three independent experiments are shown. $^{***}P < 0.001$ and $^{*}P < 0.05$ as compared with absence of DTT. (**C**) Prior phosphorylation of CaMKI with CaMKK did not affect subsequent Na_2S_3-induced inhibition. Purified CaMKI was initially treated either by CaMKK (CaMKK→Na_2S_3) or buffer alone (Na_2S_3→CaMKK) at 30 °C for 15 min, and then incubated with 30–100 μM Na_2S_3 at 30 °C for 10 min. An aliquot of the reaction mixture was treated either by CaMKK (Na_2S_3→CaMKK) or buffer alone (CaMKK→Na_2S_3) and subjected to the kinase assay (left panel) or western blotting (right panel). The means ± SE of three independent experiments are shown. $^{***}P < 0.001$ as compared with respective control in the absence of Na_2S_3.

inhibited by RSS with the concentration below 300 μM *in vitro*.

3.2. Cys^{179} is an essential site for inactivation of CaMKI by RSS

We previously showed that CaMKI was fully and reversibly inactivated by oxidative *S*-glutathionylation at Cys^{179} [18]. We constructed and purified the CaMKI C179V mutant by site-specific separation of the GST tag from the expressed protein using pGEX-6P vectors. The CaMKI C179V mutant was similar to the wild-type in terms of activation by CaMKK (300.69 ± 3.87 and 349.59 ± 15.19 nmol/min/mg protein, respectively). However, a minimal decrease in activity was noted with the CaMKI C179V mutant with increasing amounts of Na_2S_3 (30–100 μM), whereas the CaMKI wild-type was significantly inactivated (Fig. 2A). Treatment of CaMKI wild-type with Na_2S_3 (30 or 100 μM) led to a decrease in Thr^{177} phosphorylation by CaMKK. In contrast, the CaMKI C179V mutant was resistant to Na_2S_3 treatment.

Cystathionine γ-lyase (CSE) is the last key enzyme in the transsulfuration pathway for the biosynthesis of cysteine. In addition, this enzyme is capable of directly generating CysSSH using either cystine as a substrate [8]. We assessed whether this enzymatically synthesized CysSSH may be involved in the regulation of CaMKI activity. As shown in Fig. 2B, cystine alone had minimal effects on both CaMKK-induced activity and Thr^{177} phosphorylation of CaMKI. However, when CaMKI was incubated with the CSE/cystine products, there was a significant effect on these inhibitions. Conversely, the CaMKI C179V mutant was resistant to CSE/cystine-induced inhibition. To determine the target sites of polysulfidation on CaMKI, we measured CaMKI polysulfidation using Na_2S_3-treated recombinant wild-type or C179V mutant enzyme. The treatment of wild-type CaMKI with Na_2S_3 led to an increase in polysulfidation. C179V displayed a reduction in polysulfidation by Na_2S_3 relative to the wild-type enzyme, suggesting that Cys^{179} constitutes one of the major polysulfidation sites *in vitro* (Fig. 2C). The shift of lower mobility of Na_2S_3 treated wild-type or C179V CaMKI was also observed.

3.3. Cys^{179} was the RSS-sensitive site on CaMKI in cells

The above experiments clearly demonstrated the susceptibility of Cys^{179} in CaMKI to RSS *in vitro*. Next, we examined the ability of CaMKI to be inhibited within the cellular environment under RSS-loading conditions. To determine the ability of RSS to inhibit CaMKI activity, HEK293 cells transfected with a FLAG-tagged CaMKI wild-type or C179V construct were treated with a combination of Na_2S_4 and Ca^{2+} ionophore, A23187. The Ca^{2+}/CaM-dependent activity of the

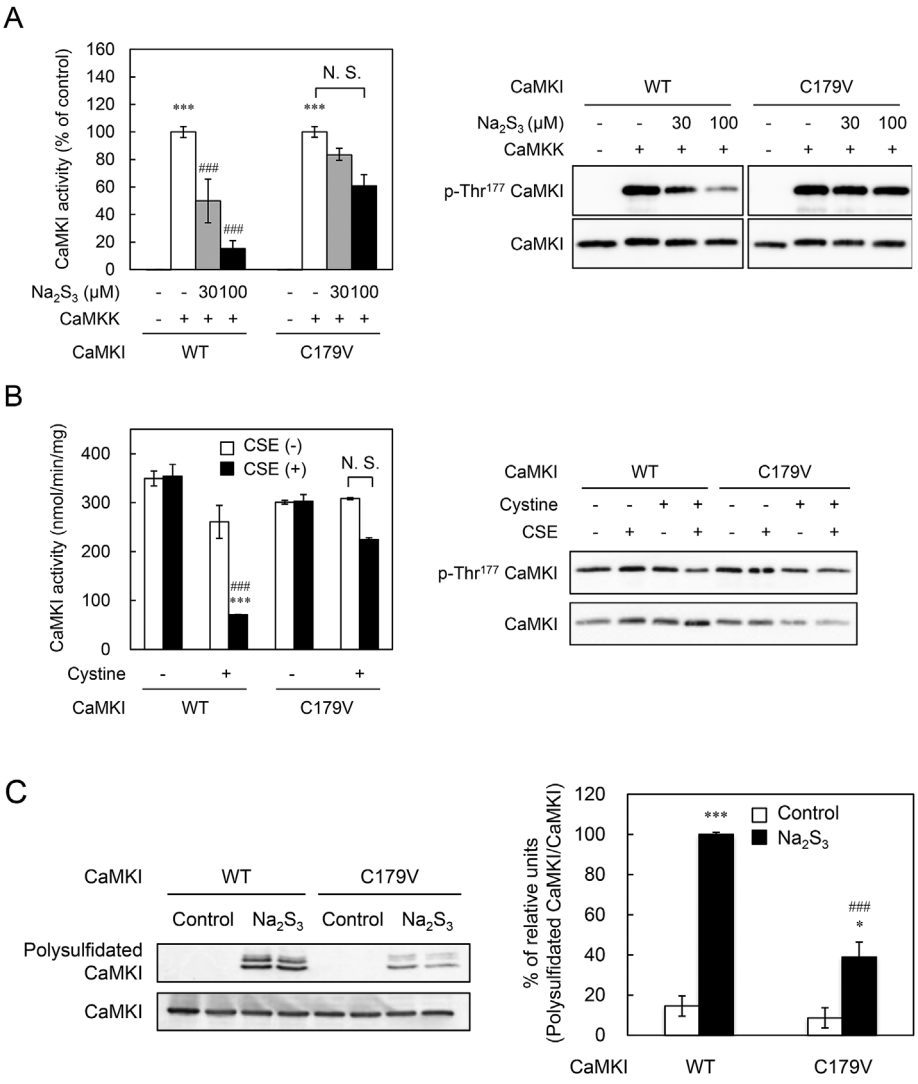

Fig. 2. In CaMKI, the Cys^{179} to Val mutation appears to confer resistance to reactive sulfur species (RSS)-induced inhibition of CaMKI activity. (A) Purified CaMKI wild-type (WT) or C179V mutant were incubated with 30–100 μM Na_2S_3 at 30 °C for 10 min, and then assayed for activity as described in Fig. 1. The means ± SE of three independent experiments are shown. $^{***}P < 0.001$ as compared with absence of incubation with CaMKK, $^{\#\#\#}P < 0.001$ as compared with respective CaMKI activity incubated with CaMKK in the absence of Na_2S_3. N. S.: no significant difference. Each sample was subjected to western blotting with *anti*-phospho-Thr^{177} CaMKI and *anti*-CaMKI antibodies (right panel). (**B**) Inhibition of CaMKI activity by an enzymatic generation of CysSSH catalyzed by CSE/cystine. Cystine (1 mM) or buffer alone was incubated either with 100 ng/mL CSE or buffer alone in 30 mM HEPES buffer (pH 7.5) containing 50 mM pyridoxal phosphate at 37 °C for 60 min. Proportional amounts of each reaction mixture were initially incubated with CaMKI at 30 °C for 30 min and then activated with CaMKK. An aliquot of reaction mixture was subjected to the kinase assay (left panel) or western blotting (right panel). The means ± SE of three independent experiments are shown. $^{***}P < 0.001$ as compared with respective the absence of cystine, $^{\#\#\#}P < 0.001$ as compared with respective the absence of CSE. N. S.: no significant difference. (**C**) Recombinant CaMKI WT or C179V mutant were treated with buffer alone (Control) or 300 μM Na_2S_3 (Na_2S_3) for 10 min. *S*-polysulfidated CaMKI was detected using a biotin-PEG-MAL-capture method. The histogram shows the amounts of polysulfidated CaMKI relative to those of expressed CaMKI. Results represent the means ± SE of three independent experiments. $^{***}P < 0.001$ and $^{*}P < 0.05$ as compared with control of each enzyme, $^{\#\#\#}P < 0.001$ as compared with CaMKI WT in the presence of Na_2S_3.

immunoprecipitated FLAG-tagged CaMKI wild-type or C179V construct was greatly enhanced by stimulation with A23187 (Fig. 3A). The A23187-induced activity of the CaMKI wild-type was suppressed by Na_2S_4. In contrast, cells expressing CaMKI C179V proved resistant to Na_2S_4. The treatment of Na_2S_3 also shows similar result to that of Na_2S_4 (Supplementary Fig. 2). The A23187-induced phosphorylation of Thr^{177} was somehow not evident in cells transfected with either of the constructs, but it is noteworthy that the treatment with Na_2S_4 resulted in remarkable increase in this phosphorylation. This finding is consistent with the results of previous studies, demonstrating that Na_2S_4 increases the phosphorylation of CaMKK-targeting AMP-activated protein kinase (AMPK) as CaMKI [23,31].

We examined whether CaMKI was polysulfidated by treatment of Na_2S_4 in cells. The treatment of cells with Na_2S_4 led to an increase in CaMKI polysulfidation (Fig. 3B). C179V displayed equal CaMKI polysulfidation by Na_2S_4 relative to the wild-type enzyme, although it is resistant to inactivation by Na_2S_4. Thus, Cys^{179} was not an only polysulfidation sites and/or RSS-induced other modifications occurred at Cys^{179} residue of CaMKI in cells.

3.4. Polysulfidation of CaMKI protected Cys^{179} from irreversible modification

The above experiments have shown that inactivation of CaMKI by polysulfidation at Cys^{179} was reversible. It has been recently proposed that protein hydropersulfide formation may represent a mechanism by which protein thiols are protected from irreversible oxidative or electrophilic modification [11,12]. It was therefore of interest to examine the ability of CaMKI to be protected from irreversible modifications. Protein succination is a stable and irreversible post-translational modification that occurs when fumarate reacts with cysteine residues to generate *S*-(2-succino)cysteine (2SC) [32]. CaMKI was incubated for 1 h with different concentrations of fumarate or the pharmacological compound dimethylfumarate (DMF). As shown in Fig. 4A, incubation of CaMKI with DMF resulted in dose-dependent inactivation, although inactivation of CaMKI with fumarate was not observed. The inactivation of CaMKI by DMF did not recover with post-treatment of DTT (Fig. 4B). In contrast, the CaMKI C179V mutant proved resistant to DMF-induced inactivation (Fig. 4C). We next examined whether polysulfidation of CaMKI at Cys^{179} (–S–$(S)_n$–H) could allow succination (–S–$(S)_n$–2SC) and thiol regeneration (–$(S)_n$–H) with post-treatment of DTT. CaMKI was pretreated either with or without Na_2S_3 and then treated with DMF. The activity of CaMKI was inhibited by DMF treatment after Na_2S_3 treatment (Na_2S_3→DMF), and its inhibition was restored by DTT (Fig. 4D). Meanwhile, the activity of CaMKI was also inhibited by Na_2S_3 treatment after DMF treatment (DMF→Na_2S_3), and its inhibition was not restored by DTT (Fig. 4E). The phosphorylation of CaMKI was inhibited by Na_2S_3 treatment alone (Na_2S_3) or DMF treatment after Na_2S_3 treatment (Na_2S_3→DMF), but not by treatment with DMF alone (DMF) or Na_2S_3 treatment after DMF treatment (DMF→

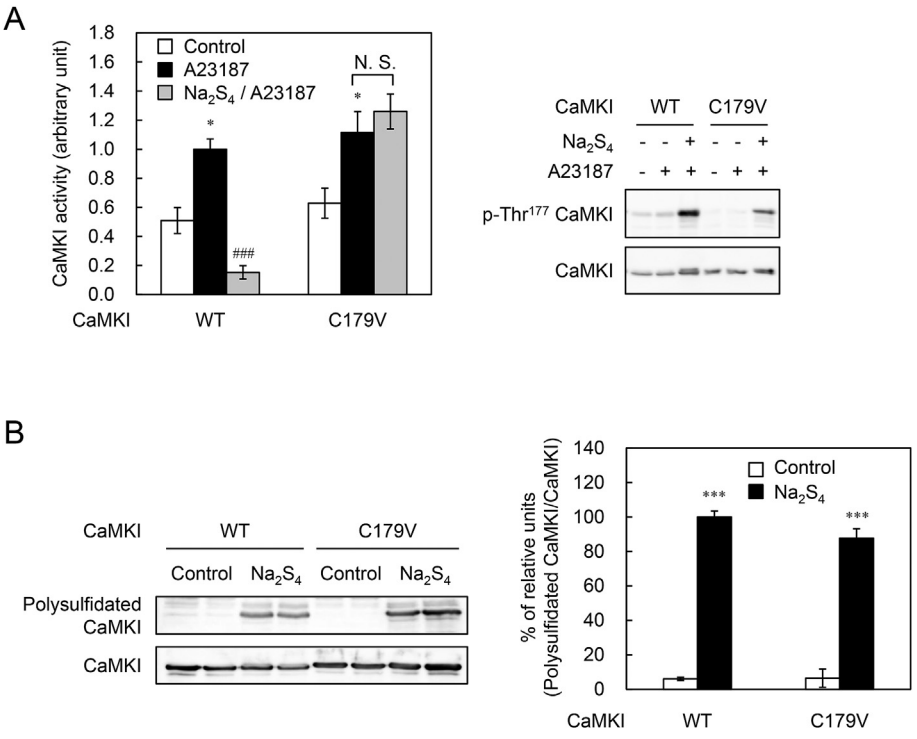

Fig. 3. Effect of reactive sulfur species (RSS) donors on the CaMKI activity in cells. (**A**) HEK293 cells expressing FLAG-tagged CaMKI wild-type (WT) or C179V mutant were treated buffer alone or 1 mM Na_2S_4 for 10 min, and then stimulated with buffer alone or 10 μM A23187 for 3.5 min. The expressed CaMKI was also affinity-purified from transfected cells by affinity chromatography using anti-FLAG agarose and then assayed at 30 °C for 3 min using 50 μM Syntide-2, 1.5 mM $CaCl_2$, 1 μM CaM, and 10 μM [γ-^{32}P] ATP (left panel). Results are the mean ± SE of three independent experiments. $^{*}P < 0.05$ as compared with the respective control, $^{\#\#\#}P < 0.001$ as compared with cells treated with A23187. N. S.: no significant difference. Lysates were immunoblotted with *anti*-phospho-Thr^{177} CaMKI and *anti*-CaMKI (right panel). (**B**) HEK293 cells expressing FLAG-tagged CaMKI WT or C179V mutant were treated with buffer alone (control) or 1 mM Na_2S_4 (Na_2S_4) for 10 min. Polysulfidated CaMKI was detected using a biotin-PEG-MAL capture method. The histogram shows the amounts of polysulfidated CaMKI relative to those of expressed CaMKI. Results represent the means ± SE of three independent experiments. $^{***}P < 0.001$ as compared with control of each transfected cells.

Na_2S_3).

4. Discussion

We showed here that CaMKI was sensitive to inhibition by polysulfidation of its Cys^{179} residue by RSS. We demonstrated that even prior phosphorylation of CaMKI at Thr^{177} with CaMKK did not prevent subsequent Na_2S_3-induced inactivation (Fig. 1C). Furthermore, although the phosphorylation of CaMKI was increased by Na_2S_4, the activity was inhibited in cells (Fig. 3A). These results indicate that inactivation of CaMKI by polysulfidation at Cys^{179} appeared to be dominant over activation of the kinase by phosphorylation at Thr^{177}. Because DTT was able to enhance the control activity of CaMKI (Fig. 1B), it is suggested that oxidation of the enzyme readily occurs during storage when the reducing agent is absent. In CaMKI, activation loop undergoes a conformational change or a disorder-to-order transition upon phosphorylation of Thr^{177} residues within the loop. This activates the kinase, by allowing ATP and substrates access to the active site. We previously studied whether CaMKI substrates (ATP, a synthetic peptide: syntide-2) and an activator (CaM) can protect against inactivation by *S*-glutathionylation via Cys^{179} [18,19]. None of them afforded competitive protection of CaMKI from inhibition. Thus, it is plausible that the inhibition of a conformational change or a disorder-to-order transition by the polysulfidation of a Cys^{179} residue within the loop. Since Cys^{179} residue is located in the catalytic domain, Cys^{179} polysulfidation inhibits the enzyme activity with a decrease of Vmax value but not of the affinities of substrates such as ATP and a synthetic peptide: syntide-2. Note that RSS increase the phosphorylation but prevent the activation of CaMKK-targeting CaMKI and the two do not appear to be causally related in cells. Interestingly, prior polysulfidation but not succination at Cys^{179} prevented subsequent CaMKK-induced Thr^{177} phosphorylation and activity (Fig. 4D and E).

We previously demonstrated that CaMKI was fully and reversibly inactivated by its *S*-glutathionylation or *S*-nitrosylation at Cys^{179} [18,19]. Thus, Cys^{179} was important for the oxidative inactivation of CaMKI, but its major modification remains unclear. A *S*-nitrosylated bond (12–20 kcal/mol) is weaker than a polysulfidated bond (60 kcal/mol) [33], and nitroso-thiol is exceptionally labile and reacts readily with other thiols by *trans*-nitrosylation or by disulfide formation [34]. Furthermore, a recent study has demonstrated that a large portion of cysteine thiols are oxidized to persulfidated thiols such as cysteine perthiosulfenic acid (CysSSOH) and perthioglutathion (CysSSSG) [35]. Quantitative data indicated that hydropersulfides and inorganic polysulfides are widespread in cells and tissues and occur at much higher physiological concentrations than ROS or RNS [8,11]. In recent years, it was revealed that extensive and prevalent cysteine polysulfidation was introduced co-translationally, sustained in the mature protein, and physiologically present even in the post-translational processes of the cells [7]. Thus, polysulfidation may be a more common regulatory mechanism than *S*-glutathionylation and *S*-nitrosylation.

The adduction of the Krebs cycle intermediate fumarate to the sulfhydryl group of certain cysteine residues in proteins during protein succination is an irreversible non-enzymatic modification that leads to the formation of 2SC [36]. Previous studies have demonstrated that succination of proteins including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), kelch-like ECH-associated protein 1 (KEAP1), and mitochondrial aconitase (ACO2) can have profound effects on cellular metabolism [37]. Aside from the enzyme inhibition, we observed that protection of CaMKI activity against its irreversible succination may be another possible role of CaMKI polysulfidation (Fig. 4D). The Cys^{179} modification of CaMKI, such as CaMKI-S-2SC or CaMKI-SS-2SC, was confirmed by mass spectrometry using the CaMKI peptide (Supplementary Fig. S3). Reversible modification including polysulfidation and *S*-glutathionylation, plays critical roles in the protection of cysteine thiols against irreversible modifications and protein damage in response to higher levels of oxidative stress [11,12,38]. Polysulfidation may also provide as a defense mechanism to protect CaMKI from irreversible damage in oxidative stress.

The cellular conditions where CaMKI is polysulfidated and inactivated has not been well studied. The CaMKK-CaMKI cascade is a significant player in neuronal development including neurite outgrowth [5]. Neurite outgrowth and differentiation also promoted polysulfide treatment in N2A cells via accelerating intracellular Ca^{2+} influx [39]. The CaMKK-CaMKI cascade might be fine-tuned by polysulfide-induced Ca^{2+} influx and/or CaMKI modification in neuronal cells. CaMKI promoted mitochondrial fission via phosphorylation of dynamin-related

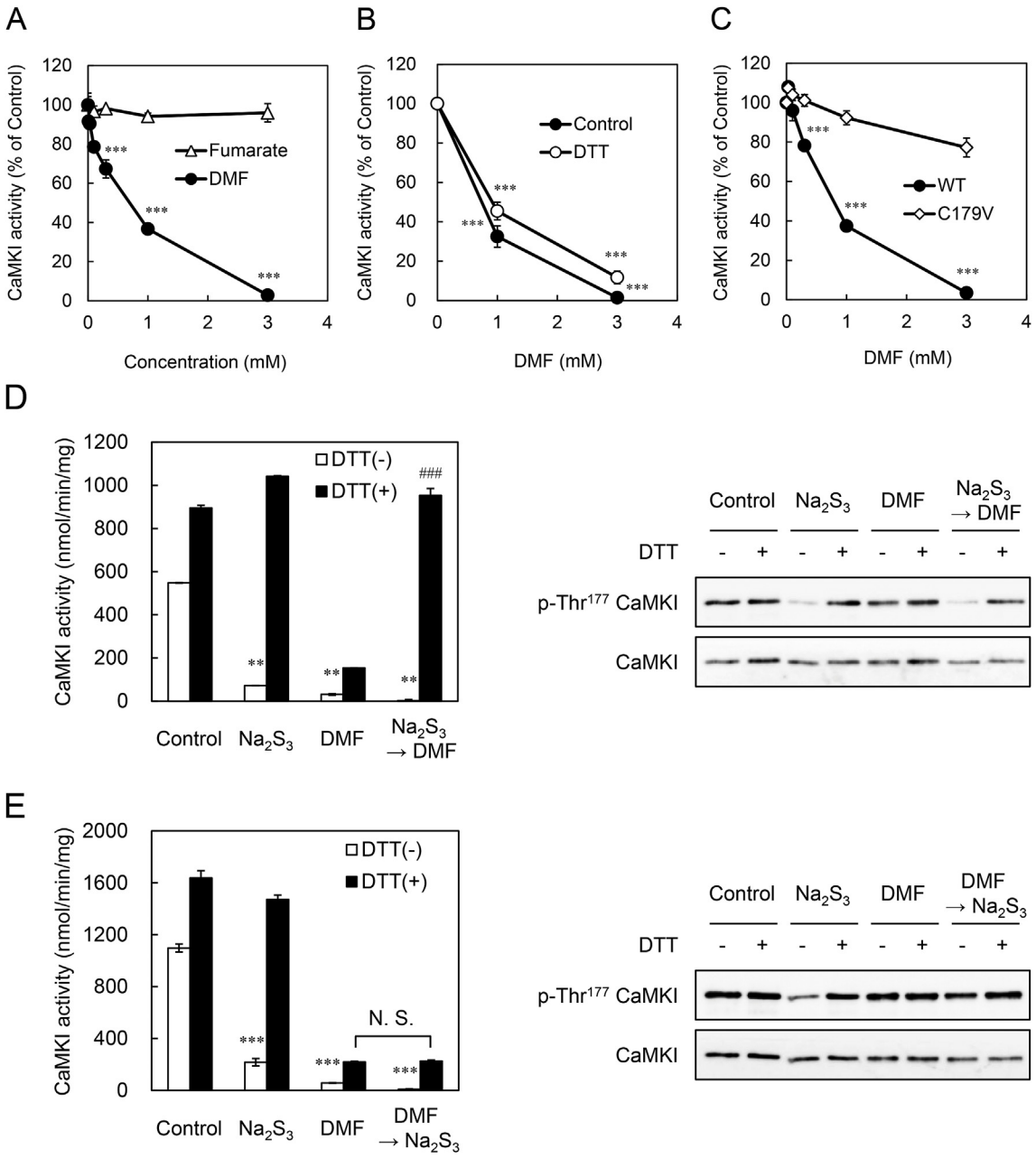

Fig. 4. Na_2S_3 protects Cys^{179} of CaMKI from dimethyl fumarate (DMF)-induced irreversible modification. (**A**) Purified CaMKI was incubated with fumarate (0.1–3 mM) or DMF (0.01–3 mM) at 30 °C for 60 min, and then activated with CaMKK and subjected to an enzymatic activity assay. (**B**) Purified CaMKI was initially incubated with 1–3 mM DMF at 30 °C for 60 min, and then an additional incubation with 20 mM DTT at 25 °C for 10 min or buffer alone. Samples were activated with CaMKK at 30 °C for 15 min and subjected to the enzymatic activity assay. (**C**) The purified CaMKI wild-type (WT) or C179V mutant was incubated with the indicated amounts of 0.03–3 mM DMF at 30 °C for 60 min and then activated with CaMKK and subjected to the enzymatic activity assay. The means ± SE of three independent experiments are shown. $^{***}P < 0.001$ as compared with respective untreated CaMKI activity. (**D**) Prior polysulfidation of CaMKI with Na_2S_3 prevented subsequent DMF-induced irreversible inhibition. Purified CaMKI was initially incubated with 100 μM Na_2S_3 at 30 °C for 10 min, and then incubated either with 3 mM DMF (Na_2S_3→DMF) or buffer alone (Na_2S_3) at 30 °C for 60 min. Purified CaMKI was also initially incubated with buffer alone at 30 °C for 10 min, and then incubated either with 3 mM DMF (DMF) or buffer alone (control) at 30 °C for 60 min. Each sample was incubated either with 20 mM DTT at 25 °C for 10 min or buffer alone, and then activated with CaMKK. An aliquot of the reaction mixture was subjected to the kinase assay (left panel) or western blotting (right panel). The means ± SE of three independent experiments are shown. $^{**}P < 0.01$ as compared with control in the absence of DTT, $^{\#\#\#}P < 0.001$ as compared with DMF alone (DMF) in the presence of DTT. (**E**) Purified CaMKI was initially incubated with 3 mM DMF at 30 °C for 60 min and then incubated either with 100 μM Na_2S_3 (DMF→Na_2S_3) or buffer alone (DMF) at 30 °C for 10 min. Purified CaMKI was also initially incubated with buffer alone at 30 °C for 60 min, and then incubated either with 100 μM Na_2S_3 (Na_2S_3) or buffer alone (control) at 30 °C for 10 min. Each sample was incubated either with 20 mM DTT or buffer alone at 25 °C for 10 min and then activated with CaMKK. An aliquot of the reaction mixture was subjected to the kinase assay (left panel) or western blotting (right panel). The means ± SE of three independent experiments are shown. $^{***}P < 0.001$ as compared with control. N. S.: no significant difference.

protein 1 (Drp1), a mediator of mitochondrial fission [40]. Meanwhile, Drp1 polysulfidation resulted in inhibition of Drp1 activity [7]. RSS might also inhibit mitochondrial fission via CaMKI inactivation.

5. Conclusions

The results presented here demonstrated for the first time that RSS can polysulfidate CaMKI at Cys^{179} and attenuate its enzymatic activity in cells. We also propose that polysulfidation of CaMKI at Cys^{179} protect against irreversible electrophilic modification. The physiological significance of CaMKI polysulfidation, including whether RSS regulated neuronal development and mitochondrial fission via CaMKI inactivation, remains to be determined in the future.

Author Contribution

T.T. and Y.W. designed the research; T.T., and A.T. performed the research; T.A. provided reagents for the polysulfide-specific biotin-labeling assay; T.T., Y.T., T.A, and Y.W. analyzed the data; and T.T. and Y.W. wrote the paper.

Conflicts of interest

The authors declare that there are no competing interests associated with the manuscript.

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Appendix A. Supplementary data

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