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Oxidation of calmodulin alters activation and regulation of CaMKII ☆

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Abstract

Increases in reactive oxygen species and mis-regulation of calcium homeostasis are associated with various physiological conditions and disease states including aging, ischemia, exposure to drugs of abuse, and neurodegenerative diseases. In aged animals, this is accompanied by a reduction in oxidative repair mechanisms resulting in increased methionine oxidation of the calcium signaling protein calmodulin in the brain. Here, we show that oxidation of calmodulin results in an inability to: (1) activate CaMKII; (2) support Thr^{286} autophosphorylation of CaMKII; (3) prevent $Thr^{305/6}$ autophosphorylation of CaMKII; (4) support binding of CaMKII to the NR2B subunit of the NMDA receptor; and (5) compete with α-actinin for binding to CaMKII. Moreover, oxidized calmodulin does not efficiently bind calcium/calmodulin-dependent protein kinase II (CaMKII) in rat brain lysates or *in vitro*. These observations contrast from past experiments performed with oxidized calmodulin and the plasma membrane calcium ATPase, where oxidized calmodulin binds to, and partially activates the PMCA. When taken together, these data suggest that oxidative stress may perturb neuronal and cardiac function via a decreased ability of oxidized calmodulin to bind, activate, and regulate the interactions of CaMKII.

Keywords: CaMKII; Calmodulin; Oxidation; Aging; Oxidative stress; Methionine oxidation; Redox state; Calcium signaling; PSD

Calmodulin (CaM) is a ubiquitous intracellular calcium (Ca^{2+}) sensor essential for rapid and coordinated responses of a variety of enzymes, channels, and receptors to both local and global Ca^{2+} transients. CaM contains two globular EF-hand domains on each terminus, able to bind a total of four Ca^{2+} ions, connected by a central flexible helix. Binding of Ca^{2+} to the EF-hands conveys a conformational change resulting in the N- and C-terminal domains exposing hydrophobic binding pockets that allow CaM to bind amphipathic α-helices in target molecules. CaM can also bind targets in the apo or partially saturated ($2Ca^{2+}$ ions bound) forms. These targets include IQ motifs, such as those found in the L-type calcium channel, neuromodulin, and many others.

Neuronal Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a ubiquitous target of CaM [1]. Under basal conditions, the kinase exists as a 12-subunit holoenzyme, with individual CaMKII subunits inhibited by binding of the autoinhibitory domain (AID) at the catalytic cleft. Ca^{2+}-dependent binding of CaM to a region overlapping the AID relieves this autoinhibition, allowing kinase activity. Ca^{2+}/CaM-dependent Thr^{286} autophosphorylation between adjacent subunits in the holoenzyme enhances the affinity for Ca^{2+}/CaM and confers autonomous (Ca^{2+}-independent) activity after CaM dissociates. $Thr^{305/306}$ autophosphorylation occurs only in the absence of bound Ca^{2+}/CaM and blocks subsequent CaM binding. The calcium-saturated carboxy-terminal lobe of CaM can partially activate CaMKII [2].

CaMKII is essential for diverse physiological processes such as Ca^{2+}-dependent long-term potentiation in the

Abbreviations: CaM, calmodulin; CaMox, oxidized calmodulin; CaMKII, calcium calmodulin dependent kinase II; PMCA, plasma membrane calcium ATPase; GST, glutathione-*S*-transferase.

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brain [3,4] and L-type Ca^{2+}-channel regulation in the heart [5]. Ca^{2+} homeostasis in these and other regions of the body are adversely affected by oxidative stress, due to ischemia, disease, or aging [6]. Multiple methionine residues within CaM can become oxidized to methionine sulfoxide (CaMox) during normal aging in the rat, causing conformational changes that alter normal association with target peptides [7]. For example, CaM isolated from the aged rat only partially activates, but still binds, the plasma membrane Ca^{2+} ATPase (PMCA) [7,8]. Thus, deleterious effects of oxidative stress may occur in part due to the inability of CaMox to properly bind and activate multiple target proteins. Previous studies show that mutation of specific methionine residues to glutamine affects CaMKII autophosphorylation [9], however the glutamine mutation does not always mimic methionine oxidation, and more detailed studies are required to look at all aspects of CaMKII activation and regulation by CaMox. Here, we show that progressive oxidation of CaM causes an inability to bind, activate, and regulate the interactions of CaMKII with various CaMKII associated proteins (CaMKAPs).

Materials and methods

Materials. H_2O_2, CaM-agarose, and glutathione agarose: Sigma (St. Louis, MO). Phenyl Sepharose CL-4B FF: Pharmacia (Piscataway, NJ). Goat α-CaMKIIα antibody was previously described [10]. Alkaline phosphatase-conjugated secondary antibody: Jackson Immunoresearch (West Grove, PA). Phospho-T^{286} CaMKII antibody: Promega (Madison, WI). Phospho-T^{305} CaMKII antibody: Gift from A.J. Silva [11].

Protein purification. Recombinant CaM, CaMKIIα, T286D CaMKII, and glutathione-*S*-transferase (GST) fusion proteins containing NR2B and α-actinin fragments were expressed and purified as described previously [12–14]. GST-NR2B contains amino acids 1260–1339 of NR2B, with Ser^{1303} mutated to alanine, and GST-actinin contains amino acids 819–894 of α-actinin-2. All protein concentrations were quantified via extinction coefficients and confirmed with Pierce BCA protein assay (GST fusions) or Bradford protein assay (CaMKII).

CaM oxidation. CaM oxidation was performed using H_2O_2 as previously described [15]. For progressive oxidation, aliquots of the oxidation reaction were removed and placed immediately into dialysis at the indicated times (see Fig. 2). CaM agarose was oxidized using the same protocol, except that H_2O_2 was removed via extensive washing of the resin with equilibration buffer.

Brain lysate preparation. Rat forebrain lysates were prepared by homogenization in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.25% Triton X-100, protease inhibitor cocktail (Sigma, St. Louis, MO), 1 mM benzamadine, 1 mM PMSF, and 1 μM microcystin. Equal aliquots of lysate were loaded onto the CaM agarose, or CaMox agarose [2 mL packed resin equilibrated with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 5 mM $CaCl_2$ (Buffer A)]. The resins were then washed with buffer A (20 mL) and eluted with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA.

CaMKII activity assays and autophosphorylation. Kinase assays were performed using the CaMKII model substrate syntide-2 [16]. CaMKIIα was autophosphorylated at T^{286} and $T^{305/6}$ as described [10,12].

GST-cosedimentation assays. GST-cosedimentation assays were performed as described [12]. Proteins on the nitrocellulose membrane were visualized by staining with Ponceau stain and identities were confirmed by Western blot (data not shown).

Results

Progressive oxidation of CaM prevents CaMKII activation

Ca^{2+}-dependent binding of CaM to CaMKII activates both T^{286} autophosphorylation and phosphorylation of exogenous substrates. In order to determine the effects of CaM oxidation on this process, CaM was exposed to H_2O_2 for times ranging from 1 min to 3 days (total). This method of CaM oxidation has been proven in the past to only target methionines and methionine oxidation is progressive under these conditions [15,17,18]. Analysis by SDS–PAGE revealed progressive increases in apparent molecular weight over the incubation time consistent with progressive oxidation (Fig. 1A) [19]. At least five distinct modified forms of CaM were visible, presumably due to the presence of mixed populations of CaM with distinct methionine residues, or combinations of residues, converted to methionine sulfoxide. The ability of partially oxidized CaM to activate Ca^{2+}-dependent CaMKII activity was determined. Increasing oxidation of CaM caused a progressive rightward shift in the CaMKII activation curve, and over-night exposure of CaM to H_2O_2 completely prevented CaM activation of CaMKII (Fig. 1B). This data contrasts data collected in the same manner using the PMCA. CaM that is fully oxidized is still capable of activating the PMCA [15].

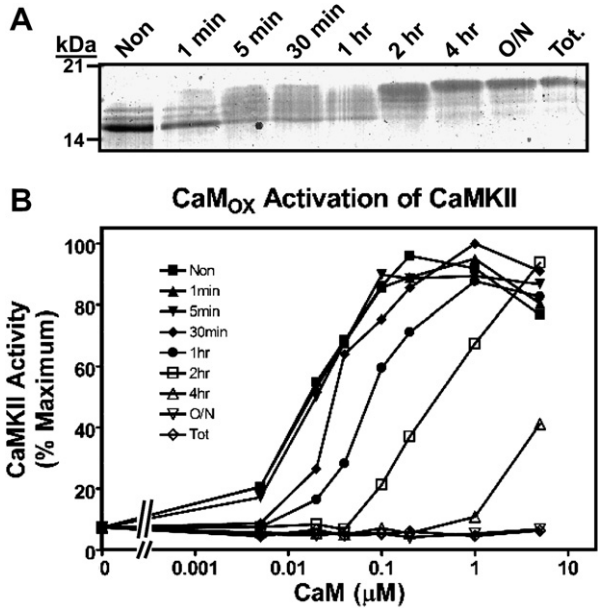

Fig. 1. Oxidation of calmodulin blocks CaMKII activation. (A) Purified calmodulin was exposed to 100 mM H_2O_2 for various times and then analyzed by SDS–PAGE on a 4–20% acrylamide gel and Coomassie stained. Calmodulin unexposed to H_2O_2 (Non) or totally oxidized (Tot.) was included as controls. (B) CaMKII was incubated with Ca^{2+}, Mg^{2+}, [γ-^{32}P]ATP, and the peptide substrate syntide-2 to examine the ability of CaM vs. CaMox to activate CaMKII in a calcium-dependent manner.

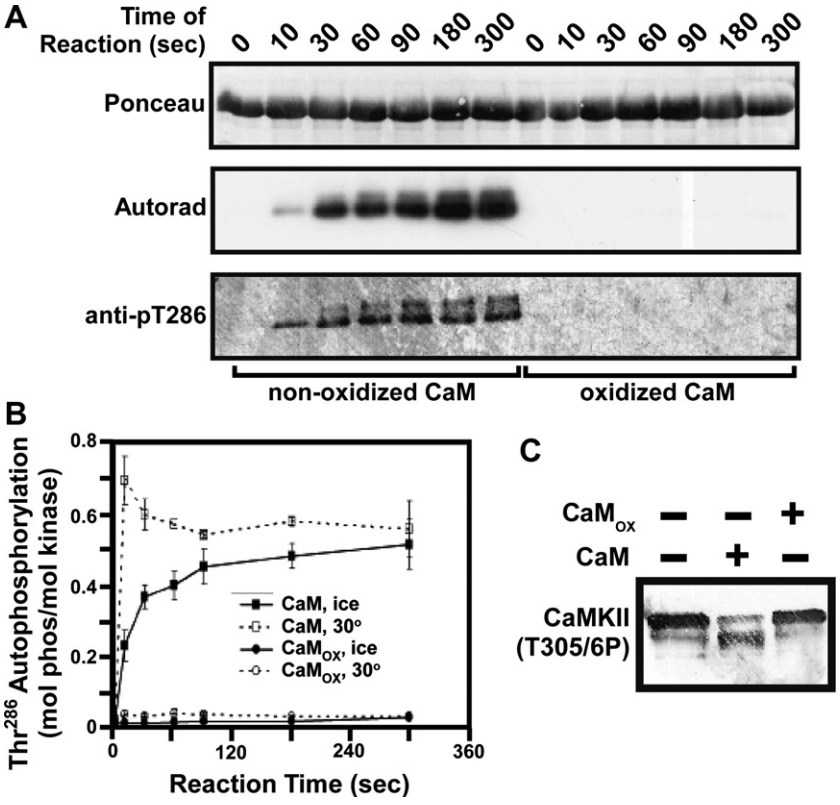

Fig. 2. Oxidized calmodulin does not regulate CaMKII autophosphorylation. (A) CaMKII was incubated with Ca^{2+}, Mg^{2+}, [γ-^{32}P]ATP, and oxidized or control calmodulin. Reactions were stopped at indicated times by the addition of SDS sample buffer. Samples were separated by SDS–PAGE and analyzed for total CaMKII by Ponceau stain (top), for total incorporation of radioactive phosphate by autoradiography (middle), and for specific autophosphorylation at Thr^{286} by Western blot with a phospho-specific antibody (bottom). (B) Samples from (A) were quantified for total incorporation of radioactive phosphate into CaMKII by scintillation counter. Error bars represent standard error of the mean, $n = 3$. (C) CaMKII [T286D] was incubated with or without oxidized or control calmodulin under conditions which stimulate $Thr^{305/6}$ autophosphorylation. Samples were separated by SDS–PAGE and analyzed for $Thr^{305/6}$ autophosphorylation by Western blot with a phospho-specific antibody.

In order to determine whether oxidized CaM supports Thr^{286} autophosphorylation, CaMKII was incubated on ice with non-oxidized or fully oxidized CaM in the presence of Ca^{2+}, Mg^{2+}, and [γ-^{32}P]ATP [10]. Incubation with oxidized CaM resulted in no detectable CaMKII autophosphorylation as assessed by autoradiography or phospho-Thr^{286}-specific immunoblotting (Fig. 2A and B). CaMKII autophosphorylation was not detected even when these incubations were repeated at 30 °C (Fig. 2B).

Ca^{2+}/CaM binding to CaMKII blocks autophosphorylation of Thr^{305} and Thr^{306} within the CaM binding domain (Fig. 2C). Even though CaMox cannot activate CaMKII, it is possible that it retains the capacity to block autophosphorylation at $Thr^{305/306}$. Therefore, we incubated CaMKIIα [T286D] with ATP, Mg^{2+}, and Ca^{2+} in the presence of either CaM or CaMox. The T286D mutant was used because constitutive activity of this kinase enhanced the CaM-dependent autophosphorylation at $Thr^{305/306}$ and other sites (not shown), resulting in a form of CaMKII with reduced mobility on SDS–PAGE and strong reactivity toward a P-Thr^{306} specific antibody. Addition of CaM substantially reduced this autophosphorylation reaction, but CaMox appears to have no detectable effect on this form of autophosphorylation (Fig. 2C).

CaMox does not properly regulate CaMKII–CaMKAP interactions

CaM differentially modulates CaMKII interaction with a variety of proteins [12,20,21]. For example, Ca^{2+}/CaM binding promotes interaction of non-autophosphorylated CaMKII with the NR2B subunit of the *N*-methyl-D-aspartate (NMDA) receptor, whereas Ca^{2+}/CaM competes with α-actinin for binding to CaMKII [21]. However, CaMox fails to support CaMKII binding to NR2B in cosedimentation assays (Fig. 3A). CaMox also failed to compete with GST-actinin for binding to CaMKII (Fig. 3B). These data indicate that CaMox is unable to properly regulate the interactions of CaMKII with neuronal binding partners.

CaMKII does not bind fully oxidized CaM

In order to directly assay whether CaMox binds CaMKII, oxidized or control CaM-agarose was incubated with purified CaMKII in the presence or absence of Ca^{2+}, washed extensively, and collected by centrifugation. Bound protein was eluted directly into SDS sample buffer, and SDS–PAGE analysis revealed purified CaMKII only in the non-oxidized CaM-agarose pellet, in a Ca^{2+}-dependent

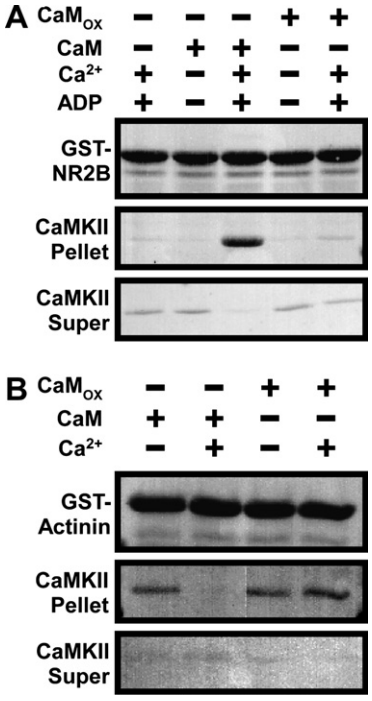

Fig. 3. Oxidized calmodulin does not properly regulate CaMKII interaction with synaptic proteins. (A) CaMKII was incubated with GST-NR2B and glutathione agarose in the presence or absence of oxidized or control calmodulin, 2 mM Ca^{2+}, or 100 μM ADP, washed extensively, and eluted in SDS sample buffer. Supernatant (Super) and eluate (Pellet) were separated by SDS–PAGE and analyzed by Ponceau stain. (B) GST pulldown of CaMKII as in (A) using GST-actinin in the absence of nucleotides.

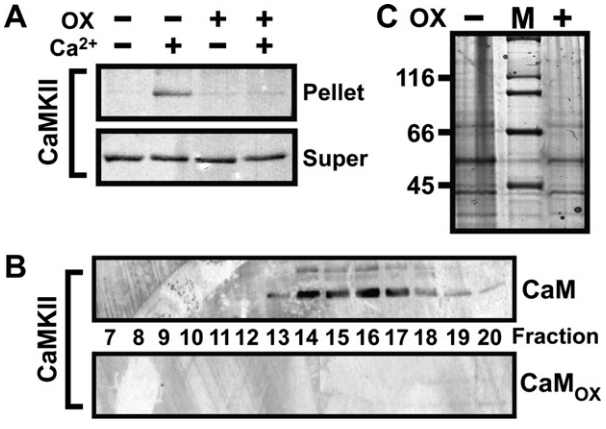

Fig. 4. Oxidized calmodulin does not efficiently bind to CaMKII. (A) Purified CaMKII was incubated with oxidized (OX, +) or control (OX, −) calmodulin agarose in the presence of 2 mM Ca^{2+} (Ca^{2+}, +) or 2 mM EDTA (Ca^{2+}, −), washed extensively, and eluted in SDS sample buffer. Supernatant (Super) and eluate (Pellet) were separated by SDS–PAGE and analyzed by Ponceau stain. (B) Control (top) or oxidized (bottom) calmodulin agarose was incubated with rat brain lysate in the presence of 2 mM Ca^{2+}, washed extensively, and eluted in 200 μl fractions with 5 mM EDTA. Fractions were separated by SDS–PAGE and analyzed by Western blot for CaMKII. (C) Rat brain lysate was passed over reduced or oxidized CaM-agarose. Resin was washed with 0.5 M NaCl buffer and eluted with EDTA. Total elution from reduced (−) or oxidized (+) resin is shown. M: broad range markers.

manner, and no stable binding at all to the oxidized CaM-agarose was observed (Fig. 4A).

CaMKII from rat brain lysate was also tested for binding to CaMox. Soluble extract was prepared from homogenized adult rat brain and passed over columns of fully oxidized or control CaM-agarose in the presence of Ca^{2+} [22]. Resin was washed and proteins stably bound to the CaM-agarose were eluted with EDTA. Fractions containing the eluted proteins were analyzed by SDS–PAGE and Western blot. CaMKII bound to the control CaM column and was eluted with EDTA. However, CaMKII was not detected in fractions eluted from the CaMox-agarose (Fig. 4B).

To demonstrate that oxidized CaM-agarose is intact and able to interact with some proteins, total elution from reduced CaM-agarose and oxidized CaM agarose after incubation with rat brain lysate are shown. Even after oxidation, several neuronal proteins are clearly capable of binding to the oxidized CaM-agarose (Fig. 4C).

Discussion

We show here that progressive oxidation of CaM prevents stable binding to CaMKII. The effects of this are fourfold: (1) Ca^{2+}-dependent activity toward exogenous substrates is not stimulated by CaMox; (2) autophosphorylation at Thr^{286} is not stimulated by CaMox; (3) autophosphorylation at $Thr^{305/6}$ is not prevented by CaMox; and (4) CaMox cannot support binding to NR2B or disrupt the interaction with α-actinin.

Previous studies have shown that all nine methionine residues of CaM can be oxidized *in vitro,* and at least two residues can be oxidized *in vivo* during normal aging. The reduction in CaMKII activation by progressively oxidized CaM could not be attributed to reduction or accumulation of any single band of CaM as visualized by SDS–PAGE. It is therefore possible that either the reduction in CaMKII activation results from the progressive oxidation of a series of methionine residues within CaM, or that shifts in apparent molecular weight of CaM represent a variety of separate, overlapping patterns of methionine oxidation, with oxidation of only one or two specific residues contributing to the reduction in CaMKII activation. Further experiments, including mutation of methionine residues to non-oxidizable amino acids individually and in groups, will be required to resolve this issue [15]. These effects of CaM oxidation on CaMKII are distinct from previous reports of the effects of oxidation on other targets of CaM. For example, the PMCA is partially activated by fully oxidized CaM with little change in apparent binding affinity [15]. In contrast, oxidation reduces the apparent affinity of CaM for CaMKII and also abolishes CaMKII activation (Fig. 1). The differential effect of CaM oxidation in binding to these targets presumably results from differences in the mechanism of interaction.

Blockade of CaMKII activity has deleterious effects in multiple physiological processes. For example, pharmaco-

logical blockade of CaMKII activity in the hippocampi of mice prevents multiple types of memory formation [23,24]. Additionally, in the mouse model, blockade of CaMKII activity in the heart can limit sarcoplasmic reticulum Ca^{2+} release, a process known to be involved in triggered arrhythmias [5,25]. Thus, physiological processes involving the generation of reactive oxygen species may result in concomitant deleterious effects on heart function or memory formation in part due to the oxidation of CaM. This oxidation in turn could result in loss of CaMKII activity as well as potential mislocalization due to improper regulation of CaMKII/CaMKAP interactions. Thus, CaM may act as a metabolic redox sensor [6], differentially affecting downstream targets, to modulate the cell's overall metabolic activity during normal aging or under ischemic conditions. In this model, one could envision CaMox acting to negatively regulate the activity of CaMKII toward targets that directly affect calcium feedback during times of disrupted calcium homeostasis.

Additional in depth experiments will be required to specifically identify individual oxidized methionines responsible for the loss of the ability of CaM to activate CaMKII. This type of information is crucial for interpreting disrupted signaling cascades during times of oxidative stress.

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