Ischemia-elicted Oxidative Modulation of Ca^{2+}/Calmodulin-dependent Protein Kinase II*

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Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) plays a critical role in neuronal signal transduction and synaptic plasticity. Here, we showed that this kinase was very susceptible to oxidative modulation. Treatment of mouse brain synaptosomes with H_{2}O_{2}, diamide, and sodium nitroprusside caused aggregation of CaMKII through formation of disulfide and non-disulfide linkages, and partial inhibition of the kinase activity. These CaMKII aggregates were found to associate with the post synaptic density. However, treatment of purified CaMKII with these oxidants did not replicate those effects observed in the synaptosomes. Using two previously identified potential mediators of oxidants in the brain, glutathione disulfide S-monoamide (GS-DSMO) and glutathione disulfide S-dioxide (GS-DSDO), we showed that they oxidized and inhibited CaMKII in a manner partly related to those of the oxidant-treated synaptosomes as well as the ischemia-elicted oxidative stress in the acutely prepared hippocampal slices. Interestingly, the autophosphorylated and activated CaMKII was relatively refractory to GS-DSMO- and GS-DSDO-mediated aggregation. Short term ischemia (10 min) caused a depression of basal synaptic response of the hippocampal slices, and re-oxygenation (after 10 min) reversed the depression. However, oxidation of CaMKII remained at above the pre-ischemic level throughout the treatment. Oxidation of CaMKII also prevented full recovery of CaMKII autophosphorylation after re-oxygenation. Subsequently, the high frequency stimulation-mediated synaptic potentiation in the hippocampal CA1 region was significantly reduced compared with the control without ischemia. Thus, ischemia-evoked oxidation of CaMKII, probably via the action of glutathione disulfide S-oxides or their analogues, may be involved in the suppression of synaptic plasticity.

Ca^{2+}/calmodulin (CaM)²-dependent protein kinase II (CaMKII) is one of the major Ca²⁺-sensing enzymes important in transducing neuronal, hormonal, and electrical signals in brain, heart, and other tissues. In the central nervous system, CaMKII plays a pivotal role in the facilitation of synaptic plasticity, learning, and memory and in activity-dependent developmental processes (1). CaMKII holoenzyme is a dodecamer composed of two stacked hexameric rings, in which each catalytic/regulatory domain from the upper ring interacts with the equivalent catalytic/regulatory domain in the lower ring by an antiparallel coiled-coil, which resides in regulatory domains (2). Binding of Ca^{2+}/CaM to the regulatory domain separates the dimer pair and causes the exposure of Thr-286 or Thr-287 (within α or β subunit) for phosphorylation by another catalytic domain in the same ring. This inter-subunit phosphorylation of Thr-286/287 converts the kinase into a high affinity binding protein for Ca^{2+}/CaM, and the kinase becomes an activator-independent autonomous enzyme (3). The autophosphorylation also leads to increased affinity of the kinase for several proteins near the sites of elevated Ca^{2+} with functional consequences (4, 5).

In the brain, translocation and clustering of CaMKII have been implicated in the NMDA receptor-dependent enhancement of synaptic plasticity (6–9) as well as in neurological disorders associated with ischemic injury (10–12) and seizure (13). The activity-dependent translocation of CaMKII from dendritic cytoplasm to the stimulated synapses may serve as a tag for long term strengthening of synaptic connection (14, 15). Stimulation of NMDA receptors by excitatory amino acids increases postsynaptic Ca^{2+} influx, which facilitates the activation and autophosphorylation of CaMKII and its interaction with the NR2B subunit of NMDA receptors and other proteins associated with the postsynaptic density (16–18). During ischemic stress and seizure-induced neuronal excitation, clustering and inactivation of CaMKII within soma and neuronal processes have been proposed as a neuroprotective mechanism, which would limit excessive kinase activity during episodes of Ca²⁺-overload (7). CaMKII holoenzyme has been shown to be capable of forming higher order complexes by self-association in the presence of Ca²⁺/CaM and slightly acidic pH (19). Using live-cell imaging, Hudmon et al. (20) showed that stimulation of glutamate receptors induced translocation of green fluorescent protein-CaMKII holoenzyme to synaptic and non-synaptic sites.

Although stimulation of NMDA receptors causes an increase in intracellular Ca^{2+} and a slight acidification of cellular cytoplasm (21, 22), a condition conducive for self-association, it also increases production of reactive oxygen species, such as nitric oxide (23) and superoxide (24). Especially during ischemic and excitotoxic stress, a sustained elevation of intracellular Ca^{2+}
and energy depletion causes uncoupling of mitochondrial oxidative phosphorylation that generates a high level of these reactive oxygen species from neurons as well as from glia cells (25, 26). It is conceivable that these reactive oxygen species could modulate CaMKII activity by direct oxidation of this protein, which constitutes 1–2% of total brain protein (27), as well as by oxidation of its activator, CaM (28). Previously, we showed that activation of NMDA receptors or direct administration of oxidants to the brain slices could trigger thionylation of neurogranin and formation of intra-molecular disulfide of this protein (29, 30). The oxidant-induced modification of protein was hypothesized as a result of oxidation by the endogenously produced glutathione disulfide S-oxides (GS-DSOs), which have a high activity to form glutathione-protein-mixed disulfide (glutathionylation) and inter- and/or intra-molecular disulfide bonds of proteins (31–33).

Although regulation of CaMKII by phosphorylation/dephosphorylation, aggregation, and association with other proteins has been extensively investigated, direct modulation of this enzyme by oxidant has not been explored. In this study we showed that CaMKII in mouse brain synaptosomes, and in the purified preparation, is very susceptible to oxidant-mediated modification. Interestingly, a similar oxidative modification of CaMKII was observed in the acutely prepared mouse hippocampal slices under ischemic conditions. Using two potential mediators of oxidants in the brain, glutathione disulfide S-monoxide (GS-DSMO) and glutathione disulfide S-dioxide (GS-DSDO), we demonstrated that the purified CaMKII oxidized by these two compounds exhibited certain characteristics resembling those modified enzymes in the oxidant-treated synaptosomes and in the ischemic hippocampal slices. The ischemia-evoked oxidation of CaMKII was not readily reversed by re-oxygenation, and this lingering oxidative modification also suppressed the autophosphorylation of the kinase and synaptic potentiation post ischemia.

**Experimental Procedures**

*Materials—*Use of animals was approved by the NICHD, National Institutes of Health Animal Care and Use Committee. The materials used in the study were obtained from the indicated sources: sodium nitroprusside (SNP), diamide, bovine serum albumin, glutathione (GSH), glutathione disulfide, iodoacetamide, and phenylmethanesulfonyl fluoride (PMSF) from Sigma-Aldrich; H2O2 from Fisher Scientific; 35S/GSH from American Radiolabeled Chemicals Inc.; Percoll, CaM-Sepharose 4B, and MicroSpin G-25 column from Amersham Biosciences/GE Healthcare; a 95% N2/5% CO2 gas cylinder from Roberts Oxygen; Bradford protein assay reagent and horseradish peroxidase-conjugated goat anti-mouse IgG from Bio-Rad; autocamtide-2 from Bachem; CaM from Calbiochem; mouse monoclonal antibody against glutathionylated protein from ViroGen; mouse antibodies against α and β CaMKII from Zymed Laboratories Inc.; and mouse antibody against Thr-286-P0, α CaMKII from Promega.

*Preparation of Mouse Brain Synaptosomes and Treatment with Oxidants—*Mouse forebrains were homogenized using a Teflon glass homogenizer with 10 volumes (v/w) of ice-cold solution containing 0.32 M sucrose, 1 mM EDTA, and 0.5 mM DTT (pH 7.4) according to Dunkley et al. (34). The homogenate was centrifuged at 1,000 × g for 10 min, and 2-ml aliquots of the supernatant (adjusted to 5 mg/ml) were layered on top of each discontinuous 4-step Percoll gradient comprising 2 ml each of 23, 15, 10, and 3% Percoll in sucrose solution made up in 10-ml polycarbonate tubes. After centrifugation at 32,500 × g for 5 min, the fractions concentrated at interface of 10/15% and 15/23% Percoll were collected, diluted 2-fold with washing buffer (5 mM Heps, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.25 mM EDTA, 0.25 mM EGTA, and 0.5 mM DTT), and centrifuged at 15,000 × g for 15 min. The pellet was washed twice with 5-ml washing buffer and suspended in oxygenated (95% O2/5% CO2) ACSF (in mM: NaCl 124, KCl 4.9, MgSO4 1.3, CaCl2 2.5, KH2PO4 1.2, NaHCO3 25.6, and d-glucose 10, pH 7.4) containing 0.1 mM DTT. After incubation at 37 °C for 30 min, synaptosomes (0.5 mg/ml) were treated with H2O2, diamide, and SNP for 2–10 min. For assay of CaMKII, samples were immediately centrifuged at 20,000 × g for 5 min at 4 °C, and the pellet was kept frozen in dry ice. Synaptosomal homogenates were prepared by sonication of the pellet in Buffer A (50 mM Heps, pH 7.5, containing 2 mM EDTA, 2 mM EGTA, 50 mM KF, 5 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, 50 mM okadaic acid, 0.1% Nonidet P-40, 0.1 mM PMSF, and 5 μg/ml each of leupeptin, aprotinin, pepstatin A, and chymostatin), and centrifuged at 20,000 × g for 5 min to collect the supernatant for activity measurement. For immunoblot analysis, iodoacetamide (10 mM) was added to the oxidant-treated samples and centrifuged to collect the pellet. Synaptosomal homogenates were prepared by sonication of the pellet in Buffer A plus 10 mM iodoacetamide. Samples (30 μg) were resolved by 8–16% SDS-gel under reducing and non-reducing condition (boiled for 5 min in SDS-PAGE sample buffer with and without mercaptoethanol, respectively), transferred to nitrocellulose membrane, and probed with the various antibodies.

*Isolation of Postsynaptic Density from Oxidant-treated Synaptosomes—*The PSD was prepared by a procedure modified from the previously described methods (35, 36). Briefly, freshly prepared synaptosomes (3 mg each, 0.5 mg/ml) were treated with H2O2 (0.5 mM), diamide (0.1 mM), or SNP (0.1 mM) for 10 min in oxygenated ACSF. The reaction was terminated with iodoacetamide (10 mM) and immediately centrifuged in Eppendorf Micro centrifuge at 7,500 rpm for 5 min at 4 °C. The pellets were suspended in 2.5 ml of Buffer B (12 mM Tris-Cl, pH 8.1, containing 32 mM sucrose and 1% Triton X-100) and stirred at 4 °C for 15 min. The solution was centrifuged at 33,000 × g for 20 min, and the resulting pellets were resuspended in 200 μl of the same buffer. The samples were layered onto discontinuous sucrose gradient containing 2 ml of 1.5 mM sucrose/1 mM NaHCO3 and 2.8 ml of 1 mM sucrose/1 mM NaHCO3 and centrifuged at 200,000 × g for 2 h at 4 °C using a Beckman SW65 rotor. The resulting pellets were suspended in 1 ml of Buffer B plus 1 ml of 1% Triton X-100/150 mM KCl and centrifuged at 200,000 × g for 60 min using a Beckman 70-Ti rotor. The resulting PSD was dispersed by sonication in 50 μl of synaptosomal homogenization buffer, and samples (5 μg of protein) were analyzed by immunoblot.
Preparation of Mouse Hippocampal Slices and Induction of Ischemic Stress—Mouse transverse hippocampal slices (400 μm) were prepared from adult mice as previously described (37) and were kept in oxygenated ACSF at room temperature for 1–2 h for recovery after slicing. To induce ischemic stress, slices were incubated in a modified ACSF containing 10 mM sucrose (to replace glucose) previously saturated with 95% N₂/5% CO₂ based on the established hippocampal slice model of ischemia (38). At timed intervals samples (4–8 slices) were removed from the medium and immediately homogenized in 400 μl of Buffer A plus 10 mM iodoacetamide. Homogenates were centrifuged at 35,000 × g for 30 min at 4 °C, and the pellet was sonicated in 200 μl of the same buffer containing 1% SDS. Equivalent amount of proteins from the soluble and pellet fractions were resolved by reducing and non-reducing 8–16% gradient SDS-gel, transferred to nitrocellulose membrane, and probed with anti-α CaMKII antibody. For measurement of the kinase activity, tissue slices were kept frozen immediately after removal of the medium. Slices were homogenized in Buffer A, and the homogenates (1–2 μg) were assayed for CaMKII activity with or without Ca²⁺/CaM with autocamtide-2 as a substrate.

Electrophysiology—Hippocampal slices were placed in a submerged recording chamber using glass electrodes (1–4 MΩ), filled with ACSF, for stimulation of Schaffer collateral/commissural fibers and for recording of field EPSP (fEPSP) from the stratum radiatum of the CA 1 region. Slices were superfused with ACSF at a flow rate of ~2 ml/min, and a test pulse was delivered at 20-s intervals with a current that evoked ~30% of the maximal response. After establishing a stable baseline with oxygenated ACSF, slices were subjected to ischemia by perfusion with modified ACSF saturated with 95% N₂/5% CO₂ for 10 min and switched back to oxygenated ACSF. After re-establishment of stable baseline for at least 10 min, the slices were subjected to high frequency stimulation (HFS) at 100 Hz for 1s. The control slices were perfused only with oxygenated ACSF and stimulated with the same HFS. Potentials were digitized by CED Power 1401 (Cambridge Electronic Design) and analyzed by Signal 2 software (Cambridge Electronic Design). The slope of the fEPSP was used to measure the responses. Potentiation after HFS was expressed as percentage of the initial baseline recording. For comparison between responses of different samples after HFS, the last 5-min blocks of recording were analyzed.

Purification and Assay of CaMKII—Fresh mouse brain, after removal of cerebellum and brain stem, was homogenized with Teflon glass homogenizer in 10 volumes (v/w) of buffer (20 mM Hepes, pH 7.5, containing 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMFSF, 5 μg/ml soybean trypsin inhibitor, and 20 μg/ml leupeptin), and the homogenate was centrifuged at 100,000 × g at 4 °C for 1 h. The resulting pellet was homogenized again in the same buffer (one-half of the original volume) and centrifuged to remove the pellet. The combined supernatant was precipitated with solid ammonium sulfate (60% saturation), centrifuged at 35,000 × g for 15 min, and the precipitated protein dissolved in Buffer C (50 mM Hepes, pH 7.5, containing 1 mM DTT, 0.5 mM CaCl₂, 100 mM NaCl, 3 mM magnesium acetate, and 0.1 mM PMFSF). The solution was clarified by centrifugation and pressed through a 0.45-μm filter, loaded onto a CaM-Sepharose column, and washed successively with five bed volumes each of Buffer C, Buffer C plus 1 M NaCl, and Buffer C again. CaMKII was eluted from the column with 50 mM Hepes, pH 7.5, containing 1 mM DTT, 2 mM EGTA, and 0.1 mM PMFSF. Fractions containing active kinase were pooled and concentrated by ultrafiltration using Amicon Ultra-4 Ultracel-10k device. The purified enzyme was kept frozen at −20 °C, and purity was checked by SDS-PAGE. CaMKII activity was measured at 30 °C for 2 min in a mixture (25 μl) containing 30 mM Hepes buffer (pH 7.5), 6 mM MgCl₂, 0.12 mM [γ³²P]ATP (1000–2000 cpm/pmol), 1 mg/ml bovine serum albumin, 2 mM EGTA, ±1.2 μM CaM/2.4 mM CaCl₂, and 80 μM autocamtide-2.

Treatment of Purified CaMKII with Oxidants—CaMKII (1 mg/ml, 50–100 μl) was incubated in 10 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol, and 2 mM DTT at room temperature for 30 min to reduce protein sulfhydryl groups. These proteins were then loaded on G-25 MicroSpin columns, which had been equilibrated with the same buffer, and centrifuged at 5000 rpm for 7 s in an Eppendorf Micro centrifuge to separate reduced kinase from DTT. Protein and residual DTT concentrations were determined before addition of oxidants to the indicated concentrations, which were the net after subtraction of the free -SH in the samples. CaMKII (1–2 μg/assay) was incubated with oxidant at room temperature for the indicated times, and the reaction was terminated by addition of iodoacetamide (final 10 mM). Samples were resolved by reducing or non-reducing SDS-PAGE and transferred to nitrocellulose membrane for immunoblot analysis. For assay of CaMKII, samples were taken at the indicated times, and the activity was determined with autocamtide-2 as a substrate. To determine the effects of oxidation on CaMKII autophosphorylation, the enzyme was incubated with oxidants at room temperature for 10 min, and then chilled on ice before initiation of reaction with ice-cold reaction mixture. CaMKII autophosphorylation was assayed on ice for 3 min in a reaction mixture containing 30 mM Hepes buffer, pH 7.5, containing 6 mM MgCl₂, 0.12 mM ATP, 2 mM EGTA, and 1.2 μM CaM/2.4 mM CaCl₂. Reaction was terminated by addition of SDS-PAGE sample buffer with or without reducing agent and analyzed by immunoblot with antibody against Thr-286-PO₄ α CaMKII.

Miscellaneous Methods—Glycerol density gradient centrifugation of CaMKII was carried out according to the procedure of Martin and Ames (39) with an SW-65 rotor. CaMKII or standard protein mixtures, including rabbit muscle aldolase (M₉ 160,000), catalase (M₉ 250,000), and ferretin (M₉ 440,000), were layered on top of a 5–ml 8–30% glycerol gradient in 50 mM potassium phosphate buffer, pH 7.5, and centrifuged at 60,000 rpm at 4 °C for 4 h in a Beckman ultracentrifuge with both the acceleration and deceleration settings at 4. Fractions of 125 μl were carefully removed from the top, and every other fraction was analyzed for CaMKII by immunoblot under reducing or non-reducing conditions. The sedimentation of standard proteins was identified by staining the SDS-gel with Coomassie Blue. Preparation and assay of GS-DMSO and GS-DSDO were carried as previously described (33). Immunoblot analysis was carried out by resolving proteins in 8–16% gradient gels containing 0.1% SDS, followed by transfer to nitrocellulose mem-
Ischemia-elicited Oxidative Modulation of CaMKII

![Graph](image)

FIGURE 1. Effect of oxidant on synaptosomal CaMKII. Mouse forebrain synaptosomes were incubated with H$_2$O$_2$ (0.5 mM), diamide (0.1 mM), SNP (0.1 mM), or without oxidant (control) at 37 °C for 5 min, and the homogenates were measured for CaMKII activity with autocamtide-2 as a substrate with or without Ca$^{2+}$/CaM (panel A). The kinase activity of the control in the presence of Ca$^{2+}$/CaM (total activity) was set at 100% (100 ± 1.7%, n = 6), and that without Ca$^{2+}$/CaM (independent activity) for the control was 17.1 ± 1.0% (n = 6). The activities (total versus independent activities) of the oxidant-treated samples were: H$_2$O$_2$, 61.7 ± 6.6 versus 11.9 ± 0.4%; diamide, 42.7 ± 5.6 versus 14.5 ± 1.2%; and SNP, 73.2 ± 5.6 versus 16.5 ± 0.7%, n = 6. The reductions in the total activity of the oxidant-treated samples were statistically significant, and that of the independent activity was significant for the H$_2$O$_2$-treated one.

Panel B, representative immunoblot analyses (n = 7) of the control (lane 1) and those treated with 0.5 mM H$_2$O$_2$ (lane 2), 0.1 mM diamide (lane 3), and 0.1 mM SNP (lane 4) using antibodies against α CaMKII, β CaMKII, and Thr-286-Po4 α CaMKII under reducing (+ MeSH) and non-reducing (-MeSH) conditions. Note that the immunoreactivities of monomeric α, β, and autophosphorylated α CaMKII were reduced in the oxidant-treated samples under non-reducing conditions, but under the reducing conditions all these species exhibited nearly uniform reactivity.

RESULTS

Oxidative Modification of CaMKII in Mouse Brain Synaptosomes—To test the oxidative modification of CaMKII, we initially employed mouse brain synaptosomal preparation for the in vitro experiments. CaMKII is known to be highly enriched in the synaptosomes and treatment of these fractions with H$_2$O$_2$ (0.5 mM), diamide (0.1 mM), and SNP (0.1 mM) caused an inactivation of the total CaMKII activity as compared with the control (Fig. 1A). The activator-independent activity, with the exception of the H$_2$O$_2$-treated one (~30% reduction), was not significantly altered by these oxidants. It appears that these oxidants preferentially inactivate the non-phosphorylated enzyme. The synaptosomal extracts derived from samples treated with H$_2$O$_2$ (Fig. 1B, lane 2), diamide (Fig. 1B, lane 3), and SNP (Fig. 1B, lane 4) along with un-treated control (Fig. 1B, lane 1) were resolved by 8–16% SDS-gel under reducing and non-reducing conditions. Immunoblot with antibody against α or β CaMKII revealed that these oxidants caused extensive oxidation of the kinase to form large molecular weight aggregates under non-reducing conditions. These aggregates were formed largely by disulfide bonds as revealed by dissolution of these high molecular weight bands under reducing conditions. The immunoreactivities of the monomeric α (50 kDa) and β (60 kDa) subunits were reduced or diminished under non-reducing conditions, and their band intensities were nearly equal under the reducing conditions (Fig. 1B). The synaptosomal extracts exhibited ~15–20% of the Ca$^{2+}$/CaM-independent kinase activity, and the Thr-286-autophosphorylated α CaMKII was also susceptible to oxidative modification. Under non-reducing conditions, the monomeric autophosphorylated α CaMKII was greatly reduced as compared with the control; yet, the activator-independent activity remained largely intact with the exception of that treated with H$_2$O$_2$. Under reducing conditions, the oxidized Thr-286-Po4 α CaMKII reappeared as monomeric form (Fig. 1B). These findings suggest that oxidative modification of CaMKII can promote its aggregation, partly, through formation of disulfide bonds.

Association of the Oxidized CaMKII with the PSD—CaMKII has been shown to associate with the PSD, where it interacts with several postsynaptic structural and signaling proteins. Oxidative modification of the kinase, which undoubtedly will cause a large conformational change, may disrupt the interaction with its binding partners. To investigate if oxidants caused...
CaMKII was incubated with H₂O₂, diamide, and SNP for was a direct effect of those reagents, purified mouse forebrain mine if CaMKII modified by the oxidant-treated synaptosomes modification of CaMKII. Because the control sample also contained this doublet in the PSD, it is indicative that oxidation of oxidation of CaMKII with diamide at 0.05 (Fig. 3A, lane 4) and 0.1 mM (lane 5) caused a more extensive formation of disulfide with no significant change in the total kinase activity, but a significant increase in the activator-independent activity (8.5 ± 0.2%, n = 4, p < 0.001 at 0.1 mM diamide). In contrast, SNAP at 0.05 (Fig. 3A, lane 6) and 0.1 mM (lane 7) exerted no significant effect on disulfide formation, although it also caused an increase in the total kinase activity (120 ± 4.0%, n = 4, at 0.1 mM). When the oxidant-treated CaMKII was reduced, all the disulfide bonds were completely reduced without any trace of 180- to 220-kDa bands, and the kinase exhibited uniform immunoreactivity toward α CaMKII antibody as the control (Fig. 3A, lane 1). These results indicate that the oxidative modifications of CaMKII by these oxidants directly do not mimic those seen in the synaptosomes.

Previously, we showed that treatment of rat brain slices with oxidants could generate reactive oxidized glutathione derivatives, GS-DSMO and GS-DSDO, which caused protein glutathionylation and formation of protein disulfide (29). Incubation of the purified CaMKII with GS-DSMO at 0.1 (Fig. 4A, lane 2) and 0.2 mM (lane 3) and GS-DSDO at 0.05 (lane 4) and 0.1 mM (lane 5) caused different modifications of the kinase when analyzed by immunoblot under non-reducing conditions (Fig. 4A). GS-DSMO was more effective than GS-DSDO at causing α CaMKII to form aggregates (Fig. 4A, center panel) but a relatively low level of glutathionylation (Fig. 4A, left panel). GS-DSDO caused thionylation of α and β CaMKII (left panel) resulting in an increase in the molecular mass as revealed by a slightly retarded electrophoretic mobility and formation of 180- to 220-kDa doublets of α CaMKII. Both GS-DSMO and GS-DSDO were relatively ineffective in causing β CaMKII to form large aggregates (Fig. 4A, right panel). When all these oxidized samples were resolved under reducing conditions, with the exception of the 180- to 220-kDa doublets, all the kinase were migrated as monomeric 50-kDa α and 60-kDa β subunits (data not shown). The stoichiometry of thionylation of the purified CaMKII mediated by 35S-labeled GS-DSDO and GS-DSMO after prolonged incubation was ~3 to 1. Assay of the kinase showed that oxidation of CaMKII by GS-DSMO caused a small reduction in activity to phosphorylate exogenous substrate, whereas GS-DSDO was a more potent inhibitor of the kinase (Fig. 4B). The low level of inhibition of CaMKII mediated by GS-DSMO could be recovered completely by incubation with 10 mM DTT. On the other hand, the GS-DSDO-mediated inhibition of CaMKII activity could only be
partially recovered (~10%) by incubation with 10 mM DTT (Fig. 4C), suggesting that an irreversible modification is largely responsible for the reduction of the kinase activity. These GS-DSOs also caused similar inhibitory effects on the autophosphorylation of CaMKII, when examined by immunoblot with antibody against Thr-286-PO4 α CaMKII, as those on the phosphorylation of autacamtide-2 (data not shown).

Characterization of the Molecular Size of Oxidized CaMKII—
Separation of the oxidized CaMKII by non-reducing SDS-PAGE provided information of the extent of disulfide bond formation without indication of the size of aggregates. To characterize the extent of aggregation and oxidation, the oxidized
CaMKII was subjected to glycerol density gradient (8–30%) centrifugation and analyzed by non-reducing SDS-PAGE and immunoblot with antibody against CaMKII. The purified kinase in the presence of reducing agent is a 500- to 600-kDa dodecamer, which sedimented slightly faster than ferritin (450 kDa) during density gradient centrifugation (Fig. 5A). Under this condition, the kinase did not form inter-subunit disulfide but still caused a small portion of the enzyme to form clusters of various sizes as evidenced by the spreading of the molecule throughout the fractions that were heavier than the dodecamer. Upon removal of DTT, the kinase formed disulfides that increased the extent of clustering to form large aggregates, which sedimented to the bottom of the gradient (Fig. 5B). Addition of Ca\(^{2+}\)/CaM in the absence of reducing agent slightly increased the aggregation (Fig. 5C).

Previously, Hudmon et al. (19) showed that CaMKII underwent self-association in the presence of Ca\(^{2+}\)/CaM (0.5 mM/2 μM), ATP (10 μM), 0.1% Tween 20, and 0.4 mM DTT at slightly acidic pH (pH 6.5) as a result of inter-subunit-interholoenzyme interactions. We demonstrated here that self-association of CaMKII at pH 6.5 was also mediated, in part, by disulfide even though the reaction mixture contained 0.4 mM DTT (Fig. 5D).

Oxidative modification of CaMKII by GS-DSMO caused a high degree of clustering that resulted in the formation of disulfide-linked aggregates, which sedimented at the bottom of the density gradient (Fig. 5E). Addition of Ca\(^{2+}\)/CaM further facilitated the clustering (Fig. 5F). In comparison, GS-DSDO apparently was less potent in causing large CaMKII aggregate formation, but it mediated the thionylation of the enzyme, which resulted in an increase in the molecular weights of the subunit (Fig. 5G). The GS-DSDO-oxidized CaMKII did not seem to be significantly affected in the presence of Ca\(^{2+}\)/CaM (Fig. 5H). These results demonstrated that these disulfide

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**FIGURE 5.** Effect of DTT, GS-DSMO, GS-DSDO, and Ca\(^{2+}\)/CaM on the aggregation of CaMKII. Purified CaMKII (0.1–0.2 mg/ml) after passing through G-25 Micro Spin column to remove DTT was incubated in 10 mM potassium phosphate buffer, pH 7.5, with 1 mM DTT (A), without DTT (B), with Ca\(^{2+}\)/CaM (0.4 mM/2 μM) (C), in 10 mM PIPES, pH 6.5, containing 0.4 mM DTT, Ca\(^{2+}\)/CaM (0.5 mM/2 μM), 10 μM ATP, 10 mM MgCl\(_2\), 20 mM KCl, 0.1 mg/ml bovine serum albumin, and 0.1% Tween 20 (D), with 0.2 mM GS-DSMO (E), 0.2 mM GS-DSDO plus Ca\(^{2+}\)/CaM (0.4 mM/2 μM) (F), 0.1 mM GS-DSMO (G), and 0.1 mM GS-DSDO plus Ca\(^{2+}\)/CaM (0.4 mM/2 μM) (H) at room temperature for 5 min, and the reaction was terminated with 10 mM iodoacetamide. Samples were layered on top of a 8–30% glycerol gradient in 50 mM potassium phosphate buffer, pH 7.5 (A, B, C, E, F, G, and H), or in 50 mM PIPES buffer, pH 6.5 (D), and centrifuged at 60,000 rpm for 4 h. Fractions were analyzed by immunoblot under non-reducing conditions using anti-CaMKII antibody. The locations of aldolase (α, 160 kDa), catalase (c, 250 kDa), and ferritin (f, 440 kDa) were revealed by protein staining after SDS-PAGE. Experiments in panel D were repeated twice and the rest three times.
oxides are potent modifiers of large CaMKII aggregate formation.

Oxidative Modification of the Autophosphorylated CaMKII—Purified mouse forebrain CaMKII exhibited very little Ca\(^{2+}/\)CaM-independent activity (<1%), and upon autophosphorylation the independent activity increased to ~70% of the total activity. Incubation of the autophosphorylated kinase with 0.2 mM GS-DSMO caused a slight increase in the activity and with 0.1 mM GS-DSDO caused nearly a 60% inhibition of both the total and independent activity (Fig. 6).

In contrast to the non-phosphorylated CaMKII, oxidation of the autophosphorylated CaMKII by GS-DSMO did not form high order aggregates, which would sediment to the bottom of the density gradient during centrifugation (Fig. 7A). Under reducing conditions, the majority of the inter-subunit disulfides were reduced to a monomer and traces of 180- to 220-kDa CaMKII-immunoreactive bands remained in this autophosphorylated kinase (Fig. 7B). Blotting with Thr-286-PO₄ CaMKII antibody confirmed that the autophosphorylated CaMKII was not prone to form large aggregates as was the non-phosphorylated enzyme (Fig. 7C). Oxidation of the autophosphorylated CaMKII with GS-DSDO caused glutathionylation and formation of low order aggregates (Fig. 7D). Under reducing conditions, the aggregates were reduced; however, two sets of doublet consisting of 180–220 kDa and >250 kDa

FIGURE 6. Effect of GS-DSMO and GS-DSDO on the kinase activity of the autophosphorylated CaMKII. Purified CaMKII (0.1 mg/ml) was autophosphorylated on ice for 10 min under the standard conditions, and the reaction was stopped with 2 mM EGTA and 6 mM EDTA. Autophosphorylated CaMKII was incubated with 0.2 mM GS-DSMO or 0.1 mM GS-DSDO for 5 min at 30 °C. Aliquots of the reaction mixtures were taken for assays of the kinase activity with Ca\(^{2+}/\)CaM (total activity) or with EGTA without activator (independent activity). The independent activity of the purified kinase was <1%, and the autophosphorylated one was ~70%. The kinase activities (pmol/µg/min, total versus independent activity) of the various samples were: CaMKII before autophosphorylation, 1732 ± 30 versus 4.6 ± 0.3; PO₄-CaMKII, 1455.6 ± 34.4 versus 1134 ± 53; PO₄-CaMKII plus GS-DSMO, 1570 ± 44 versus 1217 ± 42; and PO₄-CaMKII plus GS-DSDO, 575.4 ± 68 versus 397 ± 76 (n = 9). Compared with the autophosphorylated enzyme, the reduction of the total and independent activities was significant only for the GS-DSDO-treated one.

FIGURE 7. Density gradient centrifugation of the autophosphorylated CaMKII following treatment with GS-DSMO and GS-DSDO. Autophosphorylated CaMKII (0.5 mg/ml) was incubated with 0.2 mM GS-DSMO (left panel) or 0.1 mM GS-DSDO (right panel) at room temperature for 5 min, the reaction was terminated by iodoacetamide (10 mM), and samples were layered on top of 8–30% glycerol gradient in 50 mM potassium phosphate buffer, pH 7.5. Centrifugation was at 60,000 rpm for 4 h. Fractions were taken for analyses under non-reducing (A and D) or reducing conditions (B, C, E, and F) and immunoblot with anti-α CaMKII (A, B, D, and E) or anti-Thr-286-PO₄ α CaMKII (C and F). A set of representative blots from two experiments were shown.
Ischemia-elicited Oxidative Modulation of CaMKII

The extent of oxidation of CaMKII was studied under various conditions. Tissue slices were exposed to ischemic insults (95% N₂/5% CO₂-saturated air) for 1–2 h at room temperature for functional recovery before exposure to ischemic conditions. The increase in these doublets in the hippocampal slices under ischemic conditions (Fig. 8B) exhibited a significant increase over zero time control (58.4 ± 2.6%, n = 8/4, namely, eight measurements from four separate experiments) after 5 min (65.4 ± 1.7%), 10 (64.2 ± 1.5%), 15 (64.1 ± 1.8%), and 20 min (64.5 ± 1.4%) of ischemia (Fig. 8C, right panel). At 30 min, the increase in the oxidized form (62.1 ± 2.3%, n = 8/4) was not significant. Under the reducing conditions, the majority of the oxidized forms was reduced and, again, the characteristic 180- to 220-kDa doublets appeared (Fig. 8B, right panel). After 5 min of ischemia, these doublets increased by nearly 2-fold (from 12 ± 2.3%, n = 8/4, at zero time to 25.9 ± 2.4%, n = 8/4, at 5 min) (Fig. 8C, right panel) and gradually declined thereafter. The increase in these doublets in the hippocampal slices under ischemia mirrored those of the oxidant-treated synaptosomes (Fig. 1B). Immunoblot of the ischemic slices with antibody against glutathionylated protein failed to detect thionylation of CaMKII (data not shown), and there was no detectable electrophoretic mobility shift of α CaMKII under non-reducing SDS-PAGE (Fig. 8, A and B). These results suggest that oxidative modifications of α CaMKII do not involve thionylation, and the formation of disulfide- and non-disulfide-linked aggregates may occur under physiological conditions.

Effect of Ischemia on Basal Synaptic Response and Plasticity—

Recording of the synaptic response of the hippocampal CA1 region showed that ischemia caused a rapid depression of the basal response that reached near zero within 10 min, and re-oxygenation recovered the normal response in 10 min (Fig. 9A). Oxidation of CaMKII measured by the elevation of 180- to 220-kDa doublets reached near maximum at 5 min and was maintained at elevated levels throughout the ischemia/re-oxygenation.
Ischemia-elicited Oxidative Modulation of CaMKII

**FIGURE 9.** Ischemia/re-oxygenation mediated oxidation and phosphorylation of α CaMKII and synaptic responses. A, basal synaptic responses. fEPSPs of control (○, n = 7) slices and those slices subjected to 10-min ischemia (indicated by underline) followed by re-oxygenation (○, n = 10) were monitored by test pulse delivered at a current −30% of the maximal response. Representative traces of the fEPSP at 0, 10, and 30 min time points are shown. B, quantification of the 180- to 220-kDa bands. Tissue slices were subjected to ischemia for 5 min and returned to oxygenated ACSF for 20 (5 + 20 R) and 30 (5 + 30 R) min or 10-min ischemia and re-oxygenated for 10 (10 + 10 R) and 20 (10 + 20 R) min. The 180- to 220-kDa bands in the particulate fractions were quantified, and the levels of all the treated samples (ranging from 20 to 28%) were significantly greater than the control (8.7 ± 1.5%) (p < 0.001, n = 7/4). C, CaMKII activity and autophosphorylation. Total (plus Ca2+/CaM) and autonomous (plus EGTA) kinase activities were determined of those slices without (0 time) or subjected to 5 and 10 min of ischemia, and 10-min ischemia followed by 10 (10 + 10R) and 20-min re-oxygenation (10 + 20R). The activities (total versus autonomous, picomoles/μg/min) were: 0 time, 94.4 ± 1.85 versus 42.1 ± 1.19; 5 min, 87.1 ± 1.43 versus 28.4 ± 1.1; 10 min, 81.5 ± 3.35 versus 13.9 ± 2.42; 10 min plus 10R, 79.1 ± 1.76 versus 28.3 ± 2.18; and 10 min plus 20R, 86.8 ± 6.44 versus 30.6 ± 4.94, n = 6. The inset shows a set of representative immunoblot of PO4-α CaMKII and α CaMKII of treated tissue slices (n = 6). D, HFS-induced LTP. Both the control (○, n = 6) and those subjected to 10-min ischemia and followed by 20-min re-oxygenation (○, n = 8) were stimulated by 1 × 100 Hz for 1 s. Representative traces of the fEPSP before (1) and 30 min after (2) HFS are shown.

**DISCUSSION**

CaMKII is one of the most abundant proteins in the brain, and it functions as a protein kinase as well as an anchor for interaction with several proteins, including neurotransmitter receptors and cytoskeletal proteins. One of the most intriguing properties of this kinase is its ability to form large aggregates in neuronal dendrites and soma under a variety of conditions (6, 7, 12, 40, 41). The formation of these aggregates is isozyme-specific and is believed to be regulated by pH and ATP concentration. It was proposed that these CaMKII aggregates were formed via non-covalent inter-holoenzyme catalytic and auto-regulatory interactions that accompanied with an inhibition of the kinase activity (42, 43). In this study we demonstrated that an oxidative mechanism may also contribute to the aggregation and inhibition of CaMKII. The α and β subunits of CaMKII...
contain several (10 and 12, respectively, in mouse enzyme) cysteine residues that may be susceptible to oxidative modification. We unexpectedly found that, even under acidic conditions (pH 6.5), low ATP concentration, and in the presence of 0.4 mM DTT the aggregation of CaMKII as described by Hudmon et al. (19) occurred, in part, by disulfide formation (Fig. 5D). Generally, formation of disulfide under acidic condition is not favorable unless the local tertiary structure is concordant to such an event.

Certain cysteine residue in α CaMKII appears to be very susceptible to oxidation to form aggregates involving inter-subunit disulfides. In contrast, the cysteine residues in β isoform, although susceptible to thionylation, are not favorable in forming disulfide with its own or with the α subunit in the purified enzyme. However, this does not exclude the possibility that the β CaMKII can form mixed disulfide with other protein. This is best illustrated in the oxidant-treated synaptosomes, in which β CaMKII also participates in forming large aggregates (Fig. 1B). One of the likely candidates of the β subunit disulfide partner is actin, which has been shown to bind this subunit and is responsible for targeting CaMKII α/β hetero-oligomers to dendritic spines and in the regulation of neurite extension and synapse formation (44, 45). Similarly, although the autophosphorylated α CaMKII is less likely to form self-cluster, due to its increased affinity for several PSD-associated proteins, including NR2B subunit of NMDA receptors (5, 16, 46), it can form large aggregates containing mixed disulfides (Fig. 1B). In the acutely prepared hippocampal slices, the disulfide-linked α CaMKII accounted for nearly 30–40% in the soluble fraction (Fig. 8A) and over 50% in the particulate fraction (Fig. 8B) of the total enzyme in the hippocampus. This high percentage of the disulfide-linked α CaMKII in the control tissue suggests that several cysteine residues in this dodecameric enzyme are very susceptible to oxidation. It seems likely that generation of oxidants during normal neurotransmission is sufficient to cause CaMKII to form aggregates. Under ischemic conditions, the extent of oxidation was further increased. Thus, the magnitude of CaMKII aggregation could be regulated by changes in intracellular pH as well as in the redox potential.

Treatment of synaptosomes with H₂O₂, diamide, and SNP caused CaMKII to form disulfide- and non-disulfide-linked aggregates (Figs. 1 and 2), but these modifications could not be reproduced by the same treatment of the purified enzyme with these oxidants. In contrast, oxidation of purified CaMKII with GS-DSOs could generate those characteristic aggregates seen in the oxidant-treated synaptosomes and in the hippocampal slices subjected to ischemic stress (Fig. 8). These findings suggest that GS-DSOs or their analogues are the causative oxidants. These GS-DSOs not only cause CaMKII to form self-aggregates, and, undoubtedly, in vivo they also trigger hetero-molecular cross-linking between CaMKII and other proteins. Cross-linking of CaMKII and proteins associated with the PSD may play a critical role in neurotransmission and signal amplification. Microsequencing of the non-disulfide-linked immunoreactive bands (180- to 220-kDa species) confirmed that they were derived from CaMKII; the nature of the covalent modification, however, has yet to be determined. Serendipitously, the formation of these 180- to 220-kDa α CaMKII immunoreactive bands may serve as markers of oxidation of this enzyme in vivo.

There are at least three potential oxidative mechanisms of CaMKII, including thionylation and formation of disulfide- and non-disulfide-linked CaMKII aggregates. Treatment of purified CaMKII with diamide at concentration <0.1 mM mainly caused disulfide-linked aggregates with no inhibition of the kinase activity (Fig. 3), an indication that formation of inter-subunit disulfide is not a harmful event. Treatment of CaMKII with GS-DSMO caused aggregation and thionylation of CaMKII, and the resulting low level of inhibition could be recovered by DTT, suggesting that these disulfide-linked modifications are reversible. On the other hand, incubation of CaMKII with GS-DSDo caused thionylation and formation of irreversibly oxidized forms that resulted in a higher degree of inhibition than that by GS-DSMO. The inhibition could only be recovered partially by DTT, suggesting that formation of irreversibly oxidized species is responsible for the bulk of inhibition. In the oxidant-treated synaptosomes or hippocampal slices subjected to ischemia, there was no detectable glutathionylation determined by antibody against glutathionylated protein or by electrophoretic mobility shift assay under non-reducing SDS-PAGE. Thus, it seems plausible that the reduction in the kinase activity in the treated synaptosomes and hippocampal slices results from formation of the non-disulfide-linked species (namely, 180- to 220-kDa doublets). This later modification, which reduces the capacity of CaMKII to undergo autophosphorylation, is a likely cause of the depressed HFS-induced LTP following ischemia/re-oxygenation. It is also possible that impairment of NMDA receptors resulting from S-nitrosylation and disulfide formation (47, 48) may also contribute to the attenuated response in LTP.

Exposure of hippocampal slices to the ischemic conditions deprives the supply of oxygen and glucose, CaMKII became oxidized and dephosphorylated, and the total kinase activity was reduced. Upon re-oxygenation, the oxidized kinase persisted and the total CaMKII activity remained depressed at below the pre-ischemic level; however, autophosphorylation of CaMKII underwent dynamic changes in these processes (Fig. 9C). It seems that transient ischemia/re-oxygenation causes a dynamic change in protein phosphorylation and dephosphorylation resulting from alteration in metabolic activity depending on the supply of ATP. The ischemia/re-oxygenation-mediated dynamic changes in the basal synaptic response, which is a measure of the glutamate receptor-mediated post-synaptic depolarization, are likely caused by the alteration in metabolic activity. Thus, oxidation of CaMKII and the resulting impairment of CaMKII activity and autophosphorylation may not play a significant role in the basal synaptic response.

Autophosphorylation of CaMKII at Thr-286 within the auto-regulatory domain opens up the catalytic domain yielding autonomous activity and enhances Ca²⁺/CaM binding. The opening of the catalytic domain may prevent the formation of large aggregates. Thus, oxidative modification of the autophosphorylated CaMKII by GS-DSOs primarily caused a low order of aggregation. The reduced tendency of the autophosphorylated CaMKII to form large aggregates is similar to that of the autophosphorylation mimicking mutant, green fluorescent protein or by electrophoretic mobility shift assay under non-reducing SDS-PAGE. Thus, it seems plausible that the reduction in the kinase activity in the treated synaptosomes and hippocampal slices results from formation of the non-disulfide-linked species (namely, 180- to 220-kDa doublets). This later modification, which reduces the capacity of CaMKII to undergo autophosphorylation, is a likely cause of the depressed HFS-induced LTP following ischemia/re-oxygenation. It is also possible that impairment of NMDA receptors resulting from S-nitrosylation and disulfide formation (47, 48) may also contribute to the attenuated response in LTP.

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Ischemia-elicited Oxidative Modulation of CaMKII

protein-Thr-286Asp-CaMKII, expressed in HEK293 cells under the acidic conditions (20). Because the formation of large aggregates mediated by oxidation with GS-DSMO did not significantly inhibit the autophosphorylation of CaMKII, we predict that the disulfides between two CaMKII subunits do not directly involve the catalytic and regulatory sites. Future study to map the site(s) of oxidation by single and combinatorial mutation of the 10 cysteine residues within α-CaMKII will provide insight to delineate the mechanisms of aggregation mediated by oxidation versus that by low pH.

The animal brain, which consumes nearly 20% of the total oxygen intake, is especially active in generating oxidants during neurotransmission or encountering pathological conditions. Nitric oxide is synthesized through activation of Ca²⁺/CaM-dependent NO synthase following stimulation of NMDA receptors that causes Ca²⁺ influx. Superoxide is generated by xanthine oxidase, NADPH oxidases, and release from the mitochondria of oxygen intake, is especially active in generating oxidants during ischemia-elicited Oxidative Modulation of CaMKII actions (51) could account for the severe inactivation of the kinase under pathological conditions can be signaling molecules, but under pathological conditions they cause cellular damages. It is expected that CaMKII in the brain will be present in a normal physiological conditions can be signaling molecules, but under pathological conditions they cause cellular damages. It is expected that CaMKII in the brain will be present in a dynamic equilibrium between the oxidized and reduced form. It is expected that CaMKII in the brain will be present in a dynamic equilibrium between the oxidized and reduced form.

The autophosphorylated subunit may interact with its binding partners, including NMDA receptors and cytoskeletal proteins, to reorganize the synapse and to modify synaptic efficacy. However, under the pathological conditions, such as cerebral ischemia, over-stimulation of the neurotransmitter receptors may cause extensive oxidation of CaMKII. Thus, a combination of oxidative and intracellular acidification under these conditions (51) could account for the severe inactivation of the kinase and accumulation of both the synaptic and non-synaptic CaMKII aggregates (11, 12, 40, 52–54).

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