Nitric Oxide Induces Ca\(^{2+}\)-independent Activity of the Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II (CaMKII)\(^\ast\)

Received for publication, February 14, 2014, and in revised form, May 9, 2014 Published, JBC Papers in Press, May 22, 2014, DOI 10.1074/jbc.M114.558254

Steven J. Coultrap\(^1\) and K. Ulrich Bayer\(^2\)

From the Department of Pharmacology, Anschutz Medical Campus, University of Colorado School of Medicine, Aurora, Colorado 80045

Background: Ca\(^{2+}\)-independent autonomous CaMKII activity and nitric oxide (NO) signaling regulate neuronal function and death.

Results: NO generated autonomous CaMKII activity by Ca\(^{2+}\)/CaM-dependent S-nitrosylation, and CaMKII inhibition protected from NO-induced neuronal cell death.

Conclusion: NO-mediated regulation of CaMKII contributes to its pathological functions.

Significance: S-Nitrosylation is a novel path to CaMKII autonomy that connects Ca\(^{2+}\).- and NO signaling.

Both signaling by nitric oxide (NO) and by the Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase II \(\alpha\) isofrom (CaMKII\(\alpha\)) are implicated in two opposing forms of synaptic plasticity underlying learning and memory, as well as in excitotoxic/ischemic neuronal cell death. For CaMKII\(\alpha\), these functions specifically involve also Ca\(^{2+}\)-independent autonomous activity, traditionally generated by Thr-286 autophosphorylation. Here, we demonstrate that NO-induced S-nitrosylation of CaMKII\(\alpha\) also directly generated autonomous activity, and that CaMKII inhibition protected from NO-induced neuronal cell death. NO induced S-nitrosylation at Cys-280/289, and mutation of either site abolished autonomy, indicating that simultaneous nitrosylation at both sites was required. Additionally, autonomy was generated only when Ca\(^{2+}\)/CaM was present during NO exposure. Thus, generation of this form of CaMKII\(\alpha\) autonomy requires simultaneous signaling by NO and Ca\(^{2+}\). Nitrosylation also significantly reduced subsequent CaMKII\(\alpha\) autophosphorylation specifically at Thr-286, but not at Thr-305. A previously described reduction of CaMKII activity by S-nitrosylation at Cys-6 was also observed here, but only after prolonged (>5 min) exposure to NO donors. These results demonstrate a novel regulation of CaMKII by another second messenger system and indicate its involvement in excitotoxic neuronal cell death.

Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase II \(\alpha\) isoform (CaMKII\(\alpha\)) and its “autonomous” activity (Fig. 1) (1, 2) mediates both long-term potentiation (LTP) (3, 4) and depression (LTD) (5) of synaptic strength, as well as excitotoxic neuronal cell death during ischemia (6, 7). Several ways to generate CaMKII autonomy have been prominently described over three decades, and include several very recent additions: Thr-286 autophosphorylation (8, 9), T-site-mediated GluN2B binding (10), Met-281/282 oxidation (11), and Ser-279 glycosylation (12) (Fig. 1), the latter two for the CaMKII\(\alpha\) isoform that is dominant in the heart (13). In all cases, an initial Ca\(^{2+}\)/CaM-stimulation is required to induce the CaMKII autonomy, which then persists when the Ca\(^{2+}\) stimulus has subsided and Ca\(^{2+}\) has dissociated. However, autonomous CaMKII is by no means fully active, and is instead significantly further stimulated by Ca\(^{2+}\)/CaM, at least for normal CaMKII substrates (8, 14–16). The highest level of CaMKII autonomy (the ratio of autonomous over maximally stimulated activity) is likely generated by Thr-286 autophosphorylation (1), but with an autonomy of ~20%, even Thr-286-phosphorylated CaMKII is still ~5-fold further stimulated by Ca\(^{2+}\)/CaM (8, 14–16). This mechanism allows for a molecular memory of past Ca\(^{2+}\)-signals by the autonomous activity, but may also prevent complete uncoupling from subsequent cellular Ca\(^{2+}\) signaling. However, physiological functions for such further stimulation of autonomous CaMKII have been described only very recently, specifically in determining whether autonomous CaMKII promotes potentiation or depression of synaptic strength (5).

In CaMKII\(\alpha\), the major isoform in the brain (1), the residues homologous to Met-281/282 in the \(\delta\) isoforms are Cys-280/Met-281 (Fig. 1), and the substitution of one methionine for a cysteine raised the possibility of physiological regulation by nitric oxide (NO)-mediated S-nitrosylation (17–19), in addition to pathological regulation by oxidation. Such potential CaMKII regulation was intriguing, as nitric oxide (NO) and NO synthase (NOS) are also implicated in hippocampal LTP, LTD, and excitotoxic neuronal cell death (19–23). The results of this study show that specific S-nitrosylation generated CaMKII autonomy and that CaMKII inhibition protected from NO-induced neuronal cell death.

EXPERIMENTAL PROCEDURES

Proteins—CaMKII\(\alpha\) wt and CaM were purified after baculovirus/sf9 cell expression or bacterial expression as previously described (16, 24). For comparisons of CaMKII wt and mutants,
GFP-CaMKII fusion proteins were used (25), which were purified after expression in HEK cells. Briefly, HEK cells were homogenized with a motorized pestle (Kontes) for 10 s in 0.4 M ice-cold 50 mM PIPES pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM DTT, and complete protease inhibitor (Roche) for 20 min. The CaMKII concentration from the resulting supernatant was determined by their GFP-fluorescence measured in a spectrophotometer (Fluoromax 3; Horiba Jobin Yvon). For the comparison of CaMKII wt to T286A, these extracts were adjusted for equal CaMKII and total HEK cell protein amounts (26, 27). All other mutants were further purified as previously described (16, 24). Protein phosphatase 2A C subunit was purchased from Cayman Chemical. The substrate peptide Syntide-2 was from Genescript.

**NO Release**—NO release from the NO donor Diethylamine NONOate (DEA-NONOate) was measured spectrophotometrically in reaction buffer or deionized water by the disappearance of absorption at 250 nm due to loss of parent compound \((\epsilon = 6500 \text{ M}^{-1} \text{cm}^{-1})\).

**CaMKII Autophosphorylation, Nitrosylation, and Oxidation**—CaMKII was made autophosphorylated by reacting 200–300 nm kinase for 5 min with \(\text{Ca}^{2+}/\text{CaM} (1 \text{ mM}/1 \mu\text{M})\) and 50 mM PIPES pH 7.1 in the presence of either ATP/Mg\(^{2+}\) (0.1/10 mM) on ice (for Thr-286 autophosphorylation) or at room temperature with DEA-NONOate (3 mM; for nitrosylation; Cayman Chemical) or \(\text{H}_{2}\text{O}_{2} (3 \text{ mM}; \text{for oxidation})\). The reaction was diluted and Ca\(^{2+}\) chelated with EDTA (5 mM). Where indicated, kinase was reacted with peroxynitrite (ONOO\(^-\) Cayman Chemical) instead of NO, and/or in the absence or presence of the ONOO\(^-\) scavengers tryptophan (Sigma) or MnTMPyP (A.G. Scientific).

For experiments where kinase was both phosphorylated and nitrosylated, CaMKII was reacted with DEA-NONOate at room temperature followed by phosphorylation of Thr-286 by addition of ATP/Mg\(^{2+}\) on ice, or this was reversed and kinase was first phosphorylated at Thr-286 on ice, phosphorylation was stopped by addition of EDTA, then the kinase was reacted with NO at room temperature. Thr-305/306 phosphorylation was induced by chelating Ca\(^{2+}\) in the presence of ATP (after Thr-286 phosphorylation or nitrosylation).

**CaMKII Activity Assays**—Kinase activity was assessed by \(^{32}\text{P}\) incorporation into the peptide substrate syntide 2, either in the presence of Ca\(^{2+}/\text{CaM} (1.2 \text{ mM}/1 \mu\text{M})\) or EGTA (1.5 mM), as previously described (16, 24). To ensure that reactions are within the linear range, 1 min reaction times (at 30 °C) were used for stimulated activity and Thr-286-induced autonomy (16); reaction times of up to 5 min were used only for conditions with lower activity.

**Western Analysis**—Samples were boiled in SDS-PAGE loading buffer (2% SDS, 50 mM DTT, 67.5 mM Tris pH 6.8, 10% glycerol, 0.16 mg/ml bromphenol blue), separated on 10% acrylamide gels then transferred to PVDF membranes. Blots were blocked with 5% nonfat dry milk (or 5% BSA for the phospho-T305 detection) for 1 h at room temperature, then incubated overnight at 4 °C with either antibodies against either CaMKIIa (1:2000; C8a2, produced in-house), phospho-T286 (1:3000; Phosphosolutions), or phospho-T305 (1:1000; Assay-Biotech). Blots were imaged with a chemiimager (Alpha Innotech) after exposure to Supersignal West Femto ECL reagent (Pierce).

**Detection of S-Nitrosylation**—Detection of S-nitrosylation was assessed using the biotin-switch method (28). CaMKII WT or mutant was nitrosylated in the presence of Ca\(^{2+}/\text{CaM}\) in HEPES pH 7.1 buffer as described above, then diluted in HEN buffer (HEPES pH 7.1, 1 mM EDTA, and 0.1 mM neocuprone). Proteins were then precipitated at −20 °C with 4 volumes of cold acetone for 20 min and centrifuged at 15,000 × g for 10 min. The pellet was rinsed with ice cold acetone, resuspended in HEN buffer, then non-nitrosylated cysteines were blocked with 25 mM MMTS in 2.5% SDS for 30 min at 50 °C. Proteins were then washed with cold acetonitrile (1 ml/g) and centrifuged at 15,000 × g for 10 min. The protein was resuspended in deionized water or deionized water with TCEP (5 mM) for 1 h at room temperature. Unreacted TCEP was removed by acetone precipitating the proteins. Proteins were then run on Western blots, and biotinylation was detected using the avidin-based vectastain kit (Vector Laboratories).

**Neuronal Cell Death Assays**—Neuronal cell death was measured by release of LDH into the media using a cytotoxicity detection kit (Roche) as previously described (6). Briefly,
medium density primary dissociated hippocampal cultures were prepared from newborn Sprague-Dawley rats and plated onto poly-d-lysine-coated 24-well plates. Cultures were maintained in Neurobasal A media with B-27 supplement, 50 units/ml penicillin/streptomycin and 2 mM Glutamax at 37 °C in 5% CO₂. After 14 DIV, cell death was induced by addition of 100 μM glutamate or 300 μM DEA-NONOate to the media for 5 min. Wells with inhibitor had 100 μM APV or 5 μM tatCN21 or scrambled control peptide included during the treatment as well a 20-min pretreatment.

RESULTS

NO Generates Autonomous CaMKII Activity—Incubation of purified CaMKIIα (in the presence of Ca²⁺/CaM) either with an oxidizer (H₂O₂) or with an NO donor (DEA-NONOate) both generated autonomous activity (measured after chelation of Ca²⁺), with higher autonomy generated by the NO donor (Fig. 2A). NO release by the NO donor is well controlled, with no release under acidic conditions (as in deionized water) and with near maximal release within 5 min after addition to buffer at pH 7.2 (Fig. 2B). As expected, NO-induced autonomy was abolished by C280/M281V mutation (analogous to CaMKIIδ oxidation and M281/282V mutation; Ref. 11). However, surprisingly, autonomy was also abolished by C289V mutation (Fig. 2A), indicating that simultaneous S-nitrosylation of both Cys-280 and Cys-289 was required to induce autonomy, and that individual nitrosylation of either residue alone was not sufficient.

NO-induced CaMKII Autonomy Is Independent of Thr-286 and Blocked by tatCN21 but Not KN93—To test the possibility that generation of autonomous CaMKII activity by the NO donor was dependent on promoting Thr-286 autophosphorylation, a CaMKII T286A mutant was used. The NO donor induced autonomy also for the CaMKII T286A mutant (Fig. 2C), indicating that the effect was independent of Thr-286 phosphorylation. If any, NO-induced autonomy of the T286A mutant was even higher compared with CaMKII wild type (Fig. 2C), an effect that could be due to reduced auto-inhibitory interactions within the T286A mutant. Additionally, the effects of two mechanistically distinct CaMKII inhibitors were tested: KN93, which is Ca²⁺/CaM-competitive (29), and tatCN21, which does not act through competition with Ca²⁺/CaM (27). As expected based on these different modes of inhibition, tatCN21 completely blocked NO-induced autonomous CaMKII activity, while KN93 had no significant effect (Fig. 2C). The same differential effect of these two inhibitors has been observed previously for Thr-286-induced CaMKII autonomy (6).

Induction of NO-mediated Autonomy Requires an Initial Ca²⁺/CaM Stimulus—While a 5-min incubation with the NO donor and Ca²⁺/CaM generated autonomous CaMKII activity (Figs. 2A and 3A), it did not affect maximally Ca²⁺/CaM-stimulated activity (Fig. 3A), as measured by subsequent activity assays with or without chelation of Ca²⁺ after the initial incubation. Presence of Ca²⁺/CaM was required to induce CaMKII autonomy by the NO donor, as no autonomous activity was induced when Ca²⁺/CaM was omitted from the initial incubation (Fig. 3A). Thus, generation of this novel form of CaMKII autonomy requires coinciding NO and Ca²⁺ signals.

Prolonged Exposure to Nitric Oxide Reduces CaMKII Activity—A previous study showed that NO inhibits CaMKII activity through S-nitrosylation at C6 (30). To address this apparently conflicting finding, CaMKII was exposed to NO for varying periods of time (Fig. 3B). Indeed, while Ca²⁺/CaM-stimulated CaMKII activity was unaffected by a 5 min exposure to NO, it was significantly reduced after prolonged exposure times (Fig. 3B). A similar time-dependent reduction was also observed for

![Graph A](image)

**FIGURE 2. NO induces Ca²⁺-independent autonomous CaMKII activity by a process that requires both Cys-280 and Cys-289.** A, Incubation of CaMKII (in the presence of Ca²⁺/CaM) either with the oxidizer H₂O₂ or the NO donor DEA-NONOate for 5 min generated autonomous CaMKII activity (measured by phosphorylation of syntide 2 after chelating Ca²⁺ with EGTA). NO-induced autonomy was abolished by mutation of either Cys-280 or Cys-289. Bar graphs indicate absolute kinase activity as mean ± S.E. The numbers inserted for each bar indicate the level of autonomy (in percent of maximal Ca²⁺-stimulated activity). B, NO release from the NO donor was readily triggered in buffer at pH 7.2, but not in acidic deionized water. C, NO-induced CaMKII autonomy (measured as in panel A) was not reduced by T286A mutation or by the Ca²⁺/CaM competitive inhibitor KN93 (10 μM), but effectively blocked by the peptide inhibitor tatCN21 (5 μM). These experiments were done with CaMKII from crude HEK cell extracts, and non-transfected mock extracts were thus used as additional control. Bar graphs indicate mean ± S.E.
the NO-induced autonomous activity (Fig. 3B), indicating that the observed reduction of CaMKII activity was not mediated by interference with Ca$^{2+}$/CaM-stimulation. Thus, while NO can indeed inhibit both stimulated and autonomous CaMKII activity, this opposing effect requires prolonged NO signaling, while shorter NO signals are sufficient to generate autonomous activity.

**NO Reduces Ca$^{2+}$/CaM-induced CaMKII Thr-286 Autophosphorylation**—For other forms of Thr-286-independent forms of CaMKII autonomy, a positive cross regulation with Thr-286-induced autonomy has been suggested (10–12). By contrast, previous exposure to NO in presence of Ca$^{2+}$/CaM dramatically reduced subsequent Ca$^{2+}$/CaM-stimulated CaMKII activity, but only after prolonged exposure (>5 min). Stimulated and autonomous CaMKII activities were normalized to their respective maximums. The quantifications indicate mean ± S.E.

![FIGURE 3. NO requires Ca$^{2+}$/CaM to induce CaMKII autonomy and inhibits stimulated activity after prolonged exposure.](image)

**NO Induces CaMKII Autonomy**

![FIGURE 4. NO exposure inhibits subsequent CaMKII Thr-286 autophosphorylation. A**, pre-incubation with NO donor inhibited subsequent Ca$^{2+}$/CaM-induced CaMKII autophosphorylation at Thr-286. Western-analysis with a phospho-specific antibody is shown. **B**, pre-incubation with NO donor or H$_2$O$_2$ significantly reduced the level of CaMKII autonomy induced by a 5 min Thr-286 autophosphorylation reaction. Bar graphs indicate mean ± S.E.](image)

**NO Has No Significant Effects on CaMKII Thr-305 Autophosphorylation**—CaMKII Thr-305/306 autophosphorylation inhibits subsequent Ca$^{2+}$/CaM binding (31, 32). Vice versa, Ca$^{2+}$/CaM binding also suppresses Thr-305/306 phosphorylation. Thus, Thr-305/306 is most efficiently autophosphorylated after dissociation of CaM from Thr-286-phosphorylated autonomous CaMKII. Additional NO exposure of previously Thr-286-phosphorylated CaMKII did not reduce the Thr-305 autophosphorylation that was induced by chelating Ca$^{2+}$/CaM (Fig. 5). Thus, the NO-mediated inhibition of CaMKII autophosphorylation was specific to Thr-286 and did not extend to Thr-305.

This finding raised the possibility that generation of autonomous CaMKII activity by NO exposure may even substitute for Thr-286 phosphorylation in enabling efficient Thr-305/306 phosphorylation. However, NO exposure did not cause any significant increase in Thr-305 autophosphorylation for CaMKII that was not previously Thr-286 phosphorylated (Fig. 4) and did not extend to Thr-305.

**NO-induced CaMKII Autonomy Is Mediated by Specific S-Nitrosylation**—In addition to S-nitrosylation of cysteine residues, NO can induce oxidation of cysteine and methionine residues, the latter through formation of peroxynitrite (ONOO$^-$). However, four independent lines of evidence showed that the NO-induced CaMKII autonomy was indeed caused by S-nitrosylation of Cys-280/Cys-289 and not by ONOO$^-$-mediated oxidation:

(i) The effect of NO donors was larger compared with oxidation by H$_2$O$_2$ (see Fig. 2A), consistent with a bulkier modifica-
tion by nitrosylation causing more efficient relief from autoinhibition.

(ii) NO donors indeed caused S-nitrosylation of CaMKII (Fig. 6A), as detected by the biotin-switch method (28). This specifically included nitrosylation of Cys-280/Cys-289, as mutation of these residues clearly reduced the detected nitrosylation (Fig. 6B).

(iii) The ONOO− scavengers tryptophan (Trp) or MnTMPyP (MnTP) did not reduce the effect of NO donors, but instead even further enhanced it (Fig. 7A), consistent with suppression of Cys-280/289 oxidation allowing more nitrosylation of these residues. Comparing scavenger addition before and after the nitrosylation reaction (both before the kinase activity assays) confirmed that the scavengers affected the nitrosylation reaction and did not have any other direct effects on kinase activity (Fig. 7A).

(iv) The effect of directly adding ONOO− was much smaller compared with adding NO donors. Importantly, the effect of ONOO− was abolished by the ONOO− scavenger Trp, but dramatically enhanced by MnTP, an ONOO− scavenger that releases NO during ONOO− consumption (Fig. 7B).

**DISCUSSION**

The results of this study demonstrate that NO-induced CaMKII autonomy is a novel regulatory mechanism for an enzyme critically involved in mediating synaptic plasticity (1, 3–5) and ischemic/excitotoxic neuronal cell death (6, 7). Indeed, CaMKII inhibition protected also from neuronal cell death directly induced by NO donors. NO can cause S-nitrosylation or oxidation of proteins (the latter via formation of ONOO−); however, the results of this study showed that CaMKII autonomy was induced by specific S-nitrosylation that occurs at residues Cys-280/289. Mutation of either residue abolished NO-induced autonomy, indicating requirement of their simultaneous nitrosylation.

Generation of CaMKII autonomy by autophosphorylation at Thr-286 has been considered to be a hallmark feature of CaMKII regulation for almost 30 years, as it enables the kinase to "remember" past Ca2+ stimuli (8, 14–16) (for review see Ref. 1). Several additional ways to generate autonomous CaMKII activity have been prominently described more recently: Binding to GluN2B (10), Met-280/281 oxidation (11), and Ser-279 O-linked glucosylation (12). The novel form of autonomy that is generated by NO-induced S-nitrosylation shares several features with these previously described autonomy mechanisms. All known forms of CaMKII autonomy require an initial Ca2+ /CaM-stimulus for induction (8–12). Thus, they all provide a molecular memory of past Ca2+ stimuli. However, in case of autonomy induced by oxidation, glucosylation, or S-nitrosylation, this molecular memory is only formed in presence of a coinciding second type of signal. For oxidation and glucosylation, the second signal appears to be typically pathological (such as oxidative stress or elevated blood sugar in diabetes; Refs. 11, 12). By contrast, NO signals that are required for S-nitrosylation could additionally be generated by physiological signaling. All forms of autonomous CaMKII are also significantly further stimulated by Ca2+ /CaM (for review see Ref. 1). This can retain Ca2+ sensitivity even of "autonomous" CaMKII and may prevent complete uncoupling from subsequent Ca2+ stimuli. Indeed, recent evidence sug-

**FIGURE 5.** NO exposure does not affect subsequent CaMKII T305 auto-phosphorylation. After NO exposure and/or Thr-286 autophosphorylation (as indicated), CaMKII autophosphorylation at Thr-305 was induced by chela-
tion of Ca2+ and continued for 5 min at 30 °C. Thr-305 phosphorylation was detected by Western analysis (lower panel) and quantified by normalized immunodetection values (IDVs; upper panel). Basal background phospho-

**FIGURE 6.** NO induces CaMKII S-nitrosylation at Cys-280/289. A, NO donor induced S-nitrosylation of CaMKII, as detected by the biotin-switch method. B, nitrosylation included Cys-280/289, as the detected nitrosylation was reduced by mutation of these residues (mut).
death. Bar graphs indicate mean 

gentase (LDH) release. The NMDA receptor antagonist APV (100 
of death in hippocampal cultures, as measured 24 h later by lactate dehydro-

tifications indicate mean 

gest that autonomous CaMKII activity can have opposing effects 
on synaptic strength, depending on presence or absence of addi-
tional Ca\textsuperscript{2+}/CaM stimulation (5).
NO Induces CaMKII Autonomy

...tion of the higher level of Thr-286-dependent autonomy, an effect observed here also after CaMKII oxidation. Thus, the different forms of CaMKII autonomy can have intricate cross-regulatory effects with each other as well as with stimulated CaMKII activity. NO-induced CaMKII autonomy is a novel aspect also in the cross-talk between CaMKII and different NOS isoforms, as these NO-producing enzymes are in turn regulated by CaMKII activity (40–45).

Both CaMKII and neuronal NOS (nNOS) are localized at synapses via association with the NMDA-receptor complex, are involved in excitotoxic/ischemic cell death, and inhibition of either is neuroprotective (for review see Refs. 7, 23). The CaMKII-mediated regulation of nNOS cannot explain this effect, as CaMKII inhibits rather than activates nNOS (40, 43, 44). By contrast, the NO-induced CaMKII autonomy indicates that nNOS is instead upstream of CaMKII in excitotoxic death signaling. Indeed, CaMKII inhibition protected not only from excitotoxic/ischemic death, but also from neuronal cell death directly induced by an NO donor. Intriguingly, even disruption of nNOS from the NMDA-receptor complex provides neuroprotection (22), and it will be interesting to see if this disruption acts by preventing efficient S-nitrosylation of the co-localized CaMKII.

Two recent studies indicated NO-mediated regulation of CaMKII also in the heart (46, 47), where CaMKII is known to have crucial pathological functions (13). However, in this case, CaMKII activation by NO did not appear to be through direct S-nitrosylation, as CaMKII activation (and the downstream functions) was instead dependent on PKG (47), which is activated by cGMP after NO-induced guanylyl cyclase activation (17–19). Notably, however, one of these studies showed that NO donors did also cause a direct 2.5-fold increase of CaMKIIö activity over baseline (46). However, this rather small effect may have been caused by ONOO−-mediated oxidation (an effect that was ruled out in our experiments on the CaMKIIα isoform). Indeed, in this case, the NO effect was no larger than the effect of oxidation (46), and the CaMKIIö isoform does not have a Cys residue at the position homologous to Cys-280 in CaMKIIα (and the nitrosylation effect on CaMKIIö autonomy demonstrated here was sensitive not only to mutation of the Cys-289 that is conserved among the isoforms, but also to mutation of the unique Cys-280).

It should be noted that the absolute activity of CaMKII made autonomous by oxidation reported here (~1 μmol/min/mg) is higher than previously reported, while the level of relative autonomy (~5% of maximal stimulated activity) is lower than reported previously (~25 nmol/min/mg and ~40%, respectively) (11). The previously reported relative autonomy may be an overestimate due to kinase reactions that were beyond the linear range, for instance due to long reaction times (1). This is consistent with the low apparent activity reported after maximal stimulation and with the high apparent autonomy also after Thr-286 phosphorylation (70%) (11), even though the assays used a regular substrate described to support only lower levels of ~20% autonomy (8, 16). However, as previously discussed (1), we wish to point out that such overestimates of the relative autonomy levels after oxidation (11) or GluN2B binding (10) do not diminish the importance of these studies that elucidated new principle pathways toward CaMKII autonomy. The NO mechanisms described here add another path to CaMKII autonomy, and it will be interesting to investigate the functions in physiological CaMKII signaling, in addition to the pathological functions in neuronal cell death described here.

Acknowledgment—We thank Jacqueline Kulbe for help with the neuronal cell culture.

REFERENCES

1. Coultrap, S. J., and Bayer, K. U. (2012) CaMKII regulation in information processing and storage. Trends Neurosci. 35, 607–618
2. Chao, L. H., Stratton, M. M., Lee, I. H., Rosenberg, O. S., Levitz, J., Mandell, D. J., Kortemme, T., Groves, J. T., Schulman, H., and Kuriyan, J. (2011) A mechanism for tunable autoinhibition in the structure of a human Ca2+/calmodulin-dependent kinase II holoenzyme. Cell 146, 732–745
3. Giese, K. P., Fedorov, N. B., Filipkowski, R. K., and Silva, A. J. (1998) Autophosphorylation at Thr286 of the α-calcium-calmodulin kinase II in LTP and learning. Science 279, 870–873
4. Buord, L., Coultrap, S. J., Freund, R. K., Lee, Y. S., Dell’Acqua, M. L., Silva, A. J., and Bayer, K. U. (2010) CaMKII “autonomy” is required for initiating but not for maintaining neuronal long-term information storage. J. Neurosci. 30, 8214–8220
5. Coultrap, S. J., Freund, R. K., O’Leary, H., Sanderson, J. L., Roche, K. W., Dell’Acqua, M. L., and Bayer, K. U. (2014) Autonomous CaMKII mediates both LTP and LTD using a mechanism for differential substrate site selection. Cell Reports 6, 431–437
6. Vest, R. S., O’Leary, H., Coultrap, S. J., Kindy, M. S., and Bayer, K. U. (2010) Effective post-insult neuroprotection by a novel Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) inhibitor. J. Biol. Chem. 285, 20675–20682
7. Coultrap, S. J., Vest, R. S., Ashpole, N. M., Hudmon, A., and Bayer, K. U. (2011) CaMKII in cerebral ischemia. Acta. Pharmacol. Sin. 32, 861–872
8. Miller, S. G., and Kennedy, M. B. (1986) Regulation of brain type II Ca2+/calmodulin-dependent protein kinase by autophosphorylation: a Ca2+-triggered molecular switch. Cell 44, 861–870
9. Hansson, P. L., Meyer, T., Stryer, L., and Schulman, H. (1994) Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. Neuron 12, 943–956
10. Bayer, K. U., De Koninck, P., Leonard, A. S., Hell, J. W., and Schulman, H. (2001) Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature 411, 801–805
11. Erickson, J. R., Joiner, M. L., Guan, X., Kutschke, W., Yang, J., Oddie, C. V., Bartlett, R. K., Lowe, J. S., O’Donnell, S. E., Aykin-Burns, N., Zimmerman, M. C., Zimmerman, K., Ham, A. J., Weiss, R. M., Spitz, D. R., Shea, M. A., Colbran, R. J., Mohler, P. J., and Anderson, M. E. (2008) A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. Cell 133, 462–474
12. Erickson, J. R., Pereira, L., Wang, L., Han, G., Ferguson, A., Dao, K., Copeland, R. J., Despa, F., Hart, G. W., Ripplinger, C. M., and Bers, D. M. (2013) Diabetic hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation. Nature 502, 372–376
13. Anderson, M. E., Brown, J. H., and Bers, D. M. (2011) CaMKII in myocardial hypertrophy and heart failure. J Mol. Cell Cardiol. 51, 468–473
14. Lou, L. L., Lloyd, S. J., and Schulman, H. (1986) Activation of the multifunctional Ca2+/calmodulin-dependent protein kinase by autophosphorylation: ATP modulates production of an autonomous enzyme. Proc. Natl. Acad. Sci. U.S.A. 83, 9497–9501
15. Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1986) Reversible generation of a Ca2+-independent form of Ca2+/calmodulin-dependent protein kinase II by an autophosphorylation mechanism. J. Biol. Chem. 261, 8581–8584
16. Coultrap, S. J., Buord, I., Kulbe, J. R., Dell’Acqua, M. L., and Bayer, K. U. (2010) CaMKII autonomy is substrate-dependent and further stimulated by Ca2+/calmodulin. J. Biol. Chem. 285, 17930–17937
17. Nakamura, T., Tu, S., Akhtar, M. W., Sunico, C. R., Okamoto, S., and Lipton, S. A. (2013) Aberrant protein s-nitrosylation in neurodegenerative diseases. Neuron 78, 596–614.
18. Hess, D. T., and Stamler, J. S. (2012) Regulation by S-nitrosylation of protein post-translational modification. J. Biol. Chem. 287, 4411–4418.
19. Sen, N., and Snyder, S. H. (2010) Protein modifications involved in neurotransmitter and gasotransmitter signaling. Trends Neurosci. 33, 493–502.
20. Schuman, E. M., and Madison, D. V. (1991) A requirement for the intercellular messenger nitric oxide in long-term potentiation. Science 254, 1503–1506.
21. Stanton, P. K., Winterer, J., Bailey, C. P., Kyrozis, A., Raginov, I., Laube, G., Veh, R. W., Nguyen, C. Q., and Müller, W. (2003) Long-term depression of presynaptic release from the readily releasable vesicle pool induced by NMDA receptor-dependent retrograde nitric oxide. J. Neurosci. 23, 5936–5944.
22. Aarts, M., Liu, Y., Liu, L., Bessho, S., Arundine, M., Gurd, J. W., Wang, Y. T., Salter, M. W., and Tymianski, M. (2002) Treatment of ischemic brain damage by perturbing NMDA receptor-PSD-95 protein interactions. Science 298, 846–850.
23. Aarts, M. M., and Tymianski, M. (2004) Molecular mechanisms underlying specificity of excitotoxic signaling in neurons. Curr. Mol. Med. 4, 137–147.
24. Coultrap, S. J., and Bayer, K. U. (2012) Ca²⁺/Calmodulin-Dependent Protein Kinase II (CaMII). In Neuromethods: Protein Kinase Technologies (Mukai, H., ed) pp. 49–72, Springer.
25. Bayer, K. U., LeBel, E., McDonald, G. L., O’Leary, H., Schulman, H., and De Koninck, P. (2006) Transition from reversible to persistent binding of CaMII to postsynaptic sites and NRG2B. J. Neurosci. 26, 1164–1174.
26. Coultrap, S. J., Barcomb, K., and Bayer, K. U. (2012) A significant but rather mild contribution of T286 autophosphorylation to Ca²⁺ binding of CaMII. J. Biol. Chem. 287, 3107–3112.
27. Vest, R. S., Davies, K. D., O’Leary, H., Port, J. D., and Bayer, K. U. (2007) Nitric oxide-mediated modulation of calcium/calmodulin-dependent protein kinase II activity by regulated interactions with N-methyl-D-aspartate receptor NR2B subunits and α-actinin. J. Biol. Chem. 282, 39316–39323.
28. Rameau, G. A., Chiu, L. Y., and Ziff, E. B. (2004) Bidirectional regulation of neuronal nitric oxide synthase phosphorylation at serine 847 by the N-methyl-D-aspartate receptor NR2B. J. Biol. Chem. 279, 10141–10148.
29. Rameau, G. A., Aimola, D., Colbran, R. J., and Soderling, T. R. (2007) iNOS regulation by calcium/calmodulin-dependent protein kinase II. Trends in Protein post-translational modification. Biochem. J. 401, 267–276.
30. Song, T., Hatano, N., Kambe, T., Miyamoto, Y., Ibara, H., Yamamoto, H., Sugimoto, K., Kume, K., Yamaguchi, F., Tokuda, M., and Watanabe, Y. (2008) Nitric oxide-mediated modulation of calcium/calmodulin-dependent protein kinase II. Biochem. J. 412, 223–231.
31. Hansson, P. I., and Schulman, H. (1992) Inhibitory autophosphorylation of multifunctional Ca²⁺/calmodulin-dependent protein kinase analyzed by site-directed mutagenesis. J. Biol. Chem. 267, 17216–17224.
32. Colbran, R. J. (1993) Inactivation of Ca²⁺ /calmodulin-dependent protein kinase II by basal autophosphorylation. J. Biol. Chem. 268, 7163–7170.
33. Takao, K., Okamoto, K., Nakagawa, T., Neve, R. L., Nagai, T., Miyawaki, A., Hashikawa, T., Kobayashi, S., and Hayashi, Y. (2005) Visualization of synaptic Ca²⁺ /calmodulin-dependent protein kinase II activity in living neurons. J. Neurosci. 25, 3107–3112.
34. Hojati, M. R., van Woerden, G. M., Tyler, W. J., Giese, K. P., Silva, A. J., Pozzo-Miller, L., and Elgersma, Y. (2007) Kinase activity is not required for alphaCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses. Nat. Neurosci. 10, 1125–1127.
35. Borgesius, N. Z., van Woerden, G. M., Buitendijk, G. H., Keijzer, N., Jaarsma, D., Hoogenraad, C. C., and Elgersma, Y. (2011) betaCaMKII plays a nonenzymatic role in hippocampal synaptic plasticity and learning by targeting eCaMKII to synapses. J. Neurosci. 31, 10141–10148.
36. Lee, S. J., Escobedo-Lozoya, Y., Szatmari, E. M., and Yasuda, R. (2009) Activation of CaMKII in single dendritic spines during long-term potentiation. Nature 458, 299–304.
37. Hashimoto, Y., Hino, K., Okuno, H., Sano, Y., Takemoto-Kimura, S., Kitamura, K., Kano, M., and Bito, H. (2013) Nonlinear decoding and asymmetric representation of neuronal input information by CaMKIIα and calcineurin. Cell Rep. 3, 978–987.
38. Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B. E., and Busse, R. (2001) Phosphorylation of Thr(495) regulates Ca²⁺/calmodulin-dependent protein kinase II activity by regulated interactions with N-methyl-D-aspartate receptor NR2B subunits and α-actinin. J. Biol. Chem. 282, 39316–39323.
39. Rameau, G. A., Aimola, D., Colbran, R. J. (2007) Differential modulation of Ca²⁺/calmodulin-dependent protein kinase II activity by regulated interactions with N-methyl-D-aspartate receptor NR2B subunits. J. Biol. Chem. 282, 8051–8055.
40. Robison, A. J., Bartlett, R. K., Bass, M. A., and Colbran, R. J. (2005) Post-synaptic density-95 promotes calcium/calmodulin-dependent protein kinase II-mediated Ser847 phosphorylation of neuronal nitric oxide synthase. Biochim. J. 372, 465–471.
41. Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B. E., and Busse, R. (2001) Phosphorylation of Thr(495) regulates Ca²⁺/calmodulin-dependent protein kinase II. Circ. Res. 88, E68–E75.
42. Rameau, G. A., Tukey, D. S., Garcin-Hosfield, E. D., Titcombe, R. F., Misra, C., Khatri, L., Getzoff, E. D., and Ziff, E. B. (2007) Biphasic coupling of neuronal nitric oxide synthase phosphorylation to the NMDA receptor regulates AMPA receptor trafficking and neuronal cell death. J. Neurosci. 27, 3445–3455.
43. Jones, R. J., Jourd’heuil, D., Salerno, J. C., Smith, S. M., and Singer, H. A. (2007) Regulation of cardiac muscle. Circ. Res. 88, E68–E75.
44. Gutierrez, D. A., Fernandez-Tenorio, M., Ogrodnik, J., and Niggli, E. (2013) NO Induces CaMKII Autonomy. J. Biol. Chem. 289, H2634–H2642.
45. Zhang, D. M., Chai, Y., Erickson, J. R., Brown, J. H., Bers, D. M., and Lin, Y. F. (2014) Intracellular signalling mechanism responsible for modulation of sarcolemmal ATP-sensitive potassium channels by nitric oxide in ventricular cardiomyocytes. J. Physiol. 592, 971–990.