Substrate Localization Creates Specificity in Calcium/Calmodulin-dependent Protein Kinase II Signaling at Synapses*

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Calcium/calmodulin-dependent protein kinase II (CaMKII), a major component of the postsynaptic density (PSD) of excitatory synapses, plays a key role in the regulation of synaptic function in the mammalian brain. Although many postsynaptic substrates for CaMKII have been characterized in vitro, relatively little is known about their phosphorylation in vivo. By tagging synaptic proteins with a peptide substrate specific for CaMKII and expressing them in cultured neurons, we have visualized substrate phosphorylation by CaMKII at intact synapses. All substrates tested were strongly phosphorylated by CaMKII in HEK293 cells. However, activity-dependent phosphorylation of substrates at synapses was highly selective in that the glutamate receptor subunits NR2B and GluR1 were poorly phosphorylated whereas PSD-95 and Stargazin, proteins implicated in the scaffolding and trafficking of AMPA receptors, were robustly phosphorylated. Phosphatase activity limited phosphorylation of substrates at synapses, plays a key role in the regulation of synaptic function in the mammalian brain. Although many postsynaptic substrates for CaMKII have been characterized in vitro, relatively little is known about their phosphorylation in vivo. By tagging synaptic proteins with a peptide substrate specific for CaMKII and expressing them in cultured neurons, we have visualized substrate phosphorylation by CaMKII at intact synapses. All substrates tested were strongly phosphorylated by CaMKII in HEK293 cells. However, activity-dependent phosphorylation of substrates at synapses was highly selective in that the glutamate receptor subunits NR2B and GluR1 were poorly phosphorylated whereas PSD-95 and Stargazin, proteins implicated in the scaffolding and trafficking of AMPA receptors, were robustly phosphorylated. Phosphatase activity limited phosphorylation of substrates at synapses but not NR2B and GluR1. These results suggest that the unique molecular architecture of the PSD results in highly selective substrate discrimination by CaMKII.

Calcium-calmodulin-dependent protein kinase II (CaMKII) is a major effector for calcium-dependent signaling in neurons. It has been implicated in dendritic filopodial extension (1), presynaptic plasticity (2), retrograde signaling to the presynaptic terminal (3), and most notably, the phenomenon of long-term potentiation (LTP) (4, 5), the leading model for a synaptic mechanism underlying learning and memory (6). Although several candidates have been proposed to mediate the synaptic consequences of activating CaMKII (7), it has been difficult to characterize how synaptic proteins are phosphorylated by CaMKII when incorporated into functioning synapses. This is a particularly important and challenging issue because biochemical and proteomic approaches to identifying CaMKII substrates in the postsynaptic density (PSD), the electron dense postsynaptic specialization that contains glutamate receptors and associated signaling machinery, have found at least 28 potential substrate proteins (8). Furthermore, it is unknown whether stimulus-dependent substrate specificity is an inherent property of the PSD and whether the source of the calcium that activates CaMKII and protein phosphatase activity influence synaptic substrate phosphorylation.

Some recent evidence suggests that the intricate scaffolding of proteins within different levels of the PSD may influence CaMKII signal transduction. First, electron microscopy studies on isolated PSDs have revealed both a laminar and heterogeneous distribution of CaMKII on the cytosolic face, suggesting that CaMKII may be specifically positioned to exert its activity on nanodomains within this macromolecular complex (9). Second, CaMKII interactions with the NMDA receptor (NMDAR) subunit NR2B (10) and the Drosophila homolog of CASK, Cmg (11), a member of the MAGUK family of synaptic scaffolding proteins (12), have both been found to influence its state of activation, with NR2B resulting in calcium-independent activity and Cmg/CASK promoting inhibitory autophosphorylation (10, 11). Third, the sensitivity of CaMKII to protein phosphatases PP2A and PP1 is altered by PSD incorporation (13). Thus, it appears that CaMKII activity itself can be affected by its PSD binding partners. It is unknown, however, how CaMKII substrate selection is influenced by kinase and substrate placement at synapses, presumably within the PSD.

To begin to address this issue, we have tagged a number of prominent potential CaMKII synaptic substrates with a short peptide known to be specifically phosphorylated by CaMKII and visualized their phosphorylation in cultured neurons using a specific phosphoantibody. Surprisingly, we find remarkable discrimination between substrates that should be located within nanometer distances of one another within the PSD. NMDAR-dependent activation of CaMKII caused robust phosphorylation of the synaptic scaffold protein PSD-95 (12, 14) and Stargazin, a protein critically important for the trafficking and surface expression of AMPA receptors (AMPARs) (15). However, the NMDAR subunit NR2B and the AMPAR subunit GluR1 reporter constructs were minimally phosphorylated even though their intracellular C-terminal tails were phosphorylated when expressed alone. These results suggest that the exact manner in which substrates are scaffolded within the architecture of the PSD at synapses profoundly affects their phosphorylation by activated CaMKII. This differential phosphorylation of substrates by CaMKII has important implications for the mechanism by which CaMKII regulates synaptic function during various forms of experience-dependent plasticity.

**EXPERIMENTAL PROCEDURES**

**Gene Construction**—Vim-CFP was constructed by amplifying the initial 264-bp sequence from the vimentin head by PCR and GFP containing NES from HIV Rev (LPLERLTL) into BaMHI and EcoRI sites in lentiviral expression vector, FC1.2 under the αCaMKII promoter. PSD95-Vim-CFP was subcloned into Agel and EcoRI sites in FC1.2. The...
Vim head sequence was inserted into the Apal site in NR2B at position 4263 corresponding to amino acid 1421. GFP-NR2B-Vim was then cloned into BaMHI and EcoRI sites in Lentiviral expression vector, FUGW under the ubiquitin promoter. GFP was inserted after the GluR1 signal sequence, and the Vim head sequence was inserted by PCR at position 2688 corresponding to amino acid 880. GFP-GluR1-Vim was then cloned into BaMHI and EcoRI sites in FUGW. NR2B- and GluR1-Vim C-terminal constructs were amplified by PCR and inserted after NES-GFP (LPPLERLTL-GFP). NES-GFP-C-terminal constructs were then cloned into BaMHI and EcoRI sites in FUGW. Vim-CFP was inserted into the BglII site of Stargazin (CACNG2) at position 800 corresponding to amino acid 270. Stg-Vim-GFP was then cloned into BaMHI and EcoRI sites in FC1.2. CaMKII-HA and Lyn-cNR2B were cloned as described previously (16, 17).

Cell Culture, Transfection, and Stimulation Protocols—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and were transfected with expression plasmids using Lipofectamine Plus™ (Invitrogen) according to the manufacturer’s instructions. 24 h after the transfection, the cells were preincubated for 10 min with HBSS 25 mM Hepes, pH 7.4, and drugs as indicated. To inhibit CaMKII activity, KN-93 (10 μM) (Calbiochem) and Tat-Cntide (25 μM) (1, 18) were applied in culture medium for 1 h prior to stimulation. Stimulation was done with ionomycin (10 μM) (Calbiochem) and Ca²⁺ (2 mM) for 3 min. Dissociated neurons were prepared from p0 Sprague-Dawley pups as previously described with minor changes (19). After manufacturing problems with B-27 supplement (Invitrogen) starting May 2004, neurons were initially plated in B-27 supplemented Neurobasal media, but re-fed subsequently with N-2-supplemented MEM plus GlutaMax (Invitrogen). Glial growth was inhibited by FUDR at 3 DIV.

Neurons were infected with lentiviruses on DIV 9 and used in experiments on DIV 14–16. Lentiviruses were harvested from HEK293 cells transfected with expression vector carrying reporter constructs and lentiviral helper plasmids Δ8.9 and VSVG using Fugene 6 transfection reagent (Roche Applied Science) as previously described (20). To stimulate neurons several different protocols were used: 2-min bath applications of 100 μM NMDA (Calbiochem), 100 μM glutamate in Mg²⁺-free media or 20 μM glutamate (Sigma), 10 μM ioneysin (Calbiochem), 3-min application of 1 μM PDBU (Sigma), or 15-min application of 50 μM piicrot oxin (Tocris) in Mg²⁺-free media. To block all sources of Ca²⁺ other than NMDARs, L-type Ca²⁺ channel blocker Nifedipine (5 μM, Calbiochem), R/T-Type Ca²⁺ channel blocker NiCl₂ (100 μM), P/Q/N-type Ca²⁺ channel blocker ω-Conotoxin MVIIIC (1 μM, Tocris), mGlur antagonist LY341495 (100 μM, Tocris), and TTX (1 μM, Sigma) were simultaneously applied for 15 min prior to and during NMDA treatment. Bis-1 (10 μM, Calbiochem), FK-506 (25 μM, Fujisawa Healthcare), okadaic acid (1 μM, Calbiochem), MK-801 (10 μM, Calbiochem), were preapplied for 15 min in HBSS, 2 mM Cl⁻, 1 mM MgCl₂, and 10 mM Hepes pH 7.4 as indicated.

**Immunocytochemistry and Immunoblotting**—All cells were fixed with 4% paraformaldehyde in 100 mM phosphate buffer for 10 min, followed by 0.1% Triton-X permeabilization for 10 min. They were then incubated for 1 h at room temperature with primary antibodies in PBS with 2% NGS at the following concentrations: phosphovimentin M082 (1:1000, MBL), GFP (1:1000, Invitrogen), synaptophysin (1:150, Sigma), PSD-95 (1:100, Sigma), HA (1:2000, Covance). Immunoreactivities were visualized by incubation with species-appropriate Alexa 488-, 568-, and 647-conjugated secondary antibodies (Invitrogen), and the samples were mounted with Fluoromount medium (EMD). Data were collected with a Zeiss LSM-510 confocal microscope under a ×63, 1.4NA objective. For constructs that were not exclusively synaptically targeted (all but PSD-95), synaptic puncta were selected on Metamorph (Universal Imaging) by creating masks based on synaptophysin staining, and selecting GFP positive areas that colocalized. The pVim image was divided by the GFP image, and resulting pVim/GFP puncta were quantified. Quantification was performed on Metamorph and subsequent statistical analysis (ANOVA) on SPSS (SPSS, Inc.). Immunoblotting was performed as described previously (10), using horseradish peroxidase-conjugated secondary antibodies and the ECL Western blotting detection system (Amersham Biosciences). GluR1 (Chemicon) antibody was used at 1:1000, phospho-Ser831 (Upstate Biotechnology) at 1:500, NR2B (Zymed Laboratories Inc.) at 1:1000, phospho-Ser1300 (Upstate Biotechnology) at 1:1000. Membranes were stripped between blots with Restore Western blot stripping buffer (Pierce) per the manufacturer’s instructions. Data are presented as mean ± S.E. For immunocytochemical experiments, n refers to number of cells examined. For Western blot experiments, n refers to number of independent blots examined. Each experimental manipulation was performed in a minimum of three different culture preparations.

**RESULTS**

Previous work examining CaMKII-dependent phosphorylation of synaptic proteins has almost entirely depended on biochemical approaches, which by necessity require disruption of the native molecular architecture of the synapse. Whereas these studies have provided invaluable information about the identity of proteins that potentially can be phosphorylated by CaMKII, they do not necessarily reflect which substrates are in fact phosphorylated by CaMKII at intact, functioning synapses. To begin to address this issue we took advantage of the existence of a sequence derived from the head region of vimentin (Vim) containing a dedicated CaMKII phosphorylation site at Ser22 (21). The short sequence (88 amino acids) of Vim containing Ser82 is a highly specific substrate for CaMKII in that it is not phosphorylated by any other major Ser/Thr kinases, including CaMKI, CaMKIV, PKC, cdc2, and PKA (21–23). Importantly, in both heterologous cells and neurons, via the immunocytochemical detection of a specific antibody against the phosphorylated form of Ser82, ectopically expressed Vim has proved to be a sensitive probe for CaMKII activity (17, 21).

To test whether tagging synaptic proteins with the head region of Vim would be useful in detecting their CaMKII-dependent phosphorylation at intact synapses, we first examined the phosphorylation of a soluble Vim probe containing a nuclear export signal and fused to CFP (Fig. 1). When expressed in HEK293 cells only minimal staining with the phosphovimentin (pVim) antibody was detected (Fig. 1B). Stimulation of these cells with the calcium ionophore ionomycin caused a ~2-fold increase in the intensity of pVim staining (2.18 ± 0.09 times control levels, n = 169) and, consistent with previous work in both heterologous cells and neurons (1, 18, 21), this was completely blocked by inhibitors of CaMKII (1.02 ± 0.06, n = 178; Fig. 1, B and C).

Having confirmed that pVim staining is caused by CaMKII-mediated phosphorylation, we next expressed this same construct in cultured hippocampal neurons. Immunocytochemical measurements were limited to only those puncta that colocalized with the presynaptic marker synaptophysin and thus our analysis reflects phosphorylation specifically at postsynaptic sites. Furthermore, to compare phosphorylation between synapses expressing variable amounts of vimentin reporter proteins, the magnitude of pVim staining was normalized to the magnitude of GFP staining, which directly correlates with protein level (see “Experimental Procedures”). pVim and anti-GFP antibody staining were used to quantitate phosphorylation of all reporter constructs.
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A schematic representation of constructs used in this work. All constructs were tagged with GFP except GFP was used for PSD-95 and Vim-CFP. Endogenous CaMKII phosphorylation sites are marked in red. Ser

B. A line diagram illustrating the phosphorylation status of various proteins at synapses. The proteins include NR2B-Vim (1.04–1.14-fold increase over control, n = 81), NR2B-Vim (1.24 ± 0.03 times control levels, n = 81), and NR2B-Vim (1.19 ± 0.04, n = 81). The control group showed no significant change compared to the untreated group. The results confirm and extend previous work demonstrating that immunocytochemical detection of Vim phosphorylation at intact synapses, it is possible that endogenous NR2B behaves differently than the recombinant GFP-NR2B-Vim. We

C. A graph showing the effect of various treatments on pVim staining of Vim-CFP. NMDA, 2 min, 100 μM; DMg, 2 min, 10 μM; NMDA, FK/Oka, 25 μM FK506, and 1 μM okadaic acid was applied 15 min prior to and throughout stimulation. In this and all subsequent experiments, error bars are S.E. **, p < 0.01 by one-way ANOVA followed by Bonferroni post hoc analysis.

D. A figure showing the effect of various treatments on pVim staining of Vim-CFP. NMDA, 2 min, 100 μM DMg, picrotoxin, 0 Mg²⁺, 2 min; 1.43 ± 0.05, n = 81; 1.04–1.14-fold increase over control, n = 81. The control group showed no significant change compared to the untreated group. The results confirm and extend previous work demonstrating that immunocytochemical detection of Vim phosphorylation at intact synapses, it is possible that endogenous NR2B behaves differently than the recombinant GFP-NR2B-Vim. We

E. A graph showing the effect of various treatments on pVim staining of Vim-CFP. NMDA, 2 min, 100 μM DMg, picrotoxin, 0 Mg²⁺, 2 min; 1.43 ± 0.05, n = 81; 1.04–1.14-fold increase over control, n = 81. The control group showed no significant change compared to the untreated group. The results confirm and extend previous work demonstrating that immunocytochemical detection of Vim phosphorylation at intact synapses, it is possible that endogenous NR2B behaves differently than the recombinant GFP-NR2B-Vim. We

FIGURE 1. Visualization of CaMKII substrate phosphorylation using vimentin. A schematic representation of constructs used in this work. All constructs were tagged with GFP except GFP was used for PSD-95 and Vim-CFP. Endogenous CaMKII phosphorylation sites are marked in red. Ser

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therefore performed Western blot analysis of the endogenous CaMKII phosphorylation site on NR2B (32) using an antibody that specifically recognizes the phosphorylated form of serine 1303. Western blots of total neuron lysates revealed no increase in Ser{sup}1303 phosphorylation following preincubation of cultures with the CaMKII inhibitor KN-93 (10 μM) as indicated. E, quantification of phospho-Ser{sup}1303 signal normalized to total NR2B. No statistically significant increases detected by one-way ANOVA.

Another important synaptic membrane protein that is phosphorylated by CaMKII and has specifically been implicated in playing a critically important role in LTP is the AMPAR subunit GluR1 (33–38). Indeed, phosphorylation of GluR1 affects the biophysical properties of AMPARs in a manner similar to that seen during LTP (39). Therefore, it was of great interest to examine GFP-GluR1-Vim (Fig. 1A) and determine whether its membrane localization at synapses restricts or enhances its phosphorylation by CaMKII. Surprisingly, it behaved very similarly to NR2B. When expressed in HEK293 cells, GFP-GluR1-Vim was robustly phosphorylated in response to ionomycin treatment (Fig. 3A) and in neurons, as previously reported for GFP-GluR1 (40) it trafficked to the synaptic plasma membrane (data not shown). However, synaptic GFP-GluR1-Vim was either not phosphorylated or minimally phosphorylated when cultured neurons were activated by a number of different treatments including NMDA application (100 μM) (1.13 ± 0.04, n = 37), picrotoxin in 0 Mg{sup}2+ buffer (1.05 ± 0.04, n = 37), 200 μM glutamate in 0 Mg{sup}2+ buffer (1.18 ± 0.02, n = 21), and ionomycin (10 μM; 1.07 ± 0.05, n = 24) (Fig. 3, B and C). Furthermore, preincubation with the protein phosphatase inhibitors okadaic acid and FK-506 did not significantly affect its phosphorylation. Therefore, it was of great interest to examine the phosphorylation of other AMPAR subunits at synapses, and determine whether their membrane localization at synapses restricts or enhances their phosphorylation by CaMKII. Importantly, similar to soluble Vim-CFP, NMDA treatment increased its phosphorylation (1.81 ± 0.07, n = 40), which was further increased by pretreatment with phosphatase inhibitors (2.10 ± 0.09, n = 42; Fig. 2C). These results suggest that the exact position of proteins within the PSD at excitatory synapses can influence and even prevent CaMKII-dependent phosphorylation of otherwise eligible substrates.

FIGURE 2. Synaptic GFP-NR2B-Vim is not phosphorylated by CaMKII. A, example images of HEK293 cells co-transfected with CaMKII-HA and GFP-NR2B-Vim or GFP-cNR2B-Vim. Cells were stimulated with ionomycin (3 min, 10 μM) Note CaMKII colocalization with GFP-NR2B-Vim. Green, GFP; white, pVim; red, anti-HA. B, examples of neurons expressing GFP-NR2B-Vim or GFP-cNR2B-Vim and stimulated with NMDA (2 min, 100 μM). Left panels show anti-GFP staining, right panels anti-pVim staining. C, quantification of effects of various treatments on pVim staining of GFP-NR2B-Vim and GFP-cNR2B-Vim. NMDA, 2 min, 100 μM; 0 Mg, Picro, 15 min, 50 μM; 0 Mg, glu, 30 s, 200 μM; Iono, 2 min, 10 μM; NMDA, FK/Oka, preincubated in 25 μM FK506 and 1 μM okadaic acid. **, p < 0.01 by one-way ANOVA followed by Bonferroni post hoc analysis. D, representative Western blot from neuronal lysates showing NR2B phospho-Ser{sup}1303 (top row) and total NR2B (bottom row). Treatments were glutamate application (1 min, 100 μM) in 0 Mg{sup}2+ buffer, with or without KN-93 pretreatment (1 h, 10 μM) as indicated. E, quantification of phospho-Ser{sup}1303 signal normalized to total NR2B. No statistically significant increases detected by one-way ANOVA.
not enhance GFP-GluR1-Vim phosphorylation (1.06 ± 0.03, n = 34; Fig. 3C).

As was done for NR2B, to determine whether a soluble, non-membrane localized form of GluR1 can be phosphorylated we examined the C terminus of GluR1, which was fused to GFP and Vim. & examples of neurons expressing GFP-GluR1-Vim or GFP-cGluR1-Vim. Cells were stimulated with ionomycin (3 min, 10 μM) or NMDA (2 min, 100 μM). Left panels show anti-GFP staining, right panels anti-pVim staining. Scale bar, 2 μM. C, quantification of effects of various treatments on pVim staining of GFP-GluR1-Vim and GFP-cGluR1-Vim. **, p < 0.01 by one-way ANOVA followed by Bonferroni post hoc analysis. D, representative Western blot from neuronal lysates showing GluR1 phospho-Ser831 (top row) and total GluR1 (bottom row). Treatments were glutamate application (1 min, 100 μM) or PDBu (3 min, 1 μM) with or without KN-93 (1 h, 10 μM) or Bis-I (15 min, 10 μM) pretreatment. E, quantification of phospho-Ser831 signal normalized to total GluR1. *, p < 0.05; **, p < 0.01 by one-way ANOVA followed by Bonferroni post hoc analysis.
tioning receptors, cell adhesion proteins and signaling molecules at their appropriate synaptic locations (14). Furthermore, CaMKII phosphorylation of the Drosophila PSD-95 homologue Dlg has been reported to regulate synaptic positioning of glutamate receptors (26). PSD-95 is known to bind NR2B directly (42, 43) and is also complexed with GluR1 through its interactions with Stargazin (44). Thus synaptic PSD-95 should be positioned closely to the NMDAR and AMPAR subunits, which were minimally phosphorylated by CaMKII.

Similar to all other constructs examined, the PSD95-Vim-CFP fusion protein was robustly phosphorylated when expressed in HEK293 cells that were treated with ionomycin (Fig. 4A), confirming that it is a substrate for CaMKII. When expressed in cultured neurons, PSD95-Vim-CFP exhibited a punctate synaptic localization (Fig. 4C). Because strong overexpression of PSD-95 can dramatically alter synaptic properties (45), we estimated its magnitude of overexpression using an antibody that recognized both endogenous and recombinant PSD-95 and found only ~17% increase in the levels of PSD-95 compared with uninfected, control cells (Fig. 4B). This suggests that lentivirus-mediated gene transfer can result in a modest degree of overexpression when compared with traditional mammalian expression vectors.

When PSD95-Vim-CFP expressing neurons were stimulated with either NMDA, which will activate both synaptic and extrasynaptic NMDARs, or with picrotoxin in Mg2+ free buffer, which will activate synaptic NMDARs, there was a robust increase in pVim staining. (1.64 ± 0.05, n = 35 and 2.12 ± 0.05, n = 51 respectively; Fig. 4, C and D). Synaptically evoked increases in pVim staining were dependent on activation of NMDARs, as it was blocked by co-application of the NMDAR antagonist MK-801 (10 μM) (1.15 ± 0.04, n = 38; Fig. 4D). Although NMDA directly activates only NMDARs, it will also depolarize neurons, which may result in activation of voltage-dependent Ca2+ channels as well as the release of glutamate that can then activate metabotropic glutamate receptors (mGluRs). Therefore to test whether Ca2+ influx through NMDAR channels alone is sufficient to cause CaMKII-dependent phosphorylation of PSD-95, we applied NMDA in the presence of a mixture of Ca2+ channel blockers and mGluR antagonists.
Phosphorylation of Synaptic Stargazin by CaMKII—Stargazin, an integral membrane protein that is critical for trafficking AMPARs to synapses, interacts directly with PSD-95 and GluR1 at synapses (15) and has recently been characterized as a substrate for CaMKII and PKC (28). Phosphorylation of Stargazin at these sites also appears to be required for LTP (28). It was therefore of interest to determine whether Vim-tagged Stargazin behaves like PSD-95 and is robustly phosphorylated by CaMKII or rather like GluR1 and exhibits minimal CaMKII-dependent phosphorylation. Similar to the other Vim-tagged proteins, Stgz-Vim-GFP was phosphorylated in HEK293 cells following ionomycin treatment (Fig. 6A) and was targeted to the synaptic plasma membrane when expressed in neurons (Fig. 6B). Upon stimulation with NMDA or picROTOXin in Mg2+-free buffer, Stgz-Vim-GFP showed a marked increase in its phosphorylation (1.66 ± 0.03, n = 33 and 1.55 ± 0.02, n = 62,
respectively; Fig. 6, B and D), which was particularly prominent at synapses as evidenced by co-localization with synaptophysin (Fig. 6C). Ca$^{2+}$ entry through NMDARs was both necessary and sufficient for Stgz-Vim-GFP phosphorylation as: (1) the increase in pVim staining elicited by synaptic activity (induced by picrotoxin application) was blocked by the NMDAR antagonist MK-801 (0.84 ± 0.02, n = 30; Fig. 6D) and (2) the increased pVim staining elicited by NMDA application was not affected by including a mixture of Ca$^{2+}$ channel, mGluR and activity blockers (1.71 ± 0.08, n = 36; Fig. 6D). These results indicate that Stgz is phosphorylated by CaMKII in a manner similar to that for PSD-95 and markedly different than the glutamate receptor subunits NR2B and GluR1.

Phosphatases Limit Phosphorylation of Stargazin—We have demonstrated that the phosphorylation of PSD-95 and Stgz is specifically due to CaMKII that was activated by Ca$^{2+}$ entry through NMDARs. As both L-type and R-type Ca$^{2+}$ channels can contribute to Ca$^{2+}$ rises in dendritic spines, which can activate CaMKII (47), an important issue is what mechanisms prevented the CaMKII-dependent substrate phosphorylation at synapses by activation of voltage-dependent Ca$^{2+}$ channels. Surprisingly, we found differences in how the phosphorylation of Stgz-Vim-GFP and PSD95-Vim-CFP by CaMKII was limited to activation of NMDARs. While glutamate-induced Stgz-Vim-GFP phosphorylation was greatly augmented by application of phosphatase inhibitors (from 1.56 ± 0.03, n = 94 to 2.11 ± 0.06, n = 64; Fig. 7A), PSD95-Vim-CFP phosphorylation was not (1.96 ± 0.05, n = 35 to 2.22 ± 0.07, n = 30; Fig. 7B). Furthermore, Stgz-Vim-GFP phosphorylation in the presence of phosphatase inhibitors was no longer solely dependent on NMDAR activation as the increase in pVim staining was only partially blocked by MK-801 (10 μM) (1.51 ± 0.04, n = 45, Fig. 7A). Instead, complete inhibition of Stgz-Vim-GFP phosphorylation required a combination of MK-801 and the L-type calcium channel blocker nifedipine (5 μM) (0.93 ± 0.05, n = 26) whereas the R-type Ca$^{2+}$ channel blocker Ni$^{2+}$ had no additional effect beyond that of MK-801 (1.43 ± 0.06, n = 29). In contrast, even in the presence of phosphatase inhibitors, PSD95-Vim-CFP phosphorylation was substantially reduced by MK-801 (1.20 ± 0.03, n = 52; Fig. 7B), indicating a reduced role of phosphatase regulation in its input specificity.

Further pharmacological experiments suggested that calcineurin was particularly important in limiting the phosphorylation of Stgz-Vim-GFP. Application of the calcineurin inhibitor FK506 alone (2.10 ± 0.11, n = 31), but not the PP1/PP2A inhibitor okadaic acid alone (1.79 ± 0.10, n = 30), enhanced the stimulated increase in Stgz-Vim-GFP phosphorylation to the same degree as application of both inhibitors together (2.11 ± 0.06, n = 64; Fig. 7C). Unstimulated, basal phosphorylation was not affected by the phosphatase inhibitors (1.10 ± 0.03, n = 43; Fig. 7C) suggesting that the activation of calcineurin required stimulation.

Taken together these results suggest that the CaMKII-dependent phosphorylation of Stgz-Vim-GFP at synapses is stimulated primarily by NMDAR activation and does not occur in response to activation of Ca$^{2+}$ channels in large part because of phosphatase activity, in particular calcineurin. Consistent with a more active role for phosphatases in limiting phosphorylation of Stgz-Vim-GFP than PSD95-Vim-CFP, stimulated phosphorylation of Stgz-Vim-GFP due to synaptic activity was reversed within 5 min following the stimulation whereas PSD95-Vim-CFP phosphorylation persisted for ~20 min (Fig. 7D).

**DISCUSSION**

Traditional biochemical approaches have been invaluable in identifying many candidate neuronal substrates for CaMKII (48), some of which have been extensively characterized (e.g. GluR1, see below) and found to be phosphorylated when biochemically isolated from neurons. An important limitation of such approaches, however, is that the cellular compartment in
which the phosphorylation has taken place is often difficult to identify and there is the possibility that the isolation/fractionation procedures may not yield absolutely pure subcellular fractions and may contribute to the extent of measured phosphorylation. This is particularly relevant when considering synaptic substrates that are part of the PSD since they are embedded in a complex macromolecular protein network (49). The methodology used to visualize substrate phosphorylation in the present work provides an important complementary approach because it permits maintenance of the normal synaptic architecture throughout the assay procedure. Thus it made possible an examination of which substrates at synapses in intact, living neurons are normally phosphorylated when CaMKII is activated by synaptic activity.

Using pVim staining to specifically visualize substrate phosphorylation by CaMKII at intact synapses, we have demonstrated that CaMKII exhibits remarkable specificity among substrates localized within the postsynaptic compartment, presumably the PSD, of excitatory synapses. Both the NMDAR subunit NR2B and the AMPAR subunit GluR1, which are both well documented substrates for CaMKII in biochemical assays, were poorly phosphorylated either under basal conditions or following manipulations designed to strongly stimulate neurons. In contrast, the synaptic scaffold protein PSD-95 and the AMPAR chaperone Stargazin exhibited large increases in phosphorylation upon stimulation. Soluble Vim constructs including the C-terminal tails of NR2B and GluR1 fused to Vim also showed robust phosphorylation. Since the same reporter sequence (i.e., Vim) was assayed using identical procedures in all experiments and the degree of pVim staining was normalized to expression level, the dramatic differences between the ability of endogenous CaMKII to phosphorylate different reporter constructs cannot be attributed to a staining or kinetic artifact.

It is conceivable that the recombinant, tagged proteins used in the present study may have incorporated into the postsynaptic compartment in a manner distinct from endogenous proteins. However, this...
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seems unlikely for several reasons. The magnitude of overexpression of the GFP-labeled constructs was modest due to the use of lentiviruses and GFP-labeled versions of all the reporter proteins have been found to behave appropriately even when strongly overexpressed in neurons (15, 40, 50–52). Furthermore, intact protein-protein interaction domains are required for appropriate synaptic incorporation of PSD-95, GluR1, NR2B, and Stargazin and we found that all constructs were targeted to synapses (15, 25, 51, 53). This suggests that these interactions are retained in our reporter constructs. Finally, the lack of stimulated, CaMKII-dependent phosphorylation of recombinant NR2B and GluR1 was confirmed using biochemical assays that measured phosphorylation of CaMKII sites on the endogenous proteins. These results suggest

that the postsynaptic molecular architecture is structured so that CaMKII activity is directed toward specific substrates within a single synaptic protein complex that also contains eligible CaMKII substrates that are not phosphorylated.

An unexpected result was the lack of phosphorylation of the GluR1 reporter, which has been the focus of intense interest as a CaMKII substrate because of its importance in the expression of NMDAR-dependent LTP (7, 54). Ser831 on GluR1 is known to be phosphorylated by either CaMKII or PKC (35, 41) and phosphorylation of this residue increases channel conductance of GluR1 homomers (39) and has been associated with LTP (36, 38). Furthermore, genetic deletion of GluR1 prevents LTP (34) and its delivery to synapses appears to be required for LTP (25, 54). However, there are several more recent findings suggesting that GluR1 is not a critical CaMKII substrate during LTP. Mutation of Ser831 to alanine to prevent its phosphorylation by CaMKII does not prevent the activity- and CaMKII-dependent delivery of GluR1 to synapses (25). Furthermore, when AMPARs containing both GluR1/GluR2 are studied, a stoichiometry commonly found in endogenous AMPARs (55), phosphorylation of GluR1 Ser831 no longer affects AMPAR channel properties (56). These results taken together with our observations that our GluR1 reporter is poorly phosphorylated, and that Ser831 is primarily phosphorylated by PKC, suggest that the locus of CaMKII regulation of AMPAR trafficking lies outside the GluR1 subunit.

CaMKII can directly bind to the C-terminal tail of NR2B (10, 30, 57) and this has been suggested to be important for the translocation of CaMKII to synapses upon stimulation (10). It was therefore also surprising that our NR2B reporter at synapses was minimally phosphorylated by CaMKII, especially given that Ca2+ influx through NMDARs was critical for the CaMKII-dependent phosphorylation of other substrates. Instead, the robust phosphorylation of PSD-95 and Stzg reporters by NMDAR activation suggests that at synapses, CaMKII regulates proteins that are important for the trafficking and scaffolding of glutamate receptors and not the receptors themselves. Indeed, the phosphorylation of Stargazin has recently been implicated in being important for LTP (28). Furthermore, that PSD-95 binding to NR2B (42, 43) and Stargazin interacts with both GluR1 and PSD-95 (15) provides additional evidence that CaMKII can discriminate between direct binding partners within the PSD.

Based on our results, we propose that the relative positioning of substrates and CaMKII as well as phosphatases dictate how CaMKII activity is routed in the synapse. Our results also suggest that postsynaptic substrate phosphorylation by CaMKII is dependent on the source of Ca2+ that activates CaMKII. PSD-95 and Stargazin were only phosphorylated in response to NMDAR activation and appear insulated from the CaMKII that is activated via other sources such as voltage-dependent Ca2+ channels. For Stargazin, this specificity appears to be achieved by activation of calcineurin. In contrast, the NMDAR-dependence of PSD-95 phosphorylation was less affected by phosphatase activity suggesting that its specific localization within the PSD, perhaps its direct tethering to the C-terminal tails of NMDARs, was critical for limiting its phosphorylation due to activation of Ca2+ channels. Previous work on Stargazin has found that application of NMMA can stimulate its dephosphorylation via PP1 and calcineurin (PP2B) (28). However, this work measured the contribution of any of nine possible phosphorylation sites, which may individually have different kinetics and activity-dependent phosphorylation profiles that are not captured in bulk shifts in electrophoretic mobility. Furthermore, since phosphatase specificity between vimentin and endogenous phosphorylation sites on Stargazin may differ, our results should not be interpreted as ruling out a role for PP1 in regulating endogenous Stargazin phosphorylation.

Previous studies have suggested that AMPAR-mediated synaptic transmission and synaptic maturity are tightly correlated with synapse size (58–60). Our results reveal that these parameters are unlikely to be encoded by CaMKII-dependent substrate phosphorylation, since the mean amount of substrate phosphorylation was constant across synapse size. Instead, as phosphorylation of the PSD-95 reporter substrate responded specifically to Ca2+ through NMDARs, we would suggest that average CaMKII substrate phosphorylation depends on NMDAR content and NMDAR-dependent Ca2+ increases at individual synapses; variables that appear to be poorly correlated with synapse size (61, 62). Recent studies have also shown that smaller spines, but not large spines, selectively contain NR2B subunits that mediate high variability in NMDAR-dependent Ca2+ rises within spines (61). Such variability in Ca2+ influx through NMDARs combined with greater heterogeneity in levels of CaMKII at smaller synapses could account for the observation that smaller spines exhibit greater variance in CaMKII-dependent substrate phosphorylation. This, in turn, may correlate with either the plasticity state of individual synapses or their potential for plasticity (63).

The methodology used in the present work will be helpful in determining which other substrates are phosphorylated by CaMKII at synaptic sites. It also seems likely that other kinases exhibit similar fine-tuning of synaptic substrate phosphorylation resulting in distinct signaling profiles for each synaptic protein kinase.

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