Ca\(^{2+}\)-independent Activation of Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II Bound to the C-terminal Domain of Ca\(_V\)2.1 Calcium Channels*

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Background: Calmodulin regulation of Ca\(^{2+}\) channels mediates short term synaptic plasticity.

Results: Binding of CaMKII to Ca\(_V\)2.1 channels induces Ca\(^{2+}\)-independent kinase activity.

Conclusion: A complex of CaMKII and Ca\(_V\)2.1 channels is required for short term synaptic plasticity.

Significance: CaMKII bound to Ca\(_V\)2.1 may regulate synaptic plasticity.

Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) forms a major component of the postsynaptic density where its functions in synaptic plasticity are well established, but its presynaptic actions are poorly defined. Here we show that CaMKII binds directly to the C-terminal domain of Ca\(_V\)2.1 channels. Binding is enhanced by autophosphorylation, and the kinase-channel signaling complex persists after dephosphorylation and removal of the Ca\(^{2+}\)/CaM stimulus. Autophosphorylated CaMKII can bind the Ca\(_V\)2.1 channel and synapsin-1 simultaneously. CaMKII binding to Ca\(_V\)2.1 channels induces Ca\(^{2+}\)-independent activity of the kinase, which phosphorylates the enzyme itself as well as the neuronal substrate synapsin-1. Facilitation and inactivation of Ca\(_V\)2.1 channels by binding of CaMKII/CaM mediates short term synaptic plasticity in transfected superior cervical ganglion neurons, and these regulatory effects are prevented by a competing peptide and the endogenous brain inhibitor CaMKIIN, which blocks binding of CaMKII to Ca\(_V\)2.1 channels. These results define the functional properties of a signaling complex of CaMKII and Ca\(_V\)2.1 channels in which both binding partners are persistently activated by their association, and they further suggest that this complex is important in presynaptic terminals in regulating protein phosphorylation and short term synaptic plasticity.

P/Q-type calcium currents (1) are conducted by voltage-gated Ca\(^{2+}\) channel Ca\(_V\)2.1 (2), which is localized in high density in presynaptic nerve terminals (3, 4) and initiates synaptic transmission at most synapses in the central nervous system (1, 5, 6). The Ca\(_V\)2.1 channel protein consists of a pore-forming \(\alpha_1\) subunit associated with auxiliary \(\beta, \alpha_2\delta, \) and possibly \(\gamma\) subunits (7, 8). The function of Ca\(_V\)2.1 channels is dynamically regulated by interaction with SNARE proteins, G-protein \(\beta\gamma\) subunits, RIM and other SNARE-interacting proteins, calmodulin (CaM), and related calcium sensor proteins (8, 9). Ca\(_V\)2.1 channels form a presynaptic complex that is co-localized with nearly 100 interacting proteins at the active zone, which serves to dock neurotransmitter vesicles, modulate Ca\(_V\)2.1 channel function, and mediate exocytosis (9, 10).

Ca\(^{2+}\)/CaM-dependent protein kinase II (CaMKII) is a ubiquitous, multifunctional serine/threonine kinase (11–14). It mediates Ca\(^{2+}\)-dependent phosphorylation of a wide range of neuronal targets (15, 16). CaMKII in the brain is composed of dodecamers of 52-kDa \(\alpha\) and 60-kDa \(\beta\) subunits (17, 18). Under basal conditions, an autoinhibitory domain binds to the catalytic domain, rendering the kinase inactive. Upon Ca\(^{2+}\) influx, Ca\(^{2+}\)/CaM binds to the autoinhibitory domain, relieves the block of kinase activity, and stimulates autophosphorylation of Thr-286 and phosphorylation of other substrates. After phosphorylation of Thr-286, block of catalytic activity is re-established slowly even after the Ca\(^{2+}\) level falls and Ca\(^{2+}\)/CaM dissociates from the kinase, which allows CaMKII to integrate signals from trains of Ca\(^{2+}\) transients (19–22). On the postsynaptic side of the synapse, CaMKII phosphorylation regulates glutamate receptors in long term potentiation and has several other well established functions (15, 16).

CaMKII is also present in presynaptic terminals (23, 24), but its presynaptic functions are not well established. Previous studies implicate presynaptic CaMKII in different forms of synaptic plasticity (25–29), including modulation of paired-pulse facilitation (30–32). CaMKII forms a complex with Ca\(_V\)2.1 channels in transfected cells via a site in the proximal C-termi-
nal domain (33). Interaction with CaMKII increases CaV2.1 channel activity and enhances CaV2.1 channel facilitation by slowing inactivation and shifting the voltage dependence of inactivation to more positive membrane potentials (33). These effects of CaMKII did not require the catalytic activity of the kinase, suggesting that binding per se was sufficient for channel regulation (33). Here we report that CaMKII binds directly to a site in the C-terminal domain of CaV2.1 channels and that auto-phosphorylation of CaMKII stimulates binding to this site. Autophosphorylated CaMKII can bind to the CaV2.1 channel and synapsin-1 simultaneously. Binding of CaV2.1 to CaMKII induces CaV2.1-independent kinase activity, which mediates both autophosphorylation and phosphorylation of synapsin-1 at Ser-603. Block of this interaction with competing peptides or both autophosphorylation and phosphorylation of synapsin-1 inhibits CaMKII at the site of influx of Ca2+. Block of this interaction with competing peptides or both autophosphorylation and phosphorylation of synapsin-1 inhibits synaptic plasticity.

Binding of CaV2.1 to CaMKII at the site of influx of Ca2+ induces Ca2+-independent kinase activity, which mediates both autophosphorylation and phosphorylation of synapsin-1 at Ser-603. Block of this interaction with competing peptides or both autophosphorylation and phosphorylation of synapsin-1 inhibits synaptic plasticity.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—CaV2.1(1848–1964)pGEX-4T-2, encoding the C-terminal domain of CaV2.1 containing the IQ-like motif, was amplified via PCR using CaV2.1(1766–2212) as template. The PCR product was cloned into the BamHI/EcoR1 sites of pGEX-4T-2. CaV2.1(1959–2035)pGEX-4T-2, encoding the C-terminal domain of CaV2.1 containing the IQ-like motif in pGEX-4T-2, was amplified via PCR using CaV2.1(1766–2212) as template. The PCR product was cloned into the BamHI/EcoR1 sites of pGEX-4T-2. CaV2.1(1848–1964)pGEX-4T-2, encoding the C-terminal domain of CaV2.1 containing the IQ-like motif, was amplified via PCR using CaV2.1(1766–2212) as template. The PCR product was cloned into the BamHI/EcoR1 sites of pGEX-4T-2. CaV2.1(1848–1964)/EEDAAA, encoding the C-terminal domain of CaV2.1 containing the IQ-like motif, was cloned into BamHI/EcoR1 sites of pGEX-4T-2. The CaMKII tethering site TVGKICY, located upstream of IQ-like containing domain, was mutated to EEDAAA. N-terminal MGCALCC lipid anchor + CaV2.1(1766–2211) was amplified via PCR using the CaV2.1 α-subunit as template. The PCR product was cloned into the BamHI/EcoR1 sites of pCDNA3.1/myc-HisA. Myc-CaV2.1(1764–2211) was generated by subcloning α2.1 C-terminal fragment into CS2 + Myc with six consecutive myc tags (33).

cDNA encoding full-length CaMKII was amplified by PCR, and the product was cloned into the BamHI/EcoR1 sites of the pMALc2X vector. Synapsin-1/pEGFP c-1 encodes full-length synapsin-1 in pEGFP c-1. Syntaxin-1A pGEX-4T-2 encodes syntaxin-1A in pGEX-4T-2 (34). Na+,1.2(1848–1964)pGEX-4T-2, encoding a segment of the C-terminal domain of the Na+,1.2 channel, was amplified via PCR using full-length Na+,1.2 as template. The PCR product was cloned into the BamHI/Xho1 sites of pGEX-4T-2.

The following antibodies were diluted and used as described below: anti-myc, 1:5,000 (monoclonal, Sigma); anti-CaMKII, 1:5,000 (monoclonal, BD Transduction Laboratories); anti-GST, 1:10,000 (monoclonal, Sigma); anti-phospho-CaMKII-(Thr-286), 1:10,000 (polyclonal, PhosphoSolutions); anti-CaM, 1:5,000 (monoclonal, Millipore); anti-Hsp 90, 1:10,000 (monoclonal, BD Transduction Laboratories); anti-phospho-Synapsin-1(Ser-603), 1:10,000 (polyclonal, PhosphoSolutions); anti-Synapsin-1, 1:10,000 (polyclonal, PhosphoSolutions).

**Culture and Transfection of tsA-201 Cells**—TsA-201 cells were plated and grown at the density of 9 x 10^5 cells/100 mm dish in Hyclone DMEM/F medium and afterward used in experiments (33). The cells were transfected using trans-it-LT1 (Mirus) and 8 μg of total DNA.

**Protein Extraction and Immunoblotting**—At least 24 h post transfection, tsA-201 cells were processed to extract recombinant protein. The Petri dishes were placed on ice, and cells were harvested with a rubber scraper and sedimented at 3500 rpm at 4 °C for 20 min. Cells were washed once with 20 ml of ice-cold PBS to remove serum proteins. The cell pellet was resuspended in 500 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 20 mM β-glycerophosphate, 320 mM sucrose, complete protease inhibitor mixture (Roche Diagnostics), HalTM phosphatase inhibitor mixture (Thermo Scientific), and pipetted up and down 10 times. The preparation was sedimented at 2500 rpm at 4 °C for 5 min to remove the nuclear fraction. The supernatant was collected and sedimented at 14,000 rpm at 4 °C for 30 min. The pellet was washed with 500 μl of lysis buffer and sedimented again at 14,000 rpm at 4 °C for 30 min to obtain pure pellet devoid of contaminants from the previous supernatant fraction. The pellet was resuspended in 20 μl of 2X sample buffer and heated at 50 °C for 5 min. Proteins were resolved on 4–20% SDS-PAGE gels, and immunoblotting was performed with the indicated antibodies.

**Co-immunoprecipitation**—TsA-201 cells were cultured and transfected as described above. For co-immunoprecipitation studies, the cells were processed as described previously (33).

**Recombinant Protein Production**—Purified recombinant CaMKII was a kind gift of Dr. Thomas Soderling (Oregon Health and Science University, Portland, OR (35)). GST-tagged CaV2.1(1848–1964), CaV2.1(1959–2035), or GST alone were expressed in *Escherichia coli* (BL21) in baffled shaker flasks. Isolated single colonies were inoculated and grown overnight in 50 ml of LB medium containing 100 μl of ampicillin (100 mg/ml) at 37 °C and 220 rpm to obtain precultures. Large-scale cultures were prepared using 400 ml of LB containing 800 μl of ampicillin with the addition of 10 ml of overnight precultures. The cells were grown at 37 °C and 220 rpm until the absorbance increased to 1.0 at 600 nm. Protein expression was induced by the addition of 400 μl of 100 mM isopropyl 1-thio-β-D-galacto-pyranoside for 14 h at 11 °C. Cells were harvested by centrifugation at 3500 rpm, 4 °C for 30 min, and washed 3 times by resuspension in 50 ml of cold PBS. The 15-ml cell suspension (in PBS) was incubated with 500 μl of lysozyme (10 mg/ml) for 1 h at 4 °C with shaking and subsequently sonicated four times at 20-s intervals. Cell-free supernatant was obtained by 2 rounds of centrifugation at 13,000 rpm at 4 °C for 1 h. Glutathione-Sepharose beads (100 μl) were washed 3 times in 50 ml of cold PBS and sedimented at 1500 rpm at 4 °C for 1 min before use. Cell-free supernatant from the earlier step was incubated overnight with glutathione-Sepharose beads. Nonspecific binding was reduced by washing the bound protein six times with cold PBS. The washes were carried out for 30 min at 4 °C on a
shaker, and beads/resin were sedimented at 1500 rpm for 1 min at 4 °C. The yield of fusion protein was estimated by Coomassie Blue staining after SDS-PAGE using a calibration curve with bovine serum albumin. These proteins are pure by SDS-PAGE analysis and are native with respect to binding of CaM as expected (Fig. 1).

For some experiments, CaMKII was expressed with a maltose-binding protein (MBP) epitope tag on its N terminus (36). MBP-CaMKII or MBP alone was expressed in E. coli (BL21) in baffled shaker flasks. Isolated single colonies were inoculated, grown overnight in 50 ml of LB medium containing 100 µl ampicillin (100 mg/ml) at 37 °C and 220 rpm to obtain precultures. Large-scale cultures were prepared using 400 ml of LB containing 800 µl of ampicillin with the addition of 10 ml of overnight precultures. The cells were grown at 37 °C and 220 rpm until the absorbance increased to 1.0 at 600 nm. Protein expression was induced by the addition of 400 µl of 100 mM isopropyl-1-thio-β-d-galactopyranoside for 14 h at 11 °C. Cells were harvested by centrifugation at 3500 rpm and 4 °C for 30 min and washed 3 times by resuspension in 50 ml of cold PBS. The 15-ml cell suspension (in PBS) was incubated with 500 µl of lysozyme (10 mg/ml) for 1 h at 4 °C with shaking and subsequently sonicated 4 times at 20-s intervals. Cell-free supernatant was obtained by 2 rounds of centrifugation at 13,000 rpm and 4 °C for 1 h. Amylose resin (100 µl) was washed 3 times in 50 ml of cold PBS and sedimented at 1500 rpm and 4 °C for 1 min before use. Cell-free supernatant from the earlier step was incubated overnight with amylose resin beads. Nonspecific binding was reduced by washing the bound protein six times with cold PBS. The washes were carried out for 30 min at 4 °C on a shaker, and beads/resin were sedimented at 1500 rpm for 1 min at 4 °C. The yield of fusion protein was estimated by Coomassie Blue staining after SDS-PAGE using a calibration curve with bovine serum albumin.

**Binding Experiments**—Binding of CaMKII-GST fusion proteins and CaMKII was analyzed by GST pulldown assays using Tris binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, and 0.1% BSA). Autophosphorylation of Thr-286 in purified CaMKII was carried out by incubation on ice for 5 min in Tris binding buffer containing 0.025 mM CaCl₂, 0.125 µM CaM, 0.025 mM ATP, and 0.125 mM MgCl₂ (37). This procedure resulted in essentially complete phosphorylation of CaMKII as judged by its change in mobility in SDS-PAGE (Fig. 1C). The binding reaction was carried out for 1 h at 4 °C. After incubation, washing was performed in binding or washing buffer with the addition of 5 mM EGTA where indicated. The proteins were washed 3 times for 5 min at 4 °C by sedimentation at 500 rpm. The beads were boiled at 95 °C in 10 µl of 4X sample buffer. Proteins were resolved on 10% SDS-PAGE, and immunoblotting was performed. Binding of CaMKII was detected using anti-CaMKII (monoclonal, BD Biosciences). CaMKII phosphorylation at Thr-286 was detected using antiphospho-CaMKII (polyclonal, PhosphoSolutions). For re-probing of immunoblots, nitrocellulose membranes were stripped as described (38). Equal bait loading was confirmed by blotting with anti-GST (Sigma). Immunoblots (ECL detection) were documented with Chemidoc XRS (Bio-Rad).

Binding was quantified using densitometric measurement of band intensity using NIH ImageJ software.

**Expression and Electrophysiological Recording in Cultured Neurons**—Superior cervical ganglion (SCG) neurons were cultured as described to allow synapse formation (39). cDNAs encoding α₁.2 subunit, eGFP, and (where indicated) CaMKII was microinjected into the nuclei of SCG neurons through glass micropipettes with 5% fast-green dye (Sigma). Entry of the injected reagents into the cell nucleus was monitored by the intensity of green dye in the nucleus. The cells were maintained at 37 °C in a 95% air, 5% CO₂ humidified incubator for 2–3 days.

Excitatory postsynaptic potentials (EPSPs) were recorded from SCG neurons cultured for 6 weeks as described (39). Injected neurons were identified with an inverted microscope equipped with an epifluorescence unit. Conventional intracellular membrane potential recordings were made from two neighboring neurons using microelectrodes filled with 1 m KAc (70–90 mΩ). Paired action potentials were generated in the injected, presynaptic neuron expressing the α₁.2.1 channels and eGFP by passing 1–2 nA of current for 5 ms through the intracellular recording electrode. EPSPs were recorded from a neighboring non-injected neuron. CaMKII was co-expressed with CaMKII channels or CaMKII (1897–1912) was injected 30 min before recordings as indicated. Endogenous synaptic transmission was blocked by bath application of 3 mM ω-conotoxin GVIA in a modified Krebs solution consisting of 136 mM NaCl, 5.9 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 11 mM glucose, and 3 mM Na-HEPES, pH 7.4. For recording sub-threshold EPSPs, the membrane potential of the postsynaptic cell was held at −70 or −80 mV by passing current (0.2–0.4 nA) through the recording electrode. Each 30-s recording protocol was repeated 4 times for each inter-stimulus interval, and the ratio of the second EPSP to the first EPSP was averaged for each synapse. Data values with associated error shown in the text and figures represent the mean ± S.E.

**RESULTS**

**CaMKII Binds Directly to the C-terminal Domain of CaMKII Channels**—As shown in previous studies, we confirmed that CaMKII binds to the C-terminal domain of CaMKII channels in transfected cells, as assessed by co-immunoprecipitation (Fig. 2A) (33). However, it was not known whether this binding interaction was direct or required other intermediary proteins. To measure direct interactions between CaMKII and CaMKII, we investigated binding of CaMKII in vitro to adjacent segments of the C-terminal domain that contain the two components of the calcium sensor protein interaction site: the IQ-like motif (CaMKII(1848–1964)) and the CaM binding domain (CaMKII(1959–2035)) (40, 41). We prepared autophosphorylated CaMKII in which Thr-286 was essentially completely phosphorylated (Fig. 1C). We incubated purified preparations of nonactivated, CaMKII, and treated, and autophosphorylated CaMKII with purified GST-tagged CaMKII(1848–1964), CaMKII(1959–2035), or GST alone in vitro and detected the complex by GST pulldown assay. Binding of autophosphorylated CaMKII to GST-CaMKII(1848–1964) (Fig. 2B, lane a3) was substantially greater than binding of nonactivated CaMKII
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FIGURE 1. Expression, purification, and function of CaV2.1 and NaV1.2 proteins. A, CaV2.1(1848–1964)-GST (lane 1), CaV2.1(1959–2035)-GST (lane 2), and GST (lane 3) were expressed and purified as described under “Experimental Procedures.” Samples (7.5 µg of purified protein) were denatured, loaded, and resolved on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to show the quality of the expressed proteins, which were used for in vitro binding assays. B, expression and purification of NaV1.2(1848–1964)-GST, NaV1.2(1959–2035)-GST, and GST alone were incubated with CaMKII. Lack of any band corresponding to the nonactivated CaMKII in lane 1 suggests that CaMKII is autophosphorylated to near completion under our experimental conditions. C, extent of CaMKII phosphorylation. Nonactivated CaMKII was analyzed side by side with autophosphorylated CaMKII to estimate the extent of CaMKII autoprophorylation by comparison of their migration positions in SDS-PAGE. Autophosphorylation of CaMKII causes an upward shift in the migration of CaMKII (lane 2, arrow) as compared with the nonactivated CaMKII in lane 1 (arrowhead) when separated on 4–20% SDS-PAGE. The blot was developed using anti-CaMKII that detects both nonactivated and autophosphorylated CaMKII. Lack of any band corresponding to the nonactivated CaMKII in lane 2 (compared with lane 1) suggests that CaMKII is autophosphorylated to near completion under our experimental conditions. D, CaV2.1(1848–1964)-EEDAAA-GST (lane 1), CaV2.1(1848–1964)-GST (lane 2), and GST (lane 3) were expressed and purified as described under “Experimental Procedures.” Samples (7.5 µg of purified protein) were denatured, loaded, and resolved on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to show the purity of the expressed proteins, which were used for in vitro binding assays. E, binding of CaV2.1(1848–1964)-EEDAAA, CaV2.1(1848–1964)-GST, and GST alone to CaM. a, the blot was probed with anti-CaM; b, the same blot after stripping and re-probing with anti-GST to show loading of CaV2.1(1848–1964)-EEDAAA (lane 1), CaV2.1(1848–1964)-GST (lane 2), and GST (lane 3). c, quantitation of relative CaM binding under the indicated conditions (means ± S.E.; **p < 0.01 by Student’s t test; n = 5). Asterisks mark the primary protein band corresponding to the expressed protein of interest in panels A, B, and D.

or CaMKII incubated with Ca2+]/CaM but without ATP (Fig. 2B, lanes a1 and a2). In contrast, GST-tagged CaV2.1(1959–2035) did not bind any of the three forms of CaMKII (Fig. 2B, lanes a4–a6), and GST itself was also unable to bind CaMKII (Fig. 2B, lanes a7–a9). Quantification of the results revealed that binding of the autophosphorylated kinase to GST-CaV2.1(1848–1964) was 8-fold greater than binding to the non-
activated or Ca\(^{2+}\)/CaM-treated kinase (Fig. 2C). These results demonstrate direct and specific binding of CaMKII to CaV2.1(1848–1964) containing the IQ-like motif.

In similar experiments we used GST-tagged syntaxin 1A as a positive control ligand for specific binding to autophosphorylated CaMKII. Syntaxin 1A is a key component of the SNARE complex that initiates regulated exocytosis, and it binds only autophosphorylated CaMKII (42). We observed CaMKII-syntaxin 1A interaction only when CaMKII was autophosphorylated (Fig. 2D, lanes a4–a6). Autophosphorylated CaMKII also bound specifically to CaV2.1(1848–1964) (Fig. 2D, lanes a1–a3) in a parallel experiment, demonstrating preferential binding of autophosphorylated CaMKII to CaV2.1(1848–1964) as compared with nonactivated or Ca2\(^{++}\)/CaM-treated CaMKII.

Location of the CaMKII Binding Site—CaV2.1(1848–1964) contains an IQ-like motif as part of a bipartite regulatory site that is important for CaM-induced facilitation and inactivation of CaV2.1 channels (9). IQ-like motifs bind CaM, which could potentially serve as a docking site for CaMKII (43). On the other hand, CaM-independent docking of CaMKII to cardiac CaV1.2 channels is well described, suggesting that CaM is not required for CaMKII binding to CaV2.1 channels (37)

![Graph A](image1.png)

**Figure 3. Binding site for CaMKII on CaV2.1 channels.** A, binding of CaMKII was detected by GST pull-down assay. CaMKII (20 nM) was used as prey in the presence/absence of Ca\(^{2+}\)/CaM (5 \(\mu\)M) and ATP (1 mM) as indicated. GST alone or GST-tagged CaV2.1(1848–1964) was used as bait as indicated: WT, TVGKFIY; mutant, EEDAAA. a, the blot was probed with anti-phospho-CaMKII(Thr-286). b, the same blot after stripping and re-probing using anti-GST to show equal loading of CaV2.1(1848–1964). B, quantitation of relative CaMKII autophosphorylation using anti-phospho CaMKII(Thr-286) under the indicated conditions is shown (mean ± S.E.; **, \(p < 0.01\) by Student’s t test; \(n = 6\)).

![Graph B](image2.png)

**Figure 4. Persistence of binding of autophosphorylated CaMKII to CaV2.1(1848–1964).** A, binding of CaMKII was detected by GST pull-down assay. CaV2.1(1848–1964) was incubated with CaMKII (20 nM) and Mg\(^{2+}\) (5 mM) in the presence/absence of Ca/CaM (5 \(\mu\)M) and ATP (1 mM) as indicated. Samples were washed in the absence or presence of 5 mM EGTA as indicated. a, the blot was probed with anti-CaMKII antibody. b, the same blot after stripping and re-probing using anti-phospho-CaMKII(Thr-286). c, the same blot after stripping and reprobing using anti-CaM. d, the same blot after stripping and re-probing using anti-GST to show equal loading of CaV2.1(1848–1964). B, quantitation of relative CaMKII binding using anti-CaMKII under the indicated conditions (mean ± S.E.; **, \(p < 0.01\); ***, \(p < 0.001\) by Student’s t test; \(n = 4\)).
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![Image of a graph or diagram related to the text]

**FIGURE 5. Persistence of binding of CaMKII after dephosphorylation.** Purified recombinant protein phosphatase 1 (PP1) was added before (Pre-PP1) or after (Post-PP1) the kinase-channel complex was established as indicated. Washes were carried out in the presence or absence of 5 mM EGTA as indicated. A, binding of CaMKII to Ca\textsubscript{v}2.1(1848–1964) GST was detected by GST pulldown assay, a, the blot was probed with anti-CaMKII. b, the same blot is shown after stripping and re-probing using anti-phospho-CaMKII(Thr-286). c, the same blot after stripping and re-probing using anti-GST to show equal loading of GST.

**FIGURE 6. Activation of autophosphorylation of CaMKII by binding to Ca\textsubscript{v}2.1 channels.** Ca\textsubscript{v}2.1(1766–2212) was expressed in tsA-201 cells, and autophosphorylation of endogenous CaMKII was measured with anti- phospho-CaMKII(Thr-286). A, left to right, untreated control tsA-201 (lane 1), cells transfected with C-terminal domain of Ca\textsubscript{v}2.1 (lane 2), cells treated with 5 \textmu M ionomycin to allow Ca\textsuperscript{2+} entry for 15 min before lysis (lane 3), and cells treated with 5 \textmu M ionomycin and additionally with cyclosporin A to inhibit the protein phosphatase calcineurin (lane 4). a, autophosphorylation of CaMKII was assayed by immunoblotting with anti- phospho-CaMKII-(Thr-286). b, the same blot after stripping and re-probing with anti-Hsp90 to show equal loading of cell lysates. B, quantitation of relative CaMKII autophosphorylation using anti-phospho-CaMKII(Thr-286) under the indicated conditions (means ± S.E.; *, p < 0.01 by Student’s t test; n = 3).

EEDAAA (Fig. 3A, lanes a2 and a3), confirming the specificity of binding to phospho-CaMKII(Thr-286). These results indicate that the TGYKIY motif forms an important part of the binding site for CaMKII, but other nearby sequence elements must also contribute substantially to kinase binding when this sequence is mutated.

**Binding of Autophosphorylated CaMKII Persists after Ca\textsuperscript{2+} / CaM Dissociation—**Binding of CaMKII to Ca\textsubscript{v}1.2 and Ca\textsubscript{v}2.1 channels modulates their function (33, 37). The local Ca\textsuperscript{2+} concentration at the active zone is tightly regulated to create a Ca\textsuperscript{2+} nanodomain (9). Because of its slowly reversible autophosphorylation, activation of CaMKII can integrate repetitive Ca\textsuperscript{2+} signals and serve as a molecular memory of synaptic activity (15). Therefore, it is important to test the persistence of the kinase-channel interaction as the level of Ca\textsuperscript{2+} declines. To address this question, we chelated Ca\textsuperscript{2+} using 5 mM EGTA in the washing buffers. CaMKII was isolated by binding to Ca\textsubscript{v}2.1(1848–1964) immobilized to glutathione-Sepharose beads, and the binding of CaMKII was analyzed in the absence or presence of 5 mM EGTA in the washing buffer (Fig. 4A). CaMKII is highly phosphorylated in the presence of Ca\textsuperscript{2+}/CaM and ATP. Autophosphorylated CaMKII bound more effectively to Ca\textsubscript{v}2.1(1848–1964) (Fig. 4A, lane a3) than nonactivated or Ca\textsuperscript{2+}/CaM-treated CaMKII (Fig. 4A, lanes a1 and a2). Both Ca\textsuperscript{2+}/CaM and ATP are essential components in triggering the autophosphorylation reaction, and omission of ATP followed by chelation of Ca\textsuperscript{2+} using EGTA (5 mM) in the washing reaction reduced binding significantly (Fig. 4A, lane a4). However, binding of the autophosphorylated kinase persisted even after Ca\textsuperscript{2+}/CaM was removed from the kinase-channel complex by washes with EGTA-containing washing buffer (Fig. 4A, lane a5), suggesting that Ca\textsuperscript{2+}/CaM removal does not rapidly reverse binding of autophosphorylated CaMKII. The efficacy of chelation of Ca\textsuperscript{2+}/CaM by EGTA treatment was probed using anti-CaM. No bound CaM was detected after EGTA treatment (Fig. 4A, lanes c4–c5). We quantified the relative CaMKII binding in each of these conditions and found significant retention of bound CaMKII after EGTA treatment (Fig. 4B). Collectively, these results show that binding of CaMKII to Ca\textsubscript{v}2.1(1848–1964) is greatly enhanced by Ca\textsuperscript{2+}/CaM-dependent autophosphorylation of CaMKII, but once formed, the complex persists even after removal of Ca\textsuperscript{2+}/CaM.

**Dephosphorylation of CaMKII Does Not Reverse Binding—**To test whether autophosphorylation is required to maintain binding of CaMKII to Ca\textsubscript{v}2.1(1848–1964), we dephosphorylated the channel-bound CaMKII and tested the fate of kinase-channel interaction. Protein phosphatases PP1, PP2A, PP2B, and PP2C represent the majority of serine/threonine phosphatase activity in brain and other tissues (44). CaMKII in isolated post-synaptic densities was mostly dephosphorylated by PP1 (44). Autophosphorylated CaMKII preferentially bound to Ca\textsubscript{v}2.1(1848–1964) (Fig. 5A, lane a1). Dephosphorylation of autophosphorylated CaMKII by PP1 treatment before incuba-
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To extend these findings of increased autophosphorylation of CaMKII bound to Ca_{2.1} channels in transfected tsA-201 cells. Quantification of these results revealed an ~6-fold increase in autophosphorylation upon transfection of Ca_{2.1}(1766–2212) or treatment with ionomycin (Fig. 6B, lane a4), suggesting that calcineurin does not play a major role in control of CaMKII phosphorylation in tsA-201 cells.
cells to pure proteins, we reconstituted the kinase-channel dimeric complex in vitro and measured autophosphorylation. We activated CaMKII in the presence of Ca\(^{2+}\)/CaM, ATP, and Mg\(^{2+}\) and studied the autophosphorylation levels in the absence or presence of the Ca\(_{2.1}(1848–1964)\) peptide (Fig. 7A). Autophosphorylation of CaMKII increased in the presence of increasing concentrations of the Ca\(_{2.1}\) channel peptide (Fig. 7Aa, lanes 1–4) and then decreased at higher concentrations of the peptide (Fig. 7Aa, lanes 4–6). Quantification of the levels of CaMKII autophosphorylation using anti-phospho-CaMKII-(Thr-286) (Fig. 7B) showed that direct binding of Ca\(_{2.1}(1848–1964)\) in vitro mimics the activation of kinase autophosphorylation observed in transfected tsA-201 cells. These results confirm that direct binding of Ca\(_{2.1}(1848–1964)\) activates CaMKII autophosphorylation. As negative controls, we used a GST-tagged protein of the same size from the C-terminal of Nav1.2 channels (Fig. 7, C and D) or GST itself (data not shown), and we found no effect on CaMKII activity, further supporting the specificity of this interaction.

Phosphorylation of Synapsin-1 by CaMKII Bound to Ca\(_{2.1}(1766–2212)\) Channels

Synapsin-1 is a major presynaptic phosphoprotein that is a prominent substrate for CaMKII, and phosphorylation by CaMKII regulates the effects of synapsin-1 on synaptic vesicle trafficking (23). Phosphorylation of synapsin-1 by CaMKII substantially increases synaptic transmission at the squid giant synapse (28, 29). Expression of Ca\(_{2.1}(1766–2212)\) with synapsin-1 in tsA-201 cells led to a substantial increase in synapsin-1 phosphorylation at Ser-603 (Fig. 8A, lane a3) compared with untransfected tsA-201 cells (Fig. 8A, lane a1) or cells expressing synapsin-1 alone (Fig. 8A, lane a2). Increasing the cytosolic Ca\(^{2+}\) concentration with ionomycin, which triggers CaMKII autophosphorylation, also led to a significant increase in synapsin-1 phosphorylation at Ser-603 (Fig. 8, lane a4), and these levels are comparable to those observed when synapsin-1 is coexpressed with Ca\(_{2.1}(1766–2212)\). Ionomycin treatment of tsA-201 cells coexpressing Ca\(_{2.1}(1766–2212)\), and synapsin-1 shows further enhancement in synapsin-1 phosphorylation (Fig. 8, lane a5). These results indicate that the C-terminal domain of Ca\(_{2.1}\) channels stimulates activation and autophosphorylation of CaMKII as effectively as Ca\(^{2+}\)/CaM, and this leads to phosphorylation of synapsin-1 at Ser-603 and potentially to phosphorylation of other presynaptic substrates.

If binding of Ca\(_{2.1}\) to CaMKII can lead to phosphorylation of synapsin-1, a stable ternary complex of Ca\(_{2.1}\) and synapsin-1 bound to CaMKII may be formed. To test this possibility we expressed CaMKII in bacteria with a MBP epitope tag and purified the resulting fusion protein. MBP-CaMKII attached to amylase resin was able to bind both Ca\(_{2.1}(1848–1964)\) and synapsin-1 simultaneously (Fig. 9), whereas control experiments with MBP showed no binding (Fig. 9B). Formation of this ternary complex in presynaptic terminals would allow local phosphorylation of synapsin-1 by CaMKII bound to Ca\(_{2.1}\) channels to modulate the dynamics of synaptic vesicle function in active zones containing these proteins. As CaMKII is a dodecamer, this ternary complex may be formed within a single subunit or may reflect binding of Ca\(_{2.1}\) and synapsin-1 to different subunits in the CaMKII complex.
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terminal from the first stimulus (9). Paired-pulse facilitation of synaptic transmission in this transfected SCG neuron preparation is primarily caused by facilitation of Ca,2.1 channel activity by Ca2+/CaM binding to the Ca2+ sensor protein binding site in the C-terminal domain (48). As illustrated in Fig. 10A, Ca,2.1 channels expressed alone generate synaptic transmission in which the paired-pulse ratio is highly dependent on the inter-stimulus interval (ISI) between the paired pulses. At short ISI, synaptic depression is dominant, and paired-pulse ratio values are less than 1.0. At longer ISI, synaptic facilitation becomes dominant, peaks at ~1.75 for an ISI of 80 ms, and declines to 1.0 at long ISI (Fig. 10A). Perfusion of a competing peptide that blocks the interaction of CaMKII with Ca,2.1 channels (Ca,2.1(1897–1912)) prevented both paired-pulse facilitation and paired-pulse depression at this model synapse (Fig. 10A), suggesting that binding of CaMKII to Ca,2.1 channels is required for expression of this regulatory effect. Similarly, expression of the brain-specific CaMKII inhibitor CaMKIIN (50), which prevents CaMKII binding to Ca,2.1 channels (33), also prevented paired-pulse facilitation and depression (Fig. 10A). This is consistent with previous results showing that facilitation of Ca,2.1 channels expressed in tsA-201 cells also requires binding of CaMKII (33). It is unlikely that the basal release probability is affected by competing peptide injection or CaMKIIN expression because the mean amplitudes of the first EPSP in paired pulse experiments are shown. C, a similar experiment to that described in panel A was carried out with CaV2.1(1897–1912) injected through a whole-cell patch electrode in untransfected SCG neurons, and paired-pulse facilitation of neurotransmission initiated by the endogenous CaV2.2 channels was measured in the absence of ω-conotoxin GVIA. D, a similar experiment to that described in panel A was carried out with expression of CaMKII in untransfected SCG neurons, and paired-pulse facilitation of neurotransmission initiated by the endogenous CaV2.2 channels was measured in the absence of ω-conotoxin GVIA. Normalized paired-pulse ratios of control and CaMKII-expressing neurons are plotted.
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CaMKII by Ca\(_{\alpha}2.1\) channels is required for both up-regulation of channel activity in paired pulses and for Ca\(^{2+}\)-independent activation of CaMKII by Ca\(_{\alpha}2.1\), and one or both of these effects is necessary for normal short term synaptic plasticity.

**DISCUSSION**

**CaMKII Binds Directly to Ca\(_{\alpha}2.1\) Channels**—Previous studies showed that CaMKII can be co-immunoprecipitated with Ca\(_{\alpha}2.1\) channels from transfected cells and that binding of CaMKII *per se* was sufficient for up-regulation of Ca\(_{\alpha}2.1\) channel activity in transfected cells and neurons (33), but no evidence was provided for direct interaction of these two key Ca\(^{2+}\)-signaling proteins. Our results show that CaMKII does indeed bind directly to the C-terminal domain of Ca\(_{\alpha}2.1\) channels at an interaction site located in Ca\(_{\alpha}2.1\)(1848–1964). Autophosphorylation enhances binding of CaMKII, and autophosphorylated CaMKII remains bound to the C-terminal domain of Ca\(_{\alpha}2.1\) channels even after dephosphorylation and removal of the original Ca\(^{2+}\)/CaM stimulus for binding. Thus, Ca\(_{\alpha}2.1\) channels with bound CaMKII are likely to serve as a signaling complex in the presynaptic active zone in neurons.

**Binding to Ca\(_{\alpha}2.1\) Channels Induces Sustained Ca\(^{2+}\)-independent Activity of CaMKII**—CaMKII activation normally requires binding of Ca\(^{2+}\)/CaM, but previous studies have demonstrated Ca\(^{2+}\)/CaM-independent activation of CaMKII by interaction with NMDA-type glutamate receptors and K\(_{\text{v}}\)11 channels (45, 46). Our results show that binding of CaMKII to Ca\(_{\alpha}2.1\) channels persistently activates its catalytic activity, as measured by autophosphorylation. CaMKII remains activated even after removal of the Ca\(^{2+}\)/CaM stimulus. This is a provocative result, as it implies that CaMKII bound to Ca\(_{\alpha}2.1\) channels is poised to phosphorylate nearby substrates and thereby regulate synaptic transmission locally.

It is interesting to compare the regulatory effects of CaM and CaMKII on Ca\(_{\alpha}2.1\) channels and Ca\(_{\alpha}1.2\) channels. These two types of Ca\(^{2+}\) channels are less than 50% identical in amino acid sequence and serve different physiological roles: Ca\(_{\alpha}2.1\) in initiation of synaptic transmission versus Ca\(_{\alpha}1.2\) in initiation of excitation-contraction coupling in muscle and in postsynaptic regulation in neurons (8). Although both proteins bind CaM and CaMKII to nearby sites in their C-terminal domains, the regulatory consequences are quite different. Binding of Ca\(^{2+}\)/CaM to Ca\(_{\alpha}2.1\) channels causes facilitation followed by inactivation, whereas binding to Ca\(_{\alpha}1.2\) channels causes only Ca\(^{2+}\)-dependent inactivation (9, 51). Binding of CaMKII to Ca\(_{\alpha}2.1\) channels enhances their activity and their facilitation, whereas phosphorylation of Ca\(_{\alpha}1.2\) channels by CaMKII bound to the C-terminal domain and/or the Ca\(_{\alpha}2\)B2 subunit is required for facilitation of their activity (9, 37, 52, 53). The structural and mechanistic basis for this differential regulation of Ca\(_{\alpha}1.2\) and Ca\(_{\alpha}2.1\) channels by CaM and CaMKII bound to nearby sites in their C-terminal domains is an interesting area for further research.

**CaMKII Bound to Ca\(_{\alpha}2.1\) Channels Phosphorylates Synapsin-1**—Synapsin-1 is abundant in presynaptic terminals, where it tethers synaptic vesicles to the actin cytoskeleton and is required for normal replenishment of synaptic vesicles during periods of high synaptic activity (54). Actin surrounds clusters of synaptic vesicles in presynaptic terminals and concentrates synapsin-1 at vesicle clusters (55). It also plays an important role in the dynamics of synaptic vesicle transfer to the readily releasable pool that is poised for rapid exocytosis, and both synapsin-1 and CaM are involved in those processes (55–57). Synapsin-1 is phosphorylated at Ser-603, which regulates trafficking of synaptic vesicles in *vivo* (54). Our results show that CaMKII bound to Ca\(_{\alpha}2.1\) channels is effective in phosphorylating Ser-603 in the absence of stimulation by Ca\(^{2+}\)/CaM. In the nerve terminal, phosphorylation of Ser-603 detaches synapsin-1 from synaptic vesicles and renders the vesicles mobile (58, 59). Thus, CaMKII bound to Ca\(_{\alpha}2.1\) channels may phosphorylate synapsin-1 nearby and regulate synaptic vesicle dynamics in and near the active zones in the presynaptic terminal.

**Binding of CaMKII to Ca\(_{\alpha}2.1\) Channels Is Required for Short Term Synaptic Plasticity**—Recent results from studies of neurotransmission at the Calyx of Held, a large synapse in the auditory system, and in cultured SCG neurons show that Ca\(^{2+}\)-dependent facilitation and inactivation of Ca\(_{\alpha}2.1\) channel activity contribute substantially to short term synaptic facilitation and depression (9). Our results show that binding of CaMKII to Ca\(_{\alpha}2.1\) channels is required for short term synaptic plasticity in scG neurons. Block of CaMKII binding with a competing peptide from its Ca\(_{\alpha}2.1\) binding site inhibits short term facilitation and depression of synaptic transmission, as does binding of the brain-specific CaMKII inhibitor CaMKII-N. Evidently, CaMKII binding to Ca\(_{\alpha}2.1\) channels is a necessary prerequisite for short term synaptic plasticity mediated by Ca\(_{\alpha}2.1\) channels, as observed previously in studies of synaptic plasticity in genetically modified mouse strains (31). Binding of CaMKII to Ca\(_{\alpha}2.1\) channels may play a permissive role by enhancing the activation of Ca\(_{\alpha}2.1\) channels in response to trains of depolarizing stimuli and the resulting influx of Ca\(^{2+}\), because binding of the kinase does not activate or facilitate channel activity by itself (33). In addition to the role of CaMKII binding to Ca\(_{\alpha}2.1\) channels in short term plasticity demonstrated here, it is possible that phosphorylation by CaMKII bound to Ca\(_{\alpha}2.1\) channels may also be essential in the longer-term effects of synapsin-1 in regulating synaptic vesicle dynamics and synaptic transmission in the local environment of Ca\(_{\alpha}2.1\) channels at active zones.

**Functional Roles of the Presynaptic Ca\(_{\alpha}2.1\) Signaling Complex**—Previous studies show that Ca\(_{\alpha}2.1\) channels are regulated by binding of SNARE proteins, G proteins, CaM and CaM-like sensor proteins, and CaMKII (9). Proteomic analysis revealed a complex of ~100 proteins associated with Ca\(_{\alpha}2.1\) channels in isolated nerve terminals from the mouse brain (10). This large protein complex serves to bring the essential machinery for neurotransmitter release close to presynaptic Ca\(_{\alpha}2.1\) channels, which provide the trigger of Ca\(^{2+}\) entry to initiate rapid exocytosis. It also serves to regulate the activity of Ca\(_{\alpha}2.1\) channels in response to Ca\(^{2+}\) and other regulatory messengers. Prior binding of CaMKII to Ca\(_{\alpha}2.1\) channels is required for facilitation and inactivation of the Ca\(_{\alpha}2.1\) channel during trains of repetitive depolarizations or action potentials. In this way, CaMKII binding to Ca\(_{\alpha}2.1\) serves as a molecular switch to turn on or off the millisecond timescale modulation...
of channel activity by Ca\textsuperscript{2+}-dependent facilitation and inactivation.

The Effector Checkpoint Model for Calcium Channel Regulation—Voltage-gated Ca\textsuperscript{2+} channels are regulated by their effectors such that the channels are more active when the effectors of their Ca\textsuperscript{2+} signal are bound. Examples include regulation of the skeletal muscle Ca\textsuperscript{2+} channel by the ryanodine-sensitive Ca\textsuperscript{2+} release channel (60), its effector in excitation-contraction coupling, and regulation of presynaptic Ca\textsuperscript{2+} channels by SNARE proteins, which are the effectors for Ca\textsuperscript{2+}-dependent exocytosis (9). Regulation of Ca\textsubscript{v}2.1 channels by CaMKII also fits this regulatory theme (33). Binding of CaMKII to Ca\textsubscript{v}2.1 increases the activity of both binding partners, and their interaction is required for facilitation of synaptic transmission and perhaps for other aspects of presynaptic function. Enhancement of the activity of Ca\textsuperscript{2+} channels whose effectors are bound would focus Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+}-dependent protein phosphorylation in locations where it can effectively generate a cellular response via local Ca\textsuperscript{2+} signaling. This mechanism would enhance local signal transduction and reduce ineffective Ca\textsuperscript{2+} entry and protein phosphorylation at other sites.

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REFERENCES

1. Llinás, R., Sugimori, M., Hillman, D. E., and Cherksey, B. (1992) Distribution and functional significance of the P-type, voltage-dependent Ca\textsuperscript{2+} channels in the mammalian central nervous system. Trends Neurosci. 15, 351–355
2. Ertel, E. A., Campbell, K. P., Harpold, M. M., Hofmann, F., Mori, Y., Perez-Reyes, E., Schwartz, A., Snutch, T. P., Tanabe, T., Birnbaumer, L., Tsien, R. W., and Catterall, W. A. (2000) Nomenclature of voltage-gated calcium channels. Neuron 25, 533–535
3. Wu, L. G., Westenbroek, R. E., Borst, J. G., Catterall, W. A., and Sakmann, B. (1999) Calcium channel types with distinct presynaptic localization cooperate differently to transmitter release in single calyx-type synapses. J. Neurosci. 19, 726–736
4. Westenbroek, R. E., Sakurai, T., Elliott, E. M., Hell, J. W., Starr, T. V., Snutch, T. P., and Catterall, W. A. (1995) Immunochemicai identification and subcellular distribution of the \( \alpha_{1A} \) subunits of brain calcium channels. J. Neurosci. 15, 6403–6418
5. Wheeler, D. B., Randall, A., and Tsien, R. W. (1994) Roles of N-type and Q-type Ca\textsuperscript{2+} channels in supporting hippocampal synaptic transmission. Science 264, 107–111
6. Dunlap, K., Luebke, J. I., and Turner, T. J. (1995) Exocytotic Ca\textsuperscript{2+} channels in mammalian central neurons. Trends Neurosci. 18, 89–98
7. Liu, H., De Waard, M., Scott, V. E., Gurnett, C. A., Lennon, V. A., and Campbell, K. P. (1996) Identification of three subunits of the high affinity \( \alpha \)-conotoxin MVIIIC-sensitive Ca\textsuperscript{2+} channel. J. Biol. Chem. 271, 13804–13810
8. Catterall, W. A. (2000) Structure and regulation of voltage-gated Ca\textsuperscript{2+} channels. Annu. Rev. Cell Dev. Biol. 16, 521–555
9. Catterall, W. A., and Few, A. P. (2008) Calcium channel regulation and presynaptic plasticity. Neuron 59, 882–901
10. Müller, C. S., Haupt, A., Bildl, W., Schindler, J., Knaus, H.-G., Meissner, M., Rammber, B., Stresiing, J., Flockerzi, V., Fakler, B., and Schulte, U. (2010) Quantitative proteomics of the Ca\textsubscript{v}2 channel nano-environments in the mammalian brain. Proc. Natl. Acad. Sci. U.S.A. 107, 14950–14957
11. Soderling, T. R., Chang, B., and Brickey, D. (2001) Cellular signaling through multifunctional Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II. J. Biol. Chem. 276, 3719–3722
12. Haribabu, B., Hook, S. S., Selbert, M. A., Goldstein, E. G., Tomhave, E. D., Edelman, A. M., Snyderman, R., and Means, A. R. (1995) Human calcium-calmodulin dependent protein kinase I. cDNA cloning, domain structure and activation by phosphorylation at threonine-177 by calcium-calmodulin-dependent protein kinase I. J. Biol. Chem. 270, 14950–14957
13. Hemmings, H. C., Jr., Nairn, A. C., McGuinness, T. L., Huganir, R. L., and Greengard, P. (1989) Role of protein phosphorylation in neuronal signal transduction. FASEB J. 3, 1583–1592
14. Hudmon, A., and Schultman, H. (2002) Neuronal Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II. The role of structure and autoregulation in cellular function. Annu. Rev. Biochem. 71, 473–510
15. Lisman, J., Schultman, H., and Cline, H. (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat. Rev. Neurosci. 3, 175–190
16. Lisman, J., Yasuda, R., and Raghavachari, S. (2012) Mechanisms of CaMKII action in long-term potentiation. Nat. Rev. Neurosci. 13, 169–182
17. Miller, S. G., and Kennedy, M. B. (1985) Distinct forebrain and cerebellar isozymes of type II Ca\textsuperscript{2+}-calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. J. Biol. Chem. 260, 9039–9046
18. Hoelz, A., Nairn, A. C., and Kuriyan, J. (2003) Structural crystal of a tetradecameric assembly of the association domain of Ca\textsubscript{v}2.1/calmodulin-dependent protein kinase II. Mol. Cell 11, 1241–1251
19. Schwerer, C. M., Colbran, R. J., and Soderling, T. R. (1986) Reversible generation of a Ca\textsuperscript{2+}-independent form of Ca\textsuperscript{2+} (calmodulin)-dependent protein kinase II by an autophosphorylation mechanism. J. Biol. Chem. 261, 8581–8584
20. Lai, Y., Nairn, A. C., and Greengard, P. (1986) Autophosphorylation reversibly regulates the Ca\textsuperscript{2+}/calmodulin-dependence of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II. Proc. Natl. Acad. Sci. U.S.A. 83, 4253–4257
21. Miller, S. G., and Kennedy, M. B. (1986) Regulation of brain type II Ca\textsuperscript{2+}/calmodulin-dependent protein kinase by autophosphorylation. A Ca\textsuperscript{2+} -triggered molecular switch. Cell 46, 861–870
22. Schwerer, C. M., Colbran, R. J., Keefer, J. R., and Soderling, T. R. (1988) Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II. Identification of a regulatory autophosphorylation site adjacent to the inhibitory and calmodulin binding domains. J. Biol. Chem. 263, 13486–13489
23. Benfenati, F., Valtorta, F., Chieregatti, E., and Greengard, P. (1992) Interaction of free and synaptic vesicle-bound synapsin I with F-actin. Neuron 8, 377–386
24. Benfenati, F., Valtorta, F., Rubenstein, J. L., Gorelick, F. S., Greengard, P., and Czernik, A. J. (1992) Synaptic vesicle-associated Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II is a binding protein for synapsin I. Nature 359, 417–420
25. Greengard, P., Valtorta, F., Czernik, A. J., and Benfenati, F. (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259, 780–785
26. Luk, C. C., Naruo, H., Prince, D., Hassan, A., Doran, S. A., Goldberg, J. I., and Syed, N. I. (2011) A novel form of presynaptic CaMKII-dependent short term potentiation between Lymnaea neurons. Eur. J. Neurosci. 34, 569–577
27. Pang, Z. P., Cao, P., Xu, W., and Südhof, T. C. (2010) Calmodulin controls synaptic strength via presynaptic activation of calmodulin kinase II. J. Neurosci. 30, 4132–4142
28. Llinás, R., Gruner, J. A., Sugimori, M., McGuinness, T. L., and Greengard, P. (1991) Regulation by synapsin I and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. J. Physiol. 436, 257–282
29. Llinás, R., McGuinness, T. L., Leonard, C. S., Sugimori, M., and Greengard, P. (1985) Intratetrameric injection of synapsin I calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proc. Natl. Acad. Sci. U.S.A. 82, 3035–3039
30. Chapman, P. F., Frenguelli, B. G., Smith, A., Chen, C. M., and Silva, A. J. (1995) The \( \alpha \)-Ca\textsuperscript{2+}/calmodulin kinase II. A bidirectional modulator of presynaptic plasticity. Neuron 14, 591–597
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31. 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 CaMKII-dependent presynaptic plasticity at CA3-CA1 synapses. *Nature Neurosci.* **10**, 1125–1127

32. Lu, F. M., and Hawkins, R. D. (2006) Presynaptic and postsynaptic Ca\textsuperscript{2+} and CaMKII contribute to long-term potentiation at synapses between individual CA3 neurons. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 4264–4269

33. Jiang, X., Lautermilch, N. J., Watari, H., Westenbroek, R. E., Scheuer, T., and Catterall, W. A. (2008) Modulation of Ca\textsubscript{v}2.1 channels by Ca\textsuperscript{2+} and calmodulin-dependent protein kinase II bound to the C-terminal domain. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 341–346

34. Yokoyama, C. T., Myers, S. J., Fu, J., Mockus, S. M., Scheuer, T., and Catterall, W. A. (2005) Mechanism of SNARE protein binding and regulation of Ca\textsubscript{v}2.1 channels by phosphorylation of the synaptic protein interaction site. *Mol. Cell. Neurosci.* **28**, 1–17

35. Brickey, D. A., Colbran, R. J., Fong, Y. L., and Soderling, T. R. (1990) Expression and characterization of the \(a\)-subunit of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II using the baculovirus expression system. *Biochem. Biophys. Res. Commun.* **173**, 578–584

36. Chao, L. H., Pellicena, P., Deindl, S., Barclay, L. A., Schulman, H., and Pitt, G. S. (2005) CaMKII tethers to L-type Ca\textsuperscript{2+} channels, establishing a local and dedicated integrator of Ca\textsuperscript{2+} signals for facilitation. *J. Cell Biol.* **171**, 537–547

37. Hudmon, A., Schulman, H., Kim, J., Maltez, J. M., Tsien, R. W., and Pitt, G. S. (2006) [*Inhibition of neurotransmission by peptides containing the synaptopodin/calmodulin action site. Proc. Natl. Acad. Sci. U.S.A.* **103**, 105, 7954–7967]

38. Hudmon, A., Schulman, H., Kim, J., Maltez, J. M., Tsien, R. W., and Pitt, G. S. (2006) [*Expression and characterization of the \(a\)-subunit of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II using the baculovirus expression system. Biochem. Biophys. Res. Commun.* **173**, 578–584]

39. Hudmon, A., Schulman, H., Kim, J., Maltez, J. M., Tsien, R. W., and Pitt, G. S. (2006) [*Multiple RIBEYE-RIBEYE interactions create a dynamic scaffold for the formation of synaptic ribbons. J. Neurosci.* **28**, 7954–7967]

40. Maguppali, V. G., Schwarz, K., Alpadi, K., Natarajan, S., Seigel, G. M., and Schmitz, F. (2008) [*Multiple RIBEYE-RIBEYE interactions create a dynamic scaffold for the formation of synaptic ribbons. J. Neurosci.* **28**, 7954–7967]

41. Lee, A., Zhou, H., Scheuer, T., and Catterall, W. A. (2003) [*Molecular characterization of the \(a\)-subunit of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II with isoforms of voltage-gated calcium channel \(\beta\) subunits. Biochemistry* **47**, 1760–1767]

42. Lee, A., Zhou, H., Scheuer, T., and Catterall, W. A. (2003) [*Molecular determinants of Ca\textsuperscript{2+}/calmodulin-dependent regulation of Ca\textsubscript{v}2.1 channels. Proc. Natl. Acad. Sci. U.S.A.* **100**, 16059–16064]

43. Mohida, S., Sheng, Z. H., Baker, C., Kobayashi, H., and Catterall, W. A. (1996) [*Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca\textsuperscript{2+} channels. Neuron* **17**, 781–788]

44. Lee, A., Wong, S. T., Gallagher, D., Li, B., Storm, D. R., Scheuer, T., and Catterall, W. A. (1999) [*Ca\textsuperscript{2+}/calmodulin binds to and modulates P/Q-type calcium channels. Nature 399*, 155–159]

45. Lee, A., Zhou, H., Scheuer, T., and Catterall, W. A. (2003) [*Molecular determinants of Ca\textsuperscript{2+}/calmodulin-dependent regulation of Ca\textsubscript{v}2.1 channels. Proc. Natl. Acad. Sci. U.S.A.* **100**, 16059–16064]

46. Ohyama, A., Hosaka, K., Komiya, Y., Akagawa, K., Yamauchi, E., Taniguchi, H., Sasagawa, N., Kumakura, K., Mohida, S., Yamauchi, T., and Iga-rashi, M. (2002) [*Regulation of excitatory tension through Ca\textsuperscript{2+}/ATP-dependent binding of autophosphorylated Ca\textsuperscript{2+}/calmodulin-activated protein kinase II to syntaxin IA. J. Neurosci.* **22**, 3342–3351]

47. Bähler, M., and Rhoads, A. (2002) [*Caldemulin signaling via the IQ motif. FEBS Lett.* **513**, 107–113]

48. Colbran, R. J. (2004) [*Protein phosphatases and calcium/calmodulin-dependent protein kinase II-dependent synaptic plasticity. J. Neurosci.* **24**, 8404–8409]

49. 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

50. Sun, X. X., Hodge, J. J., Zhou, Y., Nguyen, M., and Griffith, L. C. (2004) [*The eag potassium channel binds and locally activates calcium/calmodulin-dependent protein kinase II. J. Biol. Chem.* **279**, 10206–10214]

51. Rusnak, F., and Mertz, P. (2000) [*Calcineurin. Form and function. Nature 411*, 411–422]

52. Grueter, C. E., Abiria, S. A., Dzhura, I., Wu, Y., Ham, A. J., Mohler, P. J., Anderson, M. E., and Colbran, R. J. (2006) [*L-type Ca\textsuperscript{2+} channel facilitation mediated by phosphorylation of the \(\beta\) subunit by CaMKII. Mol. Cell* **23**, 641–650]

53. Grueter, C. E., Abiria, S. A., Wu, Y., Anderson, M. E., and Colbran, R. J. (2008) [*Differential regulated interactions of calcium/calmodulin-dependent protein kinase II with isoforms of voltage-gated calcium channel \(\beta\) subunits. Biochemistry* **47**, 1760–1767]

54. Cesca, F., Baldelli, P., Valtorta, F., and Benfenati, F. (2010) [*The synapsins. Key actors of synapse function and plasticity. Prog Neurobiol.* **91**, 313–348]

55. Sankaranarayanan, S., Atluri, P. P., and Ryan, T. A. (2003) [*Actin has a molecular scaffolding, not propulsive, role in presynaptic function. Nat. Neurosci.* **6**, 127–135]

56. Lee, S. H., Ho, W. K., and Lee, S. H. (2012) [*Actin-dependent rapid recruitment of reluctant synaptic vesicles into a fast-releasing vesicle pool. Proc. Natl. Acad. Sci. U.S.A.* **109**, E765–E774]

57. Sakata, T., and Neher, E. (2003) [*Involvement of actin polymerization in vesicle recruitment at the calyx of Held synapse. J. Neurosci.* **23**, 837–846]

58. Stefani, G., Onofri, F., Valtorta, F., Vacciara, P., Greengard, P., and Benfenati, F. (1997) [*Kinetic analysis of the phosphorylation-dependent interactions of synapsin I with rat brain synaptic vesicles. J. Physiol.* **501**, 501–515]

59. Bennett, A. F., and Baines, A. J. (1992) [*Bundling of microtubules by synapsin I. Characterization of bundling and interaction of distinct sites in synapsin I head and tail domains with different sites in tubulin. Eur. J. Biochem.* **206**, 783–792]

60. Nakai, J., Dirksen, R. T., Nguyen, H. T., Pessah, I. N., Beam, K. G., and Allen, P. D. (1996) [*Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. Nature 380*, 72–75]