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Ca^{2+}-independent Activation of Ca^{2+}/Calmodulin-dependent Protein Kinase II Bound to the C-terminal Domain of $Ca_V2.1$ Calcium Channels*

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Background: Calmodulin regulation of Ca^{2+} channels mediates short term synaptic plasticity.
Results: Binding of CaMKII to $Ca_V2.1$ channels induces Ca^{2+}-independent kinase activity.
Conclusion: A complex of CaMKII and $Ca_V2.1$ channels is required for short term synaptic plasticity.
Significance: CaMKII bound to $Ca_V2.1$ 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 $Ca_V2.1$ 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 $Ca_V2.1$ channel and synapsin-1 simultaneously. CaMKII binding to $Ca_V2.1$ 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 $Ca_V2.1$ channels by binding of Ca^{2+}/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 $Ca_V2.1$ channels. These results define the functional properties of a signaling complex of CaMKII and $Ca_V2.1$ 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 $Ca_v2.1$ (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 $Ca_v2.1$ channel protein consists of a pore-forming α_1 subunit associated with auxiliary β, $\alpha_2\delta$, and possibly γ subunits (7, 8). The function of $Ca_v2.1$ channels is dynamically regulated by interaction with SNARE proteins, G-protein $\beta\gamma$ subunits, RIM and other SNARE-interacting proteins, calmodulin (CaM),[4] and related calcium sensor proteins (8, 9). $Ca_v2.1$ 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 $Ca_v2.1$ 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 α and 60-kDa β 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 $Ca_V2.1$ channels in transfected cells via a site in the proximal C-termi-

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[4] The abbreviations used are: CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinase II; CaMKIIN, CaM kinase inhibitor; MBP, maltose-binding protein; SCG, superior cervical ganglion; EPSP, excitatory postsynaptic potential; ISI, inter-stimulus interval; RIM, Rab3 interacting molecule; eGFP, enhanced green fluorescent protein.

nal domain (33). Interaction with CaMKII increases $Ca_V2.1$ channel activity and enhances $Ca_V2.1$ 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 $Ca_V2.1$ channels and that autophosphorylation of CaMKII stimulates binding to this site. Autophosphorylated CaMKII can bind to the $Ca_V2.1$ channel and synapsin-1 simultaneously. Binding of $Ca_V2.1$ to CaMKII induces Ca^{2+}-independent kinase activity, which mediates both autophosphorylation and phosphorylation of synapsin-1 at Ser-603. Block of this interaction with competing peptides or the endogenous brain-specific CaMKII inhibitor, CaMKIIN, prevents short term synaptic facilitation and depression. We present a molecular model in which the pore-forming α_1 subunit of the $Ca_v2.1$ channel serves as a platform for association of CaMKII at the site of influx of Ca^{2+}, where it is persistently activated and poised to phosphorylate synapsin-1 and other nearby substrates to regulate synaptic vesicle dynamics and synaptic plasticity.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—$Ca_V2.1$(1848–1964)pGEX-4T-2, encoding the C-terminal domain of $Ca_V2.1$ channel containing the IQ-like motif in pGEX-4T-2, was amplified via PCR using $Ca_V2.1$(1766–2212) as template. The PCR product was cloned into the BamHI/EcoR1 sites of pGEX-4T-2. $Ca_V2.1$(1959–2035)pGEX-4T-2, encoding the C-terminal domain of $Ca_V2.1$ channel containing the CaM binding domain in pGEX-4T-2, was amplified via PCR using $Ca_V2.1$(1766–2212) as template. The PCR product was cloned into the BamHI/EcoR1 sites of pGEX-4T-2. $Ca_V2.1$(1848–1964)/EEDAAA, encoding the C-terminal domain of $Ca_V2.1$ containing the IQ-like motif, was cloned into BamHI/EcoR1 sites of pGEX-4T-2. The CaMKII tethering site TVGKIY, located upstream of IQ-like containing domain, was mutated to EEDAAA. N-terminal MGALCC lipid anchor + $Ca_V2.1$(1766–2211) was amplified via PCR using the $Ca_V2.1$ α_1 subunit as template. The PCR product was cloned into the BamHI/EcoR1 sites of pCDNA3.1/myc-HisA. Myc-$Ca_V2.1$(1764–2211) was generated by subcloning $\alpha_12.1$ 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 BamH1/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). $Na_V1.2$(1848–1964)pGEX-4T-2, encoding a segment of the C-terminal domain of the $Na_V1.2$ channel, was amplified via PCR using full-length $Na_V1.2$ 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 9×10^5 cells/100 mm dish in Hyclone DMEM/F medium and afterward used in experiments (33). The cells were transfected using transit-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), Halt™ 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 2× 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 $Ca_V2.1$(1848–1964), $Ca_V2.1$(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-galactopyranoside 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 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 $Ca_V2.1$-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 $CaCl_2$,0.125 μM CaM, 0.025 mM ATP, and 0.125 mM $MgCl_2$ (37). This procedure resulted in essentially complete phosphorylation of CaMKII as judged by its change in mobility in SDS-PAGE (Fig. 1*C*). 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 4× 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 anti-phospho-CaMKII(Thr-286) (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 $\alpha_1$2.1 subunit, eGFP, and (where indicated) CaMKIIN 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% CO_2-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 $\alpha_1$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. CaMKIIN was co-expressed with $Ca_V2.1$ channels or $Ca_V2.1$(1897–1912) was injected 30 min before recordings as indicated. Endogenous synaptic transmission was blocked by bath application of 3 μM ω-conotoxin GVIA in a modified Krebs solution consisting of 136 mM NaCl, 5.9 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 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 $Ca_v2.1$ Channels—As shown in previous studies, we confirmed that CaMKII binds to the C-terminal domain of $Ca_V2.1$ channels in transfected cells, as assessed by co-immunoprecipitation (Fig. 2*A*) (33). However, it was not known whether this binding interaction was direct or required other intermediary proteins. To measure direct interactions between CaMKII and $Ca_V2.1$, 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 ($Ca_V2.1$(1848–1964)) and the CaM binding domain ($Ca_V2.1$(1959–2035)) (40, 41). We prepared autophosphorylated CaMKII in which Thr-286 was essentially completely phosphorylated (Fig. 1*C*). We incubated purified preparations of nonactivated, Ca^{2+}/CaM-treated, and autophosphorylated CaMKII with purified GST-tagged $Ca_V2.1$(1848–1964), $Ca_V2.1$(1959–2035), or GST alone *in vitro* and detected the complex by GST pulldown assay. Binding of autophosphorylated CaMKII to GST-$Ca_V2.1$(1848–1964) (Fig. 2*B*, *lane a3*) was substantially greater than binding of nonactivated CaMKII

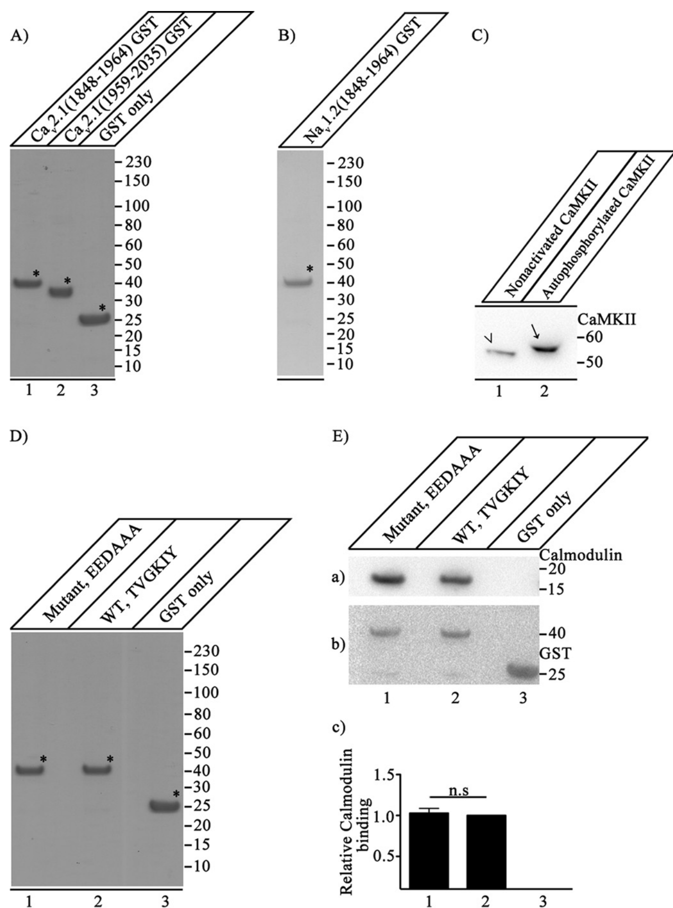

FIGURE 1. **Expression, purification, and function of $Ca_V2.1$ and $Na_V1.2$ proteins.** *A*, $Ca_V2.1$(1848–1964)-GST (*lane 1*), $Ca_V2.1$(1959–2035)-GST (*lane 2*), and GST (*lane 3*) were expressed and purified as described under "Experimental Procedures." Samples (7.5 μg of purified protein) were denatured, loaded, and resolved on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue to show the purity of the expressed proteins, which were used for *in vitro* binding assays. *B*, expression and purification of $Na_V1.2$(1848–1964)-GST. $Na_V1.2$(1848–1964)-GST was 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. *C*, extent of CaMKII phosphorylation. Nonactivated CaMKII was analyzed side by side with autophosphorylated CaMKII to estimate the extent of CaMKII autophosphorylation 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 (*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*, $Ca_V2.1$(1848–1964)-EEDAAA-GST (*lane 1*), $Ca_V2.1$(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 $Ca_V2.1$(1848–1964)-EEDAAA, $Ca_V2.1$(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 $Ca_V2.1$(1848–1964)-EEDAAA (*lane 1*), $Ca_V2.1$(1848–1964)-GST (*lane 2*), and GST (*lane 3*). *c*, quantitation of relative CaM binding under the indicated conditions (mean ± S.E.; *n.s.*, not significant 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*.

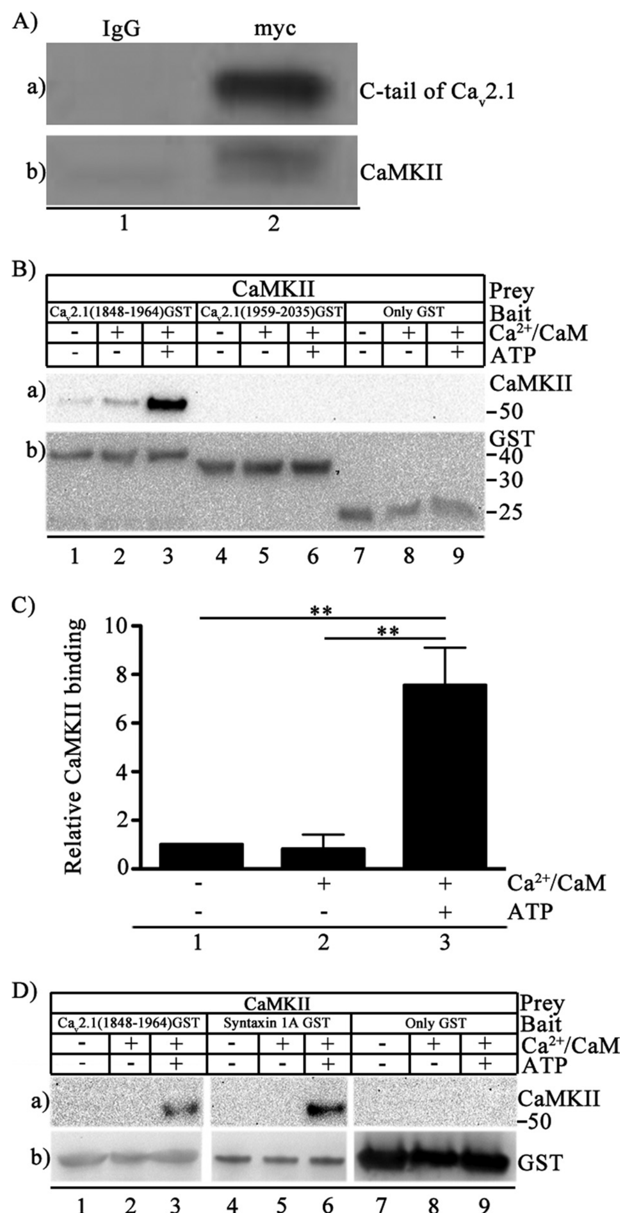

FIGURE 2. **Binding of CaMKII to $Ca_V2.1$ channels.** *A*, Myc-tagged $Ca_v2.1$(1764–2211) was expressed in tsA-201 cells, and immunoprecipitation was carried out with control IgG (*lane 1*) or anti-myc antibody (*lane 2*). *a*, immunoblotting with anti-myc. *b*, immunoblotting with anti-CaMKII antibody. *B*, binding of autophosphorylated CaMKII to $Ca_v2.1$ channel. *Left* to *right*, $Ca_V2.1$(1848–1964), $Ca_V2.1$(1959–2035), or GST alone were incubated with Mg^{2+} (5 mM) and CaMKII (20 nM) in the presence/absence of Ca^{2+}/CaM (5 μM) and ATP (1 mM) as indicated. *a*, binding of CaMKII was probed using anti-CaMKII. *b*, the same blot after stripping and re-probing using anti-GST antibody to show equal loading of $Ca_V2.1$(1848–1964), $Ca_V2.1$(1959–2035), or GST alone. *C*, quantitation of relative CaMKII binding to $Ca_V2.1$(1848–1964) using anti-CaMKII under the indicated conditions (means ± S.E.; **, $p < 0.01$ by Student's *t* test; $n = 4$). *D*, *left* to *right*, $Ca_V2.1$(1848–1964) (*lanes 1–3*), syntaxin 1A (*lanes 4–6*), or GST (*lanes 7–9*) were incubated with CaMKII (20 nM) and Mg^{2+} (5 mM) in the presence/absence of Ca^{2+}/CaM (5 μM) and ATP (1 mM) as indicated. *a*, binding of CaMKII probed using anti-CaMKII. *b*, the same blot after stripping and re-probing using anti-GST to show loading of $Ca_V2.1$(1848–1964), syntaxin 1A, or GST alone. The section of each blot containing GST-labeled proteins or GST itself was aligned for presentation even though the molecular weights are different.

or CaMKII incubated with Ca^{2+}/CaM but without ATP (Fig. 2*B*, *lanes a1* and *a2*). In contrast, GST-tagged $Ca_V2.1$(1959–2035) did not bind any of the three forms of CaMKII (Fig. 2*B*, *lanes a4–a6*), and GST itself was also unable to bind CaMKII (Fig. 2*B*, *lanes a7–a9*). Quantification of the results revealed that binding of the autophosphorylated kinase to GST-$Ca_V2.1$(1848–1964) was ~8-fold greater than binding to the non-

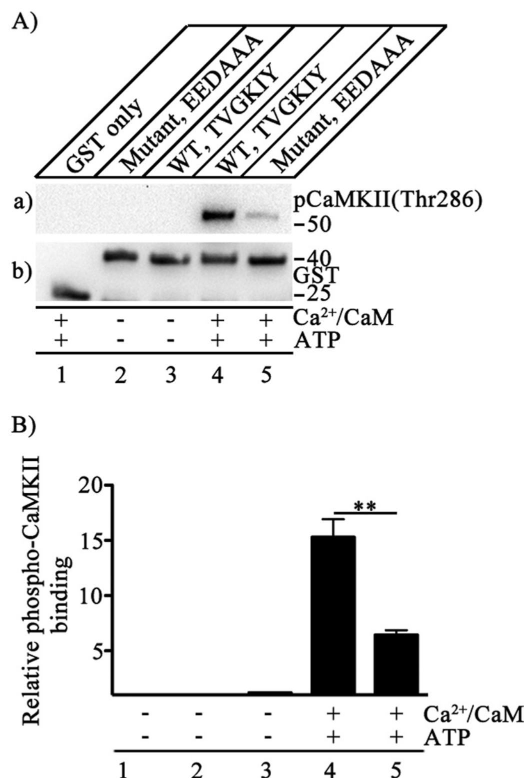

FIGURE 3. **Binding site for CaMKII on $Ca_V2.1$ channels.** *A*, binding of CaMKII was detected by GST pulldown assay. CaMKII (20 nM) was used as prey in the presence/absence of Ca^{2+}/CaM (5 μM) and ATP (1 mM) as indicated. GST alone or GST-tagged $Ca_V2.1$(1848–1964) was used as bait as indicated: WT, [1903]TVGKIY[1908]; mutant, [1903]EEDAAA[1908]. *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 $Ca_V2.1$(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; $n = 6$).

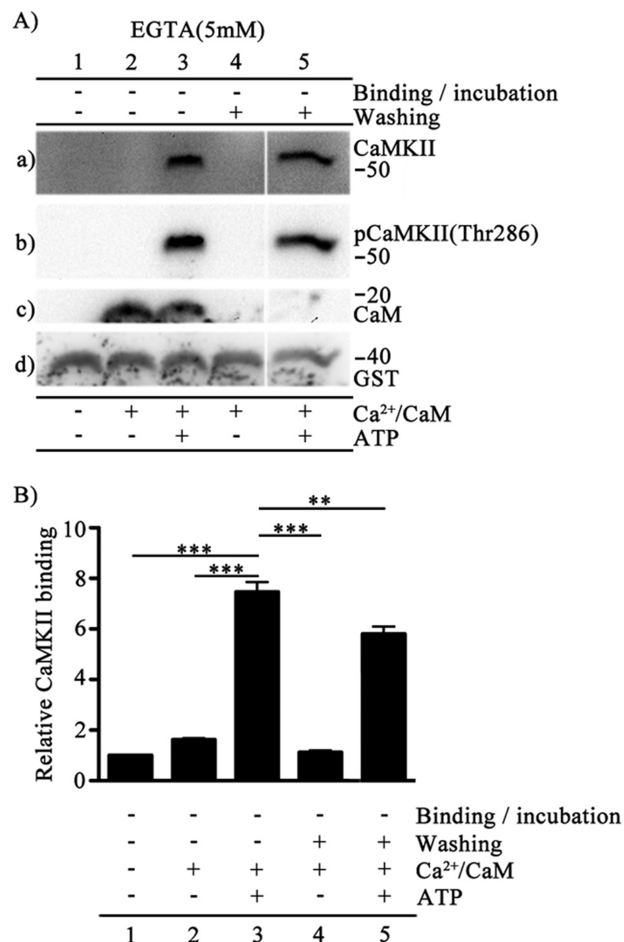

FIGURE 4. **Persistence of binding of autophosphorylated CaMKII to $Ca_V2.1$(1848–1964).** *A*, binding of CaMKII was detected by GST pulldown assay. $Ca_V2.1$(1848–1964) was incubated with CaMKII (20 nM) and Mg^{2+}(5 mM) in the presence/absence of Ca/CaM (5 μM) and ATP (1 mM) as indicated. Samples 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 $Ca_V2.1$(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; $n = 4$).

activated or Ca^{2+}/CaM-treated kinase (Fig. 2*C*). These results demonstrate direct and specific binding of CaMKII to $Ca_V2.1$(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. 2*D*, *lanes a4–a6*). Autophosphorylated CaMKII also bound specifically to $Ca_V2.1$(1848–1964) (Fig. 2*D*, *lanes a1–a3*) in a parallel experiment, demonstrating preferential binding of autophosphorylated CaMKII to $Ca_V2.1$(1848–1964) as compared with nonactivated or Ca^{2+}/CaM-treated CaMKII. GST alone did not bind non-activated, Ca^{2+}/CaM-treated, or autophosphorylated kinase (Fig. 2*D*, *lanes a7–a9*).

Location of the CaMKII Binding Site—$Ca_V2.1$(1848–1964) contains an IQ-like motif as part of a bipartite regulatory site that is important for CaM-induced facilitation and inactivation of $Ca_V2.1$ 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 $Ca_V1.2$ channels is well described, suggesting that CaM is not required for CaMKII binding to Ca^{2+} channels (37). Sequence alignment of the C-terminal domains of $Ca_v2.1$ and $Ca_V1.2$ channels revealed a conserved amino acid sequence motif in $Ca_V2.1$(1848–1964): TVGKFY in $Ca_V1.2$ channels *versus* TVGKIY in $Ca_V2.1$ channels (37). Peptides containing this motif prevented CaMKII regulation of $Ca_V2.1$ channels, suggesting an important role in CaMKII binding (33); however, intact $Ca_V2.1$ channels with this sequence mutated were inactive. To ascertain the role of the TVGKIY motif in binding to $Ca_V2.1$(1848–1964), we substituted the sequence EEDAAA used previously (37) for TVGKIY and tested CaMKII binding. Autophosphorylated CaMKII bound specifically to WT $Ca_V2.1$(1848–1964) (Fig. 3*A*, *lane a4*), but its binding to $Ca_V2.1$(1848–1964)/EEDAAA was significantly reduced to 0.42 ± 0.02 of WT when quantified using phospho-CaMKII-(Thr-286)-specific antibody (Fig. 3*A*, *lane a5*; Fig. 3*B*, $p < 0.01$). Autophosphorylated CaMKII did not bind to the GST control (Fig. 3*A*, *lane a1*), and non-activated CaMKII did not bind to either $Ca_V2.1$(1848–1964)/TGVKIY or $Ca_V2.1$(1848–1964)/

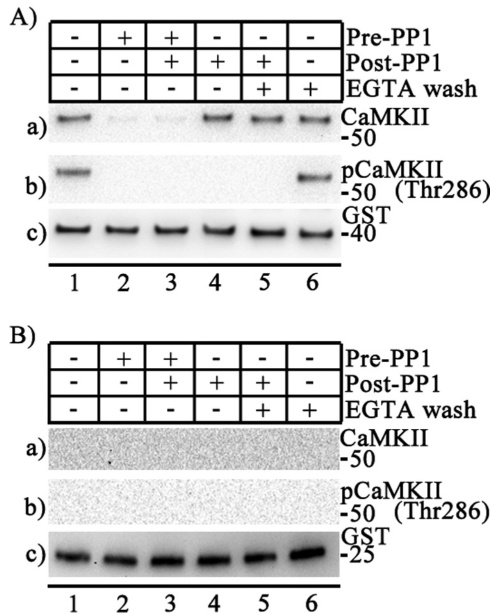

FIGURE 5. **Persistence of binding of CaMKII after dephosphorylation.** Purified recombinant protein phosphatase 1 (*PP1*) was added before (*Pre-PP1*) or after (*Post-PP1*) the kinase-channel complex was established as indicated. Washes were carried out in the presence or absence of 5 mM EGTA as indicated. *A*, binding of CaMKII to $Ca_V2.1(1848-1964)$ 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 $Ca_V2.1(1848-1964)$. *B*, binding of CaMKII to GST alone (control) was detected by GST pulldown assay. *a*, the blot was probed with anti-CaMKII. *b*, the same blot 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.

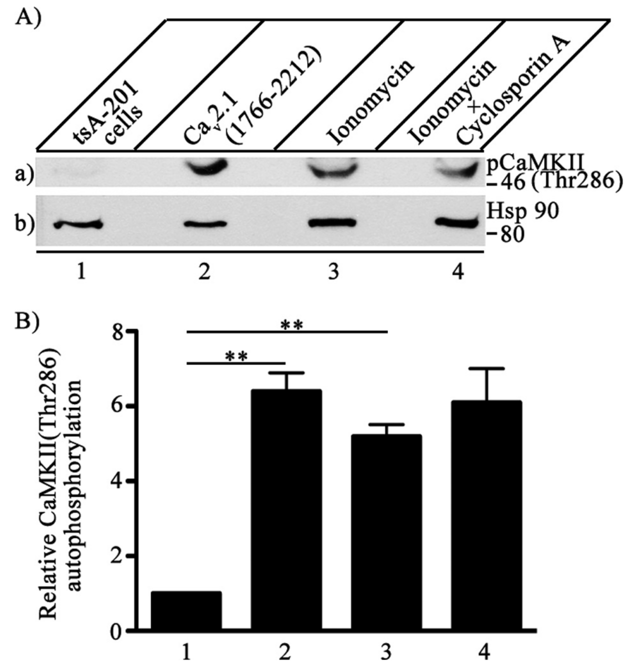

FIGURE 6. **Activation of autophosphorylation of CaMKII by binding to $Ca_V2.1$ channels.** $Ca_V2.1(1766-2122)$ was expressed in tsA-201 cells, and autophosphorylation of endogenous CaMKII was measured with anti- phospho-CaMKII(Thr-286). *A*, *left* to *right*, untreated control tsA-201 (*lane 1*), cells transfected with C-terminal domain of $Ca_V2.1$ (1766–2212) (*lane 2*), cells treated with 5 μM ionomycin to allow Ca^{2+} entry for 15 min before lysis (*lane 3*), and cells treated with 5 μM 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. 3*A*, *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^{2+}/CaM Dissociation—Binding of CaMKII to $Ca_V1.2$ and $Ca_V2.1$ channels modulates their function (33, 37). The local Ca^{2+} concentration at the active zone is tightly regulated to create a Ca^{2+} nanodomain (9). Because of its slowly reversible autophosphorylation, activation of CaMKII can integrate repetitive Ca^{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^{2+} declines. To address this question, we chelated Ca^{2+} using 5 mM EGTA in the washing buffers. CaMKII was isolated by binding to $Ca_V2.1(1848-1964)$ 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. 4*A*). CaMKII is highly phosphorylated in the presence of Ca^{2+}/CaM and ATP. Autophosphorylated CaMKII bound more effectively to $Ca_V2.1(1848-1964)$ (Fig. 4*A*, *lane a3*) than nonactivated or Ca^{2+}/CaM-treated CaMKII (Fig. 4*A*, *lanes a1* and *a2*). Both Ca^{2+}/CaM and ATP are essential components in triggering the autophosphorylation reaction, and omission of ATP followed by chelation of Ca^{2+} using EGTA (5 mM) in the washing reaction reduced binding significantly (Fig. 4*A*, *lane a4*). However, binding of the autophosphorylated kinase persisted even after Ca^{2+}/CaM was removed from the kinase-channel complex by washes with EGTA-containing washing buffer (Fig. 4*A*, *lane a5*), suggesting that Ca^{2+}/CaM removal does not rapidly reverse binding of autophosphorylated CaMKII. The efficacy of chelation of Ca^{2+}/CaM by EGTA treatment was probed using anti-CaM. No bound CaM was detected after EGTA treatment (Fig. 4*A*, *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. 4*B*). Collectively, these results show that binding of CaMKII to $Ca_V2.1(1848-1964)$ is greatly enhanced by Ca^{2+}/CaM-dependent autophosphorylation of CaMKII, but once formed, the complex persists even after removal of Ca^{2+}/CaM.

Dephosphorylation of CaMKII Does Not Reverse Binding—To test whether autophosphorylation is required to maintain binding of CaMKII to $Ca_V2.1(1848-1964)$, 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 postsynaptic densities was mostly dephosphorylated by PP1 (44). Autophosphorylated CaMKII preferentially bound to $Ca_V2.1(1848-1964)$ (Fig. 5*A*, *lane a1*). Dephosphorylation of autophosphorylated CaMKII by PP1 treatment before incuba-

tion of CaMKII with the channel peptide or both before and after incubation greatly reduced its binding (Fig. 5*A*, *lane a2* and *a3*), confirming that autophosphorylation is prerequisite for CaMKII binding to the channel. The remaining faint signal in the absence of autophosphorylation is in accordance with our earlier finding of a low level of binding of nonactivated or Ca^{2+}/CaM-treated CaMKII to the channel (Fig. 2*B*). Surprisingly, dephosphorylation of autophosphorylated CaMKII by post-PP1 treatment did not destabilize its binding (Fig. 5*A*, *lane a4*). Evidently, once kinase is bound to the channel, dephosphorylation does not reverse its binding, even when dephosphorylation is essentially complete (Fig. 5*A*, *lanes b2–b5*). Combining post-PP1 treatment and washing the kinase-channel complex with EGTA-containing buffer also did not reverse CaMKII binding (Fig. 5*A*, *lane a5*). Together these results show that once the autophosphorylated CaMKII is bound to $Ca_V2.1$(1848–1964), the interaction cannot be rapidly reversed by dephosphorylation (Fig. 5*A*, *lane a4*), Ca^{2+}/CaM removal (Fig. 5*A*, *lane a6*), or both processes carried out simultaneously *in vitro* (Fig. 5*A*, *lane a5*). GST alone does not have any effect (Fig. 5*B*), further strengthening evidence for the specificity of these interactions.

Increased Autophosphorylation of CaMKII Bound to $Ca_V2.1$(1848–1964)—Binding of CaMKII to its target sites on NMDA-type glutamate receptors and K_V11 channels causes Ca^{2+}-independent activation of the kinase (45, 46). Although there is no detectable amino acid sequence similarity between $Ca_V2.1$ channels, K_V11 channels, and glutamate receptors, expression of the C-terminal tail of $Ca_V2.1$ channels, $Ca_V2.1$(1766–2212), without the pore domain leads to enhancement of autophosphorylation of Thr-286 on endogenous CaMKII in human embryonic kidney tsA-201 cells (Fig. 6*A*, *lane a2*). These results suggest that binding of CaMKII to $Ca_V2.1$ channels *per se* may be sufficient to activate the kinase and increase its autophosphorylation substantially. The addition of ionomycin, which increases entry of Ca^{2+} into the cytosol, resulted in a comparable increase in CaMKII autophosphorylation on Thr-286 (Fig. 6*A*, *lane a3*). Increased cytosolic Ca^{2+} activates the Ca^{2+}-dependent phosphoprotein phosphatase calcineurin (47), which might limit the level of autophosphorylation induced by ionomycin. However, in the presence of ionomycin (5 μM) and the calcineurin inhibitor cyclosporin A (5 μM), there was little increase in autophosphorylation compared with ionomycin alone (Fig. 6*A*, *lane a4*), suggesting that calcineurin does not play a major role in control of CaMKII phosphorylation in tsA-201 cells. Quantification of these results revealed an ~6-fold increase in autophosphorylation upon transfection of $Ca_V2.1$(1766–2212) or treatment with ionomycin plus cyclosporin A (Fig. 6*B*).

To extend these findings of increased autophosphorylation of CaMKII bound to $Ca_V2.1$ channels in transfected tsA-201

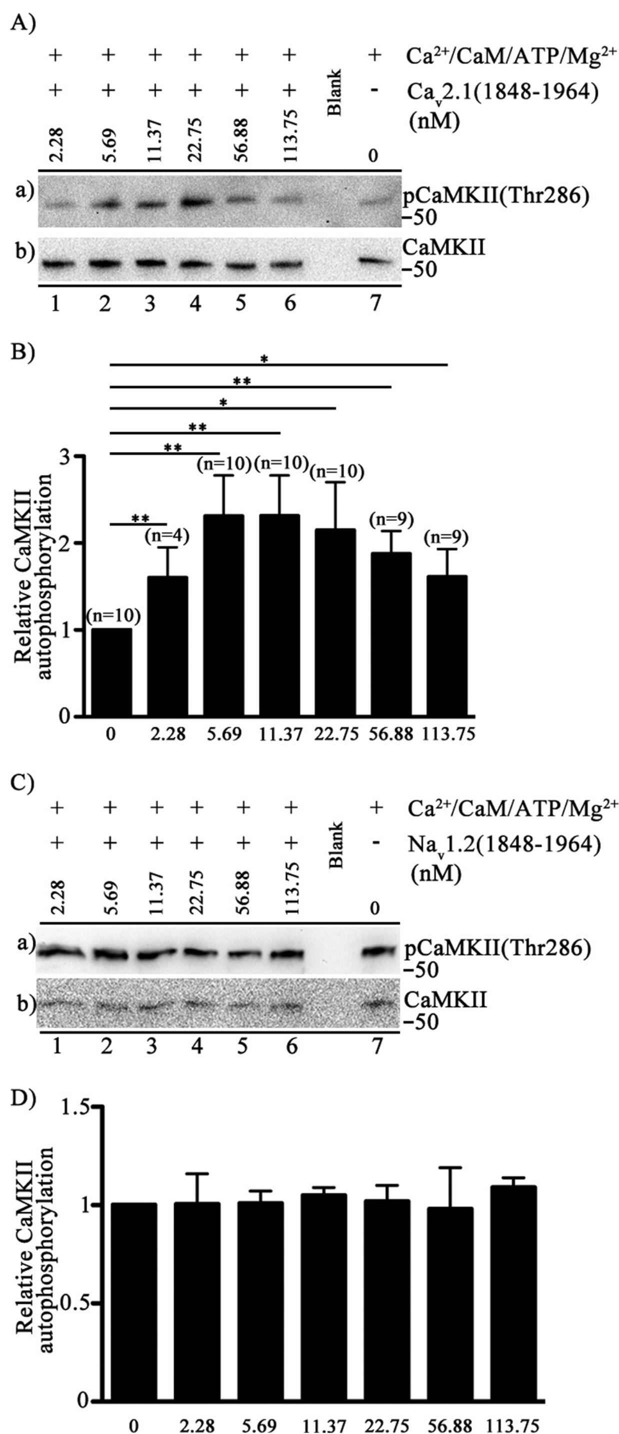

FIGURE 7. **Activation of autophosphorylation of CaMKII by binding of $Ca_V2.1$(1848–1964).** *A*, autophosphorylation of CaMKII was carried out by incubation in the presence of Ca^{2+} (0.6 μM), CaM (2.2 μM), ATP (32 μM), Mg^{2+}(2.3 mM), CaMKII (20 nM) and the indicated concentrations (0–113.75 nM) of $Ca_V2.1$ (1848–1964). Free Ca^{2+} was precisely controlled. *Left* to *right* (*lanes 1–6*), the concentration of $Ca_V2.1$(1848–1964) channel peptide ranging from 2.28 nM to 113.75 nM. *Extreme right* (*lane 7*), the control in the absence of $Ca_V2.1$(1848–1964). *a*, the blot was probed with anti-phospho-CaMKII(Thr-286). *b*, the same blot after stripping and re-probing using anti-CaMKII. *B*, quantitation of relative CaMKII autophosphorylation using anti-phospho-CaMKII(Thr-286) under the indicated conditions (mean ± S.E.; *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test). *C*, CaMKII autophosphorylation in the presence of $Na_V1.2$(1848–1964)-GST is shown. Autophosphorylation of CaMKII was carried out by incubation in the presence of Ca^{2+} (0.6 μM), CaM (2.2 μM), ATP (32 μM), Mg^{2+}(2.3 mM), CaMKII (20 nM), and the indicated concentrations (0–113.75 nM) of $Na_V1.2$(1848–1964). Free Ca^{2+} was precisely controlled. *Left* to *right* (*lanes 1–6*), concentration of $Na_V1.2$(1848–1964) channel peptide ranging from 0 to 113.75 nM. *Extreme right* (*lane 7*), control in the absence of $Na_V1.2$(1848–1964). *a*, the blot was probed with anti- phospho-CaMKII(Thr-286). *b*, the same blot after stripping and re-probing using anti-CaMKII. *D*, the quantification of relative CaMKII autophosphorylation using anti-phospho-CaMKII(Thr-286) under the indicated conditions (mean ± S.E.; not significant by Student's *t* test; $n = 3$).

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 $Ca_V2.1$(1848–1964) peptide (Fig. 7*A*). Autophosphorylation of CaMKII increased in the presence of increasing concentrations of the $Ca_v2.1$ channel peptide (Fig. 7*Aa*, *lanes 1–4*) and then decreased at higher concentrations of the peptide (Fig. 7*Aa*, *lanes 4–6*). Quantification of the levels of CaMKII autophosphorylation using anti-phospho-CaMKII-(Thr-286) (Fig. 7*B*) showed that direct binding of $Ca_V2.1$(1848–1964) *in vitro* mimics the activation of kinase autophosphorylation observed in transfected tsA-201 cells. These results confirm that direct binding of $Ca_V2.1$(1848–1964) activates CaMKII autophosphorylation. As negative controls, we used a GST-tagged protein of the same size from the C-terminal of $Na_v1.2$ 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 $Ca_V2.1$(1766–2212)—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 $Ca_V2.1$(1766–2212) with synapsin-1 in tsA-201 cells led to a substantial increase in synapsin-1 phosphorylation at Ser-603 (Fig. 8*A*, *lane a3*) compared with untransfected tsA-201 cells (Fig. 8*A*, *lane a1*) or cells expressing synapsin-1 alone (Fig. 8*A*, *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 $Ca_V2.1$(1766–2212). Ionomycin treatment of tsA-201 cells co-expressing $Ca_V2.1$(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 $Ca_V2.1$ 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 $Ca_V2.1$ to CaMKII can lead to phosphorylation of synapsin-1, a stable ternary complex of $Ca_V2.1$ 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 amylose resin was able to bind both $Ca_V2.1$(1848–1964) and synapsin-1 simultaneously (Fig. 9), whereas control experiments with MBP showed no binding (Fig. 9*B*). Formation of this ternary complex in presynaptic terminals would allow local phosphorylation of synapsin-1 by CaMKII bound to $Ca_V2.1$ 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 $Ca_V2.1$ and synapsin-1 to different subunits in the CaMKII complex.

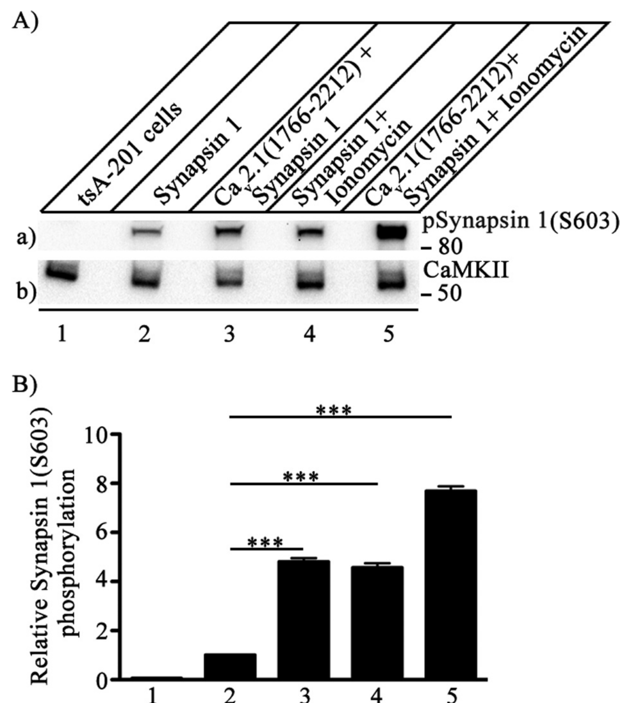

FIGURE 8. **Phosphorylation of synapsin-1 by CaMKII bound to the C-terminal domain of Cav2.1 channels.** $Ca_V2.1$(1766–2122) was co-expressed in tsA-201 cells along with synapsin-1, and phosphorylation of synapsin-1 was measured with anti-phosphosynapsin-1(Ser-603). *A*, *left* to *right*, untreated control tsA-201 cells (*lane 1*), cells single-transfected with synapsin-1 (*lane 2*), cells double-transfected with C-terminal domain of $Ca_v2.1$ (1766–2212) channel and synapsin-1 (*lane 3*), cells single-transfected with synapsin-1 and treated with 5 μM ionomycin to allow Ca^{2+} entry for 15 min before lysis (*lane 4*), and cells double-transfected with C-terminal domain of $Ca_v2.1$ (1766–2212) channel and synapsin-1 additionally treated with 5 μM ionomycin to allow Ca^{2+} entry for 15 min before lysis (*lane 5*). *a*, phosphorylation of synapsin-1 was assayed by immunoblotting with anti-phosphosynapsin-1(Ser-603) antibody. *b*, the same blot after stripping and re-probing with anti-CaMKII antibody to show equal loading of CaMKII. *B*, quantitation of relative synapsin-1(Ser-603) phosphorylation under the indicated conditions (mean ± S.E.; ***, $p < 0.001$ by Student's *t* test; $n = 5$).

Functional Role of Interaction of CaMKII with $Ca_V2.1$ Channels in Synaptic Plasticity—Binding of CaMKII to $Ca_V2.1$ channels enhances their functional activity by inhibiting their inactivation (33) and enhances the activity of CaMKII by increasing its autophosphorylation and its phosphorylation of other substrates as shown above. To critically test the potential effects of this specific interaction on synaptic transmission, it is necessary to manipulate the activity of CaMKII bound specifically to $Ca_V2.1$ channels in the presynaptic terminal without altering the functional activity of CaMKII in the postsynaptic compartment or CaMKII in other locations in the presynaptic terminal. Accordingly, we expressed $Ca_V2.1$ channels in SCG neurons in cell culture by microinjection of cDNA into the nucleus of a single cell using methods that were well defined in previous work (42, 48, 49). After 2–3 days, we impaled the injected cell and a nearby uninjected postsynaptic partner, and we measured synaptic transmission driven exclusively by the transfected $Ca_V2.1$ channels by blocking endogenous N-type Ca^{2+} currents with ω-conotoxin GVIA. We studied paired-pulse facilitation as a measure of short term synaptic plasticity. In this experimental paradigm, the synaptic response to the second stimulus in the pair is enhanced by residual Ca^{2+} remaining in the nerve

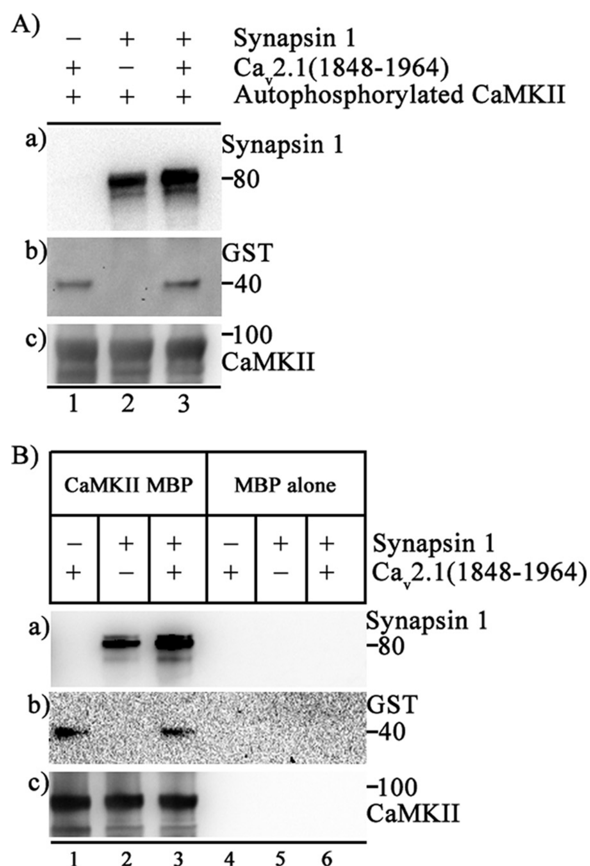

FIGURE 9. **Formation of a ternary complex of $Ca_V2.1$(1848–1964), CaMKII, and synapsin-1.** CaMKII was expressed as a MBP fusion protein and immobilized on amylose resin. *A*, incubations were carried out as depicted in the figure. *a*, binding of synapsin-1 was detected using anti-synapsin-1 antibody (PhosphoSolutions, polyclonal, 1;5000 TBST). *b*, shown is the same blot as *a* but after stripping and reprobing with anti-GST to show the binding of $Ca_V2.1$(1848–1964). *c* is the same blot as *a* and *b* but after stripping and reprobing with anti-CaMKII (Invitrogen, monoclonal, 1:1000 in TBST) to show the CaMKII bait. *B*, incubations were carried out as depicted in figure. *a*, binding of synapsin-1 was detected using anti-synapsin-1 antibody (PhosphoSolutions, polyclonal, 1;5000 TBST). *b*, shown is the same as *a* but after stripping and reprobing with anti-GST to show the binding of $Ca_V2.1$(1848–1964), and *c* is same blot as *a* and *b* but after stripping and reprobing with anti-CaMKII (Invitrogen, monoclonal, 1:1000 in TBST) to show the CaMKII bait.

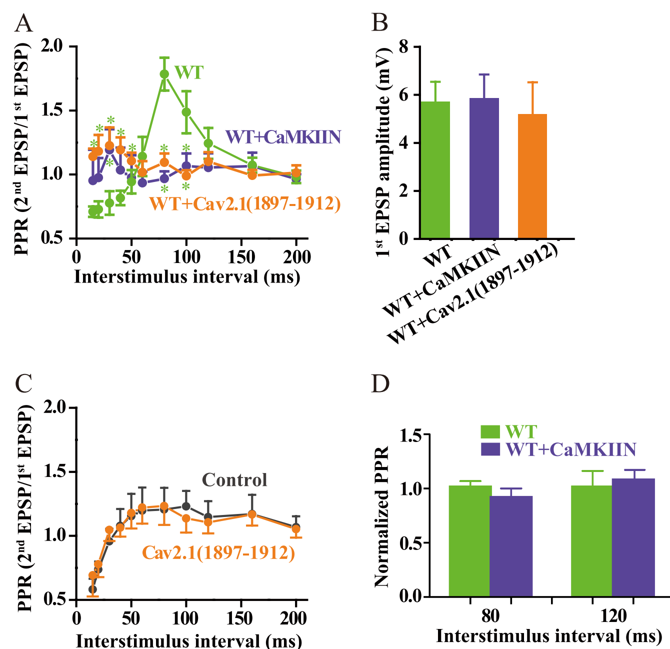

FIGURE 10. **Binding of CaMKII to $Ca_V2.1$ channels is required for short term synaptic plasticity.** *A*, SCG neurons were cultured and injected with cDNA encoding $Ca_V2.1$ channels as described under "Experimental Procedures." Where indicated, CaMKIIN was co-expressed with $Ca_V2.1$ channels, or $Ca_V2.1$(1897–1912) was injected through a whole-cell patch electrode 30 min before recording as described (39). Sharp microelectrode impalements were made in the previously transfected, presynaptic neuron and a neighboring, synaptically connected neuron. Action potentials were generated in the presynaptic cell, and EPSPs were recorded in the postsynaptic cell and analyzed as described under "Experimental Procedures." The paired-pulse ratio (*PPR*) was plotted against inter-stimulus interval (mean ± S.E.; *, $<$ 0.05, Student's *t* test, n = 6–9). WT, *green*; CaMKIIN, *blue*; $Ca_V2.1$(1897–1912), *orange*. *B*, 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 $Ca_V2.1$(1897–1912) injected through a whole-cell patch electrode in untransfected SCG neurons, and paired-pulse facilitation of neurotransmission initiated by the endogenous $Ca_V2.2$ 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 CaMKIIN in untransfected SCG neurons, and paired-pulse facilitation of neurotransmission initiated by the endogenous $Ca_V2.2$ channels was measured in the absence of ω-conotoxin GVIA. Normalized paired-pulse ratios of control and CaMKIIN-expressing neurons are plotted.

terminal from the first stimulus (9). Paired-pulse facilitation of synaptic transmission in this transfected SCG neuron preparation is primarily caused by facilitation of $Ca_V2.1$ channel activity by Ca^{2+}/CaM binding to the Ca^{2+} sensor protein binding site in the C-terminal domain (48). As illustrated in Fig. 10*A*, $Ca_V2.1$ 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. 10*A*). Perfusion of a competing peptide that blocks the interaction of CaMKII with $Ca_V2.1$ channels ($Ca_V2.1$(1897–1912)) prevented both paired-pulse facilitation and paired-pulse depression at this model synapse (Fig. 10*A*), suggesting that binding of CaMKII to $Ca_V2.1$ channels is required for expression of this regulatory effect. Similarly, expression of the brain-specific CaMKII inhibitor CaMKIIN (50), which prevents CaMKII binding to $Ca_V2.1$ channels (33), also prevented paired-pulse facilitation and depression (Fig. 10*A*). This is consistent with previous results showing that facilitation of $Ca_V2.1$ 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 EPSPs are unchanged (Fig. 10*B*). Because the competing peptide $Ca_V2.1$(1897–1912) applied acutely through the recording pipette and CaMKIIN expressed from cDNA both reduce paired-pulse facilitation of synaptic transmission, as expected from their inhibition of paired-pulse facilitation of $Ca_V2.1$ current, these results support the conclusion that these are specific effects. To further support the specificity of action of these agents, we applied $Ca_V2.1$(1897–1912) through the recording pipette and CaMKIIN by expression of injected cDNA and analyzed their effects on synaptic transmission initiated by endogenous $Ca_V2.2$ channels. We found no effect of either of these agents (Fig. 10, *C* and *D*), further supporting the specificity of their effects on facilitation of $Ca_V2.1$ currents and neurotransmission initiated by $Ca_V2.1$ channels. Evidently, binding of

CaMKII by $Ca_V2.1$ channels is required for both up-regulation of channel activity in paired pulses and for Ca^{2+}-independent activation of CaMKII by $Ca_V2.1$, and one or both of these effects is necessary for normal short term synaptic plasticity.

DISCUSSION

CaMKII Binds Directly to $Ca_V2.1$ Channels—Previous studies showed that CaMKII can be co-immunoprecipitated with $Ca_V2.1$ channels from transfected cells and that binding of CaMKII *per se* was sufficient for up-regulation of $Ca_V2.1$ 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 $Ca_V2.1$ channels at an interaction site located in $Ca_V2.1$(1848–1964). Autophosphorylation enhances binding of CaMKII, and autophosphorylated CaMKII remains bound to the C-terminal domain of $Ca_V2.1$ channels even after dephosphorylation and removal of the original Ca^{2+}/CaM stimulus for binding. Thus, $Ca_V2.1$ channels with bound CaMKII are likely to serve as a signaling complex in the presynaptic active zone in neurons.

Binding to $Ca_V2.1$ 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_V11 channels (45, 46). Our results show that binding of CaMKII to $Ca_V2.1$ 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 $Ca_V2.1$ 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 $Ca_V2.1$ channels and $Ca_V1.2$ channels. These two types of Ca^{2+} channels are less than 50% identical in amino acid sequence and serve different physiological roles: $Ca_V2.1$ in initiation of synaptic transmission *versus* $Ca_V1.2$ 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 $Ca_V2.1$ channels causes facilitation followed by inactivation, whereas binding to $Ca_V1.2$ channels causes only Ca^{2+}-dependent inactivation (9, 51). Binding of CaMKII to $Ca_V2.1$ channels enhances their activity and their facilitation, whereas phosphorylation of $Ca_V1.2$ channels by CaMKII bound to the C-terminal domain and/or the $Ca_V\beta2$ subunit is required for facilitation of their activity (9, 37, 52, 53). The structural and mechanistic basis for this differential regulation of $Ca_V1.2$ and $Ca_V2.1$ channels by CaM and CaMKII bound to nearby sites in their C-terminal domains is an interesting area for further research.

CaMKII Bound to $Ca_V2.1$ 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 $Ca_V2.1$ 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 $Ca_V2.1$ 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 $Ca_V2.1$ 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 $Ca_V2.1$ channel activity contribute substantially to short term synaptic facilitation and depression (9). Our results show that binding of CaMKII to $Ca_V2.1$ channels is required for short term synaptic plasticity in SCG neurons. Block of CaMKII binding with a competing peptide from its $Ca_V2.1$ binding site inhibits short term facilitation and depression of synaptic transmission, as does binding of the brain-specific CaMKII inhibitor CaMKIIN. Evidently, CaMKII binding to $Ca_V2.1$ channels is a necessary prerequisite for short term synaptic plasticity mediated by $Ca_V2.1$ channels, as observed previously in studies of synaptic plasticity in genetically modified mouse strains (31). Binding of CaMKII to $Ca_V2.1$ channels may play a permissive role by enhancing the activation of $Ca_V2.1$ 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 $Ca_V2.1$ channels in short term plasticity demonstrated here, it is possible that phosphorylation by CaMKII bound to $Ca_V2.1$ 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 $Ca_V2.1$ channels at active zones.

Functional Roles of the Presynaptic $Ca_V2.1$ Signaling Complex—Previous studies show that $Ca_V2.1$ channels are regulated by binding of SNARE proteins, G proteins, CaM and CaM-like calcium sensor proteins, and CaMKII (9). Proteomic analysis revealed a complex of ~100 proteins associated with $Ca_V2.1$ 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 $Ca_V2.1$ channels, which provide the trigger of Ca^{2+} entry to initiate rapid exocytosis. It also serves to regulate the activity of $Ca_V2.1$ channels in response to Ca^{2+} and other regulatory messengers. Prior binding of CaMKII to $Ca_V2.1$ channels is required for facilitation and inactivation of the $Ca_V2.1$ channel during trains of repetitive depolarizations or action potentials. In this way, CaMKII binding to $Ca_V2.1$ serves as a molecular switch to turn on or off the millisecond timescale modulation

of channel activity by Ca^{2+}-dependent facilitation and inactivation.

The Effector Checkpoint Model for Calcium Channel Regulation—Voltage-gated Ca^{2+} channels are regulated by their effectors such that the channels are more active when the effectors of their Ca^{2+} signal are bound. Examples include regulation of the skeletal muscle Ca^{2+} channel by the ryanodine-sensitive Ca^{2+} release channel (60), its effector in excitation-contraction coupling, and regulation of presynaptic Ca^{2+} channels by SNARE proteins, which are the effectors for Ca^{2+}-dependent exocytosis (9). Regulation of $Ca_v2.1$ channels by CaMKII also fits this regulatory theme (33). Binding of CaMKII to $Ca_v2.1$ 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^{2+} channels whose effectors are bound would focus Ca^{2+} entry and Ca^{2+}-dependent protein phosphorylation in locations where it can effectively generate a cellular response via local Ca^{2+} signaling. This mechanism would enhance local signal transduction and reduce ineffective Ca^{2+} entry and protein phosphorylation at other sites.

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