Calmodulin Kinase II Is Involved in Voltage-dependent Facilitation of the L-type Ca\textsubscript{v}1.2 Calcium Channel

**IDENTIFICATION OF THE PHOSPHORYLATION SITES**

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Calcium-dependent facilitation of L-type calcium channels has been reported to depend on the function of calmodulin kinase II. In contrast, the mechanism for voltage-dependent facilitation is not clear. In HEK 293 cells expressing Ca\textsubscript{v}1.2, Ca\textsubscript{\beta}2a, and calmodulin kinase II, the calcium current measured at +30 mV was facilitated up to 1.5-fold by a 200-ms-long prepulse to +160 mV. This voltage-dependent facilitation was prevented by the calmodulin kinase II inhibitors KN93 and the autocompete-2-related peptide. In cells expressing the Ca\textsubscript{v}1.2 mutation I1649E, a residue critical for the binding of Ca\textsuperscript{2+}-bound calmodulin, facilitation was also abolished. Calmodulin kinase II was coimmunoprecipitated with the Ca\textsubscript{v}1.2 channel from murine heart and HEK 293 cells expressing Cav1.2, the calcium current measured at expressing the Cav1.2 mutation I1649E, a residue critical for the up to 1.5-fold by a 200-ms-long prepulse to 25560 JOURNAL OF BIOLOGICAL CHEMISTRY

3 The abbreviations used are:
- Ca\textsuperscript{2+}: calcium ion
- Ca: calcium current
- CaM: calmodulin
- CaM-KII: calmodulin kinase II
- F: fluorescence intensity
- IQ: isoleucine–aspartate–glutamate–residue
- PKC: protein kinase C
- Ser: serine
- Thr: threonine
- Tr: truncated

These findings were not confirmed by others (8, 10–12). In contrast, it was shown that facilitation required elevated [Ca\textsuperscript{2+}]\textsuperscript{i} in the vicinity of the channel (“Ca\textsuperscript{2+}-dependent facilitation”) (13). Excessive calcium influx is toxic to cells. As a consequence, calcium channels have evolved voltage- and Ca\textsuperscript{2+}-dependent inactivation mechanisms. Both Ca\textsuperscript{2+}-dependent facilitation and inactivation require high affinity binding of calmodulin (CaM) to the IQ motif located in the carboxyl-terminal tail of Ca\textsubscript{v}1.2 channel (14–16). Zühlke et al. (14, 15) showed that mutation of the isoleucine to alanine in the IQ motif abolished Ca\textsuperscript{2+}-dependent inactivation without affecting Ca\textsuperscript{2+}-dependent facilitation. In contrast, the mutation of the same isoleucine to glutamate eliminated both forms of autoregulation. Several additional peptide sequences may be involved in the complex formation of CaM with the channel (17). The mechanisms behind this Ca\textsuperscript{2+}/CaM-dependent regulation are reviewed in Ref. 18.

Ca\textsuperscript{2+}-dependent facilitation has been attributed to the action of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM-KII) (19–22). Constitutively active CaM-KII facilitated L-type current in excised patches (23). CaM-KII was reported to phosphorylate the main subunit of an isolated L-type calcium channel (24). However, the phosphorylation site was not identified in that study. So far, the mechanism of facilitation caused by a strong depolarizing prepulse (“voltage-dependent facilitation”) is unclear.

The functional significance of CaM-KII depends on its subcellular localization (13). CaM-KII has been shown to be localized closely to the Ca\textsubscript{v}1.2 channel in cardiac muscle (25, 26). Despite this localization, activation of the enzyme apparently required release of Ca\textsuperscript{2+} from intracellular stores (27, 28), thereby allowing integration of spatially and temporally separated [Ca\textsuperscript{2+}]\textsuperscript{i}, spikes. These and other studies showed that CaM-KII is an important regulator of neuronal and muscular
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TABLE 1
Sequences of forward and reverse primers for the design of Ca$_{1.2}$ and Ca$_{\beta}$ mutants
Shaded triplets correspond to intended mutations. Underlined boldface letters correspond to mutated amino acids.

| Planned Mutation | Sequence of Designed Primer |
|------------------|-----------------------------|
| Ca$_{1.2}$ subunit |                            |
| S808A forward    | 5'-GCC AGG ACT GCC GCC CGG GAG AAG AAA C-3' |
| reverse          | 5'-GT TTC TCC TTC GGG GCA GTC TGT CC-3'  |
| S888A forward    | 5'-CT CGG CCA CTC GCC GAG CTC GAC C-3'  |
| reverse          | 5'-GTG CAT GCC GAG AGC AG-3'            |
| S1512A forward   | 5'-ACA AGG GAC TGG GCA ATC CTT GGT CCC-3' |
| reverse          | 5'-GGG ACC AAG TAT CCA GTC CCT TGT C-3' |
| S1570A forward   | 5'-CT TGC AAA GGC CCG GCC CAT GAT CAT C-3' |
| reverse          | 5'-GTT CAT CAT GGT CAT GAC TGC CAG TTT TGT C-3' |
| S1695A forward   | 5'-CGA CGG GCC ATC ACA AGA GAC TCA A-3'  |
| reverse          | 5'-C TGT CAG TCC TGC GAT GGC CGG C-3'   |
| I1649E forward   | 5'-CTT CTG TAC CAA GAG TTA TCT CCA AAA TTC-3' |
| reverse          | 5'-TA CTC TGG TCC CAG AAG GTA GCG TAG AAC-3' |
| Ca$_{\beta}$ subunit |                            |
| T500A forward    | 5'-C CTC TCT AGG CAA GAG GCA TTA GAC TCA GAA ACC-3' |
| reverse          | 5'-GGT TTA GGT AAA TGA CGC TCC TTA CAT AGA GAG G-3' |

function (29), because its activity reflects the frequency of cytosolic Ca$^{2+}$ oscillations. The kinase might, therefore, be able to integrate variable Ca$^{2+}$ signals over time.

Here we show that the Ca$_{1.2}$ protein immunoprecipitated in a complex with CaM-KII and was phosphorylated in a Ca$^{2+}$/CaM-dependent manner. Transient expression of Ca$_{1.2}$ and the cardiac Ca$_{\beta}2$ subunit in a cell line stably expressing CaM-KII allowed us to identify for the first time the two CaM-KII phosphorylated serines that are necessary for voltage-dependent facilitation. Interestingly, these two serines flank the EF-hand of Ca$_{1.2}$.

MATERIALS AND METHODS

All substances used were of the highest purity available. The Ca$_{1.2}$-specific antibody used in this study has been described previously (30). CaM was detected using a monoclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY). The CaM-KII antibody MAB8699 was from Chemicon.

cDNA Clones and Site-directed Mutations—The cDNAs of all subunits, including full-length Ca$_{1.2}$b (data base accession number X55763) (31) or truncated Ca$_{1.2}$b (Tr1728) (32), cardiac Ca$_{\beta}2$ (X64297) (Ca$_{\beta}2$a) (33), $\alpha_{\beta}6$-1 (M21948) (34), or mutants were subcloned into the pcDNA3 vector. The Ca$_{1.2}$b mutants S808A, S888A, S808A/S888A, S1512A, S1570A, S1512A/S1570A, S1695A, the IQ $\rightarrow$ EQ (I1649E) (15), and the Ca$_{\beta}2$a mutant T500A were made by the use of the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers are given in Table 1. PCR amplification was performed using pcDNA3Ca$_{1.2}$b, pcDNA3Tr1728 or pcDNA3Ca$_{\beta}2$a as template. For construction of the mutants S1512A, S1570A, S1512A/S1570A, and S1695A, the PCR products were digested with SfiI/XbaI. For construction of the mutants S808A, S888A, and S808A/S888A, the PCR products were digested with SgrAI/PmlII. The resulting PCR fragments were ligated into the corresponding sites of the digested vector. The Ca$_{1.2}$b subunit was truncated carboxy-terminally at amino acid 1728 (for details, see Ref. 32). The GST fusion proteins of the truncated COOH terminus were cloned in the pGEX6P-1 (Amersham Biosciences) vector and mutared using

overlapping PCR. Each construct was sequenced to verify the correctness of the sequence around the mutation and the ligation.

Transfection of HEK 293 Cells—Human embryonic kidney cells (HEK 293 cells) stably expressing Ca$_{1.2}$b and the cardiac Ca$_{\beta}2$a subunit were transiently transfected with the expression vector for CaM-KII (total cDNA 0.3 $\mu$g) by a lipofection method using Lipofectamine 2000 according to the manufacturer’s guide (Invitrogen). HEK 293 cells stably expressing CaM-KII were transiently transfected with the expression vectors for wild type and the various Ca$_{1.2}$b chimeras and the cardiac Ca$_{\beta}2$a subunit (0.15 $\mu$g of each). After transfection, the cells were grown for 40–48 h before beginning the electrophysiological experiments.

Electrophysiological Recordings—Whole-cell $I_{Ca}$ was measured at room temperature. Stimulation and data acquisition were performed through EPC 9 and HEKA PULSE (version 8.54; HEKA Electronic, Lambrecht, Germany). Data were sampled at 5 kHz and filtered at 1.67 kHz. The pipette had a resistance of 2–3.5 megohms when filled with intracellular solution. Series resistance and capacitive components were compensated by circuitry equipped in EPC 9. Extracellular solution was composed of 82 mM NaCl, 20 mM triethanolamine-Cl, 30 mM CaCl$_2$, 5 mM CsCl, 1 mM MgCl$_2$, 0.1 mM EGTA, 10 mM glucose, 5 mM HEPES, pH 7.4, adjusted with NaOH. Equimolar BaCl$_2$ was used instead of CaCl$_2$ in the case of barium current ($I_{Ba}$) measurement. Intracellular solution was composed of 102 mM CsCl, 10 mM triethanolamine-Cl, 10 mM EGTA, 1 mM MgCl$_2$, 3 mM NaN$_2$ATP, 5 mM HEPES, pH 7.4, adjusted with CsOH. To measure current-voltage ($I-V$) relations, the cell was stimulated with 300-ms square pulses of $-50$ to $80$ mV in increments of 10 mV from a holding potential of $-80$ mV. Facilitation of $I_{Ca}$ was measured during a triple pulse protocol with a 100-ms control pulse to $+30$ mV ($TP1$) followed by a 200-ms prepulse to $+160$ mV followed by a 100-ms test pulse to $+30$ mV ($TP2$) (Fig. 1a). The extent of voltage-dependent facilitation was calculated as the ratio of the peak current during TP2 and TP1. The extent of inacti-
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vation was evaluated by the residual current fraction at the end of a 300-ms square pulse at +30 mV (R\textsubscript{300}). 0.5 μM KN-93 (Calbiochem) was applied in extracellular solution, or 10 μM autocamtide-2-related inhibitory peptide (Calbiochem) was added to intracellular solution to inhibit CaM-KII. KN-92 (1 μM; Calbiochem) was used as control of KN-93. Data plotting and statistical analysis was carried out using ORIGIN software (version 6.1052; Microcal, Northampton, MA). Pooled data are given as mean ± S.E.

Generation of Cell Lines—The cDNA for CaM-KII\textalpha (AK083245) was cloned from a murine embryonal cDNA library using a PCR strategy and subcloned into the EcoRI site of pcDNA3 (Invitrogen). The 5'-PCR primer contains an additional consensus sequence for initiation of translation (GCC ACC). HEK 293 cells were transfected by electroporation (Gene Pulser\textsuperscript{TM}; Bio-Rad) with the expression vectors pcDNA3Ca\textsubscript{v}1.2b plus pcDNA3Ca\textsubscript{v}1.2β2a or pcDNA3CaM-KII using linearized DNA. The HEK 293 cells were grown in minimal essential medium supplemented with 5–10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml). After electroporation, the cells were grown for 48 h without selection medium. Thereafter, the medium was supplemented with 400 μg/ml G418 to select for CaM-KII or with 400 μg/ml G418 plus 200 μg/ml hygromycin B to select for Ca\textsubscript{v}1.2b plus Ca\textsubscript{v}1.2β2a. The medium was changed every third day. Three weeks after the electroporation, individual cell clones were isolated with the aid of a cloning ring. Stable expression of the calcium channel subunits was confirmed by patch-clamp experiments; stable expression of CaM-KII was confirmed by Western blotting with a monoclonal CaM-KII antibody (MA1-048; Dianova, Hamburg, Germany) and a goat secondary antibody (IgG (H + L) 112-055-062; Dianova). Untransfected HEK 293 cells expressed neither L-type calcium channels nor CaM-KII.

Preparation and Solubilization of Membranes and GST Fusion Proteins—All preparative steps were performed at 4°C using precooled solutions containing the protease inhibitor mixture P8340 (2 μl/ml; Sigma), phenylmethylsulfonyl fluoride (200 nM), calpain inhibitor I (8 μg/ml), and calpain inhibitor II (8 μg/ml). Hearts from 6–12-week-old male mice or HEK 293 cells were frozen and pulverized under liquid N\textsubscript{2} in a porcelain mortar and then resuspended in membrane preparation buffer containing 300 mM sucrose, 75 mM NaCl, 20 mM EDTA, 20 mM EGTA, 10 mM Tris-HCl, pH 7.4 (1 ml/100 mg of tissue or 3 × 10\textsuperscript{6} cells, respectively). Homogenates were centrifuged at 4,500 rpm for 10 min at 4°C to remove larger cell fragments, including nuclei. Membranes were collected by ultracentrifugation (50,000 rpm at 4°C) for 30 min, and channels were solubilized for 20 min on ice with 1% deoxycholate, 10 mM EDTA, 10 mM EGTA, 50 mM Tris-HCl, pH 7.4, containing protease inhibitors. The following buffer was used for solubilization in the presence of calcium: 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.5 mM CaCl\textsubscript{2}, 1 mM dithiothreitol, 1% Triton, and EDTA-free protease inhibitor mixture. Nonosuble material was removed by a second ultracentrifugation step (50,000 rpm at 4°C for 30 min). GST fusion proteins were expressed in BL21 E. coli according to the manufacturer’s instructions (Amersham Biosciences).
Immunoprecipitation and Immunoblotting of Cav1.2 and CaM-KII—The solubilized membranes from heart and HEK 293 cells were preincubated with protein A-Sepharose (Sigma) or protein G-Sepharose (Amersham Biosciences) to remove proteins that bind to the resin nonspecifically. After removal of the Sepharose beads by centrifugation, the supernatant was incubated on ice with antibodies. After 2.5 h, protein A-Sepharose or protein G-Sepharose was added, samples were tilted for 1 h, and the resins were washed and extracted with 1:6 (v/v) SDS sample buffer (35). Eluted proteins were separated on a 10% SDS gel. The separated proteins were immunoblotted as described in Ref. 30. All experiments were repeated three times with comparable results.

Phosphorylation with CaM-KII—For CaM-KII kinase reactions, full-length Cav1.2 was immunoprecipitated with the Cav1.2-specific antibody, and immune complexes were captured on protein A-Sepharose. GST fusion proteins were purified on glutathione-Sepharose. Precipitated complexes were resuspended in phosphorylation buffer (0.1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 0.1 mM EDTA). Samples were incubated with 250 units of CaM-KII (New England Bio-labs) (preincubated in the presence or absence of Ca2+/CaM), and phosphorylation was initiated at 30 °C with 33 µCi/µmol (PerkinElmer Life Sciences) and termi-

FIGURE 3. I-V relation and inactivation properties of the S1512A/S1570A mutated Cav1.2 channel. a, current-voltage relation of Tr1728 (truncated at amino acid 1728; open circles) and the S1512A/S1570A mutant (filled circles) co-transfected with Cavαβ2a in a CaM-KII-expressing HEK 293 cell. b, comparison of voltage-dependent facilitation between Tr1728 (open bar) and S1512A/S1570A (filled bar). c, superimposed I_{Ca} and I_{Ba} current traces of wild type (WT) and S1512A/S1570A mutant. **, p < 0.005 versus Tr1728; *, p < 0.05 S1512A/S1570A versus WT.

| Mutant of Cav1.2 | Peak current density – prepulse | n | Normalized current at 30 mV + prepulse | n |
|------------------|--------------------------------|---|--------------------------------------|---|
| With βWT        |                                |   |                                      |   |
| WT               | −8.8 ± 0.8*                    | 52| 1.45 ± 0.09                          | 47|
| Tr1728          | −14.4 ± 1.1                    | 78| 1.29 ± 0.04                          | 79|
| S808A           | −9.9 ± 1.9                     | 13| 1.38 ± 0.17                          | 11|
| S888A           | −8.6 ± 1.3                     | 11| 1.32 ± 0.12                          | 6 |
| S808A/S888A     | −8.0 ± 1.2*                    | 10| 1.26 ± 0.09                          | 5 |
| S1512A          | −13.1 ± 2.5                    | 21| 1.05 ± 0.04*                         | 19|
| S1570A          | −23.5 ± 3.9*                   | 15| 1.03 ± 0.04*                         | 15|
| S1512A/S1570A   | −13.8 ± 2.3                    | 21| 1.03 ± 0.04*                         | 22|
| S1695A          | −19.2 ± 5.9                    | 12| 1.28 ± 0.16                          | 13|
| With βT500A     |                                |   |                                      |   |
| Tr1728          | −14.7 ± 1.7                    | 18| 1.21 ± 0.09                          | 18|
| S1512A/S1570A   | −16.3 ± 2.3                    | 13| 1.04 ± 0.07*                         | 14|

*p < 0.005 versus Tr1728 with βWT.

TABLE 2
Summary of current density and facilitation obtained from each Cav1.2 mutant (see Fig. 2) co-transfected with wild type (βWT) or mutant (βT500A) Cavαβ

The cell line expressing stably CaM-KII was used.

Facilitation of Cav1,2 by CaM-KII—For CaM-KII kinase reactions, full-length Cav1,2 was immunoprecipitated with the Cav1,2-specific antibody, and immune complexes were captured on protein A-Sepharose. GST fusion proteins were purified on glutathione-Sepharose. Precipitated complexes were resuspended in phosphorylation buffer (0.1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 0.1 mM EDTA). Samples were incubated with 250 units of CaM-KII (New England Bio-labs) (preincubated in the presence or absence of Ca2+/CaM), and phosphorylation was initiated at 30 °C with 33 µM Mg-[γ-32P]ATP (54 µCi/µmol) (PerkinElmer Life Sciences) and termi-
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RESULTS

Numerous experiments have shown that the cardiac L-type $I_{\text{Ca}}$ can be facilitated by a strong prepulse to positive membrane potentials or by Ca$^{2+}$ released from intracellular stores (for a review, see Ref. 13). Initial experiments with an HEK 293 cell line stably expressing the Ca_{1.2} and the Ca_{\beta2a} subunits of the L-type calcium channel in the presence or absence of the S808A, S888A, or S1695A subunit did not result in voltage-dependent facilitation of $I_{\text{Ca}}$ (Fig. 1b). Similar findings have been reported by other groups (13, 14). The failure to induce facilitation in HEK 293 cells was not caused by a lack of CaM, which is expressed in sufficient amounts, but was rather due to a deficiency of CaM-KII, which we were unable to detect in these cells with the antibodies used. In line with this view, facilitation was readily observed following coexpression of the Ca_{1.2} and the Ca_{\beta2a} subunit with CaM-KII (Fig. 1b). Moreover, superfusion of the cells with the CaM-KII inhibitor KN-93 (0.5 \mu M) abolished $I_{\text{Ca}}$ facilitation (Fig. 1b), whereas it was not affected by the inactive analogue KN-92 (1 \mu M). The CaM-KII specificity of the reaction was substantiated further by the finding that inclusion of the CaM-KII inhibitor peptide, autocomtide-2-related inhibitory peptide (10 \mu M), in the pipette solution suppressed $I_{\text{Ca}}$ facilitation. As reported by Zühlke et al. for calcium-dependent facilitation (14, 15), voltage-dependent facilitation was abolished by mutation of the IQ motif to EQ in the Ca_{1.2} subunit (Fig. 1b). The relative small current was not unexpected (see also Ref. 36), because mutations in this region of Ca_{1.2} affect trafficking of the channel (37). The importance of an intact IQ motif for calcium-dependent inactivation of the channel has also been reported by several other groups (38–41). The depolarizing prepulse significantly inhibited $I_{\text{Ca}}$ through the mutated channel (Fig. 1, b and c), supporting the notion that voltage-dependent inactivation is intact in the I1649E mutant. Similar results have been reported by others (16, 38). The lack of facilitation of the I1649E channel was not caused by an impairment of the interaction between the channel and the CaM-KII, because CaM-KII immunoprecipitated with the mutated channel (see Fig. 4d). Complex formation of CaM-KII with the channel outside of the IQ motif has been reported also by Hudmon et al. (42) during the processing of this manuscript.

The results above strongly support the hypothesis that CaM-KII is activated during the prepulse and that activated CaM-KII phosphorylates either the Ca_{1.2} or the Ca_{\beta2a} subunit. To substantiate this hypothesis, we analyzed the sequence of both proteins for potential CaM-KII phosphorylation sites using the prediction algorithm of Refs. 43 and 44. Fifteen putative phosphorylation sites were identified in the Ca_{1.2} sequence (Fig. 2). Ser^{249}, Thr^{713}, and Thr^{1439} were not considered further, because it is rather unlikely that these residues are accessible from the intracellular space. The remaining sites were analyzed in a HEK 293 cell line stably expressing CaM-KII. Six of the sites were located carboxyl-terminally from amino acid 1728 (Fig. 2).

Truncation of the Ca_{1.2} subunit at amino acid 1728 did not affect facilitation (Table 2), ruling out that these putative phosphorylation sites were involved in the facilitation process. In agreement with previous findings (12, 32, 45), the current voltage relation of the truncated channel was also not affected (Fig. 3a).

Further experiments were carried out using the truncated channel (Tr^{1728}) as template. We investigated the role of serine residues Ser^{808}, Ser^{888}, Ser^{808/888}, or Ser^{1695} by mutating them to alanine. $I_{\text{Ca}}$ through the Ca_{1.2} subunit containing the mutation S808A, S888A, S808A/S888A, or S1695A still showed significant facilitation following the prepulse protocol (Table 2). $I_{\text{Ca}}$ facilitation was also observed in HEK 293 cells coexpressing the truncated Ca_{1.2} subunit and the Ca_{\beta2a} subunit with a mutation of its single putative phosphorylation site (T500A) (Table 2). In sharp contrast to these “silent mutations,” mutations of either S1512A or S1570A almost completely abolished...
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Figure 5. CaM-KII phosphorylates Ser\textsuperscript{1512} and Ser\textsuperscript{1570}. a and b, full-length Ca\textsubscript{1,2} immunoprecipitated from mouse heart was incubated with exogenous CaM-KII and [\gamma\textsuperscript{32P}ATP. The proteins were separated on a 10% SDS gel, transferred to nitrocellulose, and Western blotted followed by autoradiography. The specificity of the CaM-KII phosphorylation was verified by a phosphorylation reaction in the absence of CaM and Ca\textsuperscript{2+}. a, an autoradiograph of a whole gel demonstrating CaM-KII autophosphorylation and phosphorylation of the WT Ca\textsubscript{1,2} protein. b, upper panels, region between 150 and 300 kDa cut from a whole gel as shown in a and exposed five times longer to facilitate visualization of the Ca\textsubscript{1,2} signal without the very strong background of the autophosphorylated CaM-KII. Lower panels, Ca\textsubscript{1,2} immunoblot demonstrating equivalent Ca\textsubscript{1,2} input into the phosphorylation assay. c, identification of Ser\textsuperscript{1512} and Ser\textsuperscript{1570} as the CaM-KII phosphorylation sites on full-length Ca\textsubscript{1,2} by alanine substitution (S1512A/S1570A). WT Ca\textsubscript{1,2} and Ca\textsubscript{1,2}-S1512A/S1570A coexpressed with Ca\textsubscript{\beta2a} in HEK 293 cells were immunoprecipitated with anti-Ca\textsubscript{1,2} and incubated with CaM-KII (250 units) and [\gamma\textsuperscript{32P}ATP. The proteins were separated on a 10% SDS gel and Western blotted followed by autoradiography. The specificity of the CaM-KII phosphorylation was verified by phosphorylation reactions in the absence of CaM and Ca\textsuperscript{2+}. All blots are representative of three experiments. d, the GST-fused Ca\textsubscript{1,2} WT and S1512A/S1570A COOH fragments 1492–1728 were phosphorylated by CaM-KII kinase in the presence of [\gamma\textsuperscript{32P}ATP. The reaction mixture was separated on SDS gels and autoradiographed (upper panel). CaM-KII phosphorylated the wild type but not the S1512A/S1570A mutated fragment. Lower panels, loading controls using an antibody against the GST fusion part. All blots are representative of three experiments.

Voltage-dependent facilitation of \(I_{Ca}\) (Table 2), suggesting their critical function for this process. The same result was obtained when both sites were mutated concurrently. Remarkably, the two serine residues involved in facilitation flank the EF-hand of the Ca\textsubscript{1,2} subunit, a domain that has been implicated in the Ca\textsuperscript{2+}-dependent inactivation of the Ca\textsubscript{1,2} channel (46–48). It is noteworthy that the loss of facilitation by the mutation S1512A/S1570A was not accompanied by any alteration in the voltage dependence of the channel (Fig. 3, a and b) and of its voltage-dependent inactivation measured with \(I_{\text{Ba}}\) (Fig. 3, c and d). As expected, Ca\textsuperscript{2+}-dependent inactivation was not abolished but was slightly increased by this mutation (Fig. 3, c and d).

CaM-KII-dependent facilitation is well described in cardiac myocytes (21–23). In rat ventricular myocytes, facilitation requires translocation of activated CaM-KII to the inner leaflet of the transverse tubule (26), probably as a prerequisite for a close association of the kinase with the channel protein. This prompted us to investigate a physical interaction between CaM-KII and the Ca\textsubscript{1,2} calcium channel in cardiac myocytes and HEK 293 cells. Therefore, the full-length Ca\textsubscript{1,2} channel was immunoprecipitated from murine heart membranes and HEK 293 cells. Western blots of the precipitated proteins showed that CaM-KII was co-immunoprecipitated with the Ca\textsubscript{1,2} protein in the presence and absence of Ca\textsuperscript{2+} (Fig. 4, a–c). The same two proteins precipitated when an antibody against CaM-KII was used. As noted also by other groups (38, 49–52), coprecipitation of CaM with the full-length Ca\textsubscript{1,2} channel protein was variable and not always detectable.

Based upon our electrophysiological data, the two serines Ser\textsuperscript{1512} and Ser\textsuperscript{1570} within the carboxyl terminus are expected to represent authentic CaM-KII phosphorylation sites of the Ca\textsubscript{1,2} subunit. To substantiate this idea, we first sought to determine whether CaM-KII directly phosphorylates the full-length Ca\textsubscript{1,2} channel protein from cardiac tissue. In the presence of [\gamma\textsuperscript{32P}ATP, radioactive phosphate was incorporated into the Ca\textsubscript{1,2} protein in a Ca\textsuperscript{2+}/CaM-dependent manner (Fig. 5, a and b).

Having validated the specificity of our system to track kinase-dependent phosphorylation of Ca\textsubscript{1,2}, we next used it to examine the phosphorylation status of full-length recombinant WT and S1512A and S1570A Ca\textsubscript{1,2} subunits expressed in HEK 293 cells (Fig. 5c). The extent of radioactivity associated with the full-length Ca\textsubscript{1,2} protein was strongly reduced when the
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S1512A/S1570A mutant protein was used, demonstrating that these two serines in the carboxyl terminus of Ca\textsubscript{v}1.2 are phosphorylated by CaM-KII. The mutations did not interfere with the interaction between the CaM-KII and the channel, as shown by the co-immunoprecipitation of both proteins (Fig. 4, d and e). These findings support the notion that Ser\textsuperscript{1512} and Ser\textsuperscript{1570} are not involved in association of CaM-KII with the calcium channel but are true phosphorylation sites.

We confirmed these findings utilizing wild type and S1512A/S1570A-mutated GST fusion proteins containing amino acids 1492–1728 of the Ca\textsubscript{v}1.2 carboxyl terminus as substrates for in vitro CaM-KII phosphorylation. Whereas CaM-KII readily phosphorylated the wild type truncated carboxyl terminus, only very weak phosphorylation could be detected in the S1512A/S1570A mutant protein (Fig. 5d).

DISCUSSION

The cardiac Ca\textsubscript{v}1.2 Ca\textsuperscript{2+} channel shows two properties mediated through an interaction of Ca\textsuperscript{2+} /CaM with the carboxyl-terminal sequence of the channel protein (14, 39, 40): Ca\textsuperscript{2+}-dependent inactivation and facilitation. Mechanisms supporting facilitation have not been established clearly, because the site(s) phosphorylated by CaM-KII remained unknown. The present findings strongly support the view (13, 23) that facilitation can be induced by CaM-KII-dependent phosphorylation of two amino acids flanking the EF-hand motif of the Ca\textsubscript{v}1.2 channel protein.

Coimmunoprecipitation experiments with probes obtained from native tissue and HEK 293 cells suggest a close association of the kinase with the channel protein that may be functionally relevant. Phosphorylation was greatly reduced in Ca\textsubscript{v}1.2 proteins with the amino acid residue Ser\textsuperscript{1512} and/or Ser\textsuperscript{1570} mutated to alanine. During the processing of this manuscript, CaM-KII-dependent phosphorylation of Ser\textsuperscript{1512} has been also reported by others (53), strengthening the finding of this study. Mutation of the two serines eliminated facilitation but did not reduce Ca\textsuperscript{2+}-dependent inactivation of the Ca\textsubscript{v}1.2 channel. This dissociation emphasizes that Ca\textsuperscript{2+}-dependent facilitation and inactivation rely on distinct and independent structural mechanisms.

So far, only Ca\textsuperscript{2+}-dependent inactivation has been observed for Ca\textsubscript{v}1.2 channels expressed in HEK 293 cells (39). Our data suggest that this is due to the fact that HEK 293 cells do not express CaM-KII to an extent sufficient for supporting voltage-dependent facilitation. These findings fit well with data obtained in oocytes (14, 15) that express CaM-KII abundantly (54, 55).

The location of the two phosphorylated serines is intriguing, because the sequence between them includes an EF-hand motif. Initially, this EF-hand motif was implicated in the Ca\textsuperscript{2+}-dependent regulation of the Ca\textsubscript{v}1.2 channel (56), but later it was concluded that the EF-hand motif is not relevant for Ca\textsuperscript{2+}-dependent inactivation (14, 15, 39, 40, 47, 57). A recent report concluded that the EF-hand interacts with CaM-KII and, thereby, mediates channel facilitation (42). The findings of the present report that mutation of Ser\textsuperscript{1512} and/or Ser\textsuperscript{1570} does slightly increase Ca\textsuperscript{2+}-dependent inactivation but abolishes facilitation support the notion that the sequence flanking the EF-hand motif is required for the facilitation process. Facilitation also requires an intact IQ motif. Mutation of the IQ motif to EQ decreased 100-fold the affinity of the corresponding peptide for CaM (15), eliminated Ca\textsuperscript{2+}-dependent facilitation (15), and eliminated CaM-KII dependent facilitation (see Fig. 1).

These results support the hypothesis that the IQ motif is of primary importance for Ca\textsuperscript{2+}-dependent modulation of the Ca\textsubscript{v}1.2 channel. However, the signaling mechanism leading to inactivation and facilitation involves different parts of the channel sequence. Apparently, facilitation needs a conformational new positioning of the EF-hand motif triggered by CaM-KII-dependent phosphorylation. Together with two reports (42, 53) published during the processing of this manuscript, these results identify the carboxyl-terminal region around the EF-hand as important for Ca\textsuperscript{2+}-dependent regulation of I\textsubscript{Ca}.

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