Deletion of the C-terminal Phosphorylation Sites in the Cardiac β-Subunit Does Not Affect the Basic β-Adrenergic Response of the Heart and the Cav1.2 Channel

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Background: β-Adrenergic receptors stimulate cardiac $I_{Ca}$ via PKA-dependent phosphorylation.

Results: Deletion of the C-terminal phosphorylation sites in the β2 gene did not affect isoproterenol-stimulated $I_{Ca}$.

Conclusion: Phosphorylation of the C terminus of the $\beta_2$ subunit in vivo does not contribute to β-adrenergic regulation of $I_{Ca}$.

Significance: The PKA-dependent regulation of $I_{Ca}$ does not require the C terminus of the β2 subunit.

Phosphorylation of the cardiac β subunit (Ca2+/β2) of the Cav1.2 L-type Ca2+ channel complex has been proposed as a mechanism for regulation of L-type Ca2+ channels by various protein kinases including PKA, CaMKII, Akt/PKB, and PKG. To test this hypothesis directly in vivo, we generated a knock-in mouse line with targeted mutation of the Ca2+/β2 gene by insertion of a stop codon after proline 501 in exon 14 (mouse sequence Cacnb2; βStop mouse). This mutation prevented translation of the Ca2+/β2 C terminus that contains the relevant phosphorylation sites for the above protein kinases. Homozygous βStop mice were born at Mendelian ratio, had a normal life expectancy, and normal basal L-type $I_{Ca}$. The regulation of the L-type current by stimulation of the β-adrenergic receptor was unaffected in vivo and in cardiomyocytes (CMs). βStop mice were cross-bred with mice expressing the Cav1.2 subunit. The β-adrenergic regulation of the cardiac $I_{Ca}$ was unaltered in these mouse lines. In contrast, truncation of the Cav1.2 at Asp1904 abolished β-adrenergic up-regulation of $I_{Ca}$ in murine embryonic CMs. We conclude that phosphorylation of the C-terminal sites in Ca2+/β2, Ser1928, Ser1512, and Ser1570 of the Cav1.2 protein is functionally not involved in the adrenergic regulation of the murine cardiac Cav1.2 channel.

The cardiac L-type Ca2+ current ($I_{Ca}$) is regulated by a number of protein kinases including PKA, CaMKII, PKC, Akt/PKB, and PKG (1, 2). Regulation of the cardiac Cav1.2 channel by β-adrenoceptors, cAMP, and PKA has been implicated as basic mechanism of the fight or flight reaction of an animal (3, 4). Phosphorylation of some channel subunits plays a critical role in several physiological cardiac processes, e.g. excitation-contraction coupling, the regulation of positive inotropy and chronotropy, as well as pathological processes such as heart failure (for review, see 2, 5). The molecular basis of $I_{Ca}$ regulation by protein kinases could not be defined conclusively so far because expression studies suggested phosphorylation sites on the α1 subunit and the β2 subunit of the L-type calcium channel. Phosphorylation sites on the α1 subunit were invoked for PKA (6–8), CaMKII (9–11), PKG (12, 13), Akt/PKB (14, 15), and by PKC (16–19). In addition, phosphorylation sites in the Cav2β2 subunit were reported for PKA (20) at Ser479/480 (rabbit protein sequence (rbs)) (21), CaMKII (22) at Thr500 (rbs), PKG (12) at Ser496 (rbs), and Akt/PKB (14, 15, 23, 24) at Ser576 (rbs). The amino acids modified by protein kinases in Cav2 β2 or Cav1.2 in the protein sequence from rabbit, rat, and mouse are listed in supplemental Table 1.

This very impressive work of several groups missed a clear statement, if the phosphorylation of one subunit was necessary to regulate $I_{Ca}$ in vivo. Previously, we investigated whether phosphorylation of Ser1928 of the α1 subunit was a necessary step for the β-adrenergic regulation of the cardiac $I_{Ca}$ in vivo. Mutation of the Ser to Ala did not affect the β-adrenergic regulation (25), raising the possibility that phosphorylation of the Cav2β2 subunit by PKA (20) might be the requested regulatory step. Therefore, we generated a mouse line that contained a stop codon in exon 14 of the mouse Cacnb2 gene after Pro501 (βStop). This stop codon resulted in a truncated β2 subunit protein that lacked the potential phosphorylation sites for PKA, PKG, Akt/PKB, and CaMKII. Basal properties of the Cav1.2 current were unaffected as expected from the report that deletion of the Cacnb2 gene in the adult heart has minimal effects on $I_{Ca}$ (26). We crossed the βStop line with the S1928A (25) and SF (S1512A and S1570A) (27) mouse lines that contain well-characterized phosphorylation sites for PKA and CaMKII, respectively. Again, the basal properties of the Cav1.2 current were unaffected, suggesting that β-adrenergic regulation of the...
Ca_{1,2} channel may be mediated by other phosphorylation sites, e.g. Ser^{1700} of the α_{1} subunit (8).

**EXPERIMENTAL PROCEDURES**

All substances used were of the highest purity available. Amino acid numbering is according to the *M. musculus* Cacnb2 sequence (GenBank accession number Q8CC27) or to the *Oryctolagus cuniculus* (rabbit) Cacnb2 sequence (GenBank accession number X64297). The amino acids modified by protein kinases in Ca_{αβ_2} or Ca_{1,2} in the protein sequence from rabbit, rat, and mouse are listed in supplemental Table 1. Within this paper we refer to the amino acid modified in the rabbit sequence of GenBank, X64297.

**Generation of Mice Lacking the C Terminus of Ca_{αβ_2}—**To construct the targeting vector, a 7.3-kb fragment containing exons 13–14 of CACNB2 was isolated from 129/Sv mouse genomic DNA. The targeting vector included a 1.6-kb short arm and 5.7-kb long arm with PKG-neo and the thymidine kinase gene (tk) flanked by two loxP sites. The 3’-side long arm contained exon 14 with the stop codon TGA in-frame after Pro^{501} and the phosphorylation sites Ser^{529}/530 (corresponding to Ser^{479}/480 rbs), Ser^{545} (corresponding to Ser^{496} rbs), Thr^{549} (corresponding to Thr^{496} rbs), and Ser^{625} (corresponding to Ser^{574} rbs) behind the stop codon (see supplemental Table 1). All mutation procedures were carried out by QuikChange II site-directed mutagenesis (Stratagene). The targeting construct was electroporated into R1 ES cells (129/Sv×129/Sv-CP F1). Positive clones were identified by PCR and confirmed by Southern blotting using a probe on the neo gene. One positive clone was detected and injected in C57BL/6 blastocysts. Chimeras were crossed to C57BL/6 mice. By crossing with a Cre-recombinase expressing transgenic B6.C-Tg (CMV-cre)1Cgn/J mouse strain, the neo tk marker genes were excised. Heterozygous mice were bred to produce homozygotes. The intercross of heterozygotes resulted in production of wild-type, heterozygous, and homozygous offspring at almost the expected Mendelian ratio (75:131:64). For all analyses, filial generation 2 (F2) mice with 129/Sv and C57BL/6 hybrid genetic background were used. All procedures relating to animal care and treatment were authorized by the “Regierung von Oberbayern” and conformed to the institutional, governmental, Directive 2010/63/EU of the European Parliament guidelines and to the Care and Use of Laboratory Animals published by the US National Institutes of Health. Anesthetized mice (1.5% isoflurane) were used. All procedures relating to animal care and treatment were authorized by the “Regierung von Oberbayern” and conformed to the institutional, governmental, Directive 2010/63/EU of the European Parliament guidelines and to the Care and Use of Laboratory Animals published by the US National Institutes of Health. Anesthetized mice (1.5% isoflurane) were euthanized by cervical dislocation.

**Antibodies—**The anti-Ca_{1,2} and anti-Ca_{αβ_2/Ca_{1,2}} antibodies have been described previously (28). The anti-Ca_{αβ_2}, N4/1195 antibody was a kind gift from Prof. Flockerzi (Universität des Saarlandes). The antibody against MAPK (p44/42) was obtained from Cell Signaling.

**Membrane Preparation and Immunoblotting—**Frozen heart and brain tissue were minced to a fine powder and homogenized in membrane preparation buffer (20 mM EDTA, 20 mM EGTA, 10 mM Tris, 300 mM NaCl, pH 7.4, inhibitors per ml buffer: 8 μg of calpain inhibitor I (Roche Applied Science), 8 μg of calpain inhibitor II (Roche Applied Science), 1 μl of phenylmethylsulfonyl fluoride (PMSF; Fluka), and 2 μl of protease inhibitor mixture (Sigma)). Cell organelles were separated by centrifugation, the supernatant containing the membrane proteins was centrifuged at 100,000 × g for 30 min, and the pellet was solubilized in deoxycholate buffer (20 mM EDTA, 10 mM EGTA, 10 mM Tris-HCl, pH 7.4, 1% deoxycholate) for 20 min. Membrane proteins were separated by centrifugation at 100,000 × g for 30 min. The supernatant was aliquotted and stored at −80 °C, and protein concentration was measured according to the BCA method (Pierce). 50 μg of protein were separated per lane on 10% SDS-polyacrylamide gels, blotted, and probed with antibodies by using a chemiluminescence detection system.

**Cell Isolation—**Ventricular myocytes were isolated as described (AfCS Procedure Protocol PP00000125), maintained at 37 °C, and aerated with 98% O_{2}, 2% CO_{2}. Embryonic ventricular myocytes were isolated as described in Ref. 29.

**Electrophysiological Recordings—**Whole cell IC_{Ca} was measured at room temperature from rod-shaped, striated, calcium-tolerant myocytes within 1–24 h after isolation. Stimulation and data acquisition were performed as described in Refs. 27, 30, 31. Facilitation of IC_{Ca} was measured during a triple pulse protocol with a 200-ms control pulse to 0 mV (V1) followed by a 200-ms prepulse (V_{prep}) +80 mV followed by 200-ms test pulse to 0 mV (V2) (27). The extent of voltage-dependent facilitation was calculated as the ratio of the peak current during V2 and V1. Time constants of IC_{Ca} inactivation were obtained by a fit from peak current to the current value at the end of the voltage pulse by a two-exponential function using pClamp 9. Facilitation of IC_{Ca} was measured as described in Ref. 30. The stimulatory effect of isoproterenol (100 nmol/liter containing an equal concentration of ascorbic acid) on IC_{Ca} was examined after establishing a solid base line. Stimulation of IC_{Ca} by isoproterenol was measured at a membrane potential of ±0 mV and is given as percentage of control (1 = 100%) determined before superfusion with isoproterenol. All fits showed a correlation coefficient >0.98.

**Telemetric Electrocardiogram (ECG) Recordings—**Radiotelemetric ECG transmitters (ETA-F20; DSI, St. Paul, MN) were implanted into the peritoneal cavity under general anesthesia with isoflurane/O_{2}. The ECG leads were sutured subcutaneously onto the upper right chest muscle and the upper left abdominal wall muscle. The animals were allowed to recover for 2 weeks before the experiments. Isoproterenol (0.1 mg/kg mouse; Sigma) or phenylephrine (3 mg/kg mouse; Sigma) was dissolved in 0.9% NaCl. After 15 min of base-line recording, the mice were injected intraperitoneally with the drugs. The ECGs were recorded for 45 min thereafter. The animals were allowed to recover for at least 48 h between experiments. Data were acquired using the DSI acquisition system.

**Echocardiography—**Images were obtained using a Vevo 770 Visual Sonics scanner equipped with a 30-MHz probe (Visual Sonics Inc., Toronto, ON, Canada). The mice were lightly anesthetized (1.5% isoflurane) and anchored to a warming platform in dorsal position, and ECG limb electrodes were placed. The chests were shaved and cleaned to minimize ultrasound attenuation. Fractional shortening (FS, the diameter at the end of systole minus the diameter at the end of diastole divided by the diameter at the end-diastole) was assessed from the M mode of the parasternal short axis view.
and mice carrying the various mutations were studied before and after administration of isoproterenol (0.1 mg/kg mouse intraperitoneally).

**Statistics**—Data are presented as mean ± S.E. Statistical significance was tested by using a (two-tailed) unpaired Student’s t test. The null hypothesis was rejected if \( p < 0.05 \).

**RESULTS**

We report the generation of a mouse line in which the \( \beta_2 \) subunit of the Cav1.2 channel complex (Cav \( \beta_2 \)) was truncated at Pro501 (\( \beta \)Stop mice). For this purpose, we used a gene-targeting strategy that utilized a replacement vector containing a stop codon after proline 501 in exon 14 and a \( \text{neo tk} \) gene cassette flanked by loxP sites (Fig. 1A). The Cav \( \beta_2 \) C terminus was truncated to prevent the expression of several putative phosphorylation sites (PKA Ser479/480 rbs, PKG Ser496 rbs, CaMKII Thr498 rbs, and Akt/PKB Ser574 rbs; see supplemental Table 1) and to test the physiological relevance of these sites (Fig. 1B). All homozygous \( \beta \)Stop mutants analyzed were chimeric F2 mice (mixed sv129 and C57BL/6 background). \( \beta \)Stop mice were compared with litter-matched control mice (Ctr \( \beta \)Stop). Nomenclature and genotype of mouse lines are outlined in supplemental Table 2. The correct genomic localization (supplemental Fig. 1) and mutation (Fig. 1C) in \( \beta \)Stop mice was confirmed by Southern blotting and genomic sequencing. \( \beta \)Stop mice were viable, fertile, and reproduced in a 1:2:1 Mendelian ratio (WT 27.7%, heterozygous Ctr \( \beta \)Stop 48.5%, \( \beta \)Stop 23.7%) (Fig. 1D). Western blot analysis of heart and brain membrane fraction showed no alterations in Cav1.2 expression. The expression of the C-terminal truncated Cav \( \beta_2 \) protein was con-

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**FIGURE 1.** Generation and biochemical analysis of \( \beta \)Stop mice. A, gene targeting strategy of the \( \beta \)Stop mouse. Top, genomic DNA structure of CACNB2 with the relevant restriction enzyme sites; boxes represent exons 13 and 14 encoding the C terminus of Cav \( \beta_2 \). Middle, targeting vector. Neo, neomycin-resistance gene; TK, thymidine kinase gene with loxP sequence (triangles) at both sides. The insertion of the stop codon after proline 501 is shown. Bottom, knock-in locus after homologous recombination and Cre-mediated deletion of resistance markers. N, NotI; B, BamHI; C, ClaI; X, XhoI; P, PstI; E, EcoRI; A, Acc65I. B, location of PKA, PKG, CaMKII, and Akt/PKB phosphorylation sites in exon 14 of the murine Cav \( \beta_2 \) protein. The amino acid sequence is according to *M. musculus* CACNB2 sequence (GenBank accession number Q8CC27), and the nucleotide sequence is according to *M. musculus* CACNB2 sequence (GenBank accession number NM_023116.4). The phosphorylation sites (right) are those of the mouse amino acid sequence. C, genotyping of a Cav \( \beta_2 \)Stop litter (635-bp \( \beta \)Stop band, 530-bp WT band) and sequence analysis of the \( \beta \)Stop knock-in mice. The sequence shows exon 14 of a homozygous \( \beta \)Stop offspring. The stop mutation is in-frame within exon 14. PCR primers bind 5’ and 3’ of the loxP site remaining after Cre recombination. The primers amplify a 635-bp fragment in Cav \( \beta \)Stop DNA (including one loxP site after Cre recombination) or a 530-bp fragment in WT DNA (without loxP site). D, Mendelian ratio at birth of the Cav \( \beta \)Stop/WT strain. E and F, Western blot analysis of heart and brain membrane fractions on 10% SDS PAGE; 50 \( \mu \)g of protein was applied per lane. E, Cav1.2 and Cav \( \beta_2 \) protein expression. The truncated Cav \( \beta_2 \) protein is not detected by the common Cav \( \beta_2 \)v2 antibody which binds C-terminal of the Stop mutation; loading control, MAPK. F, detection of the Cav \( \beta_2 \)Stop protein with the N-terminal binding Cav \( \beta_2 \)v2-N4/1195 antibody.
firmed by the C-terminal binding Ca$_{\beta_2}$ antibody and the N-terminal binding antibody Ca$_{\beta_2}$-N4/1195. The C-terminal binding Ca$_{\beta_2}$ antibody detected the 75-kDa WT Ca$_{\beta_2}$ protein, but not the truncated Ca$_{\beta_2}$ protein (Fig. 1F), whereas the N-terminal binding Ca$_{\beta_2}$-N4/1195 antibody detected both the truncated Ca$_{\beta_2}$ protein (58 kDa) and the WT protein (75 kDa) (Fig. 1F). These results show that the $\beta$Stop mouse expressed the truncated Ca$_{\beta_2}$ protein that missed the reported phosphorylation sites.

Telemetric ECG measurement of heart rate (HR) and activity revealed no differences in WT and $\beta$Stop mice (supplemental Fig. 2). Both genotypes showed a typical cardiac response to isoproterenol and phenylephrine administration with an increase and a drop in HR, respectively (Fig. 2A). FS was identical in Ctr $\beta$Stop and $\beta$Stop mice (Fig. 2B). Isoproterenol doubled FS in both genotypes. These data indicate that the putative PKA phosphorylation sites Ser$^{797/840}$ rbs of the Ca$_{\beta_2}$ subunit are not necessary to observe the positive inotropic, $\beta$-adrenergic regulation of the heart muscle.

To further support the insignificant effect of the Ca$_{\beta_2}$ truncation for cardiac $\beta$-adrenergic regulation, patch clamp experiments were carried out on isolated cardiomyocytes (CMs). Isolated CMs of either genotype had normal size (WT, 161.2 ± 22 pF, n = 6; Ctr $\beta$Stop, 170 ± 14 pF, n = 13; $\beta$Stop, 149 ± 8 pF, n = 13), normal IC$_{Ca}$ density at +10 mV (WT, 2.99 ± 0.25 pA/pF, n = 6; Ctr $\beta$Stop, 3.39 ± 0.2 pA/pF, n = 13; $\beta$Stop, 3.01 ± 0.2 pA/pF, n = 13), half-maximal activation constants (WT, −21.7 ± 1.6 mV, n = 4; Ctr $\beta$Stop −16.3 ± 1.7 mV, n = 8; $\beta$Stop −14.1 ± 0.9 mV, n = 9) and a normal I-V relation (Fig. 2C). As expected from the results of Meissner et al. (26), further analysis showed normal half-maximal steady state inactivation (Fig. 2D) (V$_{0.5}$: Ctr $\beta$Stop, −18.2 ± 1.6 mV (n = 3); $\beta$Stop, −18.7 ± 1.2 mV (n = 5)), and normal half-maximal recovery time from inactivation (Fig. 2E) (τ$_{1/2}$ (ms): Ctr $\beta$Stop, 135.3 (n = 6); $\beta$Stop, 147.6 (n = 6)).

It is widely accepted that calcium-dependent facilitation (CDF) is caused by activation of CaMKII followed by phosphorylation of a component of the Ca$_{1.2}$ channel complex. Recently, we reported that CDF requires phosphorylation of Ca$_{1.2}$ at Ser$^{1512/1570}$ (27). In contrast, Colbran and co-workers (22) reported that phosphorylation of Ca$_{\beta_2}$ by CaMKII at Thr$^{500}$ modulated CDF. Because Thr$^{500}$ was not present anymore in the $\beta$Stop protein, we tested whether or not the truncation of the Ca$_{\beta_2}$ C terminus might affect IC$_{Ca}$ facilitation. We compared prephase facilitated IC$_{Ca}$ in CMs of both genotypes. CDF was not affected by the truncation of the Ca$_{\beta_2}$ protein (Fig. 2F), suggesting that phosphorylation of the Ca$_{\beta_2}$ subunit is not a necessary prerequisite to induce CDF under basal conditions.

In agreement with the ECG results, isoproterenol stimulated IC$_{Ca}$ of Ctr $\beta$Stop and $\beta$Stop CMs to the same level (Fig. 2G). Representative current traces for a $\beta$Stop CM are shown in Fig. 2H. Isoproterenol treatment increased IC$_{Ca}$ in Ctr $\beta$Stop CMs by 193 ± 25% and in $\beta$Stop CMs by 180 ± 24% (Fig. 2G). Furthermore, there was no change in the slow or fast component of inactivation either with or without isoproterenol stimulation (supplemental Table 3). The fast component of inactivation describes Ca$^{2+}$-dependent inactivation (CDI), the slow component the voltage-dependent inactivation. Neither inactivation pathway is affected by the C-terminal truncation of the Ca$_{\beta_2}$ protein.

These negative experiments raised the possibility that the positive inotropic effect was mediated by phosphorylation of both the Ca$_{1.2}$ and Ca$_{\beta_2}$ subunit. We tested this hypothesis by cross-breeding the $\beta$Stop line with the Ca$_{1.2}$S$^{2A}$ or the Ca$_{1.2}$S$^{2F}$ lines. The Ca$_{1.2}$S$^{2A}$ mouse line expresses a Ca$_{1.2}$ channel containing the mutation S1928A (25). Mice homozygous for the double mutation Ca$_{1.2}$S$^{2A}$/Ca$_{1.2}$S$^{2A}$, Ca$_{\beta_2}$P$_{501}$ stop/Ca$_{\beta_2}$P$_{501}$ stop (SAP$\beta$Stop) had the same size and weight as the heterozygous litters. Diurnal cardiac rhythm was not altered in these mice (supplemental Fig. 3). The cell capacitance of Ctr SAP$\beta$Stop and double knock-in SAP$\beta$Stop CMs was the same (Ctr SAP$\beta$Stop: 168.6 ± 12 pF (n = 15); SAP$\beta$Stop: 163.0 ± 18 pF (n = 6)). Inactivation time constants of IC$_{Ca}$ were not affected by this double mutation (supplemental Table 3). We did not observe an effect of the double mutation on CDF (Fig. 3A). Isoproterenol stimulated FS (Fig. 3B) and HR (supplemental Fig. 4) in both mouse lines to the same extent. No statistically significant difference was noted between the curves. Phenylephrine decreased the HR to the same extent in both genotypes (supplemental Fig. 4). Stimulation of the corresponding CMs by 100 nM isoproterenol increased IC$_{Ca}$ by 194.3 ± 19.2% (n = 6) and 205.3 ± 14% (n = 6) in heterozygous Ctr SAP$\beta$Stop and homozygous SAP$\beta$Stop, respectively (Fig. 3, C and D).

In the next series of experiments we tested the double mutation Ca$_{1.2}$S$^{1512/1570A}$/Ca$_{1.2}$S$^{1512/1570A}$, Ca$_{\beta_2}$P$_{501}$ stop/Ca$_{\beta_2}$P$_{501}$ stop (S$\beta$Stop). Mice homozygous for the double mutation SF$\beta$Stop had the same size and weight as the heterozygous litters. Diurnal cardiac rhythm was not altered in these mice (supplemental Fig. 3). The cell capacitance of Ctr SF$\beta$Stop and double knock-in SF$\beta$Stop CMs was the same (Ctr SF$\beta$Stop: 213 ± 13 pF (n = 9); SF$\beta$Stop: 195 ± 17 pF (n = 8)). Inactivation time constants of IC$_{Ca}$ were not affected by this double mutation (supplemental Table 3). As shown for the SF mice (27), CDF was also significantly decreased in the SF$\beta$Stop mice (Fig. 4A).

Isoproterenol stimulated FS (Fig. 4B) and HR (supplemental Fig. 5) in both mouse lines to the same extent. Phenylephrine decreased the HR to the same extent in both genotypes (supplemental Fig. 5). Stimulation of these CMs by 100 nM isoproterenol increased IC$_{Ca}$ by 187 ± 17% (n = 10) and 196 ± 26% (n = 8) in the heterozygous Ctr SF$\beta$Stop and homozygous SF$\beta$Stop line, respectively (Fig. 4, C and D). We concluded

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$^{3}$These results indicated to us that there is no gene dose effect through the deletion of the C terminus of one $\beta_2$ gene. Almost identical results have been reported by Meissner et al. (26), which reported the inactivation of both $\beta_2$ alleles. Comparison of WT and heterozygous animals should allow the detection of a gene dose effect more easily. However, if no different phenotype has been found between the WT and heterozygous animals, it is generally requested that the heterozygous litter-matched animals are the correct controls to the knockout mice because they carry one WT chromosome and one chromosome carrying the mutated gene. Based on these and generally accepted $\beta$Stop considerations, we tested against the heterozygous, litter-matched CTR animals. Furthermore, heart-specific inactivation of the $\beta_2$ subunit gene in adult mice yielded minimal or no effect on IC$_{Ca}$ kinetics (see Ref. 26, Figs. 4 and 5).
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from this analysis that neither double mutation affected the β-adrenergic stimulation of FS in the intact mouse nor that of $I_{Ca}$ in the CMs.

The results presented so far suggested that none of the mutated potential PKA or CaMKII phosphorylation sites was necessary for β-adrenergic stimulation of the cardiac $I_{Ca}$. PKA

FIGURE 2. βStop mutation does not prevent positive inotropic heart regulation. A, normal regulation of beating frequency by isoproterenol (0.1 mg/kg of body weight intraperitoneally) and phenylephrine (0.3 mg/kg of body weight intraperitoneally). B, FS unchanged in βStop mice before and after isoproterenol administration (0.1 mg/kg of body weight intraperitoneally). C, $I_{Ca}$ trace ± isoproterenol (0.1 μM) in a βStop CM. Number of animal/cells is given in columns or within figures. The voltage protocol is depicted above the corresponding figure.

FIGURE 3. Unchanged β-adrenergic regulation of $I_{Ca}$ in SAβStop mice. A, unchanged CDF in Ctr SAβStop and SAβStop CMs. B, FS unchanged in βStop mice before and after isoproterenol administration (0.1 mg/kg of body weight intraperitoneally). C, statistics of isoproterenol (0.1 μM) stimulation of $I_{Ca}$ in Ctr SAβStop and SAβStop CMs. D, $I_{Ca}$ trace ± isoproterenol (0.1 μM) in a SAβStop CM. Number of animal/cells is given in columns or within figures.

FIGURE 4. Unchanged β-adrenergic regulation of $I_{Ca}$ in SFβStop mice. A, CDF in SFβStop CMs decreased compared with Ctr SFβStop. B, FS unchanged in βStop mice before and after isoproterenol administration (0.1 mg/kg of body weight intraperitoneally). C, statistics of isoproterenol (0.1 μM) stimulation of $I_{Ca}$ in Ctr SFβStop and SFβStop CMs. D, $I_{Ca}$ trace ± isoproterenol (0.1 μM) in a SFβStop CM. Number of animal/cells is given in columns or within figures.
needs to bind to the L-type channel complex through a PKA-anchoring protein (AKAP) before it can phosphorylate the necessary subunit. The CMs contain several AKAPs that may be an essential part of the β-adrenergic regulation (32). Disruption of AKAP5 interfered with β-adrenergic-stimulated intracellular Ca\(^{2+}\) transients but not with \(I_{\text{Ca}}\) (33). AKAPs bind to the C terminus of Cav1.2 between amino acids 2026 and 2085. This sequence was not modified in the mouse lines studied, suggesting that PKA was still targeted to the β-adrenergic-regulated site. Truncation of the Cav1.2 sequence at Asp1904 (31) or at Gly1796 (34) leads to a channel that does not bind any more AKAPs. In contrast to a previous in vitro study (20) but in agreement with Fu et al. (34), \(I_{\text{Ca}}\) of embryonic Cav1.2Stop CMs is not stimulated anymore by isoproterenol (Fig. 5, A and C) or forskolin (Fig. 5, B and D), suggesting that the amino acids C-terminal to Asp1904 are essential for the adrenergic up-regulation of \(I_{\text{Ca}}\) in the heart.

**DISCUSSION**

Adrenergic up-regulation of the cardiac \(I_{\text{Ca}}\) is an extensively studied physiological phenomenon that was recognized in the seventies (35) to be regulated by cAMP. Since then evidence has been published that β-adrenergic stimulation requires a PKA-mediated phosphorylation step (3, 4, 7, 20, 36–39). However, the molecular mechanism of β-adrenergic regulation of Cav1.2 channel remains unsolved. Previously, it was found that the mutation S1928A of the Cav1.2 protein did not abolish β-adrenergic regulation of the heart and \(I_{\text{Ca}}\) (25), supporting the notion that phosphorylation of S1928 by PKA was not an obligatory step to allow β-adrenergic regulation of the murine heart.

The Cav\(_{1.2}\)Stop subunit has been promoted as an alternative sub-strate for PKA (20, 40, 41). Initially, it was suggested that PKA-dependent up-regulation of the expressed \(I_{\text{Ca}}\) requires truncation of the Cav1.2 protein at amino acid 1905 and the co-expression of the Cav\(_{1.2}\)β\(_2\) subunit (20). Truncation of the murine Cav1.2 channel at Asp\(_{1904}\) resulted in death around birth (31) and the inability of isoproterenol to stimulate the truncated channel (Fig. 5). Similar results have been reported, when the Cav1.2 protein was truncated at Gly\(_{1796}\) (34). These negative results are most likely caused by the deletion of the AKAP binding sequence (32). AKAPs are components that target various proteins of the β-adrenergic signaling cascade to Cav1.2 (38). These results clearly demonstrate that truncation of the Cav1.2 protein in vivo is not required for the adrenergic regulation.

Truncation of the Cav\(_{1.2}\)β\(_2\) subunit at P501 by site-directed mutagenesis removed the “classical” PKA phosphorylation sites and that for PKG, CaMKII, and PKB. Removing these reported phosphorylation sites had no effect on the basic properties of the murine cardiac \(I_{\text{Ca}}\). The Cav\(_{1.2}\)β\(_2\)Stop mice showed normal β-adrenergic regulation, CDI, CDF, and basic behavior. From these results we conclude that these reported phosphorylation sites are not necessary for the basic regulation of the channel by PKA, CaMKII, PKG, and PKB.

The reported results do not rule out the possibility that PKA modified an additional site on the truncated Cav\(_{1.2}\)β\(_2\) subunit that was necessary for β-adrenergic regulation of the channel (42). This consideration appears unlikely in view of the report that deletion of the Cav\(_{1.2}\)β\(_2\) subunit in the adult heart does not result in an severe phenotype (26). The negative results reported here are only relevant for the relative classical tests carried out in this study. It could be that removal of these phosphorylation sites may alter more subtle cardiac functions that have not been

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**FIGURE 5.** \(I_{\text{Ca}}\) is not regulated by β-adrenergic stimulation in Cav\(_{1.2}\)Stop CMs. A and B, \(I_{\text{Ca}}\) traces of WT (+/+) and Cav\(_{1.2}\)Stop (−/−) embryonic CMs ± 0.1 \(\mu\)M isoproterenol. The CMs were obtained from day 18.5 embryos as described (31). C and D, statistics of isoproterenol (C) and forskolin (D)-dependent increase in peak current of WT (open columns), heterozygous (gray columns), and Cav\(_{1.2}\)Stop (black columns) CMs. Number of cells is given in columns. *, \(p < 0.05\); **, \(p < 0.01\).
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1. Catterall, W. A., Hulme, J. T., Jiang, X., and Few, W. P. (2006) Regulation of sodium and calcium channels by signaling complexes. J. Recept. Signal Transduct. Res. 26, 577–598
2. Dai, S., Hall, D. D., and Hell, J. W. (2009) Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol. Rev. 89, 411–452
3. Osterrieder, W., Brum, G., Hescheler, J., Trautwein, W., Flockerzi, V., and Hofmann, F. (1982) Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates Ca^{2+} current. Nature 298, 576–578
4. Hartzell, H. C., Méry, P. F., Fischmeister, R., and Szabo, G. (1991) Sympathetic regulation of cardiac calcium current is due exclusively to cAMP-dependent phosphorylation. Nature 351, 573–576
5. Bers, D. M. (2002) Cardiac excitation-contraction coupling. Nature 415, 198–205
6. Kamp, T. J., and Hell, J. W. (2000) Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circ. Res. 87, 1095–1102
7. Hulme, J. T., Westenbroek, R. E., Scheuer, T., and Catterall, W. A. (2006) Phosphorylation of serine 1928 in the distal C-terminal domain of cardiac Ca_{1.2} channels during β-adrenergic regulation. Proc. Natl. Acad. Sci. U.S.A. 103, 16574–16579
8. Fuller, M. D., Emrick, M. A., Sadilek, M., Scheuer, T., and Catterall, W. A. (2010) Molecular mechanism of calcium channel regulation in the flight-or-flight response. Sci. Signal. 3, ra70
9. Lee, T. S., Karl, R., Moosmang, S., Lenhardt, P., Klugbauer, N., Hofmann, F., Kleppisch, T., and Welling, A. (2006) Calmodulin kinase II is involved in voltage-dependent facilitation of the L-type Ca_{1.2} calcium channel: identification of the phosphorylation sites. J. Biol. Chem. 281, 25560–25567
10. Hudmon, A., Schulman, H., Kim, J., Maltez, J. M., Tsien, R. W., and Pitt, G. S. (2005) CalM1KII tethers to L-type Ca^{2+} channels, establishing a local and dedicated integrator of Ca^{2+} signals for facilitation. J. Cell Biol. 171, 537–547
11. Dzhura, I., Wu, Y., Colbran, R. J., Balser, J. R., and Anderson, M. E. (2000) Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels. Nat. Cell Biol. 2, 173–177
12. Yang, L., Liu, G., Zakharov, S. I., Bellingher, A. M., Mongillo, M., and Marx, S. O. (2007) Protein kinase G phospho-tylates Ca_{1.2} α_{1c} and β_{2} subunits. Circ. Res. 101, 465–474
13. Jiang, L. H., Gawler, D. J., Hosdon, N., Milligan, C. J., Pearson, H. A., Porter, V., and Wray, D. (2000) Regulation of cloned cardiac L-type calcium channels by cGMP-dependent protein kinase. J. Biol. Chem. 275, 6135–6145
14. Sun, H., Kerfant, B. G., Zhao, D., Trivieri, M. G., Oudit, G. Y., Penninger, J. M., and Backx, P. H. (2006) Insulin-like growth factor-1 and PTEN deletion enhance cardiac L-type Ca^{2+} currents via increased PI3Kα signaling. Circ. Res. 98, 1390–1397
15. Lawlor, M. A., and Alessi, D. R. (2001) PKB/Akt: a key mediator of cell proliferation, survival, and insulin responses? J. Cell Sci. 114, 2903–2910
16. Singer-Lahat, D., Gershon, E., Lotan, I., Hullin, R., Biel, M., Flockerzi, V., Hofmann, F., and Dascal, N. (1992) Modulation of cardiac Ca^{2+} channels in Xenopus oocytes by protein kinase C. FASEB J. 6, 113–118
17. Shistik, E., Ivanina, T., Blumenstein, Y., and Dascal, N. (1998) Crucial role of N terminus in function of cardiac L-type Ca^{2+} channels and its modulation by protein kinase C. J. Biol. Chem. 273, 17901–17909
18. Yang, L., Doshi, D., Morrow, J., Katchman, A., Chen, X., and Marx, S. O. (2009) Protein kinase C isoforms differentially phosphorylate Ca_{1.2} α_{1c} subunit. Biochemistry 48, 6674–6683
19. McHugh, D., Sharp, E. M., Scheuer, T., and Catterall, W. A. (2000) Inhibition of cardiac L-type calcium channels by protein kinase C phosphorylation of two sites in the N-terminal domain. Proc. Natl. Acad. Sci. U.S.A. 97, 12334–12338
20. Bünemann, M., Gerhardstein, B. L., Gao, T., and Hosey, M. M. (1999) Functional regulation of L-type calcium channels via protein kinase A-mediated phosphorylation of the β_{2} subunit. J. Biol. Chem. 274, 33851–33856
21. Hullin, R., Singer-Lahat, D., Freichel, M., Biel, M., Dascal, N., Hofmann, F., and Flockerzi, V. (1992) Calcium channel β subunit heterogeneity: functional expression of cloned cDNA from heart, aorta, and brain. EMBO J. 11, 885–890
22. 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^{2+} channel facilitation mediated by phosphorylation of the β subunit by CalM1KII. Mol. Cell 23, 641–650
23. Catalucci, D., Zhang, D. H., DeSantiago, J., Aimon, F., Barbara, G., Chemin, J., Bonci, D., Picht, E., Rusconi, F., Dalton, N. D., Peterson, K. L.

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REFERENCES
Modulation of Ca,1.2 by Phosphorylation

Richard, S., Bers, D. M., Brown, J. H., and Condorelli, G. (2009) Akt regulates L-type Ca\(^{2+}\) channel activity by modulating Ca,\(\alpha\), protein stability. J. Cell Biol. 184, 923–933

24. Viard, P., Butcher, A. J., Hallet, G., Davies, A., Nürnberg, B., Heblich, F., and Dolphin, A. C. (2004) PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane. Nat. Neurosci. 7, 939–946

25. Lemke, T., Welling, A., Christel, C. J., Blaich, A., Bernhard, D., Lenhardt, P., Hofmann, F., and Moosmang, S. (2008) Unchanged \(\beta\)-adrenergic stimulation of cardiac L-type calcium channels in Ca,1.2 phosphorylation site S1928A mutant mice. J. Biol. Chem. 283, 34738–34744

26. Meissner, M., Weissgerber, P., Londoño, J. E., Prener, J., Link, S., Ruppenthal, S., Molkentin, J. D., Lipp, P., Nilius, B., Freichel, M., and Flockerzi, V. (2011) Moderate calcium channel dysfunction in adult mice with inducible cardiomyocyte-specific excision of the Cacnb2 gene. J. Biol. Chem. 286, 15875–15882

27. Blaich, A., Welling, A., Fischer, S., Wegener, J. W., Köstner, K., Hofmann, F., and Moosmang, S. (2010) Facilitation of murine cardiac L-type Ca,1.2 channel is modulated by calmodulin kinase II-dependent phosphorylation of Ser-1512 and Ser-1570. Proc. Natl. Acad. Sci. U.S.A. 107, 10285–10289

28. Ludwig, A., Flockerzi, V., and Hofmann, F. (1997) Regional expression and functional embryonic cardiomyocytes after disruption of the L-type Ca,1.2 channel gene. J. Biol. Chem. 272, 39193–39199

29. Poomvanicha, M., Wegener, J. W., Blaich, A., Fischer, S., Domes, K., Moosmang, S., and Hofmann, F. (2011) Facilitation and Ca\(^{2+}\)-dependent inactivation are modified by mutation of the Ca,1.2 channel IQ motif. J. Biol. Chem. 286, 26702–26707

30. Domes, K., Ding, J., Lemke, T., Blaich, A., Wegener, J. W., Brandmayr, J., Moosmang, S., and Hofmann, F. (2011) Truncation of murine Ca,1.2 at Asp-1904 results in heart failure after birth. J. Biol. Chem. 286, 33863–33871

31. Motz, E., Allaben, H., Langer, P., and Lederer, W. J. (2008) Facilitation and Ca\(^{2+}\)-dependent inactivation are modified by mutation of the Ca,1.2 channel IQ motif. J. Biol. Chem. 286, 26702–26707

32. McConnachie, G., Langeberg, L. K., and Scott, J. D. (2006) AKAP signaling complexes: getting to the heart of the matter. Trends Mol. Med. 12, 317–323

33. Nichols, C. B., Rossov, C. F., Navedo, M. F., Westenbroek, R. E., Catterall, W. A., Santana, L. F., and McKnight, G. S. (2010) Sympathetic stimulation of adult cardiomyocytes requires association of AKAP5 with a subpopulation of L-type calcium channels. Circ. Res. 107, 747–756

34. Fu, Y., Westenbroek, R. E., Yu, F. H., Clark, J. P., Marshall, M. R., Scheuer, T., and Catterall, W. A. (2011) Deletion of the distal C terminus of Ca,1.2 channels leads to loss of \(\beta\)-adrenergic regulation and heart failure in vivo. J. Biol. Chem. 286, 12617–12626

35. Reuter, H. (1974) Localization of \(\beta\)-adrenergic receptors and effects of noradrenaline and cyclic nucleotides on action potentials, ionic currents, and tension in mammalian cardiac muscle. J. Physiol. 242, 429–451

36. De Jongh, K. S., Murphy, B. J., Colvin, A. A., Hell, J. W., Takahashi, M., and Catterall, W. A. (1996) Specific phosphorylation of a site in the full-length Ca,1C but not serine 1928. J. Biol. Chem. 271, 939–946

37. Sculptoreanu, A., Rotman, E., Takahashi, M., Scheuer, T., and Catterall, W. A. (1993) Voltage-dependent potentiation of the activity of cardiac L-type calcium channel \(\alpha\) subunits due to phosphorylation by cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. U.S.A. 90, 10135–10139

38. Schroeder, T., Yatani, A., Dell’Acqua, M. L., Sako, H., Green, S. A., Dascal, N., Scott, J. D., and Hosey, M. M. (1997) cAMP-dependent regulation of cardiac L-type \(\text{Ca}^{2+}\) channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron 19, 185–196

39. Ganesan, A. N., Maack, C., Johns, D. C., Sidor, A., and O’Rourke, B. (2006) \(\beta\)-Adrenergic stimulation of L-type \(\text{Ca}^{2+}\) channels in cardiac myocytes requires the distal carboxyl terminus of \(\alpha_{1C}\) but not serine 1928. Circ. Res. 98, e11–e18

40. Perez-Reyes, E., Castellano, A., Kim, H. S., Bertrand, P., Baggstrom, E., Lacerda, A. E., Wei, X. Y., and Birnbaumer, L. (1992) Cloning and expression of a cardiac/brain \(\beta\) subunit of the L-type calcium channel. J. Biol. Chem. 267, 1792–1797

41. Burau, Z., and Yang, J. (2010) The \(\beta\) subunit of voltage-gated \(\text{Ca}^{2+}\) channels. Physiol. Rev. 90, 1461–1506

42. Gerhardstein, B. L., Puri, T. S., Chien, A. J., and Hosey, M. M. (1999) Identification of the sites phosphorylated by cyclic AMP-dependent protein kinase on the \(\beta\) subunit of L-type voltage-dependent calcium channels. Biochemistry 38, 10361–10370