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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 $Ca_v1.2$ Channel*[S]

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Julia Brandmayr, Montatip Poomvanicha, Katrin Domes, Jie Ding, Anne Blaich, Jörg W. Wegener, Sven Moosmang, and Franz Hofmann[1]

From the Forschergruppe 923, Institut für Pharmakologie und Toxikologie, Technische Universität München, 80802 München, Germany

Background: β-Adrenergic receptors stimulate cardiac I_{Ca} via PKA-dependent phosphorylation.
Results: Deletion of the C-terminal phosphorylation sites in the β_2 gene did not affect isoproterenol-stimulated I_{Ca}.
Conclusion: Phosphorylation of the C terminus of the β_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 β_2 subunit.

Phosphorylation of the cardiac β subunit ($Ca_v\beta_2$) of the $Ca_v1.2$ L-type Ca^{2+} channel complex has been proposed as a mechanism for regulation of L-type Ca^{2+} 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 $Ca_v\beta_2$ gene by insertion of a stop codon after proline 501 in exon 14 (mouse sequence *Cacnb2*; βStop mouse). This mutation prevented translation of the $Ca_v\beta_2$ C terminus that contains the relevant phosphorylation sites for the above protein kinases. Homozygous cardiac 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 $Ca_v1.2$ gene containing the mutation S1928A (SAβStop) or S1512A and S1570A (SFβStop) in the C terminus of the α_{1C} subunit. The β-adrenergic regulation of the cardiac I_{Ca} was unaltered in these mouse lines. In contrast, truncation of the $Ca_v1.2$ at Asp^{1904} abolished β-adrenergic up-regulation of I_{Ca} in murine embryonic CMs. We conclude that phosphorylation of the C-terminal sites in $Ca_v\beta_2$, Ser^{1928}, Ser^{1512}, and Ser^{1570} of the $Ca_v1.2$ protein is functionally not involved in the adrenergic regulation of the murine cardiac $Ca_v1.2$ channel.

The cardiac L-type Ca^{2+} current (I_{Ca}) is regulated by a number of protein kinases including PKA, CaMKII,[2] PKC, Akt/PKB, and PKG (1, 2). Regulation of the cardiac $Ca_v1.2$ 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 α_1 subunit and the β_2 subunit of the L-type calcium channel. Phosphorylation sites on the α_1 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 $Ca_v\beta_2$ subunit were reported for PKA (20) at $Ser^{479/480}$ (rabbit protein sequence (rbs)) (21), CaMKII (22) at Thr^{500} (rbs), PKG (12) at Ser^{496} (rbs), and Akt/PKB (14, 15, 23, 24) at Ser^{576} (rbs). The amino acids modified by protein kinases in $Ca_v\beta_2$ or $Ca_v1.2$ 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 Ser^{1928} of the α_1 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 $Ca_v\beta_2$ 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 Pro^{501} (βStop). This stop codon resulted in a truncated β_2 subunit protein that lacked the potential phosphorylation sites for PKA, PKG, Akt/PKB, and CaMKII. Basal properties of the $Ca_v1.2$ 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 $Ca_v1.2$ current were unaffected, suggesting that β-adrenergic regulation of the

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[S] This article contains supplemental Tables 1–3, Figs. 1–5, and additional references.
[1] To whom correspondence should be addressed: FOR923, Institut für Pharmakologie und Toxikologie, TU München, Biedersteiner Str. 29, 80802 München, Germany. Tel.: 49-89-4140-3240; Fax: 49-89-4140-3250; E-mail: franz.hofmann@mytum.de.
[2] The abbreviations used are: CaMKII, calmodulin kinase II; AKAP, PKA-anchoring protein; CDF, calcium-dependent facilitation; CDI, calcium-dependent inactivation; CM, cardiac myocyte; Ctr, control; FS, fractional shortening; HR, heart rate; rbs, rabbit protein sequence.

$Ca_v1.2$ 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 *Mus 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 $Ca_v\beta_2$ or $Ca_v1.2$ 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 $Ca_v\beta_2$—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 *PGK-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^{498} 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 euthanized by cervical dislocation.

Antibodies—The anti-$Ca_v1.2$ and anti-$Ca_v\beta_{2v2}$ antibodies have been described previously (28). The anti-$Ca_v\beta_2$-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 pestled 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 inhibtor 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 aliquoted 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 I_{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 I_{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_{pre}) to +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 I_{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 I_{Ca} was measured as described in Ref. 30. The stimulatory effect of isoproterenol (100 nmol/liter containing an equal concentration of ascorbic acid) on I_{Ca} was examined after establishing a solid base line. Stimulation of I_{Ca} by isoproterenol was measured at a membrane potential of ±0 mV and is given as percentage of control (= 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. Control

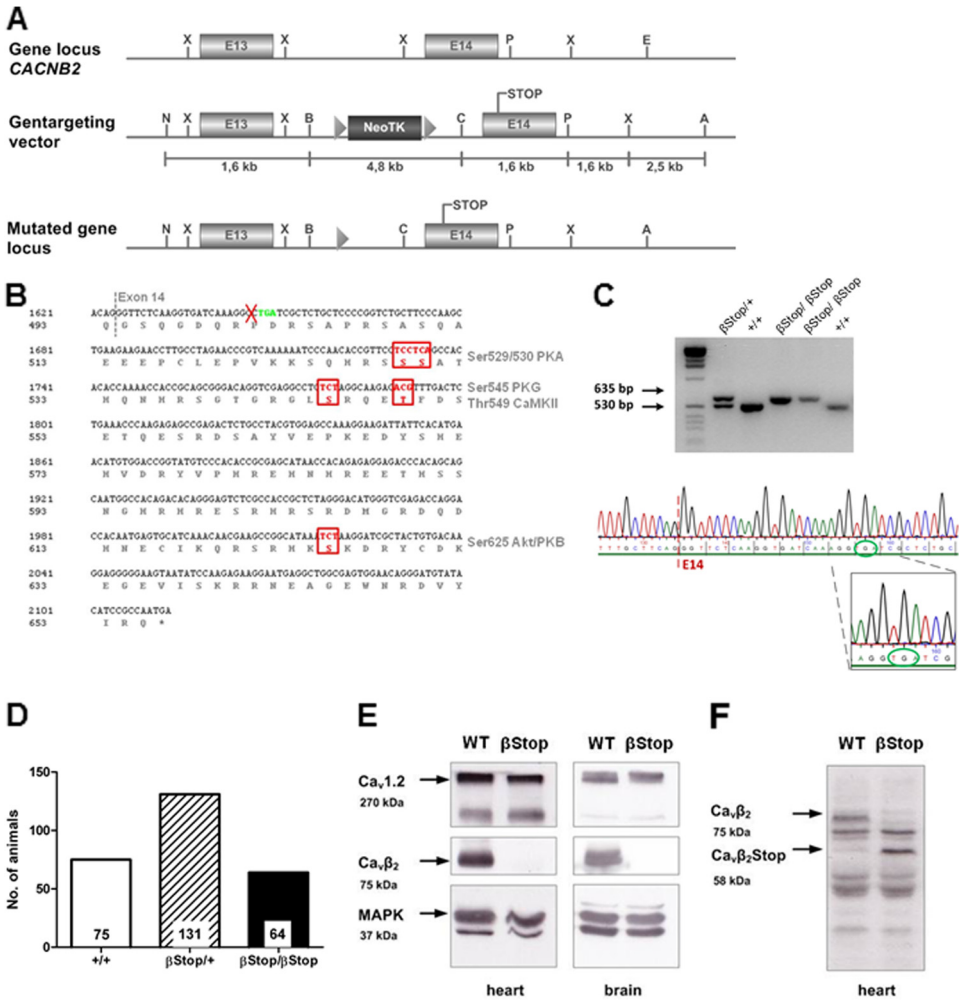


FIGURE 1. **Generation and biochemical analysis of βStop mice.** *A*, gene targeting strategy of the βStop mouse. *Top*, genomic DNA structure of *CACNB2* with the relevant restriction enzyme sites; *boxes* represent exons 13 and 14 encoding the C terminus of $Ca_v\beta_2$. *Middle*, targeting vector. *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 $Ca_v\beta_2$ 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 $Ca_v\beta_2$Stop litter (635-bp βStop band, 530-bp WT band) and sequence analysis of the βStop knock-in mice. The sequence shows exon 14 of a homozygous βStop 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 Ca_vβStop 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 $Ca_v\beta_2$Stop/WT strain. *E* and *F*, Western blot analysis of heart and brain membrane fractions on 10% SDS PAGE; 50 μg of protein was applied per lane. *E*, $Ca_v1.2$ and $Ca_v\beta_2$ protein expression. The truncated $Ca_v\beta_2$ protein is not detected by the common $Ca_v\beta_{2v2}$ antibody which binds C-terminal of the Stop mutation; loading control, MAPK. *F*, detection of the $Ca_v\beta_2$Stop protein with the N-terminal binding $Ca_v\beta_{2v2}$-N4/1195 antibody.

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 β_2 subunit of the $Ca_v1.2$ channel complex ($Ca_v\beta_2$) was truncated at Pro^{501} (βStop 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 *neo tk* gene cassette flanked by loxP sites (Fig. 1*A*). The $Ca_v\beta_2$ C terminus was truncated to prevent the expression of several putative phosphorylation sites (PKA $Ser^{479/480}$ rbs, PKG Ser^{496} rbs, CaMKII Thr^{498} rbs, and Akt/PKB Ser^{574} rbs; see supplemental Table 1) and to test the physiological relevance of these sites (Fig. 1*B*). All homozygous βStop mutants analyzed were chimeric F2 mice (mixed sv129 and C57BL/6 background). βStop mice were compared with litter-matched control mice (Ctr βStop). Nomenclature and genotype of mouse lines are outlined in supplemental Table 2. The correct genomic localization (supplemental Fig. 1) and mutation (Fig. 1*C*) in βStop mice was confirmed by Southern blotting and genomic sequencing. βStop mice were viable, fertile, and reproduced in a 1:2:1 Mendelian ratio (WT 27.7%, heterozygous Ctr βStop 48.5%, βStop 23.7%) (Fig. 1*D*). Western blot analysis of heart and brain membrane fraction showed no alterations in $Ca_v1.2$ expression. The expression of the C-terminal truncated $Ca_v\beta_2$ protein was con-

firmed by the C-terminal binding $Ca_v\beta_{2v2}$ antibody and the N-terminal binding antibody $Ca_v\beta_2$-N4/1195. The C-terminal binding $Ca_v\beta_{2v2}$ antibody detected the 75-kDa WT $Ca_v\beta_2$ protein, but not the truncated $Ca_v\beta_2$ protein (Fig. 1*E*), whereas the N-terminal binding $Ca_v\beta_2$-N4/1195 antibody detected both the truncated $Ca_v\beta_2$ protein (58 kDa) and the WT protein (75 kDa) (Fig. 1*F*). These results show that the βStop mouse expressed the truncated $Ca_v\beta_2$ protein that missed the reported phosphorylation sites.

Telemetric ECG measurement of heart rate (HR) and activity revealed no differences in WT and βStop 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. 2*A*). FS was identical in Ctr βstop and βStop mice (Fig. 2*B*). Isoproterenol doubled FS in both genotypes. These data indicate that the putative PKA phosphorylation sites $Ser^{479/480}$ rbs of the $Ca_v\beta_2$ subunit are not necessary to observe the positive inotropic, β-adrenergic regulation of the heart muscle.

To further support the insignificant effect of the $Ca_v\beta_2$ truncation for cardiac β-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 βStop, 170 ± 14 pF, $n = 13$; βStop, 149 ± 8 pF, $n = 13$), normal I_{Ca} density at +10 mV (WT, 2.99 ± 0.25pA/pF, $n = 6$; Ctr βStop, 3.39 ± 0.2 pA/pF, $n = 13$; βStop, 3.01 ± 0.2 pA/pF, $n = 13$), half-maximal activation constants (WT, −21.7 ± 1.6 mV, $n = 4$; Ctr βStop −16.3 ± 1.7 mV, $n = 8$; βStop −14.1 ± 0.9 mV, $n = 9$) and a normal I-V relation (Fig. 2*C*).[3] As expected from the results of Meissner *et al.* (26), further analysis showed normal half-maximal steady state inactivation (Fig. 2*D*) ($V_{0.5}$: Ctr βStop, −18.2 ± 1.6 mV ($n = 3$); βStop, −18.7 ± 1.2 mV ($n = 5$)), and normal half-maximal recovery time from inactivation (Fig. 2*E*) ($\tau_{1/2}$ (ms): Ctr βStop, 135. 3 ($n = 6$); βStop, 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 $Ca_v1.2$ channel complex. Recently, we reported that CDF requires phosphorylation of $Ca_v1.2$ at $Ser^{1512/1570}$ (27). In contrast, Colbran and co-workers (22) reported that phosphorylation of $Ca_v\beta_2$ by CaMKII at Thr^{500} modulated CDF. Because Thr^{500} was not present anymore in the βStop protein, we tested whether or not the truncation of the $Ca_v\beta_2$ C terminus might affect I_{Ca} facilitation. We compared prepulse facilitation of I_{Ca} in CMs of both genotypes. CDF was not affected by the truncation of the $Ca_v\beta_2$ protein (Fig. 2*F*), suggesting that phosphorylation of the $Ca_v\beta_2$ subunit is not a necessary prerequisite to induce CDF under basal conditions.

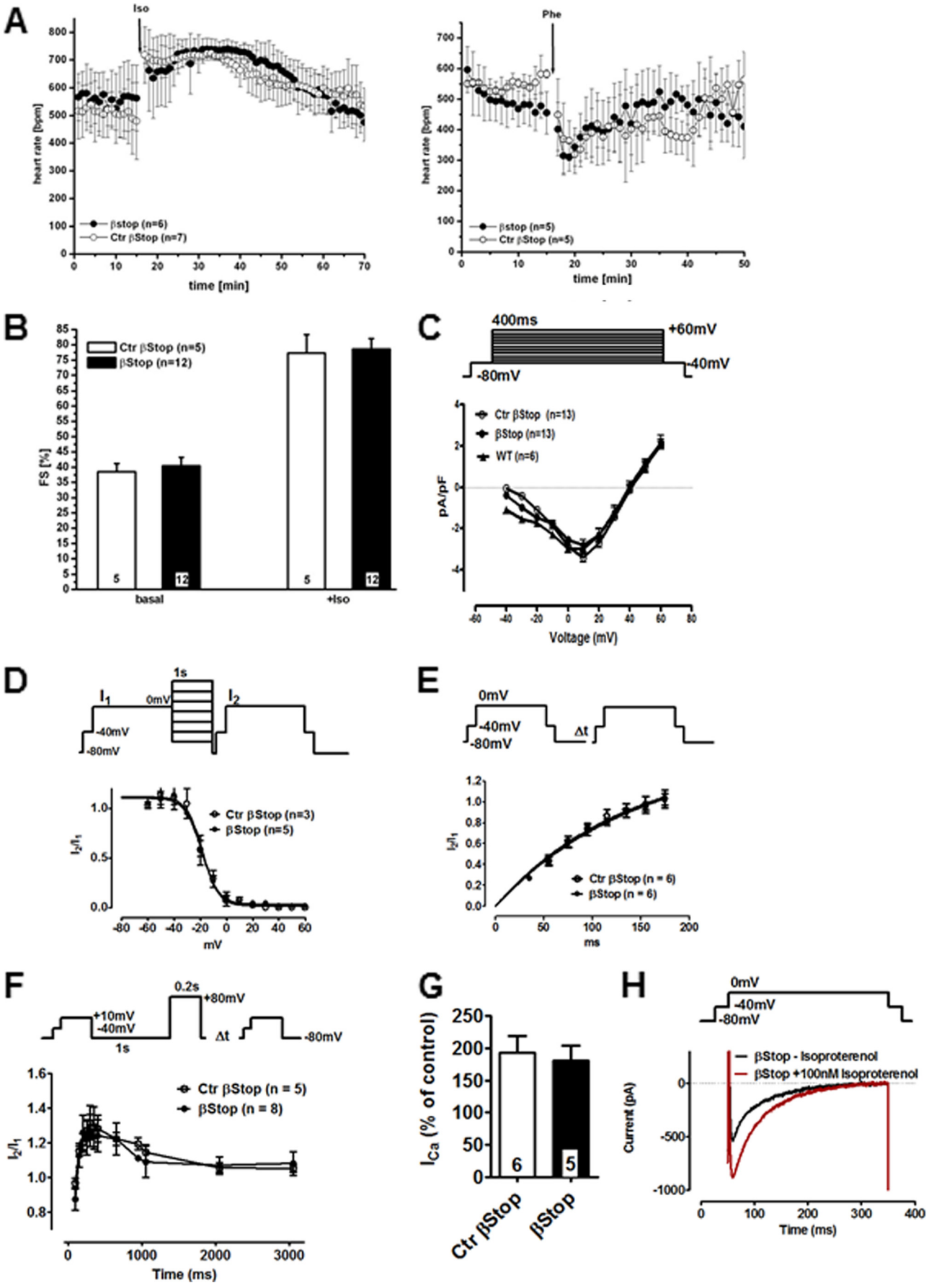
In agreement with the ECG results, isoproterenol stimulated I_{Ca} of Ctr βStop and βStop CMs to the same level (Fig. 2*G*). Representative current traces for a βStop CM are shown in Fig. 2*H*. Isoproterenol treatment increased I_{Ca} in Ctr βStop CMs by 193 ± 25% and in βStop CMs by 180 ± 24% (Fig. 2*G*). 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 $Ca_v\beta_2$ protein.

These negative experiments raised the possibility that the positive inotropic effect was mediated by phosphorylation of both the $Ca_v1.2$ and $Ca_v\beta_2$ subunit. We tested this hypothesis by cross-breeding the βStop line with the $Ca_v1.2^{SA}$ or the $Ca_v1.2^{SF}$ lines. The $Ca_v1.2^{SA}$ mouse line expresses a $Ca_v1.2$ channel containing the mutation S1928A (25). Mice homozygous for the double mutation $Ca_v1.2^{S1928A}/Ca_v1.2^{S1928A}$, $Ca_v\beta_2{}^{P501Stop}/Ca_v\beta_2{}^{P501Stop}$ (SAβStop) 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 SAβStop and double knock-in SAβStop CMs was the same (Ctr SAβStop: 168.6 ± 12 pF ($n = 15$); SAβStop: 163.0 ± 18 pF ($n = 6$)). Inactivation time constants of I_{Ca} were not affected by this double mutation (supplemental Table 3). We did not observe an effect of the double mutation on CDF (Fig. 3*A*). Isoproterenol stimulated FS (Fig. 3*B*) 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 I_{Ca} by 194.3 ± 19.2% ($n = 6$) and 205.3 ± 14% ($n = 6$) in heterozygous Ctr SAβStop and homozygous SAβStop, respectively (Fig. 3, *C* and *D*).

In the next series of experiments we tested the double mutation $Ca_v1.2^{S1512/1570A}/Ca_v1.2^{S1512/1570A}$, $Ca_v\beta_2{}^{P501Stop}/Ca_v\beta_2{}^{P501Stop}$ (SFβStop). Mice homozygous for the double mutation SFβStop 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 SFβStop and double knock-in SFβStop CMs was the same (Ctr SFβStop: 213 ± 13 pF ($n = 9$); SFβStop: 195 ± 17 pF ($n = 8$)). Inactivation time constants of I_{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 SFβStop mice (Fig. 4*A*).

Isoproterenol stimulated FS (Fig. 4*B*) 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 I_{Ca} by 187 ± 17% ($n = 10$) and 196 ± 26% ($n = 8$) in the heterozygous Ctr SFβStop and homozygous SFβStop line, respectively (Fig. 4, *C* and *D*). We concluded

[3] These results indicated to us that there is no gene dose effect through the deletion of the C terminus of one β_2 gene. Almost identical results have been reported by Meissner *et al.* (26), which reported the inactivation of both β_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 generally accepted βStop considerations, we tested against the heterozygous, litter-matched CTR animals. Furthermore, heart-specific inactivation of the β_2 subunit gene in adult mice yielded minimal or no effect on I_{Ca} kinetics (see Ref. 26, Figs. 4 and 5).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βStop (n = 8)
I2/I1
Time (ms)
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ICa (% of control)
6
5
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βStop
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-80mV
βStop - Isoproterenol
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Current (pA)
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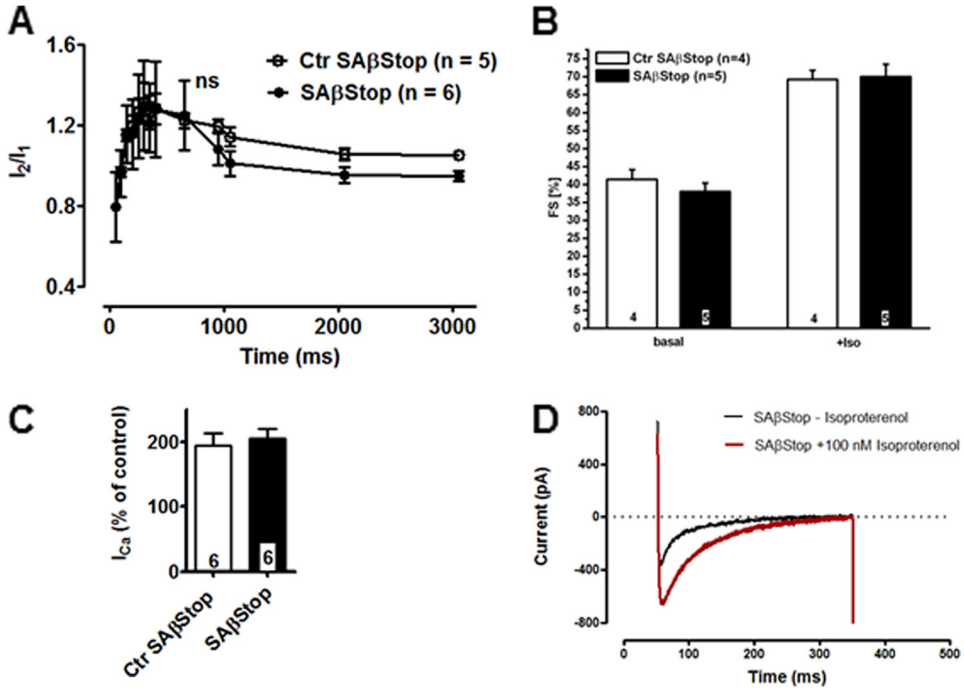

FIGURE 3. **Unchanged β-adrenergic regulation of I_{Ca} in SAβStop mice.** *A*, unchanged CDF in Ctr SAβStop and SAβStop CMs. *B*, FS unchanged in βStop mice before and after isoproterenol administration (0.1 mg/kg of body weight intraperitoneally). *C*, statistics of isoproterenol (0.1 μM) stimulation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

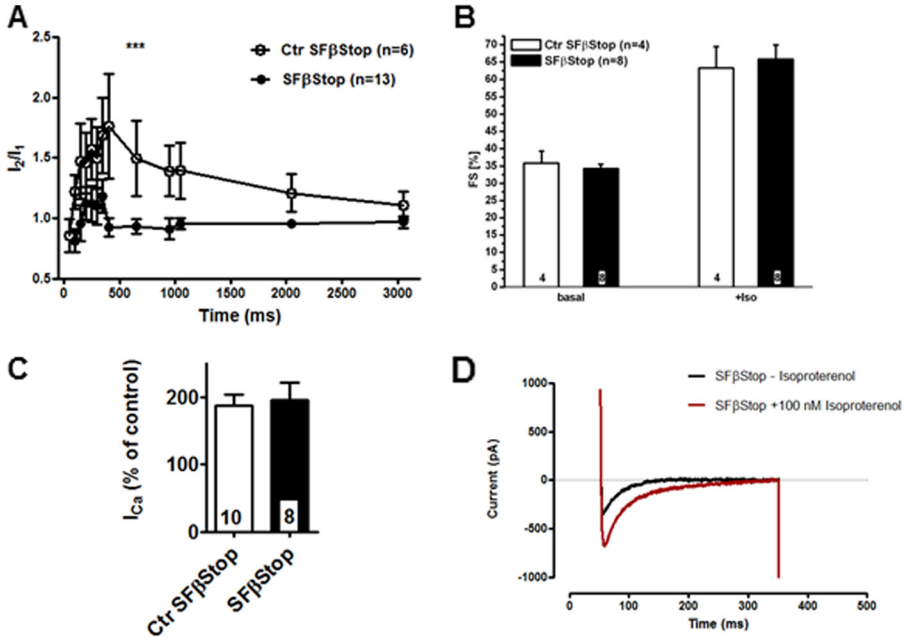

FIGURE 4. **Unchanged β-adrenergic regulation of I_{Ca} in SFβStop mice.** *A*, CDF in SFβStop CMs decreased compared with Ctr SFβStop. *B*, FS unchanged in βStop mice before and after isoproterenol administration (0.1 mg/kg of body weight intraperitoneally). *C*, statistics of isoproterenol (0.1 μM) 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.

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*/*V* relation of WT, Ctr βStop, and βStop CMs. *D*, steady-state inactivation of Ctr βStop and βStop CMs. *E*, recovery from inactivation of Ctr βStop and βStop CMs. *F*, unchanged CDF in Ctr βStop and βStop CMs. *G*, statistics of isoproterenol (0.1 μM) stimulation of I_{Ca} in Ctr βStop and βStop CMs. *H*, 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.

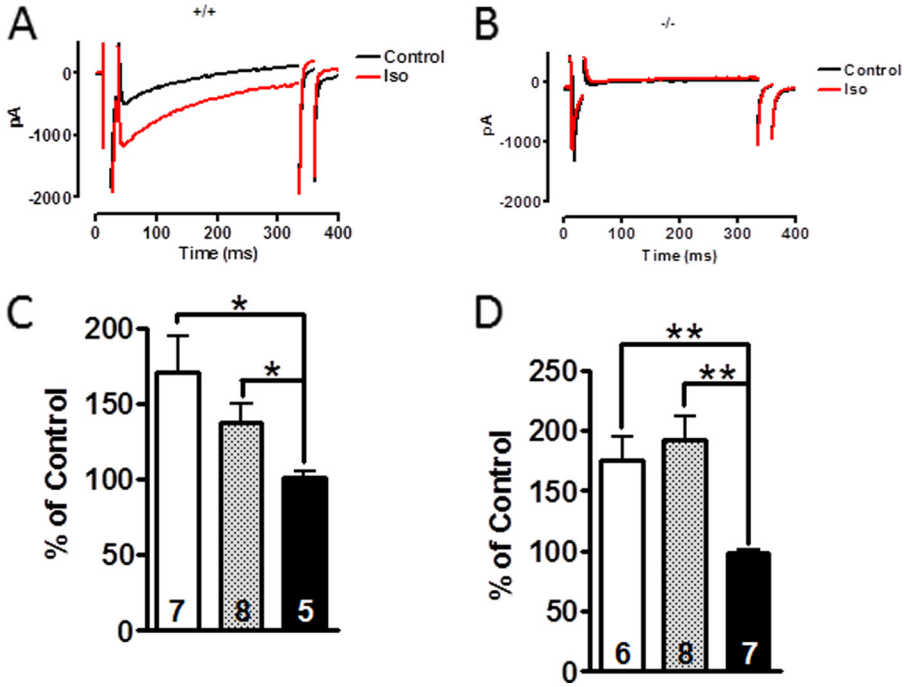

FIGURE 5. **I_{Ca} is not regulated by β-adrenergic stimulation in $Ca_v1.2$Stop CMs.** *A* and *B*, I_{Ca} traces of WT (*A*, +/+) and $Ca_v1.2$Stop (*B*, −/−) embryonic CM ± 0.1 μM isoproterenol. The CMs were obtained from day 18.5 embryos as described (31). *C* and *D*, statistics of isoproterenol (*C*) and forskolin (*D*)-dependent increase in peak current of WT (*open columns*), heterozygous (*gray columns*), and $Ca_v1.2$Stop (*black columns*) CMs. Number of cells is given in columns. *, $p = < 0.05$; **, $p = < 0.01$.

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_{Ca} (33). AKAPs bind to the C terminus of $Ca_v1.2$ 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 $Ca_v1.2$ sequence at Asp^{1904} (31) or at Gly^{1796} (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_{Ca} of embryonic $Ca_v1.2$Stop 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 Asp^{1904} are essential for the adrenergic up-regulation of I_{Ca} in the heart.

DISCUSSION

Adrenergic up-regulation of the cardiac I_{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 $Ca_v1.2$ channel remains unsolved. Previously, it was found that the mutation S1928A of the $Ca_v1.2$ protein did not abolish β-adrenergic regulation of the heart and I_{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 $Ca_v\beta_2$ subunit has been promoted as an alternative substrate for PKA (20, 40, 41). Initially, it was suggested that PKA-dependent up-regulation of the expressed I_{Ca} requires truncation of the $Ca_v1.2$ protein at amino acid 1905 and the co-expression of the $Ca_v\beta_2$ subunit (20). Truncation of the murine $Ca_v1.2$ 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 $Ca_v1.2$ 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 $Ca_v1.2$ (38). These results clearly demonstrate that truncation of the $Ca_v1.2$ protein *in vivo* is not required for the adrenergic regulation.

Truncation of the $Ca_v\beta_2$ 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_{Ca}. The $Ca_v\beta_2$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 $Ca_v\beta_2$ 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 $Ca_v\beta_2$ 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

associated so far with a phosphorylation event at the $Ca_v\beta_2$ subunit.

An alternative possibility is that PKA phosphorylates simultaneously sites at the $Ca_v\beta_2$ and the $Ca_v1.2$ subunit. This possibility was tested with two additional mouse lines. However, the combination of the $Ca_v\beta_2$ mutation with mutation at the C terminus of the $Ca_v1.2$ protein did not influence the cardiac response to β-adrenergic stimulation. However, the $Ca_v\beta_2$ mutation did not affect the reduced CDF caused by the SF mutation (27). Our results do not rule out the possibility that phosphorylation at these sites might affect parameter of the $Ca_v1.2$ channel that have not been studied under our condition. However, the physiological significance of these putative parameters appears to be restricted because deletion of the cardiac $Ca_v\beta_2$ gene in the adult mouse caused only negligible changes in the cardiac performance (26).

An alternative target for PKA and β-adrenergic regulation of the heart has been proposed recently (8). Expression studies implicated PKA-dependent phosphorylation of Ser^{1700} and Thr^{1704} in the C terminus of $Ca_v1.2$ to be important for the β-adrenergic regulation (8). The phosphorylation needs to be combined with proteolytic cleavage of the mature $Ca_v1.2$ protein close to amino acid 1800. The cleaved distal C terminus has to stay with the truncated $Ca_v1.2$ channel to allow β-adrenergic regulation. The distal part inhibits I_{Ca} of the truncated $Ca_v1.2$ channel. The inhibition is then removed by β-adrenergic stimulation (8).

For β-adrenergic regulation of the expressed I_{Ca}, an additional function of the distal part, its AKAP binding site, is required. The AKAP binding site allows the close positioning of PKA to the $Ca_v1.2$ channel. Partial verification of the AKAP concept has been given by Nichols *et al.* (33), Fuller *et al.* (8) and by the results of this report. The I_{Ca} of embryonic CMs expressing a truncated $Ca_v1.2$ channel (see Fig. 7 in Ref. 34) was not stimulated by isoproterenol or forskolin. To support further the above concept, *in vivo* mutation of Ser^{1700}/Thr^{1704} is necessary to show that β-adrenergic regulation of I_{Ca} requires the phosphorylation of Ser^{1700}/Thr^{1704}.

The reported results suggest that the C-terminal phosphorylation sites of the $Ca_v\beta_2$ subunit are not used to regulate basic properties of the murine cardiac I_{Ca}. In contrast, CaMKII-dependent phosphorylation of the C terminus of $Ca_v1.2$ is necessary for CDF. The results support again the previous notion (31, 34) that the distal C terminus of the $Ca_v1.2$ channel is necessary for β-adrenergic regulation of murine I_{Ca}.

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