Phosphorylation of serine 1928 (Ser\textsuperscript{1928}) of the cardiac Ca\textsubscript{\textit{a}},1.2 subunit of L-type Ca\textsuperscript{2+} channels has been proposed as the mechanism for regulation of L-type Ca\textsuperscript{2+} channels by protein kinase A (PKA). To test this directly \textit{in vivo}, we generated a knock-in mouse with targeted mutation of Ser\textsuperscript{1928} to alanine. This mutation did not affect basal L-type current and the characteristics or regulation of the L-type current by PKA and the β-adrenergic receptor, whereas the mutation abolished phosphorylation of Ca\textsubscript{\textit{a}},1.2 by PKA. Therefore, our data show that PKA phosphorylation of Ser\textsuperscript{1928} of Ca\textsubscript{\textit{a}},1.2 is not functionally involved in β-adrenergic stimulation of Ca\textsubscript{\textit{a}},1.2-mediated Ca\textsuperscript{2+} influx into the cardiomyocyte.

There is excellent evidence for a regulation of the cardiac L-type Ca\textsuperscript{2+} current (I\textsubscript{CaL}) by β-adrenoceptors, cAMP, and protein kinase A (PKA)\textsuperscript{2} (1, 2). This mechanism most likely plays a critical role in physiological processes in the heart, e.g. excitation-contraction coupling, the regulation of inotropy and chronotropy, as well as pathological processes such as heart failure (for review see Refs. 3–5).

The molecular basis of I\textsubscript{CaL} regulation by PKA could not be defined conclusively so far (5). This is mainly due to the fact that the extent of regulation of I\textsubscript{CaL} activity by PKA in experiments in transfected cells (10–50% increase) falls well short of the magnitude recorded in native cardiac cells (200–400% increase). It seems likely that regulatory influences not reproduced in heterologous expression systems are important for the control of the activity of cardiac Ca\textsuperscript{2+} channels \textit{in vivo} (2). It is a widely accepted finding that phosphorylation of Ser\textsuperscript{1928} of the Ca\textsubscript{\textit{a}},1.2 L-type channel subunit is necessary for β-adrenergic regulation of I\textsubscript{CaL} (5, 6).

The amino acid Ser\textsuperscript{1928}, which is located in the intracellular C terminus of Ca\textsubscript{\textit{a}},1.2, has been reported to be the only detectable \textit{in vivo} and \textit{in vitro} PKA phosphorylation site of the Ca\textsubscript{\textit{a}},1.2 subunit (6–10). Electrophysiological studies using heterologous expression of the Ca\textsubscript{\textit{a}},1.2 subunit reported contrary findings, i.e. stimulation (11) or no stimulation (12) of I\textsubscript{CaL} by PKA when only the Ca\textsubscript{\textit{a}},1.2 subunit was expressed. In contrast, the necessity of coexpression and phosphorylation of the Ca\textsubscript{\textit{a}},β\textsubscript{2}a subunit was reported (13). Recently, the functional importance of Ca\textsubscript{\textit{a}},β\textsubscript{2}a phosphorylation for β-adrenergic regulation of I\textsubscript{CaL} has been questioned (14). Regardless of these findings, mutation of Ca\textsubscript{\textit{a}},2 Ser\textsuperscript{1928} to alanine prevented phosphorylation and regulation of the channel by PKA in heterologous expression systems (15–17).

In contrast to these reports, Ganesan et al. (18) postulated that at least 70% of the β-adrenergic regulation of I\textsubscript{CaL} in virally transduced heart cells cannot be attributed to the Ser\textsuperscript{1928} phosphorylation event. However, the viral infection system by Ganesan et al. (18) only partially reconstituted the regulation of I\textsubscript{CaL} (the PKA-mediated increase in current was only 50%).

On the other hand, Hulme et al. (6) recently correlated β-adrenergic stimulation with phosphorylation of Ser\textsuperscript{1928} and functional up-regulation of I\textsubscript{CaL} in intact ventricular myocytes. In addition to these findings, Oliveria et al. (17) postulated a critical role for the Ser\textsuperscript{1928} phosphorylation in the PKA-mediated regulation of Ca\textsubscript{\textit{a}},1.2 channels in HEK293 cells and neurons.

To analyze this controversial issue in intact, untransfected, native cardiomyocytes containing the complete regulatory system, we generated a knock-in mouse line carrying the Ca\textsubscript{\textit{a}},1.2\textsuperscript{S1928A} mutation.

**EXPERIMENTAL PROCEDURES**

**Generation of Mice Lacking the Ser\textsuperscript{1928} Phosphorylation Site on Ca\textsubscript{\textit{a}},1.2**—To construct the targeting vector, a 7.4-kb fragment containing exons 44–47 of CACNA1C was isolated from 129/Sv mouse genomic DNA. The targeting vector included a 1.2-kb short arm and 6.2-kb long arm with PGK-neo and the thymidine kinase gene (tk) flanked by two loxP sites. The 3′-side long arm contained exon 45 with the phosphorylation site, serine 1928, mutated to alanine. All mutation procedures were carried out by overlap PCR mutagenesis. The targeting construct was electroporated into R1 ES cells (129/Sv×129/Sv-CP F1) (19). Positive clones were identified by PCR and confirmed by Southern blot using an outer probe (5′-probe in Fig. 1a) and an inner probe (neo/tk probe in Fig. 1a).

Two positive clones were transfected with a Cre-expressing plasmid to delete the neo/tk marker genes. Five clones with the deletion event were injected in C57BL/6 blastocysts, and chimeras were crossed to C57BL/6 mice. Heterozygous mice were bred to produce homozygotes. The intercross of heterozygous mice generated a knock-in mouse line carrying the Ca\textsubscript{\textit{a}},1.2\textsuperscript{S1928A} mutation.
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 mmol/liter EDTA, 10 mmol/liter EGTA, 50 mmol/liter Tris-HCl, pH 7.4, containing protease inhibitors. Nonsoluble material was removed by a second ultracentrifugation step (50,000 rpm at 4 °C for 30 min). GST fusion proteins were expressed in BL21 Escherichia coli according to the manufacturer’s instructions (Amersham Biosciences).

**Antibodies**—The anti-Ca,1.2 and -Ca,β2 antibodies have been described previously (20, 21). Antibodies against the catalytic (PKAc) and regulatory subunits (RIIα and RIIβ) of cAMP-dependent protein kinase were purchased from BD Biosciences. The antibodies against GST and against the β,1-adrenergic receptor were obtained from Calbiochem and Upstate, respectively. The phosphospecific antibody against Ser(P)1928 was generated by CovAlab against the peptide NH2-C-LGRRRA(pS)FHLECLK-COOH. The sensitivity and specificity of the phospho-specific antibody were confirmed utilizing GST fusion proteins/enzyme-linked immunosorbent assay/incubation with phosphorylated antigenic peptide (CovAlab).

**Immunoprecipitation and Immunoblotting**—The solubilized membranes from heart were preincubated with protein A-Sepharose (Sigma) 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 was added; samples were tilted for 1 h, and the resins were washed and extracted with 1:6 (v/v) SDS sample buffer (22). For total cellular protein analysis, hearts were homogenized in lysis buffer (2% SDS, 50 mmol/liter, Tris, pH 7.4). Proteins were separated on 10% SDS-polyacrylamide gels, blotted, and probed with antibodies by using a chemiluminescence detection system or nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as described in Ref. 20. To quantitatively evaluate Ser1928 phosphorylation, blots were first probed with anti-phospho-Ser1928 antibody and subsequently with anti-Ca,1.2 antibody to correct for variability in the amount of total Ca,1.2. The ratio of the anti-phospho-Ser1928 antibody to the anti-Ca,1.2 signal was determined for each sample by blot densitometry using Quantity One software (Bio-Rad). Ratios were normalized to the ratio of the control animals.
Phosphorylation with PKA—For PKA phosphorylation reactions, full-length Ca\textsubscript{1.2} was immunoprecipitated with the Ca\textsubscript{1.2}-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 mmol/liter HEPES-NaOH, pH 7.4, 10 mmol/liter MgCl\textsubscript{2}, 0.5 mmol/liter EGTA, 0.5 mmol/liter dithiothreitol). Phosphorylation was carried out by mixing the Sepharose pellets with 33 μmol/liter Mg\textsubscript{2+}/[γ\textsuperscript{32}P]ATP (6.6 μCi/reaction). The reaction, initiated by the addition of 5 units of PKAc (Sigma) to either the GST fusion proteins or immunoprecipitated Ca\textsubscript{1.2} complexes, was carried out at 23 °C for 5 min (GST fusion proteins) or 10 min (immunoprecipitated Ca\textsubscript{1.2} complexes) and terminated by boiling in 1:6 (v/v) SDS sample buffer. Importantly, phosphorylation without the addition of exogenous PKAc yielded no detectable autoradiography signals under these conditions. Proteins were separated on 10 or 12.5% SDS gels, and incorporated \(^{32}\)P was detected by autoradiography. To quantitatively evaluate the autoradiography signals, the ratio of the intensity of each signal was determined for each sample using a Fujix Bas1000 PhosphorImager and Aida 2.11 software. Signal intensities were normalized to the signal in the control preparations.

Cell Isolation—Ventricular myocytes were isolated as described (AFCS Procedure Protocol PP00000125), maintained at 37 °C, and aerated with 98% O\textsubscript{2}, 2% CO\textsubscript{2}.

**FIGURE 2. Serine 1928 is phosphorylated by PKA both in vitro and in murine heart.**

- **a,** top, schematic of the GST fusion proteins containing the C terminus of Ca\textsubscript{1.2}. Bottom, autoradiogram of PKA in vitro kinase reaction performed with GST-fused wild type fragments amino acids 1509–1733 (1), amino acids 1733-COOH (2), and amino acids 1733-COOH S\textsuperscript{1928A} (3). PKI, reactions in the presence (+) or absence (−) of 1 μmol/liter PKA inhibitor peptide. Anti-GST antibody was used as loading control.

- **b,** GST fusion proteins (wild type fragments amino acids 1509–1733, amino acids 1733-COOH, and amino acids 1733-COOH S\textsuperscript{1928A} fragment) were phosphorylated by PKA, size-fractionated on SDS-polyacrylamide gel, transferred to nitrocellulose, analyzed by autoradiography, and immunoblotted with a phospho-specific antibody recognizing phosphorylated Ser\textsuperscript{1928} (pS1928).

- **c,** autoradiogram of wild type and S1928A mutant Ca\textsubscript{1.2} phosphorylated by the catalytic subunit PKAc. Strong phosphorylation was only detectable in the wild type protein, whereas Ca\textsubscript{1.2} expression levels were unchanged. The graph shows quantification of the autoradiography signals (n = 5) normalized to Ctr levels. *, p < 0.001 t test. Absence of phosphorylation at Ser\textsuperscript{1928} in phosphomutants. Heart samples from wild type (Ctr) and homozygote (Ki) mice were prepared for immunoblot analysis using antibodies against phosphorylation site Ser\textsuperscript{1928} (top lane, left), total Ca\textsubscript{1.2} (middle), or Ca\textsubscript{1,2} (bottom). Quantification of the immunoblot signals (bar graph, normalized to Ctr levels) shows that phosphorylation is absent in Ca\textsubscript{1.2}\textsuperscript{S1928A-129B6F2} mice, whereas Ca\textsubscript{1.2} expression level is normal. n = 5, *, p < 0.001 t test. Preincubation with antigenic peptide (top lane, right) abolished the immunoblot signal.
Electrophysiology—Calcium currents ($I_{Ca}$) were recorded in whole-cell mode at room temperature from rod-shaped, striated, calcium-tolerant myocytes within 1–24 h of isolation. The extracellular solution contained 140 mM tetraethylammonium-Cl, 2 mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4. Patch pipettes (1–2 megohms) were filled with an intracellular solution, pH 7.4, containing 135 mM CsCl, 1 mM MgCl$_2$, 5 mM MgATP, 10 mM HEPES, and 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid. Recordings were discarded if the series resistance was over 6 megohms. Calcium current was elicited by either repeated 200-ms depolarizing pulses to 10 mV or a series of depolarizing pulses to different test potentials ($-40$ to $+80$ mV in 10-mV steps) from a holding potential of $-40$ mV. Calcium current was measured as the difference between the peak inward current and the current at the end of the test pulse. After establishing a solid baseline, the effects of either isoproterenol (100 nmol/liter) containing an equal concentration of ascorbic acid or forskolin (10 μmol/liter) on $I_{Ca}$ were examined. H89 (Sigma), ICI118551 (Tocris), and CGP20712A (Tocris) were diluted from stocks in DMSO (H89, 10 mM) and water (10 mM, respectively). Currents were recorded with a patch clamp EPC9 device (HEKA, Lambrecht, Germany) and sampled at 5 kHz. Data acquisition and command potentials were controlled by Pulse + Pulsefit software version 8.54 (HEKA, Lambrecht, Germany), and data were stored for later off-line analysis. Leak compensation was performed online in Pulse + Pulsefit when necessary. All data are expressed as the means ± S.E. Values of $p < 0.05$ were considered significant.

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 (Sigma) was dissolved in 0.9% NaCl. After 15 min of baseline recording, the mice were injected intraperitoneally with the drugs used. 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.

Open Field—The open field consisted of a transparent plastic box with a white floor ($41 \times 41 \times 41$ cm). The illumination at floor level was 150 lux. Mice were individually placed into the center of the open field, and their behaviors were tracked with an automated activity monitoring system (TSE Systems GmbH, Germany). The overall distance traveled by the mice and the vertical plane entries (rearings) was monitored for 5 min.

Beam Walking—The beam consisted of long strips of plastic (1 m) with a 1.0-cm cross-section and grooves (0.5–1.0 cm) every 5 cm. The beam was placed horizontally, 50 cm above the bench surface, with both ends mounted on a narrow support. During training mice were placed at the start of the beam and trained once to traverse the beam. 24 h later the number of times the hind feet slipped off the beam was recorded. To detect motor learning, mice had to traverse the beam after 1 h and again after 24 h.

**FIGURE 3. Modulation of calcium current in mouse ventricular myocytes by isoproterenol.** a, mean normalized (± S.E.) current-voltage relationships before (filled symbols) and after (open symbols) exposure to 100 nmol/liter isoproterenol (Iso) in wild type (Ctr, top graph) and Cav1.2S1928A-129B6F2 (Ki, bottom graph) ventricular myocytes. b, representative calcium currents elicited by 200-ms test pulses from $-40$ to 10 mV before and after exposure to 100 nmol/liter Iso. c, mean current densities of $I_{CaL}$ of wild type (squares) and Cav1.2S1928A-129B6F2 (triangles) ventricular myocytes.
**RESULTS AND DISCUSSION**

We used a gene-targeting strategy that utilized a replacement vector containing the point mutation and a neo/tk gene cassette flanked by loxP sites (Fig. 1a). All homozygous mutants analyzed were F2 mice from a cross between the chimeras (contributing 129 background) and C57BL/6 mice (Cav1.2S1928A-129B6F2). The mutants showed no overt cardiovascular phenotype and bred normally. Ca_{1.2}^{S1928A-129B6F2} animals could not be differentiated from litter-matched wild type mice in open field, and beam walking tests (data not shown), indicating no obvious changes in behavior or motor performance. We confirmed the mutation of the phosphorylation site by DNA sequencing, Southern blot (Fig. 1b), and immunoblot analysis phosphorylated at Ser^{1928}. To extend these in vitro data to the Ca_{1.2}^{S1928A-129B6F2} animal model, we next evaluated phosphorylation of cardiac Ca_{1.2} subunits from these mice.

Phosphorylation by PKA in the presence of [γ-32P]ATP and the catalytic subunit of PKA (5 units) was examined using an immunoprecipitation protocol and autoradiography. Only weak weak phosphoprotein signals (9.4 ± 4.8% of control level) corresponding to Ca_{1.2} were observed when heart preparations from Ca_{1.2}^{S1928A-129B6F2} mutants were used, whereas in controls Ca_{1.2} phosphorylation could be readily detected (Fig. 2c). In strong agreement with these results, detection of Ca_{1.2} by the phosphospecific antibody to Ser^{1928} was barely detectable in the homozygotes (4.4 ± 2.1% of control level). Specificity of the anti-Ser^{1928} antibody in Ca_{1.2} full-length preparations was additionally confirmed by preincubation with phosphorylated antigenic peptide, which abolished the immunoblot signal (Fig. 2d).

Taken together, these findings suggest that Ser^{1928} is the only easily detectable Ca_{1.2} PKA phosphorylation site. Moreover, the Ca_{1.2} subunit lacking this phosphorylation site is properly targeted to the Ca_{1.2}-associated proteins (7, 23), indicating that the phosphorylation site may not be critical for maintaining the steady-state level of channels at the membrane or the assembly of the Ca_{1.2} signaling complex.

Next, we looked at whether the lack of this phosphorylation site on Ca_{1.2} affects β-adrenergic regulation in adult litter-

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**Echocardiography**—Images were obtained using a Vevo 770 Visual Sonics scanner equipped with a 30-MHz probe (Visual Sonics Inc., Toronto, Canada). The mice were lightly anesthetized (1.5% isoflurane), 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, reduction of the length of the end-diastolic diameter that occurs by the end of systole) was assessed from the M mode of the parasternal short axis view. Ctr and Ki mice were studied before and 1 min after administration of isoproterenol (0.5 mg/kg body weight intraperitoneally).

**FIGURE 4.** Forskolin occludes further modulation of calcium current by isoproterenol in mouse ventricular myocytes. a, representative calcium currents elicited by 200-ms test pulses from −40 to 10 mV before and after exposure to 10 μmol/liter forskolin. Additional application of 100 nmol/liter isoproterenol did not alter the amplitude of the calcium currents. b, statistical analysis of the effects of Iso, forskolin, or forskolin + Iso on the calcium current in both genotypes. Sample sizes are indicated.
mate controls and homozygotes. The current-voltage relationship and expression level measured as current density of the mutant channel were similar to that of the wild type channel (Fig. 3, a and c) indicating that L-type channels show no different voltage dependence for activation or membrane expression in Cav1.2S1928A-129B6F2 mice. To quantitatively correlate phosphorylation of Ser1928 with regulation of \( I_{\text{Ca,L}} \), we measured \( \beta \)-adrenergic stimulation of whole-cell \( I_{\text{Ca,L}} \) by isoproterenol (Iso). Iso treatment increased \( I_{\text{Ca,L}} \) in control mice 280 ± 25% and in Cav1.2S1928A-129B6F2 mice 268 ± 20%, respectively (Fig. 3, a and b). In addition to the unchanged Iso-induced increase in peak inward \( I_{\text{Ca,L}} \), the 10-mV left shift in \( V_{1/2} \) of activation was preserved in cardiomyocytes from Cav1.2S1928A-129B6F2 mice (Fig. 3a).

In agreement with the Iso results, we found that the adenylate cyclase agonist forskolin enhanced \( I_{\text{Ca,L}} \) in both control and mutant cardiomyocytes to identical levels (228 ± 39% versus 240 ± 20%, see Fig. 4, a and b). Application of forskolin completely prevented further isoproterenol stimulation in both genotypes (Fig. 4, a and b), indicating that a membrane-delimited pathway involving the "direct" stimulation of \( I_{\text{Ca,L}} \) by a Gs subunit (24, 25) was not operative.
To further substantiate these findings, we characterized the functional role of additional components of the β-adrenergic pathway in $I_{\text{CaL}}$ regulation. The PKA inhibitor H89 consistently blocked the isoproterenol-induced increase in $I_{\text{CaL}}$ in litter-matched control and Ca$_{1.2}$S1928A-129B6F2 cardiomyocytes (Fig. 5, a and b).

The isoproterenol effect was also completely suppressed by application of 0.1 μM of the β$_1$-adrenergic blocker CGP20712A and partially by 0.1 μM of the β$_2$-adrenergic blocker ICI118551 in wild type and mutant mice (Fig. 5c). Taken together, these data rule out a switch of the physiological β-adrenergic regulation of cardiac Ca$_{1.2}$ in Ca$_{1.2}$S1928A-129B6F2 mice.

The results shown so far did not rule out the possibility that phosphorylation of Ser$^{1928}$ is required for the in vivo regulation of cardiac function. First, we looked at a potential role of Ca$_{1.2}$ Ser$^{1928}$ phosphorylation in the regulation of heart rate and rhythm using ECG telemetry. There were no detectable differences in basal heart rate (587 ± 24 beats/min versus 567 ± 27 beats/min, t test, $p > 0.05$). Isoproterenol infusion increased heart rate to the same level (734 ± 15 beats/min versus 741 ± 6 beats/min, t test, $p > 0.05$) in both control and Ca$_{1.2}$S1928A-129B6F2 animals (Fig. 6a).

We next tested the consequence of ablation of Ca$_{1.2}$ Ser$^{1928}$ phosphorylation for cardiac contractility. Echocardiography clearly showed that isoproterenol increased cardiac FS, an indicator of systolic performance, to the same level in both genotypes (Fig. 6, b and c) demonstrating that the positive inotropic effect of β-adrenergic stimulation in vivo is not dependent on Ser$^{1928}$.

Taken together, our results support the notion that PKA phosphorylation of Ser$^{1928}$ of Ca$_{1.2}$ is functionally not involved in β-adrenergic regulation of $I_{\text{CaL}}$ in murine ventricular cardiomyocytes. The results are in line with the previous finding that β-adrenergic stimulation requires a PKA-mediated phosphorylation step (1, 6, 9, 11, 13, 16, 18, 24). Many questions remain to understand the β-adrenergic regulation of Ca$^{2+}$ channels. What is the physiological substrate of PKA in the Ca$_{1.2}$ channel complex responsible for acute stimulation of $I_{\text{CaL}}$ if not Ser$^{1928}$? The phosphorylation target could be the Ca$_{\beta_{2a}}$ subunit (13) or the giant protein AHNAK (26). Our studies provide the basis to address these critical questions in the future. Because it has been difficult to reconstitute reproducibly $I_{\text{CaL}}$ regulation in cultured cells, it is likely that the generation and subsequent analysis of transgenic mice targeting additional components of the $I_{\text{CaL}}$ signaling complex will be necessary to understand the physiological process of β-adrenergic regulation of cardiac L-type Ca$^{2+}$ channels.

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