Overexpression of β₂-Adrenergic Receptors cAMP-dependent Protein Kinase Phosphorylates and Modulates Slow Delayed Rectifier Potassium Channels Expressed in Murine Heart

EVIDENCE FOR RECEPTOR/CHANNEL CO-LOCALIZATION*

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The cardiac slow delayed rectifier potassium channel (I_Ks), comprised of α (KCNQ1) and β (KCNE1) subunits, is regulated by sympathetic nervous stimulation, with activation of β-adrenergic receptors PKA phosphorylating I_Ks channels. We examined the effects of β₂-adrenergic receptors (β₂-AR) on I_Ks in cardiac ventricular myocytes from transgenic mice expressing fusion proteins of I_Ks subunits and hβ₂-ARs. KCNQ1 and β₂-ARs were localized to the same subcellular regions, sharing intimate localization within nanometers of each other. In I_Ks/β₂-AR myocytes, I_Ks density was increased, and activation shifted in the hyperpolarizing direction; I_Ks was not further modulated by exposure to isoproterenol, and β₂-AR signaling domain to regulate the phosphorylation of Ser27 in I_Ks, remaining responsive to cAMP. These data indicate intimate association of KCNQ1 and β₂-ARs and that β₂-AR signaling can modulate the function of I_Ks channels under conditions of increased β₂-AR expression, even in the absence of exogenous β₂-AR agonist.

I_Ks, the slowly activating component of the human cardiac delayed rectifier K⁺ current, is a major contributor to repolarization of the cardiac action potential (1). The I_Ks channel results from the co-assembly of two subunits KCNQ1 (KvLQT1) and KCNE1 (minK), (2, 3). KCNQ1, the α-subunit of I_Ks, shares topological homology with other voltage-gated K⁺ channels in that its 676 amino acids encode six transmembrane domains and a pore-forming region. KCNE1, the β subunit of I_Ks, encodes a protein containing 129-130 amino acids consisting of a single transmembrane spanning domain (4). The contribution of KCNE1 to regulation of action potential duration is augmented by the sympathetic nervous system via activation of β-adrenergic receptors (β-AR) (5, 6). Sympathetic nervous sys-

dependent protein kinase; RyR, ryanodine receptor; ISO, isoproterenol; Ab, antibody; FRET, fluorescence resonance energy transfer; ICD, intercalated disc; SSM, surface sarcosmmal membrane.

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1 The abbreviations used are: AR, adrenergic receptor; PKA, cAMP-
increases in intracellular cAMP by receptor expression because we found that basal L-type calcium channel current (\(I_{\text{Ca,L}}\)) was not up-regulated in these mice. Thus, our data reveal close coupling between \(\beta_2\)-AR signaling and the \(I_{\text{Ca,L}}\) channel and indicate that, with sufficiently high level of \(\beta_2\)-AR expression, the channel can be PKA-phosphorylated and up-regulated even in the absence of exogenous agonist.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice and Isolation of Cardiac Ventricular Myocytes**

Transgenic mice expressing hKCNQ1-hKCNE1 fusion protein in the heart have been described in detail (9). These mice express functional slow delayed rectifier potassium channel currents (IKs) normally absent from murine cardiac ventricular myocytes. Transgenic mice overexpressing \(\beta_2\)-AR (TG4) have also previously been described in detail. TG4 mice exhibit cardiac overexpression of the human \(\beta_2\)-AR at >100-fold higher than endogenous myocardial levels. Strains of TG+ mice overexpressing \(\beta_2\)-AR and hKCNQ1-hKCNE1 were crossed to produce double TG+ (DTG+) mice overexpressing both \(\beta_2\)-AR and expressing hKCNQ1-hKCNE1. Both transgene constructs were under the control of the \(\alpha\)-myosin heavy chain promoter providing cardiomyocyte specific expression. Mice were genotyped by PCR analysis with primers specific for the \(\alpha\)-myosin heavy chain promoter, \(\beta_2\)-AR, and hKCNQ1.

Mice were humanely killed by intraperitoneal injection of a lethal dose of KBr in the absence of exogenous agonist. The channel can be PKA-phosphorylated and up-regulated even in the absence of exogenous agonist.

**Immunohistochemistry**

Ventricular myocytes were isolated as above and plated onto laminin (Sigma) coated coverslip chambers and incubated at 37°C in Dulbecco's modified Eagle's medium to allow cellular attachment. The cells were then fixed and permeabilized using 100% ethanol (15 min at −20°C). The cells were rinsed (three times) in phosphate-buffered saline and then incubated for 20 min with 5% normal goat serum in phosphate-buffered saline to reduce nonspecific binding. After overnight (5°C) incubation with primary antibodies (Ab) against KCNQ1 (Santa Cruz) or \(\beta_2\)-AR (Santa Cruz) cells were rinsed four times with phosphate-buffered saline, after which secondary Abs were added for 2 h at room temperature. IgGs conjugated to Alexa 488 or Alexa 660 were used to label the primary Abs. After a further four washes, coverslips (#1) were mounted onto the microscope slides along with Slowfade™ light (Molecular Probes) S-7461 and anti-fade reagent in a glycerol buffer. The coverslips were sealed in place using nail varnish. To determine the degree of nonspecific binding, incubation with secondary Ab alone was conducted (minimal fluorescence was observed; results not shown). The cells were viewed using a Nikon PCM20000 fluorescence confocal laser-scanning microscope. Alexa 488 was excited with 488-nm light, and fluorescence emissions were measured at greater than 505 nm. Alexa 660 was excited with 635-nm light, and fluorescence emissions were measured between 650 and 750 nm. The images were optimized by adjustment of photo multiplier gain to use the full linear range of pixel intensity, and fluorophore emissions were simultaneously recorded using separate photo multiplier tubes for each wavelength.

**Co-localization**

Immunofluorescence images of each protein were obtained in the same cell. For the purposes of identifying the location of each protein, fluorescence intensity was used. All regions of the cell with fluorescence intensity equal to or greater than a threshold level were identified and marked in each image as a site of the protein as long as at least three contiguous pixels were at or above the threshold. Co-localization was marked when identical regions contained both proteins. The threshold was set using pixel intensity histograms of each immunofluorescence image. The most frequent pixel intensity after the black level peak was set as the threshold. Images were discarded for this analysis when noise in the image or saturation was significant. Analysis was carried out using Velocity software by Improvision (Coventry, UK).

**Acceptor Bleaching Fluorescence Resonance Energy Transfer**

Immunohistochemical labeling of isolated ventricular myocytes was performed as above. Secondary Abs with one of the FRET pair Alexa 555 (donor) or Alexa 647 (acceptor) were used. This FRET pair has a Förster radius of 5.1 nm (Molecular Probes). The acceptor and donor fluorophores were excited with 543- and 653-nm light, and emissions were measured at 565–620 and 650–750 nm, respectively. Firstly, separate images were acquired for the individual fluorophore emissions (Alexa 555 and 647). Following this, excitation of the acceptor fluorophore (647-nm light only) for ~5 min to photobleach the fluorophore was performed. After acceptor photobleaching, fluoroscence emission from the donor fluorophore (Alexa 555) was recorded, using identical gains to those before acceptor photobleaching. Even at increased gains no fluorescence emission was observed from the acceptor after photobleaching. Any increase in emitted fluorescence from the donor fluorophore after acceptor photobleaching indicates FRET between donor and acceptor fluorophores was occurring prior to photobleaching. The occurrence of FRET between this particular pair of fluorophores requires a distance between them of 5.1 nm or less.

**Electrophysiology**

Single ventricular myocytes were isolated as above. The membrane currents were recorded at room temperature using the whole cell patch clamp technique (16). Solutions used for recording \(I_{\text{Ko}}\) were as follows, superfusion solution 132 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 5 mM glucose, and 10 mM HEPES, pH 7.4, plus E-4031 (5 μM) to block I\(_{\text{Ks}}\) and nisoldipine (1 μM) to block I\(_{\text{CaL}}\). Patch pipettes of nominal resistance of 0.5–3 MΩ were used and were filled with an internal solution of 110 mM aspartic acid (potassium salt), 5 mM ATP-K\(_2\), 11 mM EGTA, 10 mM HEPES, 1 mM CaCl\(_2\), and 1 mM MgCl\(_2\), pH 7.3. Whole cell permeabilization (17) was used to study the effects of inhibiting cAMP dependent protein kinase, the selective and potent inhibitor of PKA, H-89 (Sigma) (5 μM) was included in the \(I_{\text{Ko}}\) superfusion solution described above. In experiments to study \(I_{\text{Ko}}\) membrane potential was held at −65 mV with prepulse depolarizations from −40 mV to +60 mV for 2 s at 0.067 Hz before a 2-s test pulse to −40 mV. To ensure \(I_{\text{Ko}}\) stabilization after the membrane rupture or drug application, \(I_{\text{Ko}}\) was monitored during prepulse depolarizations (2 s) to +60 mV followed by a return pulse (2 s) to −40 mV at 0.067 Hz. Unless otherwise noted, isochronal activation of \(I_{\text{Ko}}\) was studied by analysis of activating tail currents recorded at −40 mV after 2-s depolarizing pulses in 20-mV increments. \(I_{\text{Ko}}\) tail currents resulting from this test pulse were recorded. In experiments to study the effects of inhibiting CAMP dependent protein kinase, the selective and potent inhibitor of PKA, H-89 (Sigma) (5 μM) was included in the \(I_{\text{Ko}}\) superfusion solution described above. In experiments to study \(I_{\text{Ko}}\), membrane potential was held at −40 mV to increase m, which was measured by a series of potentials (10-mV increments) at 0.2 Hz from −40 mV to +60 mV. To examine the effects of cAMP upon \(I_{\text{Ko}}\), the membrane permeable cAMP analog (pCPT-CAMP) (Sigma) (0.5 mM) was added to the superfusion solution. To ensure \(I_{\text{Ko}}\) stabilization after membrane rupture or drug application, \(I_{\text{Ko}}\) was monitored by depolarizations (150 mV) every 20 ms at 0.2 Hz. The initial peak amplitudes were digitally subtracted by the P/N method (\(N = 6\)). Note that the representative traces shown in the figures are raw traces without subtracting the leak currents. The activation curves were fitted with Boltzmann equations to determine the influence of \(\beta\)-AR stimulation, overexpression of \(\beta_2\)-AR, or effect of drugs on the voltage for which the half of channels are available (\(V_1/2\)) and the slope factor for the curve (\(\Delta\)).

**Molecular Biology**

**Immunoprecipitation and PKA Back-phosphorylation of KCNQ1**

**Channels—Heart homogenates were prepared by homogenizing −1.0 g of cardiac tissue in 1.0 ml of a buffer containing 10 mM Tris-maleate, 10 mM NaF, 1.0 mM NaVO\(_4\), and protease inhibitors (complete tablets from Roche Applied Science), pH 7.0. The samples were centrifuged at 3,000 \(\times\) g for 10 min, and the supernatants were centrifuged at 12,000 \(\times\) g for 20 min. After detergent and protein concentration of the supernatants, the samples were aliquoted and stored at −80°C until use. The cardiac KCNQ1 channel was immunoprecipitated from heart samples by incubating 500 μg of homogenerate with anti-KCNQ1 antibody (Santa Cruz) in 0.5 ml of a modified radiomimule precipitations assay buffer (containing 50 mM Tris-HCl, 0.8% NaCl, 5.0 mM NaF, 1.0 mM NaVO\(_4\), 0.25% Triton X-100, and 10% glycerol) for 4 h at 4°C. The samples were incubated with protein A-Sepharose beads (Amersham Biosciences) at 4°C for 1 h, after which the beads were washed three times with 1x kinase buffer containing 8 mM MgCl\(_2\), 10 mM EGTA, and 50 mM Tris/piperazine-N,N′-bis(2-ethanesulfonic acid) (TPEA) (pH 7.0).
acid), pH 6.8. After resuspending the beads in 10× kinase buffer containing 5 units of PKA catalytic subunit (Sigma), back-phosphorylation of the immunoprecipitated KCNQ1 was initiated with 5 µl of 100 mM Mg-ATP containing 10% \( ^{32} \text{P} \)ATP (PerkinElmer Life Sciences). The reaction was terminated after 8 min at room temperature with 5 µl of stop solution (4% SDS and 0.25 M dithiothreitol). The samples were heated to 95°C, size-fractionated on 10% PAGE, and KCNQ1 radioactivity was quantified using a Molecular Dynamics phosphorimaging and ImageQuant software (Amersham Biosciences). The resulting value was divided by the amount of KCNQ1 protein and expressed as the inverse of the PKA-dependent \(^{32}\text{P} \) signal.

**Western Analysis**—The proteins were size-fractionated on 10% SDS-PAGE for KCNQ1, and the immunoblots were developed using anti-KCNQ1 Ab (custom made by Zymed Laboratories Inc., San Francisco, CA) (18) using (GARRGpSAGL) as the antigenic peptide diluted in 5% milk/Tris-buffered saline/Tween. Immunoblot signals were quantified by densitometry.

**Statistics**

Graphical and statistical data analysis was carried out using Origin 7.0 software (Microcal, Northampton, MA), Excel (Microsoft), and Clampfit 8.2 (Axon Instruments, Inc.). The data are presented as the mean values ± S.E. Statistical significance was assessed with Student’s \( t \) test for simple comparisons; differences at \( p < 0.05 \) were considered to be significant. Two-sample comparisons were performed using unpaired Student’s \( t \) test.

**RESULTS**

Localization of KCNQ1 and \( \beta_2 \)-AR in Murine Ventricular Myocytes—Using double labeling immunohistochemical techniques combined with laser scanning confocal microscopy KCNQ1 and \( \beta_2 \)-AR were found to be in similar subcellular localizations in ventricular myocytes. In cells isolated from the...
PKA Phosphorylation of KCNQ1

Fig. 2. Intimate localization of KCNQ1 and β2-AR shown by acceptor bleaching FRET. Immunohistochemistry performed as for Fig. 1; however, secondary antibodies used were conjugated to the fluorophores Alexa 555 or Alexa 647. A, fluorescence emissions from Alexa 555 conjugated to a secondary antibody specific for primary antibody against KCNQ1. Alexa 555 was excited with 543-nm light, and emission was measured at 565–620 nm. B, fluorescence emissions from Alexa 647 excited with 633-nm light and emission measured at 650–750 nm. C, overlay of A and B showing co-localization of KCNQ1 and β2-AR. D, fluorescence emission from Alexa 555, excited with 543 nm light and emissions measured at 565–620 nm after photobleaching of Alexa 647 fluorophore. Any increase in the intensity of fluorescence emission is indicative of FRET taking place before acceptor photobleaching (compare A and D). E, fluorescence emission from Alexa 647, excited with 633-nm light and emissions measured at 650–750 nm after photobleaching of Alexa 647 fluorophore. Emissions in D and E are acquired at the same gain as those in A and B. After continual illumination of Alexa 647– with 633-nm light for 5 min, no fluorescence emission was observed even at much higher PMT gains. F, difference image (D − A) showing subcellular location of occurrence of FRET between Alexa 555 and Alexa 647.

hKCNQ1-hKCNE1 (TG⁺) mice, KCNQ1 was localized to the intercalated disc (ICD) regions, the surface sarcolemmal membrane (SSM), and the transverse (T-) tubules (see arrows A–C in Fig. 1A, panel ib). In these myocytes β2-AR were also located in ICD, SSM, and T-tubules (Fig. 1A, panel ib). In ventricular myocytes from DTG⁺ mice, expressing hKCNQ1-hKCNE1 and overexpressing β2-ARs, a similar localization for KCNQ1-KCNE1 and β2-ARs to the ICD, SSM, and T-tubules was observed (Fig. 1B). KCNQ1 and β2-AR were determined to be highly co-localized in myocytes from both TG⁺ and DTG⁺ mice. However, the resolving power of conventional confocal microscopy is limited to ~0.2 μm. With cardiac T-tubules being up to 300 nm in diameter, it would be possible for proteins to appear co-localized without in fact having an intimate association or localization with each other. To investigate this further we made use of FRET to provide substantially increased spatial resolution.

Acceptor Bleaching FRET between Immunohistochemically Labeled KCNQ1 and β2-AR in Murine Ventricular Myocytes—Using dual labeling immunohistochemical techniques and acceptor bleaching FRET methods combined with laser scanning confocal microscopy, an intimate localization of KCNQ1 and β2-AR within nanometers of each other was seen. For the particular pair of fluorophores used here the distance between them must be 5.1 nm or less for FRET to occur. Any increase in donor fluorescence after acceptor photobleaching indicates the occurrence of FRET before photobleaching. The increased intensity of donor fluorescence seen after acceptor photobleaching (Fig. 2, compare A and D; also see Fig. 2F) indicates there is an intimate and close localization between KCNQ1 and β2-AR, within nanometers of each other (Fig. 2).

Effect of β-AR Stimulation on IKs in Ventricular Myocytes from hKCNQ1-hKCNE1 Mice—We have previously shown that IKs channels are regulated in the hKCNQ1-hKCNE1 mouse in response to PKA stimulation (9). As indicated in Fig. 3, stimulation of β-AR using the β-AR agonist isoproterenol (ISO) increases IKs in myocytes isolated from hKCNQ1-hKCNE1 mice, as reflected in the increase of outward current measured in response to depolarization and in the decay of outward current (tail) after termination of depolarizing pulses. IKs tail currents in myocytes from hKCNQ1-hKCNE1 mice were significantly (p < 0.05) increased for all prepulse potentials examined. In addition to the increase in IKs tail current, ISO induced a hyperpolarizing shift in the IKs activation curve (Fig. 3 and Table I). Thus, the hKCNQ1-hKCNE1 channel is regulated by agonist-induced β-AR stimulation in the TG⁺ mice in a manner strikingly similar to the effects of β-AR stimulation on IKs in other species, such as guinea pig, where a similar channel is endogenously expressed (6, 19). We next asked whether or not similar IKs regulation could be detected in myocytes from DTG⁺.
Fig. 3. Effect of β-AR stimulation on $I_{Ks}$ in ventricular myocytes from hKCNQ1-hKCNE1 mice. A, voltage dependence of $I_{Ks}$ current density (pA/pF). $I_{Ks}$ tail current density in response to depolarizing step to $-40$ mV after prepulse steps to potentials from $-40$ mV to $+80$ mV in ventricular myocytes from hKCNQ1-hKCNE1 mice in the absence ($n = 10$) and presence ($n = 10$) of extracellular ISO (1 μM). With the addition of ISO $I_{Ks}$ is significantly ($p < 0.05$) increased compared with control for all prepulse potentials. The sample current traces recorded after $+60$ mV prepulse are shown in the inset. B, voltage dependence of normalized $I_{Ks}$ tail currents showing the voltage dependence of channel activation. With the addition of ISO a hyperpolarizing shift in activation of $I_{Ks}$ is seen, with the channel being activated at more negative potentials. Half-maximal activation ($V_1/2$) for control is $41.0 \pm 3.8$ mV in contrast to $28.7 \pm 4.1$ mV in the presence of ISO, a hyperpolarizing shift in $V_1/2$ of 12.3 mV. The asterisks indicate $p < 0.05$. Inset scale bars represent 1 s (abscissa) and 10 pA/pF (ordinate).

### Table I

| hKCNQ1-hKCNE1 | $V_1/2$ | $I_{max}$ | $I_{tail}$ | n   |
|---------------|--------|-----------|-----------|-----|
| Control      | 41.0 ± 3.8 | 17.4 ± 0.8 | 5.5 ± 1.0 | 3.9 ± 0.7 | 10 |
| ISO          | 28.7 ± 4.1* | 20.6 ± 1.3 | 7.4 ± 1.3* | 5.8 ± 1.0* | 10 |

*p < 0.05 versus control (paired t test).

### Results

mice with overexpression of β2-AR in addition to expression of hKCNQ1-hKCNE1 channels.

**I$_{Ks}$ in Ventricular Myocytes from hKCNQ1-hKCNE1 and hKCNQ1-hKCNE1/β$_{2}$-AR Mice**—We characterized $I_{Ks}$ in ventricular myocytes from hKCNQ1-hKCNE1 and hKCNQ1-hKCNE1/β$_{2}$-AR (DTG$^+$) mice. We detected robust expression of $I_{Ks}$ in these cells and found that $I_{Ks}$ current density was consistently greater in DTG$^+$ myocytes compared with TG$^+$ (hKCNQ1-hKCNE1) myocytes. $I_{Ks}$ tail current density was significantly ($p < 0.05$) greater in ventricular myocytes from hKCNQ1-hKCNE1/β$_{2}$-AR mice at all prepulse potentials examined. In addition to a larger $I_{Ks}$ tail current, a hyperpolarizing shift in the normalized $I_{Ks}$ tail current was recorded in myocytes from hKCNQ1-hKCNE1/β$_{2}$-AR mice (Fig. 4 and Table II). Thus, similar to β-AR stimulation in hKCNQ1-hKCNE1 myocytes, overexpression of β$_{2}$-AR produces an increase in $I_{Ks}$ and altered channel gating even in the absence of exogenous agonist. Overexpression of β$_{2}$-AR resulted in significantly ($p < 0.05$) increased current density (~135%), and activation was shifted in the hyperpolarizing direction in myocytes from DTG$^+$ mice compared with TG$^+$ mice expressing the hKCNQ1-hKCNE1 transgene alone (Table III). We found no further increase in $I_{Ks}$ density or change in activation voltage dependence with β-AR stimulation in DTG$^+$ mice (Fig. 5 and Table III), suggesting that β$_{2}$-AR overexpression results in maximal activation of $I_{Ks}$. These results suggest that overexpression of β$_{2}$-AR results in maximal up-regulation of $I_{Ks}$ without the application of additional β-AR agonist.

**PKA Phosphorylation of hKCNQ1 in hKCNQ1-hKCNE1/β$_{2}$-AR Mice**—Functional data from cardiomyocytes isolated from the transgenic mice indicate that $I_{Ks}$ channels are maximally stimulated by β-ARs. These results suggest that this channel complex may be PKA-phosphorylated in the mouse model. To address this possibility we used back-phosphorylation experiments to determine the relative PKA phosphorylation of KCNQ1/KCNE1 channels in our DTG$^+$ mice. In this procedure immunoprecipitated proteins are incubated with radioactively labeled ATP. The more PKA-phosphorylated a protein, the less radioactive phosphate it will be able to incorporate. Back-phosphorylation experiments revealed greater PKA phosphorylation of KCNQ1 in DTG$^+$ mice overexpressing β$_{2}$-ARs compared with PKA phosphorylation of KCNQ1 in our TG$^+$ mice, consistent with the predictions of our functional data (Fig. 6).

**Inhibition of PKA in Myocytes from hKCNQ1-hKCNE1/β$_{2}$-AR Mice**—Results from the above back-phosphorylation experiments show KCNQ1 phosphorylation to be increased in the presence of overexpression of β$_{2}$-AR. Consistent with functional data this increased phosphorylation of KCNQ1 is thought to result from the action of PKA, which becomes activated as a...
Fig. 4. $I_{Ks}$ in ventricular myocytes from hKCNQ1-hKCNE1 and hKCNQ1-hKCNE1/β2-AR mouse hearts. A, voltage dependence of $I_{Ks}$ current density (pA/pF). $I_{Ks}$ tail current density in response to depolarizing step to $-40$ mV after pre-pulse steps to potentials from $-40$ mV to $+80$ mV in hKCNQ1-hKCNE1 ($n = 8$) and hKCNQ1-hKCNE1/β2-AR ($n = 6$) myocytes. In myocytes from DTG+ mice $I_{Ks}$ is significantly ($p < 0.05$) greater at all potentials examined above $-40$ mV. Sample currents are shown in the inset. B, voltage dependence of normalized $I_{Ks}$ tail currents showing the voltage dependence of channel activation. In addition to greater current density in myocytes from DTG+ mice a negative shift in activation of $I_{Ks}$ is also seen, with the channel being activated at more negative potentials. Half-maximal activation ($V_{1/2}$) for $I_{Ks}$ in myocytes from mice is $36.2 \pm 5.0$ mV in contrast to $15.2 \pm 2.7$ mV for hKCNQ1-hKCNE1/β2-AR myocytes. The asterisks indicate $p < 0.05$. Inset scale bars represent $1$ s (abscissa) and $20$ pA/pF (ordinate).

Effects of overexpression of β2-AR on $I_{Ks}$ channel availability and amplitude in hKCNQ1-hKCNE1 myocytes

| TG/DTG | $V_{1/2}$ | $k$ | $I_{max}$ | $I_{tail 50mV}$ | $n$ |
|--------|-----------|-----|-----------|-----------------|-----|
| hKCNQ1-hKCNE1 | $36.2 \pm 5.0$ | $15.4 \pm 1.6$ | $5.1 \pm 0.8$ | $4.0 \pm 0.7$ | $8$ |
| hKCNQ1-hKCNE1/β2-AR | $15.2 \pm 2.7$ | $16.2 \pm 1.9$ | $10.0 \pm 2.3$ | $9.2 \pm 1.7$ | $6$ |

$^a p < 0.05$ versus control (Student’s t test).

Effects of ISO on $I_{Ks}$ channel availability and amplitude in hKCNQ1-hKCNE1/β2-AR myocytes

| hKCNQ1-hKCNE1/β2-AR | $V_{1/2}$ | $k$ | $I_{max}$ | $I_{tail 60mV}$ | $n$ |
|----------------------|-----------|-----|-----------|-----------------|-----|
| Control              | $16.4 \pm 2.9$ | $16.8 \pm 2.3$ | $10.4 \pm 2.8$ | $9.4 \pm 2.5$ | $5$ |
| ISO                  | $15.2 \pm 4.3$ | $17.7 \pm 1.3$ | $9.4 \pm 2.7$ | $8.3 \pm 1.7$ | $5$ |

The maximal activation of $I_{Ks}$ observed in ventricular myocytes from hKCNQ1-hKCNE1/β2-AR mice.

Effect of overexpression of β2-AR on $I_{CaL}$ in Ventricular Myocytes—The maximal activation of $I_{Ks}$ seen with the overexpression of β2-AR may simply be the result of a global increase in intracellular cAMP or alternatively a more localized and specific increase that selectively modulates the $I_{Ks}$ channel. To examine this possibility we recorded L-type Ca2+ channel currents $I_{CaL}$ in ventricular myocytes isolated from mice overexpressing β2-AR, both with and without hKCNQ1-hKCNE1, in a cardiac-specific fashion. The first indication that $I_{Ks}$ modulation with β2-AR overexpression is localized and specific was the lack of modulation of $I_{CaL}$ seen with β2-AR overexpression. $I_{CaL}$ density and the activation voltage dependence was the same for myocytes from wild type, β2-AR overexpressing and hKCNQ1-hKCNE1/β2-AR mice (Fig. 8A). The addition of pCPT-cAMP (a membrane-permeable cAMP analog) to myocytes from hKCNQ1-hKCNE1/β2-AR mice produced a significant and characteristic increase in the magnitude of $I_{CaL}$ and hyperpolarizing shift (12 mV) in activation (Fig. 8B). Similar results were observed in ventricular myocytes from mice overexpressing β2-AR alone (data not shown). These results indicate the expression of $I_{Ks}$ does not affect modulation of $I_{CaL}$, and that $I_{CaL}$ remains fully responsive to cAMP in the presence of β2-AR overexpression. In addition these results indicate that the modulation of $I_{Ks}$ as a result of β2-AR overexpression is localized and specific and not simply the result of a global increase in cAMP.
**DISCUSSION**

**Intimate Localization of KCNQ1 and β₂-AR in TG⁻ Mice**—In ventricular myocytes isolated from TG⁻ mice expressing the fusion protein hKCNQ1-hKCNE1, KCNQ1 is localized in T-tubular membranes, at the surface sarcolemmal membrane and at the intercalated discs. This distribution throughout all...
PKA Phosphorylation of KCNQ1 in Mice Overexpressing the β2-AR—Here we extend the analysis of putative substrates for PKA phosphorylation that may be modulated in chronic activation of the stress response and show, for the first time, that like RyR2 (24), the KCNQ1-KCNE1 channel is also PKA-phosphorylated in a transgenic mouse in which the human KCNQ1-KCNE1 channel and hβ2-AR are overexpressed. Using patch clamp analysis of myocytes isolated from DTG+ mice, we found that overexpression of β2-AR increases Ik, causing a hyperpolarizing voltage shift in channel activation, and renders the channels insensitive to further modulation by addition of external β-AR agonist. Thus, overexpression of β-ARs alone can stimulate downstream signaling that results in sustained PKA phosphorylation of substrates (hKCNQ1 channel in this case). It is inter-
testing to note that the functional coupling between H9252-AR and IKs seen in adult myocytes is fundamentally different from that seen during development when IKs is not affected by overexpression of H9252-ARs (25), indicating that changes in H9252-AR coupling to effecter targets occur during developmental changes.

Localized and Specific Modulation of IKs in Mice Overexpressing H9252-AR—The maximal activation of IKs seen in myocytes overexpressing H9252-AR may simply be the result of a global increase in cAMP. To investigate this possibility we examined ICaL in ventricular myocytes from mice overexpressing H9252-ARs (both with and without hKCNQ1-hKCNE1). Using patch clamp analysis we found no basal modulation of ICaL as a result of overexpressing H9252-AR. The addition of pCTP-cAMP produced a robust increase in both the peak current and a hyperpolarizing shift in ICaL activation. This is in contrast to IKs, which was found to be maximally activated under basal conditions and not responsive to further stimulation in myocytes from DTG H11001. These results show the modulation of IKs is not simply the result of a global increase in cAMP and that IKs modulation by overexpression of H9252-AR is localized and specific. Further, we find that modulation of IKs occurs under conditions of increased H9252-AR expression, even in the absence of exogenous H9252-AR agonist.

Chronic PKA Phosphorylation of Targets in Failing Human Hearts—Heart failure has been described as a maladaptive activation of the classic “fight or flight” stress response that occurs as a consequence of a systemic response to maintain cardiac output in the face of decreasing cardiac contractile performance (26). One consequence of the chronic maladaptive response has been shown to be PKA hyperphosphorylation of fig. 8. L-type Ca²⁺ current (ICaL) in ventricular myocytes from hKCNQ1-hKCNE1/H9252-AR mouse hearts is sensitive to cAMP stimulation. A, comparison of ICaL activation between myocytes from wild type (WT) (open square), β2-AR overexpressing (filled circle), and hKCNQ1-hKCNE1/β2-AR (filled square) mice. Peak current-voltage relationships (left) measured during depolarizing pulses (150 ms) from −40 mV to +60 mV (10-mV increments) from a holding potential of −40 mV were not changed by β2-AR overexpression or expression of hKCNQ1-hKCNE1. Representative raw traces (middle panel) recorded at 0 mV test potential show similar inactivation kinetics for ICaL in all three types of myocytes. Scale bars indicate 5 pA/pF and 50 ms. The voltage dependence of ICaL activation (right panel) was plotted by normalizing the current-voltage relationship to driving force. Half-maximal activation (IV₁/₂) is −1.9 ± 2.7 mV (n = 10) for WT, −1.0 ± 1.6 mV (n = 16) for β2-AR, and −2.0 ± 1.1 mV (n = 7) for hKCNQ1-hKCNE1/β2-AR myocytes (p = not significant). B, effect of cAMP stimulation on ICaL in ventricular myocytes from hKCNQ1-hKCNE1/β2-AR mice. Peak current-voltage relationships (left panel) were obtained from seven cells as described in A before (control, open circle) and after (pCPT-cAMP, filled circle) a 5-min external application of pCPT-cAMP (0.5 mM). With the addition of pCPT-cAMP, peak ICaL amplitudes over depolarizing pulses (−30 to +60 mV) were increased (left panel). Representative raw traces (middle panel) recorded at 0 mV test potential are superimposed before and after the application of pCPT-cAMP. Capacitative currents greater than 1 pA/pF are truncated. Scale bars indicate 5 pA/pF and 50 ms. Voltage dependence of ICaL activation (right panel) shows a hyperpolarizing shift in the presence of pCPT-cAMP. Half-maximal activation (IV₁/₂) for control is −2.0 ± 1.1 mV and −14 ± 2.5 mV in the presence of pCPT-cAMP. The asterisks indicate p < 0.05 versus control.
the cardiac ryanodine receptor, RyR2, which results in altered calcium homeostasis (an aberrant diastolic sarcoplasmic reticulum calcium leak) in the heart (24, 27, 28). Selective down-regulation of β2-AR results in a relative increase in the abundance of β2-ARs (29). PKA phosphorylation of RyRs has been observed in heart failure (26). Moreover, RyRs are seen to be PKA-phosphorylated in β2-AR overexpressing mice (24). Our results complement those previously obtained for the RyR and show that a second PKA substrate, the I$_{Ks}$ channel, is also chronically PKA-phosphorylated in the β2-AR overexpressing mouse. It will be of interest to determine whether, like the RyR, the I$_{Ks}$ channel is also PKA-phosphorylated in failing human heart where, if so, it would act to counter the documented action potential prolongation in this disorder. This observation in addition to the fact that a relative increase in the abundance of β2-ARs is seen in heart failure prompts the question: are other end effectors of β-AR stimulation, namely the KCNQ1/ KCNE1 channel, PKA-phosphorylated under these conditions?

In summary, KCNQ1 and β2-AR are localized to the intercalated discs, surface sarcolemma, and transverse tubules of isolated ventricular myocytes. In these subcellular regions KCNQ1 and β2-AR are closely associated, lying within nanometers of each other. This intimate association results in a functional coupling as shown by overexpression of β2-AR in the heart, which results in PKA phosphorylation of KCNQ1 and maximal activation of I$_{Ks}$. Conversely, β2-AR overexpression did not affect I$_{Ks}$ under basal conditions with I$_{Ks}$ remaining responsive to cAMP. These data indicate intimate association of KCNQ1 and β2-AR and that β2-AR signaling can modulate the function of KCNQ1 in a localized and specific fashion with up-regulation of the slow delayed rectifier potassium channel current (I$_{Ks}$) with chronic activation of β2-AR, suggesting functional coupling between I$_{Ks}$ channels and β2-ARs with modulation of I$_{Ks}$ occurring under conditions of increased β2-AR expression, even in the absence of exogenous β-AR agonist. As such, cardiomyocytes overexpressing β2-AR may provide a good system to examine effects on ion channel function under conditions of chronic activation of β-AR. Maximal activation of I$_{Ks}$ seen under these conditions may act as a physiological brake to counter action potential duration prolongation during stress.

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Overexpression of β2-Adrenergic Receptors cAMP-dependent Protein Kinase Phosphorylates and Modulates Slow Delayed Rectifier Potassium Channels Expressed in Murine Heart: EVIDENCE FOR RECEPTOR/CHANNEL CO-LOCALIZATION

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