Remodeling of excitation-contraction coupling in transgenic mice expressing ATP-insensitive sarcolemmal K\textsubscript{ATP} channels

Thomas P. Flagg, Thomas P., Flavien Charpentier, Jocelyn Manning-Fox, Maria Sara Remedi, Decha Enkvetchakul, Anatoli Lopatin, Joseph Koster, and Colin Nichols

1Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110; 2Institut National de la Santé et de la Recherche Médicale Unité 533, Physiopathologie et Pharmacologie Cellulaires et Moléculaires, Faculté de Médecine, 44035 Nantes, France; 3Department of Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109; and 4Department of Pharmacology, University of Alberta, Edmonton T6G 2H7, Alberta, Canada

Submitted 25 July 2003; accepted in final form 2 December 2003

Flagg, Thomas P., Flavien Charpentier, Jocelyn Manning-Fox, Maria Sara Remedi, Decha Enkvetchakul, Anatoli Lopatin, Joseph Koster, and Colin Nichols. Remodeling of excitation-contraction coupling in transgenic mice expressing ATP-insensitive sarcolemmal K\textsubscript{ATP} channels. Am J Physiol Heart Circ Physiol 286: H1361–H1369, 2004. First published December 4, 2003; 10.1152/ajpheart.00676.2003.—Reducing the ATP sensitivity of the sarcolemmal ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channel is predicted to lead to active channels in normal metabolic conditions and hence cause shortened ventricular action potentials and reduced myocardial inotropy. We generated transgenic (TG) mice that express an ATP-insensitive K\textsubscript{ATP} channel mutant [Kir6.2(ΔN2–30,K185Q)] under transcriptional control of the α-myosin heavy chain promoter. Strikingly, myocyte contractile amplitude was increased in TG myocytes (15.68 ± 1.15% vs. 10.96 ± 1.49%), even though K\textsubscript{ATP} channels in TG myocytes are very insensitive to inhibitory ATP. Under normal metabolic conditions, steady-state outward K\textsuperscript{+} currents measured under whole cell voltage clamp were elevated in TG myocytes, consistent with threshold K\textsubscript{ATP} activation, but neither the monophasic action potential measured in isolated hearts nor transmembrane action potential measured in right ventricular muscle preparations were shortened at physiological pacing cycles. Taken together, these results suggest that there is a compensatory remodeling of excitation-contraction coupling in TG myocytes. Whereas there were no obvious differences in other K\textsuperscript{+} conductances, peak L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca}) density (−16.42 ± 2.37 pA/Pf) in the TG was increased compared with the wild type (−8.43 ± 1.01 pA/Pf). Isooproterenol approximately doubled both I\textsubscript{Ca} and contraction amplitude in wild-type myocytes but failed to induce a significant increase in TG myocytes. Baseline and isoproterenol-stimulated Ca\textsuperscript{2+} conductances were not different in wild-type and TG hearts, suggesting that the enhancement of I\textsubscript{Ca} in the latter does not result from elevated Ca\textsuperscript{2+} entry. Collectively, the data demonstrate that a compensatory increase in I\textsubscript{Ca} counteracts a mild activation of ATP-insensitive K\textsubscript{ATP} channels to maintain the action potential duration and elevate the inotropic state of TG hearts.

ATP-sensitive K\textsuperscript{+} channels; contractility; action potential

The coordinated action of many ion channels and transporters contributes to the generation of the cardiac action potential (AP) that triggers myocyte contraction. Derangements of any one of these channels can lead to life-threatening arrhythmias and/or heart failure. The discovery of disease-causing mutations and the use of transgenic (TG) mice has been instrumental in delineating the molecular details of cardiac ion channels. For example, mutations in a number of K\textsuperscript{+} channels have been genetically linked to the long Q-T syndrome, thereby clarifying the role of this channel in AP repolarization (8). Similarly, TG overexpression of the L-type voltage-dependent Ca\textsuperscript{2+} channel has illuminated a potential role for this essential cardiac channel in the development of hypertrophy and heart failure (16).

The physiological role of one cardiac ion channel, the ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channel in the sarcolemma (21), has thus far remained elusive. Under normal metabolic conditions, K\textsubscript{ATP} channels are not significantly open and thus do not contribute to AP repolarization and excitation-contraction coupling (5, 19, 27, 28). When myocytes are exposed to a severe metabolic stress (anoxia, hypoxia, metabolic inhibition, ischemia, etc.), K\textsubscript{ATP} channels do open, causing AP shortening and contractile failure (13, 27, 28). However, the precise trigger for K\textsubscript{ATP} channel activation, the timing of channel opening, and physiological consequences in vivo remain murky, and nature offers no clues because there are no known K\textsubscript{ATP} mutations linked with cardiac disease.

The coexpression of two gene products, Kir6.2 and SUR2A, in heterologous expression systems produces channels with the essential properties of the cardiac sarcolemmal K\textsubscript{ATP} channel (2) and, in vitro, these two proteins physically associate (15). Thus it is now widely accepted that K\textsubscript{ATP} channels are heteromeric protein complexes composed of four Kir6.2 and four SUR2A subunits. The Kir6.2 subunits form the potassium-selective pore and contain determinants of ATP inhibition, phosphatidylinositol 4,5-bisphosphate activation, and inward rectification, whereas the SUR subunits contribute additional determinants of channel regulation (24).

To further probe the function of the cardiac sarcolemmal K\textsubscript{ATP} channel, we recently engineered several TG mouse lines that express an ATP-insensitive Kir6.2 mutant [Kir6.2(ΔN30,K185Q)] under the transcriptional control of the α-myosin heavy chain promoter (10). Four independent TG lines were established, and two have been extensively characterized. In one line (line 4 TG), all isolated myocytes express mutant Kir6.2 subunits, and K\textsubscript{ATP} channels are very insensitive to inhibition by ATP (K\textsubscript{1/2} = 2.7 ± 0.01 mM) compared with...
wild-type (WT) myocytes ($K_{1/2} = 51 \pm 2 \mu M$) (10). In another line (line 2 TG), the expression level varies from cell to cell, and the sensitivity of the sarcolemmal $K_{ATP}$ channels is not as dramatically altered ($K_{1/2} = 514 \pm 5 \mu M$) (10). Because computer modeling studies (18, 25) and experimental simulations (9, 20, 29) predict that activation of only 1% of the $K_{ATP}$ channels in a ventricular myocyte should be sufficient to significantly shorten the AP duration (APD) at 90% repolarization (APD$_{90}$) and reduce contractility, it was expected that, in these TG hearts, the AP would be shortened and contractility would be reduced.

Remarkably, there is no significant electrophysiological impairment in these TG mice (10, 22). Although there is a mild reduction of heart rate and ventricular ectopy as well as an increased incidence of atrioventricular block, electrocardiographic waveforms are similar between WT and TG mice (10). In addition, the AP recorded in isolated cardiac myocytes at room temperature is not shortened (10). Contractility is also not reduced. In fact, left ventricular developed pressure is significantly elevated in isolated TG hearts compared with WT hearts (22).

In the present study, we expand on these initial findings to further examine the electrophysiological and contractile properties of these TG mice. Here, we elucidate one compensatory mechanism that may underlie the altered electrical and contractile function, demonstrating that there is stimulation of the contraction trigger, L-type Ca$^{2+}$ channel current ($I_{Ca,L}$), which can lead to increased contractility and contribute to the absence of AP shortening in TG myocytes.

**MATERIALS AND METHODS**

**Molecular biology.** cDNA mutagenesis and generation of the TG mice have been described previously (10, 12).

**Solutions.** Wittenberg isolation medium (WIM) contained the following (in mM): 116 NaCl, 5.3 KCl, 1.2 NaH$_2$PO$_4$, 11.6 glucose, 3.7 MgCl$_2$, 20 HEPES, 2.0 l-glutamine, 4.4 HACO$_3$, and 1.5 K$_2$HPO$_4$, with 1× essential vitamins (GIBCO-BRL catalog no. 12473-013) and 1× amino acids (GIBCO-BRL catalog no. 11120-052); pH 7.3–7.4.

Normal Tyrode solution contained the following (in mM): 137 NaCl, 5.4 KCl, 0.16 NaH$_2$PO$_4$, 10 glucose, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 5.0 HEPES, and 3.0 NaHCO$_3$; pH 7.3–7.5.

Kreb-Henseleit (KH) solution contained the following (in mM): 118 NaCl, 4.7 KCl, 25 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 1.2 CaCl$_2$, and 10 glucose and was saturated with a 95% O$_2$–5% CO$_2$ gas mixture; pH 7.3–7.4.

Intracellular K$^+$ solution contained the following (in mM): 140 KCl, 5 K$_2$ATP, 10 HEPES, and 10 EGTA; pH 7.3–7.4.

Intracellular Cs$^+$ solution contained the following (in mM): 120 CsCl, 20 TEA-Cl, 5 ATP, and 10 HEPES; pH 7.3–7.4.

**Isolation of cardiac myocytes.** Mice were housed at the animal facility at Washington University and fed a standard diet and water ad libitum. All animal procedures were approved by the Animal Studies Committee at Washington University School of Medicine. Mice were anesthetized by subcutaneous injection with a ketamine-xylazine-acepromazine cocktail. The heart was rapidly excised, and the aorta was cannulated. The heart was retrogradely perfused with a Ca$^{2+}$-free solution for 5 min, followed by perfusion with a digestion solution containing 0.8 mg/ml collagenase (type 2, Worthington Biochemical) and 0.01 mg/ml protease (type XIV, Sigma). Left ventricular cells were gently dispersed by manual trituration using a pasteur pipette. Cells were stored at room temperature in WIM.

**Cellular electrophysiology.** Isolated myocytes were transferred into a recording chamber containing either normal or cesium-containing (replacement of KCl with 5.4 mM CsCl) Tyrode solution. Isoproterenol (Iso; 1 μM) and tetrodotoxin (TTX; 10 μM) were added to the bathing medium where indicated. Macroscopic currents in isolated ventricular myocytes were recorded using standard whole cell voltage-clamp recording techniques. Patch-clamp electrodes (1–3 MΩ when filled with electrode solution) were fabricated from soda lime glass microhematocrit tubes (Kimble 73813). Cell capacitance and series resistance were determined using a 5- to 10-mV hyperpolarizing square pulse from a holding potential of −80 mV after establishment of the whole cell recording configuration. The series resistance was electronically compensated by 60–80%. pCLAMP 6.0 software and a DigiData 1200 converter were used to generate command pulses and collect data. The specific voltage-clamp protocols are described in the figures and text where appropriate. Data were filtered at 5 kHz.

**Single cell contraction measurements.** To assess contractility, un-loaded cell shortening of isolated ventricular myocytes was measured using a video edge detection system (Crescent Electronics). Cells were placed in the recording chamber, bathed with normal Tyrode solution, and stimulated with a bipolar stimulating electrode placed near the cell. Electrodes were constructed by immersing Ag/AgCl wires in Tyrode solution within a 0-glass capillary tube pulled to a fine tip.

**Monophasic AP recording.** Monophasic APs (MAPs) were measured from freshly isolated hearts. After excision, hearts were retrogradely perfused through the aorta with KH solution at a constant pressure of 75–85 cmH$_2$O and submerged in a glass tissue bath that was continuously bathed with KH solution, maintained at 37°C. After an initial equilibration period (15–30 min), MAPs were recorded using a DP-304 differential amplifier (Axon Instruments) and digitized at 3.3 kHz with a Digidata 1322A and pCLAMP 8.0 software. MAP electrodes were constructed from Teflon-coated silver wire (0.01 in. diameter). The exposed tip of the wire was polished with fine sandpaper and plated with AgCl. APD was determined as the time between the maximum upstroke velocity and 30% (APD$_{30}$), 60% (APD$_{60}$), or 90% of full repolarization (APD$_{90}$).

**Microelectrode studies.** Transmembrane recordings were obtained as previously described (4). Briefly, the heart was quickly removed and immersed in a cool modified Tyrode solution containing (in mM) 108 NaCl, 25 NaHCO$_3$, 1.8 NaH$_2$PO$_4$, 27 KCl, 1 MgCl$_2$, 0.6 CaCl$_2$, and 55 glucose, which was saturated with a 95% O$_2$–5% CO$_2$ gas mixture (pH 7.3). The right free ventricular wall was dissected from the heart and sliced to obtain the experimental preparations (1–1.5 mm wide and ~3 mm long). Preparations were mounted in a Lucite tissue chamber with the epicardial surface facing down, so that the endocardial surface could be impaled with a microelectrode. The preparation was superfused with oxygenated (95% O$_2$–5% CO$_2$) Tyrode solution warmed to 37 ± 0.5°C containing (in mM) 120 NaCl, 27 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 5.4 KCl, 1.2 MgCl$_2$, 1.8 CaCl$_2$, and 10 glucose (pH 7.4). The flow rate in the tissue chamber was 10 ml/min. The tissue was allowed to recover for at least 1 h before the experiment. During this period, the tissue was paced at a cycle length of 200 ms by bipolar stimulation through Teflon-coated silver wire electrodes. The stimulus pulse width was 1.5 ms, and the amplitude was twice threshold. AP characteristics were measured at the steady state for each pacing cycle length. APDs were measured at APD$_{30}$, APD$_{60}$, and APD$_{90}$.

**Radioimmunoassay to detect cAMP.** Hearts were rapidly excised and retrogradely perfused for 10 min with Ca$^{2+}$-free WIM (±1 μM Iso). After perfusion, the ventricles were immediately removed and submerged in liquid nitrogen. The tissue was homogenized in 0.3 M perchloric acid. After centrifugation, the supernatant was neutralized with K$_2$HPO$_4$, and the cAMP concentration was assessed by radioimmunoassay (Amersham) following the manufacturer’s protocols.

**Real-time RT-PCR.** The relative expression of Ca$_{1.2}$ and Kir6.2 mRNA in TG compared with WT hearts was assessed using real-time RT-PCR (3). Total RNA was isolated from cardiac ventricles using TRIzol (Invitrogen) following the manufacturer’s protocols. The iso-
lated RNA was then further purified using a silica-based column protocol (RNasey, Qiagen). The RNA concentration was determined spectrophotometrically (Nanodrop Technologies). RT and PCR were carried out in a single tube in an ABI Prism 7000 sequence detection system (Applied Biosystems); 100 ng of template RNA were used in all reactions. After the RT reaction (30 min, 48°C), 40 cycles of PCR were carried out. Double-stranded DNA was fluorescently labeled with SYBR green (Applied Biosystems). Gene-specific primers for Cav1.2 and Kir6.2 were designed using PrimerExpress software (ABI) and purchased from Integrated DNA Technologies. β-Actin control primers were obtained from Ambion. Reactions with each primer pair and template were performed in triplicate or quadruplicate. After baseline correction, a fluorescence threshold was established, and the cycle when this threshold was crossed (Ct) was determined for each reaction. To control for variability in RNA quantity, the normalized value, (ΔCt) for each sample was determined using the formula ΔCt = Ct(Actin) - Ct(Cav1.2/Kir6.2). The relative expression in TG tissue (normalized to WT tissue) was then determined using the following relationship: gene expression = 2-ΔΔCt, where ΔΔCt = ΔCt (WT) - ΔCt (TG).

Data analysis. All data were analyzed using ClampFit and Microsoft Excel software, and (except where noted in the figures) results are presented as means ± SE. Statistical tests and P values are denoted in the figures where appropriate.

RESULTS

Ventricular AP is not shortened in TG muscle. In agreement with our initial findings in isolated TG myocytes at room temperature (10), neither MAPs recorded from isolated Langendorff-perfused hearts nor transmembrane APs recorded in right ventricular muscle preparations were shortened in TG mice compared with WT controls at 37°C. Representative and averaged MAP recordings obtained in un paced WT and TG hearts are shown in Fig. 1, A and B, respectively. The duration of the AP was not shortened in the TG heart. Instead, the MAP tended to be longer at all phases of repolarization and the plateau phase may have been more pronounced in TG hearts. Figure 2A shows typical transmembrane APs recorded from right ventricular preparations obtained from either control or TG animals stimulated at a physiological (10 Hz, left) or subphysiological (2 Hz, right) frequency. Figure 2B summarizes the data from all experiments. The AP comparison was more complex in these experiments. On one hand, the early phase of repolarization (APD90) was significantly longer in TG mice at all pacing cycle lengths. In contrast, the APD90 in WT mice was markedly shorter than that in WT mice at subphysiological pacing cycle lengths. At physiological pacing rates, however, the APD90 in TG muscle did not differ from that in WT muscle. Other AP characteristics (resting membrane potential, AP amplitude, and upstroke velocity) were similar between the two groups of mice (data not shown).

Contrastility is enhanced in TG myocytes. The increased contractility detected in isolated TG hearts (22) was also observed in isolated myocytes in this study. To assess contractile function, the contraction amplitude of single isolated ventricular cells was measured using video edge detection. Typical results are shown in Fig. 3A. The fractional shortening of line 4 TG myocytes was largely independent of the stimulus frequency over the range of 0.5–2Hz, similar to previously reported studies (1, 26, 30), and was significantly greater (15.68 ± 1.15%, n = 14) than that of control cells (9.86 ± 0.96%, n = 27) at a stimulus frequency of 1 Hz. We also examined the dependence of contraction amplitude on extra-
cellular Ca2+ concentration ([Ca2+]o). The contraction amplitude was assessed in normal Tyrode solution containing 0.3–3.6 mM Ca2+, and the data were fit with a modified Hill equation (see Fig. 3). As shown in Fig. 3B, the [Ca2+]o dependence of the contraction amplitude was significantly shifted in line 4 TG myocytes (K1/2 = 0.89 ± 0.08 mM) compared with control cells (K1/2 = 1.50 ± 0.09 mM).
in line 2 TG mice, these data suggest that the magnitude of the phenotype is commensurate with the level of transgene expression and not simply an artifact of the transgene insertion point.

Compensatory increase in $I_{Ca}$ can account for increased myocardial contractility and maintained APD. The above results collectively suggest that excitation-contraction coupling is significantly altered as a consequence of the altered K$_{ATP}$ channel substrate in TG hearts. Several compensatory mechanisms could conceivably explain the remodeling of excitation-contraction coupling. We began by examining two alternative, but not mutually exclusive, hypotheses that might explain the observed consequences for both the AP waveform and myocardial contractility. First, an alteration in either the amplitude or the kinetics of voltage-dependent K$^+$ currents could prolong the AP. Such a mechanism has previously been proposed to account for the AP prolongation and increased Ca$^{2+}$ transient amplitude induced after myocardial infarction (7, 23). Alternatively, an increase in $I_{Ca}$ could produce both an increase in APD and myocyte contractility. Therefore, we assessed both outward K$^+$ conductances and $I_{Ca}$ in isolated cardiac myocytes using the whole cell voltage-clamp technique.

Representative families of macroscopic currents elicited by 4.5-s depolarizing voltage-clamp pulses (from −50 to +50 mV in 10-mV increments) and the corresponding mean current-voltage relationships for both the peak and steady-state currents are shown in Fig. 4. There were no differences in the inactivation time constants of the fast and slow components of the transient outward K$^+$ current. Both peak and steady-state K$^+$ current were slightly increased in TG myocytes at positive holding potentials. The difference current ($I_{DIFF} = I_{TG} - I_{WT}$; Fig. 4, C and D) revealed a time-independent, weakly inward rectifying current. Such a current is consistent with the predicted activation of K$_{ATP}$ channels given that K$_{ATP}$ channels in TG mice are very insensitive to inhibition by intracellular ATP (10). Because deletion of the NH$_3$ terminal of Kir6.2 abolishes the channels sulfonylurea sensitivity (12), we cannot conclusively demonstrate pharmacologically that this current is the K$_{ATP}$ channel current ($I_{K_{ATP}}$). Regardless, because net outward K$^+$ currents are not decreased, but are actually increased, changes in K$^+$ currents could not explain the lack of AP shortening and enhanced contractility. Collectively, the data suggest that voltage-gated K$^+$ conductances do not differ in WT and TG myocytes.

In contrast to the lack of effect on voltage-gated K$^+$ channels, peak $I_{Ca}$ was increased in both line 4 and line 2 TG myocytes. Representative families of macroscopic Ca$^{2+}$ currents are shown in Fig. 5A. Peak currents (membrane potential = 5 mV) were increased from $-10.46 \pm 1.01$ pA/pF in control myocytes ($n$ = 31) to $-14.72 \pm 1.94$ pA/pF in line 4 TG myocytes ($n$ = 23). The increase was significant at all voltages tested between −25 and +15 mV (Fig. 5B). Ca$^{2+}$ current in line 2 TG myocytes also tended to be elevated (Fig. 5B). As with the functional measurements of cell contractility, $I_{Ca}$ was not significantly different from either WT or line 4 TG, but again fell between the two. Collectively, the data demonstrate that there is a compensatory increase in $I_{Ca}$ in TG myocytes that likely contributes to both maintaining APD and enhancing contractility in TG myocytes.

Calcium channel mRNA expression is not elevated. We examined the possibility that the increase of $I_{Ca}$ resulted from an increased expression of the Ca$^{2+}$ channel gene using real-
time RT-PCR. The transcription of Ca\textsubscript{v}1.2, Kir6.2, and \(\beta\)-actin were assessed in RNA samples obtained from WT (\(n = 3\)) and line 4 TG hearts (\(n = 5\)). The results of these experiments are summarized in Fig. 6. In contrast to the Kir6.2 transcript, which was significantly elevated (156-fold), the expression of Ca\textsubscript{v}1.2 transcripts were not significantly different between the WT and TG hearts, suggesting that an upregulation of channel mRNA does not explain the increased \(I_{Ca}\) in TG myocytes.

\(I_{Ca}\) is prestimulated. Because the expression does not appear to be upregulated, we considered the possibility that \(I_{Ca}\) is hyperactivated in TG mice. As expected, the application of 1 \(\mu\)M Iso to non-TG myocytes caused an increase in the peak amplitude of both contraction (Fig. 7, A and B) and \(I_{Ca}\) (Fig. 7C). In contrast, there was no increase in either \(I_{Ca}\) or contraction in myocytes isolated from TG mice. Because Iso is without effect on the TG myocytes, increased PKA-dependent channel phosphorylation appears a likely mechanism for increased basal \(I_{Ca}\) in TG myocytes. To test the possibility that elevated cAMP underlies this stimulation, isolated hearts were perfused with or without Iso, and [cAMP] in tissue lysate was measured using radioimmunoassay. As shown in Fig. 7D, there was no difference in either basal or Iso-stimulated [cAMP] in WT or TG hearts. While elevated cAMP is thus excluded, the data are consistent with the notion that the chronic activation of sarcolemmal K\textsubscript{ATP} channels causes a prestimulation of \(I_{Ca}\) (through an as-yet-unknown mechanism) that maintains APD and myocardial contractility despite an increase in \(I_{K\text{ATP}}\).

**DISCUSSION**

Compensatory \(K_{\text{ATP}}-I_{Ca}\) coupling in the heart? Whereas most studies have applied a “loss-of-function” TG or knockout strategy to probe the molecular composition and cellular function of cardiac K\textsuperscript{+} channels in vivo (for a review, see Ref. 17), here we describe a “gain-of-function” approach that provides an example of the complex remodeling that can result from the TG expression of a K\textsuperscript{+} channel. In response to the cardiac-restricted overexpression of an ATP-insensitive K\textsubscript{ATP} channel subunit, there is a remodeling of the molecular substrates of
excitation-contraction coupling that produces an unexpected cellular/tissue phenotype. The principal manipulation of the genotype is to introduce a $K_{\text{ATP}}$ channel that is insensitive to inhibition by ATP ($K_i = 1.4$ vs. $25 \mu$M in WT) (10). The resulting enhancement of time-independent outward $K^+$ current in TG myocytes (due to a threshold activation of $I_{\text{K}_{\text{ATP}}}$) is counteracted by an increase in the peak inward $I_{\text{Ca}}$.

Although we cannot rule out additional undetected compensatory mechanisms in the myocardium of TG mice, the increase of $I_{\text{Ca}}$ likely contributes significantly to both of the principal phenotypic findings: the maintained APD and enhanced contractility.

The molecular mechanisms that link increased $I_{\text{K}_{\text{ATP}}}$ to $I_{\text{Ca}}$ remain elusive. RT-PCR analysis suggests that expression of the L-type $Ca^{2+}$ channel is not altered. We have not directly determined the $Ca^{2+}$ channel protein level in TG compared with WT hearts; however, the results with Iso argue against an increase in channel protein because the maximum attainable current in WT myocytes (i.e., the Iso-stimulated current) was not significantly different from the basal current in line 4 TG mice (which was not stimulated by Iso). Because the mRNA levels are certainly not increased and the protein levels are not likely to be increased, the parsimonious conclusion is that the function of the $Ca^{2+}$ channel is altered. The $\beta$-adrenergic agonist Iso failed to elicit an increase in either $I_{\text{Ca}}$ or contractility in TG myocytes, suggesting that $I_{\text{Ca}}$ is prestimulated. However, the basal and Iso-stimulated cAMP concentrations (and presumably PKA activation) are equivalent in WT and TG hearts, implying that the adrenergic-cAMP-PKA axis of signaling is unaltered in the TG heart. We cannot distinguish at this point between an elevated downstream action (e.g., enhanced PKA activity independent of cAMP) or stimulation through a separate convergent pathway.

It should also be noted that there is no evidence for cardiac hypertrophy, as assessed by the heart weight-to-body weight ratio (22) or myocyte capacitance (data not shown) in TG

### Figure 4

Outward $K^+$ currents recorded in isolated ventricular myocytes. A: representative families of macroscopic currents recorded from ventricular myocytes isolated from either control or line 4 TG hearts. Voltage ($V$)-dependent $Na^+$ channels were inactivated by a brief (50 ms) prepulse to $-20 \text{ mV}$. B: slow and fast time constants of inactivation ($\tau_{\text{slow}}$ and $\tau_{\text{fast}}$ respectively) of transient outward $K^+$ current in WT and line 4 TG myocytes. C and D: mean peak ($I_{\text{peak}}$; C, top) and steady-state current ($I_{\text{ss}}$; D, top) densities (normalized to cell capacitance) plotted as a function of voltage. In both cases, $K^+$ current is elevated in TG myocytes (*$P < 0.05$). $I_{\text{DIFF}}$ ($I_{\text{TG}} - I_{\text{WT}}$) is plotted in C and D, bottom. The weakly inward rectifying, time-independent current is consistent with chronic activation of $K_{\text{ATP}}$ channel current.
suggest a central role for $K_{ATP}$ channels in maintaining $Ca^{2+}$ homeostasis.

The striking lack of pathological consequences of cardiac $K_{ATP}$ channel gene manipulation remains in stark contrast to

animals. It has recently been demonstrated that TG mice overexpressing the L-type $Ca^{2+}$ channel $\alpha$-subunit develop an age-dependent hypertrophy, resulting from a sustained increase in $Ca^{2+}$ entry and activation of PKC$\alpha$ (16). At present, it is unknown what mechanisms protect the cell from hypertrophy despite the increased $I_{Ca}$ in our TG mice, but the absence of hypertrophy might suggest that activation of $K_{ATP}$ channels could rescue an $I_{Ca}$-dependent hypertrophy.

$K_{ATP}$ overactivity versus $K_{ATP}$ suppression in the heart and pancreas. A number of recent studies of the cardiac phenotype of Kir6.2$^{-/-}$ mice have recently been reported; however, none have demonstrated evidence of myocardial excitation-contraction coupling remodeling (14, 27, 28, 31). Neither APD nor myocardial contractility differ between WT and knockout animals (27). The principal phenotype of knockout mice is an altered electrophysiological response to metabolic inhibition and ischemia (14, 28) as well as abolition of ischemic preconditioning (28). There is some evidence that $K_{ATP}$ channels may play a role in the $Ca^{2+}$ cycling during periods of enhanced or stressful activity in both skeletal (6) and cardiac muscle (31). This finding, combined with the data presented here, may

Fig. 5. L-type $Ca^{2+}$ channel currents ($I_{Ca}$) in isolated control and TG myocytes. A: representative leak-subtracted families of macroscopic $Ca^{2+}$ current obtained from either control or line 4 TG myocytes. Na$^{+}$ channels were inactivated by a slow voltage ramp from a holding potential of $-80$ to $-45$ mV. In some experiments, tetrodotoxin (10 $\mu$M) was also added to inhibit Na$^{+}$ channels. B: peak $I_{Ca}$ density plotted as a function of voltage. There is a significant increase in peak $I_{Ca}$ in line 4 TG myocytes compared with control myocytes (*$P < 0.05$). Peak $I_{Ca}$ is also elevated in another line (line 2) of TG mice, which express the same ATP-insensitive $K_{ATP}$ channel at a lower level.

Fig. 6. $Ca_{v}.1.2$ expression is unaltered in line 4 TG mice. A: representative amplification plots using primer sets that are specific for $Ca_{v}.1.2$, Kir6.2, or $\beta$-actin. SYBR green fluorescence ($R_n$) is plotted as a function of cycle number. The threshold is marked with a dotted line. Assays for each RNA sample from WT ($n = 3$) or TG ($n = 5$) mice was repeated in triplicate or quadruplicate. B: relative (normalized to WT) expression levels of $Ca_{v}.1.2$ and Kir6.2. Consistent with TG overexpression, Kir6.2 RNA is 156-fold (range: 106–231) greater in TG mice. In contrast, $Ca_{v}.1.2$ expression is not significantly different from WT mice.
the effect of similar perturbations in the pancreas. Whereas the transgene described in the present study does not significantly impair cardiac function, a similar ATP-insensitive Kir6.2 transgene expressed in /H9252 -cells severely disrupts glucose-induced insulin secretion, causing a lethal diabetic phenotype (11). The reasons for such disparate effects of similar transgenes at present are unclear but may reflect differences in SUR subunit composition of the heteromeric channels.

In summary, we generated TG mice that overexpress a cardiac-restricted, ATP-insensitive mutant of Kir6.2. Contrary to predictions, these mice display enhanced contractile function that results from a remodeling of the molecular substrates of excitation-contraction coupling. A marked increase in peak $I_{Ca}$ density likely contributes to counteracting the effects of transgene expression, maintaining the APD and causing increased myocyte contractility. The generality of such excitation-contraction coupling remodeling, as a counterbalance to the negative inotropic effects of increasing $K^{+}$ currents, remains an open question.

ACKNOWLEDGMENTS

We are indebted to Jefferson Gomes for assistance with cell isolation, Meredith McLerie for help with isolated heart experiments, and Kamelia Markova for assistance with animal husbandry.

GRANTS

This work was primarily supported by National Institutes of Health Grant HL-45742 (to C. G. Nichols), Cardiovascular Training Grant HL-07275 (fellowship support of T. P. Flagg), Grant HL-69052 (to A. Lopatin), and Grant KO8 DK-60086 (to D. Enkvetchakul).
REFERENCES


