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Research Paper

# Calmodulin Kinase II Inhibition Enhances Ischemic Preconditioning by Augmenting ATP-Sensitive K$^+$ Current

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## KEY WORDS

calcium, calmodulin, calmodulin kinase II, ischemia, ischemic preconditioning, ATP-dependent potassium channel, therapeutics

## ABSTRACT

Mice with genetic inhibition (AC3-I) of the multifunctional Ca$^{2+}$/calmodulin dependent protein kinase II (CaMKII) have improved cardiomyocyte survival after ischemia. Some K$^+$ currents are up-regulated in AC3-I hearts, but it is unknown if CaMKII inhibition increases the ATP sensitive K$^+$ current (I$_{KATP}$) that underlies ischemic preconditioning (IP) and confers resistance to ischemia. We hypothesized increased I$_{KATP}$ was part of the mechanism for improved ventricular myocyte survival during ischemia in AC3-I mice. AC3-I hearts were protected against global ischemia due to enhanced IP compared to wild type (WT) and transgenic control (AC3-C) hearts. I$_{KATP}$ was significantly increased, while the negative regulatory dose-dependence of ATP was unchanged in AC3-I compared to WT and AC3-C ventricular myocytes, suggesting that CaMKII inhibition increased the number of functional I$_{KATP}$ channels available for IP. We measured increased sarcolemmal Kir6.2, a pore-forming I$_{KATP}$ subunit, but not a change in total Kir6.2 in cell lysates or single channel I$_{KATP}$ opening probability from AC3-I compared to WT and AC3-C ventricles, showing CaMKII inhibition increased sarcolemmal I$_{KATP}$ channel expression. There were no differences in mRNA for genes encoding I$_{KATP}$ channel subunits in AC3-I, WT and AC3-C ventricles. The I$_{KATP}$ opener pinacidil (100 μM) reduced MI area in WT to match AC3-I hearts, while the I$_{KATP}$ antagonist HMR1098 (30 μM) increased MI area to an equivalent level in all groups, indicating that increased I$_{KATP}$ and augmented IP are important for reduced ischemic cell death in AC3-I hearts. Our study results show CaMKII inhibition enhances beneficial effects of IP by increasing I$_{KATP}$.

## INTRODUCTION

Chronic inhibition of the multifunctional Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) in cardiomyocytes significantly reduces maladaptive remodeling after myocardial infarction (MI).[1] CaMKII inhibition can prevent adverse remodeling after MI by reducing cell death,[2] normalizing intracellular Ca$^{2+}$ (Ca$^{2+}_i$) homeostasis[1,3] and reducing hypertrophy.[1] Recently, we found that CaMKII inhibition significantly shortens action potential durations by up-regulating the fast component of the transient outward current (I$_{to,f}$) and the inward rectifier (I$_{K1}$) potassium currents, suggesting the possibility that CaMKII inhibition may reduce maladaptive myocardial responses to ischemic stress by increasing the activity of some K$^+$ channels.[4]

Brief ischemic episodes protect the heart from subsequent lethal ischemic injury by a process termed ischemic preconditioning (IP).[5] The ATP-sensitive K$^+$ current (I$_{KATP}$) is required for IP and increased I$_{KATP}$ activity facilitates IP to reduce MI size in response to ischemia-reperfusion injury.[6-8] Two protein subunits, Kir 6.2 and SUR2A, functionally and physically interact to produce a pharmacological and biophysical profile that closely resembles the cardiac sarcolemmal I$_{KATP}$[9] Under normal conditions, sarcolemmal I$_{KATP}$ channels are predominantly closed, however, during a metabolic insult, such as cardiac ischemia, I$_{KATP}$ channels are activated and protect the myocardium from death. We hypothesized that CaMKII inhibition might increase I$_{KATP}$ and thereby improve myocardial survival after ischemia.

Here we show that Langendorff-perfused mouse hearts with CaMKII inhibition produced by transgenic expression of a specific CaMKII inhibitory peptide (AC3-I)[1-4] were resistant to ischemia-reperfusion injury and showed enhanced protection from IP compared to transgenic animals expressing a control peptide (AC3-C) and to wild type (WT) littermate controls. Excised cell membrane patches from AC3-I ventricular myocytes had significantly increased I$_{KATP}$ current density and greater sarcolemmal

expression of Kir6.2, the pore forming subunit of the myocardial $I_{KATP}$[10] than controls. However, $I_{KATP}$ channel opening probability was equivalent in cell membrane patches from AC3-I, WT and AC3-C ventricular myocytes. CaMKII inhibition increased the sarcolemmal density of functional $I_{KATP}$ channels, without affecting the inhibitory effects of ATP on $I_{KATP}$. Treatment with the selective $I_{KATP}$ antagonist HMR 1098 increased the infarct size in AC3-I and WT littermate control mice to an equivalent level, while the $I_{KATP}$ opener pinacidil reduced MI size in WT mice to levels similar to those seen in AC3-I mice. There were no differences in total Kir6.2 expression from ventricular myocyte lysates or in the mRNA expression levels of genes encoding $I_{KATP}$ subunits from AC3-I, WT or AC3-C hearts, indicating that up-regulation of $I_{KATP}$ during CaMKII inhibition was not directly related to enhanced gene transcription or protein translation of the channel subunits. Our study shows that myocardial CaMKII inhibition confers resistance to ischemia-reperfusion injury by enhancing IP through increasing activity of $I_{KATP}$. These surprising findings provide a new mechanism to understand how CaMKII signaling helps determine myocardial survival after ischemia-reperfusion injury.

## METHODS

**Ethical approval.** All experiments were performed under the approval of the University of Iowa Institutional Animal Care Committee. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Mice with myocardial CaMKII inhibition.** The AC3-I and AC3-C mice were generated by synthesis of a minigene based on the peptide sequence of AC3-I (KKALHRQEAVDCL) or AC3-C (KKALHAQERVDCL) and the engineering and characterization of these mice was previously described.[1] Age and sex matched mice (3–6 months old) were used for all of the experiments. WT control mice were AC3-I and AC3-C littermates.

**Electrophysiology.** Mice were sacrificed after deep anesthesia with 2.5% Avertin at a dose of 20 μl/g [10 g of tribromoethanol alcohol (Aldrich) + 10 ml of tert-amyl alcohol (Aldrich) with the addition of 1 mg/ml of heparin (Sigma; 187 USP units/mg), stored at 4°C as a stock solution]. Ventricular myocytes were isolated from Langendorff-perfused hearts not subjected to ischemia, as previously described.[11] $I_{KATP}$ from ventricular myocytes was recorded using inside-out patch-clamp configuration of the patch-clamp technique as described.[12] Tip resistance was 1.4–1.8 MΩ when pipettes were filled with intracellular solution, and series resistance compensation was routinely set at >85% in all experiments. All recordings were obtained at room temperature (22–26°C). GFP fluorescence was visualized in cells excited by high-power Hg-lamp (Nikon Instruments). Standard bath (intracellular) and pipette (extracellular) solution used in patch-clamp experiments was KINT (in mM: KCl 140, K+-HEPES 10, K+EGTA 10, pH 7.3 with KOH). $I_{KATP}$ was evoked by a membrane potential of -50 mV (pipette potential +50 mV) at six different concentrations of ATP: 0, 10, 25, 50, 100 and 1000 μM. Data were filtered at 5 kHz. Off-line analysis was performed using ClampFit and Microsoft Excel programs. Steady-state dependence of membrane current on [ATP] was obtained by calculating the relative current indexed to zero ATP ($I_{rel}$). The data were fitted using the Hill equation:

$$I_{rel} = 100/(1+([ATP]/K_{1/2})^H$$

where $I_{rel}$ is the relative current, $K_{1/2}$ is the concentration causing half-maximum blockade, and H is the Hill coefficient.

Single channel $I_{KATP}$ recordings were obtained from inside out patches using fire-polished pipettes, (resistance ~7–10-MΩ), when filled with pipette solution containing (mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-KOH, pH 7.3. Cardiac cells were superfused with intracellular solution containing (mM): 140 KCl, 1 $MgCl_2$, 5 EGTA, 5 HEPES-KOH, pH 7.3, in the absence or presence of ATP, and recordings made at room temperature (20–26°C) as described.[13] Single-channel recordings in the inside-out configuration were measured, at a holding potential of +60 mV (22°C), using an amplifier (Axopatch-100B, Axon Instruments, Foster City, CA), monitored and stored pClamp-10 data acquisition system (Molecular Devices Co, Sunnyvale, CA). Signal was low-pass filtered at 1 kHz and sampled every 50-μs. The threshold for judging the open state of $K_{ATP}$ channels was set at half of the single channel amplitude. The $nP_o$, where $n$ represents the number of channels in the patch and $P_o$ the probability of each channel to open, was assessed using Clampfit-10 software.

**IP protocol.** Langendorff-perfused mouse heart models were used for measuring myocardial viability and heart function.[4] Mice were anesthetized with avertin and hearts were quickly excised and rinsed in iced Tyrode's solution containing (in mM): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.16 $NaH_2PO_4$, 3 $NaHCO_3$, 5 HEPES-NaOH, and Heparin 1 mg/10ml, which was previously adjusted to a pH of 7.4 with NaOH at room temperature. Excess tissue was dissected away. Hearts were immediately perfused retrogradely through the aorta for 1–2 minutes at room temperature with Hanks' balanced salt solution (GIBCO, Cat No. 14025-092) and mounted on a modified Langendorff apparatus (HSE-HA perfusion systems, Harvard Apparatus) for retrograde aortic perfusion at a constant pressure of 80 mmHg with carbogen (95% $O_2$, 5% $CO_2$) and Krebs-Henseleit bicarbonate (KHB) solution consisting of (in mM): 25 $NaHCO_3$, 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $NaH_2PO_4$, 2.5 $CaCl_2$, 0.5 Na-EDTA, 15 Glucose, Pyruvate 2, with pH equilibrated to 7.4. The perfused heart was immersed into the water-jacked bath and was maintained at 36°C. A polyethylene balloon filled with dd$H_2O$ was inserted into the cavity of left ventricle through a left atrial incision. After allowing the heart to stabilize for 16 minutes, IP with two 2 min cycles of global ischemia were followed by 5 min of reperfusion. Some hearts had 30 min of stabilization with no IP. All hearts were then subjected to 20 min of global ischemia and 45 min reperfusion. HMR1098 (30 μm, Aventis Pharma, Germany) or pinacidil (100 μm, Sigma) were included in the bath solution for some experiments.

**Measurement of MI size.** At the end of ischemia/reperfusion, each heart was removed from the Langendorff perfusion apparatus and immediately frozen. The frozen hearts were cut from apex to base into eight transverse slices of approximately equal thickness (~0.8 mm). The slices were placed into a small cell culture dish and then incubated in 10% triphenyl-tetrazolium chloride (TTC) in phosphate buffer ($Na_2HPO_4$: 88 mM, $NaH_2PO_4$ 1.8 mM, pH 7.8) at room temperature for 30 min by shaking the dish as described.[14] The development of the red formazan pigment in living tissues relies on the presence of lactate dehydrogenase or NADH, while failure to stain red indicates a loss of these constituents from necrotic tissue.

After staining, the TTC buffer was replaced by 10% formaldehyde, the slices were fixed for the next 4–6 h before the areas of infarct tissue were determined by computer morphometry (Magna FiRE SP, with magnification 6.3 and exposure time 10 ms). The risk area was the sum of total ventricular area. The infarct size was calculated and presented as percentage of risk area.

**Immunoblots.** Following dissociation of ventricular myocytes from adult WT, AC3-I and AC3-C mice, whole cell lysates were prepared as described.[15] Protein concentrations were determined using BCA reagent (Pierce) and 60 micrograms of total lysate from each sample were separated by SDS-PAGE and transferred to membranes for immunoblotting. Blots were probed with anti-Kir6.2 (Alomone) or anti-NCX1 (RDI) antibody (to ensure equal protein loading). Representative Western blots are illustrated and cumulative data represent results of three experiments using at least three mice of each genotype.

**Surface biotinylation assays.** Freshly isolated ventricular cardiomyocytes were incubated at 4°C to inhibit membrane protein internalization and labeled with NHS-biotin. The biotinylation reactions were quenched and cells were washed and collected. Detergent-soluble lysates were prepared and biotinylated membrane surface proteins were affinity-purified using streptavidin-conjugated sepharose. Purified membrane proteins were analyzed by SDS-PAGE and immunoblot using Kir6.2 or $Na^+/Ca^{2+}$ exchanger (NCX)-specific antibody.

**RNA preparation.** Hearts were excised from 10–12 week-old male and female WT mice and from mice expressing either the CaMKII AC3-I (AC3-I) or AC3-C (AC3-C) peptide (n = 6 in each group). All animals were sacrificed by cervical dislocation, and the hearts were rapidly removed. Ventricles were dissected from each heart and flash-frozen in liquid nitrogen for further RNA isolation. Total RNA was isolated and DNase treated using the RNeasy Fibrous Tissue Mini Kit (Qiagen). The quality of total RNA was assessed by gel electrophoresis. Genomic DNA contamination was assessed by PCR amplification of total RNA samples without prior cDNA synthesis; no genomic DNA was detected.

**TaqMan real-time RT-PCR.** TaqMan Low Density Arrays (TLDA, Applied Biosystems) were completed in a two-step RT-PCR process as described previously.[16] First strand cDNA was synthesized from 2 μg of total ventricular RNA, prepared using the High-Capacity cDNA Archive Kit (Applied Biosystems). PCR reactions were carried out in TLDA using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The 384 wells of each array were preloaded with 96 x 4 predesigned FAM-labeled fluorogenic TaqMan probes and primers. The probes were labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM®, Applera Corporation) on the 5' end and with a nonfluorescent quencher on the 3' end. The low density array included genes encoding ion channel pore-forming (α), accessory (β) and regulatory subunits (n = 68), proteins involved in calcium homeostasis (n = 11), transcription factors (n = 6), markers of vessels, neuronal tissue, fibroblasts, inflammation and hypertrophy (n = 7). These genes were analyzed simultaneously, together with (n = 4) control genes used for normalization. Two ng of cDNA from each sample was combined with 1X TaqMan Universal Master Mix (Applied Biosystems) and loaded into each well. The TLDA were thermal-cycled at 50°C for 2 min and 94.5°C for 10 min, followed by 40 cycles of 97°C for 30 seconds and 59.7°C for 1 minute. Data were collected with instrument spectral compensation using the Applied Biosystems SDS 2.2.2 software and analyzed using the comparative threshold cycle ($C_T$) relative quantification method.[17] The Hypoxanthine guanine Phosphoribosyl Transferase I (*HPRT*) gene was used as an endogenous control to normalize the data. Results in each group are expressed relative to *HPRT*.

**Data analysis.** Values are means ± SE. Data analysis was performed using Student's *t* test or ANOVA, as appropriate (Sigma Stat). Post hoc comparisons after ANOVA were performed using the Holm-Sidak test. The null hypothesis was rejected for p ≤ 0.05.

## RESULTS

**CaMKII inhibition reduces myocardial infarction after ischemia and reperfusion.** Langendorff-perfused hearts were subjected to global ischemia followed by reperfusion. Some hearts from each group (AC3-I, WT and AC3-C) were initially exposed to two episodes of IP (Fig. 1A), while others had no IP. In the absence of IP, AC3-I hearts showed significantly less infarcted tissue than AC3-C (p < 0.001), but only a nonsignificant trend (p = 0.13) toward less MI than WT hearts (Fig. 1C). Resistance of AC3-I and WT hearts to global ischemia-reperfusion injury was significantly enhanced by IP, compared to no IP. AC3-I hearts had significantly smaller MIs (p < 0.001) after IP compared to WT and AC3-C hearts after IP (Fig. 1B and C). These findings show that CaMKII inhibition can augment the efficacy of IP and enhance resistance to ischemia induced cardiomyocyte death, compared to wild-type littermates or transgenic controls. The AC3-C hearts had an unexpected vulnerability to ischemic reperfusion injury, which was not affected by IP, suggesting that transgenic protein expression alone, without CaMKII inhibition, enhanced susceptibility to ischemic stress.

**AC3-I ventricular myocytes have increased $I_{KATP}$.** $I_{KATP}$ activation can protect against MI and is required for IP.[6] Based on 1) the superior efficacy of IP in reducing MI in AC3-I hearts and 2) the recent finding that some $K^+$ currents are up-regulated in AC3-I ventricular myocytes,[4] we measured $I_{KATP}$ to test if the increased myocardial viability after ischemia-reperfusion injury in AC3-I hearts was associated with increased $I_{KATP}$ density. Ventricular myocytes isolated from AC3-I hearts exhibited significantly greater $I_{KATP}$ in excised cell membrane patches compared to WT and AC3-C (Fig. 2A and B). Single $I_{KATP}$ channel recordings revealed similar Po in AC3-I, AC3-C and WT sarcolemmal membranes (Fig. 3). $I_{KATP}$ activity is negatively regulated by ATP and the dose-response relationship of $I_{KATP}$ for ATP applied to the cytoplasmic face of excised cell membrane patches was equivalent in AC3-I, WT and AC3-C ventricular myocytes (Fig. 2C). These findings show that chronic CaMKII inhibition does not affect the $I_{KATP}$ gating response to ATP and so suggest that increased $I_{KATP}$ in AC3-I ventricular myocytes is due to an increase in the number of functional $I_{KATP}$ channels at the sarcolemma.

**Selective Increase in Sarcolemmal Kir6.2 Expression in AC3-I Ventricular Myocytes.** In order to test if AC3-I mice had increased $I_{KATP}$ channels at the sarcolemma, we measured surface expression of Kir6.2, the pore forming subunit of the sarcolemmal $I_{KATP}$ channel complex in heart (Fig. 4). Immunoblotting revealed a significant increase in Kir6.2 membrane surface expression in AC3-I cardiomyocytes, compared with WT or control cardiomyocytes (Fig. 4B). In contrast, AC3-I, WT and AC3-C myocytes had equivalent sarcolemmal expression of NCX1 (Fig. 4B), consistent with earlier findings that showed no significant differences in NCX1 current between these genotypes.[1] We observed no significant difference in
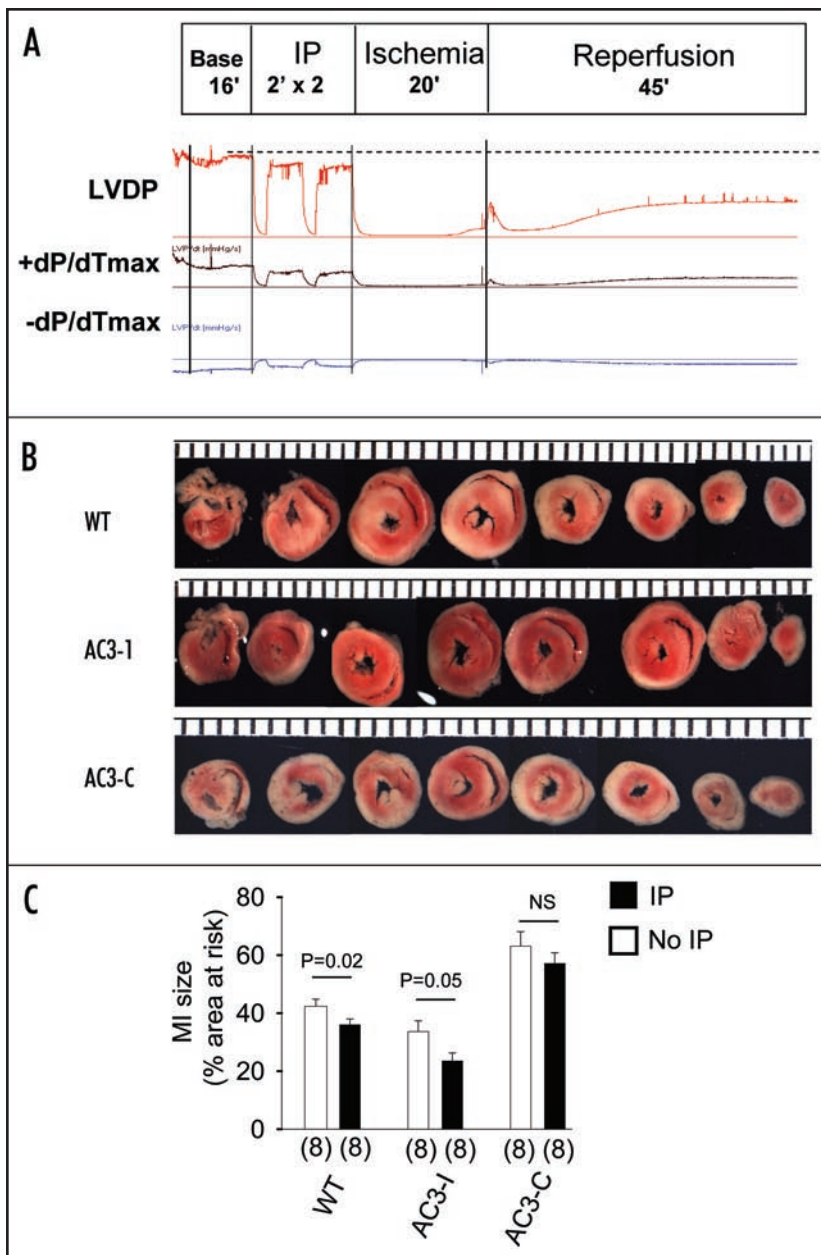
Figure 1. CaMKII inhibition enhances ischemic preconditioning. (A) Protocol for ischemia-reperfusion injury with two cycles of ischemic preconditioning (IP). Representative tracings from a wild type (WT) control heart are shown, where left ventricular developed pressure (LVDP) and the maximum contractility (+dP/dtmax) and and lusitropy (-dP/dtmax) are recorded. (B) Transverse sections from WT, AC3-I and transgenic control (AC3-C) hearts stained with TCC after IP and ischemia-reperfusion show MI as pale and viable myocardium as brown. (C) Summary data of MI size expressed as a percentage of total myocardium for WT, AC3-I and AC3-C hearts in the presence or absence of IP. The number of hearts studied in each condition is shown in parentheses. In the absence of IP both WT and AC3-I hearts had smaller MI size than AC3-C (p < 0.001), but WT and AC3-I MI were not significantly different (p = 0.13). In the presence of IP differences between all groups were significant (p < 0.001); AC3-I MI was significantly smaller than WT MI after IP (p = 0.05).

total Kir6.2 or NCX expression from cellular homogenates between genotypes (Fig. 4A). These data indicate that CaMKII inhibition enhances myocyte membrane surface expression of Kir6.2 and suggest that a previously unrecognized post-translational mechanism is important for increasing $I_{KATP}$ in AC3-I hearts.

**CaMKII inhibition does not affect mRNA levels for $I_{KATP}$ encoding genes.** CaMKII is known to participate in gene transcription in heart.[18] On the other hand, AC3-I mice have increased K+ currents by a mechanism that appears to be independent of changes in transcription.[4] Using qRTPCR, we measured mRNA expression levels of genes encoding SUR1 (*ABCC8*), SUR2 (*ABCC9*), Kir6.1 (*KCNJ8*) and Kir6.2 (*KCNJ11*), because these subunits can reconstitute functional $I_{KATP}$ channels. Specifically, *KCNJ11*[9] and *ABCC9*[19] are thought to encode the primary components of myocardial $I_{KATP}$ channels. There were no significant differences in mRNA expression for these genes between AC3-I, WT or AC3-C hearts (Fig. 5). These findings support the inference of our expression studies that CaMKII mediated increases in $I_{KATP}$ are due to a nontranscriptional mechanism (Fig. 4) and are reminiscent of other recent results, showing that voltage-gated and inwardly rectifying K+ currents are upregulated in the ventricles of AC3-I mice independent of augmented transcription of the underlying K+ channel subunit genes.[4]

**Increased $I_{KATP}$ activity is the mechanism for resistance to ischemia in AC3-I hearts.** To determine directly if the increase in $I_{KATP}$ density observed in AC3-I ventricular myocytes is responsible for ischemia resistance in AC3-I hearts, we measured MI after IP and ischemia reperfusion in the presence of the $I_{KATP}$ agonist pinacidil. We reasoned that if increased $I_{KATP}$ was a critical downstream effector for enhanced resistance to MI with IP in AC3-I hearts then an $I_{KATP}$ opener would reduce or eliminate the differences in MI resistance after IP between WT and AC3-I hearts. Pinacidil eliminated the significant difference in MI size between AC3-I over WT hearts after IP seen in vehicle treated hearts. MI sizes were equivalent between AC3-I and WT hearts treated with pinacidil (Fig. 6). These data suggested that increased $I_{KATP}$ in AC3-I hearts was necessary and possibly sufficient to account for the enhanced resistance to MI after ischemia reperfusion injury. AC3-C hearts did not show a significant change in MI size after pinacidil, consistent with earlier findings (Fig. 1C) that AC3-C hearts had increased vulnerability to ischemic injury and were unresponsive to IP.

We next tested the role of $I_{KATP}$ in IP by using an $I_{KATP}$ antagonist, HMR 1098. We hypothesized that the benefit of CaMKII inhibition for enhancing IP responses and reducing MI would be lost after $I_{KATP}$ blockade. HMR 1098 significantly increased MI area in AC3-I (p = 0.02) and WT hearts (p = 0.03) to equivalent levels (Fig. 6). In contrast, MI size in AC3-C hearts was greater than in AC3-I or WT and was not significantly responsive to HMR 1098 (p = 0.09). These data are complementary to findings with pinacidil and indicate that increased $I_{KATP}$ is a major final common pathway component of the mechanism of resistance to MI in AC3-I hearts.

**No improvement in mechanical function in AC3-I hearts after ischemia.** Baseline values were not different between AC3-I, WT and AC3-C hearts for LVDP, +dP/dtmax or -dP/dtmax (Fig. 7A–C). These
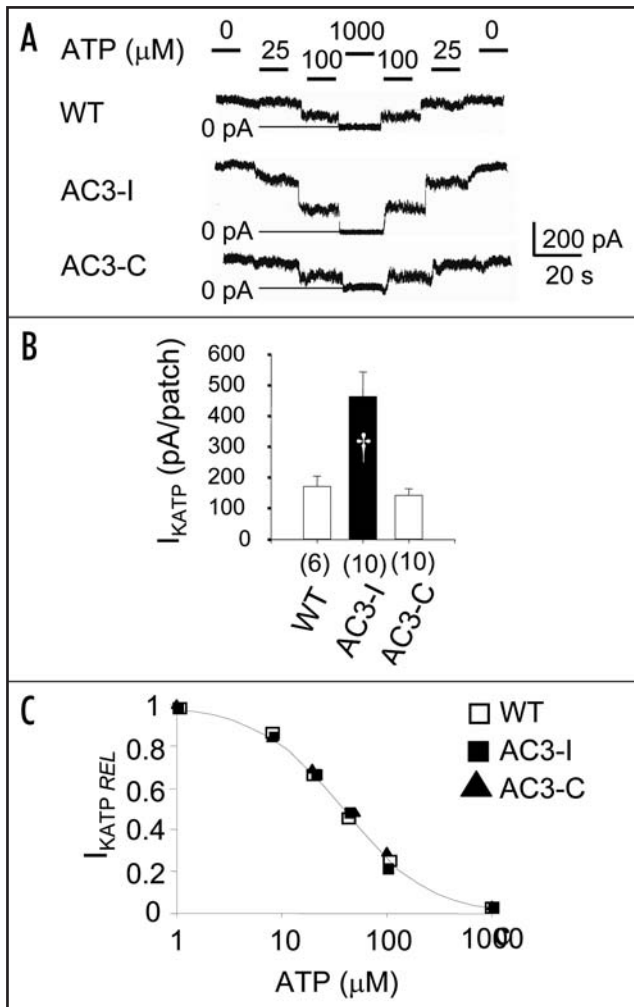
Figure 2. Upregulation of $I_{KATP}$ in AC3-I hearts. (A) $I_{KATP}$ recorded from cell membrane patches excised from AC3-I, WT and AC3-C ventricular myocytes under various ATP concentrations applied to the cytoplasmic membrane face. $I_{KATP}$ is ~0 pA at 1 mM ATP in each tracing. (B) Summary data show significantly more $I_{KATP}$ in AC3-I than WT or AC3-C ventricular myocytes ($^{‡}p < 0.001$). (C) There were no differences in concentration dependence for the negative regulation of $I_{KATP}$ by ATP in WT (n = 8), AC3-I (n = 10) and AC3-C (n = 10) ventricular myocytes. $I_{KATP}$ data were normalized to the 0 ATP condition ($IKATP_{REL}$).

data show that the isolated heart preparations were reproducible and that CaMKII inhibition did not disturb basal myocardial function, consistent with previous reports.[1] Surprisingly, the reduced MI size in AC3-I mice did not result in improved mechanical function. The IP protocol did not significantly improve any of the mechanical parameters for WT or AC3-I hearts and recovery of $^{+}dP/dt$ max and $-dP/dt$ max were significantly impaired by IP, compared to ischemia without IP, in AC3-C hearts (p = 0.03 for both). Recovery of $^{+}dP/dt$ max and $-dP/dt$ max was worse after IP in AC3-C compared to WT hearts (p < 0.05), while there was no significant difference between WT and AC3-I recovery in the presence or absence of IP (Fig. 7D–F). These data show that augmented myocyte survival in AC3-I mice is not sufficient for improved acute recovery after reperfusion.
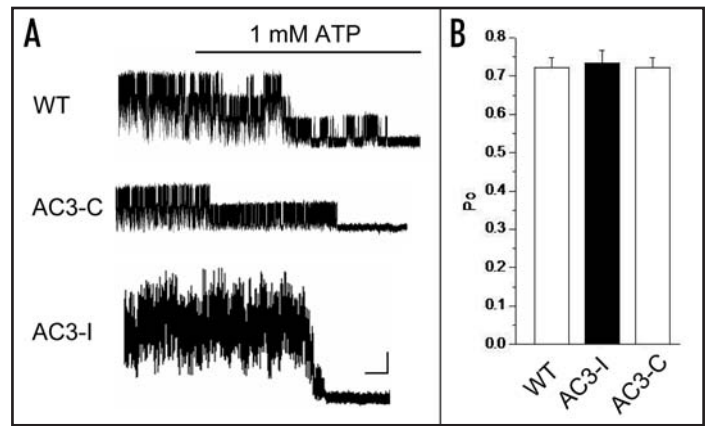


Figure 3. $K_{ATP}$ channels from AC3-I, WT and AC3-C mice exhibit equal open probability in the absence of ATP. (A) Examples of the single channel recording from the AC3-I, WT and AC3-C cardiomyocytes. Cell membrane patches excised from the AC3-I cardiomyocytes typically contained more channels compared to WT and AC3-C, even though the recording was done using pipettes with high resistance. The calibration bars indicate 100 ms (abscissa) and 5 pA (ordinate). (B) Bar graphs represent calculated single channel $I_{KATP}$ open probability in WT, AC3-C and AC3I mice. No significant differences were present between groups (n = 4/group).

## DISCUSSION

**Improved cell survival in AC3-I hearts depends upon sarcolemmal $I_{KATP}$ expression.** The central findings of our work are that chronic myocardial CaMKII inhibition augments resistance to cell death and increases the efficacy of IP by enhancing the density of sarcolemmal $I_{KATP}$. $I_{KATP}$ agonist and antagonist drugs eliminated the differences between WT and AC3-I hearts, a finding that points to $I_{KATP}$ as a critical downstream target for the beneficial effects of CaMKII inhibition in transient ischemia. The mechanism of CaMKII inhibition on $I_{KATP}$ involves enrichment of functional channels at the sarcolemma. This post-translational process increases the total $I_{KATP}$ available for IP. Our findings are consistent with cellular studies showing that simulated ischemia also increases Kir6.2 levels at the membrane surface.[20] In contrast, our data do not support a role for CaMKII in $I_{KATP}$ channel gating. We cannot exclude the possibility that some of the increased sarcolemmal Kir6.2 was not active in AC3-I cells. However, our findings that $I_{KATP}$ density was increased without a change in channel opening probability strongly suggest that most of the increased $I_{KATP}$ channels were capable of K+ conductance.

The use of the Langendorff-perfused heart preparation allows for control of the ischemic time and places the entire myocardium 'at risk' for ischemia. Interpretation of our results is also unencumbered by potentially variable adrenergic input. Our findings thus reflect intrinsic properties of myocardium to ischemia and reperfusion. On the other hand, they may not fully reveal the role of CaMKII inhibition in ischemia reperfusion injury in vivo. The absolute amount of IP in WT and AC3-I hearts was potentially reduced by the brief ischemia inherent in cardiac excision and the modestly lower temperature during Langendorff perfusion compared to in vivo. Nevertheless, our previous findings show that AC3-I mice have improved resistance to MI in vivo. AC3-I mice have reduced myocardial apoptosis after 2 hours of coronary artery occlusion,[2] while AC3-I hearts have better function and reduced LV chamber dilation from one to three weeks after fixed coronary occlusion, compared to WT and control mice.[1]
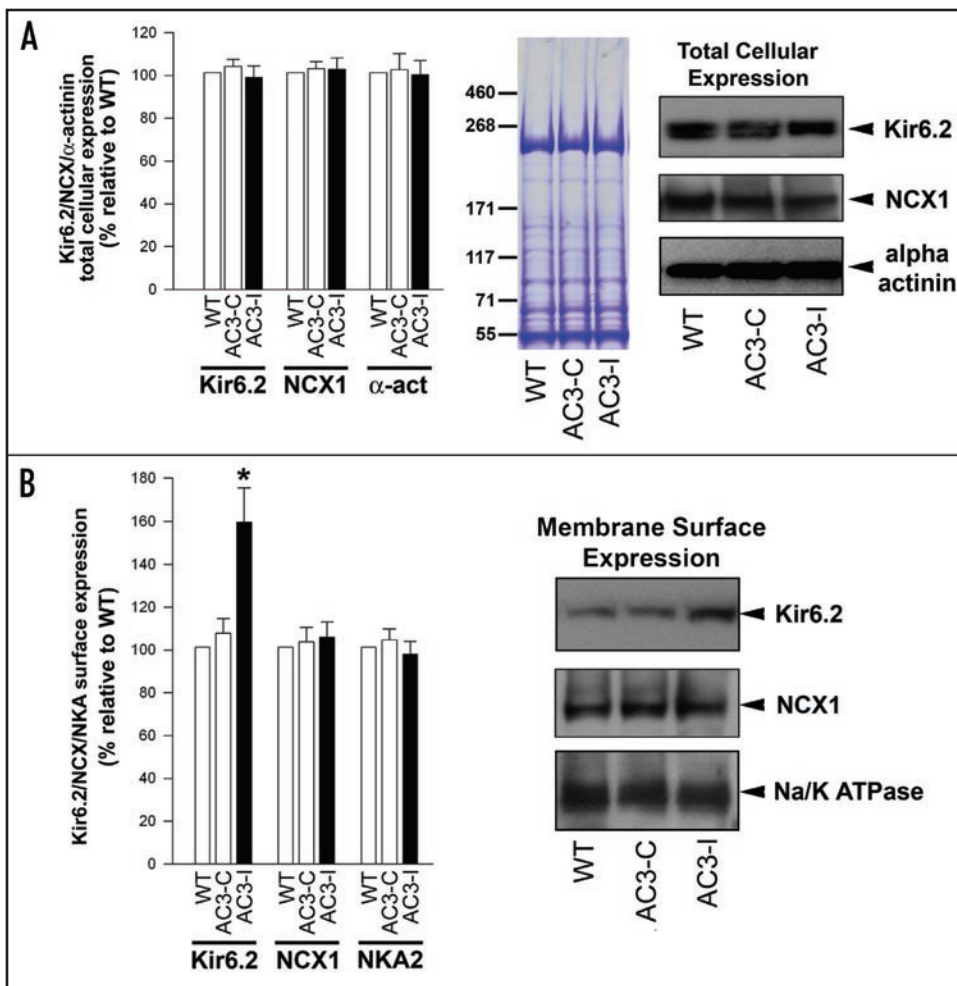
Figure 4. Cardiomyocytes derived from AC3-I mice display increased Kir6.2 membrane surface expression. (A) Whole cell cardiomyocyte lysates were prepared from freshly dissociated adult mouse cardiomyocytes. 60 µg protein from each genotype was analyzed by SDS-PAGE and immunoblot using Kir6.2, Na/Ca exchanger (NCX1) and α-actinin (α-act) antibodies. Protein levels from cardiomyocytes isolated from WT (n = 6), AC3-C (n = 5), and AC3-I (n = 7) hearts were averaged and expressed relative to WT. (B) Freshly dissociated adult cardiomyocytes were chilled to 4°C and membrane surface proteins were labeled with biotinylation reagent. Following membrane-surface protein enrichment, proteins were analyzed by SDS-PAGE and immunoblot. Protein levels from cardiomyocytes isolated from WT (n = 6), AC3-C (n = 5), and AC3-I (n = 7) hearts were averaged and are expressed as relative membrane surface expression compared to WT (p < 0.01).

The fact that cardiac function was improved in AC3-I mice one to three weeks after MI in vivo suggests that the lack of acute improvement in mechanical function in Langendorff-perfused AC3-I hearts is due to transient effects of myocardial stunning or possibly reflects a requirement for extracardiac signals, such as adrenergic stimulation, present in vivo.

Present findings in isolated hearts strongly suggest that a CaMKII-independent process(es), such as changes in myofilament Ca$^{2+}$$_i$ sensitivity,[21] is activated by ischemia and contributes to mechanical dysfunction after reperfusion using our experimental protocol. Our protocol used two cycles of IP prior to ischemia reperfusion. This protocol enhanced myocardial survival in WT and AC3-I hearts without significantly improving mechanical function, consistent with other studies using two IP cycles.[14] On the other hand, studies with four IP cycles do preserve mechanical function in mouse heart,[22] suggesting that the IP benefits to myocardial survival and mechanical recovery have different thresholds. The fact that

myocardial survival was not enhanced by IP and mechanical recovery was impaired by IP in AC3-C hearts points to an unanticipated effect of transgenic protein expression that reduces myocardial adaptation to ischemic stress. The findings in AC3-C hearts appear to indicate that the resistance of AC3-I hearts to MI after IP compared to WT may underestimate the true therapeutic efficacy of CaMKII inhibition, because of a competing negative influence of transgenic protein expression.[23]

**Ischemia, Ca$^{2+}$$_i$, CaMKII and cell death.** Myocardial ischemia is a major cause of heart failure and sudden cardiac death.[24] Identification of downstream signals activated during ischemia, therefore, is an important goal in cardiovascular medicine. CaMKII has recently emerged as a candidate for drug therapy in structural heart disease.[25] CaMKII is activated by stressors, such as isoproterenol,[1] that increase Ca$^{2+}$$_i$. It is probably not so surprising, therefore, that CaMKII may also play a role in ischemia, which is marked by loss of Ca$^{2+}$$_i$ homeostasis.[26] AC3-I mice show preserved cardiac function and Ca$^{2+}$$_i$ homeostasis after MI and in response to excessive catecholamines.[1] CaMKII is involved in determining cardiomyocyte survival and increased CaMKII activity triggers apoptosis in response to isoproterenol stimulation.[27] AC3-I mice are resistant to apoptosis initiated by isoproterenol or ischemia,[2] but CaMKII inhibition has not previously been implicated in preventing cardiomyocyte necrosis in ischemia. CaMKII may also affect factors other than I$_{KATP}$ that influence resistance to ischemic stress, such as heart rate and SR Ca$^{2+}$ content. However, our results support a model where cardiomyocyte viability after ischemia is markedly affected by CaMKII and where CaMKII inhibition potentiates the beneficial effects of IP.

**Is CaMKII a metabolic signal in heart?** I$_{KATP}$ couples the cellular energy charge to sarcolemmal repolarization[28] and is required for IP.[6,29] Our study showed that the mechanism for the benefit of CaMKII inhibition in enhancing IP and reducing MI was post-translational processing that resulted in increased density of functional I$_{KATP}$ channels in the sarcolemma. Future studies will be necessary to determine if CaMKII inhibition directly affects I$_{KATP}$ constituents or targets membrane trafficking proteins to accomplish this important task. Ca$^{2+}$$_i$ homeostasis is a significant metabolic expense in cardiomyocytes, and the reduced availability of ATP during ischemia contributes to loss of Ca$^{2+}$$_i$ homeostasis and myocardial necrosis in MI.[21] CaMKII is activated by Ca$^{2+}$$_i$ and CaMKII is also a key regulatory signal for Ca$^{2+}$ homeostatic proteins in heart.[30] Less is known about the role of CaMKII in myocardial metabolism, but transient CaMKII over-expression in cardiomyocytes does
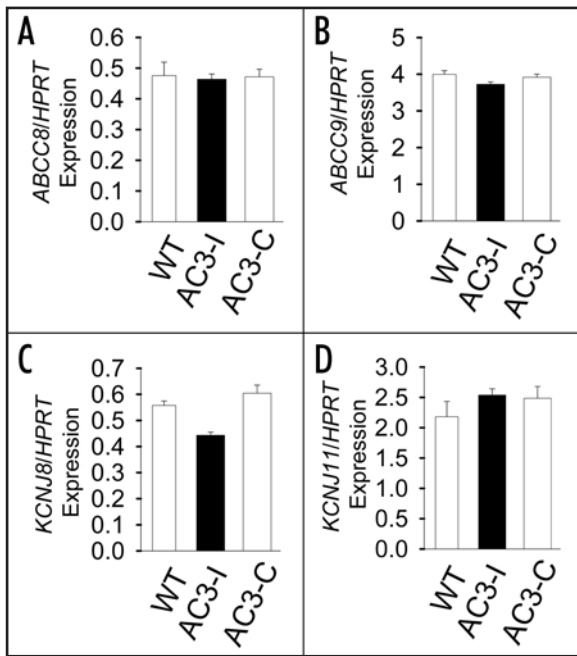
Figure 5. No changes in mRNA expression levels for genes encoding $I_{KATP}$ channel subunits. A-D) mRNA exprression levels of (A) *ABCC8* (SUR1), (B) *ABCC9* (SUR2) (C) *KCNJ8* (Kir 6.2) and (D)*KCNJ11* (Kir6.2) were not significantly different among the three groups. All data were normalized to *HPRT* mRNA and are averages from 4–6 hearts.
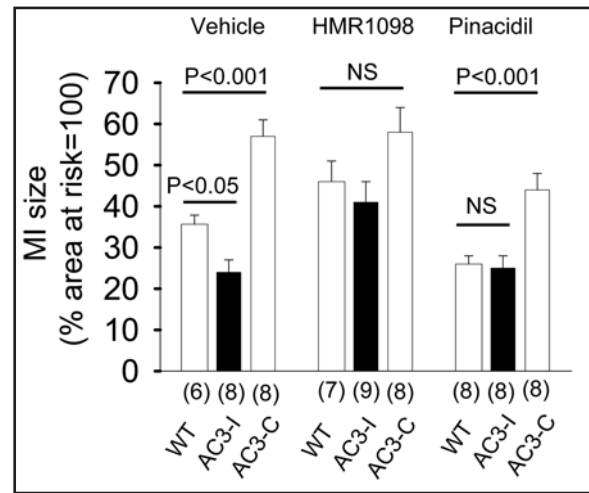
Figure 6. Increased $I_{KATP}$ is the mechanism for enhanced IP efficacy in AC3-I hearts. Summary data for MI size in WT, AC3-I and AC3-C hearts treated with the $I_{KATP}$ antagonist HMR1098 (30 μM), the $I_{KATP}$ agonist pinacidil (100 μM) or vehicle during the IP and ischemia-reperfusion protocol (Fig. 1A). The vehicle data were previously shown in Figure 1C. MI sizes were not significantly different (p = 0.09) between groups after HMR1098. Pinacidil eliminated the differences in MI size between WT and AC3-I hearts, and both WT and AC3-I MI sizes were significantly less (p < 0.001) than AC3-C after pinacidil. The numbers of hearts studied are given in parentheses.
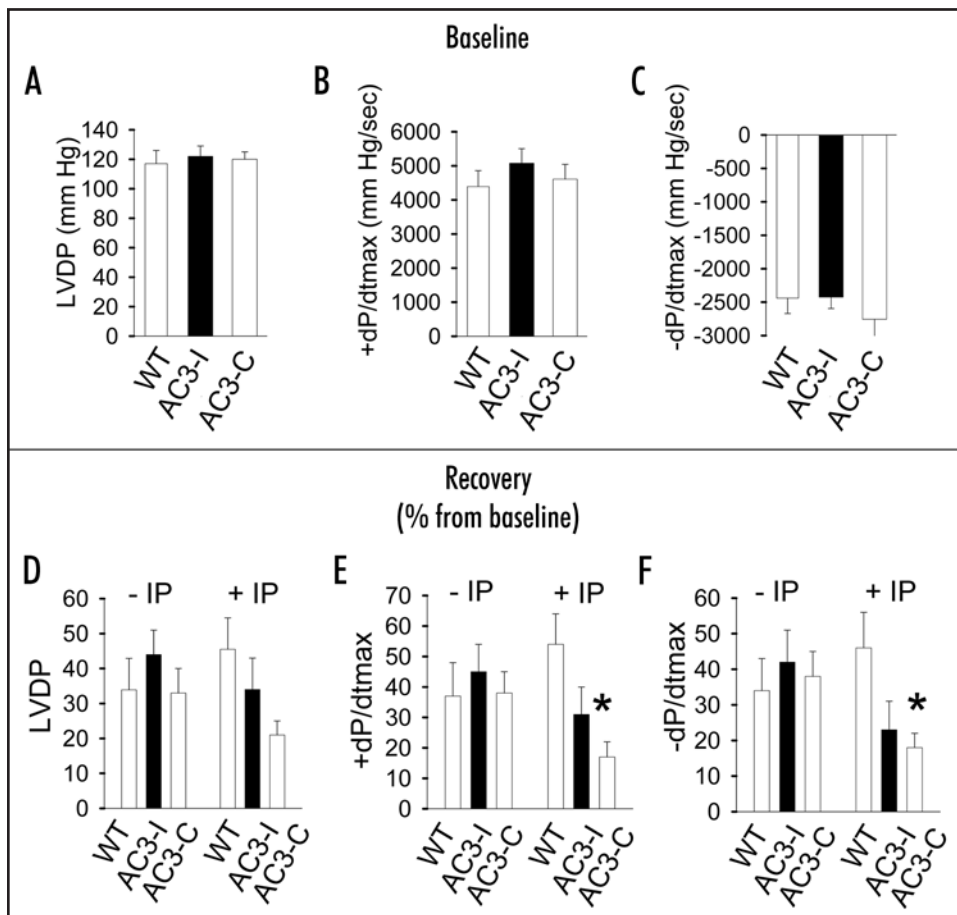


Figure 7. (At left) Mechanical recovery in AC3-I is not improved relative to WT and AC3-C hearts after ischemia-reperfusion injury. Basal mechanical properties in terms of (A). Left ventricular developed pressure (LVDP), (B) $^+$dP/dtmax and (C) -dP/dtmax were similar in WT, AC3-I and AC3-C hearts. (D) LVDP recovery was not significantly affected by IP for any group. Recovery of (E) $^+$dP/dtmax and (F) -dP/dtmax were significantly less for AC3-C, compared to WT, hearts after IP (*p < 0.05); recovery of $^+$/-dP/dtmax was paradoxically reduced (p = 0.03) in AC3-C hearts by IP. Values represent means of measurements on 6–8 hearts.

induce peroxisome proliferator-activated receptor gamma coactivator-1alpha, a transcription factor involved in multiple cellular energy metabolic pathways.[31] Thus, it is plausible that CaMKII inhibition results in a variety of other metabolic changes in heart, in addition to increasing I$_{KATP}$.

### References

1. Zhang R, Khoo MS, Wu Y, Yang Y, Grueter CE, Ni G, Price EE, Thiel W, Guatimosim S, Song LS, Madu EC, Shah AN, Vishnivetskaya TA, Atkinson JB, Gurevich VV, Salama G, Lederer WJ, Colbran RJ, Anderson ME. Calmodulin kinase II inhibition protects against structural heart disease. Nat Med 2005; 11:409-17.

2. Yang Y, Zhu WZ, Joiner ML, Zhang R, Oddis CV, Hou Y, Yang J, Price EE, Gleaves L, Eren M, Ni G, Vaughan DE, Xiao RP, Anderson ME. Calmodulin kinase II inhibition protects against myocardial cell apoptosis in vivo. Am J Physiol Heart Circ Physiol 2006; 291:H3065-75.

3. Wu Y, Shintani A, Grueter C, Zhang R, Hou Y, Yang J, Kranias EG, Colbran RJ, Anderson ME. Suppression of dynamic Ca$^{2+}$ transient responses to pacing in ventricular myocytes from mice with genetic calmodulin kinase II inhibition. J Mol Cell Cardiol 2006; 40:213-23.

4. Li J, Marionneau C, Zhang R, Shah V, Hell JW, Nerbonne JM, Anderson ME. Calmodulin kinase II inhibition shortens action potential duration by upregulation of K$^+$ currents. Circ Res 2006; 99:1092-9.

5. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124-36.

6. Suzuki M, Sasaki N, Miki T, Sakamoto N, Ohmoto-Sekine Y, Tamagawa M, Seino S, Marban E, Nakaya H. Role of sarcolemmal K(ATP) channels in cardioprotection against ischemia/reperfusion injury in mice. J Clin Invest 2002; 109:509-16.

7. Patel HH, Gross ER, Peart JN, Hsu AK, Gross GJ. Sarcolemmal KATP channel triggers delayed ischemic preconditioning in rats. Am J Physiol Heart Circ Physiol 2005; 288: H445-7.

8. Rajashree R, Koster JC, Markova KP, Nichols CG, Hofmann PA. Contractility and ischemic response of hearts from transgenic mice with altered sarcolemmal K$_{ATP}$ channels. Am J Physiol Heart Circ Physiol 2002; 283:H584-90.

9. Babenko AP, Gonzalez G, guilar-Bryan L, Bryan J. Reconstituted human cardiac K$_{ATP}$ channels: Functional identity with the native channels from the sarcolemma of human ventricular cells. Circ Res 1998; 83:1132-43.

10. Inagaki N, Gonoi T, Clement JP, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J. Reconstitution of IKATP: An inward rectifier subunit plus the sulfonylurea receptor. Science 1995; 270:1166-70.

11. Li J, McLerie M, Lopatin AN. Transgenic upregulation of IK1 in the mouse heart leads to multiple abnormalities of cardiac excitability. Am J Physiol Heart Circ Physiol 2004; 287: H2790-802.

12. Koster JC, Knopp A, Flagg TP, Markova KP, Sha Q, Enkvetchakul D, Betsuyaku T, Yamada KA, Nichols CG. Tolerance for ATP-insensitive K$_{ATP}$ channels in transgenic mice. Circ Res 2001; 89:1022-9.

13. Alekseev AE, Brady PA, Terzic A. Ligand-insensitive state of cardiac ATP-sensitive K$^+$ channels: Basis for channel opening. J Gen Physiol 1998; 111:381-94.

14. Xi L, Hess ML, Kukreja RC. Ischemic preconditioning in isolated perfused mouse heart: Reduction in infarct size without improvement of post-ischemic ventricular function. Mol Cell Biochem 1998; 186:69-77.

15. Mohler PJ, Splawski I, Napolitano C, Bottelli G, Sharpe L, Timothy K, Priori SG, Keating MT, Bennett V. A cardiac arrhythmia syndrome caused by loss of ankyrin-B function. Proc Natl Acad Sci USA 2004; 101:9137-42.

16. Marionneau C, Couette B, Liu J, Li H, Mangoni ME, Nargeot J, Lei M, Escande D, Demolombe S. Specific pattern of ionic channel gene expression associated with pacemaker activity in the mouse heart. J Physiol 2005; 562:223-34.

17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-8.

18. Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN. CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J Clin Invest 2006; 116:1853-64.

19. Chutkow WA, Samuel V, Hansen PA, Pu J, Valdivia CR, Makielski JC, Burant CF. Disruption of Sur2-containing K$_{ATP}$ channels enhances insulin-stimulated glucose uptake in skeletal muscle. Proc Natl Acad Sci USA 2001; 98:11760-4.

20. Budas GR, Jovanovic S, Crawford RM, Jovanovic A. Hypoxia-induced preconditioning in adult stimulated cardiomyocytes is mediated by the opening and trafficking of sarcolemmal KATP channels. FASEB J 2004; 18:1046-8.

21. Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. Physiol Rev 1999; 79:609-34.

22. Gumina RJ, Pucar D, Bast P, Hodgson DM, Kurtz CE, Dzeja PP, Miki T, Seino S, Terzic A. Knockout of Kir6.2 negates ischemic preconditioning-induced protection of myocardial energetics. Am J Physiol Heart Circ Physiol 2003; 284:H2106-13.

23. Khoo MS, Grueter CE, Eren M, Yang J, Zhang R, Bass MA, Lwin ST, Mendes LA, Vaughan DE, Colbran RJ, Anderson ME. Calmodulin kinase II inhibition disrupts cardiomyopathic effects of enhanced green fluorescent protein. J Mol Cell Cardiol 2007; In press.

24. Zipes DP, Camm AJ, Borggrefe M, Buxton AE, Chaitman B, Fromer M, Gregoratos G, Klein G, Moss AJ, Myerburg RJ, Priori SG, Quinones MA, Roden DM, Silka MJ, Tracy C, Smith Jr SC, Jacobs AK, Adams CD, Antman EM, Anderson JL, Hunt SA, Halperin JL, Nishimura R, Ornato JP, Page RL, Riegel B, Priori SG, Blanc JJ, Budaj A, Camm AJ, Dean V, Deckers JW, Despres C, Dickstein K, Lekakis J, McGregor K, Metra M, Morais J, Osterspey A, Tamargo JL, Zamorano JL. ACC/AHA/ESC 2006 guidelines for management of patients with ventricular arrhythmias and the prevention of sudden cardiac death. A Report of the American College of Cardiology/American Heart Association Task Force and the European Society of Cardiology Committee for Practice Guidelines (Writing Committee to Develop Guidelines for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death). Developed in Collaboration With the European Heart Rhythm Association and the Heart Rhythm Society. Circulation 2006; 114:e385-484.

25. Bers DM. Beyond beta blockers. Nat Med 2005; 11:379-80.

26. Dekker LR, Coronel R, VanBavel E, Spaan JA, Opthof T. Intracellular Ca$^{2+}$ and delay of ischemia-induced electrical uncoupling in preconditioned rabbit ventricular myocardium. Cardiovas Res 1999; 44:101-12.

27. Zhu WZ, Wang SQ, Chakir K, Yang DM, Zhang T, Brown JH, Devic E, Kobilka BK, Cheng HP, Xiao RP. Linkage of $\beta_1$-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca$^{2+}$/calmodulin kinase II. J Clin Invest 2003; 111:617-25.

28. Nichols CG. K$_{ATP}$ channels as molecular sensors of cellular metabolism. Nature 2006; 440:470-6.

29. Suzuki M, Saito T, Sato T, Tamagawa M, Miki T, Seino S, Nakaya H. Cardioprotective effect of diazoxide is mediated by activation of sarcolemmal but not mitochondrial ATP-sensitive potassium channels in mice. Circulation 2003; 107:682-5.

30. Maier LS, Bers DM. Calcium, calmodulin, and calcium-calmodulin kinase II: Heartbeat to heartbeat and beyond. J Mol Cell Cardiol 2002; 34:919-39.

31. Schaeffer PJ, Wende AR, Magee CJ, Neilson JR, Leone TC, Chen F, Kelly DP. Calcineurin and calcium/calmodulin-dependent protein kinase activate distinct metabolic gene regulatory programs in cardiac muscle. J Biol Chem 2004; 279:39593-603.