Regulation of Cardiac ATP-sensitive Potassium Channel Surface Expression by Calcium/Calmodulin-dependent Protein Kinase II*

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Background: Surface expression of cardiac ATP-sensitive potassium (K$_{ATP}$) channels impacts cellular energy homeostasis.

Results: Activation of calcium/calmodulin-dependent protein kinase II (CaMKII) results in K$_{ATP}$ channel internalization, requiring specific motifs on the Kir6.2 channel subunit.

Conclusion: CaMKII phosphorylation of Kir6.2 promotes endocytosis of cardiac K$_{ATP}$ channels.

Significance: This mechanism reveals new targets to improve cardiac energy efficiency and stress resistance.

Cardiac ATP-sensitive potassium (K$_{ATP}$) channels are key sensors and effectors of the metabolic status of cardiomyocytes. Alteration in their expression impacts their effectiveness in maintaining cellular energy homeostasis and resistance to injury. We sought to determine how activation of calcium/calmodulin-dependent protein kinase II (CaMKII), a central regulator of cardiac signaling, translates into reduced membrane expression and current capacity of cardiac K$_{ATP}$ channels. We used real-time monitoring of K$_{ATP}$ channel current density, immunohistochemistry, and biotinylation studies in isolated hearts and cardiomyocytes from wild-type and transgenic mice used to determine how activation of CaMKII triggers endocytosis of cardiac K$_{ATP}$ channels. A molecular model of the μ2 subunit of the endocytosis adaptor protein, AP2, complexed with 330YSKF333 endocytosis motif of the Kir6.2 pore-forming subunit. Activation of CaMKII predicted that phosphorylation of Thr-180 and Thr-224 would favor interactions with the corresponding arginine- and lysine-rich loops on μ2. We concluded that CaMKII results in phosphorylation of Kir6.2, which promotes endocytosis of cardiac K$_{ATP}$ channel subunits. This mechanism couples the surface expression of cardiac K$_{ATP}$ channels with calcium signaling and reveals new targets to improve cardiac energy efficiency and stress resistance.

ATP-sensitive potassium (K$_{ATP}$) channels have the unique ability to adjust membrane excitability in response to changes in the energetic status of the cell (1–8). The channel complex is formed through physical association of the pore-forming inwardly rectifying potassium channel, Kir6.x, with the regulatory sulfonylurea receptor, SUR (3, 6, 11–15). In ventricular myocytes, the primary site of cardiac energy consumption, KATP channels are composed primarily of Kir6.2 and SUR2A subunits (6, 14, 16). The metabolic sensing of these channels occurs through modulation of the ATP sensitivity of Kir6.2 by SUR (5, 11–17). The tight integration of SUR with cellular energetic networks (18–21) as well as the proximity of the channel to sites of ATP con-

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** The abbreviations used are: SUR, sulfonylurea receptor; CaMKII, calcium/calmodulin-dependent protein kinase II; DNP, 2,3 dinitrophenol; NS, not significant; Bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
sumption in the compartmentalized cellular environment (17, 22) allows $K_{\text{ATP}}$ channel opening to be regulated not only by severe metabolic stress, such as during ischemia, but also by heart rate acceleration within the range induced by normal physical activity (23, 24). When activated by a reduced ATP/ADP ratio, reflecting either increased cellular metabolic demand or reduced cellular ATP generation, $K_{\text{ATP}}$ channel-dependent potassium efflux shortens cardiac action potential duration, allowing for a longer diastolic interval that supports myocardial relaxation and restoration of ion gradients and energetic resources as well as limits sodium and calcium entry into the cell and thus reduces energy requirements for ion transport/exchange and contraction (1, 9, 10, 23–28).

There is a growing body of evidence that the ability of cardiac $K_{\text{ATP}}$ channels to affect cellular excitability and function depends on their abundance at the membrane surface (24, 25, 27, 29–31). For instance, in the heart an increase in functional sarcolemmal $K_{\text{ATP}}$ channel presence enhances the speed and degree of shortening of action potentials and limits cardiac energy consumption in response to escalating workloads, whereas a decrease in their presence has the opposite effect (24). Additionally, although even a small fraction of the normal population of $K_{\text{ATP}}$ channels can dramatically shorten the action potential duration in response to severe metabolic stress, the speed at which this myocardially protective action occurs is $K_{\text{ATP}}$ channel expression-dependent (25). Defining the mechanisms that control cardiac membrane $K_{\text{ATP}}$ channel expression and associated metabolic signaling is an important step toward understanding how to promote myocardial energy efficiency and stress resistance to prevent or treat cardiac disease.

Myocardial effects of $K_{\text{ATP}}$ channel current up-regulation are mirrored in many ways by inhibition of calcium/calmodulin-dependent protein kinase II (CaMKII), including shortened action potentials, improved resistance to cell death under metabolic stress, and normalized intracellular calcium homeostasis (32–34), suggesting a potential interaction between CaMKII and $K_{\text{ATP}}$ channels and a common calcium-related regulatory pathway. CaMKII is a multifunctional kinase, densely expressed in cardiomyocytes, that targets numerous proteins involved in excitation-contraction coupling and excitability to support short-term enhancement of cardiac performance, whereas persistent activation results in adverse cardiac remodeling and dysfunction (35–38). Indeed, inhibition of CaMKII has been shown to increase cardioprotective $K_{\text{ATP}}$ channel surface presence and current density (29). However, the molecular mechanism underlying this regulation is unknown.

Here we identify CaMKII as a critical regulator of the surface expression and consequent whole cell current capacity of cardiac $K_{\text{ATP}}$ channels. We find that CaMKII activation triggers rapid internalization of cardiac $K_{\text{ATP}}$ channels by endocytosis. This process requires phosphorylation of threonine at positions 180 and 224 and an intact YXXO endocytosis motif, $^{330}$YSKF$^{333}$ of Kir6.2. A molecular model of Kir6.2 predicts that the $\mu2$ subunit of the endocytosis adaptor protein, AP2, docks with the $K_{\text{ATP}}$ channel by interacting with $^{330}$YSKF$^{333}$ and Thr-180 on one and Thr-224 on an adjacent Kir6.2 subunit. These findings provide new insight into the regulation of cardiac $K_{\text{ATP}}$ channels and provide valuable new targets for the promotion of cardiac energy efficiency and stress resistance.

**EXPERIMENTAL PROCEDURES**

**Mouse Models**—Transgenic mice expressing a specific peptide inhibitor of CaMKII (AC3-I, which targets a conserved region of the CaMKII regulatory domain) or a scrambled non-inhibiting peptide (AC3-C), both under control of the cardiac-specific Myh6 promoter (32), were compared with wild-type littermates. The AC3-I mice have been shown to exhibit increased membrane $K_{\text{ATP}}$ channels not caused by increased transcription or elevated total protein expression (29).

**Cardiomyocyte Isolation**—Single ventricular cardiomyocytes from anesthetized (Avertin, 2.5% solution of tribromoethanol in 2-methyl-2-butanol, Sigma, 240 mg/kg intraperitoneally) male, heterozygous AC3-I and AC3-C transgenic mice (32) and their wild-type littermates aged 10–12 weeks were enzymatically isolated (24). Hearts were cannulated in situ then rapidly excised and retrogradely perfused at 90 mm Hg at 37 °C, pH 7.4, for 5 min with Hepes buffer (Medium 199, Sigma), 1 min with a “low calcium” medium (100 mmol/liter NaCl, 10 mmol/liter KCl, 1.2 mmol/liter KH$_2$PO$_4$, 5 mmol/liter MgSO$_4$, 20 mmol/liter glucose, 50 mmol/liter taurine, 10 mmol/liter HEPES supplemented with 0.13 mmol/liter CaCl$_2$, 2.1 mmol/liter EGTA), then 13 min with low calcium medium supplemented with 1% bovine serum albumin, 0.2 mmol/liter CaCl$_2$, collagenase (type IV, 22 units/ml, Worthington) and Pronase (100 µg/ml, Serva). Ventricles were dissected away, cut into pieces (~3 × 3 mm), and incubated at 37 °C for 15 min in the enzyme solution with gentle stirring until dissociated cardiomyocytes could be collected. Protocols conform to the Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Institutional Animal Care and Use Committee.

**Patch Clamp**—Studies were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) integrated with a Nikon TE2000-U microscope. Experiments were performed at 33–35 °C using a temperature controller TC2r (Cell MicroControls, Norfolk, VA).

For whole-cell recording, borosilicate glass pipettes (3–4 megahoms) were filled with internal solution: 140 mmol/liter KCl, 1 mmol/liter MgCl$_2$, 5 mmol/liter EGTA, 5 mmol/liter ATP, 5 mmol/liter HEPES-KOH, pH 7.3. Cardiomyocytes were superfused with Tyrode solution: 136.5 mmol/liter NaCl, 5.4 mmol/liter KCl, 1 mmol/liter CaCl$_2$, 0.53 mmol/liter MgCl$_2$, 5.5 mmol/liter glucose, 5.5 mmol/liter HEPES-NaOH, pH 7.4. Whole-cell current traces were obtained in response to 1-s rectangular pulses from a holding potential of −50 mV to test potentials from −100 to +40 mV. For quantification, whole cell $K_{\text{ATP}}$ channel current was measured as the difference between base line and pinacidil- and/or 2,3-dinitrophenol (DNP)-stimulated current recorded just before the end of a 1-s applied voltage step from −50 to +40 mV. For analysis, only whole cell recordings in which beginning and ending capacitance were within 10% were used.

For inside-out single channel recording, myocytes were bathed in internal solution 140 mmol/liter KCl, 1 mmol/liter MgCl$_2$, 5 mmol/liter EGTA, 5 mmol/liter HEPES-KOH, pH 7.3, supplemented with glucose (1 g/liter) and various concentra-
Krebs-Henseleit buffer bubbled with 95% O₂, 5% CO₂ at 37 °C and retrogradely perfused at 90 mm Hg with activity was analyzed as previously described (39).

Whole Heart Biotinylation—Hearts were excised from anesthetized mice and retrogradely perfused at 90 mm Hg with Krebs-Henseleit buffer bubbled with 95% O₂, 5% CO₂ at 37 °C and pH 7.4. The AV node was mechanically ablated, and hearts were paced at 150 or 100 ms cycle length (i.e. 400 or 600 beats/min, Bloom Electrophysiology, Fischer Imaging Corp., Denver, CO) using a platinum pacing catheter positioned in the right ventricle (NuMed; Hopkinton, NY). Coronary flow was measured in series with the aortic cannula (T402, Transonic Systems, Ithica, NY). After 25 min the hearts and perfusion system were cooled to 4 °C (Isotemp 3006D, Fisher Scientific, Inc., Pittsburgh, PA) and perfusion buffer was switched from Krebs-Henseleit buffer to PBS containing 1 mg/ml Sulfo-NHS-biotin (ThermoScientific) and supplemented with glucose 5 mmol/liter. During the cooled stage, hearts were asystolic, pacing was discontinued, and perfusion was changed from constant pressure to constant flow to maintain coronary flow at the level measured immediately before cooling. After 25 min of perfusion with biotin, hearts were perfused with PBS containing 15 mmol/liter glycine for 5 min at 4 °C to quench unbound biotin. Immediately after these venticles were freeze-clamped in liquid nitrogen. The tissue was lysed in buffer containing 100 mmol/liter Tris-Base, 50 mmol/liter NaCl, 5 mmol/liter EDTA, 50 mmol/liter NaF, 30 mmol/liter Na₃P₂O₇, 1.25% Triton X-100, and protease inhibitor mixture (Roche Applied Science) at pH 7.4. Biotinylated (cell surface) proteins were isolated by incubating the tissue lysate with immobilized NeutrAvidin beads (ThermoScientific) for 12 h at 4 °C. After separation by 3–8% gradient Nu-Page Tris acetate gel (Invitrogen), biotinylated Kir6.2 was detected by immunoblot with anti-Kir6.2 antibody (Novus) and HRP-conjugated secondary antibody (Santa Cruz Biotechnology).

Cell Culture and cDNA Expression—HEK293T/17 cells (ATCC; CRL-11248) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mmol/liter glutamine, and penicillin-streptomycin. Cells were grown on coverslips for immunofluorescence in a 24-well plate and in 100 ml of tissue culture dishes for immunoprecipitation and phosphorylation assays. Cells were transfected with pcDNA constructs for WT or mutant K_ATP channel subunits and CaMKII (see below) using Lipofectamine 2000 (Invitrogen). Immunofluorescence—HEK293T/17 cells were grown on 13 mm fibronectin-coated glass coverslips and transfected (Lipofectamine 2000; Invitrogen) with cDNA for 1) Kir6.2-HA or its mutants, 2) SUR2A and 3) CaMKII-GFP, 1:5:1 ratio for 48 h at 37 °C. 48 h after transfection cells were blocked with 5% BSA in PBS (30 min at 4 °C) labeled with primary anti-HA monoclonal mouse antibody (1:1000, Clone H-11, Covance) for 1 h at 4 °C. Cells were then washed with phosphate-buffered saline (PBS+/−), then incubated at 37 °C for 30 min in the presence of A23187, fixed with 4% paraformaldehyde in PBS (15 min), permeabilized with 0.1% Triton X-100 (10 min), and blocked with 1% BSA in PBS for 1 h at room temperature. Cells were labeled with Alexa 568-conjugated goat anti-mouse secondary antibody (1:500, Invitrogen) for 1 h at room temperature in a dark chamber. Cells were washed and mounted with vectashield on glass slides for confocal microscopic analysis.

Confocal Fluorescence Imaging—Cells were imaged on a Zeiss LSM 510META laser scanning confocal microscope under a 63× oil immersion lens. Middle cuts through cells were analyzed using ImageJ Software (National Institutes of Health, public domain). The ratio of intracellular-total intensity was calculated.

Western Blot—Whole cell protein extracts (24) were used for immunoblotting with CaMKII (pan), phospho-CaMKII (Thr-286, Cell Signaling), and anti-GAPDH (Santa Cruz Biotechnologies) antibodies.

Drugs—The following drugs were purchased from Sigma: isoproterenol, A23187, pinacidil, DNP, chelerythrine, and dynasore. Dominant-negative dynamin and scrambled control were synthesized (biosYNTHESIS) according to the published sequence (30).

Biotinylation—HEK293T/17 cells were transfected (Lipofectamine 2000; Invitrogen) with cDNA for Kir6.2-HA, SUR2A, and CaMKII-GFP. The cells were cultured in Dulbecco’s modified Eagle’s medium. 48 h after transfection the cells were washed with phosphate-buffered saline (PBS+/−). The cells were incubated at 37 °C for 0, 10, or 30 min in the presence of A23187 and then placed on ice. Channels remaining at the cell surface were incubated with 0.5 mg/ml Sulfo-NHS-biotin (ThermoScientific) and then incubated at 37 °C for 30 min on ice and then quenched with 100 mmol/liter glycine in PBS+/− for 10 min on ice. The cells were lysed in Nonidet P-40 lysis buffer (0.4% sodium deoxycholate, 1% Nonidet P-40, 63 mmol/liter EDTA, 50 mm Tris-HCl, pH 8, and protease inhibitor mixture). Biotinylated (cell surface) proteins were isolated by incubating the tissue lysate with immobilized NeutrAvidin beads (ThermoScientific) for 12 h at 4 °C.
Immunoprecipitation—The left ventricle was isolated from WT mice and freeze-clamped. The tissue was ground in liquid N$_2$ until powder. Tissue was added to lysis buffer containing protease and phosphatase inhibitors (Roche Applied Science), vortexed, and placed on ice for 1 h with sonication for 15 s every 15 min. After centrifugation at 14,000 rpm for 1 h, protein was quantified (Bio-Rad). The lysed ventricle tissue sample (100 µg/100 µl of total protein), and lysis buffer with protease inhibitors (100 µl) was added to Dynabeads (Bio-Rad) conjugated with anti-Kir6.2 (Santa Cruz Biotechnology) or anti-CaMKII (Cell Signaling) antibodies. After elution, Western blot with anti-CaMKII or anti-Kir6.2 antibodies was performed.

Phosphorylation—HEK cells transfected with WT and mutant Kir6.2-HA and SUR were lysed (see method under whole heart biotinylation), and the HA-tagged proteins were isolated using immunoprecipitation with agarose complexed with anti-HA antibody (ThermoScientific). Agarose beads complexed with channel proteins were resuspended in Heps/magnesium acetate buffer: 50 mM Heps, 10 mM magnesium acetate, 0.5 mM CaCl$_2$, 1 mg/ml BSA, 1 µM calmodulin, and incubated with [γ-32P]ATP (1–1.5 µCi, PerkinElmer Life Sciences) with or without activated CaMKII (250 ng). The CaMKII, purified from Sf9 insect cells (see below), was activated by 1 mM CaCl$_2$, 1 mmol/liter magnesium acetate, 0.5 mmol/liter CaCl$_2$, 0.001 mmol/liter calmodulin, 1 mg/ml BSA, 1 mmol/liter DTT, 0.4 mmol/liter [γ-32P]ATP (200–500 cpm/pmol), 0.02 mmol/liter syntide. The mixture was incubated for 10 min at 30 °C. The reaction was stopped with SDS-PAGE loading buffer or by spotting on Whatman P81 paper. 5 washes of 10 min each were performed before counts obtained by scintillator.

Homology Modeling of Kir6.2—The amino acid sequence of mouse Kir6.2 (UniProt ID Q61743) was employed as a query for homology modeling. The N-terminal segment Met1—Arg25 and the C-terminal segment Lys-345–Ser390 were deleted before any calculation. The mouse Kir3.2 channel in closed state co-crystallized with K$^+$, Na$^+$, and a phosphatidylinositol diphosphate analog (PDB code 3SYA) was used as a template for the homology modeling. The sequence of Kir6.2 was aligned to that of Kir3.2 using T-Coffee (43). The alignment was manually refined with VMD1.9.1 and the Multiseq plugin. Homology modeling was performed with Modeler 9.10. One hundred models of the Kir6.2 homotetramer were generated and ranked according to their DOPE score. The model with the lowest score was selected. The best model was minimized in five successive stages using the CHARMM27 force field and NAMD2.9: 1) minimization of the hydrogen atoms (1,000 steps); the position of non-hydrogen atoms was restrained by a force constant of 2 kcal/mol/Å$^2$; 2) minimization of the amino acid side chains (4,000 steps) with position restraints applied on carbons C$\beta$ plus the backbone; 3) minimization of the whole amino acid side chains (4,000 steps) with position restraints applied on the backbone; 4) minimization (10,000 steps) with position restraints applied on carbons C$\alpha$; 5) minimization of the whole Kir6.2 homotetramer (15,000 steps). The phosphorylation (PO$_4^-$) of Thr-180 and/or Thr-224 was introduced before stage 1. The final models were checked with the What If and the JCSG protein structure validation servers. One disulfide bridge between Cys110 and Cys142 was defined during the calculation. The coordinates of the four K$^+$ ions and the four phosphatidylinositol diphosphate analogues initially present in the x-ray structures of Kir3.2 were preserved in the Kir6.2 model. The position of all their non-hydrogen heteroatoms was restrained over all the minimization process. The phosphatidylinositol diphosphate analog was modeled using the CHARMM36 parameters. Analysis of the results was performed with VMD 1.9.1.

The model of µ2 is based on the published structure of µ2 co-crystallized with the TGN38 internalization signal peptide DYQRN (PDB code 1BXX). The peptide YQ was superimposed with the YS of YSQKF and µ2 positioned by trial and error. The position with the fewest steric conflicts was selected.
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**Statistical Analysis**—Results are expressed as the mean ± S.E. Comparisons between two groups were made using the 2-sided Student’s $t$ test and appropriate post hoc tests. A $p$ value < 0.05 was considered statistically significant.

**RESULTS**

**CaMKII Activation Reduces Whole Cell $K_{ATP}$ Channel Current and Cell Membrane Expression**—Transgenic mice expressing a specific peptide inhibitor of CaMKII (AC3-I) exhibit an increased presence of membrane $K_{ATP}$ channels not caused by increased transcription or elevated total protein expression (29). CaMKII is activated by signals that increase intracellular calcium, including catecholamine stimulation (32). Using real-time patch clamp monitoring of the $K_{ATP}$ channel, whole cell current induced by the opsin pinacidil, and metabolic inhibition by DNP, we found that application of isoproterenol significantly reduces $K_{ATP}$ current in isolated ventricular cardiomyocytes within 2–3 min (Fig. 1, A and D, fractional reduction = 0.66 ± 0.1, $p < 0.05$). A similar significant and prompt reduction was found in ventricular cardiomyocytes from transgenic mice expressing a non-CaMKII-inhibiting control peptide, AC3-C (Fig. 1, B and D, fractional reduction = 0.58 ± 0.08, $p < 0.05$, $p$ NS versus WT). In contrast, very little $K_{ATP}$ channel current reduction was observed in cardiomyocytes isolated from transgenic mice expressing the CaMKII inhibitory peptide, AC3-I (Fig. 1, C and D, fractional reduction = 0.14 ± 0.06, $p < 0.05$ versus WT), supporting the hypothesis that the isoproterenol effect on reducing whole cell $K_{ATP}$ channel current in the WT cardiomyocytes occurred through CaMKII activation. In WT and AC3-I cardiomyocytes, the reduction of stimulated $K_{ATP}$ channel whole cell current in response to isoproterenol was reversed within ~5 min upon washout of isoproterenol, indicating that the phenomenon is not related to channel rundown (Fig. 1, A and B). $K_{ATP}$ channel current may also be regulated by calcium activated forms of PKC (30, 44–49). To test for a potential role of PKC in the witnessed reduction of $K_{ATP}$ channel current, we examined the isoproterenol effect in the presence of the PKC inhibitor, chelerythrine. Despite pretreatment with chelerythrine, isoproterenol induced a significant decrease in whole cell $K_{ATP}$ channel current in isolated cardiomyocytes (Fig. 1, E–G, additional fractional reduction with isoproterenol = 51.2 ± 7.6, $p < 0.05$ versus reduction by chelerythrine alone).

The interaction of CaMKII and $K_{ATP}$ channels in cardiomyocytes was further confirmed by co-immunoprecipitation. Anti-CaMKII Ig co-immunoprecipitated Kir6.2 and anti-Kir6.2 antibodies co-immunoprecipitated CaMKII from detergent-soluble lysates generated from left ventricles of WT mice (Fig. 1, H and I).

In the presence of increased pacing rates, previously linked to CaMKII activation (50), the membrane fraction of ventricular lysates from biotinylated isolated hearts (WT and AC3-C) exhibited lower Kir6.2 membrane expression (fractional difference in ratio of Kir6.2 expression normalized to Na$^{+}$/K$^{+}$ ATPase expression at 100 compared with 150-ms pacing cycle length = −0.76 ± 0.09 for WT and −0.66 ± 0.18 for AC3-C, $p < 0.05$). This difference is similar in magnitude to the whole cell current change observed with isoproterenol treatment of isolated WT and AC3-C cardiomyocytes (Fig. 1D), indicating that the current change can be mostly accounted for by a reduction in sarcolemmal expression of $K_{ATP}$ channels. A reduction in surface channel expression at higher pacing rates as assessed by whole heart biotinylation was not seen in hearts expressing a peptide inhibitor of CaMKII (AC3-I, Fig. 1, J and K, fractional difference, 0.01 ± 0.13, $p = NS$), similar to the absence of whole cell current reduction observed in isolated cardiomyocytes from AC3-I mice (Fig. 1D). The expression of Na$^{+}$/K$^{+}$ ATPase and GDPH in membrane and total fractions, respectively, did not show significant changes with faster pacing ($p = NS$).

We next developed a heterologous cell expression system to dissect the components of the CaMKII-$K_{ATP}$ channel pathway. We examined HEK cells with and without exogenous overexpression of CaMKII and found that HEK cells had very little native CaMKII expression (Fig. 2A). We then performed a densitometric analysis of immunoblots from HEK cells with exogenous CaMKII overexpression and found that their exposure to the calcium ionophore A23187 resulted in a significant increase in phosphorylated (activated) CaMKII (0.75 ± 0.09 versus 1.29 ± 0.07, $n = 3$ each, $p < 0.05$).

To confirm the effect of CaMKII activation on the surface expression of $K_{ATP}$ channels in this model, we used a cell surface biotinylation assay. Extracted total and biotinylated proteins from HEK cells transfected with CaMKII and the cardiac $K_{ATP}$ channel subunits Kir6.2 and SUR2A with and without CaMKII activation by A23187 were assayed by Western blot. This indicated that CaMKII activation reduced the fraction of surface versus total HA-tagged Kir6.2 subunits in a time-dependent manner (Fig. 2, B and C, % of expression at time 0 = 67 ± 8 at 10 min and 17 ± 11 at 20 min, $p < 0.05$).

These changes in the presence of $K_{ATP}$ channels at the cell membrane were further confirmed by measuring whole cell $K_{ATP}$ channel current by patch clamp. A23187 dramatically reduced $K_{ATP}$ channel current (Fig. 2, D and E, $n = 4$, fractional reduction = 0.49 ± 0.04, $p < 0.05$). Similar to the finding in cardiomyocytes, the effect was reversible with washout of the ionophore (Fig. 2D). The dependence of this phenomenon on CaMKII activation was confirmed in HEK cells expressing $K_{ATP}$ channel subunits but without exogenous CaMKII overexpression. In these cells very little $K_{ATP}$ channel current reduction by A23187 was observed (Fig. 2E, $n = 5$, fractional reduction = 0.06 ± 0.03, $p < 0.05$ versus cells with CaMKII). These biochemical and biophysical data indicate that CaMKII directly interacts with cardiac $K_{ATP}$ channels and that CaMKII activation promotes a reversible reduction in the presence of $K_{ATP}$ channels at the cell membrane.

**CaMKII Activation Reduces $K_{ATP}$ Channel Membrane Expression through Endocytosis**—The above whole cell patch clamp experiments and biotinylation experiments in HEK cells expressing $K_{ATP}$ channel subunits and CaMKII indicate a reversible down-regulation of membrane $K_{ATP}$ channel subunits and $K_{ATP}$ channel current without a change in total cellular Kir6.2, suggesting endocytosis as a possible mechanism. To test this, we tracked whole cell $K_{ATP}$ channel current when endocytosis was prevented by the addition of a dominant negative dynamin peptide (30) to the patch pipette solution. We found that $K_{ATP}$ current reduction in response to CaMKII acti-
vation by A23187 was eliminated in the presence of dominant negative (DN) dynamin (Fig. 2, F and G). In contrast, an inactive scrambled peptide had no effect on the $K_{ATP}$ channel current response to A23187 (Fig. 2G, $n = 5$ each, fractional current reduction = 0.40 ± 0.05 for scrambled, $p < 0.05$, and 0.03 ± 0.04 for dominant negative dynamin, $p = $ NS versus base line). These data implicate enhanced endocytosis of channel subunits as the dominant mechanism for the witnessed CaMKII activation-dependent decrease in whole cell $K_{ATP}$ channel current capacity.

We confirmed these data using immunofluorescence to detect internalization of labeled surface Kir6.2 subunits in response to A23187. HEK cells transfected with Kir6.2-CA, SUR2A, and CaMKII-GFP were incubated with an anti-CA antibody to allow the antibodies to bind the surface channels and undergo internalization. We then treated cells with
A23187, permeabilized, and stained them with red fluorescence-conjugated secondary antibody. Thus, only channels that were initially at the cell membrane and either remained there or became internalized were labeled. When CaMKII was not overexpressed (Fig. 3, A and B) or when CaMKII was overexpressed but cells were not treated with A23187 (Fig. 3, C and D), we detected bright red fluorescence only on the cell surface (n = 16 cells from 1 transfection, fluorescence ratio 0.52 ± 0.04, p < 0.05 versus control and n = 16 cells from 1 transfection, fluorescence ratio 0.52 ± 0.03, p < 0.05 versus control, respectively). However, when cells had CaMKII overexpression and were treated with A23187 (Fig. 3, E and F), we detected distinct puncta of red fluorescence in the cytoplasm, consistent with K ATP channel subunit internalization (n = 34 cells from 2 transfections, cytosolic/total cellular intensity ratio = 0.71 ± 0.02). This effect was eliminated in CaMKII-overexpressing cells that had treatment with A23187 and the membrane-permeable endocytosis inhibitor dynasore (Fig. 3, G and H, n = 18 cells from 1 transfection, fluorescence ratio 0.48 ± 0.01, p < 0.05 versus control). Taken together, measurement of membrane currents, biotinylation of surface proteins, and immunodetection of K ATP channel subunits indicates that CaMKII activation
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Figure 3. CaMKII-dependent internalization of $K_{ATP}$ channels and Kir6.2 subunit dependence. Immunofluorescence confocal imaging was performed on HEK cells expressing HA-tagged Kir6.2, WT SUR2A, and GFP-tagged CaMKII or GFP alone without CaMKII. Anti-HA antibody was applied at the beginning of the experiment, and cells were washed. After a 30-min exposure to 10 μmol/liter A23187 to activate CaMKII, cells were cooled, fixed, and permeabilized, and secondary antibody was applied (red) to track surface Kir6.2-HA movement. Shown are cells expressing Kir6.2-HA, SUR2A, and GFP without CaMKII (A and B), Kir6.2-HA, SUR2A, and CaMKII-GFP but not stimulated with A23187 (C and D), Kir6.2-HA, SUR2A, and CaMKII-GFP with application of A23187 (E and F), and Kir6.2-HA, SUR2A, and CaMKII-GFP stimulated with A23187 in the presence of the endocytosis inhibitor, dynasore 50 μmol/liter (G and H). I and J, Immunofluorescence confocal microscopy was performed on HEK cells expressing Kir6.2Δ36-HA (red) and CaMKII-GFP following the same protocol. K, representative whole cell $I_{KATP}$ channel current was stimulated by 200 μmol/liter DNP in response to 5 μmol/liter A23187 in a HEK cell expressing Kir6.2Δ36 and CaMKII. L, shown are summary data for DNP-stimulated whole cell $K_{ATP}$ channel current from HEK cells expressing CaMKII and Kir6.2Δ26 (n = 4) or Kir6.2Δ36 (n = 5) in response to CaMKII activation by A23187 (*, p < 0.05).

reduces the cardiac $K_{ATP}$ channel current capacity of cells by enhancing endocytosis.

CaMKII-dependent Endocytosis of $K_{ATP}$ Channels Does Not Require the SUR Subunit—Because cardiac $K_{ATP}$ Channels are composed of pore-forming and regulatory subunits, we sought to determine whether both were required for CaMKII-triggered endocytosis. WT Kir6.2 requires SUR for normal assembly and trafficking to the cell membrane. To circumvent the interdependence of Kir6.2 and SUR, we transfected HEK cells with an HA-tagged Kir6.2 truncation mutant (Kir6.2Δ36) that can traffic to the membrane without the SUR subunit (51). Application of A23187 resulted in internalization of the Kir6.2Δ36-HA subunit to a similar degree as HA-tagged WT $K_{ATP}$ channels as demonstrated by immunofluorescence (Fig. 3, I and J, n = 36 cells from 3 transfections, cytosolic/total cellular intensity ratio = 0.58 ± 0.02, $p = \text{NS versus WT}$) with a significant reduction in DNP-stimulated $K_{ATP}$ channel current as demonstrated by whole cell patch clamp (Fig. 3, K and L, n = 5, fractional reduction = 0.89 ± 0.04, $p < 0.05$). Similar findings were also seen for Kir6.2Δ26, which can also traffic to the membrane without SUR (51) (Fig. 3L, n = 4, fractional reduction = 0.95 ± 0.02, $p < 0.05$), immunofluorescence not shown, n = 24 cells from 3 transfections, cytosolic/total cellular intensity ratio = 0.60 ± 0.02, $p = \text{NS versus WT}$ channels). These findings support that CaMKII-induced internalization of membrane cardiac $K_{ATP}$ channels is mediated through Kir6.2 and does not require SUR.

Phosphorylation of Thr-180 and Thr-224 on Kir6.2 Are Required for $K_{ATP}$ Channel Endocytosis—CaMKII regulates the function of proteins by targeting specific serines and threonines embedded within consensus sequences. To further investigate regions on Kir6.2 that may be required for CaMKII-mediated $K_{ATP}$ channel endocytosis, we investigated four sites identified through scanning the Kir6.2 sequence for the RX(S/T) motif and through literature reports of sites with other serine/threonine kinase interactions (44, 45, 47, 52, 53). We identified candidate sites at Ser-37, Thr-180, Thr-224, and Ser-372. In experiments to determine the impact of these sites on endocytosis of cardiac $K_{ATP}$ channels, we found no effect of S37A and S372A mutations on $K_{ATP}$ channel current or membrane expression (data not shown), and thus further results and discussion focus only on the Thr-180 and Thr-224 sites.

We probed isolated WT and mutant Kir6.2-HA-containing channels for in vitro phosphorylation by CaMKII. Mutation of
Thr-180 or Thr-224 to a non-phosphorylatable alanine (T224A and T180A) resulted in only a faint signal for incorporation of the radio-labeled \( \gamma \)H9253-phosphate from ATP in the presence of activated CaMKII, and this signal was further attenuated in the presence of double T180A/T224A mutation in Kir6.2 compared with WT (Fig. 4A).

Next, we generated segments of the Kir6.2 subunit surrounding Thr-224 and Thr-180 to further demonstrate phosphorylation of these sites by CaMKII (Fig. 4B). Mutation to T224A and T180A in these fragments eliminated incorporation of the radiolabeled \( \gamma \)H9253-phosphate from ATP in the presence of activated CaMKII (Fig. 4B).

In HEK cells expressing CaMKII, SUR2A, and Kir6.2T224A-HA or Kir6.2T180A-HA, much less internalization of \( K_{\text{ATP}} \) channels in response to treatment with A23187 was appreciated compared with cells expressing HA-tagged WT Kir6.2 (Fig. 4, C–H). The cytosolic/total cellular intensity was 0.61 ± 0.02 for WT versus 0.39 ± 0.01 for T224A and 0.37 ± 0.02 for T180A, \( p < 0.05 \) versus WT for both mutants, Fig. 4I).

Similarly, in HEK cells expressing CaMKII, SUR2A, and Kir6.2T224A-HA, the whole cell \( K_{\text{ATP}} \) channel current reduction after CaMKII activation was diminished compared with cells with HA-tagged WT Kir6.2 subunits (Fig. 4, J and K, \( n = 6 \), *p < 0.05).
phosphorylation of Kir6.2 at Thr-224 results in a reduction in $K_{\text{ATP}}$ channel ATP sensitivity. However, this decrease in ATP sensitivity of the channel would oppose, not contribute to or account for, the reduction in whole cell $K_{\text{ATP}}$ channel current in the presence of CaMKII activation.

Expression of constitutively active CaMKII caused no differences in $K_{\text{ATP}}$ single channel conductance (70.6 ± 0.5 versus 71.1 ± 0.8 picosiemens with WT CaMKII) or open probability (0.81 ± 0.05 versus 0.84 ± 0.03 with WT CaMKII).

Mutations of the Kir6.2 Thr-180 residue resulted in loss of normal channel gating. Overall activity of channels with the Kir6.2T180A mutation was rare, and these channels appeared to have dramatically increased ATP sensitivity ($n = 3$, Hill = 1.8, $IC_{50} = 40 \mu\text{M}$). No $K_{\text{ATP}}$ channel current was detected in the presence of the Kir6.2T180E mutant. These data indicate that the Thr-180 site is essential for normal regulation of channel gating.

Single channel conductance was unchanged in channels comprised of WT Kir6.2 versus Kir6.2T224A or Kir6.2T180A (71.4 ± 0.8 versus 70.9 ± 0.6 versus 70.1 ± 0.8 picosiemens, respectively, $n = 3$ each, Fig. 5C). Overall, these data show that CaMKII-dependent phosphorylation of the Thr-224 and Thr-180 residues of Kir6.2 has a role in regulating channel gating and is critical to promote endocytosis of the $K_{\text{ATP}}$ channel subunits.

The Kir6.2 Internalization Motif $330\text{YSKF}^{333}$ Is Required for CaMKII-induced $K_{\text{ATP}}$ Channel Down-regulation—Patients with a genetic form of neonatal diabetes have been found to harbor Y330C and F333I mutations of Kir6.2 that inhibit spontaneous endocytosis of $K_{\text{ATP}}$ channels at 37 °C and result in a ~2-fold increase in surface channels (54). Here, we examined whether the $330\text{YSKF}^{333}$ sequence is required for internalization of cardiac $K_{\text{ATP}}$ channels driven by CaMKII activation. In HEK cells expressing Kir6.2Y330C-HA, SUR2A, and CaMKII, pinacilid- and DNP-stimulated KATP channel current was reduced very little by application of A23187 (fractional reduction 0.05 ± 0.02, $n = 6$, $p < 0.05$ versus 0.49 ± 0.04, $n = 4$ for WT Kir6.2, Fig. 6, A and B). Similarly, there was little cytosolic appearance of labeled Kir6.2Y330C-HA after stimulation of HEK cells with A23187 (Fig. 6 C and D, $n = 44$ cells from 4 transfections, cytosolic/total cellular intensity = .20±.01, $p < 0.05$ versus WT). These findings indicate that an intact $330\text{YSKF}^{333}$ motif is required for CaMKII-induced endocytosis of cardiac $K_{\text{ATP}}$ channels.

A Molecular Model Suggests Docking of the Endocytosis Adaptor Protein $\mu_2$ Subunit Requires CaMKII Phosphorylation Sites on Neighboring Kir6.2 Subunits—The Kir6.2 C terminus including residues 178–364 (which would include the Thr-180 and Thr-224 sites and the $330\text{YSKF}^{333}$ motif) has been shown to interact directly with the $\mu_2$ subunit of the endocytosis adaptor protein, AP2 (54). Here, to investigate the effect of phosphorylation of Thr-180 and Thr-224 on this relationship, we formulated a molecular homology model of Kir6.2 based on the crystal structure of mouse Kir3.2. The model predicts that Thr-224 and Thr-180 are too distant from the $330\text{YSKF}^{333}$ sequence to directly interact and that phosphorylation of Thr-224 and Thr-180 is unlikely to induce large enough conformational changes
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FIGURE 6. Mutation of YXXØ internalization motif interferes with CaMKII-induced $K_{\text{ATP}}$ channel down-regulation. A, shown is representative whole cell $K_{\text{ATP}}$ channel current, stimulated by 50 μmol/liter pinacidil (pin) and 50 μmol/liter DNP, measured during application and wash-out of 5 μmol/liter A23187 in HEK cells engineered to express Kir6.2Y330C-HA, SUR2A, and CaMKII-GFP. B, shown are summary data from whole cell patch clamp experiments performed with Kir6.2-HA ($n = 4$) versus Kir6.2Y330C-HA ($n = 6$; *, $p < 0.05$). C and D, shown are representative confocal fluorescence images of HEK cells expressing Kir6.2Y330C-HA, SUR2A, and CaMKII-GFP after treatment with A23187.

However, docking of the known structure of the μ2 subunit of the endocytosis adaptor protein, AP2, to the modeled Kir6.2 suggests that μ2 could bind $330^\text{YSKF}^{333}$ and interact with the nearby Thr-180 via a long loop (possibly the lysine-rich Asn-217—Asp-244, Fig. 7, C and D), assuming the ATP binding pocket was unoccupied. The μ2 subunit could simultaneously interact with Thr-224 of the neighboring Kir6.2 subunit via a loop (possibly Ile-159—Asn-171, Asn-217—Asp-244, or Val-406—Ser-414, Fig. 7, C and D). The arginine-rich N terminus could be electrostatically compatible with a Thr-224 phosphate. Thus, the model suggests a possible role for the Thr-180 and Thr-224 residues in coordinating the docking of μ2 with Kir6.2 at $330^\text{YSKF}^{333}$.

DISCUSSION

Cardiac $K_{\text{ATP}}$ channels are critical for cellular energetic homeostasis and protection from injury and arrhythmia (1, 6, 26). As such, deciphering their regulation is key to understand-
of the channel, we can hypothesize that phosphorylation of Thr-180 by CaMKII would discourage ATP binding and thus allow μ2 to dock, thereby permitting endocytosis. Similarly, our model predicts that Thr-224 is unlikely to interact directly with YSKF and that phosphorylation of Thr-224 is unlikely to affect accessibility of YSKF. However, it does appear that μ2 on one Kir6.2 subunit may interact with Thr-224 on a neighboring Kir6.2 subunit. Thus, our model suggests several potential mechanisms for how Thr-180 and Thr-224, and YSKF on Kir6.2 may interface with the cellular endocytic machinery to permit CaMKII-triggered endocytosis of cardiac K<sub>ATP</sub> channels. These prospects will be the subject of future experimental studies.

Our data also indicate the significance of the Kir6.2 residues Thr-180 and Thr-224 and their phosphorylation state in the regulation of K<sub>ATP</sub> channel gating. The effect of the T180A mutant on K<sub>ATP</sub> channel function and current capacity is particularly intriguing. The immunofluorescence images clearly demonstrate bright fluorescence at the cell membrane thus indicating that the effect of the T180A mutation on reduced NPo of single channel K<sub>ATP</sub> channel recordings does not primarily occur through interference with surface presence of the
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mutant channel. Rather, it appears the mutation alters channel gating and/or ATP sensitivity. Interestingly, a previous report of the Thr-180 mutation showed abnormal biophysical properties of the channel as well (47). In that study, whole cell $K_{\text{ATP}}$ channel activity in response to openers and/or DNP, as was done in the current study, was not tested nor was the full-length Kir6.2T180A mutant in the presence of SUR2A tested in the inside-out mode as was done here, and thus a direct comparison with our results is not possible. Because we only saw rare lone channels in the full-length Kir6.2T180A mutant with SUR2A, we were unable to assess the effect of this mutation on rundown. Nonetheless, our study is in line with the findings of other investigators that indicate the Thr-180 residue is a critical determinant of channel gating behavior (47, 58).

The T224A and T224(D/E) mutations in Kir6.2 have also been previously described and found to alter protein kinase A-mediated regulation of $K_{\text{ATP}}$ channel gating (52, 53). We confirmed that mimicking phosphorylation of Thr-224, by the T224E mutation, shifts the ATP sensitivity of Kir6.2/SUR2A complexes to the right. However, this would oppose the T224A mutation witnessed here and thus can be excluded as contributor to the phenomenon.

Taken together with previous findings that changes in $K_{\text{ATP}}$ channel surface abundance have a significant impact on cardiac energetics (23–25), sarcolemmal $K_{\text{ATP}}$ channel down-regulation via CaMKII-mediated endocytosis reveals a new mechanism for control of myocardial performance and energy use. Indeed, CaMKII inhibition has been investigated as a promising mechanism for control of myocardial performance and energy use. The T224E mutation, shifts the ATP sensitivity of Kir6.2/SUR2A complexes to the right. However, this would oppose the T224A mutation witnessed here and thus can be excluded as contributor to the phenomenon.

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