Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase II-dependent Remodeling of Ca\textsuperscript{2+} Current in Pressure Overload Heart Failure*

Received for publication, April 21, 2008, and in revised form, July 8, 2008 Published, JBC Papers in Press, July 11, 2008, DOI 10.1074/jbc.M803043200

Yanggan Wang\textsuperscript{1,2}, Samvit Tandan\textsuperscript{2}, Jun Cheng\textsuperscript{3}, Chunmei Yang\textsuperscript{1}, Lan Nguyen\textsuperscript{4}, Jessica Sugianto\textsuperscript{5}, Janet L. Johnstone\textsuperscript{6}, Yuyang Sun\textsuperscript{1}, and Joseph A. Hill\textsuperscript{1,3}

From the Departments of \textsuperscript{1}Internal Medicine (Cardiology) and \textsuperscript{2}Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-8573 and the \textsuperscript{3}Department of Pediatrics, Emory University, Atlanta, Georgia 30322

Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) activity is increased in heart failure (HF), a syndrome characterized by markedly increased risk of arrhythmia. Activation of CaMKII increases peak L-type Ca\textsuperscript{2+} current (I\textsubscript{C\text{a}0}) and slows I\textsubscript{C\text{a}} inactivation. Whether these events are linked mechanistically is unknown. I\textsubscript{C\text{a}} was recorded in acutely dissociated subepicardial and subendocardial murine left ventricular (LV) myocytes using the whole cell patch clamp method. Pressure overload heart failure was induced by surgical constriction of the thoracic aorta. I\textsubscript{C\text{a}} density was significantly larger in subepicardial myocytes than in subendocardial/myocytes. Similar patterns were observed in the cell surface expression of r1c, the channel pore-forming subunit. In failing LV, I\textsubscript{C\text{a}} density was increased proportionately in both cell types, and the time course of I\textsubscript{C\text{a}} inactivation was slowed. This typical pattern of changes suggested a role of CaMKII. Consistent with this, measurements of CaMKII activity revealed a 2–3-fold increase (p < 0.05) in failing LV. To test for a causal link, we measured frequency-dependent I\textsubscript{C\text{a}} facilitation. In HF myocytes, this CaMKII-dependent process could not be induced, suggesting already maximal activation. Internal application of active CaMKII in failing myocytes did not elicit changes in I\textsubscript{C\text{a}}. Finally, CaMKII inhibition by internal diffusion of a specific peptide inhibitor reduced I\textsubscript{C\text{a}} density and inactivation time course to similar levels in control and HF myocytes. I\textsubscript{C\text{a}} density manifests a significant transmural gradient, and this gradient is preserved in heart failure. Activation of CaMKII, a known pro-arrhythmic molecule, is a major contributor to I\textsubscript{C\text{a}} remodeling in load-induced heart failure.

Patients with heart failure are at increased risk of malignant arrhythmia, which accounts for a substantial component of the mortality associated with this disease (1). Mechanisms underlying these arrhythmias are multifactorial, but they stem, at least in part, from disordered electrical currents arising from prolongation of ventricular action potentials. The resulting delay in the recovery of excitability, a consistent feature of heart failure (2), predisposes to early and delayed after-depolarizations. Heart failure is also associated with myocardial fibrosis, altered electrotonic coupling between cells, slowed conduction, and dispersion of refractoriness, all of which predispose to reentrant mechanisms of arrhythmia. Together, these “electrical remodeling” responses underlie the propensity to arrhythmia, syncope, and sudden death.

In recent years, electrical remodeling has emerged as an important pathophysiologic mechanism in many types of cardiac pathology. Whereas considerable progress has been made recently in elucidating the molecular pathogenesis of cardiac hypertrophy and failure (reviewed in Refs. 3 and 4), our understanding of mechanisms underlying disease-related action potential prolongation is limited. As a result, the means of treating heart failure-associated arrhythmias remain disappointingly ineffective. Importantly, there is evidence demonstrating that alterations in transmembrane Ca\textsuperscript{2+} fluxes, a central feature of electrical remodeling, contribute to the pathogenesis of heart failure by abnormally activating Ca\textsuperscript{2+}-responsive signaling pathways.

Among the hypertrophic signaling pathways activated by Ca\textsuperscript{2+} is one mediated by Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) (5). CaMKII is a ubiquitously expressed homo- or heteromultimer of 6–12 subunits consisting of \( \alpha, \beta, \gamma, \text{ or } \delta \) subunits, each encoded by a separate gene (6). In heart, the \( \delta \) isofrom is predominant, and the \( \gamma \) subunit is expressed at low levels. Genetic overexpression of CaMKII in ventricular myocytes increases peak L-type Ca\textsuperscript{2+} current (I\textsubscript{C\text{a}0}) and slows I\textsubscript{C\text{a}} inactivation, events that are likely pro-arrhythmic. Further, both CaMKII levels and kinase activity are increased in heart failure (7–10). However, a link between increased CaMKII activity and heart failure-associated remodeling of I\textsubscript{C\text{a}} has not been demonstrated.

In cardiac myocytes, CaMKII phosphorylates an array of protein substrates; prominent among them is the L-type Ca\textsuperscript{2+}...
channel (11). Here, we investigated heart failure-associated changes in \( I_{Ca} \) in a clinically relevant model of pressure overload heart failure. We provide evidence that \( I_{Ca} \) is a focus of robust disease-related remodeling by a mechanism involving CaMKII activation.

**EXPERIMENTAL PROCEDURES**

**Severe Thoracic Aortic Banding**—Increased pressure in the proximal aorta was induced by means of severe thoracic aortic banding (sTAB) (12) according to protocols approved by the institution’s Animal Care and Use Committee. Briefly, male mice (C57BL6, 6–8 weeks old) were anesthetized with ketamine (100 mg/kg intraperitoneally) plus xylazine (5 mg/kg intraperitoneally). The mice were then orally intubated with 20-gauge tubing and ventilated (Harvard Apparatus Rodent Ventilator, model 687) at 120 breaths/min (0.1 ml of tidal volume). A 3-mm left-sided thoracotomy was created at the second intercostal space. The transverse aortic arch was ligated (7–0 Prolene) between the innominate and left common carotid arteries with an overlying 28-gauge needle, and then the needle was removed, leaving a discrete region of stenosis. The chest was closed, and the left-sided pneumothorax was evacuated. Perioperative (24 h) mortality was <10%.

**Isolation of Individual Ventricular Myocytes**—Mouse LV myocytes were isolated enzymatically by a protocol described previously (13) with modifications. In brief, after retrograde perfusion with Krebs-Ringer solution at 2 ml/min (5 min), the heart was perfused by a fresh solution containing 0.8 mg/ml collagenase (Worthington type II) for another 15 min. Next, the apex was removed, and the LV wall was cut and put into a culture dish filled with KB solution. A small 90-degree curved forceps was used to carefully dissect a very thin layer of endomyocardium. Next, the mid-region myocardium was dissected and discarded, leaving the remainder as epimyocardium. Endomyocardium and epimyocardium were minced into small pieces in KB solution, triturated, and studied within 4–6 h. All of the steps were performed at 36 °C in solutions gassed with 95% O\(_2\) + 5% CO\(_2\). Only calcium-tolerant, quiescent, and rod-shaped cells showing clear cross-striations were studied.

**Electrophysiological Recordings**—Isolated myocytes were investigated in a continuously superfused (1.5 ml/min) recording chamber (volume 1 ml) fixed to an inverted microscope. For \( I_{Ca} \) recording, we used the whole cell voltage clamp technique (Axopatch 200B, Molecular Devices), with pipette resistances of 2–3 MΩ when filled with internal solution. The junctional potential was corrected by zeroing the potential just before the pipette tip touched the cell membrane. After the cell membrane was broken by application of additional suction, cell capacitance and series resistance were electrically compensated. After access was gained in the whole cell voltage clamp configuration, the myocytes were allowed to equilibrate for 5 min with the internal solution before the data were collected. \( I_{Ca} \) was recorded at room temperature. The cells were depolarized every 10 s from a holding potential of −50 mV to test potentials between −40 to +60 mV (10 mV steps) for 300 ms.

\( I_{Ca} \) run-down, when present, typically occurs within the first 5 min of recording. To minimize the impact of \( I_{Ca} \) run-down, we added 5 mmol/liter Mg-ATP to the pipette solution and commenced data acquisition after 5–10 min of equilibration between pipette solution and intracellular contents (14). Cells showing continuous current run-down (≥5%) were excluded from the analysis. In some experiments, active CaMKII (1 μM, 14–217; Upstate, Temecula, CA), a mixture of α, β, γ, and δ CaMKII isoforms, was infused intracellularly.

**Solutions**—The Krebs-Ringer solution for cell isolation contained 35 mmol/liter NaCl, 4.75 mmol/liter KCl, 1.19 mmol/liter KH\(_2\)PO\(_4\), 16 mmol/liter NaHPO\(_4\), 134 mmol/liter sucrose, 25 mmol/liter NaCO\(_3\), 10 mmol/liter glucose, 10 mmol/liter HEPES, pH 7.4, with NaOH. The KB solution for storage of cells contained: 10 mmol/liter tauroine, 70 mmol/liter glutamic acid, 25 mmol/liter KCl, 10 mmol/liter KH\(_2\)PO\(_4\), 22 mmol/liter glucose, 0.5 mmol/liter EGTA, pH adjusted to 7.2 with KOH. Test solution for \( I_{Ca} \) recording contained 135 mmol/liter TEA, 0.53 mmol/liter MgCl\(_2\), 1.8 mmol/liter CaCl\(_2\), 20 mmol/liter CsCl, 5 mmol/liter HEPES, pH 7.4, with CsOH. Pipette solution for \( I_{Ca} \) recording contained 110 mmol/liter CsSO\(_4\), 90 mmol/liter aspartic acid, 20 mmol/liter CsCl, 10 mmol/liter tetraethylammonium chloride, 10 mmol/liter HEPES, 10 mmol/liter EGTA, 5 mmol/liter Mg-ATP, 5 mmol/liter Na\(_2\) creatine phosphate, 0.4 mmol/liter GTP (Tris), 0.1 mmol/liter leupeptin, pH 7.2, with CsOH.

**Ca\(^{2+}\)/CaMKII Activity Measurement**—Acutely isolated myocytes were lysed in cell extraction buffer (10 mm Tris-HCl, pH 7.4, 150 mm NaCl, 20% glycerol, 1 mm dithiothreitol, 0.1% Triton X-100, Mini-Complete\(^\circ\) protease inhibitor mixture (Roche Applied Science), and phosphatase inhibitor cocktails I and II (Sigma-Aldrich)). CaMKII activity was measured using the SigmaTECT calcium/calmodulin-dependent protein kinase assay system (Promega) (15). Briefly, cell lysates were incubated in reaction buffer (250 mm Tris-HCl, pH 7.5, 50 mm MgCl\(_2\), 2.5 mm dithiothreitol, 5 mm CaCl\(_2\), 5 μM calmodulin, 0.1 mg/ml bovine serum albumin, 0.5 mm ATP) containing the biotinylated CaMKII peptide substrate and 0.5 μCi of [γ-32P]ATP. The reactions were performed at 30 °C for 2 min and terminated by the addition of 7.5 μL guanidine hydrochloride. Equal amounts of reaction products were blotted on SAM\(^\circ\) Biotin Capture Membrane (Promega), and the membrane was washed with 2 mL NaCl and 1% H\(_3\)PO\(_4\) to remove unincorporated isotope. Enzyme activity was analyzed by scintillation counting and normalized to protein concentration.

**Surface-Protein Biotinylation Assay**—To assay for cell surface protein expression, myocytes isolated from both SEPI and SEN regions of sham and sTAB LVs were washed three times on ice with K\(^+\)-reversal Tyrode buffer (140 mm KCl, 8 mm KHCO\(_3\), 2 mm MgCl\(_2\), and 0.4 mm KH\(_2\)PO\(_4\), pH 8.0). The suspended cells were then biotinylated at 4 °C with continuous agitation for 2 h using 20 mM EZ-Link Sulfo-NHS-LC-Biotin (Pierce) to specifically label surface proteins. The reaction was quenched with 10 mm glycine (20 mm Tris, 10 mm glycine, 140 mm NaCl, pH 7.4), and the cells were lysed with 1% Triton X-100 lysis buffer (1% Triton X-100, phosphate-buffered saline, and Mini-Complete\(^\circ\) protease inhibitor mixture (Roche Applied Science)). Samples were aliquoted for measurement of protein concentration, and equal amounts of lysates were incubated with 15 μL of neutralavidin protein agarose (Pierce) for 2 h to pull down biotinylated proteins. The beads were washed with
CaMKII and \textit{I}_\textit{Ca} in Heart Failure

\textbf{FIGURE 1.} A and \(B\), representative current traces (SEN myocytes) elicited by 300-ms test pulses in 20-mV increments from a holding potential of \(-50\) mV to potentials between \(-30\) to \(+50\) mV at pulse intervals of 10 s. \(I_{Ca}\) amplitude was increased, and inactivation was slowed at all test potentials in failing ventricular myocytes. \(C_{\text{Ca}}-I_{Ca}\)-voltage relationship in ventricular myocytes from sham-operated and failing LV. Current amplitudes were normalized to cell capacitance and plotted as mean values. The vertical bars represent S.E. \(D\), \(I_{Ca}\) inactivation time constants recorded in myocytes from sham-operated and failing LV. No significant differences in \(I_{Ca}\) inactivation kinetics were observed between SEN and SEP myocytes, and the results were pooled and fit by a biexponential function. The mean values were obtained by fitting currents recorded from each individual cell. \(\tau_1\) and \(\tau_2\) refer to fast and slow inactivation time constants, respectively. The vertical bars represent S.E. * denotes \(p < 0.05\).

the lysis buffer, and the proteins were eluted and resolved by SDS-PAGE.

\textit{Western Blot Analysis—}To analyze total \(\alpha_{1c}\) protein abundance, isolated epi- and endocardial myocytes were homogenized in 1% Triton X-100 buffer (50 mM Tris-HCl, pH 7.4, 4% glycerol, 1 mM dithiothreitol, 1% Triton X-100, 1 mM EDTA, Mini-Complete\textsuperscript{protease inhibitor mixture (Roche Applied Science), and phosphatase inhibitor cocktails I and II (Sigma-Aldrich)) and immunoblotted for \(\alpha_{1c}\) (Alomone Labs, ACC-003) and glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz, SC-25778). A similar Western blot protocol was used to detect cell surface \(\alpha_{1c}\) protein expression. Total CaMKII and auto-phosphorylated CaMKII (pCaMKII) were detected using anti-CaMKII H-300 (sc-13082; Santa Cruz) and anti-CaMKII Thr(P)\textsuperscript{286} (A00563; GenScript Corp.), respectively. Densitometric quantification was performed using Kodak Molecular Imaging software (version 4.0.5, Eastman Kodak). In each experiment, the mean value for SEN lysates was normalized to 100%.

\textit{Statistical Analyses—}\(I_{Ca}\) amplitude was evaluated as the peak inward current. To correct for variability in cell size, current amplitudes were expressed as current density by dividing the absolute current by cell capacitance (pA/pF). The results are expressed as the means \pm S.E. Biexponential functions were used to model current decay kinetics. Statistical analysis was performed using Sigmasat for Windows (Jandel Scientific). Paired and unpaired \(t\) tests were used in most of the comparisons, and \(p < 0.05\) was regarded as significant. Mann–Whitney Rank Sum tests were performed if tests for normality or equal variance failed.

\text{RESULTS}

\textit{Spatial Heterogeneity of \(I_{Ca}\) Density—}To test for transmural variation in \(I_{Ca}\) density (16), we isolated cells from SEN and SEP regions of mouse LV and recorded \(I_{Ca}\) by whole cell voltage clamp. In these studies, we found that \(I_{Ca}\) density was significantly larger in SEP than in SEN myocytes (Fig. 1). For instance, peak \(I_{Ca}\) (recorded at test potential of +10 mV) was 7.0 \pm 0.38 pA/pF (\(n = 19\)) for SEP and 5.1 \pm 0.4 pA/pF (\(n = 17\)) for SEN cells, respectively (\(p < 0.05\)). There were no significant differences in current decay kinetics or in the voltage dependence of \(I_{Ca}\) activation between cells isolated from these two regions of LV.

To test for differences in the surface expression of \(\alpha_{1c}\), the major pore-forming subunit of the L-type Ca\textsuperscript{2+} channel, we isolated SEN and SEP myocytes and exposed the cell exterior to an activated biotin reagent. We then lysed the cells and isolated cell surface proteins using avidin-coated beads. Western blot analysis for surface-expressed \(\alpha_{1c}\) revealed modestly higher levels of surface-expressed \(\alpha_{1c}\) protein in SEP myocytes compared with SEN myocytes (\(p < 0.05\)) (Fig. 2A). This is consistent with our observation of a modest (~30%) but consistent and statistically significant increase in \(I_{Ca}\) density in SEP relative to SEN (Fig. 1).

\textit{Increased \(I_{Ca}\) Density in Heart Failure—}To test for remodeling of \(I_{Ca}\) in heart failure, the animals were subjected to sTAB
(12), a model of pressure overload heart failure. A similar cohort of animals was subjected to sham operation. In the sTAB model, ventricular hypertrophy, dilation, and systolic dysfunction are observed along with clinical features similar to those observed in human Class IV heart failure, including lethargy, impaired mobility, and diminished appetite (12). Hearts dissected from sTAB mice were markedly enlarged and dilated with significantly increased mass (heart weight to body weight ratio: sham, 5.8 \pm 0.1 mg/g, n = 9; sTAB, 10.1 \pm 0.2, n = 13, p < 0.05). Elevated ventricular filling pressures were documented as pulmonary venous congestion (lung weight to body weight ratio: sham 5.8 \pm 0.1 mg/g, n = 9; sTAB 17.7 \pm 1.2, n = 13, p < 0.05). Together, these data are consistent with pressure overload heart failure.

In failing LV, \( I_{Ca} \) density in both SEP and SEN myocytes was significantly increased (Fig. 1). For example, peak \( I_{Ca} \) density (recorded at +10 mV) in failing myocytes was 9.2 \pm 0.6 pA/pF for SEP (\( n = 15 \)) and 7.1 \pm 0.5 pA/pF for SEN cells (\( n = 15 \)), compared with 7.0 \pm 0.4 pA/pF for SEP (\( n = 19 \)) and 5.1 \pm 0.4 pA/pF for SEN (\( n = 17 \)) in sham-operated cells (\( p < 0.05 \), comparing control with HF), respectively. Overall, increases in \( I_{Ca} \) in SEP and SEN myocytes were proportional, such that the intrinsic transmural gradient of \( I_{Ca} \) density was not altered in HF (Fig. 1C). Consistent with this, the statistically significant transmural gradient of surface-expressed \( \alpha_1C \) protein was maintained in myocytes isolated from failing hearts (Fig. 2A).

Steady state levels of total cellular \( \alpha_1C \) protein from myocyte lysates, however, were not different between SEP and SEN, nor were they altered in HF (Fig. 2B). These data, then, document the existence of a transmural gradient in L-type \( Ca^{2+} \) channel current and surface expression of the subunit protein in LV, which is not altered in HF.

**Slowed Kinetics of \( I_{Ca} \) Inactivation in Heart Failure**—To test for changes in the biophysical characteristics of \( I_{Ca} \), we evaluated the kinetics of \( I_{Ca} \) inactivation. Here, kinetics of \( I_{Ca} \) inactivation were similar in SEP and SEN myocytes, and \( I_{Ca} \) inactivation slowed significantly and similarly in SEP and SEN myocytes from failing LV (Fig. 1). To quantify these changes, \( I_{Ca} \) inactivation was modeled as a bi-exponential decay. As expected (17), the decay phase of \( I_{Ca} \) was well fit by two exponential functions (18). Mean values of fast (\( \tau_1 \)) and slow (\( \tau_2 \)) time constants in sham-operated (\( n = 18 \)) and HF myocytes (\( n = 19 \)) revealed robust differences (Fig. 1D). In both cases, no discernable voltage dependence of \( I_{Ca} \) inactivation was detected. In contrast, however, \( I_{Ca} \) inactivation was significantly slowed at all potentials in failing myocytes. For example, at +10 mV, \( \tau_1 \) and \( \tau_2 \) for control myocytes (\( n = 18 \)) were 14.2 \pm 1.1 and 68.3 \pm 2.4 ms, respectively, compared with 29.1 \pm 1.5 and 104.5 \pm 6.4 ms, respectively, for myocytes from failing LV (\( n = 18, p < 0.05 \) control versus HF).

**Voltage-dependent Activation and Inactivation of \( I_{Ca} \)—**For determination of the voltage dependence of \( I_{Ca} \) inactivation, we used a two-pulse protocol (Fig. 3, inset A) with a 300-ms conditioning pulse at different potentials ranging from –70 to +30 mV (from a holding potential of –50 mV), followed by a 300-ms test pulse to +10 mV. For determination of the voltage dependence of \( I_{Ca} \) activation, we used a holding potential of –50 mV and steps of 300-ms duration test pulses from –40 to +60 in 10 mV steps (Fig. 3, inset B). Using this approach, we detected no significant voltage-dependent differences in \( I_{Ca} \) activation between myocytes from control and failing LV (Fig. 3). Half-maximal activation of \( I_{Ca} \) current occurred at –9.1 \pm 0.4 mV (control SEN, \( n = 17 \)) and –8.4 \pm 0.3 mV (control SEP, \( n = 19, p = \text{NS} \)). Slope factors were also similar: 5.6 \pm 0.4 mV for (control SEN cells) and 5.2 \pm 0.3 mV (control SEp). In myocytes isolated from failing LV, similar values were obtained; half-maximal activation occurred at –10.2 \pm 0.4 mV (HF SEN, \( n = 15 \)) and –11.6 \pm 0.4 mV (HF SEP, \( n = 15, p = \text{NS} \)). The slope factors were also similar: 5.1 \pm 0.3 mV (HF SEN) and 5.4 \pm 0.4 mV (HF SEP, \( p = \text{NS} \)).

Similarly, no statistically significant differences in voltage-dependent inactivation kinetics of \( I_{Ca} \) were observed between SEP and SEN myocytes in control and failing LV. In control LV, the half-maximal inactivation voltages and slope factor were –19.4 \pm 0.2 and 6.1 \pm 0.2 mV for SEN cells (\( n = 13 \)), and –19.8 \pm 0.42 and 6.1 \pm 0.2 mV for SEP cells (\( n = 11, p = \text{NS} \)); in failing LV, half-maximal inactivation voltages and slope factors were –19.6 \pm 0.4 and 5.0 \pm 0.4 mV for SEN cells (\( n = 13 \)) and –20.8 \pm 0.1 and 5.9 \pm 0.1 mV for SEP cells (\( n = 15, p = \text{NS} \)). Taken together, these data reveal that \( I_{Ca} \) density is significantly increased in pressure overload heart failure, but the voltage dependence of current activation and inactivation are not altered.

**Delayed Recovery from \( I_{Ca} \) Inactivation in HF**—To evaluate differences in the time course of recovery from inactivation, we held cells at a holding potential of –50 mV and applied a 300 ms pulse to +10 mV, followed by a variable time period (\( \Delta T \)) at –50 mV and a test pulse of 300 ms to +10 mV (Fig. 4C). Recovery from inactivation was evaluated by fitting data for each cell with a mono-exponential equation. In both sham-operated and failing LV, we did not detect significant differences in recovery time constants between SEP and SEN myocytes. In failing LV (Fig. 4B), however, recovery time constants were significantly slowed in both SEP (236.5 \pm 3.9 ms, \( n = 10 \)) and SEN (238.5 \pm 3.6 ms, \( n = 10 \)) myocytes, compared with SEP (194.2 \pm 2.4 ms, \( p = \text{NS} \)).
CaMKII and $I_{\text{Ca}}$ in Heart Failure

$n = 9$, $p < 0.05$) and SEN (196.2 ± 4.8 ms, $n = 9$, $p < 0.05$) myocytes from sham-operated controls (Fig. 4A). The mean values of the percentage recovery of normalized, peak $I_{\text{Ca}}$ were similar (Fig. 4D).

**Abolished $I_{\text{Ca}}$ Facilitation in Pressure Overload Heart Failure**—Increased peak $I_{\text{Ca}}$ and slowed inactivation are suggestive of activation by CaMKII. To test this, we measured $I_{\text{Ca}}$ facilitation in pressure overload failing LV myocytes, as frequency-dependent, Ca$^{2+}$-induced $I_{\text{Ca}}$ facilitation is CaMKII-dependent (17, 19, 20). Consistent with already nearly saturated effects of CaMKII on $I_{\text{Ca}}$, Ca$^{2+}$-induced $I_{\text{Ca}}$ facilitation was significantly blunted in failing myocytes (Fig. 5A). Whereas Ca$^{2+}$-induced $I_{\text{Ca}}$ facilitation was readily demonstrable in myocytes isolated from sham-operated LV, paradoxical frequency-dependent $I_{\text{Ca}}$ suppression was observed in failing myocytes. Similar findings were observed in 23 ventricular myocytes from 13 HF mice. This frequency-dependent suppression of $I_{\text{Ca}}$ is consistent with the slowed $I_{\text{Ca}}$ recovery time course in the failing myocytes.

One possible explanation for the absence of $I_{\text{Ca}}$ facilitation in HF myocytes is that CaMKII is already highly activated in these myocytes such that frequency-dependent activation of CaMKII no longer plays a role in $I_{\text{Ca}}$ remodeling. To test this, we measured CaMKII activity in acutely isolated ventricular myocytes from control and pressure overload failing LV, conditions that exactly mimic our electrophysiological recording paradigm. In preparations of freshly isolated myocytes from three control and three HF mice, CaMKII activity in HF was significantly increased (5.0 ± 0.6 pmol/min/μg (SEN); 3.8 ± 0.3 (SEP)) relative to sham (1.6 ± 0.3 (SEP), $p < 0.01$ versus HF; 1.7 ± 0.2 (SEP), $p < 0.01$ versus HF) (Fig. 5B). Similar results were obtained on probing for CaMKII phosphorylated at Thr$^{286}$ (pCaMKII), a kinase isoform that is constitutively active and Ca$^{2+}$-independent (21) (Fig. 5C). Thus, a 2–3-fold increase in CaMKII activity in failing LV likely contributes to the disease-related alterations in $I_{\text{Ca}}$ properties that we observed. Next, we set out to evaluate this specifically.

**Exogenous CaMKII Cannot Facilitate $I_{\text{Ca}}$ in Failing Myocytes**—These findings suggest that $I_{\text{Ca}}$ is fully phosphorylated by CaMKII in failing myocytes. To test this further, we dialyzed constitutively active CaMKII into ventricular myocytes isolated from control and failing LV. Active CaMKII was incorporated in the patch pipette and allowed to dialyze into the voltage-clamped myocyte over time. After the cell membrane was ruptured, peak $I_{\text{Ca}}$ was recorded continuously until it achieved steady state (i.e. no change in amplitude for >5 min). Throughout, we observed similar effects in SEP and SEN, and for simplicity, the results were pooled. First, we found that internal application of constitutively active CaMKII did not elicit significant changes in $I_{\text{Ca}}$ in failing myocytes. Indeed, in the presence of active CaMKII, $I_{\text{Ca}}$ inactivation was similar in control and HF myocytes (Fig. 6). Further, there were no significant differences in either $I_{\text{Ca}}$ density or $I_{\text{Ca}}$ inactivation time constants in myocytes from control and failing LV with internally applied CaMKII (Fig. 7). For example, at test potential of +10 mV (peak current), $I_{\text{Ca}}$ density was 8.1 ± 0.4 pA/pF in sham and 7.9 ± 0.3 pA/pF in HF myocytes, and the fast (τ1) and slow (τ2) inactivation time constants were 15 ± 0.4 and 88 ± 0.5 for sham and 13 ± 2 and 95 ± 3 for HF myocytes, respectively ($p =$ NS). These data, then, lend support to the notions that $I_{\text{Ca}}$ is fully activated by CaMKII in failing LV and that CaMKII-
dependent Ca\(^{2+}\) channel phosphorylation is a major mechanism governing \(I_{\text{Ca}}\) remodeling in HF.

**CaMKII Inhibition Reverses HF-related \(I_{\text{Ca}}\) Remodeling**—To test directly the role of CaMKII activation on HF-related \(I_{\text{Ca}}\) remodeling, we employed a specific CaMKII inhibitor, autophosphorylation-2-related inhibitory peptide (AIP). AIP is a highly specific and potent CaMKII inhibitor, which has no effects on cyclic AMP-dependent protein kinase, protein kinase C, CaMKIV, and other known protein kinases (22). AIP was incorporated in the patch pipette, the cell membrane was ruptured, and peak \(I_{\text{Ca}}\) current was recorded continuously. After 2 min, before significant amounts of AIP had dialyzed into the cell, an \(I_{\text{Ca}}\)-voltage relation was recorded. Then peak \(I_{\text{Ca}}\) was recorded continuously until it achieved steady state (no change in amplitude for >5 min), at which time a second \(I_{\text{Ca}}\)-voltage relationship was recorded (typically 12–15 min after membrane rupture). To rule out changes resulting from \(I_{\text{Ca}}\) run-down, currents that quickly and continuously decreased during the first 5 min were excluded from analysis. AIP began to inhibit \(I_{\text{Ca}}\) starting ~5 min after the rupture of cell membrane and reached a maximum at around 15 min (Fig. 8), a time course consistent with intracellular diffusion from the patch pipette. We then studied effects of AIP in ventricular myocytes isolated from both sham-operated and failing LV. In failing myocytes, AIP significantly diminished \(I_{\text{Ca}}\) density, and the rate of \(I_{\text{Ca}}\) inactivation was significantly accelerated (Fig. 9, A and B). Intracellular dialysis with a nonspecific control peptide triggered no changes in \(I_{\text{Ca}}\) (data not shown). It is notable that the two biophysical changes observed in cells exposed to AIP, decreased \(I_{\text{Ca}}\) density and accelerated \(I_{\text{Ca}}\) inactivation, are exactly opposite to the changes seen in HF. These data, then, are consistent with AIP-mediated inhibition of CaMKII, which reverses HF-related changes in the biophysical properties of \(I_{\text{Ca}}\).

To test this further, we evaluated Ca\(^{2+}\)-induced \(I_{\text{Ca}}\) facilitation in the absence (2 min after membrane rupture, i.e. before AIP had diffused into the myocyte) and presence (15 min after membrane rupture) of AIP. In the absence of CaMKII inhibition, Ca\(^{2+}\)-induced \(I_{\text{Ca}}\) facilitation was observed, as expected (Fig. 9C). In the presence of AIP, it was abolished (Fig. 9D). These data, then, are consistent with the effects seen on \(I_{\text{Ca}}\) density and point to AIP-dependent inhibition of CaMKII under the experimental conditions employed here.

Finally, the effects of AIP were compared in sham-operated and HF myocytes. Whereas the patterns of AIP-induced changes in \(I_{\text{Ca}}\) were similar in control and HF myocytes, declines in \(I_{\text{Ca}}\) density were greater in HF myocytes (Fig. 10A), consistent with our findings of increased CaMKII activity in these cells (Fig. 5, B and C). Importantly, after inhibition of CaMKII, differences in \(I_{\text{Ca}}\) density between sham and HF myocytes were greatly diminished (Fig. 10A). Moreover, AIP accelerated both the fast and slow \(I_{\text{Ca}}\) inactivation time constants in both control and HF myocytes, with greater changes observed in HF myocytes (Fig. 10B). Thus, CaMKII inhibition reversed
both of the HF-related remodeling features of \( I_{\text{ca}} \), viz. increased \( I_{\text{ca}} \) density (Fig. 10A) and accelerated inactivation (Fig. 10B).

**DISCUSSION**

CaMKII has emerged recently as a major mechanism of pathological signaling in heart failure, a syndrome marked by adverse arrhythmic events. Here, we studied inward \( \text{Ca}^{2+} \) currents and the role of CaMKII in heart failure-related electrical remodeling. We report that transmural gradients in \( \text{L-type Ca}^{2+} \) current density and subunit expression, which exist under basal conditions, are augmented proportionally in pressure overload heart failure. In addition, biophysical properties of \( I_{\text{ca}} \) are altered in heart failure, viz. both \( I_{\text{ca}} \) inactivation and recovery from \( I_{\text{ca}} \) inactivation are slowed, changes that are suggestive of a role of CaMKII. Consistent with this, frequency-dependent \( I_{\text{ca}} \) facilitation, a CaMKII-dependent process, was abolished in failing ventricular myocytes because of extensive CaMKII-dependent activation of \( I_{\text{ca}} \). Internal application of exogenous CaMKII in control myocytes induced heart failure-like changes in \( I_{\text{ca}} \) density and inactivation; in contrast, exogenous CaMKII had no effect when dialyzed into failing myocytes. Finally, inhibition of CaMKII reversed heart failure-related changes in both \( I_{\text{ca}} \) density and inactivation. Together, these findings provide evidence that enhanced CaMKII activation plays a direct and major role in failure-related remodeling of \( I_{\text{ca}} \).

\( I_{\text{ca}} \) in Heart Failure—Differences in the electrophysiological properties of myocytes from different regions of LV are critical to normal functioning of the heart; at the same time, spatial dispersion of disease-related electrical remodeling contributes importantly to the genesis of arrhythmias (23). Transmural dispersion of \( I_{\text{ca}} \) density has been reported in canine (24) and rat (25) LV but not in guinea pig (26). To our knowledge, a transmural gradient of \( I_{\text{ca}} \) in murine LV has not been reported previously.

In several previous studies, \( I_{\text{ca}} \) has been reported to be decreased or unchanged in ventricular myocytes of failing LV (27), although there are reports to the contrary (28). In general, \( I_{\text{ca}} \) is increased or unchanged in mild-to-moderate hypertrophy and decreased in severe hypertrophy and heart failure (2). In addition, regional variation in \( I_{\text{ca}} \) remodeling has been observed, with reduced \( I_{\text{ca}} \) in subendocardial myocytes and increased \( I_{\text{ca}} \) in mid-myocardial and subepicardial myocytes (26). In human LV, similar differences have been reported, including either no change in current density (29, 30) or a decrease (31). Inconsistencies among these reports may stem from differences in species and recording methods or may be due to a lack of attention to possible transmural variations in \( I_{\text{ca}} \). In this study, we observed significant increases in \( I_{\text{ca}} \) density and slowed inactivation in myocytes isolated from failing LV without alterations in the transmural dispersion of \( I_{\text{ca}} \).

CaMKII Activation as a Mechanism of Electrical Remodeling—A number of CaMKII phosphorylation substrates have been identified in cardiac myocytes, including the ryanodine receptor, phospholamban, K\(^+\) channels, Na\(^+\) channels, and L-type Ca\(^{2+}\) channels (32). As a result, CaMKII has a plethora of effects on trans-sarcolemmal electrical events, intracellular Ca\(^{2+}\) uptake and release, activation of hypertrophic signaling pathways, and regulation of cell death. With respect to heart failure, mice with cardiomyocyte-specific overexpression of a CaMKII inhibitory peptide are resistant to electrophysiological remodeling post-myocardial infarction (33), and CaMKII inhibition can reduce arrhythmias (reviewed in Ref. 34).

The cardiac L-type Ca\(^{2+}\) channel is subject to complex regulation by multiple kinases and phosphatases, including PKA (35), protein kinase C (36), CaMKII (11), and cal-
cineur (37, 38). Indeed, complex interplay exists among these pathways, including functional association between PKA and CaMKII (40). Recent work has uncovered a binding site specific to CaMKII on the L-type Ca^{2+} channel β subunit that allows for targeting of a nearby amino acid for phosphorylation (41). Indeed, CaMKII-dependent phosphorylation of threonine 498 on the β subunit is required for facilitation (41). Other studies have demonstrated that CaMKII binds directly to the pore-forming α subunit of the channel (42, 43), with a requirement for phosphorylation of Ser^{1512} (43, 44) and Ser^{1570} (43) of the α subunit in I_{Ca} facilitation.

Evidence has grown supporting a critical role for CaMKII in the effects induced by both acute and chronic β-adrenergic receptor stimulation. Interestingly, a signaling switch from PKA to CaMKII in the phosphorylation of the L-type calcium channel and phospholamban under conditions of sustained β-adrenergic stimulation has been reported (40). CaMKII activation has been reported downstream of calcineurin signaling (45), as well, another major mechanism of heart failure pathogenesis. In line with this, our data implicate CaMKII in the regulation of I_{Ca} in chronic heart failure. Indeed, when CaMKII was inhibited, changes in I_{Ca} density and inactivation were completely reversed, indicating that PKA and other kinases contributed only a minor degree to I_{Ca} activation in our model of pressure overload HF. Together, these results demonstrate that CaMKII plays a direct and major role in HF-related remodeling of I_{Ca} in ventricular myocytes.

Slowing of recovery from I_{Ca} inactivation may be the result of intracellular Ca^{2+} overload in failing ventricular myocytes because of reduced sarcoplasmic reticulum Ca^{2+}-ATPase activity and consequent SR Ca^{2+} leak. Indeed, recent studies have shown that recovery from I_{Ca} inactivation was significantly slowed during fast stimulation (inducing intracellular Ca^{2+} accumulation), whereas buffering of intracellular Ca^{2+} with BAPTA and EGTA reversed these changes (46). Interestingly, delayed recovery from I_{Ca} inactivation has been observed previously in ventricular myocytes isolated from failing hamster heart (47).

Although the changes we report in I_{Ca} kinetics in failing LV are typical of those caused by CaMKII-dependent phosphorylation of the I_{Ca} channel (11), activation of PKA can also trigger similar changes (48). Thus, to test the role of CaMKII, we employed AIP, a peptide that has been widely used as a specific CaMKII inhibitor. Other CaMKII inhibitors, such as KN93, have been reported to have little or no influence on the activities of PKA, protein kinase C, and other important protein kinases (49). However, KN93 has been shown to directly block voltage-dependent K^+ currents (50) and Ca^{2+} currents (51).

Consistent with our studies of internally dialyzed CaMKII, cardiomyocyte-specific overexpression of CaMKII elicits increases in I_{Ca} (52). In this transgenic model, CaMKII activation is required for the initiation of early after-depolarizations, and inhibition of CaMKII by KN93 reduces early after-depolarizations and prevents ventricular arrhythmias. In addition, theoretical studies suggest that increased cardiomyocyte I_{Ca,I} steepens restitution curves for both action potential duration (APD) and conduction velocity, promoting discordant alternans and arrhythmia initiation (53). Studies such as these, then, highlight the emerging importance of CaMKII as a proarrhythmic molecule in cardiomyopathy where APD is significantly prolonged (52) and in cellular models of drug-induced APD prolongation (54, 55). Some evidence suggests that up-regulation of CaMKII expression and activity is a general feature of cardiomyopathies of diverse etiologies both in animal models (7, 56, 57) and in patients (58). The resulting increases in I_{Ca} density and slowed I_{Ca,inactivation} suggest an increase in the number of calcium channels gating in mode 2, which has been demonstrated to promote the occurrence of early after-depolarizations and ventricular arrhythmias (39). Together with decreased transient outward current (13), CaMKII-mediated facilitation of I_{Ca} may be an important contributor to the prolongation of APD in pressure overload HF.

**Perspective**—A number of recent studies have highlighted the important roles played by CaMKII in the regulation of cellular excitation-contraction coupling in heart (32). Further, evidence from both in vitro and in vivo models indicates that CaMKII can serve as a pro-arrhythmic signaling molecule (34). Findings reported here implicate increases in CaMKII activity as a direct mechanism governing heart failure-related changes in I_{Ca} and thereby lend additional credence to the emerging importance of CaMKII as an anti-arrhythmic target.

---

*REFERENCES*

1. Janse, M. J. (2004) *Cardiovasc. Res.* 61, 208–217
2. Akar, F. G., and Tomasselli, G. F. (2005) *Ann. Med.* 37, 44–54
3. Heineke, J., and Molkentin, J. D. (2006) *Nat. Rev. Mol. Cell Biol.* 7, 589–600
4. Hill, J. A., and Olson, E. N. (2008) *New Engl. J. Med.* 358, 1370–1380
5. Maier, L. S., Bers, D. M., and Brown, J. H. (2007) *Cardiovasc. Res.* 73, 629–630
6. Braun, A. P., and Schulman, H. (1995) *Annu. Rev. Physiol.* 57, 417–445
7. Colomer, J. M., Mao, L., Rockman, H. A., and Means, A. R. (2003) *Mol. Endocrinol.* 17, 183–192
8. Zhang, T., Maier, L. S., Dalton, N. D., Miyamoto, S., Ross, J., Jr., Bers, D. M., and Brown, J. H. (2003) *Circ. Res.* 92, 912–919
9. Saito, T., Fukuwaza, J., Osaki, J., Sakuragi, H., Yao, N., Haneda, T., Fujino, T., Wakamiya, N., Kikuchi, K., and Hasebe, N. (2003) *J. Mol. Cell Cardiol.* 35, 1135–1160
10. Ai, X., Curran, J. W., Shannon, T. R., Bers, D. M., and Pogwizd, S. M. (2005) *Circ. Res.* 97, 1314–1322
11. Anderson, M. E. (2004) *Trends Cardiovasc. Med.* 14, 152–161
12. Rothermel, B. A., Berenji, K., Tannous, P., Kutschke, W., Dey, A., Nolan, B., O’Callaghan, J. P., and Guilarte, T. R. (2005) *Am. J. Physiol. Heart Circ. Physiol.* 288, H982–H993
13. Yuan, W., and Bers, D. M. (1994) *Annu. Rev. Physiol.* 56, 357–370
14. Wang, Y., Wagner, M. B., Joyner, R. W., and Kumar, R. (2000) *Circ. Res.* 86, 152–161
15. Heineke, J., and Molkentin, J. D. (2006) *Circulation* 113, 1849–1856
16. Wang, Y., Wagner, M. B., Joyner, R. W., and Kumar, R. (2000) *Cardiovasc. Res.* 48, 310–322
17. Tocchetti, C. D., O’Callaghan, J. P., and Guiltarte, T. R. (2005) *Brain Res.* 1044, 51–58
18. Gaborit, N., Le Boutier, S., Szuts, V., Varro, A., Escande, D., Nattel, S., and Demolombe, S. (2007) *J. Physiol.* 582, 675–693
19. Yuan, W., and Bers, D. M. (1994) *Am. J. Physiol.* 267, H982–H993
20. Kohlihaas, M., Zhang, T., Seidler, T., Zibrova, D., Dybkova, N., Steen, A.,...
CaMKII and $I_{Ca}$ in Heart Failure

Wagner, S., Chen, L., Brown, J. H., Bers, D. M., and Maier, L. S. (2006) *Circ. Res.* 98, 235–244

21. Hudmon, A., and Schulman, H. (2002) *Biochem. J.* 364, 593–611

22. Ishida, A., Kameshita, I., Okuno, S., Kitani, T., and Fujisawa, H. (1995) *Biophys. J.* 121, 806–812

23. Antzelevitch, C. (2007) *Am. J. Physiol.* 293, H2024–H2038

24. Wang, H. S., and Cohen, I. S. (2003) *J. Cell Biol.* 157, 825–833

25. Pandit, S. V., Clark, R. B., Giles, W. R., and Demir, S. S. (2001) *Biophys. J.* 81, 3029–3051

26. Bryant, S. M., Shipsey, S. J., and Hart, G. (1997) *Cardiovasc. Res.* 35, 315–323

27. Hasenfuss, G. (1998) *Cardiovasc. Res.* 39, 60–76

28. Schroder, F., Handrock, R., Beuckelmann, D. J., Hirt, S., Hullin, R., Priebel, L., Schwinger, R. H., Weil, J., and Herzig, S. (1998) *Circulation* 98, 969–976

29. Beuckelmann, D. J., and Erdmann, E. (1992) *Basic Res. Cardiol.* 87, Suppl. 1, 235–243

30. Mewes, T., and Ravens, U. (1994) *J. Mol. Cell. Cardiol.* 26, 1307–1320

31. Ouadid, H., Albat, B., and Nargeot, J. (1995) *J. Cardiovasc. Pharmacol.* 26, 282–291

32. Maier, L. S., and Bers, D. M. (2007) *Cardiovasc. Res.* 73, 631–640

33. Zhang, R., Khoo, M. S., Wu, Y., Yang, Y., Grueter, C. E., Ni, G., Price, E. E., Wu, Y., Roden, D. M., and Anderson, M. E. (1999) *Circulation* 99, C768–C774

34. Anderson, M. E. (2002) *Cardiovasc. Res.* 55, 235–244

35. Van Coppenolle, F., Ahidouch, A., Guibault, P., and Ouadid, H. (1997) *Mol. Cell. Biochem.* 168, 155–161

36. Alden, K. J., Goldspink, P. H., Ruch, S. W., Buttrick, P. M., and Garcia, J. (2002) *Am. J. Physiol.* 282, C768–C774

37. Wang, Z., Kutschke, W., Richardson, K. E., Karimi, M., and Hill, J. A. (2001) *Circulation* 104, 1657–1663

38. Yatani, A., Honda, R., Tymitz, K. M., Lalli, M. J., and Molkentin, J. D. (2001) *J. Mol. Cell. Cardiol.* 33, 249–259

39. Tanskanen, A. J., Greenstein, J. L., O’Rourke, B., and Winslow, R. L. (2005) *Biophys. J.* 88, 85–95

40. Wang, W., Zhu, W., Wang, S., Yang, D., Crow, M. T., Xiao, R. P., and Cheng, H. (2004) *Circ. Res.* 95, 798–806

41. Grueter, C. E., Abira, S. A., Dzhura, I., Wu, Y., Ham, A. J., Mohler, P. J., Anderson, M. E., and Colbran, R. J. (2006) *Mol. Cell* 23, 641–650

42. Hudmon, A., Schulman, H., Kim, J., Maltez, J. M., Tsien, R. W., and Pitt, G. S. (2005) *J. Cell Biol.* 171, 537–547

43. Lee, T. S., Karl, R., Moosmang, S., Lenhardt, P., Klugbauer, N., Hofmann, F., Kleppisch, T., and Welling, A. (2006) *J. Biol. Chem.* 281, 25560–25567

44. Erxleben, C., Liao, Y., Gentile, S., Chin, D., Gomez-Alegria, C., Mori, Y., Birnbaumer, L., and Armstrong, D. L. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 3932–3937

45. Khoo, M. S., Li, J., Singh, M. V., Yang, Y., Kannankeril, P., Wu, Y., Grueter, C. E., Guan, X., Oddis, C. V., Zhang, R., Mendes, L., Ni, G., Madu, E. C., Yang, J., Bass, M., Gomez, R. J., Wadzinski, B. E., Olson, E. N., Colbran, R. J., and Anderson, M. E. (2006) *Circulation* 114, 1352–1359

46. Gao, J., and Duff, H. J. (2006) *J. Physiol.* 574, 509–518

47. Rossner, K. L. (1991) *Am. J. Physiol.* 260, H1179–H1186

48. Dzhura, I., Wu, Y., Colbran, R. J., Corbin, J. D., Balser, J. R., and Anderson, M. E. (2002) *J. Physiol* 545, 399–406

49. Sumi, M., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T., and Hidaka, H. (1991) *Biochem. Biophys. Res. Commun.* 181, 968–975

50. Ledoux, J., Chartier, D., and Leblanc, N. (1999) *J. Pharmacol. Exp. Ther.* 290, 1165–1174

51. Gao, L., Blair, L. A., and Marshall, J. (2006) *Biochem. Biophys. Res. Commun.* 345, 1606–1610

52. Wu, Y., Temple, J., Zhang, R., Dzhura, I., Zhang, W., Trimble, R., Roden, D. M., Passier, R., Olson, E. N., Colbran, R. J., and Anderson, M. E. (2002) *Circulation* 106, 1288–1293

53. Gong, Y., Xie, F., Stein, K. M., Garfinkel, A., Culpian, C. A., Lerman, B. B., and Christini, D. J. (2007) *Circulation* 115, 2094–2102

54. Wu, Y., MacMillan, L. B., McNeill, R. B., Colbran, R. J., and Anderson, M. E. (1999) *Am. J. Physiol.* 276, H2168–H2178

55. Wu, Y., Roden, D. M., and Anderson, M. E. (1999) *Circ. Res.* 84, 906–912

56. Currie, S., and Smith, G. L. (1999) *FEBS Lett.* 459, 244–248

57. Hagemann, D., Bohlender, J., Hoch, B., Krause, E. G., and Karczewski, P. (1999) *Basic Res. Cardiol.* 94, 1307–1320

58. Rossner, K. L. (1991) *Am. J. Physiol.* 290, 60–76

59. Tanskanen, A. J., Greenstein, J. L., O’Rourke, B., and Winslow, R. L. (2005) *Biophys. J.* 88, 85–95

60. Wang, W., Zhu, W., Wang, S., Yang, D., Crow, M. T., Xiao, R. P., and Cheng, H. (2004) *Circ. Res.* 95, 798–806

61. Grueter, C. E., Abira, S. A., Dzhura, I., Wu, Y., Ham, A. J., Mohler, P. J., Anderson, M. E., and Colbran, R. J. (2006) *Mol. Cell* 23, 641–650

62. Hudmon, A., Schulman, H., Kim, J., Maltez, J. M., Tsien, R. W., and Pitt, G. S. (2005) *J. Cell Biol.* 171, 537–547