RESEARCH ARTICLE | Cardiac Excitation and Contraction

Loss of caveolin-3-dependent regulation of $I_{Ca}$ in rat ventricular myocytes in heart failure

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Submitted 21 July 2017; accepted in final form 30 October 2017

Bryant SM, Kong CH, Cannell MB, Orchard CH, James AF. Loss of caveolin-3-dependent regulation of $I_{Ca}$ in rat ventricular myocytes in heart failure. *Am J Physiol Heart Circ Physiol* 314: H521–H529, 2018. First published November 3, 2017; doi:10.1152/ajpheart.00458.2017.—$\beta_{2}$-Adrenoceptors and L-type Ca$^{2+}$ current ($I_{Ca}$) redistribute from the t-tubules to the surface membrane of ventricular myocytes from failing hearts. The present study investigated the role of changes in caveolin-3 and PKA signaling, both of which have previously been implicated in this redistribution. $I_{Ca}$ was recorded using the whole cell patch-clamp technique from ventricular myocytes isolated from the hearts of rats that had undergone either coronary artery ligation (CAL) or equivalent sham operation 18 wk earlier. $I_{Ca}$ distribution between the surface and t-tubule membranes was determined using formamide-induced detubulation (DT). In sham myocytes, $\beta_{2}$-adrenoceptor stimulation increased $I_{Ca}$ in intact but not DT myocytes; however, forskolin (to increase cAMP directly) and H-89 (to inhibit PKA) increased and decreased, respectively, $I_{Ca}$ at both the surface and t-tubule membranes. C3SD peptide (which decreases binding to caveolin-3) inhibited $I_{Ca}$ in intact but not DT myocytes but had no effect in the presence of H-89. In contrast, in CAL myocytes, $\beta_{2}$-adrenoceptor stimulation increased $I_{Ca}$ in both intact and DT myocytes, but C3SD had no effect on $I_{Ca}$; forskolin and H-89 had similar effects as in sham myocytes. These data show the redistribution of $\beta_{2}$-adrenoceptor activity and $I_{Ca}$ in CAL myocytes and suggest constitutive stimulation of $I_{Ca}$ by PKA in sham myocytes via concurrent caveolin-3-dependent (at the t-tubules) and caveolin-3-independent mechanisms, with the former being lost in CAL myocytes.

NEW & NOTEWORTHY In ventricular myocytes from normal hearts, regulation of the L-type Ca$^{2+}$ current by $\beta_{2}$-adrenoceptors and the constitutive regulation by caveolin-3 is localized to the t-tubules. In heart failure, the regulation of L-type Ca$^{2+}$ current by $\beta_{2}$-adrenoceptors is redistributed to the surface membrane, and the constitutive regulation by caveolin-3 is lost.

caveolin-3; cAMP; heart failure; L-type Ca$^{2+}$ current; myocardial infarction

INTRODUCTION

L-type Ca$^{2+}$ current ($I_{Ca}$) plays a key role in excitation-contraction (EC) coupling in cardiac ventricular myocytes: activation of L-type Ca$^{2+}$ channels (LTCCs) during the action potential causes influx of Ca$^{2+}$ that triggers Ca$^{2+}$ release via ryanodine receptors (RyRs) in the adjacent sarcoplasmic reticulum (SR) membrane (2, 8). Previous work has shown that the function of many of the key proteins involved in EC coupling, including LTCCs and RyRs, occurs predominantly at the t-tubules: invaginations of the surface membrane that enable near-synchronous SR Ca$^{2+}$ release, and thus contraction, throughout the cell (18, 21, 28). The mechanism for the localization of $I_{Ca}$ at the t-tubules is less clear, although it has been suggested that the caveolar protein caveolin-3 (Cav-3) plays a role in the localization of $I_{Ca}$, possibly via a mechanism involving cAMP/PKA signaling pathways (1, 5, 9, 24).

Cav-3 is also involved in the localization of cAMP signaling via $\beta_{2}$-adrenoceptors to the t-tubules, and it has been proposed that LTCCs and $\beta_{2}$-adrenoceptors are colocalized in a Cav-3 signaling microdomain (1, 5, 7, 23, 30). It has been shown that Cav-3 plays a critical role in the constitutive maintenance of $I_{Ca}$ at the t-tubule (5). In heart failure, there is redistribution of $\beta_{2}$-adrenoceptors from the t-tubular to the surface membrane so that they become more uniformly distributed across the cell membrane (22, 27). This redistribution is associated with a change from localized to more diffuse signaling in response to $\beta_{2}$-adrenergic stimulation (27). We (6) have recently shown in a coronary artery ligation (CAL) model in that rat that ventricular $I_{Ca}$ is also redistributed from the t-tubules to the surface sarcolemma in heart failure.

We hypothesized that the redistribution of $I_{Ca}$ after CAL is due to loss of Cav-3-dependent localization at the t-tubules, which may be secondary to the decreased expression of Cav-3 observed in heart failure. Thus, changes in the localization of the $\beta_{2}$-signaling pathway in heart failure may be associated with a loss of constitutive regulation of $I_{Ca}$ by PKA at the t-tubules. Therefore, we further investigated the relationship between the distribution of $I_{Ca}$ and changes in Cav-3/$\beta_{2}$-adrenergic signaling observed after CAL in rats (6).

METHODS

Animals and surgical procedures. All procedures were performed in accordance with United Kingdom legislation and approved by the University of Bristol Ethics Committee. This study was conducted in parallel with other investigations using cells from the same animals to investigate ventricular and atrial cellular remodeling in heart failure and thereby conform with the reduction component of the 3Rs ("replace, reduce, refine") (3, 6, 16). Adult male Wistar rats (~250 g) were subjected to either ligation of the left anterior descending coronary artery (CAL; 10 animals) or equivalent surgery without ligation (sham; 12 animals). Operations were conducted under general anesthesia [ketamine (75 mg/kg) and medetomidine (0.5 mg/kg ip)] with appropriate analgesia [buprenorphine (0.05 mg/kg sc)], as previously described (6). Data regarding changes in cardiac morphology and function as well as in cell morphology in these groups of animals have been previously published (3, 6).
**Myocyte isolation.** Left ventricular myocytes were isolated from the hearts 18 wk after surgery as previously described (5). Animals were euthanized under pentobarbitone anesthesia, and the heart was quickly excised and Langendorff perfused at 8 ml/min (37°C), initially with Tyrode solution (see Solutions below) plus 0.75 mmol/l CaCl₂ for 4 min and then in nominally Ca²⁺-free solution for 4 min and finally plus 1 mg/ml collagenase (Worthington) for 10 min. The left ventricle was then excised and shaken in collagenase-containing solution at 37°C for 5–7 min, filtered, and centrifuged. The supernatant was discarded, and the pellet was resuspended in the Krebs solution and stored at 4°C for 2–10 h before use on the day of isolation (20). Detubulation (DT) of myocytes (physical and functional uncoupling of the t-tubules from the surface membrane) was achieved using formamide-induced osmotic shock, as previously described (21).

**Solutions.** Tyrode solution for cell isolation contained (in mmol/l) 130 NaCl, 5.4 KCl, 0.4 NaH₂PO₄, 4.2 HEPES, 10 glucose, 1.4 MgCl₂, 20 taurine, and 10 creatinine; pH 7.4 (NaOH). Krebs solution for cell storage contained (in mmol/l) 90 l-glutamic acid, 30 KCl, 10 HEPES, 1 EGTA, 5 Na pyruvate, 20 tauro, 20 glucose, 5 MgCl₂, 5 succinic acid, 5 creatine, 2 Na₂ATP, and 5 β-OH butyric acid; pH 7.4 with KOH. For patch-clamp experiments, cells were superfused with solution that contained (in mmol/l) 133 NaCl, 1 MgSO₄, 1 CaCl₂, 1 NaHPO₄, 10 glucose, 10 HEPES (pH 7.4 (NaOH)); 5 mmol/l CsCl was added to inhibit K⁺ currents. The pipette solution contained (in mmol/l) 110 CsCl, 20 TEA-Cl, 0.5 MgCl₂, 5 Mg-ATP, 5 BAPTA, 10 HEPES, and 0.4 GTP-Tris; pH 7.2 (CsOH). BAPTA was used to inhibit Ca²⁺-dependent inactivation of I₅₋₆ (33).

Selective β₂-adrenoceptor stimulation was achieved as previously described (5) using the β₂-adrenoceptor agonist zinterol (1 and 3 μmol/l) in the presence of the β₁-adrenoceptor-selective antagonist atenolol (10 μmol/l); cells were superfused with atenolol alone for at least 4 min before superfusion with zinterol in the presence of atenolol. Under these conditions, the effects of 1 and 3 μmol/l zinterol could be completely abolished by 100 nM ICI-118,551, a β₂-adrenoceptor-selective antagonist (5). The plant alkaloid forskolin (10 μmol/l) was used to activate adenyl cyclase directly (31). C3SD, a short peptide encompassing the Cav-3 scaffolding domain, was used to disrupt binding of Cav-3 to its protein partners as previously described (5, 13, 15, 23); myocytes were incubated in 1 μmol/l TAT-C3SD for at least 45 min before use. PKA was inhibited using H-89 (20 μmol/l) (11, 17).

**Recording and analysis of I₅₋₆.** Myocytes were placed in a chamber mounted on a Nikon Diaphot inverted microscope. Membrane currents and cell capacitance were recorded using the whole cell patch-clamp technique using an Axopatch 200B, Digidata 1322A analogo-to-digital converter, and pClamp 10 (Axon Instruments). Pipette resistance was typically 2–4 MΩ when filled with pipette solution, and pipette capacitance and series resistance were compensated by ~70%. Currents were activated from a holding potential of −80 mV by a 100-ms step depolarization to −40 mV (to inactivate Na⁺ current) followed by steps to potentials between −50 and +80 mV for 500 ms before repolarization to the holding potential at a frequency of 0.2 Hz. I₅₋₆ amplitude (in pA) was measured as the difference between peak inward current and current at the end of the depolarizing pulse and was normalized to cell capacitance [pF; a function of membrane area (25)] to calculate I₅₋₆ density (in pA/pF). Surface membrane current density was obtained from currents measured in DT myocytes, whereas t-tubular membrane current density was calculated by subtraction of surface from whole cell currents and corrected for incomplete DT, as previously described (5, 6, 19, 21). DT efficiency, measured from images of intact and DT cells stained with di-8-ANEPPS, was ~84% and was not different between wild-type and CAL myocytes (6). To correct for incomplete DT, the distribution of membrane capacitance and I₅₋₆ between the t-tubule and surface

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**Fig. 1.** β₂-Adrenergic potentiation of L-type Ca²⁺ current (I₅₋₆) in sham and coronary artery ligated (CAL) myocytes. A: representative I₅₋₆ traces (elicited by step depolarization to 0 mV) recorded from intact and detubulated (DT) myocytes isolated from sham hearts. Overlapping traces were from the same cell and were recorded under control conditions and after application of 1 and 3 μmol/l zinterol (in the presence of 10 μmol/l atenolol). Vertical scale bar = 1 nA; horizontal scale bar = 50 ms. B: time course of changes in mean normalized peak I₅₋₆ (±SE) of intact (n = 5) and DT (n = 6) sham myocytes during superfusion with control solution (containing 10 μmol/l atenolol) and 1 and 3 μmol/l zinterol. I₅₋₆ elicited by step depolarization to 0 mV at 0.1 Hz, was expressed as a percentage of control measured just before application of the first concentration of zinterol. C: mean changes in I₅₋₆ elicited by application of 1 and 3 μmol/l zinterol to intact (1 μmol/l: n = 7 and 3 μmol/l: n = 9) and DT (1 μmol/l: n = 7 and 3 μmol/l: n = 9) sham myocytes. Data were subjected to two-way ANOVA: β₂-agonism P < 0.001; DT P < 0.001; interaction P < 0.001, *P < 0.05 and **P < 0.001, Bonferroni post hoc test. D: representative I₅₋₆ traces (elicited by step depolarization to 0 mV) recorded from intact and DT myocytes isolated from CAL hearts. Conditions and scale were as in A. E: time course of changes in mean normalized peak I₅₋₆ of intact (n = 5) and DT (n = 4) CAL myocytes during superfusion with control solution (containing 10 μmol/l atenolol) and 1 and 3 μmol/l zinterol. F: mean changes in I₅₋₆ elicited by application of 1 and 3 μmol/l zinterol to intact (1 μmol/l: n = 5 and 3 μmol/l: n = 19) and DT (1 μmol/l: n = 4 and 3 μmol/l: n = 6) CAL myocytes. Data were subjected to two-way ANOVA: β₂-agonism P < 0.001, DT not significant, interaction not significant. **P < 0.01 and ***P < 0.001, Bonferroni post hoc test.
membrane was calculated as previously described (6). As we have previously reported, there was no statistically significant difference between sham and CAL myocytes in the degree of osmotic shock-induced DT, nor was there any relationship between the whole cell capacitance and time of recording (6).

Statistics. Data are expressed as means ± SE of n myocytes. Statistical analysis was performed using GraphPad Prism (GraphPad Software). I_Ca density-voltage relationship curves were analyzed using repeated-measures ANOVA with voltage and the corresponding intervention (i.e., DT, H-89, or C3SD) as factors. I_Ca properties elicited by a step depolarization to a single voltage were analyzed by two-way ANOVA. Post hoc tests used the Bonferroni correction. The limit of statistical confidence was taken as P < 0.05. Errors in derived variables (specifically I_Ca density at the t-tubule membrane) and the subsequent statistical analysis (unpaired Student’s t-test) were calculated using propagation of errors from the source measurements (6, 14).

RESULTS

Effect of CAL on the response to β2-adrenoceptor stimulation. In intact ventricular myocytes from sham hearts, selective activation of β2-adrenoceptors (1 and 3 μmol/l zinterol in the presence of 10 μmol/l atenolol) caused a significant, concentration-dependent increase of I_Ca, which reached ~140% of control in the steady state in the presence of 3 μmol/l of the β2-agonist (Fig. 1A. left, B, and C). In contrast, in DT cells, 3 μmol/l zinterol did not increase I_Ca (Fig. 1A. right, B, and C). In CAL myocytes, 3 μmol/l zinterol caused an increase of ~40% in intact myocytes and ~29% in DT myocytes (Fig. 1, D–F). Thus, because I_Ca recorded in DT cells represents the current at the surface membrane, it appears that in sham myocytes, the response of I_Ca to β2-adrenoceptor stimulation occurs predominantly at the t-tubule membrane. However, after CAL, the β2-adrenergic response redistributes and occurs at both the cell surface and t-tubule membranes. These data also show that the DT procedure per se is not responsible for the lack of response to zinterol observed in sham myocytes.

Effect of CAL on the response to forskolin. To investigate whether the distribution of the β2-adrenergic response was due to localization of a downstream component of the signaling pathway, we used forskolin (10 μmol/l) to activate adenylyl cyclase directly, to increase cAMP in the absence of adrenoceptor stimulation. Superfusion with forskolin (10 μmol/l) increased I_Ca in both intact and DT myocytes from sham hearts (Fig. 2A). The corresponding mean I_Ca density-voltage relationships for intact and DT myocytes are shown in Fig. 2B. Figure 2C shows the effect of forskolin on I_Ca density at a test potential of −10 mV in sham intact and DT myocytes. Forskolin also caused an increase in I_Ca in both intact and DT myocytes from CAL hearts (Fig. 2, D–F). These data show that the increase in I_Ca in response to forskolin was similar in intact sham and CAL myocytes. More importantly, these data also show that forskolin caused a significant increase in the amplitude of I_Ca in DT sham myocytes, which was similar to that observed in DT CAL myocytes. Thus, it appears that adenylyl cyclase and PKA are present at both the surface and t-tubule membranes in both sham and CAL myocytes and can stimulate I_Ca to a similar extent at either site. It is unlikely, therefore, that the lack of effect of zinterol in DT sham myocytes was due to absence of the components of the cAMP signaling pathway (i.e., adenylyl cyclase and PKA) at the cell surface but may be due to the absence of β2-adrenoceptors.

Effect of CAL on the response to C3SD. Since Cav-3 has been implicated in the localization of β2-adrenoceptor/cAMP signaling at the t-tubules, we investigated the effect of acutely inhibiting Cav-3 binding to its partner proteins by pretreatment...
of cells with C3SD peptide (5, 15). $I_{\text{Ca}}$ density was reduced in intact sham myocytes treated with the C3SD peptide (Fig. 3A, left, and B). However, treatment with C3SD had no effect on $I_{\text{Ca}}$ density in DT sham myocytes (Fig. 3A, right, and C). The effects of treatment with C3SD on $I_{\text{Ca}}$ density at 0 mV in intact and DT myocytes from sham hearts are shown in Fig. 3D. In contrast to its effect in sham myocytes, C3SD had no effect on $I_{\text{Ca}}$ density in intact CAL myocytes (Fig. 3E, left, F, and H), nor did C3SD have any effect on $I_{\text{Ca}}$ in DT CAL myocytes (Fig. 3E, right, G, and H). Thus, there appears to be no Cav-3-dependent regulation of $I_{\text{Ca}}$ at the surface membrane in either sham or CAL myocytes. However, there does appear to be Cav-3-dependent stimulation of $I_{\text{Ca}}$ at the t-tubules of sham myocytes, which is absent in CAL cells.

**Effect of CAL on the response to H-89 in the absence and presence of C3SD.** Since it has been suggested that Cav-3-dependent stimulation of t-tubular $I_{\text{Ca}}$ is via a PKA-dependent mechanism (5), we investigated the effect of the PKA inhibitor H-89 on $I_{\text{Ca}}$ density after CAL and the effect of C3SD on the response to H-89. Inhibition of PKA (20 μmol/l H-89) decreased $I_{\text{Ca}}$ in untreated and C3SD-treated sham myocytes, indicating that constitutive stimulation of $I_{\text{Ca}}$ by PKA that did not require Cav-3 (Fig. 4, A–C). Moreover, there was no difference in the $I_{\text{Ca}}$ density-voltage relations of untreated and C3SD-treated cells in the presence of H-89 (Fig. 4B), demonstrating that the effects of H-89 and C3SD treatment were not summative. Thus, in the presence of PKA inhibition, treatment with C3SD peptide was without effect on $I_{\text{Ca}}$ density, indicating that PKA activity was required for the constitutive regulation of $I_{\text{Ca}}$ by Cav-3 in sham myocytes. Similarly, H-89 decreased $I_{\text{Ca}}$ density to the same level in both untreated and C3SD-treated CAL myocytes, indicating constitutive regulation of $I_{\text{Ca}}$ by PKA (Fig. 4, D–F). C3SD was without effect on $I_{\text{Ca}}$ density in either the absence or presence of H-89 (Fig. 4, E and F). These data show that in sham myocytes, there is constitutive stimulation of $I_{\text{Ca}}$ by PKA that is mediated both via Cav-3-dependent (localized to the t-tubule membrane) and Cav-3-independent mechanisms. Although the constitutive regulation by Cav-3 was lost in CAL myocytes, constitutive regulation of $I_{\text{Ca}}$ via PKA remained.

**Effect of DT on the constitutive regulation of $I_{\text{Ca}}$ by PKA.** To further investigate the site of constitutive PKA-dependent regulation, the response to H-89 was determined in DT myocytes. $I_{\text{Ca}}$ density was reduced by H-89 in both intact and DT sham myocytes (Fig. 5, A–C). $I_{\text{Ca}}$ density was also reduced by DT in both the presence or absence of PKA inhibition (Fig. 5C). H-89 also reduced $I_{\text{Ca}}$ density in intact and DT myocytes from CAL hearts (Fig. 5, D–F). However, in contrast to sham myocytes, in CAL, $I_{\text{Ca}}$ density was similar in intact and DT myocytes in either the presence or absence of PKA inhibition (Fig. 5F).

The calculated current densities at the cell surface and in the t-tubule membrane before and after inhibition of PKA are
shown in Fig. 6. These data show that in sham myocytes without inhibition of PKA, $I_{\text{Ca}}$ density was significantly greater in the t-tubule membrane than at the cell surface, consistent with previous reports (5, 6, 16). In contrast, in CAL myocytes, there was no difference in $I_{\text{Ca}}$ density between t-tubule and surface membranes (Fig. 6B). Inhibition of PKA caused a broadly similar fractional decrease in $I_{\text{Ca}}$ at the surface membrane in both sham and CAL myocytes, so that surface membrane $I_{\text{Ca}}$ remained larger in CAL than sham myocytes. Thus, constitutive stimulation of basal $I_{\text{Ca}}$ by PKA at the cell surface was similar in the two cell types. H-89 also decreased t-tubular $I_{\text{Ca}}$ density in both cell types so that it was smaller in CAL than sham myocytes. However, after inhibition of PKA, $I_{\text{Ca}}$ density remained higher at the t-tubules than in the surface membrane in sham myocytes, whereas in CAL myocytes, $I_{\text{Ca}}$ density was smaller at the t-tubules than at the surface membrane. Thus, it appears not only that $I_{\text{Ca}}$ redistributes from the t-tubules to the cell surface in heart failure but also that constitutive stimulation of basal $I_{\text{Ca}}$ by PKA is increased in these cells (cf. Fig. 6, A and B).

**DISCUSSION**

This study presents two novel findings regarding the regulation of $I_{\text{Ca}}$ in heart failure. First, stimulation of $I_{\text{Ca}}$ by $\beta_2$-adrenoceptors, but not by adenylyl cyclase/PKA, is localized to the t-tubules in sham myocytes and redistributes to the cell surface after CAL. Second, it demonstrates constitutive stimulation of $I_{\text{Ca}}$ by PKA in sham myocytes that is mediated both via Cav-3-dependent (at the t-tubules) and Cav-3-independent mechanisms, whereas in CAL myocytes, constitutive regulation by Cav-3 is lost, although that via PKA remains at both sites. Thus, the present study advances previous findings from our laboratory that Cav-3 plays a role in the regulation of $I_{\text{Ca}}$ at the t-tubule by PKA and $\beta_2$-adrenoceptors in normal myocytes (5, 9) and that $I_{\text{Ca}}$ is redistributed from the t-tubules to the surface sarcolemma in CAL-induced heart failure (6). Interestingly, although constitutive PKA-dependent stimulation of $I_{\text{Ca}}$ at the cell surface appeared to be the same in both sham and CAL myocytes, constitutive stimulation of t-tubular $I_{\text{Ca}}$ appeared to increase in CAL myocytes, helping to maintain t-tubular $I_{\text{Ca}}$. Figure 7 shows schematic diagrams illustrating the regulation of $I_{\text{Ca}}$ by $\beta_2$-adrenoceptors, Cav-3, and PKA in normal cells (Fig. 7A) and in heart failure (Fig. 7B).

**Localization of $I_{\text{Ca}}$ regulation by PKA in sham myocytes.** The Cav1.2 pore-forming a-subunit of ventricular LTCCs has been shown to be colocalized with Cav-3, adenylyl cyclase, PKA, and the $\beta_2$-adrenoceptor (1). Stimulation of $\beta_2$-adrenoceptors in cardiac myocytes activates adenylyl cyclase, causing a local increase of cAMP, activation of PKA, and thereby phosphorylation and stimulation of colocalized LTCCs (1). The present data show that $\beta_2$-adrenoceptor stimulation of $I_{\text{Ca}}$ in sham myocytes occurs predominantly at the t-tubules (Fig. 1), although direct activation of adenylyl cyclase using forskolin increased $I_{\text{Ca}}$ at both the t-tubular and surface membranes (Fig. 2). Thus, in normal myocytes, adenylyl cyclase and the downstream pathway is present at both the t-tubular and surface membranes, but the $\beta_2$-adrenoceptor is present only at the t-tubules, consistent with previous work showing t-tubular localization of $\beta_2$-adrenoceptor signaling in normal ventricular myocytes (27). Pretreatment with C3SD peptide decreased basal $I_{\text{Ca}}$ at the t-tubules but not at the surface membrane in sham myocytes (Fig. 3), showing that Cav-3 plays a role in the constitutive stimulation of $I_{\text{Ca}}$ at the t-tubules but not at the surface membrane in normal cells (Fig. 7A). These data are
entirely consistent with our previous report (5) in which we showed that pretreatment with C3SD abolished both the constitutive regulation of $I_{Ca}$ at the t-tubule and the response to $\beta_2$-adrenoceptors in myocytes from unoperated animals. In contrast, inhibition of PKA using H-89 in the present study decreased $I_{Ca}$ at both the surface and t-tubule membranes, presumably reflecting the loss of tonic activity of the adenyl cyclase/cAMP/PKA pathway at both the surface and t-tubular membranes (Figs. 5 and 7A). Although it has been suggested that H-89 may have nonspecific effects independent of PKA inhibition (26), we (9) have previously shown that basal $I_{Ca}$ was decreased by a peptide inhibitor of PKA, PKI. Moreover, we have recently shown that H-89 was without effect on basal $I_{Ca}$ in rat atrial myocytes from the same hearts as used in the present study, demonstrating both regional differences in the role of PKA in the regulation of $I_{Ca}$ and that H-89 was without direct effect on $I_{Ca}$ per se (3). The regulation of basal $I_{Ca}$ by constitutive PKA activity has also been previously demonstrated in rat ventricular myocytes (4, 5, 9).

While the inhibitory effect of H-89 in sham myocytes was not abolished by pretreatment of the cells with C3SD, H-89 reduced basal $I_{Ca}$ to the same mean amplitude in C3SD-treated and untreated cells (Fig. 4), indicating that the effects of C3SD and H-89 were not summative. Thus, PKA is required for the constitutive regulation of $I_{Ca}$ by Cav-3 at the t-tubules in sham myocytes, but there is an additional Cav-3-independent constitutive regulation of $I_{Ca}$ by PKA. As basal $I_{Ca}$ density in DT sham myocytes was reduced by H-89 but not by C3SD, it can be concluded that PKA is also involved in the constitutive regulation of $I_{Ca}$ at the surface sarcolemma through a mechanism independent of Cav-3. Taken together, these data suggest a role for Cav-3 in coordinating a complex of signaling proteins including LTCC, PKA, and the $\beta_2$-adrenoceptor at the t-tubule membrane in normal ventricular myocytes (1, 5, 27).
Although Cav-3 is important to the constitutive maintenance of \( I_{\text{Ca}} \) by PKA at the t-tubule in normal ventricular myocytes, it does not appear to be required for localizing \( I_{\text{Ca}} \) density at the t-tubule membrane, because the difference in \( I_{\text{Ca}} \) density between t-tubule and surface sarcolemma was maintained after inhibition of PKA (Fig. 6A).

**Regulation of \( I_{\text{Ca}} \) by PKA in CAL myocytes.** In contrast to sham myocytes, \( I_{\text{Ca}} \) increased in response to \( \beta_2 \)-adrenergic stimulation in both intact and DT CAL myocytes (Fig. 1). Moreover, C3SD had no effect on \( I_{\text{Ca}} \) in CAL myocytes (Fig. 3). However, as in sham myocytes, forskolin increased (Fig. 2), and H-89 decreased (Figs. 4, 5, and 6), \( I_{\text{Ca}} \) at both the surface and t-tubular membranes. The simplest explanation of these data is that the normal Cav-3-dependent localization of \( \beta_2 \)-adrenoceptor signaling at the t-tubules is disrupted in CAL myocytes, so that the \( \beta_2 \)-adrenoceptor is distributed across both the surface and t-tubular membranes and can stimulate adenylyl cyclase/PKA and thus LTCCs at both sites, even without Cav-3 regulation; this is consistent with the redistribution of \( \beta_2 \)-adrenoceptor cAMP signaling in heart failure (27) and demonstrates that Cav-3 is not required for \( \beta_2 \)-adrenoceptor stimulation of adenylyl cyclase/PKA, which are already present at both sites (Fig. 7B). Interestingly, in the presence of H-89, \( I_{\text{Ca}} \) density was similar in the t-tubular and surface membranes of CAL myocytes, suggesting that LTCCs are also redistributed in heart failure (6). The mechanisms underlying the redistribution of \( \beta_2 \)-adrenoceptors and LTCCs away from the t-tubules, resulting in a more uniform distribution across the cell membrane, are unclear; presumably, the redistribution of Cav-3 to noncholesterol-rich membranes in heart failure leads to a loss of Cav-3 from the t-tubules and the consequent disruption of Cav-3-dependent complexes containing LTCC/adenylyl cyclase/PKA/\( \beta_2 \)-adrenoceptors (29).

Cav-3 likely plays a role in the localization of the \( \beta_2 \)-adrenoceptor to the t-tubule so that the loss of Cav-3 regulation from the t-tubule membrane in heart failure contributes directly to the redistribution of the receptor to the surface sarcolemma and the loss of localization of \( \beta_2 \)-adrenoceptor signaling to the t-tubule in failing myocytes (1, 5, 27, 32). Alternatively, in principle, it is possible that \( \beta_2 \)-adrenoceptors are more uniformly distributed between the t-tubule and surface membranes and that Cav-3 may be responsible for the localization of adenylyl cyclase/PKA signaling to the \( \beta_2 \)-adrenoceptors in the t-tubules. Consistent with either of these proposals, treatment of normal ventricular myocytes with C3SD peptide has been shown to antagonize \( \beta_2 \)-adrenoceptor-mediated increases in \( I_{\text{Ca}} \) (5). Moreover, overexpression of Cav-3 restored the localization of \( \beta_2 \)-adrenoceptor signaling to the t-tubules in failing cells, implying a direct role for Cav-3 in the localization of the receptors and/or receptor signaling to the t-tubules, presumably via binding with the scaffolding domain (32). However, the observation that zinterol stimulates \( I_{\text{Ca}} \) at the surface membrane of CAL myocytes, in which C3SD has no effect on \( I_{\text{Ca}} \), suggests that \( \beta_2 \)-adrenoceptor stimulation can stimulate adenylyl cyclase/PKA even without Cav-3 binding. The role of Cav-3 in the loss of t-tubular localization of \( \beta_2 \)-adrenoceptor signaling in heart failure might be tested in future studies by investigating the effect of C3SD peptide on the response of CAL myocytes to \( \beta_2 \) stimulation. On the other hand, Cav-3 does not seem to play a direct role in the localization of LTCCs to the t-tubule because 1) interference of Cav-3 binding to its partners in intact sham myocytes by treatment with C3SD peptide had no effect on \( I_{\text{Ca}} \) in the presence of PKA inhibition (Fig. 4), indicating that PKA activity was required for the Cav-3-dependent regulation of LTCCs, and 2) PKA was not required for the concentration of \( I_{\text{Ca}} \) at the t-tubule in sham myocytes (Fig. 4). Although Cav-3-dependent regulation of t-tubular \( I_{\text{Ca}} \) by PKA was lost in CAL myocytes, they showed an increased ratio of basal t-tubular \( I_{\text{Ca}} \) density to t-tubule \( I_{\text{Ca}} \).
density in the presence of H-89 compared with sham myocytes (CAL: basal $-5.9 \pm 1.6$ pA/pF and H-89 $-1.7 \pm 0.8$ pA/pF; sham: basal $-12.9 \pm 3.0$ pA/pF and H-89 $-7.4 \pm 1.6$ pA/pF), indicating that the contribution of PKA to the maintenance of t-tubular $I_{Ca}$ was augmented in heart failure. This is consistent with increased PKA-dependent regulation of basal whole cell $I_{Ca}$ in failing human ventricular myocytes (10). Nevertheless, the mechanism for the increased constitutive regulation of t-tubular $I_{Ca}$ by PKA in heart failure remains unclear.

Functional implications of regulation of $I_{Ca}$ by PKA. Previous work has shown that $I_{Ca}$ occurs predominantly in the t-tubules, in close proximity to RyRs in the SR membrane, allowing efficient coupling between Ca$^{2+}$ entry via $I_{Ca}$ and Ca$^{2+}$ release from the SR (21, 28). The present work shows that even in the presence of PKA inhibition, $I_{Ca}$ still occurs predominantly in the t-tubules of sham myocytes, suggesting a higher concentration of LTCCs in the t-tubules. The observation that $\beta_2$-adrenoceptor stimulation of $I_{Ca}$ is normally localized to the t-tubules is consistent with the importance of this site for the normal regulation of EC coupling and the potential detrimental effects of a whole cell increase of cAMP.

In CAL myocytes, although there was little change in whole cell $I_{Ca}$ density, there was redistribution of $I_{Ca}$ so that it was more uniformly distributed across the surface and t-tubular membranes. Unless accompanied by parallel redistribution of RyRs, which, to the best of our knowledge, does not occur, the reduced Ca$^{2+}$ entry at the t-tubules will result in less effective coupling of Ca$^{2+}$ entry and release and increased numbers of “orphaned” RyRs, resulting in a smaller, slower Ca$^{2+}$ transient and thus contraction. However, the present work shows that increased local constitutive stimulation of $I_{Ca}$ by PKA helps to maintain $I_{Ca}$ at the t-tubules, which will help ameliorate these deleterious effects.

It has been proposed that a subpopulation of LTCCs in surface membrane caveolae play a role in cardiac hypertrophy (12, 24). The observation that C3SD has little effect on $I_{Ca}$ in DT sham or CAL myocytes suggests that Cav-3 binding has little effect on LTCC function at the cell surface, although it remains possible that downstream effects of $I_{Ca}$ are altered.

Summary. The present study shows that Cav-3 plays a vital role in the coordination of PKA-dependent regulation of both basal and $\beta_2$-adrenoceptor stimulation of $I_{Ca}$ in myocytes from healthy hearts. The colocalization by Cav-3 is lost in heart failure, and both $\beta_2$-adrenoceptors and LTCCs are redistributed from the t-tubular to surface sarcolemma membranes. The role of Cav-3 in the redistribution in heart failure remains unclear, but the data are consistent with a shift in Cav-3 from cholesterol-rich to noncholesterol-rich membranes (29).

GRANTS

This work was funded by British Heart Foundation Grants PG/10/91/28644, PG/14/65/31055, and RG/12/10/29802.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.M.B. performed experiments; S.M.B. analyzed data; S.M.B., C.H.K., M.B.C., C.H.O., and A.F.J. interpreted results of experiments; S.M.B. prepared figures; S.M.B., C.H.K., C.H.O., and A.F.J. drafted manuscript; S.M.B., C.H.K., M.B.C., C.H.O., and A.F.J. edited and revised manuscript; S.M.B., C.H.K., M.B.C., C.H.O., and A.F.J. approved final version of manuscript; C.H.O. and A.F.J. conceived and designed research.

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