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Reduced effects of BAY K 8644 on L-type Ca^{2+} current in failing human cardiac myocytes are related to abnormal adrenergic regulation

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Abstract

Abnormal L-type Ca^{2+} channel (LTCC, also named Cav1.2) density and regulation are important contributors to depressed contractility in failing hearts. The LTCC agonist BAY K 8644 (BAY K) has reduced inotropic effects on failing myocardium. We hypothesized that BAY K effects on the LTCC current (I_{CaL}) in failing myocytes would be reduced because of increased basal activity. Since support of the failing heart with a left ventricular assist device (LVAD) improves contractility and adrenergic responses, we further hypothesized that BAY K effects on I_{CaL} would be restored in LVAD-supported failing hearts. We tested our hypotheses in human ventricular myocytes (HVMs) isolated from nonfailing (NF), failing (F), and LVAD-supported failing hearts. We found that 1) BAY K had smaller effects on I_{CaL} in F HVMs compared with NF HVMs; 2) BAY K had diminished effects on I_{CaL} in NF HVM pretreated with isoproterenol (Iso) or dibutyryl cyclic AMP (DBcAMP); 3) BAY K effects on I_{CaL} in F HVMs pretreated with acetylcholine (ACh) were normalized; 4) Iso had no effect on NF HVMs pretreated with BAY K; 5) BAY K effects on I_{CaL} in LVAD HVMs were similar to those in NF HVMs; 6) BAY K effects were reduced in LVAD HVMs pretreated with Iso or DBcAMP; 7) Iso had no effect on I_{CaL} in LVAD HVMs pretreated with BAY K. Collectively, these results suggest that the decreased BAY K effects on LTCC in F HVMs are caused by increased basal channel activity, which should contribute to abnormal contractility reserve.

Keywords

heart failure; L-type calcium channel; left ventricular assist device

Depressed basal myocyte contractility, heightened activation of sympathetic neurohormonal pathways, and blunted adrenergic contractile responsiveness are characteristic features of failing human hearts (25). Abnormal Ca^{2+} handling is a primary cause for the depressed myocyte contractility. Altered L-type Ca^{2+} channel [LTCC, also named Cav1.2 (15)] density and regulation are thought to contribute to the deranged Ca^{2+} handling in failing cardiac myocytes (5), but the nature of the channel dysregulation has not been clearly defined.

Contractile and adrenergic abnormalities of the failing heart are partially normalized when the failing human heart is supported with a left ventricle assist device (LVAD) (8,12,45). Since LVAD support reduces circulating catecholamines(27), it has been suggested that contractile abnormalities may be related to the heightened adrenergic state in heart failure.

Increasing cardiac contractility is one therapy to improve the function of the failing heart. One approach would be to increase Ca^{2+} entry through the LTCC. In this regard, the positive inotropic effects of the LTCC dihydropyridine agonist BAY K 8644 (BAY K) have been tested and are found smaller in failing hearts than in normal myocardium (34). We have shown that in isolated failing human trabeculae, BAY K produces smaller than normal increases in developed force, especially at higher stimulation frequencies (39). Others have found that myocytes from failing rat hearts, induced by aortic stenosis, have reduced inotropic response to BAY K, whereas myocytes from compensated hypertrophied hearts in spontaneous hypertensive rats maintained a normal inotropic response (1). These studies suggest that fundamental alterations in the density or basal activity level of the LTCC are present in many forms of heart failure. Given the central role of the LTCC in excitation-contraction coupling in cardiac myocytes (4), we hypothesize that the reduced inotropic effects of BAY K in failing human myocardium result from reduced effects of BAY K on Ca^{2+} influx through the LTCC.

The response of the LTCC to substances that induce LTCC phosphorylation, such as isoproterenol (Iso), cAMP, and phosphatase inhibitors, are reduced in failing (F) human ventricular myocytes (HVMs) (26). One possible explanation for these observations is that the basal phosphorylation state of the LTCC is increased in the failing heart (8,41) due to heightened sympathetic and/or decreased phosphatase activity.

BAY K and adrenergic agonists such as Iso and dibutyryl cyclic AMP (DBcAMP) all increase the availability, open probability, and “mode 2” gating of the LTCC (36,48). They all also shift the activation and inactivation voltage dependence to more negative voltages and accelerate the decay of the LTCC current (I_{CaL}) (33). Adrenergic stimulation of cardiac myocytes leads to the activation of protein kinase A (PKA) and subsequent phosphorylation of target proteins including the LTCC (29). Chronically heightened adrenergic activity in heart failure also activates Ca^{2+} /calmodulin-dependent kinase II (24), which is able to phosphorylate the LTCC and enhance the activity of the LTCC (13).

The objective of the present study was to further define the nature of the abnormalities of the LTCC in human heart failure and their recovery after LVAD support. We examined the idea that increased basal activity levels of the LTCC, possibly due to increased phosphorylation state, may cause decreased responses to BAY K in F HVMs. These experiments also addressed whether or not adrenergic agonists and BAY K have independent (31,42) or interactive (16,43) effects on the LTCC. Our experiments show that the effects of both BAY K and Iso on LTCCs are smaller in F HVMs than in HVMs from nonfailing (NF) and LVAD-supported hearts. Acetylcholine (ACh), which inhibits I_{CaL} , at least in part by reducing the phosphorylation of the LTCC (31), restored the response of the LTCC to BAY K in F HVMs. Exposure to either BAY K or adrenergic agonists (Iso or DBcAMP) reduced or eliminated the effects of the other drug. Our results suggest that the reduced BAY K effects on the LTCC in failing hearts are related to increased basal LTCC activity, possibly due to increased basal LTCC phosphorylation caused by chronic exposure to high catecholamines in failing hearts.

MATERIALS AND METHODS

Isolation of HVMs and I_{CaL} recording

HVMs were isolated from 7 nonfailing (NF), 15 failing (F), and 14 LVAD-supported failing human hearts as described previously (8). Failing and LVAD-supported failing human hearts were obtained from the Temple Cardiac Transplant Team at the time of cardiac transplantation. Nonfailing hearts were donor hearts unsuitable for transplantation. Our protocol was approved by Temple University Institutional Review Board. Patient characteristics are presented in Table 1. Isolated HVMs were used within 12 h postisolation.

The L-type Ca^{2+} current (I_{CaL}) was measured in a sodium-free and potassium-free solution as described previously (8). In short, isolated myocytes were placed in a chamber mounted on an inverted microscope (Nikon Diaphot) and perfused with normal Tyrode solution (composition in mM: 10 glucose, 5 HEPES, 5.4 KCl, 1.2 $MgCl_2$, 150 NaCl, and 2 Na-pyruvate, pH 7.4 with NaOH). Both the inflow solution and the chamber were water-heated to maintain the temperature at 36 ± 1 °C. A 1–4 M Ω pipette filled with a Cs^+ -containing solution [composition in mM: 130 Cs-aspartate, 10 *N*-methyl-D-glucamine (NMDG), 20 tetraethylammonium chloride, 10 HEPES, 2.5 Tris-ATP, 1 $MgCl_2$, and 10 EGTA, pH 7.2, and drugs needed specifically for experiments] was used to obtain gigaseals. Once a gigaseal was formed, the patch was ruptured and the cell was dialyzed for 10 min. The extracellular bath was then changed to a Cs^+ substitution bath solution (composition in mM: 2 4-aminopyridine, 2 $CaCl_2$, 5.4 CsCl, 10 glucose, 5 HEPES, 1.2 $MgCl_2$, and 150 NMDG, pH 7.4 with CsOH). Membrane voltage was controlled by an Axo-patch 2B voltage-clamp amplifier and digitized by Digidata 1200 using pClamp8 software (Molecular Devices). Once the signal was converted to digital format, it was stored on a personal computer for off-line analysis with Clampfit 8 (Axon Instruments). The flow of the bathing solution was ~2–3 ml/min.

For the experiments testing drug effects, myocytes were exposed to drugs and the effects were monitored by recording I_{CaL} at +10 mV from the holding potential of –70 mV every 20 s. When effects were stable, the I_{CaL} -voltage relationship was again determined. Only myocytes with minimal (<10%) rundown of I_{CaL} were included in the data sets. Whole cell LTCC conductance (G) was calculated by dividing the I_{CaL} density by the driving force for Ca^{2+} ions ($V_{test} - V_{rev}$, where V_{test} is the test potential and V_{rev} is the apparent reversible potential) and the activation curve (G plotted against V_{test}) was fitted with a Boltzmann equation to obtain the half-maximal activation voltage ($V_{0.5}$) (8). To quantify I_{CaL} decay, we fitted I_{CaL} with a double-exponential equation to obtain fast (τ_f) and slow (τ_s) time constants (8).

Drugs

Cells were exposed to the dihydropyridine LTCC agonist BAY K (1 μ M; Sigma) to increase I_{CaL} . We also tested the effect of BAY K (1 μ M; Sigma) on cells pretreated with drugs changing LTCC phosphorylation. These drugs included nonhydrolyzable DBcAMP (10 μ M in pipette solution; Sigma), Iso (1 μ M in solution; Sigma), and ACh (1 μ M in the bath solution; Sigma). These concentrations were used because they were found to produce maximal stable effects on I_{CaL} in preliminary experiments.

Statistics

Data are means $\pm$ SE. Paired or unpaired *t*-tests were used to evaluate statistics on single-factor two-group comparison. Differences among multiple groups were tested with ANOVA or repeated-measures ANOVA with SAS (SAS Institute). A *P* value of 0.05 was considered significant. *N* indicates how many hearts were used, and *n* is the number of cells measured.

RESULTS

Effects of BAY K 8644 on I_{CaL} in NF and F HVMs

Inotropic effects of BAY K are smaller in failing versus normal myocytes (1,34,39). To explore the underlying mechanism, we measured the effects of BAY K on I_{CaL} in NF ($n = 16$, $N = 4$) and F HVMs ($n = 24$, $N = 6$). I_{CaL} density, current-voltage relationship (I - V), voltage-dependent activation (G - V), and fast (τ_f) and slow time constants (τ_s) of I_{CaL} decay were measured. BAY K increased I_{CaL} density, shifted the I - V relationship and G - V , and enhanced I_{CaL} decay (Fig. 1) in all myocytes. BAY K significantly increased I_{CaL} at most voltages (-70 to $+70$ mV) and maximal I_{CaL} density in both NF (basal: -7.96 ± 0.99 pA/pF; BAY K: -15.78 ± 1.39 pA/pF; $P < 0.001$) and F HVMs (basal: -8.05 ± 0.43 pA/pF; BAY K: -12.38 ± 0.56 pA/pF; $P < 0.001$). Importantly, the BAY K effect on the peak I_{CaL} was significantly smaller in F than in NF HVMs (%increase in maximal I_{CaL} : NF, $96 \pm 12\%$; F, $59 \pm 8\%$; $P < 0.01$) (Fig. 1). The maximal I_{CaL} after BAY K was also significantly smaller in F HVMs than in NF HVMs ($P < 0.05$). Assuming that BAY K induces a similar open probability of the LTCC in both groups of myocytes, this result is consistent with the idea that LTCC density is smaller than normal in F myocytes, consistent with previous suggestions (8).

BAY K caused a leftward shift of G - V curves in NF ($V_{0.5}$: basal, 0.43 ± 1.95 mV; BAY K, -8.94 ± 1.80 mV; $P < 0.0001$) and F HVMs ($V_{0.5}$: basal, -1.78 ± 1.80 mV; BAY K, -6.65 ± 2.08 mV; $P < 0.001$). This shift was greater in NF myocytes (Fig. 1). The change of the $V_{0.5}$ for LTCC activation induced by BAY K in NF ($\Delta V_{0.5}$: -9.25 ± 1.30 mV) myocytes was significantly larger than in F myocytes ($\Delta V_{0.5}$: -4.87 ± 1.26 mV). The $V_{0.5}$ was not different among groups after BAY K ($P = 0.73$). Therefore, the difference in BAY K effects on $V_{0.5}$ in F compared with NF HVMs results from the fact that $V_{0.5}$ is already shifted leftward in F myocytes. Phosphorylation of the LTCC is known to induce a leftward shift of LTCC activation (8,41). Therefore, this result is consistent with the hypothesis that the basal phosphorylation state of the LTCC is increased in the failing human heart.

BAY K also increased the rate of I_{CaL} decay (Fig. 1, *D* and *H*). When I_{CaL} decay was fit with a double-exponential decay relationship, we found that τ_f (Fig. 1, *D* and *H*), but not τ_s (data not shown) was decreased by BAY K (10). Sanguinetti et al. (40) previously reported that BAY K decreased both τ_f and τ_s of the I_{CaL} . This acceleration of the decay of I_{CaL} by BAY K could be due to enhanced Ca^{2+} -dependent inactivation (32) and/or direct effects of BAY K on channel gating (40).

The results showing that BAY K increased the amplitude, shifted the G - V curves, and accelerated the decay of I_{CaL} in NF and F HVMs are consistent with previous studies in normal myocytes (33). However, the bases of the diminished effects of BAY K on I_{CaL} in failing myocytes are not clear. Our hypothesis is that the reduced effect of BAY K on I_{CaL} in F HVMs is due to an increased activity of the LTCC, possibly due to increased phosphorylation resulting from enhanced adrenergic stimulation and decreased phosphatase activity in the failing heart (41). We tested this idea further by measuring the effects of BAY K on the LTCC after exposure to adrenergic agonists in NF and F HVMs.

Effects of BAY K 8644 on I_{CaL} in NF HVMs Pretreated with Iso

I_{CaL} in F HVMs is less responsive to Iso stimulation than in normal myocytes, possibly due to higher basal phosphorylation state and increased open probability of the LTCC (8). We tested BAY K effects on the LTCC in NF HVMs pretreated with $1 \mu M$ Iso to explore this idea. BAY K had smaller effects on I_{CaL} after Iso treatment (basal: -10.3 ± 1.7 pA/pF; Iso: -15.0 ± 1.9 pA/pF; Iso + BAY K: -18.3 ± 1.5 pA/pF; $n = 10$, $N = 4$; $P < 0.01$, Iso vs. basal; $P < 0.001$, BAY K + Iso vs. basal; $P < 0.05$, BAY K + Iso vs. Iso; one-way repeated

ANOVA with post hoc tests, Fig. 2, A and B). The percent increase in I_{CaL} upon BAY K stimulation in Iso-pretreated NF HVMs was significantly less (F -test, $P \leq 0.05$) than in NF HVMs not pretreated with Iso (53 ± 10 vs. $96 \pm 12\%$) and was similar to the increase of I_{CaL} in F HVMs induced by BAY K. BAY K significantly shifted the $V_{0.5}$ of I_{CaL} activation in Iso-pretreated NF HVMs ($V_{0.5}$: basal, 0.75 ± 1.65 mV; Iso, -4.90 ± 3.21 mV; Iso + BAY K, -9.85 ± 1.85 mV; $P < 0.05$, basal vs. Iso; $P < 0.05$, Iso + BAY K vs. Iso), but the change produced by BAY K after Iso pretreatment was less than with BAY K alone. The $V_{0.5}$ after Iso + BAY K was not different from the $V_{0.5}$ of NF HVMs treated with BAY K alone (Fig. 2C). Iso decreased τ_f of I_{CaL} in NF HVMs and BAY K + Iso further decreased τ_f (Fig. 2D). These results support the idea that the reduced BAY K effects observed in failing human myocytes result, at least in part, from increased basal phosphorylation of the LTCC.

Effects of BAY K 8644 on I_{CaL} in NF and F HVMs pretreated with pipette DBcAMP

Adrenergic signaling is abnormal in F HVMs (38) and could be altered in our NF HVMs because they were primarily from old donors (49) (see Table 1 for average age). Therefore, a nonhydrolyzable DBcAMP (in pipette solution) was used to directly activate PKA and enhance LTCC activity. BAY K effects on these activated LTCCs were then tested. Ten minutes of cell dialysis was needed for DBcAMP to have stable, maximal effects, and then BAY K was administered via the perfusion solution. Depolarizing steps to +10 mV at 0.05 Hz were utilized to monitor DBcAMP and BAY K effects on I_{CaL} . DBcAMP significantly increased the peak I_{CaL} density in 12 F HVMs from 5 failing human hearts (-18.0 ± 2.5 pA/pF) and in 9 NF HVMs (-24.8 ± 1.8 pA/pF, $N = 3$) (Fig. 3B). The peak I_{CaL} density after DBcAMP was significantly smaller in F HVMs than in NF HVMs. DBcAMP shifted the activation of I_{CaL} in both NF and F HVMs, and after DBcAMP treatment, there was no more difference in the activation voltage dependence between NF and F HVMs, as we reported previously (8). In a subset of these F and NF HVMs, we successfully tested BAY K effects on I_{CaL} after DBcAMP exposure. BAY K did not significantly increase maximal I_{CaL} density in NF or F HVMs pretreated with DBcAMP [(DBcAMP + BAY K)/(DBcAMP): $111.9 \pm 9.8\%$ in NF, $n = 5$, $N = 2$, vs. $121.0 \pm 11.2\%$ in F, $n = 6$, $N = 2$] (Fig. 3, A, E, and F). BAY K significantly shifted the $V_{0.5}$ of I_{CaL} activation in NF ($V_{0.5}$: DBcAMP, -9.2 ± 1.5 mV; DBcAMP + BAY K, -13.3 ± 1.6 mV; $P < 0.05$) and F HVMs ($V_{0.5}$: DBcAMP, -9.2 ± 1.4 mV; DBcAMP + BAY K, -15.8 ± 2.9 mV; $P < 0.05$) pretreated with DBcAMP to more negative voltages. However, as in NF HVMs pretreated with Iso (Fig. 1C), the change in $V_{0.5}$ was significantly smaller than the change in $V_{0.5}$ induced by BAY K in the absence of Iso.

Tiaho et al. (43) showed slowing of I_{CaL} decay after BAY K when cAMP was in the pipette solution. We only observed this effect in one of five NF HVMs and one of six F HVMs tested (Fig. 3, D and H). This difference could be due to the fact that we performed our studies in human myocytes and/or that enhanced Ca^{2+} -dependent inactivation disguises a direct effect of BAY K to slow of I_{CaL} decay.

Effects of Iso on I_{CaL} in NF HVMs pretreated with BAY K

Our results show that after adrenergic stimulation, BAY K has reduced effects on the LTCC, consistent with a common mechanism of action. We tested whether or not BAY K exposure also reduces the stimulatory effects of adrenergic agonists on the LTCC. In NF HVMs ($n = 7$, $N = 3$), after exposure to BAY K, the LTCC did not respond further to Iso (Fig. 4, A and C). After BAY K, Iso had no significant effects on I_{CaL} amplitude (maximal I_{CaL} : basal, -8.9 ± 2.1 pA/pF; BAY K, -15.3 ± 2.3 pA/pF; Iso + BAY K, -20.6 ± 3.5 ; $P < 0.05$, basal vs. BAY K; $P > 0.05$, BAY K vs. Iso + BAY K; one-way repeated ANOVA). Iso did not shift the G -V curve further in NF HVMs pretreated with BAY K ($V_{0.5}$: basal, -1.0 ± 2.6 mV; BAY K, -7.7 ± 3.1 mV; Iso + BAY K, -10.8 ± 4.5 mV; $P < 0.05$, basal vs. BAY K; P

> 0.05 , Iso + BAY K vs. BAY K; one-way repeated ANOVA, $n = 7$, $N = 3$) (Fig. 4D). These results are not in agreement with the additive effects of BAY K and Iso found by Markevich et al. (31) and Sculptoreanu et al. (42). Conversely, our results support the idea that the effects of adrenergic agonists and BAY K on the LTCC are through a common mechanism.

Effects of BAY K on I_{CaL} in F HVMs pretreated with ACh

An increased basal phosphorylation state of the LTCC in heart failure would increase channel activity and shift the voltage-dependent activation. These effects could account for the diminished effects of BAY K on I_{CaL} in F HVMs (8). In this scenario, BAY K effects should be at least partially restored after dephosphorylation of the LTCC. We tested this idea by exposing F HVMs to 20 μ M ACh, which should dephosphorylate the LTCC (31). ACh decreased maximal I_{CaL} by $27.4 \pm 4.8\%$ in F HVMs, and subsequently, BAY K increased I_{CaL} by $101.0 \pm 15.7\%$ (maximal I_{CaL} : basal, -8.9 ± 1.3 pA/pF; ACh, -6.3 ± 0.5 pA/pF; ACh + BAY K, -12.3 ± 0.6 pA/pF; $P < 0.05$, basal vs. ACh; $P < 0.05$, ACh vs. ACh + BAY K, $n = 5$, $N = 2$) (Fig. 5, A–C). Effects of BAY K after ACh in F HVMs were comparable to the effects of BAY K on NF cells without any treatment. ACh did not alter $V_{0.5}$, but combined ACh and BAY K significantly shifted $V_{0.5}$ to a hyperpolarized voltage ($V_{0.5}$: basal, -4.1 ± 0.8 mV; ACh, -3.5 ± 0.9 mV; ACh + BAY K, -11.6 ± 1.7 mV; $P = 0.0006$, ANOVA) (Fig. 5D). ACh did not have significant effects on the amplitude and the voltage dependence of activation of I_{CaL} in NF HVMs (data not shown). These results support the idea that the phosphorylation state of the LTCC is increased in F HVMs, and this is responsible for the diminished effect of BAY K.

BAY K effects on I_{CaL} in LVAD HVMs are normalized

Previous studies have shown that LVAD support of the failing human heart induces reverse remodeling at the molecular, cellular, and organ levels (45). Particularly, LVAD support improves adrenergic contractile responsiveness (12). We tested whether the response of I_{CaL} to BAY K in LVAD HVMs was greater than in F HVMs. BAY K significantly increased I_{CaL} in LVAD HVMs with maximal I_{CaL} density increased by $128 \pm 39\%$ (basal: -8.1 ± 1.4 pA/pF; BAY K: -16.1 ± 1.1 pA/pF, $n = 6$, $N = 3$). These effects are comparable to those in NF HVMs (Fig. 6, A and C). BAY K tended to cause a leftward shift of the activation of I_{CaL} in LVAD HVMs ($V_{0.5}$: basal, -6.76 ± 3.17 mV; BAY K, -9.42 ± 3.23 ; $P = 0.10$; $\Delta V_{0.5}$: -2.66 ± 1.19 mV) (Fig. 6D), and $V_{0.5}$ after BAY K was not different among NF, F, and LVAD HVMs ($P = 0.73$). BAY K also significantly accelerated the decay of I_{CaL} (Fig. 6B). These results show that the effect of BAY K on I_{CaL} density is normalized by LVAD, but the effect on I_{CaL} voltage dependence of activation is not. This suggests that the mechanisms controlling the increase in I_{CaL} amplitude and shift in $V_{0.5}$ upon phosphorylation of the LTCC could be distinct, possibly controlled by different protein phosphatases (28).

Interactive effects of BAY K and adrenergic stimulation (Iso or DBcAMP) on I_{CaL} in LVAD HVMs

We showed in a previous study that LVAD support normalizes the adrenergic responsiveness of the I_{CaL} (8). In the present study we tested the interactions between the effects of BAY K and Iso or DBcAMP on I_{CaL} in LVAD HVMs. Iso increased I_{CaL} by $50 \pm 9\%$ in LVAD HVMs (basal vs. Iso: -10.7 ± 1.3 vs. -16.2 ± 2.4 pA/pF, $n = 5$, $N = 3$; $P < 0.05$). BAY K after Iso did not significantly increase I_{CaL} (-18.9 ± 2.1 pA/pF; $P = 0.25$ vs. Iso) in LVAD HVMs (Fig. 7, A and B). Iso shifted the voltage dependency of the activation of I_{CaL} to more negative voltages, and BAY K + Iso did not further shift this relationship ($V_{0.5}$: basal, -9.3 ± 1.8 mV; Iso, -14.4 ± 1.6 mV; Iso + BAY K, -18.1 ± 2.2 mV; $P < 0.05$, basal vs. Iso; $P = 0.36$, Iso vs. Iso + BAY K; one-way repeated ANOVA) (Fig. 7C). τ_f was decreased by Iso as seen in NF HVMs, but no difference in τ_f between basal and BAY K +

Iso groups or between Iso and BAY K + Iso groups was found (Fig. 7D). τ_s was not different among all three groups (data not shown).

DBcAMP increased I_{CaL} in LVAD HVMs (basal: -8.1 ± 1.4 pA/pF; DBcAMP, -29.8 ± 1.9 pA/pF; $P < 0.01$, unpaired t -test), and BAY K did not further increase I_{CaL} (DBcAMP, -31.1 ± 2.9 pA/pF, $P < 0.05$) (Fig. 7, E and F). BAY K did shift I_{CaL} activation ($V_{0.5}$: DBcAMP, -12.9 ± 1.0 mV; DBcAMP + BAY K, -18.1 ± 1.9 mV; $P = 0.01$, paired t -test) (Fig. 7G) but did not alter I_{CaL} decay in LVAD HVMs pretreated with DBcAMP (Fig. 7H).

Effect of Iso on I_{CaL} in LVAD HVMs pretreated with BAY K

For completeness, we tested whether BAY K reduced Iso effects on the LTCC. In LVAD HVMs pretreated with BAY K, Iso did not increase I_{CaL} (maximal I_{CaL} : basal, -9.84 ± 1.15 pA/pF; BAY K, -16.87 ± 1.55 pA/pF; Iso + BAY K, -17.58 ± 3.13 ; $P < 0.05$, basal vs. BAY K; $P > 0.05$, BAY K vs. Iso + BAY K; one-way repeated ANOVA, $n = 4$, $N = 2$), as in NF HVMs (Fig. 8, A and B). BAY K tended to enhance I_{CaL} decay ($P = 0.08$), but BAY K + Iso did not slow I_{CaL} decay. Neither BAY K nor BAY K with Iso shifted the activation of I_{CaL} in LVAD HVMs, indicating a partial normalization of the properties of the LTCC (8).

DISCUSSION

The present study shows that BAY K 8644 has smaller effects on I_{CaL} in failing versus nonfailing HVMs. The reduction in BAY K effects on I_{CaL} in F HVMs was related to blunted effects of adrenergic agonists and alterations in I_{CaL} properties that are consistent with increased basal channel activity like those known to be induced by LTCC phosphorylation.

Are reduced BAY K effects on I_{CaL} responsible for diminished inotropic effect of BAY K in F HVMs?

Several studies have shown a reduced inotropic effect of BAY K in failing myocardium (34,39). BAY K increases contractility of cardiac myocytes mainly by enhancing I_{CaL} , which induces Ca^{2+} release from the sarcoplasmic reticulum (18) and loads the sarcoplasmic reticulum (17). The reduced BAY K effects on I_{CaL} we observed can explain the reduced inotropic effect of BAY K in F HVMs shown in previous studies (1).

Phosphorylation of the LTCC and adrenergic stimulation on I_{CaL}

The LTCC is composed of four subunits, α_{1c} , β , γ , and α_2/δ , of which α_{1c} and β have consensus phosphorylation sites. In vitro it has been shown that the α_{1c} -subunit is a good substrate for multiple protein kinases, including PKA, PKC, and Ca^{2+} /calmodulin-dependent kinase II (CaMK II) (28,29). Conventionally, it is believed that adrenergic effects on myocyte LTCCs are mediated by the cAMP/PKA signaling pathway. Ser⁴⁷⁸ and Ser⁴⁷⁹ on the β_{2a} -subunit have also been shown to be phosphorylated by PKA (28,29). The PKA phosphorylation sites on the α_{1c} -subunit, which causes changes in channel biophysics (increased open probability) are still controversial and have been postulated to include Ser¹⁹²⁸ (11,20,37) and Ser¹⁹⁰¹ (35) on the COOH terminus. Some studies suggest that chronic adrenergic stimulation, like that seen in congestive heart failure, activates CaMK II (24,46,50), which is capable of phosphorylating the LTCC and increasing the I_{CaL} (13). We cannot rule out the involvement of CaMK II-mediated phosphorylation when human myocytes are stimulated with Iso and DBcAMP, since the local Ca^{2+} concentration near the LTCC will be increased and could activate local CaMK II. Therefore, phosphorylation of the LTCC by both PKA and CaMK II can greatly increase the availability and the open probability of the LTCC, resulting in significant increase in macroscopic whole cell I_{CaL} .

Interaction between effects of BAY K and adrenergic agonists on the LTCC

Adrenergic agonists increase LTCC availability and open probability by prolonging channel opening and inducing a highly active gating mode named mode 2 (48). It is generally believed that these effects are mediated by PKA-mediated phosphorylation (33,48). Recently, there is also evidence indicating that adrenergic stimulation can activate CaMK II, which can also phosphorylate the LTCC and increase channel activity(13). The effects of BAY K on the LTCC are very similar to these effects (36,48): increased availability, open probability, and mode 2 activity. These results suggest a commonality of BAY K and Iso effects. However, Tsien et al. (44) have suggested that Iso enhances I_{CaL} by augmenting LTCC availability and affecting fast kinetic processes like closing, whereas BAY K mainly promotes mode 2 activity of the LTCC.

BAY K binds to S5 in domain III and to S6 in domains III (23) and IV (7) of the α_{1C} -subunit, spatially distinct from the PKA and CaMK II phosphorylation sites [the COOH terminus of α_{1C} and the β -subunit (28–30)]. The fact that adrenergic stimulation and BAY K do not exert their effects at the same locations on the LTCC does not exclude the possibility that they produce a common effect. Erxleben et al. (16) have suggested that BAY K may stabilize the phosphorylated LTCC or mimic the effect of phosphorylation (16). We have measured the single-channel behavior after the application BAY K or Iso in normal feline ventricular myocytes and found that both drugs increase the availability, open probability, and mode 2 gating of the LTCC. When BAY K was included in the pipette, Iso did not have further stimulatory effects on the single-channel activities [data not shown but reported in an abstract (9)]. More detailed single-channel studies of Iso and BAY K effects on the LTCC in HVMs are needed to further define their mechanisms of actions.

Previous studies have also shown that combinations of BAY K with PKA-dependent phosphorylation produced marked slowing of I_{CaL} and tail current decay (43). These results imply that BAY K might stabilize the phosphorylated state of the LTCC by retarding dephosphorylation (3) or that PKA-dependent phosphorylation might stabilize the BAY K-induced open state of the LTCC. The present study cannot resolve these issues.

Does the LTCC have increased phosphorylation in F HVMs?

We (8) and others (41) have observed that the LTCC behaves as if it is “hyperphosphorylated” in F HVMs. To date, there is no direct biochemical evidence for enhanced phosphorylation of the LTCC in heart failure, due to the technically demanding nature of these studies: There are many unresolved issues regarding LTCC phosphorylation. The COOH terminus of the α_{1C} -subunit, where the PKA phosphorylation site (Ser¹⁹²⁸) is thought to be located, has been reported to be cleaved (21) from the protein. Phosphorylation could be mediated by both PKA and CaMK II, which has been shown to be activated in failing myocardium(24). There are PKA sites on the β -subunit (22), and there are multiple β -isoforms (β_1 , β_2 , and β_3 , each having splicing isoforms). However, there is strong indirect evidence for greater phosphorylation of the LTCC in heart failure. Schroder et al. (41) showed that there were increased open probability and availability of the LTCCs in F HVMs, which resembled the effects of 8-bromo-cAMP and a phosphatase inhibitor, okadaic acid. They concluded that there could be increased phosphorylation of the LTCC in the failing heart due to decreased phosphatase activities. Our previous study (8) of the LTCC in F HVMs is consistent with these findings.

The present results strongly support the idea that adrenergic agonists and BAY K increase I_{CaL} through a common mechanism(s) that can be saturated (summarized in Fig. 9). Therefore, when the effects of one agent are large, there is little effect of the other agent. These results suggest that both LTCC phosphorylation induced by adrenergic agonists and

BAY K can induce a maximal increase in LTCC availability, open probability, and/or mode 2 gating and the voltage dependence of activation. The effects of BAY K were smaller in F than in NF HVMs, consistent with the hypothesis that the basal phosphorylation state of the LTCC is increased in the failing human heart. BAY K had greater effects on cells pretreated with dephosphorylating agents such as ACh, which further support a higher phosphorylation state of the LTCC in failing myocytes. Several approaches were used to dephosphorylate the LTCC. These included dialysis of cells with the catalytic subunit of phosphatases, inorganic phosphatases such as 2,3-butanedione monoxime (BDM), and activation of endogenous phosphatases by ACh. Experiments with phosphatases in the pipette solution are technically demanding, since diffusion of phosphatases into myocytes is very slow. Two cells were studied with this approach, and increased BAY K effects on phosphatase-pretreated cells were observed (data not shown). The mechanism of action of BDM on I_{CaL} is controversial, with some studies showing its effect is related to its inorganic phosphatase activity (14,19) and others suggesting a direct LTCC blocking effect (2). We tested BDM on NF and F HVMs and found that its effects on I_{CaL} are very rapid, consistent with the blocker model (data not shown). Thus we used ACh as the primary approach to activate endogenous phosphatase activity, acknowledging the fact that ACh may also activate protein kinase G to inhibit I_{CaL} in F HVMs (47). Arguing against this idea is the fact that ACh did not significantly change I_{CaL} in NF HVMs. Collectively, our indirect approaches support the idea that increased basal LTCC phosphorylation is responsible for the reduced effects of Iso, BAY K, and DBcAMP on Ca^{2+} current in failing human myocytes.

Response of I_{CaL} to Iso is normalized in LVAD HVMs

LVAD support of the failing human heart at least partially normalizes cardiac function and adrenergic regulation (6). We have previously shown that abnormal adrenergic effects on contraction and Ca^{2+} transients in failing myocytes are restored toward normal after LVAD (8,12). In the present study we showed that the increase of I_{CaL} amplitude by BAY K is also normalized after LVAD. These findings are consistent with the idea that the beneficial remodeling induced by LVAD is related to the normalization of sympathetic neurohormones (6) and/or the mechanical unloading of the heart. We specifically propose that hyperphosphorylation of PKA target proteins, such as the LTCC, is reduced after LVAD support. However, the normalization of the LTCC properties is not complete, since the voltage-dependence of activation of the LTCC remains shifted to more negative voltages.

Limitations

All studies using tissue from end-stage heart failure patients suffer from the fact that there is tremendous patient-to-patient variability in disease etiologies, treatments, and a host of other factors. In addition, the limited number of available hearts leads to a small sample size in some portions of the study. Finally, the nonfailing (control) group does not necessarily reflect normal human tissue.

Conclusion

The diminished effects of BAY K 8644 on I_{CaL} in F HVMs appears to result from a higher basal phosphorylation state of the LTCC due to high adrenergic activity and/or lower phosphatase activity. LVAD support of failing heart hearts normalizes the response of I_{CaL} to BAY K 8644. These results support the idea that an increase in basal LTCC activity in failing human myocytes is responsible for the reduced inotropic effects of agonists of agents that induce their effects by increasing I_{CaL} .

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Fig. 1.

Effects of BAY K 8644 (BAY K) on L-type Ca^{2+} current (I_{CaL}) in nonfailing (NF; $n = 16$ cells, $N = 4$ hearts) and failing (F; $n = 24$ cells, $N = 6$ hearts) human ventricular myocytes (HVMs). **A** and **E**: representative recordings of I_{CaL} under control conditions and after application of BAY K in NF (**A**) and F HVMs (**E**). **B** and **F**: current-voltage (I - V) relationships in NF and F HVMs before (basal) and after BAY K. BAY K increased maximal I_{CaL} densities significantly in both NF and F HVMs, but its effect was smaller in F HVMs (% increase in maximal I_{CaL} : NF, $96 \pm 12\%$; F, $59 \pm 8\%$). **C** and **G**: voltage dependence of activation ($G_{\text{Ca}}/G_{\text{Ca,max}}$) curves fitted with Boltzmann equations in NF and F HVMs under control conditions and after application of BAY K. BAY K caused a leftward shift of voltage-dependent activation curves in NF and F HVMs. This shift was more obvious in NF myocytes. After BAY K, half-maximal activation voltage ($V_{0.5}$) values in NF and F myocytes were not different ($P = 0.73$). **D** and **H**: effects of BAY K on fast (τ_f) time constants at different test potentials in NF and F HVMs. BAY K significantly decreased τ_f , suggesting the acceleration of I_{CaL} decay. # $P < 0.05$; \$ $P < 0.001$; @ $P < 0.0001$ at the corresponding test potential. Bonferroni post hoc tests were done to compare the values at each test potential after 2-way repeated ANOVA test showed that drug had significant effects on I_{CaL} .

Fig. 2.

Effects of BAY K on I_{CaL} of NF HVMs pretreated with Isoproterenol (Iso; $n = 10$ cells, $N = 4$ hearts). **A**: representative recordings of I_{CaL} in basal conditions, with Iso, and with both Iso and BAY K. **B**: I - V relationships before (basal) and after application of Iso and Iso + BAY K. Iso significantly ($P < 0.05$, ANOVA) increased maximal I_{CaL} by $64 \pm 18\%$ (basal: -10.3 ± 1.7 pA/pF; Iso: -15.0 ± 1.9 pA/pF), and combined Iso with BAY K further increased maximal I_{CaL} by $53 \pm 10\%$ (-18.3 ± 1.5 pA/pF). **C**: after Iso-induced a leftward shift of the activation curve, BAY K further shifted the voltage dependence of activation. The change produced by BAY K after Iso pretreatment was less than that by BAY K alone on NF HVMs ($V_{0.5}$: basal, 0.75 ± 1.65 mV; Iso, -4.90 ± 3.21 mV; Iso + BAY K, -9.85 ± 1.85 mV; $P < 0.05$, basal vs. Iso; $P < 0.05$, Iso + BAY K vs. Iso) (see Fig. 1). **D**: τ_f of I_{CaL} decay at basal level, pretreatment (Iso), and combined treatment (BAY K + Iso) in NF HVMs. Iso decreased τ_f at most test potentials, and BAY K + Iso further decreased τ_f . $*P <$

0.05, basal vs. Iso; $**P < 0.01$, basal vs. Iso; $\#P < 0.05$, Iso vs. BAY K + Iso; $@P < 0.05$, basal vs. Iso + BAY K; $@@P < 0.01$, basal vs. Iso + BAY K.

Fig. 3.

Effects of BAY K on NF ($n = 5$ cells, $N = 2$ hearts) and F HVMs ($n = 6$ cells, $N = 3$ hearts) pretreated with $10 \mu\text{M}$ dibutyryl cyclic AMP (DBcAMP) in pipette. A and E: representative recordings of maximal I_{CaL} before and after BAY K in the presence of DBcAMP in the pipette in NF (A) and F HVMs (E). B: $I-V$ relationships after exposure to 10 mM DBcAMP in NF and F HVMs. Maximal I_{CaL} was significantly greater in NF HVMs. C and G: voltage dependence of activation curves before and after BAY K with pipette DBcAMP. BAY K shifted the activation of I_{CaL} to more negative voltages, but this shift was less than in NF HVMs without pretreatment. D and H: τ_f in NF and F HVMs pretreated with DBcAMP. BAY K did not significantly change τ_f in both NF and F HVMs. F: the percentage of increase in peak I_{CaL} amplitude induced by BAY K after exposure to DBcAMP. $*P < 0.05$ at the same voltage. Db, DBcAMP.

Fig. 4.

Effects of Iso on I_{CaL} in NF HVMs pretreated with BAY K ($n = 7$ cells, $N = 3$ hearts). **A:** representative recordings of maximal I_{CaL} under basal conditions, with BAY K, and then with a combination of Iso and BAY K. **B:** τ_f at different test potentials under control conditions, with BAY K, and with BAY K + Iso. BAY K significantly enhanced I_{CaL} decay, but combined BAY K + Iso did not significantly slow down the decay of I_{CaL} . **C:** BAY K significantly increased the maximal I_{CaL} in NF HVMs ($122 \pm 43\%$), and further application of Iso had no significant effect. **D:** BAY K significantly shifted the activation curve to the left, and further application of Iso did not significantly shift the activation curve. # $P < 0.05$, basal vs. BAY K; * $P < 0.05$, basal vs. BAY K + Iso; @ $P < 0.01$, basal vs. BAY K + Iso.

Fig. 5.

Effects of BAY K on F HVMs ($n = 5$ cells, $N = 2$ hearts) pretreated with $20 \mu\text{M}$ ACh. **A:** representative recordings of maximal I_{CaL} under basal conditions, with ACh, and then with ACh + BAY K. **B:** the time course of the effect of ACh and ACh + BAY K on I_{CaL} density recorded at +10 mV. **C:** $I-V$ relationships under these 3 conditions. ACh caused a decrease of $27.4 \pm 4.8\%$ in maximal I_{CaL} , and BAY K increased I_{CaL} by $101.0 \pm 15.7\%$ in cells pretreated with ACh. **D:** voltage dependence of activation curves under control conditions, ACh, and ACh + BAY K. ACh did not alter $V_{0.5}$, but ACh + BAY K significantly shifted $V_{0.5}$ to hyperpolarized voltage. # $P < 0.05$, basal vs. ACh; & $P < 0.001$, basal vs. ACh + BAY K; £ $P < 0.01$, basal vs. ACh + BAY K; * $P < 0.05$, basal vs. ACh + BAY K; @ $P < 0.01$, ACh vs. ACh + BAY K, % $P < 0.05$, ACh vs. ACh + BAY K; \$ $P < 0.001$, ACh vs. ACh + BAY K.

Fig. 6.

Effects of BAY K on I_{CaL} in left ventricular assist device (LVAD)-supported HVMs ($n = 6$ cells, $N = 3$ hearts). **A:** representative recordings of maximal I_{CaL} before (basal) and after the application of BAY K in LVAD HVMs. **B:** BAY K decreased τ_f at different test potentials in LVAD HVMs. **C:** $I-V$ relationships in LVAD HVMs before and after BAY K. **D:** activation curves fitted with a Boltzmann equation in LVAD HVMs before and after the application of BAY K. BAY K caused a leftward shift of activation curves. # $P < 0.05$; & $P < 0.01$; \$ $P < 0.001$ at the corresponding test potential. Bonferroni post hoc tests were done to compare the values at each test potential after 2-way repeated ANOVA test showed that the drug had significant effects on τ_f and I_{CaL} .

Fig. 7.

Effects of BAY K on I_{CaL} in LVAD HVMs pretreated with 1 μ M Iso ($n = 5$ cells, $N = 3$ hearts) or 10 μ M pipette DBcAMP ($n = 5$ cells, $N = 2$ hearts). **A:** representative recordings of maximal I_{CaL} under basal conditions, with Iso, and with combined BAY K and Iso in LVAD HVMs. **B:** I - V relationships under basal condition, with Iso, and with combined BAY K + Iso in LVAD HVMs. Iso increased I_{CaL} density, but further BAY K treatment did not increase I_{CaL} . **C:** I_{CaL} activation curves under basal conditions, with Iso, and with combined BAY K and Iso. Iso shifted the activation of I_{CaL} to more negative voltages, but BAY K did not further shift the activation curve. **D:** τ_f under basal conditions, with Iso, and with combined BAY K and Iso in LVAD HVMs. Iso significantly decreased τ_f , but BAY K + Iso did not slow down the decay of I_{CaL} . In **B** and **D**: $*P < 0.05$, basal vs. Iso; $\#P < 0.05$, basal vs. BAY K + Iso. **E:** representative recordings of maximal I_{CaL} in LVAD HVMs pretreated with DBcAMP and then with DBcAMP + BAY K. **F:** I - V relationships in LVAD HVMs pretreated with DBcAMP and then with DBcAMP + BAY K. DBcAMP increased I_{CaL} density, but further BAY K treatment did not increase I_{CaL} . **G:** I_{CaL} activation curves with DBcAMP and then with DBcAMP + BAY K in LVAD HVMs. BAY K shifted the activation of I_{CaL} to more negative voltages. **H:** BAY K did not significantly slow the decay of I_{CaL} in LVAD HVMs pretreated with DBcAMP ($P = 0.08$).

Fig. 8.

Effects of 1 μ M Iso on I_{CaL} in LVAD HVMs pretreated with BAY K ($n = 4$ cells, $N = 1$ hearts). **A**: representative recordings of maximal I_{CaL} under basal conditions, with BAY K, and with BAY K + Iso in LVAD HVMs. **B**: τ_f under basal condition, with BAY K, and with combined BAY K and Iso in LVAD HVMs. BAY K significantly decreased τ_f , but BAY K + Iso did not further change I_{CaL} decay. **C**: I - V relationships under basal conditions, with BAY K, and with combined BAY K and Iso in LVAD HVMs. BAY K increased I_{CaL} density, but further Iso treatment did not increase I_{CaL} . **D**: I_{CaL} activation in LVAD HVMs pretreated with DBcAMP and then DBcAMP + BAY K. * $P < 0.05$, control vs. BAY K; # $P < 0.05$, control vs. BAY K + Iso.

Fig. 9. Summary of interactive effects of adrenergic agonists (Iso and DBcAMP) and BAY K on peak I_{CaL} . *A*: changes in peak I_{CaL} (ΔI_{CaL}). *B*: relative changes.

Patient characteristics

Table 1

Patient	Etiology	Duration of LVAD, days	Age, yr	CHF Duration, mo	LVEF, %
NF (N = 7)			59±8	0	54.0±2.6
F (N = 15)	Idiopathic (3), ischemic (10), hypertension (1), valvular (1)		54±3	70±14	12.2±1.3
LVAD (N = 14)	Idiopathic (4), ischemic (8), hypertension (1), dilated (1)	164±53	53±3	51±9	12.5±0.7

Values are means ± SE. LVAD, left ventricular assist device; CHF, congestive heart failure; LVEF, left ventricular ejection fraction; NF, nonfailing heart; F, failing heart.