Title Page

Enhanced Inhibition of L-type Ca^{2+} Current by β_3-Adrenergic Stimulation in Failing Rat Heart

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Running Title Page

A. Running Title: Beta$_3$-AR Stimulation on $I_{Ca,L}$: Normal vs HF

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Abstract

β_3-adrenergic receptors (AR) have recently been identified in mammalian hearts, and shown to be up-regulated in heart failure (HF). β_3-AR stimulation reduces inotropic response associated with an inhibition of L-type Ca^{2+} channels in normal hearts, however, the effects of β_3-AR activation on Ca^{2+} channel in HF remain unknown. We compared the effects of β_3-AR activation on L-type Ca^{2+} current ($I_{Ca,L}$) in isolated left ventricular myocytes obtained from normal and age-matched rats with isoproterenol (ISO)-induced HF (4 months after 340 mg/kg, sc for 2 days). $I_{Ca,L}$ was measured using whole-cell voltage clamp and perforated-patch recording techniques. In normal myocytes, superfusion of BRL-37,344 (BRL), a β_3-AR agonist, caused a dose-dependent decrease in $I_{Ca,L}$ with maximal inhibition (21%, 1.1±0.2 vs. 1.4±0.1 nA) ($p<0.01$) at 10^{-7}M. In HF myocytes, the same concentration of BRL produced a proportionately greater inhibition (31%) in $I_{Ca,L}$ (1.1±0.2 vs. 1.6±0.2 nA) ($p<0.05$). A similar inhibition of $I_{Ca,L}$ was also observed with ISO (10^{-7}M) in the presence of a β_1- and β_2-AR antagonist, nadolol (10^{-5}M). Inhibition was abolished by the β_3-AR antagonist, L-748,337 (10^{-6}M), but not by nadolol. The inhibitory effect of BRL was attenuated by a nitric oxide synthase (NOS) inhibitor, L-NAME (10^{-4} M), and was prevented by the incubation of myocytes with pertussis toxin (PTX, 2 μg/ml, 36°C, 6h). In conclusion, β_3-AR activation inhibits L-type Ca^{2+} channel in both normal and HF myocytes. In HF, β_3-AR stimulation-induced inhibition of Ca^{2+} channel is enhanced. These effects are likely coupled with PTX-sensitive G-protein and partially mediated through a NOS-dependent pathway.

Introduction

β_1-, β_2-, and β_3- adrenergic receptors have been found to be present in mammalian hearts and shown to modulate cardiac contractility by a variety of mechanisms. β_1- and β_2-AR stimulation of L-type Ca^{2+} channel are mediated by a cAMP/PKA-signaling mechanism and coupled with G_s proteins. (Xiao, et al., 1999;Skeberdis, et al., 1997;Zhang, et al., 2001) β_2-AR also couples with G_i protein. (Xiao, et al., 1999) Recently, β_3-AR was identified in mammalian hearts, including human, dog, rat, and guinea pig. (Gauthier, et al., 1996;Cheng, et al., 2001;Dincer, et al., 2001;Kitamura, et al., 2000) β_3-AR stimulation inhibits cardiac contractility via a G_i protein pathway and by a mechanism coupled with the nitric oxide synthase (NOS) system. (Gauthier, et al., 1996;Gauthier, et al., 1998;Seppet, 2003) The negative inotropic effect of β_3-AR stimulation is associated with alterations of action potentials (Gauthier, et al., 1996) and decreased Ca^{2+} transient. (Kitamura, et al., 2000) BRL-37,344, a selective β_3 agonist, inhibits L-type Ca^{2+} channels and attenuates intracellular Ca^{2+} transients in canine ventricular myocytes with an associated dose dependent decrease in contractility. (Cheng, et al., 2001) A similar effect on basal $I_{Ca,L}$ is partly abolished by L-NAME. (Au and Kwan, 2002)

Recently, several studies have reported that β_3-ARs are up-regulated in the failing human heart, (Moniotte, et al., 2001) in the canine models of HF (Cheng, et al., 2001) as well as in diabetic rat hearts (Dincer, et al., 2001). HF is associated with selective down-regulation of β_1-AR and a marked increase in G_i protein. (Cheng, et al., 2001;Moniotte, et al., 2001) The exaggerated β_3-AR/G_i

signaling may cause alteration in the regulation of Ca^{2+} channel, thus contributing

to contractile dysfunction. However, the role and mechanism of β_3-AR activation

on $I_{Ca,L}$ in HF have not been defined.

Accordingly, the purpose of this study was to compare the effects of β_3-AR

stimulation on cardiac $I_{Ca,L}$ in LV myocytes of normal rats and rats infused with

ISO as a model of HF and to determine the underlying cellular mechanism. Our

results indicate that β_3-AR activation inhibits L-type Ca^{2+} channel in both normal

and HF myocytes. In HF, β_3-AR stimulation-induced inhibition of $I_{Ca,L}$ was

enhanced. These effects are likely coupled with a PTX-sensitive G_i protein and

partially mediated through a NOS-dependent pathway. The current findings

extend our knowledge regarding the impaired β adrenergic regulation of L-type

Ca^{2+} channel in HF and provide valuable new insight into the cellular mechanism

of the progression of functional impairment in HF.

Methods

Experimental heart failure model

This investigation conforms to the *Guide for the Care and Use of*

Laboratory Animals published by the US National Institutes of Health (NIH

Publication No 85-23, revised 1985).

As previously described, (Suzuki, et al., 1998;Kong, et al., 2004;Teerlink,

et al., 1994) HF in the rat model was induced by ISO injections with some

modification (4 months after 340 mg/kg, sc, for 2 days). Briefly, male Sprague-

Dawley rats (200-250 g) received two subcutaneous injections of 340 mg/kg ISO-

HCl at 24-hour intervals. The mortality rate was approximately 40-48% within 48 hours. The control group of rats received the same amount of sterile saline. Animals were housed and fed under identical conditions.

Four months after the injection protocol, survivors (n=16) and sham-injected rats (n=20) were lightly anesthetized with intraperitoneal Ketamine HCl (50 mg/kg) and Xylazine (10 mg/kg). Then, hemodynamic measurements were obtained using a micro-tip pressure transducer (Millar Instruments, Houston, TX) inserted into the LV through the carotid artery to verify the presence of HF in the ISO-treated rats. Heart and lung weight were obtained after the study. Consistent with previous reports, (Rona, et al., 1959;Teerlink, et al., 1994) the hearts of ISO-injected rats displayed large infarct-like necrosis involving more than one-third to one-half of the LV extending to the adjacent area of the interventricular septum and right ventricle**.** Diffuse subendocardial necrosis was also observed. In order to obtain a high yield of viable isolated myocytes, as previously described, with some modification, (Rona, et al., 1959;Pfeffer, et al., 1979) the gross lesions of infarct-like necrosis area were measured. Briefly, after Langendorff perfusion with an enzymatic buffer, the heart was weighed and LV was separated. Then the infarct-like necrosis area was carefully excised from the LV and weighed separately. Calculation of the ratio of the weights between the necrosis areas to LV was used as an approximate estimation of infarct size.

Isolation of LV myocytes. Myocytes were enzymatically dissociated by Langendorff perfusion as previously described. (Suzuki, et al., 1998). With our well established technique, more than an 80% yield of viable myocytes was

obtained from both control and ISO-treated rats. The cells were used within 10 hours.

Electrophysiological measurement. Membrane calcium current was recorded at 22-23°C with the whole-cell patch-clamp technique as previously described. (Zhang, et al., 2001;Hamill, et al., 1981) An Axopatch 200A amplifier (Axon Instruments, Forster City, CA) was interfaced with a 12-bit A/D-D/A converter (Digidata 1200, Axon Instruments). PClamp software (PClamp 6.02, Axon Instruments) was used for data acquisition and analysis. Data were filtered by 5 kHz low-pass filter and digitized at 5 kHz.

After stabilization, a drop of cell pellet containing the isolated myocytes was placed in a perfusion chamber (0.5 ml volume) mounted on the stage of an inverted microscope (IMT 2-F3, Olympus, Herndon, VA) and continuously superfused at a constant rate of 2 ml/min. Only quiescent rod-shaped cells with clear cross striations were studied. Borosilicate glass micropipette (OD.1.6 mm) was pulled with a 2-step puller (Model PP-83, Narishige, Tokyo, Japan) and heat polished with a microfuge (MT-83, Narishige, Tokyo, Japan). The tip resistances were 1.5-2.5 MΩ when filled with pipette (internal) solution.

Liquid junction potentials (<5 mV) were corrected before the pipette touched the cell. After formation of GΩ-seal, the electrode capacitance was compensated electronically. Then the cell membrane was ruptured by gentle suction to establish whole-cell configuration.

In a subgroup, a perforated-patch recording technique was used (Horn and Marty, 1988) in which nystatin stock was added to the internal solution (final

nystatin concentration of 100-200 µg/ml). The development of electrical access was monitored by the appearance of a capacitative current. The access resistance was <20 MΩ.

The membrane capacitance and series resistance were compensated to minimize the duration of the capacitative transient. The membrane capacitance was measured before compensation with a 10-mV depolarizing step from a holding potential of -80 mV to -70 mV and integrated the area under the current transient calculated with the following formula: $C_m = \tau_c I_0 / \Delta V_m [1-(I_\infty/I_0)]$, where C_m is the membrane capacitance, τ_c is the decay time constant of membrane capacitance, I_0 is the maximum capacitance current value, ΔV_m is the amplitude of the voltage step, and I_∞ is the amplitude of steady-state current. The membrane capacitance was used as an index to normalize $I_{Ca,L}$ for cell size. The series resistance was calculated as $R_s = \Delta V_m / I_0$, where R_s is the series resistance, and compensated at about 80%.

Myocytes were voltage clamped at -80mV. Ca^{2+} currents were elicited by stepping up the membrane voltage from a holding potential to 0 mV testing potential for 200 msec at 12-second intervals. To avoid contamination by fast Na-channel activation and to reduce the run-down of $I_{Ca,L}$, a brief pre-pulse (60-ms duration) was applied to -40 mV before approaching the test potential. The average peak $I_{Ca,L}$ run-down was about 10-20% for 30 minutes after initial measurement. Most (80%) of the run-down occurred within the initial 8-10 minutes. Thus, the window of time between 10 and 30 minutes after the initial recording was chosen to measure $I_{Ca,L}$ with respect to drug effects. (Xiao and

Lakatta, 1993) $I_{Ca,L}$ was measured by the standard method as the difference between peak inward current and the current at the end of a 200-msec pulse. For current-voltage relations, test potentials were from -35 to +60 mV at 5-mV increments and 0.1 Hz.

Solutions. The compositions of the pipette solution and recording bath solution were chosen to allow isolation of ion flow through the Ca^{2+} channel by blocking other ionic currents. Initially, the myocytes were superfused with a modified Tyrode's solution containing (mM): NaCl 137, KCl 5.4, $MgSO_4$ 1.2, glucose 15, HEPES 10, $CaCl_2$ 1.5. The pH was adjusted to 7.4 with NaOH at 20-22°C. After formation of giga-Ω seal, superfusion buffer was changed to a patch-recording bath solution, i.e. Na^+- K^+-free Tyrode's solution, in which NaCl was substituted by tetraethylammonium chloride and 50 μM tetrodotoxin was added to eliminate sodium current and KCl replaced by CsCl and 3 mM 4-Aminopyridine (4-AP) in order to abort the potassium current. The solution was gassed with 100% O_2. The internal solution for the pipette contained (mM): Cs Aspartate 140, $MgCl_2$ 1.0, Na_2ATP 3, GTP 0.4, EGTA 10, and HEPES 5. The pH was adjusted to 7.2 (with titrated CsOH).

For perforated patch recording, nystatin stock solution (10 mg/ml in acidified methanol) was prepared on each day and added to the internal solution at a final concentration of 100-200 µg/ml. (Horn and Marty, 1988) The pipette was dipped in nystatin-free internal solution for 2 sec and then back filled with nystatin internal solution.

Drugs. BRL 37, 344 (4-[−[2-hydroxy-(3-chlorophenyl)ethyl-amino]propyl]phenoxyacetate) was obtained from Tocris (Ballwin, MO). L-748,337, (*S*)-*N*-[4-[2-[[3-[3-(acetamidomethyl)phenoxy]-2-hydroxypropyl]ami-no]ethyl]phenyl]benzenesulfonamide was a gift from Merck Research Laboratories (Rahway, NJ). Nadolol, Isoproterenol, ICI-118,551(ICI), N^Gnitro-L-arginine methyl ester (L-NAME), Nifedipine and pertussis toxin (PTX) were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis. Data are presented as mean $\pm$ SEM. Statistical comparisons were performed with Student's *t* test or ANOVA. A *p* value of <0.05 was considered significant. Prism 3.0 (GraphPad Software) was used for the concentration–$I_{Ca,L}$ relationship nonlinear regression analysis. As previously described by Robberecht et al. (Robberecht, et al., 1983) and Lands et al., (Lands, et al., 1967) the Hill equation may allow us to model cooperativity between multiple receptor sites on each cardiomyocyte with respect to β_3-AR agonist (BRL) binding. Thus, data were fitted with the Hill equation. The best fit by the Hill equation was also compared with a fit by the one-site competition equation.

Results

Verification of experimental HF

The general hemodynamic and $I_{Ca,L}$ features in the ISO-treated rats are presented in Table 1. LV end-diastolic pressure increased 5-fold, and LV dp/dt$_{max}$, and LV dp/dt$_{min}$ were significantly decreased. The rate of LV relaxation slowed as indicated by a significant increase in the time constant of isovolumic LV pressure decay (τ, 185%) ($p<0.05$).

All ISO-treated animals had clear evidence of HF (anorexia, edema, and pulmonary congestion). In the ISO-injected rats, the total infarction area was about 43±3%. There was no significant change in body weight (596±8 vs 601±9 g) (p=NS); whereas, the heart weight (2.25±0.03 vs 1.68±0.03 g) ($p<0.05$), calculated ratio of LV to body weight (2.53±0.04 vs 1.86±0.04 g/kg) ($p<0.05$), and calculated ratio of wet lung to body weight (4.85±0.09 vs 2.89±0.08 g/kg) ($p<0.05$) were all significantly increased in ISO injected rats. In the HF rat myocytes, the membrane capacitance was significantly increased; whereas, the current density was significantly lower than that of the normal myocytes (62%) ($p<0.01$), indicating an absolute reduction of $I_{Ca,L}$.

In addition, the response of $I_{Ca,L}$ to β-AR stimulation in HF myocytes was significantly attenuated. As shown in Figure 1, in the normal myocytes, in response to the exposure to ISO (10^{-7} M), $I_{Ca,L}$ was doubled (119±9%) ($p<0.01$, n=11). However, in HF myocytes, ISO only caused half the increase in $I_{Ca,L}$ (55±4%) ($p<0.05$, n=11). The current was blocked by nifedipine (5×10^{-6} M), a

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Ca^{2+} channel blocker, consistent with the characteristics of $I_{Ca,L}$. These findings demonstrated the existence of established HF in this model.

Increased inhibition of $I_{Ca,L}$ to β_3-AR stimulation in HF myocytes. The effects of direct β_3-AR stimulation with BRL, the most potent β_3-AR agonist in rats, (Gauthier, et al., 1999) on $I_{Ca,L}$ in normal and HF myocytes are summarized in Table 2 and displayed in Figure 2. Superfusion of BRL (10^{-7} M) caused significant decreases in peak $I_{Ca,L}$ in normal myocytes (21%, 1.1±0.1 vs 1.4±0.1 nA, $p<0.01$, n=7)**.** In HF myocytes, BRL caused a greater relative and absolute decrease in $I_{Ca,L}$ (31%, 1.1±0.2 vs 1.6±0.2 nA, $p<0.05$, n=7). The absolute decrease in $I_{Ca,L}$ was greater in HF myocytes (0.3±0.03 nA) vs (0.5±0.06 nA) ($p<0.05$). In the HF myocytes, the membrane capacitance was significantly increased. After normalization of the membrane capacitance, the BRL-induced decreases in $I_{Ca,L}$ remained statistically different in both normal myocytes (21%, 5.9±0.4 vs. 7.5±0.5 pA/pF) ($p<0.01$, n=7) and HF myocytes (31%, 2.9±0.4 vs 4.1±0.3 pA/pF) ($p<0.05$, n=7). Figure 2C-D demonstrates current-voltage relations for the response of $I_{Ca,L}$ to BRL in normal and HF myocytes. BRL caused no change in the voltage dependence of peak $I_{Ca,L}$ amplitude in normal and HF myocytes.

The inhibitory effects of BRL persisted after washout. To eliminate the influence of "run-down", in a subgroup, we used nystatin perforated patch recording to examine the effect of BRL. A similar result was observed (data not shown). This is consistent with the findings of Au and Kwan. (Au and Kwan, 2002)

In a subgroup, we further examined the $I_{Ca,L}$ response to ISO (10^{-7}M) in the presence of a β_1- and β_2-AR antagonist, nadolol (Nad, 10^{-5}M). In contrast to ISO alone, we observed an inhibition of $I_{Ca,L}$ in both normal and HF myocytes. Compared with normal myocytes, this inhibition was enhanced in HF myocytes ($29\pm2\%$ vs $20\pm2\%$) ($p<0.05$, n=5).

Concentration-dependent inhibition of $I_{Ca,L}$ by BRL. Concentration-response curves of $I_{Ca,L}$ to BRL in normal and HF rat myocytes are compared in Figure 3. The maximal response of $I_{Ca,L}$ to BRL was significantly enhanced in HF myocytes. The dose-response curve was shifted downward. The half-maximal inhibition concentration (IC_{50}) was 1.1 nM for HF myocytes and 1.2 nM for normal myocytes.

The effects of β_1- and β_2-AR antagonist, β_2-AR antagonist, and β_3-AR antagonist on BRL-induced inhibition in $I_{Ca,L}$. To determine the potential mechanism, the myocytes were pre-incubated with a β_1- and β_2-AR antagonist, Nad (10^{-5} M), or a β_3-AR antagonist, L-748,337 (10^{-6} M), for 20 minutes, and BRL was given in the presence of Nad or L-748,337. To further exclude BRL action through β_2-AR, in a subgroup of three normal rats, the myocytes were pre-incubated with ICI-118,551, a β_2-AR antagonist (10^{-7} M), for 20 min, and BRL was given in the presence of ICI.

As shown in Figure 4, after using Nad to block β_1- and β_2-AR, BRL-induced decreases in $I_{Ca,L}$ still persisted (5.5 ± 0.5 vs 7.0 ± 0.6 pA/pF) ($p<0.05$, n=3) and (3.3 ± 0.4 vs 4.5 ± 0.4 pA/pF) ($p<0.05$, n=3) in normal and HF myocytes, respectively. In a subgroup of normal rats, after pre-incubation with the β_2-AR

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antagonist, ICI, the BRL-induced reductions in $I_{Ca,L}$ remained unaffected (6.5±0.5 vs 8.1±0.6 pA/pF) ($p<0.05$, n=4). In contrast, as shown in Figure 5, after pre-incubation with a β_3-AR blocker, L-748,337, BRL-induced inhibition in $I_{Ca,L}$ was abolished [(7.2±0.5 vs 7.4±0.4 pA/pF) (n=4) and (4.6±0.6 vs 4.7±0.6 pA/pF) (n=4, p=NS)] in normal and HF cells, respectively, indicating that BRL inhibited $I_{Ca,L}$ through β_3-AR, not β_1- and β_2-AR.

The effect of G_i protein blockade on BRL-induced inhibition in $I_{Ca,L}$. To define the role of G_i protein in β_3-AR inhibition of $I_{Ca,L}$, myocytes were pretreated with PTX (2 μg/ml, 36°C, 6 hours). The adequacy of the complete blockage of inhibitory G_i protein in PTX-treated cells was routinely verified by the loss of the ability of acetylcholine (ACh,10^{-5} M) to reverse the stimulatory effect of ISO on $I_{Ca,L}$ consistent with our past report (Zhang, et al., 2001) that an adequate blockade of G_i with this concentration and incubation time was achieved in the current study. PTX-treated myocytes were compared with myocytes that had been kept at 36°C in the absence of PTX for an equal amount of time. As shown in Figure 6, after PTX pre-incubation of the myocytes to block inhibitory G_i protein, there was no significant change in baseline $I_{Ca,L}$; whereas, BRL-induced inhibition in $I_{Ca,L}$ was prevented both in normal cells (8.1±0. 7 vs 8.2±0.5 pA/pF) (p=NS, n=4) and in HF cells (5.3±1.0 vs 5.3±0.9 pA/pF) (p=NS, n=4).

The effect of NOS pathway blockade on BRL-induced inhibition in $I_{Ca,L}$. We further examined the role of NO signaling in β_3-AR inhibition of $I_{Ca,L}$ by pre-incubating the myocytes with a NOS blocker, L-NAME (10^{-4} M), for 20 minutes, and BRL was given in the presence of L-NAME. Pretreatment of

myocytes with L-NAME caused no significant changes in baseline $I_{Ca,L}$, but

significantly altered myocyte $I_{Ca,L}$ response to BRL. In untreated myocytes, BRL

caused about 21% and 31% decreases of $I_{Ca,L}$ in both normal and HF myocytes,

respectively (Table 2 and Figures 2 and 3). However, comparing BRL-caused

changes of peak $I_{Ca,L}$ in myocytes without L-NAME treatment, in the presence of

L-NAME , BRL-induced $I_{Ca,L}$ inhibition was significantly attenuated and produced

only about a 12% and a 11% decrease in $I_{Ca,L}$ in both normal (6.8±0.3 vs 5.9±0.4

pA/pF) ($p<0.05$, n=5 and 7) and HF myocytes (4.1±0.1 vs 2.9±0.4 pA/pF)

($p<0.05$, n=4 and 7), respectively (Tables 2-3 and Figure 7).

Discussion

The present study demonstrates that β_3-AR stimulation with BRL causes

an enhanced inhibition of $I_{Ca,L}$ in LV myocytes of rats with HF. These effects were

coupled with PTX-sensitive inhibitory G_i protein and partly mediated through a

NOS-dependent mechanism.

Effects and possible mechanism of BRL on $I_{Ca,L}$. β-AR activation

modulating cardiac $I_{Ca,L}$ plays an important role in the positive inotropic response

to β-AR stimulation. β_1- and β_2-AR stimulation of L-type Ca^{2+} channel are

mediated by a cAMP/PKA-signaling mechanism and coupled with G_s proteins.

(Xiao, et al., 1999;Skeberdis, et al., 1997;Zhang, et al., 2001) β_2-ARs also have

the ability to activate nonclassical signaling pathways and link to inhibitory G

proteins (G_i), suggesting a function distinct from the β_1-AR subtype. (Xiao, et al.,

2003;Zhang, et al., 2001). Recent observations indicate a more complex β-AR-

mediated regulation of myocardial inotropism by catecholamines. In addition to β_1- and β_2-AR, a third β-AR, β_3-AR, was initially found to be widely expressed in fat tissues, (Krief, et al., 1993) was also found to exist in mammalian hearts and to modulate cardiac contractile function. (Gauthier, et al., 1996;Cheng, et al., 2001;Dincer, et al., 2001;Kitamura, et al., 2000) Stimulation of β_3-AR negatively modulates cardiac function through the G_i protein pathway and is coupled with the NOS system. (Gauthier, et al., 1996;Gauthier, et al., 1998;Seppet, 2003) The negative inotropic effect of β_3-AR stimulation was associated with alterations of myocardial electrophysiology and Ca^{2+} signaling. (Gauthier, et al., 1996;Cheng, et al., 2001;Kitamura, et al., 2000)

In the present study, using freshly isolated cardiomyocytes, we found that stimulation of β_3-AR with BRL resulted in a dose-dependent inhibition in $I_{Ca,L}$ with maximum inhibition (21%) at a concentration of 10^{-7} M in normal cells. This effect was completely abolished by a highly-selective β_3-AR antagonist, but not by the β_1- and β_2-AR blockade or β_2-AR blockade, indicating that the alteration of $I_{Ca,L}$ following BRL superfusion was due to β_3-AR stimulation, but not mediated by β_1-AR or β_2-AR activation.

Furthermore, as in previous studies (Gauthier, et al., 1998;Kitamura, et al., 2000;Varghese, et al., 2000), we found that in the presence of L-NAME, the BRL-induced negative response of $I_{Ca,L}$ was significantly reduced, indicating involvement of the NO pathway in β_3-AR-mediated action. However, our current observation indicates that the NO pathway may not be fully responsible for the altered LV myocyte $I_{Ca,L}$ response to BRL since we found that the $I_{Ca,L}$ response

to BRL was only partially inhibited by pretreatment myocytes with a NOS inhibitor. In the presence of L-NAME, myocyte $I_{Ca,L}$ remained significantly reduced, indicating β_3-AR activation is not mediated exclusively through the NO pathway.

Our study indicates that β_3-AR-induced inhibition of $I_{Ca,L}$ is mediated by G_i, since pretreatment of PTX completely prevented BRL-induced $I_{Ca,L}$ responses in both normal and HF myocytes. However, this is not consistent with the past report of Gauthier et al. (Gauthier, et al., 1996) In their study, it was shown that in human ventricular strips treated with PTX, the effect of BRL on contractility was attenuated, but not completely suppressed. This discrepancy may be due to species difference or incomplete blockade of G_i by PTX since a lower level of exposure to PTX (0.5 µg/ml for 2 hours) was used in that study. It has been reported that PTX treatment for 3 to 5 hours at 5 µg/ml is required to completely inactivate human cardiac G_i. (Brown and Harding, 1992) Therefore, our current study indicated that the enhanced $I_{Ca,L}$ response to β_3-AR stimulation in rat CHF myocytes may be coupled to G_i through both NO-dependent and NO-independent mechanisms.

These results are consistent with previous observations of cardiac functional response to β_3-AR stimulation made in normal hearts and cardiomyocytes of several species, including that of the human, dog, and guinea pig. (Gauthier, et al., 1996;Cheng, et al., 2001;Kitamura, et al., 2000) Gauthier and colleagues first demonstrated that in the human heart, stimulation of β_3-AR with a BRL resulted in dose-dependent, negative inotropic effects, which were

associated with decreased action potential amplitude and reduced action

potential duration. (Gauthier, et al., 1996) However, in their study, β_3-AR

transcripts were not detected in the rat ventricular strips by using a reverse

transcription-polymerase chain reaction (RT-PCR) assay. The failure to detect

functional cardiac β_3-AR expression may be attributed to the use of nonspecific

primers for rat β_3-AR mRNA. (Gauthier, et al., 1999) Recently, by using RT-PCR,

PAGE, and Western blot analysis, Dincer and colleagues (Dincer, et al., 2001)

clearly demonstrated the presence of functional β_3-AR in rat hearts and showed

significantly increased β_3-AR mRNA and protein levels in streptozotocin (STZ)-

induced diabetic rat hearts. Barbier and colleagues also detected transcripts and

cell surface expression of β_3-AR in rat hearts. They further found that LV β_3-AR

density significantly increased in female rats with treadmill training for 8 weeks.

(Barbier, et al., 2004)

A novel finding in the present study is that the inhibition of $I_{Ca,L}$ with β_3-AR

was enhanced after HF. In HF myocytes, the basal $I_{Ca,L}$ was significantly

decreased, and its response to β-AR stimulation (ISO 10^{-7} M) was also markedly

blunted (55% vs 119% increment) ($p<0.05$). However, the inhibitory response to

BRL (10^{-7} M) was significantly enhanced (31% vs 21%).

This is consistent with our past observation made in a pacing-induced

canine HF model.(Cheng, et al., 2001) In that study, we assessed the direct

effects of BRL on cardiomyocytes isolated from the dogs before and after pacing-

induced advanced HF. In these studies, we removed the effects of extracardiac

factors. We clearly demonstrated that compared with normal myocytes, BRL

caused a much greater decrease in $I_{Ca,L}$ in HF myocytes. We further found that

β_3-AR stimulation produces direct inhibition of myocyte contraction, relaxation,

and $[Ca^{2+}]_i$ transient. In pacing-induced HF, these inhibitions were increased.

These responses were coupled to G_i protein. However, our observations do not

agree with the findings by Moniotte et al. (Moniotte, et al., 2001) who reported a

similar up-regulation of β_3-AR expression, but blunted negative inotropic

response to BRL in failing human myocardium. This discrepancy might result

from several factors such as species difference in β_3-AR, (Gauthier, et al., 1999)

variation in severity of HF, or the effect of pharmacotherapy on the failing human

heart.

There is a documented different specificity for β_3-AR across species and

even tissues. It is possible that the intracellular coupling of β_3-AR may be

different in the failing rat and human heart. It is also possible that the severity of

HF may contribute to the different finding. Our rat model of HF was associated

with a 40% to 48% mortality, which is much higher than would be expected in a

population of human HF patients. The model of HF is also different. We

examined a single LV myocyte obtained from ISO-induced failing rat hearts;

whereas, Moniotte et al. studied ventricular strips obtained from dilated or

ischemia-caused failing human hearts. (Moniotte, et al., 2001) The use of

ventricular strips of multiple cell preparation vs. single LV myocyte preparation

may induce quantitatively different results due to the additional variable of

extracellular matrix and remodeling, although qualitative differences are less

likely. We feel that the most likely explanation for the discrepancy in our finding

is that the human group received multiple cardiovascular specific medications for HF. A standard regimen for HF patients consists of significant neurohormonal blockade with β-AR blockers, ACE inhibitors, and aldosterone antagonists. It would not be surprising if this regimen alters β_3-AR expression or function. Chronic treatment with cardiovascular specific medications (such as β-AR blockers and ACE inhibitors) may alter the primary defect in the contractile properties of the myocyte itself as well as the abnormalities of extracardiac factors in HF, thus, further modifying LV and cardiomyocyte functional response to β_3-AR stimulation in HF. (Lohse, et al., 2003;Spinale, et al., 1995;Spinale, et al., 1998;Bristow, 2000;Cohn, et al., 2000;Gunja-Smith, et al., 1996) It is possible that the intracellular coupling of β_3-AR may be also altered by these cardiovascular specific medications in the failing human hearts. Although we studied a rat model of HF (ISO-induced cardiomyopathy) that reproduces many of the functional and neurohormonal features of clinical HF, we cannot be certain that our results apply generally to HF of other causes. Nevertheless, the findings of Moniotte et al. (Moniotte, et al., 2001) and our past reports all demonstrated a similar, potentially detrimental, functional consequence with β_3-AR activation in HF.

The mechanism(s) for the BRL-induced enhanced decrease in $I_{Ca,L}$ for HF cells versus normal cells are unclear. We speculate that an increase in β_3-AR density on the membrane of HF cells may contribute to our current findings. As β_3-ARs are activated at higher catecholamine concentrations than β_1- and β_2-ARs (Lafontan, 1994) and β_3-ARs are relatively resistant to chronic, agonist-

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induced desensitization processes, (Liggett, et al., 1993) they could be involved in HF. Although the β_3-AR density per myocyte has not been measured in HF previously, it is possible that the enhanced inhibition of $I_{Ca,L}$ in LV myocytes of rats with HF may reflect the presence of an increase in the number of β_3-ARs per cell. Further studies are currently underway to elucidate this point.

The enhanced response to β_3-AR stimulation in HF may also be related to an altered signal transduction. Although, the intracellular pathway coupling β_3-AR stimulation is incompletely characterized, it has been reported that β_3-AR stimulation decreases cardiac contractility through activation of the NOS pathway. (Kitamura, et al., 2000;Varghese, et al., 2000) In HF, the NO-cGMP signaling may be altered, (Mohan, et al., 1996) thereby altering HF myocyte response to β_3-AR stimulation. Consistent with previous studies, we found that in the presence of L-NAME, BRL-induced inhibition with $I_{Ca,L}$ was largely attenuated, indicating an involvement of the NO pathway in β_3-AR-mediated action. In addition, we also found that the enhanced $I_{Ca,L}$ response to β_3-AR stimulation in HF myocytes couples to G_i. Thus, an up-regulation of G_i in HF may also contribute to the enhancement of the inhibitory effect of β_3-AR. The activation of G_i also has the potential to couple β_3-AR to other important signaling pathways such as the MAP kinase. (Soeder, et al., 1999). Clearly, up-regulation of cardiac β_3-AR-mediated inhibitory pathways is responsible for the enhanced BRL-induced inhibition of $I_{Ca,L}$ in HF. However the exact contribution of up-regulation of cardiac β_3-AR versus increased levels of G_i is unclear. Further

studies are needed to fully characterize the intracellular pathway coupling β_3-AR stimulation.

In conclusion, we found that β_3-AR activation inhibits L-type Ca^{2+} channel and decreases $I_{Ca,L}$ in both normal and HF myocytes. In rats with ISO-induced HF, β_3-AR activation-induced inhibition of $I_{Ca,L}$ was enhanced. These effects are likely to be partially mediated through a NOS-dependent mechanism and coupled with a PTX-sensitive inhibitory G protein. This finding is possibly an underlying mechanism for the impaired inotropic response to β-AR stimulation in the failing heart.

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Footnotes

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Legends for Figures

Figure 1

Group means (± SEM) of isoproterenol (ISO)-induced changes of peak $I_{Ca,L}$ in myocyte of normal and HF rats. Compared with normal myocytes, an ISO (10^{-7} M)-induced increase in $I_{Ca,L}$ was markedly attenuated in HF myocytes (55±4% vs 119± 9%, respectively) ($p<0.01$). In contrast, in the presence of a β_1- and β_2-antagonist, nadolol (Nad, 10^{-5}M), ISO caused an enhanced inhibition of $I_{Ca,L}$ in HF myocytes (29±2% vs 20±2%, respectively) ($p<0.01$).

Figure 2

Effects of BRL-37,344 (BRL, 10^{-7} M) on $I_{Ca,L}$. **(A)** A ventricular myocyte from normal rat was initially superfused with external solution. During the period indicated by the horizontal line, the cell was exposed to BRL, then to nifedipine (NIF, $5x10^{-6}$ M). The superimposed current tracings were recorded before **(a),** after **(b)** exposure to BRL and exposed to NIF **(c)**. $I_{Ca,L}$ was elicited by depolarization pulses from a holding potential of -80 mV to 0 mV for 200 ms with a brief pre-pulse to -40 mV (60 ms) (*inset*) at 12-second intervals. **(B)** Same experiment as in **(A)** in a HF myocyte. **(C)** Current-voltage relations of $I_{Ca,L}$ in the absence (open symbols) and presence (filled symbols) of BRL in 6 normal myocytes. * indicates that the difference between pre- and post-drug is statistically significant ($p<0.05$). The cells were depolarized from a holding potential of -40 mV to test potentials from -35 to +60 mV in 5 mV increments. **(D)** Current -voltage plots for 5 HF cells, measured under the same experimental

conditions as in **(C). (E)** Average inhibition of $I_{Ca,L}$ caused by BRL (10^{-7}M). Values

are mean $\pm$ SEM for n=7 experiments. * indicates that the difference between

normal and HF myocytes is statistically significant ($p<0.05$). The results showed

an enhanced effect of BRL on $I_{Ca,L}$ in HF myocytes.

Figure 3

Concentration-dependent inhibition of $I_{Ca,L}$ with BRL. Average percentage of

decrease of $I_{Ca,L}$ was plotted against BRL concentrations for normal (open

symbols) and HF (filled symbols) myocytes. The cell numbers for each data point

are indicated in parentheses. Smooth curves were obtained by fitting the data

with the Hill equation. Half-maximal inhibition concentration was 1.2 nM for

normal and 1.1 nM for HF myocytes.

Figure 4

The effect of BRL on $I_{Ca,L}$ is not prevented with β_1- and β_2-AR blockade in normal

and HF myocytes. The myocytes were pre-incubated with Nadolol (Nad), a β_1-

and β_2 -AR antagonist (10^{-5} M), for 20 minutes. **(A)** Superimposed current

tracings recorded before and at 5 minutes after exposure to BRL (10^{-7} M) in the

presence of Nad in a normal myocyte. $I_{Ca,L}$ was elicited by depolarization pulses

from a holding potential of -80 to 0 mV for 200 ms with a brief pre-pulse to -40

mV (60 ms). **(B)** Same experiment as in **(A)** in a HF myocyte. The results

showed the effects of BRL persisted in the presence of β_1-and β_2-AR blocker

(Nad). **(C)** Average effects of β_1- and β_2-AR blockade on BRL-induced $I_{Ca,L}$

responses. Values are mean $\pm$ SEM for n= 3 experiments. ***** indicates *p*<0.05

between control and BRL.

Figure 5

The effect of BRL on $I_{Ca,L}$ is prevented by β_3-AR blockade in normal and HF

myocytes. The myocytes were pre-incubated with L-748,337 (β_3-AR antagonist,

10^{-6} M) for 20 minutes. **(A)** Superimposed current tracings recorded before and 5

minutes after exposure to BRL (10^{-7} M) in the presence of L-748,337 in a normal

myocyte. $I_{Ca,L}$ was elicited by depolarization pulses from a holding potential of -80

to 0 mV for 200 ms with a brief pre-pulse to 40 mV (60 ms). **(B)** Same

experiment as in **(A)** in a HF myocyte. **(C)** Average effects of β_3-AR blockade on

BRL-induced $I_{Ca,L}$ responses. Values are mean $\pm$ SEM for n=4 experiments.

Figure 6

The effect of BRL on $I_{Ca,L}$ is prevented by pertussis toxin (PTX) in normal and HF

myocytes. The myocytes were pre-incubated with PTX (2 μg/ml at 36°C for 6

hours). **(A)** Superimposed current tracings recorded before and 5 minutes after

exposure to BRL (10^{-7} M) in a normal myocyte. $I_{Ca,L}$ was elicited by depolarization

pulses from a holding potential of -80 to 0 mV for 200 ms with a brief pre-pulse to

-40 mV (60 ms). **(B)** Same experiment as in **(A)** in a HF myocyte. **(C)** Average

effects of PTX on BRL-induced $I_{Ca,L}$ responses. Values are mean $\pm$ SEM for n=4

experiments. The results showed the effects of BRL were prevented by PTX

pretreatment, suggesting a PTX-sensitive G protein mechanism involved in BRL-induced $I_{Ca,L}$ response of ventricular myocytes.

Figure 7

Effect of L-NAME on BRL-induced $I_{Ca,L}$ responses of normal and HF myocytes.

(A) Superimposed current tracings recorded before and 5 minutes after exposure to BRL (10^{-7} M) in the presence of L-NAME (10^{-4} M) in a normal myocyte. $I_{Ca,L}$ was elicited by depolarization pulses from a holding potential of -80 to 0 mV for 200 ms with a brief pre-pulse to -40 mV (60 ms). **(B)** Same experiment as in **(A)** in a HF myocyte. **(C)** Average effects of L-NAME on BRL-induced $I_{Ca,L}$ responses. Values are mean $\pm$ SEM for n=4-5 experiments. The results showed the effects of BRL were attenuated in the presence of L-NAME.

Tables

Table 1. Characteristics of LV Function and Ca^{2+} Current of LV Myocytes
in Isoproterenol-Induced HF Rats

	Normal (N=20)	HF (N=16)
Heart rate (beat/min)	273±12	278±11
LVSP (mmHg)	120±4	82±14*
LV P_{ED} (mmHg)	4.4±0.4	22.0±0.8*
LV dp/dt$_{max}$ (mmHg/sec)	9116±418	6374±194*
LV dp/dt$_{min}$ (mmHg/sec)	-8081±505	-6493±228*
τ (ms)	15.4±1.5	28.5±1.7*
C_m (pF)	187±15 (n=55)	302±24* (n=47)
$I_{Ca,L}$ density (pA/pF)	7.7±0.4 (n=55)	4.8±0.2* (n=47)

Values are mean ± SEM; N, number of animals; n, number of cells; * $p<0.05$, heart

failure vs normal rats (myocytes); HF, ISO-induced heart failure model; LVSP, left

ventricular systolic pressure; LV P_{ED}, left ventricular end-diastolic pressure; LV dp/dt$_{max}$

and LV dp/dt$_{min}$, maximum and minimum time derivative of LVP; τ, time constant of left

ventricular pressure decay; C_m, membrane capacitance; $I_{Ca,L}$, L-type Ca^{2+} current.

Table 2. Effect of BRL-37, 344 (BRL 10^{-7} M) on $I_{Ca,L}$ of LV Myocytes in Normal and Isoproterenol-Induced HF Rats

	$I_{Ca,L}$ (nA)		Decrease of $I_{Ca,L}$			$I_{Ca,L}$ density(pA/pF)	
	Control	BRL	(nA)	(%)	Cm (pF)	Control	BRL
Normal (n=7)	1.4±0.1	1.1±0.1**	0.3±0.03	21±1	186±11	7.5±0.5	5.9±0.4**
HF (n=7)	1.6±0.2	1.1±0.2*	0.5±0.06†	31±3†	331±29†	4.1±0.3†	2.9±0.4*

Values are mean ± SEM; n, number of cells; *, $p<0.05$, BRL vs. control; **, $p<0.01$, BRL (BRL-37, 344, 10^{-7} M) vs. control; †, $p<0.05$, HF vs. normal myocytes; HF, ISO-induced heart failure model; $I_{Ca,L}$, L-type Ca^{2+} current; C_m, membrane capacitance.

Table 3. The Effect of NOS Pathway Blockade on BRL-induced Inhibition of $I_{Ca,L}$ in

Normal and HF Myocytes

	$I_{Ca,L}$ (nA)		Decrease of $I_{Ca,L}$			$I_{Ca,L}$ density (pA/pF)	
	L-NAME $(10^{-4}M)$	L-NAME +BRL	(nA)	(%)	Cm (pF)	L-NAME $(10^{-4}M)$	L-NAME +BRL
Normal (n=5)	1.7±0.2	1.5±0.2*	0.2±0.03‡	12±1‡	223±14	7.6±0.4	6.8±0.3*
HF (n=4)	1.8±0.2	1.6±0.1*	0.2±0.06‡	11±2‡	346±11†	4.6±0.2†	4.1±0.1*

Values are mean ± SEM; n, number of cells; *, $p<0.05$, L-NAME +BRL vs. L-NAME; †,

$p<0.05$, heart failure vs. normal myocytes; ‡, $p<0.05$, L-NAME +BRL vs. BRL; HF,

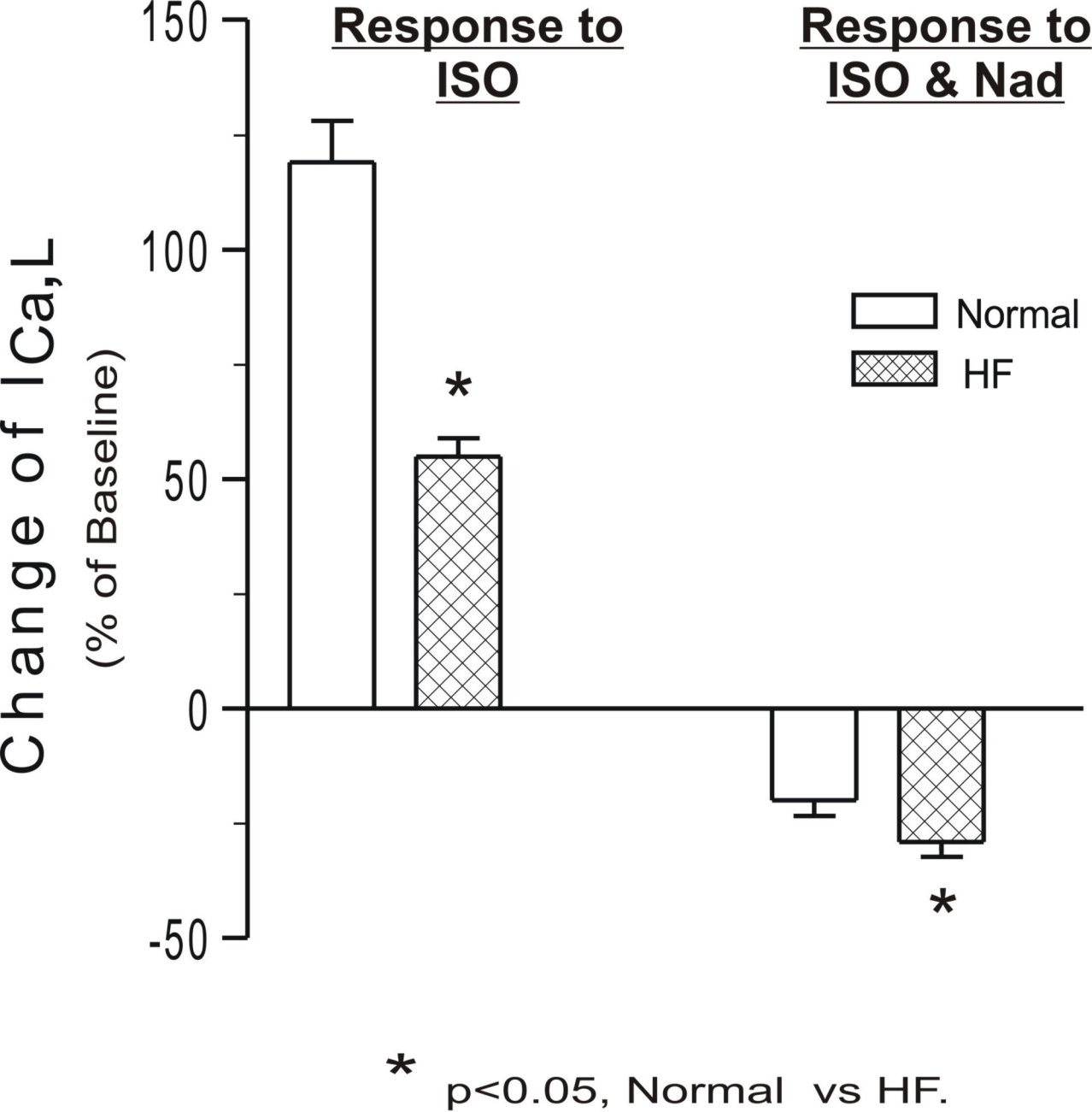ISO-induced heart failure model; $I_{Ca,L}$, L-type Ca^{2+} current; C_m, membrane capacitance.
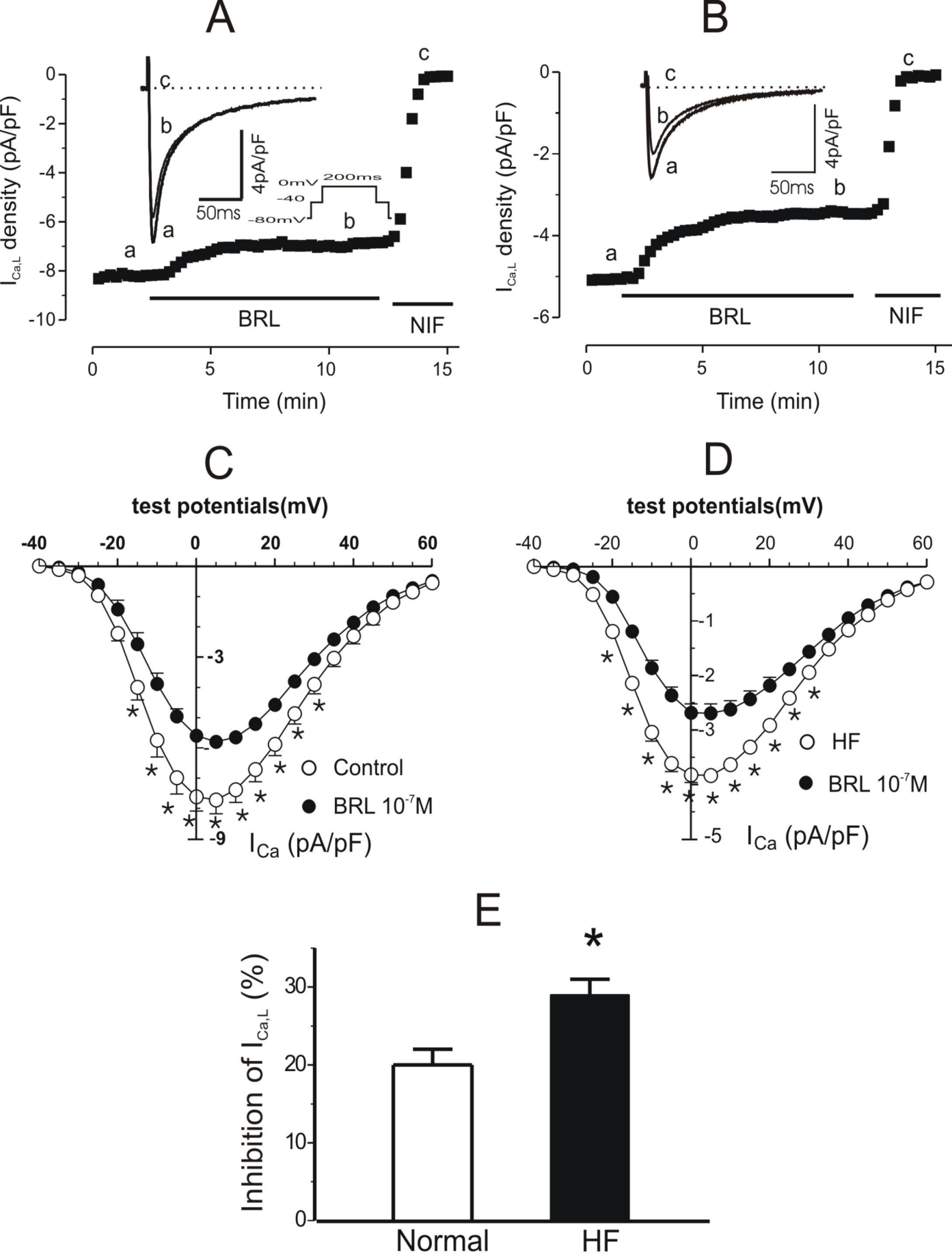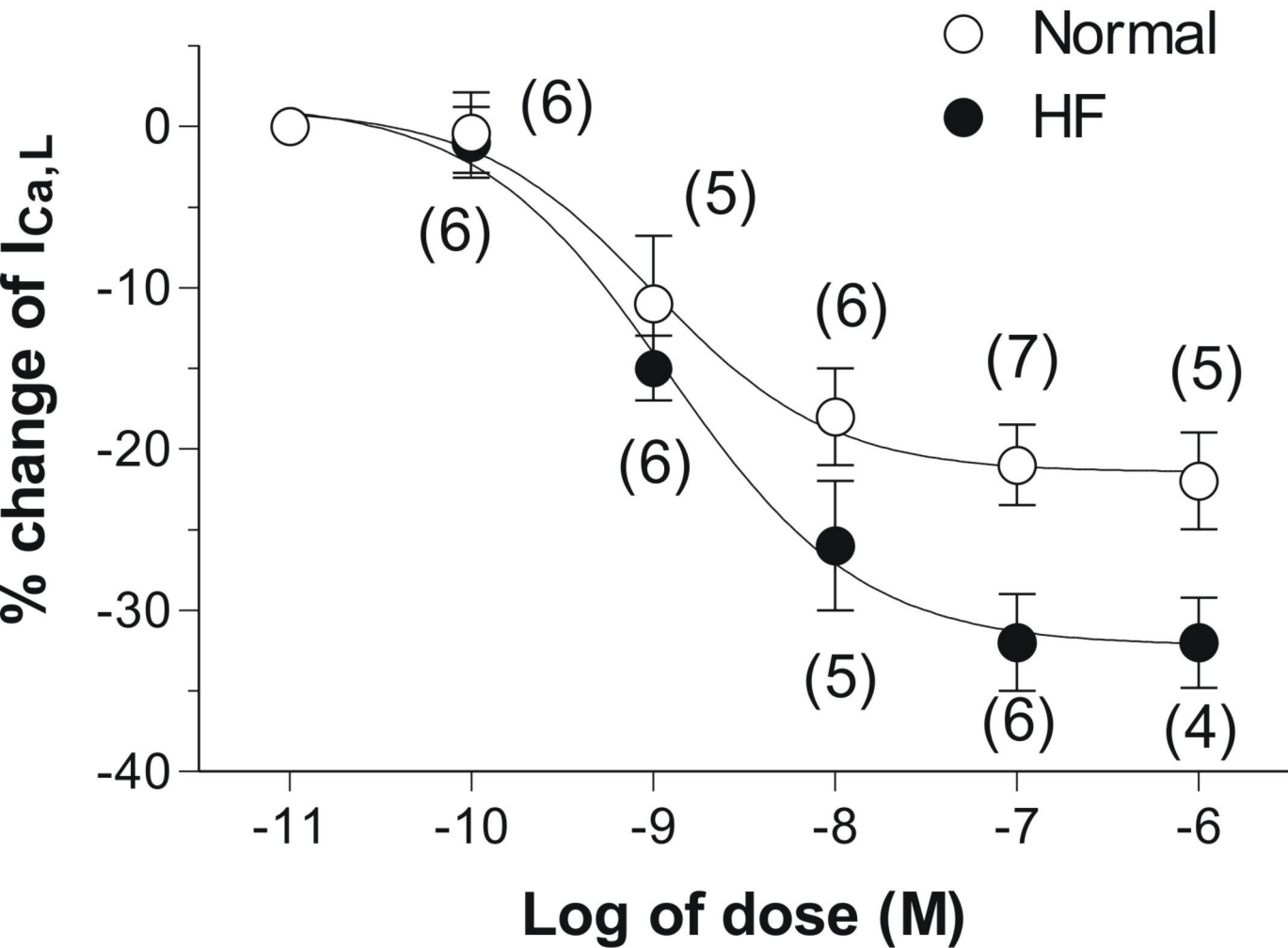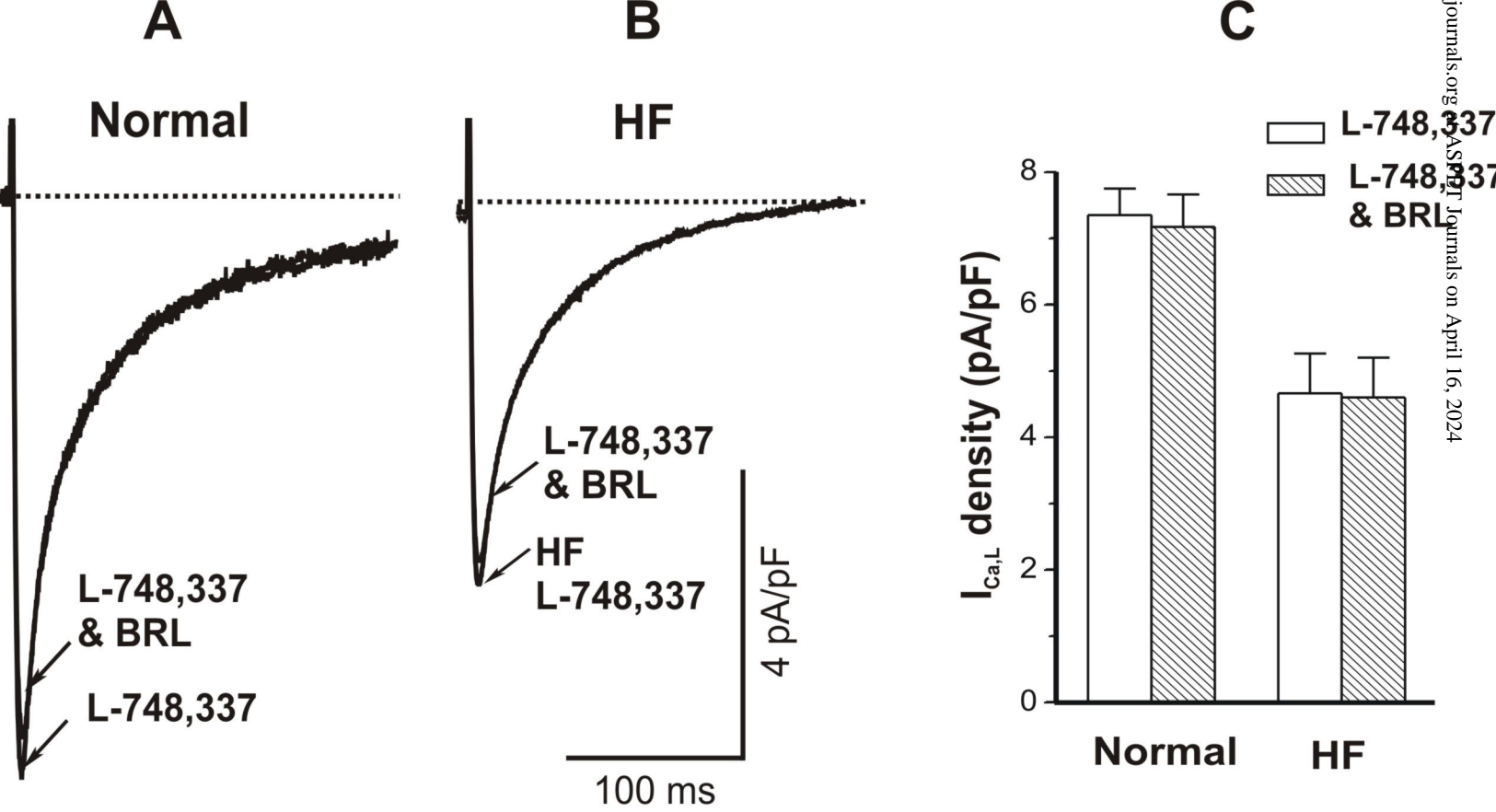
Figure 1

Figure 2

Figure 3

A

Normal

B

HF

C

Nadolol & BRL

Nadolol Control

Nadolol & BRL

HF Nadolol

4 pA/pF

100 ms

Nad

Nad & BRL

$I_{Ca,L}$ density (pA/pF)

Normal

HF

*

*

Figure 4

A
Normal

L-748,337
& BRL

L-748,337

B
HF

L-748,337
& BRL

HF
L-748,337

4 pA/pF

100 ms

C

L-748,337

L-748,337
& BRL

$I_{Ca,L}$ density (pA/pF)

Normal   HF

Figure 5

A Normal

B HF

PTX & BRL

HF PTX

4 pA/pF

100 ms

PTX & BRL

PTX

C

PTX

PTX & BRL

$I_{Ca,L}$ density (pA/pF)

Normal   HF

Figure 6

A

B

L-NAME
+BRL

HF
L-NAME

4 pA/pF

100 ms

L-NAME
+BRL

L-NAME
Control

C

10

8

6

4

2

0

$I_{Ca,L}$ density (pA/pF)

L-NAME

L-NAME+BRL

*

*

Normal

HF

Figure 7