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Up-Regulation and Functional Effect of Cardiac β_3 -Adrenoreceptors In Alcoholic Monkeys

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Abstract

Background—Recent studies link altered cardiac β -adrenergic receptor (AR) signaling to the pathology of alcoholic cardiomyopathy (ACM). However, the alteration and functional effect of β_3 -AR activation in ACM is unknown. We tested the hypothesis that chronic alcohol intake causes an up-regulation of cardiac β_3 -AR, which exacerbates myocyte dysfunction and impairs calcium regulation, thereby directly contributing to the progression of ACM.

Methods—We compared myocyte β_3 - and β_1 -AR expression and myocyte contractile, $[Ca^{2+}]_i$ transient ($[Ca^{2+}]_{iT}$), and Ca^{2+} current ($I_{Ca,L}$) responses to β - and β_3 -AR stimulation in myocytes obtained from left ventricle (LV) tissue samples obtained from 10 normal control (C) and 16 monkeys with self-administered alcohol for 12 months prior to necropsy: 6 moderate (M) and 10 heavy (H) drinkers with group average alcohol intakes of 1.5 ± 0.2 and 3.3 ± 0.2 g/kg/day, respectively.

Results—Compared with control myocytes (C), in alcoholic cardiomyocytes, basal cell contraction (dL/dt_{max} , -39% , H: 69.8 vs C: 114.6 $\mu m/s$), relaxation (dR/dt_{max} , -37% , 58.2 vs 92.9 $\mu m/s$), $[Ca^{2+}]_{iT}$ (-34% , 0.23 vs 0.35) and $I_{Ca,L}$ (-25% , 4.8 vs 6.4 pA/pF) were all significantly reduced. Compared with controls, in moderate and heavy drinkers, β_1 -AR protein levels decreased by 23% and 42% , but β_3 -AR protein increased by 46% and 85% , respectively. These changes were associated with altered myocyte functional responses to β -AR agonist, isoproterenol (ISO), and β_3 -AR agonist, BRL-37344 (BRL). Compared with controls, in alcoholic myocytes, ISO (10^{-8} M) produced significantly smaller increases in dL/dt_{max} (H: 40% vs C: 71%), dR/dt_{max} (37% vs 52%), $[Ca^{2+}]_{iT}$ (17% vs 37%), and $I_{Ca,L}$ (17% vs 27%), but BRL (10^{-8} M) produced a significantly greater decrease in dL/dt_{max} (H: -23% vs C: -11%), $[Ca^{2+}]_{iT}$ (-30% vs -11%), and $I_{Ca,L}$ (-28% vs -17%).

Conclusions—Chronic alcohol consumption down-regulates cardiac β_1 - and up-regulates β_3 -ARs, contributing to the abnormal response to catecholamines in ACM. The up-regulation of cardiac β_3 -AR signaling enhances inhibition of LV myocyte contraction and relaxation and exacerbates the dysfunctional $[Ca^{2+}]_i$ regulation and, thus, may precede the development of ACM.

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Keywords

Alcoholic cardiomyopathy; β_3 -adrenergic receptor; β -adrenergic agonists; contractility; $[Ca^{2+}]_i$ regulation

Introduction

Chronic alcohol consumption is a leading cause of dilated cardiomyopathy which produces congestive heart failure (CHF). This form of CHF is responsible for about 35% of cases of non-ischemic dilated cardiomyopathy. Despite its enormous clinical significance, the underlying mechanisms through which alcoholism results in heart damage remain poorly understood (Laonigro, et al., 2009; Lucas, et al., 2005).

Recent studies link altered cardiac β -adrenergic receptor (AR) signaling to the pathology of alcoholic cardiomyopathy (ACM). It is evident that alcohol consumption is associated with progressive activation of the sympathetic nervous system (SNS), but a diminished positive inotropic response to β -adrenergic receptor (AR) stimulation (Li, et al., 2003; McDonough and Causey, 1993; Patel, et al., 1991). Both down-regulation of β_1 -AR and up-regulation of a newly-described inhibitory pathway mediated by β_3 -ARs may be responsible for the progression of the clinical abnormalities in ACM. However, the alterations and functional effects of cardiac β -AR signal transduction systems have not been systematically evaluated during chronic alcohol intake. Although the diverse roles of β_3 -ARs, especially their negative inotropic properties, are increasingly emerging as major players in the control of normal and pathological heart function (Cheng, et al., 2001b; Gauthier, et al., 2000; Moniotte and Balligand, 2002; Morimoto, et al., 2004; Ziskoven, et al., 2007), no previous studies have specifically examined alcohol-induced changes in cardiac β_3 -AR. The functional consequence of β_3 -AR activation in ACM is not known. Whether and how the disordered cardiac β -AR regulation may worsen myocardium injury and contribute to ACM progression is unclear.

It has been well established that cardiac β_1 - and β_2 -ARs, coupled via the "stimulatory" G protein (G_s), mediate the catecholamine-induced increase in force and frequency of cardiac contractions, thereby providing a mechanism for myocardial contractile reserve. β_2 -ARs are also able to activate nonclassical signaling pathways, suggesting a function distinct from the β_1 -AR subtype (Xiao, et al., 2003; Zhang, et al., 2001). Unlike β_1 - and β_2 -ARs, the function of β_3 -AR is to inhibit ventricle contraction through its link to the inhibitory G proteins (G_i) and stimulate the endothelial nitric oxide (NO)-synthase. The activation of G_i also has the potential to couple β_3 -AR to other important signaling pathways such as the MAP kinase (Cheng, et al., 2001b; Moniotte, et al., 2001; Morimoto, et al., 2004; Ziskoven, et al., 2007).

Since alcohol consumption is a potent activator of the SNS, the β_3 -AR is activated at higher concentrations of catecholamines than β_1 - and β_2 -ARs and is relatively resistant to chronic, agonist-induced desensitization processes due to the lack of phosphorylation sites for β ARK-1 (Gauthier, et al., 2000; Liggett, et al., 1993). Therefore, it is possible that the β_3 -AR stimulation-induced cardiac depression on the normal myocardium may be amplified. The alcoholic myocardium may be more sensitive to β_3 -AR stimulation, perhaps as a result of an up-regulation of β_3 -AR-mediated, G_i -NO-coupled pathway that inhibits inotropy. This chronic alcohol-induced altered balance between opposing inotropic influences of β_1 -AR and β_3 -AR may contribute to progressive cardiac dysfunction in ACM. Accordingly, we hypothesized that up-regulation of cardiac β_3 -AR signaling, as a consequence of chronic alcohol intake-induced sympathetic overdrive (with elevated catecholamine levels), directly contributes to the progression of ACM. This augmentation of cardiac β_3 -AR activation is

proposed to exacerbate already-existing dysfunctional $[Ca^{2+}]_i$ regulation, enhance inhibition of cardiomyocyte contraction and relaxation, and contribute to progressive cardiac dysfunction in ACM.

The role of β_3 -AR could be more clearly elucidated using defined animal models of ACM. In the past, the experimental evaluation of ACM has been difficult due to the lack of reliable experimental models that resemble or mimic the human condition in terms of the amount and pattern of alcohol consumption (Lucas, et al., 2005; Noren, et al., 1983; Piano, 2002a; Thomas, et al., 1980). Previous studies of alcoholic tissue injury have been performed using involuntary administration of high amounts of ethanol and diets that were often high in polyunsaturated fatty acids. To obviate these limitations and to help evaluate this hypothesis, we assessed the consequences of alcohol consumption in a translational model that more closely mimics human exposure, nonhuman primates were allowed to self-administer ethanol and consume a diet containing only moderate amounts of lipids (Grant, et al., 2008a; Vivian, et al., 2001).

Materials and Methods

Experimental Model

Induction and Verification of Experimental Alcoholism

Induction of Oral Ethanol Self-Administration, Food and Ethanol Intake: As previously described, adult cynomolgus (*Macaca fascicularis*, n=13) and rhesus (n=3) monkeys of both sexes were housed in individual stainless steel cages in a vivarium maintained at about 21°C with 30–50% humidity and 12/12-hr light/dark cycle (Grant, et al., 2008a; Vivian, et al., 2001). Each monkey's home cage was equipped with an operant panel that supplied all of the monkey's water, food, and ethanol. Events were controlled and data were recorded with a National Instruments (Austin, TX) interface connected to a personal computer. All monkeys consumed a diet consisting of 11% lipids (of total calories) and 30% protein. Monkeys were trained to operate the drinking panel and then daily induced (with a scheduled induced polydipsia procedure) to drink water, 0.5 g/kg, 1.0 g/kg, and 1.5 g/kg ethanol (4% w/v in water) in one month epochs, respectively (see Grant et al., 2008a for more details) (Grant, et al., 2008a). Over the last 12 months of the experiments, monkeys were permitted to drink water and ethanol *ad libitum* for 22 hours per day. Following the induction procedure, the monkeys were allowed to drink water or 4% (w/v) ethanol at any time during 22-hr sessions for 12 months prior to necropsy.

Individual cynomolgus and rhesus monkeys varied greatly in their propensity to self-administer ethanol (Grant, et al., 2008b; Grant, et al., 2008a). These monkeys were phenotypically categorized as heavy (>3.0 g/kg/day), moderate (2.0–3.0 g/kg/day), or light (<1.0 g/kg/day) ethanol drinkers based on the World Health Organization classification of alcohol use (Vivian, et al., 2001). The present studies investigated 2 categories of drinking groups. On the basis of categorization, there are 6 moderate drinkers (n=2 females and 4 males) consuming an average of 1.5 ± 0.2 g/kg/day, 10 heavy drinkers (n=10 males) consuming an average of 3.3 ± 0.2 g/kg alcohol/day (and > 20% of total daily calories from alcohol), and 10 control monkeys (who lived in an identical environment as the ethanol drinkers, but who self-administered a solution containing maltose-dextrin at isocaloric levels with ethanol). All monkeys received the same daily contact with laboratory personnel except for days of training to sit in a primate-restraining chair or present their leg for awake venipuncture (Grant, et al., 2008a; Ivester, et al., 2007; Vivian, et al., 2001).

Alcohol Level Measurement and Biochemical Assay

Measurement of Blood Ethanol Concentrations: Throughout all experiments, 20- μ l blood samples were taken from the saphenous vein every 5th day from each monkey just before the lights were turned off in the room and approximately 7 hours following the onset of the session. Blood samples were assayed for blood ethanol concentration (BEC) as described previously (Grant, et al., 2008a; Vivian, et al., 2001). Additionally, in the subsets of animals, venous blood samples were collected at the onset of the study and prior to sacrificing the animals for biochemical assay on SNS activation and redox state.

Measurement of Plasma Catecholamine and 8-Isoprostane Levels: Blood was placed in chilled tubes containing EDTA and, after separation in a refrigerated centrifuge, stored at -20°C before analysis. As previously described, plasma levels of epinephrine and norepinephrine (NE) were measured by the Hypertension Center Core Laboratory as previously described. As in past reports (Fam and Morrow, 2003), plasma 8-isoprostane levels were measured in the subgroup animals (3 normal controls and 5 heavy alcohol drinkers) to reflect levels of systemic oxidative stress by using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). All assays were performed in duplicate. All procedures involving primates were conducted in compliance with institutional, state, and federal laws for the usage of primates in laboratory settings.

Experimental Protocol

Myocyte Isolation—Using techniques well established in our laboratory, reproducible, high-yield (more than 75%), and calcium-tolerant cardiomyocytes were isolated from LV samples of these animals (Cheng, et al., 1996; Cheng, et al., 2001b; Morimoto, et al., 2004). Briefly, myocardial biopsies were obtained prior to sacrificing the animals. The tissue specimens were incubated in cold (4°C) 2,3-Butanedione Monoxime (BDM, Sigma, St. Louis, MO) protective solution (pH 7.3, osmolality 285 mOsm/L) consisting of basal solution HBS-1 which contained the following (in mM): NaCl 130, KCl 5.4, NaHCO_3 2.0, glucose 15.0, and HEPES 10.0 and added $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2 plus BDM 30 and 10 μ l insulin (0.01 μ l/ml). The tissue specimens were minced into 2–3 mm^3 cubes within the protective solution and then were enzymatically digested by using collagenase (Worthington # M9b2717, Lakewood, NJ). The isolated myocyte pellets were resuspended in HBS-1 solution. After each settling (about 20 min) during calcium recovery period, the HBS-1 solution was changed stepwise to increase calcium concentrations (i.e., 250, 500, and 1000 μM). Finally, the cells were suspended in HBS-1 with 1.8 mM CaCl_2 and stored at room temperature until ready for use. At room temperature (22°C), the isolated myocytes maintained rod-shaped morphology for 18–24 hours.

Myocyte Morphology—After 2 hours of stabilization, the isolated myocytes were placed in superfused culture dishes. The myocytes were imaged with an inverted microscope using an X40 phase-contrast objective. The image was entered into the image analysis system through a high-resolution monochrome video camera. Measurement of myocyte dimensions were made in 40–50 randomly-selected rod-shaped cells from each experiment (Cheng, et al., 1996; Cheng, et al., 2001b; Morimoto, et al., 2004).

A. Myocyte Function Study

A series of experiments were performed to assess LV myocyte structure and cell size, cell functional performance, $[\text{Ca}^{2+}]_{\text{IT}}$, and $\text{I}_{\text{Ca,L}}$ responses during the following interventions.

Effects on Myocyte Contractile Response and $[Ca^{2+}]_i$ Regulation

Measurement of Contractile Response

Responses to β - and β_3 -Agonists and β_3 -Antagonist: After stabilization, isolated myocytes were placed in superfused culture dishes. The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera, which rapidly scans the image area every 8.3 ms such that the amplitude and velocity of shortening/relengthening is recorded with good fidelity. The edge detection software (IonOptix, MA) was used to measure the cell length during shortening and relengthening. Briefly, myocyte contractions were elicited by field stimulation at a frequency of 0.5 Hz. After stabilization, steady-state baseline data were recorded. To further characterize the chronic alcohol consumption-induced alterations of “contractility reserve”, we evaluated myocyte responses to β - and β_3 -AR stimulations. Myocytes were randomly exposed to a non-selective β -AR agonist, isoproterenol (ISO, 10^{-8} M), or a selective β_3 -agonist, BRL-37344 (BRL), as well as a highly selective β_3 -AR antagonist, L-748,337 (β_3 -ANT) (10^{-7} M) (Candelore, et al., 1999; Moniotte and Balligand, 2002; Morimoto, et al., 2004). Then data were acquired after 3 to 8 minutes of drug exposure and 5 to 10 minutes after drug washout. The dosing protocols of BRL, β_3 -ANT, and ISO used in this study were identified from our initial dose-response experience and verified in our previous studies (Cheng, et al., 2001b; Morimoto, et al., 2004; Zhang, et al., 2005).

In the subsets of myocytes, BRL-mediated actions were determined after pretreatment of myocytes with metoprolol (10^{-6} M, a β_1 -AR antagonist), nadolol (10^{-5} M, a β_1 - and β_2 -AR antagonist), and L-748,337 (10^{-7} M) for 10 minutes in random order.

Effect of PTX and L-NAME on β_3 -AR-Mediated Functional Response: To delineate the changes in intracellular signaling pathways, in subsets of these cells, BRL-mediated actions were determined after pretreatment of myocytes randomly with 1) Nitric oxide (NO) synthase inhibitor, *N*^G-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M) for 30 minutes, and 2) G_i inhibitor, pertussis toxin (PTX) (2 μ g/mL, 36°C) for 5 hours, to inactivate cardiac G_i (Cheng, et al., 2001b; Zhang, et al., 2005). Then the above myocyte contractile functional studies were repeated.

Simultaneous Measurement of Calcium Transient and Contractile Response

In the second series of experiments, $[Ca^{2+}]_{i,T}$ and contraction responses in a single myocyte were simultaneously measured with a dual-excitation fluorescence photomultiplier system (IonOptix, MA). When myocytes were loaded with indo-1-AM, compartmentalization of the indicator may have occurred in the mitochondria, thus the absolute value of $[Ca^{2+}]_i$ was not used. The ratio of the emitted fluorescence (410/490) was used to represent the relative changes in peak intracellular $[Ca^{2+}]_i$ before and after interventions (Cheng, et al., 2001b; Morimoto, et al., 2004).

Measurement of Calcium Current Response

To determine whether the alcohol-induced alterations in $[Ca^{2+}]_{i,T}$ might be secondary to alteration in the inward calcium current, in the third series of experiments, the $I_{Ca,L}$ response was measured by whole-cell patch-clamp technique (Zhang, et al., 2005). After baseline $I_{Ca,L}$, data were obtained during the 0 to 10 minutes of superfusion with ISO or BRL and during the period of washout of the drugs.

B. Molecular Study

Myocyte β_3 -AR mRNA Expression and β_1 - and β_3 -AR Protein Levels

Monkey Cardiomyocyte Expression of β_3 -AR mRNA: Monkey LV myocyte total RNA was extracted by RNAqueous™ Phenol-Free Total RNA Isolation Kit (Ambion, Austin,

TX) and treated with RNase-free DNase I (GIBCO BRL, Grand Island, NY). The reverse transcription (RT) reaction was performed with RETROscript™ First-Strand Synthesis Kit (Ambion, Austin, TX) using the antisense primer. The cDNA produced was amplified by polymerase chain reaction (PCR). A 317 bp DNA fragment corresponding to monkey β_3 -AR coding region (bases 1 to 317) was produced by PCR with the primers for monkey β_3 -AR: sense: 5'-ATGGCTCCGTGGCCTCAT-3' (bases 1 to 18) and antisense: 5'-CCCAACGGCCAGTGGCCAGTCAGCA-3' (bases 293 to 317). GAPDH was co-amplified as an internal control.

Western Blot Analysis of β_1 - and β_3 -AR: LV myocytes were briefly washed with pre-chilled PBS before the addition of a membrane protein-extraction reagent (Pierce, Rockford, IL) with a proteinase inhibitor cocktail. Cell membrane lysate (50 μ g) was blotted to a PVDF membrane and then incubated with polyclonal IgG to β_1 - and β_3 -AR (1:1500 dilutions, Santa Cruz Biotechnology Inc. Santa Cruz, CA) at 4°C overnight. Following washes, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:3000 dilutions, Sigma, St. Louis, MO). For normalization, the same blot was stripped and reprobed with polyclonal IgG to actin at 1:2500 dilutions (Santa Cruz Biotechnology Inc.) (Cheng, et al., 2001b).

Statistical Analysis

Data were summarized as mean $\pm$ SD. Multiple comparisons were performed by analysis of variance. When a significant overall effect was present, intergroup comparisons were performed by using a Bonferroni correction for multiple comparisons. The measurements of myocyte contraction and $[Ca^{2+}]_{iT}$ as well as $I_{Ca,L}$ were averaged from each animal and treated as a single data point. The mean differences in cell dimensions, cell dynamics, and indo-1-AM fluorescence ratios of normal and alcoholic myocytes obtained from the three groups of animals were then obtained. Significance was established as $p < 0.05$.

Results

All 3 groups of animals gained a similar amount of weight during the 12-month study period. In the experimental groups, the group average daily alcohol intakes were 1.5 ± 0.2 g/kg/day and 3.3 ± 0.2 g/kg/day (mean $\pm$ SE) in 6 moderate (5 *Macaca fascicularis* and 1 rhesus) and 10 heavy (8 *Macaca fascicularis* and 2 rhesus) drinkers, respectively. The average daily blood alcohol levels (n=58–62 observations/monkey) taken every fifth day at 7 hrs after the onset of the 22 hr session were 40.5 ± 10.7 and 116.0 ± 13.9 mg/dl (mean $\pm$ SE) in the moderate and heavy drinking monkeys, respectively. Importantly, compared with normal controls, in the heavy alcohol-fed animals, after 12 months of alcohol consumption, there were about 7.3- and 3.4-fold increases in plasma levels of NE (H: $3,928 \pm 394$ vs C: 472 ± 78 pg/ml) and 8-isoprostane (124.8 ± 11.0 vs 28.4 ± 5.1 pg/ml), respectively.

Myocyte Function and $[Ca^{2+}]_i$ Regulation with Chronic Alcohol Consumption

Effects of chronic alcohol consumption on LV myocyte cell size, basal cell functional performance, $[Ca^{2+}]_{iT}$, and $I_{Ca,L}$ are summarized in Table 1 and displayed in Figure 1. The yield of viable myocytes from the LV biopsies was similar (75–80%) in normal control and alcoholic animals. At room temperature (22°C), the isolated myocytes maintained a rod shape morphology about 18 to 22 hours. An overall viability of 70% is indicative of good-quality isolation and is consistent with previous studies (Cheng, et al., 1996; Cheng, et al., 2001a; Cheng, et al., 2001b; Morimoto, et al., 2004). Compared with the cardiomyocytes obtained from control animals, after 12 months of heavy alcohol intake, the length of myocyte was significantly increased from 120.4 ± 4.9 to $146.7 \pm 5.1 \mu$ m ($P < 0.05$), and the

length-width ratio was greater ($39 \pm 7\%$, $P < 0.05$) than the normal cells, suggesting a remodeling of myocyte shape with alcohol self-administration.

Compared with normal controls, the basal myocyte contractile functional performance, $[Ca^{2+}]_{iT}$, and $I_{Ca,L}$ were all markedly depressed in both moderate- and heavy-drinker alcoholic animals. There were more marked reductions in cell contractile performance in heavy-drinker alcoholic myocytes than that of moderate drinkers as indicated by significantly greater decreases in dL/dt_{max} (M: 89.4 ± 5.6 ; H: 69.8 ± 7.0 vs C: 114.6 ± 7.8 $\mu\text{m/s}$), and dR/dt_{max} (M: 71.2 ± 5.4 ; H: 58.2 ± 5.8 vs C: 92.9 ± 5.4 $\mu\text{m/s}$). As illustrated in Figure 3 and summarized in Table 1, heavy alcohol intake resulted in about 36% to 39% decreases in cell systolic amplitude (SA), dL/dt_{max} , and dR/dt_{max} and a 35% prolonged time to peak shortening. In these alcoholic myocytes, the peak systolic $[Ca^{2+}]_{iT}$ was significantly reduced (-34% , H: 0.23 vs C: 0.35), and the decline of $[Ca^{2+}]_i$ was slower. The membrane capacitance was increased; whereas, the current density was significantly lower than that of the normal control myocytes, indicating an absolute reduction of $I_{Ca,L}$ (-25% , H: 4.8 ± 0.7 vs C: 6.4 ± 0.6 pA/pF). Importantly, 12 months of chronic alcohol intake also altered myocyte response to an inotropic stimulus.

Altered Myocyte Functional Responses to β -AR Stimulation

Effect of Isoproterenol—As presented in Figure 2 and summarized in Table 1, compared with control myocytes, in alcoholic cardiomyocytes, not only were the basal cardiomyocyte contraction and relaxation depressed, but the ability of β -adrenergic agonist, ISO (10^{-8} M), to increase the contractility of myocytes was also progressively blunted. Compared with control myocytes, the alcoholic myocytes showed significantly reduced ISO-induced increases in SA (H: 24% vs C: 55%), dL/dt_{max} (40% vs 71%), dR/dt_{max} (31% vs 52%), peak systolic $[Ca^{2+}]_{iT}$ (17% vs 37%), and $I_{Ca,L}$ (17% vs 27%).

Effect of BRL-37344—In contrast, as shown in Figure 3 and summarized in Table 2, compared with control myocytes, in alcoholic cardiomyocytes, BRL produced significantly greater decreases in SA (H: -27% vs C: -9.6%), dL/dt_{max} (-23% vs -11%), dR/dt_{max} (-27% vs -11%), $[Ca^{2+}]_{iT}$ (-30% vs -11%), and $I_{Ca,L}$ (-28% vs -17%). These responses to BRL were not modified by pretreatment of myocytes with β_1 - or β_1 - and β_2 -AR antagonists, but were prevented by superfusion of β_3 -ANT, indicating that BRL-induced negative inotropic effect is coupled to β_3 -AR.

Effect of L-748,337—As exhibited in Figure 4, β_3 -ANT had no effect on normal myocytes. However, in alcoholic myocytes, β_3 -ANT produced slightly, but significant, increases in dL/dt_{max} (H: 11.7 vs C: 1.9%), dR/dt_{max} (H: 9.8 vs C: 2.2%), and $[Ca^{2+}]_{iT}$ (H: 7.0 vs C: 1.3%), indicating improved myocyte functional performance.

Effect of PTX and L-NAME on β_3 -AR-Mediated Functional Response—Figure 5 illustrates the contribution of NO (A) and G_i (B) in BRL-induced contractile response. Pretreatment of myocytes with L-NAME or PTX caused no significant changes in baseline SA, but significantly altered the myocyte response to BRL. As shown in Figure 5A, compared with untreated myocytes, in the presence of L-NAME, BRL-induced decreases in SA and dL/dt_{max} were significantly reduced in control and alcoholic LV myocytes. However, in this condition, BRL still significantly decreased SA and dL/dt_{max} in normal and alcoholic myocytes. The decreased SA (H: 13.6 vs C: 5.9%) and dL/dt_{max} (H: 12.1 vs C: 6.2%) in alcoholic myocytes remained more marked than that of normal myocytes, suggesting β_3 -AR activation is not mediated exclusively through the NO pathway. In contrast, as displayed in Figure 5B, myocytes pretreated with PTX prevented BRL-caused

changes in both normal and alcoholic myocytes, indicating β_3 -AR activation is coupled to G_i through both NO-dependent and NO-independent mechanisms.

Myocyte β_1 - and β_3 -AR Expression with Chronic Alcohol Consumption

Dose-Dependent Increase in β_3 -AR Gene Expression—Although β_3 -AR has been documented in human, rat, mouse, and canine myocardium, its existence and functional role in monkey hearts remain undetermined. By using RT-PCR, β_3 -AR mRNA (a single band about 317 bp) was detected in both control and alcoholic myocytes of these monkeys. Compared with normal myocytes, the signal ratios of β_3 -AR mRNA in moderate and heavy drinkers were significantly increased from 8.6% to 29.7% and 56.3%, respectively (Figure 6).

Contrasting Changes between β_1 -AR and β_3 -AR Protein Expression

Both β_3 - and β_1 -AR protein levels were quantified by using Western blot analyses in normal control and alcoholic monkey LV myocytes. Chronic alcohol consumption caused contrasting changes between β_3 - and β_1 -AR protein expressions. As displayed in Figure 7A, compared with normal control myocytes, in alcoholic myocytes, there were significant decreases in β_1 -AR protein levels. The signal ratio of β_1 -AR protein to actin in the normal myocytes was 0.57 ± 0.07 , but was decreased by 25% (0.43 ± 0.09) and 42% (0.33 ± 0.07) in the myocytes obtained from moderate and heavy drinkers, respectively. In contrast, as shown in Figure 7B, chronic alcohol intake produced a dose-dependent increase in β_3 -AR protein levels. The signal ratio of β_3 -AR protein to actin in the normal myocytes was 0.39 ± 0.06 , but significantly increased by 46% (0.57 ± 0.05) in the moderate drinkers and by about 85% (0.72 ± 0.05) in the heavy drinkers.

Discussion

We assessed the expression of β_3 -ARs and the functional effects of cardiac β_3 -AR-directed inhibitory signaling pathways in a well-established non-human primate model of alcoholism that closely mimics some tissue injuries and functional and neurohormonal changes (especially β -AR signaling) of clinical alcoholism (Grant, et al., 2008b; Grant, et al., 2008a; Grant and Bennett, 2003; Ivester, et al., 2007; Vivian, et al., 2001). The major new findings are: 1) Chronic alcohol-ingestion-produced increased activation of the SNS followed by LV myocyte structural remodeling, primary defects in cardiomyocyte contractile performance, and $[Ca^{2+}]_i$ regulation with diminished β -AR inotropic reserve. 2) Functional β_3 -ARs are expressed in normal monkey LV myocytes, and their stimulation-induced direct inhibitions on $[Ca^{2+}]_{iT}$ and $I_{Ca,L}$ produced a negative inotropic action. Importantly, chronic alcohol intake caused dose-dependent increases in β_3 -AR expression. This alteration was associated with enhanced β_3 -AR-mediated negative modulation of cardiac contractile performance and $[Ca^{2+}]_i$ regulation. These effects were expressed through PTX-sensitive inhibitory G_i protein and partly mediated through a NOS-dependent mechanism. Our observations suggest an important contributing role of β_3 -AR in the progressive nature of functional impairment in ACM. Thus, a selective β_3 -ANT could constitute a potential therapeutic agent.

Myocyte Dysfunction and Impaired $[Ca^{2+}]_i$ Regulation with Chronic Alcohol Consumption

Several clinical observations have shown that the main effect of alcohol ingestion is the loss of cardiac contractility. The direct depression of contractile function from alcohol consumption may promote long-term adaptive changes that may eventually lead to decompensation and CHF. However, the cellular and molecular defects that cause depressed myocyte contractility are not well defined. (Lucas, et al., 2005; Piano, 2002b; Ren and Wold, 2008; Thomas, et al., 1994). Past studies of the possible cellular mechanism of the

cardiodepressant effects of chronic alcohol consumption have yielded conflicting findings due to the confounding effects of tissue preparation, lack of information about alcohol levels, interspecies difference in SNS and renin angiotensin system (RAS), or the use of inadequate amounts and duration of alcohol intake. Moreover, most of the studies dealing with cellular mechanism of ACM have been performed in small animals. However, an extrapolation of studies in the rat and mouse models of alcoholism to higher mammals and humans may not be valid (Cheng, et al., 2006;Laonigro, et al., 2009;Lucas, et al., 2005;Piano, 2002b;Ren and Wold, 2008), and there is no suitable small animal model that reflects both the genotype and phenotype of the human condition.

To obviate the limitations in previous studies, in the current investigation, we used a well-characterized monkey self-administration model of drinking patterns and physiology (including similar absorption and metabolism of alcohol, SNS, RAS, and cardiac β_1 - and β_3 -AR regulation) close to that of a human (Grant, et al., 2008b;Grant, et al., 2008a;Grant and Bennett, 2003;Ivester, et al., 2007). In this investigation, we studied the cardiomyocyte functional performance in freshly-isolated single myocyte from the LV in normal and chronic alcoholic monkeys. These studies removed the effects of extracardiac factors which may influence contractility. We have demonstrated that chronic alcohol ingestion produces direct inhibition of LV myocyte contractility and relaxation along with myocyte structural remodeling. These changes are associated with depressed peak systolic $[Ca^{2+}]_T$, elevated diastolic $[Ca^{2+}]_i$, and reduced inward $I_{Ca,L}$. These findings suggest that defects in cardiomyocyte force-generating capacity and relaxation process with impaired $[Ca^{2+}]_i$ regulation may be the primary cause of ACM.

Our findings on chronic alcohol-induced decreased peak systolic $[Ca^{2+}]_T$ and elevated diastolic $[Ca^{2+}]_i$ are in agreement with past reports demonstrating the depression effects of alcohol on the capacity of sarcoplasmic reticulum (SR) to accumulate and retain Ca^{2+} and to inhibit excitation-contraction coupling in the cardiomyocytes by interfering with the calcium transient (Thomas, et al., 1994;Wu, et al., 1987), but are contrary to the findings of Figueredo et al. (Figueredo, et al., 1998) who reported no change in the cytosolic calcium transient in the heart from rats drinking 36% ethanol for 7 months. This discrepancy might result from several confounding factors such as species (monkey vs rat) difference in β_3 -ARs (Gauthier, et al., 1999), varying degrees of severity of alcohol-induced heart injury, different tissue (multiple cells preparation vs single LV myocyte) preparations, and different measurements of calcium transient.

In adult cardiomyocyte, the L-type Ca^{2+} channel is the main pathway for Ca^{2+} influx. However, chronic alcohol-induced changes in Ca^{2+} channel activity have not been well characterized, and the density of calcium channels in the sarcolemma has been variably reported to be reduced (Solem, et al., 2000) or increased (Fatjo, et al., 2007). In the current study, we observed that in alcoholic LV myocytes, the membrane capacitance was increased; whereas, the current density was significantly lower than that of the normal control myocytes, indicating an absolute reduction of $I_{Ca,L}$. This result is consistent with the observations made by Solem et al. (Solem, et al., 2000) in chronic alcohol-exposed rat cardiomyocytes, but is very different than the findings from a clinical study of radioligand-binding experiments made by Fatjo et al. (Fatjo, et al., 2007) who described a reversal of chronic alcohol-induced up-regulation of L-type receptors after ACM. They reported that the receptor density in the myocardium was greater in alcoholic subjects without cardiomyopathy; whereas, decreased when alcoholic patients developed cardiomyopathy. It is likely that the very different observations in chronic alcohol-induced changes on L-type Ca^{2+} channel between the two studies are mainly owing to the fact that we examined a single LV myocyte obtained from monkey model with heavy alcohol intake; whereas, Fatjo et al. (Fatjo, et al., 2007), studied homogenate samples of LV apex from organ donors and

control human hearts. The use of multiple cell preparation and the different measurements of myocardial L-type Ca^{2+} channel activity as well as the clinical use of multiple cardiovascular specific medications in the alcoholic cardiomyopathic patients may alter the primary defect in the contractile properties of the myocyte itself as well as the abnormalities of extracardiac factors in ACM, thus complicating the accurate assessment of chronic alcohol-induced alteration of Ca^{2+} channel activity. The different tissue distribution and functional effect of β_3 -ARs in LV and atrium may also contribute to these different observations.

Our current findings indicate that LV contractile dysfunction observed with chronic alcohol consumption is directly due to changes of LV myocytes. ACM could be attributed to the attenuation of contractile capacity of heart muscle induced by reduced $[\text{Ca}^{2+}]_{\text{IT}}$ and decreased activity of Ca^{2+} channels.

Expression and Functional Effect of Cardiac β_3 -AR with Chronic Alcohol Consumption

In the present study, we found the presence of cardiac β_3 -AR and quantified cardiac β_3 -AR mRNA and β_3 -AR protein levels by using RT-PCR and Western blot analyses in both normal and alcoholic LV myocytes of monkeys. Importantly, both the expression and the function of β_3 -AR are increased after ACM, which is a striking contrast to the down-regulation seen in β_1 -ARs. These changes were associated with significantly increased SNS activation. We found that the functional β_3 -ARs are expressed in normal monkey LV myocytes, and its stimulation by BRL produced clear depressions of myocyte contraction and relaxation with significant reductions in SA, dL/dt_{max} , and dR/dt_{max} . BRL-induced inhibitions of cell contraction and relaxation were not modified by pretreatment of myocytes with β_1 - and β_1 - and β_2 -AR antagonists, indicating that this effect was not mediated by β_1 - and β_2 -ARs. By contrast, β_3 -antagonists completely prevented the BRL-induced contractile action, indicating that the negative inotropic action of BRL is coupled to β_3 -AR.

We further found that the BRL-induced depression in myocyte contraction was accompanied by significant decreases in $[\text{Ca}^{2+}]_{\text{IT}}$ and I_{CaL} . Thus, the BRL-evoked cardiac inhibition may be at least partially mediated through $[\text{Ca}^{2+}]_{\text{i}}$ regulation. β_3 -AR-mediated negative modulation on cardiac contractile performance and $[\text{Ca}^{2+}]_{\text{i}}$ regulation was in contrast to the down-regulation of β_1 -ARs. This altered balance between opposing inotropic influences of β_1 - and β_3 -ARs in LV myocytes may also contribute to progressive cardiac dysfunction in ACM. We consistently observed that the β_3 -ANT produced no apparent inotropic effect in the normal control myocytes, but significantly increased the contraction and relaxation of cardiac myocytes obtained from ethanol-drinking monkeys. However, a definitive statement regarding the importance of β_3 -ARs in the pathogenesis of ACM cannot be drawn from the described altered β_3 -ARs in alcoholic myocytes. Whether β_3 -ARs are a contributing cause or merely a result of ventricular dysfunction remains an unresolved question. More insight will be gained from ongoing work designed to test possible beneficial actions of chronic β_3 -AR antagonists in these alcoholic animals.

In this study, we further found that β_3 -adrenergic stimulation is accompanied by a coupling of G_i proteins through both NO-dependent and NO-independent mechanisms. Our finding is in good agreement with past reports (Cheng, et al., 2001b; Ziskoven, et al., 2007), but is not consistent with the observations made by Gauthier et al. (Gauthier, et al., 1999) that β_3 -AR stimulation decreases cardiac contractility through activation of NO synthase pathway. This discrepancy may be due to species difference or incomplete blockade of G_i by PTX since a lower level of exposure to PTX was used in that study (Brown and Harding, 1992). The activation of G_i also has the potential to couple β_3 -ARs to other important signaling pathways such as the MAP kinase (Cheng, et al., 2001b; Laonigro, et al., 2009; Lohse, et al.,

2003). Further studies are needed to fully characterize the intracellular pathway coupling β_3 -AR stimulation.

Why were the cardiac responses to β_3 -AR stimulation altered as alcoholism progressed? It is possible that the enhanced β_3 -AR-mediated inhibition of LV myocyte functional performance in ACM may reflect the presence of an increase in the number of β_3 -ARs per cell. However, neither the density of β_3 -AR per cell nor the proportion of distribution between β_1 - and β_3 -ARs have been measured in the current investigation. Further studies assessing β -AR subpopulation (especially for β_3 -AR) receptor density by using receptor binding assay or flow cytometry will be necessary elucidate this point. The enhanced response to β_3 -AR stimulation in ACM may also be related to changes in β_3 -AR-mediated $[Ca^{2+}]_i$ regulation, and altered β_3 -AR-coupled signal transduction. In the present investigation both molecular and functional assessments provide evidence that the β_3 -AR is up-regulated by chronic exposure to alcohol. The prolonged stimulation by catecholamine causes β_1 -AR down-regulation and β_1 - and β_2 -AR uncoupling. In contrast, the β_3 -AR is activated at higher concentrations of catecholamines and is relatively resistant to chronic, agonist-induced desensitization processes (Gauthier, et al., 2000; Liggett, et al., 1993). Furthermore, norepinephrine (NE), the primary neurotransmitter released by SNS, has relatively high affinity for β_3 -AR (Gauthier, et al., 2000; Moniotte and Balligand, 2002). In addition, G_i , implicated in the β_3 -AR signaling, is elevated in ACM (Hoek, et al., 1988; Piano, 2002b). Thus, in ACM, changes in G_i and/or NO signaling pathways may alter alcoholic myocyte response to β_3 -AR stimulation. Therefore, we found that the β_3 -AR stimulation-induced cardiac depression on the normal myocardium is amplified. The alcoholic myocardium is more sensitive to β_3 -AR stimulation as a result of an up-regulation of β_3 -AR-mediated G_i -NO-coupled pathway that inhibits inotropy. These results are comparable with previous observations made in several diseased states where the adrenergic tone is increased. β_3 -ARs are up-regulated in failing human hearts, diabetic rat hearts (Dincer, et al., 2001), and in sepsis of human myocardium as well as murine model, a condition associated with myocardial depression (Moniotte, et al., 2007). These observations support the view that catecholamines may worsen cardiac injury by activating β_3 -AR pathway that directly depresses contractile function and exacerbates cardiac β -AR system desensitization. High adrenergic tone, characteristic of ACM and CHF, may alter cardiac contractility as a result of unmasked β_3 -AR stimulation together with reduced β_1 - and β_2 -AR function (Cheng, et al., 2006; Cheng, et al., 2001b; Gauthier, et al., 2000; Moniotte, et al., 2007; Morimoto, et al., 2004).

Summary

The nonhuman primate model of human alcoholism represents a well-defined, clinically-relevant model for the study of ACM. This model is appropriate to investigate cardiac, cellular, and molecular mechanisms of ACM. By using this model, the present study demonstrates that chronic alcohol consumption produces up-regulation of β_3 -adrenergic pathway. Thus, catecholamines may worsen cardiac injury by activating β_3 -AR pathway that directly depresses cardiomyocyte contractile function, impair $[Ca^{2+}]_i$ regulation, and exacerbate cardiac β -AR system desensitization. The implications of these findings are important to our understanding of the pathophysiology of ACM. They may also suggest several novel therapeutic strategies for the treatment of ACM, such as the use of β_3 -blockers.

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Figure 1.

Examples of analog recordings on LV myocyte (A) contractile functional performance and the peak $[\text{Ca}^{2+}]_i$ transient and (B) $I_{\text{Ca,L}}$ responses obtained from one normal control monkey and one monkey receiving heavy alcohol intake following the 12-month protocol. Compared with control, after 12 months of alcohol intake, myocyte baseline contraction and relaxation (measured as $\text{dL/dt}_{\text{max}}$ and $\text{dR/dt}_{\text{max}}$) as well as peak systolic $[\text{Ca}^{2+}]_{\text{IT}}$ and $I_{\text{Ca,L}}$ were markedly reduced, indicating LV myocyte contractile dysfunction and $[\text{Ca}^{2+}]_i$ homeostasis impairment.

Figure 2.

Examples of superimposed tracings of analog recordings on myocyte contractile functional performance and $[Ca^{2+}]_{iT}$ responses to ISO. Myocytes isolated from the heart tissue of LV obtained by biopsy from one normal and one heavy drinker monkey at the end of the 12-month study period. Myocytes were superfused with HBS solution at 22°C. Myocyte contraction and relaxation were measured as SA, dL/dt_{max} , and dR/dt_{max} . Compared with controls, after 12-months of alcohol intake, SA, dL/dt_{max} , dR/dt_{max} , and $[Ca^{2+}]_{iT}$ were markedly reduced. In the control myocytes, superfusion of ISO (10^{-8} M) caused positive inotropic responses with markedly increased SA, dL/dt_{max} , dR/dt_{max} , and $[Ca^{2+}]_{iT}$. In alcoholic myocytes, the ISO-induced positive action was attenuated.

Figure 3.

Examples of superimposed tracings of analog recordings on myocyte contractile functional performance and $I_{\text{Ca,L}}$ responses to BRL. Isolated LV myocytes obtained from one normal and one heavy drinker monkey at the end of the 12-month study period. BRL caused a negative inotropic effect with markedly decreased $\text{dL/dt}_{\text{max}}$, $\text{dR/dt}_{\text{max}}$, and $I_{\text{Ca,L}}$ in both normal and alcoholic myocytes. Of note, BRL had a greater effect in decreasing myocyte contraction, relaxation, and $I_{\text{Ca,L}}$ in the alcoholic LV myocytes, indicating myocyte contractile dysfunction and $[\text{Ca}^{2+}]_i$ homeostasis impairment as presented in ACM exacerbated by BRL.

Figure 4.

Group mean ($\pm$ SD) of β -AR agonist, isoproterenol (ISO), β_3 -AR agonist, BRL-37,344 (BRL), and β_3 -AR antagonist, L-748,337 (β_3 -ANT)-induced changes of myocyte contraction (measured as the velocity of shortening, dL/dt_{max}) and [Ca²⁺]_{iT} in normal control and chronic heavy alcohol intake monkeys. Compared with baselines, both dL/dt_{max} and [Ca²⁺]_{iT} were significantly increased during ISO superfusion, but significantly decreased with BRL in both normal and alcoholic myocytes. Of note, alcoholic myocytes showed significantly attenuated positive responses to ISO, but enhanced negative responses to BRL. In normal myocytes, β_3 -ANT caused no marked changes in dL/dt_{max} and [Ca²⁺]_{iT}; by contrast, in alcohol myocytes, β_3 -ANT significantly increased dL/dt_{max} and [Ca²⁺]_{iT}.

A. Effect of NO Blockade**B. Effect of G_i Blockade** **Figure 5.**

Representative superimposed tracings of analog recordings of contractile response to BRL in both normal and alcoholic myocytes after pretreatment with L-NAME (A) and PTX (B). After pretreatment of myocytes with L-NAME, BRL (10⁻⁸ M)-induced decreases in SA and dL/dt_{max} were greatly reduced. However, the BRL still markedly decreased SA and dL/dt_{max} in both normal and alcoholic myocytes. In contrast, pretreated myocytes with PTX prevented BRL-caused changes in both normal and alcoholic myocytes, indicating that β_3 -AR activation is coupled with G_i pathways.

Figure 6.

LV myocyte β_3 -AR mRNA expression following 12-month protocol. RT-PCR analysis of β_3 -AR mRNA expression in the normal (line 1) and alcoholic (lines 2 and 3) myocytes obtained from one normal control, one moderate (M), and one heavy drinker (H) monkey. Of the LV myocyte total RNA samples examined with RT-PCR, β_3 -AR mRNA was detected identically in both normal and alcoholic myocytes with the predicted size of 317 bp. For normalization, GAPDH was co-amplified with the predicted size of 500 bp. Compared with normal myocytes, alcoholic myocytes had a significantly higher signal ratio of β_3 -AR mRNA to GAPDH, indicating increased β_3 -AR gene expression in alcoholic monkeys.

A Effect on β_1 -AR

a) Examples

b) Group Means

B Effect on β_3 -AR

a) Examples

b) Group Means

Figure 7.

Figure 7A LV myocyte β_1 -AR protein expression following 12-month protocol. a) Examples of β_1 -AR protein levels of myocytes obtained from two normal control and 2 alcoholic monkeys. One was a moderate drinker (M) and one was a heavy drinker (H). Actin was re-probed for normalization. b) Group means of β_1 -AR protein levels of 3 groups of animals. Compared with normal control, myocyte β_1 -AR protein levels were markedly decreased in both moderate and heavy drinkers. * $P < 0.05$ vs normal; ** $P < 0.05$ moderate vs heavy drinkers.

Figure 7B LV myocyte β_3 -AR protein expression following 12-month protocol. a) Examples of β_3 -AR protein levels of myocytes obtained from two normal control and 2 alcoholic monkeys. One was a moderate drinker (M) and one was a heavy drinker (H). Actin was re-probed for normalization. b) Group means of β_3 -AR protein levels of 3 groups of animals. Compared with normal control, myocyte β_3 -AR levels were markedly increased in both moderate and heavy drinkers. Of note, compared with moderate drinkers, heavy alcohol intake caused more marked increases in cardiac β_3 -AR. * $P < 0.05$ vs normal; ** $P < 0.05$ moderate vs heavy drinkers.

Table 1

Effect of Isoproterenol (ISO) on Myocyte Contractile Performance, $[Ca^{2+}]_i$ Transient and Ca^{2+} Current in Monkeys with Chronic Alcohol Consumption

	Normal Control (C)		Heavy Alcohol Intake (H)	
	(n=10)		(n=10)	
	Baseline (C ₁)	ISO (C ₂)	Baseline (H ₁)	ISO (H ₂)
Resting length (μm)	120.4±4.9	120.2±4.4	146.7±5.1 *	147.2±3.9
Percent shortening (%)	11.5±1.9	17.8±2.2 ⁺	7.4±1.5 *	9.2±2.1 [‡] §
Velocity of shortening (μm/s)	114.6±7.8	196.4±6.7 ⁺	69.8±7.0 *	97.7±5.8 [‡] §
Velocity of relengthening (μm/s)	92.9±5.4	141.2±6.3 ⁺	58.2±5.8 *	76.4±5.0 [‡] §
Amplitude of $[Ca^{2+}]_{IT}$	0.35±0.05	0.48±0.06 ⁺	0.23±0.07 *	0.27±0.04 [‡] §
$I_{Ca,L}$ (pA/pF)	6.4±0.6	8.1±0.3 ⁺	4.8±0.7 *	5.6±0.2 [‡] §

n indicates number of monkeys. Values are mean ± SD.

* $p < 0.05$, C₁ vs. H₁

⁺ $p < 0.05$, C₁ vs. C₂

[‡] $p < 0.05$, H₁ vs H₂

§ $p < 0.05$, (C₂-C₁) vs. (H₂-H₁)

Table 2

Effect of BRL-37344 on Myocyte Contractile Performance, $[Ca^{2+}]_i$ Transient and Ca^{2+} Current in Monkeys with Chronic Alcohol Consumption

	Normal Control (C)		Heavy Alcohol Intake (H)	
	(n=10)		(n=10)	
	Baseline (C ₁)	BRL (C ₂)	Baseline (H ₁)	BRL (H ₂)
Resting length (μm)	120.8±5.4	120.0±4.8	146.3±3.1 [*]	147.0±4.7
Percent shortening (%)	11.4±2.7	10.3±0.9 ⁺	7.0±1.8 [*]	5.1±0.6 [‡] §
Velocity of shortening (μm/s)	115.2±7.3	102.8±8.0 ⁺	69.4±5.2 [*]	53.1±4.6 [‡] §
Velocity of relengthening (μm/s)	91.8±4.5	81.1±3.0 ⁺	58.2±3.8 [*]	42.4±3.6 [‡] §
Amplitude of $[Ca^{2+}]_{IT}$	0.35±0.03	0.31±0.08 ⁺	0.23±0.04 [*]	0.16±0.05 [‡] §
$I_{Ca,L}$ (pA/pF)	6.5±0.3	5.4±0.4 ⁺	4.7±0.7 [*]	3.4±0.6 [‡] §

n indicates number of monkeys. Values are mean + SD.

^{*} $p < 0.05$, C₁ vs. H₁

⁺ $p < 0.05$, C₁ vs. C₂

[‡] $p < 0.05$, H₁ vs H₂

[§] $p < 0.05$, (C₂-C₁) vs. (H₂-H₁)