Cell Stress and Chaperones (2009) 14:33–41
DOI 10.1007/s12192-008-0052-2

ORIGINAL PAPER

L-type calcium channel current up-regulation by chronic stress is associated with increased α_{1c} subunit expression in rat ventricular myocytes

Yun Zhao · Jun Xu · JingBo Gong · LingJia Qian

Received: 26 February 2008 / Revised: 7 May 2008 / Accepted: 9 May 2008 / Published online: 20 June 2008

Abstract The L-type calcium channel plays a pivotal role in the regulation of a wide range of cellular processes, including membrane excitability, Ca^{2+} homeostasis, protein phosphorylation, and gene regulation. Alterations in the density or function of the L-type calcium channel have been implicated in a variety of cardiovascular diseases. Our previous study found that acute restraint stress could cause an enhancement of the L-type calcium current ($I_{Ca\text{-}L}$), which correlated with an up-regulation of activation characters of the calcium channel. In this study, we observed the change of $I_{Ca\text{-}L}$ in rat ventricular myocytes under chronic restraint stress using the whole-cell patch-clamp technique and further explored its modulation mechanisms. The results showed that chronic restraint stress could also enhance $I_{Ca\text{-}L}$, but increased $I_{Ca\text{-}L}$ was not accompanied by an alteration of the characteristics of activation and inactivation of the L-type calcium channel. Furthermore, results from reverse-transcription polymerase chain reaction and Northern blot showed that the abundance of α_{1c} subunit messenger RNA of the L-type calcium channel in the ventricle was increased significantly after chronic stress, and Western blot analysis revealed the amount of α_{1c} subunit protein also was elevated. These results suggest that the L-type calcium channel is involved in stress-induced cardiomyocyte injury, and the up-regulated expression of the L-type calcium channel α_{1c} subunit might contribute to the $I_{Ca\text{-}L}$ change under chronic stress, which is different from the regulation mechanism of acute restraint stress that mostly relates to an alteration in protein kinase A-dependent channel activation. Thus, it would provide a new insight into the mechanism of cardiomyocyte injury induced by stress.

Keywords Calcium current · L-type calcium channel · Patch-clamp technique · Stress · Ventricular myocyte

Abbreviations

$I_{Ca\text{-}L}$	L-type calcium current
RT-PCR	reverse-transcription polymerase chain reaction
PKA/C	protein kinase A /C
$[Ca^{2+}]_i$	intracellular calcium
ER	endoplasmic reticulum
NE	norepinephrine

Introduction

Stress is defined as an adaptive physiological response to the disruption of homeostasis. It has been proven that the effects of stress on organisms are paradoxical; that is, a moderate stress load can invoke protection, but a stress overload can cause injury or can contribute to diseases, including diabetes, gastric ulcer, obesity, cancer, and Parkinson's disease. In particular, the relationship between stress and the risk of cardiovascular disease has been reported. Numerous studies have shown that the cardiovascular system is the major organ targeted by stress, and some scientists even consider that stress is the most important etiologic factor of cardiovascular diseases, such as hypertension, atherosclerosis, and even

Y. Zhao and J. Xu contributed equally to this work.

Y. Zhao · J. Xu · J. Gong · L. Qian (✉)
Laboratory of Stress Medicine,
Institute of Health and Environmental Medicine,
Da-li Road 1,
Tianjin 300050, China
e-mail: newjia@vip.sina.com

sudden cardiac failure (Das et al. 1999; Schwartz et al. 2003). Restraint is considered to be a nonspecific stressor, which can lead to a series of common biochemical and physiological changes. These changes are mediated by the neuroendocrine system including activation of hypothalamic–pituitary–adrenocortical axis and the sympathetic–adrenomedullary system and after over-secretion of corticosterone and catecholamine. Hence, the animal model of restraint stress is often used to study the influence of stress on physiological function and pathological processes. In our previous study (Zhao et al. 2007), it was demonstrated that restraint stress may lead to cardiac dysfunction and to structural injury of the heart. Moreover, severe cardiomyocyte apoptosis and necrosis were also found after restraint stress. Cardiomyocyte death is considered an important cellular basis for stress-induced cardiovascular injury and disease (Feuerstein and Young 2000). The underlying pathological mechanisms for cardiomyocyte damage caused by stress remain unclear, however.

It is well known that the L-type calcium channel plays a major role in determining the normal physiological function characteristics of cardiac myocytes. The depolarizing current through the L-type calcium channel contributes to the plateau phase of the cardiac action potential and to pacemaker activity in nodal cells. The influx of Ca^{2+} ions through the L-type calcium channel is crucial for excitation–contraction coupling in the heart (Bers 2002; Bodi et al. 2005). This influx of Ca^{2+} triggers the release of intracellular stores of Ca^{2+} from the sarcoplasmic reticulum, and the ensuing intracellular Ca^{2+} transient results in activation of the myofilaments. The L-type calcium channel can also impact other cellular processes modulated by intracellular Ca^{2+} such as gene expression and excitation–secretion coupling. Alterations in density or function of the L-type calcium channel have been implicated in a variety of cardiovascular diseases, including atrial fibrillation, heart failure, and ischemic heart disease (Yue et al. 1997; Van Wagoner et al. 1999; Balke and Shorofsky 1998; Timothy and He 2002; Aggarwal and Boyden 1995; Mukherjee and Spinale 1998). Accordingly, there may be a relationship between cardiovascular diseases and the pathophysiological changes in Ca^{2+} homeostasis regulated by the L-type calcium channel. Furthermore, several studies have shown that deregulation of Ca^{2+} through the L-type calcium channel plays a crucial role in the pathogenesis of cell death. Nevertheless, whether the L-type calcium channel is involved in cardiomyocyte damage induced by restraint stress and to what degree L-type calcium channel abundance and function are affected by stress remain largely unknown. Recently, we found that acute restraint stress could cause enhancement of the L-type calcium current ($I_{Ca\text{-}L}$), which correlated with up-regulation of the activation characters of the calcium channel. This reversible change results in transient calcium overload, then triggers apoptosis and eventually leads to cardiomyocytes injury (Xu et al. 2003). However, it is uncertain how changes in L-type calcium channels are regulated by chronic stress.

Against this background, the aims of this study were to observe the change of $I_{Ca\text{-}L}$ in rat ventricular myocytes under chronic restraint stress using the whole-cell patch-clamp technique and to explore further its modulation mechanisms. Our findings suggest that chronic restraint stress could also enhance $I_{Ca\text{-}L}$, but that the change in $I_{Ca\text{-}L}$ density might be mainly dependent on the abundance of the L-type calcium channel α_{1c} subunit expression, which is different from the regulation mechanism of acute restraint stress.

Materials and methods

Experimental animal model of restraint stress

The animal model of restraint stress was established according to the method of Galea et al. (1997), with slight modifications. Adult male Wistar rats weighing 200–250 g were divided randomly into control and stress groups. All rats were housed in a pathogen-free environment at room temperature (RT; 22–25°C) and maintained on rat chow and tap water ad libitum before restraint stress. Individual rats in the stressed group were placed in a specially built size-manipulable cabin for 6 h/day for 21 consecutive days, and control rats were not disturbed during the 21-day period. Rats were killed 24 h after the last day of restraint.

Ventricular myocytes isolation

A single ventricular myocyte from the left ventricle of adult rat heart was enzymatically isolated by langendorff retrograde perfusion of the aorta (Isenberg and Klockner 1982). In brief, hearts were quickly removed, immersed in Ca^{2+}-free Tyrode's solution (4°C), and retrogradely perfused for 5 min with Ca^{2+}-free Tyrode's solution equilibrated with 95% O_2 and 5% CO_2 at 37°C to remove any excess blood in the vessels. Thereafter, the perfusate was switched to the "enzyme medium" of low Ca^{2+} (25 μmol/l) Tyrode's solution containing collagenase type I (0.33 mg/ml, Sigma, St. Louis, MO, USA). After perfusion with the enzyme medium for 10–15 min, the hearts were perfused with the Kraft–Brühe (KB) medium for 5 min. Subsequently, the ventricles were cut out and chopped with scissors. The cells were released from the chunks by mechanical agitation and then separated by passing through a 200-μm mesh net into the KB medium. The cells were stored in KB medium for at least 1 h at 4°C before the experiments. This 1 h preincubation of the myocytes in KB medium results in a

greater yield of Ca-tolerant cells. The ventricular myocytes measured 80–90 μm in length and 20–30 μm in diameter. Only rod-shaped cells with a regular striation pattern were selected for electrophysiological studies.

Measurement of the L-type calcium channel current

The whole-cell patch-clamp technique has made it possible to measure ionic fluxes across the cell membrane and has been particularly instrumental in measuring the magnitude and kinetics of $I_{Ca\text{-}L}$ (Marty and Neher 1995).

Myocytes were placed in a chamber mounted on an inverted microscope (IX70, Olympus Inc., Tokyo, Japan) and continuously superfused with extracellular solution (1–2 ml/min). Glass microelectrodes (2–3 μm diameter) were pulled with a horizontal puller (P-87, Sutter Instrument, Novato, CA, USA), yielding a tip resistance of 3–5 MΩ when filled with pipette solution. After gigaseal was formed and the patch ruptured, an Axopatch-200B patch clamp amplifier (Axon Instruments, Union City, CA, USA) was used for voltage clamping, and $I_{Ca\text{-}L}$ was obtained by voltage clamp steps of 200-ms duration from a −90 mV holding potential to test potentials between −80 and +40 mV. During current measurements, cell capacitance and series resistance were compensated (~80%), and pCLAMP 8 software package (Axon) were used for data acquisition and analysis. Current densities were determined by dividing current amplitudes by cell capacitance. All electrophysiology experiments were performed at physiologic temperatures (35–37°C).

RT-PCR and Northern blot analysis

Voltage-gated calcium channels are multimeric protein complexes consisting of at least four subunits. The major component of the channel is the pore-forming α_1 subunit, which contains the binding site for calcium channel blockers, the voltage-sensor, and the selectivity filter. Subunit α_{1c} is the primary isoform found in the heart and gives rise to high-voltage-activated $I_{Ca\text{-}L}$ (Catterall 2000; Moosmang et al. 2005).

To determine the α_{1c} subunit of L-type calcium channel expression levels, total RNA was extracted from ventricular myocardium by using Trizol reagent (Invitrogen, San Diego, CA, USA) and then analyzed by a reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot method. RT-PCR (one step RNA PCR kit, TaKaRa, Tokyo, Japan) was performed with a primer specific for the cardiac L-type calcium channel subunit α_{1c} (forward: 5′-CTACAACCAGGAGGGCATAAT-3′, reverse: 5′-TTGAAGCGGAAGCGGTA-3′) with the myocardial RNA as a template. The RT-PCR products were analyzed by electrophoresis on 1% agarose gels.

In a Northern blot assay, an aliquot of total RNA (10 μg) sample was separated on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane by the capillary blotting. Hybridization was done using the RT-PCR products that had been radiolabeled with [α-^{32}p]-dCTP by random priming (random primer DNA labeling kit, TaKaRa). Autoradiograph of a hybridization blot was visualized on X-ray film, and its density was quantified using an image software package (ImageMaster VDS, Amersham Pharmacia Biotech, Uppsala, Sweden). The β-actin signals were used as internal controls.

Western blot analysis of L-type calcium channel protein expression

To isolate proteins, ventricular myocardium tissues were homogenized in radioimmunoprecipitation (RIPA) lysis buffer with a protease-inhibitor and centrifuged at 4°C. Protein concentrations were measured by Bradford assay with bovine serum albumin (BSA) as a standard. Proteins were denatured in sodium dodecyl phosphate (SDS) loading buffer, electrophoresed on 8% SDS-polyacrylamide gels using a mini-Protean cell (Bio-Rad, Hercules, CA, USA) and then transferred to polyvinylidine difluoride membrane (Millipore, Bedford, MA, USA) using a Trans-Blot SD cell (Bio-Rad). For immunodetection, membranes were first incubated with primary antibody (C 4980, anti-cardiac α_{1c} subunit, 1:200, Sigma) overnight at 4°C and with secondary antibody (horseradish-peroxidase-conjugated goat anti-rabbit IgG, Zhongshan, Beijing, China) for 2 h at RT. Immunoreactive bands were visualized on X-ray film using the chemiluminescence method. Band densities were quantified by densitometry, standardized to α-tubulin, and normalized to the control sample on each gel.

Solutions

For myocyte isolation, we used the following solutions: Ca^{2+}-free Tyrode's solution containing (in mmol/l) NaCl 116, KCl 5.4, NaH_2PO_4 1.4, $NaHCO_3$ 15, $MgSO_4$ 1, glucose 15, and 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) 5 (pH 7.4 adjusted with NaOH); KB medium containing (in mmol/l) L-glutamic acid 70, KCl 25, taurine 20, KH_2PO_4 10, $MgCl_2$ 3, HEPES 10, glucose 10, and ethylene glycol tetraacetic acid (EGTA) 0.5 (pH 7.4 adjusted with KOH).

The extracellular solution for $I_{Ca\text{-}L}$ studies was composed of (in mmol/l): choline chloride 120, CsCl 4, $CaCl_2$ 1.8, $MgCl_2$ 2, HEPES 10 and glucose 10 (pH 7.4 adjusted with CsOH).

The pipette solution for $I_{Ca\text{-}L}$ recording contained the following (in mmol/l): CsCl 120, $MgCl_2$ 2, HEPES 10,

EGTA 10, K_2ATP 5, and tetraethylammonium ions (TEA) 10 (pH 7.3 adjusted with CsOH).

Statistics

All data are given as mean±standard deviation (SD). Statistical significance was performed by Student's *t* test, and differences were considered significant at $P<0.05$.

Results

Detection of the L-type calcium channel current in rat ventricular myocytes

In cardiac myocytes, two types of Ca^{2+} have been identified: the T-type and the L-type calcium channels. The nomenclature of these channels is based upon their activation and current-carrying capacities. While the T-type calcium channel is characterized by transient openings and very low conductance, the L-type calcium channel is characterized by a long-lasting opening, high conductance, and activation at larger membrane depolarizations. The L-type calcium channel is activated at −40 to −30 mV, and a peak influx of Ca^{2+} ions through the L-type calcium channel has been demonstrated to occur at membrane potentials of 0 to +10 mV (Bean 1985; Bers 1991; Catterall 1995). Thus, a major determinant of the action potential plateau of the cardiac myocyte is the influx of Ca^{2+} ions through the L-type calcium channel.

The calcium current was activated by a depolarizing pulse from a holding potential of −50 to +50 mV at 10 mV step-voltage. This inward current could be completely inhibited (90%) by 5 μmol/l nifedipine, a specific L-type calcium channel blocker. The basic characteristics indicated that the major current present in rat ventricular myocytes was $I_{Ca\text{-}L}$ (data not shown). Figure 1 shows representative results. The peak of $I_{Ca\text{-}L}$ occurred at membrane potentials of 0 to +10 mV under control conditions and threshold potentials at −30 mV.

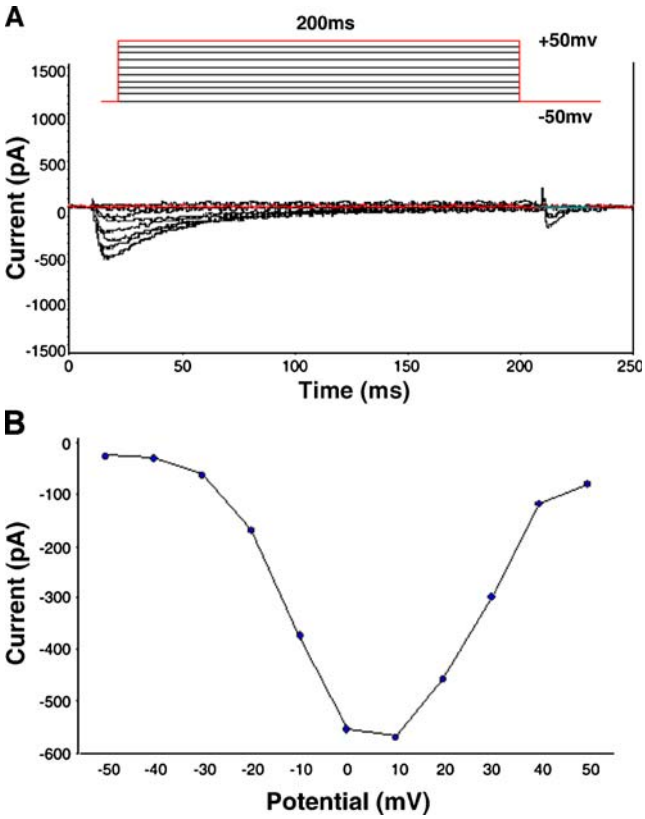
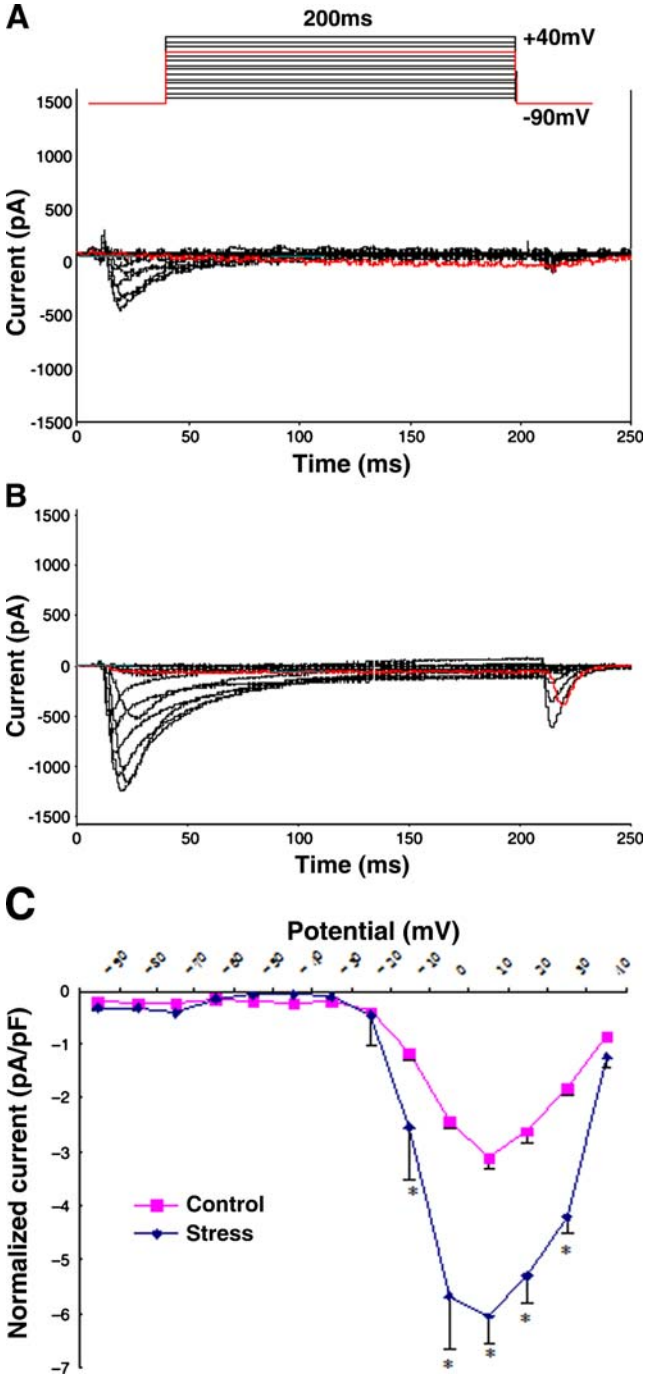


Fig. 1 Properties of basal L-type calcium current ($I_{Ca\text{-}L}$) in rat ventricular myocytes. **A** Representative traces of whole-cell $I_{Ca\text{-}L}$. Superimposed $I_{Ca\text{-}L}$ was recorded at depolarizing pulse from a holding potential of −50 to +50 mV at 10 mV step-voltage with whole-all voltage-clamp method. The duration of the applied test voltages was 200 ms. Pulse protocol was shown on the *top*. **B** Current versus voltage relationship for mean peak $I_{Ca\text{-}L}$. The peak of $I_{Ca\text{-}L}$ occurred at membrane potentials of 0 to +10 mV under control conditions and threshold potentials at −30 mV

$I_{Ca\text{-}L}$ up-regulation by chronic restraint stress

$I_{Ca\text{-}L}$ were elicited from a holding potential of −90 mV to test potentials ranging from −80 to +40 mV in 10-mV increment steps for 200 ms using the whole-cell patch clamp technique, and the effects of stress were investigated. Representative current traces in ventricular myocytes isolated from control and chronic restraint stress rats are shown in Fig. 2. The amplitude of peak $I_{Ca\text{-}L}$ at all test potential was higher in the cells isolated from stress (B) than from the control (A). The peak of $I_{Ca\text{-}L}$ recorded at each step of the voltage pulse protocol was plotted as a function of the voltage step to construct the current–voltage relationship. To account for cell size, measured $I_{Ca\text{-}L}$ is commonly normalized to the membrane capacitance, an index of cell surface area. As illustrated in Fig. 2C, in both the control and the stress groups, $I_{Ca\text{-}L}$ were activated by depolarization above −25 mV approximately and at peak current at around +10 mV. Moreover, $I_{Ca\text{-}L}$ density was markedly increased in ventricular myocytes obtained from stress (−6.04±0.98 pA/pF) compared to those from the control (−3.13±0.02 pA/pF, $n=6$, $P<0.05$). These results suggest that chronic stress could enhance $I_{Ca\text{-}L}$.

Meanwhile, the effects of stress on the steady-state activation and inactivation characters of the L-type calcium channel in ventricular myocytes were observed as described before. However, there was no significant difference in the activation and inactivation characters of the L-type calcium channel between chronic restraint stress and control ($n=6$; data not shown). That indicates that the molecular mech-



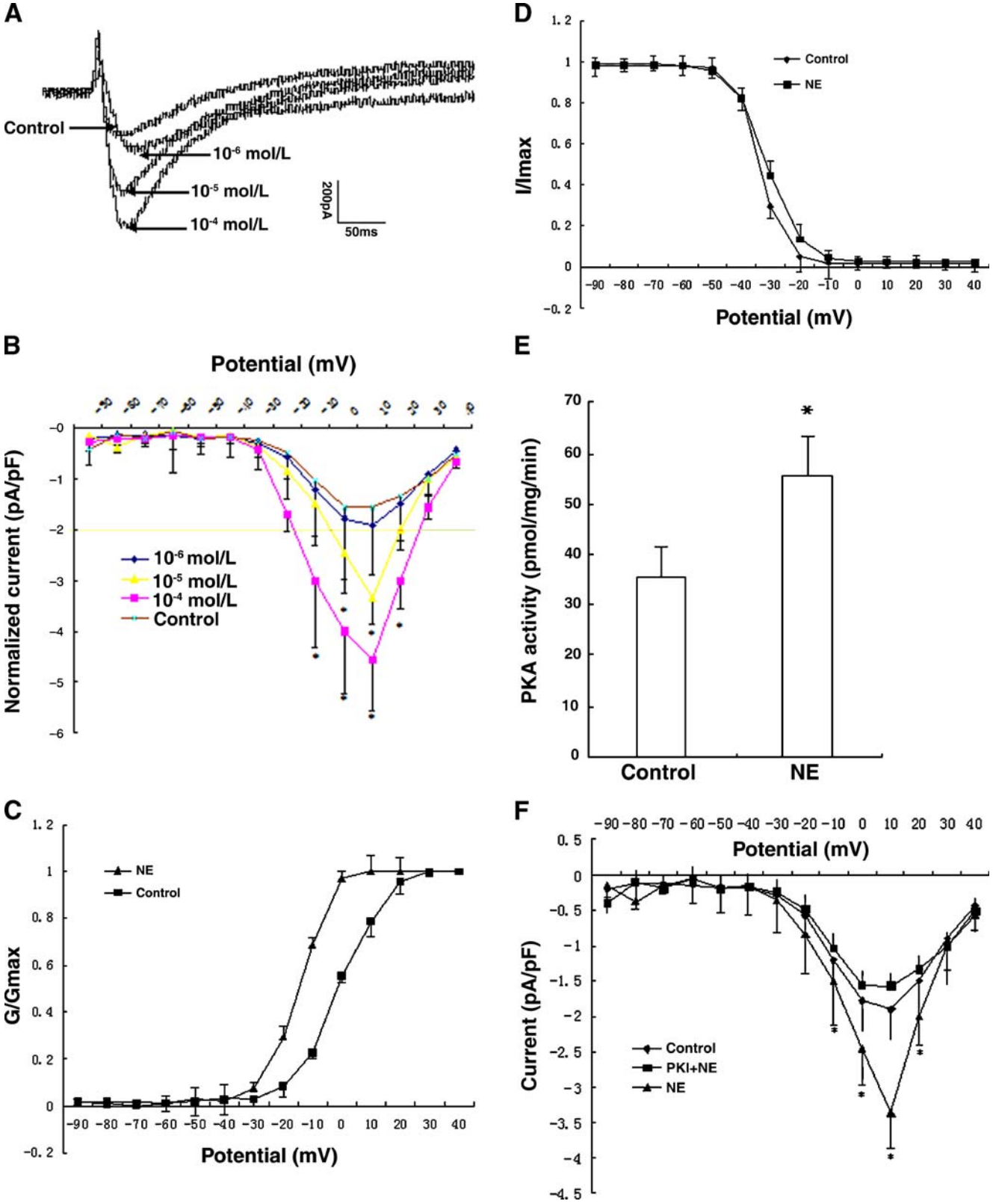
◀ **Fig. 2** Alterations of $I_{Ca\text{-}L}$ in rat ventricular myocytes under chronic restraint stress. **A**, **B**. Representative $I_{Ca\text{-}L}$ traces for control and chronic stress. Superimposed $I_{Ca\text{-}L}$ were elicited from a holding potential of −90 mV to test potential ranging from −80 to +40 mV in 10 mV increment steps for 200 ms using the whole-cell patch clamp technique. The voltage-clamp protocol is shown schematically above the traces. The amplitude of peak $I_{Ca\text{-}L}$ at all test potential was larger in the ventricular myocytes isolated from chronic stress rats (**B**) than control (**A**). **C** Current density versus voltage relationship of mean peak $I_{Ca\text{-}L}$ for control and chronic stress cells. The peak $I_{Ca\text{-}L}$ recorded at each step of the voltage pulse protocol is plotted as a function of the voltage step to construct the current–voltage relationship. To account for cell size, current density (pA/pF) was determined by dividing current amplitude by cell capacitance (an index of cell surface area). Current density increased markedly in ventricular myocytes obtained from chronic stress (−6.04±0.98 pA/pF, n=6) rats compared to control (−3.13±0.02 pA/pF, n=6, P<0.05)

anism underlying the chronic stress-induced $I_{Ca\text{-}L}$ increase may be different from that of acute stress.

In our recent findings (Fig. 3), $I_{Ca\text{-}L}$ were significantly increased (P<0.05) under acute stress (exposure of myocytes to 10^{-6}, 10^{-5}, 10^{-4} mol/l NE) in a dose-dependent manner. In addition, after treatment with 10^{-4} mol/l NE, the steady-state activation curve of the L-type calcium channel showed that the half-maximal activation voltage ($V_{1/2}$) changed from −0.69±0.36 to −14.59±0.24 mV (P<0.05, n=8), with a shift in the activation curve to more negative potentials. The alteration of a steady-state inactivation curve was not detected, indicating that the L-type calcium channels of ventricular myocytes were inclined to be activated under acute stress. The results demonstrated that up-regulation of $I_{Ca\text{-}L}$ is associated with an alteration in phosphorylation-dependent channel activation. Furthermore, activity of protein kinase A (PKA) increased markedly after acute stress, and $I_{Ca\text{-}L}$ was inhibited by protein kinase inhibitor (PKI), suggesting that acute stress-induced $I_{Ca\text{-}L}$ increase involves activation by PKA.

Expression of the L-type calcium channel α_{1c} subunit enhancement by chronic stress

The purpose of this experiment was to determine whether or not expression levels of L-type calcium channel α_{1c} subunit were regulated by chronic stress and whether or not this change was related to the up-regulation of $I_{Ca\text{-}L}$ in ventricular myocytes under chronic stress. Semi-quantitative RT-PCR analysis was first used to verify the hypothesis. As shown in Fig. 4A, the α_{1c} subunit of the L-type calcium channel was expressed in both groups and had a higher expression level in the ventricular myocardium from rats under chronic stress than those in the control. In addition, Northern blot analysis was performed to evaluate the effect of chronic stress on the mRNA levels. Consistent with the finding obtained by RT-PCR, the result from Northern blot showed that the α_{1c} subunit mRNA of the L-type calcium channel in chronic stress was more abundant and increased markedly to approximately 1.6-fold compared to the control (P<0.05, n=4; Fig. 4B). Furthermore, immunoblot analysis also revealed that the amount of α_{1c} subunit protein was increased in the heart of stressed rats, about 17% more than control (Fig. 4C). Our results, together with a previous work on L-type calcium channel, suggest that chronic stress could lead to up-regulation of α_{1c} subunit expression, and it may be a major molecular mechanism underlying the $I_{Ca\text{-}L}$ increased induced by chronic stress.


A
Control
10-6 mol/L
10-5 mol/L
10-4 mol/L
200pA
50ms
D
Control
NE
I/Imax
Potential (mV)
B
Potential (mV)
Normalized current (pA/pF)
10-6 mol/L
10-5 mol/L
10-4 mol/L
Control
E
PKA activity (pmol/mg/min)
Control
NE
C
NE
Control
G/Gmax
Potential (mV)
F
Potential (mV)
Current (pA/pF)
Control
PKI+NE
NE

◀ **Fig. 3** Effect of acute stress on the L-type calcium channels in rat ventricular myocytes. **A** Peak $I_{Ca\text{-}L}$ traces for control and NE (norepinephrine)-activated. Peak $I_{Ca\text{-}L}$ was increased under acute stress (i.e., exposure of ventricular myocytes to 10^{-6}, 10^{-5} and 10^{-4} mol/l NE for 10 min) in a dose-dependent manner. **B** Current density versus voltage relationship of mean peak $I_{Ca\text{-}L}$ for control and NE. Peak $I_{Ca\text{-}L}$ density was significantly increased in ventricular myocytes in response to 10^{-4} and 10^{-5} mol/l NE (−4.56±0.95 and −3.39±0.87 pA/pF, n=8) compared to control (−1.55±0.43 pA/pF, n=8, P<0.05). **C**, **D**. Activation and inactivation curves of the L-type calcium channel. For activation, conductance was determined by dividing the peak current amplitude at each potential by the driving force for Ca^{2+}. The continuous lines represent the best fits of Boltzmann equations to the data. The half-maximal voltage required for activation was −14.59±0.24 and −0.69±0.36 mV in ventricular myocytes with 10^{-4} mol/l NE treatment and control (n=8, P<0.05), respectively, and there is a shift in the activation curve to more negative potentials (**C**). While the two steady-state inactivation curves were nearly identical, the half-maximal inactivation voltage was −33.57±0.31 mV (control) and −31±0.48 mV (10^{-4} mol/l NE; n=7, P>0.05), respectively (**D**). It indicates that the L-type calcium channel in rat ventricular myocytes was inclined to be activated under acute stress. **E**, **F** Regulation of the L-type calcium channel by protein kinase A (*PKA*) in ventricular myocytes. PKA activity was markedly increased after10^{-4} mol/l NE treatment, from 35.50±6.34 (control) to 55.59±7.51 pmol/mg/min (n=8, P<0.05) (**E**). Then, $I_{Ca\text{-}L}$ was measured in the presence of *protein kinase inhibitor* (PKI), in contrast to NE group, $I_{Ca\text{-}L}$ was significantly decreased (**F**).It suggests that acute stress-induced $I_{Ca\text{-}L}$ increase involves activation by PKA

Discussion

The voltage-gated L-type calcium channel plays a pivotal role in the regulation of a wide range of cellular processes, including membrane excitability, Ca^{2+} homeostasis, protein phosphorylation, and gene regulation (Brosenitsch et al. 1998). In the heart, excitation–contraction coupling depends on the L-type calcium channel. The cardiac L-type calcium channel is a membranous multimer consisting of the pore-forming α_{1c} subunit and the regulatory α_2/δ and β subunits. The α_{1c} subunit contains voltage sensor and receptor sites for the different classes of calcium channel antagonists/agonists and determines the basic electrophysiological properties (Schultz et al. 1993).

In the present study, we have shown that chronic stress enhanced $I_{Ca\text{-}L}$ density and increased $I_{Ca\text{-}L}$ that was not accompanied by alteration of the characteristics of activation and inactivation, but corresponded with up-regulation in the mRNA and protein levels of the L-type calcium channel α_{1c} subunit. Past studies have demonstrated that the relation between the number of L-type calcium channels and whole-cell $I_{Ca\text{-}L}$ may be expressed as: $I=N_T\times P_f\times i\times P_o$, where I is the whole-cell current, N_T is the total number of channels, P_f is the probability that the available channels are functional, i is the total current that any single channel can carry, and P_o is the probability that the functional channel is open (Bers 1991; Klockner and Isenberg 1994; Campbell and Strauss 1995). Since $I_{Ca\text{-}L}$ density is obtained by dividing $I_{Ca\text{-}L}$ amplitude by membrane capacitance (an electrical index of membrane surface area), then, by definition, a significant correlation must exist between L-type calcium channel abundance and $I_{Ca\text{-}L}$ density. Thus, conditions that change L-type calcium channel abundance could influence whole-cell $I_{Ca\text{-}L}$ density. As our findings have shown that the characteristics of activation and inactivation were not affected by chronic restraint stress, it may be speculated that the expression alteration of the L-type calcium channel in ventricular myocytes underlies the

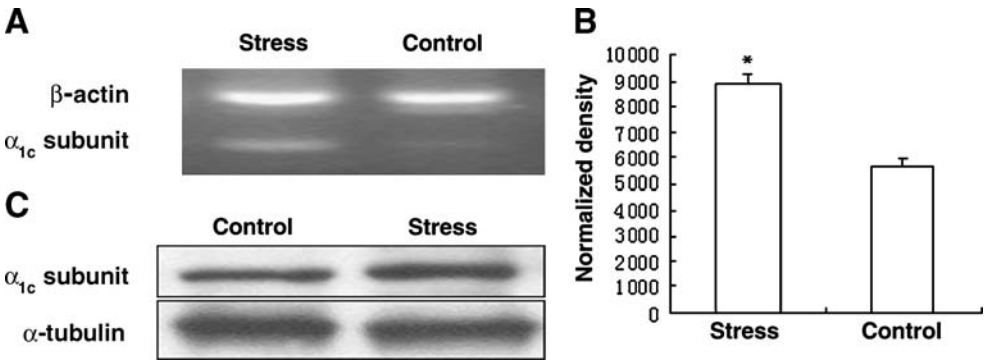


Fig. 4 Change in expression of L-type calcium channel α_{1c} subunit in rat ventricular myocardium. The cardiac L-type calcium channel is a membranous multimer consisting of the pore-forming α_{1c} subunit and the regulatory α_2/δ and β subunits. The α_{1c} subunit contains voltage sensors and receptor sites for the different classes of calcium channel antagonists/agonists, and determines the basic electrophysiological properties. **A** Semi-quantitative RT-PCR analysis shown the α_{1c} subunit of L-type calcium channel was expressed in both control and chronic stress groups and that it had a higher expression level in ventricular myocardium from chronic stress rat than control. **B** The results from Northern blot showed the α_{1c} subunit mRNA of the L-type calcium channel in chronic stress was more abundant and increased markedly to approximate 1.6-fold compared to control (P< 0.05, n=4). β-actin was used as an internal control. **C** Western blot analysis also revealed that the amount of α_{1c} subunit protein was increased in the heart of stressed rats, about 17% more than control. α-tubulin was used as the loading control. It suggests that chronic stress could lead to up-regulation of α_{1c} subunit expression

up-regulation of $I_{Ca\text{-}L}$. RT-PCR, Northern blot, and Western blot assays confirmed the hypothesis that the abundance of mRNA and protein of the L-type calcium channel α_{1c} subunit in the ventricle was increased significantly in the chronically stressed rat. Considering these results, the change in $I_{Ca\text{-}L}$ density might be mainly dependent on the abundance of the L-type calcium channel under chronic stress, i.e., the expression regulation. This modulation change is irreversible, and is different from the change induced by acute stress. Numerous studies have demonstrated that functional regulation of the calcium channel relies on phosphorylation processes, such as PKA and PKC, which all affect $I_{Ca\text{-}L}$. In the heart, phosphorylation of $I_{Ca\text{-}L}$ is counteracted by type 1 and type 2A phosphatases. Thus, the actual amplitude of basal $I_{Ca\text{-}L}$ is determined by the balanced activity of kinases and phosphatases (Kamp and Hell 2000; Herzig and Neumann 2000; Christ et al. 2004). This confirms the findings in our previous research, which found that acute stress only caused cardiac dysfunction, whereas chronic stress may lead to an organic pathological change of the heart.

Calcium ions are central to multiple signal transduction pathways that accomplish a variety of biological functions. The spatial and temporal regulation of intracellular calcium ($[Ca^{2+}]_i$) serves as a modulator of pathways involved in fertilization, proliferation, and development (Berridge et al. 2000). However, it is apparent that modulation of $[Ca^{2+}]_i$ plays a very important role in the pathogenesis of cell injury and cell death (Trump and Berezesky 1995). Several studies have shown that increased Ca^{2+} influx through the L-type calcium channel has been implicated in the apoptosis of cardiomyocytes induced by ischemia/reperfusion, catecholamines, and angiotensin II (Gao et al. 2001; Communal et al. 1998; Goldenberg et al. 2001). Moreover, a higher myocyte apoptotic rate was observed in the intact heart of a line of transgenic mice overexpressing the α_{1c} subunit (Muth et al. 2001), suggesting a role for persistent increases in Ca^{2+} influx in apoptosis in vivo. The Ca^{2+} homeostasis in the cell is maintained by the balance among the calcium channel, the endoplasmic reticulum (ER), and the mitochondria. Any deregulation among them can cause disruption of the Ca^{2+} equilibrium, such as Ca^{2+} overload, that is harmful and may trigger apoptosis (Demaurex and Distelhorst 2003; Hajnoczky et al. 2000). This was also confirmed in our previous study, but the interaction details between Ca^{2+} ions and apoptosis needed to be clarified further. A recent study reported that the transfer of Ca^{2+} from the ER to the mitochondria is required for initiation of programmed cell death by some (Scorrano et al. 2003).

In conclusion, our results show that chronic restraint stress could enhance $I_{Ca\text{-}L}$, and the up-regulated expression of the L-type calcium channel α_{1c} subunit might contribute to altered calcium handling in stress-induced cardiomyocyte injury, which is different from the regulation mechanism of acute restraint stress that mostly related to activation of the PKA signal transduction pathway. The precise regulation of the Ca^{2+} influx in response to various physiological situations is further controlled by several regulatory mechanisms, working at different levels, including channel expression, localization, or activity, via additional interactions with modulatory proteins. There are some limitations in the present study, such as the relationship between the expression level of all the subunits and the electrophysiological function of the whole channel has not yet been clarified. Therefore, a combined investigation of the mRNA levels, protein levels, histological distribution, and electrophysiological functions is required. Although limited for these reasons, the present result provides a new understanding of the mechanism of cardiomyocyte injury induced by stress and may help in the prevention and cure of cardiovascular diseases.

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China (NNSFC; 30430590) and the National High Technology Research Development Program of China (2006AA02Z4C8).

References

Aggarwal R, Boyden PA (1995) Diminished calcium and barium currents in myocytes surviving in the epicardial border zone of the 5-day infracted canine heart. Circ Res 77:1180–1191

Balke CW, Shorofsky SR (1998) Alterations in calcium handling in cardiac hypertrophy and heart failure. Cardiovasc Res 37:290–299

Bean BP (1985) Two kinds of calcium channels in canine atrial cells. J Gen Physiol 86:1–30

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1:11–21

Bers DM (1991) Ca influx via sarcolemmal Ca channels. In: Bers DM (ed) Excitation-contraction coupling and cardiac contractile force. Kluwer, Norwell, MA, pp 49–70

Bers DM (2002) Cardiac excitation–contraction coupling. Nature 415:198–205

Bodi I, Mikala G, Koch SE, Akhter SA, Schwartz A (2005) The L-type calcium channel in the heart: the beat goes on. J Clin Invest 115:3306–3317

Brosenitsch TA, Salgado Commissariat D, Kunze DL, Katz DM (1998) A role for L-type calcium channels in developmental regulation of transmitter phenotype in primary sensory neurons. Neurosci 18:1047–1055

Campbell DL, Strauss HC (1995) Regulation of calcium channels in the heart. Adv Second Messenger Phosphoprot Res 30:25–88

Catterall WA (1995) Structure and function of voltage-gated ion channels. Annu Rev Biochem 64:493–501

Catterall WA (2000) Structure and regulation of voltage-gated Ca^{2+} channels. Annu Rev Cell Dev Biol 16:521–555

Christ T, Boknik P, Wöhrl S et al (2004) L-type Ca^{2+} current downregulation in chronic human atrial fibrillation is associated with increased activity of protein phosphatases. Circulation 110:2651–2657

Communal C, Singh K, Pimentel DR, Colucci WS (1998) Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. Circulation 98:1329–1334

Das DK, Engelman RM, Maulik N (1999) Oxygen free radical signaling in ischemic preconditioning. In: Das DK (ed) Heart in stress . Ann NY Acad Sci 874:49–65

Demaurex N, Distelhorst C (2003) Apoptosis—the calcium connection. Science 300:65–67

Feuerstein GZ, Young PR (2000) Apoptosis in cardiac diseases: stress- and mitogen-activated signaling pathways. Cardiovasc Res 45:560–569

Galea LAM, Mcewen BS, Tanapat P et al (1997) Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. Neurosci 81:689–697

Gao F, Gong B, Christopher TA, Lopez BL, Karasawa A, Ma XL (2001) Anti-apoptotic effect of benidipine, a long-lasting vasodilating calcium antagonist, in ischaemic/reperfused myocardial cells. Br J Pharmacol 132:869–878

Goldenberg I, Grossman E, Jacobson KA, Shneyvays V, Shainberg A (2001) Angiotensin II-induced apoptosis in rat cardiomyocyte culture: a possible role of AT1 and AT2 receptors. J Hypertens 19:1681–1689

Hajnoczky G, Csordas G, Madesh M, Pacher P (2000) Control of apoptosis by IP3 and ryanodine receptor driven calcium signals. Cell Calcium 28:349–363

Herzig S, Neumann J (2000) Effects of serine/threonine protein phosphatases on ion channels in excitable membranes. Physiol Rev 80:173–210

Isenberg G, Klockner U (1982) Calcium tolerant ventricular myocytes prepared by preincubation in a “KB” medium. Pflugers Arch 395:6–18

Kamp TJ, Hell JW (2000) Regulation of L-type calcium channels by protein kinase A and protein kinase C. Circ Res 22:1095–1102

Klockner U, Isenberg G (1994) Intracellular pH Modulates the Availability of Vascular L-type Ca^{2+} Channels. J Gen Physiol 103:647–663

Marty A, Neher E (1995) Tight-seal whole-cell recording. In: Sakmann B, Neher E (eds) Single-channel recording, 2nd edn. Plenum, New York, pp 31–52

Moosmang S, Lenhardt P, Haider N, Hofmann F, Wegener JW (2005) Mouse models to study L-type calcium channel function. Pharmacol Ther 106:347–355

Mukherjee R, Spinale FG (1998) L-type calcium channel abundance and function with cardiac hypertrophy and failure: A review. J Mol Cell Cardiol 30:1899–1916

Muth JN, Bodi I, Lewis W, Varadi G, Schwartz A (2001) A Ca^{2+}-dependent transgenic model of cardiac hypertrophy: A role for protein kinase Calpha. Circulation 103:140–147

Schultz D, Mikala G, Yatani A et al (1993) Cloning, chromosomal localization, and functional expression of the 1 subunit of the L-type voltage-dependent calcium channel from normal human heart. Proc Natl Acad Sci USA 90:6228–6232

Schwartz AR, Gerin W, Davidson KW, Picking TG, Brosschot JF, Thayer JF, Christenfeld N, Linden W (2003) Toward a causal model of cardiovascular responses to stress and the development of cardiovascular disease. Psychosom Med 65:22–35

Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, Korsmeyer SJ (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 300:135–159

Timothy JK, He JQ (2002) L-type Ca^{2+} channels gaining respect in heart failure. Cardiovasc Res 91:451–453

Trump BF, Berezesky IK (1995) Calcium-mediated cell injury and cell death. FASEB 9:219–228

Van Wagoner DR, Pond AL, Lamorgese M, Rossie SS, McCarthy PM, Nerbonne JM (1999) Atrial L-type Ca^{2+} currents and human atrial fibrillation. Circ Res 85:428–436

Xu J, Ma Q, Duan HF, Qian LJ (2003) Effects of stress on L-type calcium channels of rat ventricular myocytes. Chin J Applied Physiol 19:216–219

Yue L, Feng J, Gaspo R, Li GR, Wang Z, Nattel S (1997) Ionic remodeling underlying action potential changes in a canine model of atrial fibrillation. Circ Res 81:512–525

Zhao Y, Wang WY, Qian LJ (2007) Hsp70 may protect cardiomyocytes from stress -induced injury by inhibiting Fas-mediated apoptosis. Cell Stress Chaperones 12:83–95