



## Determination of L-Type Calcium Current in the Primary Hypertrophied Left Ventricular Cardiomyocytes of Hypertrophy Heart Rats/HHR

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### ABSTRAK

**Background:** The molecular mechanisms responsible for electromechanical derangements found in primary left ventricular (LV) hypertrophy have not been clearly elucidated. Of particular interest is the voltage gated L-type Ca current ( $I_{CaL}$ ) which plays crucial role in the EC-coupling. This study aimed to investigate cardiomyocyte  $I_{Ca(L)}$  in newly developed line of rats namely HHR (hypertrophy heart rat) and NHR (normal heart rat).

**Methods:** A pure experimental study has been conducted using male HHR ( $n=7$  rats) and NHR ( $n=9$  rats), aged 19-25 week. Cardiomyocytes were isolated enzymatically. By using whole cell patch clamp techniques,  $I_{Ca}$  were measured and normalized (pA/pF) for cell size determined by capacitance (Cm). In Na-free superfusate, the  $I_{Ca(L)}$  of HHR ( $n=18$  cells) and NHR ( $n=12$  cells) obtained at holding potential (HP) -50 mV were analyzed using Student's t-test and one-way ANOVA (repeated measures).  $p<0.05$  values were considered significance.

**Results:** Myocyte mean Cm of HHR slightly increased but not significantly greater than that of NHR ( $364.3\pm22.33$  pF vs  $300.2\pm27.78$  pF,  $p=0.08$ ). In HHR, peak Ca current density was significantly increase, i.e. at HP -50 mV, the  $I_{Ca(L)}$  evoked at test potential (TP) -10 mV were  $-7.74\pm0.86$  (HHR) vs  $-4.68\pm1.09$  (NHR) pA/pF,  $p<0.02$ .

**Conclusions:** Peak Ca current density of HHR increases approximately 1.6 times that of the NHR ( $-7.74\pm0.86$  vs  $-4.68\pm1.09$  pA/pF). This increase is attributed primarily to L-type Ca current, best known to peak at around TP -10 mV. The dramatic increase in sarcolemmal calcium influx in the HHR provides evidence of altered EC coupling processes in primary LV hypertrophy.

**Keywords:** HHR rats, LV hypertrophy, cardiomyocyte, L-type calcium current

### ABSTRACT

Penentuan arus kalsium tipe L pada kardiomyosit tikus yang mengalami hipertrofi primer ventrikel kiri (Hypertrophy Heart Rats/HHR)

**Latar belakang:** Mekanisme molekuler yang bertanggung jawab pada munculnya kelainan elektro-mekanik pada hipertrofi primer ventrikel kiri masih belum jelas, khususnya arus kalsium tipe L,  $I_{Ca(L)}$ , yang berperan penting pada 'EC-coupling'. Tujuan penelitian ini untuk mengukur dan menganalisis  $I_{Ca(L)}$  kardiomyosit dari tikus jenis baru bernama HHR (hypertrophy heart rat) dan NHR (normal heart rat).

**Metode:** Eksperimen murni laboratorium dilakukan pada tikus jantan NHR ( $n=9$ ) dan HHR ( $n=7$ ) berusia 19-25 minggu. Kardiomyosit diisolasi menggunakan teknik enzimatik. Dengan teknik 'whole cell patch clamp'; arus kalsium  $I_{Ca(L)}$  diukur dan dinormalisasi sesuai ukuran selnya (pA/pF), yang ditentukan melalui pengukuran kapasitans membran (Cm). Pada kondisi bebas Na,  $I_{Ca(L)}$  dari kedua kelompok sel HHR ( $n=18$ ) dan NHR ( $n=12$ ), yang didapat dari 'holding potential' (HP) -50mV dianalisis dengan Student's t-test dan one-way ANOVA (repeated measures). Nilai  $p<0,05$  dianggap bermakna.

**Hasil:** Rerata kapasitans membran (Cm) dari HHR dan NHR secara statistik tidak beda secara bermakna ( $364,3\pm22,33$  pF vs  $300,2\pm27,78$  pF,  $p=0,08$ ). Pada HP -50 mV, densitas arus kalsium  $I_{Ca(L)}$  yang dihasilkan dari 'test potential' (TP)-10 mV meningkat secara bermakna pada HHR dibanding NHR  $-7,74\pm0,86$  vs  $-4,68\pm1,09$  pA/pF ( $p<0,02$ ).

**Simpulan:** Terjadi peningkatan densitas arus kalsium pada HHR yaitu 1,6 kali NHR ( $-7,74\pm0,86$  vs  $-4,68\pm1,09$  pA/pF). Peningkatan ini seluruhnya disebabkan oleh  $I_{Ca(L)}$ , karena tipe ini biasanya memuncak pada TP sekitar -10 mV. Peningkatan influx kalsium sarkolemma HHR yang sangat besar ini, merupakan bukti adanya perubahan proses 'EC coupling' pada ventrikel kiri yang mengalami hipertrofi primer.

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## INTRODUCTION

Left ventricular hypertrophy (LVH, an increase in LV mass), has been primarily considered an adaptive growth response to pressure or volume overload.<sup>1,2</sup> Clinical and experimental animal studies investigating pressure overload-induced cardiac hypertrophy have shown that hypertrophy is linked with contractile dysfunction,<sup>3-6</sup> and elevated arrhythmogenic morbidity.<sup>7,8</sup> In heart failure which is mostly a continuum of LVH, the cardiac derangements are also related to  $\text{Ca}^{2+}$  handling deficits manifesting in depression of developed force, prolongation of relaxation and blunting of the frequency-dependent facilitation of contraction.<sup>9</sup> In addition, the arrhythmic events which occur during cardiac hypertrophy and failure, may be linked with derangements in depolarizing and repolarizing currents including alterations in  $\text{Ca}^{2+}$  currents.<sup>9,10</sup>

Thus, although the fundamental changes in  $\text{Ca}^{2+}$  handling that attend ventricular failure are thought to account for the abnormalities in excitation-contraction coupling (i.e. impaired contractile function and arrhythmias), the cellular and molecular basis of the  $\text{Ca}^{2+}$  handling deficits in primary ventricular hypertrophy or cardiac hypertrophy *per se*, in the absence of hypertension is not clear. The role of calcium currents which have a crucial influence on  $\text{Ca}^{2+}$  homeostasis have also not yet been elucidated in the primary hypertrophic state. Specifically, little is known about changes in the fluxes of calcium through voltage dependent calcium channels, which are important in excitation-contraction coupling (E-C coupling). Of particular interest in non-haemodynamically loaded primary hypertrophy is the L-type calcium current, the  $I_{\text{Ca(L)}}$ .

The previous lack of availability of experimental models of primary hypertrophy has limited investigation of the cellular mechanisms that underlie the functional impairment, including for the investigation of sarcolemmal calcium (Ca) flux. For the purpose, this present study was to investigate cardiomyocyte voltage gated L-type Ca currents ( $I_{\text{Ca(L)}}$ ) in the HHR, a new rat model of LVH without any influence of hypertension, and NHR lines using whole cell patch clamp techniques.

## METHODS

### Animals and housing

All experimental procedures involving animals (NHR and HHR) were conducted in the period of 1998-2002. The procedures are in accordance with the guidelines of the NHMRC of Australia's code of practice for the care and use of animals for scientific purposes (1990) and The Prevention of Cruelty to Animals Act 1986.

Approval from the University of Melbourne's Animal Experimental and Ethics Committee was granted for the experiments conducted in this study as part of the requirement for pursuing PhD degree in the Department of Physiology, Faculty of Medicine-University of Melbourne.

Male rats of the HHR and the NHR from the newly developed strains previously described,<sup>11</sup> were obtained from the Biological Research Facility (BRF) at the University of Melbourne. The HHR and NHR were housed under standard conditions with a 12-hour light/dark cycle and room temperature maintained at 18-21°C. Animals received standard laboratory diet (Clarke King, Australia) and tap water *ad libitum*.

### Animal groups/cell groups

To evaluate  $I_{\text{Ca}}$ , myocytes were obtained from age matched male rats 19-25 week NHR and HHR (body weights 300-450 g) from generations of F17-F18. The tissue removal and cell isolation procedures are described below. All cells were studied within 8-10 hours of isolation, i.e. NHR = 12 cells (from 9 rats) and HHR = 18 cells (from 7 rats).

### Cell Isolation

Left ventricular myocytes were enzymatically dissociated by a technique previously described.<sup>11</sup> For anesthesia, rats were injected intra peritoneally with pentobarbitone sodium (Nembutal 6.5 mg/100g body weight). When the rat was unconscious the heart was rapidly excised and immersed into warm 95% $\text{O}_2$ /5% $\text{CO}_2$  (carbogen)-buffered Krebs-Hensleit (KH) Solution containing (in mM): DecaKrebs (118 NaCl, 4.8 KCl, 1.2  $\text{KH}_2\text{PO}_4$ , 1.2  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 11 glucose, 25  $\text{NaHCO}_3$  with 2.5  $\text{CaCl}_2$ , a physiologic concentration of calcium to allow spontaneous heart contraction and blood ejection to assist in perfusion of the coronary arteries. After trimming the excess fat/vascular tissue, the heart was quickly attached to a Langendorff apparatus for aortic cannulation and retrograde perfusion with 200 ml Ca-free KH solution, delivered by gravity from a height of 75 cm to clear the coronary arteries and wash out any residual blood. The perfusate was maintained at 37°C with water jacket deliver system and gassed with carbogen to maintain pH 7.4. The heart was then treated with perfusate containing 0.5 mg/ml collagenase (Worthington Type II), recycled for 20 minutes to produce enzymatic digestion. When flaccid and translucent, the heart was transferred to a Petri dish for removal of the atria and right ventricle. The left ventricle was minced and transferred to Erlenmeyer flask containing 10 ml KH-collagenase and 1 mM  $\text{CaCl}_2$  recycling solution. The flask was gently agitated in a

shaking water bath at 36°C for 2 minutes. The supernatant was discarded and two other 1 minute incubations with 10 ml aliquots of a calcium-free KH-Collagenase solution were repeated. After the last incubation, the flask contents were filtered through a 150  $\mu$ m gauge nylon mesh and poured into 10 ml tube to allow the cells settle. This cell aliquot was rinsed several times with Na-HEPES buffered solution containing DecaKrebs, 11 mM glucose and 25 mM Na-HEPES, and the  $Ca^{2+}$  concentration was progressively increased to 1 mM. The isolated cells were finally resuspended at room temperature in Na-HEPES buffered solution containing 1 mM  $Ca^{2+}$  and 0.025 mg/ml trypsin inhibitor (Worthington Biochemical Corporation). Rod shaped and striated cells were selected for patch clamping experiments.

### Cardiomyocyte whole cell patch clamp current measurement

In this study "whole cell patch" clamp technique, first described in 1981 by Hamill *et al.*,<sup>12</sup> was employed with slightly modification.<sup>13</sup> Membrane capacitance ( $C_m$ ) was measured from response to  $\pm 5$  mV steps from -90 mV holding potential (HP), using method previously described.<sup>14</sup> Sarcolemmal L type  $Ca^{2+}$  currents ( $I_{Ca(L)}$ ) were measured under whole cell voltage clamp (25°C), using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) coupled to an A-D amplifier (Digidata 1200B, Axon Instruments) with 1-3 M $\Omega$  pipette resistances (glass type TW150F-3, World Precision Instruments, Sarasota, FL). Patch pipette solution contained (mM): CsCl 100, TEA-Cl 20, MgATP 5, Li-GTP 0.2, EGTA 10, HEPES 10, and pH 7.4 (adjusted with CsOH, at 25°C).

During the formation of giga-ohm seals and initial break-in to the whole-cell voltage clamp configuration, left ventricular cells were perfused with normal Tyrode's solution containing (mM): NaCl 140, KCl 6, MgCl<sub>2</sub> 1, Glucose 10, HEPES 5, CaCl<sub>2</sub> 1, pH 7.4 (adjusted with NaOH, at 25°C). When cell access was obtained, perfusion medium was switched to the external recording solution which was Na<sup>+</sup>- and K<sup>+</sup>-free. In this recording solution, NaCl and KCl were replaced by TEA-Cl and CsCl (pH 7.4 with CsOH, 25°C) and Ca increased to 2 mM.

For  $I_{Ca(L)}$  measurement test pulses (400 ms, interval 2 s, 10 mV increments up to +60mV) were applied to identify the currents available at holding potential (HP) -50 mV.  $I_{Ca(L)}$  currents were measured as the difference between maximum inward current and baseline current ( $I_{Ca}$  inactivation complete) at the end of each test voltage.<sup>15</sup> Current analyses were performed using Clampfit in pClamp8. Currents were normalized by

membrane capacitance ( $C_m$ ) to calculate comparative current densities.<sup>15</sup>

### Data analysis and statistics

Current analysis and curve fitting procedures were performed using Clampfit in pClamp8. Peak current amplitude was measured as the difference between peak inward current and the steady state current i.e current at the end of the depolarizing step of 400 ms pulse. Manipulation of data to produce current-voltage plots was performed using Excel (Microsoft Excel 2002).

For each experimental group, currents elicited at HP were pooled for each test potential of +10mV increments. For calculation of current density calcium currents were normalized by membrane capacitance ( $C_m$ ) values. To compare the  $Ca$  currents obtained at HP -50 mV in cells of different animal types, data were analyzed by one-way ANOVA (repeated measures). Student's t-test was applied for analyzing  $C_m$ . All results are presented as mean  $\pm$  SEM. Values of  $p < 0.05$  were considered to indicate statistical significance.

## RESULTS

### Membrane capacitance ( $C_m$ )

The mean membrane capacitance ( $C_m$ ) of the HHR myocytes from which  $Ca^{2+}$  current recordings were obtained was greater but not statistically significant different to the mean capacitance value measured from NHR myocytes. HHR myocyte capacitance was about 20% greater than NHR capacitance ( $364.3 \pm 22.33$  pF;  $n=18$  cells/7 rats vs  $300.2 \pm 27.78$  pF;  $n=12$  cells/9 rats,  $p=0.08$ , student's t-test).

### Measured currents

Figure 1 shows examples of representative  $I_{Ca}$  traces obtained from NHR and HHR myocytes in response to the application of the voltage clamp protocols at each of the holding potential -50 mV with +10 mV incremental test voltage steps. After the initial fast capacitance surge, an envelope of calcium currents inactivating usually within 200 ms of the voltage clamp was observed.

Calcium current-voltage (I-V) relationships for representative single cell current sets are presented in Figure 2. It is clearly evident that the amplitude of  $I_{Ca}$  in the HHR cell is larger than in the NHR cell at HP -50 mV protocol.

The mean  $I_{Ca(L)}$  of 12 NHR cells was compared to the mean  $I_{Ca}$  of 18 HHR cells at HP -50 mV (Figure 3). Currents elicited from HP -50 mV, presumed to constitute only  $I_{Ca(L)}$  subtype, were peaked at 0 mV and

found to be significantly larger in the HHR ( $-2626.1 \pm 227.0$  vs  $-1327.0 \pm 286.3$  pA,  $p < 0.002$ ).

#### Mean peak current densities of $I_{Ca(L)}$

To determine the extent to which the observed increases in the whole cell  $I_{Ca(L)}$  in HHR are independent of myocyte size *per se*, current densities (current per unit membrane capacitance, pA/pF) were calculated. The measured  $I_{Ca(L)}$  was normalized to the cell capacitance

and the mean of these normalized calcium currents were plotted against their corresponding test voltages (Figure 4). For the 12 NHR and 18 HHR cells, the peak density of  $I_{Ca(L)}$  (pA/pF) recorded from HP -50 mV was significantly larger in the HHR LV myocytes. At the peak of the current-voltage curve (-10 mV) obtained from HP -50 mV the mean value of peak HHR  $I_{Ca(L)}$  density was increased 65% compared to those of NHR ( $-7.74 \pm 0.86$  vs NHR  $-4.68 \pm 1.09$  pA/pF,  $p < 0.02$ ).

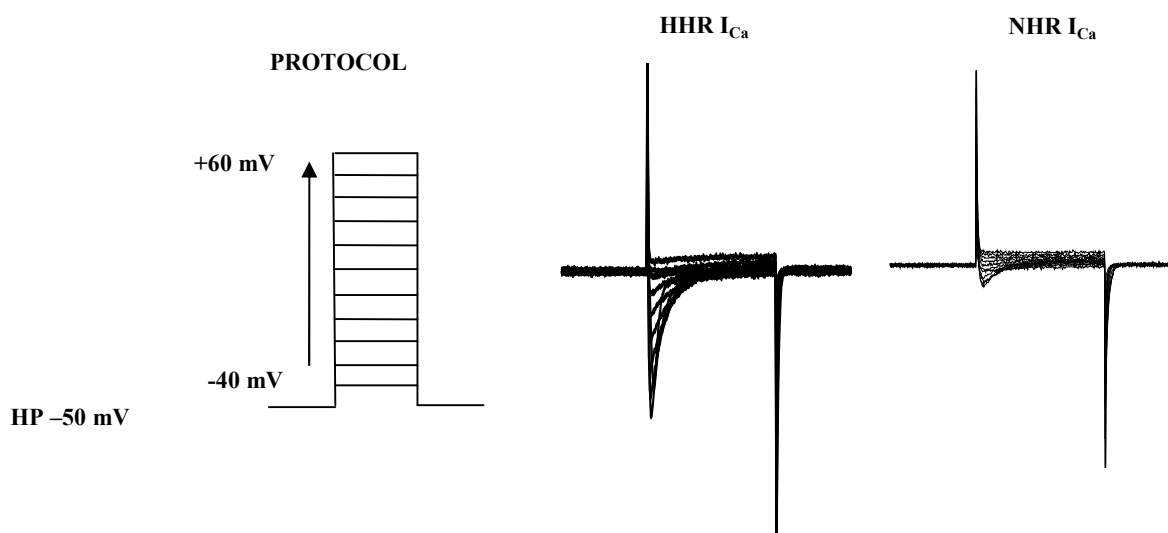


Figure 1. Voltage step protocol to measure calcium currents ( $I_{Ca}$ ) and typical resultant of  $I_{Ca}$  for NHR and HHR. Current records were obtained from representative NHR and HHR myocytes, at holding potential (HP) -50 mV. Holding potential of -50 mV activates L-type only. Calcium currents of NHR cardiomyocytes were smaller than the HHR.

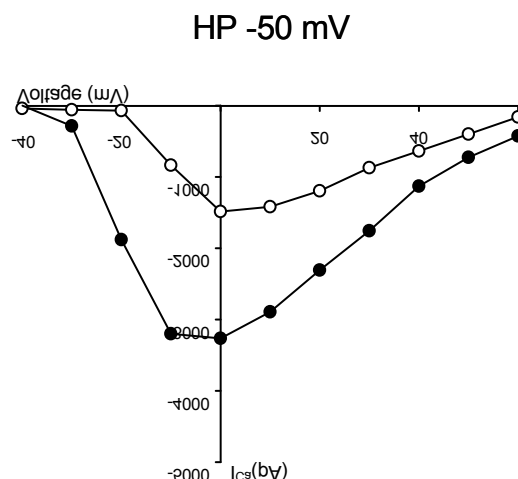


Figure 2. Calcium current-voltage plots of NHR and HHR single myocytes. Individual  $I_{Ca}$ -voltage plots of NHR (o) and HHR (•) were obtained at holding potential (HP) -50 mV.

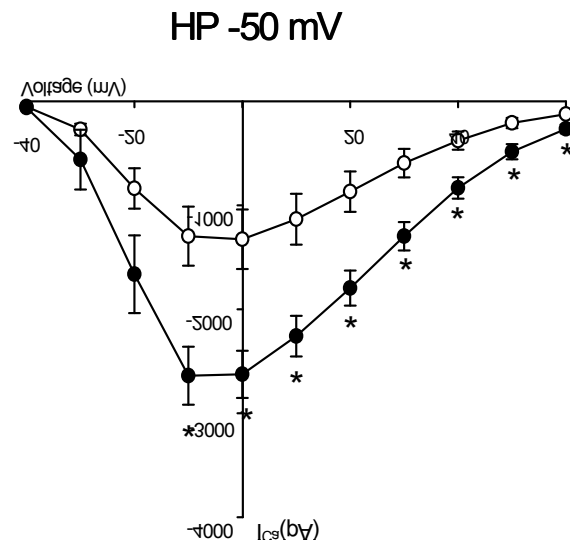


Figure 3. Mean calcium current-voltage plots of NHR and HHR myocytes. Mean cell  $I_{Ca}$ -voltage plots of NHR (o) and HHR (•) were obtained at holding potential (HP) -50 mV. \* =  $p < 0.05$  one way ANOVA repeated measures.

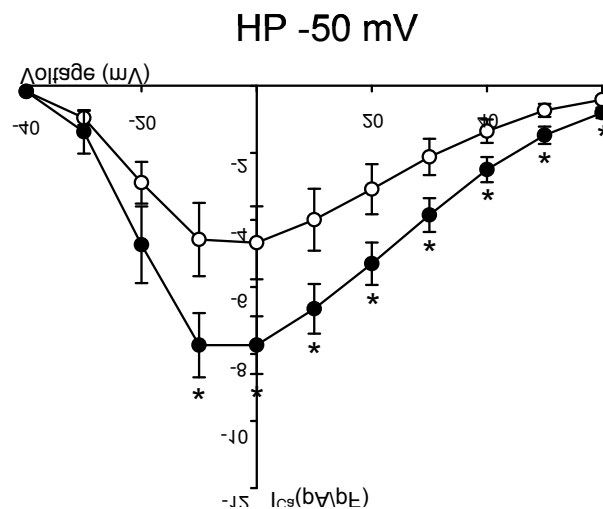


Figure 4. Mean normalised calcium current-voltage plots of NHR and HHR myocytes. Mean current density plots of NHR (o) and HHR (•) were obtained at holding potential (HP) -50 mV. \* =  $p < 0.05$  one way ANOVA repeated measures.

## DISCUSSION

### The increase in total $I_{Ca(L)}$

The observed increase in myocyte peak  $I_{Ca(L)}$  amplitude in the HHR is unlikely to be a result of an increased cell surface area because the mean membrane capacitance of the HHR cardiomyocytes in this particular series of experiments was not statistically different (although increased by 20%). Thus the increase in total  $I_{Ca}$  density in the HHR must be intrinsic to the HHR cellular

phenotype, and not an indirect consequence of cell size. Data presented previously demonstrates that HHR myocytes are larger than NHR myocytes when an unbiased sampling procedure is used.<sup>11</sup> Larger myocytes can be difficult to seal and it is not surprising that the subgroup of cells used in these experiments did not show a significant size difference. Therefore, the differences in  $I_{Ca}$  density of HHR and NHR can be related to the hypertrophic stimulus *per se* rather than to some cell size dependent phenomenon.

### The increased $\text{Ca}^{2+}$ influx in the HHR

In many animal and human models of ventricular hypertrophy, findings in relation to  $\text{Ca}^{2+}$  current levels vary, perhaps reflecting the complex and differential contributions of hemodynamic and neurohumoral disturbance,<sup>16-20</sup> and also species difference,<sup>21,22</sup> in these models. In the present study, the defined genetic contrast between the model of HHR and NHR, in the context of a matched milieu as previously observed,<sup>23</sup> demonstrates the electrophysiological effects of cardiac hypertrophy modulation without any influence of hypertension. Thus a definitive characterization of the excitation-contraction coupling defects linked with hypertrophy *per se*, independent of systemic perturbation, can be achieved. This study is the first direct evaluation of  $\text{Ca}^{2+}$  ionic fluxes in cardiomyocytes of a HHR and NHR model.

In the cardiomyocytes of HHR, the mean peak  $I_{\text{Ca}}$  measured was almost double that observed in the NHR myocytes using the protocol with HP -50 mV. Increased mean peak total  $I_{\text{Ca}}$  observed at this HP could be attributed mainly to the  $I_{\text{Ca(L)}}$ , confirmed by the peak of the current at around 0 mV in rat.<sup>24,25</sup> Thus, at the peak of the current-voltage curve the increase in mean HHR  $I_{\text{Ca}}$  density would be expected to comprise entirely  $I_{\text{Ca(L)}}$ , without any contamination of the  $I_{\text{Ca(T)}}$  or the so called  $I_{\text{Ca(TTX)}}$  or LVACC (low voltage activated calcium current) which normally appear at the HP -90 mV and -115 mV subsequently.<sup>26,27</sup>

In the cases of severe hypertrophy or failure, findings in animal models are parallel with human models particularly for the calcium currents.<sup>22,28,29,30</sup> It is likely that when signs of cardiac decompensation or failure emerge,  $I_{\text{Ca(L)}}$  density was found to be unchanged or even decreased. Thus the increase in  $I_{\text{Ca(L)}}$  density seen in the cardiomyocytes of HHR with moderate hypertrophy might reflect adaptation in the compensated state of cardiac hypertrophy for controlling or maintaining the E-C coupling, whereas reduction in  $I_{\text{Ca(L)}}$  density seen in the other models of severe hypertrophy may be an important sign in the transition from compensated hypertrophic state to congestive heart failure. Further investigation of cardiomyocyte contractile performance based on these electrophysiological findings would be informative.

Calcium entry through the L-type calcium channel is a critical first step in the  $\text{Ca}^{2+}$  activation cycle which controls the force of cardiomyocyte contraction during the action potential. Enhanced  $\text{Ca}^{2+}$  entry during depolarization arising from increased  $I_{\text{Ca(L)}}$  density in the HHR may indicate an increase in the number of channels per unit membrane, increased open probability

of individual channels and/or increase in unitary single channel conductance.

The  $I_{\text{Ca(L)}}$  is also one of inward current candidates for initiating arrhythmia-triggering after depolarizations in electrically remodelled myocardium.<sup>31,32</sup> In transgenic mice overexpressing calmodulin kinases (CaMK II and IV) increased  $I_{\text{Ca(L)}}$  density and L-type  $\text{Ca}^{2+}$  channel open probability is associated with triggered early after depolarizations, EADs.<sup>33</sup> Further investigation of the HHR action potential (AP) characteristics would be required to determine the arrhythmogenic effects of the large increase in  $I_{\text{Ca}}$  observed. In addition, HHR cardiomyocytes of older rats may offer a useful model to study  $\text{Ca}^{2+}$ -related arrhythmias, as at senescence the HHR exposure to chronically high  $I_{\text{Ca(L)}}$ , would be expected to be linked with heart failure. Longer term survival studies of HHR would be required to establish this link.

Study of a transgenic mouse overexpressing the  $\alpha 1$  subunit of L-type  $\text{Ca}^{2+}$  channel provides evidence that increased  $I_{\text{Ca(L)}}$  has profound cardiac effects, as in this model full blown cardiomyopathy and heart failure are associated with accelerated apoptosis of cardiomyocytes.<sup>34</sup> In these transgenic mice at the age of 8 weeks an increase in  $\text{Ca}^{2+}$  current was accompanied by an increase in basal contractility and activation of the  $\beta$ -adrenergic signalling pathway. In older mice, sustained  $\text{Ca}^{2+}$  increase had significant impact (ventricular fibrosis, apoptosis) on ventricular remodelling with premature progression from moderate hypertrophy to severe cardiomyopathy and cardiac failure.<sup>35</sup> Since HHR cardiomyocytes have moderate increase in cell dimensions, membrane capacitance, with unaltered basal contractility and therefore no overt sign of heart failure up to 35 weeks, as confirmed also by previous reports,<sup>11,23</sup> but yet have significant increase in  $\text{Ca}^{2+}$  current, this model may be characteristic of slowly progressing human cardiac disease. The HHR may be a valuable addition to our tools for further studying of mechanisms of cardiac hypertrophy and progression to end stage heart failure.

### CONCLUSIONS

The HHR peak Ca current density, evoked from holding potential of -50 mV, is significantly greater than the NHR. At test potential -10 mV, HHR  $I_{\text{Ca}}$  was approximately 1.6 times that of the NHR (-7.74±0.86 vs -4.68±1.09 pA/pF). This increase is attributed primarily to L-type Ca current which is known to peak at this region of the current-voltage relation. The dramatic increase in cardiomyocyte sarcolemmal calcium influx in the HHR provides evidence of altered excitation

contraction coupling processes in primary cardiac hypertrophy.

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**Synopsys**

The L-type calcium current ( $I_{Ca-L}$ ) from the primary hypertrophied left ventricular cardiomyocytes of 'HHR' rats is larger than the  $I_{Ca-L}$  from normal size left ventricular cardiomyocytes of 'NHR' rats.

**Sinopsis**

Arus kalsium tipe L ( $I_{Ca-L}$ ) dari kardiomiosit ventrikel kiri yang mengalami hipertrofi primer pada tikus 'HHR' adalah lebih besar dibandingkan dengan  $I_{Ca-L}$  dari kardiomiosit ventrikel kiri yang berukuran normal pada tikus 'NHR'.