Korean J Physiol Pharmacol
Vol 4: 197−210, June, 2000

Relatoinship between Sarcoplasmic Reticular Calcium Release and Na^+-Ca^{2+} Exchange in the Rat Myocardial Contraction

Eun-Gi Kim[1], Soon Jin Kim[2], and Chang Mann Ko[2]

Departments of [1]Thoracic Surgery, [2]Pharmacology, Institute of Basic Medical Science, Yonsei University Wonju-College of Medicine, Wonju 220−701, Korea

Suppressive role of Na^+-Ca^{2+} exchange in myocardial tension generation was examined in the negative frequency-force relationship (FFR) of electric field stimulated left atria (LA) from postnatal developing rat heart and in the whole-cell clamped adult rat ventricular myocytes with high concentration of intracellular Ca^{2+} buffer (14 mM EGTA). LA twitch amplitudes, which were suppressed by cyclopiazonic acid in a postnatal age-dependent manner, elicited frequency-dependent and postnatal age-dependent enhancements after Na^+-reduced, Ca^{2+}-depleted (26 Na-0 Ca) buffer application. These enhancements were blocked by caffeine pretreatment with postnatal age-dependent intensities. In the isolated rat ventricular myocytes, stimulation with the voltage protocol roughly mimicked action potential generated a large inward current which was partially blocked by nifedipine or Na^+ current inhibition. 0 Ca application suppressed the inward current by 39±4% while the current was further suppressed after 0 Na-0 Ca application by 53±3%. Caffeine increased this inward current by 44±3% in spite of 14 mM EGTA. Finally, the Na^+ current-dependent fraction of the inward current was increased in a stimulation frequency-dependent manner. From these results, it is concluded that the Ca^{2+} exit-mode (forward-mode) Na^+-Ca^{2+} exchange suppresses the LA tension by extruding Ca^{2+} out of the cell right after its release from sarcoplasmic reticulum (SR) in a frequency-dependent manner during contraction, resulting in the negative frequency-force relationship in the rat LA.

Key Words: Na^+-Ca^{2+} exchange, Sarcoplasmic reticulum, Ca^{2+} extrusion, Negative frequency-force relationship, Rat heart

INTRODUCTION

Na^+-Ca^{2+} exchange and sarcoplasmic reticulum (SR), two major contributors to the myocardial contraction and relaxation, work either cooperatively or competitively during cardiac cycle. They cooperate with each other, when Ca^{2+} entering the cell through Na^+-Ca^{2+} exchange in reverse-mode (Ca^{2+} entry-mode: Na^+ outflux vs. Ca^{2+} influx) triggers the Ca^{2+} release from SR to raise intracellular free Ca^{2+} ($[Ca^{2+}]_i$) during contraction (LeBlanc & Hume, 1990). While, during relaxation, the Na^+-Ca^{2+} exchange in forward-mode (Ca^{2+} exit-mode: Na^+ influx vs. Ca^{2+} outflux) competes with the SR Ca^{2+}-ATPase for the cytosolic Ca^{2+} to decrease $[Ca^{2+}]_i$ to the resting level (Bers et al, 1996).

Another possibility in the Na^+-Ca^{2+} exchange and SR interrelationship may be raised in the rat heart. It has been reported that there is a Na^+-Ca^{2+} exchange-dependent Ca^{2+} compartment in the subsarcolemmal space of the triadic region in the rat heart (Langer & Rich, 1992; Langer et al, 1995). In the compartment, Ca^{2+} may be trapped by the negatively charged phospholipid in the subsarcolemmal space after it is released from SR. Therefore, a certain fraction of the Ca^{2+} is extruded out of the cell via Na^+-Ca^{2+} exchange before it diffuses to and interacts with the myofilament to trigger the contraction (Langer & Rich, 1992; Langer et al, 1993; Wang et al, 1996;

Corresponding to: Chang Mann Ko, Department of Pharmacology, Yonsei University Wonju-College of Medicine, 162 Ilsan-Dong, Wonju 220-701, Korea. (Tel) 82-33-741-0301, (Fax) 82-33-742-4966, (E-mail) changmko@wonju.yonsei.ac.kr

Peskoff & Langer, 1998). If this is the case, the Na^+-Ca^{2+} exchange in the Ca^{2+} exit-mode is able to curtail the $[Ca^{2+}]_i$ rise and therefore the contractile tension generation during contraction in the rat heart. This may give rise to the third relationship. However, it has not been demonstrated to date that the actual current generated by Ca^{2+} exit-mode Na^+-Ca^{2+} exchange during depolarization.

The present study was aimed to clarify the suppressive role of Ca^{2+} exit-mode Na^+-Ca^{2+} exchange by interacting with SR, the third relationship, in the left atrial contraction of the rat heart. In order to pursue this aim, two series of experiments have been performed. First, the involvement of Ca^{2+} exit-mode Na^+-Ca^{2+} exchange has been tested in the frequency-dependent decrease in contractile force in the electrically stimulated left atria (LA) from postnatal developing rats. The negative frequency- force relationship (FFR) was used because the activity of Na^+-Ca^{2+} exchange is affected proportionally to the frequency increase (Harrison & Boyett, 1995; Lostan et al, 1995; Maier et al, 1997). Second, the inward current generated by the Ca^{2+} exit-mode Na^+-Ca^{2+} exchange during depolarization have been pharmacologically dissected and its frequency dependence was tested by using whole-cell mode patch-clamp technique with high concentration of intracellular Ca^{2+} buffer in the adult rat ventricular myocytes.

METHODS

Sprague-Dawley rats of either sex weighing 250 g were purchased from Jung-Ang Lab. Animal Co. (Seoul, Korea). Some were bred and mated in the animal room in Wonju-College of Medicine, Yonsei University. Post-partum neonatal rats were kept with their mothers until sacrificed by decapitation at appropriate times after birth. The others were anesthetized with pentobarbital sodium and sacrificed to isolate ventricular myocytes. Food and water were ad libitum.

Left atrial contraction

Left atrial preparation

Postnatal rats at 1-day, 1-week and 4-weeks after birth were chosen, since the rat myocardial SR undergo functional and histological maturation during the first 4 weeks after birth (Olivetti et al, 1980; Vetter et al, 1995; Koban et al, 1998). Rats were sacrificed by decapitation and the hearts were quickly excised and suspended in oxygenated Krebs-Hensleit buffer (KHB). Left atria were cautiously dissected and mounted in 8ml organ baths. KHB was bubbled with mixed gas of 95% O_2 and 5% CO_2. The compositions of gases were closely regulated by flow regulator (Cole-Palmer, USA) to maintain pH=7.40$\pm$0.03. Left atrial contraction was evoked by electrical field-stimulation delivered through platinum electrodes in square wave pulses of 3 Hz, 0.5 msec duration with supramaximal voltages by digital stimulator (STM-1000, Hansung, Korea). As the stimulation frequency affects the SR Ca^{2+} repletion (Frampton et al, 1991), the resting stimulation was applied at 3 Hz throughout the experiment in order to maintain the same level of SR Ca^{2+} store. The KHB was changed at 10 minute-intervals throughout the experiment, if not mentioned. LA twitch amplitude was recorded on Polygraph (Model 7, Grass, USA) via force displacement transducer (FT. O3, Grass, USA).

Frequency-force relationship

Before starting the series of experiment, maximal twitch amplitude attained after NE 10^{-6} M application was measured during 3 Hz stimulation. After resting for 20 minutes in normal KHB, the stimulation frequency was abruptly reduced from resting 3 Hz to the test frequency for 5 minutes and was returned to 3 Hz again. The test frequency was applied at 0.1, 0.3, 0.5, 0.7 and 1 Hz. The maximal amplitudes (PEAK) and the last amplitudes (LAST) at 5 minutes during stimulation frequency reduction to the test frequencies for 5 minutes were measured. The measured amplitudes were calculated into the percentages (%) to the maximal amplitude attained by NE 10^{-6} M at 3Hz stimulation and expressed as % tension. The FFR for the peak and the last amplitudes over the test frequencies were plotted. In the case of Na^+-Ca^{2+} exchange inhibition, the KHB with Na^+=26 mM (17% of control) and Ca^{2+}=0 mM (26 Na-0 Ca KHB) was applied rapidly two times in order to wash out the remnants and the frequency was reduced to the test frequency without any delay for minimal duration just required to recognize the maximal twitch amplitude attained (usually less than 2 minutes). Right after, normal KHB was applied and the frequency was returned to 3 Hz again. Prior to 26 Na-0 Ca KHB application at each test frequencies, its own control amplitude was measured with normal

KHB in order to calculate the % changes and to check up any critical Ca^{2+} reapplication-induced damages in the left atrial strips. The composition of KHB solution was as follows (mM); NaCl 118.8, KCl 4.70, $CaCl_2$ 2.52, $MgSO_4$ 1.16, $NaHCO_3$ 24.88, KH_2PO_4 1.18, Glucose 5.55, Na-Pyruvate 2.0. In the 26 Na-0 Ca KHB, $CaCl_2$ was just omitted and NaCl was substituted with equimolar choline chloride (As still there were $NaHCO_3$ and Na-Pyruvate, $[Na^+]$ was 26 mM which was 17% of $[Na^+]$ in control solution.).

Drug application

Ryanodine or cyclopiazonic acid was pretreated for 40 minutes prior to frequency change. In the case of other drugs, the test frequencies were applied 1 minute after the drug effects were stabilized (usually around 5 minutes). All the drugs were purchased from Sigma Chemical Co. (MO, USA).

Whole-cell mode patch-clamp experiment

Single ventricular myocytes

Adult rat ventricular myocytes were isolated according to Mitra and Morad (Mitra & Morad, 1985). Briefly, rats were deeply anesthetized with pentobarbital sodium (50 mg/kg, ip) and hearts were excised quickly and perfused at 6 mL/min in a Langendorff apparatus first with Ca^{2+}-free Tyrode solution composed of (in mM) 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$ and 10 glucose, pH=7.4 at 37°C for 8 min, then with Ca^{2+}-free Tyrode solution containing collagenase (type 2, Worthington: 250 U/ml) and protease (type XIV, Sigma: 0.55 U/ml) for 15 min, and finally with Tyrode solution containing 0.2 mM $CaCl_2$ for 8 min. The ventricle of the digested heart was then cut into several sections and subjected to gentle agitation to dissociate cells. The freshly dissociated cells were stored at room temperature in Tyrode solution containing 0.2 mM $CaCl_2$ and were used for <10 Hr after isolation. All the experiments were performed at room temperature.

Current recording

Inward current, generated by depolarization with the 10 msec rectangular pulse from holding potential of 85 mV to 50 mV and the following 100 msec ramp pulse to -50 mV, was measured using a Axopatch 200 B amplifier (Axon, CA, USA) in the rat ventricular myocytes patch-clamped in whole-cell configuration. The patch electrodes, made of borosilicate glass capillaries, were fire polished to have a resistance of 1.5~3.0 MΩ when filled with the internal solution composed of (in mM) 110 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 10 HEPES, 5 Mg-ATP 0.1 Li-GTP, 14 EGTA, and 0.2 c-AMP, and titrated to pH=7.2 with CsOH. K^+ was replaced by Cs and TEA in order to block K^+ currents. High Ca^{2+} chelator (14 mM EGTA) was used here to limit the influences of global cytosolic Ca^{2+} and to specify the interaction between SR Ca^{2+} release and Na^+-Ca^{2+} exchange in the local microdomain, since it has been reported that this situation, high concentration of intracellular Ca^{2+} buffer, can be used to study Ca^{2+} signals in the microdomain surrounding the dihydropyridine (DHP) and ryanodine receptors without significant interference from the global cytosolic Ca^{2+} concentrations (Sham et al, 1995; Adachi-Akahane et al, 1996; Song et al, 1998). Generation of voltage-clamp protocols and acquisition of data were carried out using pCLAMP software (version 5.7.1, Axon, CA, USA). The series resistance was 1.5~3.0 times the pipette resistance and was electronically compensated through the amplifier. Sampling frequency was 0.5~2.0 kHz, and current signals were filtered at 10 kHz before digitization and storage. The membrane capacitance was measured by applying +10 mV amplitude voltage step from -70 mV and calculated according to the equation:

$$C_m = \tau_c \times I_o / \delta V_m \ (1-I_s/I_o),$$

where C_m: membrane capacitance, τ_c: time constant of the membrane capacitance, I_o: capacitative current, δV_m: amplitude of voltage step, I_s: amplitude of steady-state current (Benitah et al, 1993).

RESULTS

Na^+-Ca^{2+} exchange in postnatal developing rat left atrial contraction

Negative frequency-force relationship

Fig. 1A shows the actual procedure and results of the present experiment with electrically field-stimulated LA. Right after the frequency was reduced from resting 3 Hz to 0.1 Hz for 5 minutes, the twitch amplitudes increased rapidly until to the peak (within 30 sec) and then slowly decreased in the 4-week-old LA. The LA twitch amplitudes during frequency reduction was not totally suppressed by 0 Ca KHB application, but it was totally suppressed by the very

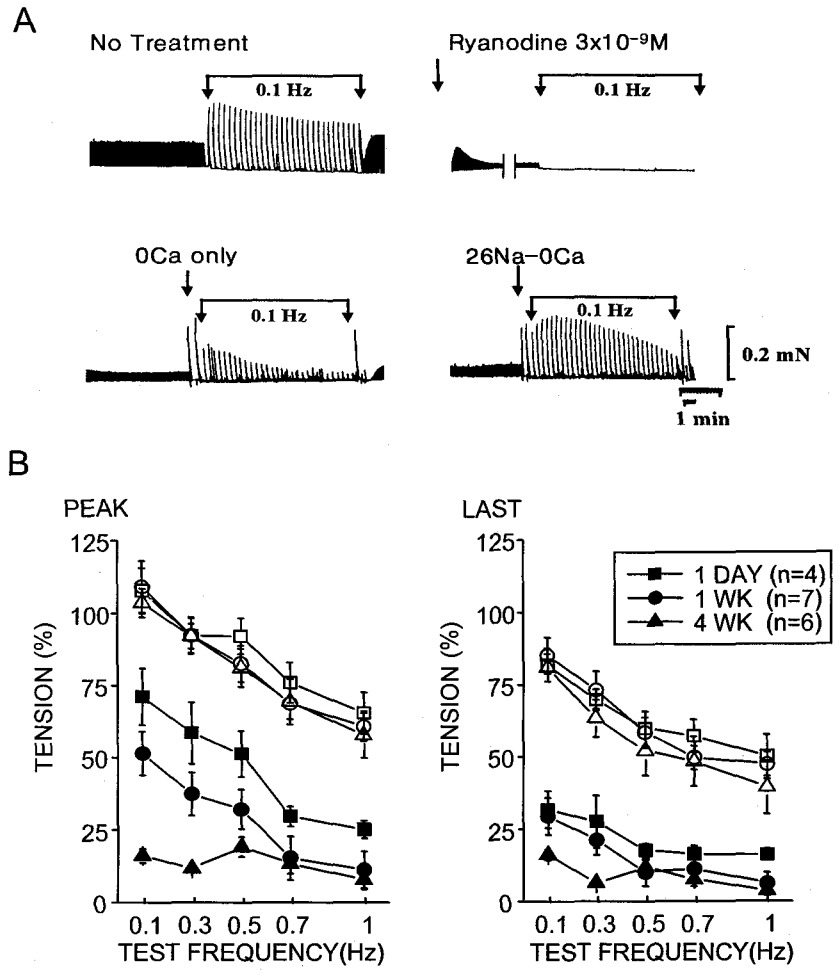

Fig. 1. Frequency reduction-induced twitch amplitude enhancement in the postnatal developing rat left atria. A. Roles of Na^+-Ca^{2+} exchange in the stimulation frequency reduction-induced twitch amplitude enhancement in the 4-week-old rat left atria. B. Postnatal age related suppression of the twitch amplitudes by cyclopiazonic acid (3×10^{-5} M) in the negative frequency-force relationship of the stimulation frequency reduction-induced twitch amplitude enhancements in the postnatal developing rat left atria. Left atrial contraction was evoked by electrical field stimulation (0.5 msec duration, supramaximal voltage) through platinum electrodes. Stimulation frequency was reduced from resting 3 Hz to test frequencies (0.1～1 Hz) for 5 minutes. Abbreviations: 0 Ca: calcium depleted Krebs Hensleit buffer (KHB), 26 Na-0 Ca: KHB with reduced Na^+ to 26 mM and Ca^{2+} depletion, Tension (%): % value of the twitch amplitude to the maximal amplitude attained after norepinephrine 10^{-6} M at 3 Hz. PEAK: maximal twitch amplitude obtained during frequency reduction. LAST: twitch amplitude at the end of 5 minute-frequency reduction. Open symbol: control, Closed symbol: cyclopiazonic acid pretreated. Values are Means±S.E.M. and parentheses are numbers of data.

low concentration of ryanodine (3×10^{-9} M) pretreatment, showing the importance of internally stored Ca^{2+} than external Ca^{2+} influx in the LA contraction during lower frequency stimulation. When 26 Na-0 Ca KHB was applied, the peak twitch amplitude was attained within 1 minute (30 sec later than control) with the same magnitude as that in control and, interestingly enough the twitch contraction never ceased for 5 minutes. Comparing with the result after 0 Ca KHB, additional reduction in Na^+ concentration

from 145 mM to 26 mM besides the Ca^{2+} depletion in the KHB caused marked twitch amplitude enhancement. The Ca^{2+} responsible for the twitch amplitude enhancement in this case should come from inside the cell, which is achievable only by inhibition of Ca^{2+} extrusion through Na^+-Ca^{2+} exchange. Therefore, these results suggest that [Na^+] reduction to 26 mM with 0 Ca in the KHB (26 Na-0 Ca KHB) applied in this experiment is enough to inhibit the Na^+-Ca^{2+} exchange, resulting in the twitch amplitude enhancement. Similar changes were obtained in 1-day- and 1-week-old LA (data not shown).

As the test frequency was gradually increased from 0.1 Hz to 1 Hz, the peak twitch amplitudes converted as % tension (to the maximal tension attained after 10^{-6} M norepinephrine during 3 Hz stimulation) decreased in inverse proportion to the test frequencies from around 100% at 0.1 Hz to 70% at 1 Hz, showing typical negative FFR in all age groups tested here without any age related differences (Fig. 1B). The last amplitudes measured at the end of the frequency reduction for 5 minutes showed similar changes with the peak amplitudes, except their magnitudes were less than 80% of their own peak amplitudes. Cyclopiazonic acid (3×10^{-6} M), a SR Ca^{2+}-ATPase inhibitor (Demaurex et al, 1992), however elicited a postnatal age-dependent-suppression of the peak twitch amplitude. At 0.1 Hz, the peak twitch amplitudes were suppressed after cyclopiazonic acid by 29%, 48%, and 84% in 1-day-, 1-week-, and 4-week-old LA, respectively. Nevertheless, the negative FFRs were still maintained after cyclopiazonic acid treatment except in the 4-week-old LA. In the last twitch amplitudes, no postnatal age-dependence in the cyclopiazonic acid-induced suppression was noted. These results apparently show that the physiological activity of SR as a Ca^{2+} source in the LA contraction increases during the first 4-weeks after birth (Olivetti et al, 1980; Vetter et al, 1995; Koban et al, 1998).

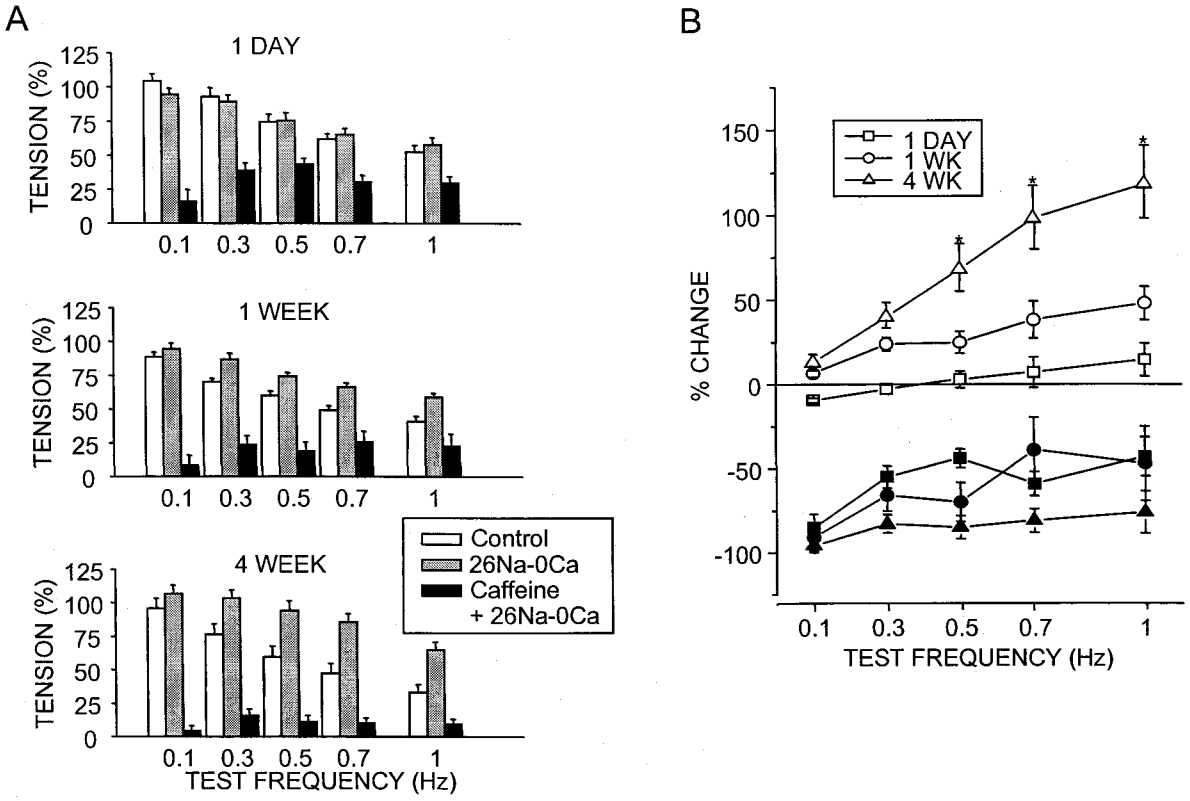

Fig. 2. Effects of Na^+-Ca^{2+} exchange inhibition on the enhancements of the twitch amplitude during stimulation frequency reduction to the test frequencies and influences of caffeine (5 mM) pretreatment on it in the postnatal developing rat left atria. Abbreviation: % change: % change of twitch amplitude attained after 26 Na-0 Ca KHB application to its own control. Numbers of data are 6. (*$p<0.05$: compare with 1-week old LA). Other legends are same as Fig. 1.

Na^+-Ca^{2+} exchange inhibition

In Fig. 2, the influences of Na^+-Ca^{2+} exchange in the FFR of the postnatal developing rat LA were tested. The magnitude of the twitch amplitude enhancements after Na^+-Ca^{2+} exchange inhibition induced by 26 Na-0 Ca KHB application increased in a postnatal age-dependent manner (Fig. 2A). The % changes of twitch amplitude enhancements to their own control were clearly in direct proportion to the test frequency and the magnitudes were remarkably increased in accordance with the postnatal age, as shown in Fig. 2B. After 5 mM caffeine pretreatment, which depletes SR Ca^{2+} store, the responses of twitch amplitudes to the 26 Na-0 Ca KHB application changed from enhancement into suppression. The intensity of caffeine-induced twitch amplitude suppression was also postnatal age-dependent (Fig. 2A). Therefore, it may be postulated fromthese results that the Ca^{2+} extrusion out of the cell through the Na-Ca^{2+} exchange increases in a stimulation frequency-dependent manner and its magnitude increases as the SR matures in postnatal developing rat LA.

Effects of positive inotropic agents

If it is the case that this frequency-dependent Ca^{2+} extrusion through Na^+-Ca^{2+} exchange increases as the SR matures, then it will curtail any $[Ca^{2+}]_i$ increase regardless of its mechanism and the resulting positive inotropy should be weakened age dependently in the postnatal developing rat heart. This possibility was pursued in Fig. 3 with norepinephrine, which increases $[Ca^{2+}]_i$ through beta-receptor stimulation. In 1-day-old LA, the negative FFR was not affected by 10^{-9} M of norepinephrine and it start to be flattened by 10^{-8} M and was completely flattened by 10^{-7} M of norepinephrine (Fig. 3A). In 4-week-old LA, however, 10^{-8} M of norepinphrine had no effect on the negative FFR. It was at 10^{-7} M that the negative FFR started to be flattened. The negative FFR was completely flattened by 10^{-6} M of norepinephrine application (Fig. 3B). Norepinephrine enhanced the twitch amplitudes in a frequency-

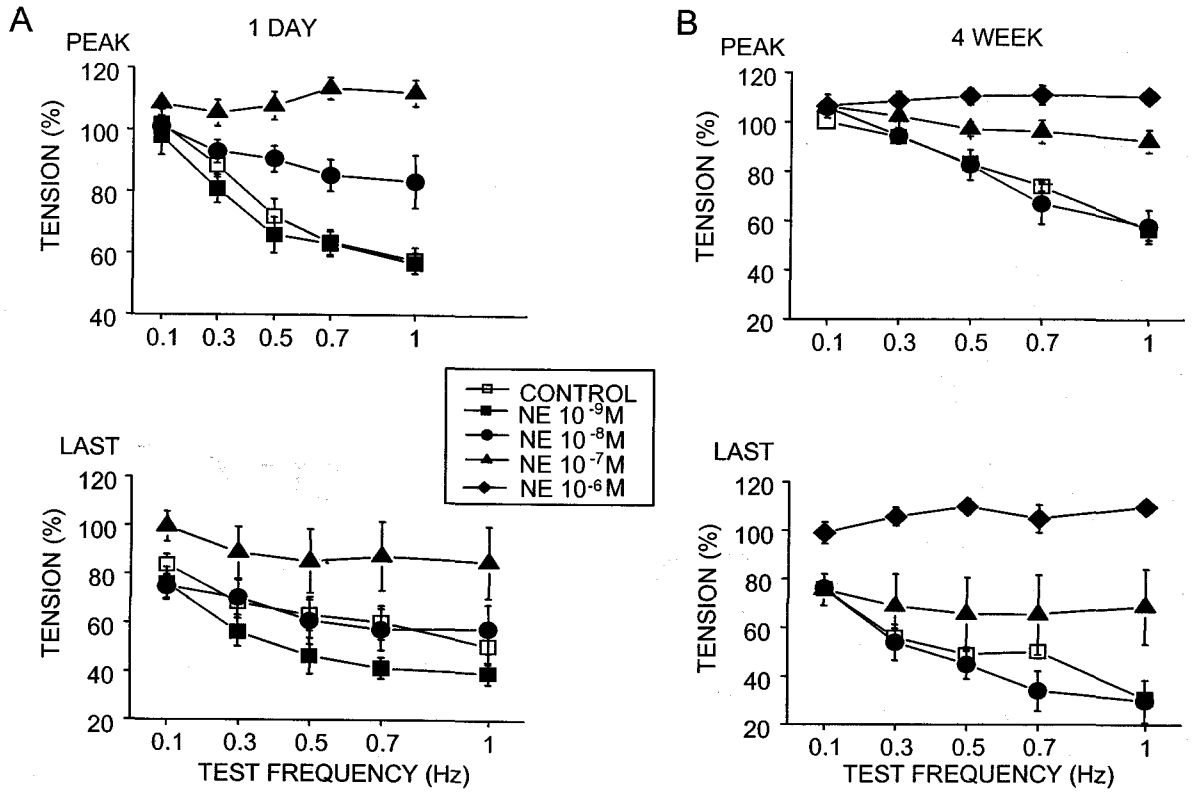

Fig. 3. Differential sensitivity of norepinephrine on the negative frequency-force relationships of the twitch amplitude enhancement induced by the stimulation frequency reduction in the 1-day- and 4-week-old rat left atria. Note 10 times higher concentrations in 4-week-old LA. Numbers of data are 6. Other legends are same as Fig. 1.

dependent manner to flatten the negative FFR. However the magnitude was weaker in 4-week-old LA so that 10 times higher concentration of norepinephrine was required in 4-week-old LA to elicit the same responses as in 1-day-old LA. Therefore, it is clear from these results that Ca^{2+} exit-mode Na^+-Ca^{2+} exchange is able to curtail the $[Ca^{2+}]_i$ increase regardless of its mechanism, and its magnitude becomes stronger as the SR matures in the postnatal developing rat LA.

Na^+-Ca^{2+} exchange in the adult rat ventricular myocyte

As the results from the postnatal developing rat LA suggested the possibility that Na^+-Ca^{2+} exchange suppresses the LA twitch tension through its Ca^{2+} exit-mode (forward mode) in stimulation frequency-dependent and SR maturity-dependent manners, the next experiment was performed to clarify the inward current generated by Ca^{2+} exit-mode Na^+-Ca^{2+} exchange and its relationship with stimulation frequency and SR Ca^{2+} release in the whole-cell clamped adult rat ventricular myocytes. High concentration of intracellular Ca^{2+} buffer (14 mM EGTA) was used to limit the Ca^{2+} diffusion time in order to exclude the influences of global Ca^{2+} and to confine the responses in the microdomain in the rat ventricular myocytes, as the high Ca^{2+} chelator (14 mM of EGTA) used in the present study does not seem to suppress the calcium-induced calcium release (CICR) significantly (Sham et al, 1995; Adachi-Akahane et al, 1996; Song et al, 1998).

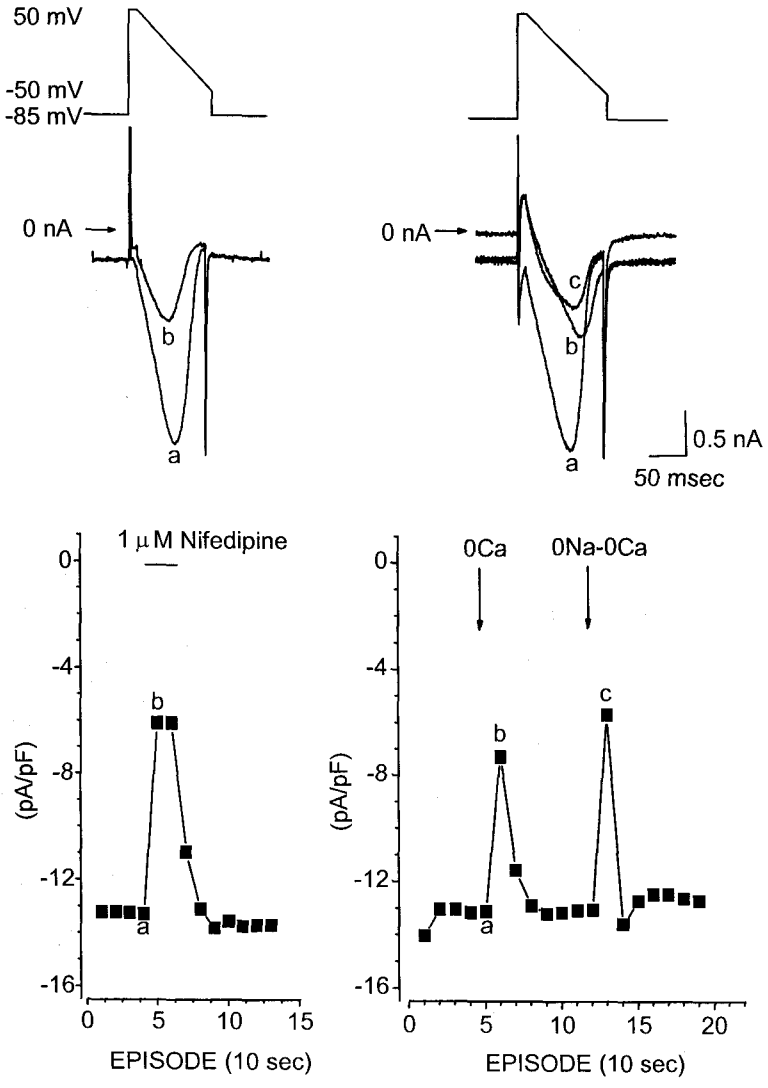

Fig. 4. Involvement of Na^+-Ca^{2+} exchange-induced inward current during depolarization with the voltage protocol roughly mimicked action potential in the rat ventricular myocytes. Rat ventricular myocytes were stimulated by the 10 msec rectangular pulse from holding potential of −85 mV to 50 mV and the following 100 msec ramp pulse to −50 mV at every 10 sec. CsCl 110 mM) and TEA (20 mM) were added in the internal solution in order to block K^+ currents and EGTA (14 mM) were added in the internal solution to limit the diffusion of Ca^{2+}.

Na^+-Ca^{2+} exchange during stimulation

When the ventricular myocyte held at 85 mV was stimulated to 50 mV by the 10 msec rectangular pulse and the following 100 msec ramp pulse to −50 mV, a large inward current was generated as shown in Fig. 4A & B. 1 mM nifedipine partially blocked this inward current by 54±2% (n=41). Nominal 0 Ca Tyrode application suppressed the inward current by 39±4% (n=10), however, the additional depletion of Na^+ besides the Ca^{2+} depletion (nominal 0 Na-0 Ca Tyrode) further suppressed the inward current by 14% reaching to 53±3% (n=6, $p<0.01$). Therefore, at least this 14% of the inward current seems to be generated purely by the Na^+-Ca^{2+} exchange. However, it cannot be ruled out that the fraction of inward current generated by the Na^+-Ca^{2+} exchange may range from 14% to 53% in the rat ventricular myocytes stimulated with the present pulse protocol. Anyway, these results clearly show that Na^+-Ca^{2+} exchange generates an inward current during depolarization in the rat ventricular myocytes.

Frequency-related changes of Na^+-Ca^{2+} exchange

The relationship between the present inward current and Na^+ influx was tested by comparing the changes in the inward current after inhibition of Na^+ current either by lidocaine (2 mM) application or by increasing the holding potential from 85 mV to 40 mV. The present inward current was suppressed by 51±3% (n=6) after lidocaine application (Fig. 5A). Similar degree of suppression was noted after holding potential change. As soon as the holding potential was increased from 85 mV to 40 mV to block the Na^+ current, the inward current decreased by 49±9% (n=7), which resumed to the previous level after returning the holding potential to 85 mV (Fig. 5B). Therefore, it is evident that a half of the inward current generated by the present protocol is dependent upon Na^+ influx. Next, the influences of $[Na^+]_i$ increase on the inward current was tested by investigating the relationship between stimulation frequency and the inward current, as $[Na^+]_i$ increases proportionally to the stimulation frequency increase

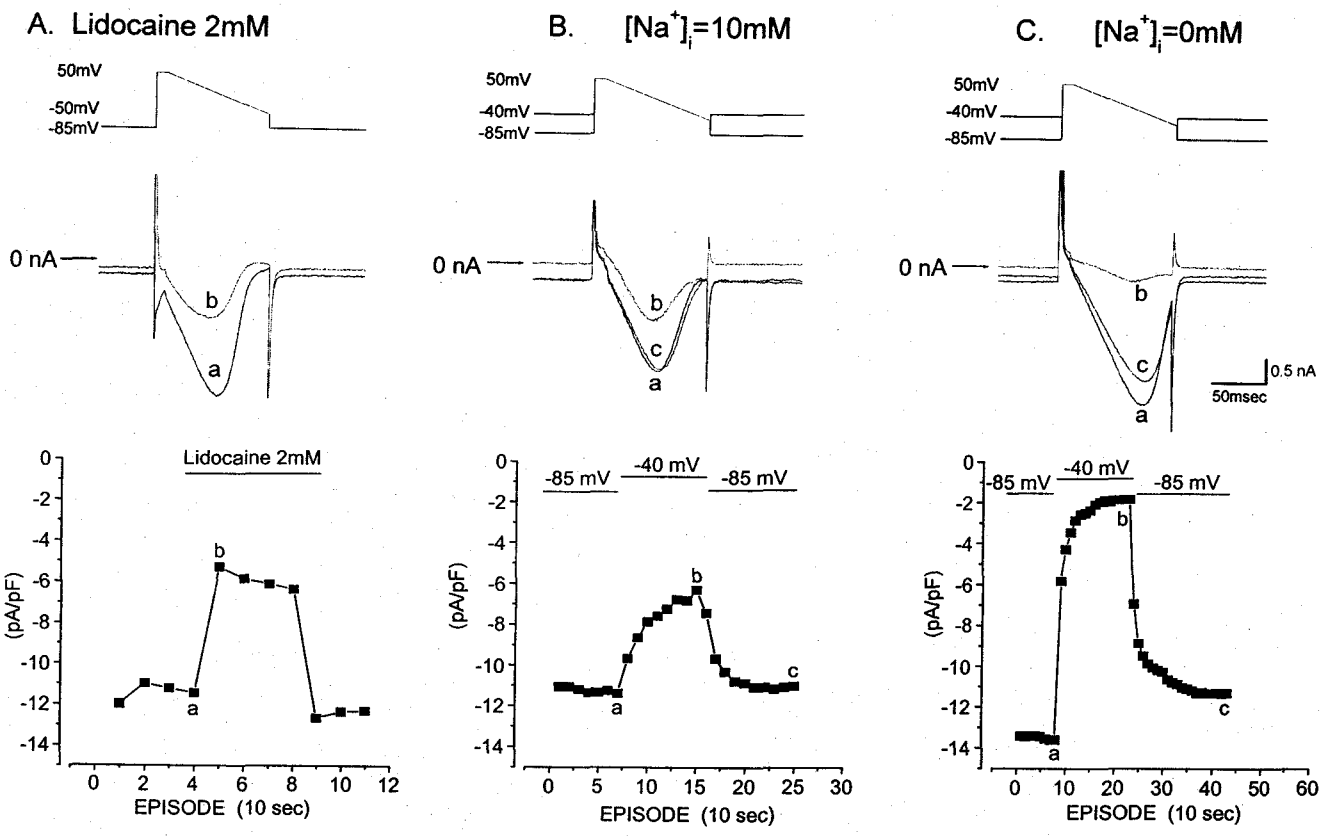

Fig. 5. Suppression of the inward current generated by the stimulation with voltage roughly mimicked action potential after Na^+ current inhibition and the influence of global Na^+ concentration on it in the rat ventricular myocytes. Na^+ current was blocked either by lidocaine or by increasing holding potential from −85 mV to −40 mV. Other legends are same as Fig. 4.

(Harrison & Boyett, 1995; Lostan et al, 1995; Maier et al, 1997). The I-V relationships were compared in between the rat ventricular myocytes held at 85 mV and 40 mV. Myocytes were stimulated using the protocol with same shape but the voltage was increased from 50 mV to 50 mV in 10 mV steps. As shown in Fig 6, the inward currents were larger in the cell held at 85 mV than in that held at 40 mV (Fig. 6A & B). Na^+ influx-dependent fraction of the inward currents, calculated as the differences between two, increased in a stimulation voltage dependent manner (Fig. 6C). Stimulation frequency-related changes in the % values of the differences to the currents held at 85 mV were plotted against the stimulation voltage in Fig 6D. Interestingly, the Na^+ influx-dependent fraction of the present inward currents increased as the stimulation frequency increased. However, when the cells were dialyzed with 0Na internal solution as shown in Fig. 5C and Fig. 6E, all these Na^+ influx-dependent inward currents were rather enhanced. These results may suggest that the $[Na^+]_i$ increase required for the stimulation frequency-dependent increase in the present Na^+ influx-dependent fraction of the inward current should be compartmentalized in the microdomain of the subsarcolemmal space in the rat ventricular myocytes, because it may be more difficult to raise the global $[Na^+]_i$ than that in micro domain when internal solution with 0 Na was applied. Therefore, it may be postulated that a half of the present inward current is dependent upon $[Na^+]_i$ increase in the subsarcolemmal space attained by the Na^+ influx during depolarization, being manipulated by stimulation frequency which affects $[Na^+]_i$ in the rat ventricular myocytes.

Influences of SR Ca^{2+} release on the inward current

The interactions of SR Ca^{2+} release and the present inward current were tested in the rat ventricular

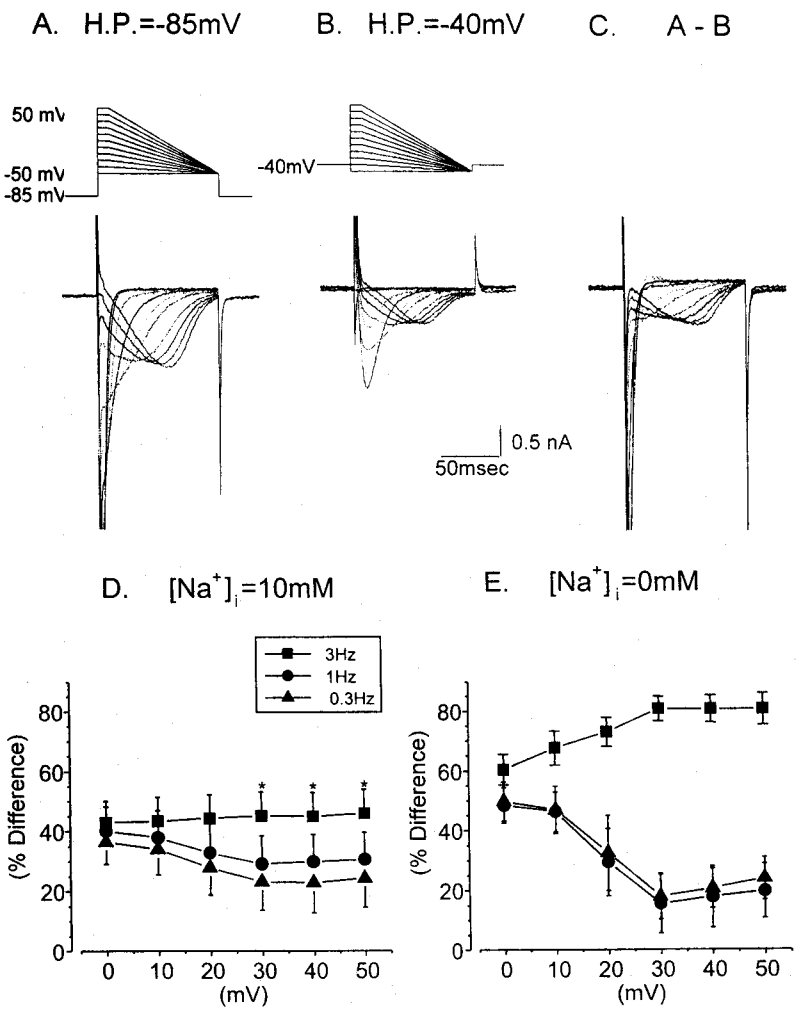

Fig. 6. Influences of stimulation frequency on the Na^+ influx-dependent inward current and the influence of global Na^+ concentration on it in the rat ventricular myocytes. Rat ventricular myocytes were stimulated using the protocol with same shape as in Fig. 4 but the stimulation voltage was increased from −50 mV to 50 mV in 10 mV steps. Other legends are same as Fig. 4.

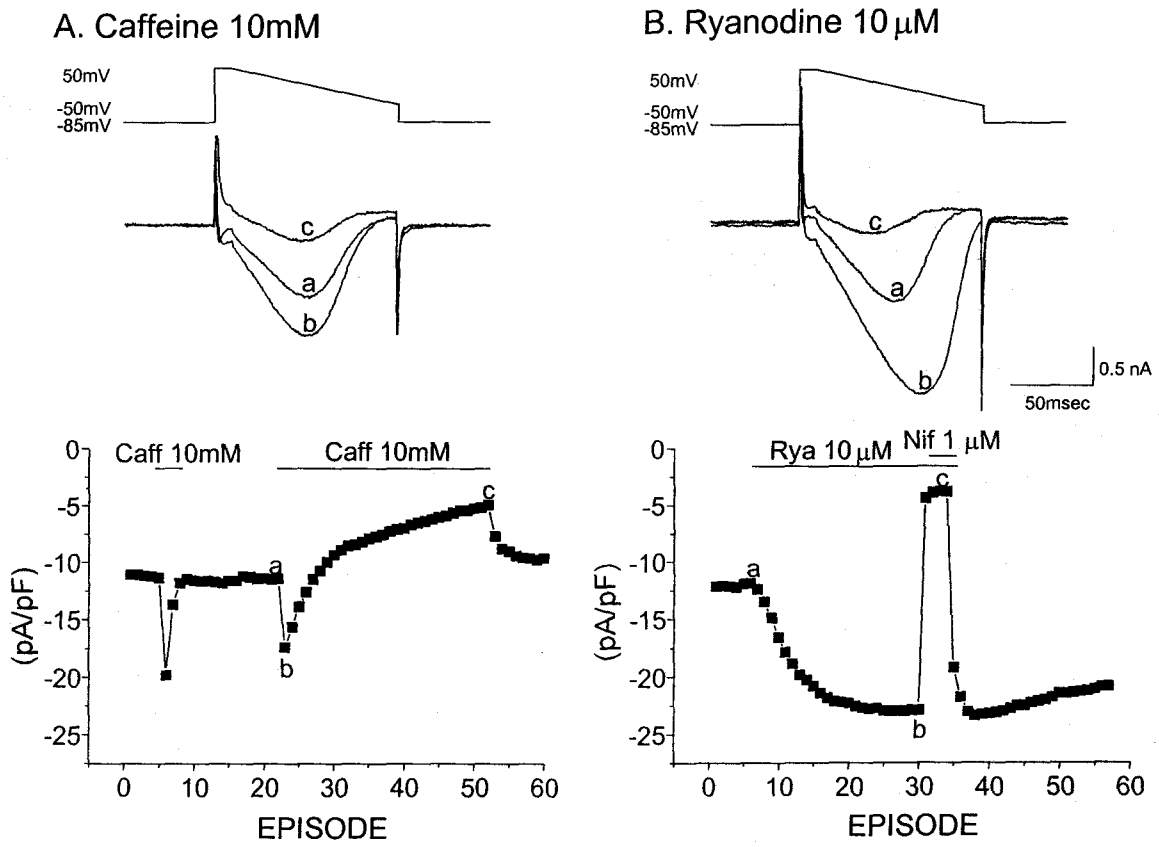

Fig. 7. Relationship between sarcoplasmic reticular Ca^{2+} release and the inward current generated by stimulation with voltage roughly mimicked action potential in the rat ventricular myocytes. Abbreviation: Caff: Caffeine, Rya: Ryanodine, Nif: Nifedipine. Other legends are same as Fig. 4.

myocytes dialyzed with internal solution containing high concentration of intracellular Ca^{2+} buffer (14 mM EGTA). As shown in Fig. 7A, application of caffeine (10 mM), which stimulates SR Ca^{2+} release and results in SR Ca^{2+} depletion, initially increased the present inward current by 44±3% (n=6), while the inward current was gradually suppressed by the prolonged application with caffeine. Therefore, these results seemed to show the dependence on the SR Ca^{2+} release of the present inward current. However, the ryanodine results conflicted this suggestion. After 10 μM ryanodine, a SR Ca^{2+} release channel (ryanodine receptor) inhibitor, the inward current was gradually increased (Fig. 7B). To solve this conflict, the possibility of I_{CaL} suppression by Ca^{2+} released from SR (Janczewski & Lakatta, 1993; Varro et al, 1993; Sham, 1997) was tested using nifedipine. As shown in Fig. 7B, nifedipine blocked the inward current enhanced by ryanodine with much stronger intensity than nifedipine alone. Therefore, it seems to be clear that the Ca^{2+} released from SR suppresses the I_{CaL} in this high Ca^{2+} buffered situation with 14 mM EGTA. Considering the results with caffeine or ryanodine, it can be postulated that a fraction of Ca^{2+} released from SR is extruded via Ca^{2+} exit-mode Na^+-Ca^{2+} exchange during depolarization in the rat ventricular myocytes, increasing the inward current by 44%.

DISCUSSION

The major finding of the present study is that additional depletion of Na^+ in the external solution besides the Ca^{2+} depletion by applying 0 Na-0 Ca Tyrode further suppressed the inward current generated by the stimulation protocol roughly mimicked the action potential of the rat ventricular myocyte by 14%, resulting in the 53% suppression of the inward current. This result clearly shows that a certain fraction of the present inward current is generated by the Na^+-Ca^{2+} exchange. As Na^+-Ca^{2+} exchange gener-

ates a current in an opposite direction of Ca^{2+} movement (Shattock & Bers, 1989; Blaustein & Lederer, 1999), the Na^+-Ca^{2+} exchange extrudes Ca^{2+} out of the cell through its Ca^{2+} exit-mode during the depolarization mimicked action potential in the rat ventricular myocytes. The present result with caffeine suggests that the Ca^{2+} released from SR can be extruded out of the cell through this mechanism, as the inward current was increased by 44% after stimulation of CICR by caffeine application (Gilbert et al, 1991; Li et al, 1998). The increase in I_{CaL} induced by c-AMP accumulation may be responsible for the inward current increase after caffeine. However, the possibility does not seem to be major, because the Ca^{2+} released from SR after caffeine will inhibit the L-type calcium channel (Janczewski & Lakatta, 1993; Varro et al, 1993; Sham, 1997) as shown in the present results with ryanodine. The high Ca^{2+} chelator (14 mM EGTA) used in the present study does not seem to suppress CICR significantly (Sham et al, 1995; Adachi-Akahane et al, 1996; Song et al, 1998). However, since the high Ca^{2+} chelator will limit the diffusion of Ca^{2+} to/from global cytosol, the responses obtained in the present study would be confined in the microdomain (subsarcolemmal "fuzzy space", Lederer et al, 1990). This suggestion is supported by the existence of the Na^+-Ca^{2+} exchange-dependent Ca^{2+} compartment in the subsarcolemmal triadic space in the rat heart (Langer & Rich, 1992; Langer et al, 1995). Therefore, it may be restated more specifically that the Ca^{2+} released from SR is extruded out of the cell via Na^+-Ca^{2+} exchange in the subsarcolemmal triadic space where calcium channel, Na^+-Ca^{2+} exchanger and ryanodine receptor (SR Ca^{2+} releasing channel) reside in close vicinity.

The present inward current was suppressed by around 50% after Na^+ influx inhibition by 2 mM lidocaine, or holding potential change from 85 mV to −40 mV. The activation of this Na^+ influx-dependent inward current also seems to take place in the subsarcolemmal microdomain. The nature of this Na^+ influx-dependent inward current cannot be confirmed by the present results alone. Nevertheless, there is no known such a big Na^+-influx dependent inward current during depolarization in the rat ventricular myocytes yet except the Na^+-Ca^{2+} exchange. Therefore, it may be reasonable to consider that the most of the present Na^+-influx-dependent inward current would be due to the Na^+-Ca^{2+} exchange. This idea would be supported by the previous reports mentioning the changes in $E_{Na\text{-}Ca}$ during depolarization. It has been reported that the depolarization rapidly activates Na^+ channels (<1 ms) and Ca^{2+} channels and rapidly increase the concentrations of Na^+ and Ca^{2+} in the subsarcolemmal space right after the initiation of depolarization in order to dramatically alter $E_{Na\text{-}Ca}$ in that space (LeBlanc & Hume, 1990; Langer & Peskoff, 1996; Soeller & Cannel, 1997; Santana et al, 1998). The present results are also in line with the recent suggestion that the general consensus about the primary role of Na^+-Ca^{2+} exchange in ventricular heart muscle is to extrude Ca^{2+} out of the cell during depolarization, which seems to be due to the changes in the Na^+ and Ca^{2+} concentration in the subsarcolemmal space (Blaustein & Lederer, 1999). Therefore, it seems to be the case that $[Na^+]_i$ increase during depolarization is required to activate the Ca^{2+} exit-mode Na^+-Ca^{2+} exchange. The present results further showed the stimulation frequency-dependence of the Na^+ influx dependent inward current, where the frequency increase results in more frequent Na^+ channel opening and eventual $[Na^+]_i$ increase (Harrison & Boyett, 1995; Lostan et al, 1995; Maier et al, 1997). Exact mechanism remains to be cleared by defining the net effect of depolarization on the $(V_m\text{-}E_{Na\text{-}Ca})$ term after measuring the local concentrations of Na^+ and Ca^{2+} in the subsarcolemmal space.

In another part of the present study, the LA from 1-day, 1-week and 4-week old rat were used, as the SRs of the neonatal rat heart in quantity have histologically immature structures and attain full maturation at 4th week after birth (Olivetti et al, 1980; Koban et al, 1998; Vetter et al, 1995). Postnatal SR development was confirmed by the cyclopiazonic acid, a SR Ca^{2+}-ATPase inhibitor (Demaurex et al, 1992; Bonnet & Leoty, 1996), in the present study. The negative FFR of the electric field stimulated LA from postnatal developing rat showed no age-related differences in the present study. This may reflect the previous suggestion that, in quality, the SR and the Na^+-Ca^{2+} exchanger in the neonatal rat heart acquired adult like activities after birth (Chin et al, 1990; Ostadalova et al, 1993; Vornanen, 1996), although those are not mature in quantity. However, after Na^+-Ca^{2+} exchange inhibition by 26 Na-0 Ca KHB application, the twitch amplitudes were enhanced in direct proportion to the test frequency increases

with the age-dependent magnitudes in the postnatal developing rat LA. And, the twitch amplitude enhancements after 26 Na-0 Ca KHB application were totally blocked by the SR Ca^{2+} depletion after caffeine pretreatment. These results may suggest that the Ca^{2+} exit-mode Na^{+}-Ca^{2+} exchange is involved in the negative FFR as a responsible mechanism, which suppresses the LA contractility in stimulation frequency-dependent manner and its magnitude increased as the SR matures in the postnatal developing rat LA. The potency of Ca^{2+} exit-mode Na^{+}-Ca^{2+} exchange seemed to become strong enough in curtailing norepinephrine-induced $[Ca^{2+}]_i$ increase in the 4-week old LA which elicit adult like SR maturity so that 4-week-old LA required 10 times higher concentration of norepinephrine than 1 day-old LA to flatten the negative FFR. These results from the LA in the present study share the common properties with those from the ventricular myocytes. The Ca^{2+} exit-mode Na^{+}-Ca^{2+} exchange in both cases elicited stimulation frequency- and SR activity-dependencies. Furthermore, the $[Ca^{2+}]_i$ increases induced by caffeine treatment in the case of ventricular myocytes and by norepinephrine treatment in the case of LA were extruded via the Ca^{2+} exit-mode Na^{+}-Ca^{2+} exchange, increasing the inward current increase and decreasing the LA sensitivity to norpienphrine, respectively. Taken together, it may be postulated that the Ca^{2+} exit-mode Na^{+}-Ca^{2+} exchange suppresses the LA tension by extruding Ca^{2+} released from SR out of the cell in a frequency-dependent manner, resulting in the negative frequency-force relationship. This conclusion is in line with the previous reports suggesting that a certain fraction of the Ca^{2+} is extruded out of the cell via Na^{+}-Ca^{2+} exchange right after its release from SR before it diffuses to and interacts with the myofilament to trigger the contraction (Langer & Rich, 1992; Langer et al, 1993; Wang et al, 1996; Peskoff & Langer, 1998).

In the case of Na^{+}-Ca^{2+} exchange, it has been reported that the maximal activity attained before birth but its activity decreased by 75% during the first 4 weeks after birth (Studer et al, 1997; Koban et al, 1998). Considering these reports, the postnatal change in Na^{+}-Ca^{2+} exchange is in contrast with the present results, which showed that the twitch amplitude suppression by Na^{+}-Ca^{2+} exchange increased according to the postnatal ages. However, other reports have suggested that the SR Ca^{2+}-ATPase mRNA level increases by 97% between day 1 and 30 after birth, while Na^{+}-Ca^{2+} exchange decreases during same period, resulting in the reciprocal changes with SR (Vetter et al, 1995). Therefore, it seems to be the amount of Ca^{2+} released from SR than the amount of Na^{+}-Ca^{2+} exchanger that determines the amount of Ca^{2+} extruded out of cell by this mechanism. Anyway, the exact reason for this discrepancy remains to be cleared.

Another question can be raised regarding the requirement of t-tubule in this mechanism. The original suggestion about this mechanism was based on the spatial closeness between Ca^{2+} channel, SR and Na^{+}-Ca^{2+} exchanger in the subsarcolemmal space in the t-tubule. The LA, which showed the suppression of contractility induced by the Ca^{2+} exit-mode Na^{+}-Ca^{2+} exchange in the present study, has been well known to be scarce in t-tubule. Furthermore, in the case of Na^{+}-Ca^{2+} exchanger, there is also a disagreement about its distribution in cardiac myocytes. Frank et al. (Frank et al, 1992) have suggested that the exchanger is found predominantly in the t-tubules, whereas Kieval et al. (Kieval et al, 1992) have reported that the exchanger is uniformly distributed in the plasma membrane of cardiac myocytes. Therefore, the requirement of t-tubule for this mechanism also needs to be cleared.

In this study, it has been found that Na^{+}-Ca^{2+} exchange generated an inward current extruding Ca^{2+} out of the cell (Ca^{2+} exit-mode) during depolarization in the whole-cell clamped rat ventricular myocytes with high Ca^{2+} chelator (14 mM EGTA), which was frequency-dependent and was enhanced by caffeine treatment. This Ca^{2+} exit-mode Na^{+}-Ca^{2+} exchange suppressed the LA twitch amplitudes in a frequency-dependent manner with the SR maturity-related magnitudes in the postnatal developing rat heart. Therefore, it is concluded that Ca^{2+} exit-mode Na^{+}-Ca^{2+} exchange suppresses the LA tension by extruding Ca^{2+} out of the cell right after its release from SR in a frequency-dependent manner, resulting in the negative frequency-force relationship in the rat LA.

ACKNOWLEDGEMENTS

This study was supported by Yonsei University Research Fund of 1998.

REFERENCES

Adachi-Akahane S, Cleemann L, Morad M. Cross-signaling between L-type Ca^{2+} channels and ryanodine receptors in rat ventricular myocytes. *J Gen Physiol* 108: 435−454, 1996

Benitah JP, Gomez AM, Bailly P, Da Ponta JP, Berson G, Delgado C, Lorente P. Heterogeneity of the early outward current in ventricular cells isolated from normal and hypertrophied rat hearts. *J Physiol (Lond)* 469: 111−138, 1993

Bers DM, Bassani JW, Bassani RA. Na-Ca exchange and Ca fluxes during contraction and relaxation in mammalian ventricular muscle. *Ann N Y Acad Sci* 779: 430−442, 1996

Blaustein MP, Lederer JW. Sodium/Calcium exchange: Its physiological implications. *Physiol Rev* 79: 763−854, 1999

Bonnet V, Leoty C. Effects of caffeine and cyclopiazonic acid on contractile proteins of adult and newborn ferret cardiac fibres. *J Mol Cell Cardiol* 28: 1287−1295, 1996

Chin TK, Friedman WF, Klitzner TS. Developmental changes in cardiac myocyte calcium regulation. *Circ Res* 67: 574−579, 1990

Demaurex N, Lew DP, Krause KH. Cyclopiazonic acid depletes intracellular Ca^{2+} stores and activates an influx pathway for divalent cations in HL-60 cells. *J Biol Chem* 267: 2318−2324, 1992

Frampton JE, Orchard CH, Boyett MR. Diastolic, systolic and sarcoplasmic reticulum [Ca^{2+}] during inotropic interventions in isolated rat myocytes. *J Physiol (Lond)* 437: 351−375, 1991

Frank JS, Mottino G, Reid D, Molday RS, Philipson KD. Distribution of the Na^+-Ca^{2+} exchange protein in mammalian cardiac myocytes: an immumofluorescence and immunocolloidal gold-labeling study. *J Cell Biol* 117: 337−345, 1992

Gilbert JC, Shirayama T, Pappano AJ. Inositol triphosphate promotes Na-Ca exchange current by releasing calcium from sarcoplasmic reticulum in cardiac myocytes. *Circ Res* 69: 1632−1639, 1991

Harrison SM, Boyett MR. The role of the Na^+-Ca^{2+} exchanger in the rate-dependent increase in contraction in guinea-pig ventricular myocytes. *J Physiol (Lond)* 482: 555−566, 1995

Janczewski AM, Lakatta EG. Buffering of calcium influx by sarcolplasmic reticulum during the action potential in guinea-pig ventricular myocytes. *J Physiol (Lond)* 471: 343−363, 1993

Kieval RS, Bloch RJ, Lindenmayer GE, Ambesi A, Lederer WJ. Immunofluprescence localization of the Na-Ca exchanger in heart Cells. *Am J Physiol* 263: C545−C550. 1992

Koban MU, Moorman AF, Holtz J, Yacoub MH. Boheler KR. Expressional analysis of the cardiac Na-Ca exchanger in rat development and senescence. *Cardiovasc Res* 37: 405−423, 1998

Langer GA, Peskoff A. Calcium concentration and movement in the diadic cleft space of the cardiac ventricular cell. *Biophys J* 70: 1169−1182, 1996

Langer GA, Wang SY, Rich TL. Localization of the Na/Ca exchange-dependent Ca compartment in cultured neonatal rat heart cells. *Am J Physiol* 268: C119−C126, 1995

Langer GA, Peskoff A, Post JA. How does the Na^+-Ca^{2+} exchanger work in the intact cardiac cell? *J Mol Cell Cardiol* 25: 637−639, 1993

Langer GA, Rich RL. A discrete Na-Ca exchange-dependent Ca compartment in rat ventricular cells: exchange and localization. *Am J Physiol* 262: C1149−C1153, 1992

LeBlanc N, Hume JR. Sodium current-induced release of calcium from cardiac sarcolplasmic reticulum. *Science* 248: 372−376, 1990

Lederer WJ, Niggli E, Hadley RW. Sodium-calcium exchange in excitable cells: fuzzy space. *Science* 248: 283, 1990

Levi AJ, Boyett MR, Lee CO. The cellular actions of digitalis glycosides on the heart. *Prog Biophys Mol Biol* 62: 1−54, 1994

Li L, Chu G, Kranias EG, Bers DM. Cardiac myocyte calcium transport in phospholamban knockout mouse: relaxation and endogenous CaMKII effects. *Am J Physiol* 274: H1335−H1347, 1998

Lostan CS, Miller SK, Simor T, Elgavish GA. Cardiac staircase and NMR-determined intracellular sodium in beating rat hearts. *Am J Physiol* 269: H332−H340, 1995

Maier LS, Pieske B, Allen DG. Influence of stimulation frequency on $[Na^+]_i$ and contractile function in Langendorff-perfused rat heart. *Am J Physiol* 273: H1246−H1254, 1997

Mitra R, Morad M. A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *Am J Physiol* 249: H1056−H1060, 1985

Olivetti G, Anversa P, Loud AV. Morphometric study of early postnatal development in the left and right ventricular myocardium of the rat. II. Tissue composition, capillary growth, and sarcolplasmic alterations. *Circ Res* 46: 503−512, 1980

Ostadalova I, Kolar F, Ostadal B, Rohlicek V, Rohlicek J, Prochazka J. Early postnatal development of contractile performance and responsiveness to Ca^{2+}, verapamil and ryanodine in the isolated rat heart. *J Mol Cell Cardiol* 25: 733−740, 1993

Peskoff A, Langer GA. Calcium concentration and movement in the ventricular cardiac cell during an excita-

tion-contraction cycle. *Biophys J* 74: 153−174, 1998

Santana LF, Gomez AM, Lederer WJ. Ca^{2+} flux through promiscuous cardiac Na^{+} channels: slip-mode conductance. *Science* 279: 1027−1033, 1998

Sham JS. Ca^{2+} release-induced inactivation of Ca^{2+} current in rat ventricular myocytes: evidence for local Ca^{2+} signalling. *J Physiol (Lond)* 500: 285−295, 1997

Sham JS, Cleemann L, Morad M. Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Proc Natl Acad Sci USA* 92: 121−125, 1995

Shattock MJ, Bers DM. Rat vs. rabbit: Calcium flux and intracellular sodium assessed by ion-selective microelectrodes. *Am J Physiol* 256: C813−C822, 1989

Soeller C, Cannel MB. Numerical simulation of local calcium movements during L-type calcium channel gating in the cardiac diad. *Biophys J* 73: 97−111, 1997

Song L-S, Sham JSK, Stern MD, Lakatta EG, Cheng H. Direct measurement of SR release flux by tracking 'Ca^{2+} spikes' in rat cardiac myocytes. *J Physiol (Lond)* 512: 677−691, 1998

Studer R, Reinecke H, Vetter R, Holtz J, Drexler H. Expression and function of the cardiac Na^{+}/Ca^{2+} exchanger in postnatal development of the rat, in experimental-induced cardiac hypertrophy and in the failing human heart. *Basic Res Cardiol* 92 (Suppl. 1): 53−58, 1997

Varro A, Hester S, Papp JG. Caffeine-induced decreases in the inward rectifier potassium and the inward calcium currents in rat ventricular myocytes. *Br J Pharmacol* 109: 895−897, 1993

Vetter R, Studer R, Reinecke H, Kolar F, Ostadalova I, Drexler H. Reciprocal changes in the postnatal expression of the sarcolemmal Na^{+}-Ca^{2+} exchanger and SERCA2 in rat heart. *J Mol Cell Cardiol* 27: 1689−1701, 1995

Vornanen M. Excitation-contraction coupling of the developing rat heart. *Mol Cell Biochem* 163-164: 5−11, 1996

Wang S-Y, Peskoff A, Langer GA. Inner sarcolemmal leaflet Ca^{2+} binding: Its role in cardiac Na/Ca exchange. *Biophys J* 70: 2266−2274, 1996