Korean J Physiol Pharmacol
Vol 9: 95–101, April, 2005

Na^+-Ca^{2+} Exchange Curtails Ca^{2+} before Its Diffusion to Global $Ca^{2+}{}_i$ in the Rat Ventricular Myocyte

Sung-Wan Ahn and Chang Mann Ko

Department of Pharmacology and Institute of Basic Medical Science, Yonsei University Wonju-College of Medicine, Wonju 220-701, Korea

In the heart, Na^+-Ca^{2+} exchange (NCX) is the major Ca^{2+} extrusion mechanism. NCX has been considered as a relaxation mechanism, as it reduces global $[Ca^{2+}]_i$ raised during activation. However, if NCX locates in the close proximity to the ryanodine receptor, then NCX would curtail Ca^{2+} before its diffusion to global $Ca^{2+}{}_i$. This will result in a global $[Ca^{2+}]_i$ decrease especially during its ascending phase rather than descending phase. Therefore, NCX would decrease the myocardial contractility rather than inducing relaxation in the heart. This possibility was examined in this study by comparing NCX-induced extrusion of Ca^{2+} after its release from SR in the presence and absence of global $Ca^{2+}{}_i$ transient in the isolated single rat ventricular myocytes by using patch-clamp technique in a whole-cell configuration. Global $Ca^{2+}{}_i$ transient was controlled by an internal dialysis with different concentrations of BAPTA added in the pipette. During stimulation with a ramp pulse from +100 mV to −100 mV for 200 ms, global $Ca^{2+}{}_i$ transient was suppressed only mildly, and completely at 1 mmol/L, and 10 mmol/L BAPTA, respectively. In these situations, ryanodine-sensitive inward NCX current was compared using 100 μmol/L ryanodine, Na^+ depletion, 5 mmol/L $NiCl_2$ and 1 μmol/L nifedipine. Surprisingly, the result showed that the ryanodine-sensitive inward NCX current was well preserved after 10 mmol/L BAPTA to 91 % of that obtained after 1 mmol/L BAPTA. From this result, it is concluded that most of the NCX-induced Ca^{2+} extrusion occurs before the Ca^{2+} diffuses to global $Ca^{2+}{}_i$ in the rat ventricular myocyte.

Key Words: Na^+-Ca^{2+} exchange, Global $Ca^{2+}{}_i$ transient, BAPTA, Ryanodine receptor, Ca^{2+}-induced Ca^{2+} release

INTRODUCTION

Na^+-Ca^{2+} exchange (NCX) is a Na^+ and Ca^{2+} antiporter with a high Ca^{2+}-carrying capacity driven by the Na^+ and Ca^{2+} concentration gradients across the plasmalemma. NCX generates an electric current against the Ca^{2+} movement as its stoichiometry is 3 Na^+ against 1 Ca^{2+}. NCX becomes bidirectional as its activity is controlled by the membrane potential as well as the Na^+ and Ca^{2+} concentration gradients across the plasmalemma (Carafoli, 1987; Bers et al, 1996; Bers & Weber, 2002; Philipson et al, 2002).

In the heart, NCX transiently transports external Ca^{2+} into myocytes during early phase of an action potential, which is also able to trigger SR Ca^{2+} release (Leblanc & Hume, 1990; Sipido et al, 1997). During the rest of an action potential, however, NCX extrudes Ca^{2+} from myocytes (Weber et al, 2002). Overall in the heart, NCX has been considered as the major Ca^{2+} extrusion mechanism that induces relaxation by reducing global $[Ca^{2+}]_i$ raised during activation (Bers et al, 1996; Bers and Weber, 2002; Philipson et al, 2002).

In order to facilitate the NCX-induced SR Ca^{2+} release (Leblanc and Hume, 1990; Sipido et al, 1997), the Ca^{2+} should diffuse to the ryanodine receptor (RyR) (Scriven et al, 2002). It has been known that NCX locates prevalently in the t-tubule in the heart (Frank et al, 1992; Frank et al, 1996; Scriven et al, 2000; Scriven et al, 2002; Yang et al, 2002; Thomas et al, 2003). In the t-tubule, however, it is still unclear whether the NCX locates inside (Yang et al, 2002; Thomas et al, 2003) or outside (Scriven et al, 2000; Scriven et al, 2002) the dyad, in which L-type Ca^{2+} channel and RyR faces each other. Nevertheless, the distance between the NCX and the RyR has been suggested to be close enough so that the Ca^{2+} entered through the NCX can diffuse to the RyR in the heart (Scriven et al, 2002).

If this suggestion were true so that Ca^{2+} entered through NCX could diffuse to the RyR, then the reverse would also be possible in the heart. In this case, NCX could curtail a fraction of Ca^{2+} from SR before its diffusion to the global $Ca^{2+}{}_i$ transient by extruding it from cell. This process will reduce global $[Ca^{2+}]_i$ rise especially during its ascending phase rather than descending phase. The influence of NCX on diastolic $[Ca^{2+}]_i$ would be minimal unless there is Ca^{2+} overload in the heart. The result will be that NCX decreases myocardial contractility rather than induces a relaxation

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

ABBREVIATIONS: NCX, Na^+Ca^{2+} exchange; $[Ca^{2+}]_i$, intracellular calcium concentration; SR, sarcoplasmic reticulum.

in the heart. This possibility is supported by the two recent contradictory reports obtained from ventricular myocytes overexpressing the cardiac NCX. Firstly, homozygous transgenic mice overexpressing the cardiac NCX exhibited unexpectedly smaller global $Ca^{2+}{}_i$ transients despite of the larger L-type Ca^{2+} current and intact SR Ca^{2+} content (Reuter et al, 2004). Secondly, partial inhibition of NCX restored the diminished global $Ca^{2+}{}_i$ transient in the NCX-overexpressing ventricular myocytes from dog due to heart failure. Interestingly, the restoration of global $Ca^{2+}{}_i$ transient never accompanied any enhancement in diastolic global $[Ca^{2+}]_i$ (Hobai et al, 2004).

Therefore, this study was planned to compare the NCX-induced Ca^{2+} extrusion after its release from SR in the presence and absence of global $Ca^{2+}{}_i$ transient in the heart. To achieve this goal, the isolated single rat ventricular myocytes were patch-clamped in a whole-cell configuration. Global $Ca^{2+}{}_i$ transient was suppressed either minimally or completely by using an internal dialysis with different concentrations of BAPTA added in the pipette. In this situation, we compared the ryanodine-sensitive inward NCX current (RSI-I_{NCX}) representing extrusion of 1 Ca^{2+} from cell against 3 Na^+ after its release from SR. The results showed that the RSI-I_{NCX} was well preserved after complete suppression of global $Ca^{2+}{}_i$ transient to 91 % of that obtained after minimal suppression of global $Ca^{2+}{}_i$ transient. Therefore, it is concluded that most of the NCX-induced Ca^{2+} extrusion occurs before the Ca^{2+} diffuses to the global $Ca^{2+}{}_i$ transient in the rat ventricular myocyte.

METHODS

Cell isolation

Ventricular myocytes from Sprague-Dawley rats of either sex weighing 250 g were isolated using 35 mg of collagenase (type A, Boehringer) and 3 mg of protease (type XIV, Sigma) according to the method reported by Mitra and Morad (1985). The compositions of the Tyrode solution (in mmol/L) were 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$ and 10 glucose, pH=7.4 at 37°C. All the experiments were performed at room temperature.

Current recording

The rat ventricular myocytes were patch-clamped in a whole-cell configuration and held at 40 mV with a superfusion of the Tyrode solution containing 2 mmol/L Ca^{2+} throughout the experiment. Myocytes were stimulated every 10 s with a descending ramp pulse from +100 mV to −100 mV (at −1 mV/ms) for 200 ms. In some experiments, myocytes were stimulated with a step pulse to +10 mV for 200 ms every 10 s in addition to the descending ramp pulse. Resistance of the patch electrodes was 2.0~3.0 MΩ when filled with an internal solution composed of (in mmol/L) 10 NaCl, 105 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 10 HEPES, 5 Mg-ATP, and 0.1 cAMP. Concentration of BAPTA added in the pipette was increased from 1 mmol/L to 10 mmol/L. $CaCl_2$ was also added in the pipette from 0.1 mmol/L to 1 mmol/L to maintain the basal $[Ca^{2+}]_i$ at 13 nmol/L by using Winmax C v2.5 (Stanford University, USA). PH was titrated to 7.2 using CsOH at 37°C. KCl was replaced with Cs and TEA in order to block the K^+ currents. cAMP was added for prevention of SR Ca^{2+} depletion by fully activating SR Ca^{2+} reuptake through phosphorylation of phospholamban (Adachi-Akahane et al, 1996; Adachi-Akahane et al, 1997; Sham, 1997). Membrane capacitance was measured using pCLAMP software (version 8, Axon Instruments, CA, USA). The inward current obtained during the test pulse was integrated (Area Under the Curve, AUC) to calculate the charge influx and expressed into the charge influx through the unit membrane (pC/pF).

$Ca^{2+}{}_i$ measurement

Isolated single rat ventricular myocytes were loaded with fura-2 (200 μmol/L) added in the pipette and placed in a chamber on the stage of a fluorescence microscope (Olympus, Tokyo, Japan). During stimulation with the test pulse, fluorescence was measured in single ventricular myocyte using a dual-wavelength fluorescence photomultiplier system (the Ratio Fluorescence system, Photon Technology International Inc., Lawrenceville, NJ, USA) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Qualitative changes in $[Ca^{2+}]_i$ were inferred from the ratio of the fluorescence intensity at the two wavelengths.

Drug application

Tyrode solution with 2 mmol/L $CaCl_2$ was used as a drug solvent after KCl was omitted and 0.1 mmol/L DIDS was added to suppress the K^+ and Ca^{2+}-activated Cl^- currents, respectively. Drugs were diluted from stock solutions to the required concentrations in the solvent. In the case of Na^+-free (0Na) solution, NaCl was replaced with equimolar LiCl in the above solvent. Ryanodine was continuously applied for more than 10 episodes until the response equilibrated. Other drugs were applied for 10 s from the end of one test pulse to the end of the next test pulse using a rapid drug exchanger (time required for exchange < 100 ms), otherwise mentioned.

RESULTS

Fig. 1 shows that internal dialysis with BAPTA suppressed the global $Ca^{2+}{}_i$ transient in a concentration-dependent manner in the rat ventricular myocytes. Each concentration was examined in different cells in this experiment. We tested two different voltage protocols, a step pulse to +10 mV for 200 ms in Fig. 1A and a descending ramp pulse from +100 mV to −100 mV (at −1 mV/ms) for 200 ms in Fig. 1B. However, no particular differences were noted in the changes in global $Ca^{2+}{}_i$ transient related with the stimulation pattern except its duration. Global $Ca^{2+}{}_i$ transient was mildly suppressed at 1 mmol/L BAPTA, and completely disappeared at 10 mmol/L BAPTA regardless of the pulse pattern. Similar responses were obtained from 3 different cells for each dose. Therefore, we chose 1 mmol/L and 10 mmol/L BAPTA for situations representing the presence and the absence of global $Ca^{2+}{}_i$ transient, respectively.

Through out the rest of this experiment, K^+ currents and Cl^- currents were suppressed in order to reduce interferences. 0.1 mmol/L cAMP was added in the pipette for prevention of SR Ca^{2+} depletion by fully activating SR Ca^{2+} reuptake through phosphorylation of phospholamban (Ada-

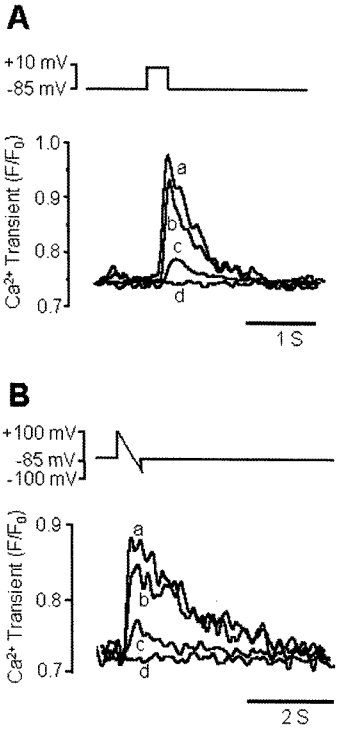

Fig. 1. Suppression of global $[Ca^{2+}]_i$ in a BAPTA concentration-dependent manner. Isolated singe rat ventricular myocytes were patch-clamped in a whole-cell configuration and held at −40 mV. Rat ventricular myocytes were internally dialyzed with different concentrations of BAPTA added in the pipette. Basal $[Ca^{2+}]_i$ was maintained at 13 nmol/L (see 'Methods'). Global $[Ca^{2+}]_i$ was measured using 200 μmol/L Fura-2 also added in the pipette during stimulation with either a step pulse to +10 mV for 200 ms or a descending ramp pulse from +100 mV to −100 mV for 200 ms every 10 s. Each signal was measured from different cells and superimposed. Representative actual global $[Ca^{2+}]_i$ signals obtained after different concentrations of BAPTA during stimulation with a step pulse (A) or with a descending ramp pulse (B). Abbreviations, a: no BAPTA, b: 1 mmol/L BAPTA, c: 5 mmol/L BAPTA, d: 10 mmol/L BAPTA.

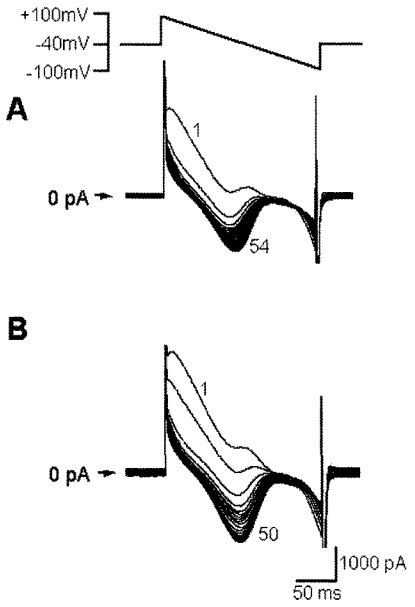

Fig. 2. Changes in membrane current after internal dialysis with BAPTA. Representative actual membrane current measured in the rat ventricular myocyte internally dialyzed with either 1 mmol/L BAPTA (A) or 10 mmol/L BAPTA (B). Isolated singe rat ventricular myocytes were held at −40 mV and stimulated with a descending ramp pulse from +100 mV to −100 mV for 200 ms every 10s after patch-clamp in a whole-cell configuration. Numbers denote episodes of stimulation.

chi-Akahane et al, 1996; Adachi-Akahane et al, 1997; Sham, 1997) (see "Methods"). During stimulation with the descending ramp pulse in this situation, rat ventricular myocytes elicited dramatic change in the membrane current immediately after a patch. As shown in Figs. 2A and B, the initial outward current gradually turned into an inward current. This change was more prominent after 10 mmol/L BAPTA than after 1 mmol/L BAPTA. It is assumed that this change may be due to the K^+ currents suppression and the L-type Ca^{2+} current (I_{CaL}) enhancement. K^+ currents were intentionally suppressed to reduce interferences as mentioned above. I_{CaL} enhancement may be caused by the BAPTA as it gradually cancelled the Ca^{2+}- induced I_{CaL} inhibition (Adachi-Akahane et al, 1996; Adachi- Akahane et al, 1997; Sham, 1997). cAMP added in the pipette would be also responsible for the I_{CaL} enhancement. After an equilibration, which required 8~12 minutes, the maximal inward current and the total charge influx (calculated by integrating the inward current) became −11.0±0.4 pA/pF and −0.654 ±0.027 pC/pF (n=30), respectively, after 1 mmol/L BAPTA. The equilibrated inward current was much enhanced after 10 mmol/L BAPTA eliciting 14.3±0.5 pA/pF in maximal inward current and 0.842±0.033 pC/pF (n=41) in total charge influx.

Fig. 3A shows that the equilibrated inward currents during stimulation with the descending ramp pulse were suppressed by ryanodine application in a concentration-dependent manner not only after 1 mM BAPTA but also after 10 mM BAPTA. In this experiment, each dose of ryanodine was examined in different cells for accuracy. Ryanodine was continuously applied for more than 10 episodes to obtain a full response. In the case with 1 mmol/L BAPTA, maximal suppression was obtained at 100 μmol/L and the magnitude was 31.8±1.6% (n=6). After 10 mM BAPTA, the ryanodine concentration required for the maximal suppression decreased to 30 μmol/L and its magnitude also decreased to 17.3±1.3% (n=9). Therefore, 100 μmol/L ryanodine was chosen to measure the RSI-I_{NCX} through out the rest of this experiment.

During stimulation with the step pulse, on the other hand, rat ventricular myocytes elicited fully activated inward current from the beginning. However, in this case, ryanodine effect was variable from mild decrease in the inward current to no effect or mild increase in the inward current (data not shown). Therefore, the descending ramp pulse was used through out the rest of this experiment.

As shown in Figs. 3B and C, 100 μmol/L ryanodine suppressed the control inward current by 0.163±0.019 pC/pF (n=6) in total charge influx after 1 mmol/L BAPTA. However, the effect of 100 μmol/L ryanodine was well preserved after 10 mmol/L BAPTA eliciting 91% (0.149±0.019 pC/pF, n=9) of that obtained after 1 mM BAPTA in total charge influx. This result suggests that SR Ca^{2+} release

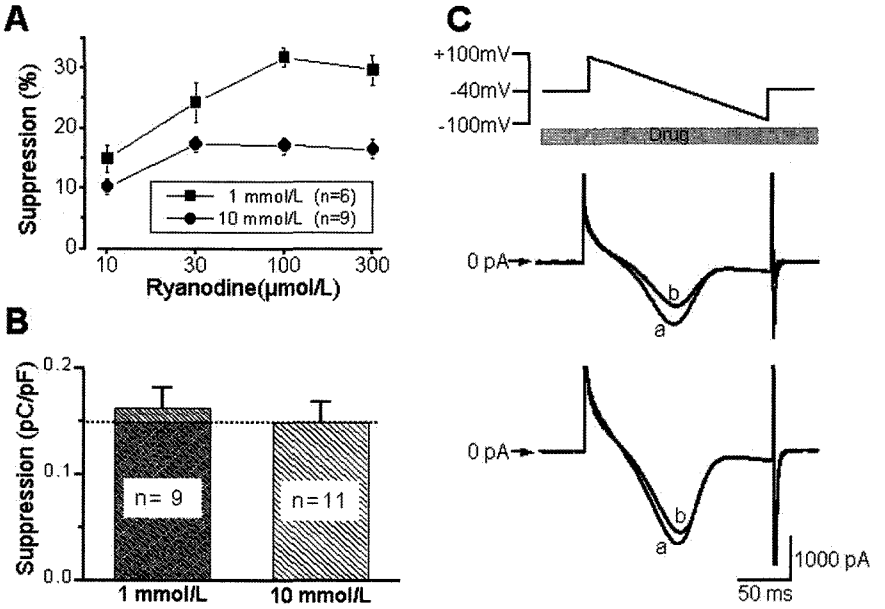

Fig. 3. Ryanodine-induced suppression of the inward current obtained after intrernal dialysis with either 1 mmol/L BAPTA or 10 mmol/L BAPTA. (A) concentration-response relationships of ryanodine obtained from the rat ventricular myocytes internally dialyzed with either 1 mmol/L BAPTA or 10 mmol/L BAPTA, (B) Comaprison of 100 μmol/L ryanodine-induced suppressions between after 1 mmol/L BAPTA and after 10 mmol/L BAPTA, (C) representative actual membrane current suppression after 100 μmol/L ryanodine in the rat ventricular mlyocyte internally dialyzed with either 1 mmol/L BAPTA (upper panel) or 10 mmol/L BAPTA (lower panel). Abbreviations, a: vehicle (Tyrode solution with KCl omission and 0.1 mmol/L DIDS addition) only, b: 100 μmol/L ryanodine. Drugs were applied for 10 s from the end of one test pulse to the end of next test pulse. Other legends are same as in Fig. 2.

caused a part of the inward current obtained during stimulation with the descending ramp pulse in the rat ventricular myocyte. It also suggests surprisingly that most of the SR Ca^{2+} release-induced inward current was well preserved even after a complete suppression of the global $Ca^{2+}{}_i$ transient.

As a next experiment, we examined the effect of L-type Ca^{2+} channel blocker and NCX blockers to characterize this ryanodine-sensitive inward current (RSIC). Firstly, 1 μmol/L nifedipine, a L-type Ca^{2+} channel blocker, was pretreated. As shown in Figs. 4A and B, pretreatment with 1 μmol/L nifedipine suppressed the control inward current in a similar magnitude after either 1 mmol/L or 10 mmol/L BAPTA, eliciting 76.8$\pm$1.7% (n=5, Fig. 4A), and 79.6$\pm$1.8% (n=6, Fig. 4B) suppressions, respectively. Pretreatment with 1 μM nifedipine also completely suppressed the effect of 100 μ mol/L ryanodine in both cases. This result suggests that the RSIC was triggered by the CICR and the CICR was still preserved after a complete suppression of the global $Ca^{2+}{}_i$ transient in the rat ventricular myocyte (Adachi-Akahane et al, 1996; Adachi-Akahane et al, 1997).

In Fig. 5A, 5 mmol/L $NiCl_2$, a most frequently used NCX blocker but has a strong L-type Ca^{2+} channel blocking action (Hobai et al, 2000), was applied to examine the involvement of NCX in the RSIC. $NiCl_2$ completely suppressed the RSIC not only after 1 mmol/L BAPTA (upper panel of Fig. 5A), but also after 10 mmol/L BAPTA (lower panel of Fig. 5A). However, in this case, we could not discern whether these suppressions were due to blocking of either NCX or L-type Ca^{2+} channel, because $NiCl_2$ sup

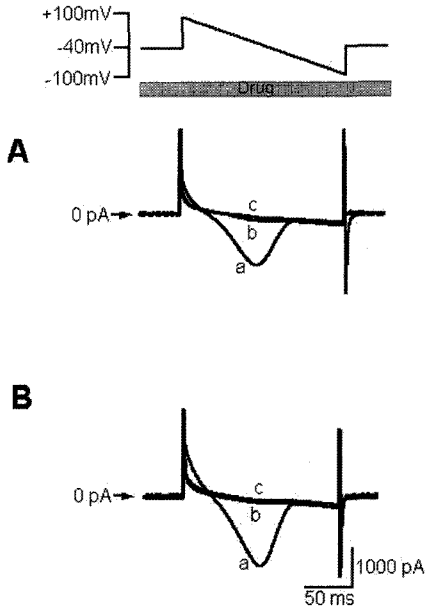

Fig. 4. Blocking of ryanodine (100 μmol/L) effect after pretreatment with nifedipine (1 μmol/L). Representative actual membrane current change in the rat ventricular myocyte internally dialyzed with either 1 mmmol/L BAPTA (A) or 10 mmol/L BAPTA (B). Abbreviations, a: vehicle only, b: 1 mol/L nifedipine, c: 1 mol/L nifedipine plus 100 mol/L ryanodine. Other legends are same as in Fig. 3.

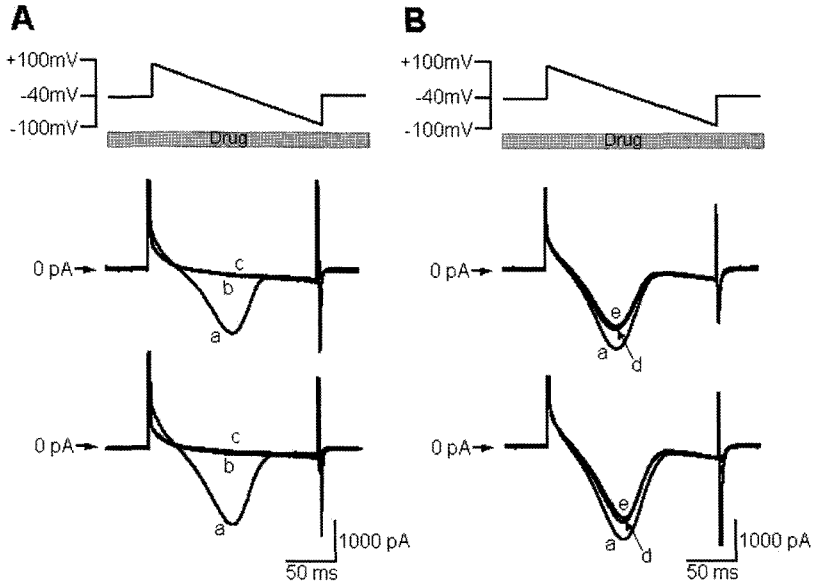

Fig. 5. Blocking of ryanodine (100 μmol/L) effect after pretreatment with either $NiCl_2$ (5 mmol/L) or Na^+-depletion (0Na). Representative actual membrane current change after pretreatment with either 5 mmol/L $NiCl_2$ (A) or 0Na (B) in the rat ventricular myocyte internally dialyzed with either 1 mmmol/L BAPTA (upper pannel) or 10 mmol/L BAPTA (lower pannel). Abbreviations, a: vehicle only, b: 5 mmol/L $NiCl_2$, c: 5 mmol/L $NiCl_2$ plus 100 μmol/L ryanodine, d: 0Na, e: 0Na plus 100 μmol/L ryanodine. Other legends are same as in Fig. 3.

pressed the control inward current in similar magnitudes with those obtained after nifedipine. Therefore, 0Na (vehicle with Na^+ omission, see Materials and Methods) was applied again to suppress the NCX. As shown in Fig. 5B, 0Na pretreatment suppressed the control inward currents in much lesser magnitudes than nifedipine or $NiCl_2$, eliciting 25.0±6.7% (n=6), and 18.4±3.7% (n=5) after 1 mmol/L (upper panel of Fig. 5B), and 10 mmol/L BAPTA (lower panel of Fig. 5B), respectively. Fig. 5B also shows that 0Na pretreatment completely suppressed the RSIC not only after 1 mmol/L BAPTA but also after 10 mmol/L BAPTA. It may be suggested from this result that the RSIC was actually an inward NCX current representing NCX-induced extrusion of Ca^{2+} from cell after its release from SR. And, 91 % of the RSI-I_{NCX} was well preserved even after a complete suppression of global $Ca^{2+}{}_i$ transient in the rat ventricular myocyte. The preservation of the RSI-I_{NCX} even after a complete suppression of global $Ca^{2+}{}_i$ transient may imply that the NCX-induced Ca^{2+} extrusion actually occurs before the Ca^{2+} diffuses to the global $Ca^{2+}{}_i$. Therefore, it may be concluded from all the results obtained in this experiment that most (91%) of the NCX-induced Ca^{2+} extrusion occurs after the Ca^{2+} is released from SR but before the Ca^{2+} diffuses to global $Ca^{2+}{}_i$ transient in the rat ventricular myocyte.

Figs. 6A and B show that total SR Ca^{2+} content measured using 20 mmol/L caffeine decreased from −3.68±0.30 pC/pF (n=3) to −1.13±0.17 pC/pF (n=7) as the concentration of BAPTA increased from 1 mmol/L (Fig. 6A) to 10 mmol/L (Fig. 6B). This result may suggest that the amount of SR Ca^{2+} release may be much higher after 1 mmol/L BAPTA than after 10 mmol/L BAPTA.

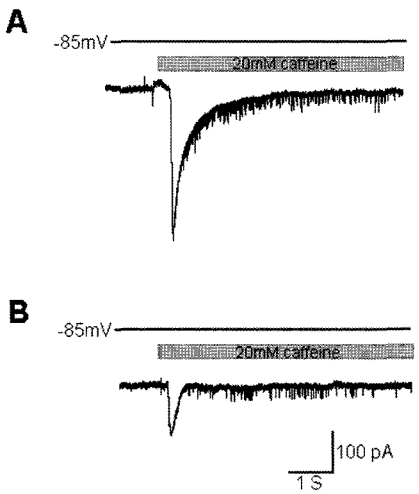

Fig. 6. Changes in the SR Ca^{2+} contents after internal dialysis with BAPTA. Representative actual membrane current change during application of 20 mmol/L caffeine in the rat ventricular myocyte internally dialyzed with either 1 mmol/L BAPTA (A) or 10 mmol/L BAPTA (B). Rat ventricular myocytes were continuously stimulated at −85 mV and continuously applied with caffeine.

DISCUSSION

The major finding of this study is that ryanodine- sensitive inward NCX current (RSI-I_{NCX}) was well preserved up to 91% after an internal dialysis with 10 mmol/L BAPTA in the rat ventricular myocyte. As internal dialysis with 10 mmol/L BAPTA completely suppressed the global $Ca^{2+}{}_i$

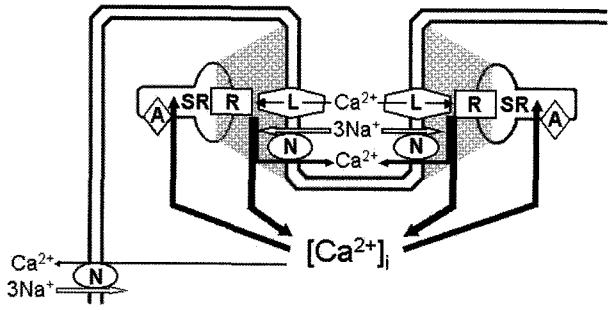

Fig. 7. Suggested diagram showing global $[Ca^{2+}]_i$ regulation in the rat ventricular myocyte. Abbreviations: A: SR Ca^{2+} pump (SERCA 2), L: L-type Ca^{2+} channel, N: Na^+-Ca^{2+} exchanger, SR: sarcoplasmic reticulum, R: ryandodine receptor.

transient in this study, this finding may suggest that the amount of Ca^{2+} that is extruded from cell via NCX from the global cytosol is minimal in the rat ventricular myocyte. Therefore, it may be concluded that most of the NCX-induced Ca^{2+} extrusion (up to 91%) occurred in a microdomain between the RyR and global cytosol in the rat ventricular myocyte.

It has been known that buffering of $[Ca^{2+}]_i$ with Ca^{2+} chelators such as EGTA and BAPTA is subject to diffusion barriers in a restricted subsarcolemmal 'fuzzy space' in the dyadic cleft in the heart (Lederer et al, 1990; Carmeliet, 1992; Convery and Hancox, 1999). Inside the 'fuzzy space', however, Ca^{2+} from SR could diffuse to the L-type Ca^{2+} channel co-locating with the RyR to inactivate it (Argibay et al, 1988; Sham, 1997; Sun et al, 1997). Considering this notion, it may also be proposed from this finding that most of NCXs co-locate with RyRs in the dyad, and therefore Ca^{2+} from SR was able to diffuse to NCX and extruded from cell after 10 mmol/L BAPTA in the rat ventricular myocyte.

Actually, the location of NCX in the heart is still under a debate. A series of reports (Frank et al, 1992; Frank et al, 1996; Yang et al, 2002; Thomas et al, 2003) have proposed that NCX prevalently locates inside the dyad in the heart. On the other hand, another reports (Kieval et al, 1992; Chen et al, 1995; Scriven et al, 2000; Scriven et al, 2002) have claimed that NCX locates rather diffusely throughout the cardiac myocyte. However, the present finding may be strong evidence that supports the prevalent of location of NCX in the dyad in the heart. The prevalent of location of NCX inside the dyad is also supported by the result shown in Fig. 6. In Fig. 6, the amount of SR Ca^{2+} content was 3 times higher in myocytes internally dialyzed with 1 mmol/L BAPTA than those with 10 mmol/L BAPTA. This result indicates that the actual amount of SR Ca^{2+} release would be much higher (may be 3 times higher) after 1 mmol/L BAPTA than after 10 mmol/L BAPTA in the rat ventricular myocyte. Nevertheless, the RSI-I_{NCX} measured in this study was not much different between two conditions eliciting 91% after 10 mmol/L BAPTA compared with that after 1 mmol/L. These findings imply that the NCX-induced Ca^{2+} extrusion was almost saturated even after 10 mM BAPTA in this study. Therefore, these findings may draw the following two explanations: 1. The Ca^{2+} concentration inside the dyad is high enough to saturate the NCX activity even after 10 mM BAPTA. 2. The total amount of Ca^{2+} extrusion via NCX was nearly attained by the NCX located inside the dyad only, which again indicates that the amount of Ca^{2+} extrusion via NCX from outside the dyad is minimal.

It may be true that the functional value obtained from this study may not directly indicate the exact amount of NCX, because the functional activity of NCX is affected by the concentration differences of Ca^{2+} and Na^+ across the sarcolemma. From this reason, the activities of NCX would be different inside and outside the dyadic cleft because of their differences in the Ca^{2+} and Na^+ concentrations (Lederer et al, 1990; Carmeliet, 1992; Trafford et al, 1995; Bers and Weber, 2002). Therefore, the actual proportions of NCX inside and outside the dyadic cleft would also be different from the functional value obtained from this study. However, the value of 91% obtained from this study may be high enough to draw a conclusion that NCX is prevalently located in the dyadic cleft closely facing RyR in the heart. After all, it may be finally concluded that most of the NCX-induced Ca^{2+} extrusion occurred after the Ca^{2+} is released from SR but before the Ca^{2+} diffuses to the global $Ca^{2+}{}_i$ transient in the rat ventricular myocyte.

The conclusion obtained from this study is in line with the previous reports, which have shown an NCX-dependent Ca^{2+} compartment in the subsarcolemmal space under a t-tubule by using ^{45}Ca in the rat heart (Langer & Rich, 1992; Langer et al, 1995; Langer & Peskoff, 1996). They also hypothesized from their conclusion that, in the compartment, Ca^{2+} from SR is trapped and extruded from cell via NCX before it diffuses to the myofilaments to induce a contraction (Langer & Peskoff, 1996; Wang et al, 1996). This study has directly visualized the actual inward I_{NCX} and clarified its proportion as much higher than 91 % of total NCX-induced Ca^{2+} extrusion in the rat ventricular myocyte. This study has also specified its location into the dyadic cleft in the rat ventricular myocyte. This location is depicted as shaded areas in Fig. 7.

Implication in myocardial excitation-contraction coupling

This study has shown that most of the NCX extrudes Ca^{2+} immediately after its release from SR but before its diffusion the cytosol to participate in the global $Ca^{2+}{}_i$ in the rat ventricular myocyte. This conclusion may indicate that NCX works especially during the ascending phase rather than the descending phase of the global $Ca^{2+}{}_i$ transient reducing the rate of rise and the amplitude of the global $Ca^{2+}{}_i$ transient in the rat heart. Therefore, it may be proposed that NCX is a negative regulator in myocardial force generation than a relaxation mechanism in the rat heart. This new paradigm in myocardial excitation-contraction (E-C) coupling is supported by the following recent contradictory findings obtained from the hearts overexprssing NCX. 1. Unexpected smaller global $Ca^{2+}{}_i$ transients in homozygous transgenic mice overexpressing the cardiac NCX despite of larger I_{CaL} and intact SR Ca^{2+} content (Reuter et al, 2004), 2. Restoration of diminished global $Ca^{2+}{}_i$ transient but without enhancement in diastolic global $[Ca^{2+}]_i$ after partial inhibition of NCX in the dog heart with heart failure (Hobai et al, 2004). This new paradigm in myocardial E-C coupling will provide new understandings in the roles of NCX in normal myocardial E-C coupling. And, hopefully, this new paradigm will provide new insights regarding the roles of NCX especially in the pathophy-

siological changes of the myocardial E-C coupling after NCX over-expression as like in heart failure (Hobai & O'Rourke, 2000; Hobai et al, 2004).

In conclusion, the NCX-induced Ca^{2+} extrusion was compared after the Ca^{2+} was released from SR either in the presence or in the absence of global $Ca^{2+}{}_i$ transient in the isolated single rat ventricular myocytes in this study. Global $Ca^{2+}{}_i$ transient was controlled by using an internal dialysis with different concentrations of BAPTA added in the pipette after a patch-clamp in whole-cell configuration. The results showed that the ryanodine-sensitive inward NCX current was well preserved after complete suppression of global $Ca^{2+}{}_i$ transient to 91% of that obtained after minimal suppression of global $Ca^{2+}{}_i$ transient. From this result, it is concluded that most of the NCX-induced Ca^{2+} extrusion occurs before the Ca^{2+} diffuses to the global $Ca^{2+}{}_i$ transient in the rat ventricular myocyte.

REFERENCES

Adachi-Akahane S, Cleemann L, Morad M. Cross-signaling between L-type Ca2+ channels and ryanodine receptors in rat ventricular myocytes. *J Gen Physiol* 108: 435–54, 1996

Adachi-Akahane S, Lu L, Li Z, Frank JS, Philipson KD, Morad M. Calcium signaling in transgenic mice overexpressing cardiac Na(+)-Ca2+ exchanger. *J Gen Physiol* 109: 717–729, 1997

Argibay JA, Fischmeister R, Hartzell HC. Inactivation, reactivation and pacing dependence of calcium current in frog cardiocytes: correlation with current density. *J Physiol* 401: 201–226, 1988

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

Bers DM, Weber CR. Na/Ca exchange function in intact ventricular myocytes. *Ann N Y Acad Sci* 976: 500–512, 2002

Carafoli E. Intracellular calcium homeostasis. *Annu Rev Biochem* 56: 395–433, 1987

Carmeliet E. A fuzzy subsarcolemmal space for intracellular Na+ in cardiac cells? *Cardiovasc Res* 26: 433–442, 1992

Chen F, Mottino G, Klitzner TS, Philipson KD, Frank JS. Distribution of the Na+/Ca2+ exchange protein in developing rabbit myocytes. *Am J Physiol* 268: C1126–C1132, 1995

Convery MK, Hancox JC. Comparison of Na+-Ca2+ exchange current elicited from isolated rabbit ventricular myocytes by voltage ramp and step protocols. *Pflugers Arch* 437: 944–954, 1999

Frank JS, Chen F, Garfinkel A, Moore E, Philipson KD. Immunolocalization of the Na(+)-Ca2+ exchanger in cardiac myocytes. *Ann N Y Acad Sci* 779: 532–533, 1996

Frank JS, Mottino G, Reid D, Molday RS, Philipson KD. Distribution of the Na(+)-Ca2+ exchange protein in mammalian cardiac myocytes: an immunofluorescence and immunocolloidal gold-labeling study. *J Cell Biol* 117: 337–345, 1992

Hobai IA, Hancox JC, Levi AJ. Inhibition by nickel of the L-type Ca channel in guinea pig ventricular myocytes and effect of internal cAMP. *Am J Physiol Heart Circ Physiol* 279: H692–H701, 2000

Hobai IA, Maack C, O'Rourke B. Partial inhibition of sodium/calcium exchange restores cellular calcium handling in canine heart failure. *Circ Res* 95: 292–299, 2004

Hobai IA, O'Rourke B. Enhanced Ca(2+)-activated Na(+)-Ca(2+) exchange activity in canine pacing-induced heart failure. *Circ Res* 87: 690–698, 2000

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

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, Peskoff A. Calcium in the cardiac diadic cleft. Implications for sodium-calcium exchange. *Ann N Y Acad Sci* 779: 408–416, 1996

Langer GA, Rich TL. A discrete Na-Ca exchange-dependent Ca compartment in rat ventricular cells: exchange and localization. *Am J Physiol* 262: 1149–1153, 1992

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: 119–126, 1995

Leblanc N, Hume JR. Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science* 248: 372–376, 1990

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

Mitra R, Morad M. A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *Am J Physiol* 249: 1056–1060, 1985

Philipson KD, Nicoll DA, Ottolia M, Quednau BD, Reuter H, John S, Qiu Z. The Na+/Ca2+ exchange molecule: an overview. *Ann N Y Acad Sci* 976: 1–10, 2002

Reuter H, Han T, Motter C, Philipson KD, Goldhaber JI. Mice overexpressing the cardiac sodium-calcium exchanger: defects in excitation-contraction coupling. *J Physiol* 554: 779–789, 2004

Scriven DR, Dan P, Moore ED. Distribution of proteins implicated in excitation-contraction coupling in rat ventricular myocytes. *Biophys J* 79: 2682–2691, 2000

Scriven DR, Klimek A, Lee KL, Moore ED. The molecular architecture of calcium microdomains in rat cardiomyocytes. *Ann N Y Acad Sci* 976: 488–499, 2002

Sham JS. Ca2+ release-induced inactivation of Ca2+ current in rat ventricular myocytes: evidence for local Ca2+ signalling. *J Physiol* 500: 285–295, 1997

Sipido KR, Maes M, Van de Werf F. Low efficiency of Ca2+ entry through the Na(+)-Ca2+ exchanger as trigger for Ca2+ release from the sarcoplasmic reticulum. A comparison between L-type Ca2+ current and reverse-mode Na(+)-Ca2+ exchange. *Circ Res* 81: 1034–1044, 1997

Sun H, Leblanc N, Nattel S. Mechanisms of inactivation of L-type calcium channels in human atrial myocytes. *Am J Physiol* 272: H1625–1635, 1997

Thomas MJ, Sjaastad I, Andersen K, Helm PJ, Wasserstrom JA, Sejersted OM, Ottersen OP. Localization and function of the Na+/Ca2+-exchanger in normal and detubulated rat cardiomyocytes. *J Mol Cell Cardiol* 35: 1325–1337, 2003

Trafford AW, Diaz ME, O'Neill SC, Eisner DA. Comparison of subsarcolemmal and bulk calcium concentration during spontaneous calcium release in rat ventricular myocytes. *J Physiol* 488: 577–586, 1995

Wang SY, Peskoff A, Langer GA. Inner sarcolemmal leaflet Ca(2+) binding: its role in cardiac Na/Ca exchange. *Biophys J* 70: 2266–2274, 1996

Weber CR, Piacentino V, 3rd, Ginsburg KS, Houser SR, Bers DM. Na(+)-Ca(2+) exchange current and submembrane [Ca(2+)] during the cardiac action potential. *Circ Res* 90: 182–189, 2002

Yang Z, Pascarel C, Steele DS, Komukai K, Brette F, Orchard CH. Na+-Ca2+ exchange activity is localized in the T-tubules of rat ventricular myocytes. *Circ Res* 91: 315–322, 2002