Korean J Physiol Pharmacol
Vol 5: 487−494, December, 2001

Forward-Mode Na^+-Ca^{2+} Exchange during Depolarization in the Rat Ventricular Myocytes with High EGTA

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During depolarization, extrusion of Ca^{2+} from sarcoplasmic reticulum through forward-mode Na^+-Ca^{2+} exchange was studied in the rat ventricular myocytes patch-clamped in whole-cell configuration. In order to confine the Ca^{2+} responses in a micro-domain by limiting the Ca^{2+} diffusion time, rat ventricular myocytes were dialyzed with high (14 mM) EGTA. K^+ current was suppressed by substituting KCl with 105 mM CsCl and 20 mM TEA in the pipette filling solution and by omitting KCl in the external Tyrode solution. Cl^- current was suppressed by adding 0.1 mM DIDS in the external Tyrode solution. During stimulation roughly mimicking action potential, the initial outward current was converted into inward current, $47 \pm 1\%$ of which was suppressed by 0.1 mM $CdCl_2$. 10 mM caffeine increased the remaining inward current after $CdCl_2$ in a cAMP-dependent manner. This caffeine-induced inward current was blocked by 1 μM ryanodine, 10 μM thapsigargin, 5 mM $NiCl_2$, or by Na^+ and Ca^{2+} omission, but not by 0.1 μM isoproterenol. The I∼V relationship of the caffeine-induced current elicited inward current from −45 mV to +3 mV with the peak at −25 mV. Taken together, it is concluded that, during activation of the rat ventricular myocyte, forward-mode Na^+-Ca^{2+} exchange extrudes a fraction of Ca^{2+} released from sarcoplasmic reticulum mainly by voltage-sensitive release mechanism in a micro-domain in the t-tubule, which is functionally separable from global $Ca^{2+}{}_i$ by EGTA.

Key Words: Na^+-Ca^{2+} exchange, Ryanodine receptor, EGTA, Caffeine t-tubule

INTRODUCTION

Na^+-Ca^{2+} exchange (NCX) is the principal mechanism by which intracellular Ca^{2+} increased during contractions is removed from cardiac myocytes through its forward-mode competing with the sarcoplasmic reticulum (SR) during relaxation (O'Neill et al, 1991; Hryshiko & Philipson, 1997; Reeves, 1998). NCX has also been suggested to trigger calcium-induced calcium release (CICR) from SR during contraction in addition to I_{CaL} by transporting Ca^{2+} into the cell through its reverse-mode (LeBlanc & Hume, 1990; Wasserstrom & Vites, 1996; Litwin et al, 1998).

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Recently, an another possibility has been raised in the roles of cardiac NCX by measuring ^{45}Ca transport in the rat heart (Langer & Rich, 1992; Langer et al, 1995; Wang et al, 1996). It has been reported that, in the subsarcolemmal space of the t-tubule in the rat heart, there is a Ca^{2+} compartment that is extruded only by the NCX (Langer & Rich, 1992). In this compartment, NCX has been proposed to extrude a certain fraction of the Ca^{2+} from SR in the compartment before it diffuses to and interacts with the myofilaments to trigger contraction (Langer et al, 1993, 1995; Langer & Peskoff, 1996; Wang et al, 1996; Peskoff & Langer, 1998). However, this idea is considered to be contradictory to the general belief that the NCX works in its reverse-mode during depolarization according to the calculated NCX equilibrium potential (E_{Na-Ca}), based on the estimated Na^+ and Ca^{2+} concentration differences across the sarcolemma as they exist in the general sarcoplasm.

Furthermore, the hypothesis has not been proven, as several other reports (Sham et al, 1995; Adachi-Akahane et al, 1996, 1997) could not identify the Ca^{2+} signal that suggests NCX and RyR coupling in the same Ca^{2+} compartment, which is distinguished from global $Ca^{2+}{}_i$ using high fura-2, although CICR is well preserved in this situation. Considering the voltage protocol of the square wave pulse to 10 mV from holding potential of −40 mV, which they used, however, a question may be raised if the $[Ca^{2+}]_i$ in the subsarcolemmal space of the t-tubule may not be high enough to activate NCX in forward-mode, since the stimulation voltage could activate CICR but not the another Ca^{2+} release mechanism, voltage sensitive release mechanism (VSRM), which is activated below 0 mV with the peak at around −40 mV (Ferrier & Howlett, 1995, 2001).

Therefore, the present study was performed to clarify the possibility that forward-mode NCX extrudes Ca^{2+} released from SR before it participates in global Ca^{2+} during activation in the rat ventricular myocytes. To achieve this goal, rat ventricular myocytes were dialyzed with high (14 mM) EGTA in order to limit the Ca^{2+} diffusion time and thereby to confine the Ca^{2+} responses in a micro-domain, and stimulated roughly mimicking action potential in order to activate both CICR and VSRM by using patch-clamp technique in whole-cell configuration. During stimulation, an inward NCX current was pharmacologically dissected from the 10 mM caffeine-induced inward current obtained in the rat ventricular myocytes, in which I_{CaL} was suppressed by 0.1 mM Cd pretreatment (Hobai et al, 1997). The results illustrated that forward-mode NCX extruded a fraction of Ca^{2+} released from SR mainly by VSRM in a micro-domain, which was functionally separable from global $Ca^{2+}{}_i$ by EGTA, during activation in the rat ventricular myocyte.

METHODS

Cell isolation

Rat ventricular myocytes were isolated according to the method of Mitra & Morad (1985). Briefly, Sprague-Dawley rats of either sex, weighing approximately 250 g, were deeply anesthetized with pentobarbital sodium (50 mg/kg, ip). Hearts were quickly excised 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 25 ml of Ca^{2+}-free Tyrode solution containing 35 mg of collagenase (type A, Boehringer) and 3 mg of protease (type XIV, Sigma) 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 used within 10 hr after isolation. All the experiments were performed at room temperature.

Current recording

Rat ventricular myocytes were patch-clamped in whole-cell configuration and held at −85 mV with superfusion of Tyrode solution containing 2 mM Ca^{2+} throughout the experiment (Other compositions were the same as described in '*Cell isolation*'.). Myocytes were depolarized every 10 s by the 10 ms-rectangular pulse to +50 mV, which was followed by 100 ms-ramp pulse from +50 mV to −50 mV to roughly mimic the action potential. The inward current generated by the test pulse was measured using Axopatch 200B amplifier (Axon, USA). 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) 10 NaCl, 105 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 10 HEPES, 5 Mg-ATP, 1 $CaCl_2$, 0.1 cAMP, and 14 mM EGTA (titrated to pH 7.2 with CsOH). KCl was replaced with Cs and TEA in order to block K^+ currents, and cAMP was added to enhance SR Ca^{2+} uptake (otherwise mentioned). The generation of voltage-clamp protocols and the 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 an 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 according to the method of Benitah et al(1993).

Drug application

Drugs were diluted from stock solutions to required concentrations in Tyrode solution, from which KCl was omitted and 0.1 mM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) was added in order to suppress the K^+ and Ca^{2+}-activated Cl^- currents (Zygmunt, 1994). In the case of Na^+- and Ca^{2+}-omitted Tyrode solution (0Na-0Ca), $CaCl_2$ was omitted and NaCl was replaced with an equimolar LiCl. Drugs were applied for 10 s until the end of the test pulse using rapid drug exchanger.

RESULTS

In this study, rat ventricular myocytes were dialyzed with 14 mM EGTA in order to confine the Ca^{2+} responses in a micro-domain by limiting Ca^{2+} diffusion time and minimizing the effect of global $Ca^{2+}{}_i$. Fig. 1 shows the effect of 14 mM EGTA-dialysis on the current generated during test pulse, 10 ms-rectangular pulse from holding potential of −85 mV to +50 mV followed by 100 ms-ramp pulse from +50 mV to −50 mV, which roughly mimicked action potential. Right after patch, rat ventricular myocytes first elicited an outward current. As the stimulation repeated every 10 s, the outward current became smaller, turned into an inward current and the inward current became larger, requiring 5~8 minutes to equilibrate (Fig. 1A). These changes might have been due to the suppression of K^+ outward currents caused by the Cs^+ and TEA^+ replaced in the pipette together with I_{CaL} enhancement caused by the cAMP and EGTA added to the pipette (Sham et al, 1995; Song et al, 1998). In Fig. 1B, the equilibrated inward current became larger and more parabolic, as the stimulation voltage increased from 0 mV to +50 mV in 10 mV steps. Therefore, +50 mV was chosen throughout the following experiments.

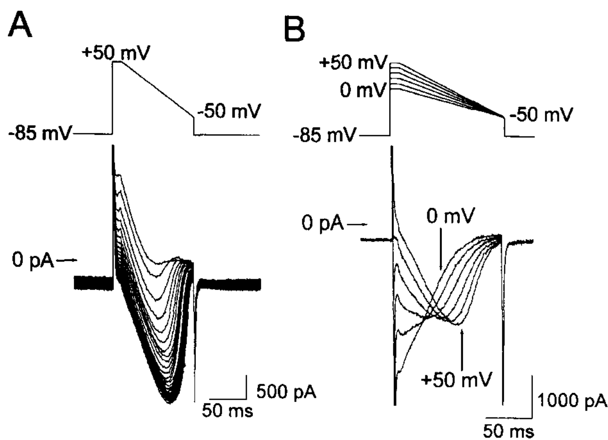

Fig. 1. Current change after dialysis with EGTA (14 mM) (A) and the I-V relationship after equilibration (B) in the rat ventricular myocytes patch-clamped in a whole-cell configuration. In the pipette, 14 mM EGTA and 0.1 mM cAMP were added and KCl was replaced with 105 mM CsCl and 20 mM TEA-Cl.

Fig. 2 shows the effect of 10 mM caffeine on the

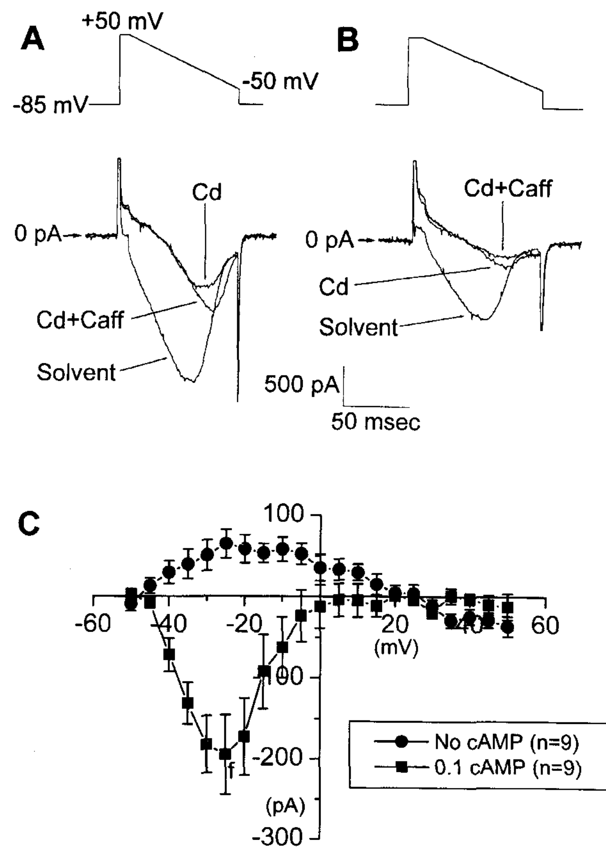

Fig. 2. Effect of caffeine (10 mM) on the remaining inward current after $CdCl_2$ (0.1 mM) pretreatment in the rat ventricular myocytes dialyzed with EGTA (14 mM) in the presence or absence of cAMP (0.1 mM). Representative superimposed actual currents in the presence (A) and absence (B) of 0.1 mM cAMP, and their I~V relationships of the net currents induced by caffeine (C). Abbreviations: Solvent: Tyrode solution without KCl but with 0.1 mM DIDS, Cd: 0.1 mM $CdCl_2$, Cd+Caff: 0.1 mM $CdCl_2$ plus 10 mM Caffeine. Drugs were applied for 10 s. Parentheses are numbers of data. Other legends are same as in Fig. 1.

remaining inward current after I_{CaL} suppression with 0.1 mM Cd pretreatment and the influence of 0.1 mM cAMP added in the pipette filling solution on it. As shown in Fig. 2A, with 0.1 mM cAMP in the pipette filling solution, the control inward current after solvent only (KCl omitted and 0.1 mM DIDS added to Tyrode solution) completely suppressed the inward holding current and elicited 11.8±0.6 pA/pF (n=9) during the test pulse. 0.1 mM Cd pretreatment suppressed the inward current to 5.0±0.4 pA/pF (43±2% of the control inward current). However, 10 mM caffeine increased the remaining inward current after Cd pretreatment (RICC) to 5.8±0.4 pA/pF (50±2% of the control, P=0.0147, paired Student's t-test with RICC) (Fig. 2A). Without cAMP, however, caffeine rather decreased the RICC, as shown in Fig. 2B. Omission of cAMP in the pipette made the inward current after solvent smaller (8.3±0.4 pA/pF, n=9, control) and 0.1 mM Cd suppressed the control inward current to 2.8±0.1 pA/pF (34±1% of the control). However, the RICC in this case tended to decrease to 2.3±0.1 pA/pF (29±2% of the control, P=0.0001, paired Student's t-test with its own RICC) after 10 mM caffeine. The I-V relationships of the net caffeine-induced current in both cases were plotted from 50 mV to +50 mV in Fig. 2C by subtracting the RICC from the inward current after caffeine treatment. With 0.1 mM cAMP in the pipette, the caffeine induced an inward current from −45 mV to +3 mV with the peak at −25 mV, which is totally relevant to the voltage range of the VSRM (Ferrier & Howlett, 1995; 2001). Without cAMP in the pipette, however, the current became outward from −45 mV to +20 mV. Therefore, the result in Fig. 2 shows that caffeine induced an inward current depending on the cAMP, which accelerated SR Ca^{2+} uptake through phospholamban phosphorylation (James et al, 1989).

The SR-dependence of the caffeine-induced inward current was further tested in Fig. 3 by directly suppressing either SR Ca^{2+} release with 10 μM ryanodine or SR Ca^{2+} uptake with 1 μM thapsigargin (Adachi-Akahane, 1996) after I_{CaL} suppression with 0.1 mM Cd in the rat ventricular myocytes dialyzed with 14 mM EGTA. Pretreatment with 10 M ryanodine decreased RICC and completely blocked the caffeine-induced inward current (n=5, Fig. 3A). The net suppression of caffeine-induced inward current after ryanodine was also increased by cAMP added in the pipette as shown in Fig. 3B. The situation in the case of 1 μM thapsigargin pretreatment was also similar to those with rynodine, but in slightly increased magnitudes, as shown in Fig. 3B (n=5) and Fig. 3C, which showed the I∼V relationships of the net suppressions of caffeine-induced inward currents after either ryanodine or thapsigargin treatment. These results confirm the SR-dependence of the caffeine-induced inward current in the rat ventricular myocyte dialyzed with 14 mM EGTA.

The involvement of NCX in the caffeine-induced

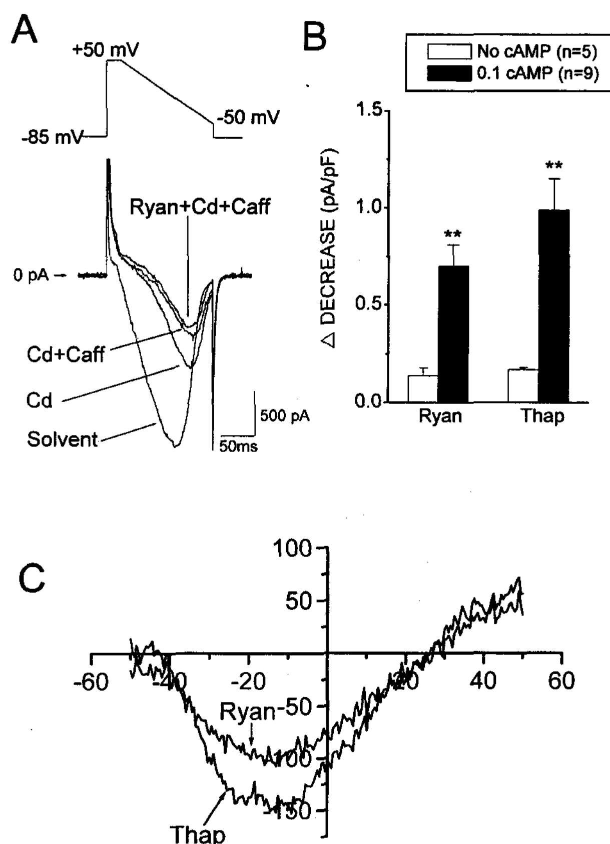

Fig. 3. Suppression of the caffeine (10 mM)-induced inward current by either ryanodine (10 μM) or thapsigargin (1 μM) in the remaining inward current after $CdCl_2$ (0.1 mM) pretreatment in the rat ventricular myocytes dialyzed with 14 mM EGTA. Representative superimposed actual currents obtained by ryanodine in the presence of cAMP (0.1 mM, A), net suppressions of caffeine-induced inward current by either ryanodine or thapsigargin in the presence or absence of 0.1 mM cAMP (B), an their I∼V relationships (C). Solvent: Tyrode solution without KCl but with 0.1 mM DIDS, Cd: 0.1 mM $CdCl_2$, Cd+Caff: 0.1 mM $CdCl_2$ plus 10 mM caffeine, Ryan+Cd+Caff: 10 M ryanodine plus 0.1 mM $CdCl_2$ and 10 mM Caffeine. Values are Means±S.E. Parentheses are numbers of data. **P<0.01, Student's t-test with No cAMP.

inward current was tested by using either 5 mM Ni or 0Na-0Ca in the 14 mM EGTA-dialyzed rat ventricular myocytes. As shown in Fig. 4A, 5 mM Ni, which blocks L-type Ca^{2+} channel and NCX[24], suppressed the control inward current by 80±5% (1.7± 0.5 pA/pF, n=9). However, 10 mM caffeine, in this case, could not increase the remaining inward current after 5 mM Ni pretreatment. Net current changes induced by caffeine treatment were compared among variable pretreatments such as 0.1 Cd, 5 mM Ni, and 0Na-0Ca (Fig. 4B). Caffeine increased the RICC after Cd pretreatment by 0.9±0.3 pA/pF (calculated from the data shown in Fig. 2B), while it had no influence on the remaining inward current after pretreatment with either 5 mM Ni or 0Na-0Ca, showing 0.1±0.1 pA/pF ($p<0.05$, unpaired t-test with Cd pretreatment) or −0.1±0.2 pA/pF ($p<0.05$, unpaired *t*-test with Cd pretreatment), respectively. Therefore, the results in Fig. 4 illustrates that caffeine induced an inward current through forward-mode NCX after 0.1 mM Cd pretreatment during depolarization in the rat ventricular myocyte dialyzed with 14 mM EGTA.

Finally, a possibility of whether the I_{CaL} activation caused the increase in RICC after caffeine was tested by applying 1 μM isoproterenol, since caffeine activates I_{CaL} through cAMP accumulation. However, as shown in Fig. 5, 1 μM isoproterenol could not increase the RICC (0.02±0.2 pA/pF, n=5), suggesting

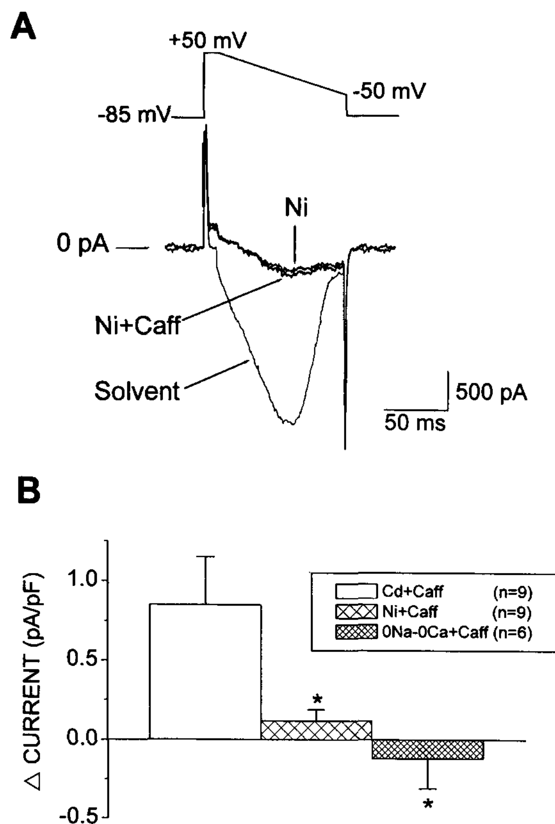

Fig. 4. Suppression of caffeine (10 mM) effect by either Ni (5 mM) or 0Na-0Ca pretreatment in the rat ventricular myocytes dialyzed with EGTA (14 mM). A: representative superimposed actual currents, B: net currents induced by 10 mM caffeine after different pretreatments. Solvent: Tyrode solution without KCl but with 0.1 mM DIDS, Ni: 5 mM $NiCl_2$, Ni+Caff: 5 mM $NiCl_2$ plus 10 mM caffeine, Cd+Caff: 0.1 mM $CdCl_2$ plus 10 mM caffeine, 0Na-0Ca+Caff: 10 mM caffeine plus Na^+ and Ca^{2+} omitted Tyrode solution. Values are Means±S.E. Parentheses are numbers of data. *$P<0.05$, unpaired Student's t-test with Cd+Caff.

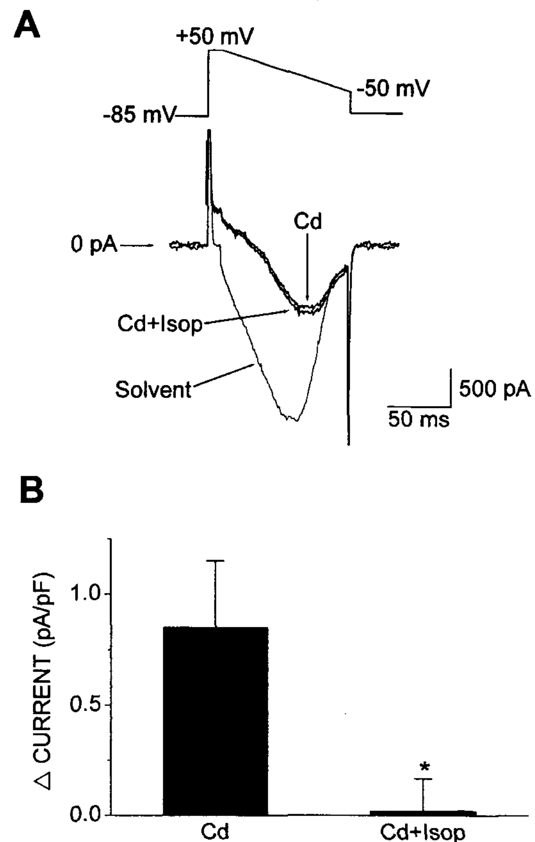

Fig. 5. Effect of isoproterenol (1 μM) on the remaining inward current after Cd (0.1 mM) pretreamtne in the rat ventricular myocytes dialyzed with EGTA (14 mM). Solvent: Tyrode solution without KCl but with 0.1 mM DIDS, Cd: 0.1 mM $CdCl_2$, Cd+Isop: 0.1 mM $CdCl_2$ plus 1 μM Isoproterenol. Values are menas±S.E.M. Parentheses are numbers of data. *$P<0.05$ unpaired Student's t-test.

that there was no relation between I_{CaL} activation and the caffeine-induced inward current after Cd pretreatment under the present condition.

DISCUSSION

The major finding of the present study is that caffeine induced an inward current after 0.1 mM Cd pretreatment through a 5 mM Ni- or 0Na-0Ca- sensitive mechanism during stimulation with the voltage roughly mimicking action potential in the rat ventricular myocytes dialyzed with high (14 mM) EGTA.

It is well known that caffeine releases Ca^{2+} from SR and caffeine also activates I_{CaL} by increasing cAMP. However, the latter mechanism seems to have no influence on the present inward current, since 1 μM isoproterenol did not increase the RICC as shown in Fig. 5. Therefore, it may be surmised that the present inward current generated by caffeine was probably caused by the Ca^{2+} released from SR. This was confirmed by the dependence of the present inward current on the cAMP (shown in Fig. 2) and ryanodine- or thapsigargin-induced blocking of the present inward current (shown in Fig. 3). The I~V relationship shown in Fig. 2C further suggested that the Ca^{2+} from SR after caffeine treatment was released mainly through VSRM, since its activation voltage range, from -45 mV to $+3$ mV with the peak at -25 mV, fitted that of VSRM (Ferrier & Howlett, 1995; 2001). Since the present inward current was completely blocked by either 5 mM Ni or 0Na-0Ca, both of which are the major experimental tools used for identification of NCX to date (Hobai et al, 2000), it may be obvious that the present inward current after caffeine treatment was generated by NCX. Taking these into consideration, therefore, it is proposed that the present inward current with caffeine was generated while the Ca^{2+} released from SR mainly by VSRM was extruded from cell through forward-mode NCX.

On the other hand, since the present experiment was carried out with the high EGTA-dialysis in order to confine Ca^{2+} responses in a micro-domain by limiting its diffusion time and minimizing the influences of global $Ca^{2+}{}_i$, the present phenomenon probably took place in the vicinity of the SR Ca^{2+} release site, the RyR, which is a micro-domain in the subsarcolemmal space in the t-tubule of the heart. This proposal is based on the previous report (Frank et al, 1992) on the prevalent location of NCXs in the T-tubular sarcolemma. Therefore, it could be finally concluded that, during activation in the rat ventricular myocyte, forward-mode Na^+-Ca^{2+} exchange extrudes a fraction of Ca^{2+} released from sarcoplasmic reticulum mainly by voltage-sensitive release mechanism in a micro-domain in the t-tubule, which is functionally separable from global $Ca^{2+}{}_i$ by EGTA.

If global $Ca^{2+}{}_i$ rather than a micro-domanial Ca^{2+} were extruded through the NCX, the above conclusion can be seriously questioned. However, this possibility seems minimal at least, since the I~V relationship of the net caffeine-induced inward NCX current (shown in Fig. 2C) was reversed at $+3$ mV even in the mildly SR Ca^{2+}-depleted myocytes. $E_{Na\text{-}Ca}=+3$ mV predicts that $[Ca^{2+}]_i$ will be 0.8 μmol/L (based on the equation $E_{Na\text{-}Ca}=3E_{Na}-2E_{Ca}$ and the Nernst equation), when $[Na^+]_o$=137 mmol/L, $[Ca^{2+}]_o$=2 mmol/L, and $[Na^+]_i$ were expected in the present study to return to the baseline of 10 mmol/L during ramp pulse (Matsuoka & Hilgemann, 1992). 0.8 μmol/L is significantly higher than the reported peak $[Ca^{2+}]_i$ achieved in ventricular myocyte after high Ca^{2+} buffer, which was 0.1 μM at the most (Adachi-Akahane, 1996).

The conclusion obtained in the present study provides important evidence of the first-line negative regulatory role of NCX upon $[Ca^{2+}]_i$ increase in the heart (Langer & Rich, 1992; Langer et al, 1995; Wang et al, 1996). If this were the case, increasing NCX activity decreases the peak global $Ca^{2+}{}_i$ transient during activation in the rat heart. Moreover, this idea may explain one of the mechanisms responsible for the heart failure, in which the peak amplitude of the $Ca^{2+}{}_i$ transient is decreased (Beuckelmann & Erdmann, 1992; O'Rourke et al, 1999) and Na^+-Ca^{2+} exchanger is upregulated by a factor of 2~3 times (Flesch et al, 1996; Pogwizd et al, 1999; Hobai & O'Rourke, 2000). However, further studies are required to clarify the situation by measuring the exact concentration of $[Ca^{2+}]_i$ in the micro-domain of the dyadic cleft in the heart, which is beyond the spatial resolution of present electron microprobe technology.

The present study also suggests that the previous studies, which employed 2 mM fura-2 (Adachi-Akahane et al, 1996, 1997; Sham et al, 1995), the peak $[Ca^{2+}]_i$ in the vicinity of the Na^+-Ca^{2+} exchanger after caffeine treatment might have been too low to activate NCX, because the myoplasmic rate constant for Ca^{2+} binding for fura-2 is 28 times that

of EGTA (Jong et al, 1995; Pape et al, 1998), and 2 mM fura-2 actually is 4 times higher concentration than 14 mM EGTA. This consideration together with our present results suggest that 14 mM EGTA may be a better choice for the functional isolation of the micro-domain containing RyR and NCX from global $Ca^{2+}{}_{i}$.

In the present study, the caffeine-induced inward NCX current was pharmacologically dissected after dialysis with 14 mM EGTA during stimulation, which roughly mimicked action potential in the rat ventricular myocytes patch-clamped in a whole-cell-configuration. We conclude that, during activation in the rat ventricular myocyte, forward-mode Na^{+}-Ca^{2+} exchange extrudes a fraction of Ca^{2+}, which is released from sarcoplasmic reticulum mainly by voltage-sensitive release mechanism, in a micro-domain in the t-tubule, which is functionally separable from global $Ca^{2+}{}_{i}$ by EGTA.

ACKNOWLEDGEMENT

Authors express special thanks to Ms. Soon-Jin Kim and Mr. Byong-Yong Ahn for their technical assistances. This study was supported by Yonsei University Wonju-College of Medicine Research Fund of 2000.

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