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Roles of Na^+-Ca^{2+} Exchange in the Negative Force-Frequency Relationship

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Frequency-force relationships (FFR) were studied in electrically field stimulated rat left atria (LA) by reducing the stimulation frequency from resting 3 Hz to test frequencies (0.1-1 Hz) for 5 minutes. The twitch amplitudes of LA elicited the typical negative staircases with 3-phased changes: the initial rapid increase, the second decrease and the following plateau at test frequencies. Verapamil (3×10^{-5} M) pretreatment elicited frequency-dependent suppression of the twitch amplitudes, exaggerating the negative staircase. Monensin pretreatment enhanced not the peak but the plateau amplitudes in a concentration-dependent manner. When the Na^+-Ca^{2+} exchange was blocked by Na^+ and Ca^{2+} depletion in the Krebs Hensleit buffer (0 Na^+-0 Ca^{2+} KHB), the twitch amplitudes increased in a frequency-dependent manner, changing the negtive staircase into the positve one. Meanwhile, the 0 Na^+-0 Ca^{2+} KHB applicationinduced enhancement was strongly suppressed by caffeine (5 mM) pretreatment. Only dibucaine among the local anesthetics increased the basal tone during frequency reduciton. There were no differences in ^{45}Ca uptakes between 0.3 Hz and 3 Hz stimulation except at 1 min when it was significantly low at 0.3 Hz than 3 Hz, illustrating net Ca^{2+} losses. Monensin pretreatment enhanced the rate of this Ca^{2+} loss. Taken together, it is concluded that Na^+-Ca^{2+} exchange extrudes more SR released Ca^{2+} out of the cell in proportion to the frequency, resulting in the negative rate staircase in the rat LA.

Key Words: Negative staircase, Na^+-Ca^{2+} exchange, Ca^{2+} extrusion, Dibucaine

INTRODUCTION

The tension development on stimulation frequency in the rat heart has long been recongnized to follow an inverse-relationship (Orchard & Lakatta, 1985; Woodworth, 1902). A gradual decrease in Ca^{2+} content of the sarcoplasmic reticulum (SR) according to the frequency increases has been suggested as a responsible mechanism for this negative staircase of forece-freqeuncy relationship (FFR) (Borzak et al, 1991; Frampton et al, 1991; Orchard and Lakatta, 1985), as the rat heart is almost totally dependent on SR Ca^{2+} to activate the myofilaments (Bers 1985). In addition, the higher level of intracellular Na^+ concentraction ($[Na^+]_i$) in the rat heart has recently been claimed to be the source of this frequency related changes in the SR calcium store, because diastolic $[Ca^{2+}]_i$ is increased by Ca^{2+} influx via reverse-mode Na^+-Ca^{2+} exchange in the presence of high $[Na^+]_i$ depending on the diastolic interval (Mubagwa et al, 1997). This might be supported by the characteristic rest potentiation which is produced by the calcium influx through reverse mode Na^+-Ca^{2+} exchanger activated during diastole in the rat heart (Shattock & Bers, 1989). However, the $[Na^+]_i$ in the rat heart has been reported to increase in proportion to the stimulation frequency increase just like in other species (Lostan et al, 1995; Maier et al, 1997). Therefore, this frequency- dependent $[Na^+]_i$ increase is believed to possibly cancel the freqeuncy related Ca^{2+} influx via reverse mode Na^+-Ca^{2+} exchange.

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On the other hand, in the rat heart, the higher $[Na^+]_i$ together with the shorter action potential duration has been claimed to make E_{Na-Ca} exceed the E_m and strongly favor the Ca^{2+} extrusion during systole (Negretti et al, 1995; Shattock & Bers, 1989). If this is the case, the further increase in $[Na^+]_i$ after freqeuncy increase will enhace the Ca^{2+} extrusion through this mechanism, resulting in the contractility decrease. However, no reports have been focused on the relationship between frequency and Ca^{2+} extrusion via Na^+-Ca^{2+} exchanger to date. Therefore, the present study was aimed to determine the rate-related changes of Ca^{2+} extrusion through Na^+-Ca^{2+} exchange as a possible explanation for the mechanism of the negative rate staircase in the rat heart.

METHODS

Left atrial strip

Sprague-Dawley rats of either sex weighing 200 g were sacrificed by cervial dislocation. The hearts were quickly excised and suspended in oxygenated Krebs-Hensleit buffer (KHB). Left atria (LA) were cautiously dissected and mounted in 8 ml organ bath filled with KHB. KHB was bubbled with mixed gas of 95% O_2 and 5% CO_2. The compositions of gases were closely regulated by gas proportioner (Cole-Parmer, USA) to maintain pH=7.40+0.03. Left atrial contraction was evoked by electrical field stimulation delivered through platinum electrodes in square wave pulses of 3Hz, 0.5 msec pulse duration with supramaximal voltages by digital stimulator (STM-1000, Hansung, Korea). Left atrial contraction was recorded on Polygraph (Model 7, Grass, USA) via force displacement transducer (FT. 03, Grass, USA). The atria were equilibrated for 30 minutes at 3 Hz. The resting stimulation was applied at 3Hz all through the experiment to maintain the same level of SR Ca^{2+} store (Frampton et al, 1991). The KHB was changed at 10 minute-interval during the rest throughout the experiment. The frequency was abruptly reduced from resting 3 Hz to test frequency for 5 minutes and then returned to 3 Hz. After 20 minute-rest, the same series of protocol was repeated. Test freqeuncy was applied on 0.1, 0.3, 0.5, 0.7 and 1 Hz. The changes in twitch amplitudes during freqeuncy reduction to the test frequencies were observed, and the maximal amplitudes attained within 30 sec (the peak) and the last twitch amplitudes obtained at the end of the 5 minute after frequency reduction (the plateau) were calculated as % of the norepinephrine 10^{-6} M-induced maximal amplitude at 3Hz stimulation. Ryanodine, a SR Ca^{2+} release channel blocker (Fleischer et al, 1985), was pretreated at a very low concentration (3×10^{-9} M) for 40 minutes prior to frequency reduction. With the other drugs, the frequency was reduced 1 minute after the drug effects were stabilized (usually around 5 minutes after drug application). In the case of the Ca^{2+} or Na^+ and Ca^{2+} depletion, the KHB with 0 Ca^{2+} or 0 Na^+-0 Ca^{2+} KHB was applied two-times rapidly in order to wash out the membrane bound Ca^{2+}. The frequency was reduced for 5 minutes without any delay. Prior to 0 Ca^{2+} or 0 Na^+-0 Ca^{2+} KHB application at each frequency, their own controls with normal KHB were held in order to confirm that there were no critical Ca^{2+} reapplication-induced damages in the LA strips. The maximal twitch amplitudes attained after 0 Ca^{2+} or 0 Na^+-0 Ca^{2+} KHB application were calculated as % contraction or net % changes to its own control. 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, and Na-Pyruvate 2.0. In the case of 0 Ca^{2+} or 0 Na^+-0 Ca^{2+} KHB, $CaCl_2$ was just omitted and NaCl was substituted with equimolar Choline Chloride.

^{45}Ca accumulation

^{45}Ca accumulation into left atrial strips during the frequency reduction was measured according to the cold lanthanum method by Karaki and Weiss (Karaki & Weiss, 1979). Briefly, 1 Ci/ml of $^{45}CaCl_2$ was introduced into the muscle chamber. After 30 seconds, the stimulation frequency was changed from 3 Hz to 0.3 Hz for 3 different periods of time, ie. 0.5, 1 and 5 minutes. The atrial strips were taken out and washed in cold KHB (4°C) and then immersed in the cold lanthanum solution for 60 minutes. The atrial strips were dried overnight at 60 C. The radioactivity was measured by liquid scintillation counter (LS3801, Beckman, USA). The ^{45}Ca accumulation was calculated as DPM/g of atrial cell H_2O. The composition of lanthanum solution was as follows (mM); $LaCl_3$ 80.0, Glucose 11, and Tris base 6 (pH=6.8 titrated with 1 N Maleic acid).

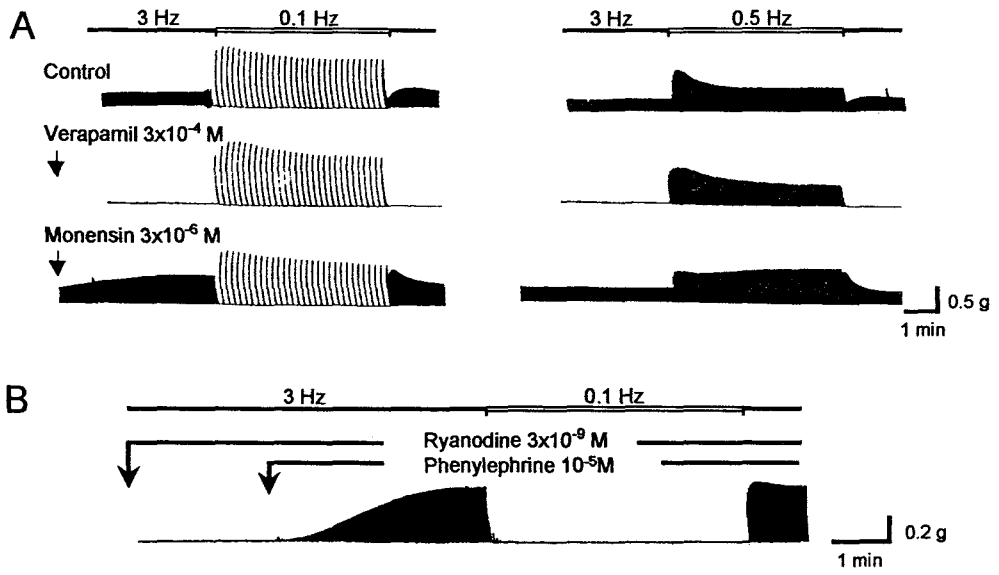

Fig. 1. Effects of Verapamil, Monensin or Ryanodine Pretreatment on the Changes in the Electrically Field Stimulated Rat Left Atrial Contractions after Frequency reduction. Left atrial contraction was evoked by electrical field stimulation (0.5 msec duration, supramaximal voltage) through platinum electrodes and the stimulation frequency was reduced from resting 3 Hz to test frequencies for 5 minutes.

RESULTS

Left atrial contraction

As shown in Fig. 1A, the rat LA elicited 3-phased changes; the initial rapid increasing phase, the rapid decreasing phase and the following plateau-phase in the electrically driven twitch amplitudes during the stimulation frequency reduction from resting 3 Hz to test frequencies. The peak amplitude was achieved within 3 beats (30 sec) after the stimulation frequency was reduced to 0.1 Hz. When the stimulation frequency returned to 3 Hz again, the twitch amplitude was dropped abruptly so that the second beat after 3 Hz reached the lowest amplitude and elicited gradual increase until the peak within 1 minute and slow decrease to the plateau. Similar changes were achieved at 0.5 Hz, except the smaller twitch amplitudes and the steeper decrease in the second phase. The FFR of the peak and plateau amplitudes of the rat LA in the range of test frequencies from 0.1 Hz to 1 Hz elicited typical negative rate staircase with the highest at 0.1 Hz and the lowest at 1 Hz. Both of the peak and plateau amplitudes achieved were 96+8%, 71+8% at 0.1 Hz and 42+2%, 31+2% at 1 Hz, respectively (Fig. 2). Ryanodine, which

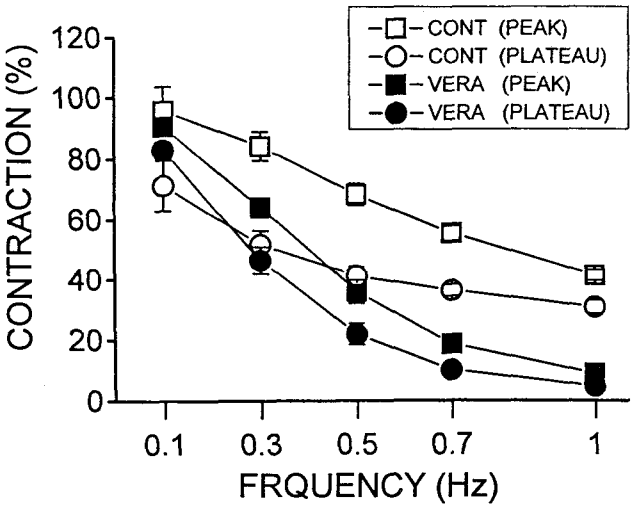

Fig. 2. Effects of Verapamil (3×10^{-5} M) on the Force-Frequency Relationships of the Peak and Plateau Amplitudes in the Electrically Field Stimulated Rat Left Atria. cont : control (n=5), vera : verapamil pretreated (n=7), peak : maximal amplitude of the first phase, plateau : the last amplitude attained at the end of the frequency reduction. Other legends are same as Fig 1.

locks the SR Ca^{2+} release channel in an open state (17), showed higher sensitivity in suppression of the twitch contraction at a lower rate. As shown in Fig.

1B, the contraction of LA was totally suppressed either at 3 Hz or 0.1 Hz after pretreatment with extremely low concentration (3×10^{-9} M) of ryanodine for 40 minutes. When isoproterenol 10^{-7} M was added, the contraction was markedly enhanced during 3 Hz stimulation. However, as soon as the stimulation frequency was reduced to 0.1 Hz, the contraction totally disappeared, and it reappeared as soon as the frequency was returned to 3 Hz. The same results were achieved with phenylephrine 10^{-5} M and monensin 3×10^{-6} M (data not shown). These results suggest that increase in $[Ca^{2+}]_i$ by isoproterenol, phenylephrine or monensin treatment is able to overcome the SR Ca^{2+} leaking caused by the ryanodine treatment (Nagasaki and Fleischer, 1988; Bull et al, 1989) only at a higher frequency (duBell et al, 1993).

Verapamil treatment

High concentration (3×10^{-5} M) of verapamil treatment totally abolished the contractions during 3 Hz stimulation. In the meantime, the contractions reappeared as soon as the frequency was reduced to 0.1 Hz. When the frequency returned to 3 Hz, it disappeared again right after the first beat. A similar pattern of change with reduced amplitude was obtained when the test frequency was increased to 0.5 Hz (Fig. 1A). As shown in Fig. 2, the peak and plateau amplitudes were 96+8% and 71+8% (of the maximal amplitude increment after NE 10^{-6} M treatment at 3 Hz) at 0.1 Hz, respectively, showing no suppression after verapamil pretreatment. On the other hand, both amplitudes became 9+2% and 5+1% at 1 Hz, respectively, eliciting almost total suppression of left atrial twitch contraction by verapamil pretreatment. Therefore, verapamil pretreatment suppressed the LA contraction in a stimulation frequency-dependent manner, exaggerating the negative staircase. Nifedipine had similar effects (data not shown). Thus, the twitch contraction at a lower stimulation frequency appeared to be resistant to the L-type Ca^{2+} channel inhibition.

Monensin treatment

When monensin (3×10^{-6} M), a Na^{+} ionophore, was pretreated, the twitch contractions during frequency reduction elicited 2-phased changes at 0.1 Hz: the initial rapid increase and the following slow and continuous decrease. When increased the test frequency, the LA twitch contraction changed in 4 phases duirng the 5 minute-frequency reduciton. The total duration of the decreasing phase after the initial rapid increasing phase was shortened, which was followed by the third phase of slow increase in amplitude and then the plateau (the data at 0.5 Hz was shown, Fig. 1A). As shown in Fig. 3A, the peak amplitudes attained at the end of the first-phase were not

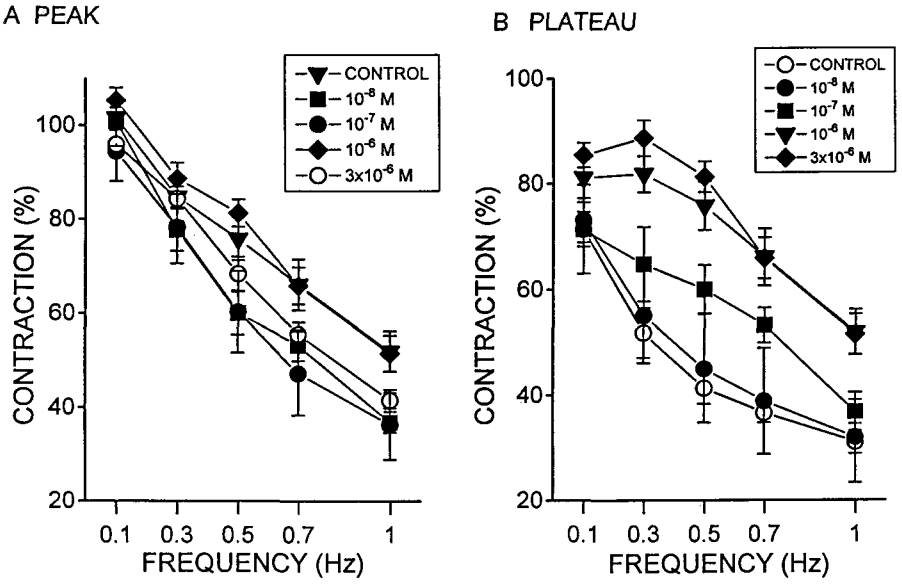

Fig. 3. Concentration-Related Effects of Monensin on the Frequency-Force Relationship of the Electrically Field Stimulated Rat Left Atria. Number of data are 4~6. Other legends are same as Fig 2.

enhanced significantly by monensin pretreatment in the doses tested (from 10^{-8} M to 3×10^{-6} M), preserving the negative rate staircase. On the other hand, the plateau amplitudes were markedly increased in a dose dependent manner, and the maximal response was shifted from 0.1 Hz to 0.3 Hz as the monensin concentration increased. With 10^{-6} M or 3×10^{-6} M of monensin pretreatment, the plateau amplitudes were much higher than the peak attained at the end of the first-phase (Fig. 3B).

0 Ca^{2+} or 0 Na^{+}-0 Ca^{2+}

In order to investigate the effects of Ca^{2+} efflux via Na^{+}-Ca^{2+} exchanger on the FFR, we examined the effects of 0 Ca^{2+} or 0 Na^{+}-0 Ca^{2+} KHB on the amplitude increments during frequency reduction. As shown in Fig. 4A, when the 0 Ca^{2+} was applied, the twitch contraction at 3 Hz disappeared within several beats (data not shown), but interestingly enough, the contractions never ceased during frequency reduction from 3 Hz to 0.3 Hz for 5 min. The twitch amplitude increased rapidly until the peak and then it was decreased continuously for 5 minutes. The peak amplitude achieved was much smaller than that of the control. When 0 Ca^{2+} was applied in the middle of frequency reduction, usually the first beat after 0 Ca^{2+} was larger than the previous twitch amplitude held with normal KHB, and the rapid decrease was followed, looking like the post-rest potentiation. Meanwhile, the application of 0 Na^{+}-0 Ca^{2+} KHB enhanced the twitch amplitudes as shown in Fig. 4B. The twitch amplitude elicited the rapid increase to the peak and the following gradual decrease. When 0 Na^{+}-0 Ca^{2+} KHB was applied in the middle of the frequency reduction, the twitch amplitude was still enhanced. There was no huge first beat, but the twitch amplitude increased gradually up to the peak and then slowly decreased, showing a totally different pattern of changes in twitch contraction from the one with 0 Ca^{2+} KHB.

The FFR of the peak amplitudes after 0 Na^{+}-0 Ca^{2+} KHB expressed as % of their own control were increased according as the test frequency increased and the negative staircase was converted into positive staircase in the freqeuncy range tested (from 0.1 Hz to 1 Hz). Pretreatment with caffeine 5 mM strongly suppressed the twitch amplitudes achieved by 0 Na^{+}-0 Ca^{2+} KHB application (Fig. 5A). In Fig. 5B, test frequency-related net % changes in twitch amplitudes after 0 Ca^{2+} or 0 Na^{+}-0 Ca^{2+} KHB application to their equivalent controls were plotted. 0 Ca^{2+} application elicited net suppression of twitch amplitudes, and the net suppression appeared to be 16+3% at 0.1 Hz and increased to 24+3% and 30+4% at 0.3 and 0.5 Hz, respectively. Then, no further suppression was elicited up to 1 Hz. Interestingly, the peak twitch amplitudes were enhanced in a test frequency-dependent manner with 0 Na^{+}-0 Ca^{2+} KHB application. At 0.1 Hz, the net increase was only 15+5%, while it became 65+6% at 1 Hz. The differences of % changes between 0Ca^{2+} and 0Na^{+}-0Ca^{2+} groups, which were Na^{+} dependent changes and therefore possibly denote the fractions of contractions that might be disappeared by Ca^{2+} extrusion via Na^{+}-Ca^{2+} exchanger, were increased in a test frequency-dependent manner from 22+6% at 0.1 Hz to 80+9% at 1 Hz. This frequency-dependent

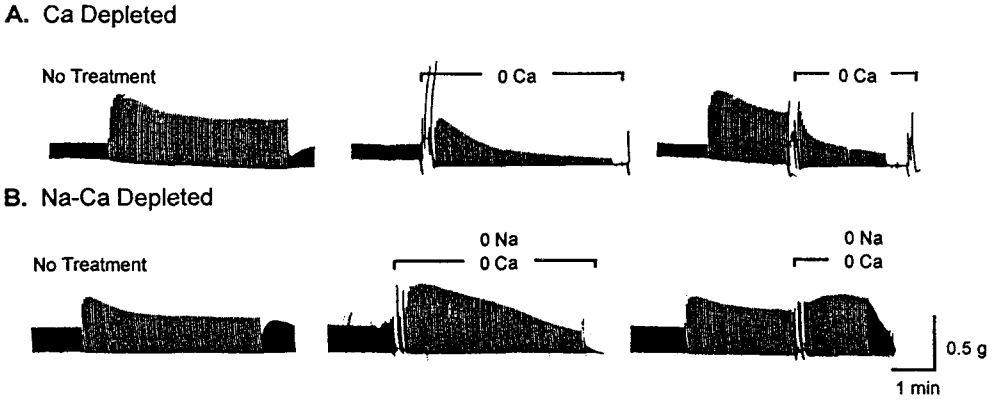

Fig. 4. Effects of Ca^{2+} or Na^{+} and Ca^{2+} Depletion on the Twitch Amplitude after Frequency Reduction in the Electrically Field Stimulated Rat Left Atria. Other legends are same as Fig 1.

enhancement after 0 Na^+-0 Ca^{2+} KHB application was totally suppressed by the caffeine pretreatment, as shown in Fig. 5A. These results may illustrate that the Na^+-Ca^{2+} exchange inhibition by 0 Na^+-0 Ca^{2+} KHB application increases the twitch amplitudes in a frequency dependent manner by using Ca^{2+} from SR store (Callewaert et al, 1989).

Dibucaine and local anesthetics

It has been reported that, in the rat heart, a certain fraction of Ca^{2+} released from SR is extruded out of the cell by the Na^+-Ca^{2+} exchanger before it interacts with myofilaments to trigger the contraction (Langer & Rich, 1992; Langer et al, 1993; Wang et al, 1996). The Ca^{2+} released from SR binds with anionic and zwitterionic phospholipids at the inner sarcolemmal leaflet in the diadic region (Post et al, 1988; Post & Langer, 1992) to increase the local concentration of Ca^{2+} in the diadic region by limiting the diffusion into the cytosol. In the long run, Ca^{2+} in this compartment is to be extruded out of the cell

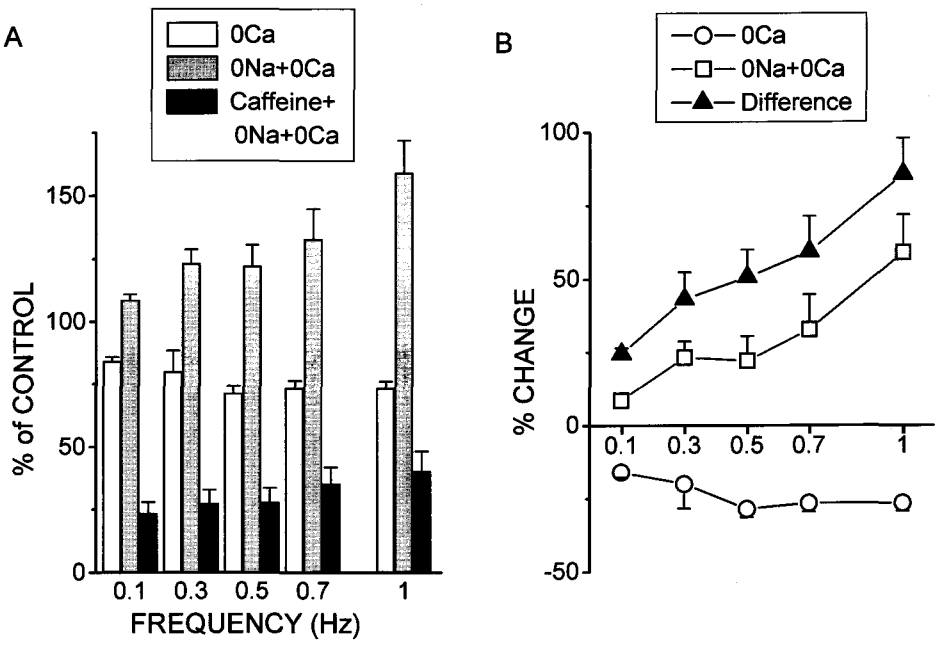

Fig. 5. Frequency-Dependent Enhancements of Twitch Amplitudes after Na^+ and Ca^{2+} Depletion in the Electrically Field Stimulated Rat Left Atria. Number of data are 6 for caffeine pretreated group and 5 for other gourps. Other legends are same as Fig. 2.

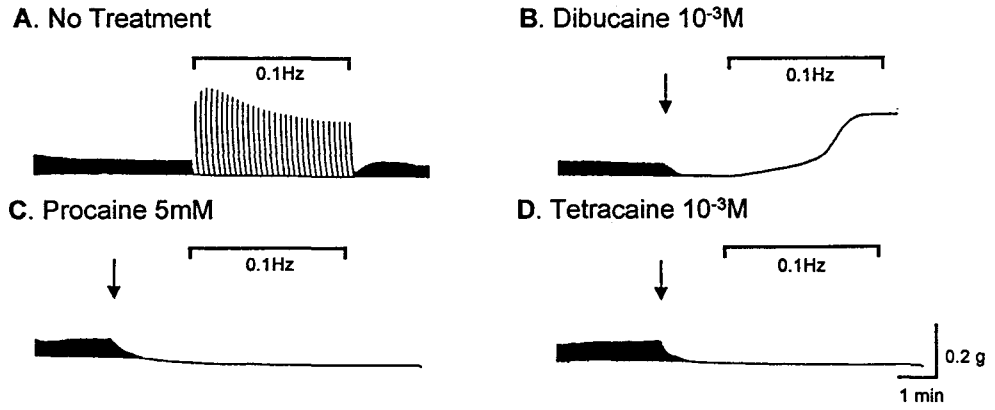

Fig. 6. Effects of Dibucaine on the Twitch Amplitudes during Frequency Reduction in the Electrically Field Stimulated Rat Left Atria. Other legends are same as Fig. 1.

by Na^+-Ca^{2+} exchanger before it diffuse to the myofilament (Wang et al, 1996). Only dibucaine among the local anesthetics has been reported to compete with Ca^{2+} in binding with these anionic and zwitterionic phospholipids at the inner sarcolemmal leaflet, because it has an higher lipid solubility than other local anesthecits (Post et al, 1988; Post and Langer, 1992). The effects of local anesthetics including dibucaine on the frequency reduction induced enhancemnets of the twitch amplitudes were examined. As shown in the Fig. 6, all the local anesthetics suppressed the contractions right after the application, because the local anesthetics is believed to suppress the Na^+ currents. However, when the stimulation frequency was reduced to 0.1 Hz, only dibucaine elicited basal tension increment. Therefore, if the previous reports mentioned above are the case, a certain fraction of Ca^{2+} seems to be extruded out of the cell via Na^+-Ca^{2+} exchange mechanism in the rat heart.

^{45}Ca accumulation

^{45}Ca accumulations in the rat left atria were measured in order to clarify the changes of Ca^{2+} fluxes during frequency reduction for 5 min. At 3 Hz, the ^{45}Ca accumulation increased rapidly during the first 1 min, and then increased slowly but continuosly for the next 5 min, the equivalent duration of the frequency reduction held in the present experiment. When the frequency was reduced to 0.3 Hz, ^{45}Ca accumulation was not different from that of 3 Hz for the first 30 sec which is equivalent to the time for the peak twitch tension. However, during the next 30 sec when the twitch tension elicited rapid decrease, ^{45}Ca accumulation was also rapidly decreased, and it was significantly lower than that of 3 Hz ($p<0.05$). Then, it was increased gradually and there was no difference in ^{45}Ca accumulation with that of 3 Hz at 5 min (Fig. 7A). In the case of monensin 3×10^{-6} M pretreatment, the ^{45}Ca accumulation during the first 30 sec was significantly higher than that of control under 0.3 Hz stimulation ($p<0.05$). However, the ^{45}Ca accumulation decreased very fast during the next 30 sec, and the ^{45}Ca accumulations at 1 min and 5 min were not different from controls of 0.3 Hz. With the ryanodine (3×10^{-9} M) treatment which totally abolished the contractions during frequency reduction, the ^{45}Ca accumulations at the first 30 sec after frequency reduction were significantly lowered ($p<0.001$). Then the ^{45}Ca accumulations were continuously increased until 5 min, showing no dif-

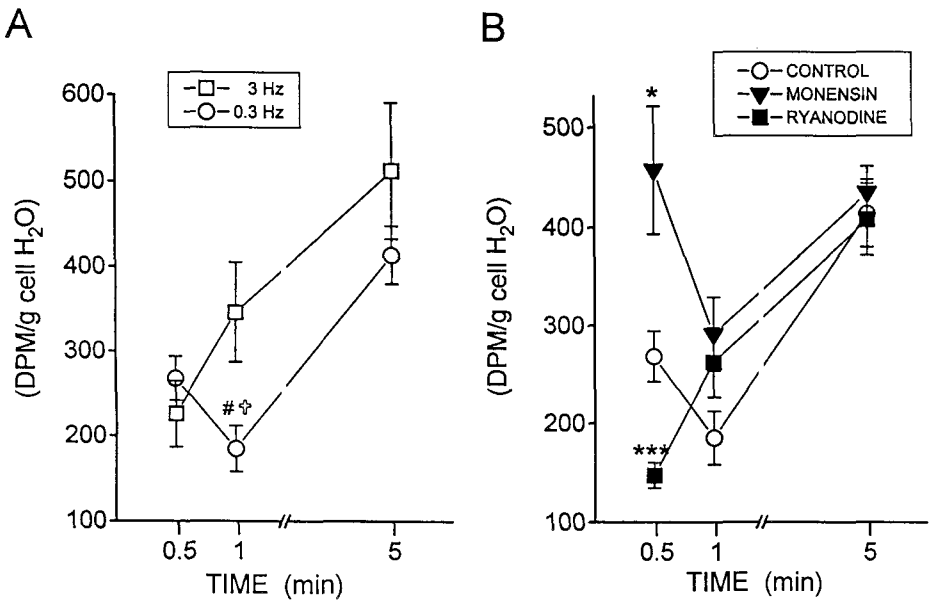

Fig. 7. Effects of Frequency Reduction on ^{45}Ca Uptake in the Electrically Field Stimulated Rat Left Atria and the Influences of Monensin (3×10^{-6} M) or Ryanodine (3×10^{-9} M) Pretreatment on It. Number of data are 8. (# $p<0.05$ compare with 3 Hz, U $p<0.05$ compare with 0.5 min at 0.3 Hz, *$p<0.05$, ***$p<0.001$ compare with control (0.3 Hz))

ferences from 0.3 Hz control at 1min and 5 min. There was no decrease at 1 minute in ryanodine pretreated group (Fig. 7B).

DISCUSSION

The major findings of the present study are; 1) The inhibition of Na^+-Ca^{2+} exchange by 0 Na^+-0 Ca^{2+} KHB application increased the twitch amplitude in a frequency-dependent manner, which was totally supperesed by caffeine-indcued SR Ca^{2+} depletion; 2) Left atrial ^{45}Ca uptake was significantly lower at 1 min than 0.5 min after frequency reduction from 3 Hz to 0.3 Hz.

Ca^{2+} accumulations during frequency reduction

The present results of left atrial ^{45}Ca uptake showed that when the stimulation frequency was lowered from 3 Hz to 0.3 Hz for 5 minutes, there were no significant changes in ^{45}Ca uptake except at 1 min when it was decreased. Thus, the marked enhancement of twitch amplitudes after frequency reduction was achieved supprisingly with no further Ca^{2+} accumulations. However, the decrements in twitch amplitudes of the second phase, during the 30 seconds from 0.5 min to 1 min after freqeuncy reduciton, were accompnied by the Ca^{2+} loss instead. As the Ca^{2+} uptake denotes the net Ca^{2+} flux (the difference between the influx and outflux of Ca^{2+}), this Ca^{2+} loss may mean increase in Ca^{2+} efflux (this will be discussed later).

3-phased changes in twitch amplitude during frequency reduction

It is interesting that although there was no further Ca^{2+} accumulation after frequency reduction, the twitch amplitude during the first phase increased to the peak which was much larger than those of plateau phase or resting twitch amplitudes. These results imply that there was redistribution of Ca^{2+} into SR during the frequency reduction (Borzak et al, 1991; Frampton et al, 1991; Orchard and Lakatta, 1985), since the rat heart is almost totally dependent on SR Ca^{2+} to activate the myofilaments (Bers, 1985). This explanation seems to be reasonable because SR Ca^{2+} store has been reported to be higher with the lower stimulation frequency than with the higher frequency (duBell et al, 1993; Field et al, 1996; Frampton et al, 1991). Therefore, as soon as the stimulation frequency was reduced to the test frequencies, the SR Ca^{2+} increased to enhance the twitch amplitudes. However, the SR Ca^{2+} store started to decrease, soon because there was the following Ca^{2+} loss. This would change the twitch amplitude into decreasing phase after the peak. After a while, the influx and outflux of Ca^{2+} would meet the balance and the twitch amplitude fell into plateau. Therefore, when the frequency is reduced from high to low, the twitch amplitudes elicit the 3-phased changes.

Ca^{2+} extrusion in frequency-force relationship

Considering the present findings of 0 Na^+-0 Ca^{2+} KHB application, the peak amplitude at a given frequency seems to be controled by the Ca^{2+} extrusion via Na^+-Ca^{2+} exchanger. When the major Ca^{2+} extrusion route-the Na^+-Ca^{2+} exchange (Bers et al, 1996)-was blocked by 0 Na^+, 0 Ca^{2+} KHB application, the peak tensions were increased without any influxes of external Ca^{2+}. In addition, the intensity of the twitch amplitudes enhancement after 0 Na^+, 0 Ca^{2+} KHB application was increased in direct proportion to the stimulation frequency increase. These results imply that the inhibition of Ca^{2+} extrusion via Na^+-Ca^{2+} exchange induces the $[Ca^{2+}]_i$ increase (Terracciano et al, 1998), and the intensity is dependent on stimulation frequency. Therefore, it may be concluded that the Ca^{2+} extrusion via Na^+-Ca^{2+} exchange increases according as the stimulation frequency increases.

Two possible mechanisms may be raised for this frequency-dependent Ca^{2+} extrusion via Na^+-Ca^{2+} exchange. It has been reported that, in the rat heart, the Ca^{2+} was extruded via Na^+-Ca^{2+} exchange during systole and that higher $[Na^+]_i$ was responsible for this (Mubagwa et al, 1997; Negretti et al, 1995; Shattock & Bers, 1989). The concentration changes of Na^+ and Ca^{2+} especially in the limited space seem to be responsible for and to potentiate the movement of the ions across the membrane (Fujioka et al, 1998). If this is the case, then, further $[Na^+]_i$ increase after stimulation frequency increase (Lostan et al 1995; Maier et al, 1997) will be able to enhance this Ca^{2+} extrusion during systole. This is the first possible mechanism for the frequency-dependent increase in Ca^{2+} extrusion in the rat heart. This may be supported by the present result that monensin pre-

treatment which increases $[Na^+]_i$ enhanced the rate of decrease in the ^{45}Ca uptake during the second phase. The second putative mechanism is based on the previous report that, in the rat heart, local concentration of Ca^{2+} in the diadic region has been suggested to be higher than the other cytosolic space since the quantity of anionic and zwitterionic phospholipids at the inner sarcolemmal leaflet could easily bind the quantity of Ca^{2+} released from SR to restrict the diffusion (Post et al, 1988; Post and Langer, 1992). This Ca^{2+} has been reported to be extruded out of the cell only by the Na^+-Ca^{2+} exchanger before it interacts with myofilaments to trigger the contraction (Langer & Rich 1992; Langer et al, 1993; Wang et al, 1996). The present dibucaine results support the existence of this Na^+-Ca^{2+} exchange-dependent Ca^{2+} compartment. As the higher the frequency increases the SR Ca^{2+} releases, the Ca^{2+} extrusion by this mechanism is expected to be increased in direct proportion to the frequency increase. Furthermore, the first putative mechanism mentioned earlier could be applied in this Ca^{2+} compartment. If the Ca^{2+} extrusion via Na^+-Ca^{2+} exchanger in the rat heart is favored during the systole because of the higher intracellular $[Na^+]_i$ and the shorter action potential duration than those of other species, this mechanism should work on the diadic Ca^{2+} compartment. However, the exact mechanism is yet to be cleared.

Roles of Ca^{2+} current (Isi) in the FFR

The relative functional contributions of complex Ca^{2+} currents across the sarcolemma and SR in myocardial E-C coupling vary from species to species (Fabiato, 1982). In particular, it has been reported that, in the rat heart, approximately 92% of Ca^{2+} removal is via SR Ca^{2+}-ATPase and only 7% via Na^+-Ca^{2+} exchange during a twitch, while the balance is more in the range of 70% SR Ca^{2+}-ATPase and 25~30% Na^+-Ca^{2+} exchange in other species (Bers et al, 1996). In rat ventricular myocytes, the SR is much more dominant (Bers, 1985) and the Ca^{2+} removal rate via Na^+-Ca^{2+} exchange is approximately 4 times less than those of other species. Therefore, the rat heart, in general, requires less Ca^{2+} influx to refill the amount of Ca^{2+} eflux in the steady state, since the amount of Ca^{2+} which enters the myocardial cell during each cycle must equal the amount extruded from the cell in order to maintain a steady state and prevent progressive Ca^{2+} overload or loss (Bers et al, 1996). In addition, the amount of Ca^{2+} influx needed to refill the loss is low during the lower rate stimulation, and it increases during the higher rate stimulation, because the rate of Ca^{2+} efflux changes in direct proportion to the frequency increases as shown in the present experiment. This would be the reason why the inhibition by verapamil of L-Ca^{2+} current which is the main route of Ca^{2+} influx elicited stronger suppression of twitch tension during higher stimulation frequency.

Proposed mechanism for the negative rate staircase in the rat heart

The changes in the stimulation frequeny affect the SR Ca^{2+} store and the extrusion rate of Ca^{2+} released from SR via Na^+-Ca^{2+} exchange in the rat heart. As the stimulation frequency increases, the SR Ca^{2+} store decreases, and more Ca^{2+} released from SR is extruded out of the cell before it interacts with myofilaments. Therefore, in the rat heart, the myocardial tension becomes smaller according as the stimulation frequency increases.

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