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Inhibition of Na^+/Ca^{2+} exchange by hexapeptide FRCRSFa in rat ventricular myocytes[1]

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KEY WORDS FRCRSFa; patch-clamp techniques; Na^+/Ca^{2+} exchanger; myocardium

ABSTRACT

AIM: To study the effect of Phe-Arg-Cys-Arg-Ser-Phe-$CONH_2$ (FRCRSFa) on Na^+/Ca^{2+} exchange and its specificity in rat ventricular myocytes. **METHODS**: Na^+/Ca^{2+} exchange current ($I_{Na^+/Ca^{2+}}$) and other currents were measured using whole-cell voltage clamp technique. **RESULTS**: A concentration-dependent inhibition of hexapeptide FRCRSFa on Na^+/Ca^{2+} exchange was observed in rat ventricular myocytes. IC_{50} of inward and outward $I_{Na^+/Ca^{2+}}$ were 2 and 4 μmol/L, respectively. FRCRSFa 5 μmol/L did not affect L-type Ca^{2+} current, voltage-gated Na^+ current, transient outward K^+ current, and inward rectifier K^+ current. **CONCLUSION**: These data indicate that FRCRSFa is an available inhibitor of Na^+/Ca^{2+} exchange with relative selectivity and may be valuable for studies of the Na^+/Ca^{2+} exchange in cardiac myocytes.

INTRODUCTION

In cardiac myocytes, the plasma membrane Na^+/Ca^{2+} exchanger plays an important role in intracellular Ca^{2+} homeostasis, the generation of electrical activity, excitation-contraction (E-C) coupling and the regulation of inotropism[1,2].

One major factor that has hindered rapid progress in the knowledge of the contribution of Na^+/Ca^{2+} exchange to the control of Ca^{2+} homeostasis, is the lack of specific inhibitors of the exchanger. Amiloride and its derivatives have been identified as relatively effective inhibitor of the Na^+/Ca^{2+} exchange. They inhibit the Na^+/Ca^{2+} exchange, but also inhibit the Na^+ channel and other Na^+-transport systems, displaying their insufficient selectivity[3]. Heavy metal ions such as nickel (Ni) and manganese (Mn) are known to inhibit the Na^+/Ca^{2+} exchange effectively, at the same time, they also inhibit the L-type Ca^{2+} channel and K^+ channels[4]. The exchanger inhibitory peptide (XIP) is more specific inhibitor but it acts upon the cytoplasmic surface only. However, the macromolecule of XIP does not seem to permeate the membrane, so that its use is significantly limited for most experimental study[5]. Therefore, the development of a new potent inhibitor that is selective for the Na^+/Ca^{2+} exchanger *in vivo* is highly desired.

In present study, a hexapeptide FRCRSFa that we synthesized on Na^+/Ca^{2+} exchange was assessed and the specificity of its effect was also examined in rat ventricular myocytes using whole-cell patch clamp technique.

MATERIALS AND METHODS

Cell isolation Single ventricular myocytes were isolated[6] from Wistar rats (250 g ± 30 g; Certificate No 070101; Grade Ⅱ) provided by Experimental Animal Center of Shanxi Medical University. Briefly, animals were killed by cervical dislocation and the hearts were rapidly removed, cannulated *via* aorta, and perfused through the coronary artery with Ca^{2+}-free Tyrode's solution for 8 min. The composition of Ca^{2+}-free Tyrode's solution was (in mmol/L) NaCl 135, KCl 5.4, $MgCl_2$ 1.0, NaH_2PO_4 0.33, glucose 10, and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 10, pH adjusted to 7.35 with NaOH at room temperature. The hearts were then perfused with 50 mL low Ca^{2+} Tyrode's solution containing $CaCl_2$ 50 μmol/L, taurine 10 mmol/L, and collagenase 0.1 g/L for 3 - 5

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min. All perfusates were gassed with 100 % O_2 and the temperature maintained at approximately 37 ℃. Hearts were perfused under the perfused pressure of 70 cm H_2O (6862.16 Pa). After completion of the perfusion, the left ventricle was removed. The cells were isolated by gentle agitation and kept in Krebs buffer (KB) solution composed of (mmol/L): KOH 85, glutamic acid 50, KCl 30, taurine 20, KH_2PO_4 30, $MgCl_2$ 1.0, HEPES 10, glucose 10, and egtazic acid 0.5, pH 7.4 adjusted by KOH.

Electrophysiologic recording Isolated cells were placed in a recording chamber (volume 0.6 mL) mounted on the stage of an inverted microscope (XDP-1, Shanghai Optical Factory, Shanghai, China). After 3 – 5 min for the cells' settling, the chamber was continuously perfused with Tyrode's solution at 30 ℃ at a rate of 1 – 2 mL/min. The resistance of patch electrode was 1 – 3 MΩ after being filled with the electrode internal solution. The pipette was connected through an Ag-AgCl wire to a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Inc, Union city, USA). The sequence of clamped pulses and holding potential, collection and save of signals, and the analysis of results were established by Pclamp 5.51 and 6.04 (Axon Instruments, Inc, Union city, USA) soft wares. The extracellular solution used for the measurement of Na^+/Ca^{2+} exchange current composed of (mmol/L) NaCl 140, $CaCl_2$ 1.8, $MgCl_2$ 2.0, HEPES 5.0, and glucose 10, pH 7.4 adjusted by CsOH. The Na^+-K^+ pump, K^+ channels, and Ca^{2+} channels were blocked by perfusing a solution containing: ouabain 20 μmol/L, $BaCl_2$ 1 mmol/L, CsCl 2 mmol/L, and nicardipine 1 μmol/L. The composition of the pipette solution was (in mmol/L): egtazic acid 42, $CaCl_2$ 29, $MgCl_2$ 13, aspartate 42, K_2ATP 10, Na_2 creatinephosphate 5, 4-aminopyridine (4-AP) 20, and HEPES 5.0, pH 7.4 adjusted by CsOH. The extracellular solution used for measuring L-type Ca^{2+} current ($I_{Ca\text{-}L}$) composed of (mmol/L) NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.33, HEPES 10, and glucose 10, pH 7.4 adjusted by NaOH. The composition of the pipette solution was (in mmol/L): KCl 140, HEPES 5.0, egtazic acid 10, Na_2ATP 2.0, $MgCl_2$ 1.0, and 4-AP 5.0, pH 7.3 adjusted by KOH. Calcium currents were evoked by depolarization of individual ventricular myocytes from a holding potential of – 40 mV to + 10 mV at a frequency of 0.2 Hz. The extracellular solution used for measuring voltage-gated Na^+ current (I_{Na}) composed of (mmol/L) NaCl 60, CsCl 5.0, $CdCl_2$ 0.1, $MgCl_2$ 2.5, 4-AP 5.0, glucose 10, sucrose 80, and HEPES 5.0, pH 7.4 adjusted by NaOH. The composition of the pipette solution was (in mmol/L): KCl 130, $MgCl_2$ 2.0, $CaCl_2$ 1.0, egtazic acid 11, HEPES 10, Na_2ATP 5.0, and 4-AP 5.0, pH 7.2 adjusted by CsOH. Sodium currents were evoked by depolarization from a holding potential of – 80 mV to 0 mV. The extracellular solution for measuring transient outward K^+ current (I_{to}) was the same as that for measuring $I_{Ca\text{-}L}$. To avoid $I_{Ca\text{-}L}$ and inward rectifier potassium current (I_{k1}) participating, $CdCl_2$ 0.1 mmol/L and $BaCl_2$ 0.2 mmol/L were added into the perfusate. The composition of the pipette solution was the same as that for measuring $I_{Ca\text{-}L}$ except that it lacked 4-AP. Transient outward K^+ currents were evoked by depolarization from a holding potential of – 40 mV to + 50 mV. The extracellular solution of measuring I_{k1} was the same as that for measuring $I_{Ca\text{-}L}$. $CdCl_2$ 0.1 mmol/L was added into the perfusate to avoid $I_{Ca\text{-}L}$ participating. The composition of the pipette solution was the same as that of measuring $I_{Ca\text{-}L}$. Inward rectifier potassium currents were evoked by hyperpolarization from a holding potential of – 40 mV to – 100 mV.

In all experiments, membrane current density was expressed as membrane current per cell capacitance. The cell capacitance was measured by the method described by Coetzee WA *et al*[7].

Drugs Phe-Arg-Cys-Arg-Ser-Phe-$CONH_2$ (FRCRSFa) was synthesized by Shanghai Biochemistry Institute (Shanghai, China). It was dissolved in distilled water and diluted to the desired final concentrations immediately before each experiment. Collagenase P was purchased from Boehringer Mannheim (Germany). Taurine, ouabain, 4-AP, and nicardipine were purchased from Sigma Chemical Co (St Louis, Mo, USA).

Statistical analysis Data were expressed as $x \pm s$, paired t-test were made, and $P < 0.05$ was considered significant.

Calculation of half inhibiting content (IC_{50}) Calculation of IC_{50} was accorded to the linear regression equation of the concentration-effect relationship[8].

RESULTS

Measurement of the Na^+/Ca^{2+} exchange current ($I_{Na^+/Ca^{2+}}$) Na^+/Ca^{2+} exchange current was measured as described by Kimura *et al*[9]. Ramp

voltage-clamp pulses (− 40 mV to + 60 mV to − 120 mV, 90 mV/s) were applied at a rate of 0.06 Hz from a holding potential of − 40 mV. The current-voltage relationship was constructed from the declining slope of the ramp pulse (Fig 1A, a). After the application of Ni^{2+} 5.0 mmol/L, the current immediately decreased, at both positive and negative potentials (Fig 1A, b). The difference between current-voltage relationships in the absence and presence of Ni^{2+} 5.0 mmol/L (Ni^{2+} sensitive current) reflected the activity of the electrogenic Na^+/Ca^{2+} exchange current (Fig 1B).

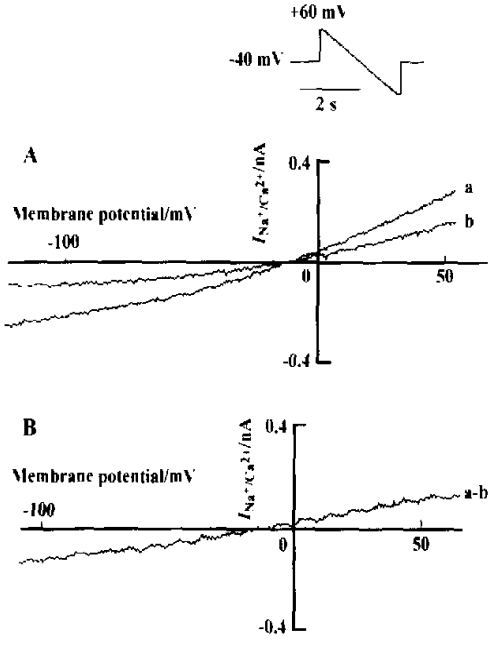

Fig 1. Measurement of Ni^{2+}-sensitive electronic Na^+/Ca^{2+} exchange current of rat ventricular myocytes. Current-voltage relationships are shown before (trace a) and after (trace b) application of Ni^{2+} (A). Numerical subtraction of these two current-voltage relationship (or Ni^{2+}-sensitive current) is shown (B).

Effect of FRCRSFa on $I_{Na^+/Ca^{2+}}$ in rat ventricular myocytes A concentration-dependent inhibition of FRCRSFa on $I_{Na^+/Ca^{2+}}$ was observed in rat ventricular myocytes (Tab 1, Fig 2). IC_{50} of inward and outward $I_{Na^+/Ca^{2+}}$ were 2 μmol/L and 4 μmol/L.

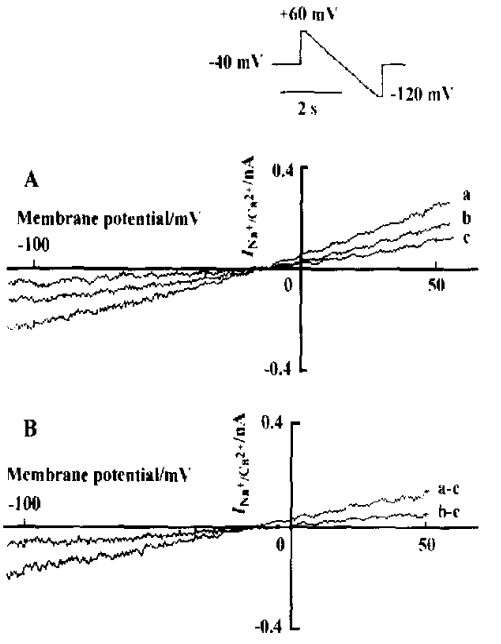

Fig 2. Effect of FRCRSFa on Na^+/Ca^{2+} exchange current in ventricular myocytes of rat. A: current-voltage relationships before (a), after application of FRCRSFa 3 μmol/L (b), and after application of $NiCl_2$ 5.0 mmol/L (c). B: Na^+/Ca^{2+} exchange current before (a − c) and after (b − c) application of FRCRSFa 3 μmol/L.

Effects of FRCRSFa on L-type Ca^{2+} current ($I_{Ca\text{-}L}$), voltage-gated Na^+ current (I_{Na}), transient outward K^+ current (I_{to}) and inward rectifier K^+ current (I_{k1}) FRCRSFa 5 μmol/L did not

Tab 1. Inhibitory effect of FRCRSFa on Na^+/Ca^{2+} exchange current in rat ventricular myocytes. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

FRCRSFa /μmol·L^{-1}	n	Na^+/Ca^{2+} exchange current (pA·pF^{-1})			
		+50 mV	Inhibition/%	−100 mV	Inhibition/%
0 (Control)	6	1.36 ± 0.11	0	1.35 ± 0.10	0
0.3	6	1.15 ± 0.06^b	16	1.13 ± 0.05^b	17
1	6	1.03 ± 0.06^b	24	0.83 ± 0.07^c	39
3	6	0.75 ± 0.05^c	45	0.59 ± 0.08^c	57
10	5	0.48 ± 0.10^c	65	0.38 ± 0.06^c	72

Membrane current density was expressed as membrane current (pA) per cell capacitance (pF).

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affect $I_{Ca\text{-}L}$, I_{Na}, I_{to}, and I_{kl} in intact rat ventricular myocytes. They were not significantly decreased after FRCRSFa 5 μmol/L treatment ($P > 0.05$). No changes was observed after washout (Fig 3).

The inhibitory effect of internal dialysis with FRCRSFa on $I_{Na^+/Ca^{2+}}$ FRCRSFa 30 μmol/L was prepared by adding 30 μL of FRCRSFa 1 mmol/L into 1 mL pipette solution. FRCRSFa 30 μmol/L was added into the glass micro-electrode. When the high sutured resistance of the electrode tip and the membrane was formed, the membrane was disrupted by negative pressure and the pipette solution was dialyzed into the cell. The densities of Na^+/Ca^{2+} exchange current under the condition of internal dialysis with FRCRSFa 30 μmol/L were significantly decreased (Tab 2) and were not further inhibited when adding FRCRSFa with same concentration into the perfused solution.

Tab 2. Inhibitory effect of intracellular dialysis with FRCRSFa on Na^+/Ca^{2+} exchange current in rat ventricular myocytes. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Group	Dose /μmol·L^{-1}	n	Na^+/Ca^{2+} exchange currents (pA·pF^{-1}) +50 mV	−100 mV
Control	0	5	0.89 ± 0.16	0.95 ± 0.13
FRCRSFa	30	4	0.202 ± 0.021^c	0.231 ± 0.022^c

Membrane current density was expressed as membrane current (pA) per cell capacitance (pF).

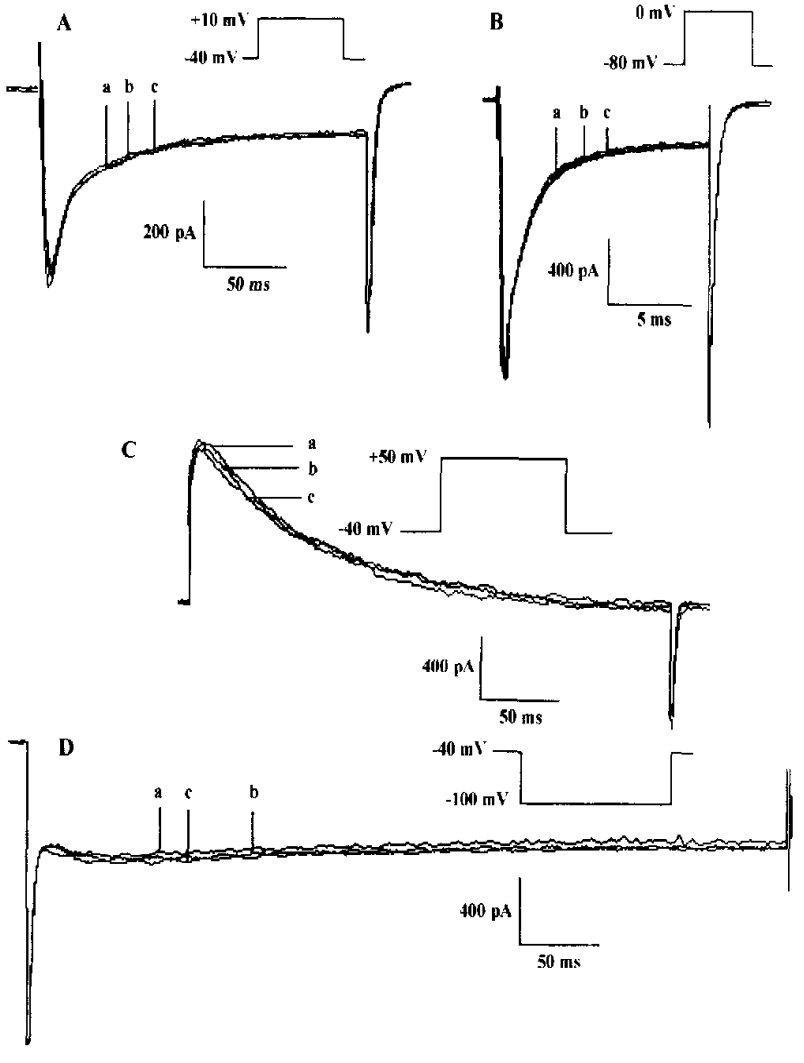

Fig 3. Effects of FRCRSFa and a series of ion currents in ventricular myocytes of rat. A, B, C, and D show the effects of FRCRSFa on $I_{Ca\text{-}L}$, I_{Na}, I_{to}, and I_{kl}, respectviely. a: control. b: FRCRSFa 5 μmol/L. c: washout.

DISCUSSION

Hobai *et al*[10] reported that the hexapeptide FRCRCFa inhibited Na^+/Ca^{2+} exchange current in cardiac myocytes from cell interior using intracellular dialysis *via* the patch-pipette. The hexapeptide FRCRSFa we synthesized was similar to FRCRCFa in amino acid sequence, but it was extracellularly effective to inhibit Na^+/Ca^{2+} exchange currents (inward and outward IC_{50} were 2 μmol/L and 4 μmol/L, respectively) as shown in our experiments. There was no detectable effect of FRCFSFa on the major ion channel currents (L-type Ca^{2+} current, voltage-gated Na^+ current, transient outward K^+ current, and inward rectifier K^+ current) in rat ventricular myocytes, showing its relatively selective inhibition on $I_{Na^+/Ca^{2+}}$. These results indicate that FRCRSFa is a relatively selective inhibitor of Na^+/Ca^{2+} exchanger.

Intracellular dialysis of FRCRSFa 30 μmol/L can notably inhibit $I_{Na^+\cdot Ca^{2+}}$ and can not induce further alteration of $I_{Na^+\cdot Ca^{2+}}$ when adding FRCRSFa with same concentration into perfused solution. This suggests that FRCRSFa perform its inhibitory action from cell interior and extracellular FRCRSFa can play its role of inhibitor to Na^+/Ca^{2+} exchange by passing through the membrance into the cell interior *via* a uncertain pathway.

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六肽 FRCRSFa 对大鼠心室肌 Na^+/Ca^{2+} 交换的抑制[1]

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关键词 FRCRSFa；膜片箝；Na^+/Ca^{2+} 交换；心肌

目的：研究六肽 FRCRSFa 对大鼠心室肌细胞 Na^+/Ca^{2+} 交换的作用及其特异性. **方法**：用膜片箝全细胞记录法测定 Na^+/Ca^{2+} 交换电流（$I_{Na\,Ca^{2+}}$）及其它离子通道电流. **结果**：六肽 FRCRSFa 对大鼠心室肌细胞 Na^+/Ca^{2+} 交换呈剂量依赖性抑制，内向和外向 $I_{Na^+/Ca^{2+}}$ 的 IC_{50} 分别是 2 μmol/L 和 4 μmol/L. FRCRSFa 5 μmol/L 对 L 型钙电流，门控钠电流、瞬时外向钾电流和内向整流钾电流均无显著抑制作用. **结论**：FRCRSFa 是一个对 Na^+/Ca^{2+} 交换选择性较高的抑制剂，对研究心肌细胞 Na^+/Ca^{2+} 交换具有较高价值.

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