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E-4031 enhanced Ca^{2+} transient and ventricular myocytes contraction via reverse mode Na^+/Ca^{2+} exchange in normal and hypertrophic rats[1]

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KEY WORDS E-4031; Fura-2; calcium; contraction; electric stimulation; Na^+/Ca^{2+} exchange; cardiomegaly

ABSTRACT

AIM: To study the effects of *N*-[4-[[1-[2-(6-methyl-2-pyridinyl)ethyl]-4-piperidinyl]carbonyl]phenyl] methanesulfonamide dihydrochloride dihydrate (E-4031), a specific I_k blocker, on Ca^{2+} transient and cell contraction of normal and hypertrophied rat ventricular myocytes. **METHODS:** Electrically triggered Ca^{2+} transient and cell shortening were measured simultaneously using the Ion Imaging System with charge coupled digital (CCD) camera. **RESULTS:** E-4031 (10 μmol/L) increased Ca^{2+} transient and cell shortening from (210±49) and (3.0±0.8) μm to (245±47) and (3.6±1.0) μm, respectively ($P<0.05$) in normal cardiomyocytes and from (196±54) and (3.0±1.3) μm to (240±49) and (3.6±1.3) μm respectively ($P<0.05$) in hypertrophied cardiomyocytes, while did not change calcium sensitivity in both groups. KB-R7943 completely blocked the activating effects induced by E-4031 in both of normal and hypertrophied cardiomyocytes. Nicardipine did not block the increasing effects of E-4031. **CONCLUSION:** E-4031 increased Ca^{2+} transient and cell contraction via stimulating reverse mode of Na^+/Ca^{2+} exchange while without influencing calcium sensitivity. These effects were more notable in hypertrophied cardiomyocytes than in normal myocytes.

INTRODUCTION

Previous studies showed that *N*-[4-[[1-[2-(6-methyl-2-pyridinyl)ethyl]–4-piperidinyl]carbonyl]phenyl] methanesulfonamide dihydrochloride dihydrate (E-4031), a specific I_k blocker, had positive inotropic action in guinea pigs and dogs[1]. It is generally accepted that this inotropic action results from prolongation of action potential duration (APD) induced by I_k blocking [2]. However, whether E-4031 has positive inotropic effect on rat cardiomyocytes in which there is no I_k remains uncertain. In general, Ca^{2+} influx during exitation-contraction coupling was through two major routes: voltage dependent L-type Ca^{2+} channels and re-

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verse-mode electrogenitic Na^+/Ca^{2+} exchange[3]. Our electrophysiological study[4] has proved that E-4031 increased Na^+/Ca^{2+} exchange current and did not affect inward calcium current. It is, therefore, a reasonable deduction that if E-4031 has positive inotropic effect on rat cardiomyocytes with the exception of alteration in calcium sensitivity, enhanced reverse mode Na^+/Ca^{2+} exchange would be a main factor. In order to demonstrate this supposition, we examined simultaneously the effect of E-4031 on Ca^{2+} transient and cell contraction of rat ventricular myocytes in normal and hypertrophied heart.

MATERIALS AND METHODS

Cell isolation Single ventricular myocytes were isolated from Wistar rats (250±30) g and rats with myocardial hypertrophy which was induced by banding the abdominal aorta at suprarenal level. The rats were provided by Experimental Animal Center of Shanxi Medical University (Grade II, Certificate № 070101). Briefly, cells were dissociated from rat hearts with Langendorff perfuse apparatus by a protocol which consisted of a constant-flow perfusion 8-10 mL/min with: 1) 5 min of Ca^{2+}-free Tyrode's solution; 2) 4-6 min of Ca^{2+}-free Tyrode's solution, containing collagenase (Type P, Boehringer Mannheim, 0.3 g/L); 3) 9 min of 0.18 mmol·L^{-1} of Ca^{2+} solution[5]. The isolated myocytes were stored in normal Tyrode's solution at room temperature (22 ℃) for 1 h before use.

Experimental protocol Cardiomyocytes were resuspended to 1×10^9/L and incubated with Fura-2/AM (5 μmol/L) at 37 ℃ for 40 min[6]. After washed out of extra fluorescence dye Fura-2/AM, cells were placed in an experimental chamber which was mounted on the stage of a Zeiss Axiovert microscope adapted for dual-wavelength excitation fluorescence microscopy. Cells were perfused continuously with Tyrode's solution (extracellular solution). Two platinum needles on opposite sides of the chamber were connected to the output of a stimulator for the electrical field stimulation. The frequency of field stimulation is 0.5 Hz with a 10-V pulse. Cells were selected for study based on their overall physical appearance, quiescence in the absence of electrical stimulation, and their ability to contract in response to electrical field stimulation.

Fluorescence and cell shortenting measurements Measurements were performed with the Imaging System. The Imaging System (Tillphotonics, Germany) consists of an air cooled CCD camera (Charge Coupled Digital camera), a camera control, an image-analysis computer, a video display monitor, and a high-speed wavelength switcher. The CCD camera is capable of collecting a series of fluorescent images at very rapid time intervals. From the same series of images, the ratio of $fluorescence_{340}/fluorescence_{380}$ (expressed as F_{340}/F_{380}) and the length of cells were measured with Tillvision v 3.3 software. Ca^{2+} transient was expressed by the ratio of F_{340}/F_{380}. Cell shortening (μm) is the quiescent length of the cell minus the shortest length of the same cell during its contraction. Calcium sensitivity was expressed by the ratio of Ca^{2+} transient/cell shortening of the same cell[7].

The extracellular solution is composed of (mmol/L): NaCl 140, $CaCl_2$ 1.0, $MgCl_2$ 2.0, HEPES 5.0, and glucose 10. The pH of the solution was adjusted to 7.4 with NaOH. The L-type Ca^{2+} channel was blocked by nicardipine (1 μmol/L), and reverse mode Na^+/Ca^{2+} exchange was blocked by a new specific blocker KB-R7943[8]. Drugs containing solution had the same composition with the extracellular solution. Solutions given to cells were switched by an equipment called BPS-4 Solution Exchange System from Scientific Instruments of New York. Cells were perfused with one solution for 3 to 4 min before they were recorded and then switched to another perfusing solution to do the next recording.

Drugs E-4031 was gifted by Eissai Pharmaceuticals (Tokyo, Japan). Fure-2/AM was the product of Sigma Company. KB-R7943 was purchased from Tocris Ltd. E-4031 and KB-R7943 were dissolved in distilled water as stock solution and diluted in perfusates to the desired final concentration just before use. Fure-2/AM was dissolved in dimethyl sulphoxide and diluted in perfusates to 5 μmol/L before use.

Statistical analysis All the data were presented as mean±SD. Paired *t* test were made and $P<0.05$ was

considered significant.

RESUITS

Ca^{2+} transient and cell contraction in normal and hypertrophied ventricular myocytes The amplitude of Ca^{2+} transient was a little lower in hypertrophied group than that in normal group (Tab 1, 2) but the difference had no statistical importance. The Ca^{2+} sensitivity of the two groups showed no significant difference.

Tab 1. Effects of E-4031 on Ca^{2+} transient and cell contraction in the presence of KB-R7943 (0.3 mmol/L) and nicardipine (1 mmol/L) in normal cardiac myocytes. *n*=7 cells from 7 rats. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* nicardipine.

Group	F_{340}/F_{380}	Shortening /μm	Ca^{2+} sensitivity/μm^{-1}
Control	210±49	3.0±0.8	71±16
E-4031 5 μmol/L	215±52	3.1±0.8	71±12
10 μmol/L	245±47^{b}	3.6±1.0^{b}	71±15
15 μmol/L	249±42^{b}	3.6±0.7^{b}	71±17
E-4031+KB-R7943	207±39	3.0±0.3	70±16
Nicardipine	78±14^{c}	1.7±0.7^{b}	55±19^{b}
Nicardipine+E-4031	141±12^{bf}	2.4±0.5^{e}	61±18

Tab 2. Effects of E-4031 on Ca^{2+} transient and cell contraction in the presence of KB-R7943 (0.3 mmol/L) and nicardipine (1 mmol/L) in hypertrophied cardiac myocytes. *n*=7 cells from 7 rats. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

Group	F_{340}/F_{380}	Shortening /μm	Ca^{2+} sensitivity/μm^{-1}
Control	196±54	3.0±1.3	70±26
E-4031 10 μmol·L^{-1}	240±49^{b}	3.6±1.3^{b}	69±22
E-4031+KB-R7943	190±49	2.9±0.8	70±11
Nicardipine	82±9^{c}	1.6±0.6^{c}	54±16^{b}
Nicardipine+E-4031	164±26^{b}	2.4±0.8	62±11

Effects of E-4031 on Ca^{2+} transient and cell contraction in normal ventricular myocytes E-4031 from 5 to 15 μmol/L enhanced Ca^{2+} transient and cell contraction concentration-dependently in normal myocytes with no change of Ca^{2+} sensitivity (Tab 1, Fig 1). KB-R7943 did not influence the baseline of Ca^{2+} transient and cell contraction (Fig 2), while completely

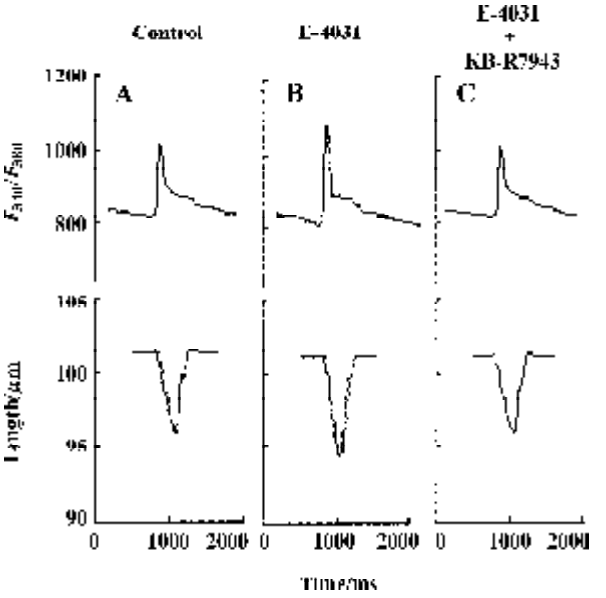

Fig 1. KB-R7943 (0.3 mmol/L) abolished the increments of Ca^{2+} transient and cell contraction induced by E-4031 (10 mmol/L) in normal cardiac myocytes (from the same cell).

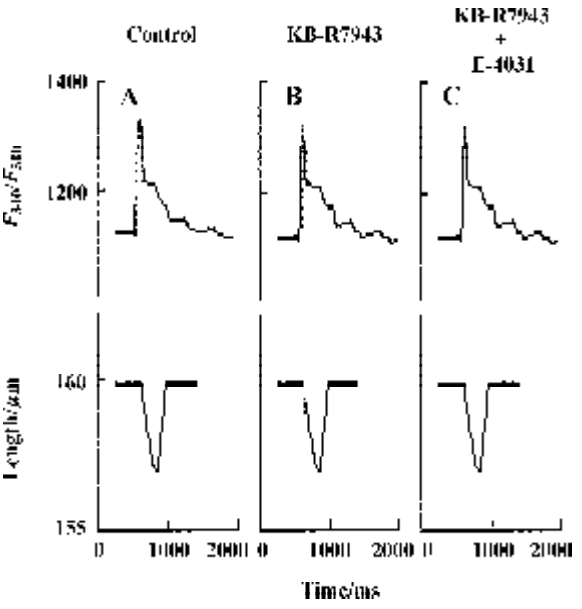

Fig 2. KB-R7943 (0.3 mmol/L) had no effect on the baseline of Ca^{2+} transient and cell contraction in normal cardiac myocytes (from the same cell).

abolished the increments of both Ca^{2+} transient and cell shortening induced by E-4031 (Fig 1, Tab 1) at concentration of 0.3 μmol/L. These results suggested that E-4031 enhanced Ca^{2+} transient and cell shortening via stimulating reverse mode Na^{+}/Ca^{2+} exchange.

Nicardipine, a L-type Ca^{2+} channel blocker, inhibited Ca^{2+} transient and cell contraction remarkably (Tab 1, Fig 3) at concentration of 1 μmol/L. The drug, however, did not block the increments of both Ca^{2+} transient and cell shortening induced by E-4031 at this concentration (Tab 1, Fig 3). These results gave further evidence that E-4031 enhanced Ca^{2+} transient and cell shortening via stimulating reverse mode Na^{+}/Ca^{2+} exchange.

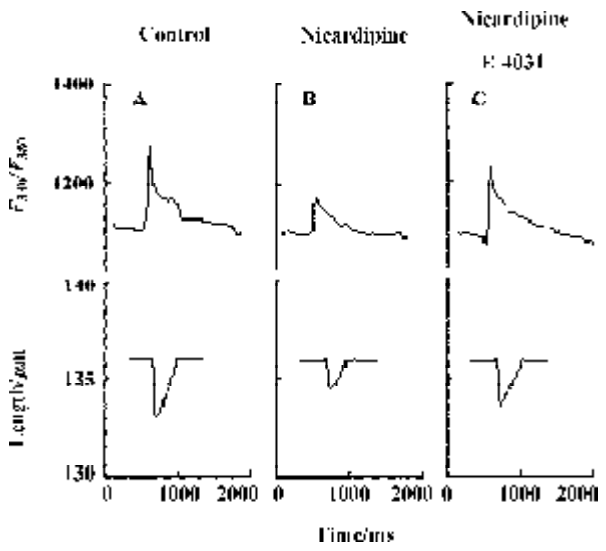

Fig 3. Effects of E-4031 (10 mmol/L) on Ca^{2+} transient and cell contraction in the presence of nicardipine (1 mmol/L) in normal cardiac myocytes (from the same cell).

Effects of E-4031 on Ca^{2+} transient and cell contraction in hypertrophied ventricular myocytes In hypertrophied cardiac cells, E-4031 10 μmol/L enhanced Ca^{2+} transient and cell contraction significantly (Tab 2, Fig 4). When cardiomyocytes were pre-treated with KB-R7943 for 3 min and then E-4031 was added in this perfuse solution, E-4031 failed to increase the amplitude of Ca^{2+} transient and cell contraction (Fig 4). KB-R7943, at concentration of 0.3 μmol/L, did not influence the baseline of Ca^{2+} transient and cell contraction

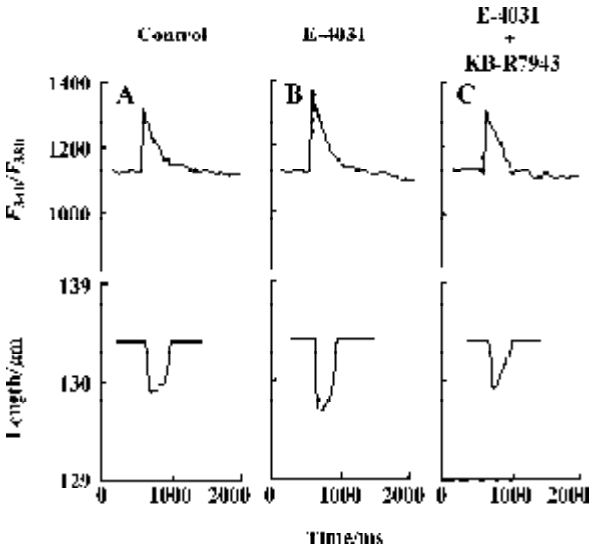

Fig 4. KB-R7943 (0.3 mmol/L) abolished the increments of Ca^{2+} transient and cell contraction induced by E-4031 (10 mmol/L) in hypertrophied cardiac myocytes (from the same cell).

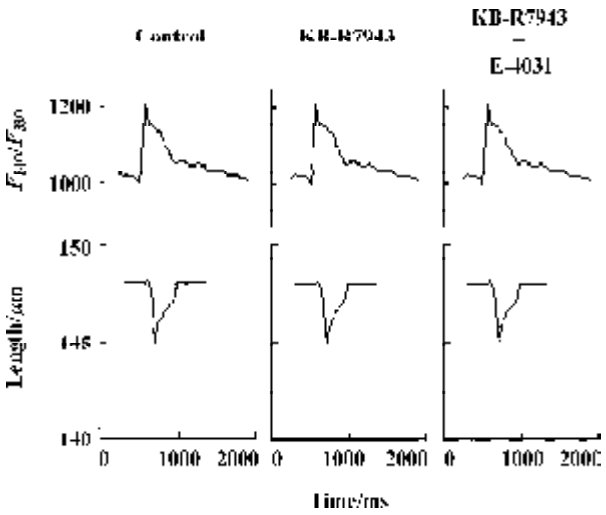

Fig 5. KB-R7943 (0.3 mmol/L) had no effect on the baseline of Ca^{2+} transient and cell contraction in hypertrophied cardiac myocytes (from the same cell).

while abolished their increments induced by E-4031 (Tab 2, Fig 5). E-4031 also increased Ca^{2+} transient and cell contraction in the presence of nicardipine (Tab 2, Fig 6), and the amplitude of Ca^{2+} transient increased by E-4031 was higher in hypertrophied myocytes than that in normal heart cells (Tab 1, 2).

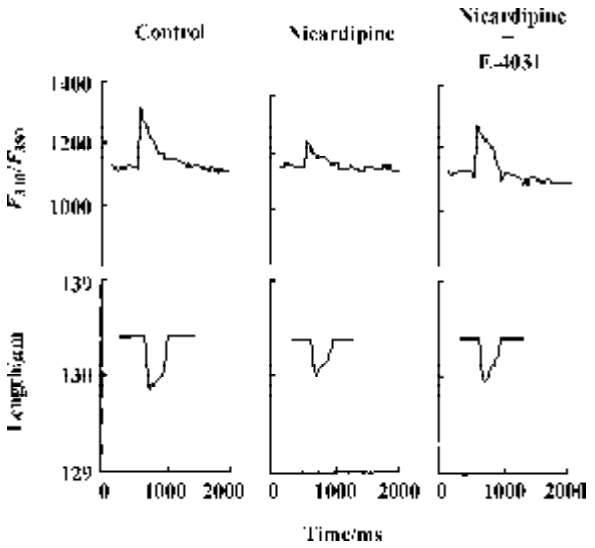

Fig 6. Effects of E-4031 (10 μmol/L) on Ca^{2+} transient and cell contraction in the presence of nicardipine (1 μmol/L) in hypertrophied cardiac myocytes (from the same cell).

DISCUSSION

In the present study, E-4031 exerted a positive inotropic effect with a significant increase in the amplitude of Ca^{2+} transient. In general, the increase of Ca^{2+} transient resulted from increase of Ca^{2+} influx via L-type Ca^{2+} channels. However, this possibility had been excluded since a more pronounced increase of Ca^{2+} transient could be induced by E-4031 when L-type Ca^{2+} channels were blocked. Recently, a number of experimental results suggested that the reverse mode Na^+/Ca^{2+} exchange might be involved in intracellular Ca^{2+} transient and cell contraction during the process of excitation-contraction coupling. Yang[8] *et al* and Fujita and Endoh[9] have reported that the activation of reverse mode Na^+/Ca^{2+} exchange may be responsible for the increase in contractility and Ca^{2+} transient induced by endothelin-1 and angiotensin II. Our results showed that the increase of Ca^{2+} transient and cell shortening induced by E-4031 were completely abolished by KB-R7943 0.3 μmol/L, a specific blocker of reverse mode Na^+/Ca^{2+} exchanger. Thus, a possible mechanism was that reverse mode Na^+/Ca^{2+} exchange might be involved.

In rats with myocardial hypertrophy, E-4031 showed a similar pharmacologic profile as in normal rats but more pronounced stimulating effects on Ca^{2+} transient. These observations are consistent with the findings that in hypertrophied myocardium, the Na^+/Ca^{2+} exchanger gene expression was enhanced[10] and the density of Na^+/Ca^{2+} exchange current was increased[4].

The Ion Imaging System with CCD camera ensures simultaneous measurement of cell length and Ca^{2+} transient to analyse the changes in calcium sensitivity. The experimental results excluded the possibility that the positive inotropic effect induced by E-4031 was produced by increase of calcium sensitivity.

In summary, the present results suggested that E-4031 enhanced Ca^{2+} transient and cell contraction via reverse mode Na^+/Ca^{2+} exchange with no significant influence on calcium sensitivity and these effects were more notable in hypertrophied rat cardiomyocytes than in normal cardiomyocytes.

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E-4031通过反向钠钙交换增加正常和心肌肥厚大鼠心肌细胞钙瞬变和收缩[1]

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关键词 E-4031；Fura-2；钙；收缩；电刺激；钠钙交换；心肥大

目的：观察 I_K 阻断剂E-4031对正常和心肌肥厚大鼠钙瞬变和细胞收缩的影响．方法：应用带CCD Camera的离子影象分析系统 (Ion Imaging System)测定离体大鼠心肌细胞钙瞬变和细胞长度．结果：E-4031 (10 μmol/L)分别使正常组钙瞬变和细胞缩短从对照组的(210±49)和(3.0±0.8) μm增加到(245±47)和(3.6±1.0)μm($P<0.05$)，使心肌肥厚组钙瞬变和细胞缩短分别从对照组的(196±54)和(3.0±1.3) μm 增加到(240±49)和(3.6±1.3)μm（$P<0.05$），而对钙敏感性无显著影响．KB-R7943可完全阻断正常组和肥厚组心肌细胞由E-4031诱导的激动作用．尼卡地平不能阻断E-4031的增加作用．结论：E-4031可通过刺激反向钠钙交换增加正常和心肌肥厚大鼠心肌细胞钙瞬变和细胞收缩，同时并不影响钙敏感性．且对肥厚心肌细胞的影响大于正常心肌细胞．

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