SEA0400, a Novel Na^+/Ca^{2+} Exchanger Inhibitor, Reduces Calcium Overload Induced by Ischemia and Reperfusion in Mouse Ventricular Myocytes

Jun WANG[1], ZhiFei ZHANG[1], YingAn HU[1], XiaoLi HOU[1], Qian CUI[1], YiMin ZANG[2*], Chen WANG[3*]

[1.] *Department of Physiology, Capital University of Medical Sciences, Beijing 100069, China*

[2.] *Department of Physiology, Fourth Military Medical University, Xi An 710032,China*

[3.] *Beijng Institute of Respiratory Medicine, Beijing 100020, China*

**:Corresponding author*

Contacted to:

Jun Wang

Department of Physiology

Capital University of Medical Sciences,

Beijing 100069,China.

Email: wangjunbw@tom.com

Tel: 86-10-63652116

Fax:86-10-83911494

Summary

Given the potential clinical benefit of inhibiting Na^+/Ca^{2+} exchanger (NCX) activity during myocardial ischemia reperfusion (I/R), pharmacological approaches have been pursued to both inhibit and clarify the importance of this exchanger. SEA0400 was reported to have a potent NCX selectivity. Thus, we examined the effect of SEA0400 on NCX currents and I/R induced intracellular Ca^{2+} overload in mouse ventricular myocytes using patch clamp techniques and fluorescence measurements. Ischemia significantly inhibited inward and outward NCX current (from –0.04±0.01nA to 0 nA at –100 mV; from 0.23±0.08 nA to 0.11±0.03 nA at +50 mV, n=7), Subsequent reperfusion not only restored the current rapidly but enhanced the current amplitude obviously, especially the outward currents (from 0.23±0.08 nA to 0.49±0.12 nA at +50 mV, n=7). $[Ca^{2+}]_i$, expressed as the ratio of Fura-2 fluorescence intensity, increased to 138±7% (P<0.01) during ischemia and to 210±11%(P<0.01) after reperfusion. The change of NCX current and the increase of $[Ca^{2+}]_i$ during I/R can be blocked by SEA0400 in a dose-dependent manner with an EC_{50} value of 31 nM and 28 nM for the inward and outward NCX current respectively. The results suggested that SEA0400 is a potent NCX inhibitor which can protect mouse cardiac myocytes from Ca^{2+} overload during I/R injuries.

Key words

SEA0400 • Ischemia/reperfusion • Patch clamp • Na^+/Ca^{2+} exchanger current

Introduction

The Na^+/Ca^{2+} exchanger (NCX) is a transmembrane protein expressed in the membrane of almost every cell type. It transports Ca^{2+} in exchange for Na^+ bi-directionally across the cell. It can catalyze electrogenic exchange of Na^+ and Ca^{2+} across the plasm membrane. The stoichiometry of NCX is 3 Na^+ per Ca^{2+}. So when it works via the forward mode (Ca^{2+} extrusion),it will produce an inward current (1 Ca^{2+} extrusion , 3Na^+ influx) , and if it works via the reverse mode(Ca^{2+} influx), it will generate an outward currents (3Na extrusion 1 Ca^{2+} influx). In general, this antiport system is involved in the homeostasis of intracellular Ca^{2+} concentration, helping to maintain the resting Ca^{2+} concentration and indirectly regulating other biological functions in many cell types. There is growing evidence that NCX is an important mechanism in mammalian cardiac cell Ca^{2+} regulation. It works as a central mechanism for Ca^{2+} extrusion and muscle relaxation (Egger *et al.*1999). So it is likely that pathophysiological factors that lead to the change of NCX activity will result in disruption to the cellular Ca^{2+} handling.

Myocardial ischemia and reperfusion (I/R) has profound effects on the function and viability of cardiac myocytes by elevation of intracellular Ca^{2+} concentration. Several mechanisms including change of NCX function have been proposed for I/R induced $[Ca^{2+}]_i$ elevation (Silerman *et al.* 1994). And there are already many reports demonstrating the beneficial effects of NCX inhibitors on myocardial I/R injuries. But as the NCX inhibitors used in those studies have other non-specific actions including Na^+/H^+ exchanger inhibition and Ca^{2+} channel blockade, a question remains as to whether their benefits were due to inhibition of cardiac NCX. Moreover, whether I/R injury per se alters NCX activity directly in mouse cardiac myocytes is still not yet completely understood

Recently,2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400), was reported to have protective effect against cell death, cardiac dysfunction and cardiac arrhythmias after myocardial I/R (Lee *et al.* 2004). But its direct effect on NCX current in mouse cardiac myocytes has not been investigated.

The aim of this study was to observe the effect of simulated I/R on NCX current and the mechanism of protective effect of SEA0400 on myocardial I/R injury in single cell models.

Methods

Cell isolation

Ventricular myocytes were enzymatically isolated from heart of C57BL/6J mouse. Briefly, the mouse was injected with heparin (100 U intraperitoneally) 30 min before sacrifice and then deeply anesthetized by pentobarbital sodium (50mg/kg i.p.). The heart was rapidly excised and arrested in ice-cold buffer. The aorta was cannulated and mounted on a langendorff apparatus. The heart was retrogradely rinsed for 8–10 min with calcium-free Tyrode solution, consisting of (in mmol/L) NaCl 137, KCl 5.4, NaH_2PO_4 1.2, $MgCl_2$ 0.5, HEPES 5, Glucose 5.5 (pH 7.4 by NaOH). The heart was enzymatically digested by perfusing with normally calcium-free Tyrode solution containing collagenase (0.1 mg /ml, wako Japan) and BSA (1 mg/ml). After 5-8 min of digestion, the heart was perfused with calcium-free Tyrode solution, again for 5 minutes. The temperature of these perfusates was maintained at 36 ± 0.5^{o}C and equilibrated with 100% O_2. The heart was then minced in 30 ml calcium-free Tyrode solution, to disperse cells and then kept at 4℃. All experimental protocols were approved in advance by the Ethics Review Committee for Animal Care and Experimentation of the Capital University of Medical Sciences.

Whole-cell voltage-clamp recordings

Whole-cell currents were recorded from isolated cardiac myocytes using nystatin-perforated patch clamp configuration (Horn *et al.*1988). Nystatin concentration was 250 μg/ml. Pipettes were pulled from borosilicate glass capillaries with a micropipette puller (P-2000: Sutter Instruments, Novato, CA). The electrode had a resistance of between 4-5 MΩ when filled with pipette solution. Experiments were initiated when the series resistance was stable and under 20 MΩ. Series resistance was monitored at 5 min intervals during an experiment, cells with high series resistance(>20 MΩ) were discarded. For NCX current, a ramp pulse was used to activate the NCX current. The ramp pulse included three phases: an initial phase of 90 mV depolarizing from the holding potential of –30 mV to +60 mV (150 ms), a second phase of 180 mV hyperpolarization to –120 mV(250 ms) and then a third phase returning to the holding potential –30 mV(150 ms). The I/V curve was measured during the second hyperpolarization phase.

Data were acquired using an EPC-9 amplifier and Pulse software (HEKA Electroniks, Lambrecht, Germany). Current signals were low-pass filtered at 2.9 kHz using a four-pole Bessel filter and digitized at 10 kHz. Sampled data were analyzed by an original software application called PulseMate, and Origin 6.1 (Origin Lab Inc.,

Northampton, MA).

For patch clamp recordings, the extracellular solution contained (in mmol/L) NaCl 140, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 0.3, HEPES 10, Glucose 5.5. (pH was adjusted to 7.4). To block Na^+/K^+ pump currents, and currents flowing through K^+ or Ca^{2+} channels, ouabain (20 μmol/L), CsCl (2 mmol/L), $BaCl_2$ (2 mmol/L) and nisoldipine (5 μmol/L) were added to the solution. The pipette solution contained (in mmol/L) CsOH 140, aspartic acid 40, NaCl 10, $CaCl_2$ 21, EGTA 42, $MgCl_2$ 2, HEPES 5. EC_{50} value was calculated by interpolation between the two data points close to 50% inhibition. The free Ca^{2+}concentrations of the internal solution for NCX current measurement was calculated to be 67 nM. Chemical ischemia solution was produced by adding 5 mmol/L NaCN and 10 mmol/L deoxyglucose in the glucose-free extracellular solution. Ischemia condition was made by perfusing the cells with chemical ischemia solution and reperfusion was achieved by changing ischemia solution to the control extracellular solution. This method is convenient, fast and severe enough to produce an ischemia single cell model. And the chemicals (NaCN and deoxyglucose) have little effect on the cell autofluorescence. The temperature of all perfusing solutions was maintained at 36± 0.5^{o}C.

Fura-2 fluorescence measurement

The Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed by Fura-2 fluorescence ratio image method as described previously(Allen et al. 1983). Cells were loaded with 5 μmol/L Fura-2/AM (Molecular Probes, Eugene, Oreg.) for 30 min at 35°C and then transferred to recording chamber. After a brief period to allow adhesion, cells were continuously perfused with pre-warmed (36°C) bath solution (same as extracellular solution for patch clamp recording). Experiments were initiated after 15 min of perfusion to wash out extracellular Fura-2/AM and to allow the conversion of intracellular dye into its non-ester form. Fluorescence images at excitation wavelengths of 340 and 380 nm were collected and digitized by an image processor (Argus 50; Hamamatsu Photonica, Hamamatsu City, Japan.). The background fluorescence was determined by removing the cell from the field after the experiment. The $[Ca^{2+}]_i$ was represented as the fluorescence intensity ratio (F_{340}/F_{380}).

Chemicals

EGTA and nystatin were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were obtained from Sigma-Aldrich Japan Inc. (Tokyo, Japan). SEA0400 was dissolved in dimethylsulphoxide (DMSO) as 1mmol/L stock solution.

Statistical analysis

Data were presented as means ± S.E. of *n* observations. Statistical differences in data were evaluated by unpaired Student's *t-test* and a probability value (*P*) less than 0.05 was considered significant.

Results

NCX currents in mouse cardiac myocytes

Mouse cardiac myocytes were voltage-clamped at –30 mV using perforated patch clamp method and NCX currents were recorded by using ramp pulse under conditions in which the currents through various ion channels and Na^+/K^+ pump were blocked. The currents activated by the ramp pulse is shown in Fig 1Aa. To validate that this current includes NCX currents further, the sensitivity of the current to a NCX blocker Ni^{2+} was tested. 5 mmol/L Ni^{2+}decreased the current obviously. (Fig 1Ab). So the electrogenic NCX currents can be measured as Ni^{2+}-sensitive current by subtracting the current in the presence of 5 mM Ni^{2+}, from the currents in the absence of Ni^{2+}.

Blocking effect of SEA0400 on NCX currents

Recently, it was reported that SEA0400 is a novel agent that preferentially

blocks the Ca^{2+} influx mode of cardiac NCX rather than the Ca^{2+} extrusion mode in guinea pig myocytes with a high affinity (Takahashi *et al.*2004, Hobai *et al.*2004). So we examined the sensitivity of NCX current to SEA0400 in mouse cardiac myocytes.

When 0.3μM SEA0400 was added to the extracellular solution, NCX current was inhibited significantly (not only outward current but also inward current) (Fig 1B). Further research was employed to observe the dose-dependency of this inhibitory effect. The results showed that 1 μM SEA0400 inhibited the NCX current by more than 95%, and the inhibition effect is dose-dependent (Fig 1C). The EC_{50} value of SEA0400 for the inward and outward NCX current was 31 nM and 28 nM, respectively.

These results indicated that NCX current in mouse cardiac myocytes was sensitive to SEA0400 for both inward and outward currents. Similar experiments were performed in 5 cells.

Fig1, near here

Effect of chemical ischemia/reperfusion on NCX currents

Till now it was not yet clear whether chemical ischemia or reperfusion has direct effect on NCX currents in mouse cardiac myocytes. So next we sought to determine the effect of chemical ischemia and reperfusion on NCX currents. After equilibration with

control extracellular solution for more than 5 minutes, cells were perfused with chemical ischemia solution. Compared with the control currents, chemical ischemia inhibited both inward and outward currents(from –0.04±0.01nA to 0 nA at –100 mV; from 0.23±0.08 nA to 0.11±0.03 nA at +50 mV,n=7). Subsequent recovery to the normal control solution (reperfusion), not only restored the current to its pre-ischemia level but also enhanced the current amplitude obviously within one minute (from –0.04 ±0.01 nA to –0.07±0.03 nA at –100 mV; from 0.23±0.03 nA to 0.49±0.12 nA At +50 mV,n=7) and the reversal potential of reperfusion current was shifted to a more negative potential. The current-voltage (I/V) relationships during control, chemical ischemia and reperfusion are shown in Fig 2A. The Ni^{2+}-sensitive component (i.e. NCX currents) during control, chemical ischemia and reperfusion which were obtained by subtraction are shown in Fig 2B.

Fig2, near here

Effect of SEA0400 on $[Ca^{2+}]_i$ during chemical ischemia/reperfusion

According to the above results, ischemia inhibited NCX current and reperfusion enhanced NCX current especially the outward current. As it is well-known that NCX is a main mechanism for extruding intracellular Ca^{2+} under physiological conditions, its

inhibition should result in an elevation of intracellular Ca^{2+} concentration and as reperfusion enhanced the outward NCX current this should also result in an obvious increase of intracellular Ca^{2+} concentration. To investigate if the change of NCX current was in accordance with the intracellular $[Ca^{2+}]_i$ during ischemia and reperfusion, $[Ca^{2+}]_i$ was measured. The measurement of $[Ca^{2+}]_i$ was made in a quiescent ventricular myocyte loaded with Fura-2 and then subjected to 8 minutes of chemical ischemia followed by 20 minutes reperfusion. This protocol was designed to provide a severe enough insult to cardiac myocytes but neither Ca^{2+} waves nor hypercontracture was induced during ischemia. In contrast, some cells showed spontaneous Ca^{2+} waves and hypercontracture during reperfusion. During ischemia, $[Ca^{2+}]_i$ slightly increased but the most profound increase in $[Ca^{2+}]_i$ occurred immediately after reperfusion(Fig 3).SEA0400 (0.3 μmol/L) blocked the increasing of $[Ca^{2+}]_i$ during ischemia, especially during reperfusion.

Fig3, near here

Discussion

NCX is a transporter protein that couples the translocation of Ca^{2+} to that of Na^{+}

in the opposite direction and contribute to the maintenance of intracellular Ca^{2+} homeostasis in a wide variety of cell types (Van Breemen *et al*.1989). The role of NCX has been considered, at least in part, as a mechanism of Ca^{2+} overload in reperfusion injury (Nishida *et al.* 1993, Li *et al*.1991).

In this study, the important findings are that NCX activity was inhibited during ischemia but enhanced after reperfusion especially the outward current, and SEA0400 could block the current in both direction. Whereas, $[Ca^{2+}]_i$ increased both during ischemia and reperfusion, this $[Ca^{2+}]_i$ increasing could be blocked by SEA0400.

The inhibition of NCX function during ischemia is consistent with previous reports (Shigematsu *et al*. 1999, Satoh *et al*. 1995). There already have been some reports suggesting that inhibition of NCX function during ischemia was caused by intracellular acidosis and decreased cytosolic level of ATP (Earm *et al.* 1986). The results in this experiment showed that during ischemia, the inward mode of NCX currents was almost inhibited completely, this might be the reason for the increase in $[Ca^{2+}]_i$ though there may also be inhibition of other $[Ca^{2+}]_i$ extrusion mechanism such as Ca^{2+} ATPase in sarcolemma or the SR. Reperfusion increased NCX current especially the outward currents significantly and the reversal potential shifted to a more negative potential in this experiment. The increase of the outward currents would results in a

sharp increase in $[Ca^{2+}]_i$. This is consistent with the results of $[Ca^{2+}]_i$ imaging experiment. The NCX current's recovery and enhancement during reperfusion seems relatively rapid (within 1 min), it might be because of the removal of ischemia inhibitory effect on NCX or could also be a results of activation of certain processes known to stimulate NCX such as protein kinase C or reactive oxygen species. During ischemia, because of the inhibition of the forward mode NCX function, the intracellular Na^+ increased. And the progressive increase of the intracellular Na^+ will lead the operation mode of NCX changing from forward mode to reverse mode and shifting the reversal potential to the more negative potential.

SEA0400 has been reported to have a protective effect against myocardial stunning in dogs and can attenuate reperfusion injury in the in vitro and in vivo cerebral ischemic models (Matsuda et al. 2001). In isolated rabbit hearts, (subjected to regional ischemia and reperfusion) SEA0400 elicited concentration-dependent reductions in infarct size (SEA0400 EC50: 5.7 nM)(William *et al.*2003). The present results demonstrat that SEA0400 is a potent inhibitor of cardiac NCX current with an EC_{50} value of 31 nM and 28 nM for both inward and outward directions and can attenuate the Ca^{2+} overload induced by I/R.

In conclusion, these results suggested that NCX current in mouse ventricular

myocytes was inhibited by simulated ischemia but enhanced by reperfusion and the change of NCX activity after I/R plays a key role in Ca^{2+} homeostasis. The hyperactivity of the reverse mode of NCX may be responsible for the $[Ca^{2+}]_i$ overload during reperfusion (Satoh *et al.* 1995, Shigematsu *et al.*1999). SEA0400 was found to be a potent and highly selective inhibitor of NCX, and would be a powerful tool for further studies on the role of NCX in the heart and the therapeutic potential of its inhibition

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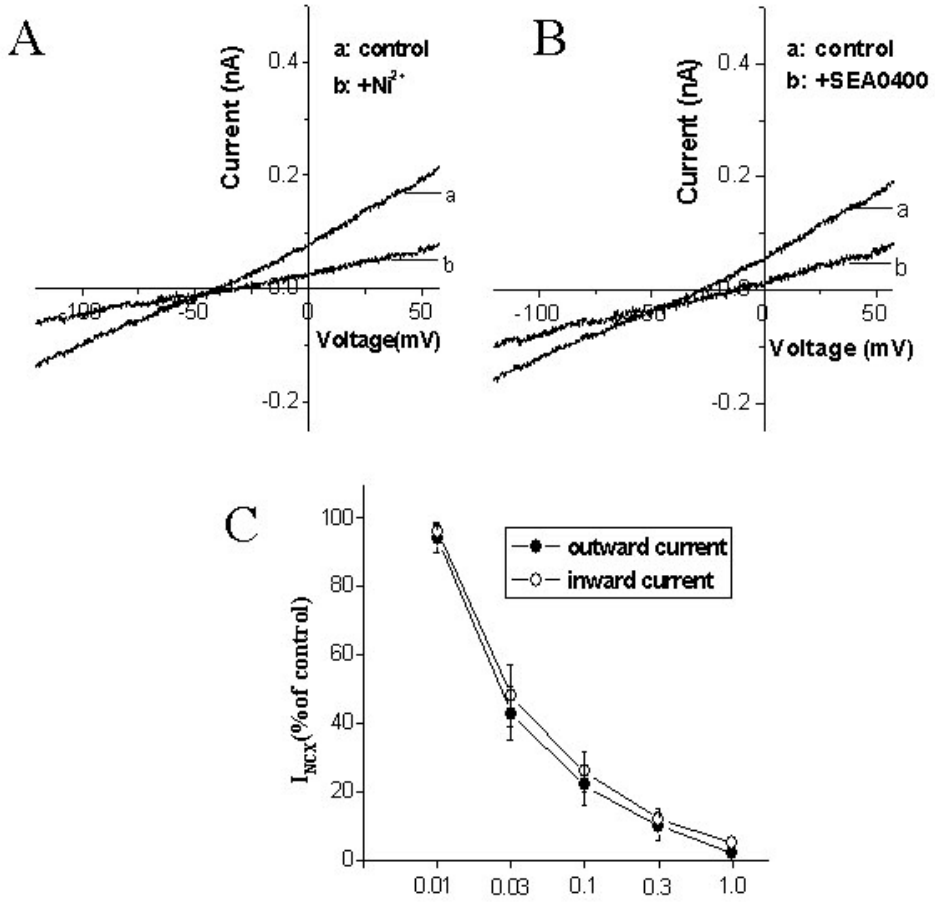

Fig.1. Na^+/Ca^{2+}exchange currents in mouse cardiac myocytes and its sensitivity to Na^+/Ca^{2+}exchange blockers. A: I-V curves of Na^+/Ca^{2+} currents before (a) and during (b) 5 mmol/L Ni^{2+} perfusion. B: I-V curves of Na^+/Ca^{2+} currents before (a) and during (b) 0.3 μmol/L SEA0400 perfusion. Similar results were achieved in 5 cells. C: Concentration -response relationships of the inhibitory effect of SEA0400 on NCX current. The outward currents was achieved at +50 mV, inward current was achieved at -100 mV (n=5).

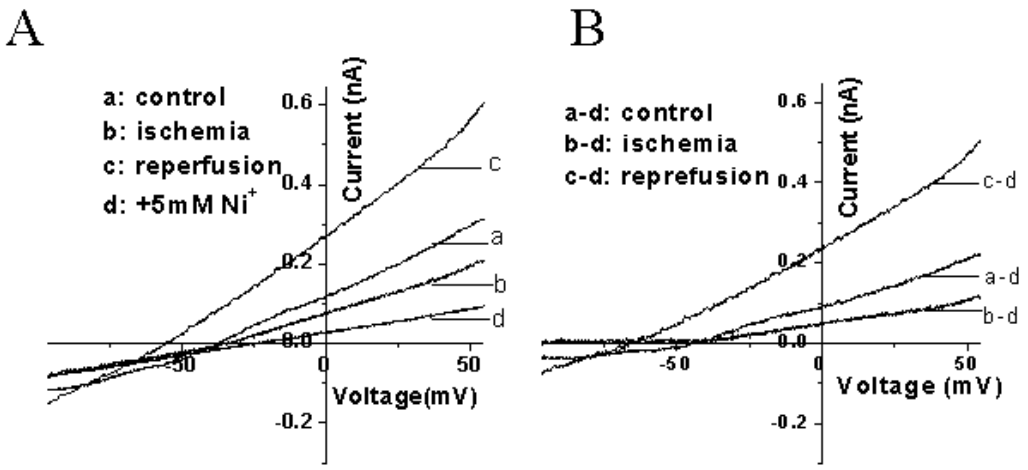

Fig.2. Effect of chemical ischemia/reperfusion on Na^+/Ca^{2+}exchange currents. A: +60 mV to –120 mV ramp pulse induced NCX currents. a: control current; b: current during chemical ischemia; c: current during reperfusion; d: current during perfusing control solution plus 5 mmol/L Ni^{2+}. B: a-d: Ni^{2+}-sensitive control NCX currents; b-d: Ni^{2+}-sensitive NCX current during ischemia; c-d: Ni^{2+}-sensitive NCX exchange currents after reperfusion.

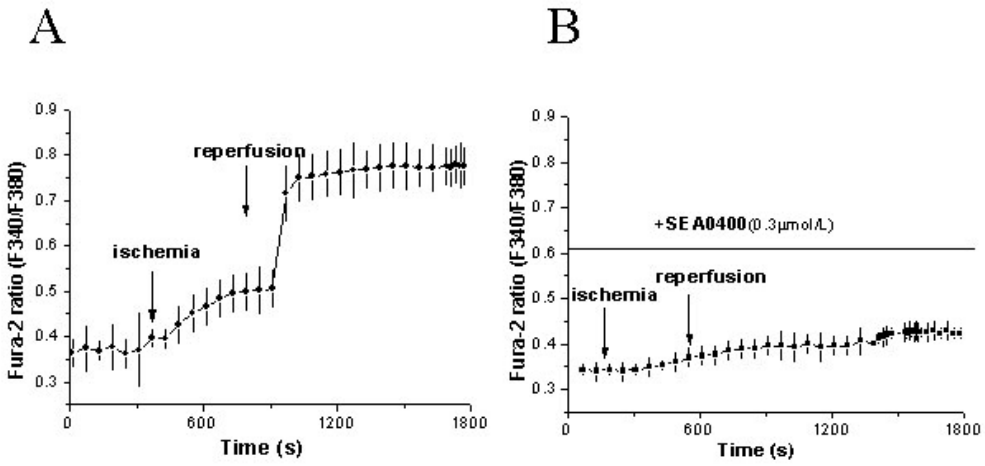

Fig.3. $[Ca^{2+}]_i$ change upon chemical ischemia and reperfusion in mouse cardiac myocytes and the blockade effect of SEA0400. A: Fura-2 measurement was performed under control, ischemia and reperfusion conditions. The radio (340/380 nm) was used as a quantitative indicator of free $[Ca^{2+}]_i$. B: Blockade effect of SEA0400(0.3 μmol/L) on $[Ca^{2+}]_i$ change during ischemia and reperfusion(n=11 from at least 5 mice).