

## Full Paper

**Involvement of the  $\text{Na}^+/\text{Ca}^{2+}$  Exchanger in Ouabain-Induced Inotropy and Arrhythmogenesis in Guinea-Pig Myocardium as Revealed by SEA0400**Hikaru Tanaka<sup>1,\*</sup>, Hideaki Shimada<sup>1</sup>, Iyuki Namekata<sup>1</sup>, Toru Kawanishi<sup>2</sup>, Naoko Iida-Tanaka<sup>1,3</sup>, and Koki Shigenobu<sup>1</sup><sup>1</sup>Department of Pharmacology, Toho University Faculty of Pharmaceutical Sciences, Funabashi, Chiba 274-8510, Japan<sup>2</sup>Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo 158-8501, Japan<sup>3</sup>Department of Food Science, Otsuma Woman's University, Chiyoda-ku, Tokyo 102-8357, Japan

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**Abstract.** Involvement of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger in ouabain-induced inotropy and arrhythmogenesis was examined with a specific inhibitor, SEA0400. In right ventricular papillary muscle isolated from guinea-pig ventricle, 1  $\mu\text{M}$  SEA0400, which specifically inhibits the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger by 80%, reduced the ouabain (1  $\mu\text{M}$ )-induced positive inotropy by 40%, but had no effect on the inotropy induced by 100  $\mu\text{M}$  isobutyl methylxanthine. SEA0400 significantly inhibited the contracture induced by low  $\text{Na}^+$  solution. In HEK293 cells expressing the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, 1  $\mu\text{M}$  ouabain induced an increase in intracellular  $\text{Ca}^{2+}$ , which was inhibited by SEA0400. The arrhythmic contractions induced by 3  $\mu\text{M}$  ouabain were significantly reduced by SEA0400. These results provide pharmacological evidence that the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger is involved in ouabain-induced inotropy and arrhythmogenesis.

**Keywords:** ouabain,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, SEA0400, inotropy, arrhythmia

**Introduction**

The  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) is involved in the physiological and pathophysiological regulation of  $\text{Ca}^{2+}$  concentration in the myocardium. It is considered to function both in the forward ( $\text{Ca}^{2+}$  extrusion) and reverse ( $\text{Ca}^{2+}$  influx) modes. The major role of myocardial NCX is to extrude  $\text{Ca}^{2+}$  from the cell through the forward mode and produce relaxation. Contribution of reverse mode NCX to  $\text{Ca}^{2+}$  entry during the early phase of normal myocardial contraction has also been postulated. The mode of NCX action possibly changes during the contractile cycle and the balance may vary with factors such as the animal species, developmental stage and the condition of the myocardium (1, 2). NCX activity is closely related to intracellular  $\text{Ca}^{2+}$  handling and is involved in normal and abnormal myocardial pacemaking (3).

NCX has also been considered to be involved in cardiac glycoside-induced positive inotropy (4). Cardiac glycosides, which inhibit the sodium-potassium pump, would increase intracellular  $\text{Na}^+$  concentration, which in turn shifts the mode of NCX towards the reverse mode, and produce positive inotropy through an increase in intracellular  $\text{Ca}^{2+}$  concentration. Extensive evidence has been presented for this view including studies with fetal myocardial tubes from NCX knockout mouse (5). However, pharmacological assessment of the role of NCX in cardiac glycoside-induced inotropy and arrhythmogenesis has been limited because of the lack of an NCX inhibitor with sufficient specificity.

SEA0400 {2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline} is a potent and selective inhibitor of NCX in cultured rat neurons, astrocytes, microglia, and myocytes and dog sarcolemmal vesicles with negligible affinities towards other transporters, ion channels, and receptors (6–9). We have previously examined the effects of SEA0400 on the myocardial NCX current using voltage clamped guinea-pig ventricular myocytes (7) and found that SEA0400 concentra-

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tion-dependently inhibits the NCX current with  $IC_{50}$  values of 40 and 32 nM for the forward and reverse modes, respectively. This was confirmed with NCX1 expressed in HEK293 cells (10). SEA0400 (1  $\mu$ M), which inhibited NCX current by more than 80%, had no effect on the  $Na^+$  current, L-type  $Ca^{2+}$  current, delayed rectifier  $K^+$  current, and inwardly rectifying  $K^+$  current (7) and the  $Ca^{2+}$  sensitivity of contractile proteins (11). KB-R7943, which has been used as an inhibitor of NCX, was revealed to have virtually no selectivity for these ion channels and transporters (7). Thus, SEA0400 can become the first specific pharmacological tool to study the role of NCX and was shown to be useful in studies on myocardial excitation-contraction mechanisms, regulation by autonomic transmitters, and ischemia-reperfusion injury (6, 11–17).

Concerning the action of cardiac glycosides, inhibition by SEA0400 of digitalis-induced arrhythmias in canine models was reported (18), but the mechanisms of cardiac glycoside-induced inotropy and arrhythmogenesis has not been examined in the isolated working myocardium with this selective inhibitor. The present study was performed to obtain pharmacological evidence for the involvement of NCX in cardiac glycoside-induced inotropy and arrhythmogenesis.

## Materials and Methods

### *Measurement of contractile force and contracture in papillary muscle preparations*

Isolated papillary muscle preparations were made, and contractile force was measured with standard techniques as described (19). Contracture induced by a low sodium solution was used as an index of reverse-mode NCX activity as described earlier (20) with a slightly modified protocol. The initial extracellular solution was of the following composition and gassed with 95%  $O_2$ –5%  $CO_2$ : 113.1 mM NaCl, 4.6 mM KCl, 2.45 mM  $CaCl_2$ , 1.2 mM  $MgCl_2$ , 21.9 mM  $NaHCO_3$ , and 10 mM glucose (pH 7.4). After the contractile force reached the steady state, stimulation was ceased. The preparation was incubated for 30 min with or without 1  $\mu$ M SEA0400, and thereafter the solution was changed to a low-sodium solution. The low-sodium solution was prepared with the equimolar substitution of tetramethyl-ammonium chloride for NaCl in a modified Krebs solution, so that the final  $Na^+$  concentration was 21.9 mM. This solution also contained 10  $\mu$ M monensin, 20 mM caffeine, and 4.9 mM  $CaCl_2$ . The amplitude of low-sodium contracture was expressed as the percentage of steady-state developed tension. SEA0400 (1  $\mu$ M) was applied from 30 min before the low-sodium perfusion and kept in the low-

sodium solution continuously.

### *Preparation of HEK293 cells expressing NCX and measurement of cytoplasmic $Ca^{2+}$ concentration*

HEK293 cells stably expressing bovine NCX1 were obtained in our previous study (10). Cytoplasmic  $Ca^{2+}$  was monitored with the fluorescent probe fura 2. The cells were loaded with 5  $\mu$ M fura 2/AM for 30 min at 37°C. They were excited at 340 and 380 nm, and emission (>500 nm) was separated with a dichroic mirror. Data acquisition and analysis were performed with the aquacosmos system (Hamamatsu Photonics, Hamamatsu). Calibration was performed *in situ* as in our previous study (17).

### *Drugs and chemicals*

SEA0400 was provided by Taisho Pharmaceutical Company, Ltd. (Saitama). The drug was dissolved in dimethyl sulfoxide (final concentration of 0.01%). Fura 2 was obtained from Dojin (Kumamoto). All other chemicals were of the highest commercially available quality.

### *Data and statistics*

Statistical significance between means was evaluated by Student's *t*-test or by the  $\chi^2$ -test, and a *P* value less than 0.05 was considered significant.

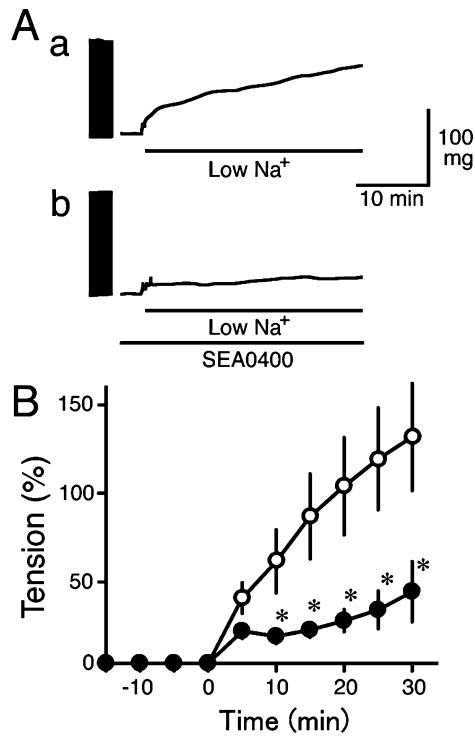
## Results

### *Effect of SEA0400 on contracture induced by low-sodium solution*

The inhibitory activity of SEA0400 on reverse mode NCX was confirmed in myocardial tissue preparations (Fig. 1). Treatment of ventricular tissue preparations with a low  $Na^+$  extracellular solution resulted in muscle contracture. SEA0400 (1  $\mu$ M) significantly decreased the contracture; the magnitude of the contracture in the absence and presence of SEA0400 at 30 min was  $134.7 \pm 34.2\%$  and  $43.4 \pm 18.4\%$  ( $n = 10$ ), respectively, of the initial contractile force.

### *Effect of SEA0400 on ouabain-induced inotropy*

Effect of SEA0400 on ouabain-induced inotropy was examined in papillary muscles isolated from guinea-pig right ventricle (Fig. 2). SEA0400 showed no significant inotropic effects; the contractile force after the application of 1  $\mu$ M SEA0400 was  $105.3 \pm 10.0\%$  ( $n = 6$ ) of that before application. Ouabain (1  $\mu$ M) induced a gradual increase in contractile force; the contractile force at 30 min after addition of ouabain was  $473.5 \pm 44.7\%$  ( $n = 6$ ) of that before addition. SEA0400 (1  $\mu$ M) significantly reduced the ouabain-induced



**Fig. 1.** Effect of SEA0400 on low Na<sup>+</sup>-induced contracture in papillary muscles. A: Typical records of contracture induced by low Na<sup>+</sup> solution in the absence (a) and presence (b) of 1  $\mu$ M SEA0400. B: Summarized results for the contracture. Increase in basal tension was expressed as a percentage of the stimulation-evoked contractile force in the same preparation. Each point with vertical bars represents the mean  $\pm$  S.E.M. of 10 experiments. Asterisks indicate significant difference ( $P < 0.05$ ) from the corresponding values in the absence (open circles) of SEA0400.

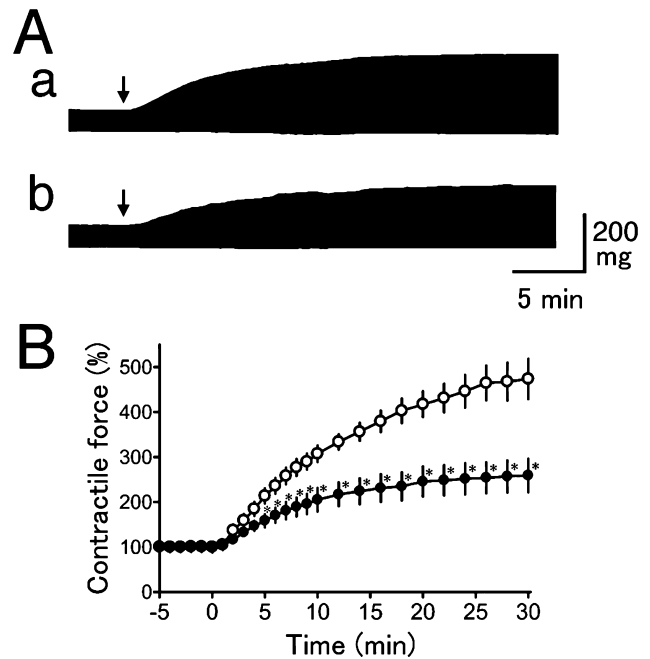
positive inotropy; in the presence of 0.3, 1, and 10  $\mu$ M SEA0400, the contractile force at 30 min after addition of ouabain was  $394.1 \pm 32.9\%$  ( $n = 6$ ),  $259.3 \pm 37.1\%$  ( $n = 6$ ), and  $279.8 \pm 32\%$  ( $n = 7$ ) of that before addition, respectively.

#### Effect of SEA0400 on IBMX-induced inotropy

Effect of SEA0400 on IBMX-induced inotropy was examined in the papillary muscles (Fig. 3). IBMX (100  $\mu$ M) induced a rapid increase in contractile force; the contractile force at 5 min after addition of ouabain was  $281.7 \pm 19.4\%$  ( $n = 6$ ) of that before addition. SEA0400 (1  $\mu$ M) did not affect the IBMX-induced positive inotropy; in the presence of SEA0400, the contractile force at 30 min after addition of IBMX was  $280.7 \pm 19.4\%$  ( $n = 6$ ) of that before addition.

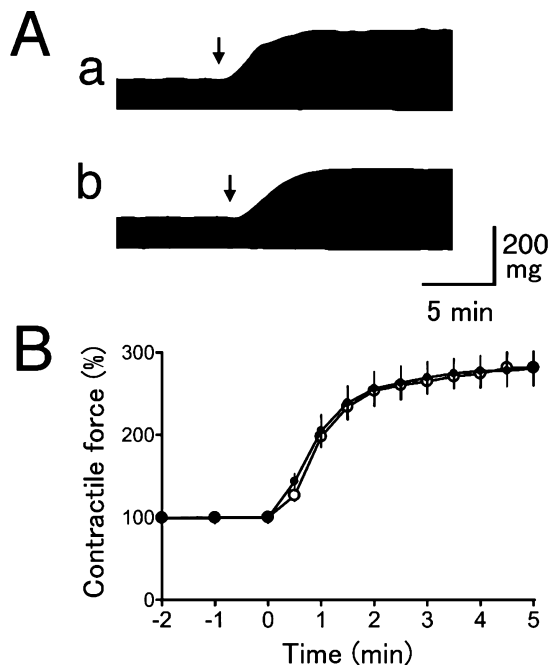
#### Dependence of ouabain effects on NCX

Dependence of ouabain effects on NCX was examined with HEK293 cells (Fig. 4). Treatment of HEK293 cells expressing the NCX1 protein with low



**Fig. 2.** Effects of SEA0400 on ouabain-induced inotropy. A: Typical records showing the effects of 1  $\mu$ M ouabain in the absence (a) and presence (b) of 1  $\mu$ M SEA0400. Arrows indicate the addition of 1  $\mu$ M ouabain. B: Summarized results in the absence (open circles) and presence (closed circles) of SEA0400. Contractile force after the addition of 1  $\mu$ M ouabain was expressed as a percentage of the contractile force before the addition. Each point with vertical bar represents the mean  $\pm$  S.E.M. of 6 experiments. Asterisks indicate significant difference ( $P < 0.05$ ) from the corresponding values in the absence of SEA0400.

Na<sup>+</sup> solution resulted in an increase in cytoplasmic Ca<sup>2+</sup> concentration that reflects reverse mode NCX activity. In such cells, ouabain induced a gradual increase in cytoplasmic Ca<sup>2+</sup> concentration. The cytoplasmic Ca<sup>2+</sup> concentration before and 20 min after the addition of 10  $\mu$ M ouabain was  $46.4 \pm 4.5$  and  $621.5 \pm 57.3$  nM ( $n = 17$ ), respectively. This ouabain-induced increase in cytoplasmic Ca<sup>2+</sup> concentration was completely inhibited by SEA0400. In the presence of 1  $\mu$ M SEA0400, the cytoplasmic Ca<sup>2+</sup> concentration before and 20 min after the addition of 10  $\mu$ M ouabain was  $58.8 \pm 8.0$  and  $37.9 \pm 7.8$  nM ( $n = 17$ ). In wild type HEK293 cells, the lack of functional NCX activity was confirmed by lack of increase in cytoplasmic Ca<sup>2+</sup> concentration by low Na<sup>+</sup> solution. Ouabain induced no significant increase in cytoplasmic Ca<sup>2+</sup> concentration in wild type HEK293 cells, indicating the dependence of ouabain action on NCX function. In wild type HEK293 cells, the cytoplasmic Ca<sup>2+</sup> concentration before and 20 min after the addition of 10  $\mu$ M ouabain was  $45.2 \pm 8.4$  and  $54.7 \pm 8.6$  nM ( $n = 24$ ) (not shown in the figure).

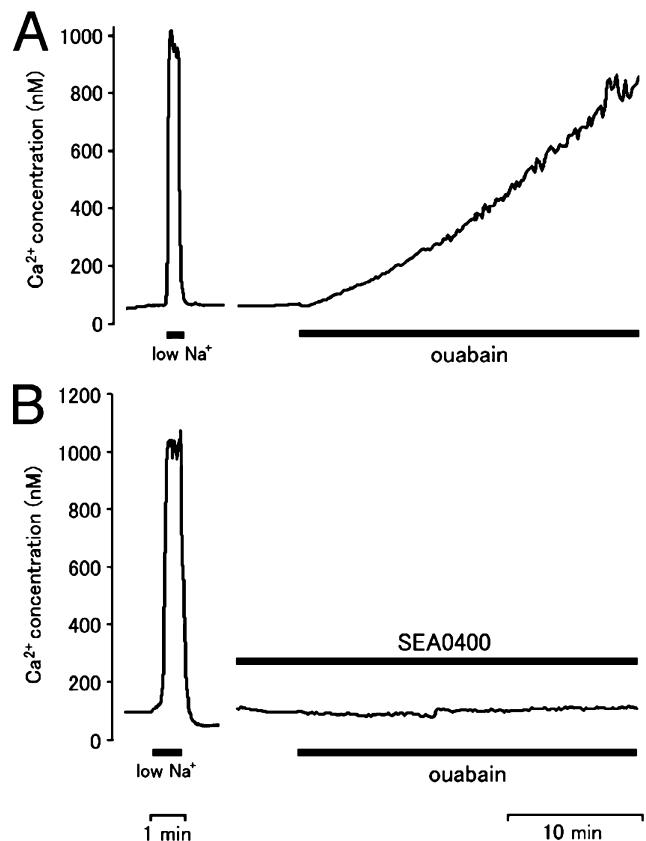


**Fig. 3.** Effects of SEA0400 on IBMX-induced inotropy. A: Typical records showing the effects of 100  $\mu$ M IBMX in the absence (a) and presence (b) of 1  $\mu$ M SEA0400. Arrows indicate the addition of 100  $\mu$ M IBMX. B: Summarized results in the absence (open circles) and presence (closed circles) of SEA0400. Contractile force after the addition of 100  $\mu$ M IBMX was expressed as a percentage of the contractile force before the addition. Each point with vertical bar represents the mean  $\pm$  S.E.M. of 6 experiments.

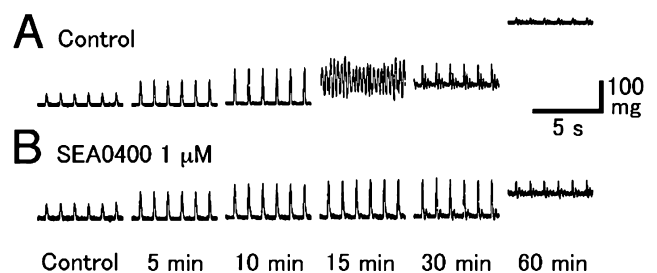
#### Effect of SEA0400 on ouabain-induced arrhythmia

Effect of SEA0400 on ouabain-induced arrhythmia was examined in papillary muscles (Fig. 5). Application of 3  $\mu$ M ouabain to papillary muscles induced positive inotropy; the contractile force at 10 min after the addition of ouabain increased to  $272.7 \pm 24.8\%$  ( $n = 26$ ) of that before the addition. This was followed by the appearance of arrhythmic contraction during the period between 10 and 60 min after ouabain application in 19 out of 26 preparations. The arrhythmic contractions were larger than the stimulation-evoked periodic contractions. After 20 min, oscillatory aftercontractions with decremental amplitude were observed instead of the large arrhythmic contractions. In addition to these changes, 3  $\mu$ M ouabain induced contracture; the basal tension at 30 min after the addition of ouabain was  $305.0 \pm 67.1\%$  ( $n = 26$ ) of the contractile force in the absence of ouabain.

In the presence of 1  $\mu$ M SEA0400, the ouabain-induced positive inotropy was smaller than that in the absence of SEA0400; contractile force at 10 min was  $239 \pm 22.7\%$  ( $n = 26$ ) of that before ouabain addition. The arrhythmic contraction during the period between 10 and 60 min was observed in 12 out of 26 preparations



**Fig. 4.** Effect of ouabain on intracellular  $Ca^{2+}$  in HEK293 cells expressing NCX loaded with fura-2. Typical records of changes in intracellular  $Ca^{2+}$  concentration in response to 10  $\mu$ M ouabain in the absence (A) and presence (B) of 1  $\mu$ M SEA0400. Expression of functional NCX in the cells observed was confirmed by the increase in intracellular  $Ca^{2+}$  in response to low  $Na^+$  solution.



**Fig. 5.** Effect of SEA0400 on ouabain-induced arrhythmogenesis. Typical records of the contractile response of papillary muscles to 3  $\mu$ M ouabain in the absence (A) and presence (B) of 1  $\mu$ M SEA0400. Note that SEA0400 inhibited arrhythmic contractions and reduced the elevation of basal tension.

in the presence of SEA0400; the incidence was significantly smaller than in the absence of SEA0400. The oscillatory aftercontractions observed after 20 min were not inhibited by SEA0400 but the elevation of basal tension was significantly reduced by SEA0400. The

basal tension at 30 min after the addition of ouabain in the presence of SEA0400 was  $184.8 \pm 55.5\%$  ( $n = 26$ ) of the contractile force in the absence of ouabain.

## Discussion

In isolated guinea-pig papillary muscles, SEA0400 reduced the contracture induced by a low-sodium solution (Fig. 1) indicating that it could inhibit  $\text{Ca}^{2+}$  influx through NCX not only in cardiomyocytes (7), but also in myocardial tissue. SEA0400 also attenuated the elevation of basal tension during late experimental ischemia (16), which is considered to reflect the inhibition of  $\text{Ca}^{2+}$  influx through the reverse mode NCX. SEA0400 produced concentration-dependent positive inotropy in mouse ventricular myocardium (11). When the effect of SEA0400 on NCX was examined in voltage clamped guinea-pig ventricular myocytes, SEA0400 inhibited both forward and reverse modes with the same potency (7). SEA0400 increased the contractile force of guinea-pig papillary preparations, suggesting that SEA0400 inhibits  $\text{Ca}^{2+}$  extrusion through the forward mode NCX in tissue preparations. However, the effect of NCX inhibition by SEA0400 on the forward and reverse modes may not be the same in tissue preparations where the membrane potential and ionic conditions change during the action potential cycle.

Concerning species difference in the positive inotropic effect, SEA0400 ( $1 \mu\text{M}$ ) increased the contractile force of papillary muscle preparations by only 5% (present study) or less (16) in the guinea pig, but by 25% in the mouse (11). In the guinea-pig ventricle, the membrane potential is more negative than the equilibrium potential of NCX only during the resting period when the intracellular  $\text{Ca}^{2+}$  is low. In contrast, the mouse has a short duration and higher intracellular  $\text{Na}^+$  concentration, which favors  $\text{Ca}^{2+}$  extrusion through the forward mode NCX during early diastole when the intracellular  $\text{Ca}^{2+}$  is still elevated (1). Thus, NCX inhibition would result in larger positive inotropy in the mouse ventricle, where the functional role of  $\text{Ca}^{2+}$  extrusion through the forward mode NCX is larger. Ouabain treatment of the guinea-pig ventricle, which increases intracellular  $\text{Na}^+$  concentration, would enhance  $\text{Ca}^{2+}$  influx from the reverse mode NCX. There is a report that inhibition of NCX by SEA0400 is more potent under higher intracellular  $\text{Na}^+$  concentration (21). This may also partially underlie the difference in positive inotropy by SEA0400 between the guinea pig and mouse, which has a higher intracellular  $\text{Na}^+$  concentration (22).

SEA0400 significantly reduced the ouabain-induced positive inotropy (Fig. 2), indicating that NCX activity is

essential for ouabain action. Inhibition of inotropy induced by a higher concentration ( $3 \mu\text{M}$ ) of ouabain was smaller (Fig. 5), probably because the inhibition of NCX is not complete (about 80%) with  $1 \mu\text{M}$  SEA0400 (7). SEA0400 had no effect on IBMX-induced inotropy (Fig. 3), which reflects its high NCX specificity. Reduction of ouabain-induced inotropy by SEA0400 also suggests that the NCX is at least partly operating in the reverse mode in the presence of ouabain. Reduction of low-sodium contracture by SEA0400 indicated that it could inhibit  $\text{Ca}^{2+}$  influx through NCX not only in cardiomyocytes (7) but also in myocardial tissue. That ouabain can activate  $\text{Ca}^{2+}$  influx through reverse mode NCX was further confirmed by induction of rise in intracellular  $\text{Ca}^{2+}$  concentration in HEK293 cells expressing the cardiac type NCX (Fig. 4). Thus, the present results provide pharmacological evidence for a contribution of the reverse mode NCX activity in the cardiac glycoside-induced inotropy. However, other mechanisms such as stimulation of sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  release have also been postulated (23). The present results do not exclude such possibilities. Rather, inhibition of the NCX pathway by the highly specific inhibitor SEA0400 would be a useful strategy to clarify the additional mechanisms for cardiac glycoside-induced inotropy.

Ouabain has been considered to increase intracellular  $\text{Na}^+$  concentration, shift the balance of the two modes of NCX to favor the reverse mode, and increase cellular  $\text{Ca}^{2+}$  load. When this  $\text{Ca}^{2+}$  load exceeds the capacity of the SR, abnormal  $\text{Ca}^{2+}$  release from the SR occurs, which in turn triggers abnormal electrical activity and arrhythmic contractions. In the present study, the ouabain-induced increase in basal tension and arrhythmic contractions were significantly reduced by SEA0400 (Fig. 5). This provides pharmacological evidence that NCX plays a crucial role in ouabain-induced arrhythmogenesis. It was also reported that SEA0400 attenuated ouabain-induced arrhythmia in a canine *in vivo* model (18) and in isolated Purkinje fibers (24). The effect of SEA0400 on other types of arrhythmia has also been investigated. Attenuation of arrhythmia induced by ischemia-reperfusion was shown to be attenuated by SEA0400 in the *in vitro* rat (13) and guinea-pig (16) model. On the other hand, SEA0400 was reported to be ineffective against various aconitine-induced arrhythmia models in the guinea pig (25). We have reported that KB-R7943, a less selective NCX inhibitor, inhibits aconitine-induced intracellular  $\text{Ca}^{2+}$  oscillations in isolated rat ventricular myocytes (26), which may reflect the action of KB-R7943 on ion channels other than the NCX (7).

In conclusion, the present results provide pharmaco-

logical evidence that the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger is involved in ouabain-induced inotropy and arrhythmogenesis. SEA0400 may be promising as a therapeutic agent against arrhythmia dependent on NCX function.

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