

**Effects of sodium-calcium exchange inhibitors, KB-R7943 and  
SEA0400, on aconitine-induced arrhythmias in guinea pigs in vivo, in  
vitro and computer simulation studies**

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Abbreviations: KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methansulphonate; SEA0400, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline; NCX, sodium-calcium exchange; PVC, premature ventricular tachycardia; TdP, torsades de pointes; VT, ventricular tachycardia; VF, ventricular fibrillation; APD<sub>50</sub> and APD<sub>90</sub>, action potential duration at 50% and 90% repolarization, respectively; LQTS, long QT syndrome; NSR, normal sinus rhythm; EAD, early afterdepolarization; DAD, delayed after depolarization.

## Abstract

The sodium-calcium exchange (NCX) plays a pivotal role in regulating contractility and electrical activity in the heart. But the effects of NCX blockers on ventricular arrhythmias are still controversial. We examined the effects of KB-R7943 (KBR) and SEA0400 (SEA), two NCX blockers, on aconitine-induced arrhythmias in guinea pigs using the ECG recordings and the current clamp method. Using Luo and Rudy's computer model for ventricular myocytes, we simulated abnormal membrane activity produced by NCX inhibition. In the whole animal model, KBR in a dose range of 1-30 mg/kg (intravenous) suppressed aconitine-induced arrhythmias dose-dependently, but 10 mg/kg of SEA did not suppress these arrhythmias. In isolated ventricular myocytes also, there was a difference. KBR (10  $\mu$ M) suppressed abnormal electrical activity induced by aconitine, but SEA (100  $\mu$ M) did not show such effects. KBR (10  $\mu$ M) significantly changed the shape of the action potential configurations (APD<sub>50</sub>), but SEA (1-100  $\mu$ M) did not change these configurations. In the computer simulation study, the aconitine-induced abnormal electrical activity was mimicked by a negative shift of the kinetics of Na<sup>+</sup> channels, and this was followed by additional suppression of NCX activity by 90% (mimicking the effect of NCX inhibitors) which enhanced abnormal

membrane activity. Our results indicate that the inhibition of aconitine-induced arrhythmias by KBR, not by SEA, might result from a mechanism other than the inhibition of NCX and thus the involvement of the NCX system plays an insignificant role in the aconitine-induced arrhythmias.

Aconitine, an alkaloid obtained from the plant, *Aconitum napellus*, is recognized for its phytomedical effects on the heart, central nervous system and skeletal muscle (Catterall, 1980; Honerjager and Meissner, 1983; Ameri, 1998). The arrhythmogenic effects of aconitine include the induction of premature ventricular contractions (PVC), ventricular tachycardia (VT), torsades de pointes (TdP), ventricular fibrillation (VF) and mortality in a dose-dependent manner (Lu and Clerck, 1993). Experimentally, aconitine-induced arrhythmias are thought to be induced by triggered activity due to delayed afterdepolarization (DAD) and early afterdepolarization (EAD). At the molecular level, aconitine binds to Na<sup>+</sup> channels and prolongs their open state favoring entry of a large quantity of Na<sup>+</sup> into cytosol, which may be accompanied by Ca<sup>2+</sup> overload via an electrogenic Na<sup>+</sup>-Ca<sup>2+</sup> exchange (NCX) system and eventually induces triggered activity (Sawanobori et al., 1987; Watano et al., 1999). Thus, the role of NCX is suspected to be important to generate triggered activity in the heart (Adaniya et al., 1994 and Sawanobori et al., 1996).

NCX inhibitors, KBR (KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiurea methansulphonate) and SEA (SEA0400, 2-[4-[(2,5-difluorophenyl)

methoxy]phenoxy]-5-ethoxyaniline), have been developed recently (Watano et al., 1996; Iwamoto et al., 1996; Kimura et al., 1999; Iwamoto et al., 1999; Matsuda et al., 2001; Shigekawa and Iwamoto 2001; Tanaka et al., 2002). SEA has been found to be about 10 times more potent than KBR in inhibiting the NCX current in guinea pig ventricular cells.

To elucidate the involvement of NCX in aconitine-induced arrhythmias and triggered activity, we tried to explore the effects of NCX inhibitors, KBR and SEA, on the aconitine-induced arrhythmias in the whole animal, single cardiac myocyte and computer simulation models.

## **Materials and methods**

The animal experiments were approved by the University of Yamanashi Interdisciplinary Graduate School of Medicine and Engineering Animal Experimentation Committee and animals were obtained through the Animal Laboratory for Research of this University.

### **Aconitine-induced arrhythmias in the whole guinea pigs**

Guinea pigs weighing 300-400 g were anesthetized with sodium pentobarbital (50 mg/kg, intra peritoneal, i.p.). Respiration was maintained with artificial ventilation (under room air, volume 1.5 ml/100 g, rate 55 strokes/min) through the cannula in the trachea to maintain PCO<sub>2</sub>, PO<sub>2</sub> and pH within the normal range. Polyethylene tubing was inserted into the right jugular vein to administer aconitine and the test drugs. The left carotid artery was cannulated to monitor systemic blood pressure. The standard limb leads of the electrocardiogram (ECG) were recorded. Blood pressure, heart rate and ECG (Lead I and II) were continuously monitored using a polygraph recorder (NEC san-ei Instruments Ltd., Tokyo, Japan). After 15 minutes of stabilization, vehicle or NCX inhibitors were administered to different groups for each dose of the drug or vehicle (i.e., 0-30 mg/kg) as i.v. bolus injection through the jugular vein. Five minutes later, 25 µg/kg of aconitine was injected to induce ventricular arrhythmias in the whole animal model (Lu and Clerck, 1993) (Figure 1, protocol I). Each animal received only one dose (treatment) of either vehicle or any of the NCX inhibitors. The doses of SEA were 1-10 mg/kg and those of KBR were 1-30 mg/kg.

### **Isolation of cardiac myocytes**

The left ventricular myocytes of guinea pigs were enzymatically isolated using a standard procedure (Isenberg and Klockner, 1982; Homma et al., 2000). Briefly, guinea pigs weighing 300-400 g were anesthetized with pentobarbital (50 mg/kg, i.p.) and sacrificed. The hearts were quickly removed and rinsed in calcium free cold Tyrode solution. Using a Langendorff retrograde apparatus, 0.2 mg/ml collagenase (type-I, Worthington, Lakewood, NJ, USA) or, 0.03 mg/ml collagenase (Blendzyme-3, Roche, Mannheim, Germany) was perfused 15-20 minutes at  $36 \pm 1$  °C. The left ventricular muscles were minced by scissors, dispersed with gentle agitation in Kraft-Brühe (KB) medium and filtered by a 200 µm-pore nylon mesh. Cells were stored in KB medium at room temperature until use. More than 80% of the myocytes were rod shaped and viable, and 65% of the total isolated myocytes were tolerant to 1.8 mM extracellular calcium. Only cells exhibiting a rod-shaped morphology and no signs of membrane damage were used for the experiments. Once isolated, the cells were used up to 12 hours. Tyrode solution for isolation contained (in mM): NaCl 135, KCl 5.4, NaH<sub>2</sub>PO<sub>4</sub> 11, MgCl<sub>2</sub> 2, glucose 20. Tyrode solution for perfusion (bath solution) contained (in mM): NaCl 135, KCl 5.4, NaH<sub>2</sub>PO<sub>4</sub> 1, HEPES (Na salt) 10, MgCl<sub>2</sub> 1, glucose 20 and CaCl<sub>2</sub> 1.8. The pH

was adjusted to 7.4 with NaOH in both solutions. The KB medium contained (in mM): glutamate K 90, taurine 10, KCl 25,  $\text{KH}_2\text{PO}_4$  20,  $\text{MgCl}_2$  3, and glucose 10. The pH was adjusted to 7.25 with KOH.

### **Electrophysiological recordings**

Cell suspension was placed on the microscopic groove and Tyrode solution containing 1.8 mmol/L of  $\text{Ca}^{2+}$  was perfused for 10 minutes for stabilization of the cells. Cells exhibiting a rod-shaped morphology and no signs of membrane damage were selected on the microscopic groove. The microelectrode was carefully touched on the membrane of the selected cell and a giga seal was made by gentle suction through the polyethylene tubing connected to a 1 ml syringe. To induce electrical abnormality, aconitine (1  $\mu\text{mol/L}$ ) dissolved in Tyrode solution containing 1.8 mmol/L of  $\text{Ca}^{2+}$ , was perfused. Electrical abnormality appeared within one minute after perfusion of aconitine solution. At this stage, the vehicle (DMSO) (control or 0  $\mu\text{mol/L}$  of NCX inhibitors) or any dose of the NCX inhibitors (1, 3, 10, 100  $\mu\text{mol/L}$  of NCX inhibitors) and aconitine (1  $\mu\text{mol/L}$ ), dissolved in Tyrode solution containing 1.8 mmol/L  $\text{Ca}^{2+}$ , was simultaneously perfused to observe the effects of the vehicle, or NCX inhibitors on aconitine-induced

triggered activity (Figure 1, protocol II). Each cell from each vehicle/drug dose group received only one dose (treatment) of aconitine, and vehicle, or NCX inhibitors. The temperature was maintained at 37 °C.

A patch clamp L/M-EPC7 amplifier (LIST-MEDICAL ELECTRONIC, D-6100, Darmstadt, Germany) was used in the current-clamp experiments for recording action potential configurations with a extracellular  $\text{Ca}^{2+}$  concentration of 1.8 mM (Hamill *et al.*, 1981). Pipettes with 2-4 Mega  $\Omega$  resistance were made from alluminosilicate capillary glass using a programmable multi-step puller (Sutter Medical, Navato, CA, USA). Pulse generation and data acquisition were controlled by pCLAMP software and by a running compaq PC computer that was interfaced with digidata 1200 interface (Axon Instruments, Foster city, CA, USA). Giga seals were made by suction. Action potentials were displayed on the computer monitor and simultaneously recorded in a recorder (Nihon Kohden Corporation, Tokyo, Japan). The temperature was controlled by a bath temperature controller (DTC 300T, Dia Medical System Co. Ltd., Tokyo, Japan) and monitored on the microscopic groove by Thermistor (Class 1.0, Shibaru Electronics Co. Ltd., Tokyo, Japan). The recording microelectrodes were filled with a solution

containing (in mM): KCl 20, K-Aspartate 120, Mg-ATP 5, HEPES (salt) 10. The pH was adjusted to 7.25 with KOH.

### **Reconstructed action potential by computer simulation**

We used the Luo and Rudy model of action potential of mammalian ventricular myocyte as the basis of this simulation study (Luo & Rudy, 1991). The source code of the Luo and Rudy model described by common computer language (C) was obtained from the website <http://www.cwru.edu/med/CBRTC/LRdOnline/>. Many of the membrane ionic currents, pumps and exchangers were incorporated in this model. At the baseline condition, the model was paced at a basic cycle length of 500 ms. The effects of aconitine on the kinetics of the sodium channels were produced by the shift of  $m$  (activation) and  $h$  (inactivation) parameters to negative membrane potential. Nilius et al. (1986) reported that aconitine shifted  $m$  to -31 mV and  $h$  to -13 mV in isolated mouse ventricular myocytes. In the Luo and Rudy model, the kinetics of sodium channels are defined using one activation parameter ( $m$ ) and two inactivation parameters ( $h$  and  $j$ ). We followed the results of Nilius et al. (1986) and shifted  $m$  to -31 mV and only  $h$  to -13 mV and mimic the condition in which aconitine was applied.

To mimic the condition in which a NCX inhibitor was applied, the contributions of the NCX in the model were simply reduced as follows:

$$\text{Modified } I_{\text{NaCa}} = a \cdot I_{\text{NaCa}},$$

where 'a' is a constant, if the value of 'a' is 0.1, the function of modified  $I_{\text{NaCa}}$  is reduced by 90% from the contribution of the original  $I_{\text{NaCa}}$ .

### **Exclusion criteria**

A total of 130 guinea pigs were used in these series of studies on the whole animal and single cell experiments. Experiments were terminated or excluded from the final data analysis, if any of the following occurred (Aye et al., 1999): arrhythmias or low mean arterial pressure (less than 15 mmHg) prior to drug or vehicle administration due to surgery. Six guinea pigs were excluded for the absence of arrhythmias after, and sudden death during drug or vehicle administration.

### **Chemicals and drugs**

KB-R7943 and SEA0400 were kind gifts from Kanebo Co. (Osaka, Japan), OrganonJapan Co. (Osaka, Japan) and Taisho Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively. KBR was dissolved in a vehicle (polyethylene glycol 10%, ethanol 10%,

DMSO 10% and saline 70%) in the whole animal experiments, and in DMSO in single cell experiments. SEA was dissolved in a vehicle supplied by Taisho. Aconitine and other agents were directly dissolved in Tyrode solution. The final concentration of DMSO was less than 0.05%. DMSO and vehicles had no significant effects on the whole animal and single cell experiments (Data not shown). All other chemicals were purchased from Sigma (St Louis, MO, USA) and Wako (Tokyo, Japan).

#### **Data analysis and statistics**

Statistical analysis was based on the guidelines for statistics (Wallenstein et al., 1980) and modified for the study of arrhythmias using guinea pig hearts (Dennis et al., 1980; Pfeiffer and Kenner, 1983). In the whole animal study, each drug dose group consisted of 10-13 animals, and in the single cell study, each drug dose group consisted of 5-8 individual cells. Data were expressed as mean  $\pm$  S.E.M. Differences in mean values between experimental groups were analyzed by one way ANOVA (analysis of variance) followed by Dunnet's multiple comparison test where applicable. A P value less than 0.01 was defined to be significant.

## Results

### **(i) Effects of KBR and SEA on aconitine-induced arrhythmias in the whole animal model**

In the whole animal experiments, injection of aconitine (25  $\mu$ g/kg, i.v. bolus) induced various types of ventricular tachyarrhythmias (Figure 2). Ventricular tachycardias were diagnosed and classified into two types, narrow QRS VT (junctional tachycardia) and wide QRS VT. The former originated from the atrioventricular nodal area because its QRS complex was followed by a P wave in regular intervals. The cellular mechanism of this arrhythmia might be enhanced automaticity in the atrioventricular node or in His-Purkinje cells close to the node (Figure 2c). The latter showed wide and rapid QRS complexes and sometimes changed to torsades de pointes. It might be caused by triggered activity in ventricular myocytes (Figure 2d).

Figure 3 shows the effects of KBR and SEA on each arrhythmia as shown in Figure 2.

In the control condition (without NCX inhibitors), the duration of normal sinus rhythm (NSR), PVC, narrow QRS VT and wide QRS VT were  $18.7 \pm 9.8$ ,  $2.6 \pm 3.7$ ,  $15.7 \pm 15.3$  and  $19.1 \pm 15.3$  (min). In the presence of KBR (10 mg/kg), those durations changed to

25.8±11.7, 8.1± 11.7, 23.5±16.6 and 2.7±6.1 (min), respectively. In the dose range of KBR from 1 to 30 mg/kg, the duration of the normal sinus rhythm was dose-dependently prolonged and that of the wide QRS VT was shortened. In the presence of SEA (10 mg/kg), those arrhythmia durations were 21.5±13.2, 4.3±4.3, 17.3±5.7 and 16.8±11.9 (min), showing no significant changes in comparison to those of the control condition. KBR suppressed the wide QRS VT, but SEA did not show such an effect.

#### **(ii) Aconitine-induced abnormal electrical activity in single ventricular myocytes**

Figure 4 shows abnormal electrical activity in isolated ventricular myocytes of guinea pigs induced by aconitine (1  $\mu$ M). The abnormal electrical activity occurred as a form of triggered automaticity within one minute after perfusion of aconitine, with spontaneous diastolic depolarization, gradually increasing its firing rate (warming up phenomenon) and finally developing to the continuous chaotic oscillatory activity, appearing similar to VT and VF (Figure 4 b-c) as in the case of the whole animal experiments. Because of the lack of automaticity in normal ventricular myocytes and the lack of syncytium in

isolated cells, this abnormal activity must have been induced by triggered activity. The abnormal activity started after the membrane potential was once repolarized, indicating the characteristics of delayed afterdepolarization.

### **Effects of KBR and SEA on the aconitine-induced abnormal electrical activity**

We examined the effects of KBR and SEA on the abnormal electrical activity induced by aconitine, as shown in Figure 5 (a) and (c). KBR (10  $\mu$ M) completely suppressed the abnormal activity (Figure 5b), but SEA (100  $\mu$ M) did not show such an effect (Figure 5 d).

### **Effects of KBR and SEA on action potential configurations of ventricular myocytes**

We investigated the effects of KBR (1-10  $\mu$ M) and SEA (1-100  $\mu$ M) on the action potential configurations in isolated ventricular myocytes. KBR (10  $\mu$ M) made the shape of the action potential (Figure 6a) almost triangular, but SEA caused no significant change of the action potential (Figure 6b). Figure 6 (c), and (d) show

dose-dependent effects of KBR and SEA on the action potential duration at 50% and 90% depolarization ( $APD_{50}$  and  $APD_{90}$ ), respectively. KBR (10  $\mu$ M) preferentially decreased  $APD_{50}$  to  $APD_{90}$ .  $APD_{50}$  at the control condition significantly decreased from  $126 \pm 51$  ms to  $64 \pm 27$  ms by 10  $\mu$ M KBR.  $APD_{90}$  changed from  $169 \pm 50$  (control) ms to  $152 \pm 43$  ms (KBR 10  $\mu$ M). SEA altered neither  $APD_{50}$  nor  $APD_{90}$ ;  $APD_{50}$  and  $APD_{90}$  were  $134 \pm 46$  ms and  $161 \pm 47$  ms at the control conditions, and were  $121 \pm 34$  ms and  $149 \pm 28$  ms in the presence of SEA (100  $\mu$ M). Washout of KBR restored the normal shape of action potentials (data not shown).

### **(iii) Reconstructed action potential modified by the kinetics of sodium channels and the NCX activity**

The contribution of changes in sodium channel kinetics and the reduction in NCX activity on membrane potential were evaluated using a mathematical model of a mammalian ventricular myocyte (Luo and Rudy model). In Figure 7a, the kinetic parameters (m and h) of sodium channels are plotted against membrane voltage (mV). After shifting the parameters to a negative value to mimic the effect of aconitine, as

described in materials and methods, the simulated membrane potential at rest became unstable and started to oscillate (Figure 7d). The oscillation occurred after deep repolarization like delayed afterdepolarization and declined spontaneously, and the mean potential at rest was higher than that of the control (Figure 7b). These changes (i.e., Figure 7d) were similar to those shown in Figure 4c. As shown in Figure 7e, both the change of sodium channel kinetics and the inhibition of NCX by 90% were incorporated in the program to mimic the effect of aconitine and NCX inhibitor. This enhanced the instability of the membrane potential at rest. The simulation study indicates that the inhibition of NCX may be ineffective to suppress the aconitine-induced activity in isolated cardiac ventricular myocytes.

## Discussion

Aconitine-induced arrhythmias or abnormal electrical activity was used to study antiarrhythmic effects in mice, rat, dog, guinea pig and rabbit models (Winslow, 1980; Honerjager and Meissner, 1983; Sawanobori et al., 1987; Lu and Clerck, 1993; Arita et al., 1996). In the whole animal models, various types of ventricular tachyarrhythmias

were induced as PVC, VT, TdP and VF. In recording action potentials, both EAD and DAD were induced (Sawanobori et al., 1987). Figure 4, that shows abnormal activity in a single ventricular myocyte, indicates that triggered activity, not re-entry or enhanced automaticity mechanism, is responsible for aconitine-induced arrhythmias.

The binding site of aconitine is voltage-dependent cardiac  $\text{Na}^+$  channels (Catterall, 1988; Catterall, 2000). Aconitine shifted the activation and inactivation kinetics of the channels towards more hyperpolarized potentials. It caused repetitive or persistent activation of the  $\text{Na}^+$  current even at the resting membrane potentials (Schmidt & Schmidt, 1974; Honerjager and Meissner, 1983; Grischenko et al., 1983). The sustained influx of  $\text{Na}^+$  ions is speculated to cause intracellular  $\text{Na}^+$  overload leading to intracellular  $\text{Ca}^{2+}$  overload through NCX. It is controversial whether or not this  $\text{Na}^+$  overload only or  $\text{Ca}^{2+}$  overload or both ( $\text{Na}^+$  and  $\text{Ca}^{2+}$  overload) is responsible for the aconitine-induced cellular activity and the whole animal triggered activity. However, in previous studies selective NCX inhibitors have not been available, so, the involvement of the NCX in aconitine-induced arrhythmia still remains uncertain.

In our whole animal experiments two types of ventricular tachycardias were observed

after i.v. injection of aconitine. Aconitine enhances automaticity in the atrioventricular node, inducing accelerated atrioventricular junctional rhythm (narrow QRS tachycardia in Figure 2c) and may induce triggered activity in ventricular myocytes (wide QRS VT in Figure 2d). KBR dose dependently decreased the duration of wide QRS tachycardia and increased that of normal and atrial rhythm as a trade off. However, SEA did not suppress either narrow or wide QRS tachycardia, even at concentrations that should maximally suppress NCX (i.e., 10 mg/kg, see Figure 2). These results indicate that KBR is potent enough to inhibit the triggered activity in ventricular myocytes.

In action potential recordings from isolated ventricular myocytes (Figure 4), aconitine induced abnormal electrical activity, known as triggered responses, that are important causes of lethal arrhythmias. KBR was effective in suppressing the triggered activity while SEA proved to be ineffective. KBR not only suppresses NCX, but also suppresses  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels simultaneously and thus prevents both  $\text{Na}^+$  and  $\text{Ca}^{2+}$  overload. SEA, on the other hand, being a relatively more potent and more selective NCX inhibitor and less effective on  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels, did not prevent these triggered responses induced by (aconitine due to) abnormal kinetics of  $\text{Na}^+$  channels.

SEA has been reported to be more potent and more selective than KBR as a NCX inhibitor (Matsuda et al., 2001; Tanaka et al., 2002). SEA was 6.5 - 14.3 times more potent than KBR, because it inhibited the outward NCX current (reverse or influx mode of  $\text{Ca}^{2+}$  by NCX) in guinea pig ventricular cells, with  $\text{IC}_{50}$  value 32-40 nM of SEA and 263-457 nM of KBR. SEA is a pure NCX inhibitor, because SEA (1  $\mu\text{M}$ ) inhibited the NCX current by 80%, reduced the  $\text{Na}^+$  current, the L-type  $\text{Ca}^{2+}$  current, the delayed rectifier  $\text{K}^+$  current and the inwardly rectifying  $\text{K}^+$  current by only 4%, 9%, 4% and 2%, respectively. However, KBR (10  $\mu\text{M}$ ) inhibited the NCX current by 80% simultaneously reducing those ionic currents by 54%, 55%, 94% and 73%, respectively (Tanaka et al., 2002). Radioligand binding study that examined the effects of SEA on neurons (Matsuda et al., 2001) showed that the  $\text{IC}_{50}$  values for SEA to inhibit NCX,  $\text{Na}^+$  current and L-type  $\text{Ca}^{2+}$  current were 33 nM, 20  $\mu\text{M}$  and 14  $\mu\text{M}$  and those for KBR were 3.8  $\mu\text{M}$ , 3  $\mu\text{M}$  and 1  $\mu\text{M}$ , respectively. In both of our experiments in which ECG was monitored and action potential was recorded, the more potent and selective NCX inhibitor, SEA, showed no suppression of aconitine-induced arrhythmia or abnormal electrical activity. Those results indicate that the role of NCX is insignificant in the

triggered activity induced by aconitine and that the effect of KBR on other channels ( $\text{Na}^+$  and  $\text{Ca}^{2+}$ ) may contribute to its inhibition of the triggered activity. These conclusions are also consistent with our results shown in Figure 3 and Figure 6 that KBR shortened APD and changed the configuration of action potentials.

The long QT Syndrome (LQTS) is a rare congenital disorder. Mutations in the cardiac sodium channel gene SCN5A are responsible for type-3 long QT disease (LQT3) that leads to sudden cardiac death. It produces a persistent sodium current in the plateau phase of cardiac action potential and results in lethal arrhythmias (Clancy and Rudy, 1999, Chern-En and Dan, 2000, Moric et al., 2003, Xiao-Li et al., 2004). It was mentioned earlier that, at the molecular level, aconitine binds to  $\text{Na}^+$  channels and prolongs their open state favoring entry of a large quantity of  $\text{Na}^+$  into cytosol and eventually induces triggered activity (Sawanobori et al., 1987; Watano et al., 1999).

KBR seems to be effective in suppressing aconitine-induced cardiac arrhythmias but KBR is a 'not so selective NCX inhibitor' and at the same time 'also blocks  $\text{Na}^+$ ,  $\text{Ca}^{+2}$  and  $\text{K}^+$  channels' significantly (Tanaka *et al.*, 2002, Amran et al., 2003). LQT3 might, therefore, be treated with multi-channel blockers including KBR that also blocks the

NCX system.

The simulation study on an integrated mathematical model for ventricular action potential has been performed to determine whether or not NCX is involved in aconitine-induced arrhythmias. The abnormal activity with membrane oscillation after aconitine in real cardiomyocytes (Figure 4c) was well mimicked by the reconstructed action potential by a computer simulation (Figure 7d) with the shift of the kinetics of  $\text{Na}^+$  channels to negative potentials following the experimental data of Nilius et al. (1986). As to the effects of the inhibition of NCX on the membrane oscillation after aconitine, there was some difference between wet tissue and simulated experiments. The oscillation was not affected by SEA in the myocytes (Figure 4c), but was aggravated in the simulation study (Figure 7e). It is difficult to explain this difference but it might suggest that the oscillation was never suppressed by the inhibition of NCX.

Our results indicate that NCX is not strongly involved in the aconitine-induced triggered activity, i.e. kinetic shift of  $\text{Na}^+$  channels themselves induced by aconitine, might be enough to cause the triggered activities.

Antiarrhythmic effects of NCX inhibitors are still controversial. Miyamoto et al. (2002)

showed that KBR did not suppress either the ischemia/reperfusion arrhythmias or the ouabain-induced arrhythmias in dog models. They showed that NCX inhibition might not be a useful strategy in suppressing those arrhythmias. On the other hand, Watano et al. (1999) found that the intravenous injection of KBR significantly increased the doses of ouabain required to induce ventricular arrhythmias (PVC, VT and VF) in anesthetized guinea pigs and concluded that KBR suppressed ouabain-induced arrhythmias through inhibition of NCX. Yeih et al. (2000) reported recently a successful treatment of aconitine induced life threatening ventricular tachyarrhythmia by amiodarone in a clinical case. As amiodarone in therapeutic concentrations bind to many membrane/channel proteins (multi-channel blocker) like KBR, their clinical results support our experimental results observed in this study.

In conclusion, our results indicate that aconitine-induced ventricular arrhythmia is induced by triggered activity due to the abnormal kinetics of the  $\text{Na}^+$  channels, and protective effects of KBR resulted from a mechanism other than the inhibition of NCX (non-NCX action), thus NCX probably has little or no role in the arrhythmias induced by aconitine. The simulation study also provided the same results, i.e., NCX is not

significantly involved in aconitine-induced arrhythmias. Comprehensive analysis with the whole animal model, recordings from single ventricular myocytes and the computer simulation study may be helpful for better understanding of the mechanism of lethal arrhythmias and for evaluation of effects of new drugs.

### **The limitations of our study**

There are a few limitations of our study that deserve to be discussed.

(1) In the aconitine-induced arrhythmias, intracellular  $\text{Ca}^{2+}$  overload was proposed to be the mechanism in many previous studies. Aconitine-modified sodium channels remain open even at the resting potential, and repetitive depolarization and continuous influx of  $\text{Na}^+$  ion results in sodium overload. Consequently the  $\text{Na}^+$  gradient may decrease and  $\text{Ca}^{2+}$  overload is followed by this  $\text{Na}^+$  overload, which might induce triggered activity. Our results indicate that triggered activity is induced by abnormal kinetics of  $\text{Na}^+$  channels. Direct measurement of the intracellular  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_i$ , during aconitine-induced activity might be necessary to elucidate this point. To the best of our knowledge, there are no studies on the change of  $[\text{Ca}^{2+}]_i$  under aconitine-induced

activity.

(2) With the development in computer simulation of cardiac action potentials the Markovian model instead of a traditional Hodgkin-Huxley model (Clancy & Rudy, 1999) has been used to describe the kinetics of  $\text{Na}^+$  channels. Because the effect of aconitine on the cardiac  $\text{Na}^+$  channels was experimentally analyzed following the Hodgkin-Huxley model (Nilius et al., 1986), and because it was difficult to apply the experimental data of Hodgkin-Huxley style to the Markovian model, we simply used the Luo and Rudy model in which the  $\text{Na}^+$  channel was described as a Hodgkin-Huxley equation. Although the membrane oscillation by aconitine (Figure 4c) was well reconstructed in the simulation (Figure 7d), further analysis might be necessary to explain why the oscillation is not affected by SEA and is aggravated only in the simulation study.

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## Figure legends

**Figure 1** Diagram of the experimental protocols. Protocol I was used in the whole animal experiments and Protocol II was used in single cell experiments. In protocol I, after stabilization, vehicle, or each dose of either KBR or SEA was pre-treated. Then aconitine (25  $\mu\text{g/kg}$ , i.v. bolus) was administered. The black column indicates the duration for observing arrhythmias. A total of 130 animals were entered in these series of experiments. In protocol I, seventy four were used but six were ruled out according to exclusion criteria. The number of animals assigned to each dose of control (vehicle), KBR, and SEA were shown in Figure 3. In protocol II, after stabilization, aconitine (1  $\mu\text{mol/L}$ ) was perfused first, then vehicle, or each dose of either KBR or SEA was post-treated. A total of 13 cells were entered in protocol II (Figure 5).

**Figure 2** Typical ECG tracings showing various types of ventricular tachyarrhythmias before (a) and after aconitine (25  $\mu\text{g/kg}$ ) (b-d). Panel (a) shows normal sinus rhythm (NSR), (b) premature ventricular contraction, (c) narrow QRS ventricular tachycardia in lead I ( $c_1$ ) and lead II ( $c_2$ ), in which P waves are regularly recognized just after QRS

complex indicating enhanced automaticity in the atrioventricular node, (d) wide and rapid QRS ventricular tachycardia, indicating triggered activity in ventricular myocytes. The vertical line indicates 0.5 mV and the horizontal line indicates 200 ms. ECG tracings are chosen from one of the thirteen (n=13) similar and representative experiments.

**Figure 3** Effects of KBR and SEA on aconitine-induced ventricular arrhythmias including premature ventricular contraction (PVC), narrow QRS ventricular tachycardia (NR QRS VT) and wide QRS ventricular tachycardia (WD QRS VT) in anesthetized guinea pigs. The sum of duration of each arrhythmia is plotted against the concentration of the drugs. Data are expressed as mean  $\pm$  S.E.M (n=10-13). The significant change against the control group ( $p<0.01$ ) was shown by asterisk.

**Figure 4** Action potentials recorded with current-clamp method in single ventricular myocytes showing abnormal electrical activity induced by aconitine (1  $\mu$ mol/L). In the absence (a) of aconitine the action potential was generated under the stimulation of 1Hz.

In the presence (b, c) of aconitine, the abnormal electrical activity during the resting period occurred and then was followed by chaotic oscillatory activity. Action potential configuration tracings are chosen from one of the 8 similar and representative experiments (n=8 individual cells).

**Figure 5** Effects of KBR and SEA on aconitine-induced abnormal electrical activity in single ventricular myocytes recorded by current-clamp method. Panel (a) and (c) show aconitine-induced abnormal electrical activity (n=5-8 cells). In panel (b) KBR (10  $\mu$ mol/L, n=8 cells) abolishes the triggered activity. In panel (d) SEA (100  $\mu$ mol/L, n=5 cells) have no inhibitory effect on it. The stimulation frequency was 1Hz.

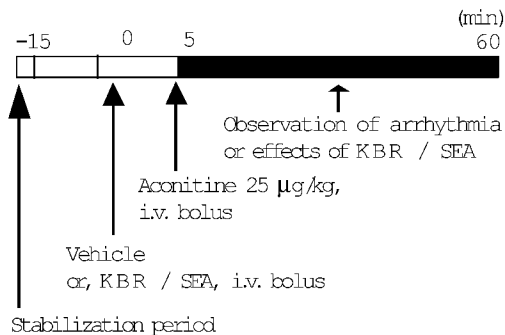
**Figure 6** Effects of KBR and SEA on the action potential configurations of the guinea pig ventricular myocytes. Panel (a) and (b) show the superimposed action potential recordings before and after applying KBR (10  $\mu$ mol/L) and SEA (10  $\mu$ mol/L). Panel (c) and (d) show the dose-dependent effects of KBR and SEA on the APD<sub>50</sub> and APD<sub>90</sub>. Data were taken from 5-8 individual cells in each treatment (n=5-8; for KBR, 1, 3 and

10  $\mu\text{mol/L}$ , and  $n=6-8$ ; for SEA, 1, 10 and 100  $\mu\text{mol/L}$ , respectively). The significant change against the control group ( $p<0.01$ ) was shown by asterisk.

**Figure 7** Computer-simulated action potentials using Luo and Rudy model. Panel (a) shows the shift of  $m$  and  $h$  gating kinetics in  $\text{Na}^+$  channels following the data from Nilius et al. (1986). The  $m'$  and  $h'$  infinity curves are generated by the shift of -31 mV and -13 mV from the  $m$  and  $h$  infinity curves in the control condition, respectively. Panel (b) shows action potentials in the control condition, (c) action potentials after the reduction of NCX current by 90%, (d) abnormal oscillatory activity after  $\text{Na}^+$  channel modification (mimicking the effect of aconitine) following the kinetic shift ( $m'$  and  $h'$ ) in panel (a), and (e) enhanced membrane activity after additional suppression of NCX activity by 90 % (mimicking the effect of NCX inhibitors).

Figure 1

Protocol-I: Whole animal experiments



Protocol-II: Single cell experiments

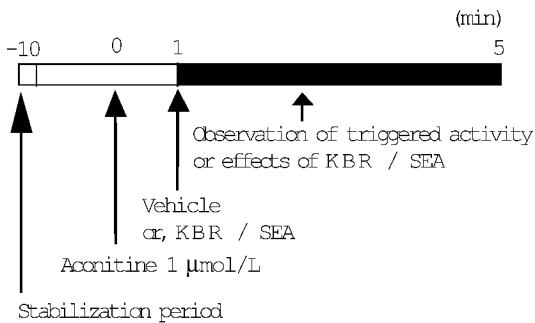


Figure 2

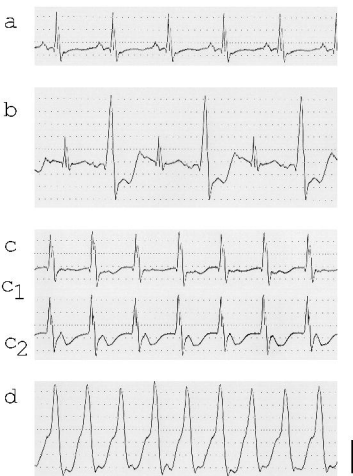


Figure 3

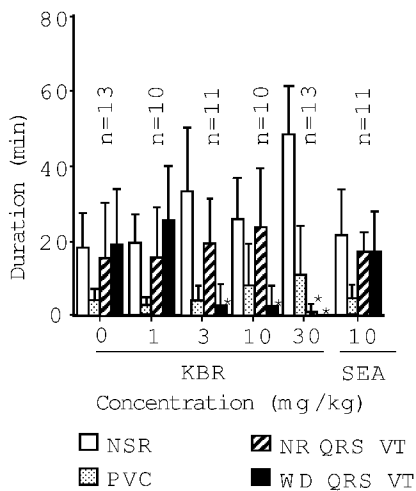


Figure 4

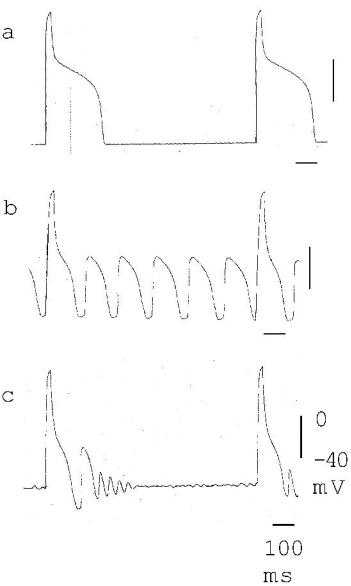


Figure 5

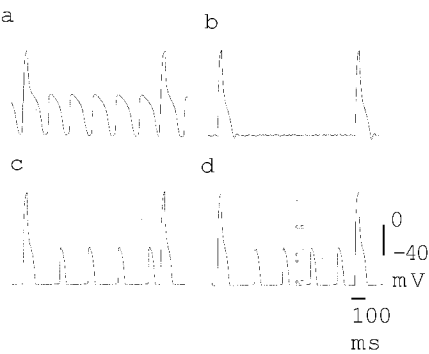


Figure 6

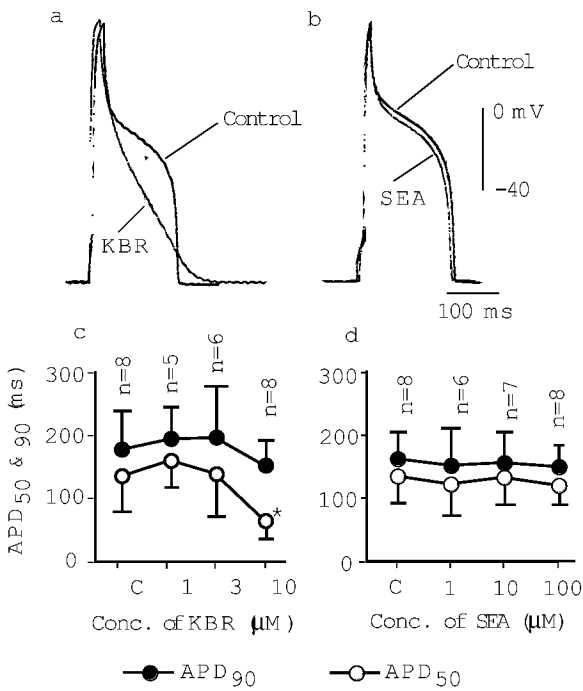


Figure 7

