Inhibition of 2-Aminoethoxydiphenyl Borate-induced Rat Atrial Ectopic Activity by Anti-arrhythmic Drugs

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Key Words

2-aminoethoxydiphenyl borate • Anti-arrhythmic drugs
• Ectopic activity • Atria

Abstract

Background/Aims: 2-aminoethoxydiphenyl borate (2-APB) provokes spontaneous mechanical activity in isolated rat left atria. The present study is to characterize 2-APB-induced ectopic activity in rat atria and to investigate the inhibition of 2-APB-induced ectopic activity by anti-arrhythmic drugs. **Methods:** 2-APB-induced ectopic activity was measured through an isometric force transducer connected to a multichannel acquisition and analysis system. Intracellular $[Ca^{2+}]_i$ was measured with fluorescence laser scanning confocal microscopy. Voltage-dependent L-type Ca^{2+} currents were recorded by using patch-clamp technique. **Results:** 2-APB dose-dependently increased the ectopic activity of left atria at 1, 5, 10, 20, 50 μM . Anti-arrhythmic drugs, quinidine (10 μM), lidocaine (10 μM), verapamil (5 μM), and amiodarone (50 μM , 100 μM) inhibited 2-APB-induced ectopic activity. 2-APB-induced ectopic activity was inhibited by Ca^{2+} -free bath, Na^+/Ca^{2+} exchanger blockers, 3',4'-dichlorobenzamil hydrochloride (DHC)

and Ni^{2+} , not by non-selective cation channel blocker Gd^{3+} . 2-APB also induced ectopic contractions in ventricular tissue strips and the ectopic contractions were inhibited by quinidine, verapamil and DHC. Lidocaine, verapamil and DHC inhibited 2-APB-induced increase of intracellular Ca^{2+} concentration in cardiomyocytes. Low molecular weight heparin inhibited phenylephrine (PE)-induced but not 2-APB-induced atria ectopic activity, and the pattern of 2-APB-induced ectopic activity was continuous, distinct from the discontinuous activity induced by PE. **Conclusion:** 2-APB-induced atria ectopic activity was inhibited by classic anti-arrhythmic drugs quinidine, lidocaine, verapamil, amiodarone, and Na^+/Ca^{2+} exchanger blockers. It can be used for testing agents able to affect any of Na^+ , Ca^{2+} channel, Na^+/Ca^{2+} exchanger without selectivity.

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Introduction

Cardiovascular diseases cause a worldwide mortality. About 25% of cardiovascular-related deaths are due to arrhythmias. Therefore, discovery of novel

anti-arrhythmic drugs has always been the emphasis in cardiovascular research field. Presently, there are several methods or technologies for screening candidates of anti-arrhythmic drugs, including the conventional patch-clamp technique, chemical models induced by acute infusion of various chemicals, e.g. aconitine, digitalis, barium [1, 2], electrical-stimulation models [3], neural stimulation models [4], ischemic arrhythmias models [1]. In general, different methods or technologies should be combined to evaluate the efficacy of anti-arrhythmic candidate drugs.

2-Aminoethoxydiphenyl borate (2-APB) is a membrane-permeable agent generally used as the inhibitor of InsP3-induced Ca^{2+} release [5-8] and Ca^{2+} entry through store-operated calcium channel (SOCs) [9, 10]. However, it has been suggested that 2-APB has multiple targets and can potentiate Ca^{2+} responses in some situations [11, 12]. Recently, Wolkowicz et al reported that 2-APB provoked sporadic mechanical activity in isolated rat left atria [13, 14] and suggested that hyperpolarization-activated current (If), chloride channel and sodium-calcium exchanger might underlie this activity. The aim of the present study is to characterize 2-APB-induced ectopic activity in rat atria and test the efficacy of the putative anti-arrhythmic drugs in this model. We suggest that 2-APB-induced ectopic activity can be an alternative way for testing agents able to affect any of Na^+ , Ca^{2+} channel, $\text{Na}^+/\text{Ca}^{2+}$ exchanger without selectivity.

Materials and Methods

All animal procedures and experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical University, P.R. China.

Isolation and superfusion of rat left atria

Wistar rats (200-240 g) were anesthetized with pentobarbital (40 mg/kg, i.v.) and the left atrial appendages were isolated, superfused at 37 °C in Krebs-Henseleit buffer. The atria contraction was measured through an isometric force transducer (Model YH-4; Institute of Space Medical-Engineering, China) connected to a multichannel acquisition and analysis system (Model BL-420E, Taimeng Technology Instrument, Chengdu, P.R. China) [15].

Cell Isolation

The cardiomyocytes dissociation procedures were performed as our previous study [16]. Briefly, hearts were removed from rats and mounted on a modified Langendorff perfusion system for retrograde perfusion via the coronary circulation. The preparation was perfused with Ca^{2+} -containing Tyrode solution (in mM: 126 NaCl, 5.4 KCl, 1 MgCl₂, 1.8 CaCl₂,

0.33 NaH₂PO₄, 10 glucose, and 10 HEPES, pH 7.4 adjusted with NaOH) at 37 °C until the effluent was blood free and then switched to Ca^{2+} -free Tyrode solution for 20 min, followed by perfusion with the same solution containing collagenase (type α , 100-150 U/mL) and 1% bovine serum albumin. The ventricle tissues were then excised from the softened hearts, dissociated into single cells and placed in a KB medium (in mM: 70 glutamic acid, 15 taurine, 30 KCl, 10 KH₂PO₄, 0.5 MgCl₂, 0.5 EGTA, 10 HEPES, 10 glucose, and 1% albumin, pH 7.4 adjusted with KOH.) at 4 °C for 1 h before experiments.

Intracellular $[\text{Ca}^{2+}]_i$ measurement

$[\text{Ca}^{2+}]_i$ was measured with fluorescence laser scanning confocal microscopy as our previous work [17]. Ventricular myocytes were loaded with the Ca^{2+} indicator fluo-3 in Tyrode solution (in mM: 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, pH 7.4) containing 20 μM fluo-3 acetoxymethyl ester (fluo-3/AM; Molecular Probes, USA) at 37 °C. Thirty minutes was allowed for deesterification of the dye. The laser scanning confocal microscope was a Fluoview FV300 (Olympus). Fluo-3 was excited with the 488 nm line of an argon ion laser and fluorescence was measured at wavelengths 520 nm. $[\text{Ca}^{2+}]_i$ transients were presented as background-subtracted normalized fluorescence (F/F_0) where F was the fluorescence intensity and F_0 was resting fluorescence recorded under steady-state. The data for $[\text{Ca}^{2+}]_i$ comparisons were presented as $(F/F_0)_{\text{Test}}/(F/F_0)_{\text{Control}}$. The composition of Ca^{2+} -free Tyrode solution for $[\text{Ca}^{2+}]_i$ measurement was same as the normal Tyrode solution except that CaCl₂ was replaced with 5mM EGTA.

Patch-clamp technique

Whole-cell voltage-clamp recordings were performed as previously described [18]. For voltage-dependent L-type Ca^{2+} currents recordings, the pipette solution consists of (in mM): CsOH 110, aspartate 110, CsCl 20, MgCl₂ 1, EGTA 10, HEPES 10, Mg-ATP 5 (pH adjusted to 7.2 with CsOH); the bath solution consists of (in mM): Tris-Cl 136, CsCl 5.4, MgCl₂ 1, CaCl₂ 2, HEPES 10, glucose 10 (pH adjusted to 7.4 with 3N Tris-OH). Recordings were made using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, USA), and data were collected using pCLAMP software (Axon Instruments, USA). Series resistance was checked regularly to ensure no variations with time. If series resistance increased during the recording, the experiment was discarded.

Chemicals

Amiodarone was purchased from Sanofi-Aventis (Shanghai, China), Lidocaine was purchased from Harbin pharmaceutical group co. ltd (China). Low molecular weight heparin was purchased from Laboratoire GlaxoSmithKline (France). All other chemicals applied in the present study were purchased from Sigma-Aldrich (USA).

Statistical Analysis

Data are presented as mean $\pm$ SEM, and significance was determined using Student *t* test directed by SigmaStat Software. $P < 0.05$ was considered significant.

Fig. 1. 2-APB dose-dependently increased the ectopic activity in rat left atria. (A) The representative recordings of the ectopic activity in rat atria induced by different concentrations of 2-APB. (B) The summarized data of 2-APB-induced ectopic activity in rat left atria. Ctr: control.

Results

2-APB dose-dependently increases the ectopic activity in rat left atria

We observed the performance of rat left and right atria under the present experimental conditions. Results showed that the left atria exhibited no spontaneous activity (n=6) but right atria contracted spontaneously at 253 ± 32 contractions/min (n=6) (Fig. 1A(a,b)). Therefore, the rat left atria was used in the present study because of no interference from spontaneous activity.

2-APB showed dose-dependent action on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [19], so 1, 5, 10, 20, 50 μM 2-APB were tested on the mechanical activity of left atria. As shown in Fig. 1A (c,d,e), 5μM 2-APB provoked slightly, but 20μM 2-APB provoked significantly mechanical activity. When 50μM 2-APB was applied, it caused rapid activation of atria contraction, then the

Fig. 2. Effects of quinidine, lidocaine, verapamil, amiodarone, 4-AP, DHC, Ni^{2+} , Gd^{3+} and Ca^{2+} -free bath on 2-APB-induced ectopic activity in rat left atria. The representative recordings of 2-APB-induced ectopic activity in left atria pretreated and aftertreated with quinidine were shown in (A) and (B). The effects of quinidine, lidocaine, and verapamil on 2-APB-induced ectopic activity were summarized in (C) and (D). (E) Amiodarone dose-dependently inhibited 2-APB-induced ectopic activity, but 4-AP(100μM, 1mM) showed no effect. (F) 2-APB-induced ectopic activity in rat atria was inhibited by DHC, Ni^{2+} , and Ca^{2+} -free bath, but not by Gd^{3+} . 4-AP, 4-aminopyridine; DHC, 3',4'-dichlorobenzamil hydrochloride. Amio, amiodarone. ** $P < 0.01$ vs 2-APB.

contractive activity gradually exhausted during the period of observation (about 15min). The frequency of contractions of 50μM 2-APB was 219 ± 9 contractions/min (n=8), more than that of 20μM 2-APB. Because 20μM 2-APB induced stable ectopic activity in left atria during 15min observation, we used this concentration level in present study. The summarized data showed that 2-APB dose-dependently induced the ectopic activity in rat left atria (Fig. 1B).

Effects of anti-arrhythmic drugs on 2-APB-induced ectopic activity

We tested the effects of two types of anti-arrhythmic drugs on 2-APB-induced ectopic activity in left atria. The two types of drugs included Na^+ channel blockers, quinidine and lidocaine, L-type Ca^{2+} channel blocker, verapamil. As shown in Fig. 2A and B, the representative original recordings showed that pretreatment of quinidine ($10\mu\text{M}$) almost abolished the actions of 2-APB, and application of quinidine ($10\mu\text{M}$) 5 min after 2-APB treatment significantly inhibited 2-APB-induced ectopic activity. Lidocaine ($10\mu\text{M}$) and verapamil ($5\mu\text{M}$) showed the similar effects as quinidine. The summarized data were shown in Fig. 2C and D.

Next, we tested the effect of amiodarone, a class III antiarrhythmic agent, on 2-APB-induced ectopic activity. $10\mu\text{M}$ amiodarone showed no effect on the 2-APB-induced ectopic activity, but $50\mu\text{M}$ amiodarone significantly inhibited and $100\mu\text{M}$ amiodarone almost abolished 2-APB-induced ectopic activity (Fig. 2E). The voltage-dependent K^+ channel blocker, 4-aminopyridine (4-AP) at $100\mu\text{M}$ and 1mM concentrations, showed no effect on the 2-APB-induced ectopic activity (Fig. 2E).

Both Na^+ and Ca^{2+} channels are voltage-gated and their activations are dependent on cell membrane depolarization. 2-APB has been reported to stimulate intracellular Ca^{2+} release [11, 20] and forward-mode sodium-calcium exchange underlies 2-APB-induced ectopic activity [14], so we speculate that 2-APB-induced ectopic activity is from abnormal cell membrane depolarization and the membrane depolarization responsible for the activation of Na^+ and Ca^{2+} channels comes from $\text{Na}^+/\text{Ca}^{2+}$ exchanger activation. Therefore, we examined the effects of $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitor, 3',4'-dichlorobenzamil hydrochloride (DCH) and Ni^{2+} , on 2-APB-induced ectopic activity. As shown in Fig. 2F, both DCH ($20\mu\text{M}$) and Ni^{2+} (5mM) inhibited 2-APB-induced ectopic activity in rat atria. It has been reported that 2-APB activated canonical transient receptor potential channel TRPC3 [20] and TRPC3 channels expressed in the heart [21], so we examined the effects of non-selective cation channel blocker Gd^{3+} at $20\mu\text{M}$, $100\mu\text{M}$ and $1000\mu\text{M}$ concentrations. Gd^{3+} at $20\mu\text{M}$, $100\mu\text{M}$ and $1000\mu\text{M}$ did not inhibit 2-APB-induced ectopic activity and the data of $100\mu\text{M}$ Gd^{3+} were shown in Fig. 2F. In addition, 2-APB could not induce atria ectopic activity in Ca^{2+} -free bath solution (Fig. 2F), indicating that 2-APB-induced ectopic activity was dependent on Ca^{2+} influxes.

Furthermore, we observed the effects of 2-APB on the ventricular tissue straps and found that 2-APB ($20\mu\text{M}$)

Fig. 3. Quinidine, verapamil and DHC inhibited 2-APB-induced ectopic activity in rat ventricular tissue straps. DHC, 3',4'-dichlorobenzamil hydrochloride. $**P<0.01$ vs 2-APB.

Fig. 4. Lidocaine, verapamil and DHC inhibited 2-APB-induced $[\text{Ca}^{2+}]_i$ increase in cardiomyocytes. (A) 2-APB ($20\mu\text{M}$) induced $[\text{Ca}^{2+}]_i$ increase in cardiomyocytes in both Tyrode and Ca^{2+} -free Tyrode solutions. Ctr, control. (B) Pretreatment of lidocaine inhibited 2-APB-induced $[\text{Ca}^{2+}]_i$ increase in cardiomyocytes. Ctr, control. (C) The summarized data showed that lidocaine, verapamil and DCH inhibited 2-APB-induced $[\text{Ca}^{2+}]_i$ increase in cardiomyocytes. $**P<0.01$ vs Control; $##P<0.01$ vs 2-APB; Ctr, control; Lid, lidocaine; Ver, verapamil; DHC, 3',4'-dichlorobenzamil hydrochloride.

Fig. 5. 2-APB had no direct effect on voltage-dependent L-type Ca^{2+} currents in rat cardiomyocytes. (A) The representative recordings of voltage-dependent L-type Ca^{2+} currents before and after 2-APB (20μM) treatment. (B) Summarized data showed that 2-APB had no effect on voltage-dependent L-type Ca^{2+} currents in rat cardiomyocytes. The peak current (at +10mV) of 10min was normalized to that of 0min. $n=4$.

also induced ectopic contractions in ventricular tissue strips. Quinidine (10μM), verapamil (5μM) and DCH (20μM) inhibited 2-APB-induced ectopic activity in ventricular strips (Fig. 3), suggesting that the mechanism of 2-APB-induced ectopic activity in atria and ventricle was similar.

Although 2-APB has been reported to induce Ca^{2+} store release, the effects of 2-APB on $[\text{Ca}^{2+}]_i$ in cardiomyocytes have not been evidenced. We found that 2-APB (20μM) induced $[\text{Ca}^{2+}]_i$ increase in cardiomyocytes in both Tyrode and Ca^{2+} -free Tyrode solutions, however, $[\text{Ca}^{2+}]_i$ of cardiomyocytes in Ca^{2+} -free tyrode solution increased, followed by a decline, which was not observed in normal Tyrode solution (Fig. 4A). Next, we examined the effects of lidocaine, verapamil and DCH on $[\text{Ca}^{2+}]_i$ increase induced by 2-APB in normal Tyrode solution. As shown in Fig. 4B, 2-APB induced $[\text{Ca}^{2+}]_i$ increase which was inhibited by pretreatment of lidocaine. Verapamil and DCH showed the similar effects as lidocaine and the summarized data were shown in Fig. 4C. We could not exclude the possibility that 2-APB directly activate voltage-dependent L-type Ca^{2+} currents.

Fig. 6. Comparisons of the ectopic activity induced by 2-APB and phenylephrine. (A) The representative recording of PE-induced ectopic activity in left atria. (B) The representative recording showed that PE-induced ectopic activity was inhibited by pretreatment with 70 IU/mL LMWH. (C) The summarized data for inhibition of PE-induced ectopic activity by pretreatment with 70 IU/mL LMWH. (D) LMWH had no effect on 2-APB-induced ectopic activity. ** $P<0.01$ vs PE. PE, phenylephrine; LMWH, low molecular weight heparin.

Therefore, we examined the effects of 2-APB on L-type Ca^{2+} currents in rat cardiomyocytes. In order to eliminate the effect of Ca^{2+} current rundown on the result judgment, we normalized the peak current (at +10mV) of 10min to that of 0min, and compared 2-APB treatment group with the control. As shown in Fig. 5, 2-APB had no effect on L-type Ca^{2+} currents, consistent with the previous work in smooth muscle cells [22].

Comparisons of the ectopic activity induced by 2-APB and phenylephrine

In addition to inhibition on InsP_3 -induced Ca^{2+} release and SOC, 2-APB has been reported to act as an agonist of InsP_3Rs [11]. We are not sure whether 2-APB-induced ectopic activity in rat left atria comes from InsP_3Rs stimulation. Therefore, we compared the effects of 2-APB with phenylephrine (PE). PE generally stimulates α -adrenoceptor to increase the second messenger inositol 1,4,5-trisphosphate which stimulates Ca^{2+} release from the sarcoplasmic reticulum. As shown in Fig. 6A, PE (50μM) induced ectopic activity in left atria; however, the pattern of 2-APB-induced ectopic activity was

obviously different from that induced by PE. 2-APB-induced ectopic activity is continuous (Fig. 1A(c)) whereas the ectopic activity induced by PE is discontinuous (Fig. 6A). Furthermore, the ectopic activity induced by PE was inhibited by pretreatment with 70 IU/mL low molecular weight heparin (LMWH) which may cross the plasma membrane and thus inhibits InsP_3Rs within intact cells [23]. The original recordings were shown in Fig. 6A and B and the summarized data were shown in Fig. 6C. LMWH (70 IU/mL) treatment showed no effect on 2-APB-induced ectopic activity (Fig. 6D). These results suggested that 2-APB-induced ectopic activity might not be through the same pathway as PE which stimulates InsP_3Rs .

Discussion

The effects of 2-APB on Ca^{2+} signaling have always been controversial. In some studies, 2-APB is generally used as the IP_3R and store-operated Ca^{2+} channel antagonist [24–26]. On the other hand, 2-APB has been reported to induce Ca^{2+} store release [11, 20]. Previous studies evidenced that 2-APB induced sporadic spontaneous mechanical activity in rat left atria [13]. In the present study, we found that 2-APB induced ectopic activity in rat atria and 2-APB-induced ectopic activity was inhibited by Na^+ channel blocker, Ca^{2+} channel blocker and $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitor. We suggest that 2-APB-induced ectopic activity can be an alternative way for testing agents able to affect any of Na^+ , Ca^{2+} channel, $\text{Na}^+/\text{Ca}^{2+}$ exchanger without selectivity.

In addition to Na^+ and Ca^{2+} channels blockers, $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors are also potential anti-arrhythmic drugs [27, 28]. At present, several $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors have been developed, including NCX inhibitory peptide (XIP), KB-R7943, SEA-0400, CGP-37157, SN-6 [26], though they are not clinically used. A potential role for the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in arrhythmogenesis lies in its modulating the action potential and generating afterdepolarizations [27, 28]. It is well accepted that intracellular Ca^{2+} store release leads to the activation of $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which induces transient inward currents leading to the generation of action potential [24, 29, 30]. Based on this understanding and the present results, the schematic illustration of 2-APB-induced atria ectopic activity was described as following (Fig. 7): 2-APB increased $[\text{Ca}^{2+}]_i$, which activated $\text{Na}^+/\text{Ca}^{2+}$ exchanger. The inward currents induced by $\text{Na}^+/\text{Ca}^{2+}$ exchanger led to membrane depolarization that

Fig. 7. The schematic illustration of 2-APB-induced ectopic activity. The inward currents induced by $\text{Na}^+/\text{Ca}^{2+}$ exchanger activation lead to membrane depolarization that activates voltage-gated Na^+ channels and subsequent induction of voltage-dependent Ca^{2+} influx through L-type Ca^{2+} channels. The further increase of $[\text{Ca}^{2+}]_i$ induces ectopic activity. 2-APB, 2-aminoethoxydiphenyl borate.

activated voltage-gated Na^+ channels and subsequent induction of voltage-gated Ca^{2+} influx through L-type Ca^{2+} channels. The further increase of $[\text{Ca}^{2+}]_i$ induced ectopic activity. $\text{Na}^+/\text{Ca}^{2+}$ exchangers, Na^+ channels and Ca^{2+} channels were three crucial steps in the pathway of 2-APB-induced ectopic activity and blocking anyone of these three targets would inhibit 2-APB-induced ectopic activity.

Previous studies explored the mechanism of 2-APB-induced mechanical activity in isolated rat left atria. Wolkowicz et al reported that 2-APB-induced mechanical activity depended on the concentration of superfusate sodium, chloride, and potassium, and was sensitive to anion transport inhibitor, hyperpolarization-activated current (If) inhibitors [13, 14]. These results indicated that the mechanism of 2-APB-induced mechanical activity in atria was complicated. In the present study, we found that Na^+ channel blocker, Ca^{2+} channel blocker or $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitor completely inhibited 2-APB-induced ectopic activity in atria and the concentrations of these anti-arrhythmic drugs used *in vitro* were nearly equivalent to those used clinically *in vivo*.

2-APB stimulates Ca^{2+} store release in Ca^{2+} -free Tyrode solution, however, in the normal Tyrode solution, 2-APB-induced $[\text{Ca}^{2+}]_i$ increase was inhibited by Na^+ channel blocker, Ca^{2+} channel blocker and $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitor. Based on these results, we thought that, on the one hand, 2-APB induced intracellular Ca^{2+} store release; on the other hand, 2-APB-induced

sustained $[Ca^{2+}]_i$ increase was dependent on Ca^{2+} influx. Generally, the pathways for Ca^{2+} influx in atria include voltage-gated Ca^{2+} channels and non-selective cation channels [31]. Our results that verapamil and Ca^{2+} -free bath solution abolished 2-APB-induced ectopic activity whereas Gd^{3+} showed no effect suggest that the Ca^{2+} influx for 2-APB-induced ectopic activity is through voltage-gated Ca^{2+} channels. However, we found that 2-APB had no direct effect on L-type Ca^{2+} currents (Fig. 5), suggesting that the involvement of L-type Ca^{2+} channels in 2-APB-induced ectopic activity is indirect.

Amiodarone is a class III anti-arrhythmic agent which inhibits voltage-gated K^+ channels, Na^+ channels, and Ca^{2+} channels [32]. In the present study, amiodarone inhibited 2-APB-induced ectopic activity at 50 μM or 100 μM , which was higher than that clinically used. We could not identify whether K^+ channel blockade is responsible for its inhibition on ectopic activity, so we further observed the effect of 4-AP, which inhibits the voltage-dependent K^+ currents involved in repolarization in rat atria and ventricle [33, 34], on 2-APB-induced ectopic activity. 4-AP up to 1 mM could not inhibit 2-APB-induced ectopic activity. However, quinidine and verapamil, which significant inhibit 2-APB-induced ectopic

activity, also block different types of K^+ channels. Therefore, the explanation of the role of voltage-dependent K^+ currents in 2-APB-induced ectopic activity should be very cautious.

2-APB induces rat atria ectopic activity *in vitro*, but it did not induce typical arrhythmias *in vivo* (data not shown). When the lethal dose of 2-APB was used, sinus bradycardia and sinus arrest occurred. 2-APB has multiple actions, for example, inhibition or activation Ca^{2+} signals [11, 20, 24-26], inhibition of TRPC channels [35, 36], activation of TRPV channels [37, 38], blocking voltage-gated K^+ channels [39]. Because that the actions of 2-APB are involved in diverse physiological functions, it is impossible to deduced the *in vivo* actions of 2-APB from the *in vitro* data.

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