Original Research

2-Aminoethoxydiphenyl borate, a inositol 1,4,5-triphosphate receptor inhibitor, prevents atrial fibrillation

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Abstract

The expression of the inositol 1,4,5-triphosphate receptor (IP_3R) is upregulated and the function of IP_3R also increases during atrial fibrillation (AF). 2-Aminoethoxydiphenyl borate (2-APB) is a membrane-permeable inhibitor of IP_3R. However, the effect of 2-APB on AF is unknown. The aim of the present study is to explore the effects of 2-APB on AF. *In vitro* rabbit heart models of ischemia-, stretch- and cholinergic agitation-induced AF were developed. Fura-2-acetoxymethyl (Fura-2-AM) and Mg^{2+}-Fura-2-AM were used to monitor alterations of intracellular Ca^{2+} and ATP, respectively, in HL-1 cells, an atrial muscle cell line, under chemical ischemia or cholinergic agitation. The results showed that inhibition of IP_3R significantly reduced the incidence and its probability of being sustained in all three types of AF. IP_3R inhibition ameliorated the cytoplasmic Ca^{2+} overload and energy compromise resulting from chemical ischemia or cholinergic agitation. Thus, IP_3R inhibition may be a novel target for AF treatment, and IP_3R may be an important molecule in the context of different kinds of AF.

Keywords: atrial fibrillation, IP_3R, 2-APB, Ca^{2+}, ATP

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Introduction

Atrial fibrillation (AF), the most frequently encountered arrhythmia in the clinical setting, has a prevalence of 0.4–1% of the general population, affecting >5% of the population >65 y of age and increasing with age to >8% in those >80 y of age.[1] It is closely associated with increased long-term risk of stroke and congestive heart failure, as well as with decreased quality of life and increased mortality.[2] Despite extensive research and advancements in antiarrhythmic therapy and myocardial protection, the incidence of these common complications has not changed, and may in fact be increasing.[3] In addition, none of the antiarrhythmic drugs available is entirely optimal in terms of efficacy and safety.[4] Therefore, strategies to prevent AF are needed.[5–7]

Inositol 1,4,5-triphosphate receptors (IP_3Rs) are involved in compartmentalized signaling, and they can alter the kinetics of calcium release and reuptake and mediate specific cellular responses to stresses.[8] Atrial tissue expresses functional IP_3R at levels 6–10 times higher than ventricular myocytes.[2,9] In the adult mammalian atrium, IP_3-dependent Ca^{2+} release enhances atrial Ca^{2+} signaling and exerts a positive inotropic effect. IP_3-dependent Ca^{2+} signaling also has a direct effect on atrial excitation–contraction coupling under physiological as well as pathological conditions.[10]

Increased IP_3R expression is a general mechanism that underlies the remodeling of Ca^{2+} signaling during heart disease.[11] IP_3R has been demonstrated to be associated with certain types of arrhythmias.[8,11] Interestingly, the expression of IP_3R is upregulated and the function of IP_3R increases during AF.[12,13] 2-Aminoethoxydiphenyl borate (2-APB) is a membrane-permeable inhibitor of IP_3R.[9] However, the effect of 2-APB on AF remained unknown.

Materials and methods

Models of ischemia-, stretch- and cholinergic agitation-induced AF

Adult male New Zealand white rabbits (2.2–2.5 kg) were anesthetized by pentobarbitone (60 mg/kg intravenously) with sodium heparin (250 U intravenously). Hearts were rapidly removed into ice-cold Krebs–Henseleit (K–H) buffer containing (mmol/L): 118.0 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 2.5 $CaCl_2$ and 11.0

glucose. The hearts were then mounted onto a Langendorff apparatus and perfused retrogradely at a constant pressure of 65 mmHg with K–H buffer gassed with 95% O_2/5% CO_2 equilibrated at pH 7.35–7.45 and maintained at 37°C for 20 min.

Atrial ischemia was induced by perfusing hearts with K–H solution containing 10 mmol/L $Na_2S_2O_4$ for about five minutes, maintaining one-third of normal perfusion pressure during the simulated ischemia. A transient pacing episode (40 Hz, cycle length 20 ms, duration 1 min) was applied at the end of ischemia to trigger AF. This protocol did not cause any macroscopically visible myocardial necrosis in our experiments, as evaluated by 2,3,5-triphenyltetrazolium chloride staining after five minutes of ischemia.

Acute atrial dilation with controlled increases in atrial pressure of up to ~16 cm H_2O was applied for about 40 min to simulate atrial stretch, and subsequent pacing (50 Hz, cycle length 10 ms, duration 1 min) allowed for the induction of stretch-induced AF.

Acetylcholine (ACh) at a concentration of 8 μmol/L was administered to create an animal model of cholinergic agitation-induced AF. ACh was infused into *in vitro* rabbit hearts for about one minute, after which a burst pacing episode (30 Hz, cycle length 10 ms, duration 1 min) was applied.

In these three models of AF, a pair of platinum pacemaker wires were implanted in the right atrium's free wall and connected to a pulse generator (AD Instruments, Castle Hill, New South Wales, Australia). Electrocardiographic electrodes were attached to the free atrial wall and connected to the chart recorder. The ablation of the atrioventricular node was performed in advance. AF longer than 60 s was defined as sustained AF.[14]

The animals used in this study were maintained in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Policy of Animal Care and Use Committee of Tongji University.

Pharmacological intervention protocols

To study ischemia-induced AF, rabbit hearts were randomly assigned to one of three groups (Figure 1a): (1) control group ($n = 10$): 25 min K–H perfusion with 1 min pacing (40 Hz, cycle length 20 ms); (2) ischemia-induced AF model group ($n = 10$): 20 min K–H perfusion, 5 min ischemia perfusion and 1 min pacing (40 Hz, cycle length 20 ms); (3) 2-APB group ($n = 10$): 20 min K–H perfusion, 5 min ischemia perfusion and 1 min pacing (40 Hz, cycle length 20 ms); the entire perfusion period except the first 10 min K–H perfusion was with 2-APB at a concentration of 2 μmol/L.

To study stretch-induced AF, rabbit hearts were randomly assigned to one of three groups (Figure 1b): (1) control group ($n = 10$): 60 min K–H perfusion with 1 min pacing (50 Hz, cycle length 10 ms); (2) stretch-induced AF model group ($n = 10$): 20 min K–H perfusion, 40 min stretch and 1 min pacing (50 Hz, cycle length 10 ms); (3) 2-APB group ($n = 10$): 20 min K–H perfusion, 40 min stretch and 1 min pacing (50 Hz, cycle length 10 ms); the entire perfusion period except the first 10 min K–H perfusion was with 2-APB at a concentration of 2 μmol/L.

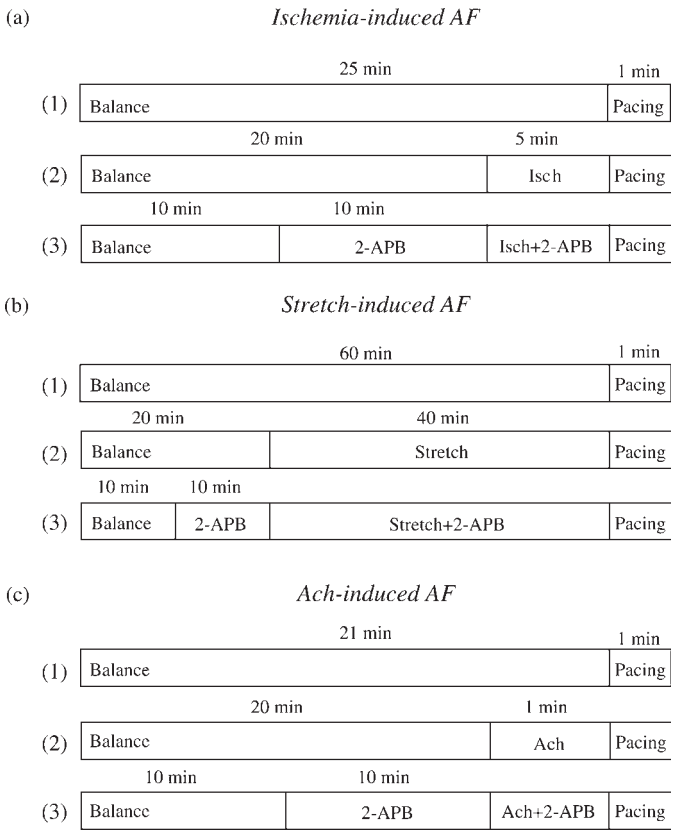

Figure 1 (a–c) Pharmacological intervention protocols. The pharmacological intervention protocol for (a) ischemia-induced AF; (b) stretch-induced AF; and (c) acetylcholine-induced AF. AF, atrial fibrillation; 2-APB, 2-aminoethoxydiphenyl borate

To study cholinergic agitation-induced AF, rabbit hearts were randomly assigned to one of three groups (Figure 1c): (1) control group ($n = 10$): 21 min K–H perfusion with 1 min pacing (30 Hz, cycle length 10 ms); (2) cholinergic agitation-induced AF model group ($n = 10$): 20 min K–H perfusion, 1 min Ach perfusion and 1 min pacing (30 Hz, cycle length 10 ms); (3) 2-APB group ($n = 10$): 20 min K–H perfusion, 1 min Ach perfusion and 1 min pacing (30 Hz, cycle length 10 ms); the entire perfusion period except the first 10 min K–H perfusion was with 2-APB at a concentration of 2 μmol/L.

2-APB was dissolved in dimethyl sulfoxide (DMSO). The exact concentration of DMSO in the solution of 2-APB is one in a thousand. This concentration does not increase the susceptibility to arrhythmias. The 2-APB dosage used in this study is the most widely used concentration and did not alter the intrinsic electrophysiological properties, including the duration of the atrial action potential, during baseline perfusion (data not shown).

Parallel ischemia and ACh agitation experiments in HL-1 cells

In parallel experiments, we simulated ischemia and ACh agitation in the murine atrial cell line HL-1 (a gift from

the laboratory of William C Claycomb, Louisiana State University Health Sciences Center, New Orleans, LA, USA), which is the only available atrial myocyte cell line that continuously divides and maintains a differentiated cardiac phenotype with spontaneous depolarization.[15,16] However, we did not simulate stretch in HL-1 cells, as it is impossible to simulate the same extent of stretch as in the Lagendorff experiments.

HL-1 cells were cultured at 37°C in a 5% CO_2 atmosphere in Claycomb medium supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 100 U/mL penicillin, 100 mg/mL streptomycin (Life Technologies, Rockville, MD, USA) and 100 μmol/L norepinephrine (Sigma, St Louis, MO, USA). Cell culture flasks were precoated with a solution of 0.02% gelatin (Difco, Sparks, MD, USA) and fibronectin (Sigma) at 12.5 mg/mL/12.5 cm^2. The medium was changed approximately every 24 h, as described previously.[15]

Ischemia in HL-1 cells was induced by 10 min of superfusion with glucose-free Tyrode's solution containing $Na_2S_2O_4$ (1 mmol/L).[17] For the assessment of the effect of Ach on $[Ca^{2+}]_i$, 8 μmol/L Ach was added to the normal Tyrode's solution and cells were superfused for five minutes. The entire superfusion period except the first five-minute Tyrode's solution superfusion was with 2-APB at a concentration of 2 μmol/L.

Intracellular $[Ca^{2+}]_i$ and $[ATP]_i$ measurements

$[Ca^{2+}]_i$ was measured from Fura-2 fluorescence signals using a dual wavelength spectrophotometric method. Fura-2 was incorporated into the cells as the acetoxymethyl (AM) ester by incubating them with 1 μmol/L Fura-2-AM at 37°C for 30 min, and then washed three times with standard Tyrode's solution. After 15–20 min to allow for de-esterification of the dye, the cells were alternately excited at 340/380 nm and the fluorescence emission was collected through a 510 ± 10 nm band pass filter (TILL Photonics GmbH, Gräfelfing, Germany). The F_{340}/F_{380} fluorescence ratio values were used to evaluate intracellular $[Ca^{2+}]_i$.

As ATP has at least 10-fold greater affinity for Mg^{2+} than does ADP or AMP, the measurable decrease in $[Mg^{2+}]_i$ is a reliable indicator of ATP increase.[18] Mg^{2+}-Fura-2 was incorporated into the cells as the AM ester by incubating them with 1 μmol/L Mg^{2+}-Fura-2-AM at 37°C for 30 min, and then washed three times with standard Tyrode's solution. After 15–20 min to allow for de-esterification of the dye, the cells were also alternately excited at 340/380 nm and the fluorescence emission was collected through a 510 ± 10 nm band pass filter (TILL Photonics GmbH, Gräfelfing, Germany). The F_{340}/F_{380} fluorescence ratio values were used to evaluate intracellular $[ATP]_i$. Data were analyzed using Image-pro plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Statistics analysis

Data are expressed as the mean ± standard error (SEM). The time-dependent data were subjected to two-way analysis of variance to determine whether there were any significant differences among the groups. If a significant difference was found, Bonferroni's *post hoc* test was conducted to determine which group differed significantly. The incidence of AF and the probability of sustained AF in different groups were analyzed using a χ^2 test. Probability values of <0.05 were considered significant.

Results

2-APB prevents three different types of AF

Ninety percent of ischemic hearts displayed AF, whereas inhibition of IP_3R with 2-APB reduced the incidence of AF to about 30% ($P < 0.05$) (Figure 2a). Seventy percent of the stretched hearts developed AF. After exposure to the IP_3R inhibitor 2-APB, the vulnerability to AF decreased significantly to 20% ($P < 0.05$) (Figure 2c). In addition, half of the high-dose Ach-treated hearts demonstrated AF, and IP_3R inhibition reduced the incidence to 10% ($P < 0.05$) (Figure 2e). Moreover, 2-APB decreased the probability of sustained AF by 45%, 70% and 60%, respectively, in the

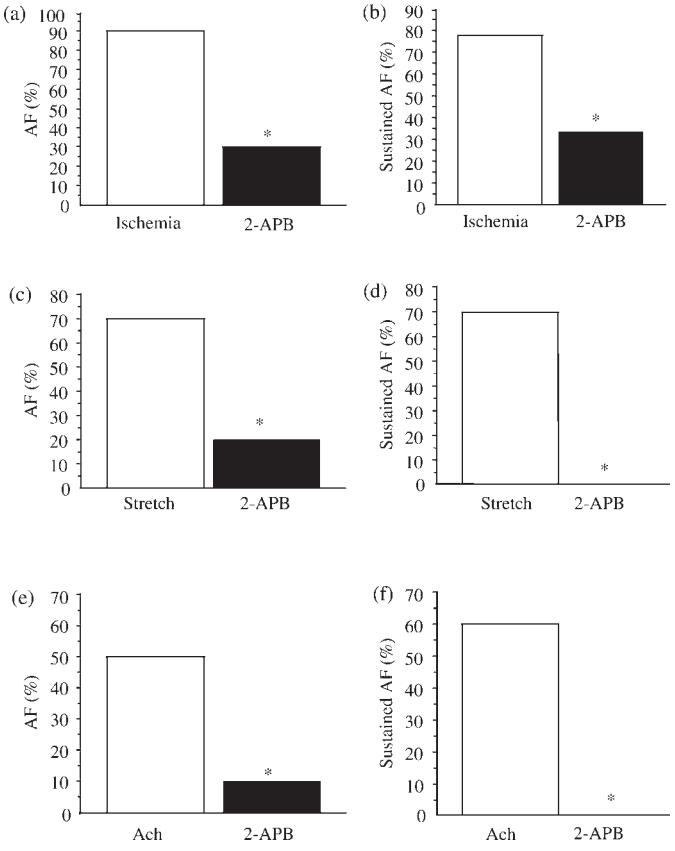

Figure 2 (a–f) 2-APB prevents three different types of AF. (a) 2-APB reduces the incidence of ischemia-induced AF. $^*P < 0.05$, compared with ischemia group. (b) 2-APB reduces the probability of sustained AF via ischemia. $^*P < 0.05$, compared with ischemia group. (c) 2-APB reduces the incidence of stretch-induced AF. $^*P < 0.05$, compared with stretch group. (d) 2-APB reduces the probability of sustained AF via stretch. $^*P < 0.05$, compared with stretch group. (e) 2-APB reduces the incidence of Ach-induced AF. $^*P < 0.05$, compared with Ach group. (f) 2-APB reduces the probability of sustained AF via cholinergic agitation. $^*P < 0.05$, compared with Ach group. AF, atrial fibrillation; 2-APB, 2-aminoethoxydiphenyl borate; Ach, acetylcholine

ischemia, stretch and cholinergic agitation models (Figures 2b, d and f).

2-APB ameliorates Ca^{2+} overload in HL-1 cells

Intracellular $[Ca^{2+}]_i$ overload is a key event triggering AF and preventing it is effective at decreasing the incidence of AF. Thus, whether the prevention of AF by 2-APB is via ameliorating intracellular Ca^{2+} overload was further explored. It was found that ischemia and high-dose Ach caused a significant increase in the cytoplasmic Ca^{2+} concentration. However, when 2-APB (2 μmol/L) was applied, the ischemia- and ACh-induced cytoplasmic Ca^{2+} overload was significantly ameliorated (Figure 3).

2-APB preserves intracellular ATP in HL-1 cells

A cellular energetic deficit was recently linked to AF. We first verified this relationship and then explored the role of IP_3R in atrial energetic homeostasis. It was found that ischemia and Ach administration decreased $[ATP]_I$, while inhibition of IP_3R with 2-APB (2 μmol/L) maintained the energetic homeostasis well in HL-1 cells (Figure 4).

Discussion

The major findings of the present study are as follows: (1) 2-APB, an inhibitor of IP_3R, significantly prevents ischemia-, stretch- and cholinergic agitation-induced AF; and (2) factors underlying AF may lead to cytoplasmic Ca^{2+} overload and ATP depletion, whereas IP_3R inhibition with 2-APB can ameliorate Ca^{2+} overload and effectively maintain energetic homeostasis.

Coronary heart diseases, hypertension, heart failure, valvular heart diseases and cholinergic agitation are regarded as the main underlying causes of AF.[19,20] To explore the effects of IP_3R inhibition, three different types of AF were developed in the present study. Ischemia, stretch and ACh were adopted as stimulators of AF in this study. Coronary heart diseases are simulated by ischemia. Hypertension, heart failure and valvular heart diseases are mimicked by stretch. Cholinergic agitation is induced by high-dose ACh administration.[20] Thus, the three AF models developed in this study are representative, which makes it possible for us to identify a common target for the treatment of AF.

Ca^{2+} is a ubiquitous intracellular signal messenger.[21] Once released from the cardiac sarcoplasmic reticulum, Ca^{2+} interacts with sarcolemmal binding sites to produce

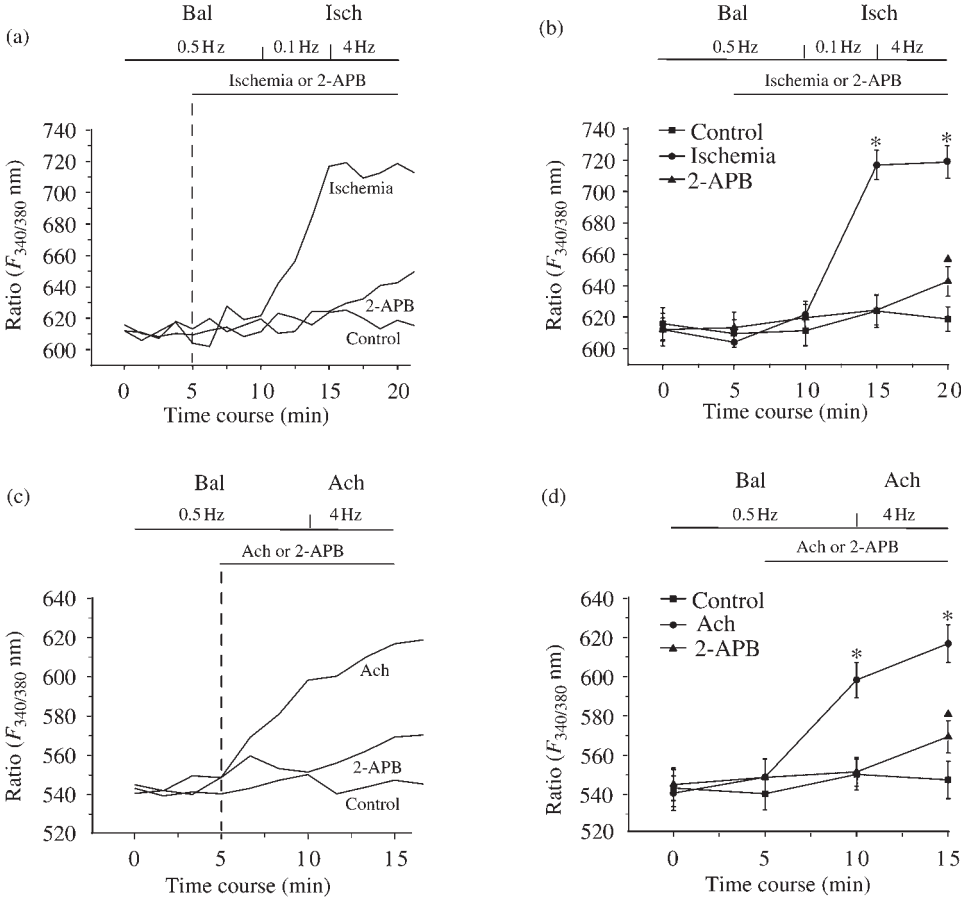

Figure 3 (a–d) 2-APB ameliorates Ca^{2+} overload in HL-1 cells. (a) Representative recording traces for the fluorescence of Fura-2-AM throughout balancing and ischemic perfusion. (b) Statistical results of the fluorescence of Fura-2-AM changes during ischemia. $^*P < 0.05$ versus control group. $^▲P < 0.05$ versus control group and ischemia group. (c) Representative recording traces for the fluorescence of Fura-2-AM throughout balancing and Ach perfusion. (d) Statistical results of the fluorescence of Fura-2-AM changes during Ach perfusion. $^*P < 0.05$ versus control group. $^▲P < 0.05$ versus control group and Ach group. Fura-2-AM, Fura-2-acetoxymethyl; Bal, balancing; Isch, ischemia; 2-APB, 2-aminoethoxydiphenyl borate; Ach, acetylcholine

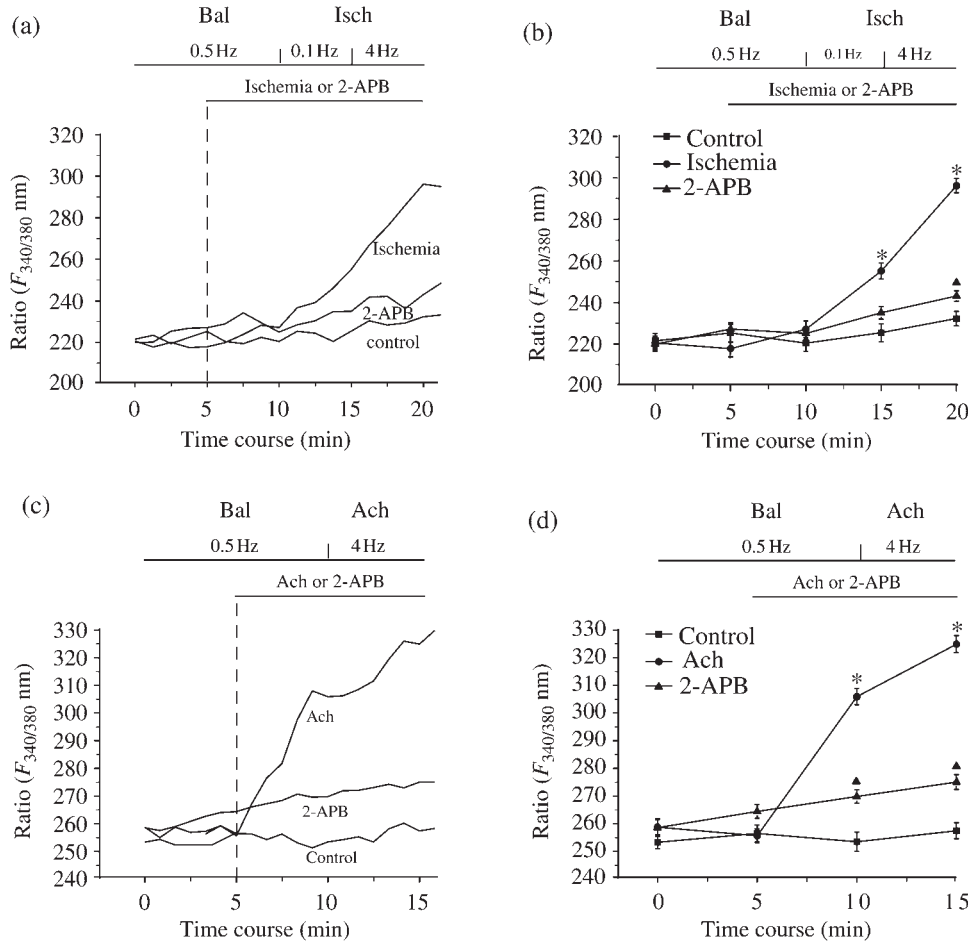

Figure 4 (a–d) 2-APB preserves intracellular ATP in HL-1 cells. (a) Representative recording traces for the fluorescence of Mag-Fura-2-AM throughout balancing and ischemic perfusion. (b) Statistical results of the fluorescence of Mag-Fura-2-AM during ischemia. $^{*}P < 0.05$ versus control group. $^{\blacktriangle}P < 0.05$ versus control group and ischemia group. (c) Representative recording traces for the fluorescence of Mag-Fura-2-AM throughout balancing and Ach perfusion. (d) Statistical results of the fluorescence of Mag-Fura-2-AM during Ach perfusion. $^{*}P < 0.05$ versus control group. $^{\blacktriangle}P < 0.05$ versus control group and Ach group. Fura-2-AM, Fura-2-acetoxymethyl; Bal, balancing; Isch, ischemia; 2-APB, 2-aminoethoxydiphenyl borate; Ach, acetylcholine

an inward current, thereby affecting the repolarization of the cardiac action potential.[22] A failure of intracellular Ca^{2+} homeostasis, with a consequent increase in membrane-triggered activity, is thought to be the primary initiating factor in AF in some circumstances.[22] An increase of intracellular Ca^{2+} concentration can produce a negative feedback effect on L-type Ca^{2+} channel activity with a decrease in the plateau phase of the action potential.[22] Therefore, cardiac cytosolic Ca^{2+} overload has been regarded as an important mediator of AF.[23,24] IP_3R-mediated Ca^{2+} release plays an important role in the modulation of excitation–contraction coupling in atrial tissue. Upregulation of IP_3R is important for modulating intracellular Ca^{2+} homeostasis and may be related to the generation of arrhythmias.[2,11] In the present study, IP_3R inhibition with 2-APB was found to be able to ameliorate Ca^{2+} overload, which likely underlies the antiarrhythmic effect of 2-APB.

Impairment in atrial energetics may contribute to AF.[25] An enhanced demand for high-energy phosphates has been indicated in AF.[26] Recent studies have indicated that a drop in the atrial phosphocreatine level occurs within 25 min of the onset of AF.[27] High atrial oxygen and energy demand during AF can result in a transient drop in phosphocreatine levels during the early stages of AF.[26] An accumulation of defects at various steps in atrial energetic signaling may compromise the ability to restore adequately electrical stability in the face of induced AF.[25] In addition, the duration of induced AF correlates with atrial ATP levels, suggesting a potential role for cellular energetic homeostasis in maintaining the electrical stability of atrial muscle.[25] In the present study, IP_3R inhibition with 2-APB was found to well preserve intracellular ATP, and this might be another important basis for the antiarrhythmic effect of 2-APB.

A recent study indicated that 2-APB was able to decrease intracellular calcium concentration and prevent thromboxane A_2-induced ventricular arrhythmias.[28] Our study also supported their data showing that AF could also be prevented by IP_3R inhibitor 2-APB. However, two other studies showed that 2-APB could provoke ectopic activity due to calcium leak in the atria contributing to AF.[29,30] This discrepancy can be explained by the different dosages of 2-APB used in these studies as 2-APB suppresses IP_3R-linked calcium signaling at low concentrations, while at higher concentrations it induces leakage from cell calcium stores.[29,30]

A potential limitation is that it is still unknown whether changes of cytosolic Ca^{2+} interfere with measurements of cytosolic Mg^{2+} in HL-1 cells. But it is reported that cytoplasmic free Ca^{2+} does not contribute significantly to Mg^{2+}-Fura-2 intensity unless the concentration exceeds 1 μmol/L.[31] A potential weakness of this study is that the exact molecular pathways of 2-APB's effects on calcium overload and energetic homeostasis still need to be explored. We cannot preclude the possibility that IP_3R inhibition may act on pathways such as regulating the distribution of gap junctions other than regulating intracellular calcium and energy metabolism to prevent AF.[12] Moreover, we also can neither differentiate the cause from the effects between Ca^{2+} overload and $[ATP]_i$ deficiency nor can we identify whether their synergetic actions contribute to all three kinds of AF. Nevertheless, our analysis of the effects of IP_3R inhibition on cytoplasmic Ca^{2+} and $[ATP]_i$ supports a predominant role of IP_3R in AF. In future studies, the concentrations of 2-APB that give the highest protective effect and that can be considered an overdose need to be determined.

Conclusions

In conclusion, the results of this study indicate that 2-APB, an IP_3R inhibitor, may prevent the initiation and/or maintenance of AF, likely by ameliorating Ca^{2+} overload and preserving energetic homeostasis. This study presents evidence that IP_3R may be a novel target for the treatment of AF.

Author contributions: Y-HC designed the experiment and wrote the manuscript. JX established the stretch-induced AF model and performed the pharmacological intervention in that model. DL and XL set up the ischemia-induced AF model and then conducted the pharmacological experiment. HZ and Yi Liu built the cholinergic agitation-induced AF model and also carried out the pharmacological study. Ying Liu and HZ cultured the HL-1 cells and measured intracellular $[Ca^{2+}]_i$. JL quantified $[ATP]_i$ and LP analyzed the data. JX, DL, HZ and Ying Liu contributed equally to this work.

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Conflict of interest: The authors declare that they have no conflict of interest.

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