Korean J Physiol Pharmacol
Vol 14: 119−125, June, 2010
DOI: 10.4196/kjpp.2010.14.3.119

A Carbohydrate Fraction, AIP1, from *Artemisia Iwayomogi* Reduces the Action Potential Duration by Activation of Rapidly Activating Delayed Rectifier K^+ Channels in Rabbit Ventricular Myocytes

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We investigated the effects of a hot-water extract of *Artemisia iwayomogi*, a plant belonging to family Compositae, on cardiac ventricular delayed rectifier K^+ current (I_K) using the patch clamp technique. The carbohydrate fraction AIP1 dose-dependently increased the heart rate with an apparent EC_{50} value of 56.1±5.5 μg/ml. Application of AIP1 reduced the action potential duration (APD) in concentration-dependent fashion by activating I_K without significantly altering the resting membrane potential (IC_{50} value of APD_{50}: 54.80±2.24, IC_{50} value of APD_{90}: 57.45±3.47 μg/ml). Based on the results, all experiments were performed with 50 μg/ml of AIP1. Pre-treatment with the rapidly activating delayed rectifier K^+ current (I_{Kr}) inhibitor, E-4031 prolonged APD. However, additional application of AIP1 did not reduce APD. The inhibition of slowly activating delayed rectifier K^+ current (I_{Ks}) by chromanol 293B did not change the effect of AIP1. AIP1 did not significantly affect coronary arterial tone or ion channels, even at the highest concentration of AIP1. In summary, AIP1 reduces APD by activating I_{Kr} but not I_{Ks}. These results suggest that the natural product AIP1 may provide an adjunctive therapy of long QT syndrome.

Key Words: Artemisia iwayomogi, Delayed rectifier K^+ current, Long QT syndrome, Ventricular myocyte

INTRODUCTION

Cardiac K^+ currents are critical for regulation of action potential (AP) repolarization. The late phase of AP repolarization in cardiomyocytes is initiated in most species by activation of the delayed rectifier K^+ current (I_K). I_K consists of two different components [1]: the rapidly activating I_K (I_{Kr}) and a slowly activating I_K (I_{Ks}), which can be distinguished based on electrophysiological kinetics, pharmacology and voltage-dependence [2-4]. Both currents play an important role in the repolarization of action potentials, and congenital or acquired prolongation of the QT interval in ECG is often brought about by a loss of function in this system [5]. Excessive AP duration (APD) prolongation causes long QT syndrome, which is associated with torsades de pointes, a ventricular tachyarrhythmia that can degenerate into ventricular fibrillation and cause sudden death [6,7]. Therefore, an activator of I_{Kr} or I_{Ks} channels may prove useful in the treatment of long QT syndrome resulting from an excessive pharmacological block of these channels or from mutations in the genes that encode the channel proteins [6].

Artemisia iwayomogi (A. messer-schmidtiana var. *viridis* Besser, Compositae), known as Haninjin or Dowijigi, is a perennial herb that is abundant in Korea and is used to treat various liver diseases, including hepatitis, in traditional medicine [8,9]. The AIP1 carbohydrate fraction has been purified from a crude water-soluble preparation from dried *A. iwayomogi* herb using size-exclusion chromatography [10]. AIP1 has various biological functions. For example, the AIP1 fraction increased antibody production and suppressed transplanted tumor cell growth [10]. The AIP1 fraction may also be involved in the survival of immune cells, either by suppressing apoptosis or by stimulating cell proliferation [11-13]. To date, however, the influence of AIP1 on cardiac function has not been elucidated. In the present study, we examined the effect of AIP1 on I_K in ventricular cardiomyocytes. Our results demonstrated that AIP1 treatment decreased APD via activation of I_{Kr}, which may provide an adjunctive therapy of long QT syndrome.

Received March 17, 2010, Revised May 4, 2010,
Accepted May 10, 2010

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ABBREVIATIONS: AP, action potential; APD, action potential duration; I_{Kr}, rapidly activating delayed rectifier K^+ current; I_{Ks}, slowly activating delayed rectifier K^+ current.

METHODS

Single cell preparation of rabbit ventricular myocytes

Single ventricular myocytes were isolated from rabbit hearts as described previously [14]. Briefly, New Zealand White rabbits (1.5~2.0 kg) of either sex were anesthetized with sodium pentobarbitone (50 mg/kg) and were injected simultaneously with heparine (100 U/kg) into the ear vein. The procedure was carried out in accordance with the guidelines of the Committee for Animal Experiments of the Inje University College of Medicine. The hearts were quickly removed and cannulated by silicon tubing and retrogradely perfused via aorta on a Langendorff apparatus. Initially the heart was perfused with normal Tyrode solution for 4~5 min to clear the blood and then perfused with Ca^{2+}-free normal Tyrode solution for 3 min. Then, the heart was perfused with enzyme solution containing 0.8 mg/ml collagenase for 20 min. Following the enzymatic treatment, the ventricle was dissected out and agitated mechanically with fire-polished Pasteur pipette in Kraft-Brühe (KB) solution. Single cells were stored in KB solution at 4°C and used on the day of preparation.

Single cell preparation of arterial smooth muscle cells

Single smooth muscle cells were isolated from rabbit coronary arteries as described previously [15]. Briefly, the coronary arteries were isolated from hearts and cleaned of connective tissue in the normal Tyrode solution under a stereomicroscope. The arteries were transferred to normal Tyrode solution without $CaCl_2$ (Ca^{2+}-free solution) for 10 min and then incubated in the Ca^{2+}-free solution containing papain (1 mg/ml) for 25 min. After the remaining enzyme was removed by washing in the Ca^{2+}-free solution, the arteries were incubated in the Ca^{2+}-free solution containing collagenase (2.8 mg/ml) for 20 min. Single smooth muscle cells were obtained by gentle agitation with a Pasteur pipette in Kraft-Brühe (KB) solution, stored at 4°C, and used on the day of preparation.

Purification of AIP1 fraction

AIP1 fraction was isolated from *Artemisia iwayomogi* as described by Koo et al. [10]. Briefly, a water-soluble crude extract was fractionated using Sephadex G-50 size exclusion chromatography with distilled water. The fractions containing carbohydrates or similar compounds were determined by the modified phenol/sulfuric acid method of total sugar determination and the fractions smaller than 1 kDa were pooled and used as the AIP1 fraction.

Electrophysiology

Whole cell patch clamp recordings were made using an Axon interface and Axopatch 1C amplifier (Axon Instruments, Union, CA). All experimental parameters, such as pulse generation and data acquisition, were controlled using PatchPro software, developed by our group. Action potentials were recorded by applying a square stimulus pulse of 1~2 nA and 3 ms duration. Patch pipettes were pulled from thin-walled borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a PP-83 vertical puller (Narishige, Tokyo, Japan). The voltage and current signals were filtered at 0.5~1.0 kHz and were sampled at a rate of 1~3 kHz. Electrode resistance before sealing was 3~4 MΩ, and after sealing it was <10 GΩ. The liquid-junction potentials between normal Tyrode and pipette solution, which were calculated based on ionic mobilities, were <5 mV. Since they were not large and liquid-junction potential between pipette solutions and intracellular solutions were not known, we did not make correction for junction potentials in presenting and analyzing data.

Measurement of arterial tone

The coronary arteries were isolated from the left anterior descending coronary artery and cleaned of connective tissue in the ice-cold Krebs-Henseleit (K-H) solution and continuously bubbled with the mixture of 95% O_2 and 5% CO_2, under a stereomicroscope. After isolation, the coronary artery was cut into rings of 5 mm length. Each ring was suspended between wires that were attached to the transducer for the measurement of isometric tension. Endothelial cells were disrupted. Successful removal of endothelium was assessed by showing that acetylcholine (10 μM) failed to relax arteries precontracted by 10 μM prostaglandin F2α. Arteries were allowed to equilibrate for at least 60 min before data collection. The rings were incubated in a chamber filled with K-H solution, continuously bubbled with the mixture of 95% O_2 and 5% CO_2, maintained at 37°C during the stabilization and experiments.

Heart rate measurements

To measure the heart rate, the methodology of Langendorff's isolated perfused heart preparation was applied. The isolated hearts were mounted on a Langendorff perfusion system, then, perfused with K-H solution continuously bubbled with mixture of 95% O_2 and 5% CO_2. The heart remained at 37°C throughout the entire experiment. Heart rate was determined by insertion of unipolar electrode in organ chamber solution to record the crude electrocardiogram (ECG) and analyzed with Powerlab (Chart 5) recording system.

Solutions and drugs

Normal Tyrode solution contained (in mM): NaCl, 140; KCl, 5.4; NaH_2PO_4, 0.33; $CaCl_2$, 1.8; $MgCl_2$, 0.5; HEPES, 5; glucose, 16.6; adjusted to pH 7.4 with NaOH. KB solution contained (in mM): KOH, 70; L-glutamate, 50; KH_2PO_4, 20; KCl, 55; taurine, 20; $MgCl_2$, 3; glucose, 20; HEPES, 10; EGTA, 0.5; adjusted to pH 7.3 with KOH. K-H solution, containing (in mM) : NaCl, 120; KCl, 4.7; $CaCl_2$, 1.8; $MgSO_4$, 1.2; KH_2PO_4, 1.18; $NaHCO_3$, 25; glucose, 16.5, continuously bubbled with the mixture of 95% O_2 and 5% CO_2; adjusted to pH 7.4 with NaOH after 1 hr of being bubbled with the gas mixture. The pipette-filled solution for recordings of I_K in ventricular myocytes and K^+ currents in vascular smooth muscle contained (in mM): K-aspartate, 115; KCl, 25; NaCl, 5; $MgCl_2$, 1; Mg-ATP, 4; EGTA, 0.1; HEPES, 10; adjusted to pH 7.2 with KOH. The ventricular Ca^{2+} channel recording solution contained (in mM): NaCl, 140; CsCl, 10; NaH_2PO_4, 0.33; $CaCl_2$, 1.8; $MgCl_2$, 0.5; HEPES, 5; glucose, 16.6; adjusted to pH 7.4 with NaOH. The pipette-filled solution for recordings of Ca^{2+} current in ventricular myocytes contained (in mM): CsCl, 106; TEA-Cl, 20; NaCl, 5; Mg-ATP, 5; EGTA, 10; HEPES, 10; adjusted to pH 7.25 using CsOH. The vascular Ca^{2+} currents were

recorded using perforated patch clamp technique. For the perforated-patch recordings of Ca^{2+} currents, the vascular smooth muscle cells bathed in solution contained (in mM): NaCl, 120; $CaCl_2$, 2; CsCl, 5; TEA-Cl, 20; $MgCl_2$, 0.5; HEPES, 10; glucose, 10; adjusted to pH 7.4 with NaOH. The pipette-filled solution contained (in mM): CsCl, 130; HEPES, 10; EGTA, 10; Mg-ATP, 5; adjusted to pH 7.2 with CsOH. Nystatin (200 μg/ml) was added to pipette solution. All pharmacological compounds were dissolved in water or dimethyl sulfoxide (DMSO) at >1,000 times to make stock solution. E-4031 (*N*-[4-[[1-[2-(6-methyl-2-pyridinyl)ethyl]-4-pi-peridinyl]carbonyl]phenyl]methanesulfonamide dihydrochroride) and Chromanol 293B were purchased from Sigma (St. Louis, MO, USA).

Statistics

Origin 6.0 software (Microcal Software, Northampton, MA) was used for data analysis. Data are presented as mean±SEM. Statistical analyses were performed by using unpaired Student's *t* test. A value of $p<0.05$ was defined as statistically significant.

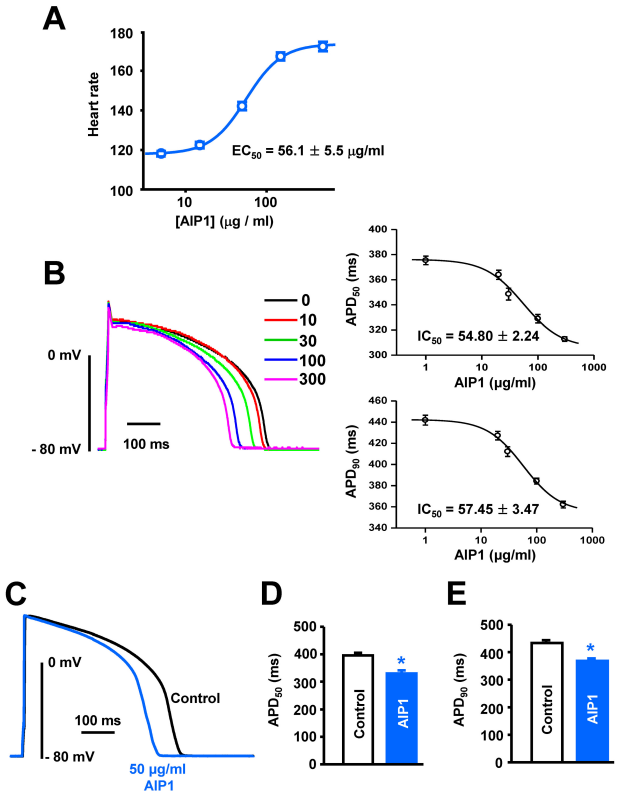

Fig. 1. Effect of AIP1 on the heart rate and action potentials of ventricular myocytes. (A) Does-dependent effect of AIP1 on the heart rate (All n=4 hearts). (B) Dose-dependent effect of AIP1 on APD. Superimposed traces of APs obtained using the patch-clamp technique under control conditions and in various concentrations of AIP1 with an apparent IC_{50} value of APD_{50} and APD_{90} (n=4 cells). (C) Representative traces of APs under control conditions in the presence of 50 μg/ml AIP1. (D, E) Summary of the APD_{50} and APD_{90} as shown in (C). APD_{50} and APD_{90} mean the time required for repolarization to 50% and to 90% of basal membrane potential, respectively. *$p<0.05$ (n=6 cells).

RESULTS

AIP1 increased the heart rate

The application of AIP1 increased the heart rate dose-dependently (Fig. 1A). A non-linear square fit of the Hill equation yielded the EC_{50} of 56.1±5.5 μg/ml for the heart rate. This suggests that AIP1 activates I_K in the sino-atrial (SA) node, which induces the reduction of APD in the SA node, resulting in the increased heart rate.

AIP1 shortened APD in ventricular cardiomyocytes

We also tested the effects of AIP1 on APs using rabbit ventricular cardiomyocytes. APs were elicited every 5s using the current clamp mode for conventional whole-cell conditions. AIP1 shortened APD in dose-dependent manner without significantly affecting resting membrane potential (IC_{50} value of APD_{50}: 54.80±2.24, IC_{50} value of APD_{90}: 57.45± 3.47 μg/ml, Fig. 1B). Considering the EC_{50} value of heart rate and IC_{50} values of APD_{50} and APD_{90}, all recordings were performed with 50 μg/ml of AIP1. 50 μg/ml AIP1 decreased APD_{50} from 396.0±9.8 to 330.0±11.3 ms (Fig. 1C and D), and APD_{90} from 433.6±10.3 to 368.0±9.3 ms (Fig. 1C and E).

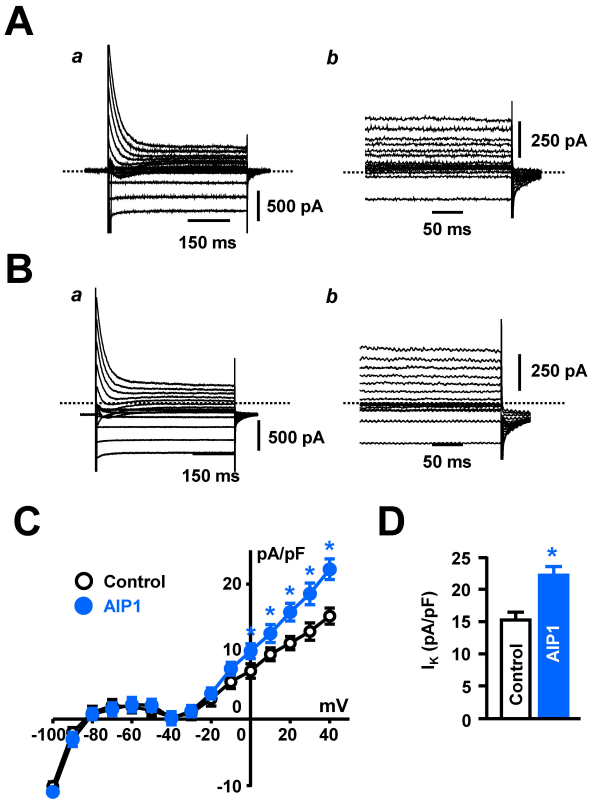

Fig. 2. AIP1 increased I_K in ventricular cardiomyocyte. (A, B) Whole-cell current of ventricular cardiomyocytes under control conditions (A) and in the presence of 50 μg/ml AIP1 (B). Panel (a) was original recording traces and panel (b) was magnified traces of the panel (a). (C) Summary of whole-cell *I-V* relationship under control conditions (○) and in the presence of 50 μg/ml AIP1 (●). (D) Statistical summary of I_K at +40 mV. *$p<0.05$ (n=5 cells).

Effect of AIP1 on I_K in ventricular cardiomyocytes

Shortening of APD is closely related to the activation of I_K or inhibition of Ca^{2+} current (I_{Ca}). Thus, we recorded whole-cell I_K current using the patch-clamp technique. I_{Ca} was inhibited by pretreatment with the *L*-type Ca^{2+} channel inhibitor, nifedipine (10 μM). I_K was elicited by 500-ms depolarizing pulses between −120 and +40 mV from a holding potential of −80 mV. As shown in Fig. 2A (control) and 2B (after application of AIP1), AIP1 (50 μg/ml) increased the I_K over the range that the current was activated (*I-V* relationship, Fig. 2C), from 15.3±1.2 to 22.2±1.5 pA/pF at +40 mV (Fig. 2D). We also confirmed the involvement of I_{Ca} in shortening of APD. I_{Ca} was elicited by one-step 400-ms depolarizing pulses from a holding potential of −50 mV (to inhibit the Na^+ and T-type Ca^{2+} currents) to 0 mV. The application of AIP1 did not affect I_{Ca} (Fig. 3). From these results, we suggest that AIP1 increased I_K, and did not inhibit I_{Ca}, which was followed by shortening of APD.

AIP1 increased activity of I_{Kr}, rather than I_{Ks} in ventricular cardiomyocytes

As described above, I_K consists of two different components, I_{Kr} and I_{Ks}. To date, many specific inhibitors have been developed to distinguish between I_{Kr} and I_{Ks}. For example, E-4031 and chromanol 293B are known to be specific inhibitors of I_{Kr} and I_{Ks}, respectively. Therefore, to examine the relative selectivity of AIP1 for I_{Kr} and I_{Ks}, we pretreated cells with 10 μM E-4031 to block I_{Kr} or 20 μM chromanol 293B to block I_{Ks} before applying AIP1. Application of E-4031 itself increased the APD_{50} from 379.3±23.0 to 591.7±25.3 ms (Fig. 4A and 4B). Under these conditions, additional application of AIP1 did not reduce the APD (593.3±19.0 ms, Fig. 4A and 4B). Previous studies have suggested that chromanol 293B-induced inhibition of I_{Ks} has only minimal effects on an *in vitro* system, when measuring APD in various species, including rabbit, without β-adrenergic stimulation [16-18]. Consistent with these findings, our recordings also showed that chromanol 293B alone had no effect on APD (Control: 381.6±15.1 ms, chromanol 293B: 380.5±18.3 ms) (Fig. 4C and 4D). However, additional application of AIP1 reduced APD_{50} to a similar level as shown in Fig. 1 (322.1±11.3 ms, Fig. 4C and 4D). These findings suggest that AIP1-induced shortening of APD is attributable to activation of I_{Kr}, rather than I_{Ks}.

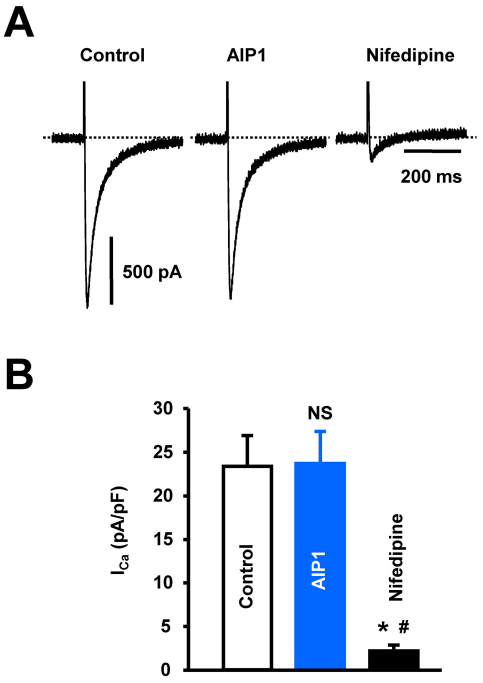

Fig. 3. The effect of AIP1 on I_{Ca} in ventricular cardiomyocyte. (A) I_{Ca} in ventricular cardiomyocytes under control conditions and in the presence of 50 μg/ml AIP1. I_{Ca} was confirmed using the I_{Ca} inhibitor, nifedipine (10 μM). (B) Statistical summary of I_{Ca} at 0 mV. NS, not significant (Control vs AIP1, n=4 cells). *p<0.05 (Control vs nifedipine, n=4 cells). #p<0.05 (AIP1 vs nifedipine, n=4 cells).

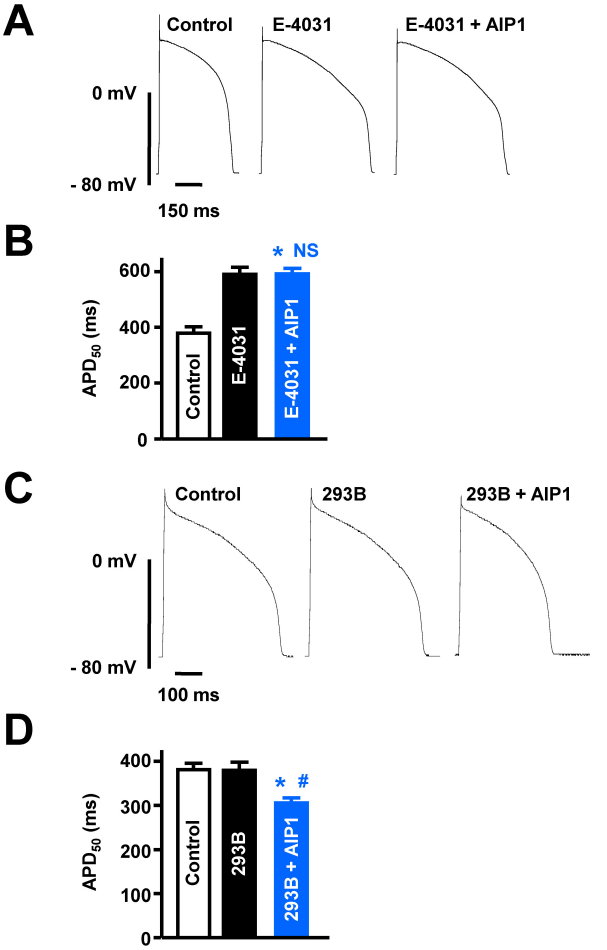

Fig. 4. Effects of AIP1 on I_{Kr} and I_{Ks}. (A) Representative traces of action potentials obtained under control conditions, in the presence of 10 μM E-4031, and with additional application of 50 μg/ml AIP1. (B) Summary of APD_{50} as shown in (A). *p<0.05 (Control vs E-4031 +AIP1, n=6 cells). NS, not significant (E-4031 vs E-4031+AIP1, n=6 cells). (C) The traces of action potentials obtained under control conditions, in the presence of 20 μM chromanol 293B, and with additional application of 50 μg/ml AIP1. (D) Summary of APD_{50} as shown in (C). *p<0.05 (Control vs chromanol 293B+AIP1, n=4 cells). #p<0.05 (chromanol 293B vs chromanol 293B+AIP1, n=4 cells).

Effect of AIP1 on coronary arteries

We also investigated the effect of AIP1 on vascular smooth muscle by measuring the changes in arterial tone and whole-cell current. AIP1 did not significantly affect arterial tone, even at the highest concentration of 500 μg/ml (Fig. 5A). Likewise, AIP1 had no effect on the activity of voltage-dependent K^+ channels in arterial smooth muscle cells, as determined by measuring the whole-cell current using a ramp (Fig. 5B) or step protocol (Fig. 5C). Furthermore, vascular I_{Ca}, which was elicited by one-step 600-ms depolarizing pulse from a holding potential of −60 mV to 0 mV, was not affected by the application of AIP1 (Fig. 5D and E). These results suggested that AIP1 was not involved in the relaxation of coronary arterial smooth muscle by activation of K^+ channels.

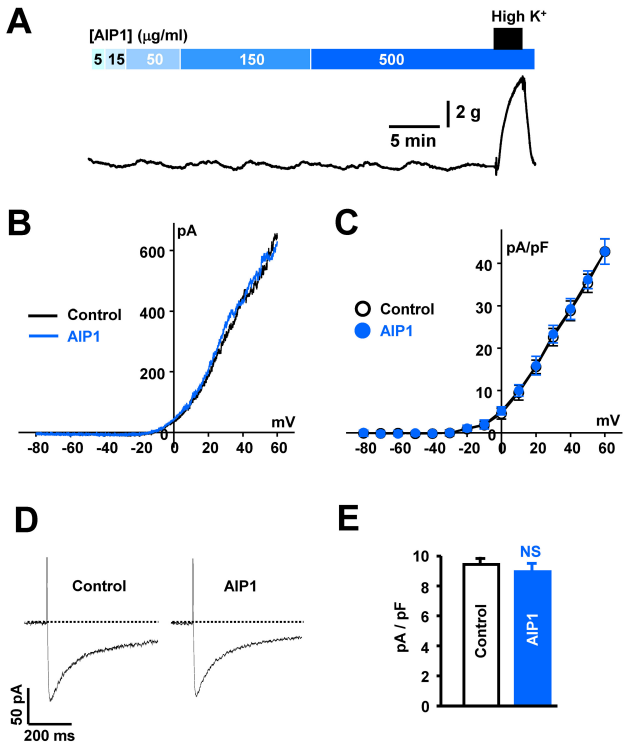

Fig. 5. AIP1 did not affect coronary vascular tone or ion channels. (A) Representative traces showing the effect of various concentrations of AIP1 on vascular tone in coronary arteries. At the end of each experiment, 60 mM high K^+ solution was applied to test the effectiveness of arteries (n=4 tissues). (B) Voltage-dependent K^+ currents recorded using the ramp-pulse protocol (from −80 to +60 mV for 280 ms) under control conditions (black line, n=5 cells) and in the presence of 50 μg/ml AIP1 (blue line, n=5 cells). (C) *I-V* relationship of voltage-dependent K^+ currents obtained by step voltages (from −80 and +60 mV in steps of 10 mV) under control conditions (○, n=5 cells) and in the presence of 50 μg/ml AIP1 (●, n=5 cells). (D) vascular Ca^{2+} currents obtained by one step voltage (−60 mV to 0 mV) under control condition and in the presence of 50 μg/ml AIP1. (E) Statistical summary of I_{Ca} at 0 mV. NS, not significant (Control vs AIP1, n=3 cells).

DISCUSSION

Our results indicate that AIP1 shortened APD by increasing the activity of I_{Kr} in rabbit ventricular myocytes, whereas I_{Ks} was largely unaffected. Furthermore, AIP1 was not involved in the relaxation of coronary arterial smooth muscle by activation of K^+ channels.

Previous studies have examined the biological function of the polysaccharide fraction AIP1 isolated from *A. iwayomogi*. For example, both AIP1 and the ethanol-soluble fraction of the hot-water extract of *A. iwayomogi* lowered serum total-cholesterol levels and inhibited fibrosis and lipid oxidation in fibrotic rats [9]. Treatment of mouse thymocytes with AIP1 resulted in the suppression of cell death and improved cell survival, by suppression of the Fas/FasL-dependent apoptotic pathway [12,13,19]. Furthermore, treatment of mouse spleen cells with the AIP1 fraction suppressed apoptosis via the modulation of apoptosis-related genes [11]. AIP1 has been shown to inhibit mast cell-derived immediate-type allergic reactions by lowering intracellular Ca^{2+}, inhibiting histamine release, and attenuating the activation of p38, a mitogen-activated protein kinase (MAPK) [20]. AIP1 also reduced allergic asthma via the down-regulation of tumor necrosis factor-alpha (TNF-α) expression [21]. AIP1 may also act as a potent anti-oxidant. Active compounds from *A. iwayomogi* are potent scavengers of $ONOO^-$, and inhibitors of inducible nitric oxide synthase [22,23]. Until now, the focus of the pharmacological effects of AIP1 has been on its anti-tumor effects, immunomodulating activity, and liver protective mechanisms.

In this study, for the first time, we demonstrated the effect of AIP1 on cardiac ion channels. Our major findings were that AIP1 reduces APD by activating I_{Kr} in rabbit ventricular cardiomyocytes. I_K consists of two components in cardiomyocytes, I_{Kr} and I_{Ks}, which have been well characterized using the patch-clamp technique, on the basis of their different electrophysiological kinetics, pharmacology and rectification properties [1-4]. Candidate genes that may encode I_{Kr} and I_{Ks} channel proteins have been identified. The human ether-a-go-go-related gene (hERG) encodes the pore-forming α-subunit of cardiac I_{Kr} channel, possibly in connection with the β-subunit KCNE2, whereas KCNQ1 and minK (KCNE1) co-assemble to form the I_{Ks} channel [24-30]. Class III antiarrhythmic drugs, notably E-4031, sotalol, and dofetilide reduce I_{Kr} activity to help prevent arrhythmia. However, excessive blocking of I_{Kr} or mutation of hERG causes long QT syndrome [31], which is linked to an increased incidence of sudden cardiac death. Therefore, if I_{Kr} blockers are used as antiarrhythmic drugs, channel activators would be helpful to reverse the effects of overdose. Furthermore, channel activators could also be used for the solution of inherited long QT syndrome. Our study clearly showed that AIP1 reduced APD by activating I_{Kr}, but not I_{Ks}. This result would provide an adjunctive therapy for treating long QT syndrome with the natural product, AIP1. Moreover, co-treatment with AIP1 may reduce the cardiac toxicity caused by hERG channel inhibition by several drugs.

The lack of effect of AIP1 on vascular smooth muscle may be the result of the different expression of Kv subtypes. I_{Kr}, which consists of hERG (Kv11.1) and auxiliary KCNE2 (MiRP1), has been identified mainly in the brain and heart, and not vascular smooth muscle. Instead, vascular smooth muscle cells express the Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv4.3, and Kv9.3 subunits, as well as the auxiliary

Kvβ1, Kvβ2, and Kvβ3 [32-34]. Therefore, the main target for increasing the activity of I_{Kr} by AIP1 might be restricted to hERG (Kv11.1) and/or auxiliary KCNE2 (MiRP1). Future experiments are planned to investigate the specific effects of AIP1 on hERG channels using cell lines over-expressing the hERG channel. Additionally, we did not address the detailed mechanism underlying the AIP1-induced increase in I_{Kr} activity; therefore further studies into drug-channel interactions or signaling mechanisms are required in the near future.

ACKNOWLEDGEMENTS

This research was supported by a Basic Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (313-2008-2-E00046, R0A-2007-000-20085-0 and R13-2007-023-00000-0).

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