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Effects of Azimilide on the Muscarinic Acetylcholine Receptor-Operated K^+ Current and Experimental Atrial Fibrillation in Guinea-Pig Hearts

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Abstract. Effects of azimilide, a class III antiarrhythmic drug, on the acetylcholine (ACh) receptor-operated K^+ current ($I_{K.ACh}$) and the delayed rectifier K^+ current (I_K) were examined in guinea-pig atrial cells using patch-clamp techniques. Effects of azimilide on experimental atrial fibrillation (AF) were also examined in isolated guinea-pig hearts. In single atrial myocytes, azimilide inhibited both the rapid (I_{Kr}) and slow component of I_K (I_{Ks}). Azimilide inhibited the $I_{K.ACh}$ induced by carbachol (CCh, 1 μM), adenosine (10 μM), and intracellular loading of GTPγS (100 μM) in a concentration-dependent manner. The IC_{50} values of azimilide for inhibiting the CCh-, adenosine-, and GTPγS-induced $I_{K.ACh}$ were 1.25, 29.1, and 20.9 μM, respectively, suggesting that azimilide inhibits $I_{K.ACh}$ mainly by blocking the muscarinic receptors. Azimilide concentration-dependently (0.3 – 10 μM) prolonged the action potential duration (APD) in the absence and presence of muscarinic stimulation. In isolated hearts, perfusion of CCh shortened the duration of the monophasic action potential (MAP) and effective refractory period (ERP) of the left atrium and lowered the atrial fibrillation threshold (AFT). Addition of azimilide inhibited the induction of AF by prolonging the duration of MAP and ERP. The $I_{K.ACh}$ inhibition by azimilide may at least in part contribute to the effectiveness to prevent parasympathetic-type AF.

Keywords: azimilide, muscarinic acetylcholine receptor-operated K^+ current, delayed rectifier K^+ current, atrial fibrillation, action potential

Introduction

Atrial fibrillation (AF) is not such a benign arrhythmia as thought for a long time, but actually is associated with considerable morbidity and mortality (1, 2). At times it is necessary to restore and maintain sinus rhythm in patients with AF. Class III antiarrhythmic drugs such as dofetilide and sotalol have been used as a pharmacotherapy for rhythm control (3). However, it has been reported that class III antiarrhythmic drugs can convert AF to and maintain sinus rhythm only in a part of the patients (4, 5). In addition, class III antiarrhythmic drugs, which selectively inhibit the rapid component of the delayed rectifier K^+ current (I_{Kr}), are known to exert proarrhythmic effects such as induction of torsades de pointes in the ventricle, especially with bradycardia. The arrhythmogenic effects have been ascribed to the reverse use-dependent prolongation of action potential duration (APD) by I_{Kr} blockers (6).

Azimilide is a new class III antiarrhythmic drug that inhibits not only I_{Kr} but also the slow component of the delayed rectifier K^+ current (I_{Ks}), although higher concentrations of azimilide are needed to inhibit I_{Ks} in ventricular cells (7). The class III drug was shown to prolong the ventricular monophasic action potential (MAP) duration without reverse frequency-dependence in anesthetized dogs (8). Recently it has been reported that azimilide is efficacious against AF with an acceptable safety profile in clinical trials (9, 10). We previously reported that several class III antiarrhythmic drugs such as amiodarone and sotalol inhibited the acetylcholine receptor-operated K^+ current ($I_{K.ACh}$) in

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atrial cells, thereby suppressing parasympathetic-type experimental AF in isolated hearts (11, 12). However, effects of azimilide on $I_{K.ACh}$, which plays an important role in the repolarization of atrial action potentials (13), have not been evaluated. This study was undertaken to examine the effect of azimilide on $I_{K.ACh}$ in guinea-pig atrial cells. Effects of azimilide on the experimental AF induced by muscarinic receptor stimulation were also evaluated in Langendorff-perfused guinea-pig hearts.

Materials and Methods

Patch-clamp study

Female Hartley strain guinea pigs weighing 200 – 450 g were used under the regulation of the Animal Research Committee of the Graduate School of Medicine, Chiba University.

Single guinea-pig atrial cells were isolated by an enzymatic dissociation method, as described previously (14). The heart was removed from the guinea pigs anesthetized with pentobarbital sodium and mounted on a modified Langendorff purfusion system to ensure the coronary circulation with a normal HEPES-Tyrode's solution. The perfusion medium was changed to a nominally Ca^{2+}-free Tyrode's solution and then to the solution containing collagenase (200 mg/L; Wako, Osaka). After digestion, the heart was perfused with high K^+, low-Cl^- solution (modified Kraft-Brühe [KB] solution) (15, 16). The atrial tissue was cut into small pieces in the modified KB solution and gently shaken to dissociate cells. The composition of the normal HEPES-Tyrode's solution was was as follows: 143 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5.5 mM glucose, and 5 mM HEPES (pH 7.40). The composition of the KB solution was as follows: 70 mM KOH, 50 mM l-glutamic acid, 40 mM KCl, 20 mM taurine, 20 mM KH_2PO_4, 3 mM $MgCl_2$, 10 mM glucose, 1.0 mM EGTA, and 10 mM HEPES (pH 7.40).

Membrane currents in the whole-cell configuration were recorded by the patch clamp method (17). Single atrial cells were placed in a recording chamber attached to an inverted microscope (IMT-2; Olympus, Tokyo) and superfused with the HEPES-Tyrode's solution at a rate of $3\ ml \cdot min^{-1}$. The temperature of the external solution was kept constant at $36.0 \pm 1.0°C$. Patch pipettes were made from glass capillaries with a diameter of 1.5 mm using a vertical microelectrode puller (PB-7; Narishige, Tokyo). They were filled with an internal solution, and the resistance was 2 – 4 MΩ. The composition of the pipette solution was as follows: 110 mM potassium aspartate, 20 mM KCl, 1.0 mM $MgCl_2$, 5.0 mM potassium ATP, 5.0 mM potassium phosphocreatine, 10 mM EGTA, and 5.0 mM HEPES (pH 7.40). In the experiments for recording of $I_{K.ACh}$, GTP (100 μM) or GTPγS (100 μM) was also added to the pipette solution. The free Ca^{2+} concentration in the pipette solution was adjusted to pCa 8 by adding 1.42 mM $CaCl_2$ according to the calculation by Fabiato and Fabiato (18) with the correction of Tsien and Rink (19). After the GΩ seal between the tip of the electrode and the cell membrane was established, the membrane patch was broken by more negative pressure to make the whole-cell voltage-clamp mode. The electrode was connected to a patch clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo). Recordings were filtered at 1-kHz bandwidth, and series resistance was compensated. Command pulse signals were generated and current signals were recorded by using pCLAMP software (Axon instruments, Inc., Foster City, CA, USA). A liquid junction potential between the internal solution and the bath solution of −8 mV was corrected. The capacitance of the membrane was calculated from the steady-state current in response to a ramp pulse (−5 mV/2.5 ms) from 0 mV.

The delayed rectifier K^+ current (I_K) was elicited by delivering the depolarizing pulses of 300 ms from a holding potential of −40 mV after the inhibition of L-type Ca^{2+} current by nisoldipine (1 μM), and effects of azimilide on I_{Kr} were examined. The amplitude of the deactivating current ($I_{K,tail}$) was measured as the difference between the holding current and the peak current that was recorded upon the clamp back to the holding potential, as described previously (16). In order to examine the effects of azimilide on I_{Kr} and I_{Ks}, effects of azimilide on the I_K were examined in the presence of chromanol 293B (100 μM), a selective I_{Ks} blocker (20), or E-4031 (10 μM), a selective I_{Kr} blocker (21), respectively. Relatively high concentrations of I_{Ks} and I_{Kr} blockers were used in this study for complete blockade of these currents.

The $I_{K.ACh}$ was activated by the extracellular application of carbachol (CCh, 1 μM) or adenosine (10 μM) in the GTP-loaded atrial cells or by the intracellular loading of GTPγS (100 μM), a nonhydrolyzable GTP analogue, in atrial cells held at −40 mV. Effects of several concentrations of azimilide on the $I_{K.ACh}$ were examined. This holding potential was selected because the contribution of I_K to the holding current was expected to be minimal.

The activated $I_{K.ACh}$ gradually decayed despite the continuing presence of CCh, adenosine, or GTPγS, probably because of desensitization (22). When the current decay became a constant, a concentration of azimilide was applied. When the inhibition of $I_{K.ACh}$ by azimilide reached a steady state, the drug was washed

out. Assuming that the current decay was a linear function of time, the percent inhibition of $I_{K.ACh}$ was calculated from the difference between the current level that was extrapolated from the current decay before the application of azimilide and that changed by a concentration of azimilide. Only one concentration of azimilide was examined in each cell.

In some experiments, we examined the effects of azimilide on the current-voltage relationship of $I_{K.ACh}$. The quasi-steady-state membrane current was recorded using a ramp pulse protocol. The membrane potential was changed from –40 to +50 mV and then to –100 mV at a rate of 1.2 mV/ms. The ramp voltage pulse was applied at appropriate timing while the membrane current at –40 mV was continuously monitored. Actually the ramp voltage pulse was delivered before, after the extracellular application of CCh, adenosine, or the intracellular loading of GTPγS and after the addition of azimilide. When the GTPγS-induced $I_{K.ACh}$ was recorded, special care was employed in delivering the first ramp voltage pulse immediately after the rupture of the patch membrane.

Current-clamp experiments were also performed in the whole-cell configuration at 36.0 ± 1.0°C. The external solution and pipette solution were the same as those used to record whole-cell membrane currents. The cells were stimulated by passing 2-ms currents through the pipette at a rate of 0.2 Hz. After stabilization of action potential configuration, effects of azimilide on the action potential in the presence or absence of CCh were examined.

Isolated heart study

The heart was removed from the guinea pigs anesthetized with pentobarbital sodium and perfused via the aorta at a constant pressure (800 mmH_2O) with normal Tyrode's solution. The composition of the normal Tyrode's solution was as follows: 125 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.8 mM NaH_2PO_4, 5.5 mM glucose, and 25 mM $NaHCO_3$. The solution was aerated with a mixture of 95% O_2 and 5% CO_2, and maintained at 36.0 ± 0.5°C.

The right atrium was stimulated at a cycle length of 160 ms with an external bipolar silver electrode. The stimuli were rectangular pulses of 2-ms duration at twice the diastolic threshold, delivered from an electronic stimulator (SEC-2102, Nihon Kohden). The left atrial MAPs were recorded using an additional bipolar suction electrode with a diameter of 2.5 mm, attached to the wall of the left atrium. Electrical signals were amplified by a bioelectric amplifier (AB-620G, Nihon Kohden) and recorded at a paper speed of 10 – 100 mm/s using a chart recorder (8K21; NEC San-ei Instruments, Ltd., Tokyo).

Atrial effective refractory period (ERP) was determined using the standard extrastimulus techniques. After every eighth basic right atrial stimulus (S_1S_1 160 ms), an extrastimulus (S_2) was delivered with a shortening of the coupling interval (S_1S_2) in 5-ms steps until the S_2 produced no atrial activity. ERP was defined as the longest S_1S_2 that failed to elicit atrial activity in response to S_2. Conduction time from the right to the left atrium was measured as the time from the pacing spike to the first upstroke of the left atrial MAP.

Atrial fibrillation threshold (AFT) was determined by rapid atrial electrical stimulation according to the method of Inoue et al. (23). The fibrillation-inducing current consisted of a train of 50 square wave pulses, 2 ms in duration at a frequency of 50 Hz for a time of 1 s. The pulse train was delivered to the right atrium after every eighth basic paced beat. The current was increased in increments of 0.1 mA from an intensity twice the diastolic threshold. AFT was defined as the minimum amount of current required to induce AF that was sustained for at least 30 s. Sustained AF was terminated spontaneously by perfusion with normal Tyrode's solution. The stimulator used in this study was unable to deliver a current greater than 20 mA. If AF could not be induced by the current as high as 20 mA, the AFT was considered as more than 20 mA.

Initial measurements were made during perfusion with Tyrode's solution (control values). The same measurements were then repeated 10 min after changing to a Tyrode's solution containing CCh (1 μM) and 10 min after the perfusion of the normal Tyrode's solution containing CCh (1 μM) and azimilide (3 or 10 μM).

Drugs

The following drugs were used: azimilide (Tanabe Seiyaku Co., Ltd., Osaka), carbachol chloride (Tokyo Kasei, Tokyo), nisoldipine (Bayer, Osaka), and E-4031 {*N*-[4-[[1-[2-(6-methyl-2-pyridiny)ethyl]-4-piperidinyl] carbonyl]phenyl] methanesulphonamide dihydrochloride dihydrate} (Eisai Co., Tokyo), chromanol 293B (Aventis Pharma, Frankfurt, Germany), and atropine sulfate monohydrate (Wako, Osaka). Nisoldipine and chromanol 293B were dissolved in ethanol and dimethyl sulphoxide, respectively. Other drugs were dissolved in distilled water.

Statistics

All values are presented the mean ± S.E.M. Student's *t*-test and analysis of variance (ANOVA) were used for statistical analyses. A *P* value of less than 0.05 was considered significant. The concentration-effects data

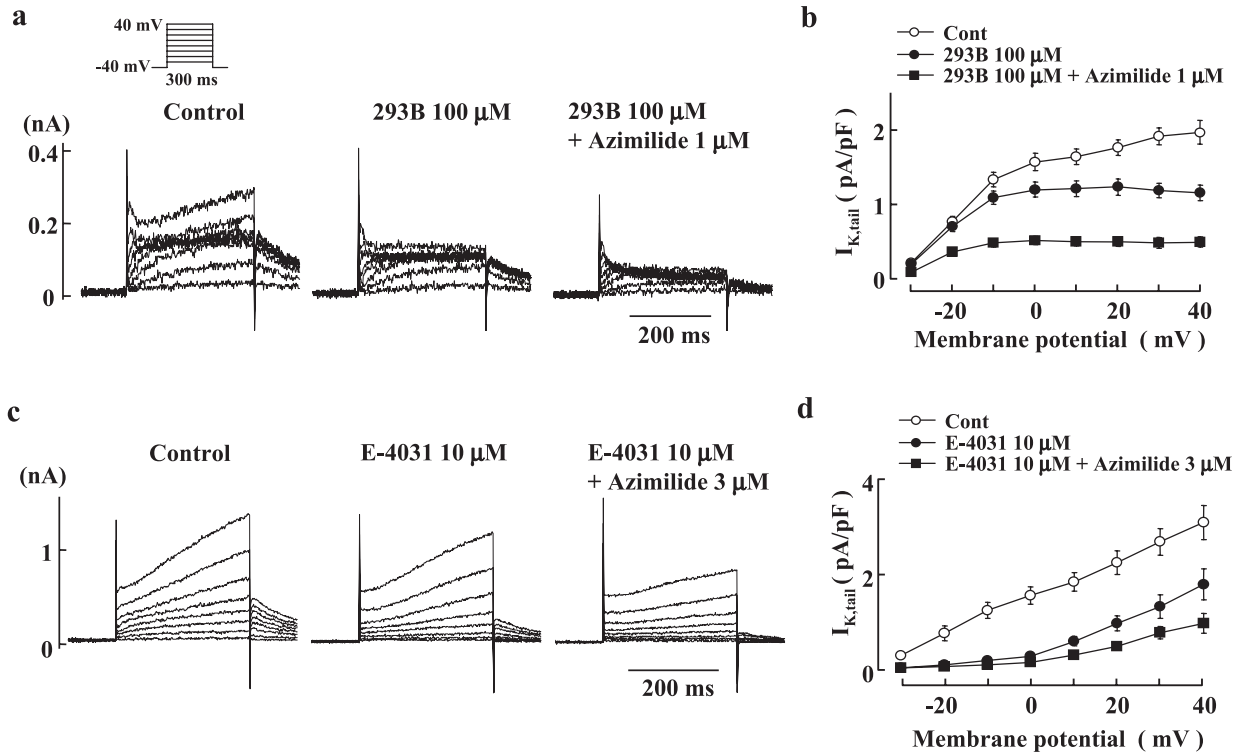

Fig. 1. Effects of azimilide on the delayed rectifier K^+ current (I_K) in guinea-pig atrial cells. Actual current traces elicited by 300-ms depolarizing pulses from a holding potential of –40 mV in the control condition (Cont), in the presence of the I_{Ks} blocker chromanol 293B (293B, 100 μM), or the I_{Kr} blocker E-4031 (10 μM) and after the addition of azimilide (1 or 3 μM) are shown in panels a and c. Pulse protocol is indicated in the upper inset. Summarized data of the I_K measured after clamp back to –40 mV from the indicated potential ($I_{K,tail}$) are shown in panels b and d. Data represent the mean ± S.E.M. of 10 cells (b) or 6 cells (d).

were fitted and the IC_{50} values were obtained using Kaleida graph (Hulinks, Tokyo).

Results

Effects of azimilide on I_{Kr} and I_{Ks} in guinea-pig atrial cells

In our preliminary study we found that in guinea-pig ventricular cells azimilide inhibited I_{Kr} and I_{Ks} with IC_{50} values of 0.7 and 2.1 μM, respectively (24). Accordingly, we selected two concentrations of azimilide (1 and 3 μM) and examined the effects of the drug at each concentration on I_{Kr} and I_{Ks} in guinea-pig atrial cells. After the blockade of the L-type Ca^{2+} current by 1 μM nisoldipine, membrane currents were elicited by 300-ms depolarizing pulses to various potentials from a holding potential of –40 mV at 0.1 Hz. Chromanol 293B (100 μM) and E-4031 (10 μM) were used to block I_{Ks} and I_{Kr}, respectively (Fig. 1). In the presence of chromanol 293B, azimilide at a concentration of 1 μM inhibited I_{Kr} at 0 mV by 57.4 ± 2.9% (n = 10) (Fig. 1b). In the presence of E-4031, azimilide at a concentration of 3 μM inhibited I_{Ks} at +40 mV by 46.0 ± 5.7% (n = 6), as shown in Fig. 1d. Thus, the concentration of azimilide needed to inhibit I_{Kr} by about half was lower than that to inhibit I_{Ks} to a similar extent in guinea-pig atrial cells.

Effects of azimilide on $I_{K.ACh}$ in guinea-pig atrial cells

We examined the effect of azimilide on the CCh-induced $I_{K.ACh}$ in GTP (100 μM)-loaded atrial cells. Upon application of CCh (1 μM) to the bath solution, an outward K^+ current was rapidly activated at a holding potential of –40 mV. After the activation, it gradually declined despite the continuing presence of CCh, perhaps because of desensitization (22) (Fig. 2a). Assuming that the current decay was a linear function of time, the percent of inhibition of $I_{K.ACh}$ was calculated. Azimilide potently depressed the CCh-induced $I_{K.ACh}$ (Fig. 2b) and recovery from the inhibition by azimilide was observed upon washout. The IC_{50} value of azimilide for depressing the CCh-induced $I_{K.ACh}$ was 1.25 μM (Fig. 3).

Although CCh and adenosine act on different membrane receptors, that is, the M_2 muscarinic receptor and A_1 adenosine receptor, adenosine can also induce $I_{K.ACh}$ through the activation of pertussis toxin-sensitive

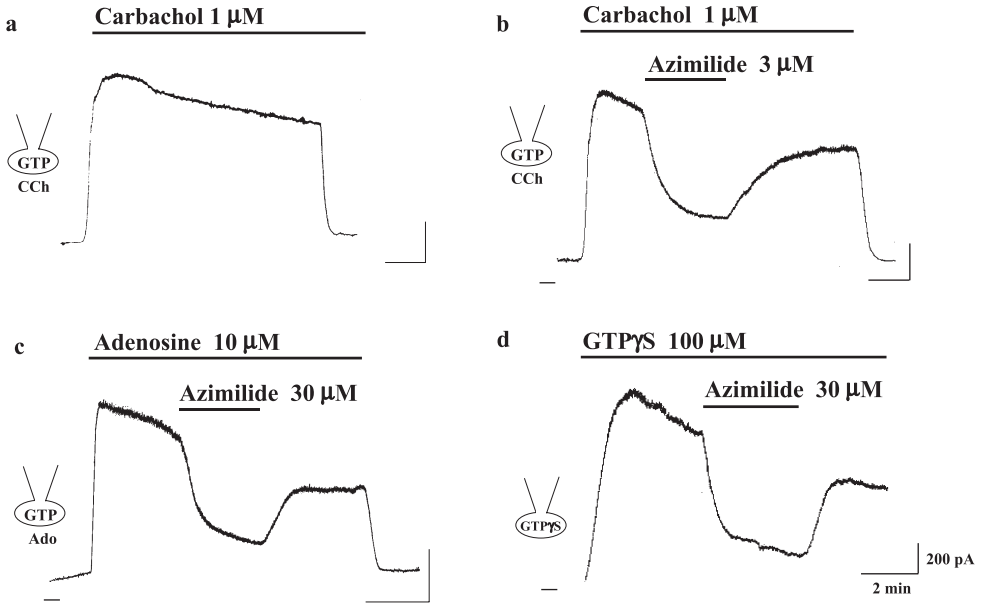

Fig. 2. Effects of azimilide on the acetylcholine receptor-operated K^+ current ($I_{K.ACh}$) in isolated guinea-pig atrial cells. The $I_{K.ACh}$ was activated by the extracellular application of 1 μM carbachol (CCh) (a and b), 10 μM adenosine (Ado) (c), or intracellular loading of 100 μM GTPγS (d). Control current trace after CCh application is shown in panel a and effects of azimilide on the $I_{K.ACh}$ activated by CCh, Ado, and GTPγS are shown in panels b – d. The holding potential was –40 mV. Intracellular loading of GTPγS, extracellular application of carbachol, adenosine, and azimilide are shown by the lines above each actual current trace.

GTP binding protein in atrial cells (25). We evaluated the effect of azimilide on the $I_{K.ACh}$ induced by extracellular application of adenosine (10 μM). Azimilide also inhibited the adenosine-induced current although higher concentrations of azimilide were needed to inhibit the adenosine-induced current compared to that to inhibit the CCh-induced $I_{K.ACh}$ (Fig. 2c and Fig. 3). Intracellular loading of GTPγS (100 μM) gradually activated an outward current at a holding potential of –40 mV, which persisted even in the absence of any agonists. The GTPγS-induced K^+ current was also inhibited by higher concentrations of azimilide (Fig. 2d and Fig. 3). Azimilide at a concentration of 10 μM inhibited the GTPγS-induced $I_{K.ACh}$ by 18.7 ± 5.8% even in the presence of 1 μM atropine (n = 4). The inhibition of the GTPγS-induced $I_{K.ACh}$ was comparable to that by the same concentration of azimilide in the absence of atropine (17.4 ± 3.3%, n = 5). The IC_{50} values of azimilide for inhibiting the adenosine- and GTPγS-induced $I_{K.ACh}$ were 29.1 and 20.9 μM, respectively (Fig. 3). These findings suggest that azimilide may produce direct inhibition of muscarinic K^+ channel itself and/or GTP-binding protein in its higher concentrations.

In order to examine the effects of azimilide on the current-voltage relationship of $I_{K.ACh}$, the quasi-steady-state current was recorded before, after the activation of $I_{K.ACh}$, and after the addition of azimilide. The inhibitory effects of azimilide on the outward component of $I_{K.ACh}$ activated by extracellular application of CCh, adenosine, or intracellular loading of GTPγS could be observed over a wide voltage range.

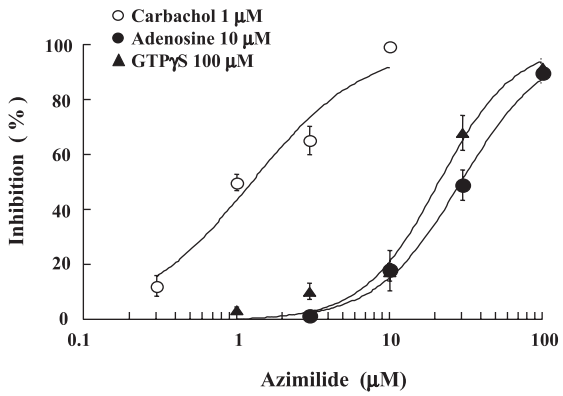

Fig. 3. Concentration-response curves for the inhibitory effects of azimilide on the $I_{K.ACh}$ activated by carbachol (1 μM), adenosine (10 μM), and GTPγS (100 μM) are shown. The IC_{50} values of azimilide for inhibiting the carbachol-, adenosine-, and GTPγS-induced $I_{K.ACh}$ were 1.25, 29.1, and 20.9 μM, respectively. The Hill coefficients for the azimilid inhibition of the carbachol-, adenosine-, and GTPγS-induced $I_{K.ACh}$ were 0.977, 0.992, and 0.998, respectively. Each point represents the mean ± S.E.M. of 5 – 6 cells.

Effects of azimilide on atrial action potentials

Effects of azimilide on atrial action potentials in the absence and presence of muscarinic receptor stimulation were examined in the current clamp mode. The baseline characteristics of action potentials recorded from single atrial myocytes stimulated at 0.2 Hz were as follows: APD at 0 mV level ($APD_{0\,mV}$), 49.2 ± 4.7 ms; APD at –60 mV level ($APD_{-60\,mV}$), 86.0 ± 4.9 ms; action potential amplitude (APA), 123.1 ± 2.9 mV; resting membrane potential (RMP), –82.5 ± 0.9 mV (n = 6). Neither APA nor RMP was significantly affected by azimilide at

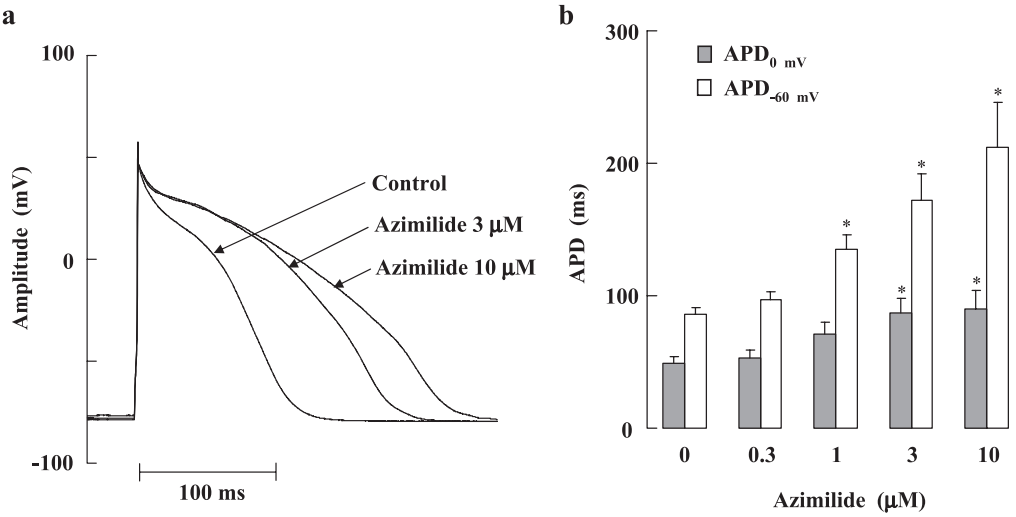

Fig. 4. Effects of azimilide on the atrial action potentials recorded in the current clamp mode. Superimposed actual action potentials obtained before and after exposure to various concentrations of azimilide are shown in panel a. Summarized data of action potential duration (APD) at 0 (hatched columns) and −60 mV (open columns) before and after application of azimilide are shown in panel b. Each column represents the mean ± S.E.M. of 6 cells. **P*<0.05 *vs* control.

these concentrations. However, $APD_{-60\,mV}$ of atrial cells was prolonged by azimilide in a concentration-dependent manner. Azimilide at concentrations of 1, 3, and 10 μM prolonged $APD_{-60\,mV}$ by 58.2 ± 13.8%, 100.3 ± 22.0%, and 148.0 ± 43.3%, respectively. $APD_{-60\,mV}$ was significantly prolonged by azimilide in concentrations higher than 1 μM (Fig. 4). Azimilide in concentrations higher than 10 μM produced repolarization failure (data not shown).

We also investigated the effect of azimilide on the CCh-induced APD shortening in guinea-pig atrial cells. The baseline characteristics of action potentials recorded from single atrial myocytes stimulated at 0.2 Hz were as follows: $APD_{0\,mV}$, 62.9 ± 7.9 ms; $APD_{-60\,mV}$, 99.7 ± 9.4 ms; APA, 130.2 ± 4.6 mV; RMP, −77.9 ± 1.6 mV (n = 7). CCh at a concentration of 1 μM markedly shortened $APD_{0\,mV}$ from 62.9 ± 7.9 to 5.4 ± 1.1 ms (P<0.05) and $APD_{-60\,mV}$ from 99.7 ± 9.4 to 10.8 ± 2.2 ms (P<0.05), respectively, with a slight but significant increase in RMP (from −77.9 ± 1.6 to −82.2 ± 0.8 mV) and an insignificant decrease in APA (from 130.2 ± 4.6 to 111.6 ± 11.9 mV) (n = 7). Azimilide reversed the CCh-induced APD shortening in a concentration-dependent manner, as shown in Fig. 5. The CCh-induced shortenings of $APD_{0\,mV}$ and $APD_{-60\,mV}$ were reversed to 94.8 ± 18.6% and 121.7 ± 20.5% of the control after 10 μM azimilide, respectively (n = 7).

Since azimilide per se can prolong APD by inhibiting I_{Kr} and I_{Ks} in the absence of muscarinic stimulation, it may be difficult to assess the contribution of $I_{K.ACh}$ inhibition to the reversal of CCh-induced APD shortenings. Therefore, we examined the effect of azimilide on the CCh-induced APD shortenings after the full blockade of I_{Kr} and I_{Ks} by 10 μM E-4031 and 100 μM chromanol 293B (Fig. 6). The baseline characteristics of action potentials recorded from single atrial cells stimulated at 0.2 Hz were as follows: $APD_{0\,mV}$, 73.1 ± 10.0 ms; $APD_{-60\,mV}$, 128.4 ± 10.1 ms; APA, 127.6 ± 5.8 mV; RMP, −77.5 ± 2.9 mV (n = 5). CCh at a concentration of 1 μM markedly shortened $APD_{0\,mV}$ and $APD_{-60\,mV}$ to 6.7 ± 2.0 (P<0.05) and 14.8 ± 3.9 ms (P<0.05), respectively, and significantly decreased APA to 104.4 ± 10.4 mV (P<0.05), although the increase of RMP to −82.0 ± 1.9 mV was insignificant. Coapplication of E-4031 and chromanol 293B significantly prolonged $APD_{0\,mV}$ and $APD_{-60\,mV}$ from 10.2 ± 3.0% to 34.0 ± 7.3% and 11.5 ± 3.2% to 35.1 ± 10.6% of the control in CCh-treated atrial cells, respectively. Addition of 10 μM azimilide further prolonged $APD_{0\,mV}$ and $APD_{-60\,mV}$ to 74.6 ± 7.7% (P<0.05) and 107.3 ± 8.7% (P<0.05) of the control, respectively. Thus, azimilide almost completely reversed the CCh-induced APD shortenings in the presence of E-4031 and chromanol 293B, indicating the significant contribution of azimilide-induced $I_{K.ACh}$ inhibition to APD prolongation in the presence of muscarinic stimulation.

Effects of azimilide on experimental AF in isolated guinea-pig hearts

In the control condition, AF could not be induced by a train of stimuli at an intensity up to 20 mA in Langendorff-perfused guinea-pig hearts. After the appli-

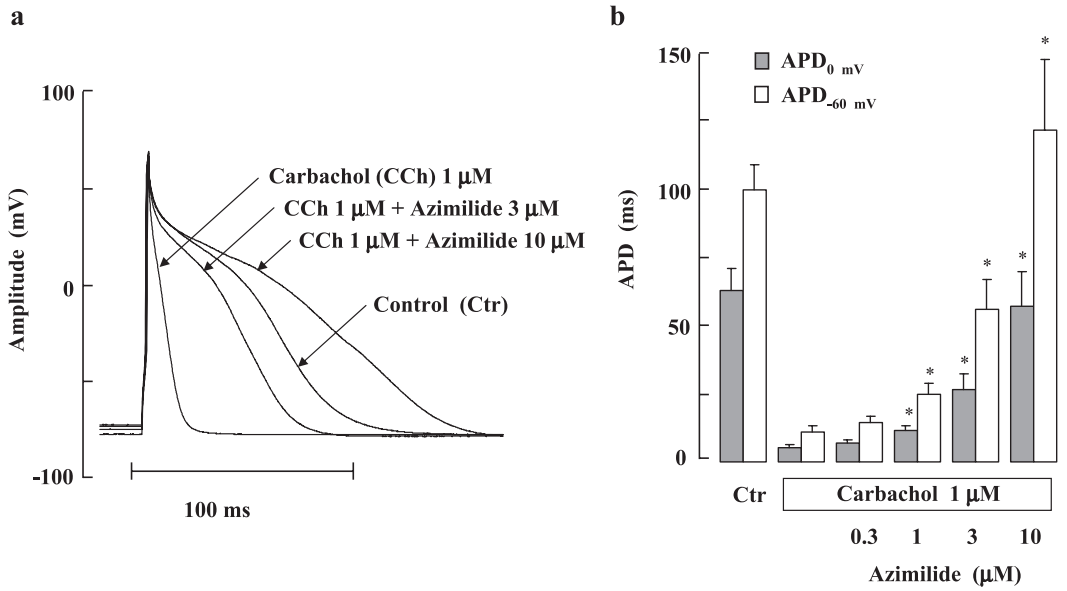

Fig. 5. Effects of azimilide on the atrial action potentials in the presence of 1 μM carbachol. Superimposed actual action potentials before (Ctr) and after exposure to carbachol (CCh) in combination with various concentrations of azimilide are shown in panel a. Summarized data of action potential duration (APD) at 0 (hatched columns) and −60 mV (open columns) before (Ctr) and after application of cabachol alone and carbachol plus azimilide are shown in panel b. Each column represents the mean ± S.E.M. of 7 cells. **P*<0.05 *vs* carbachol alone.

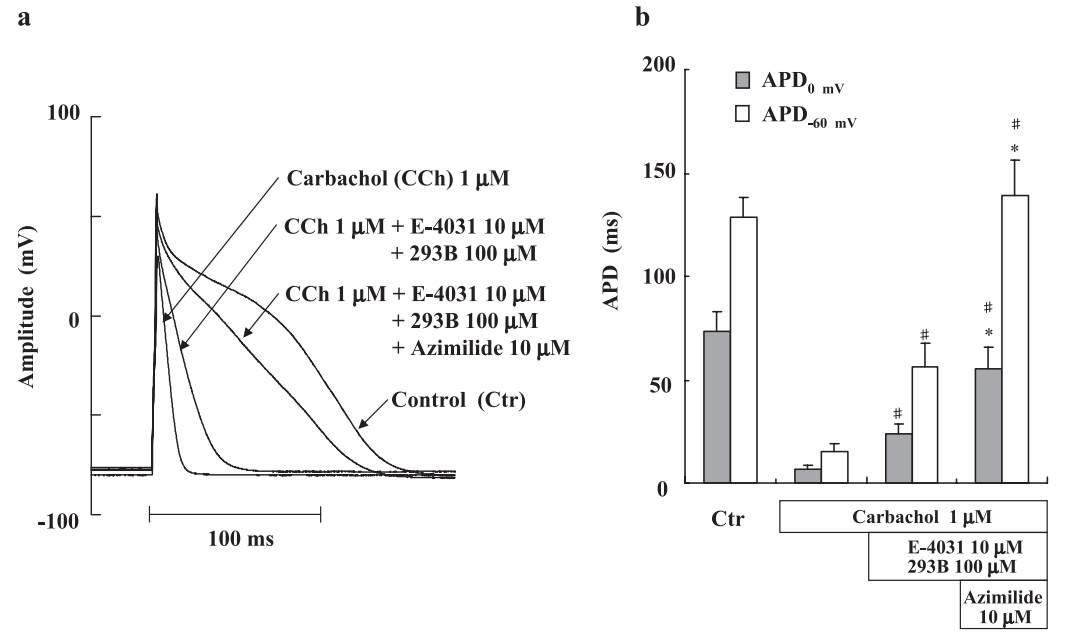

Fig. 6. Effects of azimilide on the atrial action potentials in the presence of 1 μM carbachol, 10 μM E-4031, and 100 μM chromanol 293B. Superimposed actual action potentials before (Ctr), after application of carbachol (CCh), additional full blockade of I_{Kr} (by 10 μM E-4031) and I_{Ks} (by 100 μM chromanol 293B), and addition of azimilide (10 μM) are shown in panel a. Summarized data of action potential duration (APD) at 0 (hatched columns) and −60 mV (open columns) before and after various drugs are shown in panel b. Each column represents the mean ± S.E.M. of 5 cells. $^{\#}P$<0.05 *vs* carbachol alone, **P*<0.05 *vs* carbachol + E-4031 + chromanol 293B.

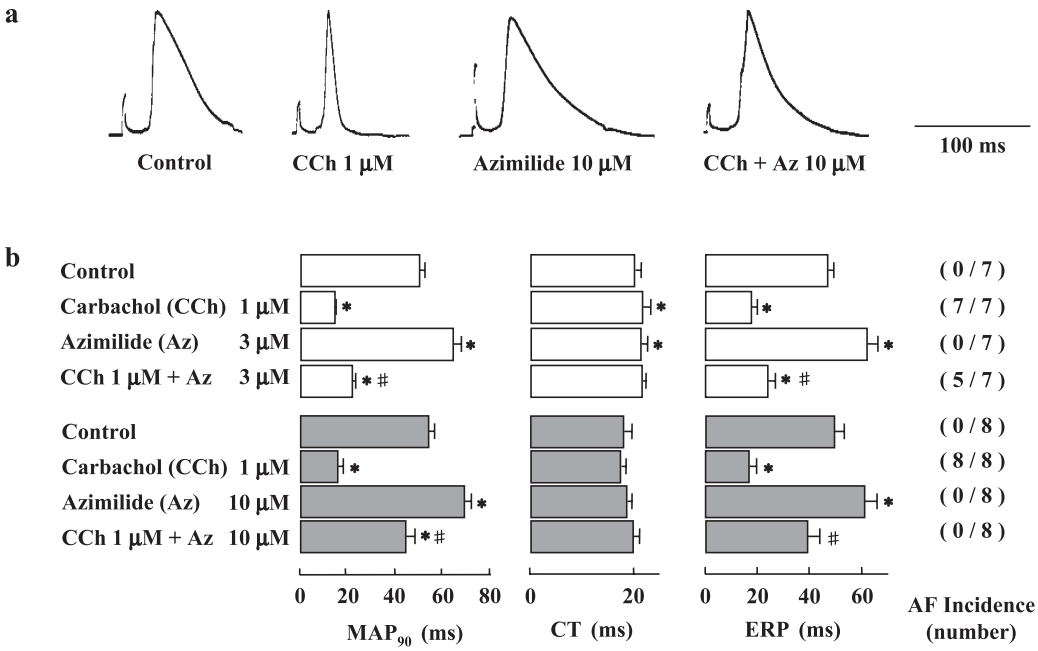

Fig. 7. Effects of azimilide on the carbachol-induced changes in monophasic action potential at 90% repolarization (MAP_{90}), conduction time (CT), effective refractory period (ERP), and incidence of atrial fibrillation (AF) in isolated guinea-pig hearts. Actual records of MAP before (control) and after carbachol (CCh) alone, azimilide (AZ) alone, and combination of these are shown in panel a. Summarized data of change of MAP, CT, ERP, and AF incidence are shown in panel b. Each column represents the mean ± S.E.M. of 7 or 8 hearts of 3 μM (open columns) and 10 μM azimilide groups (hatched columns). AF incidence represents the number of hearts showing AF occurrence in each condition. *P<0.05 *vs* control, $^{\#}P$<0.05 *vs* carbachol alone.

cation of 1 μM CCh, monophasic action potential at 90% repolarization level (MAP_{90}) was significantly decreased from 52.6 ± 1.6 to 15.5 ± 1.0 ms (to 29.6 ± 1.8% of the control) (n = 15) (Fig. 7). Concomitantly, ERP was decreased from 48.3 ± 2.2 to 17.3 ± 1.8 ms (to 36.4 ± 3.6% of the control) (P<0.05). AFT was changed to 5.3 ± 1.3 mA in the presence of 1 μM CCh. In the absence of CCh, azimilide in concentrations of 3 and 10 μM significantly increased MAP_{90} and ERP (Fig. 7). Azimilide at a concentration of 3 μM slightly but significantly reversed the CCh-induced shortenings of MAP_{90} and ERP to 44.0 ± 2.7% and 52.9 ± 6.8% of the control, respectively (n = 7). However, azimilide at a concentration of 3 μM could prevent the induction of AF in only 2 of 7 hearts and failed to prevent AF in the other 5 hearts. Azimilide at a concentration of 10 μM reversed the CCh-induced shortenings of MAP_{90} and ERP to 82.4 ± 6.3% (P<0.05) and 81.7 ± 9.5% of the control (P<0.05), respectively (n = 8). Azimilide failed to increase the conduction time measured from the right to the left atrium. In spite of 100% incidence of AF in the absence of azimilide, AF could not be induced any longer even in the presence of CCh after the treatment with 10 μM azimilide. Thus, azimilide suppressed the electrically-induced AF under muscarinic stimulation in isolated guinea-pig hearts.

Discussion

It is well-known that $I_{K.ACh}$ plays an important role in the repolarization of atrial action potential. Many class I antiarrhythmic drugs such as quinidine, disopyramide, flecainide, propafenone, cibenzoline, pirmenol, and aprindine were reported to inhibit $I_{K.ACh}$ in isolated guinea-pig atrial cells (16, 26 – 29). In terms of effects of class III antiarrhythmic drugs on $I_{K.ACh}$, we previously reported that sotalol, nifekalant, E-4031, and amiodarone inhibit $I_{K.ACh}$ (11, 12). Two mechanisms by which antiarrhythmic drugs inhibit $I_{K.ACh}$ have been proposed: some drugs block the muscarinic receptors and others inhibit the muscarinic K^+ channel itself and/or G proteins. Sotalol belongs to the former group, whereas amiodarone belongs to the latter group (11, 12). In this study, azimilide inhibited not only the current induced by CCh but also that induced by GTPγS or adenosine, although higher concentrations of the drug were needed to inhibit the GTPγS- or adenosine-induced current. Adenosine is known to induce $I_{K.ACh}$ through the activation of pertussis toxin-sensitive G proteins coupled with adenosine A_1 receptor in atrial cells (25). Since azimilide inhibited the adenosine- and GTPγS-induced $I_{K.ACh}$ with a similar potency (Fig. 3), the drug might inhibit the muscarinic K^+ channel itself and/or

G proteins in its high concentrations. These findings suggest that azimilide may interact with the muscarinic M_2 receptor dominantly and can inhibit the K^+ current by depressing the function of the muscarinic K^+ channel itself and/or G proteins in its high concentrations. It has been demonstrated by a radioligand binding study that nifekalant and *d,l*-sotalol can interact with cardiac M_2 and peripheral M_3 receptors (30), thereby inhibiting $I_{K.ACh}$ in atrial cells (11). In this context, it has been recently indicated that azimilide interacts with various receptors including muscarinic M_2 receptors (31).

Azimilide prolonged APD even in the absence of the muscarinic receptor agonist in this study. The APD prolongation may be ascribed mainly to inhibition of I_{Kr} and partly to that of I_{Ks} by azimilide. Indeed, azimilide inhibited both I_{Kr} and I_{Ks} although the inhibitory effect on I_{Kr} was more potent than that on I_{Ks} in guinea-pig atrial cells. Since azimilide inhibited the muscainic K^+ channel itself and/or G protein in its higher concentrations, the inhibitory effect on constitutively active $I_{K.ACh}$ might be also involved in the APD prolongation in the absence of muscarinic receptor agonist. Azimilide at a high concentration (10 μM) was also reported to inhibit the L-type Ca^{2+} current in guinea-pig ventricular cells (7). Therefore, azimilide might affect various ion channels in the heart.

Azimilide effectively antagonized the CCh-induced APD shortening in concentrations comparable to those to inhibit I_{Kr} and I_{Ks}. Since azimilide effectively reversed the CCh-induced APD shortening even after full blockade of I_{Kr} and I_{Ks}, the inhibitory effect on $I_{K.ACh}$ seemed to play an important role in APD prolongation in the presence of muscarinic stimulation. As already discussed, the inhibitory effect of azimilide on $I_{K.ACh}$ would be mainly attributable to muscarinic M_2 receptor blockade. However, azimilide at its higher concentrations inhibited $I_{K.ACh}$ through the direct effect on the muscainic K^+ channel itself and/or G protein. Molecular cloning has revealed the presence of many types of K^+ channel pore forming subunits in the heart (32). Voltage-gated K^+ channels such as ERG1 and KvLQT1, which underlie I_{Kr} and I_{Ks}, are proteins with six transmembrane domains that assemble as tetramers to form pores. In contrast, the $I_{K.ACh}$ is considered to arise from heteromeric assembly of Kir3.1 and Kir3.4. These inward rectifiers are generated by Kir pore subunit genes encoding proteins with two transmembrane domains. It is interesting to note that azimilide inhibits structurally distinct K^+ channels in the heart although there are some differences in the effective concentrations.

In isolated guinea-pig hearts, AF could be easily induced by a rapid atrial stimulation in the presence of cholinergic agonist, although it could not be induced under control conditions. As the mechanism involved in the establishment of AF, the wavelet hypothesis has been proposed from mapping studies in experimental AF (33). It is considered that several wavelets and the shortened wavelength are required for perpetuation of AF. The wavelength is designated as the product of the refractory period and conduction velocity. The shortening of ERP resulting from the activation of $I_{K.ACh}$ seemed to underlie the shortening of atrial wavelength and the induction of AF in this study. Addition of azimilide in concentrations of 3 and 10 μM reverted the MAP duration and ERP toward the control without affecting conduction time, and the increase in ERP paralleled that in MAP duration. Therefore, the inhibitory effect of azimilide on the experimental AF might be mainly due to the increase in ERP resulting from the inhibition of $I_{K.ACh}$.

Azimilide at a concentration of 3 μM slightly but significantly reversed the CCh (1 μM)-induced shortenings of MAP and ERP, as shown in Fig. 7. This concentration of azimilide produced incomplete prevention of experimental AF. Complete prevention of AF by 10 μM azimilide was associated with moderate prolongation of MAP and ERP. Therefore, prolongation of ERP to some critical point appears to be prerequisite for prevention of the burst stimulation-induced AF in CCh (1 μM)-treated hearts. There was some difference in the potency of the antagonizing effect of azimilide against the CCh-induced shortenings of APD between single cells and Langendorff-perfused hearts. Azimilide reversed the CCh (1 μM)-induced shortenings of APD more effectively in single cells than in isolated hearts when the same concentration was compared (Figs. 5 and 7). One possible explanation may be that azimilide might have access to the cell membrane more easily in isolated cardiomyocytes than in isolated hearts. Another explanation may be that a difference of stimulation frequency between these studies might affect the efficacy of azimilide. Another possibility may be that rapid stimulation might release intrinsic ACh from parasympathetic nerve terminals, producing an additive effect to CCh and an antagonizing effect against azimilide. Further studies may be needed to clarify the precise mechanism of the different effectiveness of azimilide in these two types of experiments.

Brooks et al. (31) reported that clinical efficacy of azimilide occurs at plasma concentrations of 1 – 5 μM. It was also reported that azimilide doses of 100 and 125 mg in once daily regimens were effective for controlling AF (9, 10). A pharmacokinetic study (34) indicated that a single, oral dose of 100 mg azimilide produced a plasma peak level corresponding to about 0.2 μM. Therefore, effective concentrations of azimilide

in the treatment of AF would be around 1 μM, at least less than 3 μM. The concentrations of azimilide examined in this study might be higher than those encountered in clinical settings. However, the concentration of the muscarinic agonist CCh used to evoke $I_{K.ACh}$ in the present study was very high. Lower concentrations of azimilide may effectively inhibit $I_{K.ACh}$ when the current is induced by a small amount of ACh released from parasympathetic nerve terminals of intact human hearts.

Recent studies indicated that in atrial cells of the patients with chronic AF, the density of carbachol-induced $I_{K.ACh}$ was smaller than that in atrial cells of the patients with sinus rhythm (35), although the $I_{K.ACh}$ was constitutively active without muscarinic receptor stimulation in atrial cells with chronic AF (36). Therefore, effectiveness of azimilide may be not so marked in patients with chronic AF. In addition, a multicenter clinical study has demonstrated that management of AF with rhythm control strategy using antiarrhythmic drugs offers no survival advantage over a rate-control strategy using digoxin, β-adrenoceptor antagonist, and/or Ca^{2+} channel blockers (37). However, rhythm control may be needed to improve quality of life by improving hemodynamics, enhancing exercise capacity, and relieving symptoms. In addition, the rhythm control with antiarrhythmic drugs is recommended for the AF patients with heart failure or coronary artery disease (3). Azimilide was reported to inhibit the recurrences of AF in a variety of patients including those with ischemic heart disease and/or congestive heart failure (9, 10). The inhibitory effect of azimilide on $I_{K.ACh}$, observed in this study, may be not so potent in clinical settings, but it may in part contribute to the efficacy against the recurrences of AF especially when parasympathetic tone prevails. When class III antiarrhythmic drugs are used for the treatment of AF, careful monitoring of QT interval in the electrocardiogram may be required. However, it was reported that the rate of torsades de pointes at the effective dose of azimilide was 0.9% (10), which was comparable to or less than those reported with dofetilide (4, 38).

In conclusion, the $I_{K.ACh}$ inhibition by azimilide may at least in part contribute to the effectiveness to prevent parasympathetic-type AF.

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