Comparative effects of azimilide and ambasilide on the human ether-a-go-go-related gene (HERG) potassium channel

Bruce D. Walker, Cameron B. Singleton, Hui Tie, Jane A. Bursill, Ken R. Wyse, Stella M. Valenzuela, Samuel N. Breit, Terence J. Campbell

Abstract

Objective: To evaluate the effects of azimilide and ambasilide on the biophysical properties of the human-ether-a-go-go-related (HERG) channel. Methods: HERG was stably transfected into Chinese hamster ovary (CHO-K1) cells and currents were measured using a whole cell, voltage-clamp technique. Results: Azimilide had a ‘dual effect’, inhibiting current at voltage steps above −40 mV and augmenting current at −40 and −50 mV. Tail current inhibition following a step to +30 mV did not vary with temperature (IC50 610 nM at 22°C and 560 nM at 37°C). The agonist effect at −50 mV was concentration-dependent and correlated with a hyperpolarizing shift in the V1/2 of activation (r=0.98, P<0.05). Time constants of inactivation were faster and there was a −10 mV shift in the V1/2 of steady state inactivation suggestive of open and inactivated state binding. By comparison, ambasilide inhibited HERG channels with lower potency (IC50 3.6 μM), in a voltage- and time-dependent but frequency-independent manner (0.03–1 Hz). Ambasilide had no effect on activation or inactivation gating but prolonged both fast and slow components of deactivation consistent with unbinding from the open state. The net effect of both drugs was similar during a voltage ramp which simulated a cardiac action potential. Conclusions: Inhibition of HERG channels by azimilide and ambasilide exhibits a similar time and voltage-dependence. While both exhibit affinity for the open state, azimilide also binds to inactivated channels. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Antiarrhythmic agents; Ion channels; K-channel; Long QT syndrome; Membrane currents; Repolarization

1. Introduction

Class III antiarrhythmic drugs prolong repolarization principally via inhibition of the rapidly activating component of the delayed rectifier channel (Ikr). The reverse frequency-dependent prolongation of action potential duration (APD) and effective refractory period (ERP), exhibited by a number of these agents may reduce efficacy at fast heart rates as well as predispose to QT prolongation and torsades de pointes at slow rates. There is no doubt that proarrhythmia is a major clinical concern with class III agents (as highlighted by the SWORD study [1]) and it has been postulated that multichannel blocking drugs (e.g. amiodarone, azimilide and ambasilide) may confer greater efficacy and safety [2,3].

Azimilide is an antiarrhythmic drug with predominant class III activity, which is chemically unrelated to the widely-studied methanesulphonanilide Ikr-blocking drugs. It is an equipotent inhibitor of Ikr and If, and has significant activity against other cardiac ion channels (Table 1). In animal models, azimilide has well-established efficacy in the treatment of canine ischaemic and post-myocardial infarction ventricular tachycardia and fibrillation [4–6] as well as atrial flutter and vagally-induced atrial fibrillation [7,8]. Azimilide is now being evaluated in several large clinical trials for the treatment of supraventricular arrhythmias, as well as prevention of sudden cardiac death in post-MI patients with impaired left ventricular function [2,9,10].

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Table 1
Inhibition of cardiac ion channels by azimilide and ambasilide

<table>
<thead>
<tr>
<th>Ion channel</th>
<th>Azimilide</th>
<th>Ambasilide</th>
</tr>
</thead>
<tbody>
<tr>
<td>I_{Kr}</td>
<td>0.2–1 [24,32–34,48]</td>
<td>2–5.6 [12,14]</td>
</tr>
<tr>
<td>I_{Kr}</td>
<td>0.7–3 [33,37,48,49]</td>
<td>32 [12]</td>
</tr>
<tr>
<td>I_{Kr}/minK</td>
<td>1.9–5.4 [49,50]</td>
<td>–</td>
</tr>
<tr>
<td>HERG</td>
<td>5.2 [24]</td>
<td>–</td>
</tr>
<tr>
<td>I_{Ks}</td>
<td>&gt;10 [33]</td>
<td>6.0 [11]</td>
</tr>
<tr>
<td>I_{K,Na,K}</td>
<td>–</td>
<td>1.6–2.3 [11,14]</td>
</tr>
<tr>
<td>I_{Ks}</td>
<td>–</td>
<td>34 [51]</td>
</tr>
<tr>
<td>I_{K1}</td>
<td>–</td>
<td>44–46 [11,52]</td>
</tr>
<tr>
<td>I_{K1}</td>
<td>75 [32]</td>
<td>5.7–23 [11,51,52]</td>
</tr>
<tr>
<td>I_{K1}</td>
<td>10–17.8 [33,48]</td>
<td>–</td>
</tr>
<tr>
<td>I_{K1}</td>
<td>19 [48]</td>
<td>–</td>
</tr>
</tbody>
</table>

* Numbers represent IC_{50} concentrations in μM.

Ambasilide is an aminobenzoic acid derivative with a chemical structure similar to E-4031, which also lacks a methanesulphonamide group [11,12]. It prolongs APD and ERP in the canine, rabbit and human atrium [11,13,14] as well as in the canine, guinea pig and human ventricle [15–18]. These effects are independent of stimulation frequency and extracellular [K\(^+\)] [11,13,15–17]. The cellular mechanism for this activity is block of I_{Kr} and I_{Ksr}, as well as less potent inhibition of other cardiac K\(^+\) channels (Table 1).

Human I_{Kr} is encoded by the human ether-a-go-go-related gene (HERG) [19] and mutations of this gene may cause the congenital long QT syndrome [20]. In this study, we compared the effects of these newer class III antiarrhythmic agents on the HERG channel. Initial experiments focused on the effects of azimilide at room and physiological temperature, with particular attention to the kinetics of channel binding, modulation of channel gating and of the inactivated state. Since the effects of ambasilide on human I_{Kr} have not previously been described, we next examined the frequency and state-dependence of HERG channel inhibition by ambasilide.

2. Methods

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). A detailed summary of experimental methods has previously been published [21].

2.1. Molecular biology

Eukaryotic expression of HERG (sequence as per [19]) was performed by directionally cloning the coding region of the HERG gene (gift from Dr. G. Robertson, Department of Physiology, University of Wisconsin Medical School, Madison, WI, USA) into the expression vector pRc/CMV (Invitrogen, San Diego, CA, USA), which also carries the G418 resistance gene. This construct was then transfected into Chinese hamster ovary (CHO-K1) cells using Lipofectamine Reagent (Gibco). Stably transfected cells were then selected with 1000 μg/ml G418 (Boehringer, Mannheim) and subcloned. Individual subclones were maintained long-term in tissue culture and used for the patch clamping experiments to be described below.

2.2. Electrophysiology

Currents were recorded from CHO-K1 cells at room temperature (22°C) and at 37°C, using a standard whole-cell patch-clamp technique. CHO-K1 cells plated on coverslips were placed at the bottom of a 0.5-ml perfusion chamber mounted on the stage of an inverted phase contrast microscope (Nikon Diaphot, Tokyo, Japan). Cells were patched using micropipettes fabricated from thin-walled borosilicate glass (Vitrex Microhematocrit Tubes, Modulohm II/S, Denmark) with a vertical pipette puller (Model 720, David Kopf Instruments, California, USA). Currents were amplified and filtered at 2 kHz with a 4-pole Bessel filter (–3dB point) using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA). Stimulation protocols and data acquisition were carried out using a microcomputer (IBM pentium), running commercial software and hardware (pCLAMP 6.0/DIGIDATA 1200, Axon Instruments and Scientific Solutions). Whole-cell capacitance was determined from capacitative transient decay in current recordings following voltage steps of ±10 mV from the holding potential. At least 80% series resistance compensation was achieved in all reported experiments.

2.3. Solutions and drugs

The intracellular pipette solution contained (mM): K gluconate 120, KCl 20, MgATP 1.5, EGTA 5, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) 10, adjusted to pH 7.3. The superfusion solution contained (mM): NaCl 130, KCl 4.8, MgCl₂ 1.2, NaH₂PO₄ 1.2, HEPES 10, glucose 12.5, CaCl₂ 1.0, adjusted to a pH of 7.4. Ambasilide was obtained from Procter and Gamble (Cincinnati, OH, USA) and prepared as stock solution using the above superfusion solution. Ambasilide was kindly provided by Knoll (Ludwigshafen, Germany) and prepared as stock solution in dimethylsulfoxide (DMSO), then subsequently diluted as required with superfusate (maximum final DMSO concentration of 0.1%, v/v). In preliminary experiments, we determined that 0.1% DMSO (v/v) had no effect on the parameters under study.

2.4. Statistics and analysis

Current analysis was performed using the CLAMPFIT module of the pCLAMP software. Statistical analyses were performed using PRISM 2.0 (Graphpad Software, San Diego, CA, USA). Data are expressed as mean±standard error for
n experiments. Unpaired t-tests were used for comparisons of two groups and a repeated measures ANOVA with post-hoc comparison of means using Bonferroni’s test was used for multiple group comparisons. A P value <0.05 was considered significant.

The amplitude of the activating current was calculated as the difference between the initial current recorded just after the step depolarization and the maximum reached at the end of the step. Similarly, the tail current amplitude was recorded as the difference between the peak and steady state current after repolarization to −60 mV. In experiments where tail currents did not fully relax to steady state, the peak tail current amplitude was used in the analysis.

The voltage-dependence of current activation was determined by fitting the values of the normalised tail currents to a Boltzmann function

\[ I = I_{\text{max}} \left( \frac{1}{1 + \exp \left( \frac{(V_1/2 - V)/k}{n} \right)} \right) \]

where \( I \) represents the tail current, \( V_{1/2} \), the voltage at which the current was half activated, \( V \), the test potential and \( k \), the slope factor. The relationship between drug concentration and current blockade was determined by fitting values to a Hill equation after normalisation of post-drug current to control current

\[ I_{\text{drug}} / I_{\text{control}} = \frac{I}{1 + 10^{\log (IC_{50} - D)}} \]

where \( I \) represents the tail current, \( IC_{50} \) the concentration required for 50% channel blockade, \( D \) the drug concentration and \( n \) the Hill coefficient.

3. Results

3.1. Concentration- and voltage-dependence of HERG channel inhibition

We have previously reported that HERG channels stably transfected into a Chinese hamster ovary (CHO-K1) cell line produce currents with similar biophysical characteristics to HERG expressed in *Xenopus* oocytes and other mammalian cell lines [19,21–23]. Currents were elicited using step depolarizations to potentials between −50 and +30 mV from a holding potential of −80 mV at room temperature (22°C). In Fig. 1A and B, representative currents are demonstrated at baseline, then during steady state blockade maintained by stimulation at 0.1 Hz in the presence of 1 μM azimilide or 3 μM ambasilide. In experiments using 1 μM azimilide, current returned to 92±10% of baseline after a 10-min washout period (\( n = 6 \)), whereas washout of 3 μM ambasilide was less complete (77±2% of baseline current, \( n = 5 \)). Azimilide inhibited tail currents at −60 mV following a voltage step to +30 mV with an IC\(_{50}\) of 610 nM (95%CI 510–730 nM) and Hill coefficient of −1.0±0.1 (\( n = 4–10 \), Fig. 1C). There was no difference in the potency of current inhibition at 37°C (IC\(_{50}\) 560 nM, 95%CI 470–670 nM, Hill coefficient −0.9±0.1, \( n = 4–7 \)), hence all remaining experiments were performed at room temperature. Using an identical protocol, ambasilide inhibited tail currents less potently with an IC\(_{50}\) of 3.6 μM (95%CI 3.1–4.3 μM) and Hill coefficient of −0.8±0.2 (\( n = 4–11 \), Fig. 1D). The potency of block by ambasilide was voltage-dependent, since the IC\(_{50}\) for tail current block at −30 mV was 16.1 μM (95%CI 9.0–28.8 μM) with a Hill coefficient of −0.8±0.2 (\( n = 4–11 \)).

Currents elicited during step depolarizations (‘activating currents’) were inhibited by 1 μM azimilide at potentials greater than −40 mV, but were augmented at −40 and −50 mV (\( n = 12 \), Figs. 1A and 2A). Similarly, azimilide inhibited tail currents at voltages above −40 mV, but augmented current at more negative potentials (Fig. 2B). This ‘agonist effect’ was use-dependent, since it was only observed when cells were stimulated with 500 ms pre-pulses to +30 mV after application of drug. Blockade of activating and tail currents was not voltage-dependent between −30 mV and +30 mV, although there was a trend towards increased block at positive potentials (\( n = 12 \), Fig. 2C). The voltage of half-maximal activation (\( V_{1/2} \)) was shifted from −18.7±1.1 mV to −25.6±1.5 mV (\( P < 0.01 \), \( n = 12 \)), whereas the slope factor did not change (8.2±0.1 in controls and 10.2±1.2 after azimilide, \( P = \text{ns} \)). Current augmentation at −50 mV was concentration-dependent, increasing by a factor of 1.9±0.2 in 100 nM azimilide, compared with 3.6±0.5 in 1 μM azimilide (\( P < 0.05 \), \( n = 9 \)). Fig. 2D demonstrates that there was also a strong correlation between the shift in voltage-dependence of activation and the degree of current augmentation for a given drug concentration (\( V_{1/2} = 21.0±0.3 \) mV with 100 nM versus −25.6±1.5 mV with 1 μM azimilide, \( P < 0.05 \), \( n = 9 \)).

The \( I–V \) relationships for both activating and tail currents at baseline and after azimilide 3 μM are shown in Fig. 3A and B. There was no shift in the voltage-dependence of activation in the presence of azimilide (\( V_{1/2} = 22.8±0.2 \) mV in controls, versus −23.9±0.6 mV after ambasilide, respectively, \( n = 8 \)), although there was a small difference in the slope factors (9.7±0.2 versus 12.2±0.5 mV, \( P < 0.01 \), \( n = 8 \)). We further assessed the voltage-dependence of block by plotting relative tail current (\( I_{\text{amb}} / I_{\text{con}} \)) at each test potential. Current inhibition increased from 20±6% at −40 mV to 49±4% at +30 mV (\( P < 0.05 \), \( n = 8 \), Fig. 3C).

3.2. Effect of pulse duration on inhibition

Using an envelope of tails protocol, peak tail currents were recorded after steps to +30 mV of increasing duration (20–400 ms) before and during exposure to azimilide 1 μM (Fig. 4A). We used small incremental time-steps based on a previous report that binding reaches
steady state relatively rapidly after channel activation [24]. HERG tail currents were normalised to the maximum control current and fitted with exponential functions. The time constant of channel activation determined using this method was shortened by azimilide (τ 96±8 ms in controls, versus 59±7 ms after azimilide, P<0.05, n=9, Fig. 4B) but not changed by ambasilide (τ 144±18 ms for controls and 103±16 ms after ambasilide, P=ns, n=12, Fig. 4D). Inhibition by both drugs increased with pulse duration and the development of block was fitted with a
Fig. 2. Voltage-dependence of HERG channel modulation by azimilide. (A) $I-V$ relationship for activating current in the presence of 1 μM azimilide. Inhibition occurred at potentials greater than $-40$ mV, but an agonist effect was apparent at $-40$ and $-50$ mV ($n=12$). (B) Tail currents were normalised to the amplitude of the maximal control current and fitted with Boltzmann functions ($-7$ mV shift with azimilide, $n=12$). (C) Voltage-dependence of tail current blockade. Relative current was plotted for each potential in (B). Inhibition by azimilide was independent of voltage between $-30$ mV and $+30$ mV, but an agonist effect was apparent at $-40$ and $-50$ mV compared with $-130$ mV ($*P<0.01$, $n=12$). Activating currents displayed an identical voltage-dependence. (D) Correlation of $V_{1/2}$ and agonist effect at $-50$ mV. Points fitted with a linear regression equation ($V_{1/2}=a(I_{az}/I_{con})+b$, where $a=-1.4\pm0.2$ and $b=-16.3\pm0.4$). Numbers in brackets represent azimilide concentration (μM).

3.3 Frequency-dependence of HERG channel block

A previous study showed that HERG channel inhibition by azimilide exhibited reverse frequency-dependence for stimulation rates between 0.04 and 1 Hz [24]. The frequency-dependence of channel blockade by 3 μM ambasilide was assessed using a protocol in which peak tail currents at $-60$ mV were recorded after each of a train of 0.5 s voltage steps to $+30$ mV, delivered at frequencies between 0.03 and 1 Hz. There was no significant change in the control current during this protocol and inhibition by ambasilide 3 μM did not differ after 2.5 min of stimulation (37±1, 36±4, 33±3 and 41±5% block with 0.03, 0.1, 0.5 and 1 Hz stimulation, respectively, $n=4-12$).

3.4 Modulation of activation and deactivation kinetics

Time constants of activation were calculated by fitting a single exponential function to activating currents in Fig. 1A. In agreement with the findings in Fig. 4, there was a significant increase in the rate of channel activation following azimilide at most potentials (Fig. 6A), whereas there was no change following ambasilide (Fig. 6B).

We studied the deactivation of tail currents produced by a 3.9 s pulse to $+30$ mV followed by a 10-s repolarization
to \(-60\) mV (Fig. 7). While azimilide did not affect tail current kinetics, both fast and slow components of deactivation were significantly slower with ambasilide \((\tau_{\text{ambasilide}} = 889 \pm 50 \text{ ms and } 4257 \pm 304 \text{ ms for controls versus } \tau_{\text{ambasilide}} = 1437 \pm 121 \text{ ms and } 7120 \pm 520 \text{ ms with ambasilide, } P < 0.01, n = 6, \text{ Fig. 7B}). The relative contribution of the fast component of deactivation was not modified by ambasilide.

### 3.5. Modulation of channel inactivation

Mutations at the S631 residue significantly weaken inactivation and abolish current augmentation by azimilide [24]. We therefore expected that azimilide itself may have effects on inactivation gating and voltage-dependence. A dual pulse protocol was used to assess the time course of channel inactivation (Fig. 8A). A 500-ms depolarization to +30 mV was followed by a 40-ms hyperpolarizing step to -80 mV to relieve rapid inactivation. A second pulse to potentials between -100 and +40 mV was then used to elicit large currents which underwent rapid reinactivation. Time constants of inactivation were determined by fitting a single exponential function to these currents. Fig. 8B demonstrates that inactivation was accelerated in the presence of 1 \(\mu\)M azimilide at most potentials (e.g. \(\tau_{\text{azimilide}} = 14.2 \pm 1.1 \text{ ms before and } 11.2 \pm 0.7 \text{ ms after azimilide at } -40 \text{ mV, } P < 0.05, n = 7). Time constants of inactivation were modestly accelerated by ambasilide at potentials between -40 and -20 mV, and not significantly affected at other potentials (data not shown, \(n = 9).\)

Recovery from inactivation was determined by fitting a single exponential function to the initial ‘hook’ preceding slower deactivation of tail currents at potentials between -120 and 0 mV (elicited by a 500-ms step to +30 mV). Azimilide \((n = 12, \text{ Fig. 8C})\) had minimal, and ambasilide \((n = 5, \text{ data not shown})\) had no discernible effect on the time constants of recovery from inactivation.

The voltage dependence of steady-state inactivation was assessed using the following protocol. Following a 1-s step to +30 mV, 20 ms pulses were applied to potentials between -120 and +40 mV, followed by a second step to +30 mV (Fig. 8D). Peak currents (measured 3 ms after the onset of the pulse to allow for capacitance artefact) elicited by the second step to +30 mV were plotted as a function of the potential of the preceding 20 ms step (Fig. 8E). After 1 \(\mu\)M azimilide, \(V_{1/2}\) shifted from -60.4 \(\pm\) 1.1 to...
Fig. 4. Envelope of tails protocol. (A) Currents were recorded after steps to +30 mV of increasing duration before and after superfusion with 1 µM azimilide (see inset). Although not shown, an identical protocol was used in experiments with 3 µM ambasilide. (B) Peak tail currents were normalised to the maximum control current and fitted with either a single (control) or double (azimilide) exponential function. Activation was faster in the presence of azimilide (n=9). (C) Relative current plotted as a function of pulse duration. Block increased monoexponentially with pulse duration (n=9). (D) Normalised peak tail currents were plotted for each pulse and fitted with a single exponential function. There was no change in the time course of activation with ambasilide (n=12). (E) Block by ambasilide increased monoexponentially with pulse duration (n=12).
Fig. 5. Inhibition during a prolonged depolarization. (A) Representative traces elicited by a 15-s depolarization to +10 mV before and after addition of 1 μM azimilide. Inhibition by both drugs was maximal within the first 2 s of the depolarization with little further time-dependent decay. (B) Time course of block development by azimilide over initial 2 s (boxed area in (A)). Relative current (I\text_{rel}/I_{con}) was fitted by a single exponential function (dotted line). (C) Currents elicited by identical protocol in the presence of ambasilide 3 μM. (D) Block development by ambasilide occurred monoexponentially (dotted line).

Fig. 6. Modulation of activation kinetics. (A) Time constants of activation obtained by single exponential fitting of activating currents from the protocol in Fig. 1A (steps to −40 and −50 mV excluded due to inaccurate fitting). Time constants were significantly accelerated after addition of azimilide at voltages between −50 and +10 mV (n=6). (B) Time constants of activation were unchanged by ambasilide (n=7).
Fig. 7. Modification of tail current deactivation by ambasilide. (A) Tail currents at $-60 \text{ mV}$ following a step to $+30 \text{ mV}$ were fitted with double exponential functions. (B) Both fast and slow components of deactivation were significantly slower in the presence of ambasilide ($**$, $P<0.01$, $n=6$).

Fig. 8. Effect of azimilide on channel inactivation. (A) A dual-pulse protocol was used to assess the time course of inactivation (see inset). Current traces from time period represented by boxed area on inset. (B) Inactivation time constants plotted as a function of membrane potential. Inactivation was accelerated in the presence of 1 $\mu$M azimilide at voltages between $-40$ and $0 \text{ mV}$ ($n=7$). (C) Recovery from inactivation was determined by fitting a single exponential function to the initial ‘hook’ preceding slower deactivation of tail currents elicited by a 500 ms depolarization to $+30 \text{ mV}$. Time constants of recovery from inactivation were only different between $-50$ and $-30 \text{ mV}$ ($n=12$). (D) Voltage dependence of steady state inactivation was assessed using a different dual pulse protocol (see text). Current traces show time period during and shortly after the brief hyperpolarizing step. (E) Peak currents elicited by the second step to $+30 \text{ mV}$ were plotted as a function of the potential of the preceding step and fitted with Boltzmann functions. After 1 $\mu$M azimilide, $V_{1/2}$ was shifted by approximately $-10 \text{ mV}$ ($P<0.01$, $n=9$).
−70.3±1.6 mV (P<0.01, n = 9), while the slope factor remained unchanged. There was no change in the voltage dependence of inactivation in the presence of ambasilide 3 μM (V_{1/2} and slope −58.5±1.0 mV and −18.9±0.9 in controls versus −57.3±1.3 mV and −20.5±1.1 after ambasilide, n = 11).

3.6. Voltage ramp protocol

The use of a ventricular action potential as the voltage clamp command protocol has shed more light on the in vivo characteristics of the HERG channel [25,26]. It has been shown that very similar current traces and I–V relationships are produced by a 0.2–0.5 s descending voltage ramp [25], and, for convenience, we have used this approach here. A 500-ms voltage ramp from +40 to −80 mV was used to elicit currents such as those demonstrated in Fig. 9A. At baseline, the HERG current peaked at 370 ms into the ramp, corresponding to a membrane potential of −48 mV (n = 10). After exposure to 1 μM azimilide, the current peaked at 350 ms (membrane potential −44 mV, n = 6, Fig. 9B). When relative current (derived from the data in Fig. 9B) was plotted as a function of membrane potential there was a non-significant trend towards increased block at negative membrane potentials (P = ns, Fig. 9C).

The same ramp protocol was applied to cells at baseline and in the presence of ambasilide (Fig. 9D). There was a modest shift in peak current from −44 mV at baseline to −47 mV after 3 μM ambasilide (n = 6, Fig. 9E). As with
Fig. 9. Voltage ramp protocol. (A) A 500-ms voltage ramp was used to simulate the repolarization during a cardiac action potential. Currents were recorded at baseline and during superfusion with 1 µM azimilide. Both increased steadily reaching a peak at 370 ms (control) and 350 ms (azimilide) and then declined more rapidly at more negative voltages due to deactivation. (B) I–V relationship for ramp experiments (n = 6). Currents were corrected for cell capacitance and every tenth point was plotted for clarity. After azimilide there was a small (14 mV) shift in peak current. (C) Relative current during voltage ramp protocol. Block was not strictly voltage-dependent, although there was a trend towards increased block at negative membrane potentials. (D) An identical voltage ramp was applied in experiments with 3 µM ambasilide. (E) I–V relationship for capacitance-corrected currents (n = 6). After the addition of 3 µM ambasilide, there was a modest (23 mV) shift in peak current. (F) Block increased during the ramp from 7±4% at the onset of the ramp to 41±9% at the end of the ramp (P<0.01, n = 6).
azimilide, block increased as the ramp potential became more negative from 7±4% at the onset of the ramp to 41±9% at the end of the ramp (P<0.01, Fig. 9F).

4. Discussion

Selective I_Kr blockade is associated with potent antiarrhythmic effects in the atria, but has not proven beneficial in the prevention of sudden death in post-myocardial infarction patients [1,27,28]. Interest in multichannel blocking drugs such as azimilide and ambasilide stems from clinical studies showing that amiodarone has at least equal or greater efficacy than other antiarrhythmic drugs with a low incidence of torsades de pointes [29,30]. Preliminary studies with azimilide suggest at least equivalent efficacy to other class III antiarrhythmic drugs with a low incidence of adverse cardiac events [2,9,10]. Although not in clinical use, ambasilide is a useful investigational agent since it blocks both components of I_K and has in vivo effects similar to amiodarone [13,31].

Inhibition of I_Kr by azimilide (IC_{50} 0.2–1.0 μM) is more potent than block of HERG channels in Xenopus oocytes (IC_{50} 5.2 μM), but equivalent to our findings (IC_{50} 610 nM) [24,32–34]. Differences between the properties of the oocyte membrane and mammalian cells may account for this discrepancy [26,35]. We found that extra-cellular application of ambasilide blocked HERG channels with an IC_{50} of 3.6 μM, which is similar to the potency of I_K block reported in guinea pig ventricular myocytes (IC_{50} of 2–5.6 μM) [12,14].

Changes in temperature are known to affect the ERP and frequency-dependence of class III antiarrhythmic activity in papillary muscle preparations [36]. Azimilide clearly exhibits forward frequency-dependence at 37°C (1–4 Hz), but this effect is much less apparent at 32°C [36]. Since the temperature at which recordings are made significantly modifies HERG channel properties and the blocking potency of class III agents [26], we repeated experiments at 37°C, finding little difference in the IC_{50} value. As expected, drug binding by azimilide was faster at 37°C, but unbinding was slow and difficult to evaluate. We performed experiments with ambasilide at room temperature and it is possible that the inhibitory potency, frequency-dependence and binding kinetics may be different at 37°C, since the greatest effect of temperature is on HERG activation kinetics followed by inactivation and deactivation [26].

4.1. Mechanism for ‘agonist effect’ of azimilide

Azimilide augments I_Kr in guinea pig ventricular myocytes at low concentrations (50–100 nM), in particular at near threshold potentials [37]. A recent report described a use-dependent HERG channel agonist effect at −50 mV in the presence of 5 μM azimilide [38]. This ‘prepulse potentiation’ was lost when the same protocol was applied to HERG channels with mutations at the outer mouth (S631C) or S5-P loop regions (H587P), suggesting that the agonist site is in the extracellular domain [38]. Our studies confirmed that the agonist effect is present at membrane potentials negative to −30 mV. The mechanism appears to be a concentration-dependent, hyperpolarizing shift in the voltage-dependence of channel activation induced by azimilide. The changes in activation kinetics may also be explained by this shift in activation gating, since after correction for the shift in the activation curve, we found that only the time constants at −20 and −30 mV were significantly different from controls. Similar findings have been reported for the experimental class III agent almokalant, in which augmentation of I_Kr at near threshold potentials was attributed to a hyperpolarizing shift in the voltage-dependence of activation [39]. These findings provide further evidence for an interaction between the outer mouth and nearby extracellular domain and activation gating (S4 region) [38].

4.2. State-dependence of channel block

HERG channel block by azimilide was not voltage-dependent between −30 and +30 mV, consistent with previous reports of HERG and I_Kr inhibition in canine ventricular myocytes [24,34]. Figs. 4 and 5 show that inhibition required channel activation and exhibited time-dependence (τ 258 ms). Azimilide is known to increase the rate of I_Kr activation in AT-1 cells [24]. In agreement, we found that time constants of channel activation obtained using the envelope of tails protocol were significantly accelerated in the presence of 1 μM azimilide, as were activation time constants obtained by direct exponential fitting of HERG currents elicited by a series of membrane depolarizations.

Since recent data suggest that the HERG channel agonist effect correlates with the degree of inward rectification [38,40], we examined the effect of azimilide on the time course and voltage-dependence of channel inactivation. There was a significant acceleration in channel inactivation at most potentials in the presence of 1 μM azimilide, but recovery from inactivation was minimally affected, and, in addition, there was a −10-mV hyperpolarizing shift in the voltage-dependence of inactivation. This shift represents a reduction in channel availability although such an effect would be significantly attenuated by the hyperpolarizing shift in the voltage-dependence of activation. It also suggests that azimilide has affinity for the inactivated channel state. In this regard, our findings contrast with those of Busch et al. and Jiang et al., who found that azimilide did not alter the rate of channel inactivation, and that affinity for the S631A mutant (a point mutation which greatly reduces HERG channel inactivation), did not differ from that for wild-type HERG channels [24,38]. The reason for this disparity is not clear but may relate to
differences in methodology or properties of the expression systems. Finally, elevation of [K+]o is known to slow inactivation kinetics and shift the steady state inactivation curve to more positive potentials, thus considerably weakening inactivation [41]. Block of HERG channels and Ik in AT-1 cells is much less potent in the presence of high [K+]o (10 mM) [24], which supports a conclusion that azimilide binds to both open and inactivated states.

Inhibition of HERG channels by ambasilide exhibited voltage-and time-dependence similar to azimilide, but was not frequency-dependent. Block was greater at positive potentials consistent with binding of a positively charged component of the drug to a site within the transmembrane electrical field. The pKd of ambasilide is 6.5 (personal communication: E. Schneider, Knoll, Milan, Italy) which means that ~13% is positively-charged at pH 7.4. The time course of HERG channel activation was not modified by ambasilide, however, time constants of deactivation were significantly prolonged in comparison with controls. This is also observed with Ik;i inhibition by ibutilide and flecainide as well as block of Ik and the cloned human K+ channel, HK2, by quinidine [42–45]. It is thought to represent partial unbinding of drug from open channel states, allowing brief conduction before channel closure [45]. Since the kinetics and voltage dependence of inactivation were not modified, these findings suggest that ambasilide has predominant affinity for the open state.

4.3. Effect of azimilide and ambasilide during a voltage ramp

Previous studies have shown that HERG current during a voltage ramp closely resembles that produced when a ventricular action potential is used as the voltage clamp protocol [25,26,46]. Azimilide caused only a small (+4 mV) shift in the voltage of peak current, with no significant variation in the degree of blockade throughout the ramp. This 4-mV shift wasprobably due to the change in the voltage-dependence of steady state inactivation (~10 mV). Block developed quickly during the ramp, reaching steady state before negative potentials were reached, as predicted from the time constants for block in Figs. 4 and 5. Current augmentation was not observed at potentials negative to ~30 mV, suggesting that this phenomenon may not be clinically relevant.

After application of ambasilide, the current was simply scaled down, but with a modest (~3 mV) shift in peak current, which is probably due to slower deactivation in the presence of drug. Block increased as the ramp progressed, consistent with the time course of open channel block described in the envelope of tails test.

4.4. Clinical relevance of HERG channel blockade

Following standard dosing, the human plasma concentration of azimilide is approximately 1 μM (personal communication: Dr. R.R. Brooks, Procter and Gamble Pharmaceuticals, [2]). At this concentration one would expect approximately 60% suppression of the HERG current, however, since the drug is 94% plasma protein bound, the concentration available to interact with the channel would be much lower. While azimilide causes QT prolongation in animal models to an equivalent or greater extent than other class III antiarrhythmic drugs [5,47], its propensity to cause QT prolongation and torsades de points in humans remains undefined. While an agonist effect was not apparent during a single voltage ramp it may have significance in the setting of a delayed after-depolarization or in partially depolarized tissue (e.g. ischaemia) in which Ik;i potentiation may occur [38].

The human therapeutic plasma concentration of ambasilide has not been reported, however, in a dog model, it was approximately 15 μM [13]. This concentration would clearly inhibit Ik;i, but would also have significant effects on other cardiac K+ channels (see Table 1). The complex electrophysiological effects of ambasilide in the human atrium and ventricle are thus explained by its inhibitory effects on multiple cardiac ion channels.

In conclusion, evidence presented in this study suggests that azimilide binds to both open and inactivated states. Current augmentation at near-threshold potentials is due to a concentration-dependent shift in the voltage-dependence of activation, but does not influence the pattern of channel block during a single action potential. Like other class III agents, ambasilide binds with greatest affinity to the open channel state but does not exhibit steady-state frequency-dependence. Both drugs have similar binding kinetics and inhibitory effects during a voltage ramp.

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