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Blockade of I_{Ks} by HMR 1556 increases the reverse rate-dependence of refractoriness prolongation by dofetilide in isolated rabbit ventricles

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1 The rate-dependent contributions of the rapid and slow components of the cardiac delayed rectifier K^+ current (I_{Kr} and I_{Ks}, respectively) to repolarization are not fully understood. It is unclear whether the addition of I_{Ks} block will attenuate reverse rate-dependence seen after I_{Kr} block.

2 The individual and combined electrophysiological effects of selective I_{Kr} and I_{Ks} blockers, dofetilide and HMR 1556, respectively, were evaluated using Langendorff-perfused rabbit hearts. Monophasic action potential duration at 90% repolarization ($MAPD_{90}$) and ventricular effective refractory period (VERP) were determined at cycle lengths (CLs) of 200–500 ms (at 50 ms intervals).

3 Dofetilide (1–100 nM) prolonged $MAPD_{90}$ in a concentration-dependent manner ($P<0.001$, $n=6$) with reverse rate-dependence ($P<0.0001$). In contrast, HMR 1556 (10–240 nM) alone did not prolong $MAPD_{90}$. However, in the presence of 7.5 nM dofetilide, HMR 1556 (100 nM) increased the extent of reverse rate-dependence by further prolonging $MAPD_{90}$ at CLs of 400, 450 and 500 ms ($P<0.05$, $n=9$) and, to a lesser extent, at shorter CLs (e.g. by 17 ± 4 ms at CL 500 vs 2 ± 3 ms at CL 200 ms).

4 Effects of dofetilide and HMR 1556 on VERP were similar to those on $MAPD_{90}$. The slope of the VERP vs CL relation was steeper after the combination (0.081 ± 0.013) than after dofetilide alone (0.028 ± 0.018, $P<0.01$, $n=9$).

5 Blockade of rabbit I_{Ks} increased reverse rate-dependence of I_{Kr} block.
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Abbreviations: APD, action potential duration; APD_{90}, APD at 90% repolarization; CL, cycle length; I_K, delayed rectifier potassium current; I_{Kr}, the rapid component of I_K; I_{Ks}, the slow component of I_K; MAP, monophasic action potential; $MAPD_{90}$, MAP duration at 90% repolarization; TdP, Torsades de Pointes; VERP, ventricular effective refractory period

Introduction

The delayed rectifier K^+ current (I_K), a key outward current for cardiac repolarization, is comprised of rapid (I_{Kr}) and slow (I_{Ks}) components with distinct rectification characteristics, kinetics and drug sensitivities (Sanguinetti & Jurkiewicz, 1990; Li *et al*., 1996; Cheng & Kodama, 2004). I_{Kr} block has been the major target for most class III antiarrhythmic agents (e.g. dofetilide, sotalol, E-4031), which exert their effects by prolonging cardiac action potential duration (APD) and refractoriness. However, the usefulness of these agents is limited by their undesirable 'reverse rate-dependent' profile: excessive drug effects at slow heart rate or long diastolic interval, but loss of efficacy at fast heart rate (Hondeghem & Snyders, 1990; Dorian & Newman, 2000). Bradycardia-induced excessive APD prolongation with such agents has been associated with the risk of acquired long QT syndrome, leading to a polymorphic ventricular tachyarrhythmia called Torsades de Pointes (TdP) (Gowda *et al*., 2004). Therefore, modification of the reverse rate-dependent frequency profile of I_{Kr} block should favorably widen the therapeutic window of I_{Kr} blockers.

There are considerable species differences in the expressions and kinetics of I_{Kr} and I_{Ks} (Lu *et al*., 2001; Cheng & Kodama, 2004), such that extrapolation of the results from animal studies to humans is difficult. It has been suggested that dogs and rabbits resemble humans in terms of I_{Kr} and I_{Ks} kinetics, while guinea pigs have larger I_{Ks} amplitude and slower deactivation kinetics than humans (Virag *et al*., 2001). Although it is controversial whether rabbit I_{Ks} has a functional role in cardiac repolarization since selective I_{Ks} blockers failed to prolong QT interval and APD in isolated rabbit ventricles (Lengyel *et al*., 2001), various studies have demonstrated the existence of rabbit I_{Ks} with similar kinetics to humans (Salata *et al*., 1996; Lengyel *et al*., 2001). Similarly, the role of I_{Ks} in human and canine ventricular tissues is hard to demonstrate unless under the condition of diminished repolarization reserve, such as after I_{Kr} block (Biliczki *et al*., 2002; Jost *et al*., 2005).

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The reverse rate-dependence of I_{Kr} block has been suggested to be caused by a compensatory increase in I_{Ks} during rapid heart rates, since the slow deactivation kinetics of I_{Ks} may lead to its incomplete deactivation and accumulation (Jurkiewicz & Sanguinetti, 1993). Additive class III effects of combined I_{Kr} and I_{Ks} block have been demonstrated in a guinea pig isolated heart model (Geelen *et al*., 1999) and recently in an *in vivo* canine model (Nakashima *et al*., 2004). However, these studies did not show that I_{Ks} block would favorably alleviate the reverse rate-dependence of I_{Kr} block.

The purpose of the present study was to investigate whether I_{Ks} block by HMR 1556 can attenuate the reverse rate-dependence of I_{Kr} block by dofetilide in perfused rabbit hearts, and to assess the functional presence of I_{Ks} in rabbit ventricles under the condition of diminished repolarization reserve (Roden & Yang, 2005). Furthermore, computer-simulated action potentials of rabbit ventricular myocytes using LabHEART (Puglisi & Bers, 2001) under the condition of combined I_{Kr} and I_{Ks} block can allow qualitative comparison of the theoretically predicted and experimentally observed results.

Methods

Langendorff-isolated rabbit hearts

All animal procedures were approved by the Animal Care Committee of St Michael's Hospital and performed according to the guiding principles of the Canadian Council on Animal Care. In all, 37 New Zealand White rabbits (3.0–4.2 kg, male) were heparinized (1000 IU i.v.) and anesthetized with sodium pentobarbital (40–50 mg kg^{-1} i.v.). The hearts were quickly excised and immersed in ice-cold Tyrode's solution. Each heart was retrogradely perfused with Tyrode's solution *via* the aorta at a constant pressure of 90 cm H_2O, which was maintained by recirculating the solution to the top reservoir by a peristaltic pump (Manostat, New York, NY, U.S.A.) in a closed system. The Tyrode's solution contained the following (mM): NaCl (130), $NaHCO_3$ (24.2), NaH_2PO_4. H_2O (1.2), $MgCl_2 \cdot 6H_2O$ (0.6), KCl (5.6), $CaCl_2$ (2.2), dextrose (12) (Sigma-Aldrich, St Louis, MO, U.S.A.). Bovine serum albumin (40 mg l^{-1}, fraction V, Sigma-Aldrich, St Louis, MO, U.S.A.) was added to the perfusate to improve the stability of the hearts by minimizing cellular edema (Kates *et al*., 1989). The solution was filtered with a 0.45 μm filter (Gelman Laboratory, Ann Arbor, MI, U.S.A.) to remove contaminant particles, and continuously aerated with a mixture of 95% O_2 and 5% CO_2 to obtain a pH of 7.40 $\pm$ 0.05. The solution temperature was maintained at 37.0 $\pm$ 0.5°C by a circulating water bath.

A latex balloon was inserted into the left ventricle through an incision in the left atrial appendage. The balloon was filled with distilled water to establish an end diastolic pressure of 5 $\pm$ 1 mmHg to maintain tension at a physiological range. The balloon was connected to a pressure transducer (Cobe CDX3, Lakewood, CA, U.S.A.) to measure left ventricular pressure with signals amplified by a pressure amplifier (Hewlett Packard 78205D, U.S.A.). A 6F quadripolar contact Ag–AgCl monophasic action potential (MAP) catheter (EP technologies Inc., Sunnyvale, CA, U.S.A.) was inserted into the left ventricle for pacing and measurement of MAP and refractory period. Epicardial unipolar and bipolar electrograms were recorded using two pairs of custom-made bipolar electrode hooks (hook-to-hook distance $\sim$2 mm) inserted into the epicardial surface of the left and right ventricle ($\sim$1 cm apart). A single hook electrode was inserted into the left atrial appendage to obtain a unipolar atrial electrogram. A reference silver electrode was placed on the aortic root. All signals were sampled at 1000 Hz and amplified using a custom-made amplifier (Cartesian Labs, Toronto, ON, Canada). The left ventricular pressure signal was filtered at 100 Hz and the electrograms were filtered at 1–100 Hz. The MAP signal was filtered at DC – 500 Hz. Signals were displayed on a monitor with multiple channels and recorded using a custom-made computer software program (Aqui2, Cartesian Labs, Toronto, ON, Canada). All recordings were archived on CD-ROM and data analysis was performed offline.

As the intrinsic sinus RR interval was shorter than 500 ms, the atrioventricular node was ablated by crush using forceps. The ventricles were immediately paced at a cycle length (CL) of 500 ms from the MAP catheter using a programmable stimulator (Medtronic 5325, Minneapolis, MN, U.S.A.) with 2 ms pulse width and twice the diastolic threshold. The heart was allowed to equilibrate for 15 min before the baseline electrophysiological measurements.

Electrophysiological study

Endocardial MAP at various CLs (500, 450, 400, 350, 300, 250 and 200 ms) was measured by the quadripolar MAP catheter inserted in the left ventricle. MAP duration at 90% repolarization ($MAPD_{90}$) was measured offline at the end of a 20 s conditioning train at various CLs. Ventricular effective refractory period (VERP) was measured at various CLs in the following sequence: 500, 450, 400, 350, 300, 250, and 200 ms. After 20 s of a conditioning train at the respective CL, the S1–S2 incremental extrastimulus technique was used to determine VERP with 2 ms precision by delivering an S2 extrastimulus following eight S1 stimulations. Ventricular effective refractory period was defined as the longest S1–S2 interval that failed to result in ventricular capture. QRS durations of the left ventricular unipolar electrogram were measured at CL 500 ms.

Coronary flow rate and left ventricular systolic pressure measurement

Coronary flow rate (ml min^{-1}) was determined by measuring the volume of perfusate flowing out of the heart per minute. Left ventricular systolic pressure was measured as the peak pressure generated when the ventricles were paced at CL 500 ms.

Drug administration

After the baseline electrophysiological measurements, increasing concentrations of dofetilide (1, 3, 10, 30 and 100 nM, $n = 6$) (Pfizer Canada Inc., QC, Canada) or HMR 1556 (10, 30, 100, and 240 nM, $n = 7$) (Aventis Pharma, Frankfurt am Main, Germany) were cumulatively administered to the circulatory perfusate. After each administration, a 15-min equilibration period was allowed before the electrophysiological measurements were repeated. Dofetilide was dissolved in saline as a stock solution of 22.7 μM. HMR 1556 was dissolved in

dimethylsulfoxide (DMSO, 0.1%), polyethylene glycol 400 (PEG-400, 0.3%) and saline as a stock solution of 25 μM. The highest DMSO concentration in the perfusate was $<0.001\%$. Same amounts of DMSO and PEG-400 were added to saline as control (drug free) solutions to investigate any potential electrophysiological effects ($n=6$). This group also served as the time control to evaluate the electrophysiological stability over time.

Concentration–response curves for dofetilide and HMR 1556 were constructed, and the inhibitory concentration for 50% inhibition of maximum (IC_{50}) of dofetilide on VERP was determined to be 15 nM at CL 500 ms. In the cumulative concentration–response study for dofetilide, no arrhythmias were observed up to the highest concentration of 100 nM, and all hearts were able to be paced at the longest CL of 500 ms. However, acute administration of 15 nM dofetilide caused frequent premature ventricular contractions precluding accurate measurement of $MAPD_{90}$ and VERP at long CLs. Since the incidence of premature beats may be greater during acute administration of high concentration of dofetilide, 7.5 nM (50% of the IC_{50}) was chosen for further combined drug studies, where no premature beats were observed at this concentration. Since HMR 1556 administration alone did not result in observable electrophysiological effects, a low and high concentration of HMR 1556 (10 and 100 nM) were chosen for the subsequent combination drug study. In the presence of 7.5 nM dofetilide, cumulative additions of two concentrations of HMR 1556 (10 and 100 nM) were performed ($n=9$). Electrophysiological measurements were repeated 15 min after each administration. In order to evaluate the stability of effects of dofetilide (7.5 nM) over time, time controls were performed by adding control (drug free) solutions in place of 10 and 100 nM of HMR 1556 ($n=9$), with measurements repeated at the same time interval.

Computer simulation of action potentials using LabHEART

Computer-simulated APD at 90% repolarization (APD_{90}) under the condition of I_{Kr}, I_{Ks} and combined I_{Kr} and I_{Ks} block at various stimulation rates was generated using the LabHEART software version 4.9.5 – computer simulation of a rabbit ventricular myocyte (Puglisi & Bers, 2001). The program is available at the following website: http://www.meddean.luc.edu/templates/ssom/depts/physio/labheart.cfm.

The computer-simulated APD_{90} was determined at various stimulation rates in the following sequence: 2, 2.2, 2.5, 2.9, 3.3, 4 and 5 Hz, which correspond to CL 500, 450, 400, 350, 300, 250 and 200 ms, respectively, in the isolated rabbit heart experiment. The APD_{90} was measured after 20 beats (following either changes in stimulation rates or channel conductance), giving the system of ordinary differential equations time to return to steady state (See Supplementary Information for the detailed settings and equations). After baseline APD_{90} simulation, complete I_{Ks} block was simulated by setting the conductance of the I_{Ks} to 0 mS μF^{-1}. I_{Kr} block was simulated by decreasing the conductance from 0.04 to 0.028 mS μF^{-1} (decreased by 30%) to achieve similar amounts of APD prolongation by 7.5 nM dofetilide, as observed experimentally. The combined I_{Kr} and I_{Ks} block was simulated by implementing both of these settings. The APD tracings were exported from LabHEART to Microsoft Excel 2002, where APD_{90} was measured with 1 ms precision.

Data analysis and statistics

Results are expressed as mean $\pm$ standard error of the mean (s.e.m.). Multiple comparisons of drug effects at various concentrations and CLs were performed by two-way analysis of variance (ANOVA) with repeated measures. When there was a significant concentration-dependent effect for the single drug studies, a sigmoidal concentration–response curve was fitted to determine the IC_{50}. Time effects on coronary flow rate, left ventricular systolic pressure and QRS duration at multiple time points were analyzed by one-way ANOVA with repeated measures. Bonferroni *post hoc* corrections were performed when *P*-values were <0.05. All statistical analysis and curve fitting were performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA, U.S.A.).

Results

Electrophysiological changes following application of I_{Kr} or I_{Ks} blockers

Baseline $MAPD_{90}$ and VERP for the control group at various CLs are shown in Table 1, and were not significantly different from the baseline values of the single or combined drug groups. Control solutions did not produce electrophysiological changes on VERP or $MAPD_{90}$, and these measurements were stable over time (e.g. change in $MAPD_{90}$ ranged from 1 ± 10 to 7 ± 5 ms at CL 200 ms, $P=0.79$ and -12 ± 7 to -1 ± 2 ms at CL 500 ms, $P=0.25$, $n=6$).

Figure 1a illustrates representative MAP recordings before and after cumulative additions of dofetilide (10 and 100 nM) at CLs 200 and 500 ms. Dofetilide prolonged $MAPD_{90}$ at both CLs in a concentration-dependent manner, but with greater effects at CL 500 ms than CL 200 ms. Concentration–response curves of dofetilide for the change in $MAPD_{90}$ from baseline at various CLs are shown in Figure 1b. Dofetilide (1–100 nM) prolonged $MAPD_{90}$ compared to control group ($P<0.001$, $n=6$). Moreover, 100 nM dofetilide increased $MAPD_{90}$ by 20 ± 5 ms at CL 200 ms vs 74 ± 9 ms at CL 500 ms with reverse rate-dependence ($P<0.0001$, $n=6$).

Table 1 Baseline [a]$MAPD_{90}$ and [b]VERP for control rabbit hearts at various cycle lengths

Cycle length (ms)	*$MAPD_{90}$* (ms)	*VERP* (ms)
200	104 ± 5	104 ± 2
250	118 ± 4	119 ± 1
300	127 ± 5	130 ± 2
350	137 ± 4	135 ± 2
400	143 ± 4	140 ± 3
450	148 ± 4	144 ± 4
500	150 ± 3	148 ± 5

Values are mean $\pm$ s.e.m., $n=6$.
[a]MAP duration at 90% repolarization.
[b]Ventricular effective refractory period.

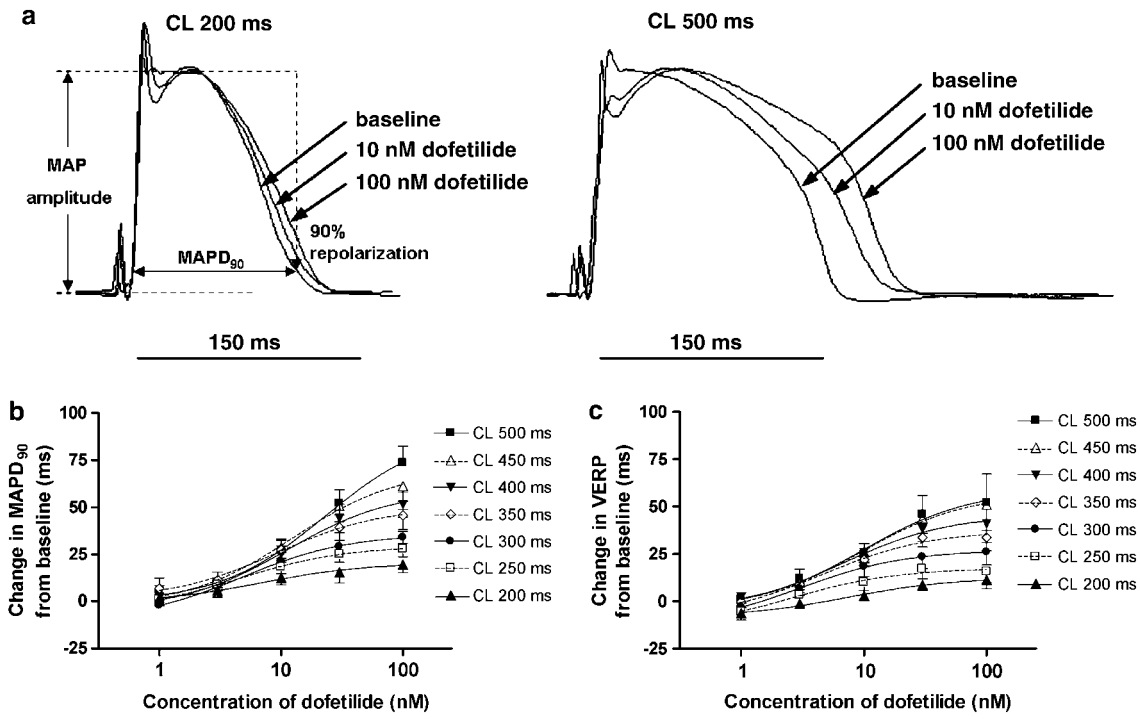

Figure 1 Representative MAP recordings before and after cumulative additions of dofetilide (10 and 100 nM) at CLs 200 and 500 ms (a). Concentration–response curves for the effects of I_{Kr} block by dofetilide (1, 3, 10, 30 and 100 nM) on the change in $MAPD_{90}$ (b) and VERP (c) from baseline at various CLs. Dofetilide concentration in nM is plotted on a log scale. Mean ± s.e.m., $n=6$. Endocardial MAP signals and VERP were obtained from the left ventricular apex of the perfused rabbit hearts. After baseline measurement, cumulative addition of dofetilide was performed. Measurements were repeated 15 min after each administration. Amplitude of the MAP recordings in (a) ranged from 5.1–9.9 mV, and was rescaled for the figure. The variation in MAP morphology and amplitude can be due to changes in contact pressure between the MAP catheter and tissue, and do not necessarily reflect changes in specific ionic currents. Such variability is consistent with the known reported feature of MAP (Franz, 1999).

Figure 1c demonstrates the effects of dofetilide on VERP at various CLs. Consistent with its effect on $MAPD_{90}$, dofetilide (1–100 nM) prolonged VERP compared to control ($P<0.01$, $n=6$). At 100 nM dofetilide, VERP was prolonged by 11 ± 5 ms at CL 200 ms vs 52 ± 15 ms at CL 500 ms with reverse rate-dependence ($P<0.0001$, $n=6$).

In contrast to the clear effects of dofetilide, cumulative addition of HMR 1556 (10–240 nM) did not elicit significant electrophysiological changes in $MAPD_{90}$ (Figure 2a) or VERP (Figure 2b) at any CLs measured.

Electrophysiological effects of combined I_{Kr} and I_{Ks} block by dofetilide and HMR 1556

In order to evaluate the stability of the effects of dofetilide over time, time controls were performed by applying control (drug free) solutions in place of 10 and 100 nM of HMR 1556 in the presence of dofetilide (7.5 nM). Prolongation of $MAPD_{90}$ and VERP by 7.5 nM dofetilide remained constant after the addition of control solutions (e.g. change in $MAPD_{90}$ beyond the post dofetilide value ranged from 1 ± 6 to 5 ± 3 ms at CL 200 ms, $P=0.60$, and −3 ± 3 to 1 ± 5 ms at CL 500 ms, $P=0.74$, $n=9$).

Figure 3a illustrates representative MAP recordings before and after the administration of dofetilide (7.5 nM) followed by HMR 1556 (10 and 100 nM) at CLs 200 and 500 ms. Dofetilide (7.5 nM) prolonged $MAPD_{90}$ to a greater extent at CL 500 ms than CL 200 ms, consistent with its reverse rate-dependent effect in the single drug study. In the presence of dofetilide, HMR 1556 (10 and 100 nM) further prolonged $MAPD_{90}$ in a concentration-dependent manner at CL 500 ms. Figure 3b summarizes the data for the combined I_{Kr} and I_{Ks} block on the change in $MAPD_{90}$ at various CLs. Dofetilide (7.5 nM) prolonged $MAPD_{90}$ by 18 ± 3 ms at CL 200 ms and 31 ± 3 ms at CL 500 ms with reverse rate-dependence ($P<0.001$, $n=9$). In the presence of 7.5 nM dofetilide, HMR 1556 increased the extent of reverse rate-dependence by further prolonging $MAPD_{90}$ at CLs 400, 450 and 500 ms ($P<0.05$, $n=9$), while to a lesser extent at shorter CLs.

Consistent with the effects on $MAPD_{90}$, HMR 1556 increased reverse rate-dependence of dofetilide by further prolonging VERP at long CLs (Figure 3c). Dofetilide (7.5 nM) prolonged VERP by 17 ± 4 ms at CL 200 ms and 26 ± 6 ms at CL 500 ms with reverse rate-dependence ($P<0.01$, $n=9$). In the presence of 7.5 nM dofetilide, HMR 1556 increased the extent of reverse rate-dependence by further prolonging VERP at long CLs of 300, 400, 450 and 500 ms ($P<0.05$, $n=9$). When the results were analyzed with two-way ANOVA with repeated measures, the effects of HMR 1556 on VERP were significantly more pronounced at long than short CL (i.e. a treatment by CL interaction; $P<0.0001$, $n=9$). When the change in VERP vs CL relation was fitted with linear regression, the slope of the line for 100 nM HMR 1556 (added to 7.5 nM dofetilide) was significantly steeper than that of 7.5 nM dofetilide alone (0.081 ± 0.013 vs 0.028 ± 0.018, $P<0.01$, $n=9$), establishing a greater degree of reverse rate-

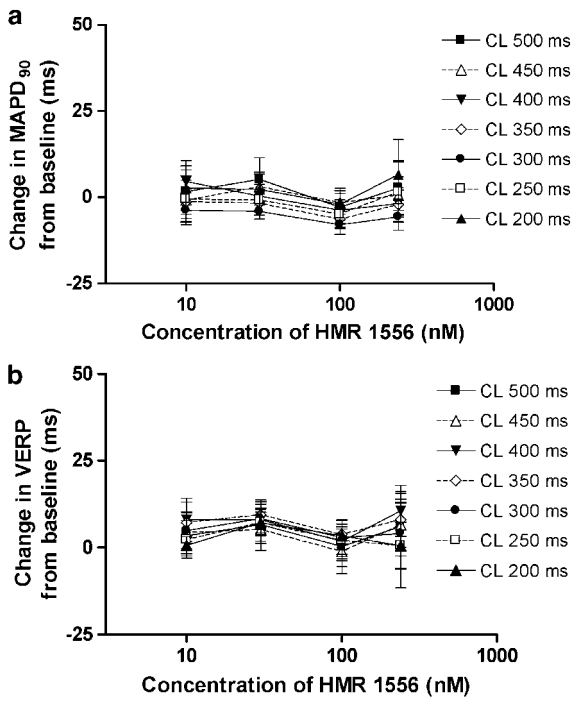

Figure 2 Effects of I_{Ks} block by cumulative addition of HMR 1556 (10, 30, 100 and 240 nM) on the change in $MAPD_{90}$ (a) and VERP (b) from baseline at various CLs in isolated rabbit ventricles. HMR 1556 concentration in nM is plotted on a log scale. Mean ± s.e.m., $n = 7$ for baseline, 10, 30 and 100 nM and $n = 4$ for 240 nM.

dependence for combined I_{Kr} and I_{Ks} block compared to I_{Kr} block alone. No spontaneous TdP-like arrhythmias were observed in any single or combined drug study.

Effects of dofetilide, HMR 1556 and their combination on coronary flow rate, left ventricular systolic pressure and QRS duration

As any potential drug-induced alterations in cardiac hemodynamics, contractility and conduction might affect the observed electrophysiological effects, coronary flow rate, left ventricular systolic pressure and QRS duration were monitored respectively. Although there was a decrease in coronary flow rate from baseline of $44 \pm 6\,ml\,min^{-1}$ to final of $38 \pm 4\,ml\,min^{-1}$ ($P < 0.05$, $n = 6$) and left ventricular systolic pressure from baseline of 108 ± 13 mmHg to final of 69 ± 7 mmHg ($P < 0.001$, $n = 6$) over time in control hearts, these time-dependent changes were not significantly affected by drug additions (single or combined). In addition, QRS duration remained constant in all groups (e.g. from baseline of 42 ± 3 ms to final of 42 ± 4 ms in the control group, $P = 0.16$, $n = 6$ and from baseline of 38 ± 1 ms to final of 40 ± 2 ms in the combined drug group, $P = 0.30$, $n = 9$).

Computer-simulated I_{Ks}, I_{Kr} and combined I_{Kr} and I_{Ks} block

Figure 4a demonstrates the computer-simulated action potential recordings during baseline, I_{Ks} block alone, I_{Kr} block alone and combined I_{Kr} and I_{Ks} block at CL 500 ms. Summary data for the computer-simulated APD_{90} at various conditions and

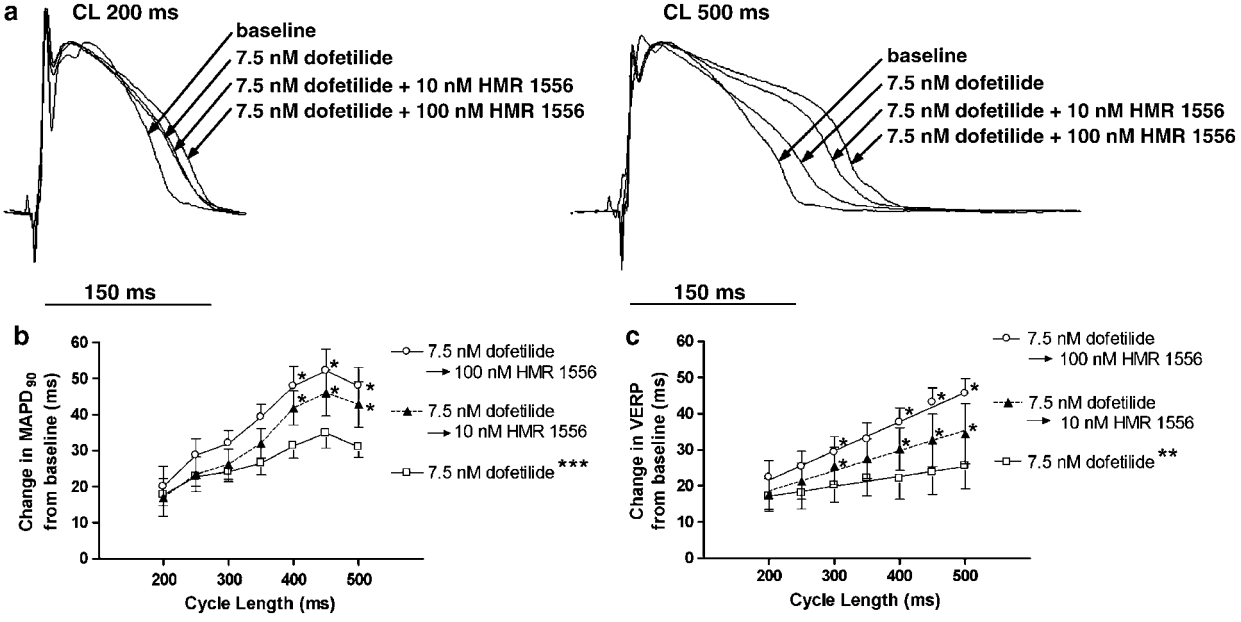

Figure 3 Representative MAP recordings before and after the administration of 7.5 nM dofetilide followed by 10 and 100 nM HMR 1556 at CLs 200 and 500 ms (a). Effects of combined I_{Kr} and I_{Ks} block by dofetilide and HMR 1556 on the change in $MAPD_{90}$ (b) and VERP (c) from baseline at various CLs. Mean ± s.e.m., $n = 9$. *$P < 0.05$ vs 7.5 nM dofetilide alone, **$P < 0.01$ and ***$P < 0.001$ vs untreated control. Endocardial MAP signals were obtained from the left ventricular apex of the perfused rabbit hearts. After baseline measurement, 7.5 nM dofetilide and cumulative addition of HMR 1556 (10 and 100 nM) were performed. Measurements were repeated 15 min after each administration. Amplitude of the MAP recordings in (a) ranged from 2.9 to 7.0 mV, and was rescaled for the figure. The variation in MAP morphology and amplitude can be due to changes in contact pressure between the MAP catheter and tissue, and do not necessarily reflect changes in specific ionic currents. Such variability is consistent with the known reported feature of MAP (Franz, 1999).

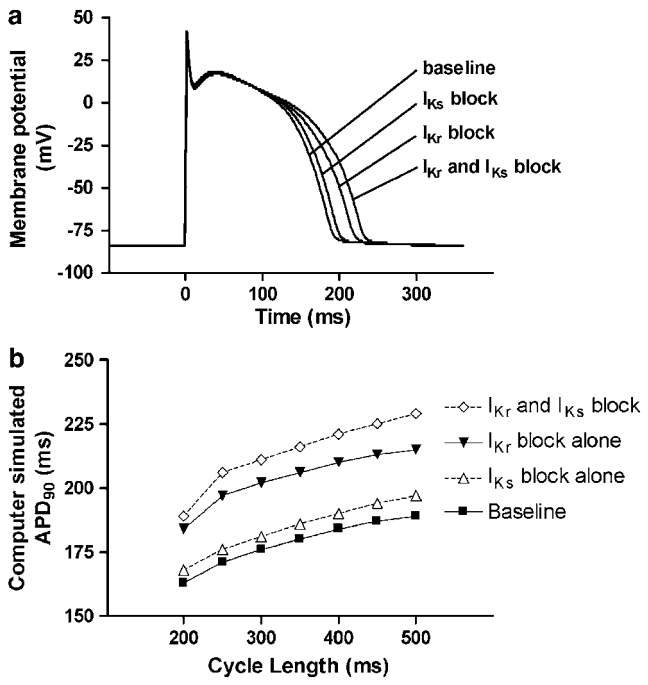

Figure 4 Representative recordings of computer-simulated action potential in rabbit ventricular myocytes during baseline, I_{Ks} block alone, I_{Kr} block alone and combined I_{Kr} and I_{Ks} block at 2 Hz (a). Summary data of the computer-simulated APD_{90} of rabbit ventricular myocytes under the above conditions (b). Complete I_{Ks} block was simulated by decreasing the conductance from 0.02 to 0 mS μF^{-1}. I_{Kr} block was simulated by decreasing the conductance from 0.04 to 0.028 mS μF^{-1}. The combined I_{Kr} and I_{Ks} block was simulated by implementing both of these settings. Results were generated by LabHEART version 4.9.5 (Puglisi & Bers, 2001).

CLs are illustrated in Figure 4b. Complete block of I_{Ks} resulted in a modest increase in APD_{90} by 4–8 ms at CL 200–500 ms, respectively. I_{Kr} block alone prolonged APD_{90} by 21–26 ms with little reverse rate-dependence. In the presence of I_{Kr} block, I_{Ks} block further prolonged APD_{90} by 7–13 ms at CL 200–500 ms, respectively, with increased reverse rate-dependence.

Discussion

The primary objective of this study was to test the ability of I_{Ks} block to attenuate the reverse rate-dependence of I_{Kr} block. The principal finding is that I_{Ks} block increased rather than reduced the reverse rate-dependence of I_{Kr} block. Rabbit I_{Ks} did not contribute significantly to cardiac repolarization unless under the condition of diminished repolarization reserve by I_{Kr} block, particularly at slow heart rates. Such novel rate-dependent interaction between I_{Kr} and I_{Ks} was qualitatively reproduced by computer simulations using a previously published computer model of rabbit ventricular myocyte (Puglisi & Bers, 2001).

Rate-dependent profile of combined I_{Kr} *and* I_{Ks} *block*

Reverse rate-dependence of I_{Kr} block was first suggested to be due to an increase in I_{Ks} during rapid heart rates in guinea pig myocytes (Jurkiewicz & Sanguinetti, 1993). However, the deactivation kinetics of I_{Ks} in guinea pig myocytes are very slow (deactivation time constant, $\tau \sim 756$ ms) compared to those of dog ($\tau \sim 89$ ms), rabbit ($\tau \sim 157$ ms) and human ($\tau \sim 122$ ms) (all at -40 mV) (Chinn, 1993; Varro *et al*., 2000; Lengyel *et al*., 2001; Virag *et al*., 2001). Thus, the role of I_{Ks} in reverse rate-dependence may not necessarily apply to species with rapid I_{Ks} deactivation kinetics, where no significant I_{Ks} accumulation occurs at physiological rapid rates (Stengl *et al*., 2003). In the present study, I_{Ks} block by HMR 1556 increased the reverse rate-dependence of I_{Kr} block, suggesting that I_{Ks} accumulation at rapid heart rates is an unlikely mechanism for the reverse rate-dependence of dofetilide in rabbit ventricles. Reverse rate-dependence of I_{Kr} block has also been suggested to be due to the greater inward L-type Ca^{2+} current at slow rates, and Ca^{2+} channel activation and blockade were shown to increase and decrease reverse rate-dependence of I_{Kr} block, respectively (Gjini *et al*., 1998). The reverse rate-dependent nature of I_{Kr} block is expected to promote more complete I_{Ks} activation at long CLs, but result in little I_{Ks} activation at short CLs. As I_{Ks} activation is greater in situations of diminished repolarization reserve, one would expect less I_{Ks} activation, even in the presence of I_{Kr} block, at short CLs. The steep reverse rate-dependence of the I_{Kr}–I_{Ks} blocker combination is thus likely a consequence of the brief time available for both I_{Kr} and I_{Ks} activation at short CLs.

Comparison with previous studies

The role of I_{Ks} in cardiac electrophysiology remained largely unknown until the recent development of selective I_{Ks} blockers (Gogelein *et al*., 2000; Gerlach *et al*., 2001; Thomas *et al*., 2003). For example, HMR 1556 is a highly selective I_{Ks} blocker which only starts to block I_{Kr}, I_{K1}, I_{to} and I_{Ca} at concentrations that are 300-fold higher than the IC_{50} of I_{Ks} (Gogelein *et al*., 2000). The contribution of I_{Ks} to cardiac repolarization was first shown to be increased during rapid heart rate as a result of its incomplete deactivation and accumulation in guinea pig myocytes (Jurkiewicz & Sanguinetti, 1993). Therefore, I_{Ks} block is expected to prolong APD at high heart rates, and to be associated with high therapeutic efficacy towards ventricular tachyarrhythmias and a lower risk of drug-induced proarrhythmia. I_{Ks} block by chromanol 293B was shown to prolong APD in human and guinea pig ventricular myocytes in a rate-independent manner (Bosch *et al*., 1998). However, chromanol 293B and the benzodiazepine I_{Ks} blocker L-735,821 failed to prolong QTc in perfused rabbit ventricles and papillary muscle APD (Lengyel *et al*., 2001). Similarly, chromanol 293B and HMR 1556 did not markedly prolong canine (by $<7\%$) or human ventricular APD (by $<3\%$), respectively, unless it was in the presence of I_{Kr} blockers (Varro *et al*., 2000; Biliczki *et al*., 2002, Jost *et al*., 2005). These studies agree with our observations that HMR 1556 alone did not significantly prolong $MAPD_{90}$ and VERP in perfused rabbit ventricles unless in the presence of dofetilide. In a recent *in vivo* canine study, HMR 1556 alone prolonged ventricular refractoriness without reverse rate-dependence, and its effects were potentiated in the presence of dofetilide, independent of frequency (Nakashima *et al*., 2004). However, dofetilide alone did not demonstrate reverse rate-dependence in Nakashima *et al*.'s study (2004), so how HMR 1556 modulated the frequency profile of dofetilide remained unclear. In contrast, we observed that HMR 1556 alone did not prolong rabbit ventricular refractoriness unless it was in the presence of dofetilide. Our

observation that HMR 1556 accentuated the reverse rate-dependence of dofetilide represents a novel view on the rate-dependent interaction between I_{Kr} and I_{Ks} in rabbits.

Our data suggest that combined blockade of I_{Kr} and I_{Ks} leads to increased reverse rate-dependent prolongation of APD, a condition under which arrhythmias related to early afterdepolarizations are likely to occur. However, no TdP was observed in any of the experiments. It is possible that the drug concentrations used in the combined drug study were not high enough to cause TdP, or that the 'I_{Kr} block-induced I_{Ks}' might still be small under the condition of sympathetic denervation in the *in vitro* perfused rabbit ventricles, as β-adrenergic stimulation activates I_{Ks} (Marx *et al*., 2002; Stengl *et al*., 2003; Volders *et al*., 2003).

Previous studies have shown that HMR 1556 is a potent I_{Ks} blocker with an IC_{50} of 34 nM in guinea pig ventricular myocytes (Gogelein *et al*., 2000), and 100 nM HMR 1556 was able to inhibit over 90% of canine I_{Ks} (Thomas *et al*., 2003). Therefore, the HMR 1556 concentrations used in our single drug study (10–240 nM) were likely high enough to block I_{Ks} significantly. Moreover, 100 nM HMR 1556 clearly prolonged $MAPD_{90}$ and VERP in the presence of dofetilide in the present study. Therefore, the lack of effects by HMR 1556 alone is likely due to little functional baseline I_{Ks} in rabbits. Another potential explanation is that sympathetic denervation in the perfused rabbit ventricles would lead to incomplete I_{Ks} activation. This may also explain the apparent discrepancies between the discernible I_{Ks} blocking effects in Nakashima *et al*.'s (2004) *in vivo* canine study and the lack of I_{Ks} blocking effects in various *in vitro* studies (Varro *et al*., 2000; Biliczki *et al*., 2002, Jost *et al*., 2005), including the present one.

Earlier reports have suggested that I_{Ks} is absent with no measurable current in rabbit ventricles (Carmeliet, 1992; Veldkamp *et al*., 1993; Howarth *et al*., 1996). However, later studies confirmed the existence of rabbit I_{Ks} (Salata *et al*., 1996; Cheng *et al*., 1999; Lu *et al*., 2001) with relatively slow activation but rapid deactivation kinetics (Lengyel *et al*., 2001). It is likely that I_{Ks} contributes to cardiac repolarization only under conditions which favor its activation and accumulation. When I_{Kr} is suppressed resulting in a prolonged APD, I_{Ks} becomes more fully activated and contributes to a greater extent to cardiac repolarization (Jurkiewicz & Sanguinetti, 1993). Short APD at shorter CLs might only allow a little I_{Ks} activation due to its slow activation. On the other hand, long APD at longer CLs would favor I_{Ks} activation, but the longer diastolic interval might lead to substantial I_{Ks} recovery with no significant accumulation due to its rapid deactivation. Therefore, I_{Ks} recruitment may not be solely dependent on the absolute APD, but the diastolic interval should also be considered. The following observation illustrates this consideration. Although the baseline $MAPD_{90}$ at CL 500 ms and the post dofetilide $MAPD_{90}$ at CL 300 ms were similar at $\sim$150 ms, HMR 1556 only further prolonged repolarization in the presence of dofetilide. At CL 300 ms, the diastolic interval was shorter ($\sim$150 ms) than at CL 500 ms ($\sim$350 ms). Thus, at CL 300 ms, the combination of prolonged $MAPD_{90}$ by dofetilide and short diastolic interval would favor I_{Ks} activation and accumulation, which contributed significantly to repolarization.

Azimilide, a class III antiarrhythmic drug with combined I_{Kr} and I_{Ks} blocking effects, is currently being evaluated clinically in humans. Although the rate-dependent effects of azimilide are not entirely clear, numerous studies indicate that the effects of azimilide on ventricular APD and refractoriness are in fact reverse rate-dependent (Fermini *et al*., 1995; Groh *et al*., 1997; Bril *et al*., 1998; Gintant, 1998), which are consistent with our experimental observations.

To further understand our experimental results, we utilized a computer model of rabbit ventricular myocytes (Puglisi & Bers, 2001) to simulate combined I_{Kr} and I_{Ks} block. We qualitatively confirmed our findings that I_{Ks} block alone produced little APD prolongation, but exerted a greater effect in the presence of I_{Kr} block with enhanced reverse rate-dependence. Although the computer simulations predicted that complete I_{Ks} block would prolong APD by a modest 4–8 ms, this was not observed experimentally at the highest HMR 1556 concentration applied (240 nM). This discrepancy could originate from incomplete I_{Ks} block experimentally, although previous studies reported that 100 nM HMR 1556 blocked canine I_{Ks} by more than 90% (Thomas *et al*., 2003). Moreover, computer models of myocytes represent functional properties of ion currents that may not accurately reflect the true channel characteristics. For example, currents in our rabbit model assumed Hodgkin and Huxley behavior, which is a simplification of the true gating features of biological channels (Aldrich *et al*., 1983). In addition, other currents not previously identified in rabbit ventricular myocytes are not incorporated into the model.

Clinical implications

Our data highlight the importance of I_{Ks} as an important reserve current that prevents the excessive prolongation of repolarization by I_{Kr} block, particularly at slow heart rates. In long QT syndrome type 1 (LQT1) caused by mutations in *KvLQT1* which encodes for I_{Ks}, some patients have 'non-manifest' phenotype with little or no baseline QTc prolongation, likely because I_{Ks} does not contribute significantly to repolarization unless in the presence of increased sympathetic activation or decreased function of other repolarization currents. These patients are likely to be affected by the impairment of repolarization reserve, which may not cause changes in the resting ECG. In addition, downregulation of K^+ currents and action potential prolongation are consistently observed in myocytes isolated from various heart failure animal models and heart failure patients. The diminished repolarization reserve in patients with heart failure may account for excessive APD prolongation, with increased propensity to develop TdP, following the use of class III antiarrhythmic agents (Nabauer & Kaab, 1998). The observation that I_{Ks} block increased the reverse rate-dependence of I_{Kr} block suggests that the use of combined I_{Kr} and I_{Ks} blocking agents is potentially proarrhythmic, especially under the pathological condition of heart failure with diminished repolarization reserve.

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