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Electrophysiological effects of azimilide in an in vitro model of simulated-ischemia and reperfusion in guinea-pig ventricular myocardium

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Abstract

There are few investigations on azimilide effects during ischemia/reperfusion. We have therefore investigated low concentrations of azimilide (0.1 and 0.5 μmol/l) versus Controls on action potential parameters and occurrence of repetitive responses during simulated ischemia and reperfusion. An in vitro model of "border zone" in guinea-pig ventricular myocardium ($n=30$) was used. Azimilide 0.5 μmol/l lengthened action potential duration in normoxic but not in ischemic-like conditions. Therefore an increased dispersion of action potential duration at 90% of repolarization during simulated ischemia in presence of azimilide was seen. Upon reperfusion, both normal and reperfused myocardium showed azimilide-induced action potential duration increase. There was a neutral effect on the occurrence of arrhythmias during simulated ischemia; however azimilide showed significant ($P=0.033$) antiarrhythmic properties following reperfusion. To mimic I_{Kr} and I_{Ks} blocking properties of azimilide we further used dofetilide 10 nmol/l with HMR 1556 1 nmol/l ($N=9$), which was accompanied by less severe shortening ($P<0.05$) of action potential duration at 90% of repolarization at 30 min of ischemic-like conditions ($-43\pm9\%$), as compared with azimilide 0.5 μmol/l ($-64\pm5\%$) but similar to what seen with azimilide 0.1 μmol/l ($-53\pm5\%$) and Controls ($-52\pm6\%$). During reperfusion, 2/9 (22%) preparations had sustained activities, which was less than what observed in Controls (5/10, 50%) and with azimilide 0.5 μmol/l (0/10, 0%), although not statistically different (respectively, $P=0.35$ and $P=0.21$). Lack versus homogenous class III effects of azimilide in respectively simulated ischemia and reperfusion may explain its different efficacy on arrhythmias, although prevention of reperfusion arrhythmias calls for other than just its I_{Kr} and I_{Ks} blocking properties.

Keywords: Azimilide; Dofetilide; HMR 1556; Ischemia–reperfusion; Delayed outward rectifier; (Guinea-pig)

1. Introduction

Azimilide has shown antiarrhythmic efficacy in animal models of atrial (Nattel and St-George, 1998) and ventricular arrhythmias both in vivo (Black et al., 1993; Drexler et al., 1996; Qi et al., 1999) and in vitro (McIntosh et al., 1994; Mittelstadt et al., 1997; Dong et al., 2004) and against atrial fibrillation in man (Pritchett et al., 1998). It is a chlorophenylfuranyl compound differing from other class III antiarrhythmic agents by *i*) lack of the chemical methylsulfamide group (Salata and Brooks, 1997) as dofetilide and sotalol have and *ii*) its ability to block both components of the delayed outward rectifier current, I_{Kr} and I_{Ks}, although a more potent inhibition of I_{Kr} as compared to I_{Ks} has also been reported along with other blocking properties on I_{CaL} and both α- and β-receptors (Fermini et al., 1995; Salata and Brooks, 1997; Yao and Tseng, 1997). As a result, definite action potential prolongation (Tatla et al., 1993; McIntosh et al., 1994; Groh et al., 1997) and

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concomitant increase of the effective refractory period (McIntosh et al., 1994; Qi et al., 1999), similar to other class III agents, are seen with azimilide in normoxic conditions.

Previous reports from our Laboratory have shown that pure I_{Kr} blockers such as D-sotalol and dofetilide exhibited proarrhythmic effects in an in vitro model aimed to mimic the "border zone" existing between normal and ischemic-like ventricular regions in the guinea-pig (Picard et al., 1998; Rouet et al., 2000). Ventricular arrhythmias were explained by differential effects of these compounds on action potential durations, in normal versus ischemic-like zones, leading to an increased dispersion of action potential's duration. With the exception of dofetilide at very low concentration (5 nmol/l), both compounds had minimal effects on action potential duration in ischemic-like conditions, which is a known characteristic of pure I_{Kr} blockers in these experiments (McIntosh et al., 1994; Dong et al., 2004) and which was interpreted as a possible consequence of overwhelming I_{KATP} and net increase of potassium conductance resulting in action potential shortening anyhow (Picard et al., 1998; Rouet et al., 2000). Furthermore, whenever an antiarrhythmic action was observed during reperfusion this was due to β-receptor blocking properties, if any (Picard et al., 1998).

In the present investigation, electrophysiological effects of azimilide on the action potential time course and the initiation of ventricular arrhythmias in the abovementioned in vitro model of ischemic-like and reperfused right ventricular myocardium were studied, based on the hypothesis that concomitant I_{Kr} and I_{Ks} blockade properties of azimilide may have different effects on arrhythmogenesis as compared to pure I_{Kr} blocking compounds such as dofetilide or D-sotalol. We also performed complementary experiments to see whether combining I_{Kr} and I_{Ks} blockade by use of dofetilide (Kiehn et al., 1994) and HMR 1556 (Gögelein et al., 2000), it was possible to mimic azimilide effects.

2. Materials and methods

Care of the animals conformed to the recommendations of the Helsinki Declaration, and the study was performed in accordance 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) and in accordance with the regulations of the official edict of the French Ministry of Agriculture.

2.1. Materials

Guinea-pigs of either sex weighing 300 to 400 g were euthanized under anesthesia with sodium pentobarbital 125 mg/kg i.p. The hearts were quickly removed and a standard strip (16 × 8 mm) of right ventricular myocardium was dissected from the free wall and placed in a special perfusion chamber bath (volume of 5 ml) partitioned into 2 compartments by a thin latex membrane. This latex membrane is perforated at its bottom allowing the ventricular strip to be passed through and so to be divided into two zones, called the Normal Zone and the Altered Zone, respectively. The preparation was pinned, endocardial surface upward, to the silicon base of the bath.

This double compartment allowed the two parts of the same ventricular strip to be independently superfused at a rate of 2 ml min^{-1}. The continuity of the partition was tested at the end of each experiment by means of dye injection (methylene blue) into one of the compartments.

Temperature at the level of the double chamber, including that of incoming fluids, was controlled and maintained to 36.5 ± 0.5 °C by a circulating thermostat-controlled bath (Polystat 5HP, Bioblock, France).

2.2. Superfusion solutions

Studies were performed in Tyrode's solution oxygenated with a mixture of 95% oxygen and 5% carbon dioxide (pO_2 and pCO_2 at 510 ± 20 and 34 ± 2 mmHg, respectively). Studies were performed in Tyrode's solution oxygenated with a mixture of 95% oxygen and 5% carbon dioxide (pO_2 and pCO_2 at 510 ± 20 and 34 ± 2 mmHg, respectively). The composition of the Tyrode's solution was (in mmol/l): Na^+, 135; K^+, 4; Ca^{2+}, 1.8; Mg^{2+}, 1.0; $H_2PO_4^-$, 1.8; HCO_3^-, 25; Cl^-, 117.8; and glucose, 5.5. The pH was 7.35 ± 0.05 (fitted with diluted HCl). Modified Tyrode's solution mimicking ischemia were also used. Ischemic-like solution differed from the normal one by 1) increased extracellular potassium concentration (12 mmol/l), 2) decreased HCO_3^- concentration (9 mmol/l) leading to decreased pH (6.90 ± 0.05), 3) decreased pO_2 (80 mmHg instead

Table 1
Electrophysiological effects of azimilide (0.1 and 0.5 μmol/l) on action potential parameters in normoxic conditions

Normal zone	Controls (n = 10)	Azimilide 0.1 μmol/l (n = 10)	Azimilide 0.5 μmol/l (n = 10)
RMP (mV)			
Baseline	−88 ± 1	−85 ± 1	−86 ± 1
Ischemia 30 min	−86 ± 2	−85 ± 1	−86 ± 1
Reperfusion 30 min	−87 ± 2	−86 ± 1	−86 ± 1
V_{max} (V/s)			
Baseline	222 ± 28	284 ± 25	255 ± 21
Ischemia 30 min	185 ± 16	228 ± 15	261 ± 15
Reperfusion 30 min	202 ± 16	247 ± 17	283 ± 49
APA (mV)			
Baseline	119 ± 1	121 ± 1	120 ± 1
Ischemia 30 min	117 ± 3	120 ± 1	122 ± 1
Reperfusion 30 min	119 ± 2	120 ± 2	122 ± 1
APD_{50} (ms)			
Baseline	116 ± 5	91 ± 4	95 ± 3
Ischemia 30 min	116 ± 9	92 ± 4	100 ± 4
Reperfusion 30 min	120 ± 9	99 ± 5[a]	109 ± 5[b]
APD_{90} (ms)			
Baseline	141 ± 6	113 ± 4	119 ± 3
Ischemia 30 min	140 ± 10	114 ± 4[a]	127 ± 4
Reperfusion 30 min	145 ± 10	121 ± 5	138 ± 6[b]

RMP: resting membrane potential; V_{max}: maximal upstroke velocity; APA: action potential amplitude; APD_{50}: action potential duration at 50% of repolarization; APD_{90}: action potential duration at 90% of repolarization. Values expressed as mean ± S.E.M.

[a] $P < 0.05$.

[b] $P < 0.01$ versus baseline values (Analysis of variance for repeated measures followed by Dunnet's test).

Table 2
Electrophysiological effects of azimilide (0.1 and 0.5 μmol/l) on action potential parameters in ischemic-like and reperfusion conditions

Altered zone	Controls ($n=10$)	Azimilide 0.1 μmol/l ($n=10$)	Azimilide 0.5 μmol/l ($n=10$)
RMP (mV)			
Baseline	−88±1	−85±1	−86±1
Ischemia 30 min	−66±3[b]	−57±2[b]	−56±3[b]
Reperfusion 30 min	−89±1	−87±1	−86±1
V_{max} (V/s)			
Baseline	277±37	214±16	265±21
Ischemia 30 min	126±22[b]	92±21[b]	100±16[b]
Reperfusion 30 min	241±22	258±34	271±24
APA (mV)			
Baseline	119±2	120±1	121±1
Ischemia 30 min	89±5[b]	76±7[b]	79±5[b]
Reperfusion 30 min	122±1	121±2	123±2
APD_{50} (ms)			
Baseline	113±5	101±4	99±5
Ischemia 30 min	52±8[b]	40±7[b]	26±5[b]
Reperfusion 30 min	119±2	116±6	116±3[b]
APD_{90} (ms)			
Baseline	134±5	124±5	122±7
Ischemia 30 min	65±10[b]	58±6[b]	45±6[b]
Reperfusion 30 min	145±4	143±5[a]	144±4[b]

RMP: resting membrane potential; V_{max}: maximal upstroke velocity; APA: action potential amplitude; APD_{50}: action potential duration at 50% of repolarization; APD_{90}: action potential duration at 90% of repolarization. Values expressed as mean±S.E.M.
[a] $P<0.05$.
[b] $P<0.01$ versus baseline values (Analysis of variance for repeated measures followed by Dunnet's test).

of 510 mmHg) by replacement of 95% O_2 and 5% CO_2 by 95% N_2 and 5% CO_2, and 4) complete withdrawal of glucose. These modifications of Tyrode's solution are believed to reproduce in vitro the electrophysiological abnormalities induced in vivo by ischemia (Morena et al., 1980).

Azimilide dihydrochloride (kindly provided by Procter and Gamble Pharmaceuticals, United States), was first diluted in water and then in Tyrode's final solution at 0.1 and 0.5 μmol/l. Dofetilide (gift of Pfizer Central Research, Sandwich, UK), was first diluted in ethanol–HCl (0.05N) and then in Tyrode's solution at 10 nmol/l. HMR 1556 (gift of Aventis, Frankfurt am Main, Germany) was prepared by initial dilution in DMSO and then into Tyrode's solution at 1 nmol/l. The two compartments, at appropriate time intervals, were superfused with drug containing solutions during both ischemic-like and reperfusion periods.

2.3. Data acquisition and analysis

The preparations were stimulated at a frequency of 1 Hz via bipolar Teflon-coated steel wire electrodes positioned near the two ventricular strip extremities either in the Normal Zone or the Altered Zone. Stimulation was applied either in one or the other half of the muscle preparation with a home-built commutator. Stimuli were rectangular pulses, 2 ms in duration and twice the diastolic threshold intensity (around 2–2.5 V) delivered by a programmable stimulator (SMP-310, Biologic, France). During the protocol, stimulation was stopped whenever spontaneous repetitive responses occurred, and an extrastimulus, 2 ms in duration and twice the intensity of the basic stimulus, was applied every four stimulations in an attempt to elicit triggered repetitive responses by a progressive increase in 5 ms steps of the time interval between the stimulus and the extrastimulus.

Transmembrane action potentials were recorded simultaneously in both ventricular regions by intracellular glass microelectrodes filled with KCl 3 mol/l (tip resistance 10 to 30 MΩ) coupled to Ag/AgCl microelectrode holders leading to the double input stage of a high impedance capacitance-neutralizing amplifier. The two reference silver–silver chloride electrodes were positioned in the superfusate of each chamber, close to the preparation. Action potentials were monitored on a digital memory oscilloscope (Gould Instrument Systems Inc, USA) and digitized by a device of automatic acquisition and processing of the action potential (DATAPAC, Biologic, France). The following action potential parameters were automatically recorded and measured: resting membrane potential, maximal upstroke velocity of action potential, action potential amplitude, and action potential duration measured respectively at 50% and 90% of full repolarization.

2.4. Experimental protocol

During a 120 min equilibration period, the two compartments were superfused with normal Tyrode's solution and the right ventricular muscle was stimulated at a frequency of 1 Hz. Thereafter, one chamber (called Altered Zone) was superfused during 30 min with the modified Tyrode's solution (ischemic-like period) and then submitted for 30 min to normal Tyrode's solution superfusion (reperfusion period), while the second compartment (Normal Zone) remained in normoxic conditions. During these two

Table 3
Effects of azimilide (0.1 and 0.5 μmol/l) on the incidence of electrical disturbances and arrhythmias during simulated ischemic conditions

Ischemic-like period	Conduction blocks	Triggered repetitive responses	Spontaneous repetitive responses			
			Total	Sustained activities (>10 APs)	Salvos (4–10 APs)	Extrasystoles (1–3 APs)
Controls ($n=10$)	40	30	70	40	30	50
Azimilide 0.1 μmol/l ($n=10$)	30	10	70	10	10	60
Azimilide 0.5 μmol/l ($n=10$)	10	10	60(+)	20	30	60

APs: action potentials.
Values are percent of preparations presenting disturbances.
(+) In order to detect a significant difference versus Controls with an α-error=0.05 and a power=0.75, $n=670$ (corrected for continuity).

phases of ischemia and then reperfusion, azimilide 0.1 or 0.5 μmol/l was superfused simultaneously in both Normal and Altered zones. Thus, the electrophysiological effects of azimilide at each concentration were investigated on: *i*) action potential time course parameters simultaneously in normal and ischemic-like conditions and *ii*) the incidence of electrical disturbances occurring around the border zone between normal and ischemic-like and reperfused myocardial regions. These effects were compared with results obtained in control conditions (ischemia-like and reperfusion in absence of azimilide).

Both during ischemia and reperfusion, electrical disturbances were recorded of the following categories: (1) myocardial conduction blocks, (2) triggered repetitive responses induced by a single extrastimulus, (3) spontaneous repetitive responses such as extrasystoles (1 to 3 spontaneous action potentials), salvos (4 to 9 spontaneous action potentials) and sustained arrhythmias (>10 spontaneous action potentials), as previously described (Rouet et al., 2000).

The same protocol was used to investigate the effects of dofetilide and HMR 1556 which were superfused at 10 and 1 nmol/l, respectively. These concentrations were selected based on previous experience (Rouet et al., 2000) and in order to mimic action potential prolongation seen with azimilide 0.5 μmol/l in normoxic conditions.

2.5. Statistical analysis

Data were expressed as mean±S.E.M. and percentages of variations with respect to initial values measured before initiation of the ischemic-like phase. Preparations were discarded if, before the onset of simulated ischemia, the following characteristics were not at least obtained: resting membrane potential −80 mV, maximal upstroke velocity 150 V/s and action potential duration at 90% of repolarization 100 ms.

Results were expressed as means±standard error of the mean (S.E.M.). In the two compartments, each cell served as its own control and significance of differences in absolute values was determined using analysis of variance (ANOVA) for repeated measures followed by Dunnet's test as compared to initial values. Significance of differences between groups was determined using 2-factor ANOVA. The Fisher's exact test was used for comparison of nonparametric categorical data. Differences were considered significant when $P<0.05$. The action potential parameters and the ratio of experiments exhibiting arrhythmias in azimilide study were analyzed from 10 experiments in each group. Power was calculated (see Tables 1–4). The action potential parameters and the ratio of experiments exhibiting arrhythmias in dofetilide-HMR 1556 study were analyzed from 9 experiments. Power was not considered. The dispersion of action potential duration was analyzed in the following way: the action potential duration at 90% of repolarization values were firstly normalized in each experiment to exclude the influence of individual differences at the beginning of each experiment. In this respect, the action potential duration at 90% of repolarization in each compartment at 0, 10, 20 and 30 min of ischemia-like conditions was expressed as a percentage of the value observed before initiation of ischemia-like conditions. Then, the resulting action potential duration at 90% of repolarization in Altered Zone was subtracted from the corresponding data in Normal Zone.

3. Results

3.1. Effects of azimilide on the action potential parameters in normoxic conditions

Main results are illustrated in Fig. 1A and reported in Table 1. In the Normal Zone, a 60 min Tyrode's superfusion did not affect any action potential parameters. Azimilide was devoid of significant effects on resting membrane potential, maximal upstroke velocity and action potential amplitude. Action potential durations were not significantly modified by 0.1 μmol/l azimilide except a slight decrease in action potential duration at 90% of repolarization (−7%, $P<0.05$) at 10 min. They were significantly increased by 0.5 μmol/l after 60 min superfusion (+15±4%, $P<0.01$), which was accompanied by an increase of action potential duration at 50% of repolarization (+16±5%, $P<0.01$).

3.2. Effects of azimilide on the action potential parameters in simulated ischemic conditions

Main results are illustrated in Figs. 1B and 2 and reported in Table 2. Following 30 min of simulated ischemia there was a significant membrane depolarization (−25±3%, $P<0.01$) and decreases in maximal upstroke velocity (−43±14%, $P<0.01$), action potential amplitude (−26±4%, $P<0.01$) and action potential duration at 50% (−55±6%, $P<0.01$) and 90% of repolarization (−52±6%, $P<0.01$) in Controls. Changes in azimilide groups were similar; in particular, after 30 min of

Table 4
Effects of azimilide (0.1 and 0.5 μmol/l) on the incidence of electrical disturbances and arrhythmias during reperfusion

Reperfusion period	Triggered repetitive responses	Spontaneous repetitive responses			
		Total	Sustained activities (>10 APs)	Salvos (4–10 APs)	Extrasystoles (1–3 APs)
Controls ($n=10$)	20	90	50	50	70
Azimilide 0.1 μmol/l ($n=10$)	10	40[a]	20	20	40
Azimilide 0.5 μmol/l ($n=10$)	0	50	0[b]	10	50

APs: action potentials.
Values are percent of preparations presenting disturbances.
[a] $P=0.057$ versus Controls (Two-tail Fisher's exact test; α-error=0.05; $n=20$; power=0.66).
[b] $P=0.033$ versus Controls (Two-tail Fisher's exact test; α-error=0.05; $n=20$; power=0.75).

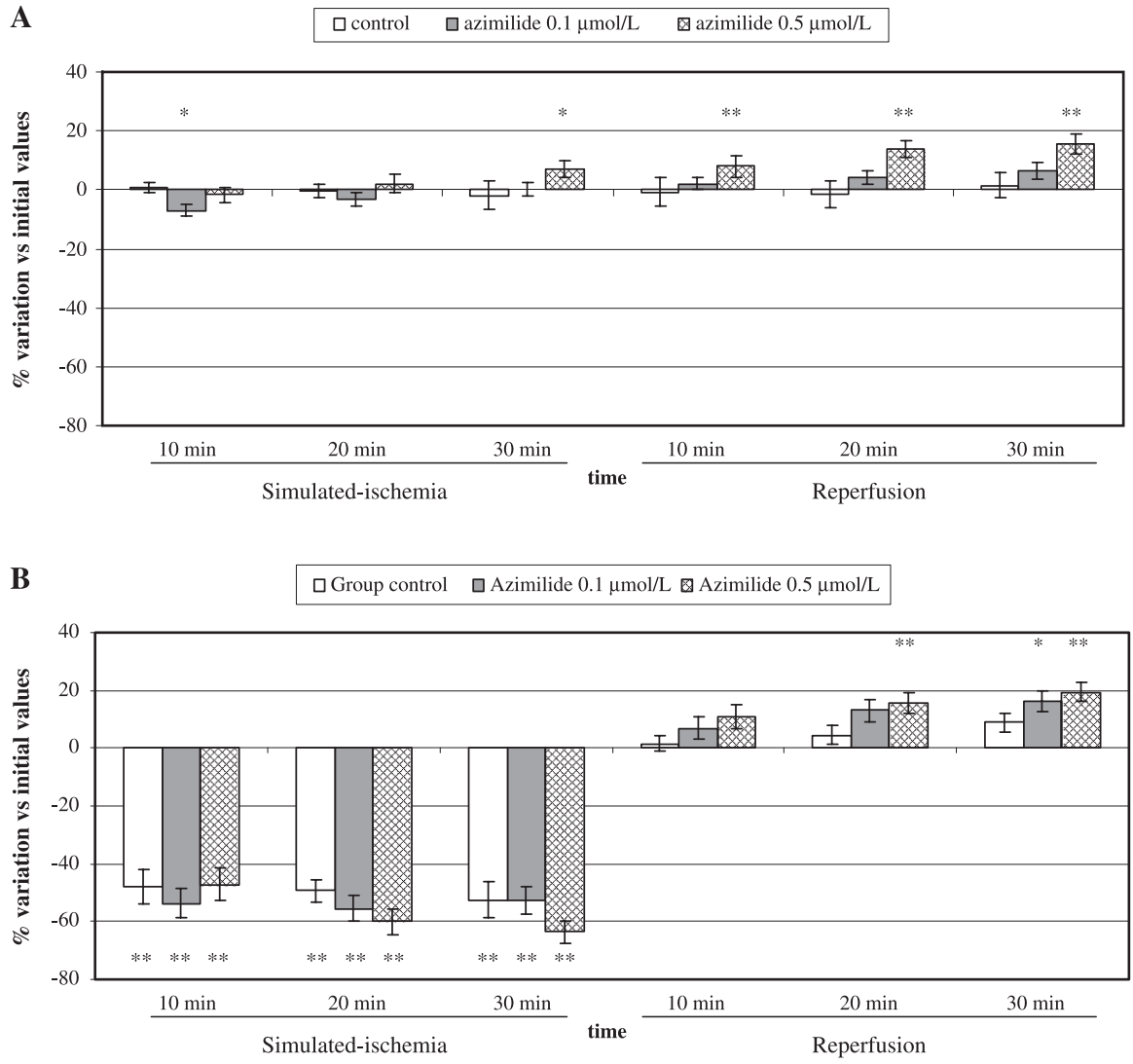

Fig. 1. Effects of azimilide 0.1 and 0.5 µmol/l on action potential duration at 90% of repolarization. Values were measured simultaneously in Normal Zone (Panel A) and Altered Zone (Panel B) during 30 min of simulated ischemia and during 30 min of reperfusion. Statistical analysis was performed on absolute values in each zone but results are presented as percent changes at each time-interval versus initial values. *$P<0.05$ and **$P<0.01$ (Analysis of variance for repeated measures followed by Dunnet's test). $n=10$ for each group.

simulated ischemia, action potential duration at 90% of repolarization was significantly ($P<0.01$) reduced as compared to baseline, respectively at $-53\pm5\%$ and $-64\pm5\%$, in presence of azimilide 0.1 and 0.5 µmol/l, which were not different from changes seen in Controls.

3.3. Effects of azimilide on action potential parameters during reperfusion

As shown in Table 2, Figs. 1B and 2, in the control group, the reperfusion period allowed a return of all action potential parameters in Altered Zone towards values observed in Normal Zone. Indeed, the percent variations of resting membrane potential, action potential amplitude, maximal upstroke velocity and action potential duration at 50% and 90% of repolarization following 30 min of reperfusion were respectively $+2\pm1\%$, $+2\pm1\%$, $-3\pm11\%$, $+7\pm4\%$ and $+9\pm3\%$ in Altered Zone and $-1\pm2\%$, $0\pm2\%$, $0\pm13\%$, $+3\pm6\%$ and $+2\pm4\%$ in Normal Zone (all NS versus baseline).

Moreover, after 30 min of reperfusion, the presence of azimilide induced a significant action potential lengthening, similar to that obtained in Normal Zone. Thus action potential duration at 50% and 90% of repolarization were respectively $+16\pm5\%$ (NS) and $+16\pm4\%$ ($P<0.05$) with azimilide 0.1 µmol/l, and $+19\pm4\%$ ($P<0.01$) and $+19\pm3\%$ ($P<0.01$) with azimilide 0.5 µmol/l.

3.4. Effects of azimilide on the dispersion of action potential duration at 90% of repolarization between the normoxic and the ischemic-like myocardium

As shown in Fig. 3, ischemic-like superfusion induced a significant increase in the dispersion of action potential duration ($P<0.0001$ for the time-factor of the analysis of variance). The

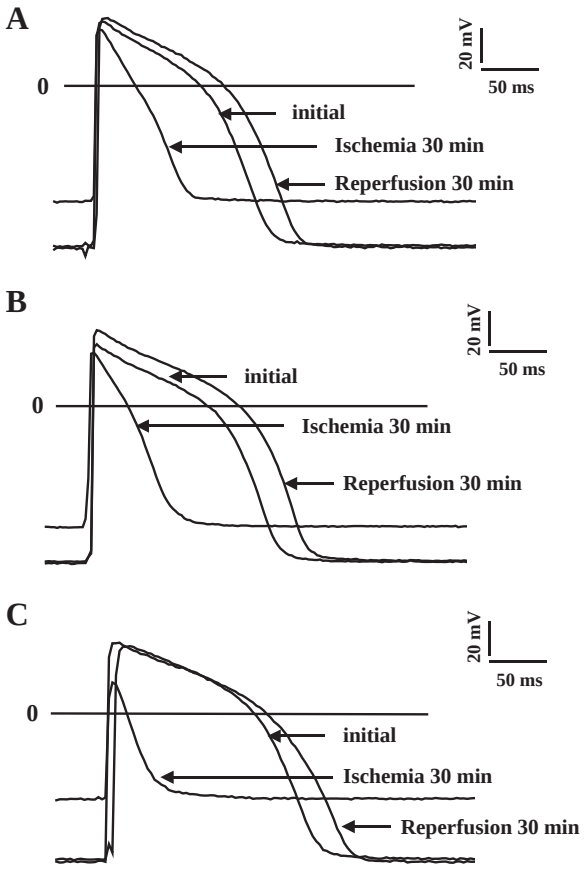

Fig. 2. Representative action potentials recorded in Altered Zone before the start of simulated ischemia, after 30 min of ischemic-like conditions and following 30 min of reperfusion. A: Controls; B: azimilide 0.1 μmol/l; C: azimilide 0.5 μmol/l.

dispersion tended to be worsened by azimilide 0.5 μmol/l although the difference from Controls was not statistically significant.

3.5. Effects of azimilide on simulated ischemia and reperfusion-induced arrhythmias

The different types of electrical disturbances and arrhythmias induced by simulated ischemia and reperfusion are illustrated in Figs. 4 and 5 and reported in Tables 3 and 4. There was a neutral effect of azimilide on both arrhythmogenesis and the occurrence of myocardial conduction blocks.

In Controls, arrhythmias occurred more frequently during reperfusion than during ischemic-like superfusion, yet these incidences were not statistically different. Thus, 9/10 control experiments exhibited spontaneous repetitive responses during reperfusion versus 7/10 during simulated ischemia. In detail, during reperfusion there were respectively 5/10, 5/10 and 7/10 control preparations with sustained activities, salvos and extra-systoles (Table 4) whereas during simulated ischemia these ratio were respectively 4/10, 3/10, and 5/10 (Table 3).

Azimilide was useful to prevent reperfusion-induced arrhythmias (Table 4) and particularly at 0.5 μmol/l. It was effective against sustained activities: whereas 5/10 control experiments exhibited sustained activities these were seen in 2/10 with azimilide 0.1 μmol/l (NS) and none with azimilide 0.5 μmol/l ($P=0.033$).

3.6. Effects of dofetilide and I_{Ks} block with HMR 1556 in this model

We concentrate here only on the effects on action potential duration at 90% of repolarization and, among spontaneous repetitive responses, on sustained activities observed during reperfusion. Baseline action potential durations at 90% of repolarization were respectively 147±3 and 153±9 ms in Normal Zone and Altered Zone, which was not significantly different from data observed in Controls. After 30 min of simulated ischemia action potential durations at 90% were respectively 150±9 (+2±6% versus baseline, NS) and 87±15 ms. Following 30 min of reperfusion they were respectively 167±5 (+14±2% versus baseline, $P<0.01$) and 179±8 ms. Thus, in the Altered Zone as compared to baseline, after 30 min of simulated ischemia, action

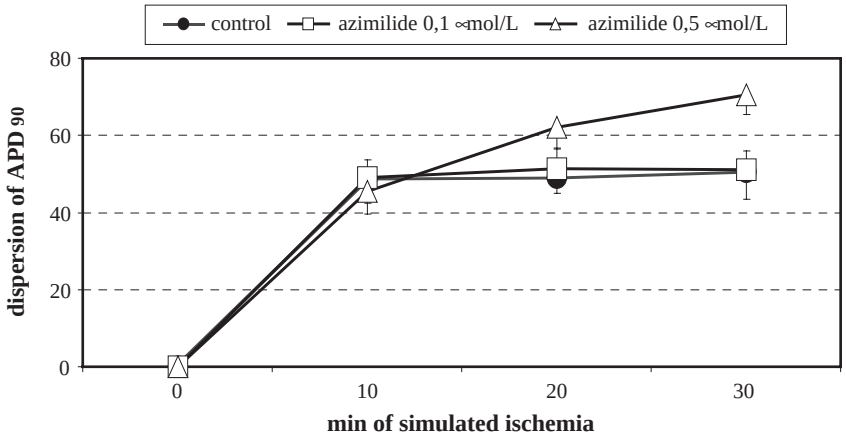

Fig. 3. Effects of azimilide 0.1 and 0.5 μmol/l on dispersion of action potential duration at 90% of repolarization between Normal Zone and Altered Zone during simulated ischemia. Dispersions are represented by subtraction of normalized action potential duration at 90% of repolarization in Altered Zone to normalized action potential duration at 90% of repolarization in Normal Zone. In all groups, simulated ischemia increased dispersion of action potential duration at 90% of repolarization (Analysis of variance for repeated measures: $P<0.0001$ for time in each group). $n=10$ for each group. APD_{90}= action potential duration at 90% of repolarization.

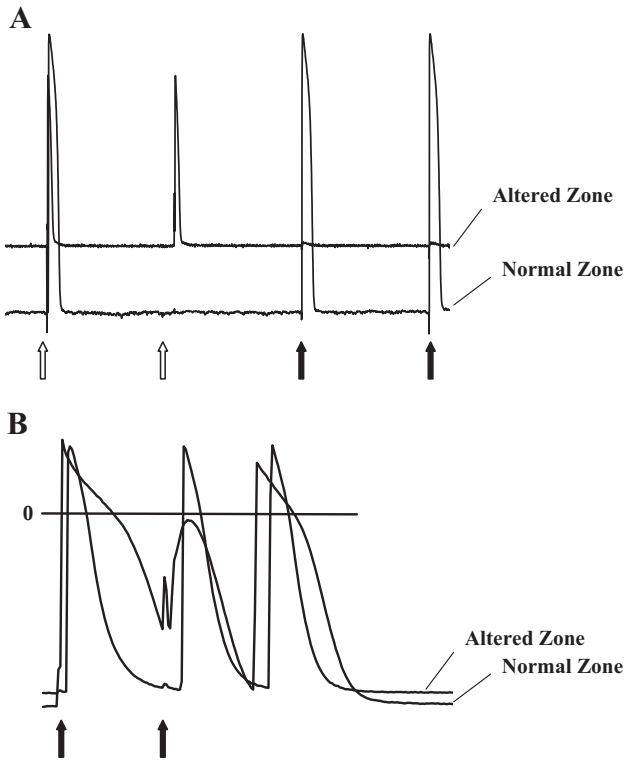

Fig. 4. Representative recordings of electrical disturbance and arrhythmias occurring during simulated ischemia or reperfusion. A: Conduction block between Normal Zone and Altered Zone. B: triggered repetitive responses induced by a single extrastimulus. Stimulation was applied in Normal Zone (solid arrows) or in Altered Zone (open arrows). Traces show action potentials recorded simultaneously in Normal Zone and Altered Zone.

potential duration at 90% of repolarization was significantly ($P<0.01$) reduced ($-43\pm9\%$,), which was significantly ($P<0.05$) less than what observed with azimilide 0.5 μmol/l ($-64\pm5\%$) but similar to what seen with azimilide 0.1 μmol/l ($-53\pm5\%$) and Controls ($-52\pm6\%$). Following 30 min of reperfusion, action potential duration at 90% of repolarization was significantly increased ($+17\pm1\%$, $P<0.01$) in the Altered Zone as compared to baseline, which was significantly more ($P<0.05$) than what observed in Controls ($+9\pm3\%$), but similar to what seen with azimilide 0.1 ($+16\pm4\%$) and 0.5 μmol/l ($+19\pm3\%$). Finally, during reperfusion, 2/9 (22%) preparations had sustained activities, which was less than what observed in Controls (50%) and with azimilide 0.5 μmol/l (0%), although not statistically different (respectively, $P=0.35$ and $P=0.21$).

4. Discussion

Azimilide concentration dependently increased action potential duration at 90% of repolarization in normoxic guinea-pig ventricular myocardium, an effect that was not evident during simulated ischemia, leading to a dispersion of action potential durations much similar to those of control experiments. Therefore, disappearance of azimilide-induced class III effects in simulated ischemic conditions, even at higher concentration, failed to prevent arrhythmias during ischemic-like conditions. Upon reperfusion, there was a more homogenous effect on action potential durations and sustained activities were prevented. It is unclear, however, whether this was due to I_{Kr} and I_{Ks} blocking properties of azimilide since dofetilide plus HMR 1556 experiments were not able to mimic its potent antiarrhythmic action.

4.1. Electrophysiological effects of azimilide on action potential parameters and arrhythmias in ischemic-like conditions

While in normoxic ventricular myocytes, at physiological $[K^+]_o$, I_{Kr} and I_{Ks} play a major role to determine action potential duration (Lu et al., 2001; Clancy et al., 2003), unitary conductances of K_r and K_s channels are relatively

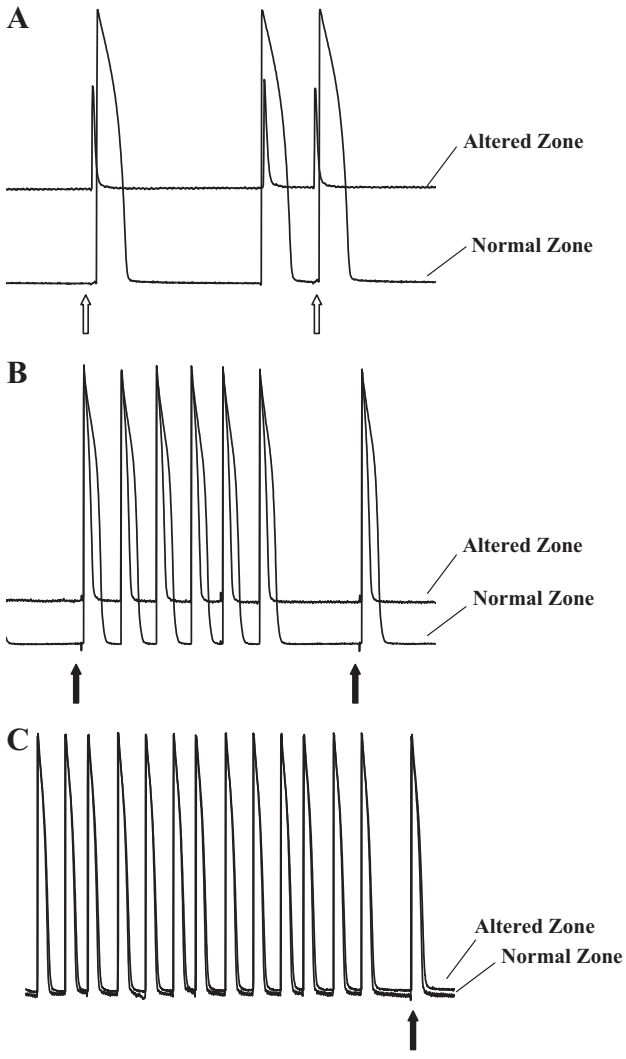

Fig. 5. Representative recordings of spontaneous repetitive responses occurring during simulated ischemia or reperfusion. A: extrasystoles (1 to 3 spontaneous action potentials). B: Salvos (4 to 6 spontaneous action potentials). C: sustained activities (more than 10 spontaneous action potentials). Stimulation was applied in Normal Zone (solid arrows) or in Altered Zone (open arrows). Traces show action potentials recorded simultaneously in Normal Zone and Altered Zone.

low [respectively in the order of 2 and 16 pS (Kiehn et al., 1996; Sesti and Goldstein, 1998)] and their distribution relatively scarce [overall there is one delayed rectifier K channel/μmm^2 of cell membrane (Coetzee, 1992)]. On the other hand, K_{ATP} channels which are activated in ischemic-like conditions, are abundant [around 10/μmm^2 of cell membrane (Coetzee, 1992; Hamada et al., 1998)] and have a much higher unitary conductance [around 90 pS (Shinbo et al., 1997)]. It is understandable that when I_{KATP} occurs and does effectively reduce action potential duration (Coetzee, 1992) this may overshadow the role of both I_{Kr} and I_{Ks} block during ischemic-like conditions (Billman, 1994; Rouet et al., 2000). It is therefore possible that the lack of class III effect of azimilide seen here during simulated ischemia, at $[K^+]_o$ of 12 mmol/l, may relate to overwhelming I_{KATP} in presence of I_{Kr} and I_{Ks} block insufficient to compensate for increased potassium conductance due to the opening of K_{ATP} channels. Alternatively, acidic conditions of our experiments may have weakened azimilide effects on I_{Kr} (Dong et al., 2004).

The differential effects of azimilide on action potential duration under normoxic versus ischemic-like conditions led to a dispersion of action potential duration that was similar to that seen in control experiments. One can notice that at the highest azimilide concentration, the dispersion of action potential duration increased (Fig. 3). Since arrhythmias seen in our model have been interpreted (Picard et al., 1998) as the consequence of re-entrant movements (Wit et al., 1974) around the border zone between normal and ischemic-like tissues, there might be a cause and effect relationship between abolished class III effect of azimilide in ischemic-like conditions and its neutral effect on arrhythmogenesis.

This neutral effect of azimilide on arrhythmogenesis was clearly different from those obtained in our model with D-sotalol (Picard et al., 1998) and dofetilide (Rouet et al., 2000) which were pro-arrhythmic. IC_{50} of I_{Kr} block due to D-sotalol, dofetilide and azimilide are respectively 10, 11, and 0.4 μmol/l, yet azimilide also blocks (with IC_{50} around 3 μmol/l) the slow component of the delayed outward rectifier potassium current I_{Ks} (Carmeliet, 1985; Kiehn et al., 1994; Fermini et al., 1995; Yao and Tseng, 1997; Rouet et al., 2000). Drugs which inhibit I_{Ks} are suspected to prolong action potential duration and effective refractory period preferentially at elevated heart rate (Lu et al., 2001). By this way, in the "normal" myocardium located near the "border zone", azimilide may prevent tachyarrhythmias linked with re-entrant mechanisms. However, it is important to point out that in the present study, where azimilide was given at 0.1 and 0.5 μmol/l, one may see these concentrations as insufficient to unveil full potency of the compound since they are much (30- to 6-fold) lower concentrations than needed to obtain 50% of I_{Ks} block in normoxic conditions (Fermini et al., 1995). Nevertheless, based on a previous experience with dofetilide which, at the highest (50 nmol/l) concentration (5-fold higher than that required for IC_{50} block of I_{Kr} in normoxia), had a proarrhythmic action as compared to the lowest (5 nmol/l) concentration which had an antiarrhythmic effect (Rouet et al., 2000), it is unlikely that higher concentrations of azimilide in ischemic-like conditions, although worthwhile studying, may have an antiarrhythmic potential in this model.

4.2. Effects of azimilide on reperfusion induced arrhythmias

Two distinctive mechanisms have been proposed in the onset and maintenance of reperfusion-induced arrhythmias (Pogwizd and Corr, 1987). The first mechanism relates to the onset of re-entrant movements probably due to the heterogeneous electrical recovery during the early reperfusion phase (Corr and Witkowski, 1984). The second one consists in abnormal automaticity which may be enhanced in the early reperfused myocardium by calcium overload (Ponce Zumino et al., 1997), accumulation of cyclic AMP (Yoshida et al., 2000) and, in vivo, by sympathetic activity (Corr and Witkowski, 1984), all known to induce delayed afterdepolarizations which may in turn be a triggering mechanism of ventricular arrhythmias.

Azimilide had antiarrhythmic effects on reperfusion-induced arrhythmias, which was different from findings obtained with D-sotalol at 5 and 10 μmol/l and dofetilide at 10 and 50 nmol/l in this model, where the last drugs were arrhythmogenic (Picard et al., 1998; Rouet et al., 2000). It is possible that the prolonging effects of azimilide on action potential duration, much evident on the normoxic side of the preparation, similar to dofetilide at the lowest concentration of 5 nmol/l (Rouet et al., 2000), may have been instrumental to interrupt re-entry circuits and/or the propagation of abnormal automaticity from its initiation site to other parts of the ventricular myocardial strip.

The study undertaken to mimic azimilide effects by using dofetilide 10 nmol/l and HMR 1556 1 nmol/l may shed some light on the abovementioned considerations on leftward shift of the dose-related curve in the antiarrhythmic potential of I_{Kr}/I_{Ks} blocking drugs. As these agents were not as effective as azimilide to prevent arrhythmias during reperfusion and action potential duration decreased less during ischemic-like conditions, it is possible that: i) the selected concentrations were insufficient to effectively block I_{Kr}/I_{Ks} and/or fully mimic azimilide action, yet this is unlikely if the effects are considered on action potential durations at 90% of repolarization; ii) not only I_{Ks} and I_{Kr} block, may have improved the antiarrhythmic profile of azimilide, but other pharmacodynamic characteristics may have helped. It is important to note that in this model DL-sotalol (10 μmol/l) had significant antiarrhythmic properties during reperfusion similar to propranolol (10 μmol/l) and that they both have β-receptor blocking actions (Picard et al., 1998). On the other hand, IC_{50} of azimilide to block α- and β-receptors is 10 μmol/l (Salata and Brooks, 1997) whereas IC_{50} to block I_{CaL} is 1 μmol/l (Yao and Tseng, 1997). It is therefore possible, and will need further study, that other properties than only I_{Kr} and I_{Ks} azimilide blocking ones did cooperate to its potent antiarrhythmic effect upon reperfusion in our investigation. This

conclusion reinforces therefore previous studies in the rat (devoid of delayed rectifier potassium current) where azimilide showed potent antifibrillatory effects although these were unrelated to its β-blocking effects (Mittelstadt et al., 1997). It is finally of note that reported I_{CaL} blocking properties of azimilide (Yao and Tseng, 1997) have not been comparatively investigated in ischemia/reperfusion models.

4.3. Study limitations and clinical relevance

The results of the present study may not be immediately extrapolated to the clinical level and may rather be viewed as speculative. Inasmuch as clinical results with azimilide have been disappointing in reducing life threat due to ventricular arrhythmias (Louis et al., 2002) as were those with other class III agents, devoid of ancillary properties such as block of I_{Ks}, I_{CaL}, and α- and β-receptors, which overall may explain why at present there is a reduced clinical interest on these compounds (Picard et al., 1998; Rouet et al., 2000).

In addition to interspecies differences, this in vitro model does not reflect exactly the electrophysiological changes occurring during ischemia and reperfusion in the human heart since: i) the composition of the extracellular bath is continuously controlled by a constant perfusion of normal or modified Tyrode's solution whereas, in the early stage of myocardial infarction in humans, the ionic alterations may differ among patients; *ii*)other components of the extracellular environment such as lysophosphatidylcholine and arachidonic acid, which were not taken into account in the present work, may accumulate during ischemia; *iii*) sympathetic and parasympathetic drives are absent; *iv*) action potentials were recorded in only one cell in each zone even when electrical disturbances such as conduction blocks may occur in other myocardial areas than those including the recorded cells.

Bearing in mind the limits of in vitro studies, the present results demonstrate, for the first time, that blockade of I_{Kr} and I_{Ks} by azimilide failed to affect the ischemic-like action potential duration and the associated arrhythmias. However, different from other I_{Kr} blocking agents, azimilide showed antiarrhythmic activity during the reperfusion phase, an effect that deserves further study.

References

Billman, C.E., 1994. Role of ATP sensitive potassium channel in extracellular potassium accumulation and cardiac arrhythmias during myocardial ischaemia. Cardiovasc. Res. 28, 762–769.

Black, S., Butterfield, J., Lucchesi, B., 1993. Protection against programmed electrical stimulation-induced ventricular tachycardia and sudden death by NE-10064, a class III antiarrhythmic drug. J. Cardiovasc. Pharmacol. 22, 810–818.

Carmeliet, E., 1985. Electrophysiologic and voltage clamp analysis of the effects of sotalol on isolated cardiac muscle and Purkinje fibres. J. Pharmacol. Exp. Ther. 232, 817–825.

Clancy, C.E., Kurokawa, J., Tateyama, M., Wehrens, X.H.T., Kass, R.S., 2003. K+ channel structure-activity relationships and mechanisms of drug-induced QT prolongation. Annu. Rev. Pharmacol. Toxicol. 43, 441–461.

Coetzee, W.A., 1992. ATP-sensitive potassium channels and myocardial ischemia: why do they open? Cardiovasc. Drugs Ther. 6, 201–208.

Corr, P.B., Witkowski, F.X., 1984. Arrhythmias associated with reperfusion: basic insights and clinical relevance. J. Cardiovasc. Pharmacol. 6, S903–S909.

Dong, D.L., Li, Z., Wang, H.Z., Du, Z.M., Song, W.H., Yang, B.F., 2004. Acidification alters antiarrhythmic drug blockade of the ether-a-go-go-related gene (*HERG*) channels. Basic Clin. Pharmacol. Toxicol. 94, 209–212.

Drexler, A.P., Micklas, J.M., Brooks, R.R., 1996. Suppression of inducible ventricular arrhythmias by intravenous azimilide in dogs with previous myocardial infarction. J. Cardiovasc. Pharmacol. 28, 848–855.

Fermini, B., Jurkiewicz, N.K., Jow, B., Guinosso, P.J., Baskin, E.P., Lynch, J.J., Salata, J.J., 1995. Use dependent effects of the class III antiarrhythmic agent NE-10064 (azimilide) on cardiac repolarization : block of delayed rectifier potassium and L-type calcium currents. J. Cardiovasc. Pharmacol. 26, 259–271.

Gögelein, H., Brüggermann, A., Gerlack, U., Brendel, J., Busch, A.E., 2000. Inhibition of IKs channels by HMR 1556. Naunyn-Schmiedeberg's Arch. pharmacol. 362, 480–488.

Groh, W.J., Gibson, K.J., Maylie, J.G., 1997. Comparison of the rate-dependent properties of the class III antiarrhythmic agents azimilide (NE-10064) and E-4031: considerations on the mechanism of reverse rate-dependent action potential prolongation. J. Cardiovasc. Electrophysiol. 8, 529–536.

Hamada, K., Yamazaki, J., Nagao, T., 1998. Shortening of monophasic action potential duration during hyperkalemia and myocardial ischemia in anesthetized dogs. Jpn. J. Pharmacol. 76, 149–154.

Kiehn, J., Villena, P., Beyer, T., Brachmann, J., 1994. Differential effects of the new class III agent dofetilide on potassium currents in guinea pig cardiomyocytes. J. Cardiovasc. Pharmacol. 24, 566–572.

Kiehn, J., Lacerda, A.E., Wible, B., Brown, A.M., 1996. Molecular physiology and pharmacology of HERG. Circulation 94, 2572–2579.

Louis, A.A., Manousos, R., Coletta, A.P., Clarck, A.L., Cleland, J.G.F., 2002. Clinical trials update: the Heart Protection Study, IONA, CARISA, ENRICHD, ACUTE, ALIVE, MADIT II and REMATCH. Eur. J. Heart Fail. 4, 111–116.

Lu, Z., Kamiya, K., Opthof, T., Yasui, K., Kodama, I., 2001. Density and kinetics of I_{Kr} and I_{Ks} in guinea pig and rabbit ventricular myocytes explain different efficacy of I_{Ks} blockade at high heart rate in guinea pig and rabbit. Circulation 104, 951–956.

McIntosh, M.A., Tanira, M., Pacini, D., Kane, K.A., 1994. Comparison of the cardiac electrophysiologic effects of NE-10064 with sotalol and E-4031 and their modification by simulated ischaemia. J. Cardiovasc. Pharmacol. 23, 653–657.

Mittelstadt, S.W., Maynard, A.E., Benn, D.R., Lowe, E.R., Kostreva, D.R., 1997. Effects of azimilide and D,L-sotalol on the heart rate and blood pressure response to isoproterenol in anesthetized rats. Cardiovasc. Drugs Ther. 11, 591–598.

Morena, H., Janse, M.J., Fiolet, J.W.T., Krieger, W.J.G., Crijns, H., Durrer, D., 1980. Comparison of the effects of regional ischemia, hypoxia, hyperkalemia and acidosis on intracellular and extracellular potentials and metabolism in isolated porcine heart. Circ. Res. 46, 634–646.

Nattel, S., St-George, D., 1998. Effects of the novel antiarrhythmic agent azimilide on experimental atrial fibrillation and atrial electrophysiologic properties. Cardiovasc. Res. 37, 627–635.

Picard, S., Rouet, R., Monti, F., Puddu, P.E., Ducouret, P., Flais, F., Gerard, J.L., 1998. Proarrhythmic effects of DL- and D-sotalol on the "border zone" between normal and ischemic regions of isolated ventricular myocardium and antiarrhythmic effects on reperfusion. J. Cardiovasc. Pharmacol. 31, 126–139.

Pogwizd, S.M., Corr, P.B., 1987. Electrophysiologic mechanisms underlying arrhythmias due to reperfusion of ischemic myocardium. Circulation 76, 404–426.

Ponce Zumino, A., Risler, N.R., Schanne, O.F., Ruiz Petrich, E., Carrion, A., 1997. Magnesium: effects on reperfusion arrhythmias and membrane potential in isolated rat. Mol. Cell. Biochem. 171, 85–93.

Pritchett, E., Page, R., Connolly, L., Marcello, S., Schnell, D., Wilkinson, W., ASAP investigator,, 1998. Azimilide treatment of atrial fibrillation. Circulation 98, I-633.

Qi, X., Newman, D., Dorian, P., 1999. Azimilide decreases defibrillation voltage requirements and increases spatial organization during ventricular fibrillation. J. Interv. Cardiol. Electrophysiol. 3, 61–67.

Rouet, R., Picard, S., Libersa, C., Ghadanfar, M., Alabaster, C., Gerard, J.L., 2000. Electrophysiological effects of dofetilide in an in vitro model of "border zone" between normal and ischaemic/reperfused myocardium. Circulation 101, 86–93.

Salata, J.J., Brooks, R.R., 1997. Pharmacology of azimilide dihydrochloride (NE-10064), a class III antiarrhythmic agent. Cardiovasc. Drug Rev. 15, 137–156.

Sesti, F., Goldstein, S.A.N., 1998. Single channel characteristics of wild-type IKs channels and channels formed with two minK mutants that cause long QT syndrome. J. Gen. Physiol. 112, 651–663.

Shinbo, A., Ono, K., Iijima, T., 1997. Activation of cardiac ATP-sensitive K^+ channels by KRN 4884, a novel K^+ channel opener. J. Pharmacol. Exp. Ther. 283, 770–777.

Tatla, D.S., David, B.C., Malloy, K.J., Moorehead, T.J., 1993. In vitro electrophysiology of NE-10064, a novel and highly selective class III antiarrhythmic agent. FASEB J. 7, A107.

Wit, A.L., Rosen, M.R., Hoffman, B.F., 1974. Electrophysiology and pharmacology of cardiac arrhythmias. II. Relationship of normal and abnormal electrical activity of cardiac fibers to genesis of arrhythmias b. Re-entry. Section II. Am. Heart. J. 88, 798–806.

Yao, J., Tseng, G., 1997. Azimilide (NE-10064) can prolong or shorten the action potential duration in canine ventricular myocytes. J. Cardiovasc. Electrophysiol. 8, 184–198.

Yoshida, Y., Hirai, M., Yamada, T., Tsuji, Y., Kondo, T., Inden, Y., Akahoshi, M., Murakami, Y., Tsuda, M., Tsuboi, N., Hirayama, H., Okamoto, M., Ito, T., Saito, H., Toyama, J., 2000. Antiarrhythmic efficacy of dipyridamole in treatment of reperfusion arrhythmias. Evidence for cAMP-mediated triggered activity as a mechanism responsible for reperfusion arrhythmias. Circulation 101, 624–630.