Potassium and calcium current blocking properties of the novel antiarrhythmic agent H 345/52: implications for proarrhythmic potential

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Abstract

**Objectives:** To study the blocking effects of H 345/52 on ionic currents of rabbit ventricular myocytes and how these features translate into a proarrhythmic potential. **Methods:** The single electrode voltage clamp technique was used to study the effects of H 345/52 on the rapid component of the delayed rectifying potassium current, \(I_{Kr}\), and the L-type calcium current \(I_{Ca}\). Differential effects of H 345/52 and almokalant on APD prolongation were studied in a rabbit Purkinje fibre/ventricular muscle preparation. The temporal variability of the action potential duration (APD) and its relation to proarrhythmias was examined in Langendorff-perfused rabbit hearts administered H 345/52 or almokalant. Anaesthetised, methoxamine-sensitised rabbits were used to assess the propensity of intravenous H 345/52 and ibutilide to induce torsades de pointes (TdP).

**Results:** H 345/52 potently blocked \(I_{Kr}\) (IC\(_{50}\) = 40 nM) without consequential use-dependency. The \(I_{Ca}\) was also blocked, but at higher concentrations (IC\(_{50}\) = 1.3 \(\mu\)M). Block of \(I_{Ca}\) was markedly frequency-dependent (positive) and influenced by membrane potential, such that H 345/52 was more effective following clamp steps from plateau potentials than from \(-80\) mV. In the Purkinje fibre–ventricular muscle preparation, almokalant prolonged the Purkinje fibre APD preferentially, whereas H 345/52 homogeneously prolonged APD in both tissue types. In the perfused rabbit heart, H 345/52 (1 \(\mu\)M) and almokalant (0.3 \(\mu\)M) prolonged APD to a similar degree but increased the temporal variability of APD differently, from 8±1.2 ms to 38±7.5 ms (\(P<0.001\) vs. H 345/52), respectively. Unequivocal early after-depolarisations were seen in 5/6 almokalant-perfused hearts but in no heart administered H 345/52 (\(P<0.05\)). In anaesthetised rabbits, H 345/52 (17.4 \(\mu\)mol/kg) or ibutilide (2.6 \(\mu\)mol/kg maximum), maximally lengthened the QT interval from 133±4.5 to 177±8.0 ms and from 125±5.1 to 166±9.3 ms (\(P<0.001\), \(n=8\)). However, whereas ibutilide induced TdP in all animals at 0.06±0.009 \(\mu\)mol/kg, H 345/52 did not induce TdP (\(P=0.0002\)) at up to 17.4 \(\mu\)mol/kg. **Conclusions:** H 345/52 blocks \(I_{Kr}\) with high potency and \(I_{Ca}\) with somewhat lower potency and was found to delay ventricular repolarisation without substantially increasing temporal or spatial dispersion and without inducing early after-depolarisations or TdP.

Keywords: Antiarrhythmic agents; K-channel; Long QT syndrome; QT dispersion; Ventricular arrhythmias

1. Introduction

Ventricular and atrial tachyarrhythmias remain a leading cause of mortality and morbidity, claiming a large number of victims every year. Unfortunately, the objective of developing antiarrhythmic agents to curb these arrhythmias while retaining a low side-effect profile has been difficult to realise. After the negative outcome of the cardiac arrhythmia suppression trials, the major focus in antiarrhythmic drug development has been on agents that selectively delay myocardial repolarisation by blocking the rapid component of the delayed rectifying potassium current \(I_{Kr}\) i.e. the class III antiarrhythmic drugs.

Despite emerging evidence of favourable antiarrhythmic efficacy in the clinical setting [1,2], proarrhythmic complications (mainly torsades de pointes, TdP [3,4]) continue to
remain a serious problem, restricting widespread use of class III antiarrhythmics. The increased propensity for TdP development is thought to be a direct consequence of the repolarisation-delaying activity. The general consensus is that $I_{kr}$ block produces a more pronounced prolongation of the action potential duration (APD) in midmyocardial and Purkinje cells, especially at slower heart rates, resulting in increased spatial and temporal dispersion of repolarisation within the ventricular wall [5]. In addition, prolonging the plateau duration may lead to early afterdepolarisations (EADs), probably due to the reactivation of the L-type calcium current, which leads to triggered activity. The increased dispersion of action potential repolarisation and EAD-triggered activity are both thought to play an important role in the genesis of TdP [5,6].

H 345/52 is a novel antiarrhythmic agent developed with the specific aim to delay myocardial repolarisation without inducing TdP. The present study comprises a series of in vitro and in vivo experiments primarily undertaken to characterise the blocking effects of H 345/52 on $I_{kr}$ and the L-type calcium current ($I_{ca}$) in rabbit cardiomyocytes and to assess the proarrhythmic potential of H 345/52 in various models of the acquired long QT syndrome.

2. Methods

The 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) and was approved by the local ethics committees on animal experiments in Göteborg, Sweden and Oostende, Belgium.

2.1. Cell isolation

Ventricular myocytes were isolated from male New Zealand White (NZW) rabbits (body weight between 1 and 2 kg). Following anaesthesia (sodium pentobarbital, 60 mg/kg i.v.), the heart was excised and ventricular myocytes isolated according to Tytgat [7]. The cells were stored at 13°C and all experiments were performed within 36 h of isolation.

2.2. Single electrode voltage clamp experiments

The single-electrode, whole-cell, continuous voltage clamp technique was used to observe membrane currents. Voltage control was achieved using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Axodata (Axon Instruments) running on a Macintosh Quadra 700 computer connected via an A/D converter (ITC-16 Computer Interface, Instrutech, Elmont, NY, USA) was used for amplifier control and data acquisition. Current recordings were filtered at 1 KHz using a custom-made lowpass Bessel filter. Non-filamented borosilicate glass electrodes (Clark Electromedical Instruments, Reading, UK, I.D. 0.69 mm, O.D. 1.2 mm), with a resistance of 1–3 MΩ when filled with electrode solution were used to establish the whole cell configuration. Cells were allowed to settle in an experimental bath mounted on an inverted microscope. Bath perfusion was continuous throughout the experiment, complete exchange of solution requiring approximately 60 s. After establishment of the whole cell configuration, cell capacitance was estimated using a depolarizing ramp (1 mV/ms) from −40 to −35 mV. The mean series resistance was 5.28±0.37 MΩ ($n=28$). This was reduced to 1.28±0.12 MΩ with compensation. Cell capacitance (mean of 123±7 pF) was also compensated. Leak subtraction was not attempted. Specifics of the voltage clamp protocols are described in the appropriate section in Results.

For measurements of $I_{kr}$, the electrodes were filled with the following electrode solution (in mM: KCl 120, MgCl$_2$ 6.0, Na$_2$ATP 5, Heps 10, EGTA 5, CaCl$_2$ 0.15, pH 7.2 adjusted with KOH). The perfusion medium contained (in mM): NaCl 140, KCl 5.4, MgCl$_2$ 0.5, CaCl$_2$ 1.8, Heps 10, glucose 5.5, pH adjusted to 7.4 with NaOH, and was warmed to 35°C. Sodium currents were inactivated by a 50 ms prepulse to −40 mV. Calcium currents were blocked by 500 nM nisoldipine. Following establishment of the whole cell configuration, a 3-min period was allowed for current amplitude stabilization before starting an experiment. Time-matched control experiments demonstrated marginal rundown following this initial 3-min period (at 40 min, by 17±8% of control amplitude, $n=3$).

To isolate the L-type calcium current, potassium currents were eliminated by substituted CsCl for KCl in the electrode and bath solutions, and adding 20 mM tetraethylammonium (TEA) to the electrode solution. Sodium currents were inactivated by using a holding potential of −50 mV, or by a 50-ms prepulse to −40 mV when the holding potential was −80 mV. The electrode solution contained: (in mM) CsCl 125, MgCl$_2$ 0.5, Na$_2$ATP 5, Heps 10, EGTA 15, TEA 20, pH 7.2 adjusted with CsOH. The perfusion solution contained: (in mM): NaCl 140, CsCl 20, KCl 5.4, MgCl$_2$ 0.5, CaCl$_2$ 1.8, Heps 10, glucose 5.5, pH adjusted to 7.4 with NaOH. All experiments were performed at 23°C. An initial 20-min period following cell membrane rupture was allowed for current amplitude stabilization before beginning an experiment. Time-matched controls with vehicle indicated that rundown was by 20±8% of control ($n=8$) during the subsequent 20-min period in which all experiments on $I_{ca}$ were performed.

2.3. Effects of H 345/52 and almokalant on action potential duration in the rabbit Purkinje fibre and ventricular muscle

Twelve male rabbits were anaesthetised with pentobarbitone sodium (60 mg/kg, i.v.). After excision of the heart, the right ventricular anterior papillary muscle with Pur-
kinje fibres running across its base was dissected out, mounted in a 2-ml organ bath and superfused (11 ml/min, 37°C) with medium containing (in mM): NaCl 130, NaHCO₃ 18, CaCl₂ 1.8, MgSO₄ 0.5, NaH₂PO₄ 1.8, KCl 4 and glucose 5.5, and stimulated at a frequency of 2 Hz (50% above threshold). The preparation was then left to stabilise for 2 h. Two microelectrodes (filled with 3 M KCl) connected via Ag/AgCl junctions to high impedance amplifiers (type 309, Hugo Sachs Elektronik, Germany) were used to make simultaneous recordings of action potentials from Purkinje fibres and ventricular muscle cells. The signals were sampled (1000 Hz) and analysed by using the PC-lab system [8]. The amplified transmembrane potentials were also recorded on a strip chart recorder at a speed of 100 mm/s.

After a period of control recordings, the lowest concentration of H 345/52 or almokalant was added to the superfusion medium. The preparation was exposed to each concentration for 30 min, and three escalating concentrations, 0.03, 0.1 and 0.3 μM were examined.

2.4. Effects of H 345/52 and almokalant on action potential duration, variability of action potential duration and repolarisation-related rhythm abnormalities in the Langendorff-perfused rabbit heart

Details of this methodology have previously been described [9]. Albino rabbits of either sex (body weight approximately 2.5 kg, n = 26) were used in this study. Following cervical dislocation, the thorax was opened and the heart removed and mounted on a cannula for immediate perfusion with a buffer solution (34°C, pH 7.4, bubbled with 95% O₂, 5% CO₂) of the following composition (in mM): NaCl 118, KCl 3, NaHCO₃ 22, MgCl₂ 1.1, NaH₂PO₄ 0.4, CaCl₂ 1.8, dextrose 5, pyruvate 2, creatine 0.038. After atrial removal, the interatrial septum was dissected away close to the aorta to enable His bundle sectioning and to suture two stimulation electrodes close to the distal part of the His bundle. An electrode was positioned in the septum of the left ventricle for subendocardial recording of monophasic action potentials using isotonic potassium depolarisation at the reference electrode [9]. Action potential duration was measured at 60% repolarisation. The preparation was then transferred to the experimental station where the hearts were stimulated at a basic cycle length of 1000 ms. The experimental protocols were initiated approximately one h after surgery. All experiments were executed and analysed fully automatically by a set of computers [9].

The study consisted of two series of experiments. In the first series, the concentrations of H 345/52 and almokalant (four hearts in each group) causing equivalent prolongation of the APD were determined by ramping the concentrations from 0 to 5 μM or from 0 to 1 μM, respectively. Based on results from those experiments, concentrations of 1 μM (H 345/52) and 0.3 μM (almokalant) were used for further electrophysiological characterisation in a second series of experiments. In these experiments, the effects of H 345/52 and almokalant on APD prolongation and on the temporal variability of APD and its relation to proarrhythmic activity were examined in groups of six animals randomised with an equal number of control experiments. The perfusion schedule included a 30-min control perfusion period followed by 30-min drug perfusion and a 30-min washout period. Drug-related effects on the following parameters were measured: APD, temporal variability of APD, conduction, excitability and coronary flow. The variability of APD was quantified by summating the absolute differences between all action potentials in every minute. If all the APDs were identical this would yield a sum of 0 ms. However, if one action potential was e.g. 25 ms longer than all the others, then this would yield a value of 50 ms, since the action potential would be 25 ms longer than the preceding action potential as well as the succeeding action potential, i.e. the sum of the absolute deviation will yield a number that is twice the sum of the actual variation.

2.5. Proarrhythmic potential of H 345/52 in vivo

This experimental model of TdP has been described in detail elsewhere [10,11]. Briefly, male NZW rabbits (body wt 2.9±0.11 kg, n = 16) were anaesthetised with methohexitol-sodium (5 mg/kg i.v.) and α-chloralose (90 mg/kg i.v.) and prepared for artificial respiration, recording of arterial blood pressure and ECGs, for infusion of drugs and for arterial blood sampling. The ECGs (leads I-III, aVR, aVL and aVF) were recorded on a Mingograph ink-jet recorder (Siemens-Elema, Solna, Sweden), and arterial blood pressure and heart rate were recorded on a Grass polygraph (Grass Instruments, Quincy, MA, USA). In addition, ECGs (leads I and II) and blood pressure were recorded at predetermined intervals on a personal computer. The signals were sampled at a frequency of 500 Hz, and each sampling period lasted for 5 s. Finally, data were processed using PC-lab [8].

After baseline measurements (10 min), a continuous infusion of the α₁-adrenoceptor agonist methoxamine (70 nmol/kg/min) was started. Two doses of H 345/52 or ibutilide were continuously administered 10 min later as sequential 30-min infusions. The second dose was ten times higher than the first dose and administered only if TdP was not induced during infusion of the first dose. Hence, the first dose of H 345/52 was 0.053 μmol/kg/min, followed by a second dose of 0.53 μmol/kg/min (cumulative dose 17.4 μmol/kg). For ibutilide, the corresponding doses were 0.008 and 0.08 μmol/kg/min (maximal cumulative dose 2.6 μmol/kg). After the start of the infusion, ECGs were continuously monitored and sampled on the computer once a minute, and the appearance of premature ventricular systoles and TdP was noted. The RR and QT intervals were measured from the computer-sam-
pled ECG signal. TdP was defined as a tachyarrhythmia (seen in the setting of a prolonged QT interval) with a typical initiation (‘short—long—short sequence’) and more than five consecutive undulating peaks of sequential QRS complexes observed in at least two leads. At the end of the experiment, the rabbit was sacrificed by injecting an overdose of pentobarbital and ethanol.

2.6. Drugs

H 345/52 (3,7-Diazabicyclo[3.3.1]nonane-3-carboxylic acid, 7-[(2S)-3-(4-cyanophenoxy)-2-hydroxypropyl]-1,1-dimethylethyl ester), almokalant and ibutilide were synthesised in house. Methoxamine hydrochloride was purchased from Sigma (St. Louis, MO, USA). Nisoldipine was kindly provided by Bayer Leverkusen (Leverkusen, Germany).

H 345/52, almokalant and ibutilide were dissolved in an equimolar amount of 0.1 M tartaric acid and diluted with saline to the final concentration. Methoxamine hydrochloride was dissolved in distilled water and further diluted with saline. Nisoldipine was prepared as a 10 mM stock solution in DMSO and stored frozen at −20°C in small aliquots. All solutions were freshly prepared on the day of use and all doses refer to bases of the compounds.

2.7. Data analysis and statistics

All results are presented as means±S.E.M., and n indicates the number of observations. Student’s t-test and the Bonferroni procedure were used to test the significance of differences as appropriate. The Fisher exact probability test was used to compare the frequency of TdP induction in the anaesthetised rabbits. A two-tailed value of P<0.05 was considered as statistically significant.

For voltage clamp experiments, Axograph (Axon Instruments) was used for analysis of experiments. Current amplitudes are expressed as current density (pA/pF) unless otherwise stated. Non-linear regression analysis of experimental data was carried out using Axograph and GRAPHPAD PRISM (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Effects of H 345/52 on the delayed rectifier

The delayed rectifier \( I_{K_{\alpha}} \) was activated using a 2000-ms clamp step to +10 mV at a frequency of 0.1 Hz. Tail currents were elicited using a 2000-ms clamp step to −40 mV. The holding potential was −75 mV. A large transient outward current obscured early activation of \( I_{K_{\alpha}} \) in most cells thus tail current amplitudes were used to indicate \( I_{K_{\alpha}} \) amplitudes. Low concentrations of H 345/52 reduced the amplitude of \( I_{K_{\alpha}} \) tail current (Fig. 1A). Tail current density was reduced from 1.1±0.22 pA/pF to 0.5±0.13 pA/pF (n=4, P<0.01) by 50 nM H 345/52. Current density returned to 1.0±0.22 pA/pF with washout. A cumulative concentration–effect curve for H 345/52 was determined by exposing cells to increasing drug concentrations (10–500 nM), each for 5 min. The concentration for half-maximal block (IC\(_{50}\)) was 40 nM, and the Hill slope was 1.08 (Fig. 1B).

Use dependence of block was tested using a protocol derived from Ohler et al. [12]. \( I_{K_{\alpha}} \) currents were activated with an identical protocol to that used in determining the concentration–effect curve. Following control measurements, 50 nM H 345/52 was washed in while the cells were held continuously at −75 mV for 5 min (sufficient time for block to reach steady-state when obtaining concentration–effect curves). Current activation was recommenced with a chain of ten pulses. Tail currents following the first pulse after drug washin were already...
significantly reduced, and block did not develop further with the subsequent pulses (Fig. 2A and C). Following the tenth pulse, current activation was halted, and H 345/52 washed out for 5 min. The holding membrane potential during washout was −75 mV. Upon recommencing current activation, the tail current amplitude was still markedly suppressed during the first pulse (Fig. 2B) but increased with subsequent activation reaching control amplitudes after seven to eight pulses (Fig. 2C).

3.2. Effects of H 345/52 on the L-type calcium current

The L-type calcium current was activated using a 250-ms test step to 0 mV from a holding potential of −50 mV (frequency 0.1 Hz). A large transient inward current consistent with \( I_{\text{Ca}} \) was reversibly blocked by 10 \( \mu \)M H 345/52 (Fig. 3A). Peak \( I_{\text{Ca}} \) was reduced from −9.5±0.8 pA/pF to −4.0±0.8 pA/pF (\( n = 5, P < 0.01 \)). With 5-min washout, the current density returned to −7.4±0.6 pA/pF. A concentration–effect curve for H 345/52 (1–10 \( \mu \)M) was determined at test pulse frequencies of 0.1 and 1.0 Hz. To minimize confounding effects due to run-down, each cell was exposed to only one concentration for 10 min. H 345/52 was more potent at 1.0 Hz than at 0.1 Hz (Fig. 3B). The IC\(_{50}\) value was 1.3 \( \mu \)M at 1.0 Hz, and 6.0 \( \mu \)M at 0.1 Hz. A Hill slope of 1.0 was obtained for both frequencies.

To determine the effects of the membrane potential on block of \( I_{\text{Ca}} \), current density–voltage relations were constructed for 10 \( \mu \)M H 345/52 (protocol: holding potential −50 mV, 250 ms activating clamp steps to membrane potentials between −35 mV and +30 mV, frequency 0.1 Hz). Drug effects were evaluated following 10-min exposure. Peak \( I_{\text{Ca}} \) current amplitudes were reduced at all potentials tested (Fig. 3C). To further quantify any voltage dependence, residual current fraction was calculated for each membrane potential and then plotted against the membrane potential (Fig. 3D). The degree of \( I_{\text{Ca}} \) block increased with greater depolarization indicating voltage dependence of block. To determine the effects of holding potential on block, the holding potential was changed to −80 mV. This necessitated using a 50-ms prepulse to −50 mV to inactivate sodium currents. Interestingly, no significant block by 10 \( \mu \)M H 345/52 was observed with a holding potential of −80 mV at a test pulse frequency of 0.1 Hz (current density at 0 mV: control −8.5±1.7 pA/pF, H 345/52 −8.2±1.6 pA/pF, \( n = 5, P > 0.05 \)). Significant block was observed, however, at higher test pulse frequencies of 0.2 and 1.0 Hz. The residual current fraction progressively decreased from 1.0±0.16 at 0.1 Hz, 0.82±0.06 at 0.2 Hz, to 0.43±0.05 at 1.0 Hz. Hence, the marked frequency-dependence of block observed at −50 mV was conserved at the more negative holding potential.

From the observed dependence of block on holding potential and frequency, H 345/52 should be more effective at reducing \( I_{\text{Ca}} \) following the plateau phase of the action potential than following the initial upstroke phase. To confirm this, plateau \( I_{\text{Ca}} \) amplitudes were measured using a double pulse protocol (protocol: holding potential −80 mV, 200 ms clamp step to +10 mV, a plateau interval at potentials between −40 and −10 mV of 200 or 600 ms duration, and finally a 200-ms test pulse to +10 mV to activate plateau \( I_{\text{Ca}} \), frequency 0.1 Hz) and the effects of 10 \( \mu \)M H 345/52 evaluated after 10 min exposure. Plateau \( I_{\text{Ca}} \) was defined as the peak inward current during the second activating clamp step. Mean residual current fractions calculated for all plateau potentials and intervals are shown in Fig. 4. Plateau \( I_{\text{Ca}} \) was significantly attenuated by H 345/52 and depending on the plateau potential and duration tested, the reductions varied between 20 and 50%.

3.3. Effects of H 345/52 and almokalant on action potential duration in the rabbit Purkinje fibre and ventricular muscle

The effects of H 345/52 and almokalant on APD (at 75% repolarisation, APD\(_{75}\)) are illustrated in Fig. 5.
Fig. 3. Block of the cardiac calcium current $I_{Ca}$ by H 345/52. (A) Original recordings of $I_{Ca}$ under control conditions (C), after 5-min exposure to 10 μM H 345/52 and 10-min washout (W). Scaling as indicated, arrow head shows zero current level, the cell capacitance was 230 pF. Currents were activated by 400 ms clamp steps to 0 mV, holding potential −50 mV, frequency 0.1 Hz. A reversible block was observed. (B) Concentration–effect curve for H 345/52 on $I_{Ca}$ at test pulse frequencies of 0.1 Hz (○) and 1.0 Hz (●). $I_{Ca}$ was activated by 400 ms clamp steps to 0 mV from a holding potential of −50 mV. Ordinate: mean residual current fraction calculated by normalizing $I_{Ca}$ amplitude in the presence of drug to the amplitude under control conditions (1.0), abscissa: logarithm of the corresponding concentration of H 345/52, C, control. The general equation for receptor occupancy (see legend to Fig. 1) was fitted to the data to aid determination of the IC50 and Hill slope values. The potency of H 345/52 was clearly dependent on the frequency of the test pulses. (C) Current density–voltage ($I/V$) relations for $I_{Ca}$ under control conditions (□) and following a 10-min exposure to 10 μM H 345/52 (●). Ordinate: mean current density (pA/pF) of five cells, abscissa: membrane potential during the activating test step. (D) Voltage dependence of 10 μM H 345/52 block of $I_{Ca}$. Ordinate: mean residual current fraction (control amplitude = 1.0), abscissa: membrane potential during the activating clamp step. The degree of block increased with greater depolarization.

Before drug superfusion commenced, the APD24 did not differ statistically ($P>0.05$) between the two groups of preparations (neither in the Purkinje fibre nor in the ventricular muscle). The ratio of the APD24 in PF and VM was calculated and used as an index of dispersion of repolarisation between the two tissue types. At baseline these ratios were 1.8±0.09 and 2.0±0.08 ($P>0.05$) in preparations subsequently superfused with H 345/52 and almokalant.

Drug superfusion was associated with a concentration-dependent and statistically significant lengthening of APD24 in both PF and VM (Fig. 5A and B). However, in the almokalant-superfused preparations the prolongation was much more pronounced in PF than in VM, an effect not seen with H 345/52. This disparity was reflected in the PF to VM ratio, which increased to 2.6±0.22 ($P<0.05$ vs. baseline) in the almokalant group (at 0.3 μM), whereas it remained unchanged (1.7±0.08, $P>0.05$) after H 345/52.

3.4. Effects of H 345/52 and almokalant on action potential duration, variability of action potential duration and repolarisation-related rhythm abnormalities in the Langendorff-perfused rabbit heart

Compared to the hearts perfused with drug-free medium throughout the experiment, neither H 345/52 nor almokalant significantly influenced coronary flow, conduction or excitability (data not shown). Only a small, progressive decline in APD was observed in experimental groups perfused with drug-free medium. At the end of the control period (i.e. after 30 min of perfusion) the APD was 240±6.0 ms, and after 60 and 90 min of perfusion it had declined to 222±6.5 and 213±5.8 ms, respectively. During perfusion with H 345/52 or almokalant, however, the APD promptly increased from 264±9.4 to 412±19.2 ms and from 251±6.9 to 436±29.4 ms. Although the absolute prolongation of the APD induced by H 345/52 and
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Fig. 4. Block of the plateau calcium current \( I_{\text{ca}} \) by H 345/52. Mean residual current fraction of plateau \( I_{\text{ca}} \) following 10 min exposure to 10 \( \mu \)M H 345/52 obtained at plateau intervals of 200 ms (○) and 600 ms (●) and at plateau potentials between −40 and −10 mV. Residual current fraction was obtained by dividing the peak plateau \( I_{\text{ca}} \) amplitude following substance by the respective plateau \( I_{\text{ca}} \) amplitude under control conditions. The protocol: holding potential −80 mV, 200 ms clamp step to +10 mV, a plateau interval at potentials between −40 and −10 mV of 200 or 600 ms duration, and finally a 200-ms test pulse to 0 mV to activate plateau \( I_{\text{ca}} \), frequency 0.1 Hz. Ordinate: mean residual current fraction (4 cells), abscissa: membrane potential during the plateau interval. Plateau \( I_{\text{ca}} \) was significantly blocked at all plateau potentials and durations.

almokalant did not differ significantly, the evolution of the APD changes was clearly different. Whereas for H 345/52 the lengthening of the APD could be anticipated minute by minute, such a prediction would have been highly inaccurate for the almokalant-perfused heart, since the minute-by-minute changes in APD became quite variable (see below for measurement of APD variability). During the period of washout, the APD progressively returned towards control values (254±10.2 ms and 224±6.5 ms in the H 345/52 and almokalant-perfused hearts, respectively).

The influence of drug-free medium, H 345/52 and almokalant on the variability of APD (see Methods for details) is illustrated in Fig. 6. During drug-free perfusion, the sum of the APD deviations was usually below 10 ms and only occasionally did the sum exceed 10 ms. For the 30 min of perfusion with drug-free medium, the mean deviation of APD was 3±0.4 ms. For H 345/52, the sum deviation of APD increased somewhat (Fig. 6A) but only rarely did it exceed 10 ms and for the 30 min of perfusion the mean deviation of APD was 8±1.2 ms. In the hearts perfused with almokalant, however, the sum of deviations of APD increased dramatically and, furthermore, markedly fluctuated from minute to minute. Only once during the 30-min perfusion period (Fig. 6B) was it below 10 ms. As the concentration of almokalant was progressively increased, the APD variability initially increased because of beat-to-beat changes of the action potentials (with consistent morphology). Only later on did EADs and TdP develop. For the 30 min of perfusion with almokalant, the median deviation of APD increased to 38±7.5 ms (\( P<0.001 \) vs. H 345/52). During this period, unequivocal EADs constituting 35±4.8% of the action potential amplitude (corresponding to 42±5.7 mV) were observed in 5/6 almokalant-perfused hearts but in no heart perfused with H 345/52 (\( P<0.05 \) vs. almokalant) or drug-free medium.

3.5. Proarrhythmic potential of H 345/52 in vivo

Baseline characteristics were measured after 10 min of methoxamine infusion in anaesthetised rabbits subsequently administered H 345/52 or ibutilide. No statistically significant differences were found in the QT interval, RR interval, mean arterial blood pressure or potassium levels in arterial blood between the two groups of rabbits.

Infusion of the first dose of H 345/52 (0.053 \( \mu \)mol/kg/min) was associated with a dose-dependent increase in the
Fig. 7. Dose-dependent effects of H 345/52 on the QT interval in the anaesthetised rabbit. The animals were given a concomitant infusion of methoxamine and two consecutive doses (0.053 μmol/kg/min for 30 min followed by 0.53 μmol/kg/min for an additional 30 min, respectively) of H 345/52. Values are means ± S.E.M. for eight experiments. The solid line indicates the cumulative dose of H 345/52 administered over time.

was similar to that observed before the infusion of H 345/52 was initiated (97 ± 3.3 vs. 97 ± 5.1 mmHg, P > 0.05). Ventricular extrasystoles were induced in all rabbits at a mean cumulative dose of 2.5 ± 1.07 μmol/kg. However, in no case did the extrasystoles degenerate into TdP.

In all eight rabbits administered ibutilide, ventricular extrasystoles were induced at a mean cumulative dose of 0.03 ± 0.060 μmol/kg, and in all cases the extrasystoles degenerated into TdP (at a mean cumulative dose of 0.06 ± 0.009 μmol/kg, P = 0.0002 vs. TdP incidence of H 345/52). The induction of proarrhythmias in the ibutilide-treated rabbits was preceded by a significant prolongation of the QT interval from 125 ± 5.1 to 166 ± 9.3 ms (33 ± 3.8%, P < 0.001), a prolongation not statistically different, however, from the maximal QT prolongation seen in the H 345/52-treated animals.

Fig. 6. Effects of H 345/52 (A) and almokalant (B) on temporal variability of action potential duration (APD) in the Langendorff-perfused rabbit heart (cycle length 1000 ms). The temporal variability of APD was quantified by summing the absolute differences in APD (sum Δ APD) between all action potentials in every minute of the perfusion. The perfusion schedule included a 30-min control perfusion period followed by 30-min drug perfusion and a 30-min washout period with drug-free medium. The panels present the mean sum Δ APD of six hearts within each group.

4. Discussion

The present study has demonstrated that H 345/52 potently blocks the rapid component of the delayed rectifier (I_Kr). This is the most likely mechanism underlying the slowing of ventricular repolarisation demonstrated in vitro and in vivo. At higher concentrations, H 345/52 blocked the L-type calcium current (I_{Ca}) in a use-dependent and voltage-dependent manner. No effect of H 345/52 was observed on the fast sodium current, the inward rectifier potassium current, or the transient outward potassium current at pharmacologically relevant concentrations (data not shown). The present series of experiments also demonstrated that H 345/52 is an agent that delays ventricular repolarisation without causing excessive QT lengthening, without increasing spatial and temporal dispersion of repolarisation, without inducing EADs and without provoking TdP. Hence, in these respects H 345/52
is clearly discernible from conventional class III antiarhythmics that selectively prolong the APD, a conclusion supported by the findings in the comparative studies with almokalant and ibutilide. Almokalant is an agent selectively targeting \( I_{Kr} \) [13], whereas ibutilide has been suggested to act preferentially by activating a slow inward sodium current and/or by blocking \( I_{Kr} \) [1].

Blockage of \( I_{Kr} \) was observed at concentrations comparable to the concentrations where APD prolongation was observed, suggesting this action likely underlies the APD prolongation. An unusual use dependence of block was observed. With washin, complete block development was already observed within the first 2 s of an activating clamp step. These results contrast other \( I_{Kr} \)-blocking agents, where activation of the current for 4–5 s was required for full block development [13,14]. These results for H 345/52 could be explained by selective affinity for the open channel state with rapid binding kinetics or that the drug binds to the closed channel. H 345/52 was clearly ‘trapped’ by closure of the \( I_{Kr} \)-channel, as current activation was essential to alleviate block [13,14]. Trapping behavior suggests that H 345/52 binds selectively to the open channel, as access from (and thus presumably to) the binding site was restricted by the closed state. An open channel block mechanism is supported by studies on HERG-conducted \( K^+ \) currents, where H 345/52 binds selectively to the open channel state of these channels, and that binding is extremely rapid (G.J. Amos, unpublished observation). Importantly, extrapolation of the kinetics of block suggests that the magnitude of block of \( I_{Kr} \) in vivo would not be dependent on heart rate. Further investigation is required to determine whether these unusual kinetics of \( I_{Kr} \)-block contribute to the low proarrhythmic risk.

An additional property of H 345/52 was the concentration-dependent block of the L-type calcium current, \( I_{Ca} \). This action was observed at concentrations approximately 30 times higher than those required for block of \( I_{Kr} \), thus calcium current block would be expected to become of significance at concentrations producing a near maximal block of \( I_{Kr} \) and APD prolongation. Calcium channel block has been suggested to prevent class III-related proarrhythmia by (1) directly inhibiting EAD development [15], (2) reducing the amplitude of any EADS which do develop, hence reducing their ability to trigger activity in surrounding tissue [16], (3) limiting the maximum prolongation of APD and consequently the increase in spatial dispersion of repolarisation [17], (4) reducing the degree of reverse use dependence of APD prolongation [17]. The 30-fold difference in the \( IC_{50} \) concentrations may at first speak against the blockade of \( I_{Ca} \) as a likely mechanism contributing to the observed low proarrhythmic potential of H 345/52. However, simulations using a guinea pig ventricular model support the notion that a protective effect of a mixed \( I_{Kr}/I_{Ca} \)-blocking profile is possible despite large differences in potency [18]. In the model, a 90% block of \( I_{Kr} \) excessively prolonged the APD, eventually leading to the appearance of EADs. However, addition of only 20% block of \( I_{Ca} \) abolished the EADs while maintaining APD prolongation. In addition, the L-type calcium channel blocking potency of H 345/52 may be underestimated in this study as it was assessed at room temperature. This is implied by a recent study demonstrating that the dose–response curve for the negative inotropic and chronotropic effects of verapamil assessed in blood-perfused dog atria was shifted to higher concentrations by hypothermia [19]. Reactivation of \( I_{Ca} \) following the plateau phase is generally accepted to be the mechanism responsible for EADs generated in the setting of prolonged repolarization [15]. Interestingly, H 345/52 (10 \( \mu M \)) was observed to markedly block \( I_{Ca} \) following clamp steps at plateau membrane potentials (−10 to −40 mV), but not to reduce \( I_{Ca} \) following clamp steps at the resting membrane potential (−80 mV). Thus, H 345/52 would be expected to exhibit EAD-suppressing effects at lower concentrations than required to produce negative inotropic effects. Such a profile may have an advantage in the clinical setting.

In the present in vivo studies, the plasma concentration of H 345/52 was not analysed making an assessment how \( I_{Ca} \) inhibition contributes to the drug’s in vivo profile and low proarrhythmic potential difficult. However, in conscious rabbits administered H 345/52 according to an identical dose regimen as in this study, peak plasma concentrations in the range of 10–15 \( \mu M \) was obtained (without the induction of torsades, unpublished observations). Assuming a protein binding of 90% (as seen in rats and dogs) free plasma concentrations of \( \approx 1–1.5 \) \( \mu M \) are possible, concentrations that most likely will influence \( I_{Ca} \).

Interestingly, H 345/52 lengthened the APD in PF and VM to an equal degree, in sharp contrast to what has previously been observed for many other class III antiarrhythmics [5,16,20]. An increased spatial dispersion of repolarisation has been suggested as an important and critical factor for imminent drug-induced TdP in experimental models as well as in the clinical setting [5,6,11,21]. In contrast, drugs with an electropharmacological profile causing less preferential APD prolongation in PF and M cells, thereby attenuating transmural repolarisation gradients and the vulnerable window for reentry, seem to be less proarrhythmic [5].

Whereas changes in spatial (transmural, inter- or intraventricular) dispersion of repolarisation or QT interval dispersion have been suggested as being of predictive value both in the congenital and the acquired long QT syndrome, the importance of temporal dispersion of repolarisation has been much less studied. Spatial as well as temporal non-uniformity of APD will result in co-existing brief and long effective refractory periods and therefore potential differences leading to electrotonic interactions between adjacent myocardial cells. Consequently, conditions supporting reentry and/or propagation of EAD-triggered arrhythmias such as TdP may be created. In the
present study using the Langendorff-perfused rabbit heart (paced at a constant cycle length) superfused with almokalant, we observed that a sequence of events proceeding from APD prolongation, slowing of repolarisation, increased temporal variability of APD and the appearance of EADs lead to TdP. However, despite a similar degree of APD lengthening, H 345/52 did not markedly alter APD variability, did not initiate EADs and, consequently, did not induce TdP. Clinical evidence in favour of APD instability preceding repolarisation-related rhythm abnormalities was recently been put forward by Svernhage et al. who demonstrated that an increased intralead QT interval variability characterised patients who developed TdP when exposed to almokalant [21].

In conclusion, this study demonstrated that the new repolarisation-delaying antiarrhythmic agent H 345/52 possessed a dual potassium–calcium current-blocking profile. At low concentrations, block of $I_{Kr}$ predominated and was the mechanism for APD prolongation. At higher concentrations, ancillary $I_{Ca}$ block becomes apparent. Block of $I_{Ca}$, by reducing the risk of EAD development with excessive APD prolongation, attenuating excessive repolarisation delay and spatial and temporal dispersion of repolarisation may be the contributing mechanism for the reduced proarhythmic potential of this compound.

References