

# Accumulation of slowly activating delayed rectifier potassium current ( $I_{Ks}$ ) in canine ventricular myocytes

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In guinea-pig ventricular myocytes, in which the deactivation of slowly activating delayed rectifier potassium current ( $I_{Ks}$ ) is slow,  $I_{Ks}$  can be increased by rapid pacing as a result of incomplete deactivation and subsequent current accumulation. Whether accumulation of  $I_{Ks}$  occurs in dogs, in which the deactivation is much faster, is still unclear. In this study the conditions under which accumulation occurs in canine ventricular myocytes were studied with regard to its physiological relevance in controlling action potential duration (APD). At baseline, square pulse voltage clamp experiments revealed that the accumulation of canine  $I_{Ks}$  could occur, but only at rather short interpulse intervals (< 100 ms). With action potential (AP) clamp commands of constant duration (originally recorded at rate of 2 Hz), an accumulation was only found at interpulse intervals close to 0 ms. Transmembrane potential recordings with high-resistance microelectrodes revealed, however, that at the fastest stimulation rates with normally captured APs (5 Hz) the interpulse interval exceeded 50 ms. This suggested that no  $I_{Ks}$  accumulation occurs, which was supported by the lack of effect of an  $I_{Ks}$  blocker, HMR 1556 (500 nM), on APD. In the presence of the  $\beta$ -adrenergic receptor agonist isoproterenol (isoprenaline, 100 nM) the accumulation with AP clamp commands of constant duration was much more pronounced and a significant accumulating current was found at a relevant interpulse interval of 100 ms. HMR 1556 prolonged APD, but this lengthening was reverse rate dependent. AP clamp experiments in a physiologically relevant setting (short, high rate APs delivered at a corresponding rate) revealed a limited accumulation of  $I_{Ks}$  in the presence of isoproterenol. In conclusion, a physiologically relevant accumulation of  $I_{Ks}$  was only observed in the presence of isoproterenol. Block of  $I_{Ks}$ , however, led to a reverse rate-dependent prolongation of APD indicating that  $I_{Ks}$  does not have a dominant role at short cycle lengths.

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Among the many ionic currents contributing to cardiac ventricular repolarization, the slowly activating delayed rectifier potassium current,  $I_{Ks}$ , is unique in its extremely slow activation kinetics. The activation occurs in the order of seconds and is quite constant in various species: guinea-pig (Bosch *et al.* 1998), rabbit (Lengyel *et al.* 2001), dog (Varro *et al.* 2000) as well as human (Virag *et al.* 2001). The deactivation, on the other hand, is species specific, slow in guinea-pig (time constant of 800 ms, Chinn 1993), but relatively fast in rabbit (Lengyel *et al.* 2001), dog (Volders *et al.* 1999a; Varro *et al.* 2000) and human (Virag *et al.* 2001; time constant of ~100 ms for all).

Such kinetics potentially allow accumulation of the current during rapid pacing. Slow activation on the one hand ensures that  $I_{Ks}$  activated by a single pulse (action potential, AP) does not reach the maximal value and can be further increased. Short interpulse intervals during rapid pacing on the other hand allow only an incomplete deactivation.

Therefore some of the channels remain in active, open state for the next beat and the current can gradually, from beat to beat, increase.

Increased levels of current at high rates due to this accumulation contribute, at least in guinea-pig, to the rate-dependent decrease in AP duration (APD; Jurkiewicz & Sanguinetti, 1993). Block of  $I_{Ks}$  could, therefore, result in a prolongation of APD that will be most pronounced at these high rates, which is considered an ideal protection against re-entry-based tachyarrhythmias and against the potentially dangerous reverse rate-dependent effects (greater APD prolongation at slower rates) of  $I_{Kr}$  blockers (Hondeghe & Snyders, 1990).

In guinea-pig ventricular myocytes, in which the deactivation of  $I_{Ks}$  is slow, accumulation was shown at physiologically relevant rates of 3 Hz (Lu *et al.* 2001) or 4 Hz (Jurkiewicz & Sanguinetti, 1993) and it was attributed to the shortening of the diastolic interval (Rocchetti *et al.*

2001). In large mammals including humans, however, the deactivation is much faster and accumulation at physiologically relevant rates (1–4 Hz) is therefore questionable. No significant accumulation was found in rabbit at a rate of 3 Hz (Lu *et al.* 2001). In dogs the accumulation was suggested to be unlikely at physiologically relevant rates (Gintant, 1996). These experiments, however, were performed in the absence of  $\beta$ -adrenergic stimulation, which is of crucial importance for the role of  $I_{Ks}$  in repolarization (Walsh & Kass, 1988; Volders *et al.* 2003).

In this study, the accumulation of  $I_{Ks}$  in canine ventricular myocytes was studied in detail and particular attention was paid to the physiological relevance of this phenomenon in controlling APD in different circumstances.

## METHODS

Animal handling was in accordance with the Dutch Law on Animal Experimentation and the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU). The experiments were approved by the Maastricht University Committee for Experiments on Animals.

Seventeen adult mongrel dogs of either sex ( $28 \pm 1$  kg) were used for the experiments. To induce anaesthesia, dogs received premedication (1 ml (5 kg)<sup>-1</sup>: 10 mg oxycodone HCl, 1 mg acepromazine and 0.5 mg atropine i.m.) and sodium pentobarbital (20 mg kg<sup>-1</sup> i.v.), and subsequently they were artificially ventilated with a mixture of oxygen, nitrous oxide (40:60 %) and halothane (0.5–1 % vapour concentration). Upon thoracotomy heparin was administered i.v. The hearts ( $229 \pm 9$  g) were quickly excised and washed in cold cardioplegic solution. Left anterior descending and right coronary arteries were cannulated and simultaneously perfused. After ~30 min of collagenase perfusion and subsequent washout of the enzyme, the digested part of the free ventricular wall was cut out and myocytes were isolated from most of it except a rim (< 1.5 mm) of epicardial and endocardial tissue. Myocytes were stored at room temperature in standard buffer solution and only quiescent rod-shaped cells with clear cross-striations were used for experiments.

Transmembrane potentials were recorded using the glass microelectrode technique and the microelectrodes had resistances of 30–60 M $\Omega$  when filled with 3 M KCl. Intracellular pacing was carried out at various cycle lengths (CL). Whole-cell currents were measured using patch pipettes with resistances of 1.0–3.0 M $\Omega$  when filled with internal solution. The square pulse voltage clamp protocols used are shown in the Results section. In AP clamp experiments, APs recorded with high resistance microelectrodes, either at baseline or in the presence of isoproterenol, were used as the command waveforms and interpulse intervals were determined as periods from the time point of 95 % completion of repolarization to the upstroke of the next AP. Experiments were performed at  $37 \pm 1$  °C. Cell capacitance was  $190 \pm 6$  pF ( $n = 54$ ).

The standard buffer solution used for the experiments was of the following composition (mM): NaCl 145, KCl 4.0, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1.0, glucose 11 and Hepes 10, pH 7.4 with NaOH at 37 °C. In most patch clamp experiments KCl was omitted from the external

solution to increase  $I_{Ks}$  amplitude. Zero external K<sup>+</sup> increases  $I_{Ks}$  magnitude (in our conditions by  $129 \pm 24$  %,  $n = 12$ ) indirectly through changes in the concentration gradient. The kinetics of  $I_{Ks}$  activation and deactivation are not influenced (Sanguinetti & Jurkiewicz, 1992). The patch-pipette solution contained (mM): potassium aspartate 125, KCl 20, MgCl<sub>2</sub> 1.0, MgATP 5, Hepes 5 and EGTA 10, pH 7.2 with KOH. L-type Ca<sup>2+</sup> current was blocked with nifedipine (5  $\mu$ M). Almolant was dissolved in the superfusate (2  $\mu$ M) to block  $I_{Kr}$ . Isoproterenol was first dissolved in distilled water containing 30  $\mu$ M ascorbic acid and then kept in the dark at 5 °C until use. HMR 1556 ((3R,4S)-(+)-N-[3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorobutoxy)chroman-4-yl]-N-methylmethanesulfonamide, Aventis Pharma Deutschland GmbH, Frankfurt am Main, Germany) was used to identify  $I_{Ks}$ . This chromanol is highly selective for  $I_{Ks}$ ; at a concentration of 500 nM,  $I_{Ks}$  is completely blocked (Volders *et al.* 2003) and other currents are unaffected (Gögelein *et al.* 2000; Thomas *et al.* 2003). HMR 1556 was dissolved in dimethyl sulfoxide (DMSO) as a 100 mM stock solution and diluted in the superfusate to achieve a final concentration immediately before each application. The final concentrations of DMSO (maximally 0.1 %) in the superfusate had no measurable effects on ion currents and AP.

The data are expressed as means  $\pm$  S.E.M. Intergroup comparisons were made with Student's *t* test for unpaired and paired data groups, after testing for the normality of distribution. Multiple groups were analysed by one-way ANOVA. Differences were considered significant if  $P < 0.05$ .

## RESULTS

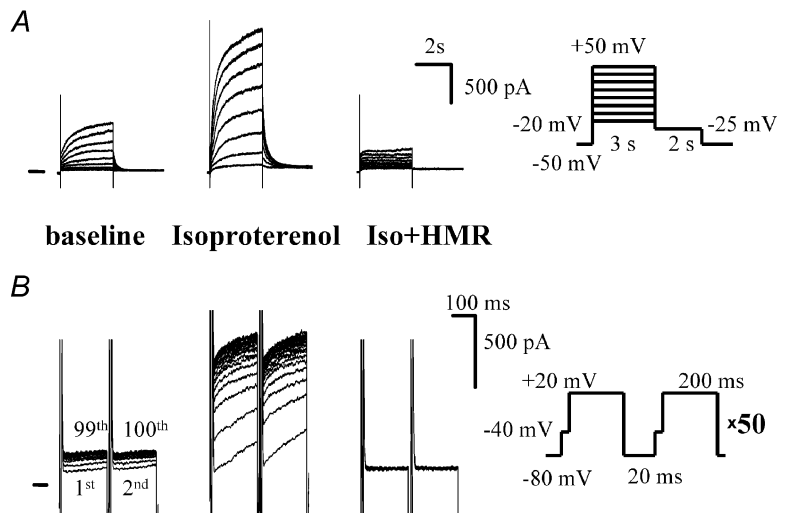
### $I_{Ks}$ accumulation at baseline assessed with square voltage clamp pulses

Figure 1A shows the fundamental properties of  $I_{Ks}$ : slow activation occurring in the order of seconds,  $\beta$ -adrenergic stimulation of the current and block by a selective  $I_{Ks}$  blocker, HMR 1556. Repetitive depolarizing pulses (200 ms long, from  $-80$  to  $+20$  mV) with a short coupling interval (20 ms) revealed an accumulating outward current (Fig. 1B). The accumulating current was markedly increased in the presence of isoproterenol (100 nM), the  $\beta$ -adrenergic agonist, and completely blocked by HMR 1556 (500 nM; Fig. 1B) indicating that  $I_{Ks}$  is the underlying current.

Increased  $I_{Ks}$  accumulation in the presence of isoproterenol could simply be a result of the  $\beta$ -adrenergic enhancement of  $I_{Ks}$ ; however, changes in  $I_{Ks}$  kinetics could also contribute. Therefore the kinetics of  $I_{Ks}$  activation and deactivation were investigated in the absence and presence of isoproterenol (100 nM). The time course of full  $I_{Ks}$  activation could be measured during a 5 s pulse to  $+20$  mV (Fig. 2C, inset). The half-maximal activation time at baseline was  $511 \pm 63$  ms, and in the presence of isoproterenol it decreased to  $412 \pm 53$  ms ( $n = 9$ ,  $P < 0.05$ ). Half-times for deactivation were voltage dependent, being, at baseline,  $128 \pm 13$  ms on repolarization to  $-25$  mV and  $29 \pm 2$  ms at  $-80$  mV. In the presence of isoproterenol, the deactivation half-times increased to  $185 \pm 15$  ms at  $-25$  mV and  $46 \pm 4$  ms at  $-80$  mV ( $P < 0.05$  for both).

**Figure 1. The accumulating current is  $I_{Ks}$**

*A*,  $I_{Ks}$  activated by conventional seconds-long (3 s) depolarizations was increased by isoproterenol (Iso, 100 nM); administration of HMR 1556 (HMR, 500 nM) completely suppressed the current. The right panel shows the voltage protocol (applied at 0.1 Hz). *B*, repetitive depolarizations (voltage protocol on right) induced accumulation of an outward current that shows the same properties (increase by isoproterenol, block by HMR 1556) as  $I_{Ks}$ . The numbers in the baseline panel mark the 1st, 2nd, 99th and 100th depolarizations.



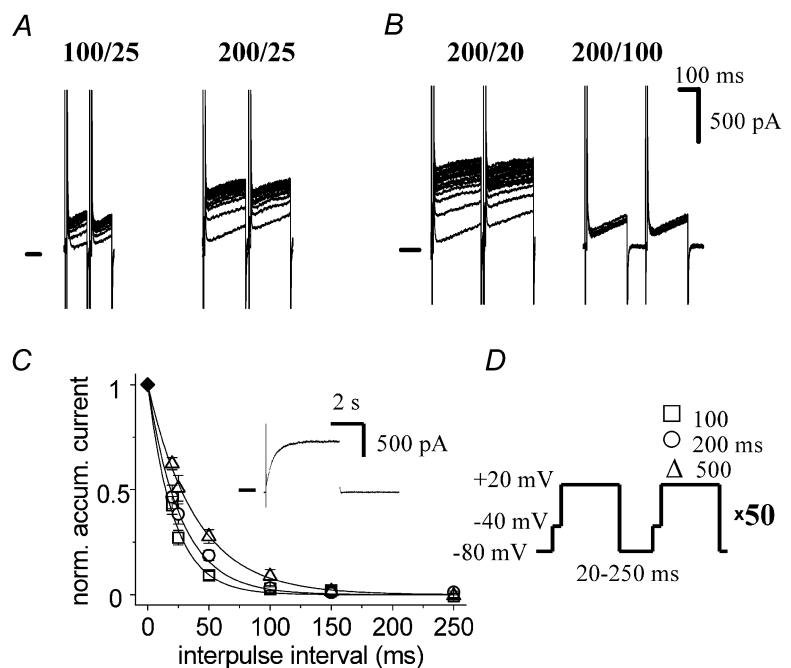
Prolonging the depolarization duration (from 100 to 200 or 500 ms) increased current accumulation (Fig. 2*A* and *C*, accumulated  $I_{Ks}$  measured as the difference between currents at the end of the 1st and 100th pulses). On the other hand, prolongation of the interpulse interval (from 20 up to 250 ms) decreased the accumulation (Fig. 2*B* and *C*). Accumulation only occurred at interpulse intervals shorter than 100 ms (Fig. 2*C*); at longer interpulse intervals there was no accumulation irrespective of depolarization duration. The maximal level of the accumulated current ( $I_{Ks}$ ) was always smaller than the amplitude of fully activated  $I_{Ks}$  ( $486 \pm 88$  pA,  $n = 13$ , activated by a sustained 5 s-long depolarization from  $-80$  to  $+20$  mV, Fig. 2*C*, inset).

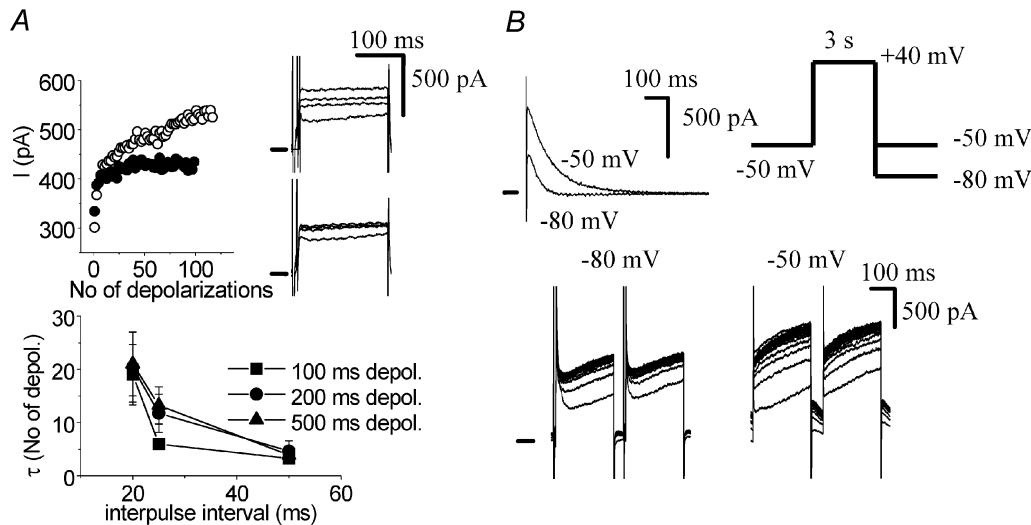
The current accumulation was relatively fast and the time course was dependent on the interpulse interval (Fig. 3*A*).

Irrespective of depolarization duration, the exponential time constant was 20 depolarization pulses for an interpulse interval of 20 ms and four depolarization pulses for an interval of 50 ms. As already mentioned in the Introduction, the accumulation is determined by deactivation kinetics. With slower deactivation more accumulation is expected, since fewer channels deactivate during a given interpulse interval and therefore more channels remain open for the next depolarization. To verify this hypothesis, we employed the voltage dependence of  $I_{Ks}$  deactivation, which is faster at more negative membrane potentials. The half-times of  $I_{Ks}$  deactivation at  $-50$  and  $-80$  mV were  $94 \pm 14$  and  $29 \pm 2$  ms ( $n = 9$ ), respectively (Fig. 3*B*). The accumulation was measured at holding potentials of  $-80$  and  $-50$  mV and, indeed, a larger accumulation was found at  $-50$  mV (Fig. 3*B*), when the deactivation was slower.

**Figure 2.  $I_{Ks}$  accumulation is dependent on interpulse interval and on depolarization duration**

*A*, increasing the depolarization duration increases current accumulation. *B*, increasing the interpulse interval diminishes current accumulation. *C*, dependence of accumulation on interpulse interval and depolarization duration. Accumulated  $I_{Ks}$  was measured as the difference between currents at the end of the 1st and 100th pulses. Data ( $n = 6$ ) are expressed relative to the  $I_{Ks}$  fully activated at  $+20$  mV (measured as  $I_{Ks}$  activated during a 5 s-long pulse to  $+20$  mV, example shown in inset). Y-axis label in full: normalized accumulated current. *D*, voltage protocol; interpulse interval varied from 20 to 250 ms, depolarization duration being 100, 200 or 500 ms.





**Figure 3. Time course and voltage dependence of  $I_{Ks}$  accumulation**

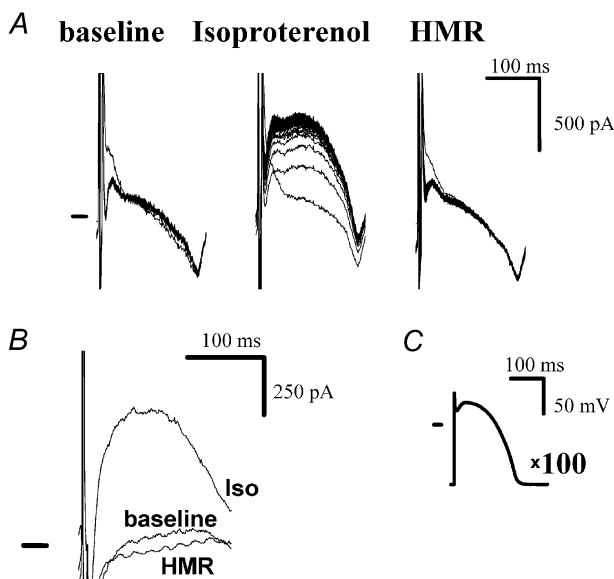
**A**, time course of accumulation. Upper panel, time course of accumulation at an interpulse interval of 20 ms (open symbols) and 50 ms (filled symbols). Only odd depolarization pulses are shown. Insets, 1st, 5th, 20th and 100th traces of the accumulating  $I_{Ks}$  at interpulse intervals of 20 ms (upper inset) and 50 ms (lower inset). Bottom panel, exponential time constant (expressed in numbers of depolarization pulses) of accumulation at various interpulse intervals (20–50 ms) and depolarization durations (100, 200 and 500 ms). Pooled data ( $n = 6$ ). **B**, the magnitude of accumulation is dependent on the rate of deactivation. Upper panel, deactivation of  $I_{Ks}$  at  $-50$  mV is slower than at  $-80$  mV. Inset, voltage protocol. Bottom panel, accumulation of  $I_{Ks}$ , is larger at a holding potential of  $-50$  mV than at  $-80$  mV, a consequence of the slower deactivation kinetics at less negative potentials.

### $I_{Ks}$ accumulation assessed with AP clamp pulses at baseline and during $\beta$ -adrenergic stimulation

The physiological pulse, i.e. the AP, is a much more complicated waveform than the square pulses used in our experiments described so far. To gain more insight into the physiological relevance of accumulation, the technique of AP clamp was employed. An AP recorded originally with high resistance glass microelectrodes at a CL of 500 ms (Fig. 4C) was used as the command waveform and current accumulation was measured under various conditions.

With an interpulse interval of 20 ms, an accumulating outward current was found, which was again increased by isoproterenol (100 nM) and completely blocked by HMR 1556 (500 nM) as expected for  $I_{Ks}$ . The accumulated current measured as the difference current between the last (100th) and the first AP pulse is shown in Fig. 4B.

When considering the physiological relevance of  $I_{Ks}$  (accumulation), the adrenergic regulation of  $I_{Ks}$  is of crucial importance (Volders *et al.* 2003).  $\beta$ -Adrenergic



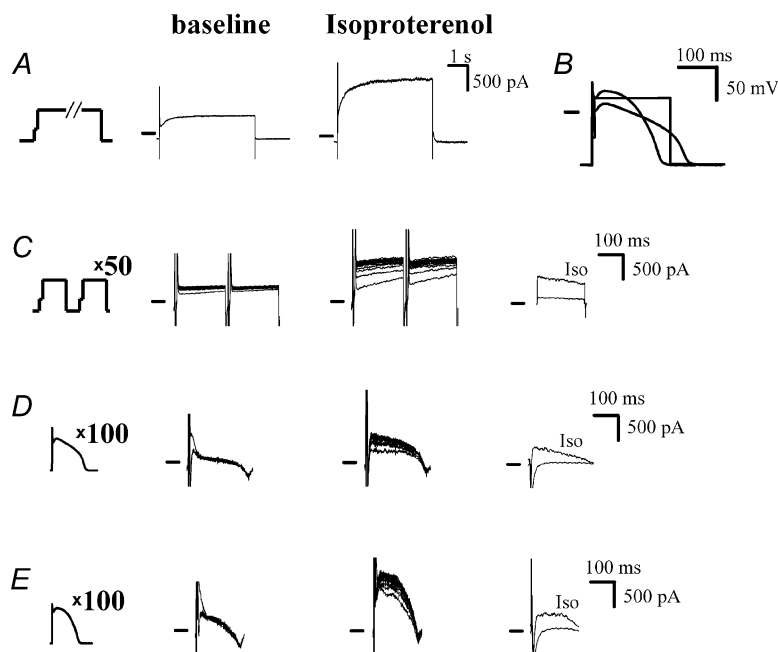
**Figure 4.  $I_{Ks}$  accumulates with AP-like pulses**

**A**, with AP pulses (interpulse interval of 20 ms) an accumulating current appeared at baseline, was increased by isoproterenol (100 nM) and was blocked by HMR 1556 (500 nM), consistent with  $I_{Ks}$ . **B**, accumulated current (obtained as the difference current of the 100th and the 1st traces shown in **A**) at baseline, in the presence of isoproterenol and in the presence of HMR 1556. **C**, command waveform AP.

agonists increase  $I_{Ks}$  directly (Marx *et al.* 2002), but also indirectly by changing the AP waveform (Volders *et al.* 2003). Plateau membrane potential ( $V_m$ ) is increased and APD at  $V_m > 0$  mV is prolonged. APD measured at 95% completion of repolarization (APD<sub>95</sub>), however, shortens (Fig. 5B). Such changes of the AP profile are favourable for  $I_{Ks}$  activation, since longer periods of time are spent at potentials relevant for  $I_{Ks}$  activation ( $> 0$  mV). Therefore, in the AP clamp experiments we used two different command waveforms: (1) baseline AP and (2) isoproterenol-shaped AP (shown, together with the 200 ms-long square pulse, in Fig. 5B). The accumulation accomplished using these AP clamp protocols (Fig. 5D and E) is shown and compared with a square pulse experiment (Fig. 5C) in Fig. 5 (interpulse interval of 20 ms for all). With regard to AP clamp experiments, at baseline the accumulation was negligible regardless of the AP shape. To reach a significant accumulation, isoproterenol (100 nM) had to be present. Both at baseline and with isoproterenol present, the

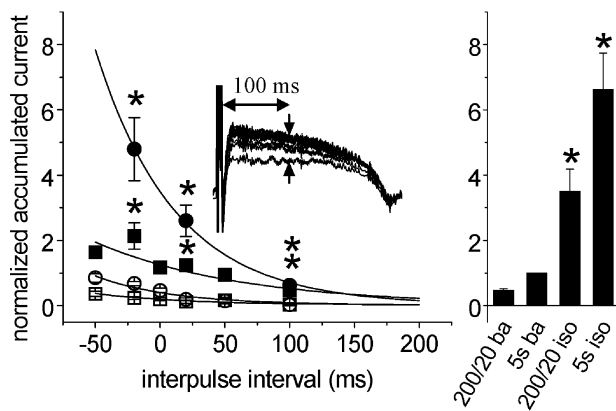
accumulation was most prominent with a square pulse protocol.

AP clamp experiments with varying interpulse intervals are summarized in Fig. 6. The accumulated current ( $I_{Ks}$ ) was determined as the difference between the last (100th) and first traces at 100 ms from the start of the pulse (Fig. 6, inset). The analysis was also performed at 50 and 150 ms with qualitatively similar results. At baseline, regardless of AP shape, accumulation occurred only at interpulse intervals  $< 0$  ms, i.e. the next AP started earlier than the time at which 95% repolarization of the previous AP was reached. With isoproterenol (100 nM) present an accumulation was observed at interpulse intervals up to 100 ms and it was more pronounced with isoproterenol-shaped APs. The column graph allows comparison with fully activated  $I_{Ks}$  and  $I_{Ks}$  accumulated by square pulses (same voltage protocols as in Fig. 5, first column) in the absence and the presence of isoproterenol.



**Figure 5. Dependence of  $I_{Ks}$  accumulation on the shape of the depolarization pulse and on isoproterenol**

A,  $I_{Ks}$  activated by a 5 s-long pulse to +20 mV (1st panel) at baseline (2nd panel) and in the presence of isoproterenol (100 nM, 3rd panel). B, comparison of command waveform pulses: square 200 ms-long pulse to +20 mV, baseline AP (long, low plateau), isoproterenol-shaped AP (short, high plateau). The APs were originally recorded with high-resistance 3 M KCl-filled microelectrodes at a cycle length (CL) of 500 ms, at baseline and in the presence of isoproterenol. C, accumulation with square pulses (1st panel, interpulse interval of 20 ms), at baseline (2nd panel) and in the presence of isoproterenol (3rd panel). Accumulated currents (measured as difference currents between the 100th and 1st traces in previous panels) at baseline and with isoproterenol (4th panel). D, accumulation with baseline AP pulses (1st panel, interpulse interval of 20 ms), at baseline (2nd panel) and in the presence of isoproterenol (3rd panel). Accumulated currents (measured as the difference currents between the 100th and 1st traces in previous panels) at baseline and with isoproterenol (4th panel). E, accumulation with isoproterenol-shaped AP pulses (1st panel, interpulse interval of 20 ms), at baseline (2nd panel) and in the presence of isoproterenol (3rd panel). Accumulated currents (measured as the difference currents between the 100th and 1st traces in previous panels) at baseline and with isoproterenol (4th panel).



**Figure 6.**  $I_{Ks}$  accumulation with AP pulses is dependent on interpulse interval, shape of AP pulse and isoproterenol

The magnitude of accumulation was determined as the difference between the 100th and the 1st traces at 100 ms from the start of the pulse (see inset) and normalized to the  $I_{Ks}$  activated during a 5 s-long pulse to +20 mV (see Fig. 2C). Open symbols represent values at baseline, filled symbols represent values in the presence of isoproterenol (100 nM), squares correspond to values obtained with baseline AP pulses, circles correspond to values recorded with isoproterenol-shaped AP pulses. The right panel enables comparisons to be made with fully activated  $I_{Ks}$  ( $I_{Ks}$  activated during a 5 s-long pulse to +20 mV) at baseline (5s ba) or in the presence of isoproterenol (5s iso) as well as with  $I_{Ks}$  accumulated with 200 ms-long square pulses (interpulse interval of 20 ms) at baseline (200/20 ba) or in the presence of isoproterenol (200/20 iso).  $n = 7$ , \* $P < 0.05$ , isoproterenol vs. baseline.

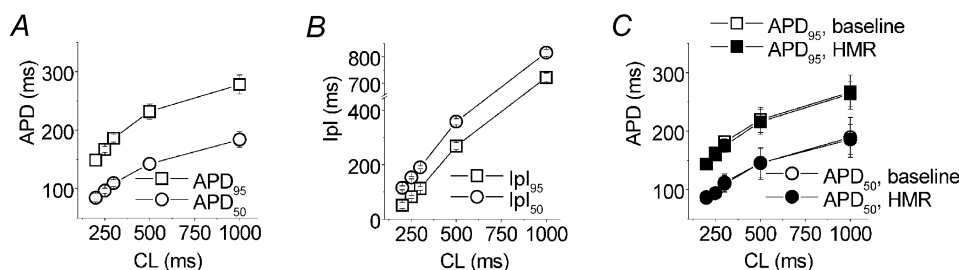
### Physiological relevance of $I_{Ks}$ accumulation

To understand the physiological relevance, we examined APD and interpulse interval at relevant (high) stimulation rates. This was done with high-resistance microelectrodes to prevent the cell dialysis and  $Ca^{2+}$  buffering. In each cell we tried to find the highest rate (shortest CL) with normal capturing: CL was gradually decreased in 50 ms steps, until capturing was lost (not all stimuli followed by an AP) or electrical alternans (pattern of a long AP followed by a short AP with APD<sub>95</sub> difference > 10 ms) was encountered, then the CL was increased by 25 ms. This allowed us to determine the shortest CL captured with a resolution of

25 ms. At baseline the shortest CL captured was 200 ms (reached in 5 of 12 cells). APD<sub>95</sub> at this CL was  $149 \pm 10$  ms (Fig. 7A) leaving an interpulse interval of ~50 ms (Fig. 7B). It should be realized, however, that  $I_{Ks}$  deactivation does not start at the level of 95% repolarization (approximately -75 mV in our experiments), but much earlier (at approximately -10 mV, though at these less negative potentials the deactivation is slower). Therefore we also measured APD<sub>50</sub> (50% repolarization corresponds to approximately -20 mV), and it was  $85 \pm 7$  ms (Fig. 7A) leaving ~115 ms for  $I_{Ks}$  deactivation. Comparison of these values with data presented in Fig. 6 suggests that, at baseline,  $I_{Ks}$  accumulation is very unlikely. To verify this, HMR 1556 (500 nM) was administered and, regardless of CL, it showed no effect on APD (Fig. 7C), which, besides confirming lack of accumulation, strongly suggests that  $I_{Ks}$  does not significantly contribute to baseline APD (Volders *et al.* 2003).

Isoproterenol (100 nM) induced shortening of APD<sub>95</sub> (Fig. 8A, left panel), most notably at long CL (e.g. 1000 ms). At short CLs (e.g. 200 ms) the effect became negligible. In contrast, APD<sub>50</sub> was prolonged at most CLs except the longest one (1000 ms; Fig. 8B, left panel), consistent with the change in AP configuration described above. Besides changes in AP duration and shape, isoproterenol also decreased the shortest CL with normal capturing (Fig. 8D), on average from  $242 \pm 14$  ms to  $175 \pm 13$  ms ( $n = 6$ ,  $P < 0.05$ ). Application of HMR 1556 (500 nM) in the presence of isoproterenol prolonged both APD<sub>95</sub> and APD<sub>50</sub> (Fig. 8A and B, right panels). The effect of HMR 1556 was CL dependent, being most pronounced at long CLs (Fig. 8C). The AP-prolonging effects were manifested at the end of the plateau and the overall AP shape was not changed. Consistent with the diminished effect on APD at short CLs, HMR 1556 did not influence the shortest CL with normal capturing (Fig. 8D).

A diminished effect of HMR 1556 at short CLs (high rates) argues against accumulation in the presence of isoproterenol. The interpulse intervals (~60 ms at CL of 200 ms, ~30 ms at CL of 150 ms, Fig. 8A), however, are,



**Figure 7.** APD at baseline

A, APD<sub>95</sub> (squares) and APD<sub>50</sub> (circles),  $n = 12$ . AP was measured with high resistance 3 M KCl-filled microelectrodes. B, interpulse intervals corresponding to APD in A. C, HMR 1556 (500 nM) failed to prolong baseline APD<sub>95</sub> and APD<sub>50</sub>.

according to AP clamp data (Fig. 6), short enough to ensure a substantial accumulation. One reason for this discrepancy could be that the AP clamp data were obtained with a constant AP (originally recorded at a CL of 500 ms) and only the interpulse interval varied. Normally, however, shortening of CL is associated with a shortening of the AP. Therefore at short CLs the AP could become so short that a sufficient activation of  $I_{Ks}$  (and subsequently accumulation) is precluded despite the presence of isoproterenol. To address this issue, we performed an AP clamp experiment, where APs recorded in the presence of isoproterenol at a CL of 175 ms served as the command waveform. Such an AP is significantly shorter than the 500 ms CL isoproterenol-shaped AP used in previous experiments (APD<sub>95</sub> 121 vs. 188 ms, Fig. 9A). In the presence of isoproterenol (100 nM), experiments with the 500 ms CL AP revealed a substantial accumulation at an interpulse interval of 20 ms (Fig. 9B). A significant  $I_{Ks}$  (identified as HMR 1556-sensitive current) was detected as early as during the first pulse (Fig. 9B, bottom panel, lower trace). When the 175 ms CL APs were delivered at a CL of 175 ms, accumulation, although much smaller than in the previous case, was also observed (Fig. 9C). HMR 1556-sensitive  $I_{Ks}$  detected during the first pulse (40 pA, Fig. 9C, bottom panel, lower trace) was smaller than  $I_{Ks}$  activated during the first 500 ms CL AP (94 pA).

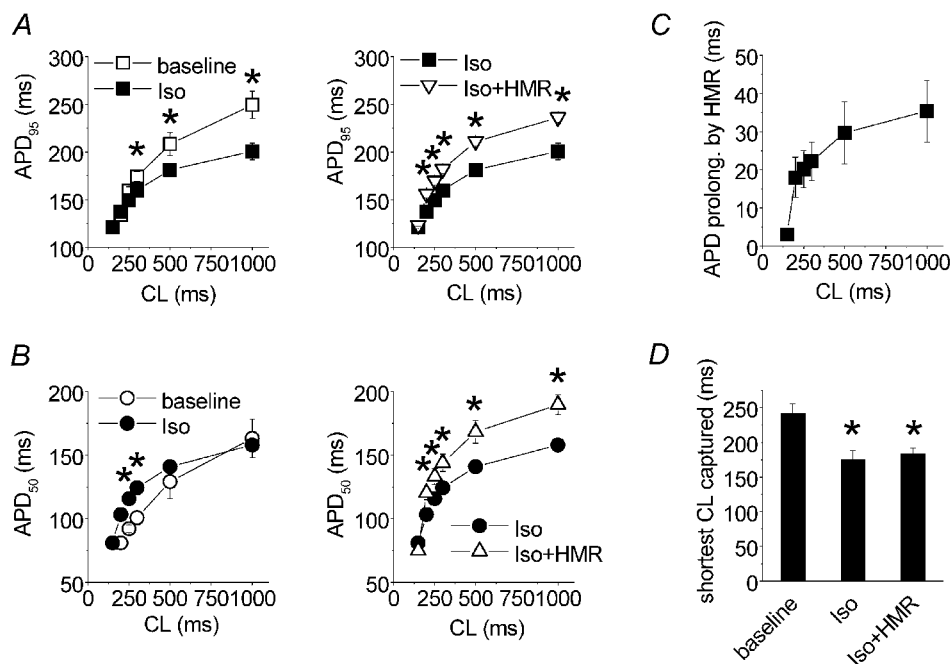
Nevertheless, the steady state  $I_{Ks}$  at a CL of 175 ms (205 pA, determined as HMR 1556-sensitive current during the 100th AP) was still twice the magnitude of  $I_{Ks}$  activated during the first 500 ms CL AP (steady state  $I_{Ks}$  at a CL of 500 ms). Similar results showing a significant accumulation at CLs of 175 and 150 ms were obtained in four other cells. This demonstrates that a significant accumulation of  $I_{Ks}$  is present at the shortest CL, even if HMR 1556 has a negligible effect on APD.

## DISCUSSION

The results of the present study show that a physiologically relevant accumulation of  $I_{Ks}$  in canine ventricular myocytes only occurs in the presence of a  $\beta$ -adrenergic agonist, isoproterenol (100 nM in our experiments). Block of  $I_{Ks}$  by HMR 1556 in the presence of isoproterenol led, however, to a reverse rate-dependent prolongation of APD. This indicates that despite the accumulative increase the relative functional contribution of  $I_{Ks}$  to the repolarization at fast rates decreases, probably as a result of the involvement of other repolarizing currents.

### Role of $I_{Ks}$ accumulation in repolarization

Experiments with conventional glass microelectrodes and a selective  $I_{Ks}$  blocker, HMR 1556, revealed that block of  $I_{Ks}$  in the presence of isoproterenol in canine ventricular cells



**Figure 8. APD in the presence of isoproterenol**

A, isoproterenol (100 nM) shortened APD<sub>95</sub> at most CLs tested, except at short CLs (left panel). Addition of HMR 1556 (500 nM) on top of isoproterenol prolonged APD<sub>95</sub> (right panel),  $n = 6$ ,  $*P < 0.05$ . B, isoproterenol prolonged APD<sub>50</sub> at short CLs (left panel). Addition of HMR 1556 (500 nM) on top of isoproterenol prolonged APD<sub>50</sub> (right panel),  $n = 6$ ,  $*P < 0.05$ . C, HMR 1556-induced prolongation of APD<sub>95</sub> in the presence of isoproterenol. D, the shortest CL with normal capturing at baseline, in the presence of isoproterenol and after addition of HMR 1556,  $*P < 0.05$  vs. baseline.

led to a prolongation of APD, which was most pronounced at low stimulation rates and decreased with increasing the rate. The impact of block of  $I_{Ks}$  (or of any other current) on APD depends on the magnitude of the current blocked and also on the magnitude of the total repolarization current. On the one hand, if the total (net) repolarization current is much larger than  $I_{Ks}$ , the relative change of the net current by block of  $I_{Ks}$  will be negligible and consequently APD prolongation will also be minor. On the other hand, if the net current is of similar amplitude to  $I_{Ks}$ , block of  $I_{Ks}$  will shift the net repolarization current close to zero, which will result in a considerable prolongation of APD. Thus, the proportion of total repolarization current accounted for by  $I_{Ks}$  will determine the degree of APD prolongation.

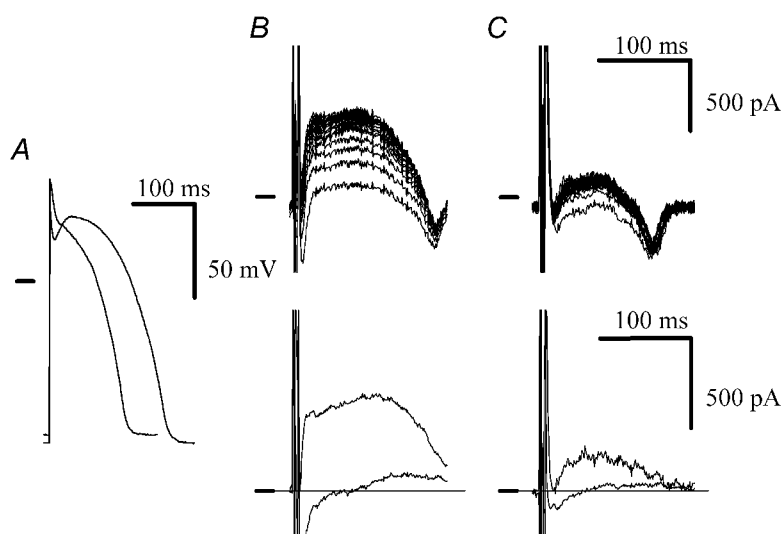
Based on these arguments, it may be suggested that the relative contribution of total  $I_{Ks}$  (single AP  $I_{Ks}$  plus accumulated  $I_{Ks}$ ) to the repolarization at (physiologically relevant) high rates is smaller than at low rates, where no accumulation occurs. Fast rate APs are much shorter than APs at low rates (Figs 8 and 9), therefore  $I_{Ks}$  activated by single fast rate AP is significantly smaller than  $I_{Ks}$  during low rate APs (Fig. 9). However, because of the accumulation, the total (or steady-state)  $I_{Ks}$  was larger at fast rates (Fig. 9). For the relative contribution of total  $I_{Ks}$  to the repolarization to be smaller at fast than at low rates, the net outward current during repolarization at fast rates must increase more than  $I_{Ks}$  alone. In other words, outward currents other than  $I_{Ks}$  have to be increased (or inward currents decreased). Boyett and Fedida investigated the rate-dependent changes of membrane currents in dog (Boyett & Fedida, 1984) and sheep (Boyett & Fedida, 1988) Purkinje fibres and, interestingly, their results are consistent with this hypothesis: at high rates (above 1 Hz) there was an increase in the background outward current, which was attributed to the  $\text{Na}^+ - \text{K}^+$  pump, and simultaneously, there was a decrease in the inward  $\text{Ca}^{2+}$  current. In our experimental setting we were unable to verify these results, since in order to

maximally visualize  $I_{Ks}$  in the voltage clamp experiments (both square pulses and AP clamp),  $\text{K}^+$  was omitted from extracellular solutions (i.e. the  $\text{Na}^+ - \text{K}^+$  pump was inhibited) and  $\text{Ca}^{2+}$  current was blocked by nifedipine (and  $I_{Kr}$  blocked by almokalant). Another rate-dependent current is the transient outward current ( $I_{to}$ ). In contrast to  $I_{Ks}$ ,  $I_{to}$  decreases at fast rates because of incomplete recovery from inactivation. In our experiments,  $I_{to}$  could be detected during the first pulse only (e.g. Figs 4 and 5). In subsequent pulses it remained completely inactivated.  $I_{to}$  did not interfere with accumulation measurements (measured at 100 ms from the start of an AP), since in canine ventricular myocytes it inactivates with a time constant of  $\sim 10$  ms (Volders *et al.* 1999b).

A possible intrinsic reverse rate dependency of  $I_{Ks}$  block by HMR 1556 could also explain the discrepancy between AP clamp data and transmembrane potential recordings. However, such a reverse rate dependency is very unlikely, since in our square pulse patch clamp experiments the stimulation rates varied between 0.1 and  $> 5$  Hz and HMR 1556 (500 nM) always fully suppressed  $I_{Ks}$  (both time-dependent outward current during depolarization and tail current upon repolarization; e.g. Fig. 1).

### Mechanism of isoproterenol-induced increase of $I_{Ks}$ accumulation

Isoproterenol enhances  $I_{Ks}$  conductance and negatively shifts the voltage dependence of  $I_{Ks}$  activation (Walsh & Kass, 1988; Sanguinetti *et al.* 1991; Han *et al.* 2001; Volders *et al.* 2003). In addition, changes in  $I_{Ks}$  kinetics, such as acceleration of  $I_{Ks}$  activation (Han *et al.* 2001; Volders *et al.* 2003; this study) and slowing down of deactivation (this study), were found in the presence of isoproterenol. These changes can significantly add to the accumulation. Accelerated activation results in an increased number of activated channels during a single pulse and subsequently can lead to a higher level of the total (steady-state) current. Analogous to this, when an increased activation was



**Figure 9.**  $I_{Ks}$  accumulation in the presence of isoproterenol at a CL of 175 ms

A, comparison of the isoproterenol-shaped AP used in the preceding AP clamp experiments (originally recorded at a CL of 500 ms) with an AP recorded in the presence of isoproterenol at a CL of 175 ms (the shorter AP). B, upper panel, accumulation with 500 ms CL AP as command waveform and interpulse interval of 20 ms in the presence of isoproterenol (100 nM). Lower panel, HMR 1556 (500 nM)-sensitive currents during the 1st and 100th AP pulses. C, upper panel, accumulation with 175 ms CL AP as command waveform at CL of 175 ms in the presence of isoproterenol (100 nM). Lower panel, HMR 1556 (500 nM)-sensitive currents during the 1st and 100th AP pulses.



reached by prolongation of the depolarization pulse in our experiments (Fig. 2), increased accumulation occurred. Slower deactivation results in more accumulation, since fewer channels deactivate during a given interpulse interval, thus leaving more channels in an open state for the next depolarization. The experiments in Fig. 3B show that slower deactivation (at  $-50$  versus  $-80$  mV) is indeed associated with increased accumulation.

### Reverse rate-dependent prolongation of APD by $I_{Kr}$ blockers

$I_{Kr}$  blockers prolong APD more at low than at high stimulation rates. In guinea-pig ventricular myocytes this reverse rate dependence was attributed to incomplete deactivation of  $I_{Ks}$  at fast rates, which partially offsets the rate-independent block of  $I_{Kr}$  (Jurkiewicz & Sanguinetti, 1993). In guinea-pigs, however, the deactivation of  $I_{Ks}$  is very slow. In other species including dog it is much faster (see Introduction). Despite the rapid deactivation of canine  $I_{Ks}$ , block of  $I_{Kr}$  still causes reverse rate-dependent prolongation of APD, which, according to Gintant (1996), is inconsistent with the postulated role of  $I_{Ks}$  in reverse rate dependence. Our data further argue against this role by showing that the  $I_{Ks}$  block itself (in the presence of isoproterenol) causes a reverse rate-dependent prolongation of APD (Fig. 8).

### Role of $I_{Ks}$ in canine ventricular repolarization

This issue remains rather controversial. On the one hand,  $I_{Ks}$  is suggested to be an important contributor, since congenital and acquired  $I_{Ks}$  deficiencies in both experimental and clinical conditions are often associated with abnormally long QT intervals. Some studies show AP prolongation after the application of  $I_{Ks}$ -blocking drugs such as chromanol 293B in a ventricular wedge preparation (Shimizu & Antzelevitch, 1998). On the other hand, some reports indicate that pharmacological  $I_{Ks}$  block does not prolong APD under baseline conditions (Varro *et al.* 2000).

Recent studies in various cardiac preparations (canine cardiac Purkinje cells, Han *et al.* 2001; rabbit sinoatrial node cells, Lei *et al.* 2002; canine ventricular cells, Volders *et al.* 2003) have shown that despite the lack of a contribution of  $I_{Ks}$  to APD at baseline, this current becomes important during  $\beta$ -adrenergic stimulation. Consistently, in conscious dogs, in which some level of adrenergic stimulation must be assumed, ventricular repolarization was found to be importantly dependent on  $I_{Ks}$  (Volders *et al.* 2003). These findings are in good agreement with the present data.

### Methodological considerations

The concentration of isoproterenol in most of our experiments was 100 nM.  $IC_{50}$  for isoproterenol-induced stimulation of  $I_{Ks}$  was found to be in the nanomolar range (0.4–18 nM, Yazawa & Kameyama, 1990; Kathöfer *et al.*

2000) in guinea-pig ventricular myocytes and in *Xenopus* oocytes coexpressing KvLQT1/MinK channels and the human  $\beta_3$ -adrenoreceptor subunit (8 nM, Kathöfer *et al.* 2000). In preliminary experiments we also found similar values for canine ventricular myocytes. Therefore, the stimulation of  $I_{Ks}$  in our experiments is maximal or close to maximal.

In most AP clamp experiments only the interpulse (diastolic) interval varied, whereas AP waveform was kept constant. This is an obvious limitation, since normal behaviour is much more dynamic and a change in diastolic interval is associated with a change in AP shape and duration. Because of this, our AP clamp experiments with a constant AP waveform will overestimate  $I_{Ks}$  accumulation. Even so,  $I_{Ks}$  accumulation only occurred in the presence of isoproterenol; unstimulated  $I_{Ks}$  did not accumulate at all. To account for the changes in AP waveform at fast rates in the presence of isoproterenol, we repeated the  $I_{Ks}$  accumulation experiments with rate-related short AP command waveforms, and some accumulation was found (Fig. 9).

### *In vivo* implications

Translation of our cellular data to the *in vivo* situation is not straightforward. A serious limitation is that in cellular experiments the concentration of the  $\beta$ -adrenergic agonist was constant and the rate was changed artificially by pacing, whereas *in vivo* the rate changes in response to changing concentrations of catecholamines. This makes the *in vivo* situation much more complex and it would be very difficult to predict the effects of  $I_{Ks}$  block *in vivo*. On the one hand, with increasing rate, the APD-prolonging effects of  $I_{Ks}$  block decrease. On the other hand, *in vivo*, the transition from a low to a high rate is associated with increased  $\beta$ -adrenergic stimulation, during which the importance of  $I_{Ks}$  (and also the APD-prolonging effect of  $I_{Ks}$  block) increases. From the point of view of arrhythmology and if  $I_{Ks}$  blockers are considered as potential antiarrhythmic drugs it will be very important to determine which mechanism prevails and subsequently what kind of response (rate-dependent, reverse rate-dependent, rate-independent APD prolongation) can be expected for  $I_{Ks}$  block *in vivo*. For this, *in vivo* experiments employing the application of  $I_{Ks}$  blockers together with interventions to the adrenergic system (exercise,  $\beta$ -blocking therapy, etc.) will be necessary.

### Conclusions

Our data indicate that at baseline (i.e. unstimulated conditions)  $I_{Ks}$  (accumulation) does not contribute to repolarization, and it only becomes important during  $\beta$ -adrenergic stimulation.

The prolongation of APD by  $I_{Ks}$  block during  $\beta$ -adrenergic stimulation was reverse rate-dependent, indicating that  $I_{Ks}$  accumulation does not have a dominant role at short CLs.

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