

RESEARCH PAPER

The plateau outward current in canine ventricle, sensitive to 4-aminopyridine, is a constitutive contributor to ventricular repolarization

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Background and purpose: I_{Kur} (Ultra-rapid delayed rectifier current) has μM sensitivity to 4-aminopyridine (4-AP) and is an important modulator of the plateau amplitude and action potential duration in canine atria. Kv1.5 encodes I_{Kur} and is present in both atria and ventricles in canines and humans. We hypothesized that a similar plateau outward current with μM sensitivity to 4-AP is present in canine ventricle.

Experimental approach: We used established voltage clamp protocols and used 4-AP (50 and 100 μM) to measure a plateau outward current in normal canine myocytes isolated from the left ventricular mid-myocardium.

Key results: Action potential recordings in the presence of 4-AP showed significant prolongation of action potential duration at 50 and 90% repolarization at 0.5 and 1 Hz ($P < 0.05$), while no prolongation occurred at 2 Hz. Voltage clamp experiments revealed a rapidly activating current, similar to current characteristics of canine atrial I_{Kur} , in $\sim 70\%$ of left ventricular myocytes. The IC_{50} of 4-AP for this current was 24.2 μM . The concentration of 4-AP used in our experiments resulted in selective blockade of an outward current that was not I_{to} or I_{Kr} . β -Adrenergic stimulation with isoprenaline significantly increased the 4-AP sensitive outward current density ($P < 0.05$), suggesting a role for this current during increased sympathetic stimulation. *In silico* incorporation into a canine ventricular cell model revealed selective AP prolongation after current blockade.

Conclusions and implications: Our results support the existence of a canine ventricular plateau outward current sensitive to micromolar 4-AP and its constitutive role in ventricular repolarization.

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Abbreviations: APD, action potential duration; 4-AP, 4-aminopyridine; DPO-1, (2-isopropyl-5-methylcyclohexyl diphenylphosphine oxide); I_{Kr} , rapid delayed rectifier K^+ current; I_{Ks} , slow delayed rectifier current; I_{Kur} , ultra-rapid delayed rectifier current; I_{to} , transient outward potassium current

Introduction

Cardiac action potential repolarization occurs through a multitude of voltage-gated K^+ channels with differing activation and inactivation patterns (Nerbonne and Kass, 2005). The transient outward potassium current (I_{to}) activates and inactivates rapidly; thereby contributing to early phase 1 repolarization (Patel and Campbell, 2005). The rapid (I_{Kr}) and slow (I_{Ks}) delayed rectifier K^+ currents activate towards the end of the action potential plateau (phase 2) and contribute significantly to phase 3 repolarization. In rat, canine and human atria, and in rodent and guinea pig ventricle, an ultra-rapid delayed rectifier current (I_{Kur}) has

been described (Yue and Marban, 1988; Fedida *et al.*, 2003; Brouillette *et al.*, 2004; Nerbonne and Kass, 2005). I_{Kur} is a determinant of the normal canine atrial action potential duration (APD). I_{Kur} , like I_{to} activates very rapidly, but in contrast to I_{to} inactivates very slowly; I_{Kur} along with I_{to} contributes to phase 1 repolarization in the atria (Nerbonne and Kass, 2005).

Despite the abundant Kv1.5 protein expression in canine and human ventricle, previous studies have not demonstrated functional ventricular I_{Kur} in canine or human ventricular myocytes (Yue *et al.*, 1996; Li *et al.*, 1996b). More recently, I_{Kur} has received increased attention due to the 'atrial-specific' expression of the current (in canines and humans), providing a novel therapeutic target for the treatment of atrial arrhythmias without the risk of ventricular proarrhythmia (Page and Roden, 2005).

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I_{Kur} is sensitive to micromolar concentrations of 4-aminopyridine (4-AP), while I_{to} is inhibited only at millimolar concentrations (Nattel *et al.*, 2000; Patel and Campbell, 2005). This difference in sensitivity to 4-AP has been used to define I_{Kur} in canine and human atria, and in rodent ventricle. We elicited a current that is sensitive to micromolar concentrations of 4-AP, which is activated during the plateau voltages of the action potential in canine left ventricular mid-myocardium. This outward current has a constitutive role in ventricular repolarization.

Materials and methods

Animal procedures and myocyte isolation

All animal procedures were approved by the Institutional Lab Animal Use and Care Committee of The Ohio State University.

Twenty-three adult hound-type dogs (age 9 months to 5 years) weighing between 8 and 20 kg were used for the experiments. Dogs were verified to have normal cardiac function by routine electrocardiograms and echocardiographic examinations during butorphanol tartarate (0.5 mg kg^{-1} intramuscularly) sedation. On the day of the experiment, dogs were killed by intravenous injection of pentobarbital sodium (120 mg kg^{-1} for the first 4.5 kg and 60 mg kg^{-1} for every 4.5 kg thereafter) via the cephalic vein. Following this, the hearts were rapidly excised via thoracotomy and perfused with cold cardioplegic solution (containing 5% glucose, 0.1% mannitol, 22.4 mM NaHCO_3 and 30 mM KCl) injected into the coronary ostia. The left circumflex artery was cannulated for myocyte isolation as described previously (Kubalova *et al.*, 2005). Following the washout of blood from the heart, collagenase (Worthington type II, 0.65 mg ml^{-1}) and protease-free bovine serum albumin (BSA) (0.65 mg ml^{-1}) were added to the perfusate (100 ml). After 30–45 min of collagenase perfusion, the digested mid-myocardial section of the lateral wall of the left ventricle was separated from the epicardial and endocardial sections; digested tissue was shaken in a water bath at 37°C for an additional 5–10 min. This typically yielded 70–90% of rod-shaped myocytes with staircase ends and sharp margins. The myocytes were stored at room temperature in a standard incubation buffer solution containing (in mM) NaCl 118, KCl 4.8, MgCl_2 1.2, KH_2PO_4 1.2, glutamine 0.68, glucose 10, pyruvate 5, CaCl_2 1, along with $1 \mu\text{mol l}^{-1}$ insulin, and 1% BSA until use.

Electrophysiological protocols

Myocytes were placed in a laminin-coated cell chamber (Cell Microcontrols, Norfolk, VA, USA) and superfused with bath solution containing (in mM) 135 NaCl, 5 MgCl_2 , 5 KCl, 10 glucose, 1 CaCl_2 , 5 HEPES, pH 7.40 with NaOH, at a temperature of $36 \pm 0.5^\circ\text{C}$. For action potential recordings, the same bath solution was used with CaCl_2 increased to 1.8 mM. During voltage clamp experiments to measure potassium currents, L-type calcium current was blocked by $2 \mu\text{M}$ nifedipine. Solutions were changed with a six-port gravity flow system ($\sim 1 \text{ ml min}^{-1}$). Borosilicate glass micropipettes

(tip resistance between 1.5–3 M Ω) were filled with pipette solution containing (in mM) 100 K^+ -aspartate, 40 KCl, 5 MgCl_2 , 5 EGTA, 5 HEPES, pH adjusted to 7.2 with KOH. Perforated whole-cell patch clamp (using amphotericin B) was used to minimize alterations in intracellular milieu. For voltage clamp experiments, only recordings with an access resistance $< 20 \text{ M}\Omega$ were included in the analyses. For determination of drug-sensitive currents, only cells with less than a 20% change in access resistance were included in the analyses. Series-resistance compensation ($\sim 70\%$) was used for current recordings. All 4-AP-sensitive currents were recorded after 3–5 min of 4-AP superfusion.

Action potentials were recorded with perforated whole-cell patch techniques, as described above. Action potentials were measured as the average of the last 10 (steady-state) action potentials, recorded during a train of 25 action potentials at each stimulation rate. To analyse beat-to-beat variability in the action potential recordings, standard deviation and coefficient of variation (CV) of the APD at 90% repolarization was calculated. The amplitude of phase 2 was measured as the maximum potential following phase 1 of the action potential.

I_{to} and the rapid component of the I_{Kr} were elicited using voltage protocols as shown in the insets of Figure 2. Currents were recorded both in the presence and absence of $100 \mu\text{M}$ 4-AP to examine potential inhibition of I_{to} and I_{Kr} by 4-AP.

Sustained outward potassium current was elicited from a holding potential of -40 mV with an 80 ms prepulse to $+30 \text{ mV}$ to inhibit I_{to} , followed by 300 ms voltage steps from -20 mV to $+50 \text{ mV}$. The interval between each voltage step was 3 s. 4-AP-sensitive plateau outward current was measured as the steady-state difference current after a minimum of 4 min of superfusion with 4-AP. The activation time constant of the 4-AP-sensitive current was determined by fitting the activation to a monoexponential function in Clampfit (v 8.0, Axon Instruments, Union City, CA, USA).

We tested two concentrations of 4-AP: 50 and $100 \mu\text{M}$, based on previously published observations (Yue *et al.*, 1996; Brouillette *et al.*, 2004). An envelope-of-tails test was adapted from a previously published method used to evaluate I_{Kur} in canine atria (Yue *et al.*, 1996). The envelope-of-tails protocol started from a holding potential of -40 mV with a prepulse to $+30 \text{ mV}$, followed 30 ms later by a variable-duration test pulse (60–240 ms) to $+20 \text{ mV}$, followed by a step to -30 mV to elicit the tail current. The interval between test pulses was 3 s. The constancy of the ratio of the step current to tail current was evaluated as discussed below.

Data acquisition was performed with Clampex 8.0 software (Axon Instruments) and an Axopatch 200A patch clamp amplifier (Axon Instruments).

Statistical analysis

Acquired data were analysed using Clampfit 8.0 software (Axon Instruments) and Origin 6.1 software (OriginLab, Northampton, MA, USA). Currents were normalized to cell capacitance in picofarads (pF) and are expressed as pA pF^{-1} . All data are presented as mean \pm s.e.

APDs obtained at baseline and during drug exposure were analysed by one-way analysis of variance (SAS for Windows

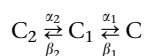
v9.1, SAS Inc., Cary, NC, USA). 4-AP-sensitive, isoprenaline-modulated and baseline 4-AP-sensitive current densities at each test voltage were statistically compared using Student's *t*-tests. Dose-response curves for 4-AP were constructed using the Hill equation in Origin 6.1 (OriginLab). To test the envelope-of-tails data, a linear regression was performed (SAS for Windows, v9.1, SAS Inc.) to calculate the slope and to test whether the slope was statistically different from zero. $P < 0.05$ was the criterion for statistical significance for all tests.

Chemicals and solutions

All chemicals for buffer and stock solution preparation were purchased from Fisher Scientific (Pittsburgh, PA, USA), Sigma-Aldrich (St Louis, MO, USA) and Invitrogen Inc. (Carlsbad, CA, USA). Stock solutions of nifedipine, amphotericin B and 4-aminopyridine were prepared fresh on the day of each experiment. DPO-1, (2-isopropyl-5-methylcyclohexyl diphenylphosphine oxide, Tocris Bioscience, Ellisville, MD, USA), a relatively new, selective I_{Kur} blocker (Lagrutta *et al.*, 2006) was used for a separate set of experiments, and was prepared from a stock solution (10 mM) in dimethylsulphoxide prepared on the day of each experiment. Isoprenaline solutions were prepared daily from commercially available injectable solutions (0.2 mg ml^{-1}), which were stored at 4°C until use. All nifedipine, isoprenaline, DPO-1 and amphotericin B solutions were protected from exposure to light.

Computational methods

Because the patch clamp data suggested little inactivation of the current, we used a three-state Markov model:



with two closed states (C_1 and C_2), and one open state (O) to fit the data. All state transition rates (α_1 , α_2 , β_1 , β_2) are of the form $P_x \cdot \exp(P_y \cdot V)$, where P_x and P_y are parameters and V is the membrane potential in mV.

From the recorded difference current, a training data set for the model fitting was constructed. First, data during the pre-pulses and data after the 300 ms pulses were discarded. Second, data demonstrating a capacitance transient were excluded to remove any artifactual changes in the current.

Optimization of the model parameters began with a set of randomly selected parameters. The same voltage clamp protocol that was used in the experiment was applied to the model. The resulting simulated current was compared to the training data. A cost function, $CF(M)$, was defined where M is the vector of the parameters in the model that was to be optimized. This cost function is defined by

$$CF(M) = \frac{\sum_i (Train_data_i - Sim_data_i(M))^2}{N}$$

Here $Train_data_i$ is the training data at the i th time point, $Sim_data_i(M)$ is the simulation value at the i th time point using the parameters given by M . The sum is taken over $i = 1$

to N , the total number of time points in the recording. The goal of the optimization process was to find the parameter values that minimize the cost function $CF(M)$ by iteratively changing the parameters, simulating the model and evaluating the cost function until the simulation nearly matched the data.

The optimization routine used a combination of global and local methods. The global method, differential evolution (Storn, 1996), used the following strategy: a population of parameter sets was generated randomly, and the cost of each parameter set was evaluated. New parameter sets were generated by first adding a weighted difference between two randomly selected parameter sets to a third random parameter set, and then by exchanging a fraction of the resulting parameter set with a member of the population according to a cross-over probability. The cost of the new parameter set was then evaluated and compared to the cost of a randomly chosen set in the population. The lower cost set was kept in the next generation of the population. The optimization strategy consisted of using differential evolution for a total of 4000 generations. After each thousand generations, the Levenberg-Marquardt (Marquardt, 1963; Press *et al.*, 1992) local method was applied to each parameter set in the population to quickly take each parameter vector to a local minimum.

All simulations and optimizations were run on a Dell Inspiron 9100 computer and a 16-node Linux cluster of Intel Xeon dual processors using custom written C++ computer code. Each model is represented by a set of differential equations of the form $dx/dt = f(x, t, p)$, where x is a vector describing the current state of the system, t is time and p is a vector of parameters. The corresponding differential equations are usually quite stiff in the sense that they have widely separated time scales: some variables change rapidly under small perturbations, while others change slowly. To improve the accuracy of our simulations, we used the CVODES package from Lawrence Livermore National Laboratories (Cohen and Hindmarsh, 1994) with the backward differentiation formula, designed for stiff systems. We also used automatic differentiation to calculate the Jacobian derivative of the function for use with the dense Newton-based solver that is included as part of CVODES.

Results

Myocyte capacitance was $163.8 \pm 7.7 \text{ pF}$ ($n = 56$). Figures 1a and b show representative action potentials recorded at 0.5 and 1 Hz at baseline, in the presence of $100 \mu\text{M}$ 4-AP and during washout. Superfusion with $100 \mu\text{M}$ 4-AP decreases the net outward current evident during phase 1. Consistent with $100 \mu\text{M}$ 4-AP block of an outward current (activated at plateau potentials), phase 2 amplitude was increased from baseline values (Table 1). Figure 1c summarizes the APD data obtained at baseline and after 4-AP superfusion at all tested stimulation frequencies. There was a statistically significant prolongation of APD at 50% (APD_{50}) and 90% (APD_{90}) repolarization seen at 0.5 and 1 Hz (Figures 1c and d). No significant change in the APD was seen at a stimulation rate of 2 Hz during 4-AP superfusion. This effect was reversible

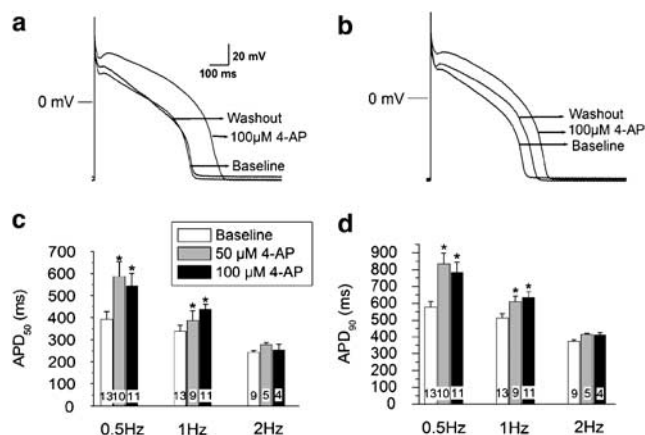


Figure 1 Blockade of I_{Kur} using 50 or 100 μM 4-AP causes reverse use-dependent action potential prolongation. Representative action potential recordings are shown at 0.5 Hz (a) and 1 Hz (b). (c) Summary data for APD_{50} plotted as a function of stimulation frequency. (d) Summary data for APD_{90} plotted as a function of stimulation frequency. The numbers on each bar indicate the number of cells tested. (* $P < 0.05$ compared to baseline recordings). 4-AP, 4-aminopyridine; APD, action potential duration; I_{Kur} , ultra-rapid delayed rectifier current.

Table 1 4-AP at 100 μM prolongs APD and increases phase 2 amplitude at 0.5 and 1 Hz

	Baseline	100 μM 4-AP	Washout
0.5 Hz			
APD ₅₀ (ms)	392.8 \pm 34.6	543.8 \pm 56.6* [†]	367.8 \pm 90.5
APD ₉₀ (ms)	576.3 \pm 33.9	784.6 \pm 60.5* [†]	518.9 \pm 106.2
Phase 2 amplitude (mV)	27.5 \pm 2.6	36.3 \pm 3.0* [†]	27.4 \pm 2.0
1 Hz			
APD ₅₀ (ms)	328.9 \pm 26.6	434.8 \pm 23.7* [†]	328.7 \pm 36.9
APD ₉₀ (ms)	498.4 \pm 21.7	633.3 \pm 34.6* [†]	483.4 \pm 51.9
Phase 2 amplitude (mV)	26.4 \pm 2.7	33.1 \pm 3.1*	29.4 \pm 1.3
2 Hz			
APD ₅₀ (ms)	243.7 \pm 9	253.1 \pm 26.8	242.5 \pm 16.4
APD ₉₀ (ms)	373.8 \pm 9.5	412.1 \pm 14.2	386.6 \pm 22.2
Phase 2 amplitude (mV)	21.6 \pm 4.4	31.4 \pm 5.2*	22.4 \pm 4.7

Abbreviations: APD, action potential duration; 4-AP, 4-aminopyridine.

* $P < 0.05$ vs baseline, [†] $P < 0.05$ vs washout ($n = 11$ –13 cells in 0.5 and 1 Hz, $n = 4$ –9 cells in 2 Hz group).

after prolonged washout of 7–10 min (Table 1). The resting membrane potential was not affected by 50 or 100 μM 4-AP (-76.5 ± 0.5 mV at baseline, -75.7 ± 0.8 mV and -75.2 ± 0.4 mV, with 50 and 100 μM 4-AP, respectively, $P = \text{NS}$). The baseline CVs of the APD_{90} values were 4.2, 5.9 and 5.8% at 0.5, 1 and 2 Hz respectively. Superfusion with 100 μM 4-AP did not change the CVs of the APD_{90} values (3.4, 5.8 and 6.7% at 0.5, 1 and 2 Hz respectively).

K^+ current-dependent action potential prolongation in the canine ventricle has been attributed to two currents: I_{to} , where blockade with 2 mM 4-AP prolongs both APD_{50} and APD_{90} (Litovsky and Antzelevitch, 1989) or I_{Kr} , where blockade selectively prolongs APD_{90} (Gintant, 2000; Varro *et al.*, 2000). Therefore, we sought to exclude these

possibilities and determine whether the 4-AP concentrations used in our experiments affected either I_{to} or I_{Kr} .

Figures 2a and b show I_{to} and I_{Kr} recorded at baseline and during superfusion with 100 μM 4-AP, respectively. There was no significant inhibition of either I_{to} or I_{Kr} amplitude by this concentration of 4-AP, confirming that the observed AP prolongation did not result from blockade of either I_{to} or I_{Kr} .

The 100 μM 4-AP-sensitive difference current, which was obtained by digital subtraction, shows rapid current activation following membrane depolarization (Figure 3a). The 4-AP-sensitive current does not display significant inactivation during the 300 ms test pulse. The current density–voltage relationship of the 100 μM 4-AP-sensitive current is shown in Figure 3b. The outward current begins to activate at -10 mV and increases with increasingly positive test potentials. The threshold for activation (Figure 3b) is consistent with the voltage range occurring during phase 1 of the action potential. The activation time constant was 16.7 ± 11 ms at -10 mV, 4.7 ± 0.81 ms at $+10$ mV and 3.96 ± 1.44 ms at $+50$ mV.

Notably, we were unable to measure a 4-AP-sensitive current (100 μM) in 12 of the 41 myocytes tested with this protocol. This finding was evaluated further in secondary experiments. Action potentials were recorded first, followed by voltage clamp experiments (with nifedipine exposure of 3–4 min) in the same cells, to record baseline currents ($n = 4$). Then, 4-AP superfusion (perfusate calcium at 1.8 mM) was performed to washout nifedipine and record action potentials during 4-AP exposure in the same myocytes. Following the second action potential recordings, voltage clamp experiments were repeated to determine the presence of any 4-AP-sensitive current. No action potential prolongation during 4-AP treatment was seen in myocytes lacking a 4-AP-sensitive current. The converse was also true; in myocytes ($n = 3$) exhibiting 4-AP-dependent action potential prolongation, we were able to consistently elicit a 4-AP-sensitive sustained outward current. These results argue against either a nonspecific effect of 100 μM 4-AP on APD or current rundown during the duration of our experiments.

We analysed the concentration–response data to evaluate the inhibition of the sustained potassium current by 4-AP (Figure 3c). This analysis revealed an IC_{50} value of 24.2 μM . At 50 and 100 μM 4-AP, this fitted curve predicts 78.7 and 96.4% inhibition, respectively. The tested concentrations were therefore close to the maximal blocking concentration, which explains the lack of a significant difference when comparing results from the two concentrations (Figure 1c). We tested only up to a concentration of 500 μM , as a recent publication (Ridley *et al.*, 2003) has shown inhibition of I_{Kr} with millimolar concentrations of 4-AP, and I_{to} is known to be blocked at 1–1.5 mM (Nattel *et al.*, 2000).

Figure 3d shows a representative envelope-of-tails test using the protocol shown. The ratio of the peak tail current to the average steady-state step current as a function of the step duration is shown in Figure 3e. Visual examination of the data revealed a constant ratio as a function of time. This was confirmed statistically by linear regression analysis, which revealed a slope of 0.021 ± 0.015 , which did not differ significantly from a slope of zero ($P = 0.17$); this is consistent with a single current component in the 100 μM 4-AP-sensitive current.

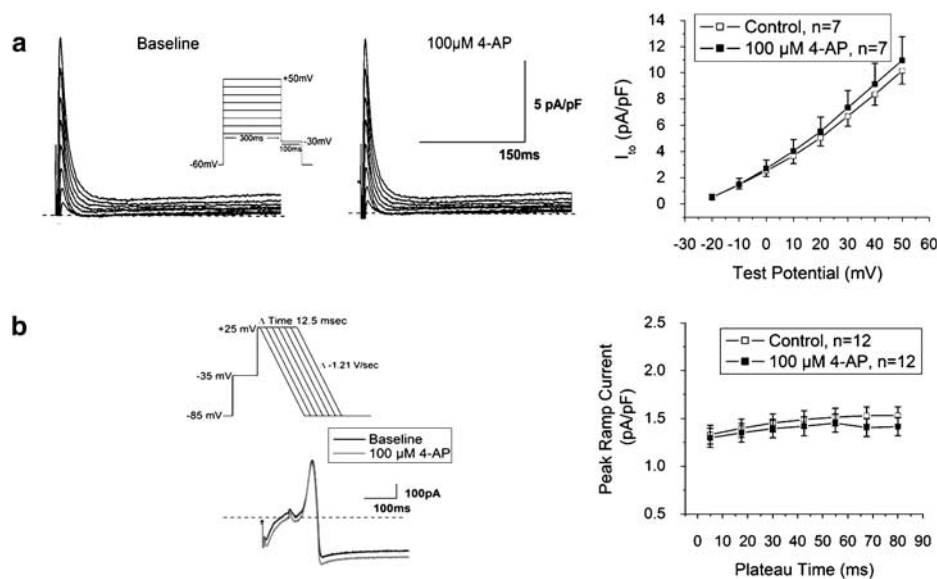


Figure 2 4-AP at 100 μ M does not block I_{to} or I_{kr} . (a) Representative I_{to} traces from a ventricular myocyte (cell capacitance—154 pF) recorded at baseline (left), after superfusion with 100 μ M 4-AP (middle, voltage protocol is inset) and the averaged I_{to} I-V curves (right). (b) The peak ramp I_{kr} from a ventricular myocyte (cell capacitance—148 pF) recorded at baseline and 100 μ M 4-AP using the ramp protocol (inset). Current was measured as the peak outward current during the voltage ramp. For purposes of clarity, only the traces obtained at the last test pulse (plateau duration = 80 ms) are shown. The current densities plotted as function of the plateau duration are shown on the right. The dashed lines represent the zero current line. The interpulse interval for I_{to} and I_{kr} was 2 and 7 s respectively. 4-AP, 4-aminopyridine; I_{to} , transient outward potassium current; I_{kr} , rapid delayed rectifier K^+ current.

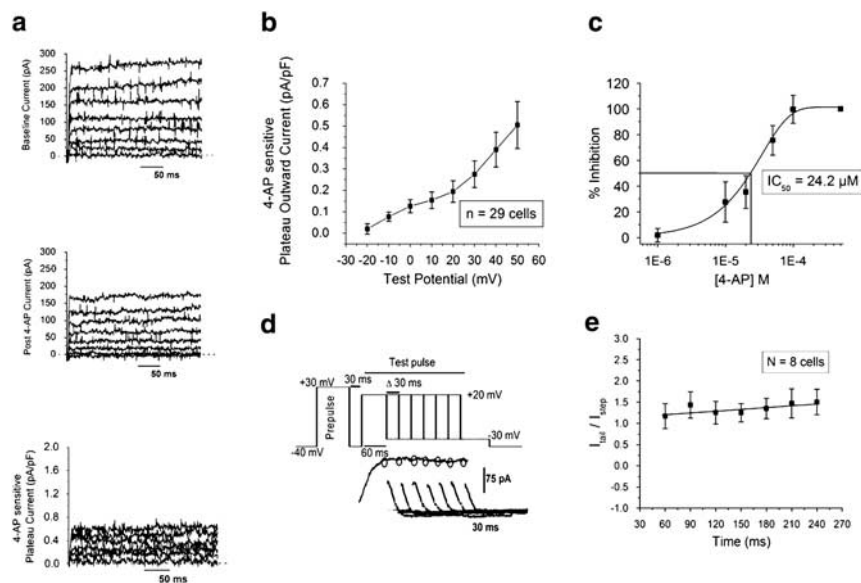


Figure 3 4-AP at 100 μ M inhibits canine ventricular-sustained outward K^+ current. (a) The baseline (top), 100 μ M 4-AP (middle) and difference currents from a ventricular myocyte (cell capacitance—166 pF) using the voltage clamp protocol shown in the inset. The dashed lines represent the zero current line. (b) The averaged I-V curve for the 4-AP-sensitive sustained outward K^+ current (from myocytes exhibiting measurable 4-AP-sensitive plateau outward current), with current measured as the average during the last 20 ms of the test pulse. (c) Dose-response relationship for 4-AP inhibition of sustained outward K^+ current, fitted to the Hill equation ($r^2 = 0.99$, $n = 5-8$ myocytes at each concentration). (d) A representative 4-AP-sensitive current trace from a ventricular myocyte using the voltage clamp protocol shown in the inset. Circles on the step current trace indicate the time at which the tail currents were elicited. (e) The mean envelope-of-tails test data where the ratio of tail to step current amplitude is plotted as a function of step pulse duration. 4-AP, 4-aminopyridine.

In canine atria, I_{Kur} is augmented by β -adrenergic stimulation (Yue *et al.*, 1999). To test the β -adrenergic modulation of the 4-AP-sensitive plateau outward current in the ventricle, we used 100 nM and 1 μ M isoprenaline (a nonspecific β_1 - and β_2 -adrenergic receptor agonist), followed by isoprenaline and

4-AP to obtain the 100 μ M 4-AP-sensitive current. Data were obtained after 6–8 min of exposure, which we found in preliminary experiments to result in steady-state activation of the sustained outward K^+ current. The baseline 100 μ M 4-AP-sensitive current recorded in the absence of isoprenaline

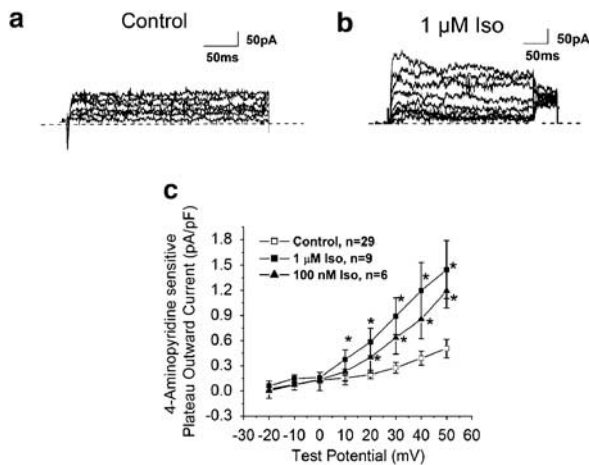


Figure 4 Canine ventricular plateau outward current is augmented by isoprenaline (Iso). Representative 100 μ M 4-AP-sensitive current traces from myocytes recorded at baseline (a; 166 pF) and after exposure to isoprenaline (b; 154 pF). The dashed lines represent the zero current line. Summary I–V curves are shown in (c). (* $P < 0.05$ vs baseline control values). 4-AP, 4-aminopyridine.

(Figure 4a) and isoprenaline stimulated, 4-AP-sensitive current is shown in Figure 4b. Isoprenaline (1 μ M) significantly ($P < 0.05$) increased the measured current amplitude at +50 mV from 0.51 ± 0.10 pA pF $^{-1}$ to 1.53 ± 0.37 pA pF $^{-1}$ (Figure 4c). Isoprenaline did not significantly alter the activation time constant of the current measured at +50 mV, which was 3.96 ± 1.4 ms at baseline vs 1.3 ± 0.9 ms with 1 μ M isoprenaline ($P = 0.09$).

In separate experiments, action potentials were recorded in the presence and absence of DPO-1 (0.3 and 1 μ M). We observed significant prolongation of APD₅₀ with DPO-1 ($P < 0.05$) at 1 and 2 Hz (Figure 5b), while significant prolongation of APD₉₀ was evident at all three stimulation frequencies (Figure 5c).

Results from computer simulations

A Markov model structure was used to develop a computer simulation of the 4-AP-sensitive 'I_{Kur-like}' current. Parameters in the model were estimated by using the nonlinear optimization routine, described above, to fit the model output to experimental data from patch clamp recordings. Multiple parameter vectors with low cost were obtained after the optimization. The lowest cost parameter vector (Table 2) was used to generate the results below. No significant differences were observed for the other low-cost parameter vectors (data not shown). Figure 6a compares the resulting ion current model and the experimental patch clamp data. The lowest cost ion current model was then incorporated into a canine action potential model (Fox *et al.*, 2002) by replacing the I_{Kp} current originally in that model. Action potentials were generated from the model at the same cycle lengths as in the experiment (basic cycle lengths of 2000, 1000 and 500 ms). To study the effect of the 'I_{Kur-like}' current on APD, control action potential simulations were compared with simulations of 'I_{Kur-like}' current block (Figures 6b–d), and to simulations of I_{to} block (Table 3). Table 3 summarizes

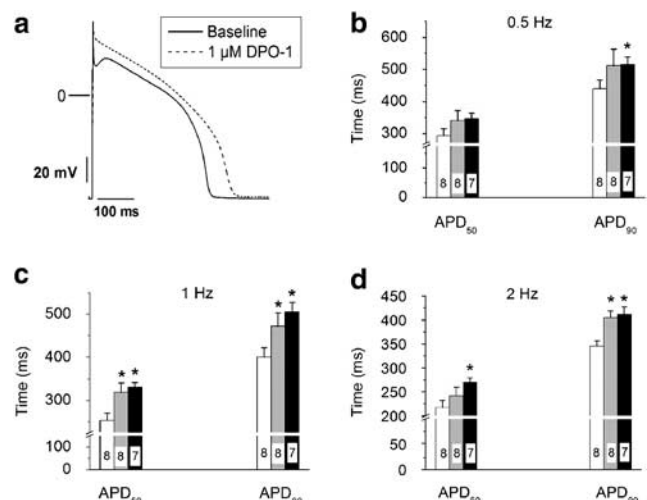


Figure 5 DPO-1, an I_{Kur}-selective blocker, prolongs APD in canine left ventricular myocytes. (a) Representative action potential trace recorded at 1 Hz before and after superfusion of 1 μ M DPO-1. Average data, for APD₅₀ and APD₉₀ measured at 0.5 (b), 1 (c) and 2 Hz (d) at baseline (open bar) or after superfusion with 0.3 μ M (grey bar), or 1 μ M (black bar) of DPO-1. (* $P < 0.05$ vs control). APD, action potential duration; DPO-1 (2-isopropyl-5-methylcyclohexyl diphenylphosphine oxide; I_{Kur}, ultra-rapid delayed rectifier current).

Table 2 Parameter values from the lowest cost fit for the I_{Kur}-like current model

Parameter	Value
E _K	−94.88 mV
C _{Kur}	0.1453 mS/uF
α_{1x}	0.04957 ms $^{-1}$
α_{1y}	0.007138 mV $^{-1}$
α_{2x}	0.002703 ms $^{-1}$
α_{2y}	0.0001 mV $^{-1}$
β_{1x}	0.2348 ms $^{-1}$
β_{1y}	−1.708 mV $^{-1}$
β_{2x}	0.02055 ms $^{-1}$
β_{2y}	−0.0001 mV $^{-1}$

the effects of I_{to} blockade vs 'I_{Kur-like}' current blockade in two different canine action potential models (Fox *et al.*, 2002; Hund and Rudy, 2004). Blockade of 'I_{Kur-like}' current in the action potential models results in significantly more APD prolongation than that of I_{to} blockade.

Discussion

We found that a plateau outward current sensitive to 4-AP exists in the majority of left ventricular mid-myocardial canine myocytes. Based on pharmacological response and activation and inactivation properties (Yue *et al.*, 1996; Nerbonne and Kass, 2005), we suggest that the observed 4-AP-sensitive current has canine 'I_{Kur-like}' properties. Furthermore, this current plays a functional role in AP repolarization in normal canine left ventricle mid-myocardial myocytes, as assessed both *in vitro* and *in silico*.

I_{Kur} has been found in rodent atria and ventricle, and canine and human atria (Wang *et al.*, 1993; Yue *et al.*, 1996).

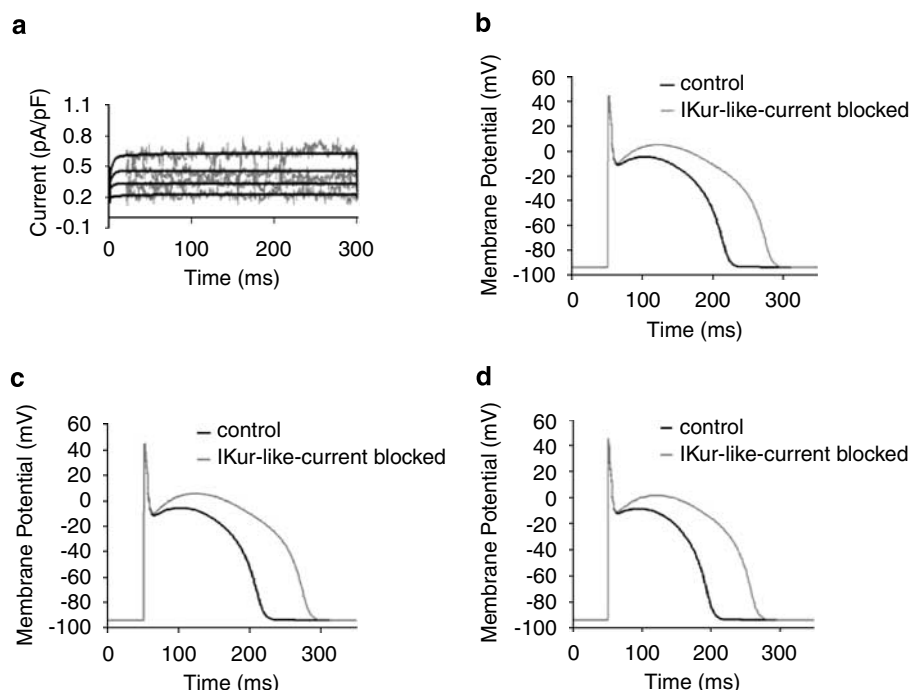


Figure 6 'I_{Kur}-like' current blockade prolongs canine ventricular action potential *in silico*. (a) Fitting of a computer model of the 4-AP-sensitive ('I_{Kur}-like') current to current data from patch clamp experiments. For purposes of clarity, current fitting to test steps to -10, 0, 20 and 40 mV are shown. Model parameters after fitting are provided in Table 2. The fitted model was incorporated into the Fox-Gilmour and Hund-Rudy canine action potential models. Steady-state action potential tracings from the Fox-Gilmour model obtained at 2000 (b), 1000 (c) and 500 ms (d) basal cycle lengths. Control action potential tracings are shown in black and action potentials in the presence of 'I_{Kur}-like' current blockade are shown in grey. 4-AP, 4-aminopyridine; I_{Kur}, ultra-rapid delayed rectifier current.

Table 3 Effects of I_{to} blockade versus 'I_{Kur}-like' current blockade on APD₉₀ and plateau amplitude in *in silico* models

Model	Fox-Gilmour			Hund-Rudy		
	2000 ms	1000 ms	500 ms	2000 ms	1000 ms	500 ms
Basic cycle length						
Control APD (ms)	166	163	148	219	211	150
Control plateau (mV)	-4.4	-5.3	-8.7	12.8	3.4	-2.5
I _{to} blocked APD (ms)	161	163	156	168	163	149
I _{to} blocked plateau (mV)	7.0	8.0	8.0	10.0	8.0	7.5
I _{Kur} -like current blocked APD (ms)	227	227	212	243	228	192
% increase to control	36	39	43	11	8	28
I _{Kur} -like current blocked plateau (mV)	5.4	5.8	1.6	21.2	16.1	11.5

Abbreviations: APD, action potential duration; I_{Kur}, ultra-rapid delayed rectifier current; I_{to}, transient outward potassium current.

This current has been shown to initially activate in phase 1 of atrial action potential, and blockade of this current results in prolongation of APD₅₀ and APD₉₀ in canine atria (Yue *et al.*, 1996). In addition, guinea-pig ventricular myocytes exhibit a similar outward K⁺ current which activates at plateau voltages (Yue and Marban, 1988). This current was named I_{Kp} due to its unique open probabilities at plateau potentials, with no detectable inactivation. Similarities between I_{Kp} and I_{Kur} in canine and human atria have been noted (Nerbonne and Kass, 2005).

Low concentrations of 4-AP (50–100 μM) have been previously used to block I_{Kur} in canine and human atria, and in rodent ventricle (Brouillette *et al.*, 2004), and the similar sensitivities to micromolar 4-AP concentrations suggests blockade of the same channel subunit (Nerbonne and Kass, 2005). The IC₅₀ for 4-AP on the plateau outward 'I_{Kur}-like' current that we measured from canine left ventricle

was 24.2 μM, which is similar to that previously reported for I_{Kur} in canine (5.31 ± 0.74 μM) and human (49 μM) atria (Wang *et al.*, 1993; Yue *et al.*, 1996) and in Kv1.5 expression systems (50 μM) (Bouchard and Fedida, 1995). As reported for canine atrial I_{Kur} (Yue *et al.*, 1996), we observed that the plateau outward current is rapidly activating, and did not show significant inactivation during the 300 ms test pulse. While previous reports (Amos *et al.*, 1996; Yue *et al.*, 1996) on I_{Kur} demonstrate that the current inactivates more rapidly at physiological temperatures (compared to room temperature), there is still sufficient I_{Kur} at physiologically relevant temperatures to contribute to action potential repolarization.

Kv1.5, the protein encoding I_{Kur} is present in canine atria and ventricle (Fedida *et al.*, 2003; Szuts *et al.*, 2004). While Kv1.5-encoded current has not been thought to be normally expressed in the ventricles of large mammals, we note one report of a C9356-sensitive current (a Kv1.5 blocker) in

ventricular myocytes isolated from a post-infarction canine model (Dun *et al.*, 2004). In this canine post-infarction model, Kv1.5 has been shown to lateralize from the intercalated disks in the peri-infarct region (Mays *et al.*, 1997). Due to the 'I_{Kur}-like' nature of the 4-AP-sensitive plateau outward current observed, we suggest that the 4-AP-sensitive current may be carried by Kv1.5 channels. Further studies are required to address this issue.

A role for I_{Kur} in canine and human ventricle?

We tested two different concentrations of 4-AP on APD at 0.5, 1 and 2 Hz. Both 50 and 100 μ M 4-AP produced a reverse use-dependent prolongation of APD₅₀ and APD₉₀ consistent with a previous study (Bouchard and Fedida, 1995), where block of Kv1.5 by 4-AP was relieved at faster stimulation rates, which was attributed to rate-dependent relief of closed channel block. In addition, we evaluated the effects of DPO-1, reportedly a use-dependent, selective I_{Kur} blocker (Stump *et al.*, 2005; Lagrutta *et al.*, 2006) to provide additional confirmatory evidence, of the existence of an outward plateau ('I_{Kur}-like') current in canine mid-myocardial left ventricular myocytes. We observed similar effects on the APD with both DPO-1 and 4-AP, and these were quantitatively similar at a stimulation frequency of 1 Hz. In contrast to a previous report, we did not observe use-dependent effects of DPO-1 on APD (Lagrutta *et al.*, 2006). The reported use-dependent properties of DPO-1 are in contrast to the known reverse use-dependent effects of 4-AP. Our simulations revealed a similar degree of frequency-independent prolongation of APD₉₀ during I_{Kur}-like current block (Table 3). This suggests that it is the block of the outward plateau current by 4-AP which is reverse-use dependent, as has been reported for I_{Kur} (Bouchard and Fedida, 1995), rather than the current itself being fundamentally reverse use-dependent.

Our data are in contrast to data obtained in an *in vivo* study on the effect of I_{Kur} blockade on canine ventricular repolarization, where dogs were anaesthetized with pentobarbital (Stump *et al.*, 2005). Pentobarbital is a known blocker of repolarizing currents, and also prolongs canine ventricular APD₅₀ and APD₉₀ at concentrations required for general anaesthesia (Nattel *et al.*, 1990). Thus, any potential effect of I_{Kur} blockade in this study of *in vivo* canine ventricular repolarization may have been obscured by anaesthesia. The *in vivo* effects of I_{Kur} blockers on *in vivo* canine ventricular repolarization therefore remain poorly defined.

A study in myocytes from failing explanted human hearts (Li *et al.*, 1996b) used 50 μ M 4-AP to detect I_{Kur}, which could be detected in atrial, but not in right ventricular myocytes. The authors suggested that the absence of I_{Kur} in ventricular myocytes may have resulted from limited sampling ($n=5$ myocytes) or possible differences in regional distribution. A recent report with a mixed I_{Kur} and I_{to} blocker did not find significant effects on the canine right ventricular APD (Schotten *et al.*, 2007); the difference between this report and our observations could potentially result from differences in the selectivity of the drugs, or differences between right and left ventricular electrophysiology (Volders *et al.*, 1999).

DPO-1, is reported to cause rate-dependent blockade of I_{Kur} and action potential prolongation in human atrial myocytes, with no action potential prolongation noted in a human ventricular myocyte, obtained from an end-stage heart at the time of transplantation (Lagrutta *et al.*, 2006). However, the source of the ventricular myocyte (right vs left ventricle) was not stated, and the results are again limited by a small sampling of myocytes. In summary, the contribution of I_{Kur} to normal human left ventricular repolarization is undefined at the present time. Notably, recent reports confirm the expression of Kv1.5 mRNA in the ventricle of normal, undiseased, human hearts, suggesting a potential role for Kv1.5 encoded currents in the human ventricle (Szuts *et al.*, 2004; Ordog *et al.*, 2006). In both human atria and ventricle, immunohistochemical studies reveal high expression of Kv1.5 which is localized to the intercalated disks, similar to the distribution in canine atrial myocytes (Mays *et al.*, 1995; Fedida *et al.*, 2003).

4-Aminopyridine and blockade of other K⁺ currents

Human ether-a-go-go gene current and canine ventricular I_{to} are blocked by 4-AP, with an IC₅₀ of 4.4 and 1.15 mM respectively (Nattel *et al.*, 2000; Ridley *et al.*, 2003). We found no inhibition of either I_{to} or I_{Kr} at the 4-AP concentrations used in our experiments. Our I_{to} measurements are consistent with a previous report (Nattel *et al.*, 2000), where a concentration of 50 μ M 4-AP only blocked ~1% of ventricular I_{to} (Nattel *et al.*, 2000). The following lines of evidence: (1) the previously reported lack of significant inhibition of ventricular I_{to} with concentrations up to 200 μ M 4-AP (Nattel *et al.*, 2000); (2) the characteristics of the 4-AP-sensitive current we recorded; (3) the use of I_{to} inactivating prepulses and (4) the absence of I_{to} inhibition with 100 μ M 4-AP in our myocytes, all suggest that the 4-AP-sensitive current that we report was not contaminated by I_{to}. Similarly, the results of the envelope-of-tails test and the absence of 4-AP block of I_{Kr} both suggest that the 4-AP-sensitive plateau current was not contaminated by I_{Kr}.

β -Adrenergic modulation of 'I_{Kur}-like' current

β -Adrenergic modulation of I_{Kur} has been previously reported in human atrial myocytes (Li *et al.*, 1996a). Kv1.5 has been shown to interact with the accessory channel subunits, Kv β 1.2 and Kv β 1.3, which alter the adrenergic responsiveness of the channel (Kwak *et al.*, 1999). Using the β -adrenergic agonist, isoprenaline, we found that the ventricular 4-AP-sensitive plateau current was also modulated by β -adrenergic stimulation. These results suggest a possible role for the current in modulating ventricular repolarization during sympathetic stimulation.

Limitations

We did not examine the presence of this current in either right ventricular or atrial myocytes, and our measurements were confined to one region of the left ventricle myocardium. The purpose of the current study was to establish the presence and functional role of this 'I_{Kur}-like'

current in the canine left ventricle, and thus we did not examine myocytes from other regions in these experiments.

The model used in the *in silico* experiments is based on experimental data derived from canine right and left endocardial tissues (Koller *et al.*, 1998), and therefore the model does not emulate the action potentials from the left ventricular lateral mid-myocardium. The modelling results are therefore more qualitative than quantitative and serve to confirm that block of a small amplitude plateau current increases action potential plateau voltage and prolongs the APD.

The length of our recorded action potentials is longer than that previously reported in canine ventricular muscle (Jost *et al.*, 2005). One possible explanation for this difference is the use of isolated myocytes, rather than multi-cellular preparations, since the APD is modulated by intercellular coupling (Zaniboni *et al.*, 2000). Alternatively, the APD may vary with site of origin of the myocytes (Litovsky and Antzelevitch, 1989). We isolated myocytes from the left lateral mid-myocardium of the left ventricle-free wall, and previous reports have primarily focused on myocytes of anterior left ventricle origin (Kaab *et al.*, 1996; Volders *et al.*, 1999). Notably, while our control APD₉₀ values are longer than those in some previous reports, they are shorter than control values previously published in isolated canine myocytes (Kaab *et al.*, 1996). Lastly, there is the possibility of alterations in APD due to the myocyte isolation procedure, which cannot be excluded in our experiments.

We did not examine the effects of 4-AP on I_{Ks}. However, in control myocytes (in the absence of β -adrenergic agonists), there is no contribution of I_{Ks} to repolarization of canine or human ventricle (Varro *et al.*, 2000; Stengl *et al.*, 2003; Volders *et al.*, 2003; Jost *et al.*, 2005). Our experimental protocols (250–300 ms test pulses) were designed to minimize the role of I_{Ks} during the current (Jost *et al.*, 2005). Furthermore, we did not observe any time-dependent increases in the amplitude of the rapidly activating, 4-AP-sensitive current, as would be expected if I_{Ks} were present.

In summary, our study demonstrates the presence of a plateau outward current in canine ventricle, which has several 'I_{Kur}-like' properties. This current activates rapidly at depolarized potentials, and block of the current causes prolongation of both APD₅₀ and APD₉₀. In addition, the β -adrenergic-mediated increase in current density suggests a potential role in rate adaptation of the APD during periods of increased sympathetic stimulation. Our findings suggest that I_{Kur} blockers, such as DPO-1, may affect ventricular as well as atrial repolarization. This may have implications for the use of I_{Kur} blockers for the treatment of atrial tachyarrhythmias.

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Conflict of interest

The authors state no conflict of interest.

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