

## Rapid Report

# Inhibition of HERG K<sup>+</sup> current and prolongation of the guinea-pig ventricular action potential by 4-aminopyridine

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4-Aminopyridine (4-AP) has been used extensively to study transient outward K<sup>+</sup> current ( $I_{TO,1}$ ) in cardiac cells and tissues. We report here inhibition by 4-AP of HERG (the human *ether-à-go-go*-related gene) K<sup>+</sup> channels expressed in a mammalian cell line, at concentrations relevant to those used to study  $I_{TO,1}$ . Under voltage clamp, whole cell HERG current ( $I_{HERG}$ ) tails following commands to +30 mV were blocked with an  $IC_{50}$  of  $4.4 \pm 0.5$  mM. Development of block was contingent upon HERG channel gating, with a preference for activated over inactivated channels. Treatment with 5 mM 4-AP inhibited peak  $I_{HERG}$  during an applied action potential clamp waveform by ~59%. It also significantly prolonged action potentials and inhibited resurgent  $I_K$  tails from guinea-pig isolated ventricular myocytes, which lack an  $I_{TO,1}$ . We conclude that by blocking the  $\alpha$ -subunit of the  $I_{Kr}$  channel, millimolar concentrations of 4-AP can modulate ventricular repolarisation independently of any action on  $I_{TO,1}$ .

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4-Aminopyridine (4-AP) has been used extensively in cardiac electrophysiology to study the physiological roles of transient outward potassium current ( $I_{TO,1}$ ; Wang *et al.* 1991; Gillis *et al.* 1998; Elizalde *et al.* 1999; Lei *et al.* 2000; Kocic *et al.* 2002) and, in recent years, of the ‘ultra-rapid’ delayed rectifier current ( $I_{Kur}$ ; Li *et al.* 1996; Yue *et al.* 1996). 4-AP is generally considered selective for these currents in the heart, with micromolar concentrations being used to block  $I_{Kur}$  (Li *et al.* 1996) and millimolar concentrations to block  $I_{TO,1}$  (typically 0.5 to 10 mM; e.g. Wang *et al.* 1991; Gillis *et al.* 1998; Elizalde *et al.* 1999; Lei *et al.* 2000; Kocic *et al.* 2002). However, limited published data raise the possibility that 4-AP may not be entirely selective for  $I_{TO,1}$  over the rapid delayed rectifier K<sup>+</sup> current ( $I_{Kr}$ ) at concentrations used to study  $I_{TO,1}$  (Mitcheson & Hancox, 1999; Liu *et al.* 1999; Zhang *et al.* 2000). Given the importance of  $I_{Kr}$  in regulating action potential repolarization and duration (Mitcheson & Sanguinetti, 1999), a non-selective effect of 4-AP on  $I_{Kr}$  would significantly influence the interpretation of data from experiments in which 4-AP is applied to cardiac muscle cells or tissues. The aim of this study was to determine whether or not 4-AP inhibits ionic current ( $I_{HERG}$ ) carried by K<sup>+</sup> channels encoded by *HERG* (the human *ether-à-go-go*-related gene, which encodes the  $\alpha$ -subunit of the  $I_{Kr}$  channel; Warmke & Ganetzky, 1994). 4-AP was found both to inhibit  $I_{HERG}$  recorded from human embryonic

kidney cells and to prolong action potentials from guinea-pig ventricular myocytes, which lack  $I_{TO,1}$ . These findings have widespread implications for the use of 4-AP as an investigative tool in cardiac muscle electrophysiology.

## METHODS

### Maintenance of a mammalian cell line stably expressing HERG

Measurements of  $I_{HERG}$  were made from human embryonic kidney (HEK 293) cells stably expressing HERG (cell line generously donated by Professor Craig January, University of Wisconsin; Zhou *et al.* 1998). Cells were passaged using a non-enzymatic agent (Splittix, AutogenBioclear) and plated out onto fragments of sterilized glass coverslips in 30 mm petri dishes containing a modification of Dulbecco’s modified Eagle’s medium with Glutamax-1 (DMEM; Gibco), supplemented with 10% fetal bovine serum, 400  $\mu\text{g ml}^{-1}$  gentamicin (Gibco) and 400  $\mu\text{g ml}^{-1}$  geneticin (G418; Gibco). Cells were incubated at 37°C (5% CO<sub>2</sub>) for a minimum of 2 days prior to any electrophysiological study.

### Isolation of guinea-pig ventricular myocytes

Male adult guinea-pigs were killed by cervical dislocation (a Schedule 1 procedure according to the Home Office UK Animals (Scientific Procedures) Act 1986) and ventricular myocytes were enzymatically dispersed from both ventricles using a method described previously (Levi & Issberner, 1996). Prior to use, the isolated cells were kept at 4°C in Kraft-Brühe medium (Isenberg & Klockner, 1982).

### Electrophysiological recordings

Cells were superfused with a normal Tyrode solution, which contained (mM): 140 NaCl, 4 KCl, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose, 5 Hepes, (titrated to pH 7.45 with NaOH). Experimental solutions were applied using a home-built, warmed solution delivery system capable of changing the bathing solution surrounding a cell in <1 s. Patch-pipettes were fire-polished to a resistance of 3.5–5 MΩ. The pipette solution for  $I_{\text{HERG}}$  measurement contained (mM): 130 KCl, 1 MgCl<sub>2</sub>, 5 EGTA, 5 MgATP, 10 Hepes (titrated to pH of 7.2 with KOH). A K<sup>+</sup>-based, EGTA-free pipette solution containing 10 mM NaCl was used for action potential recordings. The 'pipette-to-bath' liquid junction potential was small (−3.2 mV) and was uncorrected. Series resistance ( $R_s$ ) values lay typically between 4–7 MΩ and were compensated by ≥ 70%. The expected voltage drop across residual uncompensated  $R_s$  during  $I_{\text{HERG}}$  measurements was therefore only small (~3.5 mV or less) and no correction was made for this. Measurements were made at 37 ± 1 °C.

### Drugs

4-AP powder (Sigma) was dissolved directly in normal Tyrode solution to make test solutions of different concentrations referred to in the Results; pH was corrected to 7.45 with HCl.

### Voltage protocols and analysis

Measurements were made using an Axopatch 200 or 200A amplifier (Axon Instruments) and a CV-201/2A headstage. Voltage-clamp commands were generated using 'WinWCP' (John Dempster, Strathclyde University, UK) or pCLAMP (v 6.0, Axon Instruments). Data were recorded via a Digidata interface (Axon Instruments) and stored on the hard disk of a personal computer.

### Concentration–response relationships

$I_{\text{HERG}}$  tails were measured on repolarization to −40 mV after 1 s depolarizing voltage commands to +30 mV. 4-AP concentrations between 100 μM and 100 mM were each applied to a minimum of five different cells. The mean fractional blockade was obtained as:

$$\text{Fractional block} = 1 - \left( \frac{I_{\text{HERG}(4\text{-AP})}}{I_{\text{HERG}(\text{CONTROL})}} \right), \quad (1)$$

where  $I_{\text{HERG}(4\text{AP})}$  and  $I_{\text{HERG}(\text{CONTROL})}$  represent tail current amplitudes in the presence and absence of 4-AP, respectively. The agent was rapidly acting (maximal effects of a given concentration typically being observed within ~2–4 pulses), and its effects could be observed without any marked overlying current run-down. Concentration–response data were fitted by the equation:

$$\text{Fractional block} = \frac{1}{\left( 1 + \left( \frac{\text{IC}_{50}}{[4\text{-AP}]} \right)^h \right)}, \quad (2)$$

where IC<sub>50</sub> is [4-AP] producing half-maximal inhibition of the  $I_{\text{HERG}}$  tail and  $h$  is the Hill coefficient for the fit.

### $I_{\text{HERG}}$ inhibition at different voltages

From −80 mV, 2 s depolarizations were applied to potentials between −40 and +40 mV.  $I_{\text{HERG}}$  tails were measured on repolarization to −40 mV. Current–voltage ( $I$ – $V$ ) relations (not shown) were constructed for each of eight cells in the absence and presence of 5 mM 4-AP. Half-maximal activation voltages were

obtained by fitting each  $I$ – $V$  relation with a modified Boltzmann equation:

$$I = \frac{I_{\text{max}}}{1 + \exp\left(\frac{(V_{1/2} - V_m)}{k}\right)}, \quad (3)$$

where  $I = I_{\text{HERG}}$  tail amplitude following test potential  $V_m$ ,  $I_{\text{max}}$  is the maximal  $I_{\text{HERG}}$  tail current observed,  $V_{1/2}$  is the potential at which  $I_{\text{HERG}}$  was half-maximally activated and  $k$  is the slope factor describing  $I_{\text{HERG}}$  activation.

Voltage-dependent activation curves for  $I_{\text{HERG}}$  were obtained by calculating activation variables at 2 mV intervals between −80 and +40 mV. Values for  $V_{1/2}$  and  $k$  derived from fits to experimental  $I$ – $V$  data with eqn (3) were inserted into the following equation:

$$\text{Activation parameter} = \frac{1}{1 + \exp\left(\frac{(V_{1/2} - V_m)}{k}\right)}, \quad (4)$$

where the 'activation parameter' at any test potential,  $V_m$ , occurs within the range 0 to 1 and  $V_{1/2}$  and  $k$  have similar meanings to those in eqn (3).

### Envelope of tails

From −80 mV, membrane potential was stepped to +40 mV for varying time periods between 12.5 and 600 ms and was repolarized to −40 mV in order to monitor  $I_{\text{HERG}}$  tail amplitude. After control measurements, each cell was equilibrated in 4-AP-containing solution while at the holding potential and the protocol was then reapplied. For each cell, the fractional block of  $I_{\text{HERG}}$  tails at each test pulse duration was determined using eqn (1). The plot of mean fractional block *versus* test pulse duration was then fitted with an equation of the form:

$$\text{Fractional block} = A - \left( A \exp\left(\frac{-x}{\tau}\right) \right), \quad (5)$$

where  $A$  represents maximal fractional block,  $x$  represents pulse duration and  $\tau$  is the time constant of development of blockade (ms).

### Action potential voltage clamp and measurements from guinea-pig ventricular myocytes

For action potential clamp measurements of  $I_{\text{HERG}}$  from HEK cells, the command waveform used was the same as that employed in previous studies of  $I_{\text{HERG}}$  from our laboratory (Hancox *et al.* 1998a,b).

Action potentials were elicited from guinea-pig ventricular myocytes by brief suprathreshold current pulses at 15 s intervals. For measurements of guinea-pig  $I_K$  tails, membrane potential was held at −80 mV, depolarized to −40 mV for 20 ms, then depolarized to +20 mV for 500 ms.  $I_K$  tails were measured during subsequent repolarization to −40 mV.

### Data analysis and presentation

Data are presented as means ± s.e.m. Statistical comparisons were made using Student's paired  $t$  test or one-way analysis of variance (ANOVA) with a Bonferroni *post hoc* test.  $P$  values of less than 0.05 were taken as significant.

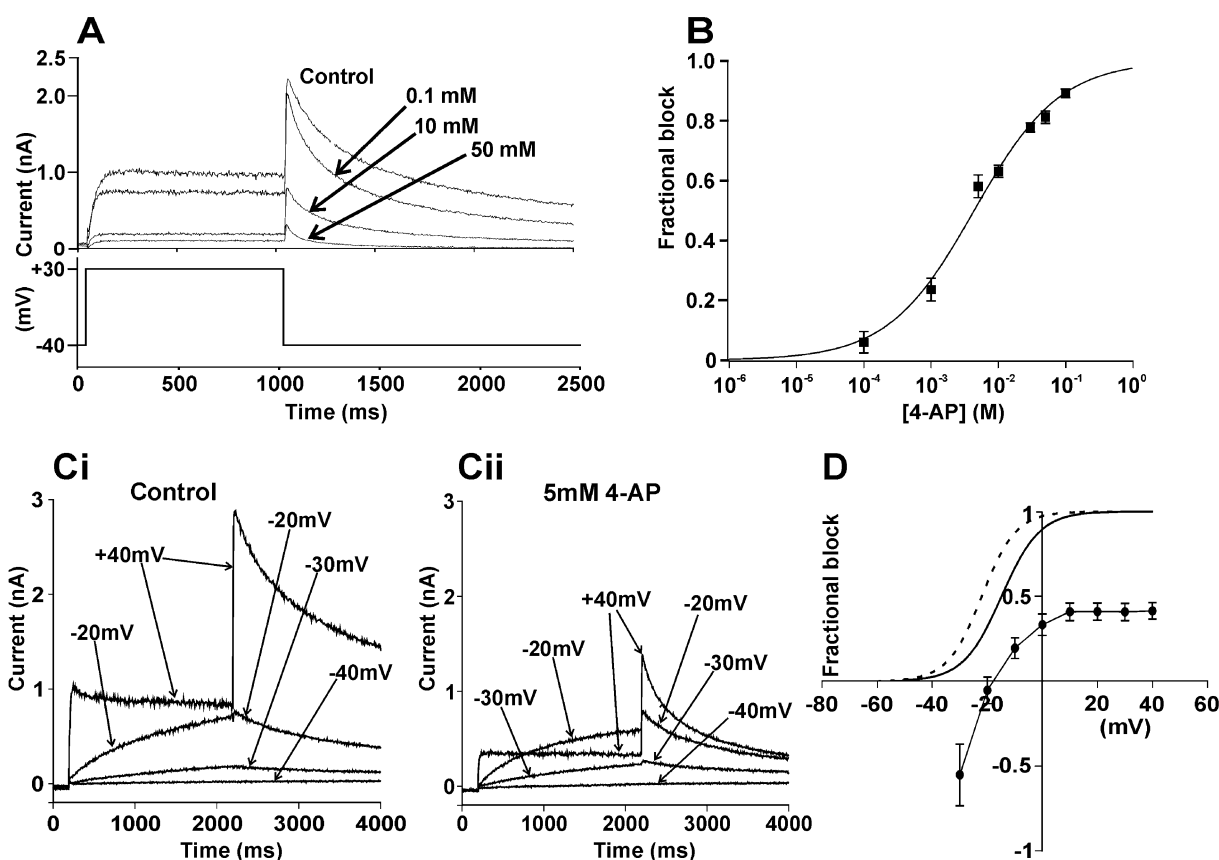
## RESULTS

Figure 1A shows that 4-AP produced a concentration-dependent inhibition of  $I_{\text{HERG}}$  and Fig. 1B shows the mean concentration–response relationship for this effect. The  $\text{IC}_{50}$  derived from the data was  $4.4 \pm 0.5$  mM (Hill coefficient  $0.7 \pm 0.1$ ). The voltage dependence of the effect was assessed by applying commands to a range of test potentials (Fig. 1Ci and Cii). No  $I_{\text{HERG}}$  tail inhibition was evident after pre-pulses to  $-30$  mV and  $-20$  mV, whilst at more positive potentials marked inhibition of  $I_{\text{HERG}}$  was observed (Fig. 1Ci and Cii). Mean data are shown in Fig. 1D, which also contains mean activation curves in control (continuous line) and 4-AP (dashed line). In the presence of 4-AP a leftward shift in the half-maximal activation voltage ( $V_{1/2}$ ; by  $-7.4 \pm 0.9$  mV,  $n = 8$ ;  $P < 0.0001$ ,  $t$  test) was observed. The activation shift may account for the apparent augmentation/ lack of blockade of by 4-AP observed at  $-30$  and  $-20$  mV. The voltage range over which inhibition was voltage dependent corresponded with that over which voltage-dependent activation of  $I_{\text{HERG}}$

developed (Mergenthaler *et al.* 2001; Paul *et al.* 2001). Treatment with 5 mM 4-AP did not significantly alter the mean deactivation time course of tails measured at  $-40$  mV, following a voltage command to  $+30$  mV.

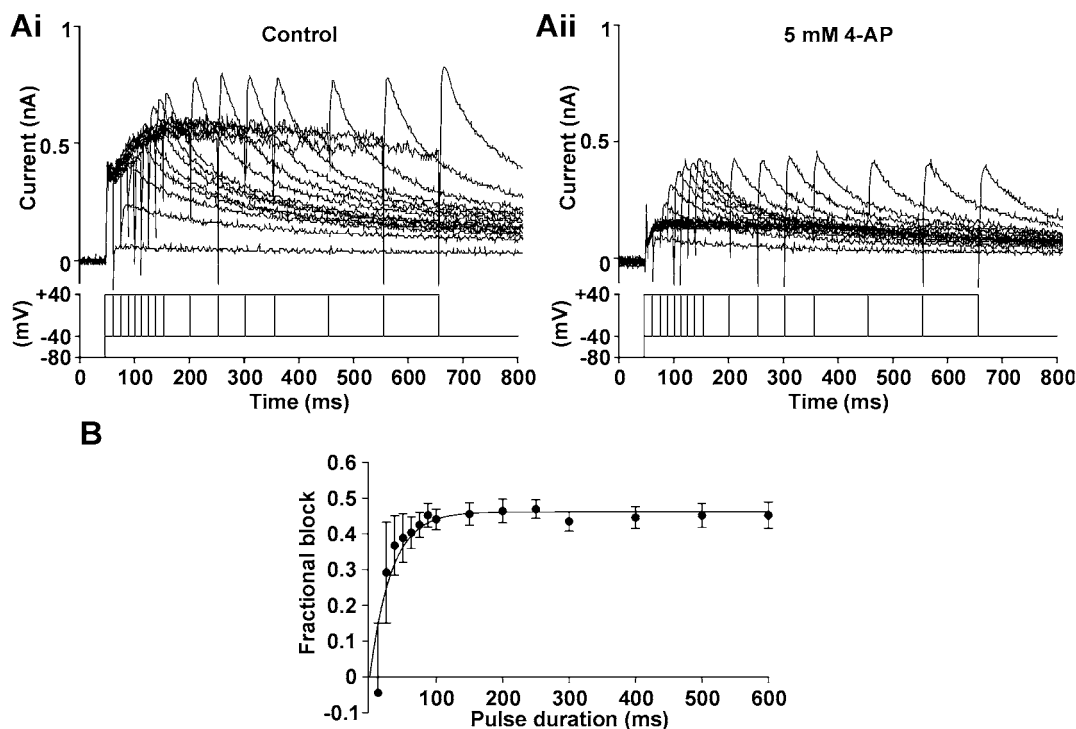
The dependence of  $I_{\text{HERG}}$  inhibition by 4-AP upon duration of depolarization was studied using an ‘envelope of tails’ protocol (Trudeau *et al.* 1995) (see Fig. 2A and B). Figure 2A shows representative current traces obtained in control (Fig. 2Ai) and in 4-AP (Fig. 2Aii); Fig. 2B shows a plot of the resulting mean levels of fractional block against pulse duration. The time course of development of blockade was described by a mono-exponential curve that intersected the origin, with a time constant of  $32.3 \pm 6.4$  ms ( $n = 5$ ). Considered collectively with Fig. 1D these data demonstrate that inhibition of  $I_{\text{HERG}}$  by 4-AP requires channel gating to occur.

An additional protocol (see Fig. 3A, lower trace) was employed to ascertain whether or not 4-AP showed a preference for activated or inactivated HERG channels. In



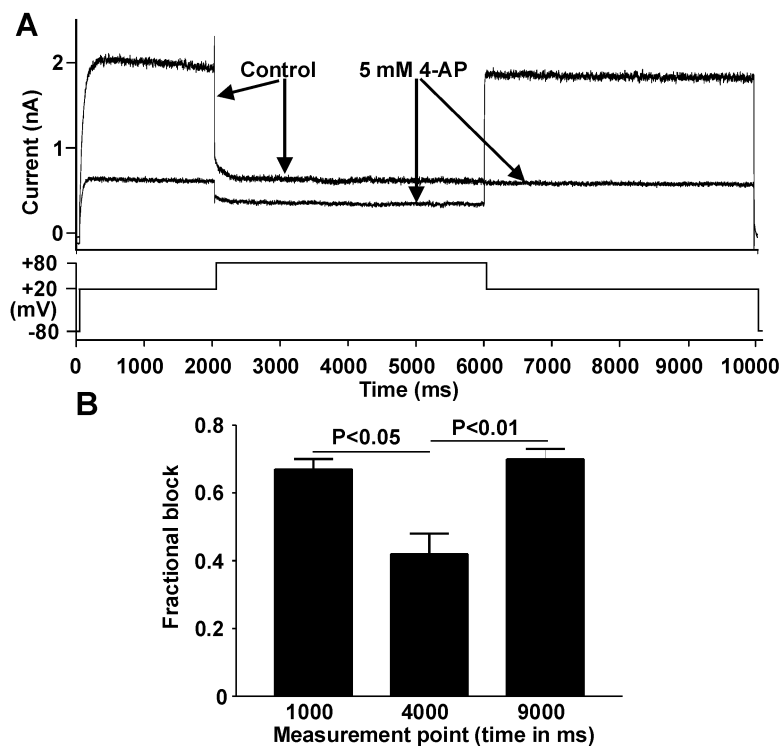
**Figure 1. Concentration and voltage-dependent inhibition of  $I_{\text{HERG}}$  by 4-AP**

A, upper traces show representative current traces illustrating the effect of different concentrations of 4-AP. Lower trace represents voltage protocol used. B, concentration response relation for  $I_{\text{HERG}}$  tail blockade by 4-AP ( $n = 5$ –8 cells at each concentration). C, representative records in the absence (Ci) and presence (Cii) of 5 mM 4-AP. D, the mean ( $\pm$  s.e.m.) fractional block of  $I_{\text{HERG}}$  by 5 mM 4-AP over a range of potentials ( $n = 8$  cells).  $I_{\text{HERG}}$  activation curves are shown in the absence (continuous line) and presence (dashed line) of 5 mM 4-AP.



**Figure 2. Time dependence of  $I_{\text{HERG}}$  block by 4-AP**

A, traces from an 'envelope of tails' protocol, in the absence (Ai) and presence (Aii) of 5 mM 4-AP. Lower traces represent the voltage protocol used. B, the mean ( $\pm$  s.e.m.) fractional block of  $I_{\text{HERG}}$  with varying pulse duration ( $n = 5$  cells). The  $\tau$  for development of inhibition was  $32.3 \pm 6.4$  ms ( $n = 5$ ).



**Figure 3. Effect of strong depolarisation on the action of 4-AP**

A, current records (upper traces) elicited by the voltage protocol shown (lower trace), applied from a holding potential of  $-80$  mV. Arrows indicate traces obtained in control and 4-AP. B, bar charts ( $n = 5$  cells) compare the mean level of blockade at 1000 ms into protocol (at  $+20$  mV), at 4000 ms (at  $+80$  mV) and at 9000 ms (after membrane potential was returned to  $+20$  mV).

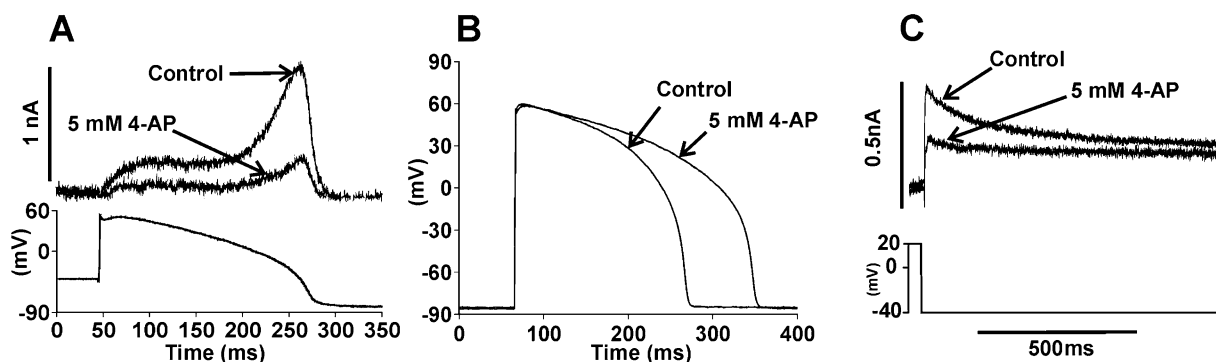
control solution,  $I_{\text{HERG}}$  amplitude decreased markedly during a depolarisation from +20 to +80 mV (Fig. 3A, upper traces, Control) as a consequence of increased  $I_{\text{HERG}}$  inactivation. In the presence of 5 mM 4-AP, blockade of  $I_{\text{HERG}}$  at +20 mV was greater than that at +80 mV (Fig. 3A, upper traces, 4-AP). Mean data shown in Fig. 3B indicate that blockade during the +80 mV step ('4000 ms') was significantly lower than that at +20 mV ('1000 ms' and 9000 ms'). Thus, when  $I_{\text{HERG}}$  was reduced by extensive  $I_{\text{HERG}}$  inactivation the blocking effect of 4-AP was diminished. This observation suggests that 4-AP binds preferentially to activated over inactivated HERG channels. In further experiments we observed an acceleration of the rate of development of  $I_{\text{HERG}}$  inactivation by 4-AP (data not shown). This effect is in marked contrast with that reported for externally applied tetraethylammonium ions (TEA), which slow the distinctive, rapidly developing C-type inactivation of  $I_{\text{HERG}}$  (Smith *et al.* 1996). This action of 4-AP could have resulted either from a genuine modification of inactivation gating, or from the rapid development of open-channel block during the protocol used to study inactivation.

The effect of 4-AP on  $I_{\text{HERG}}$  elicited by an applied ventricular action potential waveform was also determined (Fig. 4A). In control solution,  $I_{\text{HERG}}$  amplitude increased progressively through the action potential plateau and was maximal near -30 mV ( $-30.2 \pm 3.4$  mV;  $n = 5$ ; cf. Hancox *et al.* 1998a). Treatment with 5 mM 4-AP attenuated current throughout the action potential command, inhibiting maximal  $I_{\text{HERG}}$  by  $58.5 \pm 4.1\%$  ( $n = 5$ ). Also, the voltage at which  $I_{\text{HERG}}$  peaked was shifted by  $-10.6 \pm 4.1$  mV, consistent with the voltage shift in current activation described in Fig. 1. These data suggest that 4-AP might increase the action potential duration in native cardiac tissue through blockade of native  $I_{\text{Kr}}$ , independent of any inhibitory action on  $I_{\text{TO,1}}$ . Therefore, we measured action potentials from guinea-pig

ventricular myocytes, which lack  $I_{\text{TO,1}}$  (Coraboeuf *et al.* 1998). 4-AP exerted little effect immediately after the action potential peak, but the times to reach both 50% and 90% repolarization ( $\text{APD}_{50}$  and  $\text{APD}_{90}$ , respectively) were significantly prolonged by 4-AP (Fig. 4B; in control: mean  $\text{APD}_{50} = 162.2 \pm 12.2$  ms and  $\text{APD}_{90} = 184.3 \pm 12.2$  ms; in 4-AP:  $\text{APD}_{50} = 210.9 \pm 14.6$  ms and  $\text{APD}_{90} = 243.1 \pm 14.8$  ms,  $n = 8$ ;  $P < 0.01$  and  $P < 0.001$ , respectively). In complementary voltage-clamp experiments, resurgent  $I_{\text{K}}$  tails (sensitive to the  $I_{\text{Kr}}$  blocker E-4031) from guinea-pig myocytes were also inhibited by 4-AP (Fig. 4C).

## DISCUSSION

The dependence of HERG blockade by 4-AP upon channel gating observed in this study differs from the mechanism underlying its blockade of cardiac  $I_{\text{TO,1}}$ , which depends considerably upon the agent binding to the closed channel (Castle & Slawsky, 1993; Wang *et al.* 1995).  $I_{\text{TO,1}}$  from rat, rabbit and human have been reported to be blocked by 4-AP with  $\text{IC}_{50}$  values of 0.14, 0.33 and 0.67 mM, respectively, whilst Kv 4.3 current exhibits an  $\text{IC}_{50}$  of 1.54 mM (Xu *et al.* 1998; Faivre *et al.* 1999; Lei *et al.* 2000). Therefore, whilst 4-AP is clearly a low affinity HERG blocker, its  $\text{IC}_{50}$  for  $I_{\text{HERG}}$  inhibition falls within a 4-AP concentration range used to block  $I_{\text{TO,1}}$  selectively in previous studies (typically 0.5–10 mM; Wang *et al.* 1991; Gillis *et al.* 1998; Elizalde *et al.* 1999; Lei *et al.* 2000; Kocic *et al.* 2002). The fact that some level of  $I_{\text{Kr}}$  blockade may also have occurred in such experiments is supported by the present data from guinea-pig myocytes, which lack  $I_{\text{TO,1}}$ , and by previous data regarding reduction of  $I_{\text{K}}$  tails from rabbit nodal cells (Mitcheson & Hancox, 1999). On the basis of our findings, we conclude that millimolar concentrations of 4-AP can block  $I_{\text{HERG}}/I_{\text{Kr}}$  and, thereby, prolong cardiac action potential repolarization independently of any action on  $I_{\text{TO,1}}$ . In light of these observations, it may be necessary to consider carefully the



**Figure 4. Effect of 4-AP under action potential conditions**

A, representative records of  $I_{\text{HERG}}$ , after P/4 leak subtraction, (upper traces) activated by ventricular action potential command (lower trace), in control and in the presence of 5 mM 4-AP. B, effect of 5 mM 4-AP on action potentials from guinea-pig ventricular myocytes. C, effect of 4-AP on resurgent  $I_{\text{K}}$  tails from a guinea-pig ventricular myocyte (upper traces; lower trace shows a section of the voltage protocol. A mean inhibition of  $71.1 \pm 4.3\%$  was observed ( $n = 6$ ).

interpretation of experiments in which millimolar 4-AP has been used as a pharmacological tool to study cardiac  $I_{TO,1}$  in species that also exhibit  $I_{Kr}$ . Of course, *in vitro* data must only be extrapolated to the *in vivo* situation with caution and there is evidence that 4-AP infusion regimens used to study the role of  $I_{TO,1}$  in the dog (del Balzo & Rosen, 1992) may actually attain submillimolar plasma levels (Nattel *et al.* 2000). Our data are concordant with the notion that micromolar 4-AP may justifiably be used to study  $I_{Kur}$  in cells that exhibit  $I_{Kr}$ , as comparatively little  $I_{Kr}$  blockade would be expected at the low concentration levels (e.g. Yue *et al.* 1996) required to inhibit  $I_{Kur}$ .

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