British Journal of Pharmacology (2008) 154, 1619–1630

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Pathology-specific effects of the $I_{Kur}/I_{to}/I_{K,ACh}$ blocker AVE0118 on ion channels in human chronic atrial fibrillation

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Background and purpose: This study was designed to establish the pathology-specific inhibitory effects of the $I_{Kur}/I_{to}/I_{K,ACh}$ blocker AVE0118 on atrium-selective channels and its corresponding effects on action potential shape and effective refractory period in patients with chronic AF (cAF).
Experimental approach: Outward K^+-currents of right atrial myocytes and action potentials of atrial trabeculae were measured with whole-cell voltage clamp and microelectrode techniques, respectively. Outward currents were dissected by curve fitting.
Key results: Four components of outward K^+-currents and AF-specific alterations in their properties were identified. I_{to} was smaller in cAF than in SR, and AVE0118 (10 μM) apparently accelerated its inactivation in both groups without reducing its amplitude. Amplitudes of rapidly and slowly inactivating components of I_{Kur} were lower in cAF than in SR. The former was abolished by AVE0118 in both groups, the latter was partially blocked in SR, but not in cAF, even though its inactivation was apparently accelerated in cAF. The large non-inactivating current component was similar in magnitude in both groups, but decreased by AVE0118 only in SR. AVE0118 strongly suppressed AF-related constitutively active $I_{K,ACh}$ and prolonged atrial action potential and effective refractory period exclusively in cAF.
Conclusions and implications: In atrial myocytes of cAF patients, we detected reduced function of distinct I_{Kur} components that possessed decreased component-specific sensitivity to AVE0118 most likely as a consequence of AF-induced electrical remodelling. Inhibition of profibrillatory constitutively active $I_{K,ACh}$ may lead to pathology-specific efficacy of AVE0118 that is likely to contribute to its ability to convert AF into SR.
British Journal of Pharmacology (2008) **154,** 1619–1630; doi:10.1038/bjp.2008.209; published online 9 June 2008

Keywords: human atrium; $I_{Kur}/I_{to}/I_{K,ACh}$; AVE0118; action potentials; atrial fibrillation

Abbreviations: AF, atrial fibrillation; AP, action potential; AVE0118, (2′-{[2-(4-methoxy-phenyl)-acetylamino]-methyl}-biphenyl-2carboxylic acid (2-pyridin-3-yl-ethyl)-amide); cAF, chronic atrial fibrillation; CCh, carbachol; hKv1.5, human voltage-dependent potassium channel; I_{K1}, inwardly rectifying potassium current; $I_{K,ACh}$, acetylcholine-activated potassium current; I_{Kr}, rapidly activating potassium outward current; I_{Ks}, slowly activating potassium current; I_{Kur}, ultrarapidly activating potassium outward current; I_m, membrane current; I_{to}, transient outward current; KChIP2, potassium channel-interacting protein 2; Kv1.5, voltage-dependent potassium channel 1.5; Kv4.3-L, long isoform of voltage-dependent potassium channel 4.3; Kv4.3-S, short isoform of voltage-dependent potassium channel 4.3; Ltk^- cells , leukocyte tyrosine kinase-negative cells; RMP, resting membrane potential; SR, sinus rhythm

Introduction

Atrial fibrillation (AF) inflicts an increasing burden of morbidity and mortality mainly due to heart failure and thromboembolic complications (Chugh *et al.*, 2001). The efficacy of current therapy in controlling ventricular rate, restoring sinus rhythm (SR) and preventing AF recurrence is limited probably because of AF-related electrical and structural remodelling (Nattel, 2002; Dobrev and Ravens, 2003). Prolongation of atrial repolarization and refractoriness by blocking K^+ channels can terminate AF; however, many commonly used antiarrhythmic drugs prolong ventricular repolarization leading to an enhanced risk of the development of torsades de

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Received 17 January 2008; revised 6 March 2008; accepted 24 April 2008; published online 9 June 2008

pointes arrhythmias. This potentially lethal complication can be avoided if atrial refractoriness is prolonged selectively by blocking K^+ channel populations that reside exclusively in the atria. The criterion of 'atrial-selectivity' (Ehrlich *et al.*, 2007) is met by the ultra-rapid delayed rectifier potassium current I_{Kur} and by the acetylcholine-activated current $I_{K,ACh}$ (Wang *et al.*, 1993; Amos *et al.*, 1996; Nerbonne and Kass, 2005). However, electrical and structural remodelling in AF impacts the function of drug targets, which may result in pathology-specific efficacy of the therapeutic interventions.

Several I_{Kur} blockers have been developed for potential therapeutic use in AF, including the biphenyl derivative 2′-{[2-(4-methoxy-phenyl)-acetylamino]-methyl}-biphenyl-2carboxylic acid (2-pyridin-3-yl-ethyl)-amide (AVE0118). In heterologous expression systems, AVE0118 also blocks the transient outward current (I_{to}) and $I_{K,ACh}$, but not inwardly rectifying potassium current (I_{K1}) (Gogelein *et al.*, 2004; Decher *et al.*, 2006). Delayed rectifier currents rapidly activating potassium outward current (I_{Kr}) and slowly activating potassium current (I_{Ks}) are only inhibited at suprapharmacological concentrations of AVE0118. When applied in different animals, AVE0118 increases atrial effective refractory period without prolonging QT interval (Wirth *et al.*, 2003; Oros *et al.*, 2006; Blaauw *et al.*, 2007), prevents induction of AF in normal pigs (Wirth *et al.*, 2003) and converts AF to SR in a goat model of 'lone' AF (Blaauw *et al.*, 2007). Despite these encouraging results, it is unclear whether AVE0118 preserves its effectiveness in AF patients with electrically and structurally remodelled atria. On the other hand, atrial tachycardia or chronic AF (cAF) leads to agonist-independent, constitutively active $I_{K,ACh}$ channels that contribute to the abbreviation of atrial action potential (AP) (Dobrev *et al.*, 2005; Cha *et al.*, 2006; Voigt *et al.*, 2007). As AVE0118 strongly inhibits receptor-activated $I_{K,ACh}$ in pig atria (Gogelein *et al.*, 2004), concomitant suppression of profibrillatory, constitutively active $I_{K,ACh}$ channels may preserve its overall efficacy in patients with cAF.

This study was designed to establish whether and how cAF influences the inhibitory effects of AVE0118 on human I_{to}, I_{Kur} and $I_{K,ACh}$ and on corresponding changes of AP and atrial effective refractory period.

Methods

Human tissue samples

The ethics committee of Dresden University of Technology approved the study (no. EK790799). Each patient gave written, informed consent.

Right atrial appendages were obtained from 52 SR and 38 cAF patients (AF > 6 months; Table 1). Significant differences between the two groups were found for underlying heart disease and size of left atrium. Most of the AF patients received digitalis and diuretics, whereas the SR patients were more likely to be taking lipid-lowering drugs.

Electrophysiological recordings

Myocytes were isolated from atrial appendages of patients undergoing open-heart surgery. Conventional voltage-clamp techniques were used to measure I_{Kur}, I_{to}, $I_{K,ACh}$ and I_{K1} as previously described (Dobrev *et al.*, 2001; Christ *et al.*, 2004). About 70% of series resistance and up to 100 pF of membrane capacitance were compensated electronically. Mean cell capacitance was 70.8 ± 4.4 pF ($n = 26/11$) in SR and 85.5 ± 6.1 pF ($n = 41/11$) in cAF ($P < 0.05$). To control for variations in myocyte size, all currents are expressed as densities (pA pF^{-1}). Double-pulse protocols were used to separate I_{to} from I_{Kur} current components by exploiting fast I_{to} recovery from inactivation at 37 °C. Three exponential functions were fitted to outward current traces with ANA-3 software (MFK, Niedernhausen, Germany):

$$I_m = A * \exp(-t/\tau_A) + B * \exp(-t/\tau_B) + C * \exp(-t/\tau_C) + D$$

where A, B, C are amplitudes, τ_A, τ_B and τ_C time constants of the exponentially inactivating outward current I_m, D is the amplitude of the non-inactivating current component. Goodness of fit was tested by least square regression analysis; r^2 values were significantly better with a three-exponential than a two-exponential fit in SR and AF cells, $P < 0.05$. Outward current in the presence of AVE0118 and in human voltage-dependent potassium channel 1.5 (hKv1.5) expression systems were best fitted with two-exponential functions.

Atrial APs were recorded with standard intracellular microelectrodes in atrial trabeculae (Wettwer *et al.*, 2004). Bath solution contained (in mM): NaCl 127, KCl 4.5, $MgCl_2$ 1.5, $CaCl_2$ 1.8, glucose 10, $NaHCO_3$ 22, NaH_2PO_4 0.42, equilibrated with O_2–CO_2 (95:5) at 36.5 ± 0.5 °C, pH 7.4.

Table 1 Characteristics of the patients

	SR	*cAF*
n	*52*	*38*
Gender, m/f	37/15	*29*/8
Age (years)	67 ± 1	69 ± 2
BMI (kg m^{-2})	28 ± 1	27 ± 1
CAD (*n*)	29	7*
AVD/MVD (*n*)	9	19*
CAD + AVD/MVD (*n*)	14	12
Hypertension (*n*)	43	31
Diabetes (*n*)	18	9
Hyperlipidaemia (*n*)	28	13
LVEF (%)	57 ± 2	54 ± 2
LVEDP (mm Hg)	16 ± 1	15 ± 1
LA (mm)	42 ± 1	50 ± 1*
LVEDD (mm)	50 ± 1	52 ± 2
Cardiovascular medication (n)		
Digitalis	2	11*
ACE inhibitors/AT_1-blockers	31	21
β-blockers	42	32*
Calcium channel blockers	5	4
Diuretics	19	20*
Nitrates	9	8
Lipid-lowering drugs	25	5*

Abbreviations: AT, angiotensin receptor; AVD, aortic valve disease; CAD, coronary artery disease; cAF, chronic atrial fibrillation; LA, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; LVEDP, left ventricular end-diastolic pressure; LVEF, left ventricular ejection fraction; MVD, mitral valve disease; SR, sinus rhythm.

*$P < 0.05$ from Student's unpaired *t*-test for continuous variables and from χ^2 test for categorical variables.

Preparations were regularly stimulated for at least 1 h before data acquisition with a custom-made computer programme (University of Szeged, Hungary) that also generated electrical stimuli. Refractory period was determined by applying an extra stimulus (2 × threshold voltage) at increasingly smaller intervals every tenth regular stimulus until initiation of an extra AP with a clear fast upstroke AP failed.

Heterologous expression of Kv1.5 in mouse fibroblasts
Mouse leukocyte tyrosine kinase-negative (Ltk^-) cells stably transfected with voltage-dependent potassium channel 1.5 (Kv1.5) plasmid (Snyders *et al.*, 1993) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% glutamax, 1% penicillin/streptomycin and 250 μg mL^{-1} of the antibiotic G418 in a humidified 5% CO_2 atmosphere at 37 °C. Before experimentation, cells were treated with trypsin, incubated in dexamethasone-containing (1 μM) medium for 24 h, gently scraped off and left at room temperature until use within 8 h.

Determination of mRNA expression
Atrial samples were immediately stored in liquid nitrogen. Total RNA was isolated and quantitative real-time reverse transcriptase-PCR was performed as previously described (Sambrook, 2007). Primer pairs specific for human Kv4.3-L (long isoform of voltage-dependent potassium channel 4.3) and Kv4.3-S (short isoform of voltage-dependent potassium channel 4.3), potassium channel-interacting protein 2 (KChIP2) and Kv1.5 were intron spanning (Table 2). Nomenclature of channels conforms with BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Chemicals and drugs
AVE0118 was kindly provided by Sanofi-Aventis Germany GmbH (Frankfurt, Germany). All other compounds were purchased from Sigma (Steinheim, Germany).

Statistical analysis
Differences between continuous data were compared by Student's unpaired *t*-test or one-way ANOVA. Frequency data were analysed with χ^2 statistics. Data are presented as means ± s.e.mean. $P<0.05$ was considered statistically significant.

Results

Effects of AVE0118 on I_{to} and I_{Kur} in SR and CAF
In human atrial myocytes, peak outward current amplitude was higher in SR than in cAF (25.8 ± 3.6 pA pF^{-1}, $n=9/4$ and 13.1 ± 1.4 pA pF^{-1}, $n=23/7$, respectively); late outward current at the end of the clamp pulse was comparable in both groups (7.9 ± 1.3 versus 6.2 ± 0.6 pA pF^{-1}, respectively; Figure 1a). In SR, AVE0118 reduced amplitude of peak and late current in a concentration-dependent manner and apparently accelerated their inactivation. The AVE0118-sensitive currents at 10 μM were 9.5 ± 1.7 and 5.3 ± 1.2 pA pF^{-1} for peak and late current, respectively; the corresponding concentrations for half-maximum inhibition (IC_{50}) were 1.8 μM for peak and 220 nM for late current (Figure 1a).

In cAF, AVE0118 reduced peak current by only 2.2 ± 0.8 pA pF^{-1} ($IC_{50}=1.9$ μM) and late current by 1.8 ± 0.5 pA pF^{-1} ($IC_{50}=1.1$ μM) suggesting that AVE0118 induces a smaller blockade of I_{to} and I_{Kur} in cAF than in SR. Conventionally, the difference between peak and late current is taken as a surrogate for I_{to}, late current at the end of the clamp pulse as I_{Kur} (Van Wagoner *et al.*, 1997; Bosch *et al.*, 1999). Recent work using this definition revealed that I_{to} is reduced in human cAF, but provided inconsistent (reduced or unchanged) findings for I_{Kur} (reviewed in Dobrev and Ravens, 2003). However, at peak outward current, the rapidly inactivating components of I_{to} and I_{Kur} overlap, whereas the late current consists of slowly inactivating I_{Kur} and unknown outward current component(s). This does not allow a clear separation of the specific effects of AVE0118 on I_{to} and I_{Kur}. Although the rapidly inactivating I_{Kur} current component is a major and atrium-specific determinant of atrial repolarization, previous studies

Table 2 Primers and conditions for PCR

Gene	*Accession no.*	*Primer sequence (5′-3′)*	*Position (bp)*	*Size (bp)*	*T_A (°C)*	*$MgCl_2$ (mM)*
Kv4.3 long	AF205857	S:TCCACCATCAAGAACCACG	1507–1525	133	58	2.5
		A:AGCAGGTGGTAGTGAGGCC	1621–1639			
Kv4.3 short	AF205856	S:GGAAAAAACCACTAACCACGAGT	1372–1390	211	63	6.0
		A:AGCAGGTGGTAGTGAGGCC	1564–1582			
KChIP2	AF199598	S:ATGCTTGACATCATGAAGTCC	547–572	162	58	2.5
		A:TTGACAAGACTCAATGAATTC	687–708			
Kv1.5	M83254	S:CATTGCCCTGCCTGTGCC	1677–1695	158	60	4.0
		A:TGCTCCCGCTGACCTTCC	1817–1835			
T7	A32834	S:TAATACGACTCACTATAGGGCGGCCGCGG	12–40		58	2.5

Abbreviations: KChIP2, potassium channel-interacting protein 2; Kv1.5, voltage-dependent potassium channel 1.5; Kv4.3, voltage-dependent potassium channel 4.3.
The table specifies sense (S) and antisense (A) primers and reaction conditions used for reverse transcriptase-PCR of α- and β-subunits and cRNA standard generation. T_A: annealing temperature.

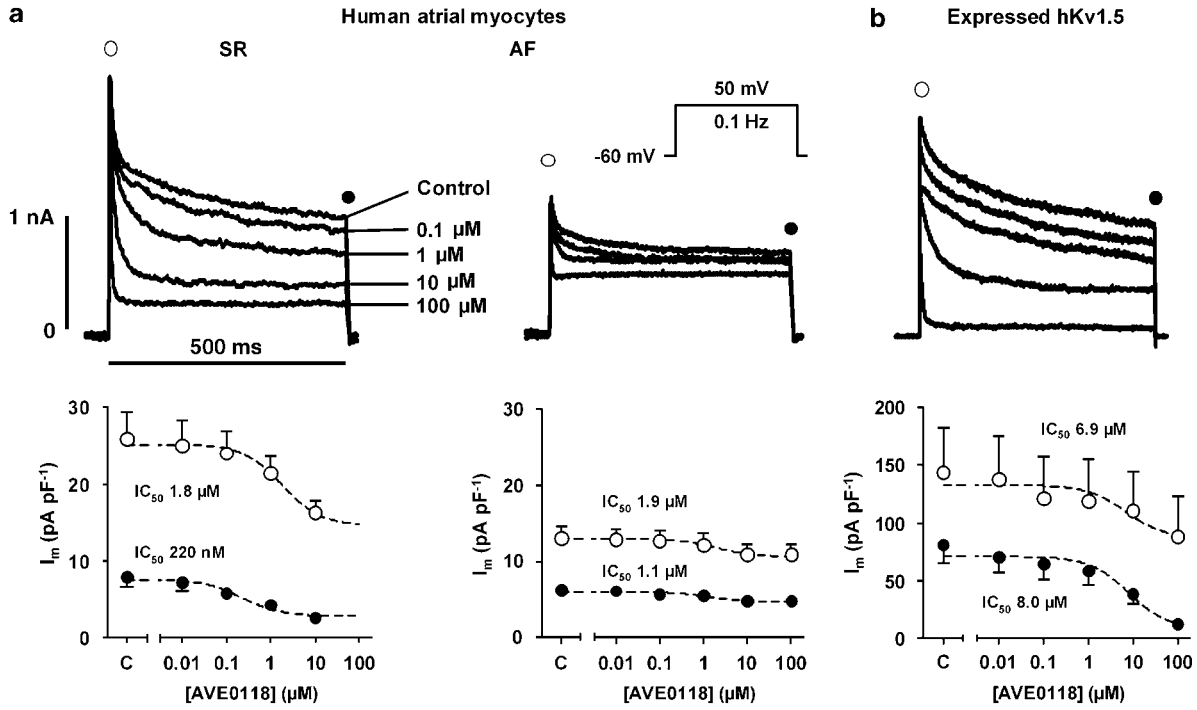

Figure 1 Effects of AVE0118 on outward currents in human atrial myocytes (**a**) and Kv1.5-expressing mouse fibroblast (**b**). Top, current traces in response to 500-ms long clamp steps (inset) under control conditions and in the presence of increasing AVE0118 concentrations. Bottom, concentration–response curves for AVE0118 effects on peak and late current at end of clamp step. Points shown are means ± s.e.mean; $n = 9/4$ (cells/patients) for SR and $n = 23/7$ for AF; 12 cells for Kv1.5. AF, atrial fibrillation; Kv1.5, voltage-dependent potassium channel 1.5; SR, sinus rhythm.

have not separated the rapidly inactivating I_{to} and I_{Kur} components when investigating specific AF-related alterations and drug responses (Van Wagoner *et al*., 1997; Bosch *et al*., 1999).

Effects of AVE0118 on heterologously expressed hKv1.5 channels

To examine specifically the effects of AVE0118 on I_{Kur}, hKv1.5 was heterologously expressed in mouse fibroblasts. This resulted in peak current by substantial inactivation, clearly suggesting that I_{Kur} possesses a rapidly inactivating peak and a slowly inactivating late component. The former was partially and the latter completely blocked by AVE0118; IC_{50} was 6.9 µM for peak and 8.0 µM for late current (Figure 1b). These data show that AVE0118 inhibits both I_{Kur} current components with comparable efficacy and raise the possibility that the reduced efficacy of AVE0118 to block peak and late current in cAF myocytes may result from reduced I_{Kur} function or channel subunit expression.

Differentiation of AVE0118 effects on current components in SR and cAF

To differentiate I_{Kur} from I_{to}, we fitted three-exponential functions to the current traces (Figure 2), assuming that I_{to} (component A) inactivates most rapidly, I_{Kur} exhibits biphasic inactivation as in the expression system (components B and C) and non-inactivating current (component D) persists. The time constant τ_A for the rapidly inactivating I_{to} was similar in both groups, yet amplitudes were significantly smaller in cAF than in SR (Figures 2b and c). The time constants (τ_B and τ_C) for the rapidly and slowly inactivating I_{Kur} components were comparable to those obtained for heterologously expressed hKv1.5 channels (Figure 2d) with no significant differences between SR and cAF. The corresponding current amplitudes (B and C) were significantly smaller in cAF than in SR; the non-inactivating current components (D) were similar in size (Figures 2b and c).

Next, we also applied the curve-fitting procedure to the current traces in the presence of AVE0118 (10 µM, Figure 2). In the expression system, AVE0118 did not reduce the rapidly inactivating I_{Kur} component B, whereas the slowly inactivating and the non-inactivating components C and D were strongly decreased. The corresponding time constants τ_B and τ_C were reduced suggesting open channel block. In atrial myocytes, AVE0118 significantly accelerated initial current inactivation (τ_A) without reducing the amplitude of I_{to} (component A; Figure 2). The rapidly inactivating I_{Kur} (component B) was abolished by AVE0118 in both SR and cAF, the slowly inactivating I_{Kur} (component C) was reduced in SR, but unchanged in cAF, with corresponding time constants τ_C being significantly decreased. AVE0118 strongly inhibited the non-inactivating current in SR suggesting a fraction of Kv1.5-mediated current in component D. Most strikingly, in cAF myocytes, the non-inactivating current component was not sensitive to AVE0118 (Figure 2c). For comparison, all curve-fitting data are summarized in Table 3.

The effects of the drug on rapidly inactivating I_{to} and I_{Kur} were separated with a second approach based on their distinctly different recovery kinetics. This allowed us to

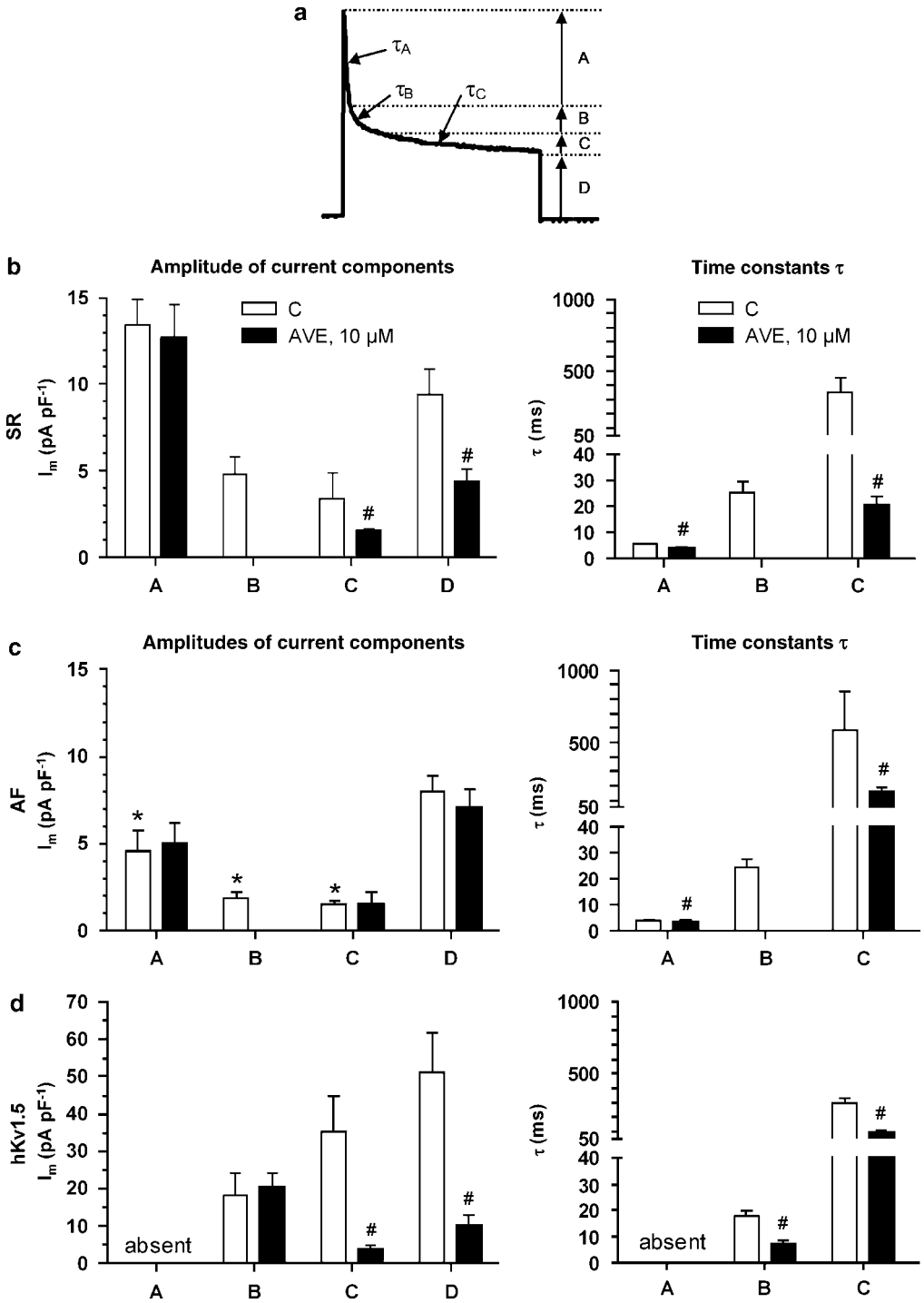

Figure 2 Separation of outward current components by curve fitting. (**a**) At clamp step to +50 mV outward current inactivated with three distinct time constants, a rapid (τ_A), an intermediate (τ_B) and a slow one (τ_C), the respective components corresponding to I_{to} (**a**), rapidly (**b**) and slowly inactivating I_{Kur} (**c**), respectively (indicated by upright arrows in (**a**)). Component D did not inactivate at all. (**b–d**) Amplitudes of current components (left) with distinct inactivation time constants (right) in human atrial myocytes from SR (**b**) and AF (**c**) and in hKv1.5 channels expressed in mouse fibroblasts (**d**). C: pre-drug controls, AVE: in the presence of 10 μM AVE0118. Columns represent means ± s.e.mean; $n = 9/4$ (cells/patients) for SR and $n = 17/7$ for AF; 12 cells for Kv1.5. *$P < 0.05$ versus corresponding SR values; $^{\#}P < 0.05$ versus corresponding control values. AF, atrial fibrillation; I_{Kur}, ultrarapidly activating potassium outward current; I_{to}, transient outward current; hKv1.5, human voltage-dependent potassium channel 1.5; SR, sinus rhythm.

study the specific effects of AVE0118 on I_{to} amplitude. Outward current was activated with a 500-ms clamp step to +50 mV. During a short 25-ms recovery interval at –60 mV, I_{to} fully recovered from inactivation with time constants <10 ms in both SR and AF, producing a transient peak current during the second clamp step to +50 mV (Figure 3). Due to the slow recovery from inactivation of Kv1.5 (Rich and Snyders, 1998), rapidly inactivating I_{Kur} is absent during

Table 3 Parameters from three exponential curve fitting to averaged ($n=5$) outward current traces in response to clamp steps to +50 mV (holding potential of −60 mV) (compare Figure 2)

Control	*SR* (n = 9/4)		*AF* (n = 17/7)		*hKv1.5* (n = 8)	
Parameter	*Pre-drug control*	*AVE0118*	*Pre-drug control*	*AVE0118*	*Pre-drug control*	*AVE0118*
A (pA pF^{-1})	13.4 ± 1.5	12.7 ± 1.9	4.6 ± 1.2*	5.0 ± 1.2	—	—
τ_A (ms)	5.7 ± 0.4	3.8 ± 0.4$^{\#}$	4.1 ± 0.3*	3.4 ± 1.1$^{\#}$	—	—
B (pA pF^{-1})	4.8 ± 1.0	0	1.9 ± 0.3*	0	18.4 ± 5.9	20.2 ± 4.1
τ_B (ms)	25.4 ± 4.4	0	24.6 ± 3.1	0	18.0 ± 1.6	7.2 ± 1.1$^{\#}$
C (pA pF^{-1})	3.4 ± 0.8	1.5 ± 0.2	1.0 ± 0.2*	1.5 ± 0.7	35.3 ± 9.5	3.7 ± 0.8
τ_C (ms)	347 ± 102	20.7 ± 3.1	582 ± 273	155 ± 39	297 ± 32	87 ± 24$^{\#}$
D (pA pF^{-1})	9.4 ± 1.5	4.4 ± 0.7$^{\#}$	8.0 ± 0.9	7.1 ± 1.0	51.3 ± 10.7	9.9 ± 3.2$^{\#}$

Abbreviation: hKv1.5, human voltage-dependent potassium channel 1.5.
Human atrial myocytes from patients in sinus rhythm (SR) or atrial fibrillation (AF); and hKv1.5 channels stably expressed in a mouse fibroblast cell line. 'Pre-drug control', in the absence and 'AVE0118' in the presence of 10 μM AVE0118.
*$P<0.01$ vs SR, $^{\#}P<0.01$ vs pre-drug control.

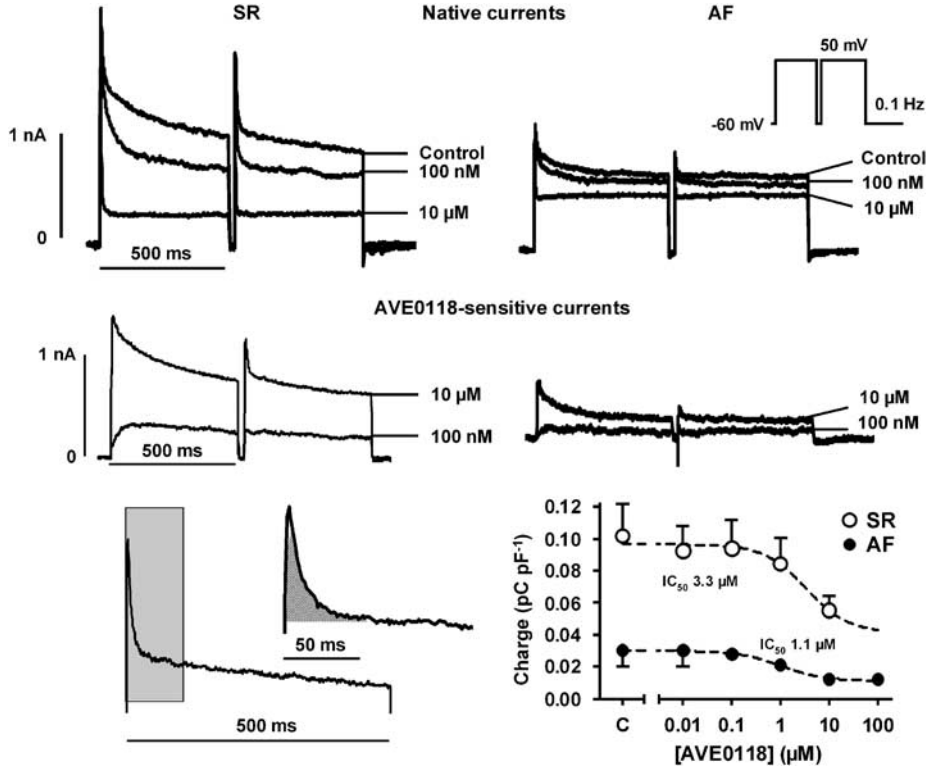

Figure 3 AVE0118-sensitive currents in human atrial myocytes of SR and AF. Double-pulse protocol to separate I_{to} from I_{Kur}: I_{to} was allowed to recover during a brief (25 ms) repolarizing clamp step to −60 mV, and was analysed as area under the curve of the voltage transient during initial 50 ms of second clamp step (hatched area of inset in lower left). Top: typical current traces in SR and AF myocytes at control and in the presence of 100 nM and 10 μM AVE0118. Middle: resulting difference currents (AVE0118-sensitive currents). Bottom: analysis of I_{to} and concentration–response curves of AVE0118 effects on I_{to} in SR and AF myocytes. Means ± s.e.mean are shown; $n=9/4$ (cells/patients) for SR and $n=17/7$ for AF. AF, atrial fibrillation; $I_{to,}$ transient outward current; I_{Kur}, ultrarapidly activating potassium outward current; SR, sinus rhythm.

the second clamp step, and only slowly and non-inactivating current components remain (Figure 3). Assuming rapid and complete recovery from inactivation of I_{to}, we took the area under the transient current component within the initial 50 ms of the second test pulse as a measure for I_{to} (Figure 3). This analysis confirmed the strong AF-related reduction of I_{to}. Expressed as charge corrected for cell size, I_{to} was 0.03 ± 0.01 pC pF^{-1} ($n=9/4$) in AF as compared with 0.10 ± 0.02 pC pF^{-1} ($n=9/4$, $P<0.01$) in SR; IC_{50} were 3.3 μM in SR and 1.1 μM in cAF, respectively ($P>0.05$) (Figure 3). Subtracting current traces in the presence of AVE0118 from pre-drug control traces revealed drug-sensitive currents (Figure 3).

Atrial expression of Kv4.3 and Kv1.5 mRNA in SR and cAF

Electrical remodelling in AF modifies the expression of K^+ channels targeted by AVE0118 (Dobrev and Ravens, 2003). The long and short splice variants of Kv4.3, $Kv4.3_{long}$ and $Kv4.3_{short}$ (Radicke *et al.*, 2006), and the β-subunit KChIP2

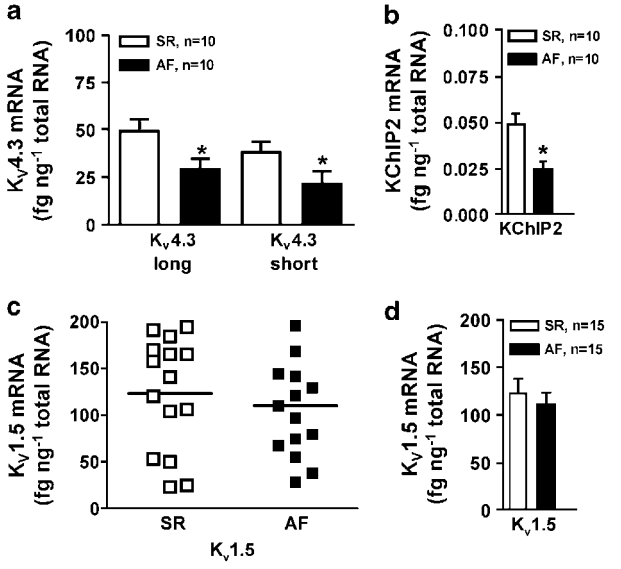

Figure 4 Real-time PCR of K^+ channel subunits in atrial tissue from SR and AF patients. (**a** and **b**) mRNA levels of long and short Kv4.3 splice variants and KChIP2. (**c** and **d**) Expression of Kv1.5 mRNA; means ± s.e.mean from 15 individual tissues in each group. *$P<0.05$ versus SR. AF, atrial fibrillation; KChIP2, potassium channel-interacting protein 2; Kv1.5, voltage-dependent potassium channel 1.5; SR, sinus rhythm.

were reduced in cAF (Figures 4a and b), which supports the loss of efficacy of AVE0118 to block I_{to}. We found similar mRNA levels of Kv1.5 in SR and cAF (Figures 4c and d). This suggests that the loss of efficacy of AVE0118 to block slowly inactivating and non-inactivating current components probably results from abnormal channel function rather than downregulation of Kv1.5 subunits.

Effects of AVE0118 on $I_{K,ACh}$ *in SR and cAF*

In previous studies, we showed that muscarinic receptor-mediated $I_{K,ACh}$ activation is smaller in cAF than in SR patients but is accompanied by the development of agonist-independent constitutive $I_{K,ACh}$ activity (Dobrev *et al*., 2005; Voigt *et al*., 2007). Subsequent studies in dogs with atrial tachycardia-induced remodelling demonstrated the profibrillatory effects of constitutively active $I_{K,ACh}$ (Cha *et al*., 2006). As AVE0118 inhibits muscarinic receptor-activated $I_{K,ACh}$ in pig atria (Gogelein *et al*., 2004), we tested whether AVE0118 blocks constitutively active $I_{K,ACh}$ channels in cAF patients. Current traces in response to ramp pulses from −100 to +40 mV showed strong inward rectification (compare Figure 5a). In addition to inward rectifying currents, I_{Kur}, but not I_{to}, is also activated by the ramp pulse. AVE0118 (10 μM) significantly reduced outward current, but this effect was smaller in cAF than in SR (Figure 5b), confirming the principal results in Figure 2.

In SR, basal current in the inward branch contains I_{K1} only, whereas cAF myocytes possess increased I_{K1} and constitutively active $I_{K,ACh}$ (Dobrev *et al*., 2005; Voigt *et al*., 2007). As AVE0118 does not block I_{K1}, any effect of this drug on inward current in cAF must involve inhibition of constitutively active $I_{K,ACh}$. Current density at –100 mV was

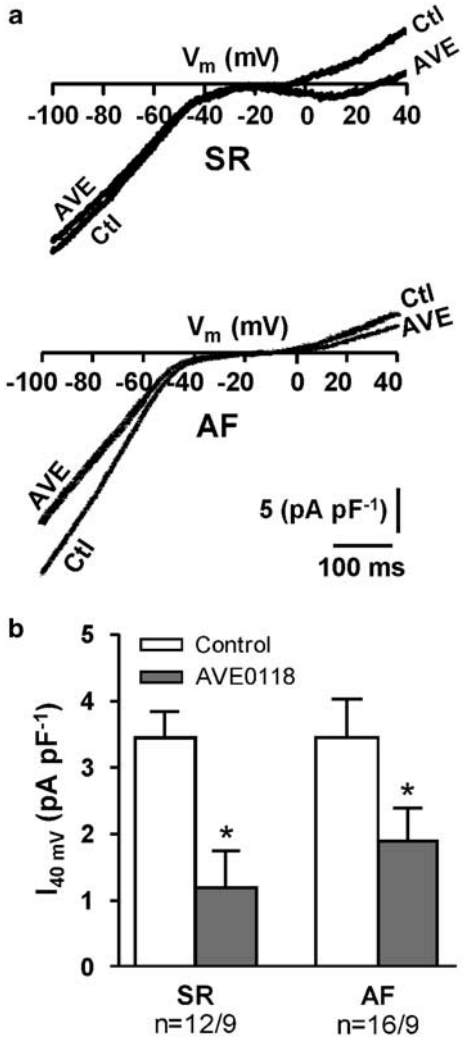

Figure 5 Effects of AVE0118 on the outward branch of currents in response to ramp pulses from −100 to +40 mV (1250 ms, 0.5 Hz) in SR and AF. (**a**) Original recordings under control conditions (Ctl) and in the presence of 10 μM AVE0118 (AVE). (**b**) Current amplitude at +40 mV in SR and AF at control and after 10 μM AVE0118. Means ± s.e.mean are shown (n = cells/patients), *$P<0.05$ versus corresponding control. AF, atrial fibrillation; SR, sinus rhythm.

13.8 ± 1.8 pA pF^{-1} in SR and 30.5 ± 2.9 pA pF^{-1} in cAF, and as expected, AVE0118 reduced basal current in cAF only (Figure 6e).

To examine whether AVE0118 inhibits human atrial $I_{K,ACh}$, we studied the effects of AVE0118 on muscarinic receptor-activated current, conventionally defined as $I_{K,ACh}$, in SR and cAF. To allow each myocyte to serve as its own control, $I_{K,ACh}$ was activated by two subsequent exposures to carbachol (CCh, 2 μM), with a period of drug-free superfusion in between (Figures 6a and b). The difference between basal current at −100 mV and in the presence of CCh was defined as $I_{K,ACh}$. During 2 min of exposure to CCh, $I_{K,ACh}$ declined from peak to a quasi steady-state value because of desensitization. In control myocytes, the second activation of $I_{K,ACh}$ was lower than the first one both at peak and quasi steady-state due to incomplete recovery from desensitization

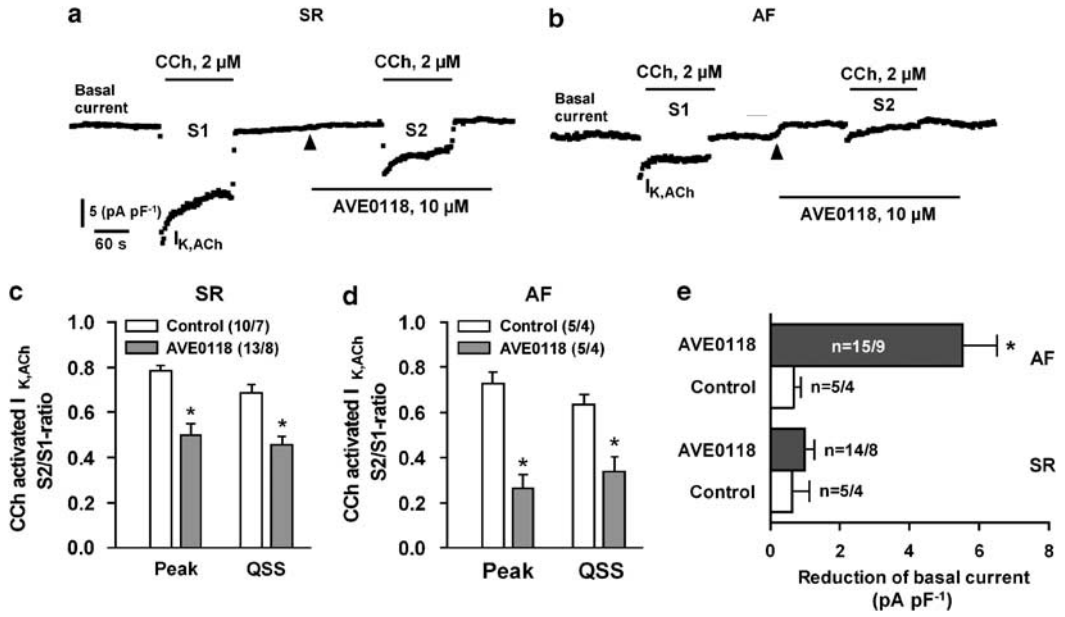

Figure 6 Time course of basal current and repeated activation of $I_{K,ACh}$ by 2 μM carbachol (CCh; $I_{K,ACh}$ defined as CCh-sensitive current). (**a** and **b**) Activation of $I_{K,ACh}$ during two successive applications of CCh (S1, S2, 4-min apart) in SR (left) and AF (right) with S2 in the presence of 10 μM AVE0118. During activation, the initial increase (peak) faded (rapid desensitization) to a quasi steady-state level (QSS). (**c** and **d**) Mean S2/S1 ratios of peak and QSS $I_{K,ACh}$ under control conditions and in the presence of AVE0118 in SR and AF. (**e**) Block of basal current with 10 μM AVE0118 in SR and AF. n = myocytes/patients. *$P<0.05$ versus corresponding control values. AF, atrial fibrillation; $I_{K,ACh}$, acetylcholine-activated potassium current; SR, sinus rhythm.

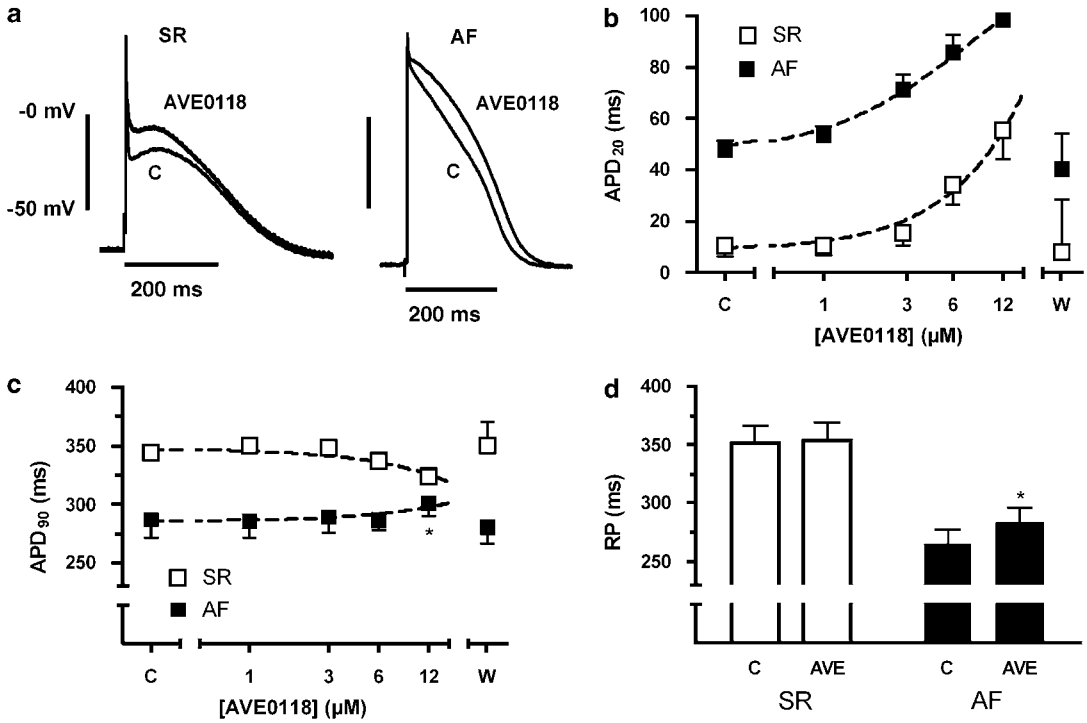

Figure 7 Effect of AVE0118 on action potentials in SR and AF. (**a**) Original AP registration in SR and AF under control conditions (C) and after 30 min of exposure to AVE0118 (12 μM). (**b** and **c**) Concentration-dependent effects of AVE0118 on APD_{20} and APD_{90}, respectively, in SR ($n=16/8$) and AF ($n=6/3$). (**d**) Effect of AVE0118 (AVE, 6 μM) on refractory period (RP) in SR ($n=8/8$) and AF ($n=6/3$). Means ± s.e.mean are shown (n = trabeculae/patients). *$P<0.05$. AF, atrial fibrillation; AP, action potential; SR, sinus rhythm.

(Voigt *et al*., 2007). AVE0118 (10 μM) significantly reduced CCh-activated $I_{K,ACh}$ in SR and cAF (Figures 6c and d), but decreased basal current in cAF only (see arrow heads in Figures 6a, b and e). Thus, it is likely that AVE0118 inhibits constitutively active $I_{K,ACh}$ and that this effect contributes to its efficacy to convert AF into SR.

Effects of AVE0118 on atrial AP and refractoriness in SR and cAF
We next examined whether the AF-specific effects of AVE0118 on atrial ion channels produce corresponding differences in early and late phase of repolarization or refractory period. Despite variability in shape, atrial APs typically exhibited spike-and-dome configuration in SR and triangular configuration in cAF (Figure 7a). Resting membrane potential (RMP) was more negative in cAF (-78.9 ± 1.1 mV, $n = 6/3$) than in SR (-75.0 ± 0.4 mV, $n = 16/8$; $P < 0.05$). AP duration at 20% of repolarization (APD_{20}) was significantly longer in cAF than in SR and was prolonged by AVE0118 (12 μM) in both groups (Figure 7b).

Consistent with increased basal inward rectifier current, APD_{90} was shorter in cAF than in SR. AVE0118 (12 μM) produced a small shortening of APD_{90} without affecting the refractory period in SR (Figures 7c and d), but consistently prolonged APD_{90} and refractory period in cAF, an effect that probably involves inhibition of constitutively active $I_{K,ACh}$. The effect of AVE0118 on APD_{90} did not depend on cycle length in either group (diastolic intervals between 1 and 2000 ms, data not shown). AVE0118 did not affect RMP, dV/dt_{max} or conduction time in SR and cAF (data not shown).

Discussion

Here we provide evidence for pathology-specific efficacy of the $I_{Kur}/I_{to}/I_{K,ACh}$ blocker AVE0118 on human atrial K^+ channels. We detected four different components of outward K^+ currents and identified AF-specific alterations in their properties. I_{to} was smaller and inactivated more rapidly in cAF than in SR. AVE0118 apparently accelerated I_{to} inactivation in SR and cAF without reducing the current amplitude indicating open channel block. Late current, which is conventionally considered to represent I_{Kur}, was not significantly different between SR and cAF; however, both inactivating components of I_{Kur} were distinctly reduced in cAF without any difference in inactivation kinetics. In the presence of AVE0118, the components B and C could no longer be distinguished probably because the time constants of components A and B converge. This interpretation is supported by effects of AVE0118 on expressed hKv1.5, accelerating component B to values within the same range as for component A of native myocytes in control as well as in the presence of AVE0118. Therefore, in the presence of AVE0118, we performed a two-exponential fit and the slower component was attributed to C with an accelerated time course instead of B with a slowed time course, and it was assumed that AVE0118 induced open channel block of all components.

The large non-inactivating current component was similar in magnitude in both groups, but was decreased by AVE0118 in SR only, suggesting apparent loss of efficacy of the drug in cAF. Finally, AVE0118 strongly suppressed AF-related constitutively active $I_{K,ACh}$ and prolonged atrial AP and refractory period, and these effects may underlie a pathology-specific efficacy of AVE0118 to convert AF into SR.

Comparison with previous studies
Electrical remodelling in AF involves complex regulatory mechanisms of ion channel function (Nattel *et al*., 2007). We observed AF-related downregulation of the long and short splice Kv4.3 variant and KChIP2 with concomitant decrease in I_{to}. The ratio between long and short splice variant of Kv4.3 was preserved in cAF suggesting no proportional change in the abundance of the long variant that contains inhibitory PKC phosphorylation sites (Radicke *et al*., 2005). This renders an AF-specific abnormal regulation of I_{to} by PKC unlikely. Although a reduction in I_{to} at the mRNA, protein and current level is a consistent finding in all studies of human AF (Grammer *et al*., 2000; Van Wagoner and Nerbonne, 2000), the available information about I_{Kur} is conflicting: mRNA levels of Kv1.5 have been found to be unaltered (Grammer *et al*., 2000; Brundel *et al*., 2001a, b; this study), whereas the protein levels are decreased in cAF (Van Wagoner *et al*., 1997; Brundel *et al*., 2001a, b). Current amplitude has been found to be decreased (Van Wagoner *et al*., 1997; Brandt *et al*., 2000; this study) or unchanged (Bosch *et al*., 1999; Grammer *et al*., 2000; Workman *et al*., 2001). These inconsistent results about I_{Kur} function in AF are not unexpected because the studies published used different strategies for identification of I_{to} and I_{Kur}, that is peak and late current, (Van Wagoner *et al*., 1997; Workman *et al*., 2001) I_{to} -inactivating prepulses (Amos *et al*., 1996; Yue *et al*., 1996) or separation by channel-selective blockers (Brandt *et al*., 2000).

Here we have used two different approaches (Figures 2 and 3) to separate I_{to} and I_{Kur} components in human atrium and to explore their AF-specific behaviours. The time constants for rapidly and slowly inactivating I_{Kur} were similar to those of expressed hKv1.5 suggesting that the estimation of these parameters in atrial myocytes provides reliable results. A substantial fraction of outward current did not inactivate at all; the composition of this current is unclear. It contains an AVE0118-sensitive non-inactivating I_{Kur} and additional still unknown non-inactivating current component(s). To the best of our knowledge, our dissection of four different components of human atrial outward K^+ currents is the first to distinguish distinct I_{Kur} current components and to identify their cAF-specific changes. The specific separation of I_{to} and I_{Kur} revealed an $\sim$70% reduction of I_{to}. In cAF, the rapidly inactivating I_{Kur} component showed an $\sim$60% decrease without any difference in inactivation kinetics. The similarity of the changes between I_{to} and rapidly inactivating I_{Kur} in cAF can explain the consistent finding in all recent publications of a 50–70% reduction of peak outward current (usually defined as I_{to}) (Van Wagoner *et al*., 1997; Bosch *et al*., 1999). However, we clearly show that there is a distinct rapidly inactivating I_{Kur} current component that is a major determinant of early repolarization, but is substantially reduced in remodelled atria. The slowly inactivating I_{Kur} component was also reduced by 70% during AF, whereas the large non-inactivating current component was of similar size in both groups. The nature of the latter current component is not known but its different sensitivity to AVE0118 in cAF (see below) suggests that atrial remodeling probably changes its composition. The impaired I_{to} and I_{Kur} are in good

agreement with slowed early repolarization as evidenced by prolonged APD_{20}.

The underlying mechanisms of reduced I_{Kur} function are only partially understood. The reduced protein Kv1.5 levels probably involve post-translational modifications that may result from increased proteolytic activity of Ca^{2+}-dependent calpains (Brundel *et al*., 2001b, 2002). There is also evidence that Ca^{2+}/calmodulin-dependent protein kinase phosphorylation accerelates the inactivation of I_{to} but increases the amplitude of I_{Kur} (Tessier *et al*., 1999). As cAF is associated with increased function of the counterbalancing protein phosphatases (Christ *et al*., 2004), the lower amplitude of the rapidly and slowly inactivating I_{Kur} components may also result from enhanced channel dephosphorylation. Further studies are required to identify the precise molecular mechanisms.

We found that AVE0118 potently blocked heterologously expressed hKv1.5 channels, and its IC_{50} of 8.0 μM was comparable to that found in a previous study (Gogelein *et al*., 2004). Consistent with open channel block (Gogelein *et al*., 2004; Decher *et al*., 2006), AVE0118 (10 μM) accelerated inactivation of the rapidly and slowly inactivating I_{Kur} components and reduced the fraction of non-inactivating current. AVE0118 suppressed outward currents of human atrial myocytes with ~10-fold greater potency for late (I_{Kur}; $IC_{50} = 220$ nM) than peak current (mixture of I_{to} and I_{Kur}; $IC_{50} = 1.8$ μM). The separate examination of AVE0118 effects on I_{to} (Figure 3f) yielded an IC_{50} of ~3.3 μM, which is in good agreement with the effect on peak current. In SR and cAF, AVE0118 accelerated the inactivation of I_{to} without a corresponding reduction of current amplitude and abolished the rapidly inactivating I_{Kur}. AVE0118 apparently accelerated the inactivation of the slowly inactivating I_{Kur} in both groups but reduced current amplitude in SR only. It partially reduced the large non-inactivating current in SR, but not in cAF, suggesting apparent loss of specific drug efficacy on major I_{Kur} components. Outward current in the ramp-pulse protocol was also less sensitive to block with AVE0118 in cAF compared to SR. Thus, the non-inactivating current must contain additional components. Although the nature of the AVE0118-insensitive current component is not known, its larger magnitude in cAF may weaken the efficacy of AVE0118 in the clinical setting.

AVE0118 reduced CCh-activated $I_{K,ACh}$ in both SR and cAF suggesting that this drug is able to inhibit human atrial $I_{K,ACh}$. Although CCh-activated $I_{K,ACh}$ was smaller in cAF, AVE0118 produced a larger fraction of inhibition (Figures 6c and d). In SR, inward basal current was not sensitive to AVE0118, and this is consistent with the inability of AVE0118 to block I_{K1} (Gogelein *et al*., 2004). In cAF, however, basal current contains a fraction of profibrillatory constitutively active $I_{K,ACh}$, and AVE0118 suppressed this current component to an extent that is comparable in magnitude to that induced by the selective $I_{K,ACh}$ blocker tertiapin (Dobrev *et al*., 2005; Voigt *et al*., 2007). I_{K1} and constitutively active $I_{K,ACh}$ channels are major determinants of the final phase of atrial repolarization and of setting the RMP. Accordingly, APD_{90} was shorter and RMP was more negative in cAF than in SR. Due to pathology-specific constitutively active $I_{K,ACh}$, AVE0118 prolonged APD_{90} and refractory period exclusively in cAF. RMP was not changed by AVE0118, and this suggests that $I_{K1,}$ which is enhanced in cAF but not targeted by AVE0118, is the major determinant of RMP during AF. Taken together, it is likely that the inhibition of constitutively active $I_{K,ACh}$ by AVE0118 underlies the prolongation of refractory period, which probably contributes to its efficacy to convert AF into SR. However, the strong efficacy of AVE0118 to convert AF in goats is accompanied by large prolongation of refractoriness (Blaauw *et al*., 2007). Thus, the efficacy of AVE0118 in AF patients is difficult to predict and requires prospective clinical testing.

Potential clinical implications

Conversion of AF into SR with available therapeutic strategies is successful in most cases, but maintenance of regular SR is achieved in less than 50% of patients. Drug efficacy is profoundly influenced by the presence of underlying heart disease and by the duration of AF. In cAF, AVE0118 may lose its efficacy due to the appearance of a large drug-resistant outward current of unknown nature. Alternatively, AF-induced electrical remodelling is accompanied by the development of the constitutively active $I_{K,ACh}$, which is likely to present a major site for antiarrhythmic drug action (Dobrev *et al*., 2005; Voigt *et al*., 2007). AVE0118 blocks these channels with a similar efficacy as that of tertiapin. Thus, studies in patients are clearly warranted to prove the efficacy and safety of AVE0118 in the clinical setting.

Study limitations

Although the perforated patch-clamp technique is preferable to ruptured patches to prevent intracellular dialysis, we were not successful in recording currents with this technique in human atrial myocytes. Therefore, the possibility that specific alterations in the intracellular milieu, as for instance redox potential, may have influenced our results cannot be excluded.

The differentiation of current components by curve fitting is an indirect method to define the distinct I_{Kur} components. Selective knockdown of Kv1.5 using small interfering RNA is needed to uncover the magnitude and nature of the distinct I_{Kur} components and their changes in AF-remodelled myocytes, but these approaches are not feasible because of difficulties in maintaining human atrial myocytes in culture. We cannot distinguish between the two major possibilities underlying the reduced efficacy of AVE0118 to block I_{Kur} in cAF, that is diminished I_{Kur} itself or loss of AVE0118 efficacy due to atrial remodelling. However, reduced Kv1.5 protein and I_{Kur} in AF patients (Van Wagoner *et al*., 1997) render it more likely that reduced channel function rather than limited AVE0118 efficacy *per se* is responsible for this effect.

Conclusion

The atrium-selective occurrence of I_{Kur} has attracted substantial interest in this channel as a potential drug target for AF treatment. In human atrial myocytes, we detected

reduced function of distinct I_{Kur} components during AF that possessed decreased component-specific sensitivity to AVE0118 most likely as a consequence of AF-induced electrical remodelling. Inhibition of profibrillatory constitutively active $I_{K,ACh}$ may lead to pathology-specific efficacy of this drug that is likely to contribute to its antifibrillatory mechanism of action in AF.

Acknowledgements

We thank Trautlinde Thurm, Ulrike Heinrich, Constanze Fischer, Sabine Kirsch and Annegret Häntzschel for technical assistance and Heinz Gögelein, Aventis Pharma AG, for critically reading the article. This work was supported by Sächsisches Staatsministerium für Wissenschaft und Kunst (Az.: 4-7531.50-04-0370-01/2, -02/4 and –03/8), Roland Ernst Stiftung für Gesundheitswesen, German Atrial Fibrillation Competence Network (Grant 01Gi0204) and European Union ('NORMACOR', Grant LSHM-CT-2006-018676). Deutsche Forschungsgemeinschaft (Do 769/1-1,2).

Conflict of interest

The authors state no conflict of interest.

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Supplementary Information accompanies the paper on British Journal of Pharmacology website (http://www.nature.com/bjp)