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Does the small conductance Ca^{2+}-activated K^+ current I_{SK} flow under physiological conditions in rabbit and human atrial isolated cardiomyocytes?

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ABSTRACT

Background: The small conductance Ca^{2+}-activated K^+ current (I_{SK}) is a potential therapeutic target for treating atrial fibrillation.
Aim: To clarify, in rabbit and human atrial cardiomyocytes, the intracellular $[Ca^{2+}]$-sensitivity of I_{SK}, and its contribution to action potential (AP) repolarisation, under physiological conditions.
Methods: Whole-cell-patch clamp, fluorescence microscopy: to record ion currents, APs and $[Ca^{2+}]_i$; 35–37°C.
Results: In rabbit atrial myocytes, 0.5 mM Ba^{2+} (positive control) significantly decreased whole-cell current, from −12.8 to −4.9 pA/pF ($P < 0.05$, $n = 17$ cells, 8 rabbits). By contrast, the I_{SK} blocker apamin (100 nM) had no effect on whole-cell current, at any set $[Ca^{2+}]_i$ (~100–450 nM). The I_{SK} blocker ICAGEN (1 μM: ≥2 x IC_{50}) also had no effect on current over this $[Ca^{2+}]_i$ range. In human atrial myocytes, neither 1 μM ICAGEN (at $[Ca^{2+}]_i$ ~ 100–450 nM), nor 100 nM apamin ($[Ca^{2+}]_i$ ~ 250 nM) affected whole-cell current (5–10 cells, 3–5 patients/group). APs were significantly prolonged (at APD_{30} and APD_{70}) by 2 mM 4-aminopyridine (positive control) in rabbit atrial myocytes, but 1 μM ICAGEN had no effect on APDs, versus either pre-ICAGEN or time-matched controls. High concentration (10 μM) ICAGEN (potentially I_{SK}-non-selective) moderately increased APD_{70} and APD_{90}, by 5 and 26 ms, respectively. In human atrial myocytes, 1 μM ICAGEN had no effect on APD_{30-90}, whether stimulated at 1, 2 or 3 Hz (6–9 cells, 2–4 patients/rate).
Conclusion: I_{SK} does not flow in human or rabbit atrial cardiomyocytes with $[Ca^{2+}]_i$ set within the global average diastolic-systolic range, nor during APs stimulated at physiological or supra-physiological (≤3 Hz) rates.

1. Introduction

The small conductance Ca^{2+}-activated K^+ current (I_{SK}) has been shown, in a variety of cell types and experimental conditions, to increase in amplitude upon increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), e.g. [1–7]. As such, I_{SK} has the potential to change the cardiac action potential (AP) shape in response to physiological and/or pathophysiological changes in $[Ca^{2+}]_i$. This, and reports that I_{SK} may be more prominent in atria than ventricles, e.g. [8–10], has led to the consideration of I_{SK} as a potential therapeutic target in the treatment of atrial fibrillation (AF) [11,12]. In animal models of AF, I_{SK} blockers have demonstrated efficacy in preventing new-onset AF, i.e. in physiologically normal (un-remodelled) atria [6,13–15], as well as AF resulting from atrial remodelling by chronic atrial tachypacing [6,16]; although atrial remodelling can diminish I_{SK} blocker effects [10,17]. It is important, therefore, to establish both the $[Ca^{2+}]_i$-sensitivity of atrial I_{SK} and the contribution of I_{SK} to atrial AP repolarisation, under physiological conditions, in un-remodelled atrium.

The $[Ca^{2+}]_i$ producing half-maximal activation (EC_{50}) of transfected SK channel current in inside-out patches from Xenopus oocytes or HEK-293 cells, was 310–740 nM [1–4]. In isolated myocytes, with I_{SK} measured as an apamin- or NS8595-sensitive, time-independent, inwardly-rectifying current, EC_{50} was 553 nM Ca^{2+} in rabbit ventricle [5], 250 nM in dog atrium [6], and 337 nM in human atrium [7]. In each of these studies, the desired free $[Ca^{2+}]$ was set by using high [EGTA] + variable $[CaCl_2]$; with their respective values calculated from various published constants and software programs. The resulting recording solution $[Ca^{2+}]$ or whole-cell global $[Ca^{2+}]_i$ was not then measured

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experimentally, except in one study [4], in which HEK cell inside-out patch pipette solution [Ca^{2+}] was measured (when ≥500 nM), with a Ca^{2+}-sensitive electrode; and the [Ca^{2+}] EC_{50} for I_{SK} was 610 nM.

The physiological global average $[Ca^{2+}]_i$ has been measured (with [Ca^{2+}]-sensitive dyes and fluorescence microscopy) in atrial myocytes of various species, e.g. human: diastolic range 168–325 nM and systolic 413–600 nM [7,18,19]; rabbit: diastolic 170 nM, systolic 453 nM [20] and these values, at least systolic, are within the range of I_{SK} EC_{50}s above. Furthermore, since the Ca^{2+} sensed by SK channels may be located in the sub-sarcolemmal space [21], which may reach 10–20 μM during systole [22], then it is reasonable to argue that I_{SK} should activate, to some degree, under physiological conditions and could therefore influence AP shape and duration (APD) in un-remodelled atrium. Additionally, the supra-physiological rates from AF paroxysms could elevate $[Ca^{2+}]_i$ further (as suggested by studies which increased stimulation rate to 2–3 Hz in human atrial trabeculae [23,24]) with the potential, therefore, to enhance I_{SK} blocker effects on APD.

However, reports of the contribution of I_{SK} to APD are equivocal. In a study comparing animal species and cardiac chambers, apamin had no effect on intact tissue APD in ventricle of rat, dog or human, or in atrium of rat or dog [25]. Absence of APD change [6], as well as shortening [26] or lengthening [27], by I_{SK} inhibitors was reported in dog atrial myocytes or tissues. In human atrial myocytes, APD was increased [9,10,28,29], or unaffected [26], by I_{SK} inhibitors. No rabbit atrial studies were found; a species also used for understanding AF mechanisms and their prevention [30]. The reasons for such reported disparity amongst these studies are unclear, but could include the variety of species and cardiac preparations/chambers studied, experimental conditions such as $[Ca^{2+}]_i$-buffering, stimulation rate, temperature, and also differing selectivities and/or concentrations of various I_{SK} blockers used.

The bee venom toxin, SK channel pore blocker, apamin, is the conventionally used inhibitor of I_{SK} (the apamin-sensitive K^+ current termed I_{KAS} [4,5,7]), usually at 100 nM [4,5,7,25,26,28,29]. This concentration should not affect Na^+ current (I_{Na}), transient outward K^+ current (I_{TO}), L-type Ca^{2+} current (I_{CaL}), rapid (I_{Kr}) or slow (I_{Ks}) delayed rectifier K^+ currents, or inward rectifier K^+ current (I_{K1}) [31] and will be used here for comparison, along with another SK pore blocker, ICAGEN, which showed high selectivity for I_{SK} over I_{Na}, I_{TO}, I_{Kur} (ultra-rapid delayed rectifier K^+ current), I_{CaL}, I_{CaT} (low voltage-activated Ca^{2+} current), I_{Kr}, I_{Ks}, I_{K1} and I_f (funny current) [10].

The aims of the present study, therefore, are to: 1) measure whole-cell I_{SK} in human and rabbit atrial cardiomyocytes, using the I_{SK} blockers apamin and ICAGEN, with $[Ca^{2+}]_i$ measured and set to various levels in the global average diastolic-systolic range; 2) investigate whether I_{SK} flows during the atrial AP, when sub-sarcolemmal $[Ca^{2+}]_i$ should exceed global average levels, at physiological and supra-physiological rates.

2. Methods

2.1. Patients, rabbits, ethics

Right atrial tissues were obtained from 29 adult patients who were undergoing cardiac surgery, predominantly for coronary artery bypass grafting. Procedures and experiments involving human atrial myocytes were approved by West of Scotland Research Ethics Service (REC: 17/WS/0134). Written, informed consent was obtained from all patients. The investigation conformed to the principles outlined in the Declaration of Helsinki. All patients were in sinus rhythm on the day of surgery. See Table 1 for patients' clinical characteristics and drug treatments. Rabbits (n = 37; strain: New Zealand White; supplier: Envigo UK; sex: male; age (mean ± SE [range]): 21.1 ± 0.6 [14.0–30.4] weeks; weight: 3.00 ± 0.06 [2.12–3.88] kg; feeding: ad libitum) were humanely killed by intravenous injection of anaesthetic (100 mg/kg Na^+-pentobarbital, via the left marginal ear vein) and removal of the heart, which was retrogradely perfused via the aorta before isolating cardiomyocytes. Procedures and experiments involving rabbit left atrial myocytes (UK Project Licence: 70/8835) were approved by Glasgow University Ethics Review Committee, and conformed to the guidelines from: Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Table 1
Patients' clinical characteristics.

Patient characteristic	Average, n (Total n = 29)
Age	66 ± 2 years (range 40–82), 29
Sex	86% male, 29
Cardiac rhythm on operation day	100% sinus rhythm, 29
Cardiac rhythm preceding operation day	93% sinus rhythm (7% paroxysmal AF), 29
Operation	
Coronary artery bypass graft surgery	86%, 29
Aortic valve replacement	34%, 29
Mitral valve replacement	10%, 29
Ventricular septal defect repair	3%, 29
Cardiac drugs	
β_1-blocker	79%, 28
Angiotensin-converting enzyme inhibitor	57%, 28
Angiotensin receptor blocker	11%, 28
Calcium channel blocker	32%, 28
Digoxin	4%, 28
Nicorandil	21%, 28
Nitrate	59%, 29
Statin	89%, 28
Disease	
Angina	66%, 29
History of myocardial infarction	41%, 29
History of hypertension	79%, 29
Diabetes	24%, 29
Left ventricular function	
Left ventricular ejection fraction	56 ± 3% (range 38–81), 24

2.2. Cardiomyocytes, electrophysiological & intracellular Ca^{2+} recording techniques

Human and rabbit atrial cardiomyocytes were isolated by enzymatic dissociation (Collagenase Type 1, Lorne Laboratories, Lower Earley, UK) and mechanical disaggregation [32,33], and stored (≤9 h, ~20°C) in cardioplaegic solution (mM): KOH (70), KCl (40), L-glutamic acid (50), taurine (20), KH_2PO_4 (20), $MgCl_2$ (3), glucose (10), HEPES (10), EGTA (0.5), pH 7.2.

The whole-cell-patch clamp technique (ruptured-patch mode; 2–5 MΩ microelectrodes) was used to record membrane currents, action potentials, or $[Ca^{2+}]_i$, with an AxoClamp 2B amplifier (Axon Instruments, Foster City, CA, USA) and WinWCP software (J Dempster). Cardiomyocytes were superfused at 35–37°C with a physiological salt solution containing (mM): NaCl (140), KCl (4), $CaCl_2$ (1.8), $MgCl_2$ (1), glucose (11), HEPES (10); pH 7.4. All action potentials (i.e. in Figs. 4–6), and currents in Figs. 1*A* and 3*A*, were recorded using a pipette solution containing (mM): K-aspartate (130), KCl (15), NaCl (10), $MgCl_2$ (1), HEPES (10), EGTA (0.1); pH 7.25. The resulting liquid-liquid junction potential (+9 mV; bath relative to pipette) was compensated [34]. In cells designated for fixed, buffered $[Ca^{2+}]_i$ of 100–500 nM (i.e. in Figs. 1*B* and *C*, 2, 3*B* and *C*), this pipette solution was modified by replacing EGTA with BAPTA (5 mM) and titrating with added $CaCl_2$ as required. Current-voltage relationships were measured using an

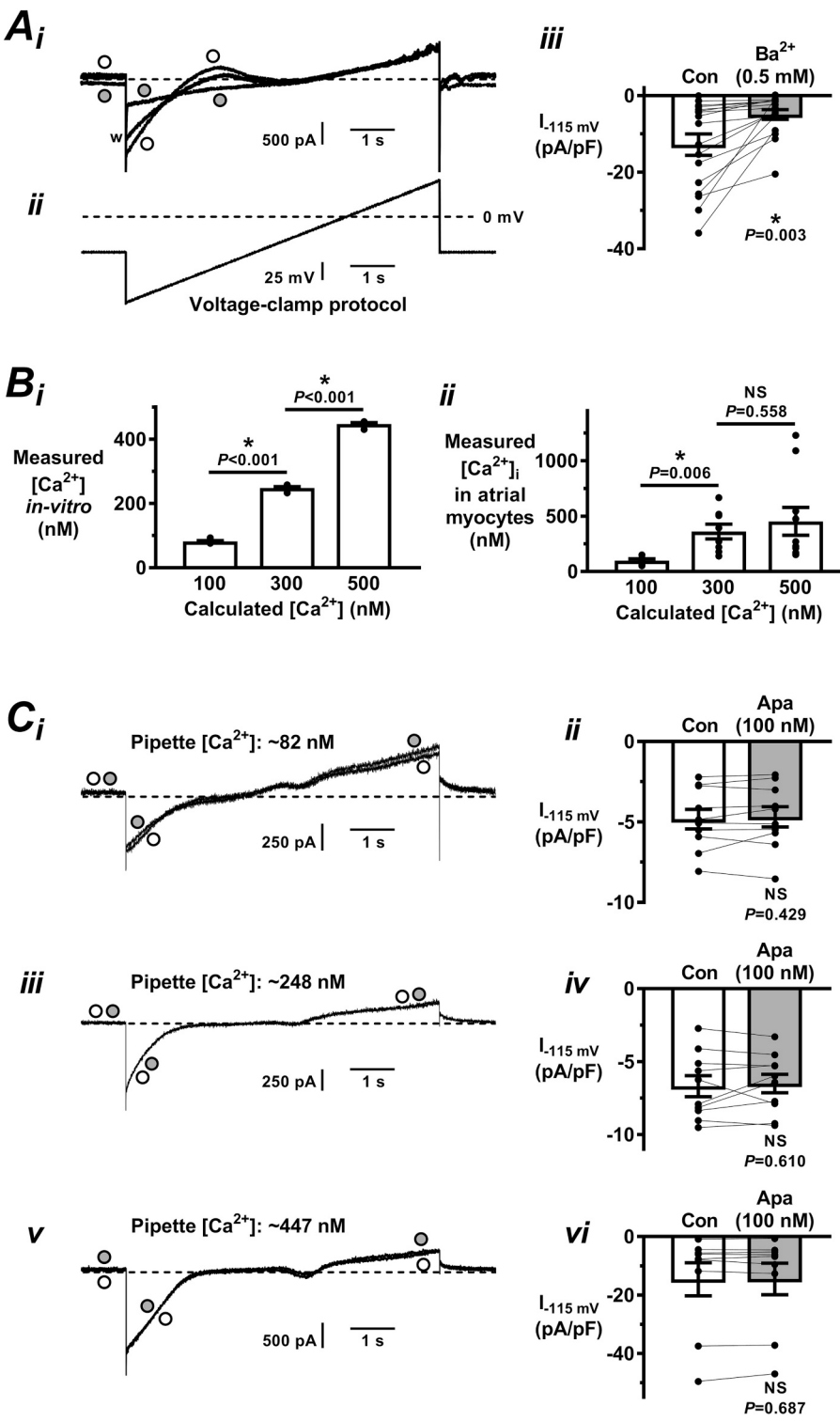

Fig. 1. Effect of K^+ channel blocker Ba^{2+}, but not I_{SK} blocker apamin (over a range of $[Ca^{2+}]_i$) on whole-cell current in rabbit atrial isolated myocytes. ***A***: +ve control: superimposed original representative quasi-steady-state current-voltage relationship traces ***(i)*** recorded from a single atrial cell with ramp voltage-clamp protocol ***(ii)*** in control (○), 50 s after starting superfusion with 0.5 mM Ba^{2+} (●), then 7 min after starting Ba^{2+} washout ("w"); ***iii***: corresponding average (mean ± SE; with individual points and connecting lines shown) current density (at −115 mV) before (□) and after (■) Ba^{2+}. * = $P < 0.05$ (paired *t*-test); $n = 17$ cells, 8 rabbits. ***B***: Comparison of average $[Ca^{2+}]$ as measured with fluorescence microscopy in-vitro ***(i)*** and in isolated myocytes ***(ii)***, against calculated (theoretical) values of 100, 300 and 500 nM, respectively. $n = 6$ samples/group in *i*; and 6 atrial cells (2 rabbits), 8c (3r), and 10c (3r)/group, respectively, in *ii*. ***C***: Absence of effect of 100 nM apamin (Apa: ● & ■) vs control (○ & □) on whole-cell currents recorded in rabbit atrial myocytes using estimated pipette $[Ca^{2+}]$ (labelled in *Ci*, *iii* & *v*; taken from *Bi*) of ~82 nM (10c, 8r), ~248 nM (10c, 5r) and ~ 447 nM (9c, 6r). NS = not significant.

ascending voltage-clamp ramp protocol (see Fig. 1*Aii*). $[Ca^{2+}]_i$ was measured using epi-fluorescence microscopy, with ratiometric quantification of $[Ca^{2+}]_i$ using Fura-2: cells were dialysed with 5 μM of the pentapotassium salt (Thermo Fisher Scientific, Inchinnan, UK) and fluorescence was measured every 15 ms using a Cairn Optoscan monochromator (Cairn Research, Faversham, UK). The relationship between $[Ca^{2+}]$ and fluorescence ratio, R (340/380 nm) was measured using a series of calibration procedures [35]. Cytoplasmic $[Ca^{2+}]$ was calculated

as K_d x S_{f2}/S_{b2} x (R-R_{min})/(R_{max}-R), where K_d is the dissociation constant for Fura-2, S_{f2} = fluorescence (upon excitation at 380 nm) of Fura-2 in its Ca^{2+}-free form and S_{b2} = fluorescence in the Ca^{2+}-bound form, R_{min} is R at nominally zero [Ca^{2+}], and R_{max} is R at saturating [Ca^{2+}]. Measured values for the quantity K_d x S_{f2}/S_{b2}, and for R_{min} and R_{max} were, respectively, 0.57 ± 0.13 μM, 0.90 ± 0.10 and 2.36 ± 0.38 (n = 6 cells, 2 rabbits for each). Action potentials were recorded by current-clamping in bridge mode, with continuous stimulation at either 1, 2 or 3 Hz frequency with current pulses of 1.5–2.0 nA amplitude, 3 ms duration, +/− a small holding current (max 1.5 pA/pF) to gain initial resting V_m of ~ −80 mV; parameters kept constant thereafter in each cell. All signals were low-pass filtered at 10 kHz and digitised (Digidata 1200 A-D converter, Axon instruments).

2.3. Drugs & reagents

All reagents for storage, pipette and superfusion solutions were supplied by Merck Life Science (Glasgow, UK), unless otherwise stated. Ba^{2+} was used at 0.5 mM, to inhibit K^+ currents including I_{TO} and I_{K1} [8,36]. 4-aminopyridine (4-AP) was used at 2 mM, to inhibit I_{TO} (IC_{50} 1.96 mM) and I_{Kur} (IC_{50} 49 μM) [37]. Apamin (Alomone Laboratories, Jerusalem, Israel), to inhibit I_{SK}, was used at 100 nM as previously [7,21,25,26,28]; ICAGEN (a gift from Acesion Pharma, Copenhagen, Denmark) was used at 1 μM to inhibit I_{SK}; i.e. a concentration ≥ 2-fold higher than IC_{50} for I_{SK} (≤0.5 μM [10]). ICAGEN was also used at 10 μM, i.e. ≥20 x IC_{50} for I_{SK} and approaching IC_{50} for I_{TO} (21 μM) and I_{Kr} (>32 μM) [10].

2.4. Data and statistical analysis

Data are expressed as means±SEM. Continuous data were compared using 2-sided, 2-sample Student's *t*-tests; paired or un-paired as appropriate. $P < 0.05$ was regarded as statistically significant. All statistical analyses were done using Graphpad Prism 7.00 or 8.00 software (Graphpad, San Diego, CA, USA).

3. Results

3.1. An I_{SK} blocker, apamin (by contrast with Ba^{2+}), had no effect on rabbit atrial whole-cell current over a range of measured [Ca^{2+}]$_i$

In rabbit atrial isolated myocytes, Ba^{2+} (0.5 mM), used as a positive control for K^+ current inhibition, markedly decreased whole cell current, as shown by the original current traces in Fig. 1*Ai* and average data in 1*Aiii*. The effect was rapid and reversible, peaking typically 30–60 s after starting Ba^{2+} superfusion. The mean current density measured at −115 mV, where current change was large but avoiding any artifact at the ramp start (−120 mV), was significantly decreased, by 62% (Fig. 1*Aiii*).

Since I_{SK} is [Ca^{2+}]$_i$-sensitive, before repeating these experiments with the conventional I_{SK} blocker apamin, pipette solutions with three different [Ca^{2+}] were prepared, anticipated to approximate 100 nM, 300 nM and 500 nM free [Ca^{2+}], i.e. substantially overlapping the global average diastolic and/or systolic [Ca^{2+}]$_i$ range. Each [Ca^{2+}] was measured optically using Fura-2, and calculated firstly by titrating against EGTA-buffered solutions, then by replacing EGTA with the faster binding buffer BAPTA as would be required in the myocytes. The resulting average free [Ca^{2+}] with BAPTA, as measured in glass tubes, was 82 nM, 248 nM and 447 nM, i.e. lower (and within 18%) than the respective anticipated values (Fig. 1*Bi*). The [Ca^{2+}]$_i$ subsequently measured (with Fura-2) in rabbit atrial isolated myocytes using these Ca^{2+}/BAPTA solutions in the patch pipettes, averaged 99 nM, 361 nM, and 453 nM, i.e. within 20% of the anticipated values (Fig. 1*Bii*).

In different groups of myocytes, using these same Ca^{2+}/BAPTA solutions and repeating the voltage clamp experiment in Fig. 1*A*, except with apamin (100 nM) instead of Ba^{2+}, it was shown (Fig. 1*C*) that apamin had no significant effect on whole-cell current at any [Ca^{2+}] tested (i.e. over the [Ca^{2+}]$_i$ range of ~100 to 450 nM), as shown by the representative traces (panels *i*, *iii* & *v*) and average data (*ii*, *iv* & *vi*).

3.2. Lack of substantial or consistent effect of the I_{SK} blocker ICAGEN on rabbit atrial whole-cell current

In rabbit atrial myocytes, the I_{SK} blocker ICAGEN, superfused at 1 μM (a concentration deemed selective for I_{SK}), under the same conditions, including the same pipette [Ca^{2+}] range, as for the above apamin experiments, had no substantial or consistent effect on whole cell current: see Fig. 2, panels (*i*) for representative traces, and panels (*ii*) for the average data. There was no significant effect of ICAGEN when using pipette [Ca^{2+}] of either ~82 nM or ~ 447 nM (Fig. 2*A* and *C*); with [Ca^{2+}] ~ 248 nM, there was a small increase in whole cell current, by 15%.

3.3. Human atrial whole-cell current: absence of effect of I_{SK} blockers

In human atrial isolated myocytes, whereas 0.5 mM Ba^{2+} (positive control) markedly and significantly decreased whole-cell current (Fig. 3*Ai* and *ii*), the I_{SK} blocker ICAGEN (1 μM) had no significant effect on whole-cell current whether using pipette [Ca^{2+}] of ~82 nM (Fig. 3*Bi* and *ii*), ~248 nM (*iii* & *iv*), or ~ 447 nM (*v* & *vi*). Apamin (100 nM) was also tested, using pipette [Ca^{2+}] ~ 248 nM, and this I_{SK} blocker also had no significant effect (Fig. 3 *Ci* and *ii*).

3.4. Effect of I_{TO}/I_{Kur} blocker 4-AP, but not I_{SK} blocker ICAGEN, on action potentials in rabbit atrial myocytes

To test whether I_{SK} flows in the rabbit atrial isolated myocytes during the action potential (AP), i.e. when local (sub-sarcolemmal) [Ca^{2+}]$_i$ is expected to exceed global average levels, drugs were superfused during the continuous recording of APs, which were stimulated at 1 Hz. First, the K^+ channel blocker 4-AP at 2 mM (inhibits I_{TO} and I_{Kur}) was tested (Fig. 4*A*). The representative superimposed AP traces in Fig 4*Ai* show that 4-AP substantially prolonged the APD, particularly around phase 1 (~APD_{20}-APD_{50}) and the AP plateau (~APD_{50}-APD_{80}). Fig 4*Aii* shows the typical time-course of this effect; with the onset of increase in APD_{30} occurring within 30 s of starting 4-AP superfusion, and the peak drug effect occurring ~30–45 s later. Corresponding average APD data (panel *iii*) confirmed a marked and significant increase by 4-AP, in APD_{30} (by 70%) and in APD_{70} (by 28%); and with no significant effect on APD_{90}.

By contrast with this positive control, ICAGEN (1 μM) did not significantly alter the AP waveform, as shown by the AP traces in Fig 4*Bi*, by the representative time course of change in APD_{30}, APD_{70} and APD_{90} in Fig 4*Bii*, and by the corresponding mean APD data in Fig 4*Biii*. The control APD may undergo a small degree of time-dependent shortening during the course of an experiment, often around the AP plateau region, e.g. APD_{70} (see Fig 5*Bii*), probably from rundown of I_{CaL}. Therefore, a separate group of contemporaneous, time-matched controls (TMC), was used (Fig. 4*C*) to compare the degree of change in APD in the presence of ICAGEN with that in its absence over a matched period, in order to avoid missing a potential small APD-lengthening effect of ICAGEN which would otherwise be obscured by such APD rundown. The TMCs showed a small degree of APD-shortening at APD_{30} and APD_{70} (Fig. 4*C*), and there was no significant difference in the mean degree of APD change at any measured level of repolarisation between the ICAGEN and TMC groups (Fig. 4*C*).

3.5. High concentration ICAGEN prolonged action potentials in rabbit atrial myocytes

The I_{SK} blocker ICAGEN was tested at high concentration (10 μM), for the dual purpose of investigating potential non-selective effects on atrial APs and, by replicating the AP recording conditions as in Fig. 4 (in

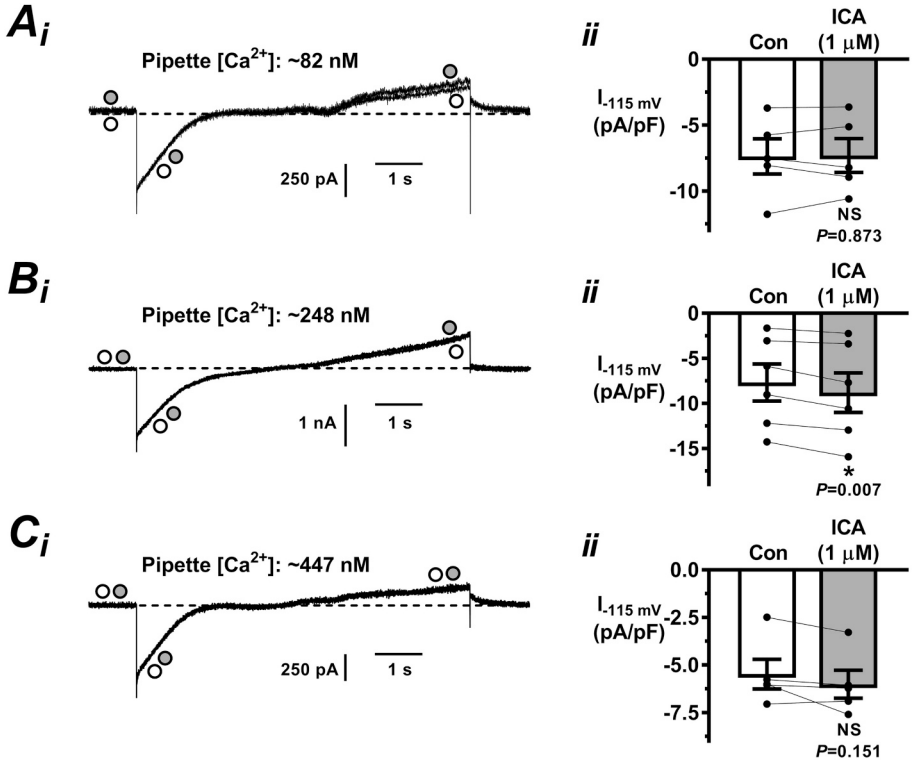

Fig. 2. Lack of substantial or consistent effect of I_{SK} blocker ICAGEN on rabbit atrial whole-cell current. ***Ai***: Representative whole-cell currents recorded from a single rabbit atrial cell, using the same voltage ramp as Fig 1*Aii*, in control (○), then with 1 μM ICAGEN (ICA; ●), using pipette $[Ca^{2+}]$ ~ 82 nM; ***ii***: corresponding mean current density before (□) and after (■) ICA (5 cells, 4 rabbits). Comparative experiments using pipette $[Ca^{2+}]$ of **(*B*)** ~ 248 nM (6c, 4r), and **(*C*)** ~ 447 nM (5c, 3r).

which 1 μM ICAGEN was tested), confirming the ability of this drug to exert any electrophysiological effects in these isolated myocytes under the same conditions; thus adding qualification of the earlier absence of effects at the lower (I_{SK}-selective) concentration. Fig. 5*A* shows the representative effect of 10 μM ICAGEN on the AP waveform: APD-prolongation around phase 2–3, i.e. ~APD_{70}-APD_{90}. The time course of this effect was moderately slower than for 4-AP, as shown for APD_{70} (Fig 5*Bi*) and APD_{90} (*Ci*). The increase in APD_{70} with ICAGEN occurred despite the corresponding continuous decrease in the TMC (Fig 5*Bii*). The mean APD_{70} and APD_{90} after ICAGEN were not significantly different from the pre-ICAGEN values (Fig. 5*D*), despite a small increase in 6 of 7 cells. However, when comparing against the TMCs, thus negating the compounding effects of APD rundown, a small but significant increase in mean APD_{70} and APD_{90} by ICAGEN was revealed, of 5 and 26 ms, respectively (Fig. 5*E*).

3.6. Absence of effect of I_{SK} blocker (1 μM ICAGEN) on human atrial action potentials, stimulated at various rates

In human atrial myocytes stimulated at physiological rate (1 Hz), 1 μM ICAGEN had no significant effect on AP configuration (Fig 6*Ai*) or duration ($APD_{30–90}$), whether measured against pre-ICA values (*Aii*) or against TMCs (*Aiii*). Supra-physiological rate stimulation, i.e. with the potential to elevate $[Ca^{2+}]_i$ and thus I_{SK}, was then used. Fig. 6*B* and *C* show that 1 μM ICAGEN had no significant effect on AP configuration or duration at either 2 Hz or 3 Hz stimulation.

4. Discussion

The study of I_{SK} blocker effects on whole-cell currents first required a positive control for verification that, under our experimental conditions and recording protocols, changes in a K^+ current of potentially small magnitude (e.g. ~1–2 pA/pF [4,5,21,29,38]) would be detectable. Our results with the K^+ channel blocker Ba^{2+} [8,36], provided such verification; with acute, marked, stable and reversible current inhibition consistently observed in both rabbit and human atrial myocytes. For the subsequent investigation of effects of apamin on whole-cell current at different $[Ca^{2+}]_i$s, we chose the Ca^{2+} buffer BAPTA (over EGTA) to set and keep constant the pipette and myocyte $[Ca^{2+}]_i$, owing to its fast binding kinetics and low pH-sensitivity [21,39]. The amount of $CaCl_2$ added to the fixed, saturating concentration (5 mM) of BAPTA to achieve the desired pipette $[Ca^{2+}]$ values (100–500 nM) was determined by first measuring the relationship between $[Ca^{2+}]$ and variable $CaCl_2$ with fixed and saturating (10 mM) EGTA, since EGTA has high and accurately known purity [40]; using the ratiometric dye Fura-2. It was important to then verify (measure) the $[Ca^{2+}]$ in the BAPTA-buffered pipette solutions, and ultimately the $[Ca^{2+}]_i$ in the isolated myocytes. We found the mean measured pipette $[Ca^{2+}]$ values to be moderately lower than the desired values, by a maximum of 18%, and with low inter-sample variation at each $[Ca^{2+}]$. Disparity between calculated and measured free $[Ca^{2+}]$ is well recognised, and can be substantial [39], and emphasises the need to verify the $[Ca^{2+}]$ by measurement. We then measured the myocyte global $[Ca^{2+}]_i$ resulting from dialysis with these solutions, in groups of rabbit atrial cells, which indicated that average $[Ca^{2+}]_i$ in the subsequent apamin experiments should range ~ 100–450 nM (i.e. within 20% of the desired values, albeit with relatively larger inter-cell variation; see Fig 1*Bii*); and close to the physiological global average diastolic-systolic range [7,18–20], and the $[Ca^{2+}]_i$ EC_{50}s for I_{SK} in isolated myocytes [5–7].

We found that apamin clearly had no effect on whole-cell current, over the voltage range tested (−120 to +60 mV), at any of the three $[Ca^{2+}]_i$ used, indicating that I_{SK} was not activated under these conditions in rabbit atrial myocytes. No previous studies of apamin on rabbit atrial whole-cell current could be found, but there are several atrial or ventricular studies in other species [4,7,9,25,29,38,41–43]. Six of those [4,7,9,29,38,43] measured an apamin-sensitive (subtraction) current, often of relatively low density (e.g. ~1–2 pA/pF [4,29,38,43]), to study effects of various interventions on this current. In the other three, a

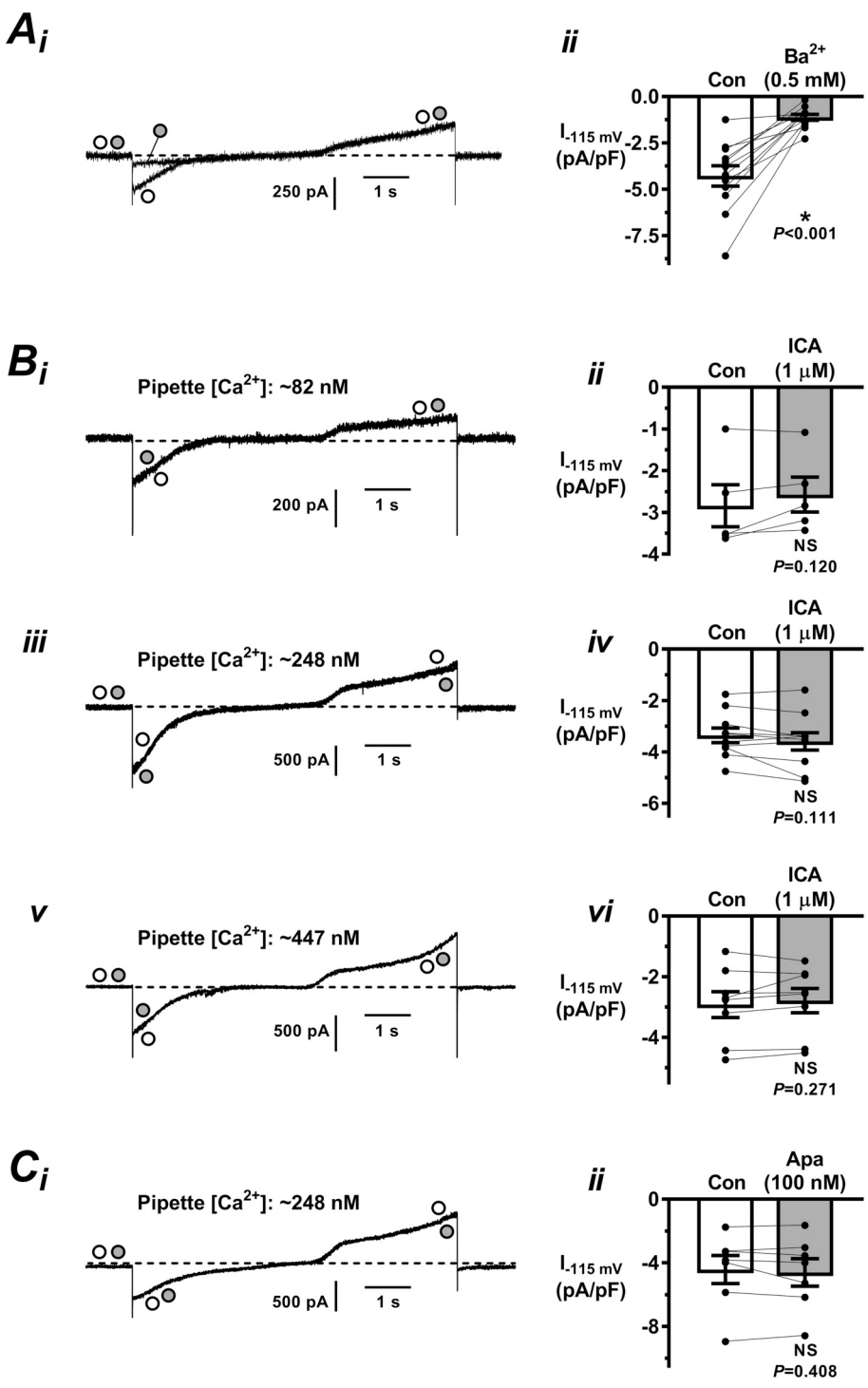

Fig. 3. Human atrial whole-cell current: absence of effect of I_{SK} blockers. ***A***: +ve control: representative whole-cell currents **(*i*)** from a single human atrial myocyte, before (○) and after (●) 0.5 mM Ba^{2+}; ***ii***: corresponding mean current density before (□) and after (■) Ba^{2+} (12 cells, 6 patients). ***B***: Absence of effect of 1 μM ICA (● & ■) vs control (○ & □) on whole-cell current, whether using pipette [Ca^{2+}] of **(*i* & *ii*)** ~ 82 nM (5c, 3p); **(*iii* & *iv*)** ~ 248 nM (10c, 4p); or **(*v* & *vi*)** ~ 447 nM (8c, 5p). ***Ci* & *ii***: Absence of effect of 100 nM apamin (Apa: ● & ■) vs control (○ & □) on whole-cell current, using pipette [Ca^{2+}] ~ 248 nM (7c, 3p).

statistical comparison of apamin vs control was provided, which showed no significant effect in rat or dog ventricular myocytes [25], and a small inhibitory effect (maximum 14%) in mouse [41] and human [42] atrial myocytes. To further investigate whole-cell I_{SK} (or its potential absence of activation) in rabbit atrial myocytes, we used the more recently developed, potent and putatively selective small molecule SK channel inhibitor ICAGEN [10]. Using the same conditions as for apamin, including pipette [Ca^{2+}]s, we found ICAGEN to have no effect on whole-

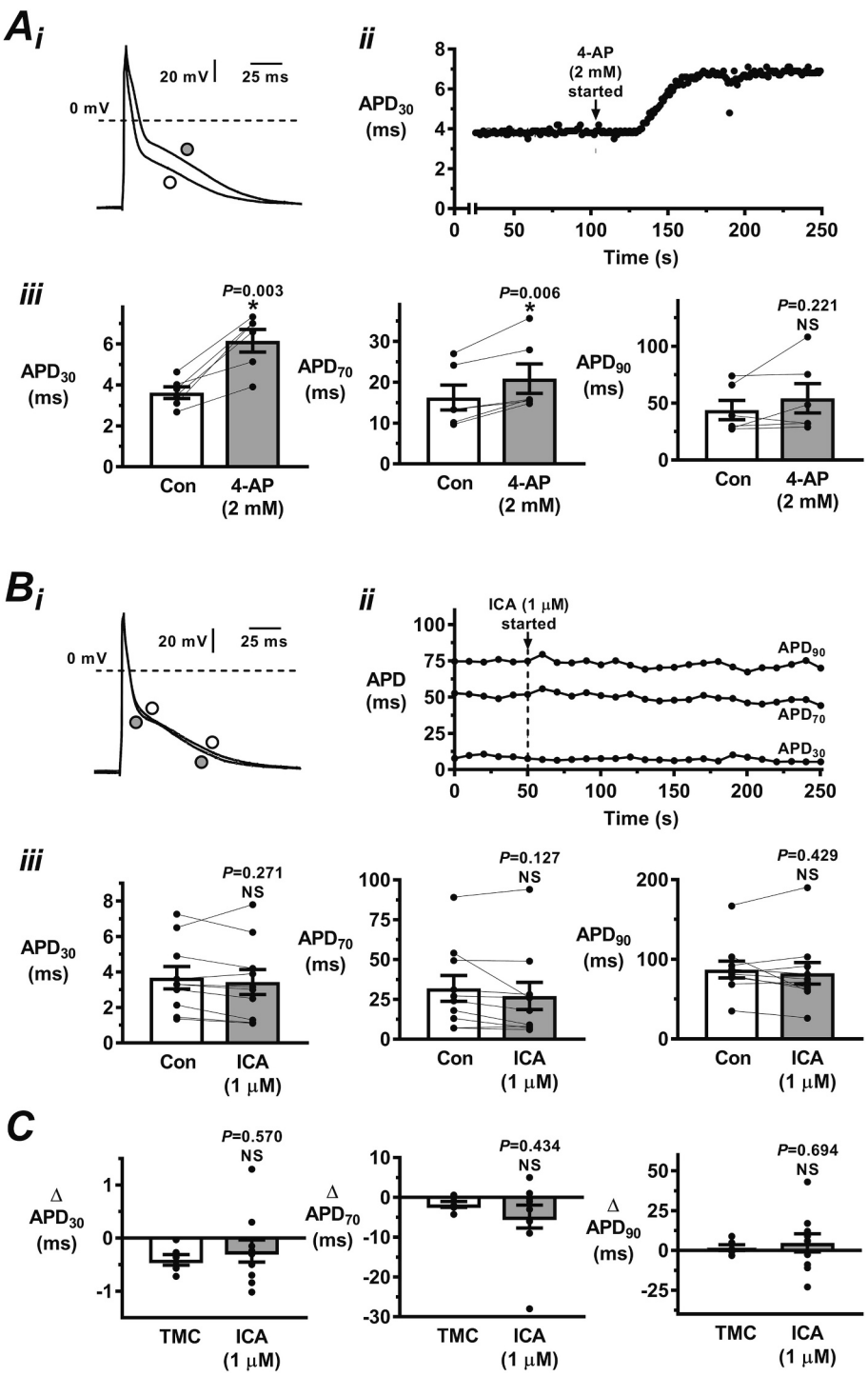

Fig. 4. Effect of I_{TO}/I_{Kur} blocker 4-AP, but not I_{SK} blocker ICAGEN, on action potentials in rabbit atrial isolated myocytes. ***A***: +ve control: ***i***: superimposed original representative action potential (AP) traces recorded (at 1 Hz stimulation) from a single atrial cell, in control (○) and after 2 mM 4-aminopyridine (4-AP; ●); ***ii***: corresponding time course of change in AP duration at 30% repolarisation (APD_{30}); ***iii***: mean APD_{30}, APD_{70} & APD_{90} before (□) and after (■) 4-AP ($n = 6$ cells, 2 rabbits). ***B***: Absence of effect of 1 μM ICA on APs: ***i***: representative traces, before (○) and after (●) ICA; ***ii***: APD time course during ICA superfusion; ***iii***: corresponding mean APDs before (□) and after (■) ICA (10c, 4r). ***C***: Mean magnitude of change (Δ) in APD_{30}, APD_{70} & APD_{90} after ICA (■; 10c, 4r) vs time-matched controls TMC (□; 6c, 3r [see Fig 5*Bii* & *Cii* for typical TMC APD time courses]); NS = non-significant (un-paired *t*-test).

cell current, at either the highest or lowest $[Ca^{2+}]_i$. At the intermediate $[Ca^{2+}]_i$, there was a statistically significant effect of ICAGEN at −115 mV, albeit it small (15%); and since it was an increase (rather than a decrease as would result from any K^+ current inhibition), does not indicate that I_{SK} was active. In a detailed ion current selectivity profiling study of ICAGEN using a heterologous expression system (HEK293 and CHO cells) [10], the IC_{50} for I_{SK2} and I_{SK3} (SK2 and SK3 being the most abundantly expressed isoforms in human atrium [10]) was 0.3 μM and 0.5 μM, respectively. The ICAGEN concentration used in the present study (1 μM) was ~2–3-fold greater, re-enforcing, along with our apamin data, the absence of I_{SK} flowing in these rabbit atrial myocytes. No reported studies of ICAGEN on currents in rabbit atrial cells could be

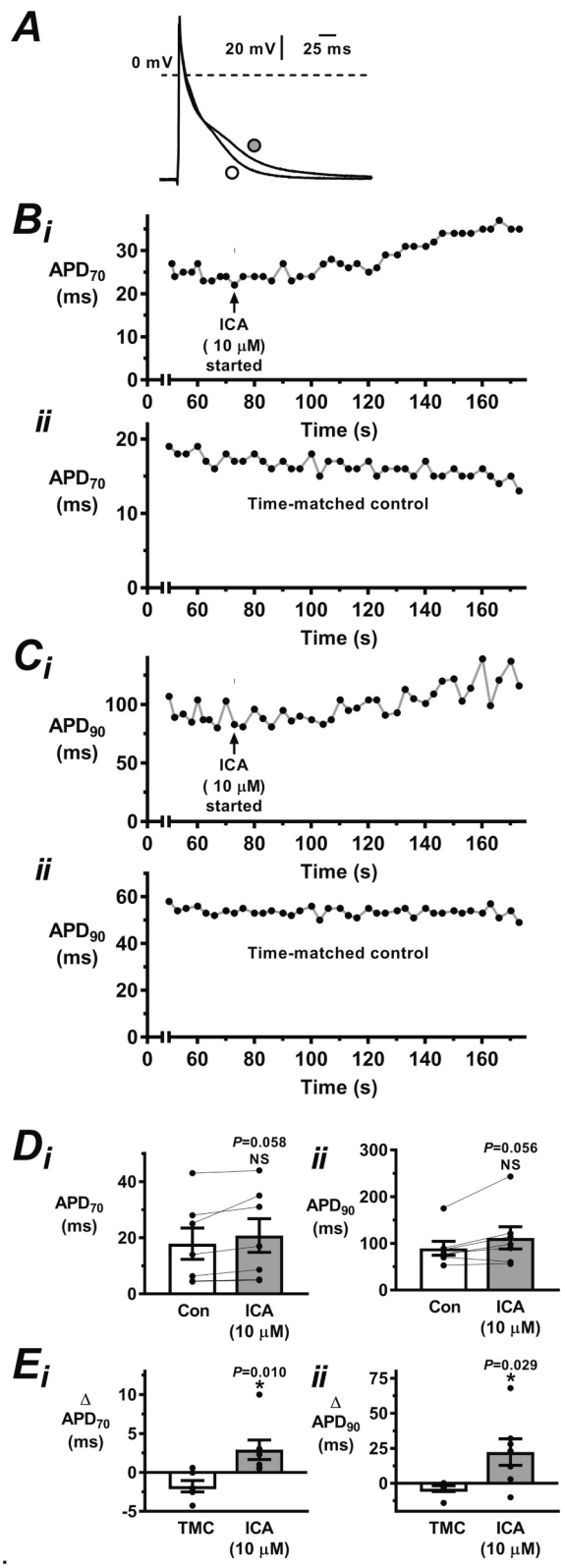

Fig. 5. High concentration ICAGEN prolongs action potentials in rabbit atrial myocytes. ***A***: Effect of 10 μM ICA (●) on AP waveform vs control (○). Corresponding time courses of change in APD_{70} ***(B)*** and APD_{90} ***(C)*** during ICA superfusion ***(i)*** and during comparative TMCs ***(ii)***. ***D***: Mean APD_{70} ***(i)*** & APD_{90} ***(ii)*** before (□) and after (■) ICA (7 cells, 3 rabbits). ***E***: Mean change (Δ) in APDs after ICA (■; 7c, 3r) vs TMCs (□; 6c, 3r). * = $P < 0.05$ (un-paired t-test).

found.

In the human atrial myocytes, we also found ICAGEN (1 μM) to have a clear absence of effect on whole-cell current, over the voltage and $[Ca^{2+}]_i$ ranges tested. In the only other study using ICAGEN in human atrial myocytes [10] (in which pipette free $[Ca^{2+}]$ was calculated as 300 nM, and high $[K^+]_o$ (20 mM) was used), a small but significant decrease in whole-cell current (by ~9%) was produced by 1 μM ICAGEN. The IC_{50} of this effect was marginally lower (at 143 nM) than in the heterologous expression system. This further supports the use of 1 μM ICAGEN for I_{SK} inhibition, and our data indicate that, as with the rabbit atrial cells, I_{SK} did not flow in the human atrial myocytes, with $[Ca^{2+}]_i$ up to ~450 nM.

During the cardiac AP, the $[Ca^{2+}]$ in the sub-sarcolemmal space may reach 10–20 μM [22], i.e. ~1–2 orders of magnitude greater than either the reported calculated $[Ca^{2+}]_i$ EC_{50}s for I_{SK} activation [1–7] or the highest measured pipette $[Ca^{2+}]$ used here. Moreover, SK channels may preferentially sense this localised Ca^{2+} source, arising both from I_{CaL} [21] and sarcoplasmic reticulum [44]. Therefore, a higher degree of I_{SK} activation (and thus responses to apamin and/or ICAGEN) might be expected during the recording of APs (with minimal $[Ca^{2+}]_i$-buffering, as used here: 0.1 mM EGTA) than of whole-cell-current with strongly buffered and constant $[Ca^{2+}]_i$. Nevertheless, we found that, by marked contrast with K^+ current inhibition using 4-AP (I_{TO}/I_{Kur} blocker) which rapidly and substantially prolonged the APs, ICAGEN at 1 μM had no significant effect on APD at either 30, 70 or 90% repolarisation in the rabbit atrial myocytes, thus indicating absence of I_{SK} activation in these cells.

We considered it prudent to check for any effects of ICAGEN at a 10-fold higher concentration, i.e. with the potential to partially inhibit I_{TO} (IC_{50}: 21 μM [10]) and possibly also I_{Kr} (IC_{50}: >32 μM [10]) and thus to prolong APD. In this case, we found a significant, albeit moderate, increase in both APD_{70} and APD_{90} by ICAGEN (10 μM) in rabbit atrial cells. Whilst we cannot exclude the possibility of a contribution from a potentially stronger I_{SK} inhibition with 10 μM ICAGEN, the more selective 1 μM is already 2–7 times higher than reported IC_{50}s [10], and since prolongation of both APD_{70} and APD_{90} results from partial I_{TO} inhibition in rabbit atrial cells under similar conditions [45], the possibility that the APD prolongation by 10 μM ICAGEN resulted from partial I_{TO} inhibition also cannot be excluded. No previous studies of effects of ICAGEN on APs in rabbit atrial myocytes or tissues could be found, but there are two studies on rat atrial tissues, in which high concentration ICAGEN was tested (10–30 μM) and found to prolong APD_{90} [46,47]; consistent with the present (10 μM ICAGEN) data. Furthermore, apamin (100 nM) has also been studied in rat atrial tissues, and had no effect on APD [25], in line with our lower concentration (1 μM) ICAGEN results.

In the human atrial myocytes, stimulated at physiological rate (1 Hz), our data indicate that I_{SK} did not flow during the AP since ICAGEN, at the I_{SK}-selective 1 μM, had no significant effect on $APD_{30–90}$. In a single previous human atrial study using ICAGEN [10], myocytes were stimulated at 0.5 Hz, and 1 μM ICAGEN caused a significant (though moderate: ~13%) increase in APD_{90}. A higher concentration (10 μM) was used in intact atrial muscle strips in the same study, which prolonged APD_{90} at 1 Hz stimulation. Other I_{SK} blockers have also been studied in human atrial myocytes, with 100 nM apamin producing either no significant effect on APD_{50} or APD_{90} (in atrial cells from patients with heart failure [26]) or an increase (of ~30% [9], ~18% [28], or ~ 11% [29]). An AP-clamp study in human atrial myocytes demonstrated a lack of apamin (100 nM)-sensitive current flowing during the course of the AP [42] whereas, in the same study, the non-peptidic SK pore blocker UCL-1684 [48] decreased outward, repolarising, current. Finally, NS8593 (a negative allosteric I_{SK} modulator) caused a moderate (~13%) increase in APD_{90} in human atrial cells (stimulated at 0.5 Hz) [10], although non-selectivity of this drug for I_{SK} was noted [10,41]. The variety of I_{SK} blockers and protocols used and AP responses observed so far in human atrial myocytes, would suggest the utility of a future study to directly compare such drugs in these cells including, along with

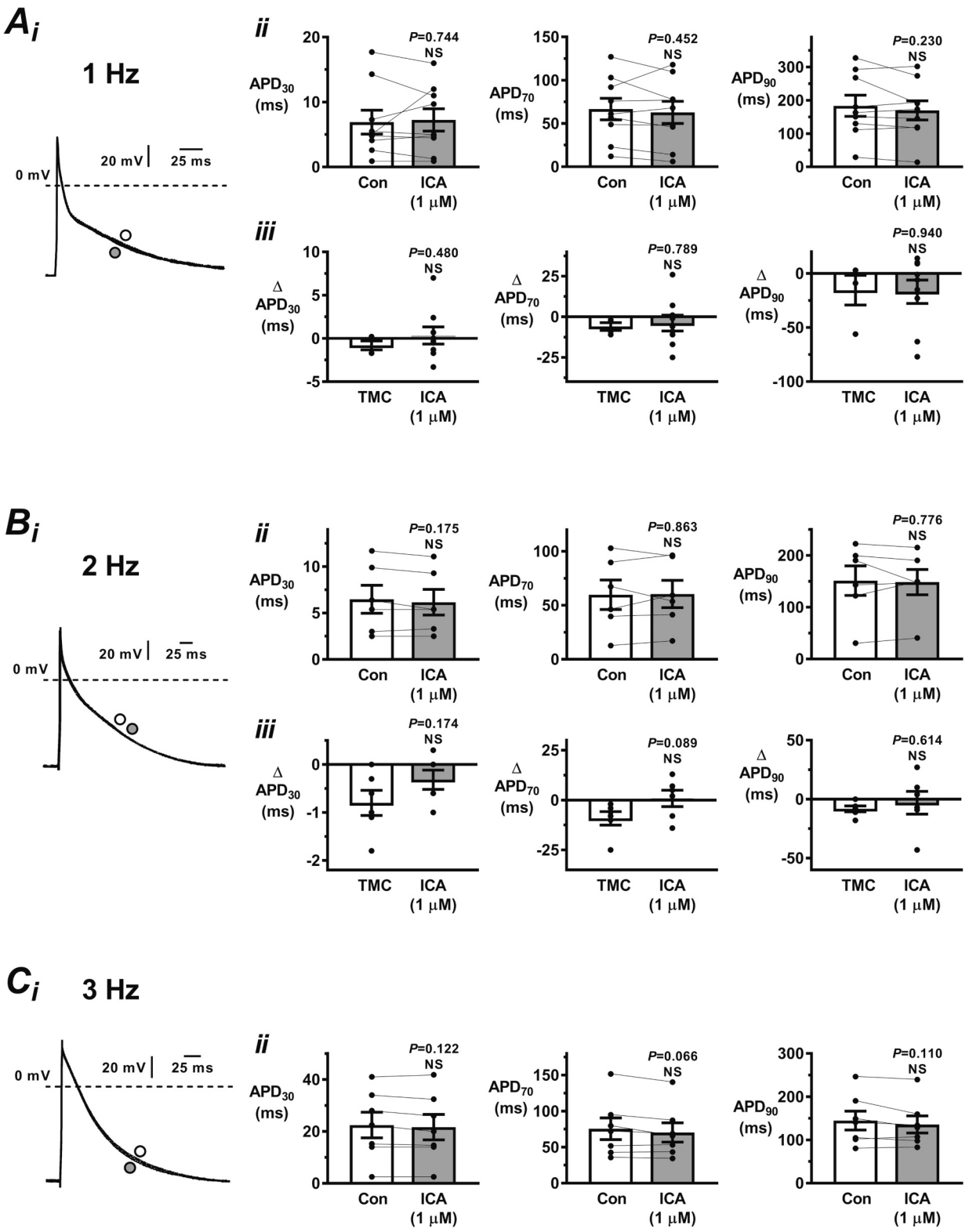

Fig. 6. Absence of effect of I_{SK} blocker (1 μM ICAGEN) on action potentials at various stimulation rates, in human atrial myocytes. APs stimulated at 1 Hz ***(A)***, 2 Hz ***(B)*** and 3 Hz ***(C)***, respectively; panels ***(i)*** show representative AP traces before (○) and after (●) 1 μM ICA, respectively; panels ***(ii)*** show mean APDs before (□) and after (■) ICA: $n = 9$ cells (4 patients), 6c (2p) and 7c (3p), respectively; panels ***(iii)*** show mean change (Δ) in APDs vs time-matched controls: TMC $n = 4$c (2p) in *Aiii*, and 6c (3p) in *Biii*.

apamin and ICAGEN, UCL-1684, the negative allosteric I_{SK} modulator AP14145 [3], and also the I_{SK} activator NS309 [49].

Increasing the AP firing rate (as would occur with new-onset, or paroxysmal, AF) has the potential to enhance I_{SK} activation (and thus APD-responses to I_{SK} inhibition), if it were to increase $[Ca^{2+}]_i$ sufficiently. In human atrial isolated trabeculae, increasing the stimulation rate from 1 Hz to 2 Hz caused an ~1.6-fold increase in systolic $[Ca^{2+}]_i$ [23]; although only diastolic $[Ca^{2+}]_i$ was increased (at 3 Hz) in another

study [24]. Our data, in human atrial isolated myocytes, indicate that increasing the stimulation rate from 1 Hz to 2 Hz or 3 Hz did not significantly activate I_{SK} during the AP, since 1 μM ICAGEN had no significant effect on $APD_{30–90}$. This is in agreement with a human atrial study using apamin [26]; which had no significant effect on APs whether stimulated at 0.5, 1 or 2 Hz. With high concentration ICAGEN (10 μM) in human atrial tissues [10], APD_{90} was increased at 1 Hz stimulation, but not at 3 Hz. Furthermore, in rat atrial tissues, 20 μM and 30 μM ICAGEN prolonged APD_{90} at 5 Hz stimulation, but the effect was not potentiated by increasing the rate to 9 Hz or 11 Hz [46]. Rate-dependent effects of I_{SK} inhibition were also investigated in dog and rat ventricular muscles (0.3–3 Hz); apamin having no effect on AP configuration at any rate [25]. In a recent study in human atrial myocytes, 5 Hz stimulation for 10 mins promoted localisation of SK2 channels to the sarcolemma, increased I_{SK} and shortened APD; effects attenuated by apamin [29].

Elevated atrial $[Ca^{2+}]_i$ can also result from acute adrenergic stimulation; shown (using isoprenaline [50,51]) to reveal I_{SK} which was absent in the un-stimulated cells. Furthermore, chronic hyper-adrenergic drive (e.g. associated with ventricular hypertrophy [50]) may enhance I_{SK}, by PKA-dependent phosphorylation and altered channel rectification [50,52]. Neither acute nor chronic adrenergic activation were studied here, but their complex signalling pathways, potentially involving a wide range of I_{SK}-associated multi-protein complexes (e.g. calmodulin and various protein kinases and phosphatases), warrant future investigation.

Taken together, the present data indicate, in line with some of the studies above as discussed, absence of I_{SK} activation in un-remodelled, non-adrenergically-stimulated, atrial myocytes, including at supra-physiological stimulation rates. However, it should be appreciated that substantial changes in I_{SK}, and in myocyte AP-responses to I_{SK} blockers, may result from pathological atrial remodelling; another aspect currently under debate. For example, chronic AF in patients has been associated with either increased [7,29,53] or decreased [10,38] atrial I_{SK} or I_{KAS}, and either potentiated [29] or attenuated [10] APD-responses to I_{SK} blockers; and chronic atrial tachy-pacing in dogs increased atrial I_{SK} and APD-responses to I_{SK} inhibition [6]. Atrial [29] or pulmonary vein [54] tachy-pacing studies have revealed the rapid onset of remodelling of I_{SK} and associated proteins, e.g. within hours [54] or even minutes [29]; and also highlight both the functional importance of SK channel location (with remodelling promoting trafficking from perinuclear regions to the plasmalemma) and the complexity of the associated signalling pathways. Remodelling from heart failure or hypertrophy features increased ventricular I_{SK} [5,50,55], whereas in atrium, both I_{SK} responses [26] and tissue expression of SK channel protein [29] were unaffected; either by heart failure or by heart failure with concurrent AF.

Study limitations: we used isolated myocytes and, whilst allowing ion current and AP data to be compared under similar experimental conditions, the possibility of damage to certain ion channels (potentially including SK) by the requisite cell isolation enzymes, cannot be excluded. However, countering that possibility, both absence [6,25] and presence [10,27] of I_{SK} blocker effects on APD were reported whether in intact tissues (not requiring enzymes) or in isolated myocytes. Also, we did not perform immuno-detection of SK channels in our myocytes. However, SK channel proteins have previously been shown, using immuno-histochemical staining techniques, to occur in the plasmalemma of human atrial myocytes, whether the cells were enzymatically-isolated [29,42] or fixed in intact tissue sections [10,29,38], as well as other cell types as above [25,50,51,54]. Finally, we used the ruptured-patch configuration, whereas several studies have used perforated-patch [9,25,26,28] which, by limiting intracellular dialysis may help retain cytoplasmic (and potentially I_{SK}-sensitive) signalling molecules. However, since both absence [25,26] and presence [9,28] of effect of I_{SK} blockers on both whole cell current and APs were reported in these perforated-patch studies, this suggests the patch configuration also should not be a confounder.

In conclusion, the present findings add weight to the argument that substantial I_{SK} does not flow in rabbit or human atrial isolated cardiomyocytes under physiological conditions.

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Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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