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Chronic atrial fibrillation up-regulates β1-Adrenoceptors affecting repolarizing currents and action potential duration

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Aims β-adrenergic stimulation has profound influence in the genesis and maintenance of atrial fibrillation (AF). However, the effects of β-Adrenoceptor stimulation on repolarizing currents and action potential (AP) characteristics in human atrial myocytes from left (LAA) and right atrial appendages (RAA) obtained from sinus rhythm (SR) and chronic atrial fibrillation (CAF) patients have not been compared yet.

Methods and results Currents and APs were recorded using whole-cell patch-clamp in RAA and LAA myocytes from SR and CAF patients. Isoproterenol concentration-dependently decreased the Ca^{2+}-independent 4-aminopyridine-sensitive component of the transient outward current (I_{to1}) and the inward rectifying current (I_{K1}). CAF significantly enhanced this inhibition, this effect being more marked in the left than in the right atria. CAF dramatically enhanced β-Adrenoceptor-mediated increase in the slow component of the delayed rectifier current (I_{Ks}), whose density was already markedly increased by CAF. Conversely, the ultrarapid component of the delayed rectifier current (I_{Kur}) of both SR and CAF myocytes was insensitive to low isoproterenol concentrations. As a consequence, stimulation of β1-Adrenoceptors in SR myocytes lengthened, whereas in CAF myocytes shortened, the AP duration. Quantitative PCR revealed that CAF up-regulated β1-Adrenoceptor expression, preferentially in the left atria.

Conclusion The present results demonstrate that CAF increases the effects of β1-Adrenoceptor stimulation on repolarizing currents by means of a chamber-specific up-regulation of the receptors. This, together with the ion channel derangements produced by CAF, could contribute to the long-term stabilization of the arrhythmia by shortening the AP duration.

Keywords Chronic atrial fibrillation • Voltage-dependent and inward rectifying potassium channels • β-Adrenoceptors • Electrical remodelling • Human myocytes • Right and left atria

1. Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and contributes to population morbidity and mortality.[1,2] Presently available therapeutic approaches have major limitations,[1,2] therefore, an improved understanding of the mechanistic insights of the disease is needed for the development of novel therapeutic approaches.

Acute and chronic atrial fibrillation (CAF) are characterized by shortening of the action potential (AP) duration (APD) and atrial refractory period (ARP), increased ARP dispersion, and loss of APD adaptation to changes in frequency.[1,2] Such alterations in atrial electrical properties (*electrical remodelling*) are caused by derangements in ion channel expression.[1–3] Importantly, it has been demonstrated that AF-induced electrical remodelling promotes AF maintenance (AF begets AF).[1–3] Indeed, the shorter the ARP the faster and

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more stable the re-entry of electrical impulses that sustain the arrhythmia. Among changes of atrial ion channels there are some that do not apparently contribute to the shortening of the ARP, for instance, the expression decrease in the channels generating the ultrarapid component of the delayed rectifier current (I_{Kur}), and the Ca^{2+}-independent 4-aminopyridine (4-AP)-sensitive component of the transient outward current (I_{to1}).[3,4] Conversely, there are other changes that seem to critically contribute to the abbreviation of APD and ARP. Such is the case of the expression decrease in the channels that generate the depolarizing L-type Ca^{2+} current (I_{CaL}).[5] Moreover, CAF increases the expression of channels that underlie the repolarizing inward rectifier (I_{K1}) and the constitutively active acetylcholine-dependent (I_{KACh}) currents.[6,7] In the same line, we recently demonstrated that the slow component of the delayed rectifier current (I_{Ks}) is markedly increased in both left (LA) and right (RA) atrial myocytes from CAF patients.[8] Since I_{Ks} contributes to AP final phase repolarization more at fast than at slow rates,[9] we proposed that I_{Ks} increase would also critically contribute to the abbreviation of APD and ARP, and, thus, to AF maintenance and recurrence.

Several data suggest that adrenergic stimulation may be involved in the initiation and/or maintenance of AF.[10] However, it is unclear how adrenergic stimulation affects the various electrophysiological mechanisms that could underlie AF. Previous data demonstrated that CAF is associated with an enhanced effect of isoproterenol to increase I_{CaL}.[5] Conversely, data on the effects of β-adrenergic stimulation on K^+ repolarizing currents and atrial AP characteristics in patients with CAF are currently unavailable. Thus, in this work we analysed the effects of β-Adrenoceptor stimulation on I_{to1}, I_{Kur}, I_{Ks}, and I_{K1} recorded in myocytes obtained from left (LAA) and right (RAA) atrial appendages from sinus rhythm (SR) and CAF patients. We also analysed the effects of isoproterenol on the characteristics of the APs recorded in SR and CAF myocytes. The results demonstrated that CAF markedly enhanced β-Adrenoceptor-mediated effects by increasing the expression of β1-Adrenoceptors. As a consequence of AF-induced changes in channels and receptors stimulation of β-Adrenoceptors in CAF myocytes significantly shortened the APD, an effect that could contribute to the perpetuation of the arrhythmia.

2. Methods

The study was approved by the Investigation Committee of the Hospital Universitario Gregorio Marañón (CNIC-13) and conformed to the principles outlined in the Declaration of Helsinki. Each patient gave written informed consent.

2.1 Electrophysiological analysis

2.1.1 Human atrial myocyte isolation

Myocytes were enzymatically isolated from RAA and LAA samples, obtained from 36 patients in SR and 32 patients with CAF that underwent cardiac surgery, following methods previously described.[8] Clinical data of patients are summarized in Supplementary material online, *Table S1*.

2.1.2 Recording of ionic currents

Currents were recorded at room temperature using the whole-cell patch-clamp configuration (micropipette resistance <3.5 MΩ). Series resistance was compensated manually and usually $\geq$80% compensation was achieved. Under our experimental conditions no significant voltage errors (<5 mV) due to series resistance were expected with the micropipettes used. Currents were filtered at half the sampling frequency.

2.1.3 Pulse protocols

The protocol to record I_{to1} and I_{sus} consisted of 250 ms pulses from -80 mV to potentials ranging -90 and $+50$ mV.[8] A 25 ms prepulse to -40 mV was applied to inactivate the inward Na^+ current (I_{Na}). I_{Ks} was recorded by applying 4 s pulses from -40 mV to potentials ranging -40 and $+60$ mV and tail currents were elicited upon repolarization to -30 mV in cells previously superfused with 2 mM 4-AP.[8] I_{K1} was recorded by applying 250 ms pulses from -120 to $+20$ mV or by applying 50 ms pulses from -80 mV to -100 mV followed by depolarizing ramps to $+20$ mV (800 ms).[8] Finally, $I_{Ca,L}$ was recorded by applying 500 ms pulses that were imposed in 5 mV increments between -40 and $+50$ mV from a holding potential of -80 mV. I_{Na} was inactivated by the application of a 50 ms prepulse to -30 mV.

2.1.4 Recording of action potentials

APs were recorded at room temperature using the current clamp configuration.[11] Tip resistance of the micropipettes used was >7 MΩ to ensure high quality gigaseals and minimal depolarization of membrane potential. APs were elicited by 2 ms depolarizing current pulses at 1.5 times the current threshold at a frequency of 1 Hz.

2.2 Mathematical model of a human atrial action potential

Effects of isoproterenol on SR human atrial APs driven at 1 Hz were simulated using the mathematical model of 'type 3' AP developed by Courtemanche *et al.*[12] by incorporating the experimentally measured isoproterenol effects on I_{to1}, I_{Ks}, and I_{CaL} (Supplementary material online). CAF-remodelled type 3 APs were obtained considering a 70% reduction in I_{CaL}, a 50% reduction in I_{to1} and I_{Kur},[12] and a 100% increase in I_{K1} and I_{Ks}. Finally, we also modelled CAF APs without considering the I_{Ks} increase.

2.3 Drugs

Drugs were dissolved as appropriate to yield 0.01 M stock solutions. Stock solutions of isoproterenol were prepared fresh daily in deionized water containing 0.04% ascorbic acid.

2.4 Analysis of the mRNA expression

Total RNA was isolated from human atrial appendages obtained from 5 SR and CAF patients, respectively (Supplementary material online, *Table S2*),[8,11] and real-time quantitative PCR (qPCR) using Taqman-based ABI gene expression assays was performed. The cycle to threshold (Ct) values corresponding to mRNA levels were normalized to 18S rRNA. To compare expression differences, the respective data were transformed from ΔCt values to equivalent fold differences as described in the Supplementary material online.

2.5 Statistical methods

Results are expressed as mean ± SEM. An unpaired *t*-test or one-way ANOVA followed by the Newman–Keuls test were used to assess statistical significance. Comparisons between categorical variables were done using Fisher's exact test. A value of $P < 0.05$ was considered significant. A multiple linear regression was used to identify putative associations between isoproterenol-induced effects and some clinical characteristics or pharmacological treatments of the patients. To make comparisons between two different concentration–response curves, an *F*-test was used. All experimental procedures are exhaustively described in the Supplementary material online.

3. Results

For electrophysiological experiments, we analysed 36 and 32 samples obtained from SR and CAF patients (Supplementary material online,

Table S1), respectively. The mean size of CAF myocytes was greater than that of SR myocytes as assessed by cell capacitance measurement (112 ± 4.7 vs. 78.9 ± 3.5 pF, $n = 234$, $P < 0.001$). In both SR and CAF patients, cell capacitance of LAA myocytes was indistinguishable from RAA myocytes (Supplementary material online, *Figure S1A*).

As previously demonstrated[8] randomized selection of healthy rod-shaped LAA and RAA myocytes obtained from SR patients resulted in three types of cells according to the voltage-dependent K^+ currents elicited at plateau potentials (Supplementary material online, *Figure S1B*). I_{to1}-predominant cells accounted for ~30% of the cells and exhibited an outward current mainly composed of a fast-activating and inactivating I_{to1}. I_{to1} was completely absent in ~30% of the cells which presented a fast-activating non-inactivating current (I_{sus} predominant). The most abundant cells were those that exhibited both I_{to1} and sustained currents (intermediate pattern). CAF modified the distribution of these cell types mainly by decreasing the percentage of I_{to1}-predominant cells in both atria (Supplementary material online, *Figure S1C*).

3.1 Effects of isoproterenol on I_{to1}

Figure 1A and *B* shows the effects of isoproterenol (1 nM), a β-Adrenoceptor agonist, on currents elicited by 250 ms pulses from −80 mV to potentials ranging from −90 to +50 mV in RAA cells with an intermediate pattern obtained from an SR and a CAF patient. At very positive potentials, an outward current was composed of I_{to1} and I_{sus}. In both SR and CAF myocytes, isoproterenol decreased peak current amplitude, whereas it did not modify the I_{sus} measured at the end of the test pulses.

Supplementary material online, *Figure S2* shows I_{to1} density–voltage relationships for LAA and RAA myocytes from SR and CAF patients in the absence and presence of isoproterenol 1 nM. I_{to1} was measured, in cells with I_{to1}-predominant and intermediate patterns, as the difference between the peak and the current at the end of the 250 ms pulse. Our results confirmed that CAF reduced I_{to1} density in both atria, the effect being significantly more marked in the LAA than in the RAA.[8] As can be observed, in SR and CAF myocytes isoproterenol significantly decreased I_{to1} density at potentials >+20 mV in both LAA and RAA cells (Supplementary material online, *Figure S2*).

We further analysed the isoproterenol-induced decrease in I_{to1} density at +30 mV, a positive membrane potential easily reached under physiological conditions (*Figure 1C*). In SR cells isoproterenol similarly decreased I_{to1} density in both atria. Compared with SR, CAF significantly enhanced the isoproterenol-induced I_{to1} inhibition, an effect that was significantly greater in the LAA than in the RAA ($n = 16$, $P < 0.05$). Importantly, isoproterenol effects were prevented in both SR and CAF cells by atenolol (1 μM), a β1-selective adrenoceptor antagonist (*Figure 1D*), suggesting that I_{to1} inhibition was mediated by β1-Adrenoceptor stimulation.

Since isoproterenol-induced I_{to1} inhibition has never been reported before, we explored the underlying signalling pathway. The results demonstrated that the isoproterenol I_{to1} inhibitory effects were abolished in the presence of either 9-cyclopentyladenine, a selective inhibitor of adenylyl cyclase (AC), or by the intracellular dialysis with PKI, a protein kinase A (PKA) inhibitor (Supplementary material online, *Figure S3*). Thus, our preliminary results suggested that the I_{to1} inhibition was mediated by the canonical signalling pathway of β1-Adrenoceptors.

The concentration that produces the half-maximum effect (EC_{50}), the maximum effect (E_{max}), and the Hill coefficient (n_H) were calculated by fitting the Hill equation to the I_{to1} inhibition produced by different isoproterenol concentrations in LAA and RAA cells from SR and CAF patients (*Figure 2A* and *B*). Comparison of the EC_{50} confirmed that in both atria isoproterenol more potently inhibited I_{to1} in CAF than in SR cells (*Figure 2C*) the effect being significantly more marked in the LAA than in the RAA. Isoproterenol produced

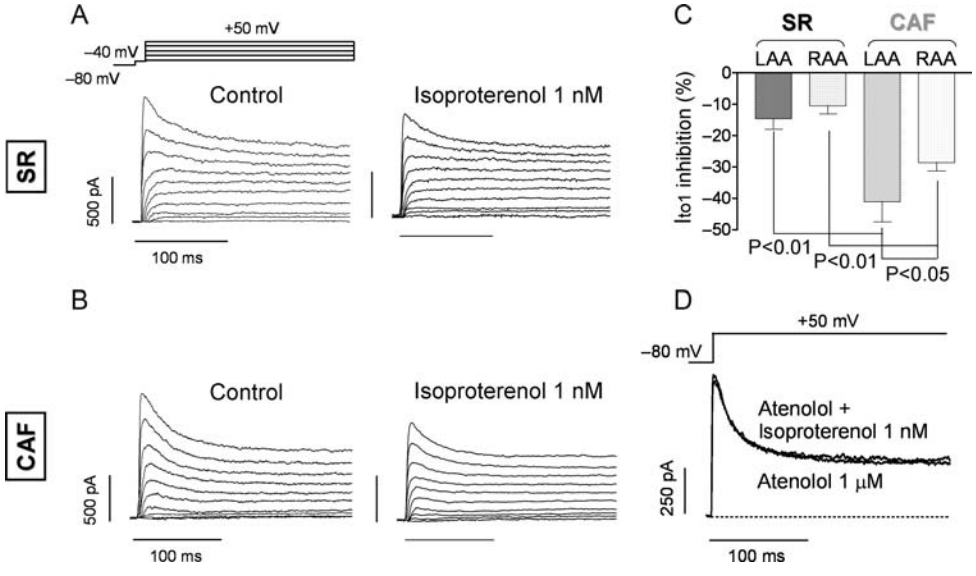

Figure 1 Effects of isoproterenol on K^+ currents elicited in two RAA cells obtained from an SR (*A*) and a CAF (*B*) patient. (*C*) Percentage of isoproterenol-induced I_{to1} inhibition at +30 mV in LAA and RAA myocytes from SR and CAF patients. Each bar represents the mean ± SEM of $n > 8$. (*D*) Effects of isoproterenol in the presence of atenolol on K^+ currents recorded in an RAA myocyte from a CAF patient.

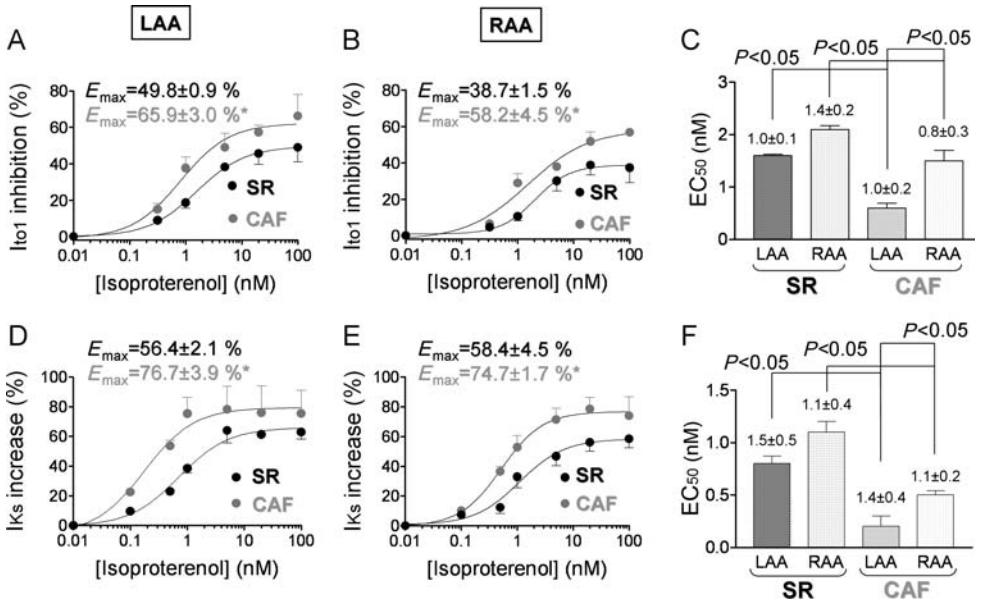

Figure 2 I_{to1} density reduction at +30 mV as a function of isoproterenol concentration in LAA (*A*) and RAA (*B*) myocytes from SR and CAF patients. Isoproterenol-induced I_{Ks} increase at +30 mV in LAA (*D*) and RAA (*E*) myocytes from SR and CAF patients. Continuous lines represent the fit of a Hill equation to the data. *$P < 0.05$ vs. SR. (*C* and *F*) EC_{50} values for the isoproterenol-induced I_{to1} inhibition (*C*) and I_{Ks} increase (*F*) in LAA and RAA myocytes from SR and CAF patients. In (*C*) and (*F*), Hill coefficients appear over the data bar. Each point/bar represents the mean ± SEM of $n > 8$.

a voltage- and time-independent I_{to1} inhibition since it did not modify the midpoint of the inactivation curve or the time constant of I_{to1} inactivation either in SR or CAF myocytes (Supplementary material online, *Figure S4* and *Table S3*).

3.2 Effects of isoproterenol on I_{sus}

Supplementary material online, *Figure S5* shows the effects of isoproterenol (1 nM) in I_{sus}-predominant RAA cells obtained from an SR and a CAF patient, respectively. Supplementary material online, *Figure S6* shows I_{sus} density–voltage relationships obtained in LAA and RAA cells from SR and CAF patients. I_{sus} amplitude was measured at the end of 250 ms pulses in intermediate pattern and I_{sus}-predominant cells. The results confirmed that I_{sus} density decreased in CAF myocytes, this effect being more marked in the RAA than in the LAA.[8] Supplementary material online, *Figures S5* and *S6* show that isoproterenol did not modify I_{sus} recorded either in SR or CAF cells at any of the voltages tested. I_{sus} is mainly (~70%) carried by the I_{Kur}[8] and treatment of I_{sus}-predominant and intermediate pattern cells with 10 mM tetraethylammonium (TEA) and 1 μM dofetilide leaves I_{Kur} unaffected.[8,13] Thus, we further analysed the effects of isoproterenol on I_{Kur} measured as the TEA + dofetilide-resistant current (Supplementary material online, *Figure S5D*). Under these conditions, isoproterenol did not modify the I_{Kur} recorded in SR and CAF myocytes.

3.3 Effects of isoproterenol on I_{Ks}

4-AP at 2 mM simultaneously blocks I_{Kur} and I_{to1}.[13] *Figure 3A* and *B* shows the effects of isoproterenol on 2 mM 4-AP-resistant currents elicited in SR and CAF myocytes from the RAA. Supplementary material online, *Figure S7* shows I_{Ks} density–voltage relationships obtained in LAA and RAA cells from SR and CAF patients in the presence and absence of isoproterenol. The 4-AP-resistant component amplitude (measured as the difference between the amplitude at the end and the beginning of the pulse) was small in SR cells, its density being similar in RAA and LAA cells (0.2 ± 0.03 pA/pF at +30 mV, $n = 16$) (Supplementary material online, *Figure S7*).[8] Importantly, in both LAA and RAA cells obtained from CAF patients I_{Ks} amplitude increased 2.5-fold compared with LAA and RAA cells from SR patients (0.5 ± 0.06 pA/pF at +30 mV, $n = 30$, $P < 0.01$ vs. SR). Here, we confirm,[8] that in both SR and CAF myocytes, the 2 mM 4-AP-resistant voltage-dependent K^+ current is I_{Ks}, since it is sensitive to TEA (10 mM) and HMR-1556 (1 μM) (Supplementary material online, *Figure S8*),[8] exhibits very slow activation kinetics (Supplementary material online, *Figure S8* and *Table S4*) and a voltage dependence also congruent with those of I_{Ks} (Supplementary material online, *Table S4*).[8] Isoproterenol significantly increased I_{Ks} density at potentials > +20 mV and > −20 mV in SR and CAF myocytes, respectively (Supplementary material online, *Figure S7*).

Figure 3C analyses the isoproterenol-induced increase in I_{Ks} density at +30 mV in SR and CAF cells. Importantly, in CAF cells I_{Ks} augmentation was significantly more marked than in SR cells and significantly greater in LAA than in RAA cells. Moreover, I_{Ks} augmenting effects produced by isoproterenol were abolished in the presence of atenolol (*Figure 3D*).

Figure 2D and *E* shows the increase in I_{Ks} density at +30 mV as a function of isoproterenol concentration. The results demonstrated that isoproterenol was significantly more potent for increasing I_{Ks} in CAF than in SR cells this effect being more marked in LAA than in RAA cells (*Figure 2F*). In CAF myocytes, effects of isoproterenol on I_{Ks} were voltage- and time-dependent, since they were accompanied by a leftward shift of the midpoint of the activation curve and an acceleration of the activation kinetics (Supplementary material online,

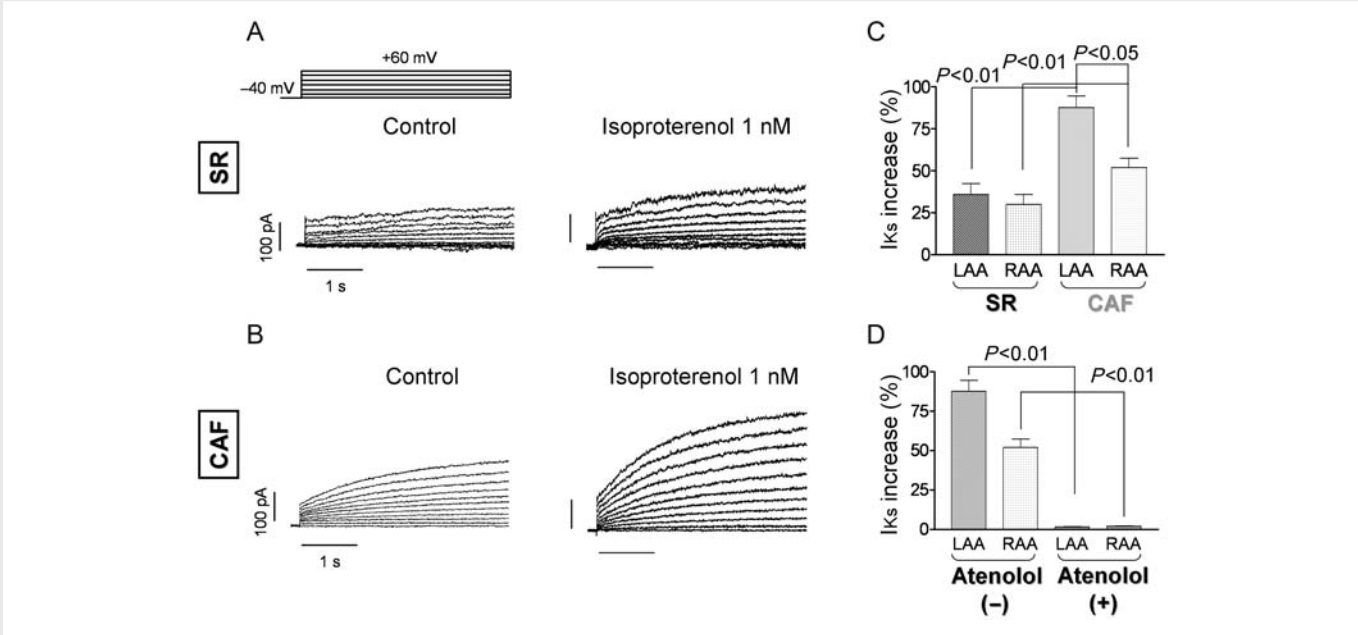

Figure 3 Effects of isoproterenol on 2 mM 4-AP-resistant K^+ currents elicited in two RAA cells obtained from an SR (*A*) and a CAF (*B*) patient. (*C*) Percentage of isoproterenol-induced I_{Ks} increase at +30 mV in LAA and RAA myocytes from SR and CAF patients. (*D*) Percentage of isoproterenol-induced I_{Ks} increase at +30 mV in LAA and RAA myocytes from CAF patients in the absence and presence of atenolol. Each bar represents the mean ± SEM of $n > 8$.

Table S4). Conversely, channel deactivation was not significantly modified (Supplementary material online, *Table S4*).

3.4 Effects of isoproterenol on I_{K1}

Figure 4A shows I_{K1} traces recorded by applying voltage-ramps in an LAA myocyte from a CAF patient in the absence and presence of isoproterenol. Inward rectifier currents were recorded in the presence of glibenclamide (10 μM) and atropine (1 μM) and were inhibited by $BaCl_2$ (100 μM), thus suggesting that the current recorded under these conditions was actually I_{K1}.[11] *Figure 4C* and *D* shows the effects of isoproterenol on I_{K1} density–voltage curves obtained in LAA and RAA cells from SR and CAF patients. I_{K1} amplitude was measured at the end of 250 ms pulses applied from −80 mV to potentials ranging from −120 to +20 mV. The results confirmed data previously reported, i.e. I_{K1} density significantly augments in CAF myocytes[7] and demonstrated that isoproterenol only inhibited I_{K1} at potentials negative to the K^+ reversal potential (E_K). The percentage of I_{K1} block at −100 mV (*Figure 4B*) and the analysis of the concentration dependence (*Figure 4E* and *F*) demonstrated that CAF significantly enhanced the isoproterenol inhibitory effects on I_{K1} in both atria.

3.5 β1-Adrenoceptor remodelling

We next investigated the expression level of AC V (ACV) and VI (ACVI), phosphodiesterase 3A (PDE3A) and 4D (PDE4D), and β1-Adrenoceptors. Ct values corresponding to β1-Adrenoceptor mRNA levels normalized to 18S rRNA (ΔCt) were significantly lower in CAF than in SR samples ($n = 20$) (*Figure 5A*). Transformation of ΔCt to fold differences demonstrated that β1-Adrenoceptor expression was 27% greater in CAF than in SR (*Figure 5B*). Conversely, no significant differences were found in mRNA levels of ACV, ACVI, PDE3A, and PDE4D between CAF and SR samples. Furthermore, the increase in β1-Adrenoceptor expression was greater in the LAA than in the RAA (*Figure 5C*).

3.6 Effects of isoproterenol on APD

We analysed the effects of β-Adrenoceptor stimulation on APs recorded in SR and CAF myocytes from the RAA. *Figure 6A* and *B* shows examples of APs recorded in RAA cells obtained from an SR and a CAF patient, respectively. Under our experimental conditions a single type of AP was recorded either in SR or CAF cells. However, as is evident in *Figure 6*, APs recorded in CAF myocytes were quite different from those recorded in SR cells since they were significantly shorter in duration.[3] This APD shortening reached statistical significance when measured at 50% (APD_{50}) and 90% (APD_{90}) of repolarization (*Figure 6C*). Moreover, resting membrane potential (RMP) was significantly more hyperpolarized in CAF (−74.8 ± 2.3 mV) than in SR myocytes (−68.3 ± 1.5 mV, $P < 0.05$). Isoproterenol 1 nM did not significantly modify AP amplitude or RMP either in SR or CAF cells. In SR cells, isoproterenol significantly lengthened the APD measured at 20% (APD_{20}) of repolarization, as well as APD_{50} and APD_{90} (*Figure 6C* and *D*). Conversely, in CAF myocytes isoproterenol significantly lengthened the APD_{20} and, more importantly, it shortened the APD_{90} (*Figure 6C* and *D*). Thus, these results suggest, for the first time, that β1-Adrenoceptor stimulation could differentially modify APD in SR and CAF myocytes, by promoting, in the latter, the shortening of the APD_{90}.

3.7 Effects of isoproterenol on I_{CaL}

Since I_{CaL} plays a key role in determining APD, we analysed the effects of isoproterenol on I_{CaL} recorded in RAA SR and CAF myocytes. Our results confirmed previous observations[5] demonstrating that CAF

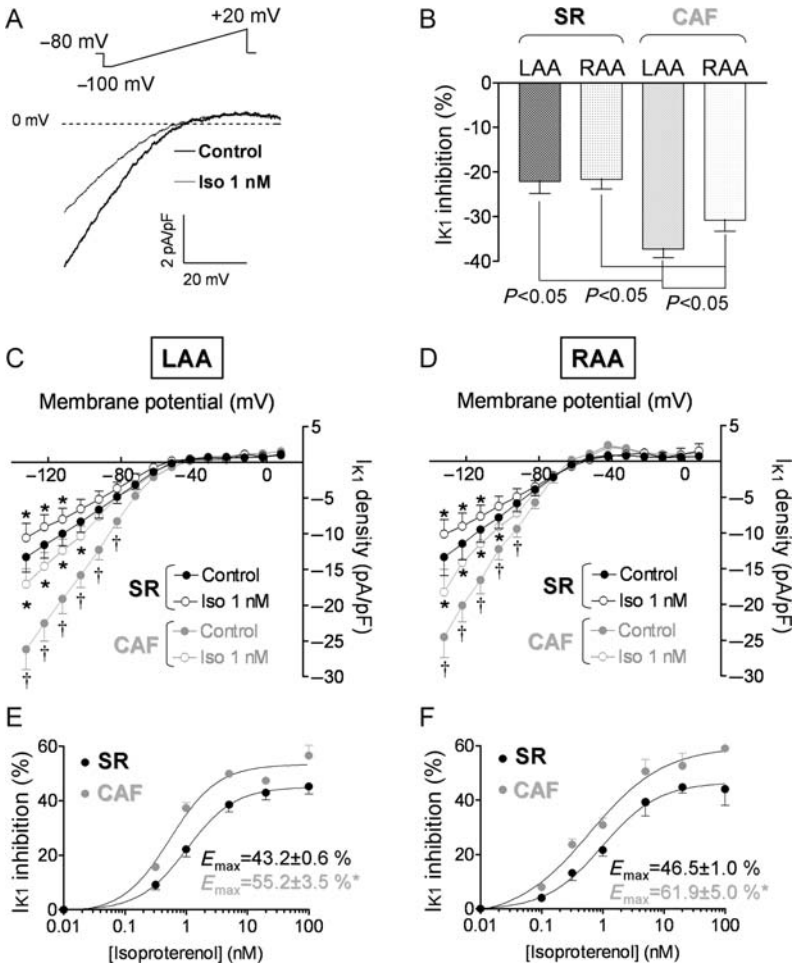

Figure 4 (*A*) Effects of isoproterenol on I_{K1} recorded by applying a voltage-ramp (800 ms) in a LAA myocyte from a CAF patient. (*B*) Isoproterenol-induced I_{K1} inhibition at −100 mV in LAA and RAA myocytes from SR and CAF patients. Effects of isoproterenol on I_{K1} density–voltage curves obtained in LAA (*C*) and RAA (*D*) myocytes from SR and CAF patients. Concentration-dependent I_{K1} inhibition produced by isoproterenol at −100 mV in LAA (*E*) and RAA (*F*) myocytes from SR and CAF patients. Continuous lines represent the fit of a Hill equation to the data. Each point/bar represents the mean ± SEM of $n > 8$. $^{*}P < 0.05$ vs. control. $^{\dagger}P < 0.05$ vs. SR.

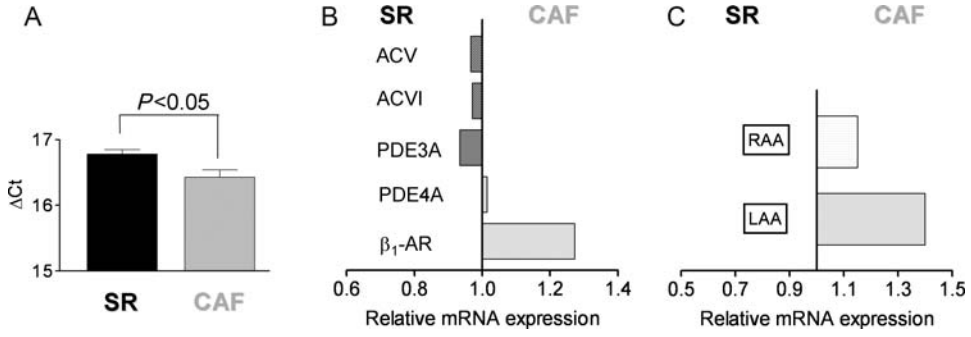

Figure 5 (*A*) ΔCt values of β1-Adrenoceptor mRNA measured by qPCR in samples obtained from SR and CAF patients (pooled data). (*B*) Relative expression level of ACV, ACVI, PDE3A, PDE4A, and β1-Adrenoceptor (β_1-AR) mRNA in SR and CAF samples. (*C*) Relative expression level of β1-adrenergic receptor mRNA in SR and CAF samples when considering LAA and RAA separately. Each bar represents the mean ± SEM of $n > 5$.

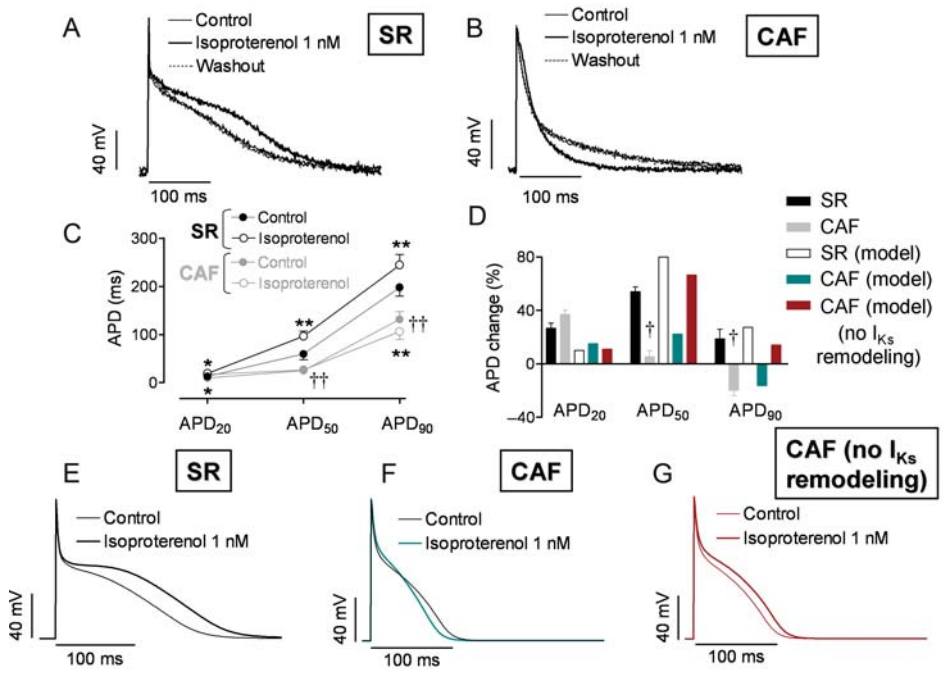

Figure 6 Representative AP traces recorded in two RAA myocytes obtained from an SR (*A*) and a CAF (*B*) patient in control conditions and in the presence of 1 nM isoproterenol. (*C*) APD measured at 20, 50, and 90% of repolarization in RAA myocytes from SR and CAF patients in the absence and presence of isoproterenol. $^{*}P < 0.05$ vs. control. $^{**}P < 0.01$ vs. control. $^{\dagger\dagger}P < 0.01$ vs. SR. (*D*) Percentage of change in the APD produced by isoproterenol in SR and CAF myocytes. $^{\dagger}P<0.05$ vs. SR. In (*C*) and (*D*), each point/bar represents the mean ± SEM of $n > 8$. Mathematically modelled steady-state APs obtained in SR (*E*) and CAF (*F*) myocytes in the absence and presence of isoproterenol at a frequency of 1 Hz. (*G*) Effects of isoproterenol on a CAF-remodelled AP simulated without considering the CAF-induced increase on I_{Ks}.

markedly decreased I_{CaL} density (from -2.1 ± 0.4 to 0.97 ± 0.2 pA/pF at +5 mV, $n = 10$, $P < 0.01$). Supplementary material online, *Figure S9A* demonstrated that 1 nM isoproterenol slightly but significantly increased I_{CaL} ($13.5 \pm 1.6\%$ at +5 mV, $n = 10$, $P < 0.05$), the EC_{50} and the E_{max} for this effect being 18.2 ± 1.9 nM and $156 \pm 3.5\%$, respectively (Supplementary material online, *Figure S9C*). CAF significantly enhanced the isoproterenol increasing effects on I_{CaL}, the EC_{50} and E_{max} for this effect being 4.8 ± 0.7 nM and $300 \pm 21.3\%$, respectively (Supplementary material online, *Figure S9C*). In fact, 1 nM isoproterenol increased I_{CaL} by $24.1 \pm 3.1\%$ (at +5 mV, $n = 10$, $P < 0.05$ vs. SR) (Supplementary material online, *Figure S9B*).

3.8 Mathematical model of SR and CAF-remodelled AP

To get further insight on the mechanism responsible for the differential effects of isoproterenol on APD in SR and CAF myocytes, we ran a mathematical model of 'type 3' human atrial AP developed by Courtemanche *et al.*[12] Among the three types described by the model we selected 'type 3' AP because its triangular shape is almost identical to that of the APs we recorded experimentally (*Figure 6E*). To simulate the effects of 1 nM isoproterenol in RAA SR myocytes, I_{to1} conductance was reduced by 10.6%, whereas I_{Ks} and I_{CaL} conductance were increased by 30.0, and 13.5%, respectively. The effects of isoproterenol on I_{K1} were not considered since they only appeared at potentials negative to the E_K. Isoproterenol produced an APD prolongation (*Figure 6E*), whose magnitude was similar to that obtained experimentally (*Figure 6D*).

To simulate 'type 3' AP under CAF conditions I_{CaL}, I_{to1}, and I_{Kur} conductances were decreased by 70, 50, and 50%, respectively, as described by Courtemanche *et al.*[12] Additionally, we included a 100% increase in I_{Ks} and I_{K1} to take into consideration CAF-induced modifications that have been described after the model had been published (*Figure 6F*).[7,8] To simulate the effects of 1 nM isoproterenol in CAF-remodelled AP, I_{to1} conductance was reduced by 28.7%, whereas I_{Ks} and I_{CaL} conductance were increased by 52.0 and 24.1%, respectively. Moreover, the effects of isoproterenol on the activation kinetics and the voltage-dependence of the activation of I_{Ks} were also considered. Under these conditions, the model reproduced the isoproterenol-induced shortening of APD_{90} (*Figure 6F*) which was of similar magnitude to that experimentally obtained (*Figure 6D*). Finally, we simulated CAF-remodelled APs by incorporating the reduction in I_{CaL}, I_{to1}, and I_{Kur} and the increase in I_{K1}, while omitting the increase in I_{Ks} (control in *Figure 6G*). Under these conditions, isoproterenol did not shorten but lengthened the APD_{90} (*Figure 6D* and *G*).

4. Discussion

The present results demonstrated that CAF upwardly regulated β1-Adrenoceptor expression, this effect being more marked in the LAA than in the RAA. As a consequence, in CAF myocytes isoproterenol-mediated I_{to1} and I_{K1} inhibition and I_{Ks} augmentation were significantly enhanced. Moreover, in SR myocytes isoproterenol lengthened whereas in CAF myocytes it further shortened APD_{90}.

4.1 Effects of isoproterenol on human atrial repolarizing currents

In SR myocytes isoproterenol inhibited I_{to1} in a concentration-dependent manner, an effect completely prevented by atenolol. Our preliminary results suggested that this β1-Adrenoceptor-mediated effect is due to the sequential stimulation of AC and PKA. There is a previous report showing that in SR myocytes I_{to1} was unaffected by 1 μM isoproterenol.[14] The reasons for this discrepancy are unknown; however, and besides the difference in the concentrations tested, we hypothesized that it could be due to the effects on membrane proteins of the enzymatic treatment used for myocyte dissociation.

We also demonstrated that β1-Adrenoceptors stimulation inhibited inward I_{K1} generated at potentials negative to the E_K, whereas it did not modify the outward component generated at potentials positive to it. This fact limits the putative physiological relevance of the effect since atrial RMP is generally more depolarized than the E_K. We further confirmed that I_{Kur} is weakly sensitive to β1-Adrenoceptor stimulation[15] since I_{Kur} was insensitive to isoproterenol concentrations <10 nM. Finally, the results indicated that human I_{Ks} is extremely sensitive to the increasing effects produced upon β1-Adrenoceptor stimulation since EC_{50} values were 0.5–1 nM. It could be argued, however, that isoproterenol increasing effects were only apparent when I_{Ks} is generated by pulses of long unphysiological duration (4 s) (*Figure 3*), while they were not present on the sustained current generated at the end of 250 ms pulses (Supplementary material online, *Figure S6*), whose duration resembles better the atrial APD. This could raise a doubt about the physiological relevance of the data here presented, particularly considering that CAF APD_{90} was ~100 ms. However, it should be stressed that I_{Ks} density, and thus its contribution to cardiac repolarization, markedly increases at increasing driving frequencies particularly following β-Adrenoceptor stimulation.[9,16] Therefore, data obtained with pulses of similar duration to that of human AP applied at very low frequencies are less representative of the I_{Ks} activated during APs driven at 1 Hz than those obtained with long pulses. To confirm this hypothesis, we ran a previously described mathematical model of I_{Ks}[16] and compared steady-state I_{Ks} amplitude reached in SR and CAF cells in the absence and presence of 1 nM isoproterenol (Supplementary material online, *Figure S10*). As can be observed, there was an increased activation of I_{Ks} during repetitive stimulation with 150 ms pulses, which suggests that I_{Ks} accumulated during brief atrial APs will contribute to AP repolarization.

Effects of isoproterenol on I_{Kur}, I_{Ks}, I_{K1}, and I_{CaL} on cardiac myocytes from different species have been attributed to the sequential activation of AC and PKA,[10,13,17,18] and thus, signalling pathways were not further studied here. In SR the effects produced by β1-adrenergic stimulation on these currents were similar in LAA and RAA myocytes. This could be explained because transcriptional levels of β1-Adrenoceptors, AC, and PDE did not exhibit differences between LAA and RAA.

In CAF myocytes, the effects produced by isoproterenol on I_{to1}, I_{Ks}, I_{K1}, and I_{CaL} were significantly greater than those produced in SR myocytes and this enhancement was more marked in the LAA than in the RAA. Quantitative analysis of the β1-Adrenoceptor mRNA level suggested that CAF produces an up-regulation of β1-Adrenoceptors which was also greater in the LA than in the RA. Conversely, mRNA levels of those AC and PDE that critically determine the intensity of the β1-Adrenoceptor stimulation in cardiac myocytes[19] remained unchanged. Thus, our results suggest that CAF-induced enhancement of β1-Adrenoceptor-mediated effects on ionic channels could be mainly attributed to a CAF-induced up-regulation of atrial β1-Adrenoceptors. However, CAF-induced changes in other proteins/enzymes also involved in β1-Adrenoceptor stimulation cannot be ruled out.

4.2 Functional impact

An important goal of this study was to compare the functional impact of β1-Adrenoceptor stimulation on the characteristics of APs recorded in SR and CAF myocytes. In fact, this was the reason why we selected a low isoproterenol concentration (1 nM), i.e. in an attempt to reproduce as much as possible the physiological setting, in which natural catecholamines reach plasma concentrations of 0.4–1.6 nM.[20] We demonstrated that in SR myocytes isoproterenol mainly prolonged the APD_{50} leaving RMP and AP amplitude unaffected. These results agree with previous reports[21] and can be interpreted in the light of isoproterenol effects on each individual current. Indeed, plateau duration is determined by I_{CaL} amplitude whose depolarizing effects are counterbalanced by the repolarizing I_{to1} and I_{Kur}.[5] Therefore, isoproterenol increasing effects on I_{CaL}, together with its inhibiting effects on I_{to1}, can account for the APD_{50} prolongation observed in SR cells. This hypothesis was confirmed by the results obtained when incorporating the experimentally observed effects of isoproterenol on a mathematical simulation of SR APs.

Interestingly, in CAF myocytes, isoproterenol prolonged APD_{20}, did not modify APD_{50}, and shortened the APD_{90}. This suggested that in CAF myocytes the role of I_{CaL} in determining the height and duration of the plateau decreases so much that the I_{to1} inhibition produced by isoproterenol resulted in a prolongation of APD_{20}. On the other hand, here we also confirmed that CAF markedly increases I_{Ks}.[8] Furthermore, CAF markedly enhanced the isoproterenol-increasing effects of I_{Ks}, which is mainly involved in terminal repolarization.[18,22] The combination of these two CAF-induced changes could account for the APD_{90} shortening produced by isoproterenol in CAF myocytes. To confirm this hypothesis, we ran the mathematical model of 'type 3' CAF-remodelled AP as described by Courtemanche *et al.*[12] However, we additionally included a 100% increase in I_{K1} and I_{Ks}. The incorporation of the isoproterenol effects in currents recorded in CAF myocytes produced an APD_{90} shortening of similar magnitude to that observed experimentally. However, this result did not discard that the decrease in I_{CaL} density itself was responsible for the observed effects. Thus, to answer this question we ran the model of a CAF-remodelled AP in which the 100% I_{Ks} increase was not included. Under these conditions, isoproterenol did not shorten but lengthened APD_{90}. Therefore, the results of the mathematical model strongly suggest that the CAF-induced increase on I_{Ks} is critical to account for the β1-Adrenoceptor-induced shortening of APD_{90} in CAF myocytes.

These are exciting results considering that the duration of terminal repolarization, which is actually shortened by CAF, is a major determinant of ARP and that it is generally accepted that shortening of ARP favours re-entry.[1,2] Importantly, the role of I_{Ks} in determining the APD rises in prominence at increasing beating frequencies.[9] Indeed, fast pacing causes channel accumulation in closed states near the open state, and adrenergic stimulation alters I_{Ks} gating to further promote this accumulation.[18,22] Therefore, it could be possible that the shortening of the APD_{90} here reported in CAF cells

driven at 1 Hz was greater in cells stimulated at high frequencies such as those reached during CAF. Moreover, there are data indicating that stable fast re-entry sources (rotors) occur with significantly higher rotation frequencies, lower conduction velocities, and shorter AP in cells with prominent I_{Ks}.[23] Finally, the frequency-dependent accumulation of I_{Ks} promotes post-repolarization refractoriness and fibrillatory conduction of waves emanating from rotors.[23] Therefore, it seems reasonable to propose that CAF-induced increase in β1-Adrenoceptor-mediated effects on I_{Ks} could contribute to the long-term stabilization of the arrhythmia by shortening the ARP. The fact that β-adrenergic stimulation of I_{Ks} could contribute to AF was previously suggested by Sampson *et al.*[24] using a transgenic I_{Ks} channel mouse model. These investigators demonstrated that *in vivo* administration of isoproterenol predisposed I_{Ks} channel transgenic mice but not wild-type littermates that lack I_{Ks} to prolonged atrial arrhythmias. Furthermore, computational modelling revealed that β-Adrenoceptor stimulation-dependent accumulation of open I_{Ks} channels accounted for the pro-arrhythmic substrate.

On the other hand, CAF also produces an heterogeneous increase in sympathetic innervation[25] which, in turn, might heterogeneously increase β1-Adrenoceptor stimulation, thus favouring repolarization heterogeneities within the atria. Additionally, up-regulation of β1-Adrenoceptors in CAF myocytes would probably imply the enhancement of most of the β1-Adrenoceptor-mediated deleterious effects including oxidative stress and fibrosis.[10,19,26] Moreover, it probably also alters intracellular Ca^{2+} concentration and handling which in turn exert pro-arrhythmic (i.e. promoting triggered focal activity), pro-apoptotic, and pro-necrotic effects.

4.3 Study limitations

All samples came from atrial appendages which could not be representative of the rest of the atria. Moreover, due to the limited availability of LA samples, effects of isoproterenol on AP were only assessed in RAA cells. However, since CAF increases I_{Ks} density similarly in both atria[8] and isoproterenol more markedly increases I_{Ks} in the LA than in the RA, it seems reasonable to assume that isoproterenol also shortens APD in the LA. Furthermore, atrial remodelling may be influenced by pharmacological treatment, gender, and/or underlying cardiac diseases of the patients.[1,2] Patients with valvular and valvular combined with ischaemic heart disease or under beta-blocker therapy were equally distributed in both groups (Supplementary material online, *Table S1*). Moreover, multiple linear regression analysis confirmed that the only variable that influences the potentiation of the β1-Adrenoceptor effects is the presence of CAF (Supplementary material online, *Table S5*). Furthermore, all experiments were carried out at room temperature, APs were recorded in isolated myocytes under current clamp conditions, and β1-Adrenoceptors were stimulated using a synthetic catecholamine.

5. Conclusions

The present results demonstrate, for the first time, that CAF increases the effects of β1-Adrenoceptor stimulation on human repolarizing currents preferentially in the LA. In CAF cells, isoproterenol further shortens the APD_{90} as a consequence of the CAF-induced up-regulation of β1-Adrenoceptors and derangements in ion channels. Therefore, it could be hypothesized that sympathetic stimulation further promotes re-entry, thus favouring AF maintenance.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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