Chronic atrial fibrillation up-regulates \( \beta_1 \)-Adrenoceptors affecting repolarizing currents and action potential duration

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Aims

\( \beta \)-adrenergic stimulation has profound influence in the genesis and maintenance of atrial fibrillation (AF). However, the effects of \( \beta \)-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 \( \beta \)-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 \( \beta_1 \)-Adrenoceptors in SR myocytes lengthened, whereas in CAF myocytes shortened, the AP duration. Quantitative PCR revealed that CAF up-regulated \( \beta_1 \)-Adrenoceptor expression, preferentially in the left atria.

Conclusion

The present results demonstrate that CAF increases the effects of \( \beta_1 \)-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 • \( \beta \)-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
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 ultra-rapid component of the delayed rectifier current (\(i_{\text{Kur}}\)), and the Ca\(^{2+}\)-independent 4-aminopyridine (4-AP)-sensitive component of the transient outward current (\(i_{\text{to1}}\)).\(^3\)\(^4\) 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 (\(i_{\text{Ca,L}}\)).\(^5\) Moreover, CAF increases the expression of channels that underlie the repolarizing inward rectifier (\(i_{\text{Ks}}\)) and the constitutively active acetylcholine-dependent (\(i_{\text{KCa}}\)) currents.\(^6\)\(^7\) In the same line, we recently demonstrated that the slow component of the delayed rectifier current (\(i_{\text{Ks}}\)) is markedly increased in both left (LA) and right (RA) atrial myocytes from CAF patients.\(^8\) Since \(i_{\text{Ks}}\) contributes to AF final phase repolarization more at fast than at slow rates,\(^9\) we proposed that \(i_{\text{Ks}}\) 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 \(i_{\text{Ca,L}}\).\(^5\) Conversely, data on the effects of \(\beta\)-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 \(\beta\)-Adrenoceptor stimulation on \(i_{\text{Kur}}, i_{\text{Ks}}\) and \(i_{\text{KCa}}\) 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 \(\beta\)-Adrenoceptor-mediated effects by increasing the expression of \(\beta_1\)-Adrenoceptors. As a consequence of AF-induced changes in channels and receptors stimulation of \(\beta\)-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 MΩ). Series resistance was compensated manually and usually ≥80% compensation was achieved. Under our experimental conditions no significant voltage errors (<0.5 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 \(i_{\text{to1}}\) and \(i_{\text{Ks}}\) consisted of 250 ms pulses from −80 mV to potentials ranging −90 and +50 mV.\(^5\) A 25 ms prepulse to −40 mV was applied to inactivate the inward Na\(^+\) current (\(i_{\text{Na}}, i_{\text{K1}}\) 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.\(^8\) \(i_{\text{K1}}\) 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, \(i_{\text{Ca,L}}\) 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. \(i_{\text{Na}}\) 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 was used >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 isoproteonol on SR human atrial APs driven at 1 Hz were simulated using the mathematical model of ‘type 3’ AP developed by Courtelmann et al.\(^12\) by incorporating the experimentally measured isoproterenol effects on \(i_{\text{to1}}, i_{\text{Ks}}\) and \(i_{\text{Ca,L}}\) (Supplementary material online). CAF-remodelled type 3 APs were obtained considering a 70% reduction in \(i_{\text{to1}}, i_{\text{Ks}}, \text{and } i_{\text{Ca,L}}\). Finally, we also modelled CAF APs without considering the \(i_{\text{Ks}}\) 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 RNA. 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,
As previously demonstrated⁸ randomized selection of healthy rod-shaped LAA and RAA myocytes obtained from SR patients resulted in the absence and presence of isoproterenol 1 nM. Three types of cells according to the voltage-dependent Kn shaped LAA and RAA myocytes obtained from SR patients resulted in the absence and presence of isoproterenol 1 nM. Figure S1A).

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

**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 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_{to1}) 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).

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_{tw}. In both SR and CAF myocytes, isoproterenol decreased peak current amplitude, whereas it did not modify the i_{tw} 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. 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).

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...
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cells at any of the voltages tested.

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. 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} and treatment of I_{sus}-predominant and intermediate pattern cells with 10 mM tetraethylammonium (TEA) and 1 μM dofetilide leaves I_{Kur} unaffected. 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_{KCl}. 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). 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, 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), 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). 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,
Table S4). Conversely, channel deactivation was not significantly modified (Supplementary material online, Table S4).

### 3.4 Effects of isoproterenol on $I_{K_1}$

Figure 4A shows $I_{K_1}$ 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 $\mu$M) and atropine (1 $\mu$M) and were inhibited by BaCl$_2$ (100 $\mu$M), thus suggesting that the current recorded under these conditions was actually $I_{K_1}$.11 Figure 4C and D shows the effects of isoproterenol on $I_{K_1}$ density—voltage curves obtained in LAA and RAA cells from SR and CAF patients. $I_{K_1}$ amplitude was measured at the end of 250 ms pulses applied from 280 mV to potentials ranging from $-120$ to $+20$ mV. The results confirmed data previously reported, i.e. $I_{K_1}$ density significantly augments in CAF myocytes7 and demonstrated that isoproterenol only inhibited $I_{K_1}$ at potentials negative to the $K^+$ reversal potential ($E_K$). The percentage of $I_{K_1}$ 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 $I_{K_1}$ 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 ($\Delta$Ct) were significantly lower in CAF than in SR samples ($n = 20$) (Figure 5A). Transformation of $\Delta$Ct 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 β1-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 ($-74.8 \pm 2.3$ mV) than in SR myocytes ($-68.3 \pm 1.5$ 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$_{20}$) and 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 $I_{Ca,L}$

Since $I_{Ca,L}$ plays a key role in determining APD, we analysed the effects of isoproterenol on $I_{Ca,L}$ recorded in RAA SR and CAF myocytes. Our results confirmed previous observations5 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. †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 (β1-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 $n > 5$. 
markedly decreased $I_{\text{cal}}$ density (from $-2.1 \pm 0.4$ to $0.97 \pm 0.2$ pA/pF at $+5$ mV, $n = 10$, $P < 0.01$). Supplementary material online, Figure S9A demonstrated that 1 nM isoproterenol slightly but significantly increased $I_{\text{cal}}$ (13.5 $\pm$ 1.6% at $+5$ mV, $n = 10$, $P < 0.05$), the $E_{50}$ and the $E_{\text{max}}$ for this effect being 18.2 $\pm$ 1.9 nM and 156 $\pm$ 3.5%, respectively (Supplementary material online, Figure S9C). CAF significantly enhanced the isoproterenol increasing effects on $I_{\text{cal}}$, the $E_{50}$ and $E_{\text{max}}$ for this effect being 4.8 $\pm$ 0.7 nM and 300 $\pm$ 21.3%, respectively (Supplementary material online, Figure S9C). In fact, 1 nM isoproterenol increased $I_{\text{cal}}$ 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, $I_{\text{to1}}$ conductance was reduced by 10.6%, whereas $I_{\text{ks}}$ and $I_{\text{cal}}$ conductance were increased by 30.0, and 13.5%, respectively. The effects of isoproterenol on $I_{\text{ks}}$ 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 $I_{\text{cal}}$, $I_{\text{to1}}$, and $I_{\text{kur}}$ conductances were decreased by 70, 50, and 50%, respectively, as described by Courtemanche et al.12 Additionally, we included a 100% increase in $I_{\text{ks}}$ and $I_{\text{to1}}$ 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, $I_{\text{to1}}$ conductance was reduced by 28.7%, whereas $I_{\text{ks}}$ and $I_{\text{cal}}$ 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 $I_{\text{ks}}$ were also considered. Under these conditions, the model reproduced the isoproterenol-induced shortening of $\text{APD}_{90}$ (Figure 6F) which was of similar magnitude to that experimentally obtained (Figure 6D).

Finally, we simulated CAF-remodelled APs by incorporating the reduction in $I_{\text{cal}}$, $I_{\text{to1}}$, and $I_{\text{kur}}$ and the increase in $I_{\text{ks}}$, while omitting the increase in $I_{\text{ks}}$ (control in Figure 6G). Under these conditions, isoproterenol did not shorten but lengthened the $\text{APD}_{90}$ (Figure 6D and G).

4. Discussion

The present results demonstrated that CAF upwardly regulated $\beta$-Adrenoceptor expression, this effect being more marked in the LAA than in the RAA. As a consequence, in CAF myocytes isoproterenol-mediated $I_{\text{to1}}$ and $I_{\text{kur}}$ inhibition and $I_{\text{ks}}$ augmentation were significantly enhanced. Moreover, in SR myocytes isoproterenol lengthened whereas in CAF myocytes it further shortened $\text{APD}_{90}$.
4.1 Effects of isoproterenol on human atrial repolarizing currents

In SR myocytes isoproterenol inhibited \( I_{lo1} \) in a concentration-dependent manner, an effect completely prevented by atenolol. Our preliminary results suggested that this \( \beta_{1}-\)Adrenoceptor-mediated effect is due to the sequential stimulation of AC and PKA. There is a previous report showing that in SR myocytes \( I_{lo1} \) was unaffected by 1 \( \mu \)M isoproterenol. 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 \( \beta_{1}-\)Adrenoceptor 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 \( \beta_{1}-\)Adrenoceptor stimulation 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 \( \beta_{1}-\)Adrenoceptor stimulation since \( E_{KS0} \) 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 APD90 was \( \sim 100 \) 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 \( \beta_{1}-\)Adrenoceptor stimulation. 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 \( I_{ks} \) 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 \( I_{kur}, I_{k1}, I_{kk}, \) and \( I_{cal} \) on cardiac myocytes from different species have been attributed to the sequential activation of AC and PKA, and thus, signalling pathways were not further studied here. In SR the effects produced by \( \beta_{1}-\)adrenergic stimulation on these currents were similar in LAA and RAA myocytes. This can be explained because transcriptional levels of \( \beta_{1}-\)Adrenoceptors, AC, and PDE did not exhibit differences between LAA and RAA.

In CAF myocytes, the effects produced by isoproterenol on \( I_{lo1}, I_{k1}, I_{k1}, \) 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 \( \beta_{1}-\)Adrenoceptor mRNA level suggested that CAF produces an up-regulation of \( \beta_{1}-\)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 \( \beta_{1}-\)Adrenoceptor stimulation in cardiac myocytes remained unchanged. Thus, our results suggest that CAF-induced enhancement of \( \beta_{1}-\)Adrenoceptor-mediated effects on ionic channels could be mainly attributed to a CAF-induced up-regulation of atrial \( \beta_{1}-\)Adrenoceptors. However, CAF-induced changes in other proteins/enzymes also involved in \( \beta_{1}-\)Adrenoceptor stimulation cannot be ruled out.

4.2 Functional impact

An important goal of this study was to compare the functional impact of \( \beta_{1}-\)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. an attempt to reproduce as much as possible the physiological setting, in which natural catecholamines reach plasma concentrations of 0.4–1.6 nM. We demonstrated that in SR myocytes isoproterenol mainly prolonged the APD50, leaving RMP and AP amplitude unaffected. These results agree with previous reports 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 \( I_{lo1} \) and \( I_{kur} \). Therefore, isoproterenol increasing effects on \( I_{cal} \), together with its inhibiting effects on \( I_{lo1} \), can account for the APD50 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 APD50 did not modify APD90, and shortened the APD90. 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 \( I_{lo1} \) inhibition produced by isoproterenol resulted in a prolongation of APD50. On the other hand, here we also confirmed that CAF markedly increases \( I_{ks} \). Furthermore, CAF markedly enhanced the isoproterenol-increasing effects of \( I_{ks} \), which is mainly involved in terminal repolarization. The combination of these two CAF-induced changes could account for the APD50 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. 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 APD50 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 APD50. Therefore, the results of the mathematical model strongly suggest that the CAF-induced increase on \( I_{ks} \) is critical to account for the \( \beta_{1}-\)Adrenoceptor-induced shortening of APD50 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 AR and that it is generally accepted that shortening of AR favours re-entry. Importantly, the role of \( I_{ks} \) in determining the APD rises in prominence at increasing beating frequencies. 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. Therefore, it could be possible that the shortening of the APD50 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 $I_{Ks}$. Finally, the frequency-dependent accumulation of $I_{Ks}$ promotes post-repolarization refractoriness and fibrillatory conduction of waves emanating from rotors. Therefore, it seems reasonable to propose that CAF-induced increase in $\beta$-Adrenoceptor-mediated effects on $I_{Ks}$ could contribute to the long-term stabilization of the arrhythmia by shortening the ARP. The fact that $\beta$-adrenergic stimulation of $I_{Ks}$ could contribute to AF was previously suggested by Sampson et al. using a transgenic $I_{Ks}$ channel transgenic mice but not wild-type littersmates that lack $I_{Ks}$ to prolonged atrial arrhythmias. Furthermore, computational modelling revealed that $\beta$-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 which, in turn, might heterogeneously increase $\beta$-Adrenoceptor stimulation, thus favouring repolarization heterogeneities within the atria. Additionally, up-regulation of $\beta_1$-Adrenoceptors in CAF myocytes would probably imply the enhancement of most of the $\beta_1$-Adrenoceptor-mediated deleterious effects including oxidative stress and fibrosis. Furthermore, computational modelling revealed that $\beta$-Adrenoceptor stimulation-dependent accumulation of open $I_{Ks}$ channels accounted for the pro-arrhythmic substrate.

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 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. 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 $\beta_1$-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 $\beta_1$-Adrenoceptors were stimulated using a synthetic catecholamine.

5. Conclusions
The present results demonstrate, for the first time, that CAF increases the effects of $\beta_1$-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 $\beta_1$-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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