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Beta-blocker treatment of patients with atrial fibrillation attenuates spontaneous calcium release-induced electrical activity

Verónica Jiménez-Sábado [a,b,1], Sergi Casabella-Ramón [b,c,d,1], Anna Llach [b], Ignasi Gich [b,e], Sandra Casellas [f], Francisco Ciruela [g,h], S.R. Wayne Chen [i], José M. Guerra [a,b,j], Antonino Ginel [f], Raúl Benítez [k], Juan Cinca [j], Carmen Tarifa [b,c,2], Leif Hove-Madsen [a,b,c,*,2]

[a] CIBER Enfermedades Cardiovasculares (CIBERCV), Instituto de Salud Carlos III, Madrid, Spain
[b] IIB Sant Pau, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
[c] Instituto de Investigaciones Biomédicas de Barcelona (IIBB-CSIC), Barcelona, Spain
[d] Department of Cell Biology, Physiology and Immunology and Neuroscience Institute, Universitat Autònoma de Barcelona, Barcelona, Spain
[e] CIBER Epidemiología y Salud Pública (CIBERESP), Instituto de Salud Carlos III, Madrid, Spain
[f] Servicio de Cirugía Cardíaca, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
[g] Pharmacology Unit, Dept. Pathology and Experimental Therapeutics, School of Medicine and Health Sciences, Institute of Neurosciences, University of Barcelona, L'Hospitalet de Llobregat, Spain
[h] Neuropharmacology and Pain Group, Neuroscience Program, Institut d'Investigació Biomèdica de Bellvitge, IDIBELL, L'Hospitalet de Llobregat, Spain
[i] Department of Physiology and Pharmacology, The Libin Cardiovascular Institute, University of Calgary, Canada
[j] Servicio de Cardiología and Univ. Autònoma de Barcelona, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
[k] Dept. d'Enginyeria de Sistemes, Automàtica i Informàtica Industrial, Univ. Politècnica de Catalunya, Barcelona, Spain

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ABSTRACT

Aims: Atrial fibrillation (AF) has been associated with excessive spontaneous calcium release, linked to cyclic AMP (cAMP)-dependent phosphorylation of calcium regulatory proteins. Because β-blockers are expected to attenuate cAMP-dependent signaling, we aimed to examine whether the treatment of patients with β-blockers affected the incidence of spontaneous calcium release events or transient inward currents (I_{TI}).

Methods: The impact of treatment with commonly used β-blockers was analyzed in human atrial myocytes from 371 patients using patch-clamp technique, confocal calcium imaging or immunofluorescent labeling. Data were analyzed using multivariate regression analysis taking into account potentially confounding effects of relevant clinical factors

Results: The L-type calcium current (I_{Ca}) density was diminished significantly in patients with chronic but not paroxysmal AF and the treatment of patients with β-blockers did not affect I_{Ca} density in any group. By contrast, the I_{TI} frequency was elevated in patients with either paroxysmal or chronic AF that did not receive treatment, and β-blocker treatment reduced the frequency to levels observed in patients without AF. Confocal calcium imaging showed that β-blocker treatment also reduced the calcium spark frequency in patients with AF to levels observed in those without AF. Furthermore, phosphorylation of the ryanodine receptor (RyR2) at Ser-2808 and phospholamban at Ser-16 was significantly lower in patients with AF that received β-blockers.

Conclusion: Together, our findings demonstrate that β-blocker treatment may be of therapeutic utility to prevent spontaneous calcium release-induced atrial electrical activity; especially in patients with a history of paroxysmal AF displaying preserved I_{Ca} density.

* Correspondence to: Cardiac Rhythm and Contraction Group, Biomedical Research Institute Barcelona, Hospital de la Santa Creu i Sant Pau, St Antoni Mª Claret 167, 08025 Barcelona, Spain.
E-mail address: leif.hove@iibb.csic.es (L. Hove-Madsen).
[1] Authors contributed equally
[2] Co-senior authors.

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1. Introduction

Atrial fibrillation (AF) has been associated with structural, molecular and electrophysiological abnormalities [1,2]. Moreover, the autonomic nervous system has been reported to play an important role in the initiation and maintenance of AF [3–5] and an increase in sympathetic nerve density has been observed in atrial samples from patients with AF [6,7]. This increase in sympathetic activity is mainly produced by the activation of G-protein coupled β-adrenergic receptors [3,8] that stimulate adenylyl cyclase, which increases cyclic AMP (cAMP) levels and activates protein kinase A (PKA) [3,8,9] that then phosphorylates calcium regulatory proteins such as phospholamban (PLB) and the cardiac ryanodine receptor (RyR2) [10,11]. Several studies have shown that hyperphosphorylation of the RyR2 increases spontaneous calcium release from the sarcoplasmic reticulum (SR) and this has been proposed to contribute to the induction of atrial arrhythmias [3,12–15].

Interestingly, β-adrenergic receptor blockers (β-blockers) are commonly used for the treatment of patients with atrial arrhythmias [8,16], although mostly intended for rate control management [8,17]. Among the different β-blockers used in clinical practice, carvedilol also modifies RyR2 gating directly [18], and some studies have shown that carvedilol is effective in suppressing spontaneous RyR2-mediated calcium waves and preventing calcium release-induced triggered ventricular arrhythmias in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) or heart failure (HF) [18,19].

These findings suggest that β-blocker treatment might prevent excessive spontaneous calcium release reported to induce afterdepolarizations in patients with AF [20]. Because studies of human atrial myocytes almost exclusively have been focusing on the effects of acute pharmacological treatments of isolated myocytes, the present study aimed to assess how β-blocker treatment of patients affects spontaneous calcium release and currents in atrial myocytes from these patients, in order to determine the net impact of this treatment on intracellular calcium homeostasis by taking into account potentially confounding effects of concurrent disease and other relevant clinical factors.

2. Methods

2.1. Study population

We analyzed 371 consecutive patients undergoing cardiac surgery in our institution. All patients gave written consent to obtain the right atrial tissue samples that would otherwise have been discarded during the surgical intervention. The study protocol was approved by the Ethics Committee at Hospital de la Santa Creu i Sant Pau (Spain). Patients were divided into four groups with or without reported episodes of AF that did or did not receive treatment with β-blockers. Clinical characteristics, echocardiographic data, and treatment of the four patient groups are summarized in Table 1.

2.2. Study protocol

Right atrial myocardial samples were collected from these patients, processed for cell isolation by enzymatic digestion as previously described [21], and subjected to one or several of the experimental protocols described below, depending on the yield of the myocyte isolation (see Supplementary Figure 1).

2.3. Patch-clamp technique

Electrophysiological recordings (using a HEKA EPC-10 amplifier) were performed using the amphotericin (250 μg/ml) perforated voltage-clamp configuration at room temperature as previously described [22]. Briefly, the L-type calcium current (I_{Ca}) was measured using a 50 ms

Table 1
Clinical characteristics of the study population. The chronic AF group includes patients with persistent and permanent AF. Categorical values are given as number of patients with the condition and % of patients in parenthesis. Continuous values are given as mean ± standard error. The statistical significance of differences among the four patient groups is given on the right for each factor and was evaluated using Chi-Square test for categorical data or using one-way ANOVA for continuous values. Smoking was divided into three groups (No-, Ex- and smokers). ACE inhibitor, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; AVR, aortic valve replacement; CABG, coronary artery bypass graft; LAD index, left atrial diameter index; LVEF, left ventricular ejection fraction. *Factors included as confounders in the linear regression analysis.

	NoAF, Untreated (n = 170)	NoAF, β-blockers (n = 97)	AF, Untreated (n = 60)	AF, β-blockers (n = 44)	p-value
Chronic AF	-	-	36	31	
Paroxysmal AF	-	-	24	13	
***Age, years**	65.36 ± 1.02	66.94 ± 1.07	69.87 ± 1.44	67.82 ± 1.31	0.078
***Sex**					< 0.05
Female	60 (35.3%)	24 (24.7%)	28 (46.7%)	21 (47.7%)	
Male	110 (64.7%)	73 (75.3%)	32 (53.3%)	23 (52.3%)	
Echocardiographic characteristics					
LAD index	2.30 ± 0.04	2.34 ± 0.05	2.98 ± 0.09	2.66 ± 0.09	< 0.001
LVEF, %	58.14 ± 1.08	56.33 ± 1.46	60.34 ± 1.84	58.95 ± 2.04	0.361
Cardiovascular risk factors					
Smoking	24 (14.1%)	14 (14.4%)	3 (5.0%)	8 (18.2%)	0.052
Ex-smoking	60 (35.3%)	41 (42.3%)	21 (35.0%)	8 (18.2%)	
***Arterial hypertension**	95 (55.9%)	63 (64.9%)	35 (58.3%)	26 (59.1%)	0.549
***Diabetes**	39 (22.9%)	41 (42.3%)	8 (13.3%)	14 (31.8%)	< 0.001
Dyslipidemia	92 (54.1%)	48 (49.5%)	22 (36.7%)	22 (50.0%)	0.144
Surgical treatment					
***AVR**	105 (61.8%)	32 (33%)	40 (66.7%)	20 (45.5%)	< 0.001
***MVR**	20 (11.8%)	9 (9.3%)	31 (51.7%)	19 (43.2%)	< 0.001
CABG	88 (51.8%)	71 (73.2%)	16 (26.7%)	18 (40.9%)	< 0.001
Pharmacological treatment					
***ACE-inhibitor**	50 (29.4%)	36 (37.1%)	29 (48.3%)	16 (36.4%)	0.066
***ARB**	28 (16.5%)	15 (15.5%)	9 (15.0%)	4 (9.1%)	0.682
Calcium antagonists	35 (20.6%)	10 (10.3%)	7 (11.7%)	4 (9.1%)	0.057
***Dicoumarin**	7 (4.1%)	4 (4.1%)	33 (55.0%)	31 (70.5%)	< 0.001
Digoxin	2 (1.2%)	1 (1.0%)	28 (46.7%)	9 (20.5%)	< 0.001
Acetyl salicylic acid	66 (38.8%)	59 (60.8%)	9 (15.0%)	9 (20.5%)	< 0.001
Statins	82 (48.2%)	71 (73.2%)	20 (33.3%)	19 (43.2%)	< 0.001

prepulse from − 80 to − 45 mV (to inactivate I_{Na}), followed by a 200 ms depolarization to 0 mV. Transient inward currents (I_{TI}) activated by spontaneous calcium waves were recorded at − 80 mV. The caffeine releasable SR calcium content was estimated from the time-integral of the transient inward current elicited by rapid exposure to 10 mM caffeine at rest. The current-voltage relationship for the L-type calcium current (I_{Ca}) was obtained using test potentials between - 40 and + 50 mV.

2.4. Confocal calcium imaging

Myocytes were loaded with 2 μM CAL-520 AM for 20 min at room temperature, followed by wash and de-esterification for at least 30 min. Confocal images (512 ×140 pixels) were recorded at a sampling frequency of 90 Hz, using a resonance-scanning confocal microscope (Leica SP5 AOBS, Wetzlar, Germany) with a 63x glycerol-immersion objective. CAL-520 was excited at 488 nm and fluorescence emission was measured between 500 and 650 nm with a Leica Hybrid Detector. Laser power was set to 20% of 100 mW and attenuated to 4%. Calcium sparks were recorded at room temperature and detected using custom-made algorithms developed with MATLAB (Mathworks Inc., Boston, MA) as previously described [23].

2.5. Immunofluorescent labeling

Human atrial myocytes were plated onto laminin coated FluoroDish™ (World Precision Instruments) and fixed with 5% paraformaldehyde for 10 min at room temperature. Subsequently, cells were first incubated with PBS / Glycine 0.1 M during 10 min and then with PBS / 0.2% Triton X-100 for 15 min to permeabilize the cells. To block the non-specific sites, the cells were incubated with PBS / 0.2% Tween 20, and 10% Horse serum, for at least 30 min. Total and Ser-2808 phosphorylated RyR2 were labeled using the primary antibodies mouse anti-RyR2 (1:1200; C3–33 NR07, Calbiochem) and rabbit anti-Ser-2808 P (1:1200; A010–30, Badrilla). Antibodies AlexaFluor 488 anti-mouse (1:2000; A21200, Molecular Probes) and AlexaFluor 594 anti-rabbit (1:1500; A11012, Molecular Probes) were used to stain total RyR2 green, and Ser-2808 phosphorylated RyR2 red. Images were acquired with a confocal microscope (Leica AOBS SP5, Wetzlar, Germany) and a 63x glycerol immersion objective.

2.6. Western blot

Right atrial samples were pulverized in liquid nitrogen and homogenized in ice-cold lysis buffer containing (in mM): 50 HEPES, 100 NaCl, 2.5 EGTA, 10 glycerol-2-phosphate 1 DTT, protease inhibitors (Roche), 0.1% (v/v) Tween 20%, and 10% (v/V) glycerol at pH= 7.4. Proteins were separated by SDS-PAGE (10% acrylamide:bisacrylamide) and electrotransferred onto Immobilon polyvinylidene diflouride membranes (Millipore). Membranes were incubated with primary antibodies against total phospholamban (PLB) (A010–14, Badrilla), PLB phosphorylation at ser-16 (A010–12AP, Badrilla) and PLB phosphorylation at thr-17 (A010–13AP, Badrilla). Detection was performed with horseradish peroxidase-labeled IgG and SupersignalTM detection system (Supersignal West DuraTM, Pierce). Molecular-mass standards (Bioline) were used to estimate protein size and alpha-actinin was used as a loading control. Immunoblots were digitized (GS-800 Calibrated Densitometer; Bio-Rad) and analyzed with the Quantity One 4.6.3 software (Bio-Rad).

2.7. Data analysis

Data collection and analysis. Analyses of biological experiments were performed without knowledge about clinical data and, unless otherwise stated, values for quantitative variables were averaged for each patient and given as mean ± standard error of the mean (SEM). Medical records were analyzed by clinicians that had no knowledge about the experimental results.

Statistical analysis. To assess the influence of clinical factors on calcium currents, patch-clamp recordings were performed in human atrial myocytes from 371 consecutive patients and data are represented with violin plots to reflect the distribution of the experimental data. For calcium spark analysis, patients without AF but with diabetes and/or mitral valve disease were excluded from the study population in order to 1) avoid confounding effects of these factors and 2) limit the size of the two groups without AF and avoid excessive weight of these groups in the statistical analysis of calcium spark frequency and properties. For this data and subsequent analyses, results are represented in bar graphs with individual data points. Statistical significance for categorical data was evaluated using Chi-Square test. For normally distributed quantitative variables, statistical significance was evaluated using analysis of variance (ANOVA) or a multiple linear regression model taking into account confounding factors indicated in Table 1 with an asterisk. For variables with clear asymmetry, statistical significance was evaluated using a Kruskal-Wallis test, ANOVA test with Welch correction or a general linear model with negative binomial distribution, taking into account confounding factors indicated in Table 1 with an asterisk. The specific statistical test is indicated in text or figure legends. Analyses were performed using IBM SPSS Statistics for Windows (V26.0) or R Studio 1.4.1717.

3. Results

3.1. β-blocker treatment does not modify the L-type calcium current

Classification of myocytes according to the atrial rhythm and the treatment with β-blockers revealed that in patients with chronic AF (long-standing persistent or permanent AF) the I_{Ca} amplitude was significantly smaller (Fig. 1A-B) and decayed more slowly (Fig. 1C). The treatment with β-blockers did not modify the amplitude or decay of I_{Ca} in patients with or without AF. Statistical analysis of the data using a linear regression model, taking into account potentially confounding effects of multiple clinical factors showed that only age ($p = 0.003$) affected the L-type calcium current amplitude. Fig. 1D shows the influence of the confounding factors on the I_{Ca} amplitude. Furthermore, β-blocker treatment did not affect the shape of the current-voltage relationship (Fig. 1E) or the voltage-dependent inactivation of I_{Ca} (Supplementary Figure 2). Patients with paroxysmal AF were not included in the I_{Ca} analysis because comparison of these patients and those with chronic AF revealed that only chronic AF affected the I_{Ca} amplitude.

3.2. β-blocker treatment reduces the incidence of I_{TI}s in atrial fibrillation

β-blocker treatment did not affect the I_{TI} frequency in patients without AF. However, the high incidence of I_{TI}s in patients with AF was significantly reduced in those receiving β-blocker treatment, to levels observed in patients without the arrhythmia (Fig. 2A-B). A linear regression analysis taking into account potentially confounding effects of multiple clinical factors and assuming a negative binomial distribution of the I_{TI} frequency, confirmed the capacity of β-blocker treatment to independently restore the elevated I_{TI} frequency observed in AF patients ($p < 0.001$) to that observed in those without AF ($p < 0.001$). Fig. 2C summarizes the influence on the I_{TI} frequency of the clinical factors included as confounders in the linear regression model. Supplementary Figure 3 shows that the I_{TI} amplitude was not modified by β-blocker treatment, suggesting that the treatment does not modify the spontaneously released calcium that is extruded by electrogenic Na^+-Ca^{2+} exchange (NCX) during the I_{TI}. Moreover, we found no significant effect of β-blocker treatment on the caffeine releasable SR calcium content (Fig. 2D-E). Fig. 2F summarizes the influence on the SR calcium load of clinical factors included as confounders in the linear regression

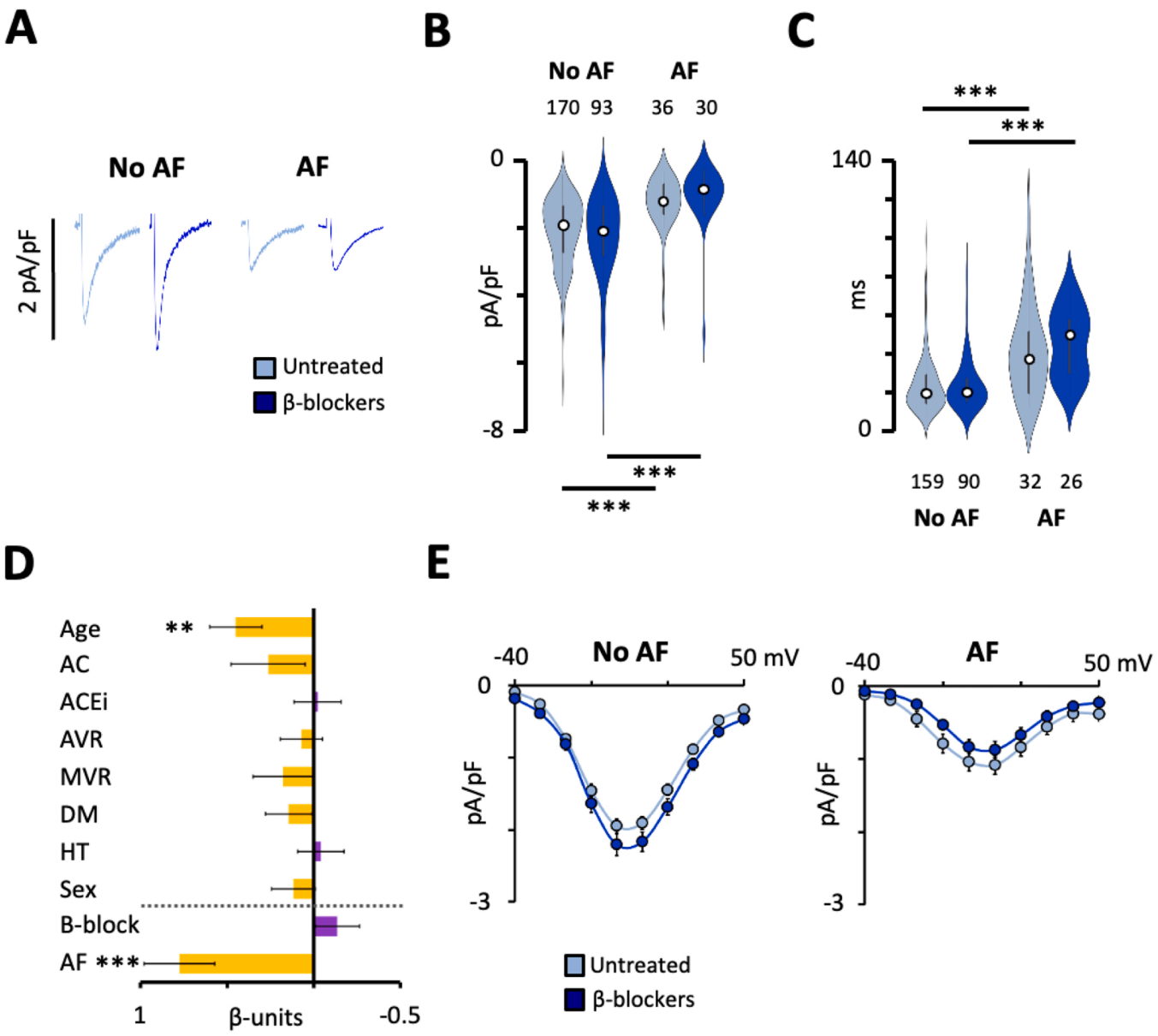

Fig. 1. Effect of β-blocker treatment on L-type calcium current. A. Representative I_{Ca} recordings from patients without AF (left traces) and patients with AF (right traces), untreated (light blue traces) and treated with β-blockers (dark blue traces). B. I_{Ca} densities in the four patient groups. C. Time constants for fast I_{Ca} inactivation. Statistical significance in B-C was determined using a linear regression model. D. Beta-values for the impact of confounding factors on the I_{Ca} density. The effects of β-block and AF are shown for comparison. Pathological effects are yellow and protective effects purple. AC, anticoagulants; ACEi, angiotensin converting enzyme inhibitor; AVR, aortic valve replacement; MVR, mitral valve replacement; DM, diabetes mellitus; HT, hypertension; AF, atrial fibrillation. E. Current-voltage relationship for myocytes from panel B. Statistically significant effects are indicated with **: $p < 0.01$; *** : $p < 0.001$. Number of patients is given above or below each group.

model.

Comparison of patients with paroxysmal and chronic AF showed that the I_{TI} frequency was similar for the two types of AF and that β-blocker treatment reduced the I_{TI} frequency to levels observed in patients without AF (Fig. 3A). Interestingly, the opposite was true for the I_{Ca} density, which was unaffected by β-blocker treatment but significantly higher in the patients with paroxysmal than permanent AF (Fig. 3B). Furthermore, division of the electrophysiological experiments performed in patients with AF, according to the type of β-blocker they were being treated with, showed that treatment with the most commonly used β-blockers reduced the I_{TI} frequency ($p = 0.04$), with significant effects in those treated with bisoprolol or carvedilol (Fig. 3C-D).

3.3. *β-blocker treatment attenuates spontaneous calcium release in atrial fibrillation*

Confocal calcium imaging analysis showed that β-blocker treatment also reduced the higher incidence of calcium sparks in patients with AF (Fig. 4A-B). Linear regression analysis confirmed a significant interaction between spark frequency and β-blocker treatment ($p = 0.001$) or atrial rhythm combined with β-blocker treatment ($p = 0.003$). Analysis of individual spark sites revealed that the reduction of spark density was due to a reduction of the number of sparks per site rather than the density of spark sites (Fig. 4C-D). Thus, linear regression analysis showed no significant interaction between spark site density and β-blocker treatment ($p = 0.97$) or atrial rhythm combined with β-blocker treatment ($p = 0.53$) while there was a significant interaction

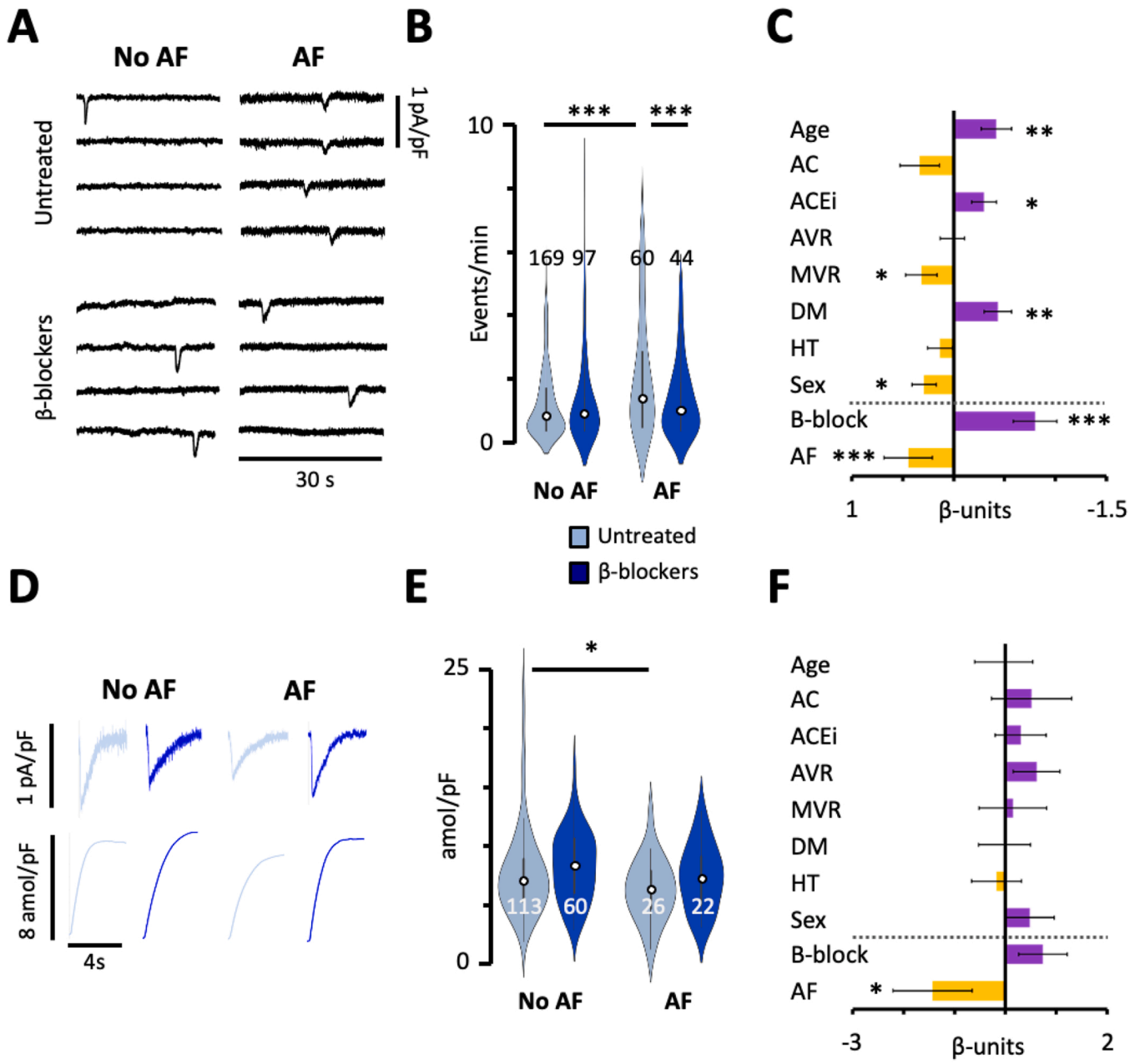

Fig. 2. Effect of β-blocker treatment on I_{TI} frequency and SR calcium load. A. Recordings of I_{TI} currents from patients without AF (left traces) and patients with AF (right traces), untreated and treated with β-blockers. B. Mean I_{TI} frequencies. Statistical significance was determined using a linear regression model with negative binomial distribution. C. Beta-values for the impact of confounding factors on the I_{TI} frequency. D. Recordings of caffeine induced NCX-currents (top) and the corresponding time integrals (bottom) corresponding to the SR calcium load released by caffeine. **E.** Mean SR calcium load for patients with AF and without (No AF). **F**. Beta-values for the impact of confounding factors on SR calcium load. The effects of β-block and AF are shown for comparison. Pathological effects are yellow and protective effects purple. AC, anticoagulants; ACEi, angiotensin converting enzyme inhibitor; AVR, aortic valve replacement; MVR, mitral valve replacement; DM, diabetes mellitus; HT, hypertension; AF, atrial fibrillation. Statistical significance was determined using a linear regression model. Statistically significant effects are indicated with *: $p < 0.05$, **: $p < 0.01$; *** : $p < 0.001$. Number of patients is given above or below each group.

between sparks per site and β-blocker treatment ($p = 0.04$). Analysis of the spark kinetics revealed that β-blocker treatment did not affect any spark property in patients without AF, while β-blocker treatment reversed the impact of AF on spark amplitude (Fig. 4E), duration (Fig. 4F) and decay (Fig. 4G) to values observed in patients without AF.

To determine if the observed effects of β-blocker treatment on SR calcium homeostasis were caused by alterations in the phosphorylation of calcium regulatory proteins, we measured phosphorylation of RyR2 clusters at Ser-2808 as well as PLB phosphorylation at Ser-16 and Thr-17. Supplementary Figure 4 shows that the treatment with β-blockers did not affect the RyR2 density and Fig. 5A-B shows that RyR2 phosphorylation at the Ser-2808 residue was not different among patients without AF that did or did not receive β-blockers. However, Ser-2808 phosphorylation was significantly higher in patients with AF that did not receive treatment with β-blockers than in AF patients treated with β-blockers. Accordingly, two-way ANOVA analysis showed significant effects of atrial rhythm ($p = 0.004$), β-blocker treatment ($p = 0.008$) and β-blocker treatment combined with atrial rhythm ($p = 0.01$) on Ser-2808 phosphorylation. In line with these findings, analysis of PLB phosphorylation at Ser-16 showed a significant ($p = 0.03$) interaction with β-blocker treatment (Fig. 5C) whereas PLB phosphorylation at Thr-17 was not affected by atrial rhythm or β-blocker

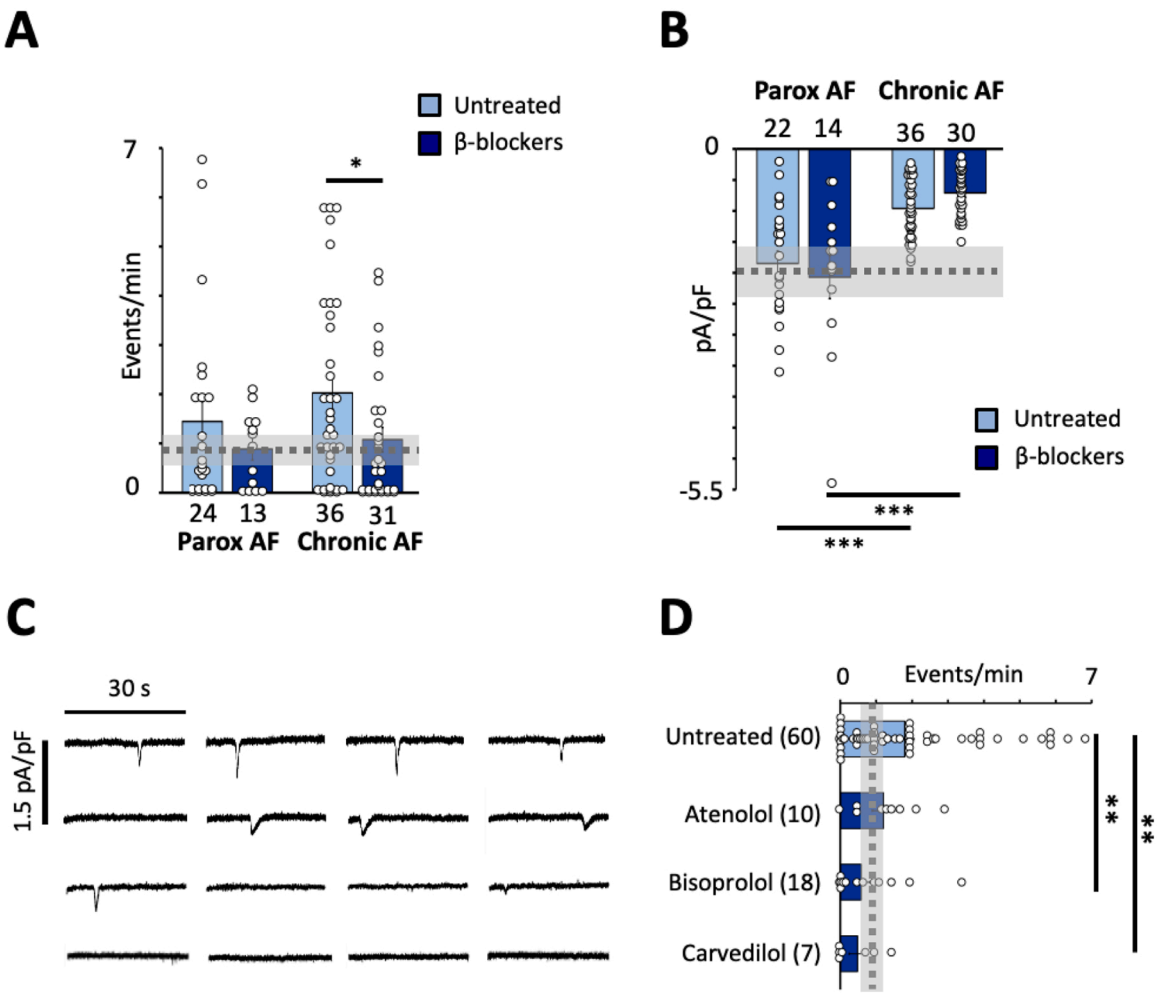

Fig. 3. Effect of the β-blocker and AF-type on I_{Ca} density and I_{TI} frequency. A. Comparison of the impact of β-blocker treatment on the I_{TI} frequency in patients with paroxysmal (Parox) and chronic AF. B. Comparison of the impact of β-blocker treatment on the I_{Ca} density in patients with paroxysmal and chronic AF. Statistical significance in panel A and B were evaluated using two-way ANOVA followed by Bonferroni adjusted multiple pairwise comparisons. C. Recordings of I_{TI} currents in myocytes from patients with AF receiving different β-blocker treatments. D. Mean I_{TI} frequency for each treatment. Statistical significance in panel D was evaluated using ANOVA test with Welch correction followed by Bonferroni adjusted multiple comparisons. Dotted lines and shaded areas indicate mean values and 95% confidence intervals for patients without AF and no β-blocker treatment. The number of patients is given next to each bar. Statistically significant effects are indicated with *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$.

treatment (Fig. 5D).

4. Discussion

4.1. Main findings

This study demonstrates that ambulatory treatment with β-blockers has a distinctive impact on intracellular calcium homeostasis in atrial myocytes from patients with and without reported episodes of AF. Thus, in patients with AF, β-blocker treatment has little impact on the L-type calcium current but reduces RyR2 phosphorylation at Ser-2808, and concurrently, lowers the incidence of calcium sparks and I_{TI}s to levels observed in patients without AF. This effect was most prominent in patients treated with bisoprolol or carvedilol. As outlined in Fig. 6, these results suggest that β-blockers may be of therapeutic utility to prevent spontaneous calcium release-induced atrial electrical activity; especially in patients with a history of paroxysmal AF that do not display the reduction in I_{Ca} density observed in patients with chronic AF.

4.2. Multivariate regression analysis of the impact of beta-blocker treatment on L-type calcium current

By triggering calcium release from the sarcoplasmic reticulum through calcium induced calcium release, the L-type calcium channels plays a crucial role in the regulation of the calcium available for the activation of contraction and β-adrenergic receptors, in turn, modulate the L-type calcium current amplitude via cAMP-dependent signaling [24]. Since AF has been associated with increased sympathetic nerve density [6,7], this would be expected to increase both spontaneous calcium release from SR and the I_{Ca} density. The latter is not easy to reconcile with a reduction in L-type calcium current shown in Fig. 1 and previously reported in patients with AF [25,26]. However the higher spark frequency in AF will increase baseline calcium and cause calcium-dependent inactivation of I_{Ca}. This effect may be very prominent in AF, where the spark density is 40-fold higher at the sarcolemma [27]. Multivariate linear regression analysis of the current data, taking into account the confounding effects of key clinical factors, confirms a reduction of the I_{Ca} density in patients with chronic but not paroxysmal AF. This analysis also revealed that atrial rhythm and age are the key factors contributing to a reduction in I_{Ca} in the study population. The latter is in line with a previous study showing that age reduces the I_{Ca}

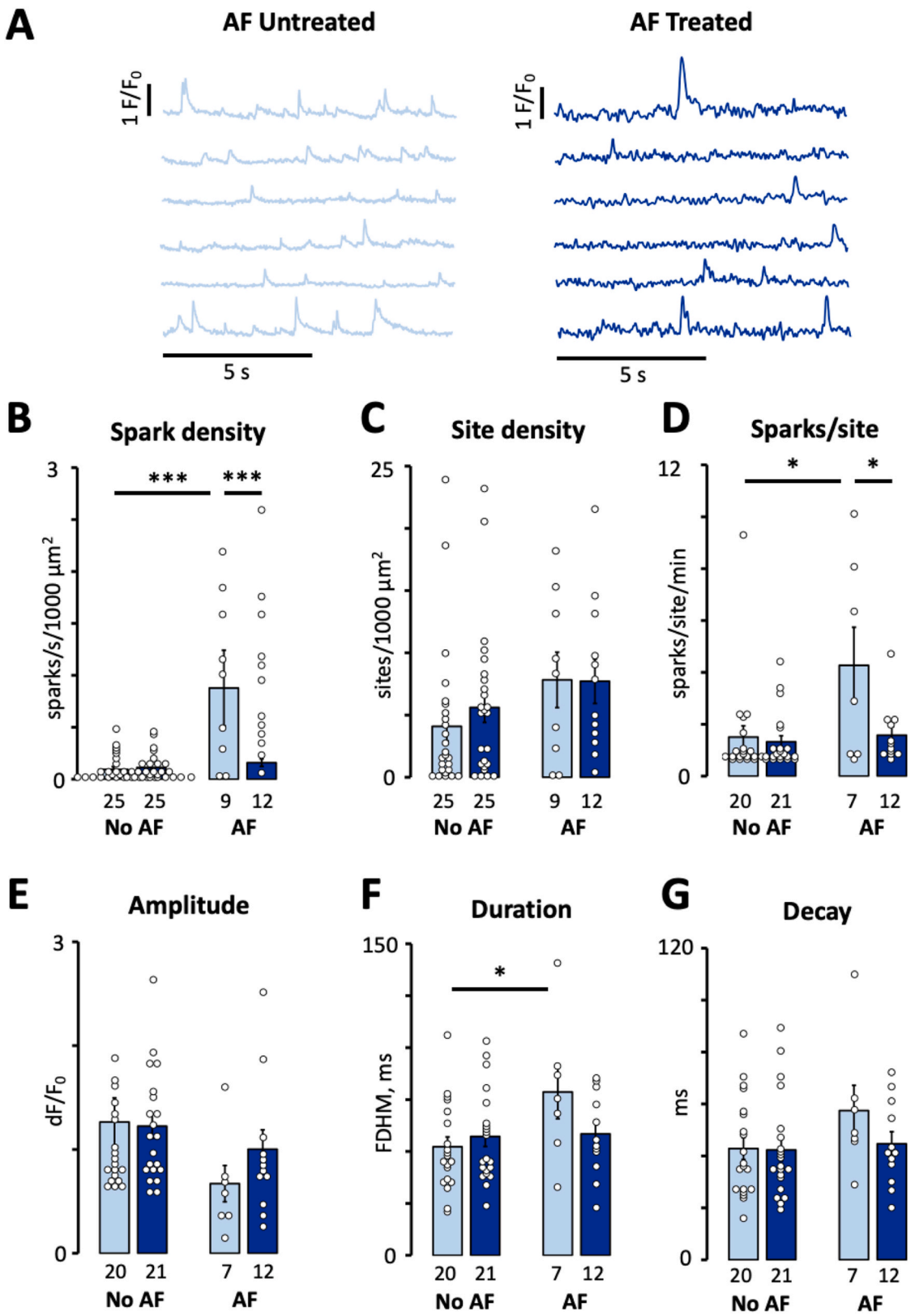

Fig. 4. Effect of β-blocker treatment on calcium spark frequency and properties. A. Calcium spark recordings in a patient with AF that did not receive β-blocker treatment (AF Untreated) or that did receive treatment (AF treated). B. Mean spark densities. C. Mean spark site density. D. Mean number of sparks per site. Statistical significance in B-D was determined using a general linear model with negative binomial distribution. E. Mean spark amplitude. F. Mean spark duration. G. Mean spark decay constant. Statistical significance in E-G was determined using a linear regression model. Statistically significant effects are indicated with *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$. Number of patients is given below each bar.

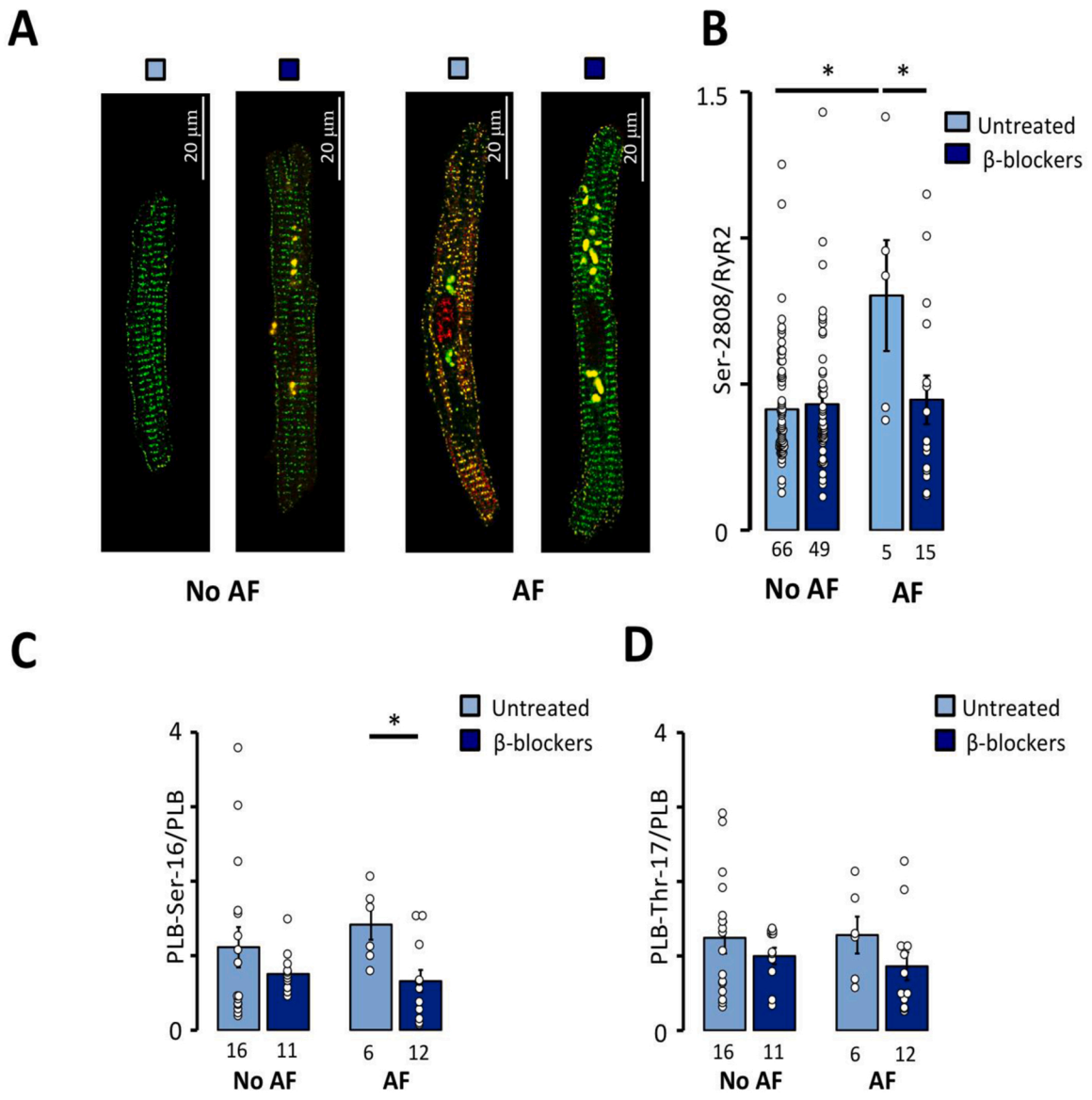

Fig. 5. Effect of β-blocker treatment on RyR2 and PLB phosphorylation. A. Overlay of total RyR2 (in green) and Ser-2808 phosphorylated RyR2 (in red) for patients without AF (left) and patients with AF (right) untreated (light blue square) and treated with β-blockers (dark blue square). B. Mean Ser-2808 phosphorylated RyR2 measured as the fluorescence intensity ratio (Ser-2808/RyR2) for all RyR2 clusters. C. Serine-16 (Ser-16) phosphorylated PLB levels normalized to total PLB expression in patients without and with AF. D. Threonine-17 (Thr-17) phosphorylated PLB levels normalized to total PLB expression in patients without and with AF. Statistical significance in panels B to D were determined using a two-way ANOVA followed by Bonferroni adjusted multiple pairwise comparisons. Statistical differences between pairs of bars are indicated with *: $p < 0.05$. Number of patients is given below each bar.

density in patients without a record of AF [22]. On the other hand, the regression analysis showed no significant impact of β-blocker treatment on the I_{Ca} density, suggesting that even though the treatment with β-blockers would be expected to prevent the impact of an increased sympathetic activity in AF, other factors such as age or oxidative stress [28–30] underlie a smaller I_{Ca} density in AF. Hence, β-blocker treatment is not suitable to prevent AF caused by reduction in I_{Ca}, but it might be suitable in those with AF that do not display reduced I_{Ca} density if it can reduce the elevated incidence of I_{TI}s observed in these patients.

4.3. Multivariate regression analysis of the impact of beta-blocker treatment on sarcoplasmic reticulum calcium homeostasis

Multivariate analysis of the impact of AF and β-blocker treatment on the I_{TI} frequency in human atrial myocytes confirmed previous findings that were performed without taking into account potentially confounding factors [20,21,31], showing that both paroxysmal and chronic AF independently increase the I_{TI} frequency. This analysis also confirmed that age and sex affect the I_{TI} frequency [22,23] and showed that other factors like diabetes and ACE inhibitors also affect it significantly. Moreover, the treatment with β-blockers significantly reduced the incidence of I_{TI}s in patients with AF to levels observed in patients without the arrhythmia. In accordance with previous studies, suggesting that carvedilol is the only β-blocker that can also modify RyR2 opening [18,19], we found differences in the ability of distinct β-blocker treatments to attenuate the incidence of spontaneous I_{TI}s. Thus, carvedilol and bisoprolol reduced the I_{TI} frequency significantly to levels observed in patients without AF while atenolol had no significant effect. While the effect of carvedilol on I_{TI} frequency might be explained by the ability the R-carvedilol enantiomer to directly modify RyR2 activity in human atrial myocytes [32]; the differences between the three β-blockers is possibly explained by their ability to block the stimulatory effect of (−)-isoprenaline, which is ten-fold lower for atenolol than for both bisoprolol and carvedilol [33].

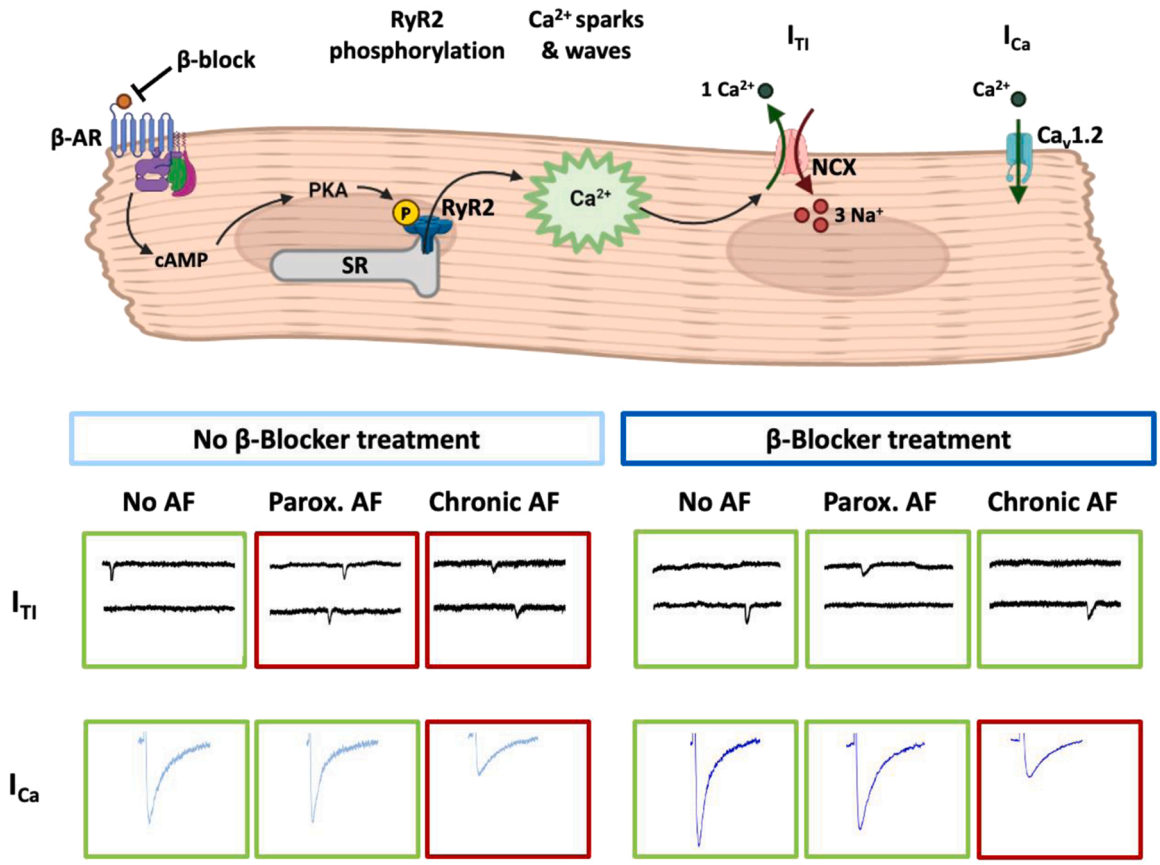

Fig. 6. Impact of β-blocker treatment on calcium homeostasis in human atrial myocytes. The top panel shows how β-blocker treatment modifies cyclic AMP (cAMP)-dependent modulation of SR calcium homeostasis and the incidence of transient inward currents (I_{TI}) but not the L-type calcium current (I_{Ca}). Consequently, β-blocker treatment normalizes SR calcium homeostasis in patients with paroxysmal AF (Parox. AF) without depression of the I_{Ca}, but not in patients with chronic AF where the I_{Ca} density remains depressed.

From a mechanistic point of view, β-adrenergic receptors have been reported to be located in macromolecular clusters with a spatial distribution that is different for the β-receptors associated with the RyR2 and those associated with PLB and SERCA2a [34,35], suggesting that β-blockers might affect RyR2 and PLB phosphorylation differently. However, our findings show that β-blocker treatment of patients with AF reduced both the phosphorylation of PLB at Ser-16 and RyR2 at Ser-2808 to levels observed in patients without AF. Accordingly, the decay of calcium sparks, which is expected to depend on PLB phosphorylation, and the spark frequency that is modulated by RyR2 phosphorylation were both reduced in AF patients treated with β-blockers to levels observed in patients without AF.

Considering that the treatment with β-blockers diminished both calcium spark and I_{TI} frequencies without affecting the SR calcium load, it is conceivable that β-blockers diminishes calcium loss from the SR by reducing RyR2 opening and counteracts the PLB-Ser-16 mediated reduction of SERCA2a activity, which is expected to diminish SR calcium loading. In support of this notion, we have recently shown that the spark frequency is proportional to RyR2 phosphorylation and that RyR2 clusters with the highest spark activity have the lowest caffeine releasable SR calcium content [36].

Finally, we found only marginal differences in calcium currents, spontaneous calcium release events and phosphorylation of calcium regulatory proteins among patients without AF that received β-blockers and those that did not, suggesting that calcium homeostasis in resting myocytes is affected minimally by the β-adrenergic tone in normal physiological conditions.

4.4. Limitations

Phosphorylation of the RyR2 at Ser-2808, Ser-2814 or Ser-2030 have all been associated with increased RyR2 opening or a higher incidence of spontaneous calcium release events. In the present study, we did not measure RyR2 phosphorylation at Ser-2814 because previous studies from ours and other laboratories did not find a significant elevation of RyR2 phosphorylation at Ser-2814 in resting myocytes [37,38]. Similarly, we were unable to detect RyR2 phosphorylation at Ser-2030 in human atrial myocytes with commercially available antibodies. Thus, we cannot rule out that RyR2 phosphorylation at Ser-2030 could also contribute to the observed increase in spontaneous calcium release in resting myocytes.

We have recently shown that myocytes from patients with AF display sex-dependent differences in L-type calcium current density and especially the incidence of spontaneous calcium release-induced electrical activity [23]. The present study confirms the latter finding, and consequently the impact of β-blockers on RyR2 phosphorylation at Ser-2808 and calcium spark frequency is presumably more pronounced in females. However, determination of the interactions between sex, β-blocker treatment and AF, and their impact on RyR2 phosphorylation and activity will require a study dimensioned specifically to address this issue.

Because left atrial tissue samples from patients without left atrial dilation or structural alterations are sparse, the current study has been conducted in right atrial myocytes to achieve a large sample size. Therefore, we cannot rule out that some of the findings in the present study may be more prominent in the right atrium.

4.5. Conclusions and clinical translation

Our findings point to β-blocker treatment as an efficient therapeutical approach to reduce spontaneous calcium-release induced electrical activity in patients with AF and the results afford a physiological foundation for testing the ability of β-blockers to prevent ectopic and triggered activity in patients with history of AF. In support of this notion, we have recently shown that activation of G_s-protein coupled receptors may contribute to a higher incidence of spontaneous calcium release at the sarcolemma and potentiate spontaneous membrane depolarizations in myocytes from patients with atrial fibrillation [27]. Our findings also underscore the importance of separating patients prone to present spontaneous calcium release-induced electrical activity from those suffering from reduction in L-type calcium current or other electrophysiological alterations that favor electrical re-entry. In this regard, patients with paroxysmal AF might be especially responsive to β-blocker treatment since they have elevated I_{TI} frequency but preserved I_{Ca} density. Basic clinical factors such as age and sex also display differential effects on I_{Ca} amplitude and I_{TI} frequency [22,23] and we have recently documented that myocytes from patients that carry a risk variant on chromosome 4q25 associated with risk of AF display higher Ser-2808 phosphorylation, spark, I_{TI} and DAD frequency [38]. Thus, combining clinical factors affecting calcium homeostasis with genetic screening for risk SNPs located in regions expected to modify β-adrenergic signaling, RyR2 activity or the resting membrane potential might provide a means to identify patient groups expected to be highly responsive or unresponsive to β-blocker treatment.

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CRediT authorship contribution statement

Verónica Jiménez-Sábado: Designed Research; Performed Research; Analyzed Data; Wrote draft. **Sergi Casabella-Ramón:** Performed Research; Analyzed Data. **Anna Llach:** Performed Research, Analyzed Data; Acquired Funding. **Ignasi Gich:** Analyzed Data. **Sandra Casellas:** Analyzed Data. **Francisco Ciruela:** Designed Research, Analyzed Data; Acquired Funding. **S.R. Wayne Chen:** Acquired Funding; Edited & Revised Draft. **José M. Guerra:** Acquired Funding; Edited & Revised Draft. **Antonino Ginel**: Analyzed Data. **Raúl Benítez:** Designed Research; Analyzed Data; Acquired Funding. **Juan Cinca:** Acquired funding; Edited & Revised Draft**. Carmen Tarifa**: Designed Research; Performed Research; Analyzed Data; Edited & Revised Draft. **Leif Hove-Madsen:** Designed Research; Analyzed Data; Wrote Draft; Acquired Funding; Edited & Revised Draft.

Conflict of interest statement

None declared.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.114169.

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