Left-to-Right Atrial Inward-Rectifier Potassium Current Gradients in Patients with Paroxysmal Versus Chronic Atrial Fibrillation

Running title: *Voigt et al.; Human Inward-Rectifier Current Gradient in AF*

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Abstract

Background - Recent evidence suggests that atrial fibrillation (AF) is maintained by high-frequency reentrant sources with a left-to-right dominant-frequency gradient, particularly in patients with paroxysmal AF (pAF). Unequal left-to-right distribution of inward-rectifier K^+-currents have been suggested to underlie this dominant-frequency gradient, but this hypothesis has never been tested in man.

Methods and Results - Currents were measured with whole-cell voltage-clamp in cardiomyocytes from right (RA) and left (LA) atrial appendages of SR- and AF-patients undergoing cardiac surgery. Western blot was used to quantify protein expression of I_{K1} (Kir2.1 and Kir2.3) and $I_{K,ACh}$ (Kir3.1 and Kir3.4) subunits. Basal current was ~2-fold larger in chronic AF (cAF) vs. sinus rhythm (SR) patients, without RA-LA differences. In pAF, basal current was ~2-fold larger in LA vs. RA, indicating a left-to-right atrial gradient. In both atria, Kir2.1 expression was ~2-fold greater in cAF but comparable in pAF vs. SR. Kir2.3 levels were unchanged in cAF and RA-pAF, but showed a 51% decrease in LA-pAF. In SR, carbachol (2 µmol/L)-activated $I_{K,ACh}$ was 70% larger in RA vs. LA. This right-to-left atrial gradient was decreased in pAF and cAF due to reduced $I_{K,ACh}$ in RA only. Similarly, in SR Kir3.1 and Kir3.4 proteins were greater in RA vs. LA and decreased in RA of pAF and cAF. Kir3.1 and Kir3.4 expression was unchanged in LA of pAF and cAF.

Conclusions – Our results support the hypothesis that a left-to-right gradient in inward-rectifier background current contributes to high-frequency sources in LA that maintain pAF. These findings have potentially-important implications for development of atrial-selective therapeutic approaches.

Key words: atrium; fibrillation; ion channels; remodeling

Introduction

There is experimental and clinical evidence suggesting that certain cases of paroxysmal (pAF) and chronic (cAF) atrial fibrillation (AF) are maintained by high-frequency reentrant sources (rotors) with a consistent left-to-right dominant-frequency gradient, particularly in pAF.[1-7] AF-maintaining waves emanating from the left atrium (LA) undergo complex, spatially-distributed conduction-patterns, with wavefront fractionation in the right atrium (RA) manifesting as "fibrillatory conduction".[8] In a large proportion of patients, ablation of dominant-frequency sites terminates pAF,[6] consistent with the notion that LA sites "drive" AF. LA-to-RA dominant-frequency gradients are much clearer in patients with pAF than those with persistent/permanent cAF.[1,2,6]

Sustained AF leads to ionic-current remodeling, including up-regulation of the inward-rectifier I_{K1}, down-regulation of the muscarinic-receptor-activated $I_{K,ACh}$ and up-regulation of a constitutively-active $I_{K,ACh}$ component ("constitutive $I_{K,ACh}$").[9-12] Inward-rectifier K^+-currents abbreviate action potential duration (APD) and hyperpolarize atrial cardiomyocytes, thereby enhancing sodium-channel availability. Therefore, increased I_{K1} and constitutive $I_{K,ACh}$ may be more effective in stabilizing and accelerating AF-sustaining rotors than changes in other currents (e.g. L-type Ca^{2+}-current downregulation) that produce similar APD-abbreviation.[13-15] In a sheep model of acetylcholine-mediated, pacing-induced AF the left-to-right dominant-frequency gradient parallels a LA-to-RA $I_{K,ACh}$ gradient,[4,16] supporting the hypothesis that an unequal LA-RA distribution of inward-rectifier K^+-currents contributes to AF maintenance. Although AF-associated changes in I_{K1} and $I_{K,ACh}$ may be critical for rapidly-firing LA driver-sources, there are no published studies directly comparing I_{K1} and $I_{K,ACh}$ in LA vs. RA in AF. The present study was designed to test the hypothesis that AF-patients show chamber-specific differences in inward-rectifier current function, providing a potential molecular basis for AF-maintaining LA drivers.

Methods

Human Tissue Samples

Experimental protocols were approved by the ethics committees of Dresden University of Technology (#EK790799) and the Cleveland Clinic (IRB#4286). Each patient gave written informed consent. RA and/or LA appendages were obtained from 43 SR, 22 pAF and 30 cAF patients undergoing open heart surgery for either coronary artery bypass grafting and/or valve replacement (Table 1), but matched samples of RA and LA appendages were obtained from only two SR and two cAF patients. After excision, samples were used for myocyte isolation and/or immediately snap-frozen in liquid nitrogen for biochemical measurements. All diseased-heart samples were collected in Dresden, except for 6 LA samples from pAF patients collected in Cleveland, used for biochemistry. Anesthesia was performed similarly in both centers; tissue handling followed the same protocol. There were no differences in results for tissues from the 2 sampling-centers (Online Figure 1).

RA and LA samples from non-diseased human hearts that were technically unusable for transplantation based on logistical, not patient-related considerations were obtained from organ donors to serve as non-diseased controls. Procedures were approved by the ethics committee of the University of Szeged (#51-57/1997OEJ). Before cardiac explantation, patients did not receive medication except dobutamine, furosemide and plasma expanders (Online Table).

Electrophysiological Recordings

Atrial cardiomyocytes were isolated with a standard protocol[17] and suspended in storage solution (mmol/L: KCl 20, KH_2PO_4 10, glucose 10, K-glutamate 70, β-hydroxybutyrate 10, taurine 10, EGTA 10, albumin 1, pH=7.4). Currents were measured with voltage-clamp-technique using ISO-2-Software (MFK, Niedernhausen, Germany) for data acquisition and analysis.[9] Borosilicate glass microelectrodes had tip-resistances of 2-5 MΩ when filled with pipette solution (mmol/L: K-aspartate 80, NaCl 8, KCl 40, $CaCl_2$ 2, Mg-ATP 5, EGTA 2, GTP-Tris 0.1,

HEPES 10, pH=7.4). Seal-resistances were 4-8 GΩ. Series-resistance and cell-capacitance were compensated.

Myocytes were superfused with bath solution containing (mmol/L): NaCl 120, KCl 20, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, HEPES 10, pH=7.4. As in previous studies[9-11,17,18] we used high (20 mM) extracellular potassium concentration because this shifts the reversal potential to more positive values and allows us to record much larger (more easily measurable/comparable) inward currents (Figure 1). We recorded the currents at room temperature because current amplitudes at room temperature are comparable to those at 37°C (Online Figure 2) and the success rate of experiments is much higher at room temperature. Drugs were applied via a rapid solution-exchange-system (ALA-Scientific-Instruments, Long Island, USA). Agonist-independent basal current was measured with a ramp-pulse from -100 to +40 mV (Figure 1A). Similar to previous studies[9-11,17,18] we analysed inward currents at -100 mV. Outward currents were analysed at -10 mV because confounding by other currents is negligible at this potential. Agonist-inducible $I_{K,ACh}$ was stimulated with carbachol (CCh, 2 µmol/L) and defined by total current in the presence of CCh minus basal current. Previous concentration-response experiments showed that 2 µmol/L CCh is a saturating concentration for $I_{K,ACh}$ activation with no differences in CCh sensitivity between SR and AF patients.[10] We used a ramp-pulse protocol, which is much better tolerated by human atrial myocytes. A direct comparison between results obtained with ramp-pulses vs. clamp-steps (Online Figure 3) indicates their similarity and supports the validity of results obtained with ramp-pulse protocols. The basal inward-rectifier-current and $I_{K,ACh}$ were specifically assessed based on Ba^{2+}(1-mmol/L)-sensitive currents. The contribution of agonist-independent $I_{K,ACh}$ to basal current was assessed with the selective Kir3-blocker tertiapin (10 nmol/L, Peptides-International, Louisville, USA). Data were not corrected for liquid-junction-potential (~-12 mV, JPCalc-Software). All drugs were from Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

Immunoblots

Due to small sample-size (usually <200 mg) and low isolated cell-yield, immunoblotting is not feasible for isolated atrial cardiomyocyte-samples from individual patients. Therefore, primary antibodies against Kir2.1 (1:200; Santa-Cruz, Santa Cruz, USA), Kir2.3 (1:200; Alomone, Jerusalem, Israel), Kir3.1 (1:1000; Alomone) and Kir3.4 (1:200, Santa-Cruz) were used to quantify corresponding proteins in atrial-tissue homogenates only.[19] Peroxidase-conjugated goat anti-rabbit (1:5000 for Kir2.3, 1:2500 for Kir3.1; Sigma-Aldrich) and donkey anti-goat (1:2000 for Kir2.1, 1:5000 for Kir3.4; Santa-Cruz) were used as secondary antibodies and visualized by chemifluorescence (GE-Healthcare, Chalfont StGiles, UK). Quantity-One-Software (Bio-Rad, Hercules, USA) was used for quantification. After primary-antibody removal with stripping-buffer (Carl-Roth, Karlsruhe, Germany), calsequestrin expression (1:2500 anti-CSQ, Dianova, Hamburg, Germany; 1:30000 goat anti-rabbit) was quantified as loading control.

Statistical Analysis

Differences between group means were compared by unpaired Student's *t*-test or by one-way ANOVA with Bonferroni-corrected *t*-test. Two-way ANOVA was applied to assess the independent contribution of atrial chamber and arrhythmia type to ionic-current densities. Frequency-data were analyzed with Fisher's exact test. Data are mean±SEM. $P<0.05$ was considered statistically significant.

Results

Patient characteristics

The pAF group included patients in SR at surgery with a history of at least one episode of self-terminating AF lasting less than 7 days. The cAF group included patients with sustained AF for at least 6 months prior to surgery. Patient-group characteristics are shown in Table 1. In general,

pAF and cAF patients were older and had larger LA-diameters than SR patients. Coronary artery disease was present more often in patients with SR and pAF, whereas cAF patients had higher incidence of valvular heart disease. Patients with cAF more frequently received digitalis than SR and pAF patients. All patients were euthyroid. SR patients who provided LA tissue had lower left-ventricular ejection fraction than those providing RA-samples, but had no clinical evidence of heart failure.

Basal Inward-rectifier Current

There were no significant differences in membrane capacitances between groups, except for a lower membrane capacitance in LA-pAF vs. LA-cAF (Online Figure 4). To control for myocyte-size variability, currents are expressed as densities (pA/pF).

Representative current-recordings in SR, pAF and cAF are shown in Figure 1B-D. Overall, inward basal current in RA was greater in cAF than in SR and pAF (Figure 2A). Inward basal current in LA was 2-fold higher in both pAF and cAF than in SR, with a left-to-right gradient of basal current in pAF only. The greater rotation-speed and persistence of rotors associated with increased inward-rectifier current have been attributed to larger outward-current components.[14] Outward basal current differences paralleled inward-current differences: pAF showed a left-to-right gradient and cAF had larger currents in both LA and RA. The enhanced basal currents in RA and LA of cAF were associated with a more negative RMP (Online Figure 5), without significant RA-LA differences. Mean RMP was larger in LA-cardiomyocytes from pAF vs. SR and vs. RA-cardiomyocytes from pAF, but the differences were not statistically-significant.

Inward-rectifier Current in the Presence of CCh

Application of the muscarinic-receptor agonist CCh led to an increase in total current density (Figure 2B and Figure 3) due to activation of $I_{K,ACh}$. Figure 2B shows total inward-rectifier

current density in the presence of 2 μmol/L CCh. In RA, the inward and outward components of total current were significantly smaller in pAF than in cAF. As for basal current, pAF patients had a significant LA-RA gradient. Compared to SR, cAF cells showed larger total current amplitudes in both atria with no RA-LA difference (Figure 2B).

Previous studies in RA showed reduced agonist-activated $I_{K,ACh}$ in pAF and cAF patients.[9-11] Figure 4A shows mean current-density of maximum CCh-activated $I_{K,ACh}$ in RA and LA of SR, pAF and cAF. In RA, the inward and outward components were lower for pAF and cAF compared to SR. In LA, however, CCh-induced $I_{K,ACh}$ was similar in SR, pAF and cAF for both inward and outward components. CCh-induced $I_{K,ACh}$ was greater in SR-RA than in SR-LA, indicating a clear right-to-left gradient. Qualitatively similar but statistically non-significant RA-LA differences were present for pAF and cAF (Figure 4A). Consistent with AF-related differences for CCh-induced $I_{K,ACh}$ in RA, the CCh-induced hyperpolarization of RMP was smaller in pAF and cAF vs. SR (Online Figure 5).

$I_{K,ACh}$ currents are well-known to show rapid "desensitization".[9-11,17] Figure 3 illustrates this phenomenon for CCh-induced $I_{K,ACh}$: rapid rises to a maximum (Peak-$I_{K,ACh}$) were followed by decreases to quasi-steady-state levels (QSS-$I_{K,ACh}$). The ratio of QSS-$I_{K,ACh}$/Peak-$I_{K,ACh}$ values, an index of desensitization, was not affected by AF (Online Figure 6). Like the Peak-$I_{K,ACh}$ responses illustrated in Figure 4A, QSS-$I_{K,ACh}$ was smaller in RA of pAF and cAF compared to SR, was comparable in each group of LA, and showed a right-to-left gradient in SR (Figure 4B).

To determine potential contributions of underlying clinical conditions and/or medication (Table 1) to variations in basal current and CCh-activated $I_{K,ACh}$, we performed univariate ANOVA analysis with rhythm status-atrial chamber (left vs. right), selected clinical parameters and medication as independent variables. The density of basal current was significantly associated with rhythm status-atrial chamber, body mass index and valvular heart disease, whereas CCh-activated $I_{K,ACh}$ was significantly associated with rhythm status-atrial chamber

only. A test of interaction effects between rhythm status-atrial chamber vs. body mass index and valvular heart disease with two-way ANOVA showed that rhythm status-atrial chamber may independently associate with basal current because no significant interaction was detected (P=0.581 for body mass index; P=0.214 for valvular heart disease).

To assess whether the RA-LA differences seen in the grouped data were consistent with RA-LA differences from individual patients we analyzed separately the current data available from matched RA-LA samples of SR and cAF patients. Online Figure 7 shows that the results obtained in myocytes from matched RA-LA samples are consistent with the overall trend in these groups (compare Figures 2 and 4), supporting the validity of the mean overall-group data.

Agonist-independent Constitutive $I_{K,ACh}$ in LA of cAF Patients

In RA of cAF patients agonist-independent constitutive $I_{K,ACh}$ likely contributes to AF maintenance.[9] To unmask constitutive $I_{K,ACh}$ in LA we used the selective $I_{K,ACh}$ blocker tertiapin (Figure 5). In LA, the tertiapin-sensitive component of basal current was higher in cAF compared to SR, indicating that cAF increased constitutive $I_{K,ACh}$.

Protein Expression of I_{K1}-channel Subunits

We analyzed expression-changes in ion-channel subunits as a potential mechanism of chamber-specific differences in current density. Kir2.1 immunoblots showed greater Kir2.1-expression in RA and LA of cAF vs. SR, without chamber-specific differences (Figure 6A and Online Figure 8). Compared to SR, pAF showed unchanged Kir2.1 expression in RA and LA. We observed no change in Kir2.3 proteins in RA of either group and in LA from SR vs. cAF. However, Kir2.3 expression was 51% lower in LA of pAF vs. SR (Figure 6B).

Protein Expression of $I_{K,ACh}$-channel Subunits in SR, pAF and cAF Patients

As in previous publications,[10,20-22] we found reduced protein-levels of Kir3.1 and Kir3.4 in RA of

both pAF and cAF (Figure 7 and Online Figure 8). LA expression of Kir3.1 and Kir3.4 was unchanged in either group. Kir3.1 and Kir3.4-subunit expression was significantly greater in RA vs. LA from SR patients. LA-RA gradients were absent in pAF and cAF, consistent with functional results shown in Figure 4.

To exclude the possibility that right-to-left gradients of Kir3.1 and Kir3.4 expression in SR were due to heart disease, we analyzed their expression in normal (non-diseased) atria (Online Figure 9). Similar to SR-patient results, Kir3.1 and Kir3.4 expression in normal atria was greater in RA compared to LA.

Discussion

Experimental studies and computer simulations point to the importance of inward-rectifier currents in AF dynamics, relating the faster rotation and greater stability of high-frequency reentrant sources to the expression levels and function of inward-rectifier currents.[13,16] Clinical evidence suggests a left-to-right dominant-frequency gradient in AF.[1,2,5-7] Differences in inward-rectifier K^+-current function, which could explain this dominant-frequency gradient, have not previously been investigated. Here, we addressed this issue for the first time in the literature. We found that pAF patients had inward-rectifier current densities that were 2-fold larger in LA than in RA cardiomyocytes. In cAF patients, despite greater basal currents, there were no significant LA-RA differences. CCh-activated $I_{K,ACh}$ was larger in RA than in LA for SR-patients, suggesting a right-to-left gradient in the absence of AF.

Comparison to Previous Studies

Emerging evidence suggests that AF is maintained by high-frequency sources (drivers), causing a hierarchical organization in activation rates for different regions of the atria.[2,5,6] The presence of a left-to-right atrial-frequency gradient has been demonstrated in a sheep model of AF and in some studies of clinical AF.[1-7] Enhanced inward-rectifier K^+-currents promote reentry by

abbreviating APD as well as by accelerating and stabilizing rotors by hyperpolarizing atrial cardiomyocytes (thereby removing voltage-dependent sodium-current inactivation).[13-15] We and others recently reported larger basal inward-rectifier current in RA and LA of cAF compared to SR patients.[9-11,23-26] Here, we noted greater basal currents in cAF, without significant LA-RA differences. These findings are in agreement with the lack of consistent left-to-right dominant-frequency gradients that has been reported in patients with persistent/permanent AF.[1,2,6] The inward-rectifier basal current increases, observed in both atria, could contribute to arrhythmia persistence by providing multiple potential regions of high-frequency rotor activity or by promoting reentrant-wavelets by shortening refractoriness. Basal current of pAF patients was 2-fold larger in LA compared to SR patients, but was similar in RA, implicating LA inward-rectifier current enhancement as a potential contributor to high-frequency LA rotors underlying AF. In addition, these results suggest that the left-to-right inward-rectifier current gradient in pAF may contribute to their left-to-right dominant-frequency gradient.[6] A comparable mechanism has been shown in experimental ventricular fibrillation, where larger left-ventricular I_{K1} critically underlies high-frequency sources.[14]

Previous studies on inward-rectifier current remodeling consistently showed that right-atrial Kir2.1 expression is increased in cAF, likely contributing to enhanced basal current.[10,22] A recent study reported increased basal current in LA of cAF patients that is associated with enhanced Kir2.1 but unaltered Kir2.3,[24] supporting the upregulation of I_{K1} as a key mechanism. Here, we provide evidence that, in addition to increased I_{K1}, agonist-independent constitutive $I_{K,ACh}$ also contributes to the larger basal inward-rectifier current in the LA during cAF. These results supplement previous findings[9,11] obtained in RA of cAF patients, underscoring the notion that constitutive $I_{K,ACh}$ may contribute, along with increased I_{K1}, to reentrant sources maintaining AF.

In contrast to increased Kir2.1 expression in both atria of cAF patients, Kir2.1 expression was unchanged in the LA of pAF patients, despite their larger basal currents. Although Kir2.3

expression was unchanged in cAF, it decreased in the LA of pAF patients. Whether decreased Kir2.3 expression contributes to the functional increase of basal current in LA of pAF patients is unclear, but heteromeric Kir2.1 channels containing less Kir2.3 subunits would be expected to have higher I_{K1} amplitude because of reduced tonic Kir2.3 inhibition by βγ-subunits of G-protein-coupled receptors,[27] protein kinase C-mediated phosphorylation[28] and transforming-growth-factor β_1 modulation.[29] In addition, constitutive $I_{K,ACh}$ may contribute to the larger basal current in LA of pAF patients. Further work is needed to verify these hypotheses and to identify the precise mechanisms of the chamber-specific increases of basal current in pAF.

Chamber-specific differences in agonist-activated $I_{K,ACh}$ show species-variation: whereas experiments in dogs and sheep suggest larger agonist-activated $I_{K,ACh}$ in LA,[16,30] murine studies indicate greater agonist-activated $I_{K,ACh}$ in RA.[31] To our knowledge, the present study is the first to address this issue in humans, showing a right-to-left gradient of agonist-activated $I_{K,ACh}$ and corresponding Kir3.1/3.4 expression differences. $I_{K,ACh}$-subunit (Kir3.1/3.4) protein expression was greater in RA vs. LA of undiseased atria, supporting the consistency of the finding and indicating that the right-to-left gradient in $I_{K,ACh}$-channel expression in SR patients was not a function of underlying heart disease. Differential post-translational channel subunit modifications may also contribute to $I_{K,ACh}$ chamber- and disease-related gradients. Because of a selective $I_{K,ACh}$ reduction in RA, AF-related remodeling strongly attenuated the right-to-left gradient of CCh-activated $I_{K,ACh}$. The lack of RA-dominant agonist-activated $I_{K,ACh}$ may play a permissive role for LA-dominant drivers in pAF and cAF, particularly in vagal contexts.

Novel Findings and Potential Significance

The precise mechanisms underlying AF-maintenance in humans are not fully understood. Consistent with the more rapid AF activity in the LA than in the RA[1-4,6,32] we noted a left-to-right inward-rectifier gradient in pAF patients. pAF patients with inducible AF after PV isolation often have localized high-frequency sources outside the PVs.[6,33,34] This observation may relate to

the left-to-right atrial gradient of inward-rectifier K^+-currents we observed in pAF. Targeting inward-rectifier K^+-currents may prove a new therapeutic approach for pAF.

Several studies suggest a complex activation pattern in cAF, with loss of spatiotemporal organization[1,6,35] and higher dominant frequencies than pAF in all atrial regions.[1,6] The comparable inward-rectifier currents in LA and RA of cAF patients, may provide a cellular explanation for the lack of left-to-right dominant-frequency gradients in cAF. The larger K^+-currents in RA of cAF vs. pAF patients may contribute to AF persistence by favoring more widespread driver-regions in cAF, or alternatively by promoting reentrant waves via abbreviated refractoriness. In addition, the fibrotic structural changes commonly seen in both atria with cAF[36] likely favor AF stabilization, potentially accounting for the greater chronicity in cAF patients compared to pAF patients.

Potential Limitations

We studied atrial myocytes from RA and LA appendages only. Therefore, our results cannot be directly extrapolated to other RA and LA regions.

We noted a right-to-left gradient of agonist-activated $I_{K,ACh}$ in SR that was attenuated in pAF and cAF patients. In vivo regulation of $I_{K,ACh}$ is more complex and chamber-specific regulation of $I_{K,ACh}$ may occur on various levels. The LA-RA distribution of parasympathetic neurons, choline acetyltransferase, acetylcholine content, and muscarinic receptors in human atria is unknown and should be addressed in future work.

We would have liked to examine the mechanistic basis of increased basal current in LA of pAF patients. Unfortunately, because of small sample size and limited access to LA biopsies we were not able to study channel expression in membrane fractions or to perform single-channel recordings to determine whether enhanced I_{K1} and/or constitutive $I_{K,ACh}$ contributes to increased basal current in pAF. The data we obtained in LA cardiomyocytes from cAF patients (Figure 5) suggest that constitutive $I_{K,ACh}$ increases in LA of cAF patients, but that it accounts for

a relatively small part of the large increases in basal current seen in cAF. Thus, most of the increase observed is likely due to I_{K1} up-regulation. While I_{K1} is also probably responsible for increased basal current in the LA of pAF patients, this idea remains to be tested in subsequent work.

The present study focused on RA-LA differences in inward-rectifier K^+-currents, which are only one factor contributing to the left-to-right dominant-frequency gradient underlying AF maintenance. LA-RA differences in distribution of sodium currents, delayed rectifier K^+-currents or other atrial currents may also contribute. In addition, we did not measure dominant-frequency gradients in the patients in which we recorded properties of inward-rectifier currents. Thus, direct correlations between distribution of dominant frequencies and properties of atrial currents in individual patients, which would be desirable to define the putative role of inward-rectifier currents for dominant-frequency gradients, are unavailable. Nevertheless, inward-rectifier currents remain an important determinant of atrial refractoriness and AF-promoting reentrant sources, and the left-atrial increases of inward rectifier currents noted in the present study are likely to shorten LA refractoriness, contributing significantly to the role of the LA in maintaining pAF.

We used CSQ as an internal standard for normalization of protein-expression data. Selection of internal standards for protein-expression correction in remodeled states must be judicious and requires that the standard is unaffected by the remodeling paradigm studied. In a previous investigation, we found atrial CSQ expression to be slightly but significantly lower in dogs with CHF compared to control dogs.[37] However, this difference is not seen in samples from AF vs. SR patients. We used CSQ for normalisation because we in previous publications protein levels of human atrial CSQ did not change during cAF[38] and these results were confirmed in samples from the present study (Online Figure 10A). GAPDH is often used as an internal standard. We measured GAPDH in samples from the present study (Online Figure 10B) and found that protein levels of GAPDH are strongly regulated in atria of AF patients, with reduced

expression in both atria of cAF patients and higher expression in the LA vs. RA of pAF patients. These data confirm the appropriateness of CSQ rather than other alternatives like GAPDH as an internal control for normalisation.

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Table 1: Characteristics of patients

	RA-SR	RA-pAF	RA-cAF	LA-SR	LA-pAF	LA-cAF
Patients, n	35	13	17	10	9	15
Gender, m/f	24/11	7/6	10/7	5/5	6/3	6/9
Age, y	67.9±1.5	73.9±2.0	70.4±1.8	53.5±3.6§	65.6±0.9*	66.3±1.8*
Body mass index, kg/m^2	27.0±0.6	27.5±1.3	27.0±0.8	26.9±1.4	26.4±2.0	27.7±1.3
CAD, n	16	7	2*#	3	1	3
MVD/AVD, n	14	2	12*#	4	6	10
CAD+MVD/AVD, n	5	4	3	3	2	2
Hypertension, n	28	10	16	5§	5	11
Diabetes, n	11	6	2#	1	1	6
Hyperlipidemia, n	26	9	12	7	3	13#
LVEF, %	55.8±1.7	45.6±3.5*	53.5±2.5	42.0±4.3§	53.3±2.9	43.1±4.5
LAD, mm	41.3±0.9	47.1±1.8	48.9±2.2*	45.6±2.0	48.3±2.7	50.8±1.6
LVEDD, mm	50.2±2.5	45.2±6.7	52.7±2.4	59.4±4.1	54.8±2.7	54.5±2.2
IVS, mm	11.6±0.3	13.9±1.4	13.0±0.7	10.2±0.6	12.9±0.9	11.2±0.6
LVPW, mm	11.2±0.3	12.9±0.9	12.3±0.6	10.2±0.6	11.7±0.6	11.2±0.4
Digitalis, n	1	3	7*	4§	1	8#
ACE-inhibitors, n	22	6	10	10§	5*	9
AT1-blockers, n	4	4	3	0	0	3
β-Blockers, n	28	9	15	9	6	14
Dihydropyridines, n	6	3	3	1	1	2
Diuretics, n	17	7	14*	8	5	10
Nitrates, n	9	5	6	1	7*	3#
Lipid-lowering drugs, n	22	9	9	7	3	10

CAD, coronary artery disease; MVD/AVD, mitral/aortic valve disease; LVEF, left-ventricular ejection fraction; LVEDD, left-ventricular end-diastolic diameter; LAD, left-atrial diameter; IVS, interventricular-septum thickness; LVPW, left-ventricular posterior-wall thickness; ACE, angiotensin-converting enzyme; AT, angiotensin-receptor.

*$P<0.05$ vs. SR, $^{\#}P<0.05$ vs pAF and §$P<0.05$ vs corresponding values in RA from ANOVA followed by Bonferroni-multiple comparisons procedure for continuous variables and from Fisher's exact test for categorical variables.

Figure legends:

Figure 1. A, Ramp protocol from -100 to +40 mV. **B-D**, Representative current recordings in right (RA) and left (LA) myocytes of SR, pAF and cAF. Basal current and carbachol (CCh, 2 µmol/L)-sensitive current increase ($I_{K,ACh}$) were analyzed at inward (I_{in}) and outward (I_{out}) branch at -100 mV and -10 mV, respectively.

Figure 2. Inward-rectifier currents in right and left atrial myocytes from SR, pAF and cAF at -100 mV and -10 mV, respectively. Mean±SEM. **A,** Basal current in absence of carbachol (CCh, 2 µmol/L). **B,** Total current (basal current+CCh-mediated current increase) in presence of CCh. Numbers indicate myocytes/patients. *P<0.05 and $^{\#}$P<0.05 vs. corresponding values in SR and pAF, respectively. §P<0.05 vs. corresponding values in right atrium.

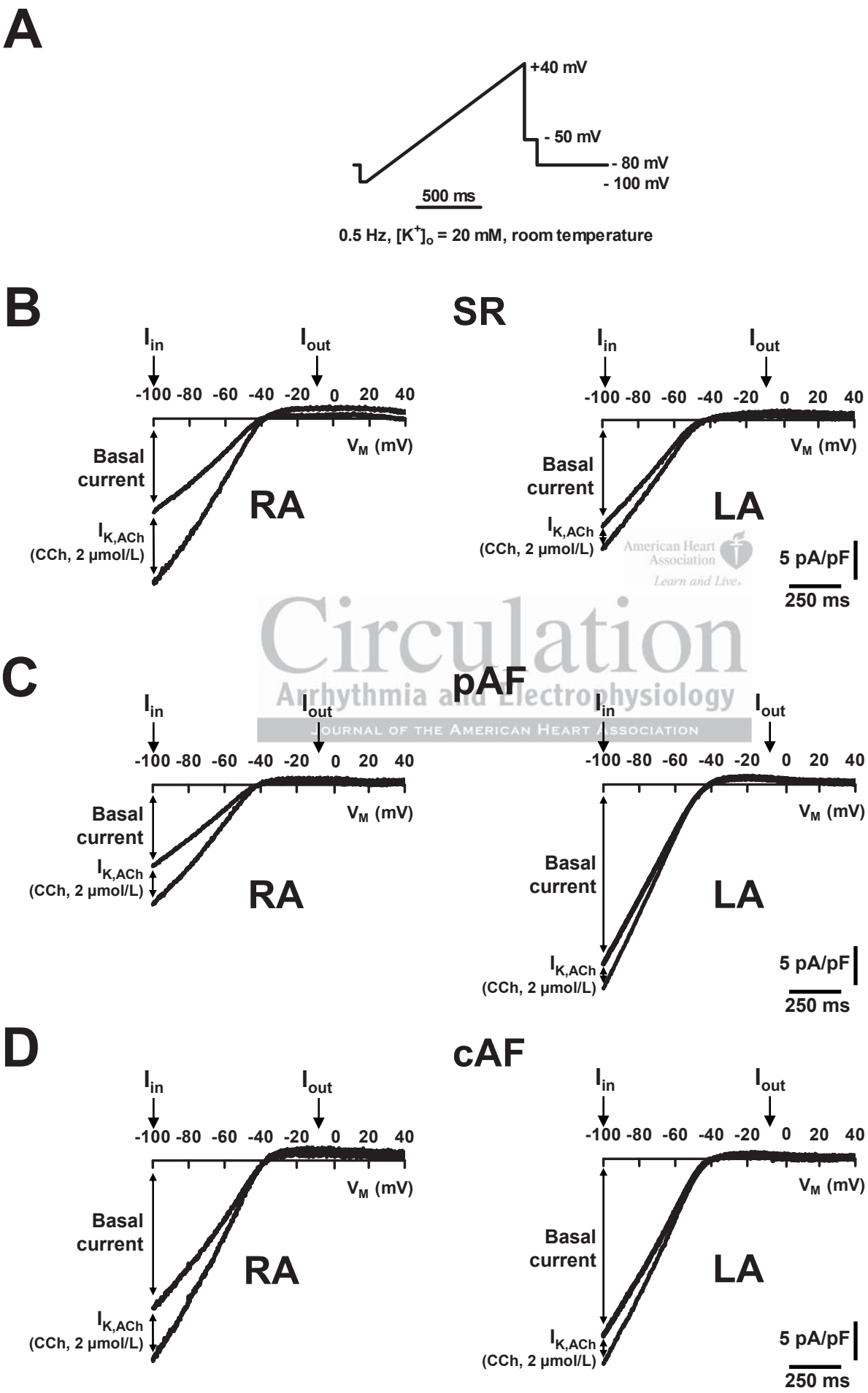
Figure 3. Representative time-course recordings of $I_{K,ACh}$ in right (RA) and left (LA) atrial myocytes from patients with SR (**A**), pAF (**B**) and cAF (**C**). Currents recorded during ramp-protocol (Figure 1) were analyzed continuously (0.5 Hz) at -100 mV. $I_{K,ACh}$ was activated with the muscarinic receptor agonist carbachol (CCh, 2 µmol/L) and was defined as CCh-sensitive current component. Despite the continuous presence of CCh during 2 min the initial increase (Peak-$I_{K,ACh}$) faded to a quasi-steady-state level (QSS-$I_{K,ACh}$) due to a process termed "desensitization".

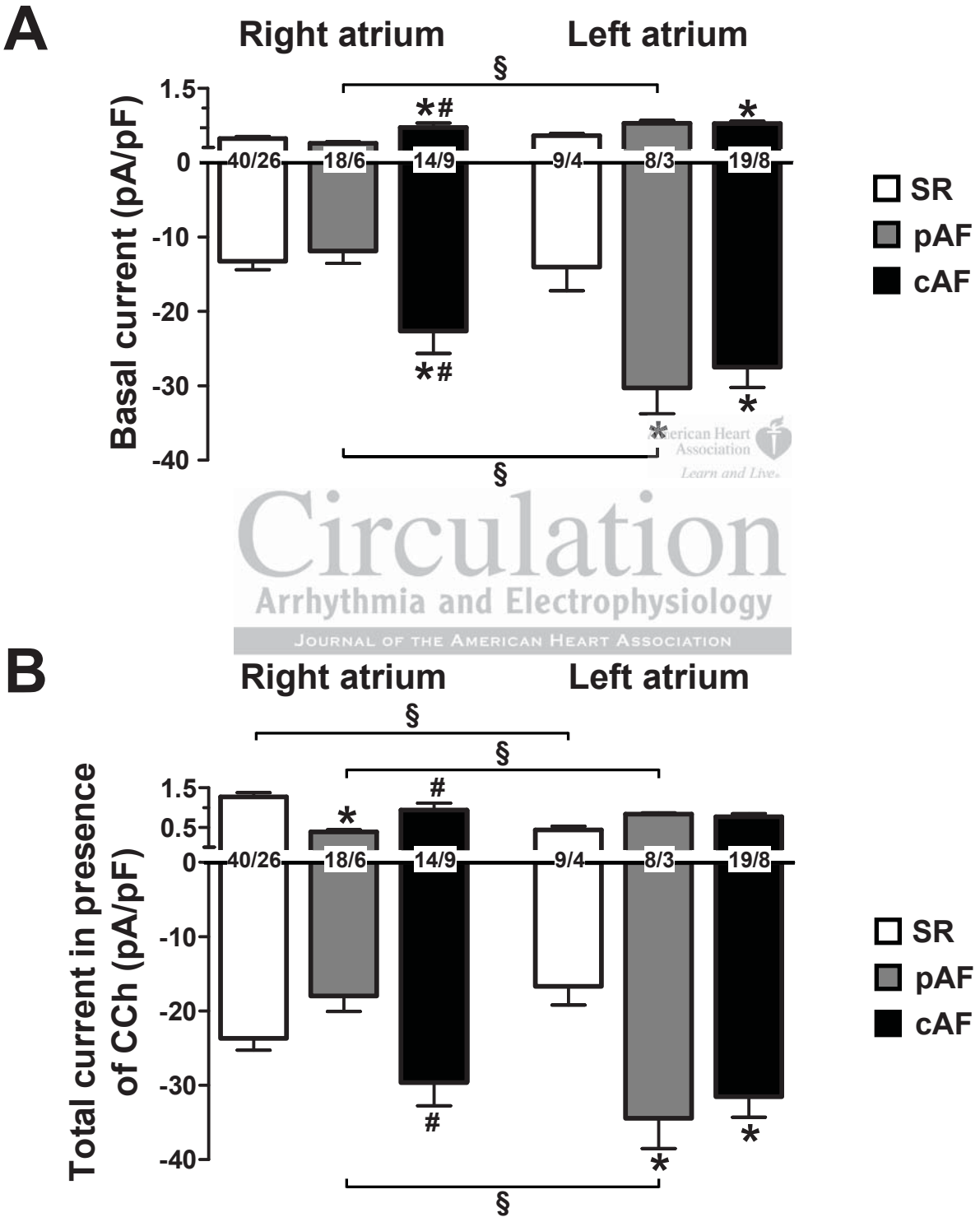
Figure 4. Carbachol (CCh, 2 µmol/L)-sensitive current increase ($I_{K,ACh}$) in right and left atrial myocytes from SR, pAF and cAF at -100 mV and -10 mV, respectively. Mean±SEM of "Peak" (**A**) and quasi-steady-state (QSS, **B**) current levels. Numbers indicate myocytes/patients. *P<0.05 vs. corresponding values in SR. §P<0.05 vs. corresponding values in right atrium.

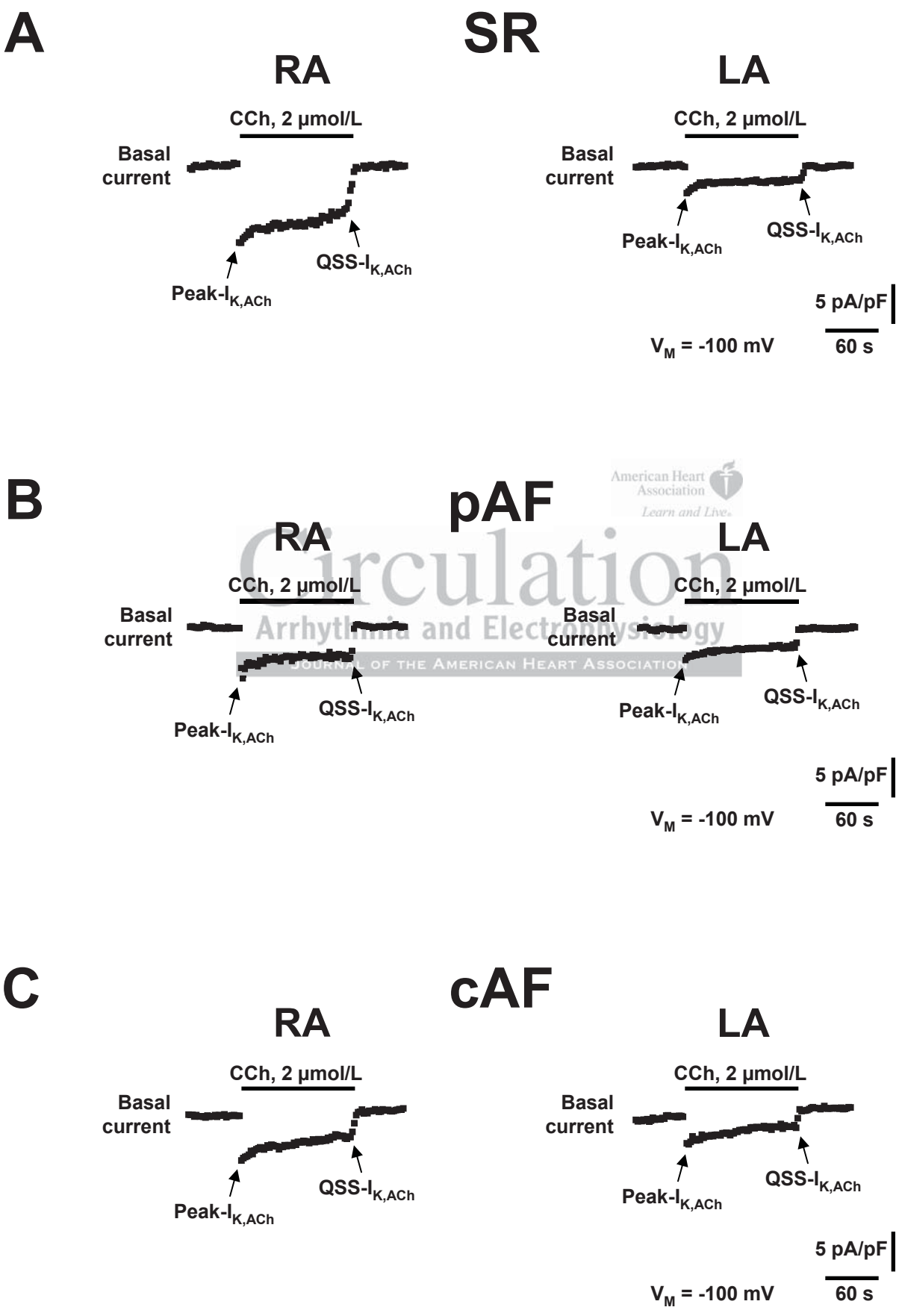
Figure 5. Effect of the $I_{K,ACh}$-channel blocker tertiapin (10 nmol/L) on basal current in left-atrial myocytes. Left-atrial basal current at -100 mV before (baseline) and during tertiapin application in SR and cAF. Numbers indicate myocytes/patients. *P<0.05 vs. baseline.

Figure 6. Expression of I_{K1}-channel subunits in right and left atria from SR, pAF and cAF. Representative immunoblots and densitometric analysis of Kir2.1 (**A**) and Kir2.3 (**B**) subunits. Numbers indicate tissue samples. *P<0.05 and $^{\#}$P<0.05 vs. corresponding values in SR and pAF, respectively. §P<0.05 vs. corresponding values in right atrium.

Figure 7. Expression of $I_{K,ACh}$-channel subunits in right and left atria from SR, pAF and cAF. Format as in Figure 6. *P<0.05 vs. corresponding values in SR. §P<0.05 vs. corresponding values in right atrium.







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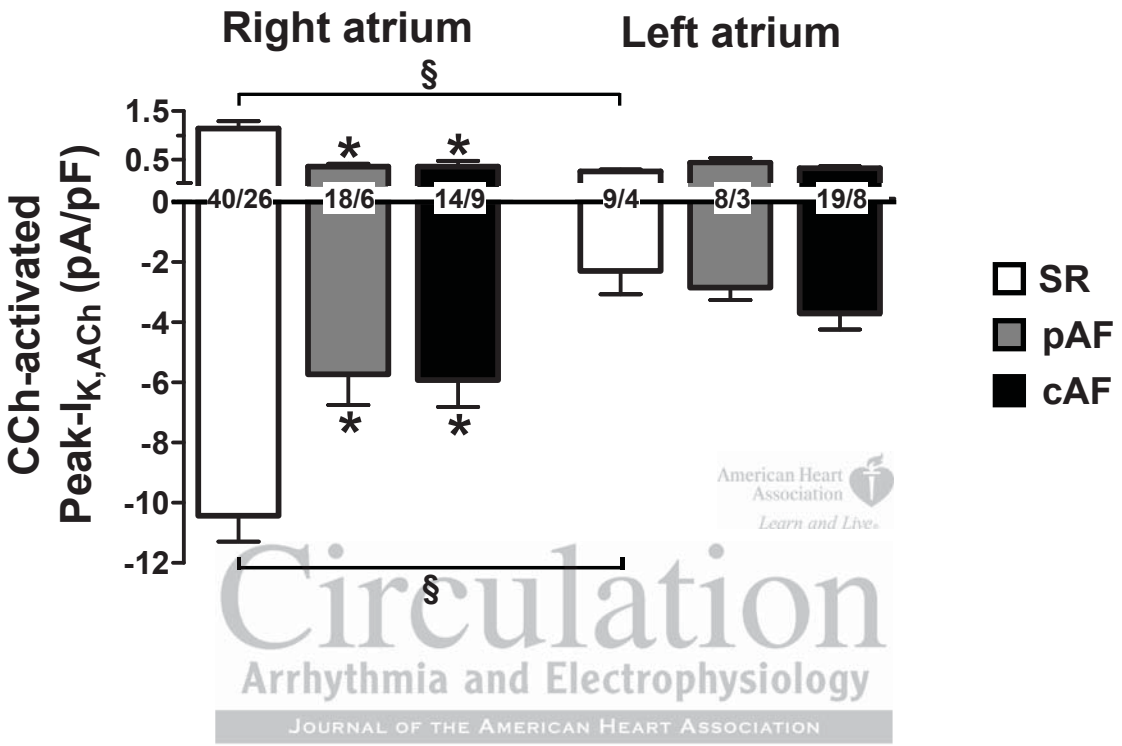

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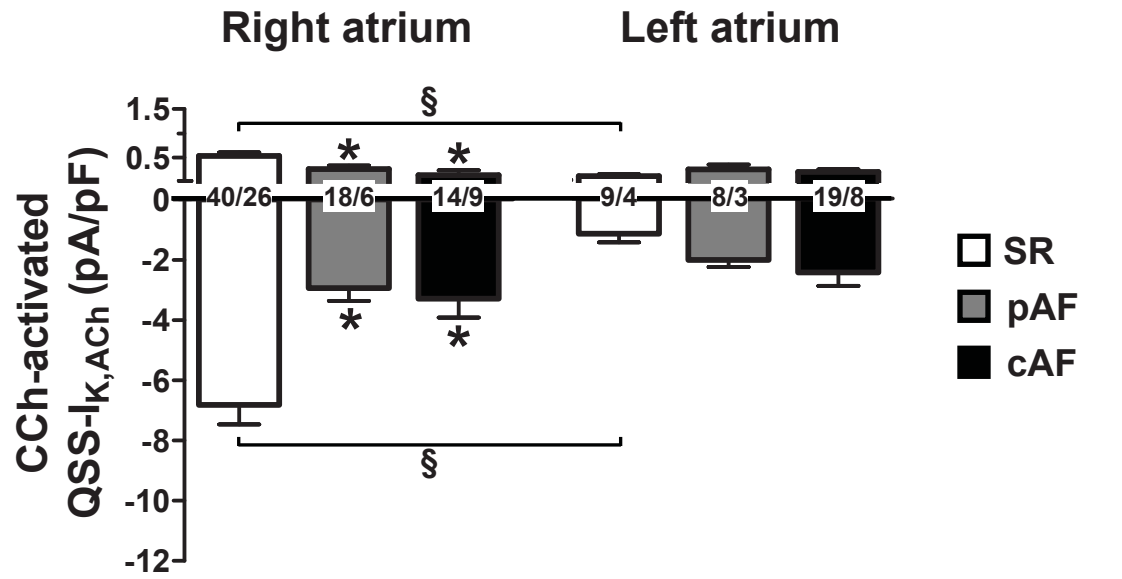

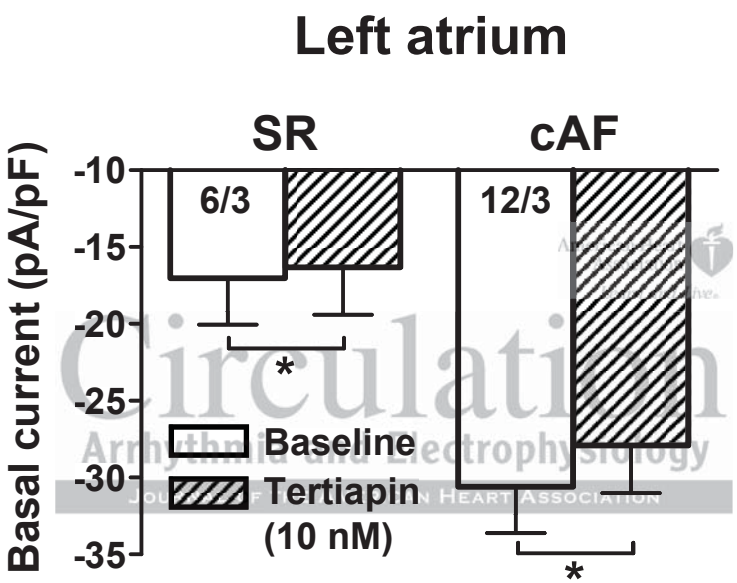

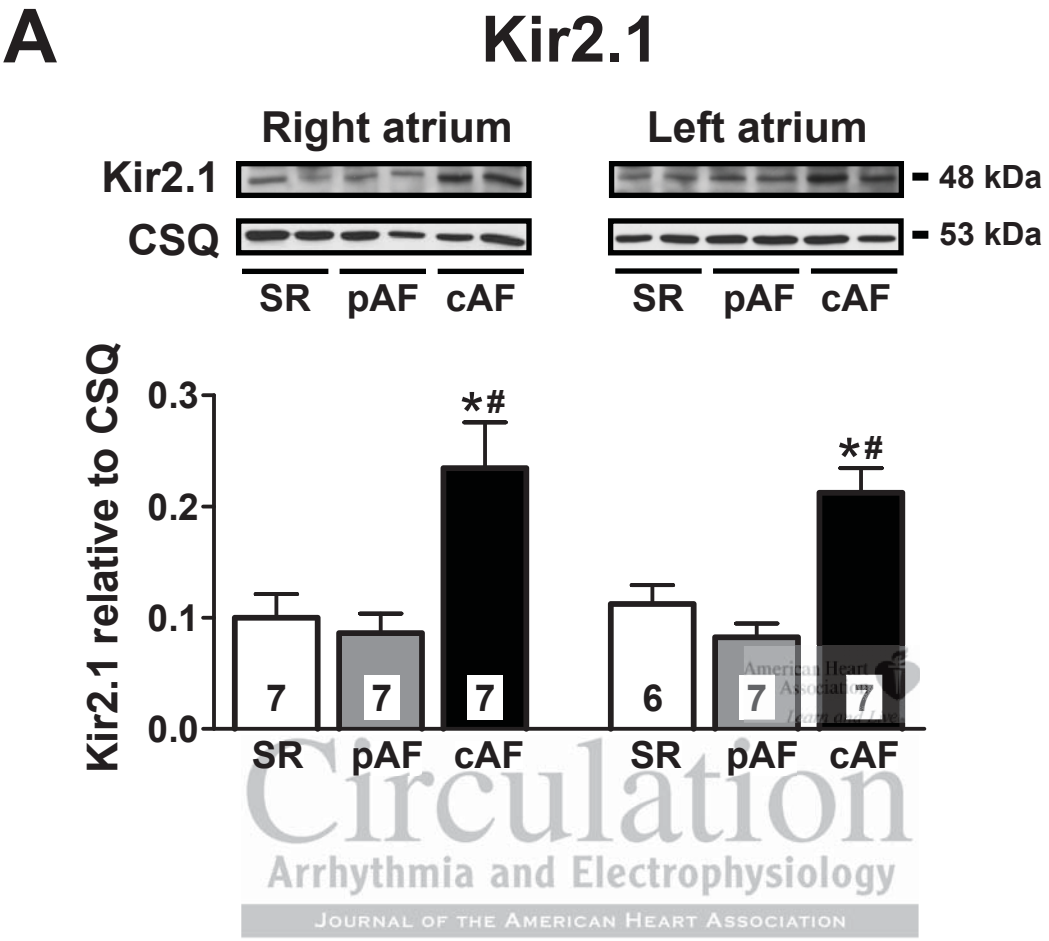

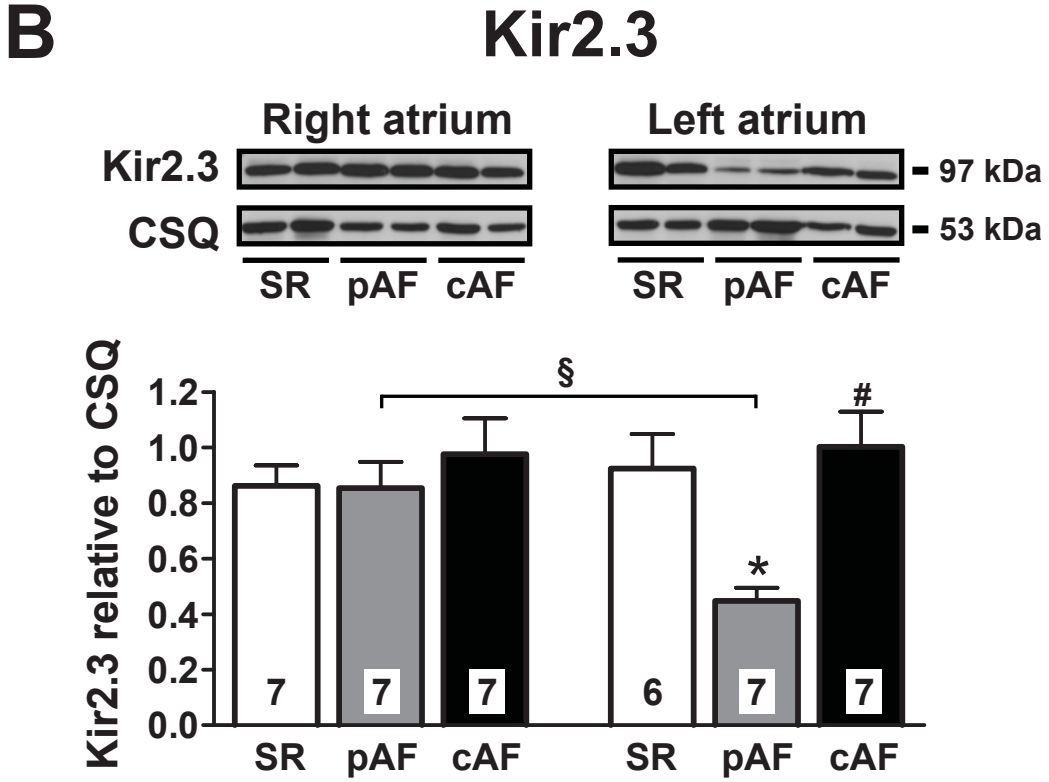

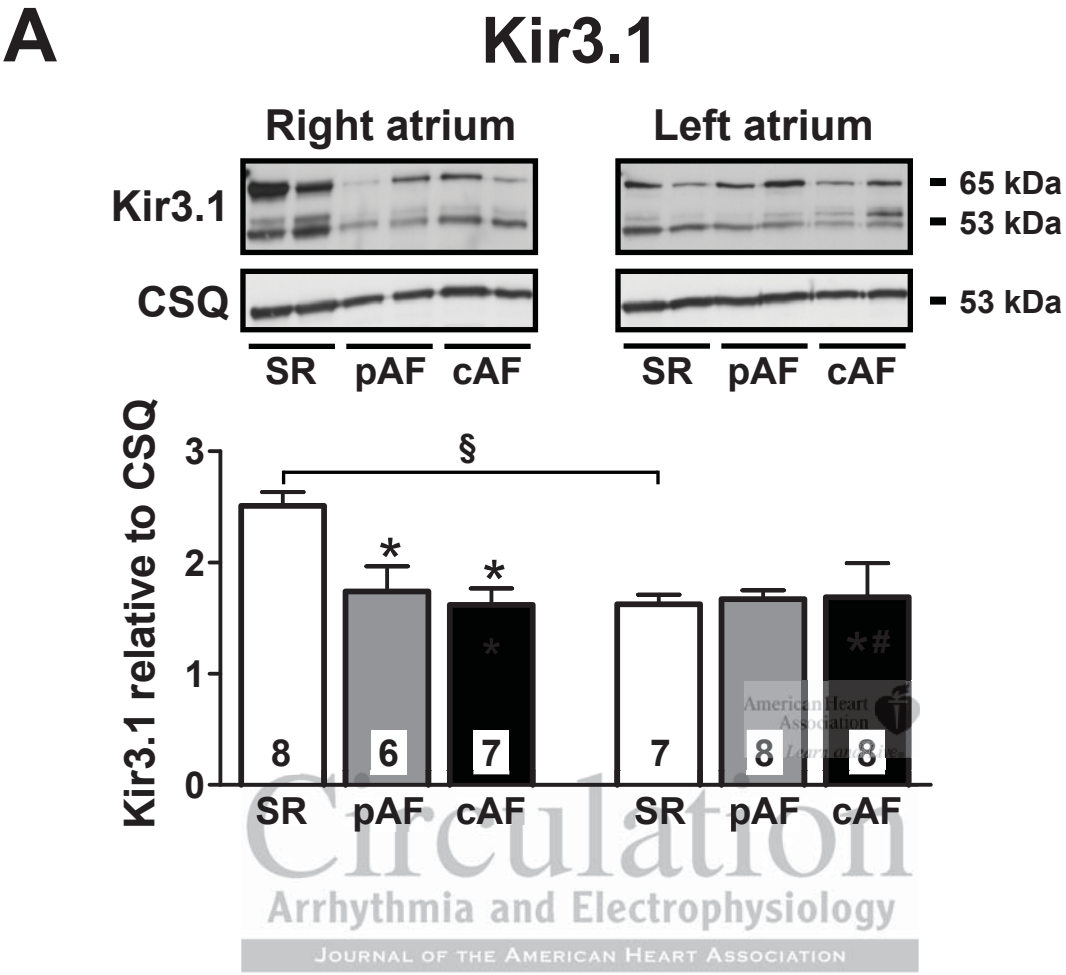

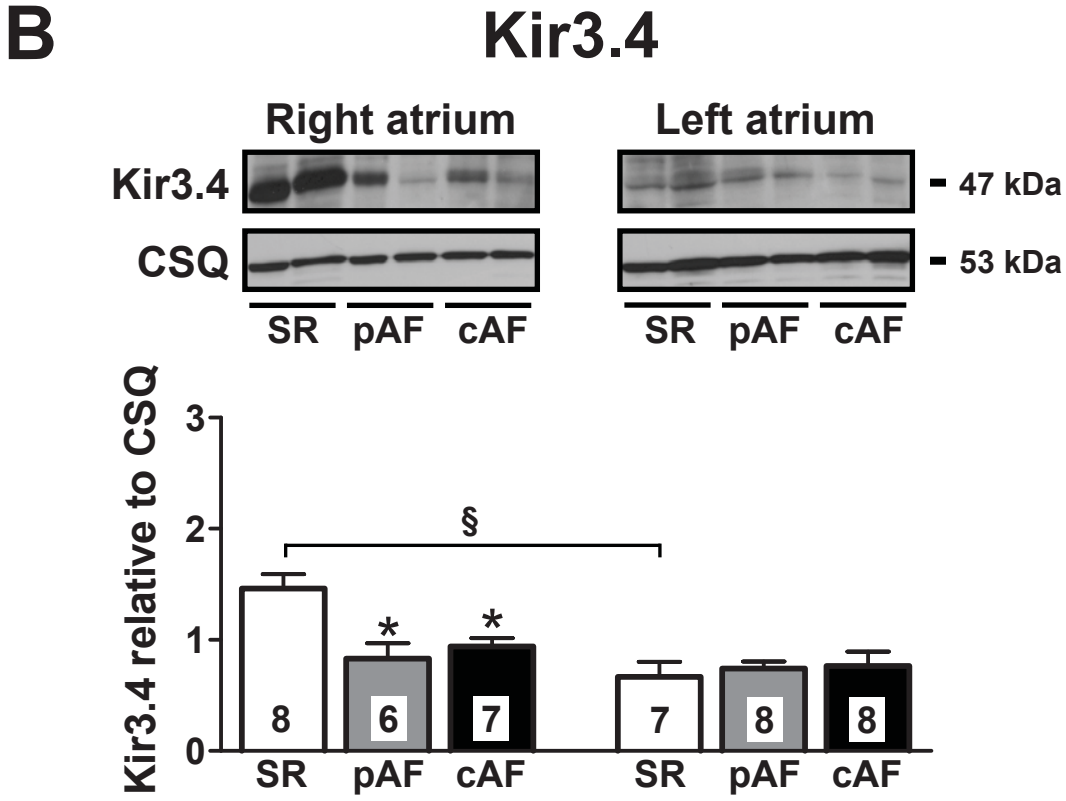

Left-to-Right Atrial Inward-Rectifier Potassium Current Gradients in Patients with Paroxysmal Versus Chronic Atrial Fibrillation

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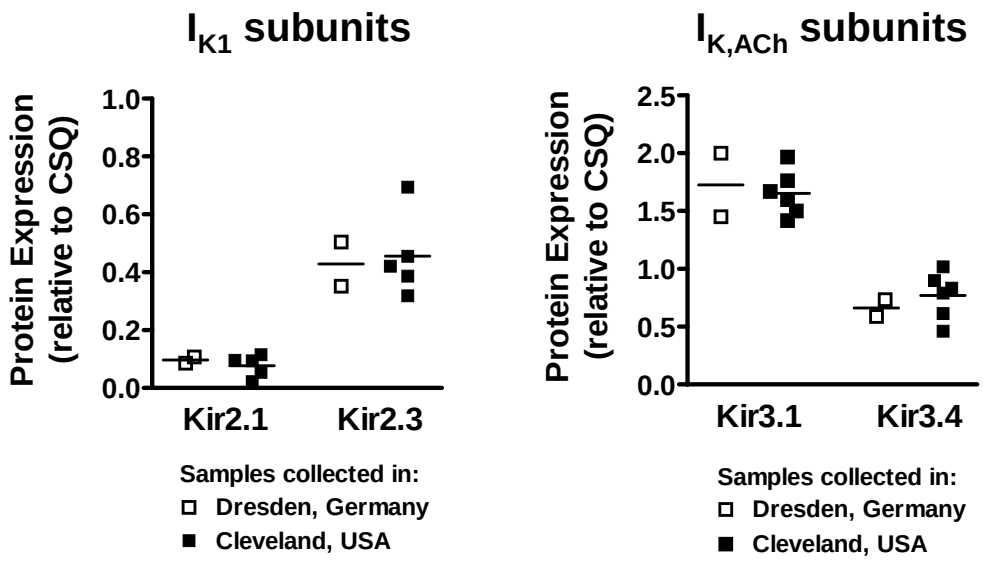
SUPPLEMENTAL MATERIAL

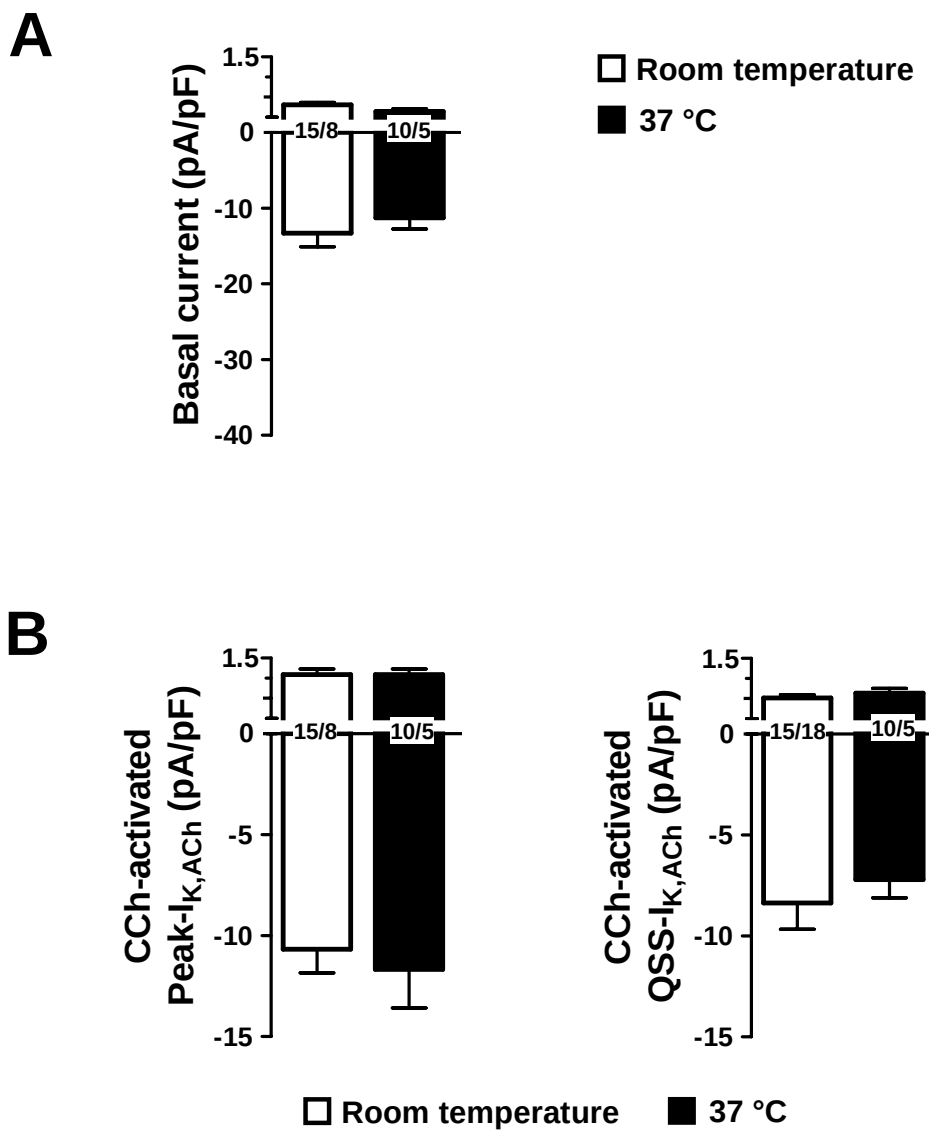
Online Table: Characteristics of patients with normal (non-diseased) atria

	Atrium	Gender	Age	Cause of death	Drugs
1.	RA+LA	female	41	*unknown*	Dopamine
2.	RA+LA	female	18	Open skull fracture	Dopamine, Diuretics
3.	RA+LA	male	18	Cerebral contusion	Dopamine
4.	RA	female	50	Ruptured cerebral aneurysm	Dopamine, Antibiotics
5.	LA	female	42	SAH	Dopamine, Diuretics

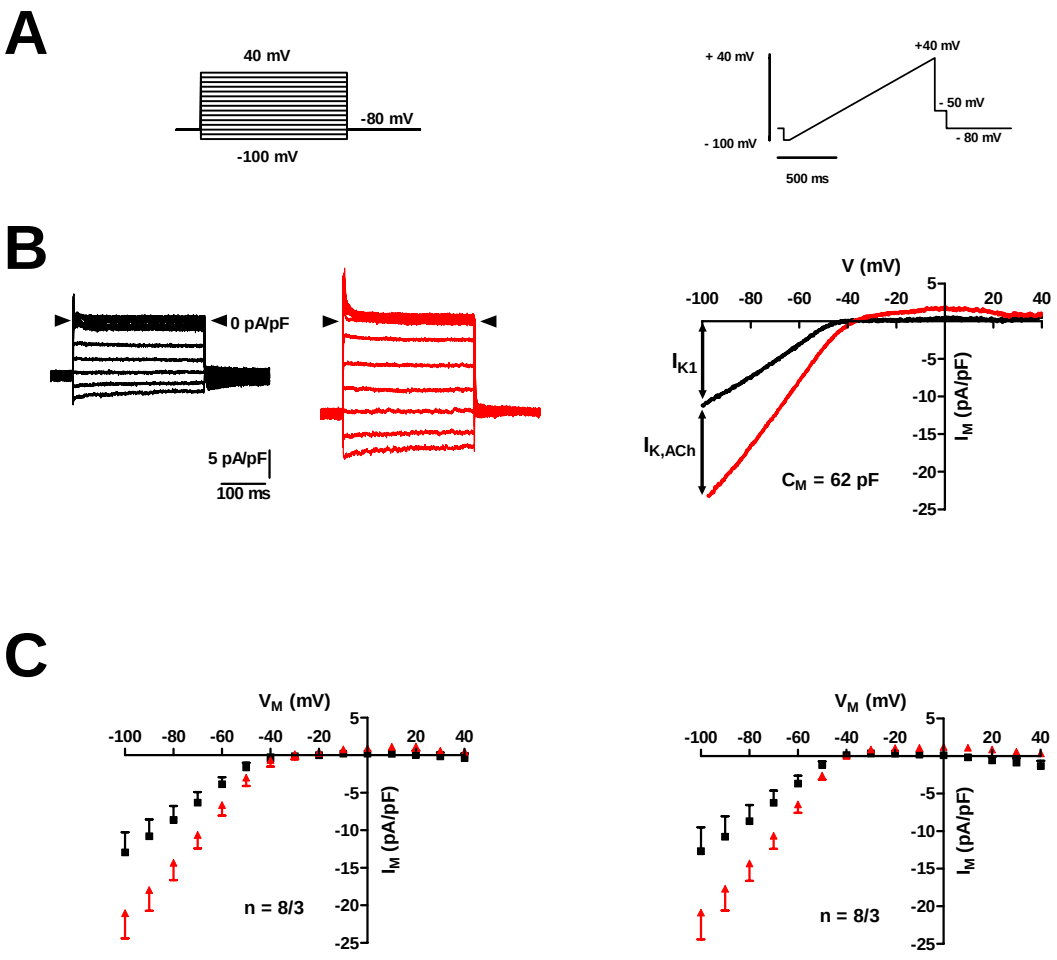
RA, right atrium; LA, left atrium; SAH, Subarachnoid haemorrhage



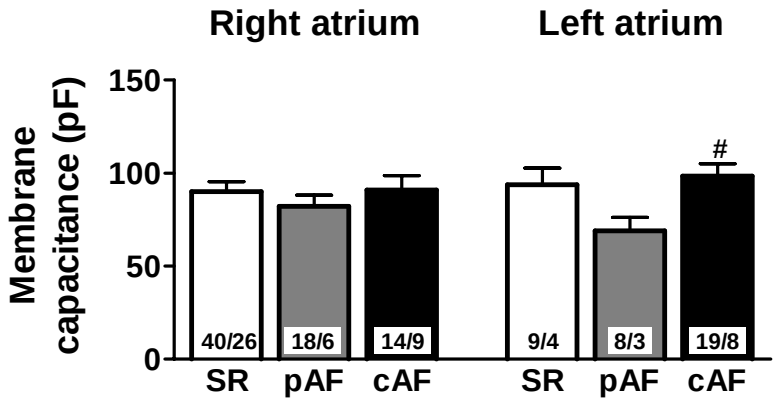
Online Figure 1. Center-related comparison of protein expression in left atrial samples from patients with pAF.



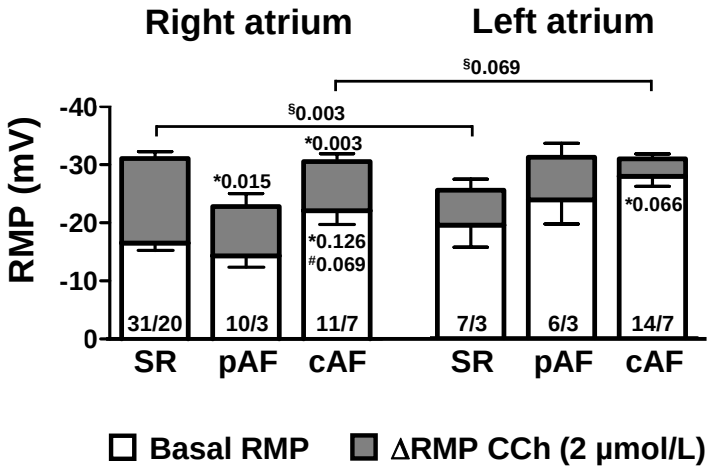
Online Figure 2. Temperature dependence of basal (**A**) inward rectifier current and CCh-activated $I_{K,ACh}$ (**B**) in SR patients. Mean±SEM. Numbers indicate myocytes/patients.



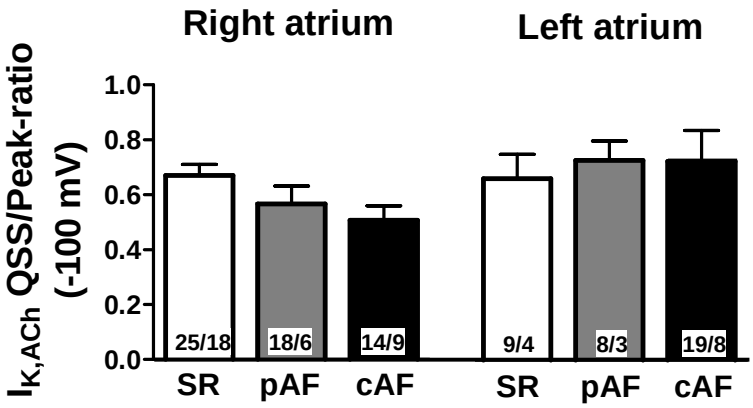
Online Figure 3. Comparison of current-voltage relation curves obtained with step pulse (**A, left panel**) and ramp-pulse (**A, right panel**) protocol respectively. **B,** Corresponding currents in absence (black) and presence (red) of CCh (2 µM) to activate $I_{K,ACh}$. **C,** Corresponding current voltage relationship. Mean±SEM. Numbers indicate myocytes/patients.



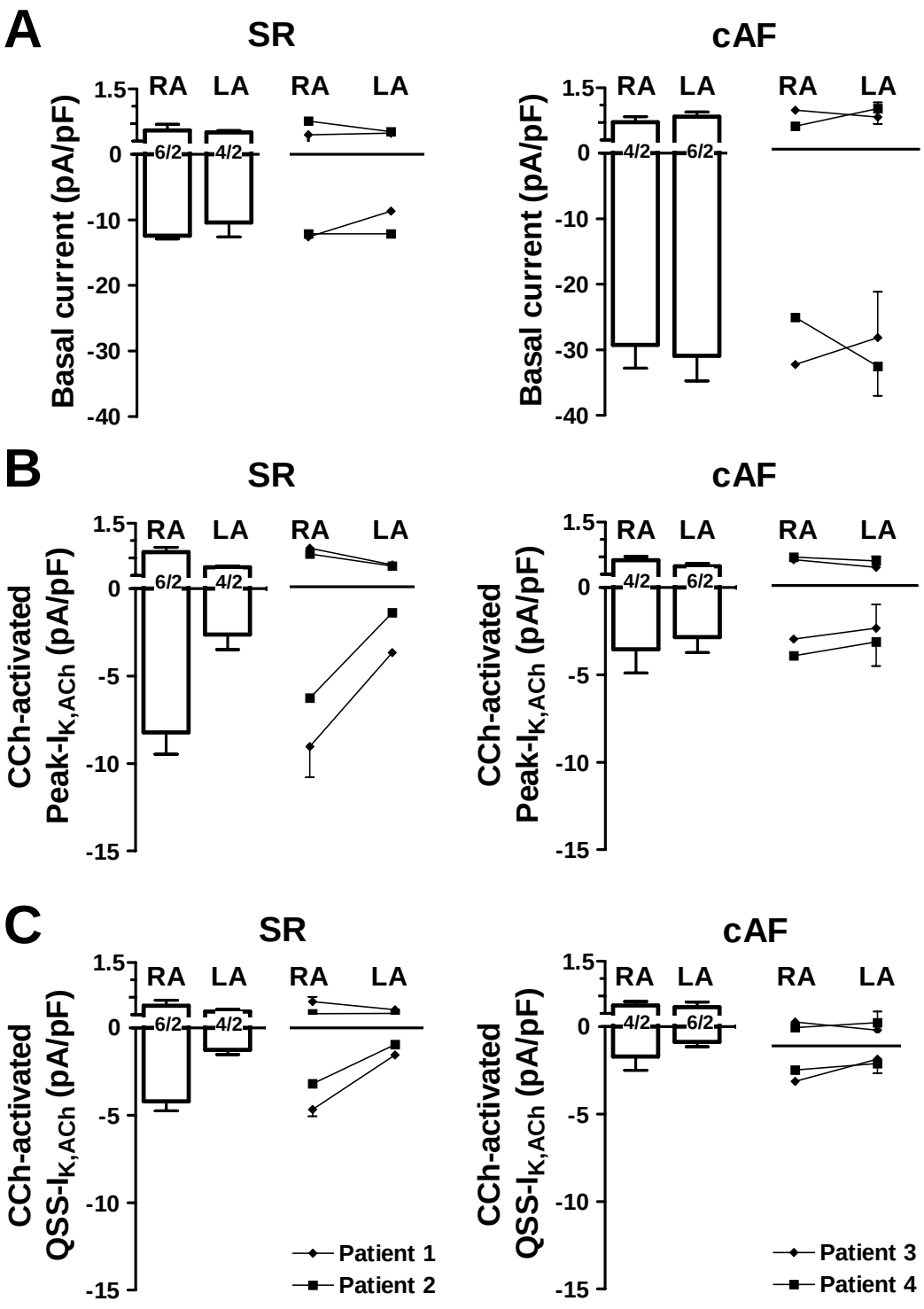
Online Figure 4. Membrane capacitance measurements in right and left atrial appendages from patients with SR, pAF and cAF. Mean±SEM. Numbers indicate myocytes/patients. $^{\#}P<0.05$ vs. corresponding values in pAF.



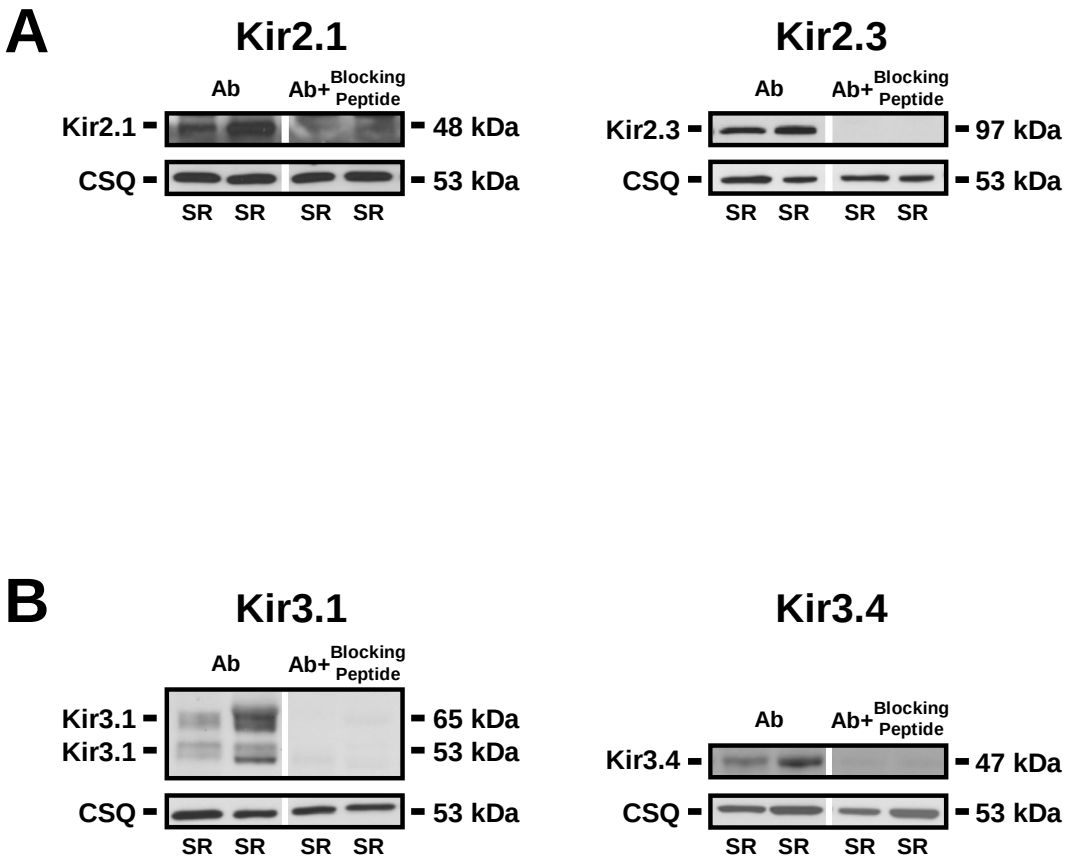
Online Figure 5. Basal resting membrane potential (basal RMP) and CCh-induced hyperpolarisation (ΔRMP CCh) in right and left atrial myocytes from patients with SR, pAF and cAF. Numbers indicate myocytes/patients. Mean±SEM. *P<0.05 and #P<0.05 vs. corresponding values in SR and pAF. §P<0.05 vs. corresponding values in right or left atrium, respectively. P-values within columns refer to basal RMP, whereas all other P-values refer to ΔRMP CCh.



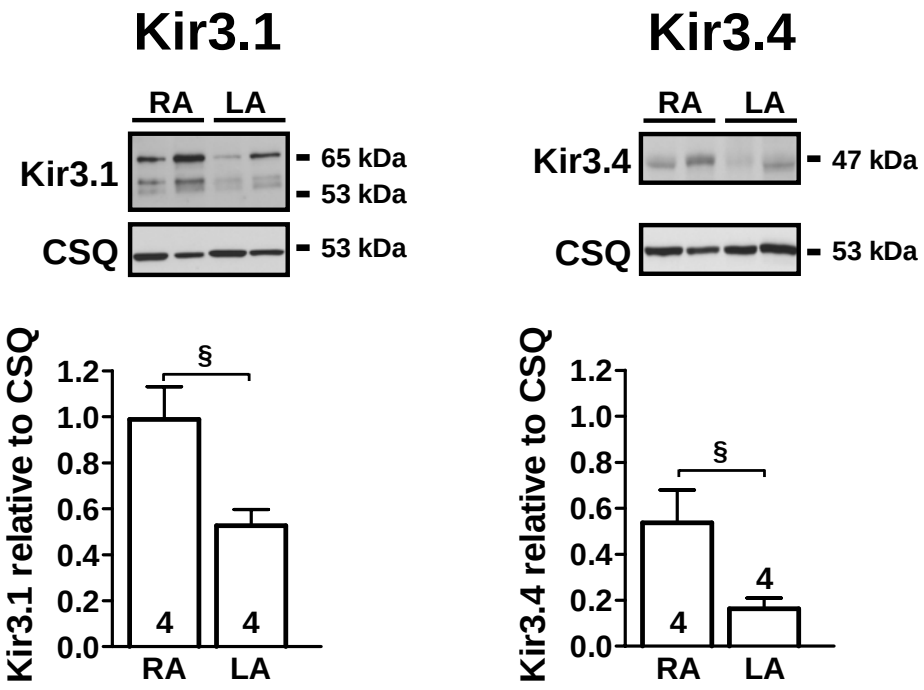
Online Figure 6. Mean±SEM of $I_{K,ACh}$ Peak/QSS-ratio as an index of $I_{K,ACh}$ desensitization (see Figures 3 and 4). Numbers indicate myocytes/patients.



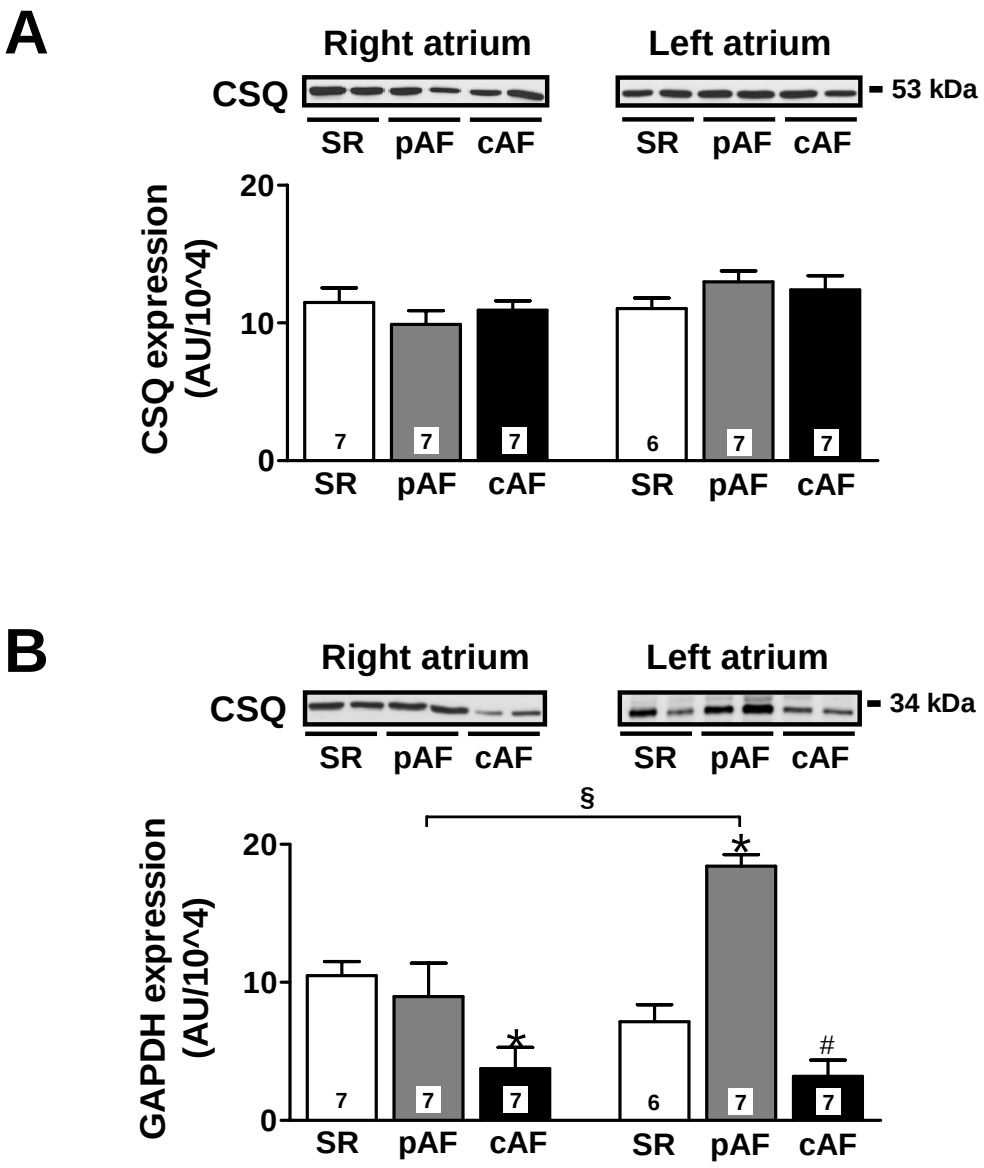
Online Figure 7. Basal inward rectifier current (**A**) and $I_{K,ACh}$ (**B, C**) from matched RA and LA samples obtained from the same SR (left, n=2) and cAF (right, n=2) patients. Columns represent Mean±SEM. Numbers indicate myocytes/patients. Symbols indicate corresponding mean values of each patient.



Online Figure 8. Specificity of used antibodies for Western blot. Specificity of bands recognized by the specific antibodies against Kir2.1, Kir2.3 **(A)**, Kir3.1 and Kir3.4 **(B)** is demonstrated by incubation of the individual antibodies (Ab) with their corresponding immunizing peptides (Ab+Blocking Peptide) which prevents binding of the antibodies to their protein targets. Calsequestrin (CSQ) levels were used as internal (loading) controls. Non-contiguous lanes are separated by white lines.



Online Figure 9. Expression of $I_{K,ACh}$-channel subunits in right (RA) and left (LA) atrial appendages from normal (non-diseased) atria. Representative immunoblots and densitometric analysis of Kir3.1 (left) and Kir3.4 (right) subunits. Numbers indicate tissue samples. $^{§}P<0.05$ vs. corresponding values in right atrium.



Online Figure 10. Comparison of CSQ (**A**) and GAPDH (**B**) protein levels (arbitrary units, AU) in right (RA) and left (LA) atria from SR, pAF and cAF patients. *P<0.05 vs. corresponding SR and pAF; #P<0.05 vs. corresponding pAF; §P<0.05 vs. pAF in RA.