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Phosphodiesterase 8 governs cAMP/PKA-dependent reduction of L-type calcium current in human atrial fibrillation: a novel arrhythmogenic mechanism

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Abstract

Aims Atrial fibrillation (AF) is associated with altered cAMP/PKA signaling and an AF-promoting reduction of L-type Ca^{2+}-current ($I_{Ca,L}$), the mechanisms of which are poorly understood. Cyclic-nucleotide phosphodiesterases (PDEs) degrade cAMP and regulate PKA-dependent phosphorylation of key calcium-handling proteins, including the $I_{Ca,L}$-carrying Cav1.2$_{\alpha 1C}$ subunit. The aim was to assess whether altered function of PDE type-8 (PDE8) isoforms contributes to the reduction of $I_{Ca,L}$ in persistent (chronic) AF (cAF) patients.

Methods and results mRNA, protein levels, and localization of PDE8A and PDE8B isoforms were measured by RT-qPCR, western blot, co-immunoprecipitation and immunofluorescence. PDE8 function was assessed by FRET, patch-clamp and sharp-electrode recordings. PDE8A gene and protein levels were higher in paroxysmal AF (pAF) *vs.* sinus rhythm (SR) patients, whereas PDE8B was upregulated in cAF only. Cytosolic abundance of PDE8A was higher in atrial pAF myocytes, whereas PDE8B tended to be more abundant at the plasmalemma in cAF myocytes. In co-immunoprecipitation, only PDE8B2 showed binding to Cav1.2$_{\alpha 1C}$ subunit which was strongly increased in cAF. Accordingly, Cav1.2$_{\alpha 1C}$ showed a lower phosphorylation at

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Ser1928 in association with decreased $I_{Ca,L}$ in cAF. Selective PDE8 inhibition increased Ser1928 phosphorylation of Cav1.2$_{\alpha 1C}$, enhanced cAMP at the subsarcolemma and rescued the lower $I_{Ca,L}$ in cAF, which was accompanied by a prolongation of action potential duration at 50% of repolarization.

Conclusion Both PDE8A and PDE8B are expressed in human heart. Upregulation of PDE8B isoforms in cAF reduces $I_{Ca,L}$ via direct interaction of PDE8B2 with the Cav1.2$_{\alpha 1C}$ subunit. Thus, upregulated PDE8B2 might serve as a novel molecular mechanism of the proarrhythmic reduction of $I_{Ca,L}$ in cAF.

Structured Graphical Abstract

Key Question

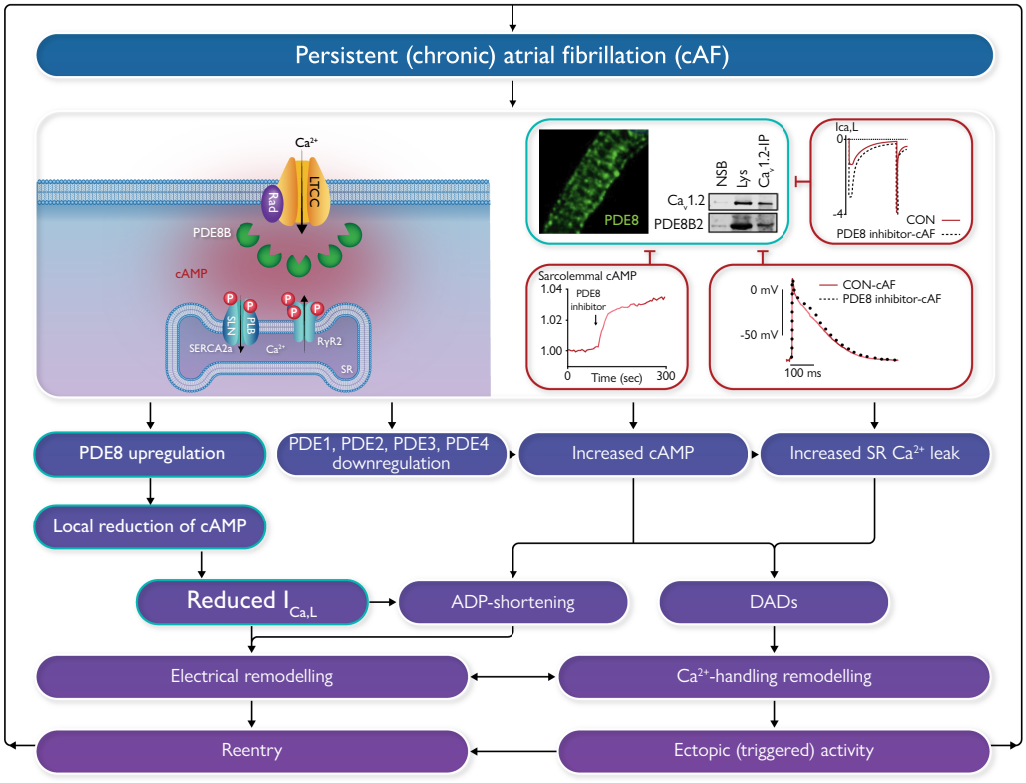
What is the molecular mechanism of reduced L-type calcium current that promotes persistent atrial fibrillation?

Key Finding

Cyclic-nucleotide phosphodiesterase 8B (PDE8B) was expressed in the human atria and upregulated in persistent atrial fibrillation, contributing to the proarrhythmic reduction of L-type calcium current by decreasing sarcolemmal cAMP levels.

Take Home Message

Targeting PDE8B may represent a new atrial-selective therapeutic strategy for atrial fibrillation.



The reduction of L-type Ca^{2+}-current in atrial myocytes promotes atrial fibrillation. Phosphodiesterase type 8B binds L-type Ca^{2+} channels and reduces local cAMP levels and PKA-dependent channel phosphorylation, thereby decreasing L-type Ca^{2+}-current and abbreviating atrial action potential that promotes atrial fibrillation persistence. ADP, adenosine diphosphate; cAF, persistent (chronic) atrial fibrillation; cAMP, cyclic adenosine monophosphate; DAD, delayed afterdepolarization; $I_{Ca,L}$, L-type Ca^{2+} current; LTCC, L-type Ca^{2+} channel; PDE, phosphodiesterase; PLB, phospholamban; RyR2, ryanodine receptor type 2; SERCA2a, sarcoplasmic reticulum Ca^{2+} ATPase type 2a; SLN, sarcolipin; SR, sinus rhythm.

Keywords Phosphodiesterases • PDE8 • cAMP • Atrial fibrillation • Calcium handling • $I_{Ca,L}$

Translational perspective

The mechanism of the proarrhythmic reduction of L-type calcium current ($I_{Ca,L}$) in patients with persistent (chronic) atrial fibrillation (cAF) remains elusive. Here we show that patients with cAF exhibit a stronger binding of phosphodiesterase 8B2 (PDE8B2) to the pore-forming Cav1.2$_{\alpha 1C}$ subunit of $I_{Ca,L}$. Selective PDE8 inhibition enhanced Ser1928 phosphorylation of Cav1.2$_{\alpha 1C}$, increased subsarcolemmal cAMP, and rescued the reduced $I_{Ca,L}$, leading to action potential prolongation in cAF atria. We thus discovered a new molecular mechanism for the proarrhythmic reduction of $I_{Ca,L}$ in cAF and established PDE8B2 as a novel atrial-selective target for cAF treatment.

Introduction

Atrial fibrillation (AF) is a common disease with poorly understood pathophysiology.[1,2] Remodeling of membrane receptors and alterations in 3′,5′-cyclic adenosine monophosphate (cAMP)-dependent regulation of Ca^{2+} handling mechanisms[3,4] are considered important contributors to AF promotion. For instance, upregulated sarcoplasmic reticulum Ca^{2+} ATPase type 2a (SERCA2a)[3,5] and PKA-dependent hyperphosphorylation of ryanodine receptor type 2 (RyR2),[6–8] are hallmarks of AF-related remodeling. Indeed, cAMP levels are increased in long-standing persistent (chronic) AF (cAF),[3] probably due to a reduction of cAMP-hydrolyzing phosphodiesterases (PDEs).[9] Decreased L-type Ca^{2+} current ($I_{Ca,L}$) density[3,10] linked to PKA-dependent dephosphorylation of L-type Ca^{2+} channel (LTCC) macromolecular complex[11] is another feature of AF. Although type 2A protein phosphatase (PP2A) activity in AF is globally increased,[12] the inhomogeneous AF-associated changes of protein phosphorylation point to a local regulation of PKA within distinct subcellular compartments.

cAMP levels, and thus the degree of PKA activation, are tightly regulated by PDEs that degrade cAMP in subcellular nanodomains.[13] So far, most of our knowledge of cAMP compartmentation was predominantly obtained in the ventricular myocytes where PDE3 and PDE4 are considered to be the main contributors to total cAMP-hydrolysis.[14,15] PDE3 is predominant in microsomal fractions in large mammals,[16] with PDE4 accounting for only ≈20% of the total PDE activity in canine ventricles.[17] Our previous studies demonstrated that although all the classical cardiac PDEs (PDE1–4) are expressed in human atria,[9,18] PDE3 and PDE4 account for only 45% and 15% of total cAMP-hydrolysis, respectively, with a large portion of cAMP-hydrolysis remaining unexplained.[9] In rodent[19–21] and human[20,22] ventricles, PDE4 was found to be associated with β-adrenoceptors, LTCC, RyR2 and phospholamban (PLN)-SERCA2 complexes. Previously, we could show that pharmacological inhibition of PDE4 increased the amplitude of $I_{Ca,L}$ and frequency of potentially proarrhythmic Ca^{2+}-sparks and Ca^{2+}-waves in human atrial myocytes (HAMs) of sinus rhythm (SR) patients.[7] PDE3 and PDE2 may also contribute to $I_{Ca,L}$ regulation of HAMs.[23] The total cAMP-hydrolytic activity in response to the unselective PDE inhibitor IBMX, which inhibits PDE1–4, is reduced in AF compared to SR patients,[9] suggesting that a reduction in PDE1–4 activity may contribute to RyR2 hyperphosphorylation and the related RyR2-mediated Ca^{2+}-dependent triggered activity in AF.[6,7] However, this global reduction of PDE activity is inconsistent with the postulated cAF-associated reduction of LTCC,[11,12] pointing to a contribution of additional PDE families. Since PDE8 is not inhibited by IBMX,[24] an increase in PDE8 activity could underlie the decrease of $I_{Ca,L}$ in cAF. The PDE8 family is encoded by the two genes, PDE8A and PDE8B, of which only PDE8A was found in human ventricles.[25,26] We recently showed that PDE8A is also expressed in human atria.[18] A previous study demonstrated that PDE8A is involved in the regulation of Ca^{2+} homeostasis in mouse ventricular myocytes.[27] Whether PDE8B is expressed in human atria and how PDE8 isoforms may contribute to the proarrhythmic reduction of $I_{Ca,L}$ in cAF is unknown and was the object of this study.

Here we show that both PDE8A and PDE8B subfamilies are present in HAMs. Patients with cAF exhibited a stronger binding of a specific PDE8B2 isoform to the pore-forming Cav1.2$_{\alpha 1C}$ subunit of LTCC. Selective PDE8 inhibition enhanced Ser1928 phosphorylation (pS1928) of Cav1.2$_{\alpha 1C}$, increased subsarcolemmal cAMP, and rescued the lower $I_{Ca,L}$, leading to action potential (AP) prolongation in cAF atria. This provides a new molecular mechanism for the proarrhythmic reduction of $I_{Ca,L}$ in cAF with PDE8B2 as a potential novel atria-selective target for cAF treatment.

Methods

Study design and human atrial samples

Right atrial (RA) tissues were obtained from patients in SR, with paroxysmal AF (pAF), or with cAF who had undergone open-heart surgery. All available RA appendages were used consecutively. Of the 165 RA samples obtained, 32 were used for qPCR, 69 for biochemical experiments, 47 for HAM isolation (thereof 19 for immunofluorescence, 18 for live cell imaging, and 10 for patch-clamp), and 17 for AP recordings (see Supplementary data online, *Tables S1–S8*). Experimental protocols were approved by ethical boards of University Hospital Essen (#12-5268-BO) and Medical Chamber Hamburg (WF-088/18) and were conducted in accordance with the Declaration of Helsinki. Each individual provided written informed consent. In addition, we included RA and left ventricular (LV) tissues from eight healthy (Ctl) and eight heart failure (HF) patients undergoing heart transplantation. This study was approved by the Scientific Board at the Hungarian Ministry of Health (ETT-TUKEB:4991-0/2010-1018EKU). Patient characteristics are provided for all samples, samples used for qPCR, immunoblotting, immunostaining, FRET, patch-clamp, and AP recordings (see Supplementary data online, *Tables S1–S8*).

RNA isolation, cDNA synthesis, real-time qPCR and quantification

Frozen tissue samples from 48 patients (16 SR, 8 pAF, 8 cAF, 8Ctl and 8 HF patients) were used for real-time PCR assays performed as previously described[18] to study the expression of 2 target genes (*PDE8A*, *PDE8B*) related to specifically chosen[28] reference genes (*POLR2A*, *YWHAZ*, *GAPDH*, *IPO8*, *PPIA*). Further experimental details are available in the online Supplement.

Western blot analysis and co-immunoprecipitation

54 snap-frozen atrial samples were used to perform western blot analysis. From those, 12 samples were, initially, incubated for 5 min with control buffer (CON), 30 nM PF-04957325 (PF), or 100 nM β-adrenergic agonist isoprenaline (ISO), and then snap-frozen. Another 15 snap-frozen samples were homogenized in immunoprecipitation buffer and used for co-immunoprecipitation experiments. Further experimental details are available in the online Supplement.

Isolation and culture of HAMs

After surgical excision, tissue samples were placed into Custodiol® solution (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) for the transport to the laboratory. Cell isolations from 47 patients were carried out as previously described.[7] Please see also Supplementary material online.

Perforated patch-clamp in freshly-isolated and cultured HAMs

Whole-cell perforated patch-clamp configuration was used to record $I_{Ca,L}$ in 16 HAMs from 10 patients as previously described.[4,11]

Live cell imaging of sarcolemmal cAMP in HAMs

Förster resonance energy transfer (FRET) based measurements of cAMP were performed on 38 living HAMs isolated from 18 patients and transduced with an adenovirus encoding for the pmEpac1-camps biosensor to measure cAMP at the membrane.[29] See online Supplement.

Immunocytochemistry and confocal imaging

HAMs from 7 SR patients, 5 pAF patients and 7 cAF patients were used for immunostaining as described in online Supplement.

Sharp-electrode AP recordings

APs were recorded as previously described using standard intracellular microelectrodes in atrial trabeculae from 5 SR, 5 pAF and 7 cAF patients.[30] Further experimental details are available in the online Supplement.

Data analysis and statistics

Results are presented as mean ± SEM. $P < 0.05$ was considered statistically significant. Normal distribution was tested by Kolmogorov–Smirnov or by Shapiro–Wilk tests. Statistical differences between groups with normally distributed data were analyzed using mixed ANOVA followed by Wald $\chi 2$ test, Student's *t*-test for two-group comparison, or one-way ANOVA and Sidak multiple comparison test for multiple groups. Non-normally distributed continuous data or data for which normality could not be assessed were compared using Mann–Whitney tests or one-way ANOVA for multiple groups with a Kruskal–Wallis test. To investigate potential influences of individual clinical variables on specific parameters, two-way ANOVA subanalyses with factors rhythm and either age, sex, bypass surgery, hyperlipidemia, use of AT1-blockers, ACE inhibitors, diuretics or amiodarone were performed (see Supplementary data online, *Figures S4–S8*).

Results

Patients

Patients with pAF had more often hyperlipidemia, diabetes and cardiopulmonary bypass surgery and were younger in some subgroups. pAF and cAF patients were more often taking digitalis, beta-blockers and diuretics, with cAF patients being more frequently treated with amiodarone (see Supplementary data online, *Table S1*). There were no significant differences between groups in sex, body mass index, comorbidities, degree of valvular regurgitation, left ventricular ejection fraction. Multivariate analysis for hyperlipidemia, cardiopulmonary bypass, digitalis, beta-blockers, and amiodarone showed no alterations in the results (see Supplementary data online, *Figures S4–S8*). Although use of diuretics was associated with higher mRNA levels of PDE8B in SR (see Supplementary data online, *Figure S5*), this association was not observed in any of the other data sets. Other medication was not different between groups.

Expression and localization of PDE8 isoforms and Cav1.2 in human atrium

To determine if both PDE8 isoforms are present in human atria and altered in AF, we assessed gene expression (*Figure 1A*) and protein levels (*Figure 1B*) of PDE8A and PDE8B in atria from SR, pAF and cAF patients. PDE8A mRNA was present in human atria and increased in both AF groups, whereas PDE8B was increased in cAF only (*Figure 1A*).

The full-length human PDE8A1 isoform has 829 amino acids with a calculated molecular weight of 93 kDa, whereas the PDE8A2 isoform consists of 783 amino acids with a calculated molecular weight of 88 kDA.[31] Similarly, the full-length human PDE8B1 isoform has 885 amino acids and an expected molecular mass of approximately 99 kDa, whereas the PDE8B2 variant has 838 amino acids and an expected molecular mass of 87 kDa.[32] Previous work could detect differential protein expression of different PDE8A and PDE8B isoforms in rat brain, mouse ovary and bovine testis.[33,34] In human atria of SR, pAF, and cAF patients we could detect two specific bands between 75 and 100 kDa, which were in reasonable agreement with the expected molecular masses predicted for PDE8A1, PDE8A2, PDE8B1, and PDE8B2 (*Figure 1B*). The protein levels of PDE8A1 and PDE8A2 were significantly higher in pAF but unaltered in cAF. Conversely, PDE8B1 and PDE8B2 were upregulated in cAF only (*Figure 1B*). In contrast, human LV do not express *PDE8B* and expression of *PDE8A* was similar between control (Ctl) and HF patients (*Figure 1C*). Most important, although human atria express both *PDE8A* and *PDE8B*, their mRNA levels were not changed during HF, indicating that the *PDE8B* increases were AF-specific (*Figure 1C*).

In addition, PDE8A was more abundant in the cytosol in HAMs and its abundance was increased in pAF *vs.* SR and cAF (*Figure 2A and C* and Supplementary data online, *Figure S1*). In contrast, PDE8B was preferentially localized at the plasma membrane, especially in cAF where its expression was increased compared to SR and pAF (*Figure 2B and C* and Supplementary data online, *Figure S1*).

Since PDEs degrade cAMP[13,15,22] and impaired PKA-dependent LTCC regulation was suggested to be the main mechanism for $I_{Ca,L}$ reduction in cAF,[11,35] we examined whether PDE8B, which we found upregulated in cAF, co-localizes with LTCC in the macromolecular complex (*Figure 3A*). In co-immunoprecipitations PDE8B was the only partner of Cav1.2, with PDE8B2 being the major isoform (*Figure 3A*). PDE8B2 showed a stronger binding to Cav1.2$_{\alpha 1C}$ subunit in cAF (*Figure 3A*).

Subsequently, we studied the consequences of selective PDE8 inhibition on cAMP/PKA-regulation of LTCC by assessing the phosphorylation state of Cav1.2$_{\alpha 1C}$ at Ser1928 (Cav1.2-pS1928), a validated PKA phosphorylation site of Cav1.2$_{\alpha 1C}$,[36] in atrial tissue lysates incubated with control buffer (CON), the selective PDE8 inhibitor PF-04957325 (PF) or the β-adrenoceptor agonist isoprenaline (ISO) from SR and cAF patients. Although phosphorylation of Cav1.2-pS1928 is not required for β-adrenoceptor stimulation of LTCC in mouse ventricular myocytes and HEK293 cells,[37–39] it is a well-established Cav1.2 PKA phosphorylation site[36] and phosphorylation of its α_{1C} subunit is relevant for channel function by increasing trafficking to the plasma membrane.[40] In CON, the relative Cav1.2-pS1928 phosphorylation was significantly lower in cAF compared to SR and increased significantly after incubation with ISO or PF to levels seen in corresponding CONs (*Figure 3C* and Supplementary data online, *Figure S2*), pointing to significant contribution of PDE8 to LTCC dephosphorylation. In agreement with previous studies,[12,41] no significant difference in total Cav1.2$_{\alpha 1C}$ protein expression was observed in cAF compared to SR (see Supplementary data online, *Figure S2*).

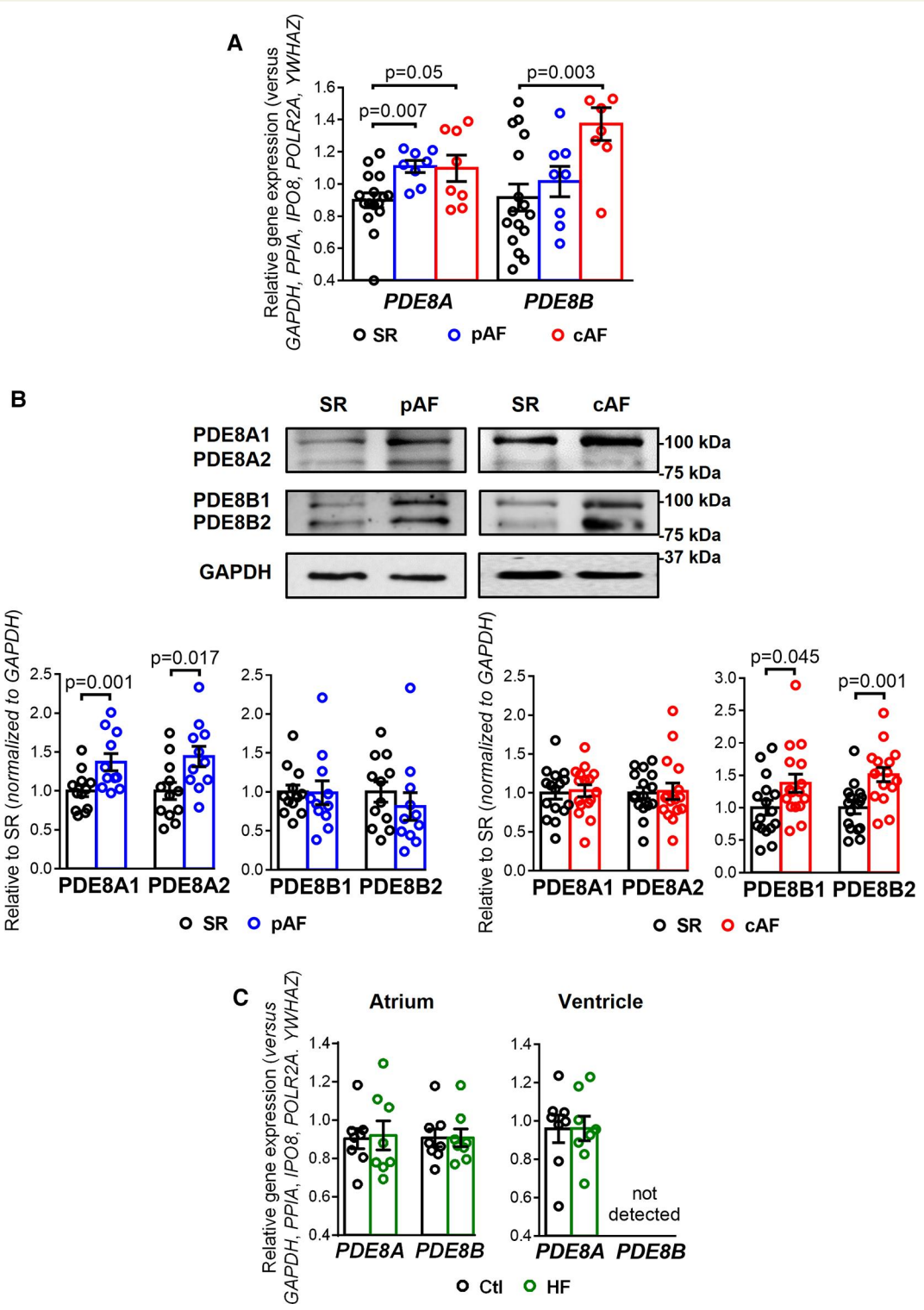


Figure 1 PDE8A and PDE8B expression in human atrium. *(A)* Plot of the individual and mean ± SEM gene expression ratio of *PDE8A* and *PDE8B* normalized to a set of five housekeeping genes (*POLR2A*, *YWHAZ*, *GAPDH*, *IPO8*, *PPIA*) in atrial tissue homogenates from 16 sinus rhythm (SR), 8 paroxysmal atrial fibrillation (pAF) and 8 persistent (chronic) atrial fibrillation (cAF) patients. **P* < 0.05 *vs.* sinus rhythm SR based on analysis of variance (ANOVA with a Kruskal–Wallis test). *(B)* Representative western blot (top) and protein expression quantification (bottom, mean ± SEM) of PDE8A and PDE8B in atrial tissue homogenates from 16 SR, 10 pAF and 16 cAF patients. GAPDH was used as loading control. **P* < 0.05 *vs.* SR based on unpaired Student's *t*-test analysis. *(C)* Plot of the individual and mean ± SEM gene expression ratio of *PDE8A* and *PDE8B* normalized to the set of five housekeeping genes in right atrial and left ventricular tissue homogenates from eight healthy control (Ctl) and eight end-stage heart failure (HF) patients. **P* < 0.05 *vs.* Ctl based on ANOVA and Mann–Whitney test.

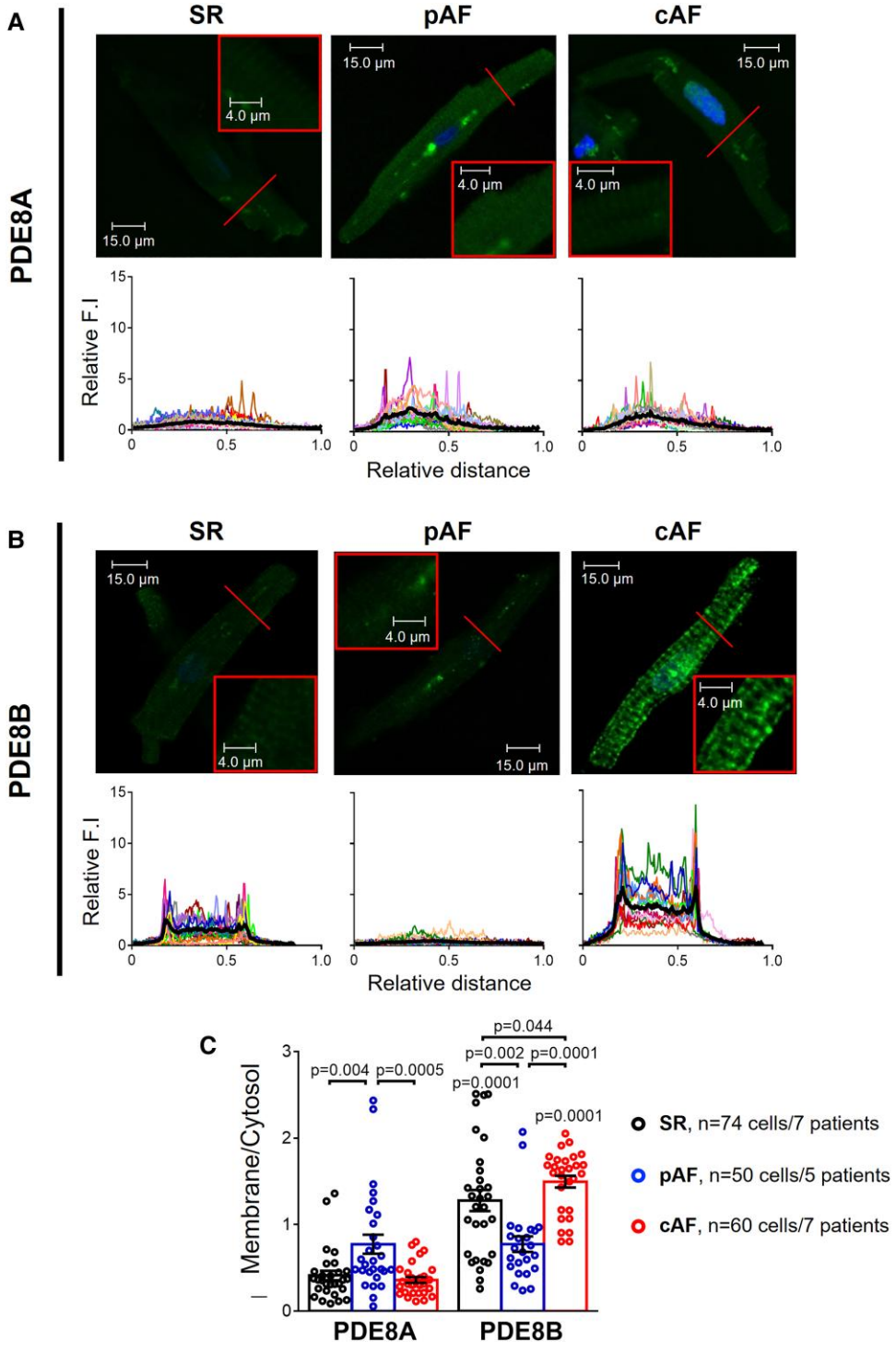


Figure 2 PDE8A and PDE8B localization in human atrial myocytes. *(A)* Representative immunocytochemistry confocal images of PDE8A showing its cytosolic distribution in 36 isolated human atrial myocytes (HAMs) from 7 sinus rhythm (SR), 25 HAMs from 5 paroxysmal atrial fibrillation (pAF) and 29 HAMs from 7 persistent (chronic) atrial fibrillation (cAF) patients. Lower panels show transversal line profiles of fluorescence intensity (F.I.) of PDE8A staining for all the myocytes analyzed (thin colored lines) and the average within all cells (thick black lines). *(B)* Similar representative immunocytochemistry confocal images and fluorescence intensity profiles of PDE8B showing mainly plasma membrane localization of this PDE isoform, in 38 HAMs from 7 SR, 25 HAMs from 5 pAF and 31 HAMs from 7 cAF patients. *(C)* Average F.I. of PDE8A and PDE8B at the plasma membrane (first and last 10% of the cell width) related to the F.I. in the cytosol (middle), in SR, pAF, and cAF. $*P < 0.05$ between groups of patients, $\# P < 0.05$ *vs.* PDE8A, by mixed ANOVA followed by Wald χ^2 test and Sidak multiple comparison test. All images were captured under the same conditions in order to obtain comparable fluorescence intensities.

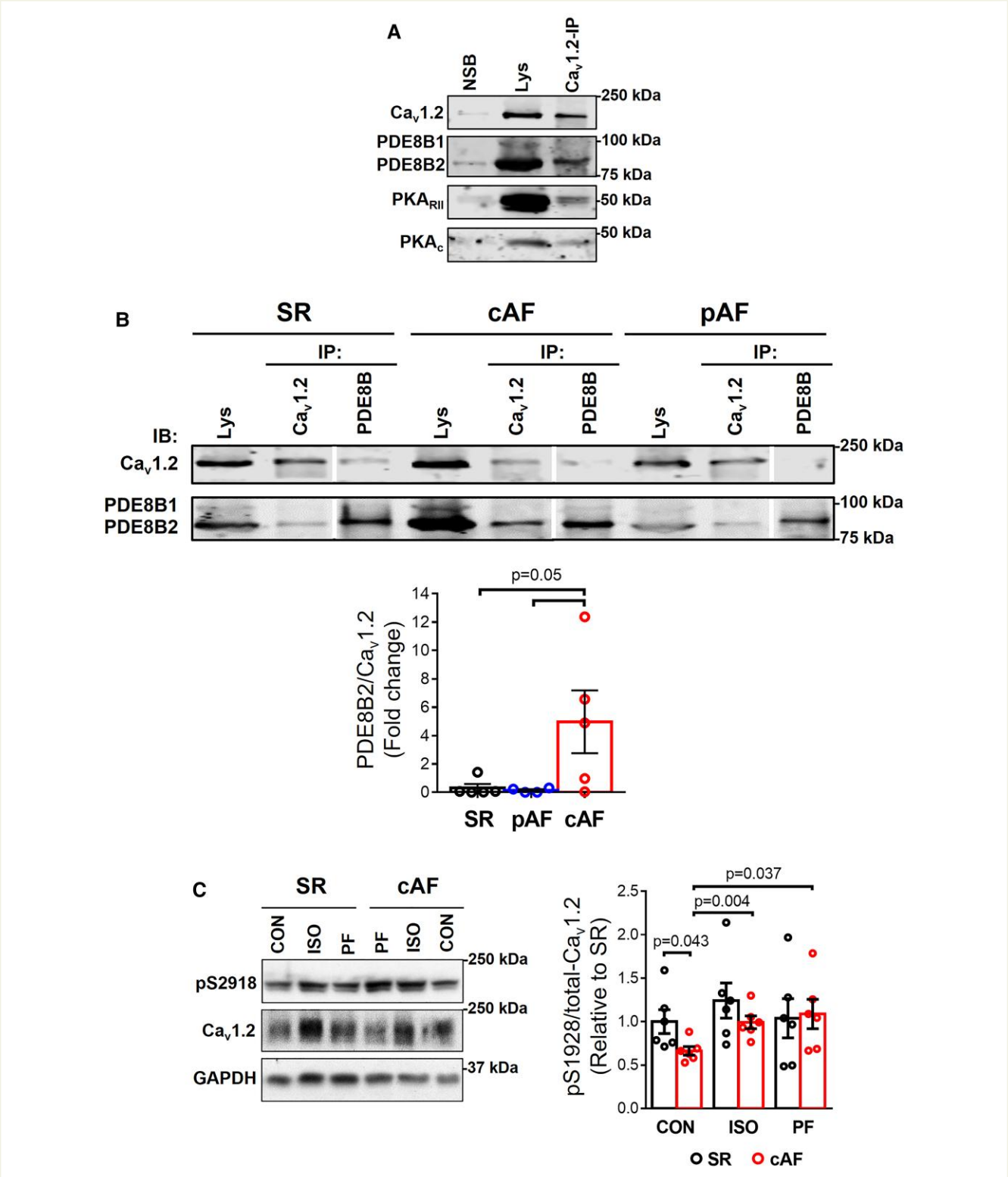


Figure 3 PDE8 co-localizes with LTCC. (*A*) Co-immunoprecipitation (Co-IP) assay followed by immunoblotting (IB) confirmed that with L-type Ca^{2+} channel (Cav1.2) binds to PDE8B, PKA_{RII} and PKA_C in human atria from patients with persistent (chronic) atrial fibrillation (cAF). NSB, non-specific binding. (*B*) Top, representative Co-IP and IB of Cav1.2 binding to PDE8B in human atrial samples from patients in sinus rhythm (SR), paroxysmal AF (pAF) and cAF. Bottom, mean data of PDE8B binding to Cav1.2 for all groups ($n = 6$ SR, $n = 4$ pAF, and 5 cAF patients). Lys, Lysate. Vertical white lines separate non-adjacent lanes on the same blot. (*C*) Representative western blot (left) and quantification (right, mean ± SEM) of relative steady-state LTCC phosphorylation (pSer1928/total $Ca_v1.2$) in atrial tissue homogenates from 6 SR and 6 cAF patients, at baseline and after 5 min stimulation with the selective PDE8 inhibitor PF-04957325 (30 nM) or the β-adrenoceptor agonist isoprenaline (ISO, 100 nM). GAPDH was used as loading control. *$P < 0.05$ based on ANOVA with a Kruskal–Wallis test.

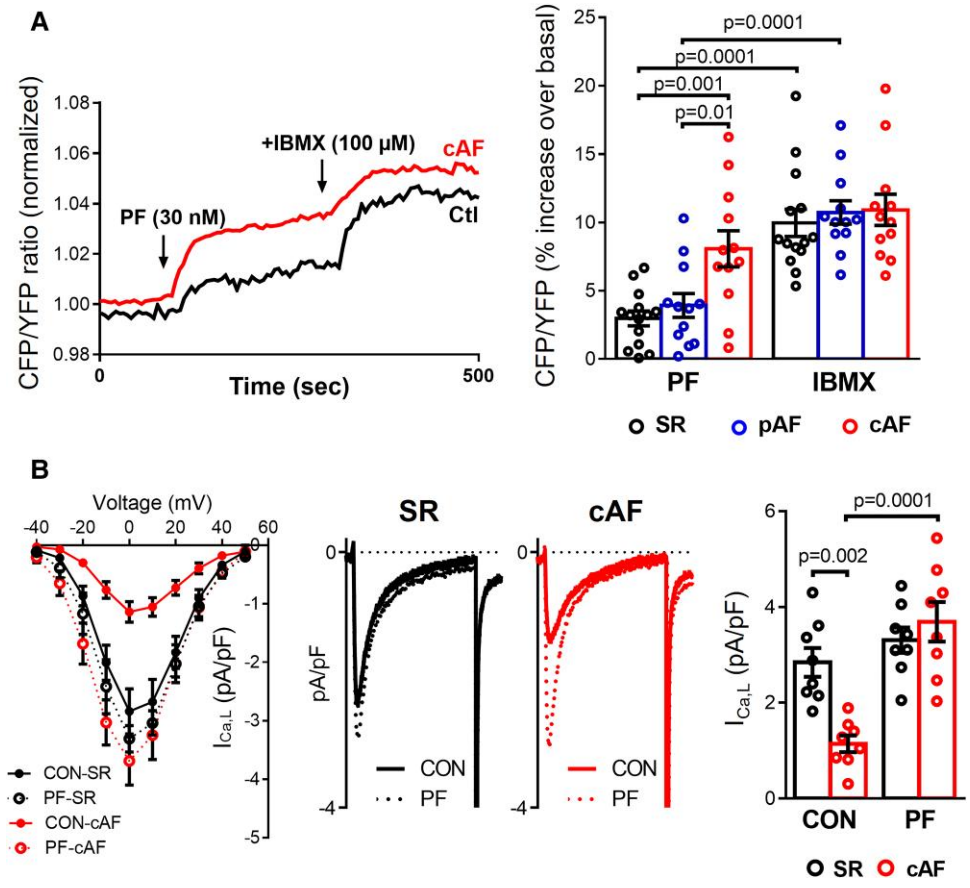


Figure 4 Functional consequences of selective PDE8 inhibition in human atrial myocytes (HAMs). *(A)* Left panel, representative experiments showing the time course of the FRET signals indicating cAMP changes in HAMs from sinus rhythm (SR) and in persistent (chronic) atrial fibrillation (cAF) patients exposed to selective PDE8 inhibition with PF-04957325 (30 nM). Right panel, effects of the selective PDE8 inhibitor and the non-selective PDE blocker IBMX (100 mM) on the individual and mean values of cAMP levels expressed as percentage of increase of CFP/YFP over its control value measured in 14 HAMs from 6 SR, 12 HAMs from 6 paroxysmal AF (pAF), and 12 HAMs from 6 cAF patients. The changes in FRET ratio were expressed as a percentage change *vs.* basal. **P* < 0.05 compared with SR, # *P* < 0.05 compared with PF. *(B)* Left panel, current-voltage (I-V) relationship for peak inward $I_{Ca,L}$ density in HAMs from SR and cAF patients before (CON) and after exposure to PF. Middle panels, representative L-type Ca^{2+} current ($I_{Ca,L}$) recordings measured at 0 mV in HAMs from SR and cAF patients before (CON) and after exposure to PF. Right panel, average current densities before and after exposure to PF (*n* = 8 cells/5 patients SR and 8/5 cAF). **P* < 0.05 *vs.* sinus rhythm SR, # *P* < 0.05 *vs.* CON. Statistical differences analyzed by mixed ANOVA followed by Wald χ^2 test and Sidak multiple comparison test.

Altogether, these results show that PDE8B localizes at the plasma membrane of HAMs and accumulates in the LTCC complex of cAF patients, whereby it causes LTCC dephosphorylation. This positions PDE8B as a potential molecular mechanism of the reduced $I_{Ca,L}$ that promotes AF.

PDE8 controls subsarcolemmal cAMP concentration in HAMs

To directly test whether PDE8 controls cAMP levels near the LTCC, cAMP concentration at the plasma membrane was measure in living HAMs from SR, pAF and cAF patients using the FRET-based targeted biosensor pmEpac1-camps.[9,29] Application of PF strongly increased basal levels of cAMP in the subsarcolemmal compartment in cAF compared to SR (*Figure 4A*), reaching approximately 74% of the respective maximal response elicited by the non-selective PDE inhibitor IBMX (100 μM). These results indicate that PDE8 controls basal cAMP levels at the plasma membrane of HAMs in cAF, proving that the observed increase of PDE8B2 in the LTCC complex could contribute to the dephosphorylation of LTCC and the reported reduction in $I_{Ca,L}$ in cAF.

No changes in $I_{Ca,L}$ density or cell capacitance were observed when comparing freshly-isolated and 48-h cultured HAMs from the same patients at baseline and after ISO stimulation (see Supplementary data online, *Figure S3*).

PDE8 controls $I_{Ca,L}$ function

We then studied the direct consequences of increased PDE8B2 for $I_{Ca,L}$ function with perforated patch-clamp recordings. $I_{Ca,L}$ amplitude was lower in cAF, but was rescued by selective PDE8 inhibition with PF (*Figure 4B*) and this effect was fully reversible. These findings validate the novel concept that enhanced PDE8B is a major molecular mechanism for the reduction of $I_{Ca,L}$ in cAF.

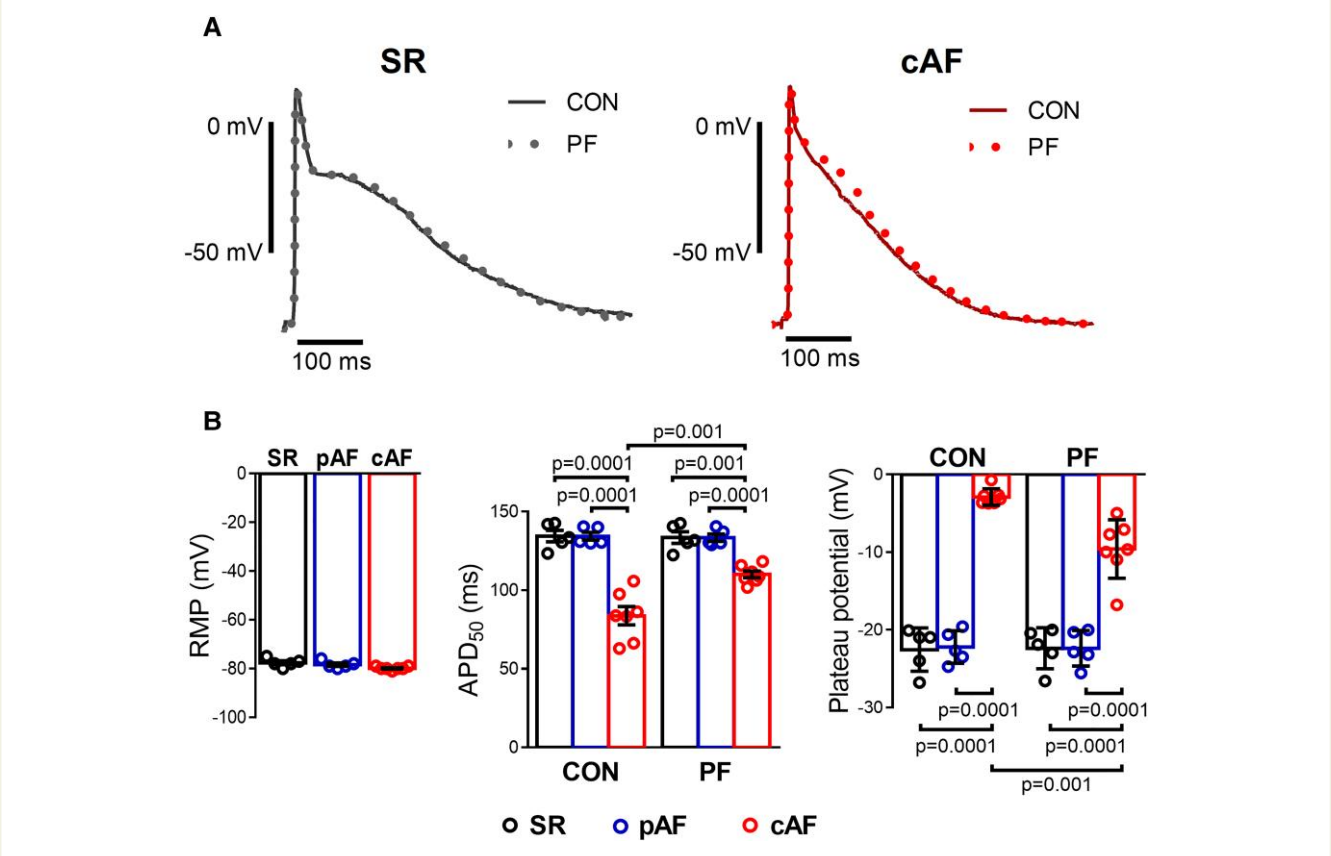


Figure 5 Effect of selective PDE8 inhibition on action potential (AP) properties of human atrial trabeculae. *(A)* Representative traces of AP at baseline (CON) and after 20 min of exposure to the selective PDE8 inhibitor PF-04957325 (PF, 100 nM) recorded in atrial trabeculae from patients in sinus rhythm (SR) and in persistent (chronic) atrial fibrillation (cAF). *(B)* Left panel, average resting membrane potentials in trabeculae from five SR, five paroxysmal AF (pAF), and seven cAF patients. Middle panel, individual and mean values of the effects of the selective PDE8 inhibitor (PF) on AP duration at 50% of repolarization (APD_{50}) in the three groups of patients. Right panel, plateau potential before (CON) and after exposure to the selective PDE8 inhibitor (PF). *$P < 0.05$ *vs.* SR, # $P < 0.05$ *vs.* CON based on ANOVA with a Kruskal–Wallis test.

Effects of PDE8 inhibition on AP in cAF

To assess the consequences of PDE8 inhibition and the related upregulation of $I_{Ca,L}$ for AP properties, we performed sharp-electrode AP recordings in atrial trabeculae from SR, pAF and cAF patients. Resting membrane potentials were similar between all groups. In cAF PDE8 inhibition shifted the plateau potential, calculated as the average voltage during a time window of 20 to 90 ms after AP upstroke, to more positive values. This shift leads to a slight prolongation of the AP duration at 50% of repolarization (APD_{50}) in cAF vs. SR and pAF (*Figure 5*), which is consistent with a stimulation of $I_{Ca,L}$. The PF effect was reversible on washout.

Discussion

Our study demonstrates that PDE8A and PDE8B are expressed in human atria and HAMs. PDE8B preferentially localizes at the plasma membrane in close proximity to Cav1.2$_{\alpha 1C}$, and the PDE8B2 isoform is enriched in the LTCC complex in cAF. Furthermore, PDE8B is central in controlling cAMP at the plasma membrane which provides a new molecular mechanism of the reduced $I_{Ca,L}$ in cAF (*Structured Graphical Abstract*). These novel insights suggest that selective PDE8B inhibition may constitute a novel atrial-selective anti-AF strategy.

Although a reduction in $I_{Ca,L}$ amplitude has been consistently reported in HAMs from cAF patients,[3,10,11,42–44] the underlying molecular mechanism has been poorly understood. Reduced $I_{Ca,L}$ is considered a main cause of the reentry-promoting APD shortening,[45] and atrial contractile dysfunction in AF.[46] Different mechanisms have been suggested to contribute to the reduced $I_{Ca,L}$ in AF. Although expression of the accessory subunit α2δ seems to be reduced,[47] protein expression of the pore-forming Cav1.2 subunits α_{1C} or β_2 are unchanged in AF,[41] suggesting that the reduced $I_{Ca,L}$ might be due to altered regulation of single-channel properties. In addition, we and others have demonstrated a higher sensitivity of $I_{Ca,L}$ to β-adrenoceptor stimulation in AF compared to SR[10,11,44] suggesting that, opposite to the hyperphosphorylation of RyR2 or PLN,[3,7,8] basal phosphorylation of the LTCC complex appears reduced.[12] The stronger stimulatory effect of isoprenaline on $I_{Ca,L}$ in AF[11] indicates that the alterations in Ca^{2+}-handling in AF are likely due to a specific alteration of cAMP-dependent protein phosphorylation in discrete subcellular nanodomains. These results are in agreement with recent studies reporting atrial-specific cAMP nanodomains in the sarcolemmal axial tubule dyadic junctions with the SR in association with RyR2 hyperphosphorylation and clustering,[35] and in the caveolar structures linked to extradyadic LTCC subpopulations.[48] Since protein phosphorylation states depend on the dynamic balance between protein kinases and protein phosphatases, it has been suggested

that a local regulation of PP2A activity may contribute to these compartmental differences in cAMP/PKA-dependent phosphorylation.[12] However, although increased PP2A may contribute to the reduced $I_{Ca,L}$, the strong decrease in $I_{Ca,L}$ in the face of hyperphosphorylated RyR2 and PLN in AF[3,7,8] likely results from different molecular mechanisms. Since in ventricular cardiomyocytes local PKA activity highly depends on the local availability of cAMP, which in turn depends on its degradation by PDEs at each discrete nanodomain,[13,15,22] PDEs could contribute to the unique LTCC complex dephosphorylation in AF. Although the classical cardiac PDE2–4 were associated with $I_{Ca,L}$,[9,23] their activity decreases in AF,[9] suggesting that the decreased $I_{Ca,L}$ in AF cannot result from altered PDE2–4 activities. Here we show that a novel PDE8 isoform, PDE8B, is present in human atria, but unlike PDE8A,[25] PDE8B is not present in human ventricles,[26] and constitutes an atrial-selective PDE isoform. Importantly, our results demonstrate that PDE8B1 and PDEB2 proteins are upregulated in cAF. Furthermore, PDE8B localizes at the sarcolemma of HAMs, with PDE8B2 strongly binding to Cav1.2$_{\alpha 1C}$ in cAF. Functionally, PDE8B inhibition increases Cav1.2-pS1928 phosphorylation, cAMP levels in the subsarcolemma, and rescues $I_{Ca,L}$, thereby prolonging APD_{50} in cAF.

We speculate that the PDE8B2 upregulation in the LTCC complex, the subsequent Cav1.2$_{\alpha 1C}$ dephosphorylation at pS1928 and the related $I_{Ca,L}$ reduction in cAF may be a protective mechanism to counteract the cytotoxic Ca^{2+} overload and related negative consequences for cardiac metabolic activity due to the high atrial rate and energy demand, although this occurs at the expense of reentry-promoting APD shortening.[49] The PDE8B upregulation could promote Rad-mediated inhibition of LTCC[37,50] and consequently reduce Ca^{2+} influx to compensate the increase in diastolic intracellular [Ca^{2+}] that causes AF-promoting remodeling.[1] Although Ca^{2+}-channel blockers can be used to control the ventricular rate in AF, they could promote AF and cause ventricular dysfunction.[51,52] Since PDE8B and particularly PDE8B2 occurrence appear atrial-selective, selective inhibition of PDE8B2 could constitute a viable approach to normalize the abnormal LTCC function and may constitute a novel atrial-selective anti-AF target. However, $I_{Ca,L}$ is not downregulated in patients with pAF.[53] Thus, the reduction of $I_{Ca,L}$ in cAF could be a consequence of the persistency of the arrhythmia and/or the sustained high atrial rate during AF. The related shortening of ERP/APD, which promotes AF-maintaining reentry, promotes the progression and the stabilization of AF. Thus, restoration of physiological $I_{Ca,L}$ by selective inhibition of PDE8B is expected to prevent the progression of AF to persistent forms rather than the initiation of AF and might also constitute a rhythm control strategy after cardioversion. These possibilities require direct verification in large animal models and suitable patient populations.

Our results clearly show that both PDE8A and PDE8B are expressed in human atrial tissue and myocytes. However, the immunoblot experiments showed multiple bands (*Figure 1B* and *Figure 3A and B*) and although the molecular weights of these bands were comparable to specific PDE8 splicing variants in previous studies,[31–34] their identities as PDE8A and PDE8B isoforms should be further validated in future work.

Because PDE8 isoforms are important for the regulation of steroidogenesis,[54–56] it remains to be determined whether selective PDE8B inhibition could be used for AF treatment without serious effects on adrenal gland or other organs.

Because of limited accessibility to human left atria at our centers, experiments were restricted to right atrial appendages. Different atrial regions may undergo distinct remodeling and further work should be directed to assess whether the observed changes in PDE8 in the right atria also apply to left atrial cells.

pAF patients had similar clinical parameters to SR patients except that they had more often hyperlipidemia and cardiopulmonary bypass surgery, were younger in some subgroups, and were more often taking digitalis, beta-blockers, and diuretics. cAF patients were more frequently treated with AT1-blockers, diuretics and amiodarone. The levels of *PDE8B* were higher in treated *vs.* non-treated SR patients with diuretics (see Supplementary data online, *Figure S5*). However, this was not observed at the protein level. Overall, controlling for individual comorbidities and medication did not alter the results of the individual sets of experiments.

A large number of atrial samples were collected, but each sample could only be used for a limited subset of experiments. Therefore, we performed extensive analyses to assess the influence of factors like patient age, sex, the influence of comorbidities and medications (see Supplementary data online, *Figures S4–S8*), we cannot fully exclude contributions from other unrecognized factors. The clinical profile of the patient subgroups used for biochemical experiments, FRET/patch-clamp, and multicellular AP recordings were comparable. The patient cohort used for qPCR and immunofluorescence showed differences in distribution of some clinical parameters; this needs to be considered when relating the differences that we noted in SR and AF patients to the other findings in our manuscript.

We observed an upregulation of PDE8A in pAF, but not cAF, and an upregulation of PDE8B in cAF, but not pAF. The underlying mechanisms for this differential regulation are still unclear. The increase of PDE8A in pAF could result from a specific atrial cardiomyopathy and might alter the regulation of targets other than $I_{Ca,L}$, although it might also constitute a transient event during the progression to persistent AF. Once persistent AF is established, the duration of the arrhythmia or/and the sustained high atrial rate could cause adaptative changes that may lead to a switch from PDE8A to PDE8B, along with a reduction in $I_{Ca,L}$, which is consistent with the selective increase in PDE8B in AF, but not in HF atria. Future work should directly address whether tachypacing upregulates atrial PDE8B.

Ser1928 is a well-established PKA phosphorylation site of Cav1.2$_{\alpha 1C}$[36] relevant for the trafficking of the channel to the membrane.[40] In agreement, we found that both ISO and PDE8 inhibition increased Ser1928 phosphorylation. However, the phosphorylation of this residue is not required for $I_{Ca,L}$ modulation in mouse ventricular myocytes and HEK293 cells.[37–39] Previous works have identified Rad as the channel inhibitor responsible of the modulation of $I_{Ca,L}$ via PKA phosphorylation in mice,[37,50] but the functional role of Ser1928 and Rad in HAMs and in AF has not been addressed here and warrants additional studies. Finally, we studied the effects of PDE8 on $I_{Ca,L}$ only. Whether and how PDE8 affects the function of other ionic currents that contribute to the atrial AP remains to be determined in future studies.

In summary, we establish PDE8B as a novel regulator of atrial Ca^{2+} handling and arrhythmogenesis and a new integral component of the LTCC macromolecular complex, which importantly contributes to the reentry-promoting reduction of $I_{Ca,L}$ in cAF.

Author contribution

Istvan Baczko, Anne Garnier (Investigation: Supporting), Markus Kamler (Formal analysis: Supporting; Resources: Supporting), Renate B. Schnabel (Investigation: Supporting), Dobromir Dobrev (Formal analysis: Supporting; Funding acquisition: Supporting; Writing – original draft: Supporting), Cristina E. Molina, Ph.D. (Conceptualization: Lead; Data curation: Lead; Funding acquisition: Lead; Investigation:

Supporting; Methodology: Lead; Supervision: Lead; Writing – original draft: Lead), Hermann Reichenspurner (Resources: Supporting), Viacheslav O. Nikolaev (Conceptualization: Supporting; Data curation: Supporting), Niels Voigt (Conceptualization: Supporting), Kira Beneke (Investigation: Lead; Writing – original draft: Supporting), Franziska Reinhardt (Formal analysis: Supporting), Nefeli Grammatika Pavlidou (Investigation: Lead; Writing – original draft: Supporting), Shokoufeh Dobrev (Formal analysis: Supporting; Investigation: Supporting), Issam H. Abu-Taha, Constanze Schmidt (Investigation: Supporting), Simon Pecha (Resources: Equal), and Eric Jacquet (Investigation: Supporting)

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Supplementary data

Supplementary data is available at *European Heart Journal* online.

Data and materials availability

All data are available in the manuscript or the supplementary materials.

Conflict of interest

The authors have no competing interests. R.B.S. reports grants from European Union Horizon 2020, the German Ministry of Research and Education, ERACOSysMed3 and personal fees from BMS/Pfizer, outside this work.

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