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Attenuated response of L-type calcium current to nitric oxide in atrial fibrillation

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Aim Nitric oxide (NO) synthesized by cardiomyocytes plays an important role in the regulation of cardiac function. Here, we studied the impact of NO signalling on calcium influx in human right atrial myocytes and its relation to atrial fibrillation (AF).

Methods and results Right atrial appendages (RAAs) were obtained from patients in sinus rhythm (SR) and AF. The biotin-switch technique was used to evaluate endogenous *S*-nitrosylation of the α_{1C} subunit of L-type calcium channels. Comparing SR to AF, *S*-nitrosylation of Ca^{2+} channels was similar. Direct effects of the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) on L-type calcium current ($I_{Ca,L}$) were studied in cardiomyocytes with standard voltage-clamp techniques. In SR, $I_{Ca,L}$ increased with SNAP (100 μM) by 48%, $n/N = 117/56$, $P < 0.001$. The SNAP effect on $I_{Ca,L}$ involved activation of soluble guanylate cyclase and protein kinase A. Specific inhibition of phosphodiesterase (PDE)3 with cilostamide (1 μM) enhanced $I_{Ca,L}$ to a similar extent as SNAP. However, when cAMP was elevated by PDE3 inhibition or β-adrenoceptor stimulation, SNAP reduced $I_{Ca,L}$, pointing to cGMP–cAMP cross-regulation. In AF, the stimulatory effect of SNAP on $I_{Ca,L}$ was attenuated, while its inhibitory effect on isoprenaline- or cilostamide-stimulated current was preserved. cGMP elevation with SNAP was comparable between the SR and AF group. Moreover, the expression of PDE3 and soluble guanylate cyclase was not reduced in AF.

Conclusion NO exerts dual effects on $I_{Ca,L}$ in SR with an increase of basal and inhibition of cAMP-stimulated current, and in AF NO inhibits only stimulated $I_{Ca,L}$. We conclude that in AF, cGMP regulation of PDE2 is preserved, but regulation of PDE3 is lost.

Keywords Nitric oxide • Atrial fibrillation • L-type calcium current • Cyclic nucleotides • Phosphodiesterases

1. Introduction

Nitric oxide (NO) is an important signalling molecule in the cardiovascular system.[1] NO causes relaxation of vascular smooth muscle cells,[2] but its effects on cardiomyocytes are more complex.[3] For instance, in human atrial cells biphasic changes of L-type calcium current ($I_{Ca,L}$) in response to the NO-donor 3-morpholino-syndonimine (SIN-1) have been reported, where low concentrations enhanced $I_{Ca,L}$ more than higher concentrations.[4] However, other groups found $I_{Ca,L}$ to be either enhanced[5,6] or depressed by NO[7,8] in cardiomyocytes isolated from different species. This paper focuses on the role of NO signalling as a modulator of human atrial calcium currents.

The mechanisms by which NO modulates $I_{Ca,L}$ are not yet clearly understood. Both cGMP-dependent and cGMP-independent NO signalling pathways have been reported.[4–6,9–11] Stimulation of soluble guanylate cyclase (sGC) by NO increases cGMP levels, thus activating cGMP-dependent protein kinase (PKG) which enhances $I_{Ca,L}$[5] and activates phosphodiesterase 5 (PDE5), thereby producing a negative feedback on cGMP signalling.[9] In addition, by stimulating cGMP production, NO can promote bidirectional regulation of PDEs,[9] providing an indirect cGMP-dependent modulation of PKA activity via cAMP hydrolysis.[4,11] There is evidence from the literature that $I_{Ca,L}$ can also be affected via cGMP produced by particulate guanylate cyclase. Atrial natriuretic peptide, for instance, inhibits $I_{Ca,L}$ via this pathway including

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decrease in intracellular cAMP by cGMP-mediated activation of PDE.[12] The cGMP-independent effects of NO include *S*-nitrosylation, which may modify the function of numerous proteins, including ion channels and contractile proteins.[6,10]

Therefore, the role of NO is controversial under physiological conditions, but it is even less well defined under pathological conditions. To the best of our knowledge, no information about cGMP-dependent NO signalling and $I_{Ca,L}$ is available for atrial fibrillation (AF). As for the cGMP-independent pathway in AF, *S*-nitrosylation of L-type Ca^{2+} channels was reported to be enhanced and this was associated with reduction of current amplitude.[10] Glutathione is required for neutralization of NO, and in AF, the glutathione content was found to be reduced.[10] *S*-nitrosylation was postulated to contribute to the reduction of $I_{Ca,L}$ in human AF.[10] Others have reported that the amount of free NO in a porcine experimental AF model is lower than in SR due to reduced expression of endocardial nitric oxide synthase (eNOS).[13] Some of the discrepant findings may be due to species or chamber differences, or to the specific experimental approaches used to manipulate NO availability such as transgenic knock-out of NOS, chemical inhibition of NOS, or the use of different NO donors.

To elucidate *the signalling pathways by which NO modulates* $I_{Ca,L}$ in SR and AF, we have studied right atrial (RA) myocytes from both groups of patients under identical experimental conditions. Our major findings are that NO enhances basal $I_{Ca,L}$, but reduces cAMP-stimulated current in sinus rhythm (SR), whereas in AF, the NO-induced stimulation of basal $I_{Ca,L}$ is *diminished*, while the reduction of cAMP-stimulated $I_{Ca,L}$ is maintained.

2. Methods

A detailed description of all methods is given in Supplementary material online.

2.1 Patients

The study was approved by the local ethics committee (No. EK114082202). The investigation conforms the principles outlined in the Declaration of Helsinki (*Cardiovascular Research* 1997;35:2–4). Each patient gave written informed consent. Right atrial appendages (RAAs) were obtained from 115 patients with no history of AF (SR) and 76 with persistent AF (>6 months) who underwent open heart surgery. Patient characteristics for each group of experiments are summarized in Supplementary material online, *Table S1*a–c. Biopsies were used for: (i) snap-freezing in liquid nitrogen directly in the operation theatre and later analysis of *S*-nitrosylation with the biotin-switch assay[14] or *cyclic nucleotide content*, (ii) isolation of RA myocytes to be studied with a conventional whole-cell voltage-clamp technique,[15] (iii) determination of cyclic nucleotide content with radioimmunoassay (RIA) or (iv) western blot and immunocytochemistry. All atrial tissue samples were quiescent after removal from the heart.

2.2 Cell isolation and whole-cell voltage-clamp experiments

RA myocytes were isolated with a standard protocol as described previously.[15] In all experiments (unless mentioned otherwise), $I_{Ca,L}$ was measured at 37°C during a 200 ms test pulse from a holding potential of −80 mV to +10 mV (Axopatch 200, Axon Instruments, Foster City, CA, USA) in the ruptured patch mode. Calcium current amplitude was determined as the difference between peak inward current and current at the end of the depolarizing step. Solution compositions are described in the Supplementary material online. From available NO donors (*S*-nitrosothiols, sydnonimines, or diazeniumdiolates),[16] we chose the commonly used *S*-nitroso-*N*-acetylpenicillamine (SNAP). SNAP has a half-life of 1.15 h[17]; its decomposition involves cleavage of an S–NO bond with release of NO.[18]

2.3 Statistical analysis

Statistical analysis was performed using either Student's *t* test, analysis of variance or Bonferroni's multiple comparison test, as appropriate. Patients' categorical data were analysed with Fisher's exact test. Data are presented as means ± SEM. $P < 0.05$ was considered statistically significant.

3. Results

3.1 *S*-nitrosylation of α_{1C} subunit of Ca^{2+} channels in human atria

S-nitrosylation of Ca^{2+} channels was suggested to attenuate $I_{Ca,L}$ in AF.[10] To test the hypothesis of direct effects of NO on Ca^{2+} channels, endogenous *S*-nitrosylation of proteins from SR and AF patients was studied with the biotin-switch assay. In RAA tissue, basal levels of *S*-nitrosylation of L-type Ca^{2+} channels were high, with no detectable difference between SR ($n = 7$) and AF ($n = 6$) (see Supplementary material online, *Figure S1*). Therefore, we investigated the acute effects of application of NO on $I_{Ca,L}$ in RAA myocytes.

3.2 Effect of SNAP and isoprenaline on L-type Ca^{2+} currents

To evaluate the impact of NO on $I_{Ca,L}$ in human RAA myocytes, we measured $I_{Ca,L}$ before and 2 min after the addition of SNAP to the bath solution. In total, the effect of 100 μM SNAP on basal $I_{Ca,L}$ was studied in 116 cells from 57 SR patients and in 75 cells from 45 AF patients. The average capacitances of SR and AF cells were 84.5 ± 2.9 and 94.8 ± 4.0 pF, respectively ($P < 0.05$). At a test potential of +10 mV, basal peak $I_{Ca,L}$ density was significantly lower in AF than SR (3.1 ± 0.2 vs. 5.6 ± 0.26 pA/pF, $P < 0.001$). Average run-down of $I_{Ca,L}$ was 9% of pre-drug control within 2 min (the typical time for drug exposure).

In the SR group, SNAP (100 μM) increased $I_{Ca,L}$ by 51% to 8.5 ± 0.4 pA/pF ($P < 0.001$ vs. baseline, $n/N = 116/57$). In contrast, in AF the effect of 100 μM SNAP was attenuated, with $I_{Ca,L}$ only increased by 12% to 3.5 ± 0.2 pA/pF ($P = 0.1518$ vs. baseline, $n/N = 75/45$) *(Figure 1A)*. In SR cells, SNAP increased $I_{Ca,L}$ in a concentration-dependent manner (*Figure 1B*) that was reversible upon wash-out. Relative to SNAP, stimulation of β-adrenoceptors with (−)-isoprenaline (Iso) produced a more robust increase in $I_{Ca,L}$ (in matched cell pairs from the same patient). With 1 μM Iso, $I_{Ca,L}$ increased by 210% (to 12.2 ± 3.0 pA/pF, $n/N = 8/8$) in SR and by 433% (to 14.3 ± 2.5 pA/pF, $n/N = 6/6$) in AF cells (*Figure 1C*). The $I_{Ca,L}$ response to 100 μM SNAP was smaller than the response to Iso and highly variable in SR, and was even more variable in AF where most cells were non-responsive *(Figure 1A)*. For better discrimination of effects, the responses to SNAP (100 μM) were plotted as histograms (*Figure 1D*). In SR the largest fraction of cells was found in bin size '30–49% increase', whereas in AF the largest fraction was in size '−10–9% increase'.

In SR but not in AF, SNAP (100 μM) *significantly* increased $I_{Ca,L}$ in the range from −10 mV to +25 mV. Moreover, the peak of the IV curves in SR tended to shift to less positive potentials (*Figure 1E*). This shift was confirmed by fitting the data points with a Boltzmann function which yielded voltages of half-maximum activation ($V_{0.5}$) of 5.2 ± 0.9 mV before and 2.4 ± 1.2 mV ($P < 0.05$) after exposure to SNAP. The respective $V_{0.5}$ values in AF were 8.0 ± 1.6 and 6.9 ± 1.4 mV ($P = 0.34$).

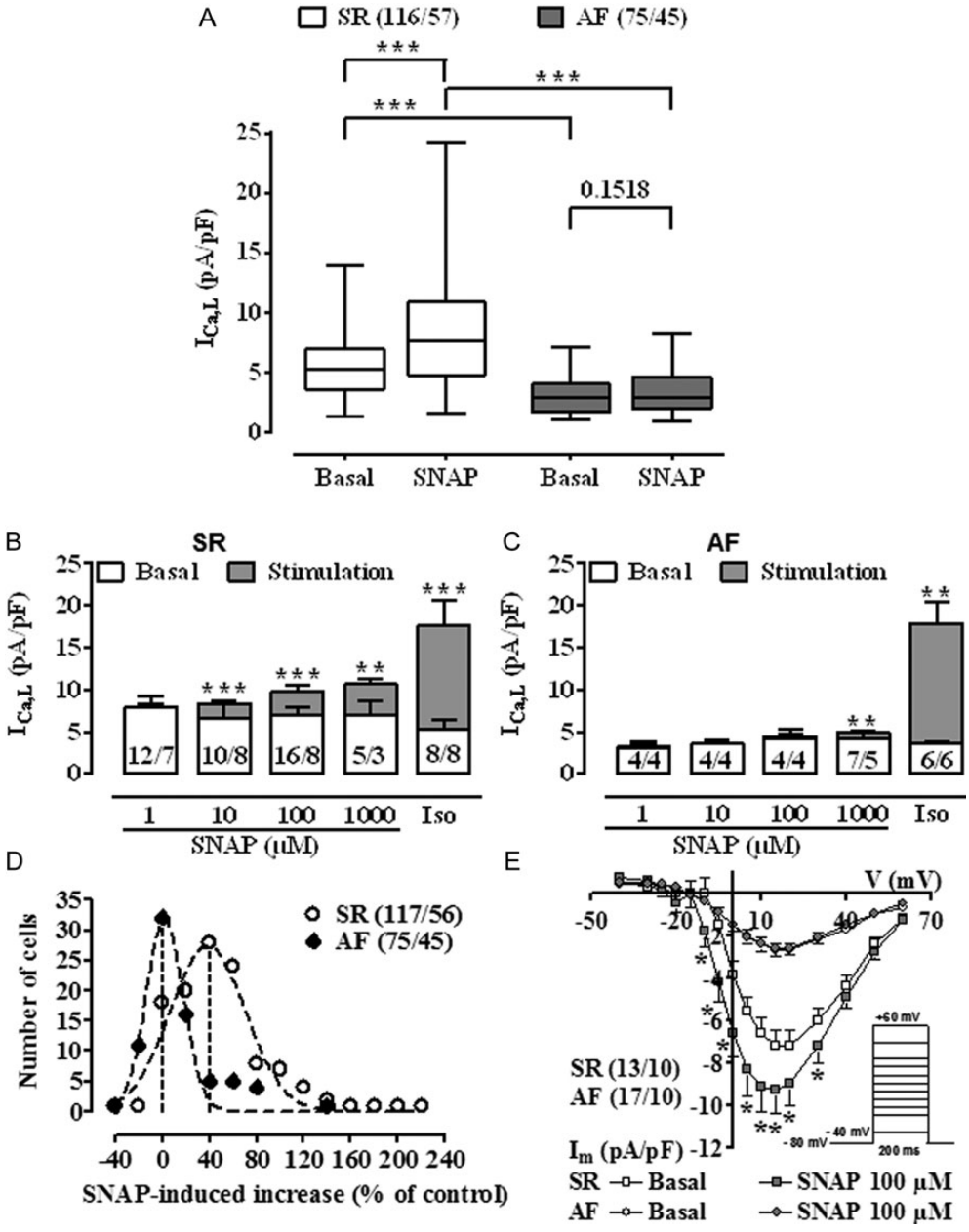

Figure 1 Effect of NO donor SNAP on $I_{Ca,L}$ in human RA cardiomyocytes. (*A*) The average $I_{Ca,L}$ before and after the addition of SNAP (100 μM) to the bath solution in RA cardiomyocytes from SR patients and AF patients. (*B* and *C*) Concentration-dependent manner of the SNAP effect and the Iso (1 μM) effect on $I_{Ca,L}$ of SR (*B*) and AF (*C*) cardiomyocytes. Open bars: basal $I_{Ca,L}$, closed bars: $I_{Ca,L}$ stimulated with various concentrations of SNAP (single concentration per cell) or Iso (1 μM). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. basal $I_{Ca,L}$. (*D*) Distribution of SNAP (100 μM)-induced increase in $I_{Ca,L}$ in SR (circles) and AF (diamonds). (*E*) IV curves in SR (squares) and AF (circles) before (open) and after exposure (closed) to 100 μM SNAP. Means ± SEM.

Since there was no response to the non-NO-releasing SNAP analogue *N*-acetyl-penicillamine (NAP, 100 μM) (*Figure 2A*), we infer that the effects of SNAP were mediated by NO. To determine whether the effects of SNAP on $I_{Ca,L}$ were related to sGC activity, SR cells were pre-treated with the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (*Figure 2B*). Exposure to 10 or 30 μM ODQ had no significant effect on basal $I_{Ca,L}$. The SNAP-induced increase of $I_{Ca,L}$ tended to be reduced with 10 μM ODQ ($P = 0.075$), just failing to reach the level of significance. The increase was significantly attenuated by 30 μM ODQ. To confirm that sGC activity is involved the $I_{Ca,L}$ increase, sGC was stimulated directly with BAY 41-2272. Indeed, this compound increased basal $I_{Ca,L}$ in SR and the effects were enhanced in the presence of 100 μM SNAP (*Figure 2C*). A similar synergism between BAY 41-2272 and the NO-donor DEA-NO was previously reported.[19] In contrast, in AF responses to BAY 41-2272 were attenuated (*Figure 2D*). Because of the intrinsic variability of responses to SNAP in AF, all AF experiments with the sGC stimulator were performed in pairs including a patient-matched control in order to estimate whether we were dealing with responders or non-responders. In the dataset of *Figure 2D*, the control cells from the three patients with AF responded on average to SNAP with a statistically significant increase in basal current. Nevertheless, the responses to BAY 41-2272 in AF were attenuated when compared with SR cells.

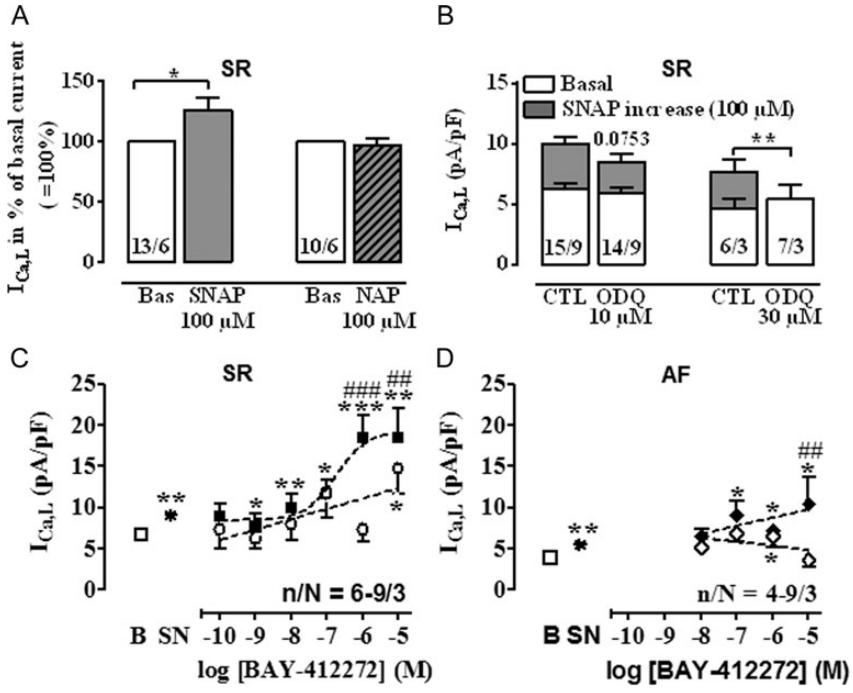

Figure 2 The effect of SNAP is due to release of NO and depends on sGC. (*A*) Patient-matched comparison of effects of SNAP and *N*-acetyl-penicillamine (NAP) on $I_{Ca,L}$. Bas, basal current, i.e. pre-drug control. (*B*) Effects of the sGC inhibitor ODQ on SNAP-induced $I_{Ca,L}$ increase. Open bars, basal current; grey bars, SNAP-stimulated $I_{Ca,L}$. CTL, patient-matched control experiments; ODQ, experiments with two concentrations of the ODQ. (*C* and *D*) Effects of sGC stimulator BAY 41-2272 alone (1 min exposure, open circles/diamonds) and together with SNAP (100 µM, additional 1 min exposure, closed squares/diamonds) on $I_{Ca,L}$ in SR (*C*) and AF (*D*) cardiomyocytes. Bas (open squares), basal current, means of all pre-drug control $I_{Ca,L}$ values; SN (stars), maximum $I_{Ca,L}$ amplitude after 100 µM SNAP only. Means ± SEM; *n*/*N* = cells/patient. Statistics: Stars compare any particular value to pre-drug control (basal), crosses compare effects of Bay 41-2272 plus SNAP to Bay 41-2272 alone. *$P < 0.05$, **$P < 0.01$, ***symbols $P < 0.001$.

To check whether the cells were generally responsive to cAMP increase, Iso (1 µM) was added immediately after the removal of Bay 41-2272 and SNAP from the bath solution. The $I_{Ca,L}$ amplitudes were 18.0 ± 2.0 pA/pF in SR and 14.2 ± 3.8 pA/pF in AF cells, indicating significant increases over the respective basal values in both groups.

Since it has been previously reported that cGMP signalling can affect the compartment-selective cAMP response to Iso,[20] we tested whether SNAP-treated cells were capable of additional increase in $I_{Ca,L}$ with Iso (*Figure 3A*). Iso (1 µM) further enhanced $I_{Ca,L}$ in the presence of SNAP. Interestingly, $I_{Ca,L}$ increased even further when SNAP was removed from the superfusion solution, suggesting that SNAP had reduced the effect of Iso. To quantify the SNAP-induced attenuation of the Iso response, we measured the effects of Iso alone or in combination with SNAP (patient-matched cells, *Figure 3C*). Iso (1 µM) added on the top of SNAP increased $I_{Ca,L}$ in SR cells to a smaller extent than Iso alone. In AF cells, despite the diminished effect of SNAP on basal $I_{Ca,L}$, SNAP-induced reduction of the Iso response was maintained (*Figure 3B* and *D*). Moreover, SNAP was able to reduce Iso-stimulated $I_{Ca,L}$ after cell exposure to Iso (*Figure 3E*).

Since SNAP activates sGC whereas Iso activates adenylate cyclase,[21] we examined the impact of the cGMP- and cAMP-dependent protein kinases on the SNAP-induced $I_{Ca,L}$ increase. While inhibition of PKG by intracellular application of R_P-8-Br-PET-cGMP (5 µM) did not prevent the SNAP effect (*Figure 4A*), the SNAP effect was completely abolished by the PKA inhibitor R_P-8-Br-cAMP (1 mM) (*Figure 4B*). These results argue against direct activation of L-type Ca^{2+} channels by PKG, but suggest that an interaction between cGMP and cAMP is involved in the NO-mediated increase in $I_{Ca,L}$.

3.3 PDE2- and PDE3-mediated signalling in $I_{Ca,L}$ regulation

As discussed above, intracellular levels of cyclic nucleotides are under tight control of PDEs.[22] Subtype-selective inhibitors were used to test the effects of the most abundant human cardiac PDEs (PDE3 and PDE2)[9] on atrial $I_{Ca,L}$ (*Figure 4*). EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine, 10 µM), a PDE2-selective inhibitor did not affect basal $I_{Ca,L}$ or the SNAP-induced effects (*Figure 4C* and *D*). In contrast, selective inhibition of PDE3 with cilostamide (1 µM) increased basal $I_{Ca,L}$ in SR (from 6.6 ± 1.4 to 11.6 ± 1.7 pA/pF, 7/4; $P < 0.001$, *Figure 4E*), and to a comparable extent in AF (from 5.0 ± 0.8 to 9.3 ± 2.1 pA/pF; 11/5; $P < 0.05$, *Figure 4F*).

Interestingly, cAMP and cGMP may cross-talk at the level of PDEs: cGMP inhibits PDE3-mediated cAMP hydrolysis by competing with cAMP but stimulates PDE2-catalysed hydrolysis of cAMP. Such cross-talk indirectly affects PKA activity with an influence on $I_{Ca,L}$ amplitude. Here, we showed that SNAP significantly decreased the impact of cilostamide on $I_{Ca,L}$ in both SR and AF to 8.6 ± 1.4 pA/pF (7/4, $P < 0.01$) and to 7.4 ± 1.5 pA/pF (11/5; $P < 0.05$), respectively, similar to the effects of SNAP on $I_{Ca,L}$ enhanced by Iso (*compare Figure 3*). We thus hypothesized that cGMP produced during SNAP application preferentially activates PDE2 when intracellular cAMP level is increased (in the presence of

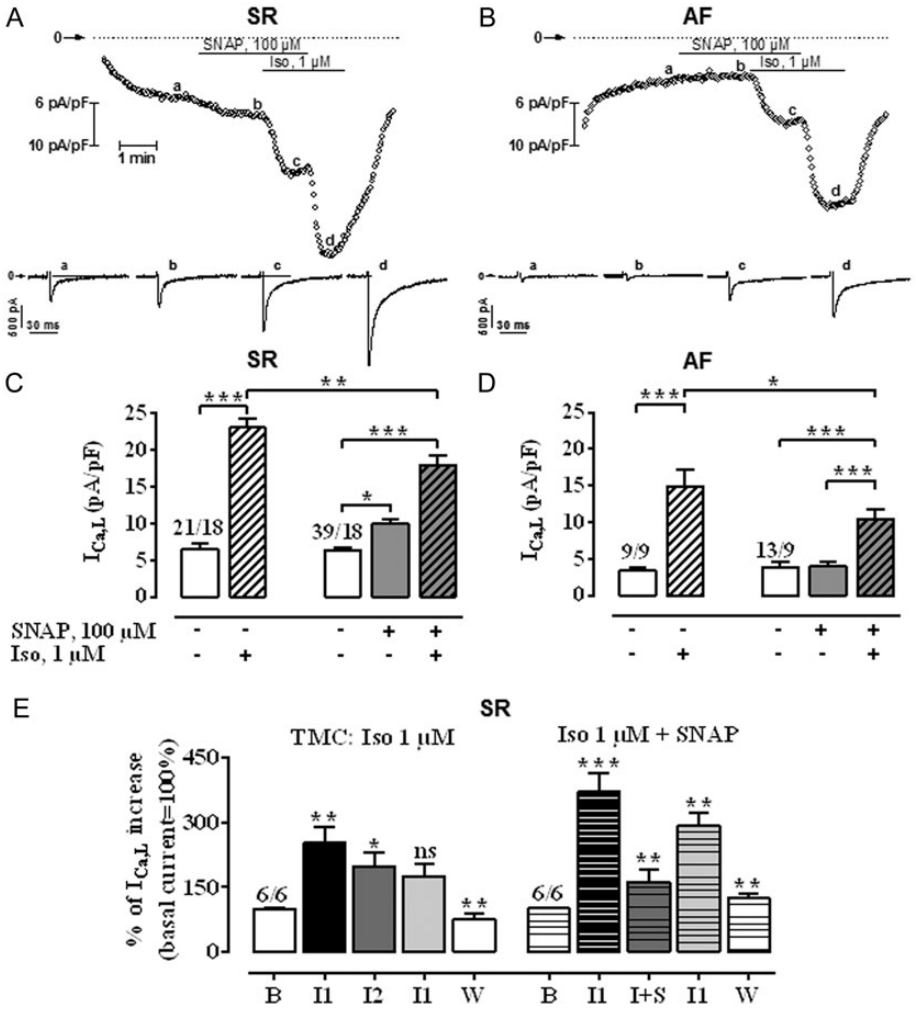

Figure 3 Effects of SNAP and Iso on $I_{Ca,L}$ in human RAA cardiomyocytes. (*A* and *B*) Diary plots of $I_{Ca,L}$ in from SR (*A*) and AF (*B*) cardiomyocytes. SNAP and (−)-Iso application as indicated by bar, same time scale in both groups. (*C* and *D*) Effects of Iso (1 μM), SNAP (100 μM), and SNAP plus Iso on basal $I_{Ca,L}$, in cells from matched SR (*C*) and AF (*D*) patients. Means ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. basal or Iso-stimulated current (Bonferroni's multiple comparison test). (*E*) Calculated mean values ± SEM of basal $I_{Ca,L}$ (*B*), after exposure to Iso (*I1; I2*) or to Iso + SNAP (*I+S*), and wash-out of all drugs (*W*). TMC, time-matched controls: two different tubes with 1 μM Iso were used at the corresponding time points. Right set of columns: Experiments, where SNAP was added on top of Iso (Iso + SNAP) for 2 min, followed by Iso only and then complete washed out of drugs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. previous current value.

cilostamide or Iso). This assumption was tested by adding EHNA in the presence of cilostamide and SNAP. *Figure 5A* illustrates an experiment in which an SR cardiomyocyte was first exposed to cilostamide, resulting in increased $I_{Ca,L}$. Addition of SNAP in the continuous presence of cilostamide decreased $I_{Ca,L}$, but the inhibitory effect of SNAP was reversed by further addition of PDE2 inhibitor EHNA (10 μM). All effects were reversible upon wash-out of the individual drugs. In an AF cell, the inhibitory effect of SNAP on cilostamide-stimulated $I_{Ca,L}$ was more pronounced, and 30 μM EHNA was required to reverse it (*Figure 5B*).

These findings suggest that NO exposure induces cross-talk between the cGMP and cAMP signalling pathways in human RA myocytes.

3.4 Cyclic nucleotide levels in RAA

Basal- and drug-stimulated tissue cAMP and cGMP content was measured in SR and AF atrial tissues using the RIA assay. Basal levels of cAMP or cGMP in tissues frozen directly in the operation theatre were not different between SR ($n = 8$) and AF ($n = 8$) (*Figure 6A*). SR and AF samples exposed to SNAP (1 mM) or to Iso (1 μM) for 10 min were compared with samples exposed to SNAP (1 mM) for 2 min followed 10 min of Iso (1 μM). All experiments were compared with the appropriate time-matched controls (CTL in *Figure 6B* and *C*). The tissue content of cAMP increased after exposure to Iso in both groups (*Figure 6B*), but was not changed after exposure to SNAP only. In contrast, cGMP content significantly increased after SNAP only and levels were similar in SR and AF (*Figure 6C*). The combination of SNAP and Iso in the bath solution led to an increase in cGMP content higher than SNAP alone in both SR and the AF groups. This finding may be explained by the competition between cGMP and cAMP for PDE3 binding. High concentrations of cAMP likely displace cGMP from PDE3 and prevent its hydrolysis, thus explaining why higher concentrations of cGMP are detected. No change of cGMP content was observed with Iso alone (*Figure 6C*).

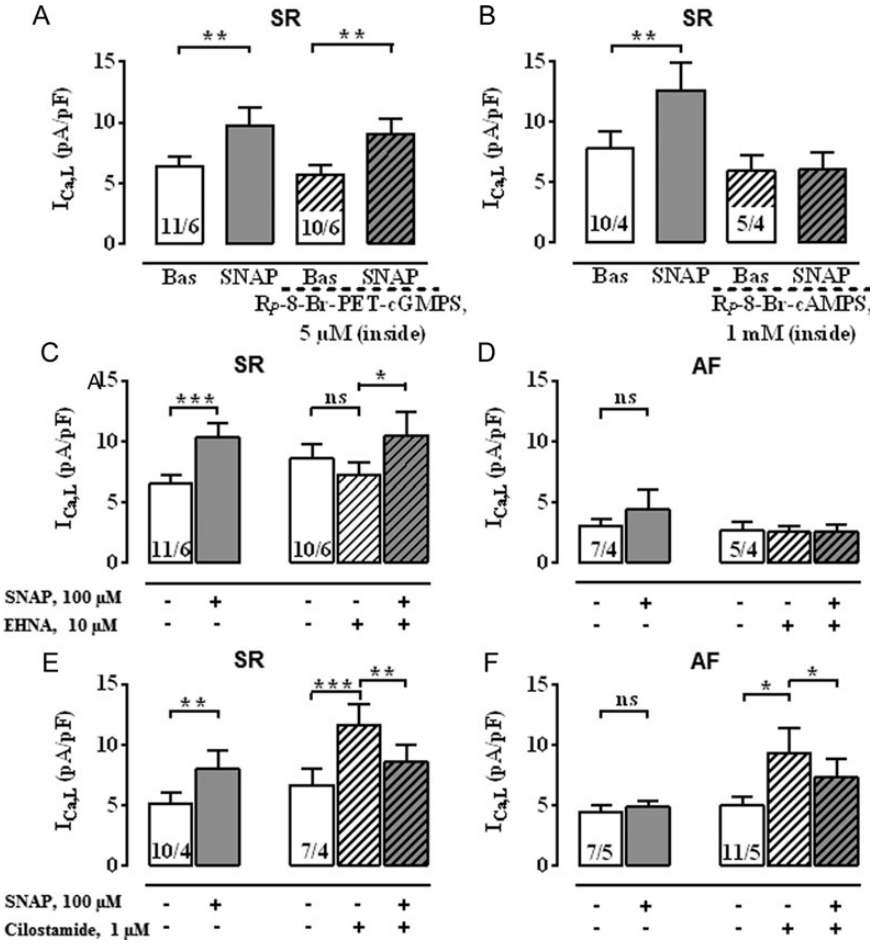

Figure 4 Effects of PKA, PKG, PDE2, and PDE3 inhibition on basal or SNAP-stimulated $I_{Ca,L}$. (*A*) Basal (Bas) and SNAP-stimulated $I_{Ca,L}$ under control conditions and with PKG inhibitor R_P-8-Br-PET-cGMP (5 μM) inside the cells (SR cells only). $^{**}P < 0.01$ vs. control. Patient-matched cells. (*B*) Effects of the PKA inhibitor R_P-8-Br-cAMP (1 mM) on basal (Bas) and SNAP-stimulated $I_{Ca,L}$, same lay-out as in (*A*). (*C* and *D*) Effects of SNAP alone (100 μM) or together with the PDE2 inhibitor EHNA (erythro-9-(2-hydroxy-3-nonyl)adenine, 10 μM) on $I_{Ca,L}$ in SR (*C*) and AF cells (*D*). (*E* and *F*) The effect of 100 μM SNAP alone or together with the PDE3 inhibitor, cilostamide (1 μM) on $I_{Ca,L}$ in SR cells (*E*) and AF cells (*F*). Means ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. basal or cilostamide-stimulated current.

3.5 Expression of PDE3A and sGC in SR and AF

Western blot analysis did not reveal any difference in the protein expression level of the three isoforms of PDE3A in RAA between SR ($n = 10$) and AF ($n = 10$). However, in 2 out of 10 AF samples the longest PDE3A isoform was barely present, but this was not reflected in the mean values (indicated by arrow in the Supplementary material online, *Figure S2*). In addition, the expression of the two subunits of sGC α_1 and β_1 was compared in RAA between SR ($n = 7$) and AF ($n = 7$). Differences in protein level expression were observed for sGCβ_1 only. Unexpectedly, it was higher in AF samples (see Supplementary material online, *Figure S2*).

4. Discussion

We have investigated the impact of NO signalling on L-type calcium currents in RA myocytes from patients with either no history of AF (SR) or with persistent AF. The NO donor SNAP increased $I_{Ca,L}$ in SR, but this effect was attenuated in AF. In contrast, SNAP reduced the Iso/cilostamide-mediated increase in $I_{Ca,L}$ independent of rhythm status. Since SR and AF patients differ in more parameters than just rhythm status, subgroup analysis according to underlying heart disease or haemodynamic parameters was performed. No correlation was found between the SNAP responses and haemodynamic conditions or type of underlying cardiomyopathy (Supplementary material online, *Figure S5*).

Our analysis suggests that (i) NO enhances production of cGMP by sGC with indirect activation of PKA, (ii) that cGMP and cAMP signalling may have cross-talk via PDE2/PDE3, and (iii) that attenuated sGC-PDE3 interaction in AF may influence NO-mediated regulation of $I_{Ca,L}$.

4.1 Cyclic-GMP-independent effects of NO

Cyclic-GMP-independent NO signalling involves *S*-nitrosylation of proteins including L-type Ca^{2+} channels, which has been considered to inhibit $I_{Ca,L}$.[8] In LAA samples from patients with chronic AF, increased *S*-nitrosylation of L-type Ca^{2+} channels was associated with a reduction of the calcium current.[10] However, here we found that RAA L-type Ca^{2+} channels were strongly *S*-nitrosylated under basal conditions, with no

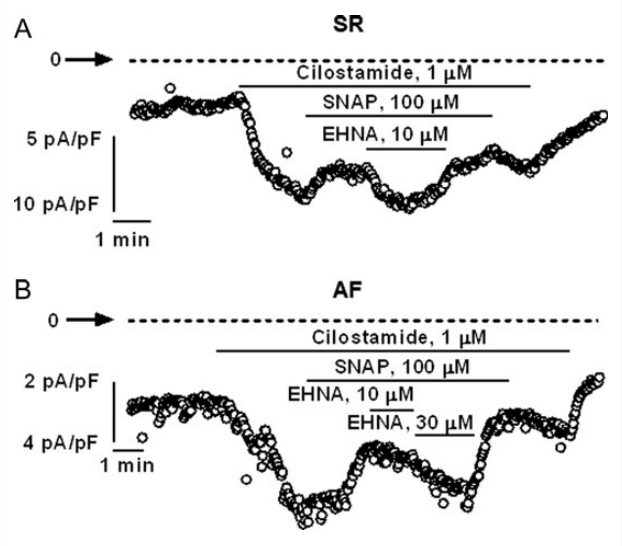

Figure 5 Inhibitory effect of SNAP on cilostamide-induced increase is due to PDE2 activation. Diary plot of $I_{Ca,L}$ in SR (*A*) and AF (*B*) cardiomyocytes. Bars indicate exposure to cilostamide, SNAP, and EHNA.

difference between SR and AF, and increasing NO via the NO donor SNAP did not further reduce, but actually enhanced $I_{Ca,L}$, suggesting that *S*-nitrosylation cannot be the dominant mechanism for the reduction in current in AF.

4.2 Cyclic-GMP-dependent effect of NO

The responses of atrial $I_{Ca,L}$ to SNAP were clearly due to NO release, as the non-NO-releasing compound NAP was ineffective. Because the selective inhibitor of sGC ODQ abolished the SNAP effect and because direct stimulation of sGC with BAY 41-2272 and SNAP was additive on $I_{Ca,L}$, we confirmed that sGC is involved. These findings support a role of cGMP in atrial NO signalling.

The most prominent effect of NO on SR atrial myocytes was a concentration-dependent increase in $I_{Ca,L}$, similar to observations in rabbit atria[5] and guinea-pig ventricle.[6] However, knock-out of the neuronal NOS isoform in mouse heart was associated with an increase of $I_{Ca,L}$,[7] suggesting a primary inhibitory effect of NO. Reasons for these apparently discrepant findings are unclear, but species differences in heart rate may contribute.

In human atria, biphasic changes of $I_{Ca,L}$ were reported in response to the NO-donor SIN-1[4] or intracellular application of cGMP,[23] where low concentrations enhanced $I_{Ca,L}$ to a larger extent than high concentrations. However, in our study the effect of SNAP on $I_{Ca,L}$ increased linearly a concentration-dependent manner. Such discrepancy might be related to the fact that various NO-releasing compounds have different potencies when it comes to producing comparable levels of cGMP.[24,25] Vandecasteele *et al.*[23] explained their dual effect of cGMP by opposite actions of cGMP on PDE2 and PDE3. Therefore, we assume that SNAP alone does not increase intracellular cGMP to a sufficiently high level for inducing PDE2–PDE3 cross-talk. Moreover, SIN-1, unlike SNAP, can release both NO and superoxide anion, the precursors of peroxynitrite ($ONOO^-$), and may therefore have additional actions on $I_{Ca,L}$.[26]

4.3 Signalling transduction pathways of NO in human atrial myocytes and importance of PDEs for cGMP–cAMP communication

Cyclic-GMP-mediated signalling involves activation of protein kinases and modulation of PDEs. Besides activating PKG, cGMP may also directly activate PKA, at least at very high concentrations (which may never be reached *in vivo*).[27] Involvement of PKG is likely excluded, because the PKG inhibitor R_P-8-Br-PET-cGMP was ineffective during the SNAP-induced increase in $I_{Ca,L}$. In contrast, PKA activity was essential for the NO-induced $I_{Ca,L}$ increase because the effect was completely blocked in the presence of the PKA inhibitor R_P-8-Br-cAMP. Thus, cGMP must influence PKA activity indirectly, by modulating cAMP levels via regulation of PDEs.[9]

Supplementary material online, *Figure S6* schematically summarizes the likely pathways involved in NO-induced regulation $I_{Ca,L}$ in human atrial myocytes. NO released by SNAP stimulates cGMP production by sGC. Under basal conditions, cGMP inhibits PDE3 promoting cAMP elevation, PKA activation, phosphorylation of L-type Ca^{2+} channels leading to increased channel open probability.[28]

Inhibition of PDE3 by cilostamide increases cAMP locally, whereas stimulation of β-adrenoceptors by Iso causes a readily detectable global increase in cAMP. At high concentrations, cAMP competes with cGMP for PDE3 binding, displacing cGMP from the enzyme, and preventing its hydrolysis. Under these conditions, the total concentration of cGMP in response to NO may not be different, but a larger fraction of the cGMP formed will become available for allosteric stimulation of PDE2, leading to accelerated hydrolysis of cAMP and a reduction of $I_{Ca,L}$ (*Figures 3* and *5*).

Thus, PDEs likely serve as switches between cAMP and cGMP signalling[29] due to their differential sensitivity to cyclic nucleotides.[30] In the present study, we focussed on PDE2–PDE3, because selective inhibitors for PDE1 are unavailable. Even though ~15% of total PDE activity in human atria is due to PDE4,[31] we did not study this isoform in detail, because the observed SNAP effects were clearly cGMP dependent, whereas PDE4 is cGMP independent.[9] PDE5 seems unlikely to be involved due to lack of NO-induced PKG activation.[32]

Further evidence for regulatory feedback between cAMP and cGMP is provided by the following finding. SNAP-induced PKA activation (see Supplementary material online, *Figure S6*) is supported by the observed shift of peak $I_{Ca,L}$ activation to more negative potentials (*Figure 1E*). However, the addition of Iso in the presence of SNAP caused a smaller increase in $I_{Ca,L}$ than the addition of Iso alone. Thus, further increases in cAMP may promote cGMP-mediated PDE2 activation, counterbalancing the Iso effect by enhancing cAMP breakdown. After SNAP wash-out, the full Iso effect is observed because cGMP-mediated negative feedback is removed. Similar interactions between cAMP and cGMP can explain the effects observed with direct inhibition of PDE3. Cilostamide elevates basal cAMP levels locally[33] and increases $I_{Ca,L}$ (albeit to a smaller extent than Iso), and the addition of SNAP reduces the cilostamide effect on $I_{Ca,L}$.

The role of PDE2 in basal cAMP regulation was evaluated using EHNA, a PDE2 inhibitor that neither affected basal $I_{Ca,L}$ nor modulated the SNAP effects on $I_{Ca,L}$. This observation contrasts with a previous report in human atria[11]; different experimental conditions (e.g. temperature, *composition of solutions, voltage-clamp protocols*) may account for the

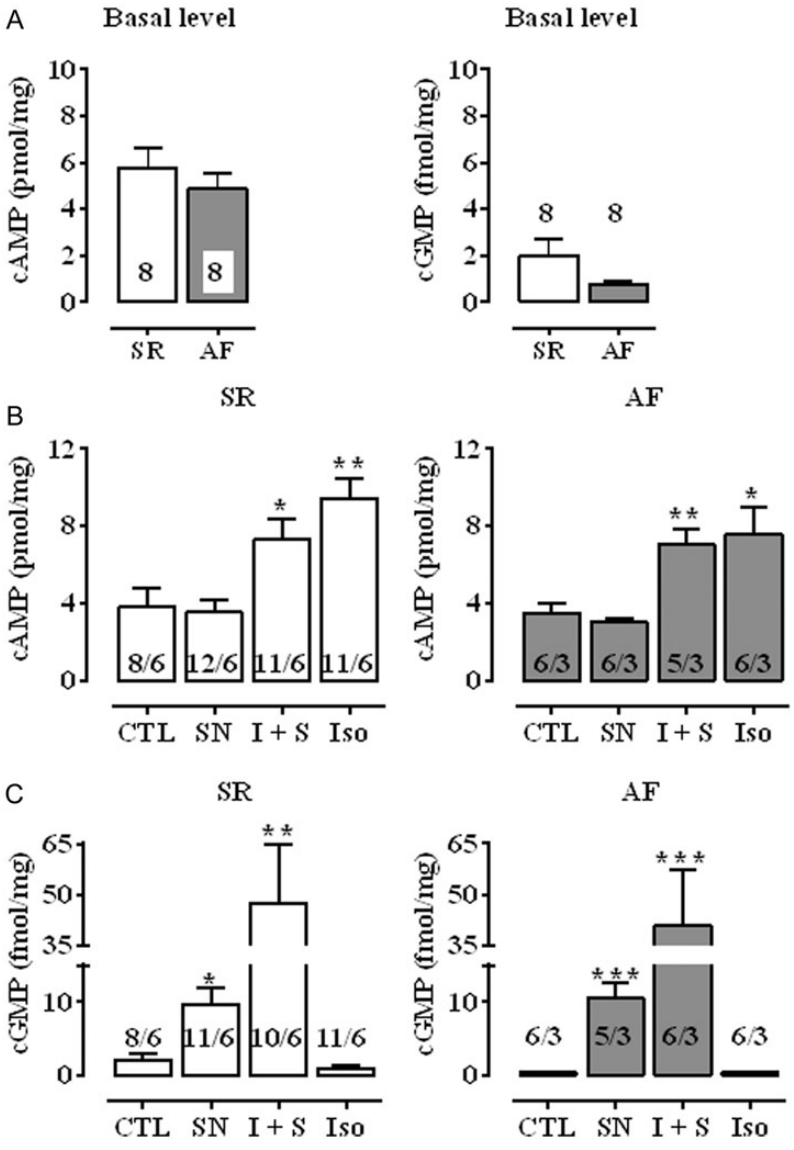

Figure 6 Cyclic nucleotide levels in human RAA trabeculae (SR vs. AF). (*A*) Basal cAMP (pmol/mg of tissue) and cGMP (fmol/mg of tissue) content in freshly frozen tissue (directly in the operation theatre) in SR and AF samples. Please note the difference in the scale for cAMP and cGMP. (*B* and *C*) Effects of SNAP (1 mM), SNAP (1 mM) plus Iso, (1 μM), and Iso (1 μM) alone on concentration of cAMP (pmol/mg of tissue) (*B*) and cGMP (fmol/mg of tissue) (*C*). Numbers represent trabeculae/patients. CTL, time-matched control trabeculae.

difference. Our results suggest that basal cAMP levels are primarily regulated by PDE3, and PDE2 is recruited only when cAMP is elevated. This hypothesis is supported by the finding that the negative effect of SNAP on cilostamide-induced $I_{Ca,L}$ increase was rescued by the PDE2 inhibitor EHNA (*Figure 5*).

These results overlap with previously published data by Vandecasteele, where strong stimulation of basal $I_{Ca,L}$ was observed during intracellular application of 0.5 μM cGMP and a 2-fold smaller increase in $I_{Ca,L}$ with a 10-fold higher cGMP concentration. Application of a selective PDE2 inhibitor (EHNA, 30 μM) fully reversed this secondary inhibitory effect of 5 μM cGMP on $I_{Ca,L}$.[23] Moreover, the cAMP–cGMP cross-talk as observed in our experiments is consistent with fluorescence resonance energy transfer analysis of cyclic nucleotides after Iso and SNAP application, which showed compartmentalization of cyclic nucleotide signalling due to differences in location of PDEs and PKA-regulatory subunits isoforms (PKA-RI and PKA-RII). Thus after NO-induced cGMP elevation, the cAMP pool in the compartment where the PKA-RI isoform is located increases during PDE3 inhibition, but cAMP in the compartment where the PKA-RII isoform resides decreases as a result of PDE2 activation.[20,34] Moreover, PDE2 was shown to be tightly coupled to the pool of adenylyl cyclases activated by β-adrenoceptor stimulation providing local control of cAMP in a stimulus-specific manner.[34]

4.4 Attenuated effect of NO in atrial myocytes from patients with AF

The diminishing or absence of SNAP effects observed in AF cells suggests that cGMP-dependent NO signalling is disrupted in AF, despite the fact that NO signalling interferes with Iso-induced $I_{Ca,L}$ as in SR. Preservation of the latter NO effect indicates that cGMP production and therefore

sGC activity is not impaired in AF. This notion is supported by similar increases in cGMP levels after SNAP exposure in SR and AF tissue. Moreover, PDE3 inhibition with cilostamide leads to an increase in $I_{Ca,L}$ in AF cells, which is comparable with the $I_{Ca,L}$ increase in SR cells, suggesting that PDE3 activity remains intact. There was no difference in the expression of any of the three PDE3A protein isoforms or in the expression of $sGC\alpha_1$ between SR and AF. However, the expression of $sGC\beta_1$ was slightly increased in AF, which may be a compensatory mechanism.

Thus, a combination of electrophysiological and biochemical approaches leads us to hypothesize that in the right atria *from AF patients, functional* interactions between sGC and PDE3 *are compromised.* One possibility could be a change in the sub-membrane distribution of sGC as has been reported in the case of pressure-induced overload by transverse aortic constriction in mice;[35] however, this hypothesis should be tested in future studies.

While cAMP–cGMP cross-talk and cellular signal compartmentalization has been extensively studied, most data have been obtained from studies of animal rather than human myocytes. Species differences may affect the expression and function of cardiac proteins, thus such studies require careful interpretation.[36] Future investigations are needed to analyse species-specific isoforms of PKGs, PKAs, and PDEs and their co-localization with cyclic nucleotide signalling proteins in live human cardiomyocytes.

In general, recent works on cyclic nucleotides signalling and PDE function as the regulator of cyclic nucleotides in various heart diseases, including AF (this work and Molina *et al.*[31]) provide a promising scientific direction towards understanding of the development of heart pathophysiology.

5. Clinical implications

Since NO-releasing drugs are widely used for treatment of angina pectoris,[37] our results may have important clinical implications. NO may limit excessive sympathetic stimulation of $I_{Ca,L}$ in the SR and AF atria, thereby alleviating the pro-arrhythmic effects of endogenous catecholamines. Attenuation of the $I_{Ca,L}$-enhancing effects of NO in AF may also limit excessive cellular Ca^{2+} loading during high-frequency activity. However, these speculations will need to be experimentally validated.

6. Limitations of study

An important limitation of our study is that only RA myocytes were available for $I_{Ca,L}$ measurements. A challenge for such studies is that, while left atrial tissue can be frequently obtained from AF patients undergoing Maze surgery, control left atrial tissues (SR) are seldom available. In addition, we cannot match patients for underlying heart disease, because patients in SR will be affected more often by coronary heart diseases CHD, whereas AF is more often associated with valvular diseases.

Moreover, the limited selectivity of available pharmacological compounds must be recognized.

7. Conclusions

Our data document a critical role for cAMP–cGMP cross-talk in the regulation of $I_{Ca,L}$ in human atrial cells and provide novel evidence for attenuation of cGMP–cAMP signalling in atrial cells associated with AF.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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