Ranolazine prevents $I_{\text{NaL}}$ enhancement and blunts myocardial remodelling in a model of pulmonary hypertension

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Received 14 March 2014; revised 4 July 2014; accepted 28 July 2014; online publish-ahead-of-print 18 August 2014

Time for primary review: 24 days

Aims Pulmonary arterial hypertension (PAH) reflects abnormal pulmonary vascular resistance and causes right ventricular (RV) hypertrophy. Enhancement of the late sodium current ($I_{\text{NaL}}$) may result from hypertrophic remodelling. The study tests whether: (i) constitutive $I_{\text{NaL}}$ enhancement may occur as part of PAH-induced myocardial remodelling; (ii) ranolazine (RAN), a clinically available $I_{\text{NaL}}$ blocker, may prevent constitutive $I_{\text{NaL}}$ enhancement and PAH-induced myocardial remodelling.

Methods and results PAH was induced in rats by a single monocrotaline (MCT) injection (60 mg/kg intraperitoneally (i.p.)); studies were performed 3 weeks later. RAN (30 mg/kg bid i.p.) was administered 48 h after MCT and washed-out 15 h before studies. MCT increased RV systolic pressure and caused RV hypertrophy and loss of left ventricular (LV) mass. In the RV, collagen was increased; myocytes were enlarged with T-tubule disarray and displayed myosin heavy chain isoform switch. $I_{\text{NaL}}$ was markedly enhanced; diastolic $\text{Ca}^{2+}$ was increased and $\text{Ca}^{2+}$ release was facilitated. $K^+$ currents were down-regulated and APD was prolonged. In the LV, $I_{\text{NaL}}$ was enhanced to a lesser extent and cell $\text{Ca}^{2+}$ content was strongly depressed. Electrical remodelling was less prominent than in the RV. RAN completely prevented $I_{\text{NaL}}$ enhancement and limited most aspects of PAH-induced remodelling, but failed to affect in vivo contractile performance. RAN blunted the MCT-induced increase in RV pressure and medial thickening in pulmonary arterioles.

Conclusion PAH induced remodelling with chamber-specific aspects. RAN prevented constitutive $I_{\text{NaL}}$ enhancement and blunted myocardial remodelling. Partial mechanical unloading, resulting from an unexpected effect of RAN on pulmonary vasculature, might contribute to this effect.

Keywords Remodelling • Hypertrophy • Late sodium current • Pulmonary hypertension • Ranolazine

1. Introduction

Pulmonary hypertension may develop as a consequence of chronic enhancement of pulmonary flow (e.g. in cardiac defects) or systemic hypoxia. Nevertheless, its most common and aggressive form (pulmonary arterial hypertension, PAH) results from ‘primary’ wall thickening in small pulmonary arteries, of uncertain aetiology, but often observed in association with systemic diseases (autoimmune, etc.). A widely accepted experimental model of PAH is generated by systemic administration of monocrotaline (MCT), a vascular toxin that selectively affects the pulmonary microcirculation.1 PAH imposes a high pressure load on the right ventricle (RV) and leads to progressive remodelling of this...
chamber. Albeit initially compensatory, the process becomes maladaptive, thus making the development of RV dysfunction the turning point in PAH prognosis. Although PAH-induced mechanical overload mainly affects the RV, left ventricular (LV) involvement has been reported by experimental and clinical studies. In a variety of experimental models other than RV hypertrophy, myocardial hypertrophy/failure is associated with enhancement of the late Na\(^+\) current (\(i_{\text{Na}}\)). This may, in turn, contribute to many of the functional (electrical and contractile) derangements associated with remodelled myocardium. Furthermore, because of its impact on homeostasis of intracellular Ca\(^{2+}\), which is pivotal in initiating hypertrophic transcriptional regulation, \(i_{\text{Na}}\) enhancement might also contribute to myocardial remodelling itself. 

Recent work reports that pressure-induced RV remodelling can be opposed by ranolazine (RAN) and trimetazidine. The effect has been attributed to partial inhibition of fatty acid oxidation (FAO), an action of RAN previously reported. Nevertheless, RAN ability to inhibit FAO at therapeutic concentrations has been disputed. More recent reports attribute cardioprotective effects of RAN to \(i_{\text{Na}}\) blockade.

The present study evaluates the hypothesis that (i) constitutive \(i_{\text{Na}}\) enhancement may occur as part of PAH-induced myocardial remodelling; (ii) RAN may prevent \(i_{\text{Na}}\) enhancement, and (iii) RAN can prevent PAH-induced myocardial remodelling.

2. Methods

The investigation conforms to the Guide of the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and to the guidelines for animal care endorsed by the hosting institution.

An expanded Methods section is available in Supplementary material online.

2.1 PAH model

Adult male Sprague–Dawley rats (110 g) were injected intraperitoneally (i.p.) with 2% MCT solution at a dose of 60 mg/kg (MCT group). A subset of MCT-treated rats received RAN (30 mg/kg, bid i.p.) for 20 days (MCT–RAN group); to avoid interference with MCT metabolism, RAN treatment was started 2 days after MCT injection. RAN vehicle was administered to the MCT group and to untreated littersmates, which served as controls (CTRL group). To evaluate remodelling-dependent effects, all measurements had to be carried out in the absence of RAN. Thus, the time between the last RAN injection and sacrifice (blood sample collection) was 15–16 h in all cases, i.e. adequate for complete RAN washout. RAN plasma level measured during treatment in a separate group of animals was 15–16 h in all cases, i.e. adequate for complete RAN washout. RAN plasma level was measured at sacrifice and, as expected, was found negligible. RAN plasma level measured during treatment in a separate group of animals was 6.07 ± 0.8 μM (N = 6).

2.2 Echocardiography

Transthoracic echocardiography was performed on sedated rats [ketamine 80 mg/kg and midazolam 2.5 mg/kg body weight (BW) i.p.]. RV systolic function was evaluated by measurement of the fractional area contraction (FAC) and the tricuspid annular plane systolic excursion (TAPSE).

LV volumes (end-diastolic volume and end-systolic volume) and LV ejection fraction (EF) were measured from the parasternal long-axis view and calculated through the modified single-plane Simpson’s rule.

RV and LV diastolic function was evaluated through isovolumic relaxation time (IRT). IRT was measured by tissue Doppler imaging of tricuspid annulus in the RV and by pulsed wave Doppler in the LV.

The operator was blinded to the treatment group during both echo recording and analysis. Further detail on echo measurements is provided in Supplementary material online.

2.3 Invasive haemodynamic monitoring

RV systolic pressure (RVSP) was measured with a tip-transducer catheter (Millar SPR671) introduced in the RV through the right jugular vein under anaesthesia (pentobarbital 50 mg/kg i.p.), allowing for spontaneous breathing. After ruling out pulmonary valve and RV outflow tract stenosis by echocardiography, RVSP was considered representative of pulmonary artery systolic pressure.

2.4 Quantitative RT-PCR

Relative expression of mRNAs encoding α- and β-myosin heavy chain (MHC) and Kv4.2 was measured using quantitative RT-PCR (RTq-PCR) in RV and LV of each experimental group. Specific primers are shown in Supplementary material online, Table S1.

2.5 Myocyte isolation and patch-clamp measurements

Rats were sacrificed by cervical dislocation under ketamine/xylazine (50 and 5 mg/kg) anaesthesia 3 weeks after MCT injection. Ventricular myocytes were isolated from LV- and RV-free walls as previously reported with minor modifications; the interventricular septum (IVS) was discarded. Rod-shaped, Ca\(^{2+}\)-tolerant myocytes were used for patch-clamp measurements within 6–8 h from dissociation.

Single-cell electrical activity was evaluated by action potential (AP) recordings in isolated myocytes during steady-state pacing at 2 Hz. For steady-state currents (\(i_{\text{Na}}\) and inward rectifier K\(^{+}\) current, \(i_K\)), IV relationships were obtained by applying slow voltage ramps (56 mV/s), \(i_{\text{Na}}\) was measured as tetrodotoxin (TTX, 30 μmol/L)-sensitive current (\(i_{\text{Na}}\text{TTX}\)). According to preliminary evidence (see Supplementary material online, Figures S1 and S2), \(i_{\text{Na}}\text{TTX}\) at 0 mV was taken as representative of \(i_{\text{Na}}\) and peak \(i_{\text{Na}}\text{TTX}\) value, occurring at more negative potentials, was assumed to reflect the Na\(^+\) window component (\(i_{\text{NaW}}\)). \(i_K\) was isolated by subtraction of recordings in K\(^+\)-free solution.

Transient currents (transient outward current \(I_o\) and L-type Ca\(^{2+}\) current \(I_{\text{CaL}}\) were measured by standard voltage-clamp protocols. To isolate \(I_o\) (see Supplementary material online, Figure S3), cadmium (0.5 mmol/L) and ethylene glycol tetracetic acid (4 mmol/L) were added to extracellular and intracellular solutions, respectively. \(I_{\text{CaL}}\) was measured in Na\(^+\)- and K\(^+\)-free solution.

To take into account the variations in cell size, current amplitude was normalized to membrane capacitance (\(C_m\)) in all measurements.

2.6 Calcium handling

Fluo-4-loaded intact myocytes were field-stimulated at 2 Hz (see Supplementary material online, Figure S4). Fluorescence signal was converted to free Ca\(^{2+}\) concentration by an indirect method, as detailed in Supplementary material online, Figure S5. Ca\(^{2+}\) transient amplitude ([Ca\(^{2+}\)]\(T\)) and diastolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(d\)) were measured at steady-state. Sarcoplasmic reticulum (SR) Ca\(^{2+}\) content ([Ca\(^{2+}\)]\(SR\)) was estimated by an electronically timed 10 mmol/L caffeine pulse after 10 s at resting. SR fractional release was obtained as the ratio between [Ca\(^{2+}\)]\(T\) and [Ca\(^{2+}\)]\(SR\).

2.7 T-tubule (TT) analysis

Sarcolemmal membranes were stained by incubating isolated ventricular myocytes with di-3-ANEPPDHQ (20 μmol/L). Eight-bit grayscaled images were subjected to spatial Fast Fourier Transform analysis allowing to quantify periodic and aperiodic components of pixel variance (see Supplementary material online, Methods). Whereas the periodic component is generated by transverse TT, the aperiodic one may reflect disarray of transverse TT, but can also be generated by longitudinal TT.
2.8 Statistical analysis
Data are presented as mean ± SE. A chi-squared test was used for the comparison of categorical variables. Comparison between multiple means was performed by one- or two-way ANOVA and post hoc Bonferroni’s correction. A repeated measurement model was applied whenever internal comparisons were involved. The Mann–Whitney test was used for multiple comparisons of categorical variables. Statistical significance was defined as \( P < 0.05 \) (NS, not significant). The main text reports percent changes; unless otherwise specified, a ‘change’ is statistically significant. The respective absolute values are shown in the figures. The number of animals (\( N \)) and cells (\( n \)) are reported in figure legends.

3. Results
For all measurements, values obtained from RV and LV in the CTRL group are compared in Supplementary material online, Table S2. Only the changes induced by MCT and by concomitant RAN administration are described here. In the whole population of animals, RAN stands for ‘chronic RAN’ treatment.

3.1 MCT model: general parameters
The haematocrit was similar between CTRL and MCT rats, thus suggesting that systemic hypoxia or significant reduction in plasma volume was not present in MCT rats. MCT rats had a lower BW (−27%). The lung-to-BW ratio was increased (LW/BW, +98%), almost entirely due to an increased water content (wet-to-dry ratio, +93%, \( N = 5 \), data not shown). The heart-to-BW ratio was increased (HW/BW, +54%), due to a substantial enhancement of the RV mass (RV/HW, +43%), partially balanced by a decrease in the LV mass (LV/HW, −19%). Overall, RV contribution to HW was markedly increased [RV/(LV + IVS), +77%; Table 1].

As summarized in Table 1, RAN partially prevented RV hypertrophy. BW, lung weight content, and LV weight were unchanged by RAN.

During 3 weeks of treatment none of 47 animals died in the CTRL group, 5 of 46 animals died in the MCT group and 6 of 50 animals died in the MCT–RAN group (\( P = 0.05 \) for overall significance).

3.2 Cardiac structure and function in vivo
Heart rate (measured under sedation) was significantly lower in MCT than in CTRL rats; in MCT–RAN rats, heart rate did not differ from that of CTRL rats (Table 2).

In the MCT group, RV anatomical and functional parameters were indicative of free wall hypertrophy (wall thickness, +95%; see Supplementary material online, Figure S6), cavity enlargement (basal diameter, +27%), and systolic dysfunction (FAC −63% and TAPSE −30%). Moreover, the RV IRT was significantly prolonged (+155%). LV function was also altered in the MCT group. All LV volumes were reduced, probably due to IVS bulging towards the LV (see Supplementary material online, Videos S1 and S2); as a result, LV stroke volume and cardiac output (CO) decreased significantly (CO −49%). LV diastolic function was also impaired, as indicated by prolonged LV IRT (+55%) and decreased E-wave deceleration time (DT, −28%). Despite the marked changes in LV geometry, the LVEF remained unchanged.

RAN significantly opposed RV wall hypertrophy (wall thickness) and chamber enlargement (basal diameter; Table 2). Indexes of RV systolic (FAC and TAPSE) and diastolic (IRT) function were not significantly affected by RAN. Impairment of LV systolic function persisted in the RAN group. For what concerns LV diastolic function, RAN failed to affect the isovolumic phase (IRT) but sharply prevented changes in diastolic filling (DT). This pattern is consistent with prevention of septal bulging by RAN (see Supplementary material online, Video S3), likely to affect the filling phase more than the isovolumic one.

To assess the time-course of MCT and RAN effects, RV wall thickness and basal diameter were evaluated at three time-points (1, 2, and 3 weeks after MCT) in a separate group of animals (see Supplementary material online, Figure S7). Most of MCT-induced hypertrophy ensued between Weeks 2 and 3, and its development was blunted by RAN at all time-points. Notably, MCT effects were null at 1 week, thus confirming that RAN administration (at Day 2) had a preventive significance.

3.3 Fibrosis and cellular hypertrophy
Myocyte cross-sectional area (CSA) and interstitial fractional collagen content were measured to assess the relative contribution of myocyte and interstitial remodelling to muscle hypertrophy.

RV interstitial collagen content was increased in the MCT group (+270% vs. CTRL; Figure 1A), a change almost prevented by RAN. LV collagen content was similar in the three experimental groups.

Myocyte CSA was increased in the RV free wall of the MCT group (+68%), and it did not change in the LV free wall, but it was slightly reduced (−22%) in the IVS (see Supplementary material online, Figure S8). RAN completely prevented myocyte CSA enhancement in the RV and partially in the IVS (see Supplementary material online, Figure S8).

### Table 1  General parameters

<table>
<thead>
<tr>
<th></th>
<th>CTRL (( N = 18 ))</th>
<th>MCT (( N = 17 ))</th>
<th>+ RAN (( N = 16 ))</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( M vs. ) CTRL</td>
<td>RAN vs. CTRL</td>
<td>RAN vs. MCT</td>
<td></td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>41.5 ± 0.8</td>
<td>45 ± 1.6</td>
<td>44 ± 1.9</td>
<td>NS</td>
</tr>
<tr>
<td>BW (kg)</td>
<td>0.29 ± 0.005</td>
<td>0.21 ± 0.007</td>
<td>0.22 ± 0.005</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LW/BW (g/kg)</td>
<td>5.65 ± 0.16</td>
<td>11.1 ± 0.45</td>
<td>10.5 ± 0.52</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HW/BW (g/kg)</td>
<td>4.37 ± 0.11</td>
<td>6.73 ± 0.27</td>
<td>5.87 ± 0.25</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RV/(LV + IVS)</td>
<td>0.39 ± 0.02</td>
<td>0.69 ± 0.04</td>
<td>0.58 ± 0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RV/HW (g/g)</td>
<td>0.23 ± 0.008</td>
<td>0.33 ± 0.011</td>
<td>0.29 ± 0.007</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(LV + IVS)/HW (g/g)</td>
<td>0.59 ± 0.01</td>
<td>0.48 ± 0.01</td>
<td>0.52 ± 0.01</td>
<td>&lt;0.05</td>
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</tbody>
</table>

BW: body weight; LW, lung weight; HW, heart weight; RV, right ventricle; LV, left ventricle; IVS, interventricular septum.
### Table 2 In vivo echocardiography

<table>
<thead>
<tr>
<th></th>
<th>CTRL (N = 15)</th>
<th>MCT (N = 13)</th>
<th>+ RAN (N = 10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MCT vs. CTRL</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>429 ± 15</td>
<td>346 ± 16</td>
<td>373 ± 27</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RV Wall thickness (mm)</td>
<td>0.39 ± 0.02</td>
<td>0.76 ± 0.03</td>
<td>0.56 ± 0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Basal diameter (mm)</td>
<td>3.17 ± 0.1</td>
<td>4.03 ± 0.1</td>
<td>3.19 ± 0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>FAC (%)</td>
<td>68 ± 3</td>
<td>25 ± 4</td>
<td>38 ± 5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TAPSE (mm)</td>
<td>4.0 ± 0.1</td>
<td>2.8 ± 0.2</td>
<td>3.1 ± 0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IRT (ms)</td>
<td>14.8 ± 0.73</td>
<td>37.8 ± 0.73</td>
<td>31.6 ± 3.75</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LV EDV (mL)</td>
<td>319 ± 14</td>
<td>157 ± 10</td>
<td>193 ± 20</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ESV (mL)</td>
<td>55 ± 6</td>
<td>24 ± 2</td>
<td>21 ± 3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SV (mL)</td>
<td>368 ± 22</td>
<td>230 ± 30</td>
<td>220 ± 30</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>155 ± 9</td>
<td>79 ± 11</td>
<td>88 ± 17</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EF (%)</td>
<td>82 ± 2</td>
<td>85 ± 1</td>
<td>90 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>IRT (ms)</td>
<td>20 ± 1</td>
<td>31 ± 4</td>
<td>30 ± 2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DT (ms)</td>
<td>31 ± 2</td>
<td>23 ± 1</td>
<td>37 ± 3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

RV, right ventricle; FAC, fractional area contraction; TAPSE, tricuspid annular plane systolic excursion; IRT, isovolumic relaxation time; LV, left ventricle; EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; CO, cardiac output; EF, ejection fraction; DT, transmitral flow E-wave deceleration time.

### Figure 1
Fibrosis and cellular hypertrophy. (A) Interstitial collagen content and myocyte CSA in each study group; comparison between RV and LV. Left: representative sections stained with 0.1% Sirius red (top) or Alexa Fluor 488-conjugated wheat germ agglutinin (bottom) for collagen content and CSA measurements, respectively. Right: average results for free wall CSA and collagen content. N = 5 for each group. (B) Cell membrane capacitance (C_m) in each experimental group. N ≥ 15 and n ≥ 117 for each group. (C) Relative mRNA expression (vs. GAPDH) of cardiac hypertrophy-related genes (α- and β-MHC, Kv4.2) in each study group by RTq-PCR. N ≥ 4 for each group. *P < 0.05 vs. CTRL, #P < 0.05 vs. MCT.
Electrical and molecular indexes of myocyte hypertrophy were also evaluated. In the MCT group, \( C_m \) was increased in RV myocytes (+34%), but remained unchanged in LV ones (Figure 1B). RAN prevented RV \( C_m \) enhancement only partially, which contrasts with the complete prevention of CSA changes. Discordant changes in CSA and \( C_m \) were also seen in LV myocytes, in which RAN reduced \( C_m \) but did not affect CSA (Figure 1B).

In the MCT group, RV \( \alpha \)-MHC expression decreased (−0.5 times), whereas \( \beta \)-MHC strongly increased (+13.8 times) (Figure 1C). A similar pattern was observed in the LV (\( \alpha \)-MHC −0.76 times, \( \beta \)-MHC +27 times), thus suggesting that MCT also caused remodelling of the LV. Kv4.2 expression, a marker of overload-induced hypertrophy, was down-regulated in the RV, but not in the LV (Figure 1C). In the MCT–RAN group, \( \alpha \)-MHC down-regulation was completely prevented in RV only and \( \beta \)-MHC up-regulation was blunted in the LV only. Thus, although RAN counteracted MCT-induced changes in MHC in both chambers, modulation of the isoform pattern differed between RV and LV. RAN did not prevent Kv4.2 down-regulation in the RV.

### 3.4 T-tubule remodelling

Disarray of the T-tubular (TT) system has been described in several hypertrophy/failure models and was generally characterized by loss of the transverse component, alone or associated with an increment of the longitudinal one. Furthermore, in the present study, changes in TT were suggested by the discrepancy between myocyte CSA and \( C_m \) changes (above). Thus, the TT pattern of the three experimental groups was analysed in isolated ventricular myocytes of both chambers (Figure 2).

A sharp pattern of transverse striations was observed in CTRL myocytes of both chambers; accordingly, in these myocytes, pixel variance was largely represented by the periodic component, whose period (0.5 \( \mu \text{m} \)) was consistent with transverse TT arrangement.

RV disarray of the transverse TT was visually obvious in MCT myocytes; the power under the periodic component was decreased (−49%), accompanied by an increase in the aperiodic one (+83%). The former was decreased also in the LV (−29%), but no changes were detectable in the latter.

**Figure 2** T-tubule (TT) remodelling. (A) Confocal images of di-3-ANEPPDHQ (20 \( \mu \text{mol/L} \)) loaded myocytes; regions of interest of 100 × 500 pixels are shown. (B) Mean power spectrum of TT obtained in each experimental group. (C) Average results of periodic and aperiodic components of TT. \(* P < 0.05\) vs. CTRL, \#P < 0.05 vs. MCT. N ≥ 2 and n ≥ 9 for each group.
RAN partially prevented the loss of the periodic component in RV myocytes only. Changes in the aperiodic component were completely prevented by RAN in both chambers. Overall, these results point to remodelling-induced loss of transverse TT, prevailing in the RV and effectively countered by RAN. Changes in the aperiodic component might reflect a tendency of RAN to minimize membrane structures not associated with transverse TT.

3.5 Remodelling of membrane currents and potential

Persistent $I_{\text{Na}}$ components were evaluated as TTX-sensitive current during slow depolarizing ramps (Figure 3). The ’late’ ($I_{\text{NaL}}$) and window ($I_{\text{NaW}}$) components were discriminated as detailed in the Methods section. MCT enhanced $I_{\text{NaL}}$ in both chambers, but more prominently in RV myocytes (RV +149% and LV +93%). In the RV, $I_{\text{NaW}}$ also showed an increment of borderline significance ($P = 0.05$ vs. CTRL). The contribution of the observed MCT-induced $I_{\text{NaL}}$ enhancement to repolarization was assessed by action potential duration (APD) measurement during acute RAN exposure (10 μmol/L). Acute RAN slightly but significantly shortened APD at 50% repolarization (APD$_{50}$ = 6.1 ± 2.2%) in RV myocytes of the MCT group only (see Supplementary material online, Figure S9).

RAN completely prevented $I_{\text{NaL}}$ enhancement in both chambers. It should be stressed that RAN was completely washed-out before $I_{\text{NaL}}$ measurement; therefore, this observation indicates that chronic $I_{\text{NaL}}$ blockade may prevent remodelling-induced, or ’constitutive’, enhancement of $I_{\text{NaL}}$.

$I_{\text{NaL}}$ enhancement is expected to induce Ca$^{2+}$ overload, secondary to reduced forward operation of Na/Ca exchanger (NCX). In RV MCT myocytes, [Ca]$_{\text{diast}}$ and [Ca]$_{\text{T}}$ were increased (+15 and +39%, respectively), but [Ca]$_{\text{SR}}$ was unchanged, thus yielding a larger fractional release (+53%). In LV myocytes, Ca$^{2+}$ handling was markedly depressed; [Ca]$_{\text{T}}$ and [Ca]$_{\text{SR}}$ were both reduced (−31 and −38%, respectively), but fractional release was still increased (+38%); [Ca]$_{\text{diast}}$ was not significantly affected (Figure 4A).

RAN blunted [Ca]$_{\text{diast}}$ and [Ca]$_{\text{T}}$ enhancement in RV myocytes; however, fractional release remained significantly higher than in CTRL myocytes. In LV myocytes, MCT-induced Ca$^{2+}$ handling abnormalities were not affected by RAN (Figure 4A).

Intracellular Ca$^{2+}$ overload may lead to spontaneous Ca$^{2+}$ release from the SR; this may occur because of SR overload or, even in the presence of reduced SR Ca$^{2+}$ content, by ryanodine receptors (RyRs) destabilization.

Propensity to spontaneous Ca$^{2+}$ release was evaluated by measuring the proportion of cells with delayed afterdepolarizations (DADs, Figure 4C). In RV myocytes, the increase in DADs incidence induced by MCT did not achieve significance; nevertheless, DADs incidence was significantly lower in the RAN-treated group than in the MCT one. Unexpectedly, MCT-induced increment in DADs incidence was more consistent in LV myocytes, but, unlike in the RV, it was not prevented by RAN.
Figure 4: Intracellular Ca\(^{2+}\) handling and \(I_{\text{CaL}}\). (A) Steady-state Ca\(^{2+}\) transients evoked in field-stimulated myocytes (2 Hz) and during caffeine superfusion (shown by horizontal bars; see protocol in Supplementary material online, Figure S4). Fluorescence signals were converted in free cytosolic Ca\(^{2+}\) (see Supplementary material online, Figure S5); dotted lines refer to RV diastolic Ca\(^{2+}\) ([Ca\(_{\text{diast}}\)] of the CTRL group (∼80 nM) to highlight intergroup changes. (B) Statistics for [Ca\(_{\text{diast}}\]), amplitude of Ca\(^{2+}\) transient ([Ca\(_i\)]), SR Ca\(^{2+}\) content [Ca\(_{\text{SR}}\)], and fractional release. \(N \geq 3\) and \(n \geq 21\) for each group. (C) Proportion of cells with DADs in patch-clamped myocytes paced at 2 Hz. (D) \(I_{\text{CaL}}/V\) relationships in each experimental group. \(N \geq 3\) and \(n \geq 11\) for each group. *\(P < 0.05\) vs. CTRL, #\(P < 0.05\) vs. MCT.
Increments in intracellular Ca\textsuperscript{2+} and, in particular, in fractional release might suggest up-regulation of I\textsubscript{CaL} in the MCT group. However, in this group, I\textsubscript{CaL} properties (see Supplementary material online, Figure S10 and Table S3), including inactivation kinetics (see Supplementary material online, Table S4), remained unchanged in RV myocytes, and only a small reduction in peak density was observed in LV myocytes (Figure 4D). RAN failed to affect I\textsubscript{CaL} in myocytes from both chambers. 

I\textsubscript{NaL} enhancement is expected to prolong repolarization, particularly at the plateau level (APD\textsubscript{50}). In the MCT group, RVAPD was prolonged and the APD\textsubscript{50}/APD\textsubscript{90} ratio was increased (+33%), to indicate that prolongation prevailed at the plateau level. LV APD was also prolonged, but changes in APD\textsubscript{50}/APD\textsubscript{90} ratio did not achieve significance (Figure 5B). Because of these changes, physiological APD differences between RV and LV were minimized in the MCT group. Notably, the dispersion of APD\textsubscript{90} values within each ventricle, expressed as coefficient of variation (CV), was increased in the MCT group, particularly in RV myocytes. This observation led to evaluate the distribution of APD\textsubscript{90} values. In the RV, MCT broadened the originally unimodal APD\textsubscript{90} distribution and added a cluster of very long APD\textsubscript{90} values. In LV myocytes, APD\textsubscript{90} distribution was originally bimodal; under MCT, all LV APD\textsubscript{90} values were clustered under a single larger mode (Figure 5A).

Unexpectedly, RAN failed to prevent mean APD\textsubscript{90} prolongation by MCT (Figure 5B). Nevertheless, consistent with a reduction in I\textsubscript{NaL} contribution, RAN prevented the increase in APD\textsubscript{50}/APD\textsubscript{90} induced by MCT in the RV. Furthermore, in the RAN group, APD distributions were restored to their control shape and very long APD\textsubscript{90} values were absent (Figure 5A). Overall, in the RAN group, albeit clustered around a higher mean, APD\textsubscript{90} values were less dispersed, as reflected by the lower CV.

Diastolic potential (E\textsubscript{diast}) was only marginally depolarized in the MCT group and in the RV only (−2 mV; P < 0.05, Figure 5C); nonetheless, AP amplitude and dv/dt\textsubscript{max} remained unchanged. The change in E\textsubscript{diast} was prevented by RAN.

Modulation of K\textsuperscript{+} currents might contribute to MCT-induced changes in the AP. In the MCT group, I\textsubscript{K1} conductance was reduced in RV myocytes only (−48.7%), without changes in the rectification pattern (Figure 6A). Unlike the change in E\textsubscript{diast}, I\textsubscript{K1} down-regulation was not prevented by RAN (Figure 6A).

In the MCT group, I\textsubscript{to} density was reduced in both chambers (Figure 6B), but to a larger extent in RV myocytes (−46% vs. −28% at
+40 mV); thus, MCT minimized physiological chamber difference in $I_{to}$. Neither the relative weight of fast and slow $I_{to}$ components, nor their time-constants, were significantly changed by MCT (see Supplementary material online, Table S5); thus, MCT did not affect $I_{to}$ kinetics. RAN partially opposed $I_{to}$ down-regulation (Figure 6B) in the RV; moreover, RAN accelerated its inactivation ($t_{\text{slow}} -27\%$ vs. CTRL; see Supplementary material online, Table S5), thus limiting the impact of $I_{to}$ on APD. Qualitatively similar effects were observed in LV myocytes, where $I_{to}$ density down-regulation was completely prevented by RAN (Figure 6B).

**Figure 6** $K^+$ currents down-regulation. (A) $K^+$ free-sensitive current ($I_{K1}$) activated during slow voltage ramps (56 mV/s); mean traces and confidence intervals are shown. Statistics for $I_{K1}$ maximal conductance ($G_{\text{max}}$) and rectification index (RI) between 0 and $-100$ mV are shown. $N \geq 3$ and $n \geq 14$ for each group. *$P < 0.05$ vs. CTRL. (B) $I_{to}$ $I/V$ relationships in each experimental group. $N \geq 4$ and $n \geq 14$ for each group.
3.6 Pulmonary vascular resistance and structural remodelling

To assess whether MCT-induced pressure overload persisted in RAN-treated animals, RVSP was measured through right jugular vein catheterization (Figure 7A). RVSP was significantly increased in MCT rats (+45% vs. CTRL). In the RAN group, RVSP did not differ from control values.

To provide preliminary information on the mechanism accounting for RAN prevention of MCT-induced increment in RVSP, small pulmonary artery wall thickness (SPAWT) was measured. As shown in Figure 7B, relative medial SPAWT was increased in the MCT group (+20%), a change prevented by RAN.

4. Discussion

The unexpected effect of RAN on pulmonary vasculature makes prevention of mechanical overload a potential contributor to the myocardial effects of treatment; nonetheless, direct anti-remodelling effects of RAN have been previously demonstrated in the LV19 and might theoretically result from IhNa blockade. Although the role of mechanical unloading should not be overlooked, the following discussion focuses on whether the complex pattern of remodelling modulation we observed can be reconciled with RAN prevention of IhNa enhancement.

MCT-induced PAH caused substantial hypertrophy and fibrosis of the RV. Cellular structural remodelling consisted of a large increased CSA, associated with a smaller Ctr increment, likely mitigated by a substantial loss of transverse TT. All structural aspects of RV structural remodelling, including fibrosis, myocyte hypertrophy, and TT disarray, were countered by RAN treatment.

Consistent with previous studies, also the LV was affected by PAH, possibly because of altered interventricular interaction resulting from RV prevalence. LV abnormalities were, under many aspects, opposite to those of the RV. Loss of LV mass occurred without changes in collagen content or myocyte size. PAH has been recently reported to increase apoptosis in the LV, which might account for the present findings.

Structural remodelling was associated with bradycardia, loss of BW, and CO. This, together with the presence of initial chamber dilation, suggests that MCT-induced changes were approaching the decompensation stage.

4.1 RV remodelling

MCT significantly increased IhNa, particularly in the pressure-overloaded RV. IhNa enhancement has been extensively described as a consequence of LV remodelling. It may prolong repolarization, perturb Ca2+ homeostasis, and thus contribute to evolution of the remodelling process.8,10 IhNa enhancement may cause Ca2+ overload by reducing the driving force for Ca2+ extrusion through the NCX. The significant IhNa enhancement observed in the RV was associated with increased [Ca]i, as expected; however, [Ca]j, was unchanged. Moreover, [Ca]j, was increased in spite of unchanged IhNa, thus indicating a larger fractional release. Altogether, this points to facilitation of RyRs opening, which may dominate to set Ca2+ dynamics at this stage. This pattern might represent the evolution from an initial condition of true Ca2+ overload, followed by Ca2+-mediated activation of RyRs destabilizing pathways (e.g., CaMKII).21 RyRs destabilization is also consistent with the appearance of DADs. Overall, the observed changes in Ca2+ handling are such as to provide partial compensation to the increased mechanical load, but also to account for the deterioration of diastolic function.

RAN completely abrogated IhNa enhancement. As the drug was absent when IhNa was measured, this implies that IhNa blockade may prevent initiation of a remodelling loop, eventually leading to a constitutive abnormality of Na+ channel inactivation.10 Such remodelling loop might be represented by the positive feedback reciprocally linking IhNa enhancement to CaMKII activation.10

RAN countered MCT-induced increments of [Ca]j and [Ca]j,; thus, suggesting that they were at least partially dependent on IhNa enhancement. RAN also prevented MCT-induced TT disarray and this may contribute to its effect on Ca2+ handling.18,22 RAN efficacy in preventing overload-induced changes in TT organization has been recently reported.23 RAN action on TT organization may be relevant to modulation of long-term response to increased load and its mechanism deserves further investigation.

RAN abolished DADs in the RV but not in the LV and this correlates with the extent of IhNa enhancement in the two chambers. Thus, albeit IhNa enhancement likely contributed to DAD generation in the RV, IhNa independent mechanisms must be postulated for the LV.
MCT increased APD and its dispersion. This is consistent with the observed down-regulation of outward currents (\(I_{\text{Na}}\) and \(I_{\text{K}}\)) and enhancement of \(I_{\text{NaL}}\), whose direct contribution to APD became significant (see Supplementary material online, Figure 5). RAN completely prevented \(I_{\text{NaL}}\) enhancement, partially opposed \(I_{\text{Na}}\) density down-regulation, but it failed to prevent reduction of \(I_{\text{K}}\) conductance. Partial preservation of \(I_{\text{Na}}\) by RAN was not mirrored by changes in Kv4.2 mRNA levels (Figure 1C), thus suggesting either the contribution of other channel transcripts (Kv4.3, Kv1.4, or KChiP2), or post-transcriptional modulation.

APD distributions reveal that RAN prevented extremely long APDs, as expected from \(I_{\text{NaL}}\) normalization; in spite of this, mean APD was still prolonged in the RAN group, potentially contributing to the residual excess of Ca\(^{2+}\) content. This is consistent with failure of RAN to shorten the rate-corrected QT interval in a previous study on overload-induced RV remodelling.\(^{11}\) This requires to postulate an additional factor delaying repolarization in the RAN group whose nature remains unclear. RAN was absent during measurements, thus making its ancillary \(I_{\text{Na}}\) blockade\(^{24}\) irrelevant during APD measurement. Nevertheless, an effect of chronic \(I_{\text{Na}}\) blockade on channel expression cannot be ruled out.

Upstroke velocity (dV/dt\(_{\text{max}}\)) was unchanged, thus suggesting that Na\(^{+}\) current enhancement was limited to its steady-state component. Previous works in MCT-induced PAH reported normal RV SCN5A transcript\(^5\) and \(I_{\text{NaL}}\) density,\(^3\) but shifts of the activation curve suitable to increase \(I_{\text{NaL}}\).\(^{3,4}\)

RAN significantly countered the development of RV hypertrophy both at the organ and cellular levels. This finding is similar to what recently reported in a pulmonary artery banding model.\(^{11}\) In the latter study, RAN and trimetazidine were found to inhibit FAO and their anti-remodelling effect was attributed to this metabolic action. Concomitance with either \(I_{\text{NaL}}\) inhibition (present data), or switch in substrate utilization,\(^{11}\) is of course inadequate to establish which mechanism may prevail in RAN anti-remodelling effect. More conclusive in this respect are the findings of Wang et al.\(^{11}\) who reported that functional recovery during reperfusion, while improved by RAN, was insensitive to FAO inhibition by a selective and more powerful agent. Even similarity between RAN and trimetazidine effects is not conclusive for a ‘metabolic’ mechanism in prevention of RV remodelling. Indeed, trimetazidine was found to improve function in hypertrophied hearts without inhibiting FAO utilization.\(^{24}\) On the other hand, improvement of cell energy balance and mitochondrial function may also be expected from \(I_{\text{NaL}}\) inhibition.\(^{25}\)

Notably, in the present study, prevention of hypertrophy and Ca\(^{2+}\) dynamics abnormalities by RAN was not associated with significant changes in echocardiographic indexes of RV systolic and diastolic function, which showed only ‘trends’ towards improvement (FAC, TAPSE, and IRT in Table 2). This contrasts with a significant improvement in cardiac index and exercise capacity reported by Fang et al.\(^{11}\) Although differences in the experimental model (proximal arterial banding vs. microvascular constriction) might account for this discrepancy, the objective difficulty in echocardiographic assessment of RV dynamics in small animals might account for failure to detect significant changes in the present study.

### 4.2 LV remodelling

LV output was markedly reduced because of matching decreases in systolic and diastolic volumes, with preserved EF. This ‘restrictive’ pattern likely reflected distortion of LV geometry secondary to RV hypertrophy and increased RV pressures (IVS bulging); nevertheless, intrinsic LV myocyte function was also altered.

\(I_{\text{NaL}}\) enhancement, although still significant, was less prominent in the LV, possibly explaining why myocyte Ca\(^{2+}\) content was more depressed than in the RV. TT disarray was present, but less obvious than in RV myocytes. Overall, changes in Ca\(^{2+}\) handling point to decreased cell Ca\(^{2+}\) content, which was nonetheless associated with DADs occurrence to suggest marked RyRs instability.

In the LV of MCT myocytes, APD changes were similar to those observed in the RV, but less prominent. Notably, APD distribution became unimodal due to loss of the lower cluster; since \(I_{\text{Na}}\) expression underlies transmural differences in LV repolarization, this might be related to \(I_{\text{Na}}\) down-regulation (Figure 6B). At variance with the RV, MCT did not reduce Kv4.2 mRNA in the LV (Figure 1C), possibly reflecting a different type of remodelling. \(I_{\text{K}}\) was unaffected in the LV.

RAN completely prevented \(I_{\text{NaL}}\) enhancement and \(I_{\text{Na}}\) down-regulation, but, as occurred in the RV, it did not affect average APD (Figure 5). Nevertheless, RAN abolished very long APDs and reinstated the physiological bimodal distribution of APD, consistent with the prevention of \(I_{\text{Na}}\) down-regulation. RAN failed to affect Ca\(^{2+}\) handling abnormalities in the LV, which may be interpreted in light of the smaller extent of \(I_{\text{NaL}}\) enhancement in this chamber.

### 4.3 Pulmonary resistance and microvascular remodelling

MCT unexpectedly increased pulmonary vascular resistance. As reported in previous studies,\(^1,2\) medial thickening occurred in small pulmonary arteries, thus providing a structural mechanism for the increase in resistance. MCT also induced pulmonary oedema, suggesting increased capillary permeability.

RAN partially prevented the increase in RVSP; this was associated with less arteriolar thickening, thus suggesting a structural basis for this haemodynamic effect. Nevertheless, although failing to affect systemic vascular resistance to a significant extent, RAN has been recently reported to exert relaxing effect on vascular preparations;\(^{26,27}\) thus, the contribution of functional pulmonary vasodilatation cannot be ruled out. RAN failed to affect pulmonary oedema.

Concerning the possibility that \(I_{\text{NaL}}\) blockade might account for RAN effect on pulmonary vasculature, only conjectures are possible thus far. Recent studies indicate that TTX-sensitive voltage-gated Na\(^{+}\) channel isoforms are expressed in human pulmonary artery smooth muscle (PASM) cells and are blocked by RAN.\(^{28}\) Although their physiological role remains unresolved, their expression is enhanced in pathological states.\(^{29,30}\) Moreover, a recent cDNA microarray study found SCN1B (the Na\(^{+}\) channel β1 subunit) transcript increased in lung tissue from patients with PAH.\(^{31}\) Several studies suggest that PASM remodelling is related to intracellular Ca\(^{2+}\) overload and reactive oxygen species signalling,\(^{32}\) components of the vicious circle supporting \(I_{\text{NaL}}\) enhancement in tissue remodelling. Thus, a role of \(I_{\text{NaL}}\) inhibition in RAN effect on pulmonary vasculature can be envisioned.

### 4.4 Study limitations

The finding that RAN also reduced RVSP raises the possibility that its effects on cardiac remodelling might be due to partial prevention of RV mechanical overload. Although this possibility cannot be ruled out, it seems unlikely that it may represent the sole mechanism underlying the effects of RAN. Indeed, whereas the prevention of pulmonary vascular resistance enhancement was incomplete, drug-induced changes in myocardial structure and function were rather dramatic at the organ,
tissue, and myocyte levels. Moreover, RAN anti-remodelling effect has been previously reported in a fixed-load model. Although demonstrating that I_{Na}L enhancement is part of PAH-induced remodelling, the present results do not allow to discriminate between I_{Na}L blockage and FAO inhibition as the mechanism underlying anti-remodelling effect of RAN. Based on the work of Fang et al., we consider FAO inhibition an unlikely mechanism; nevertheless, evaluation of I_{Na}L blockage by agents devoid of effect on FAO may be required to solve the controversy on this issue.

4.5 Conclusions and implications

The present observations point to a role of I_{Na}L enhancement in the maladaptive cardiac response to PAH and, unexpectedly, in the development of PAH itself. Taking this to justify the expectation of a benefit from I_{Na}L blockage in human PAH requires caution. The first consideration, which applies to RAN effect at the pulmonary level, concerns the relevance of MCT-induced damage to human PAH. Furthermore, although countering myocardial structural remodelling, RAN was less effective in preventing cardiac function derangement. While this might depend on the extent of cardiac damage, substantial in the present model, a potentially compensatory role of I_{Na}L enhancement should also be considered. Furthermore, RAN was administered shortly after MCT administration; thus, the present results are relevant to prevention only. Specifically designed studies are required to evaluate the efficacy of I_{Na}L blockage in reversing PAH consequences and to weigh the long-term balance between benefits and drawbacks.

Acknowledgements

We thank Luiz Belardinelli and John T. Liles (Gilead Sciences) for insightful comments on the manuscript.

Conflict of interest

Gilead Sciences, Inc. (Foster City, CA, USA) is a patent holder for RAN.

Funding

This work was supported by a grant from Gilead Sciences, Inc. (Foster City, CA, USA) and NEDD (Network Enabled Drug Design) funding to A.Z.

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