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Abstract

Atrial fibrillation (AF) has been associated with increased spontaneous calcium release from the sarcoplasmic reticulum and linked to increased adenosine A_{2A} receptor ($A_{2A}R$) expression and activation. Here we tested whether this may favor atrial arrhythmogenesis by promoting beat-to-beat alternation and irregularity. Patch-clamp and confocal calcium imaging was used to measure the beat-to-beat response of the calcium current and transient in human atrial myocytes. Responses were classified as uniform, alternating or irregular and stimulation of Gs-protein coupled receptors decreased the frequency where a uniform response could be maintained from 1.0 ± 0.1 to 0.3 ± 0.1 Hz; $p < 0.001$ for beta-adrenergic receptors and from 1.4 ± 0.1 to 0.5 ± 0.1 Hz; $p < 0.05$ for A_{2A}Rs. The latter was linked to increased spontaneous calcium release and after depolarizations. Moreover, $A_{2A}R$ activation increased the fraction of non-uniformly responding cells in HL-1 myocyte cultures (19 ± 3–51 ± 9 %; $p < 0.02$), and electrical mapping in perfused porcine atria revealed that adenosine induced electrical alternans at longer cycle lengths, doubled the fraction of electrodes showing alternation, and increased the amplitude of alternations.

Importantly, protein kinase A inhibition increased the highest frequency where uniform responses could be maintained (0.84 ± 0.12–1.86 ± 0.11 Hz; $p < 0.001$) and prevention of $A_{2A}R$-activation with exogenous adenosine deaminase selectively increased the threshold from 0.8 ± 0.1 to 1.2 ± 0.1 Hz; $p = 0.001$ in myocytes from patients with AF. $A_{2A}R$-activation promotes beat-to-beat irregularities in the calcium transient in human atrial myocytes, and prevention of $A_{2A}R$ activation may be a novel means to maintain uniform beat-to-beat responses at higher beating frequencies in patients with atrial fibrillation.

Keywords (separated by '-') Adenosine receptor - Atrial myocyte - Electrophysiology - L-Type calcium current - Sarcoplasmic reticulum

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ORIGINAL CONTRIBUTION

Prevention of adenosine A_{2A} receptor activation diminishes beat-to-beat alternation in human atrial myocytes

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Abstract Atrial fibrillation (AF) has been associated with increased spontaneous calcium release from the sarcoplasmic reticulum and linked to increased adenosine A_{2A} receptor ($A_{2A}R$) expression and activation. Here we tested whether this may favor atrial arrhythmogenesis by promoting beat-to-beat alternation and irregularity. Patch-clamp and confocal calcium imaging was used to measure the beat-to-beat response of the calcium current and transient in human atrial myocytes. Responses were classified as uniform, alternating or irregular and stimulation of Gs-protein coupled receptors decreased the frequency where a uniform response could be maintained from 1.0 ± 0.1 to 0.3 ± 0.1 Hz; $p < 0.001$ for beta-adrenergic receptors and from 1.4 ± 0.1 to 0.5 ± 0.1 Hz; $p < 0.05$ for $A_{2A}Rs$. The latter was linked to increased spontaneous calcium release and after depolarizations. Moreover, $A_{2A}R$ activation increased the fraction of non-uniformly responding cells in HL-1 myocyte cultures (19 ± 3–51 ± 9 %; $p < 0.02$), and electrical mapping in perfused porcine atria revealed that adenosine induced electrical alternans at longer cycle lengths, doubled the fraction of electrodes showing alternation, and increased the amplitude of alternations. Importantly, protein kinase A inhibition increased the highest frequency where uniform responses could be maintained (0.84 ± 0.12–1.86 ± 0.11 Hz; $p < 0.001$) and prevention of $A_{2A}R$-activation with exogenous adenosine deaminase selectively increased the threshold from 0.8 ± 0.1 to 1.2 ± 0.1 Hz; $p = 0.001$ in myocytes from patients with AF. $A_{2A}R$-activation promotes beat-to-beat irregularities in the calcium transient in human atrial myocytes, and prevention of $A_{2A}R$ activation may be a novel means to maintain uniform beat-to-beat responses at higher beating frequencies in patients with atrial fibrillation.

Keywords Adenosine receptor · Atrial myocyte · Electrophysiology · L-Type calcium current · Sarcoplasmic reticulum

Introduction

Electromechanical alternans has been observed in different pathological settings [16, 35], preceding the occurrence of atrial fibrillation (AF) [15, 16] and the identification of molecular mechanisms that regulate the stability of the beat-to-beat response could help preventing the induction or recurrence of AF.

In physiological conditions, alternation in action potential shape can be induced by artificially increasing the heart rate [8, 16]. Furthermore, mechanical alternans is modulated by the plasmatic calcium level [8], and episodes

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can be reversed by calcium administration in humans [35]. In isolated mammalian myocytes, alternations in the calcium transient (calcium alternans) can be induced by lowering calcium entry through L-type calcium channels [10, 22, 29, 36], by metabolic inhibition [19] or by increasing stimulation frequencies [1, 36], and has been ascribed to inter- and/or intra-cellular inhomogeneity in calcium handling [1, 10, 22, 29, 36]. In human atrial myocytes, calcium alternans can also be induced by elevating the stimulation frequency [26]. Moreover, human atrial myocytes with large L-type calcium current (I_{Ca}) and frequent sarcoplasmic reticulum (SR) calcium release at rest were found more prone to present calcium alternans upon elevation of the stimulation frequency while myocytes with less frequent SR calcium release and smaller I_{Ca} could maintain a uniform beat-to-beat response at higher stimulation frequencies [26]. Interestingly, atrial myocytes from patients with AF have a higher frequency of spontaneous calcium release [17] but smaller I_{Ca} density [11, 27, 39], which would have opposite effects on the beat-to-beat response.

The higher frequency of spontaneous calcium release in myocytes from patients with AF has been linked to phosphorylation of the SR calcium release channel/ryanodine receptor (RyR2) mediated by protein kinase A (PKA) or calmodulin kinase II (CaMKII) [27, 32, 41]. Moreover, activation of the Gs-protein coupled adenosine A_{2A} receptor ($A_{2A}R$) induce a PKA-mediated stimulation of spontaneous calcium release in human atrial myocytes [18] that is more pronounced in myocytes from patients with AF and linked to a concurrent increase in $A_{2A}R$ expression. The above-mentioned findings may not only promote arrhythmogenic calcium release and afterdepolarizations in patients with AF [27] but could also promote atrial arrhythmia by favoring alternating and irregular beat-to-beat responses. However, this hypothesis has never been tested and the aim of the present work was to test whether $A_{2A}R$-activation reduces the ability of human atrial myocytes to maintain a uniform beat-to-beat response.

Methods

A total of 275 atrial myocytes were isolated from the right atrial appendix from 191 patients as previously described [17]. Patients treated with Ca^{2+} antagonists were excluded from the study. Table 1 in the supplementary material summarizes the clinical parameters at baseline and pharmacological treatments for the patients included in this study. Patients with AF included those that had a previous history of AF, i.e. paroxysmal or chronic AF (see Table 1 in the supplementary material). Permission to use the tissue samples was obtained from each patient, and the study was approved by the Ethical Committee of our institution and conducted in accordance with the Declaration of Helsinki principles. The study also conforms to the guidelines for the Care and Use of Laboratory Animals, and was approved by the Institutional Animal Care and Use Committee at our institution. Specific experimental protocols and conditions used in the study are described in the supplementary material. Values are expressed as mean ± SEM. For human atrial myocytes, the number of cells and patients are indicated as $n =$ (cells/patients). Data sets were tested for normality. Student's t test was used to assess significant differences when testing a specific effect. Differences were considered significant at $p < 0.05$. Two-way ANOVA and Holm-Sidak post-test was used for comparison of multiple effects in perfused porcine atrial preparations. For multiple comparisons of beat-to-beat responses in human atrial myocytes, a Mixed-effects logistic regression analysis was performed using the Stata 12 program (StataCorp, USA). The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written. Experiments were performed without knowledge about clinical data.

Results

Protein kinase A inhibition increases the stability of the beat-to-beat response in human atrial myocytes

To determine if baseline activation of Gs-protein coupled membrane receptors, modulate the rate-dependent beat-to-beat response in human atrial myocytes through PKA activation, we first examined the effects of the PKA-inhibitor H-89. As shown in Fig. 1a, PKA inhibition increased the frequency where a stable I_{Ca} amplitude (blue squares) and the time integral of the tail current (orange squares) could be maintained, resulting in a strong increase in the fraction of uniform responses at all stimulation frequencies examined (Fig. 1b). Statistical analysis revealed that H-89 protected against non-uniform beat-to-beat responses by increasing the fraction of uniform responses ($p < 0.001$) and decreasing alternating ($p < 0.001$) and irregular responses ($p < 0.001$). Consequently, the maximal frequency where a uniform beat-to-beat response could be maintained was doubled by H-89 (Fig. 1c).

Beta-adrenergic stimulation promotes beat-to-beat alternation in human atrial myocytes

To test if activation of Gs-protein coupled beta-adrenergic receptors had the opposite effect of H-89, myocytes were

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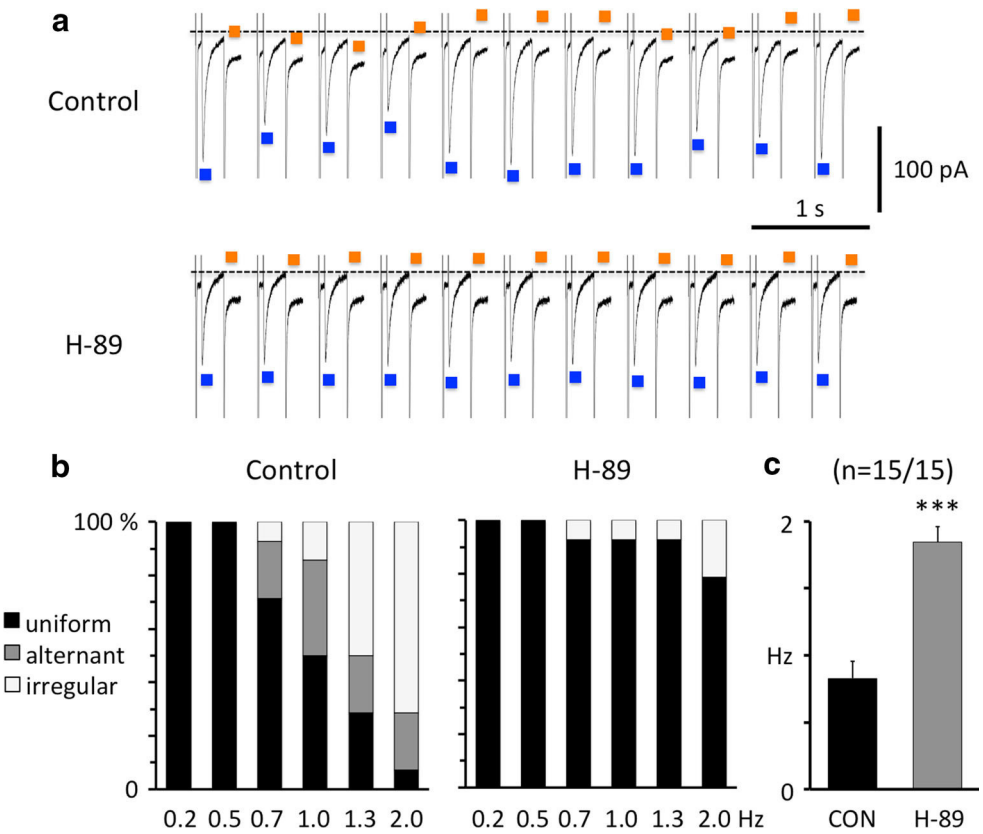

Fig. 1 PKA inhibition favors uniform beat-to-beat responses. **a** Consecutive current traces recorded in a human atrial myocyte paced at 2 Hz before (*top panel*) and after exposure to 1 μM H-89 (*lower panel*). *Blue squares* indicate I_{Ca} and *orange squares* the tail current elicited upon repolarization. The first inward peak of each current trace is the Na^+-current elicited by a prepulse to −50 mV. **b** Frequency-dependent distribution of uniform, alternating, and irregular beat-to-beat responses among 15 myocytes from 15 patients before (*control*, *left panel*) and after PKA inhibition with 1 μM H-89 (*right panel*). **c** Maximal frequency where a uniform response could be maintained. ***$p < 0.001$

stimulated with the agonist isoproterenol (ISO). As shown in Fig. 2a, this induced a pronounced alternation in the time integral of the tail current, causing a strong increase in alternating ($p < 0.001$) and irregular ($p < 0.05$) responses and consequently a reduction in the fraction of uniform responses ($p < 0.001$; Fig. 2b). Accordingly, ISO strongly reduced the threshold for the induction of non-uniform responses (Fig. 2c). Subsequently, we determined whether myocytes from patients treated with beta-blockers had a different response than myocytes from patients receiving no treatment. As shown in Fig. 2d, e there were neither differences in the response of myocytes from the two patient groups nor any difference in the maximal frequency where a uniform response could be maintained.

Adenosine A_{2A} receptors regulate beat-to-beat changes in the calcium transient

Since $A_{2A}R$ activation induces PKA-dependent stimulation of spontaneous SR calcium release without affecting I_{Ca}, we used confocal calcium imaging (see supplementary material figure S1) to investigate how activation of this receptor with its natural ligand adenosine (ADO) affected beat-to-beat changes in the calcium transient and calcium fluxes across the sarcolemma. Inclusion of 30 μM ADO in the patch pipette promoted beat-to-beat changes in the calcium transient in a time-dependent manner (Fig. 3a, b). Thus, the highest frequency where uniform calcium transients could be maintained was 1.40 ± 0.12 Hz at the beginning of ADO infusion (2–4 min after patch break) and only 0.45 ± 0.08 Hz after 18–24 min of ADO infusion ($p = 0.002$, $n = 9/6$). Analysis of local calcium transients revealed that ADO infusion gradually changed the beat-to-beat response from uniform and synchronized calcium transients to synchronized local non-uniform responses (panel 3c) that eventually degraded into non-uniform responses with un-synchronized spontaneous calcium waves. Figure 3d illustrates how the fraction of synchronized non-uniform responses and calcium waves increases with the time ADO is infused into myocytes stimulated at 1 Hz.

Simultaneous measurements of intracellular calcium transients and ionic currents were used to investigate the mechanisms underlying this adenosine-mediated effect, and revealed that elevation of the stimulation frequency induced concurrent alternation (see supplementary material figure S4) or non-uniform responses in the calcium transient, the I_{Ca} amplitude and the tail current elicited upon repolarization (Fig. 4a).

Moreover, this promotion of non-uniform beat-to-beat responses was linked to a concurrent increase in spontaneous calcium waves during ADO infusion (from

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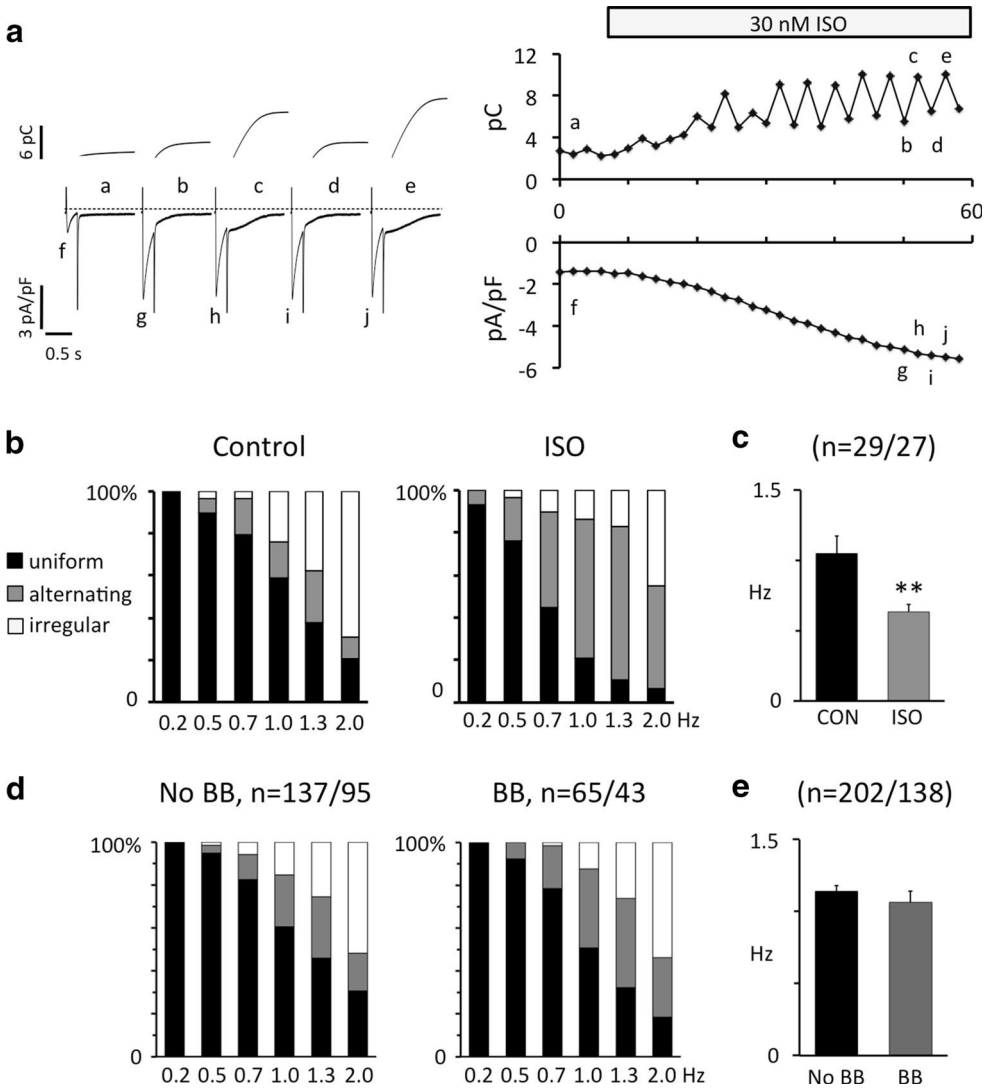

Fig. 2 Beta-adrenergic stimulation favors the induction of beat-to-beat alternation. **a** Representative recordings showing the effects of 30 nM ISO on the time integral of the tail current (*upper panel*) and I_{Ca} (*lower panel*) in a human atrial myocyte paced at 0.5 Hz. The time-dependent changes in the time integral and I_{Ca} are shown on the *right*. *Letters* denote the time point where currents shown on the *left* were recorded. **b** Frequency-dependent distribution of uniform (*black*), alternating (*grey*), and irregular (*white*) beat-to-beat responses among 29 myocytes from 27 patients before (*control*) and after beta-adrenergic stimulation with 30 nM ISO. The stimulation frequency is indicated below *each bar*. **c** Maximal frequency for maintenance of a uniform response with and without ISO. **d** Frequency-dependent distribution of uniform (*black*), alternating (*grey*), and irregular (*white*) beat-to-beat responses among 65 myocytes from 43 patients treated with beta blockers (BB) and 137 myocytes from 95 patients without beat-blocker treatment (*no BB*). **e** Maximal frequency for maintenance of a uniform response with and without BB treatment

1.1 ± 0.2 events/min at the onset to 13.7 ± 3.2 events/min after 18–24 min). Transient inward currents (I_{TI}) elicited by these calcium waves occurred both at rest and during electrical stimulation and had similar kinetics (Fig. 4b). Moreover, the I_{TI} frequency during stimulation was proportional to the I_{TI} frequency at rest (Fig. 4c). Consequently, there was an inverse relationship between the I_{TI} frequency and the highest frequency where a uniform response could be maintained (Fig. 4d). By contrast, the I_{Ca} density was not changed by ADO infusion (1.5 ± 0.2 pA/pF at the onset vs. 1.6 ± 0.2 pA/pF after ADO infusion) or depletion (1.5 ± 0.2 pA/pF at the onset vs. 1.6 ± 0.3 pA/pF after infusion of ADO free solution), and there was no correlation between the I_{Ca} amplitude and the highest frequency where a uniform response could be maintained (Fig. 4e).

Figure 5 analyzes how prolonged infusion (18–24 min) of ADO-containing and ADO-free solution affected the beat-to-beat response, and revealed that myocytes infused with ADO-free solution were able to maintain uniform responses at the higher stimulation frequencies. By contrast ADO infusion elicited irregular responses with numerous

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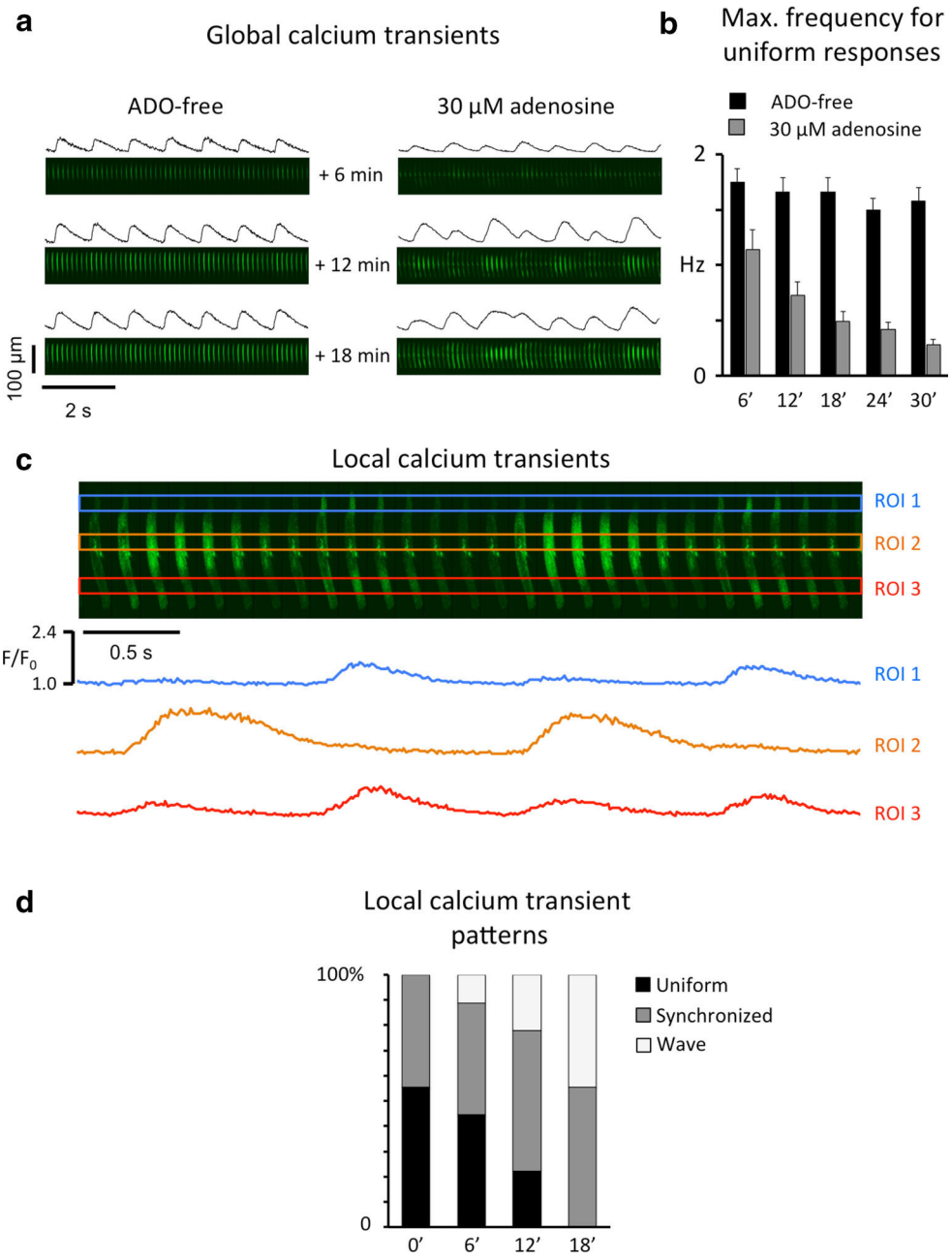

Fig. 3 Effect of the intracellular adenosine level on the beat-to-beat response. **a** Representative example of the effect of adenosine (ADO) infusion on a sequence of 55 consecutive calcium images recorded after 6 min (*top*), 12 min (*middle*), and 18 min (*lower panels*) with adenosine. Stimulation frequency was 1 Hz. Frame rate was 90 Hz and each of the 55 images in a panel is the average of 11 frames. Recordings were obtained with ADO-free (*left panels*) or 30 µM ADO (*right panels*). **b** Maximal frequency where a uniform response could be maintained with ADO-free solution (*black bars*, $n = 9/6$) or with 30 µM ADO (*grey bars*, $n = 9/7$). The duration of the treatment is given *below bars*. **c** Local calcium transients in the myocyte exposed to ADO for 12 min in *panel* **a**. Myocyte images were obtained using a binning of 11 frames. Local transients below show concordant alternans in ROI 1 and ROI 3 while alternation in ROI 2 is out of phase with ROI 1 and ROI 3. **d** Distribution of uniform responses (*black*), synchronized non-uniform responses (*grey*), and calcium waves (*white*) in myocytes stimulated at 1 Hz and infused with 30 µM ADO for the time indicated *below bars* ($n = 9/6$)

calcium waves even at the lower stimulation frequencies (Fig. 5a, b). Statistical analysis revealed that ADO significantly increased irregular responses ($p < 0.001$) and decreased uniform responses ($p < 0.001$). As a result, the maximal frequency for maintenance of uniform responses was 3.5-fold higher (1.63 ± 0.15 Hz) with ADO-free solution (Fig. 5c, $p < 0.001$, $n = 18/13$). To verify that the promotion of non-uniform beat-to-beat responses was caused by $A_{2A}R$-activation, myocytes perfused with ADO through the patch-pipette for ~15 min were exposed to the selective $A_{2A}R$ inhibitor ZM-241385 in the bath solution. As shown in Fig. 5d, e, ADO infusion reduced the threshold for the maintenance of uniform responses from 1.15 ± 0.17 to 0.61 ± 0.13 Hz ($p = 0.002$, $n = 11/10$) and subsequent addition of ZM241385 reversed the effect of ADO, increasing the threshold frequency back to 1.03 ± 0.21 Hz.

$A_{2A}R$ activation promotes beat-to-beat variations in calcium transient and T-wave alternans in cell cultures and perfused porcine atria, respectively

Since the observed modulation of the beat-to-beat response in isolated atrial myocytes could potentially be absorbed by

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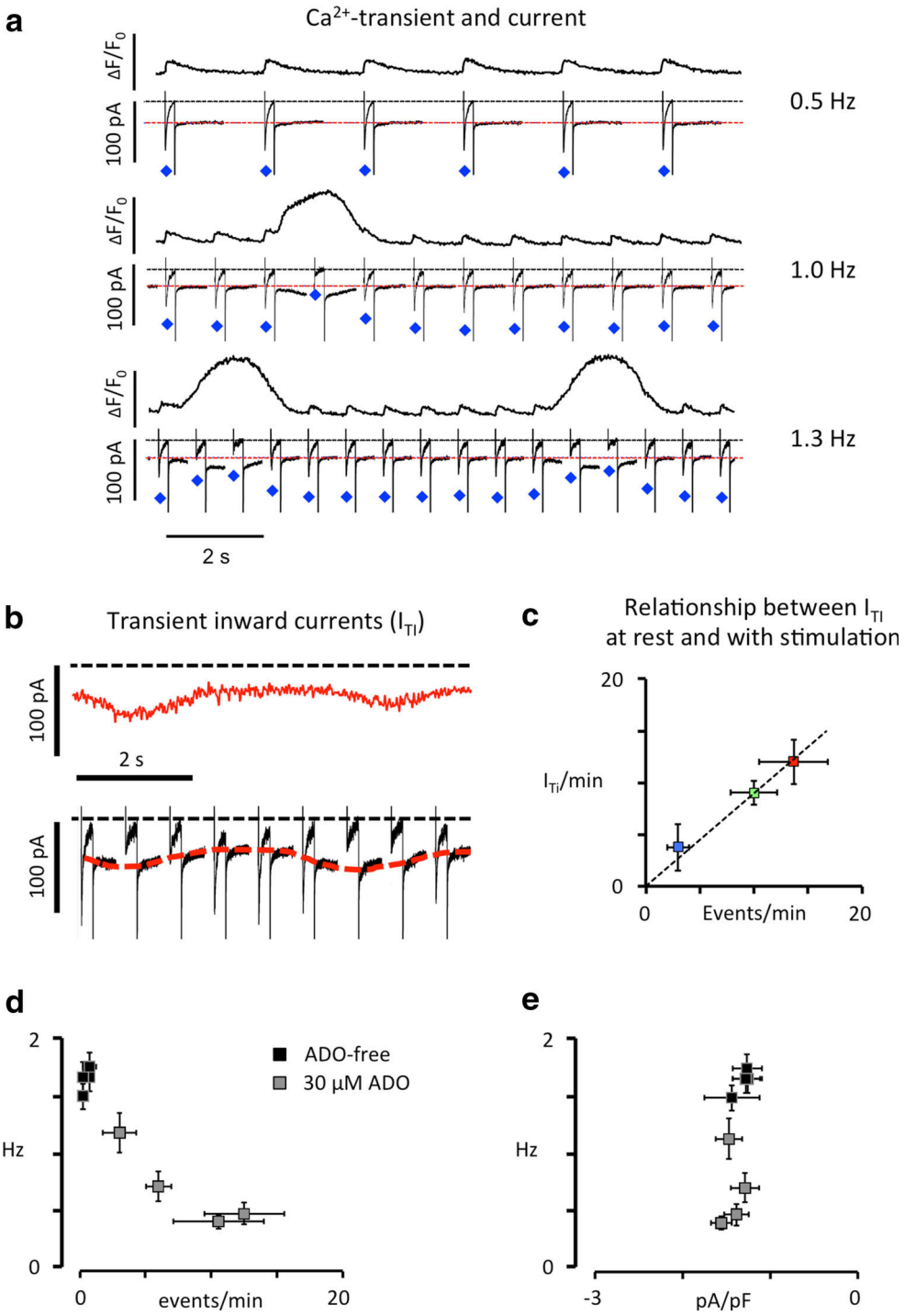

Fig. 4 Adenosine increases spontaneous calcium release during stimulation and at rest. **a** Simultaneous recordings of calcium transients and membrane currents with 30 μM adenosine (ADO) at stimulation frequencies of 0.5, 1.0 and 1.3 Hz (indicated *above traces*). *Dashed black lines* indicate 0 pA and *dashed red lines* indicate the holding current at steady state. Each I_{Ca} is indicated with a *closed diamonds*. Notice the transient inward deflection of the holding current during calcium waves resulting from Ca^{2+} extrusion by the Na^{+}–Ca^{2+} exchanger. **b** Recordings of transient inward currents (I_{TI}) recorded in the same cell at rest (*top*) and during stimulation at 1.3 Hz (*bottom*). *Red traces* indicate the I_{TI}s elicited by calcium waves. **c** Relationship between the I_{TI} frequency recorded at 2 Hz (*Y*-axis) and at rest (*X*-axis). Data were recorded after ADO infusion for 0–6 min (*blue*), 6–12 min (*green*) or 12–24 min (*red*). **d** Relationship between the frequency for the induction of non-uniform responses and the frequency of spontaneous calcium waves recorded in the absence (ADO-free) or the presence of 30 μM ADO ($n = 18$). **e** Relationship between the frequency for the induction of non-uniform responses and the I_{Ca} amplitude recorded in the absence (ADO-free) or the presence of 30 μM ADO ($n = 18$)

electrotonic effects in multicellular or in intact atrial preparations, we used calcium imaging in cultured atrial HL-1 myocytes to test how A_{2A}R-activation affected the beat-to-beat response in a multicellular myocyte preparation (see supplementary material figure S2). The HL-1 cell cultures were stimulated at 0.67 Hz where the effect of the selective A_{2A}R agonist CGS21680 on the beat-to-beat response was clear. At this frequency all fields examined under control conditions had a uniform response while the global response became irregular upon exposure to CGS21680 in 5/11 fields examined. Figure 6a shows that cells with uniform responses (blue label) predominated under control conditions while the fraction of irregularly responding cells (red label) increased after exposure to CGS21680. The corresponding global calcium transients recorded before and after exposure to CGS21680 are

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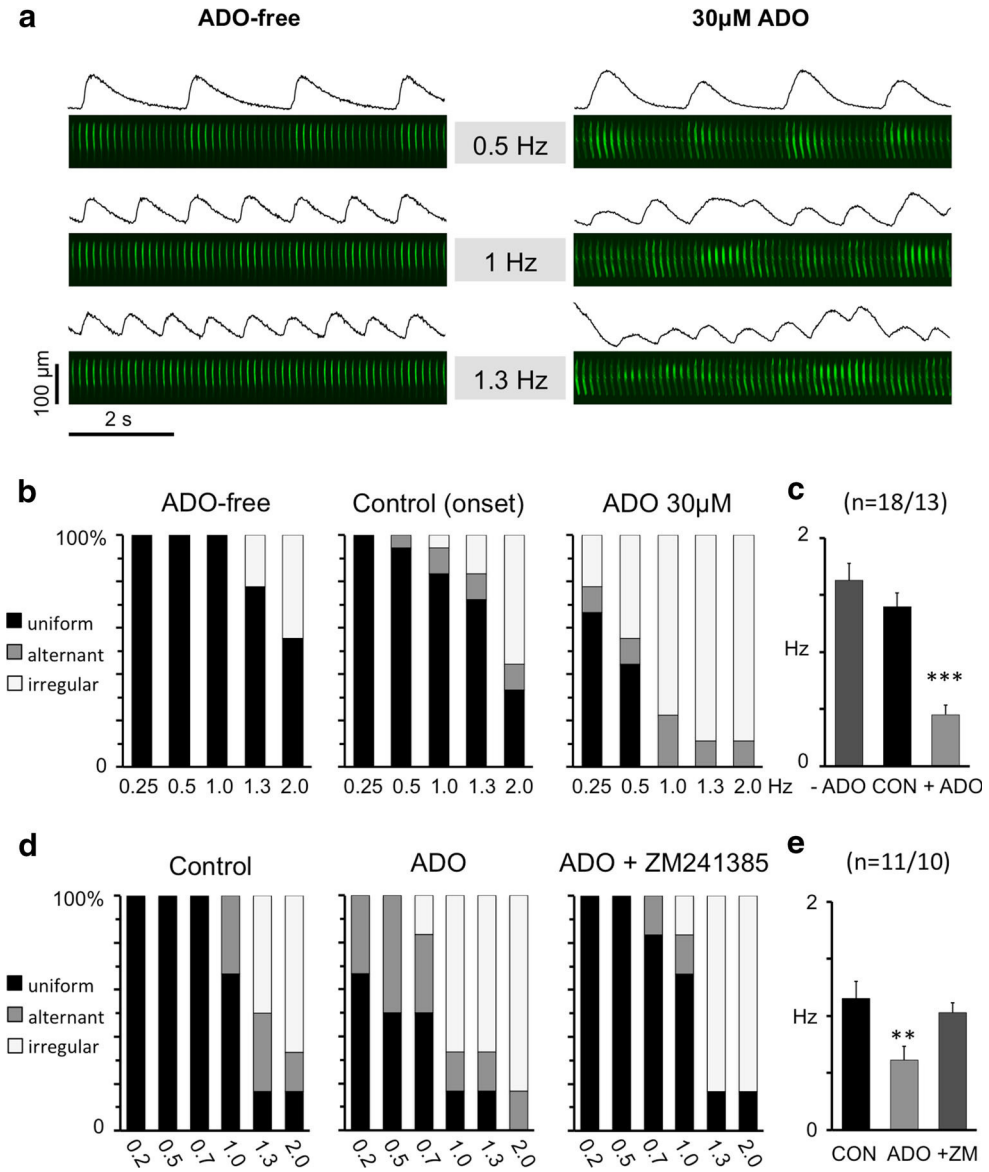

Fig. 5 Effect of adenosine A_{2A} receptor activation on the beat-to-beat response. **a** Effect of adenosine (ADO) infusion on calcium transients recorded with continuous stimulation at 0.5 Hz (*top*), 1 Hz (*middle*), and 1.3 Hz (*lower panels*). Frame rate was 90 Hz and each of the 54 images in a panel is the average of 11 frames. Recordings were obtained after 18–24 min perfusion with ADO-free (*left panels*) or 30 μM ADO (*right panels*) pipette solution. **b** Frequency-dependent distribution of uniform (*black*), alternating (*grey*), and irregular (*white*) beat-to-beat responses in myocytes perfused with ADO-free ($n = 9/7$) or with 30 μM ADO ($n = 9/6$). For reference, the distribution of responses at the onset of the infusion of ADO-free or 30 μM ADO is shown (*control*, *middle panel*). **c** Maximal frequency for maintenance of a uniform response. **d** Frequency-dependent distribution of uniform (*black*), alternating (*grey*), and irregular (*white*) beat-to-beat responses in 11 myocytes from 10 patients before (*control*, *left panel*) and 15 min after infusion of 30 μM ADO (*ADO*, *middle panel*). The effect of 150 nM extracellular ZM241385 after infusion of ADO (ADO + ZM241385) is shown in the *right panel*. **e** Maximal frequencies for maintenance of a uniform response in control (CON), with 30 μM ADO, and with 30 μM ADO + ZM241385 (+ZM)

shown in Fig. 6b. On average, uniform responses were observed in 79 ± 3 % of all myocytes when the global response was uniform while 19 ± 3 % of the myocytes had an irregular response. In the presence of CGS21680 the fraction of myocytes with irregular responses increased to 51 ± 9 % ($p = 0.03$) in five fields where the global response was irregular while the fraction of myocytes with uniform responses decreased to 45 ± 11 % of all cells ($p = 0.02$). Figure 6c shows a paired analysis of the fraction of myocytes presenting uniform, alternating and irregular responses before and after exposure to CGS21680 in all the 11 image fields analyzed.

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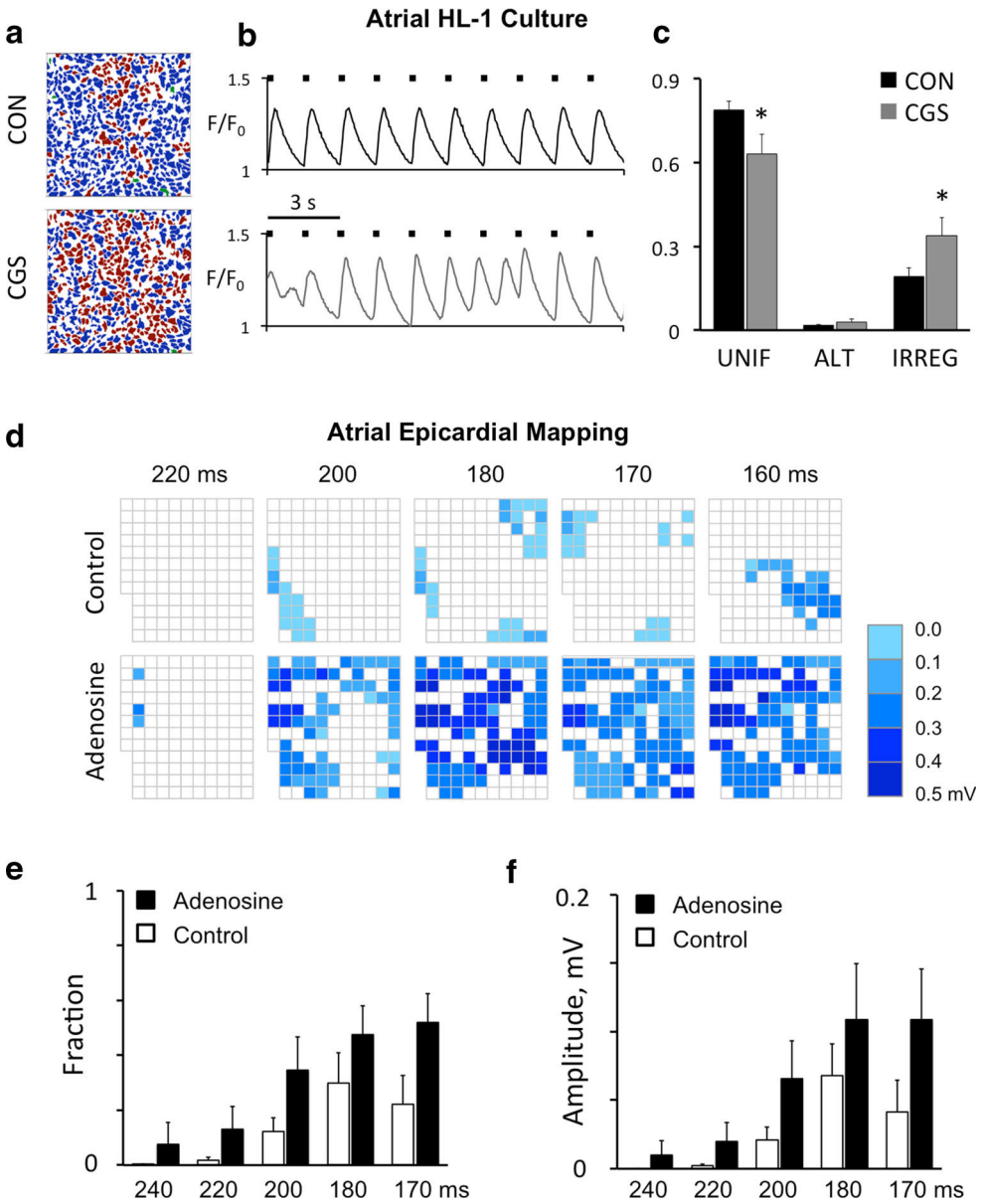

Fig. 6 $A_{2A}R$ activation favor non-uniform beat-to-beat responses in multicellular and perfused atrial preparations. **a** Mapping of the beat-to-beat response in cultured atrial HL-1 myocytes before (CON) and after exposure to 100 nM CGS21680 (CGS). Myocytes with uniform responses are *blue*, alternating responses *green*, and irregular responses *red*. Image field is 1 × 1 mm; stimulation frequency 0.7 Hz. **b** Global response of the culture shown in *panel* **a** before (*top*) and after exposure to CGS (*bottom*). **c** Fraction of uniform (UNIF), alternating (ALT), and irregular (IRREG) beat-to-beat responses among all myocytes before and 10 min after exposure to CGS ($n = 11$; $*p < 0.05$). **d** Electrical mapping in perfused porcine atria performed before and after ADO infusion. Electrodes with alternating responses are *blue*. The *color code* indicates the amplitude of the alternation. **e** Fraction of electrodes with alternating responses at different pacing intervals (given *below bars*) before and after adenosine infusion. ANOVA analysis showed that both pace rate ($p < 0.001$) and ADO ($p < 0.001$) significantly increased the fraction of electrodes with alternans. **f** Amplitude of the alternation at different pacing intervals before and after ADO infusion. Both pace rate ($p < 0.001$) and ADO ($p < 0.01$) significantly increased the amplitude ($p < 0.001$, ANOVA)

To test if the observed effects of adenosine receptor activation in isolated and cultured myocytes translate into an impact on the electrical atrial activity, we performed electrical mapping in arterially perfused porcine atria (see supplementary material figure S3). Figure 6d shows electrical mapping performed before and 10 min after the onset of adenosine infusion. When the pacing interval was shortened, there was a significant increase in the fraction of electrodes with T-wave alternans (indicated with blue squares; $p < 0.001$), as well as the amplitude of the alternation ($p < 0.001$). Perfusion with adenosine exacerbated this effect and significantly increased the fraction of

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electrodes with alternans ($p < 0.001$) as well as the amplitude of the alternation ($p < 0.01$; Fig. 6e, f).

Inhibition of $A_{2A}R$ activation selectively increases uniform beat-to-beat responses in myocytes from patients with atrial fibrillation

As elevation of spontaneous calcium release in AF has been associated with excessive RyR2 activation linked to increased $A_{2A}R$ expression and activation, we separated myocytes from patients with and without AF to test whether $A_{2A}R$ activation would promote non-uniform responses and prevention of $A_{2A}R$ activation would favor uniform beat-to-beat response in human atrial myocytes from patients with AF.

Exposure of myocytes to the selective $A_{2A}R$ agonist CGS21680 confirmed that $A_{2A}R$ activation significantly decreased the uniform responses ($p < 0.001$) and increased the alternating ($p < 0.001$) and irregular ($p < 0.05$) responses in patients with AF ($p < 0.05$, Fig. 7a). A similar effect was observed in patients without AF (Fig. 7b). Accordingly, CGS21680 significantly reduced the threshold frequency for the induction of non-uniform responses from 0.97 ± 0.12 to 0.67 ± 0.11 ($p < 0.01$) in patients with AF and from 1.11 ± 0.10 to 0.80 ± 0.08 ($p < 0.001$) in patents without AF (Fig. 7c). These effects of CGS21680 concurred with an increase in spontaneous I_{TI}s at rest in myocytes from AF (Fig. 7d), resulting in a correlation between the spontaneous I_{TI}-frequency and the frequency-threshold where uniform responses could be maintained (Fig. 7e). Comparison of frequency of I_{TI}s and spontaneous membrane depolarizations (DAD) in the same cells from five patients revealed that CGS21680 caused a parallel and significant increase in both I_{TI} and DAD frequency (Fig. 7f, g). Furthermore, CGS21680 strongly increased the DAD-amplitude (Fig. 7h). As a consequence, the afterdepolarizations persisted in 3/5 myocytes exposed to CSG21680 when they were subjected to electrical stimulation (see supplementary material Fig. 5) whereas no afterdepolarizations were recorded in the same cells before $A_{2A}R$ activation. CGS21860 neither did modify the SR calcium load nor did it change the I_{Ca} density (Supplementary material figure S6a, b); and the threshold for the maintenance of uniform responses was therefore not correlated with SR calcium load or I_{Ca} density (Supplementary material figure S6d, e).

Opposite to the effects of CGS21680, addition of exogenous adenosine deaminase (ADA) to the bath solution, to degrade extracellular adenosine, prevented the induction of non-uniform responses (Fig. 8a, middle traces). Furthermore, the non-degradable $A_{2A}R$ agonist CGS21680 was able to reverse the effect of ADA (traces on the right). Interestingly, statistical analysis revealed a more pronounced effect of ADA in myocytes from patients with than without AF. Thus, ADA significantly increased the uniform responses ($p < 0.001$) and decreased both alternating ($p < 0.01$) and irregular ($p < 0.05$) responses in patients with AF (Fig. 8b). By contrast, ADA did not significantly change the beat-to-beat response in patients without AF (Fig. 8c). Consequently, ADA significantly increased the threshold for the maintenance of uniform beat-to-beat responses in 12 patients with AF from 0.80 ± 0.12 to 1.16 ± 0.1 Hz ($p < 0.001$), while no difference was observed in the threshold for the induction of non-uniform responses in 18 patients without AF before (0.96 ± 0.12 Hz) and after exposure to ADA (1.14 ± 0.12 Hz; $p = 0.3$; Fig. 8d) These effects of ADA concurred with a reduction of spontaneous I_{TI}s in myocytes from AF patients stimulated at 1.3 Hz (from 2.9 ± 0.8 to 0.9 ± 0.4 events/min; $p < 0.05$) to rates observed in patients without AF before and after ADA (0.7 ± 0.3 vs. 0.8 ± 04 events/min). Similar results were obtained for the spontaneous I_{TI}-frequency in resting myocytes (Fig. 8e), yielding a linear relationship between the spontaneous I_{TI} frequency and the threshold for maintenance of uniform responses (Fig. 8f). By contrast, ADA did not modify the SR calcium load or the I_{Ca} density (Supplementary material figure S7a, b). Therefore, the threshold for the maintenance of uniform responses was neither correlated with SR calcium load nor with I_{Ca} density (Supplementary material figure S7d, e).

Discussion

Adenosine A_{2A} receptor-dependent regulation of the beat-to-beat response

Endogenous and interstitial adenosine levels are intimately linked to the cellular energy balance and interstitial adenosine levels have been reported to rise dramatically during ischemic episodes in the heart [3]. Moreover, $A_{2A}R$ expression is upregulated in patients with AF and promote both local (sparks) and global (waves) spontaneous calcium release events [18, 27], suggesting that elevation of adenosine levels during local atrial ischemia or hypoxia could favor spontaneous calcium release, triggering afterdepolarizations [43] and arrhythmia. In addition to this, we here report that $A_{2A}R$ activation by ADO infusion gradually reduces synchronicity of the intracellular calcium transient from uniformly synchronized responses at the onset of infusion to discordant subcellular transients (see Fig. 3c, d) that eventually degrade into asynchronous calcium waves superimposed on electrically triggered calcium transients (see the response at 1.3 Hz in Fig. 5a). Functionally, subcellular heterogeneity in the calcium transient may impair

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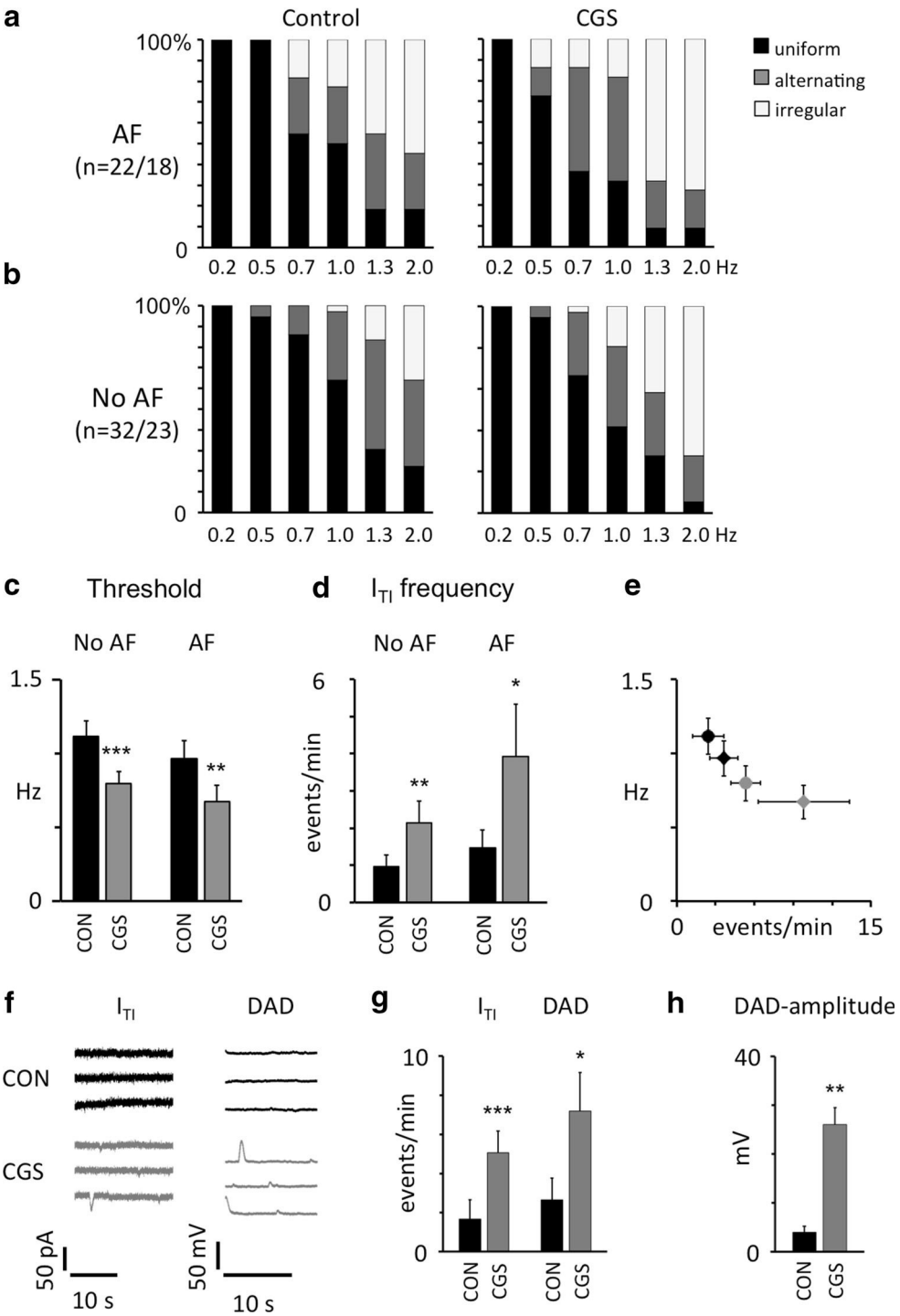

Fig. 7 $A_{2A}R$ activation favors irregular beat-to-beat responses in AF patients and is, linked to a higher frequency of I_{TI}s and afterdepolarizations. **a** Frequency-dependent distribution of uniform (*black*), alternating (*grey*), and irregular (*white*) beat-to-beat responses among 22 myocytes from 18 patients with AF recorded in control and with CGS21680 (CGS). **b** Frequency-dependent distribution of uniform, alternating, and irregular beat-to-beat responses among 32 myocytes from 23 patients without AF recorded in control and with CGS. **c** Threshold frequency for maintenance of a uniform response. $^{***}p < 0.001$. **d** Frequency of calcium release induced I_{TI}s recorded in patients without (no AF) or with AF. Measurements were done before (CON; *black bars*) and after exposure CGS (*grey bars*). **e** Relationship between the I_{TI} frequency and threshold for the maintenance of a uniform beat-to-beat response. **f** Calcium release induced I_{TI}s and spontaneous membrane depolarizations (DAD) recorded in the same myocytes before (*black traces*) and after exposure to CGS (*grey traces*). **g** I_{TI} and DAD frequencies recorded before (CON) and after exposure to CGS ($n = 5/5$). **h** DAD amplitude before (CON) and after exposure to CGS. $^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$

propagation of the electrical signal from cell to cell, but as shown in Fig. 4, it is the global calcium transient that determines the amplitude and timing of the ionic currents. Importantly, infusion of adenosine through the patch-pipette demonstrated that only minor changes in the cytosolic adenosine level within a pathophysiologically relevant range (0–30 μM) [3, 4] have a considerable impact on the beat-to-beat response.

Concurrent recordings of calcium currents and intracellular calcium revealed that the beat-to-beat stability was inversely proportional to the frequency of spontaneous calcium waves, suggesting that $A_{2A}R$-mediated stimulation of spontaneous SR calcium release through the RyR2 [18, 27] is an underlying mechanism. In support of this, spontaneous calcium waves superimposed on electrically elicited calcium transients before, during or after a

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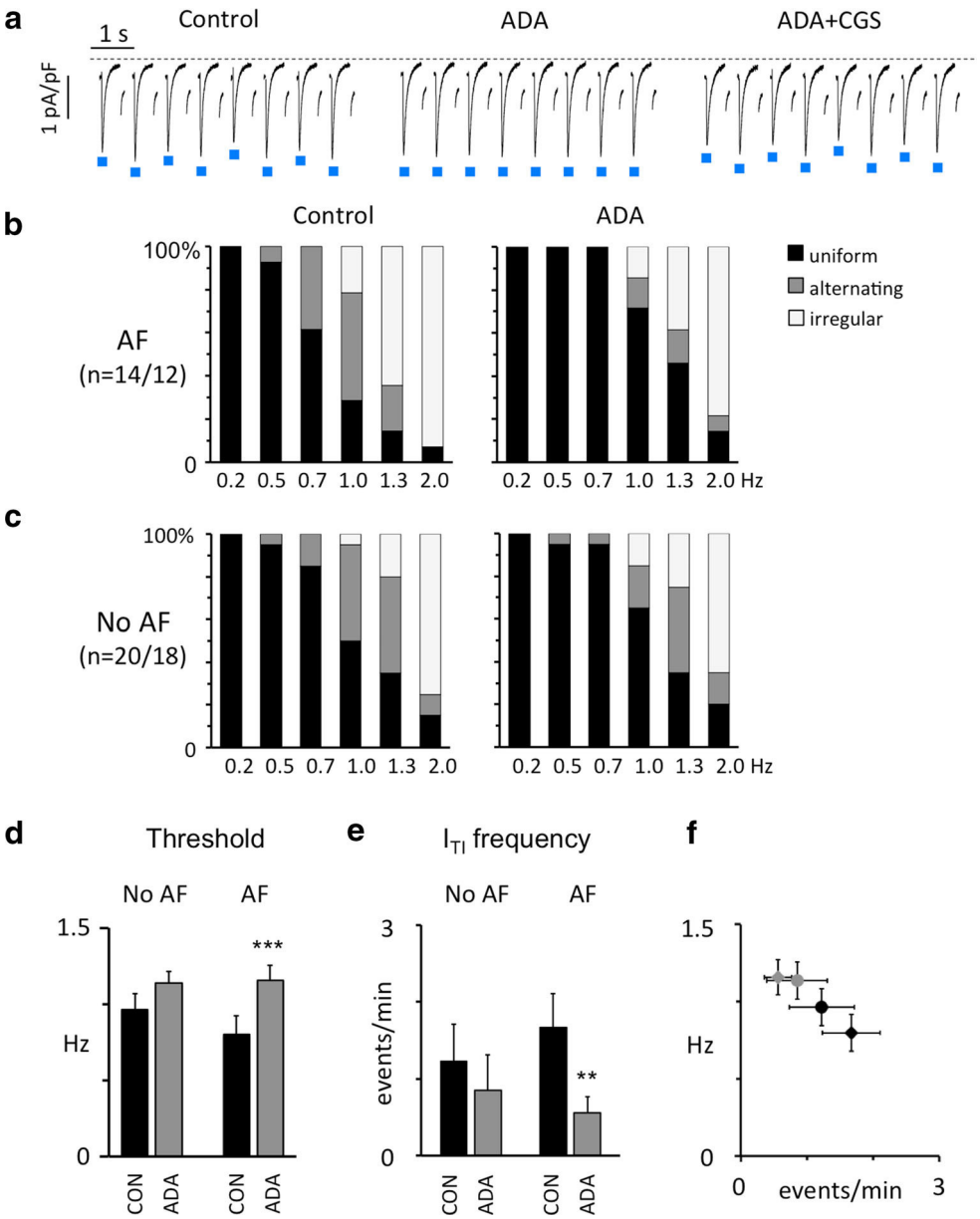

Fig. 8 Prevention of $A_{2A}R$ activation favors uniform beat-to-beat responses in myocytes from patients with AF. **a** Representative current recordings from a human atrial myocyte before (*control*) and after exposure to ADA or ADA + the non-degradable ADO analog CGS21680 (ADA + CGS). **b** Frequency-dependent distribution of uniform (*black*), alternating (grey), and irregular (*white*) beat-to-beat responses among 14 myocytes from 12 patients with AF recorded in control and with ADA. **c** Frequency-dependent distribution of uniform, alternating, and irregular beat-to-beat responses among 20 myocytes from 18 patients without AF recorded in control and with ADA. **d** Threshold frequency for maintenance of a uniform response $***p < 0.001$. **e** Frequency of calcium release induced I_{TI}s recorded in patients without (*no AF*) or with AF. Measurements were done before (CON; *black* bars) and after exposure to ADA (*grey bars*). **f** Relationship between the I_{TI} frequency and threshold for the maintenance of a uniform beat-to-beat response. $**p < 0.01$ $***p < 0.001$

stimulation pulse, ruling out that these waves are triggered events. Moreover, the $A_{2A}R$ agonist CGS21680 caused a parallel increase in calcium release-induced I_{TI}s and spontaneous membrane depolarizations (Fig. 7f, g). CGS21680 also strongly increased the amplitude these membrane depolarizations (Fig. 7h) and they persisted when myocytes were stimulated, demonstrating that $A_{2A}R$ activation stimulates spontaneous calcium release that favors electrical instability. L-Type calcium channels did not appear to be a major target for $A_{2A}R$-mediated regulation since neither adenosine nor the selective $A_{2A}R$ agonist CGS21680 had any significant effect on I_{Ca} density. Accordingly, there was no correlation between I_{Ca} density and the threshold frequency for loss of a uniform response (see Fig. 4).

The ability of $A_{2A}R$-activation to promote spontaneous calcium release has previously been linked to PKA-dependent phosphorylation of the RyR2 at ser2808 [27]. In line with this, the PKA inhibitor H-89 dramatically reduced the incidence of non-uniform responses at all stimulation frequencies studied, supporting the notion that PKA-dependent signaling intervene in the regulation of the beat-to-beat stability at baseline.

This is also compatible with previous work suggesting the presence of a cyclic adenosine monophosphate-tonus at baseline in human atrial myocytes [43]. Mechanistically, $A_{2A}R$s are coupled to G_s proteins [33] and linked to cAMP production and PKA-activation [34] and could stimulate phosphorylation of phospholamban at the residue serine16 (s16) or the RyR2 at the residues s2808 or s2030. This

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would be in accordance with previous studies linking dysfunctional calcium handling to increased phospholamban phosphorylation at s16 in chronic and paroxysmal AF [12, 42] and RyR2 phosphorylation at s2808 in chronic AF [27, 43]. In line with this, the PKA-inhibitor H-89 has been shown to reverse $A_{2A}R$-mediated stimulation of spontaneous calcium release [18]. However, $A_{2A}R$-dependent cAMP production could also activate calmodulin kinase II (CamKII)-dependent phosphorylation of the RyR at s2814, which would be in agreement with other studies linking spontaneous calcium release in AF to s2814 phosphorylation [32, 42, 43]. Moreover, irregular rhythm has been shown to increase diastolic $[Ca^{2+}]$ and activation of CaMKII and AMP-activated protein kinase [25] that could create a vicious cycle favoring irregular beating.

Beta-adrenergic stimulation with ISO also reduced the threshold for the induction of non-uniform responses and preferentially induced alternating responses. This is opposite to the reported ability of ISO to rescue calcium alternans in cat atrial myocytes [13], but in accordance with the notion that concurrent stimulation of I_{Ca} and SR calcium release, as reported for ISO here and in other studies [20, 45], promotes alternans in human atrial myocytes [26]. Similarly, stimulation of SR calcium release but not I_{Ca} by adenosine, as shown in Fig. 4 and reported by Llach et al. [27] is more likely to induce irregular beat-to-beat responses [26]. Interestingly, it has been reported that ISO promotes and propranolol decreases the amplitude and incidence of T-wave alternans in human atria from patients suffering from supraventricular tachyarrhythmia when the atria were paced at 110 bpm (1.8 Hz) [24], underscoring the physiological relevance of our findings. Indeed, our results would suggest that the mechanism underlying the ability of ISO to induce T-wave alternans in human atria is a PKA- or CaMKII-dependent stimulation of SR calcium release that reduces the threshold frequency for induction of beat-to-beat alternation in the calcium transient. The opposite effects of ISO in cat and human atrial myocytes are possibly due to different experimental conditions or species-dependent differences in the cellular calcium homeostasis. In favor of the latter possibility, mathematical models have shown that small changes in SR calcium uptake or RyR2 gating properties can profoundly affect the atrial beat-to-beat response [7, 28].

Regulation of the beat-to-beat response in atrial fibrillation

AF has previously been linked to PKA-mediated phosphorylation of the RyR2 at s2808 in patients with permanent AF [41] and phospholamban at s16 [12, 42], which would both favor elevation of spontaneous SR calcium release events at baseline [17]. Moreover, increased $A_{2A}R$ expression in patients with AF has been shown to increase RyR2 phosphorylation at s2808 and proposed to account for the higher rate of spontaneous calcium release [27]. These observations, combined with present finding of an inverse relationship between spontaneous calcium release events and the ability to maintain a uniform beat-to-beat response (see Figs. 4, 7, 8), suggest that pharmacological control of $A_{2A}R$ activation could be a means to regulate the beat-to-beat response at high stimulation frequencies in myocytes from AF patients. Indeed, we here show that infusion of adenosine-free solution through the patch-pipette or application of exogenous ADA increases the threshold frequency for induction of non-uniform responses. The latter was more pronounced in myocytes from AF patients, demonstrating that inhibition of $A_{2A}R$ activation stabilizes calcium handling in patients with AF. This stabilizing action is even more pronounced if the effect of temperature on beating rate is taken into account. Thus, a beating rate near 0.5 Hz at 22 °C would correspond to 1.25 Hz at 37 °C [5], and be representative for myocytes in patients with a normal beating rate. At this frequency more than 90 % of the myocytes have a uniform response and inhibition of $A_{2A}R$ activation with ADA has little effect. By contrast, beating rates of 1 and 1.3 Hz at 22 °C would correspond to rates around 2.5 and 3.3 Hz at 37 °C, corresponding to atrial arrhythmia. At these frequencies only 15 and 25 % of the myocytes respond uniformly, and ADA increases the number of uniformly responding myocytes to 45 and 70 % at 1.3 and 1 Hz, respectively.

As illustrated in Fig. 6a–c, such a strong reduction in the fraction of myocytes with irregular responses could potentially revert the overall response of an irregularly beating multicellular myocyte preparation to a uniform response.

It is therefore conceivable that physiologically relevant fluctuations in the cytosolic adenosine level such as those induced by stress, deficient circulation of the atrial appendices [4] or ischemia [3] have the potential to promote non-uniform beat-to-beat responses by reducing the threshold frequency for their induction. In support of this notion, electrical mapping in perfused porcine atria revealed that adenosine infusion induces T-wave alternans at lower beating rates and increases the alternans amplitude (see Fig. 6).

Importantly, selective $A_{2A}R$ inhibition was able to reverse the effects of massive increases in the cytosolic adenosine level (see Fig. 5) and exogenous adenosine deaminase increased the threshold frequency for induction of non-uniform responses in myocytes from patients with

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AF by reducing spontaneous calcium release events in both resting and beating myocytes, demonstrating that pharmacological control of $A_{2A}R$ activation can be used to counteract arrhythmogenic effects of pathological increases in the cellular adenosine level.

Study limitations

A challenge encountered with human cardiomyocyte models is that clinical or therapeutical heterogeneity among patients with and without AF can potentially bias the results. To minimize this issue, patients treated with calcium antagonists were excluded from the study. We also ruled out that there were potentially confounding effects of reduced left ventricular ejection fraction (<40 %), gender, and beta-blocker treatment (see Fig. 2d, e). Moreover, the observed changes in I_{Ca} and spontaneous calcium release are consistent with previous reports on human AF confirming that the model can faithfully reproduce observations from isolated human atrial myocytes [17, 27, 39].

The use of fluorescent dyes, such as fluo-4, to monitor intracellular calcium transients has previously been reported to favor irregular responses at the expense of alternating responses in human atrial myocytes [11], and we therefore only used calcium imaging in experiments specifically addressing effects of $A_{2A}R$-mediated effect on spatio-temporal changes in the calcium transient and the beat-to-beat response.

While pharmacological tools used to manipulate $A_{2A}R$ activation are highly selective, the selectivity of H-89 for PKA inhibition is controversial and depends on the presence of other kinases [9]. Nevertheless, unpublished data from our laboratory show that H-89 and KT5720, considered a more selective PKA inhibitor, have similar abilities to prevent spontaneous and triggered calcium release in mouse cardiomyocytes where confounding effects of concurrent cardiovascular disease and pharmacological treatments are avoided.

Functionally, adenosine also activates adenosine A_1 receptors and is associated to shortening of the atrial refractory period [44] and electrical re-entry [2], which likely affects the atrial electrical signals in the perfused porcine atria. However, it is not clear that this would promote the observed t-wave alternans. Instead, our results are consistent with findings in humans [24, 30] and in animal models [1, 21] where atrial action potential alternans and spontaneous depolarizations associated to abnormal calcium handling have been proposed to underlie arrhythmic vulnerability and increased susceptibility to AF.

Finally, regional differences are known to exist in human atrial physiology, and we cannot exclude that the present results from right atrial appendages will differ from the response of left atrial preparations.

Clinical implications

The induction or perpetuation of AF has been ascribed to remodeling of several ionic currents and calcium handling mechanisms [6, 14, 17, 39, 40], including increased spontaneous calcium release from the SR [17], hyperphosphorylation of the RyR2 at s2808 [27, 41] and s2814 [32, 42], as well as increased $A_{2A}R$ expression and activation [27]. The present study shows that remodeling of $A_{2A}R$-mediated signaling in AF can also facilitate alternating or irregular beat-to-beat responses, a phenomenon that has been reported to precede and promote the onset of AF [23, 31, 37, 38]. Importantly, prevention of $A_{2A}R$ activation significantly improved the beat-to-beat response in myocytes from AF patients. This, could prevent a massive induction of irregular beat-to-beat responses upon elevation of the beating frequency, and potentially prevent re-initiation of the arrhythmia in patients with paroxysmal AF exposed to tachycardic stress or after cardio version in patients with AF. On the other hand, the effect of prevention of $A_{2A}R$ activation is smaller at the highest stimulation frequency examined, suggesting that its efficacy may be smaller in patients with permanent AF. Inhibition of PKA-dependent signaling also promotes uniform beat-to-beat responses at high stimulation frequencies, but the ubiquitous nature and multiple functions of PKA make it difficult to envisage PKA inhibitors as a means to stabilize calcium handling in AF. Instead, pharmacological control of $A_{2A}R$ activation may be a key to selectively reduce spontaneous calcium release and promote uniform beat-to-beat responses in atrial myocytes from patients with AF without compromising the L-type calcium current, which is critical for the activation of contraction.

In summary, we show for the first time that $A_{2A}R$ activation reduces the frequency threshold for induction of non-uniform beat-to-beat changes in the calcium transient in human atrial myocytes and T-wave alternans in perfused porcine atrial preparations. Importantly, the $A_{2A}R$-mediated effect was more pronounced in patients with AF, and prevention of $A_{2A}R$ activation favored uniform responses and significantly increased the threshold for the induction of non-uniform responses in myocytes from these patients. This proposes pharmacological inhibition of $A_{2A}R$ activation as a novel therapeutical approach to prevent beat-to-beat alternation or irregular responses in atrial myocytes from patients with AF during stress-induced elevation of the beating rate or pathological conditions promoting elevation of cellular adenosine levels such as hypoxia or ischemia.

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Compliance with ethical standards

Conflict of interest None.

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