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Integrative human atrial modelling unravels interactive protein kinase A and Ca²⁺/calmodulin-dependent protein kinase II signalling as key determinants of atrial arrhythmogenesis

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Aims

Atrial fibrillation (AF), the most prevalent clinical arrhythmia, is associated with atrial remodelling manifesting as acute and chronic alterations in expression, function, and regulation of atrial electrophysiological and Ca²⁺-handling processes. These AF-induced modifications crosstalk and propagate across spatial scales creating a complex pathophysiological network, which renders AF resistant to existing pharmacotherapies that predominantly target transmembrane ion channels. Developing innovative therapeutic strategies requires a systems approach to disentangle quantitatively the pro-arrhythmic contributions of individual AF-induced alterations.

Methods and results

Here, we built a novel computational framework for simulating electrophysiology and Ca²⁺-handling in human atrial cardiomyocytes and tissues, and their regulation by key upstream signalling pathways [i.e. protein kinase A (PKA), and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII)] involved in AF-pathogenesis. Populations of atrial cardiomyocyte models were constructed to determine the influence of subcellular ionic processes, signalling components, and regulatory networks on atrial arrhythmogenesis. Our results reveal a novel synergistic crosstalk between PKA and CaMKII that promotes atrial cardiomyocyte electrical instability and arrhythmogenic triggered activity. Simulations of heterogeneous tissue demonstrate that this cellular triggered activity is further amplified by CaMKII- and PKA-dependent alterations of tissue properties, further exacerbating atrial arrhythmogenesis.

Conclusions

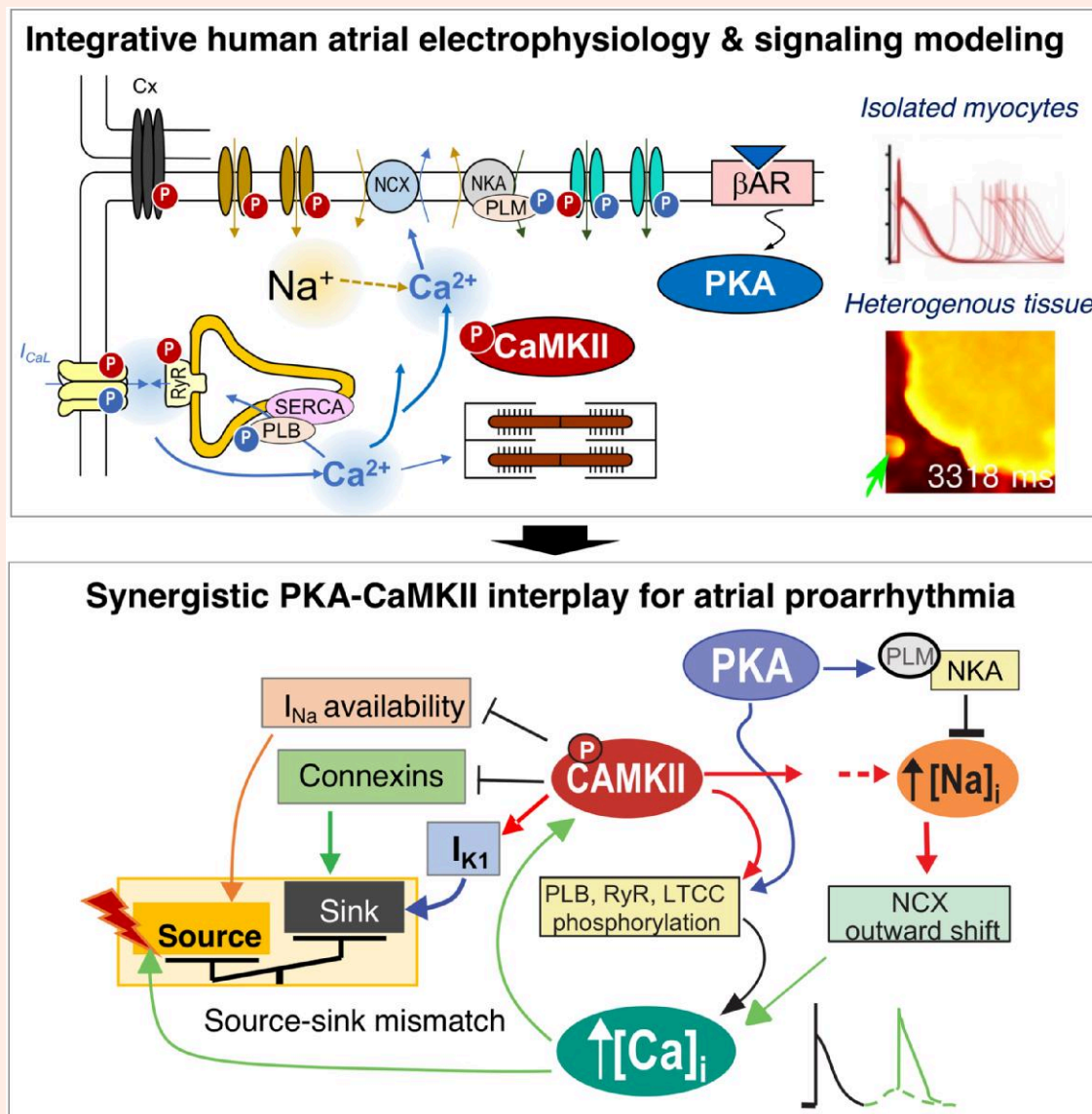
Our analysis reveals potential mechanisms by which the stress-associated adaptive changes turn into maladaptive pro-arrhythmic triggers at the cellular and tissue levels and identifies potential anti-AF targets. Collectively, our integrative approach is powerful and instrumental to assemble and reconcile existing knowledge into a systems network for identifying novel anti-AF targets and innovative approaches moving beyond the traditional ion channel-based strategy.

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Graphical Abstract



A novel integrative human atrial electrophysiology and signalling model reveals synergistic PKA and CaMKII interactions promoting delayed after depolarizations and triggered action potentials in both atrial myocytes and tissues. CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; Cx, connexin; I_{K1} , inward rectifier K^+ current; I_{Na} , fast Na^+ current; I_{CaL} , L-type Ca^{2+} current; NCX, $\text{Na}^+/\text{Ca}^{2+}$ exchanger; NKA, Na^+/K^+ ATPase; PLB, phospholamban; PLM, phospholemman; RyR, ryanodine receptor 2; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; βAR , β adrenergic receptor; PKA, protein kinase A.

Keywords

Systems biology • Computational biology • Physiology • Population modelling • Electrophysiology • Upstream signalling • Arrhythmias • Atrial fibrillation

1. Introduction

Atrial fibrillation (AF), characterized by irregular and rapid activation of the upper chambers of the heart, is the most frequently encountered arrhythmia, and its prevalence is increasing worldwide.¹⁻³ AF is associated with underlying cardiac comorbidities and with increased risks of stroke, heart failure, and mortality, thus posing significant health and socio-economic burden.¹⁻⁶ Existing strategies for treating AF, such as rate control and rhythm control through anti-arrhythmic drugs (typically, ion channel blockers) or catheter-based ablation,² suffer from unsatisfactory efficacy and adverse effects.⁴ The challenges and obstacles hampering the development of novel therapeutic approaches are underscored by the complex pathological mechanisms

underlying AF, which are multi-factorial and involve electrical remodelling, Ca^{2+} -handling abnormalities, structural, and neurohormonal changes.^{3,7-9}

These complex multi-level alterations have been mechanistically linked to ectopic (triggered) activity and impulse re-entry in cardiac tissue, which can initiate and sustain arrhythmia in the atria, thus leading to AF.^{3,7,9,10}

Cardiomyocyte and tissue responses to stressors are mediated by an intricate signalling network that allows the heart to adapt and meet physiological needs. Among these signals, protein kinase A (PKA) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) are two protein kinases that phosphorylate a vast array of ion channels and Ca^{2+} -handling and regulatory proteins and play critical roles in fine-tuning atrial cardiomyocyte stress responses.¹¹⁻²⁷ These involve changes in transmembrane

potential homeostasis via both direct influences on sarcolemmal ion channels and transporters, as well as indirect changes in Ca^{2+} signalling that acutely regulate transmembrane fluxes and can lead to remodelling in the chronic (pathologic) setting.^{13,18,20–23,25,28} Indeed, dysregulated PKA and CaMKII signalling have emerged as key transducers of myocardial stress responses to increased arrhythmia propensity via both acute and chronic regulation of cardiac structure and function and as potential novel targets for ‘upstream’ anti-arrhythmic therapy.^{29,30} Since PKA and CaMKII signalling share several downstream targets in the heart, and PKA-mediated Ca^{2+} elevation is a well-established mode of CaMKII activation, these two signalling pathways are strongly interrelated and may crosstalk to collectively promote arrhythmogenesis.³¹ However, most previous experimental work investigated these signals in isolation, studied their separate contribution to promote arrhythmia triggers, and led to controversies on the relative roles of PKA- and CaMKII-dependent processes in cardiac dysfunction and arrhythmogenesis.³² In addition, most of these studies are performed in isolated cardiomyocytes, and whether and how PKA and CaMKII signalling affect tissue properties to cause arrhythmia remain poorly understood. Therefore, we contend that developing ‘upstream’ therapeutic strategies focusing on these two signalling pathways requires (i) untangling the complex temporal regulations of PKA and CaMKII signalling cascades and dissecting contributions from modifications of each signalling target and (ii) integrating the observations across spatial scales to reveal how subcellular and cellular-level alterations interact with complex cardiac tissue dynamics.

While these are challenging experimental goals, mechanistic computational modelling of cardiomyocytes has proven instrumental to understand the complex interplay of membrane potential and Ca^{2+} -dependent signalling, by not only integrating functional and structural experimental (and clinical) data but also revealing experimentally unrecognized mechanistic underpinnings of physiological and pathophysiological processes.^{31,33–36} Indeed, extensive previous studies have constructed biochemically detailed models of PKA or CaMKII signalling and integrated these formulations with ventricular electrophysiology and Ca^{2+} -signalling models to provide insights into their individual roles in regulating ventricular excitation-contraction (EC) coupling and arrhythmia in health and disease, namely heart failure.^{31,37–40} However, cell ultrastructure, subcellular Ca^{2+} -signalling, and EC coupling differ between atrial and ventricular cardiomyocytes,^{36,41–43} and appropriate computational models and studies are conspicuously absent in atrial physiology and pathophysiology. Integrating detailed descriptions of PKA and CaMKII signalling with biophysical models of electrophysiology and Ca^{2+} handling allows to study the independent and combined effects of these two important signals. Furthermore, the existing models describe average behaviours and do not typically account for parameter variabilities that may otherwise reflect key intercellular and inter-subject heterogeneities and do not include tissue level simulations, which may lend important insight into PKA and CaMKII interactions in affecting cardiac tissue parameters, ultimately causing arrhythmia.

In this study, we integrated contemporary knowledge of atrial electrophysiology, Ca^{2+} handling, and PKA and CaMKII signalling into a comprehensive multi-scale model framework to uncover novel mechanistic insights into atrial arrhythmogenesis by addressing the following questions:

- (1) Do PKA and CaMKII signalling act synergistically to create arrhythmogenic triggered activity in human atrial cardiomyocytes and tissue?
- (2) What are the mechanistic determinants of increased triggered activity at the subcellular, cellular, and tissue level?
- (3) What is the contribution of subcellular and cellular variability to triggered activity?

Early and delayed afterdepolarizations (EADs and DADs, respectively) underlie ectopic (triggered) activity, which is a major arrhythmogenic mechanism of AF.^{3,10,44} Here, we specifically focused on DADs, since DAD-induced triggered activity appears key to the spontaneous initiation of AF, whereas the contribution of EADs to atrial arrhythmogenesis is less clear,³ except of mutations hampering repolarization reserve as with long-QT syndrome.⁴⁵

2. Methods

We coupled our well-established model of human atrial cardiomyocyte electrophysiology and Ca^{2+} handling^{46,47} with biochemically detailed systems models of CaMKII and adrenergic receptor (β AR)/cyclic adenosine monophosphate (cAMP)/PKA signalling pathways^{31,39,40,48–50} to build a novel integrative model of human atrial cardiomyocytes (see [Supplementary material online, Figure S1](#)). Each of the modules was updated to recapitulate new and human atrial-specific features. Namely, the human atrial electrophysiology and Ca^{2+} -handling model was modified to incorporate a new Markovian formulation of L-type Ca^{2+} current (I_{CaL} , [Supplementary material online, Figure S22](#)) based on previous work,⁵¹ add descriptions of atrial-predominant 2-pore and small-conductance Ca^{2+} -dependent K^+ currents (I_{K2P} and I_{KCa}), and update model formulations of rapidly and slowly activating delayed rectifier K^+ currents (I_{Kr} and I_{Ks}), inward rectifier K^+ current (I_{K1}), background Cl^- current (I_{ClB}), fast and late Na^+ current (I_{Na} and I_{NaL}). CaMKII and PKA signalling models were also extended to incorporate dynamic functional effects on additional downstream targets/substrates, including dynamic descriptions of CaMKII-dependent regulations of gap junction conductance^{52,53} and atrial-predominant I_{Kur} (see [Supplementary material online, Table S4](#)). The resulting human atrial cardiomyocyte integrative model was parameterized (model maximum ion channel conductances and transport rates were adjusted, see [Supplementary material online, Table S5](#)) to recapitulate key dynamic behaviours of human atrial cardiomyocyte AP and Ca^{2+} at various pacing rates (see [Supplementary material online, Figure S2A–C](#)) and rigorously validated by demonstrating its capability to reproduce characteristic responses of human atrial cardiomyocytes to a wide range of stressors and physiological challenges (see [Supplementary material online, Figures S3–S8](#)). We constructed populations of models^{39,54–58} of atrial myocytes and heterogeneous atrial tissue (see [Supplementary material online, Supplementary Methods](#)) to assess the precise contribution of PKA and CaMKII signalling to triggered activity in both atrial and pulmonary vein (PV)-like myocytes. We applied multivariate linear regression-based sensitivity analysis^{39,56–59} to gain quantitative understanding of influences of model parameters on key dynamic properties of atrial cardiomyocytes and performed logistic regression analysis⁵⁵ to link model parameters to the occurrence of triggered activity. Detailed descriptions of model extensions, sensitivity analysis, tissue modelling, simulation protocols, and model implementation are provided in [Supplementary material online, Supplementary Methods](#).

2.1 Code availability

All our source codes and related parameter perturbations for building populations as well as sensitivity analyses used in this study are available for download at elegrandi.wixsite.com/grandilab/downloads and github.com/drgrandilab.

3. Results

3.1 Integrative systems models of electrophysiology, Ca^{2+} handling, and CaMKII and PKA signalling recapitulate key dynamics of human atrial electrophysiology and Ca^{2+} handling

To facilitate the quantitative assessment of the interplay between PKA and CaMKII in human atria, we constructed a novel integrative model that couples our well-established systems model of electrophysiology and Ca^{2+} signalling⁴⁶ with biochemically detailed systems models of CaMKII and PKA signalling cascades^{31,40,49,50} (see [Supplementary material online, Figure S1](#)). This integrative framework has been previously established for rabbit,⁴⁰ mouse,³¹ and human^{39, 60} ventricular cardiomyocytes but is currently lacking in contemporary models of human atrial cardiomyocytes.^{46,61–63}

Our newly integrated model recapitulates the morphological characteristics and a wide range of physiological behaviours of action potential (AP)

and Ca^{2+} transient (CaT) of human atrial cells documented in previous experiments (see [Supplementary material online, Figure S2](#)). Typical APs, CaT, and L-type Ca^{2+} -current (I_{CaL}) during the AP stimulated at various frequencies are shown in [Supplementary material online, Figure S2Ai–iii](#). Our integrative cell model displays a typical Type-1 human atrial AP morphology that was most frequently encountered in experiments in human atria⁶⁴ (see [Supplementary material online, Figure S2Ai](#)). Notably, simulated AP morphology and duration (APD) and systolic and diastolic Ca^{2+} levels, associated with cardiomyocyte contractile function, are markedly rate-dependent over a wide range of physiological pacing frequencies and well match with experimental recordings^{65–72} (see [Supplementary material online, Figure S2Ai–ii](#) and [Figure S3A top row](#)). Specifically, our model displays a hallmark positive APD–pacing cycle length (PCL) relationship (see [Supplementary material online, Figure S2B](#)), which is well established despite large variabilities among experimental findings, and the computed CaT amplitude vs. PCLs displays a biphasic relationship that closely resembles the documented intra-cellular CaT measurements by aequorin light signals⁷¹ and twitch force measurements⁷² at various pacing rates (see [Supplementary material online, Figure S2C](#)). Our model shows that activating PKA signalling by simulated application of 100 nM isoprenaline (ISO) slightly prolonged the AP, in agreement with experimental reports from human atrial myocytes.^{73,74} Our simulations also recapitulate the well-known effects of PKA signalling on cardiomyocyte Ca^{2+} handling, in that application of ISO increased I_{CaL} and CaT amplitude, while accelerating CaT decay in both current (see [Supplementary material online, Figure S2D](#)) and voltage (see [Supplementary material online, Figure S7](#)) clamp settings,²² and the measured effects of CaMKII inhibition on the AP upstroke velocity and CaT characteristics (see [Supplementary material online, Figure S8Ai and Bi](#)). We validated our model by verifying the ability of our model to recapitulate characteristic dynamic responses of human atrial cardiomyocytes to various physiological stressors, pharmacological perturbation, and stimulation protocols (see [Supplementary material online, Figures S3–S8](#)). Detailed descriptions of model validation are provided in [Supplementary material online, Supplementary Text](#).

3.2 Populations of human atrial cardiomyocyte models reveal a synergistic interplay between PKA and CaMKII in promoting cellular triggered activity

Originally proposed for neuroscience research,^{75,76} the populations-of-models approach has been widely applied to understand the uncertainty of modelling outcomes, calibrate the populations to physiologically relevant variabilities, and gain mechanistic understanding through sensitivity analyses.^{39,54–58,77} Using our validated integrative model, we generated populations of models to assess the roles of PKA and CaMKII signalling in promoting the propensity of atrial cells to develop DADs and triggered APs (tAPs). We created three different populations (size of 600) by randomly perturbing the model parameters (log-normal distribution of $\sigma = 0.1$) describing: (i) maximum ion channel conductances or transporter rates (*Population-1*, see [Supplementary material online, Table S1](#)), (ii) steady-state phosphorylation levels of PKA or CaMKII downstream targets (*Population-2*, see [Supplementary material online, Table S2](#)), and (iii) concentrations of proteins (e.g. protein phosphatases, phosphodiesterases, etc.) that are intermediates within the two signalling cascades and fine-tune the target phosphorylation (*Population-3*, see [Supplementary material online, Table S3](#), similar to⁵⁴). Building these populations allows for regression-based sensitivity analyses to understand how AP and CaT characteristics are affected by the model parameters (see [Supplementary material online, Supplementary Text and Figure S9](#)), providing solid foundations for applying these populations of models to investigate the precise dependence of AP- and CaT-related abnormalities on each model parameter.

To quantify the contributions of PKA and CaMKII to atrial cardiomyocyte propensity to triggered activity, we subjected the three populations to various CaMKII settings (normal CaMKII, or increased CaMKII expression by two-fold, *i.e.* analogous to AF settings,^{12,18,20,22} and CaMKII

inhibition) with (ISO, 100 nM) or without PKA activation. We investigated the propensity for DADs following a 2 Hz pacing-pause protocol ([Figure 1A–C11](#); [Supplementary material online, Figure S10](#)). Our simulations in the control conditions rarely displayed DADs or abnormal $[\text{Ca}^{2+}]_i$ (see [Supplementary material online, Figure S10](#)), whereas DAD incidence became substantial following simulated ISO application and further increased with CaMKII over-expression (by two-fold, *i.e.* CaMKII $\times 2$, [Figure 1A–C](#)). Importantly, CaMKII inhibition dramatically suppressed the cellular arrhythmic events, uncovering a critical role of CaMKII in determining the propensity to cellular triggered activity. Importantly, our simulated effects of CaMKII inhibition on DAD incidence match well with experimental observations ([Figure 1Aii inset](#)), further validating our integrative model as a reliable tool for CaMKII studies.

Quantification of DAD events in the various populations and groups reveals a synergistic interplay between the PKA and CaMKII signalling in promoting cellular triggered activity ([Figure 1Aii–Cii](#)). Indeed, PKA signalling potentiated the pro-arrhythmic effects of CaMKII in atrial cardiomyocytes. The synergistic crosstalk between PKA and CaMKII resulted in the occurrence of DADs in 43–51% of cells in simulations with ISO and CaMKII $\times 2$. Furthermore, CaMKII inhibition generally abolished DADs even in the presence of ISO, suggesting that CaMKII activation is required for the induction of cellular triggered activity, consistent with previous studies in engineered ventricular tissue.⁷⁹

3.3 Logistic regression uncovers the key subcellular determinants of cellular triggered activity

Experimental investigations of triggered activity in human⁸⁰ and rabbit⁸¹ atrial myocytes show a large degree of cell-to-cell variability. Likewise, our simulations uncovered intrinsic variabilities in the cellular susceptibility to develop triggered activity within each model population, whereby a fraction of cells displayed arrhythmic events, whereas many others did not. To quantitatively dissect the mechanisms underlying the variable susceptibility to triggered activity and arrhythmia, we performed logistic regression analyses⁵⁵ to link the arrhythmic events to the parameters describing subcellular processes and signalling. Specifically, we focused on the simulations with both ISO application and two-fold CaMKII expression, where the size of cell subpopulation displaying DADs was comparable to that of the stable cell subpopulation, and applied binary coding to represent the presence/absence of cellular triggered activity. The sensitivity coefficients from the logistic regression analyses shed light on the influences of model parameters on arrhythmia propensity, with pro-arrhythmic processes being associated with positive coefficients and anti-arrhythmic processes with negative coefficients, and the coefficient magnitude informing the degree of parameter influence on the arrhythmic outcome.⁵⁵ The values of the sensitivity coefficients provide quantitative understanding of how changing each individual process to the same extent affects the probability of the event of interest without assuming specific disease states. While the coefficients should not be interpreted as contributions in AF, the actual pro-arrhythmic impact in disease can be approximated by the product combining the coefficient and the disease-associated change of any given process, as further explained in [Supplementary material online, Supplementary Text](#).

Logistic regression analysis uncovers the effects of altering each underlying ionic and signalling process on the cellular propensity to DADs and tAPs ([Figure 1D](#)). Comparing the outcome from the three distinct populations allows to cross validate and corroborate modelling insights. Our results show a previously under-recognized positive association between larger I_{CaL} and DAD propensity; in that, increasing the conductance (G_{CaL}) of L-type Ca^{2+} channels (LTCCs) or the PKA- or CaMKII-dependent phosphorylation levels (PKA-LTCC and CaMKII-LTCC) augments DAD probability, suggesting that augmenting I_{CaL} can substantially contribute to spontaneous Ca^{2+} releases (SCRs) and thus to DADs and tAPs, by increasing the intracellular Ca^{2+} loading during the pre-conditioning pacing beats. Notably, when removing the cells displaying tAPs from the analysis, G_{CaL} remained the parameter that most positively correlates with the presence of sub-threshold

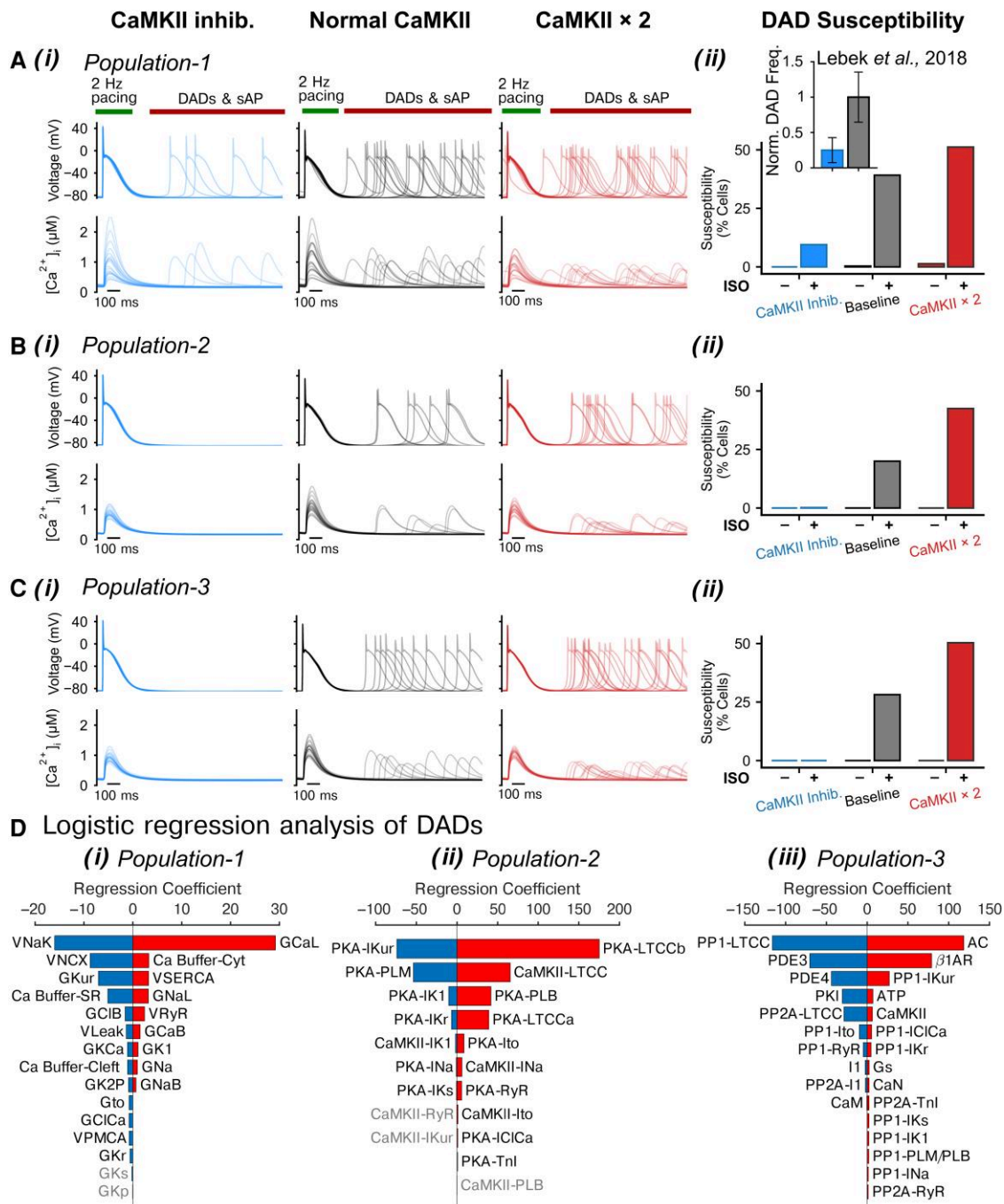


Figure 1 Populations of models uncover synergistic interplay between PKA and CaMKII signaling in promoting DADs. (A–C) (i) APs and CaTs simulated using (A) Population-1, (B) Population-2, and (C) Population-3 under CaMKII inhibition, normal CaMKII, or with two-fold CaMKII expression conditions after application of ISO following a 2 Hz pacing and pause protocol; (ii) fraction of cells developed DADs. Inset in (A) (ii): normalized frequency of DADs for human atrial cardiomyocytes under control vs. CaMKII inhibition conditions reported in experiments (Lebek et al.⁷⁸). (D) Logistic regression analysis of DAD incidence under two-fold CaMKII expression conditions and after ISO application for (i) Population-1, (ii) Population-2, and (iii) Population-3, respectively. Please refer to [Supplementary material online, Tables S1–S3](#) for abbreviation descriptions for panel (D).

DADs (Figure 3C), confirming the notion that increasing I_{CaL} contributes to sub-threshold DAD generation. Also, increasing sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) and I_{NaL} activity by elevating either velocity/conductance (V_{SERCA} or G_{NaL}) or the associated regulatory phosphorylations [PKA-phospholamban (PLB) that enhances SERCA Ca^{2+} uptake and CaMKII- I_{NaL}] promotes DADs. Conversely, increasing I_{NaK} [via increasing V_{NaK} or PKA-pho

spholamban (PLM) which enhances I_{NaK} , I_{NCX} , or atrial-predominant I_{Kur} (G_{Kur} or PKA- I_{Kur}) attenuates DAD susceptibility via directly or indirectly reducing intracellular Ca^{2+} . Consistently, elevating the expression of PP1 that targets I_{Kur} channels (PP1- I_{Kur}) increases DAD susceptibility. Finally, Population-3 uncovered the roles of the signalling cascade intermediates in producing cellular DADs. Both AC and $\beta 1AR$ expressions (thus PKA activity)

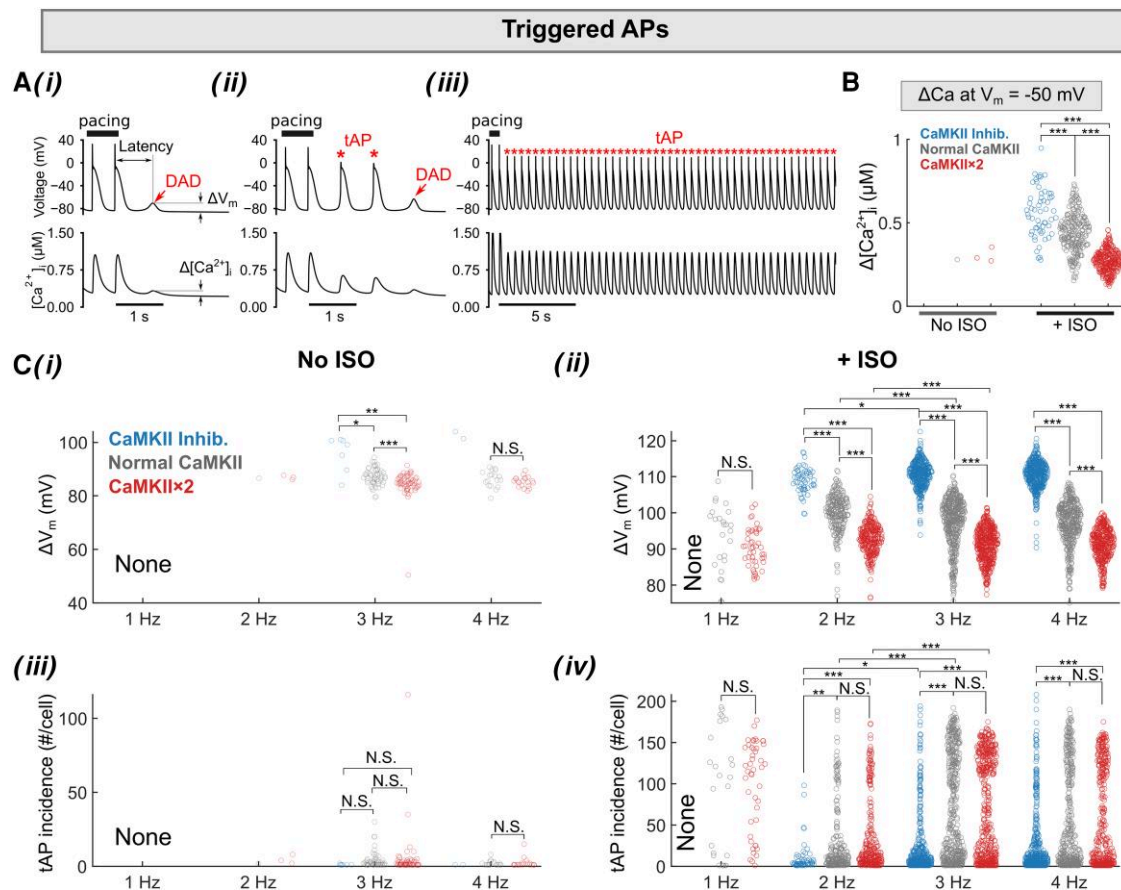


Figure 2 Populations of models reveal rate dependence of tAPs in human atrial cardiomyocytes following a pace-pause protocol. (A) Illustration of three types of membrane V_m instabilities following a pace-pause protocol: (i) sub-threshold DAD only, (ii) both sub-threshold DAD and tAPs, and (iii) long-lasting tAPs. (B) Threshold Ca^{2+} amplitude causing tAPs is measured as the $[Ca^{2+}]_i$ when V_m depolarizes to -50 mV. (C) Rate-dependent characterization of tAPs without (i and iii) and with (ii and iv) ISO treatment: (i and ii) mean voltage amplitude and (iii and iv) number of tAPs for each cardiomyocyte that developed tAPs. Statistical analysis was performed using Kruskal–Wallis test followed by planned comparisons with Wilcoxon rank sum test and Bonferroni correction. **** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; N.S., not significant.

are positively linked to DAD probability, whereas increasing the levels of phosphodiesterases (PDE3 and PDE4, which decrease active cAMP), protein phosphatases targeting LTCC (PP1-LTCC and PP2A-LTCC), or applying a PKA inhibitor peptide (PKI) all suppress DAD propensity. Collectively, these simulations provide novel and coherent mechanistic insights into the precise roles and the specific contributions of key ionic processes and upstream signalling systems to the propensity of human atrial cardiomyocytes to pro-arrhythmic triggered activity.

4. PKA and CaMKII impact the pacing rate-dependence of the incidence and amplitude of SCR events and the coupling with the resulting DADs and tAPs

Due to corresponding elevation of $[Ca^{2+}]_i$ (see [Supplementary material online, Figure S11](#)), CaMKII activity is a key mediator of cardiomyocyte responses to changes in pacing frequency. Our model captures the dynamic changes of CaMKII activity during the cardiac cycle at varying pacing rates (see [Supplementary material online, Figure S11](#)) and recapitulates both the

beat-to-beat changes in CaMKII activation and target phosphorylation, as well as their frequency dependence (see [Supplementary material online, Figure S11](#)). We further examined the frequency dependence of triggered activity in our simulations with *Population-1* ([Figures 2 and 3](#)), which displays the larger phenotypic variability when simulating the same range of perturbations in model parameters (i.e. larger standard deviation in AP and CaT parameters as shown in [Supplementary material online, Figure S9](#)). Depending on the amplitude of membrane depolarization, triggered activity manifests as sub-threshold DADs or tAPs ([Figure 2Ai–iii](#)). Examining the underlying ryanodine receptor 2 (RyR2), Na^+/Ca^{2+} exchanger (NCX), and other ion channel activities confirms that these triggered activity events are initiated by SCRs that potentiate I_{NCX} and cause subsequent membrane depolarization (see [Supplementary material online, Figure S12](#)). [Supplementary material online, Figure S13](#) illustrates the relationship between the amplitude of SCR ($\Delta[Ca^{2+}]_i$) and the resulting DAD or tAP: the ΔV_m increases with increasing $\Delta[Ca^{2+}]_i$, until it is large enough to trigger a tAP; after this threshold, the tAP amplitude barely increases even though $\Delta[Ca^{2+}]_i$ continues to grow. Both PKA and CaMKII negatively shift this relationship, whereby smaller $\Delta[Ca^{2+}]_i$ causes the greater depolarization increasing V_m - Ca^{2+} coupling gain ($\Delta V_m/\Delta[Ca^{2+}]_i$). We further assessed the frequency dependence of the amplitude and the incidence of tAPs ([Figure 2B and C](#)), SCRs, DADs, and $\Delta V_m/\Delta[Ca^{2+}]_i$ for sub-threshold DADs for each atrial cardiomyocyte ([Figure 3A and B](#)) and determined

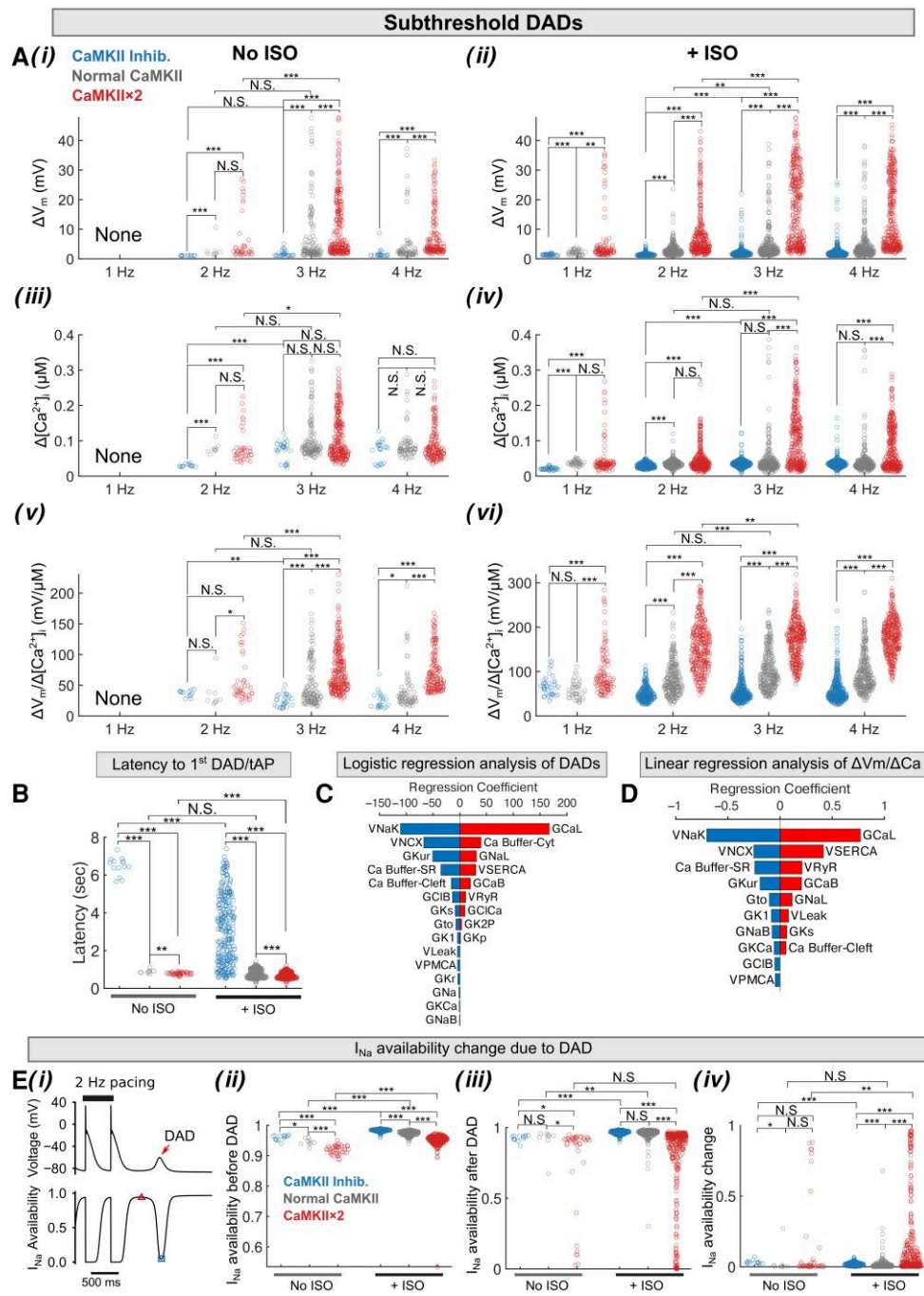


Figure 3 Populations of models reveal rate dependence of sub-threshold DADs in human atrial cardiomyocytes following a pace-pause protocol. (A) Rate-dependent characterization of sub-threshold DAD: (i and ii) amplitudes of V_m instability (ΔV_m) and (iii and iv) associated $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$), and (v and vi) V_m to $[Ca^{2+}]_i$ coupling ($\Delta V_m/\Delta[Ca^{2+}]_i$); the left and right columns display data for no ISO conditions and after ISO treatment, respectively. (B) Latency to developing first sub-threshold DAD or tAP after cessation of 2-Hz stimulation. (C) Logistic regression analysis of sub-threshold DAD incidence in a sub-population of Population-1 comprising model variants displaying only sub-threshold DADs and those showing no membrane instability for CaMKII $\times 2$ + ISO application. (D) Linear regression analysis reveals the influence of subcellular parameters on the $\Delta V_m/\Delta[Ca^{2+}]_i$ of sub-threshold DADs. (E) CaMKII activation reduces I_{Na} availability following a sub-threshold DAD. (i) Illustration of tAPs and sub-threshold DADs after 2 Hz pacing, and the accompanying I_{Na} availability. The maximum I_{Na} availability before a sub-threshold DAD is indicated with a triangle, and its minimum after a sub-threshold DAD is marked with a square. (ii) Comparison of maximum I_{Na} availability before sub-threshold DADs. (iii) Comparison of minimum I_{Na} availability after sub-threshold DADs. (iv) The I_{Na} availability change due to sub-threshold DAD (computed as the difference between maximum and minimum availability). Statistical analysis was performed using Kruskal–Wallis test followed by planned comparisons with Wilcoxon rank sum test and Bonferroni correction. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; N.S., not significant.

the impact of PKA and CaMKII on these parameters. We found that ISO treatment and increasing pacing frequency markedly enhanced the propensity for tAPs, by increasing both the number of cells developing tAPs (Figure 2B and C*i–ii*) and the incidence per cell (Figure 2C*iii* and *iv*). The burden of tAPs was markedly reduced with CaMKII inhibition (Figure 2C*iii* and *iv*), although the amplitude was increased (Figure 2C*ii*) due to CaMKII effects to reduce I_{Na} availability. Furthermore, we found that CaMKII reduced the threshold Ca^{2+} amplitude that triggers an AP (Figure 2B), measured as the $[Ca^{2+}]_i$ value at $V_m = -50$ mV.

Our data demonstrate that pacing frequency, ISO administration, and CaMKII over-expression increase the incidence and amplitude of sub-threshold DADs (Figure 3A*i* and *ii*) and SCRs (Figure 3A*iii* and *iv*) and promote $\Delta V_m/\Delta[Ca^{2+}]_i$ (Figure 3A*v–vi*). Notably, CaMKII inhibition increases the latency to first DAD/tAP (Figure 3C) and decreases the amplitude of the SCR and DAD, and $\Delta V_m/\Delta[Ca^{2+}]_i$, either in the absence or presence of ISO simulation (Figure 3A). Linear regression analysis revealed that the parameters that most strongly correlated with changes in $\Delta V_m/\Delta[Ca^{2+}]_i$ (Figure 3D) are the same that allow distinguishing between cells displaying DADs and those without DADs when applying logistic regression (Figure 3C). Interestingly, CaMKII hyper-activation also reduces I_{Na} availability following a sub-threshold DAD (Figure 3E), suggesting a critical role for sub-threshold DADs for increasing dispersion of refractoriness by impacting I_{Na} availability.

4.1 PKA and CaMKII synergistically promote DADs and triggered action potentials in electrically coupled heterogeneous tissue

Although we could establish a synergistic interplay between PKA and CaMKII that promotes triggered activity at the cellular level, it remains unclear whether triggered activity at the single-cell level persists in electrically coupled tissue, where the electrical coupling could dampen the triggered activity due to the current sink by surrounding cardiomyocytes.^{79,82,83} Indeed, to facilitate in-tissue tAP propagation, the source current density of depolarizing myocytes must be sufficiently strong to overcome the sink by the surrounding repolarized tissue.^{82,83} Besides, CaMKII may further modify the in-tissue arrhythmogenesis through a direct modulation of gap junctions⁵² and function of other ion fluxes that impact the source–sink relationship. Finally, the arrhythmia propensity at the tissue level may be affected by the degree of electrophysiological heterogeneity, which is a hallmark of the atria and critically governs arrhythmia dynamics.

To assess the contributions of PKA and CaMKII to triggered activity in tissue, we built an electrically heterogeneous and coupled tissue model (Figure 4) by creating a 'grid-like' mosaic tissue pattern and mapping the populations of our integrate models (Population-1) to the tissue, as detailed in Supplementary material online, [Supplementary Methods and Figure S23](#). The two-dimensional (2D) atrial tissue was paced following a 2 Hz pacing (five beats) and pause protocol, allowing for characterizing AP conduction properties and membrane voltage instability events. Figure 4A and [Supplementary material online, Movie S1](#) illustrate the tissue voltage map and extracted single cell APs following the last pacing stimulation. The spatial distributions and density of DADs in tissue are quantified in Figure 4B. Our simulations show normal AP wave propagation and repolarization in the control conditions + normal CaMKII (Figure 4A*i* and *Bi left column*). However, DADs emerged across the tissue after ISO application (Figure 4A*ii* and *Bi 2nd left column*) and were largely suppressed with CaMKII inhibition (Figure 4A*iii* and *Bi 2nd right column*). Remarkably, with increased CaMKII expression (two-fold, mimicking the CaMKII up-regulation in chronic AF patients^{12,18,20,22,84}), ISO exacerbated the post-repolarization membrane instabilities (Figure 4B*i right column*), which degenerated into tAPs propagating throughout the tissue (Figure 4A*iv*). These tAPs originated from the edge/border of the tissue, where the electrical coupling was much weaker. Similar observations were reported in a previous study of engineered human heart tissue from induced pluripotent stem cell-derived ventricular cardiomyocytes.⁷⁹ Quantified

densities of DAD incidence are again consistent with a synergistic interplay between PKA and CaMKII in promoting DADs in the electrically coupled tissue (Figure 4B*ii*). Furthermore, PKA activation increased the conduction velocity (CV) of AP propagation in tissue, in agreement with reports from literature.⁸⁵ Increasing CaMKII slowed CV with or without PKA activation, whereas the opposite effects were observed following CaMKII inhibition (see [Supplementary material online, Figure S14](#)). These results suggest that both PKA and CaMKII activation promote the propensity to develop transmembrane potential instabilities and tAPs in tissue. In addition, the CaMKII-dependent slowing of CV may create a substrate for AF-maintaining re-entry, another pivotal arrhythmic mechanism, by causing a conduction block and by reducing the wavelength of tissue electrical excitation.

Since triggered activity is often associated with structural remodelling, we assessed the PKA- and CaMKII-dependent propensity of tissue for tAPs with various degree of structural remodelling (e.g. as with atrial enlargement and fibrosis) and gap junction abnormalities³ (Figure 4C). Specifically, we varied the cell-to-cell electrical coupling strength between 100 and 25% and quantified the number of tAPs in tissue by examining the number of triggered activations from computed electrograms (EGMs) (Figure 4C*i*). In the absence of ISO, tAPs were not detected in any of the CaMKII expression settings, even when considering the most severe electrical decoupling (to 25% of the basal value) (see [Supplementary material online, Figure S15](#)). Following application of ISO, simulations with normal CaMKII displayed a tissue coupling disruption-dependent increase in the incidence of tAPs and progressively shortened cycle length (CL) of the spontaneous activity. The tAP number was further increased with abbreviated CLs for CaMKII $\times 2$ vs. the normal CaMKII groups (Figure 4C). Interestingly, this increase is more evident with scaling of tissue conductivity between 100 and 75% (Figure 4C*ii*), suggesting that the CaMKII-dependent propensity to triggered activity persists even with normal tissue conductivity. Thus, whereas in regions with preserved tissue conductivity, only the pathologically up-regulated CaMKII, as seen in chronic AF patients,^{12,18,20,22,84} was associated with tAP generation after ISO, in regions with strongly reduced tissue conductivity, which mimics AF-related structural remodelling, ISO produced tAP in the presence of physiological CaMKII levels. Overall, no tAPs were detected with ISO application after CaMKII inhibition in either group, suggesting that CaMKII is required and indispensable for PKA-induced triggered activity in tissue, as noted in a previous experimental study in human-engineered ventricular tissue.⁷⁹ Interestingly, acute elimination of CaMKII actions on RyR2 for CaMKII $\times 2$ + ISO could also abolish tAPs while reducing DAD occurrence in tissue (see [Supplementary material online, Figure S16](#)), confirming a crucial contribution of CaMKII-dependent regulation of RyR2 to triggered activity in experimental AF paradigms and AF patients.^{14,22,86–88}

4.2 CaMKII increases propensity to triggered activity in tissue by acting on the determinants of the source–sink relationship

Although our simulations demonstrate a clear requirement of CaMKII for ISO-induced tAP in tissue, it is unknown whether and how CaMKII modulate the determinants of electrotonic load, thereby causing triggered activity. Indeed, previous experimental studies report that Ca^{2+} -CaM reduces the connexin (Cx) conductivity,⁵³ while inhibiting CaMKII increases tissue CV.⁵² Also, CaMKII causes a hyperpolarizing shift in the voltage-dependence of I_{Na} channel availability,⁸⁹ thereby decreasing I_{Na} channel availability and limiting cellular excitability.^{90,91} Furthermore, the I_{K1} current is augmented by CaMKII,⁹² potentially elevating the sink of the tissue to be depolarized. We applied our model to dissect the mechanisms by which the CaMKII-dependent modifications to tissue parameters (namely, Cx function, I_{K1} activity, and voltage-dependence of I_{Na} availability, [Supplementary material online, Figure S17 and Table S4](#)) impact the propensity to triggered activity in tissue. Of note, these parameters are also

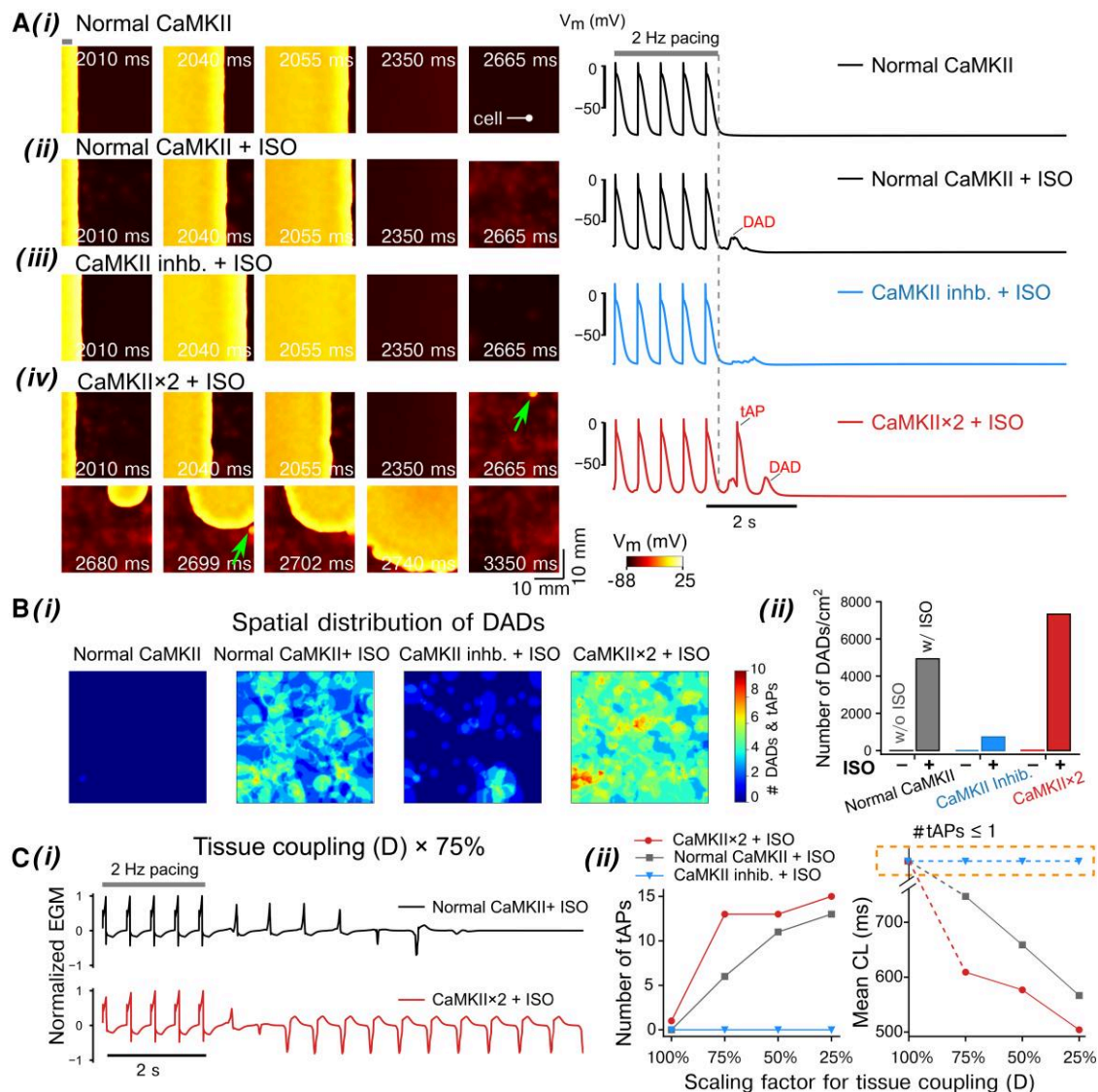


Figure 4 Effects of PKA and CaMKII activation on the membrane instabilities in simulated 2D atrial tissue. The 2D tissue slab was paced from the left side [site width indicated with a bar in top left of panel (i)] using a 2 Hz pacing (five beats) and pause protocol. The total simulated time was 10 s. (A, left) Time-stamped snapshots of tissue cell membrane voltage map featuring membrane voltage changes following the last pacing at $t = 2000$ ms. (A, right) Time courses of single-cell AP extracted from the tissue. The cell location is indicated in (A) (i), left. Panels (i–iv) illustrate (i) normal CaMKII without ISO, (ii) normal CaMKII with ISO, (iii) CaMKII inhibition with ISO, and (iv) two-fold CaMKII expression with ISO. Triggered AP initiation is indicated by arrows. (B) (i) Spatial distribution and (ii) density of DAD incidences in the tissue slab following the cessation of the pacing protocol. (C) (i) Simulated electrograms (EGMs) from the tissue simulations at reduced gap junction conductance (scaled to 75%, $D \times 75\%$) for the normal vs. CaMKII $\times 2$ with ISO application. (ii) Effects of CaMKII inhibition or two-fold CaMKII expression on the number of tAP propagation and cycle length (CL) for normal and reduced gap junction conductances (D scaled from 100 to 25%).

important determinants of re-entry.^{3,10,93} We repeated the simulations of ISO + CaMKII $\times 2$ group, but with removal of CaMKII-dependent modulations on each target individually (i.e. no CaMKII-Cx, no CaMKII-NaV, or no CaMKII- I_{K1} , respectively). Simulated time courses of tissue voltage maps and extracted single-cell APs are shown in [Figure 5A](#) and [Supplementary material online, Movie S2](#). Interestingly, excluding the CaMKII-dependent effect on Cx prevented the degeneration of DADs to propagating tAPs ([Figure 5A\(i\)](#)), while increasing tissue CV (see [Supplementary material online, Figure S18](#)). This result suggests that CaMKII-dependent reduction of Cx conductance promotes the propensity to tAPs in tissue. In contrast, removing the CaMKII-dependent modulation on I_{K1} strongly increased the

incidence of tAP ([Figure 5A\(ii\)](#)). The number of tAPs was highest when the CaMKII effect on I_{Na} availability was excluded: these tAPs originated from two distinct ectopic sites and sustained throughout the duration of simulation ([Figure 5A\(iii\)](#)). Interestingly, tissue CV was not substantially modified in either of the last two groups, though in general a slight increase was noted (see [Supplementary material online, Figure S18](#)).

We finally determined the contribution of tissue coupling to CaMKII- and PKA-dependent pro-arrhythmia ([Figure 5B](#)). Simulated tissue EGMs ([Figure 5B\(i\)](#)) were computed to quantify the number and CL of the tAP ([Figure 5B\(ii\)](#)). Remarkably, the tAP-suppressing effect of excluding CaMKII-Cx was preserved for scaling of $D \geq 50\%$, suggesting a major

contribution of CaMKII-Cx effects to the generation of tAPs in the absence of severe tissue uncoupling. Conversely, removing CaMKII- I_{K1} or CaMKII-NaV shortened the tAP CLs and increased the number of tAPs. These effects were most pronounced with normal tissue coupling, suggesting a protective role of CaMKII- I_{K1} and CaMKII-NaV against tAPs, particularly in the absence of tissue conductance disturbances. Collectively, our models provide new insights into the key roles and precise contributions of each CaMKII-dependent tissue parameter to arrhythmia propensity and highlight a critical role for CaMKII modulation of gap junctions in promoting tAPs in tissue.

4.3 PKA and CaMKII synergistically promote DADs and tAPs in PV-like myocytes and tissue

Triggered activity often occurs at the PV regions, where the PV cardiomyocytes have depolarized resting membrane potential (RMP)⁹⁴ than the atrial cardiomyocytes due to a lower I_{K1} .^{94,95} Given the interesting interactions between source and sink giving rise to triggered activity emerged at the tissue level, we investigated whether and how the source–sink balance are altered by PKA and CaMKII signalling in PV myocytes. We built a large (20 000) population of atrial cell models and selected a PV-like myocyte population as those exhibiting depolarized RMP⁹⁴ and reduced upstroke velocity,⁹⁵ but comparable $[Ca^{2+}]_i$ ⁹⁶ with respect to the average atrial model (see [Supplementary material online, Supplementary Methods; Figure 6Ai and ii](#)). Analysing the parameters of PV-like population revealed that multiple subcellular processes significantly differed between the PV-like and the atrial cells ([Figure 6Aiii](#)), including lower I_{K1} and I_{CaL} in the PV-like cells that have been documented in previous experimental studies.^{94–96} We characterized triggered activity in the PV-like population following 2-Hz pacing-pause protocol and found that the actions of PKA and CaMKII in the PV-like myocytes are consistent with those in atrial cells: both PKA and CaMKII activation augmented DAD amplitude and $\Delta V_m/\Delta[Ca^{2+}]_i$ ([Figure 6Bi](#)) while PKA activation also increased the number of cells exhibiting tAPs, whereas CaMKII inhibition reduced the number of tAPs in each PV-like myocyte ([Figure 6Bii](#)).

Lastly, we determined the contribution of CaMKII-dependent modulations on I_{Na} , I_{K1} , and Cx to propensity to triggered activity in a heterogeneous PV-like tissue ([Figure 6Ci–iv](#)). As seen in atrial tissue simulations, removing CaMKII actions on I_{Na} substantially increased the incidence of triggered activity, whereas excluding CaMKII modulation of Cx strongly decreased the propensity to triggered activity; these effects persist over a wide range of tissue conductivity ([Figure 6Civ](#)). However, the incidence of triggered activity remained unaffected when removing CaMKII effects on I_{K1} , due to the lower I_{K1} amplitude in the PV-like cells. Of note, our simulations revealed substantially greater incidence of triggered activity both in PV-like tissue and single cells compared to the atrial populations, in agreement with clinical observations of AF-promoting triggered activity often arising from the PV region.⁹⁷

5. Discussion

We have constructed a novel multi-scale human atrial model integrating electrophysiology and Ca^{2+} handling with PKA- and CaMKII-signalling pathways by assembling the currently available knowledge in the field. Through simulations of populations-of-models, we uncovered a synergistic interplay between PKA and CaMKII that promotes triggered activity at both the single cell and tissue scales in human atria. Logistic regression analyses dissected anti- from pro-arrhythmic ionic processes and signalling components, providing the foundation for informing novel therapeutic approaches against AF. Our simulations uncovered a previously unrecognized critical role of CaMKII in modifying the source–sink mismatch to favour pro-arrhythmic tAPs in atrial tissue. Overall, our study establishes key mechanistic roles of CaMKII in the generation of pro-arrhythmic triggered activity in the atria by acting on both subcellular and inter-cellular (tissue) determinants of atrial function.

5.1 Working model of interactive signalling that promotes triggered activity

Our integrative computational model uncovered complex crosstalk patterns between PKA and CaMKII signalling in the promotion of DADs at both the cell and tissue levels of human atria ([Figure 7](#)). At the single-cell level, activated CaMKII and PKA both phosphorylate key Ca^{2+} handling proteins (PLB, RyR2, LTCC) involved in EC coupling, thereby increasing $[Ca^{2+}]_i$ ([Figure 7](#)). The CaMKII-dependent augmentation of I_{NaL} function increases $[Na^+]_i$, promoting the outward shift of NCX that favours the elevation of $[Ca^{2+}]_i$ ([Figure 7](#)). Concomitant PKA-dependent modifications of PLM enhance the activity of Na^+/K^+ ATPase (NKA), which decreases both $[Na^+]_i$ and $[Ca^{2+}]_i$. Nevertheless, the CaMKII effects prevail, and the increased $[Ca^{2+}]_i$ further enhances CaMKII activity, creating a vicious cycle of Ca^{2+} /CaMKII/ Na^+ / Ca^{2+} promoting DADs ([Figure 7](#)). Accordingly, previous experimental^{16,98} and computational⁹⁵ studies revealed that CaMKII-dependent enhancement of I_{NaL} promoted Ca^{2+} overload and arrhythmogenesis. Likewise, increased Na^+ influx *per se* promoted atrial arrhythmias in mice expressing human Nav1.5 with augmented persistent Na^+ current.¹⁰⁰ In addition, CaMKII activation could increase $\Delta V_m/\Delta[Ca^{2+}]_i$ coupling, further promoting triggered activity. This vicious arrhythmogenic cycle is amplified by PKA stimulation that targets the same Ca^{2+} -handling proteins, thereby creating synergistic pro-arrhythmic effects. We propose that increased heart rates further drive this vicious cycle and $\Delta V_m/\Delta[Ca^{2+}]_i$ coupling. Furthermore, the factors promoting DADs at the single-cell level cause source–sink mismatch at the tissue level by directly augmenting the source, while CaMKII-dependent modifications to I_{Na} , connexins, and I_{K1} alter the source–sink relationship ([Figure 7](#)). For instance, CaMKII-dependent phosphorylation up-regulates I_{K1} and increases the current sink (counteracted by PKA-dependent I_{K1} decrease), while reducing the source by counteracting the depolarizing peak I_{Na} current. CaMKII activation also diminishes I_{Na} availability and thus cell excitability, thereby reducing the source. Both effects mitigate the mismatch between the source and sink. On the other hand, the reduced electrotonic coupling due to CaMKII phosphorylation of Cx substantially weakens the sink and synergizes with the increased current source (caused by the vicious cycle) to outweigh the CaMKII effects on I_{K1} and I_{Na} , thus exacerbating the source–sink mismatch that promotes triggered activity in tissue. Importantly, CaMKII inhibition abolishes triggered activity in both single cells and tissue, suggesting that CaMKII activity is required for human atrial triggered activity and arrhythmogenesis. Collectively, our simulations uncover a novel network mechanism of a synergistic pro-arrhythmic crosstalk between PKA and CaMKII and dissect mechanistically the specific roles and precise contributions of CaMKII targets at both the cell and tissue scales.

5.2 Heterogeneous tissue simulations offer novel insights for tissue-level atrial arrhythmia

We constructed a heterogeneous 2D tissue to account for the hallmark electrophysiological heterogeneities in atrial tissue, while allowing randomly mapping our single-cell population of models onto the tissue. This approach allowed us to investigate whether triggered activity seen at the single-cell level persisted in tissue. Our results show that the increased DAD propensity at the single-cell level due to the synergistic PKA–CaMKII crosstalk further synergizes with CaMKII-dependent *functional* decoupling of cell–cell electrotonic communication to induce tAP in atrial tissue. Although our tissue models were constructed from single-cell model populations, in which a substantial fraction of cells display DADs, the tAPs in tissue often originated from a limited number of sites that are frequently close to the tissue border with weaker electrotonic coupling. Interestingly, analogously localized tAP sites were observed in engineered human ventricular tissue.⁷⁹ Likewise, weakly coupled cardiomyocytes within the failing hearts can depolarize membrane voltage to trigger APs by overcoming a weakened current sink of surrounding tissue.¹⁰¹ Furthermore, decreasing the basal tissue coupling strength

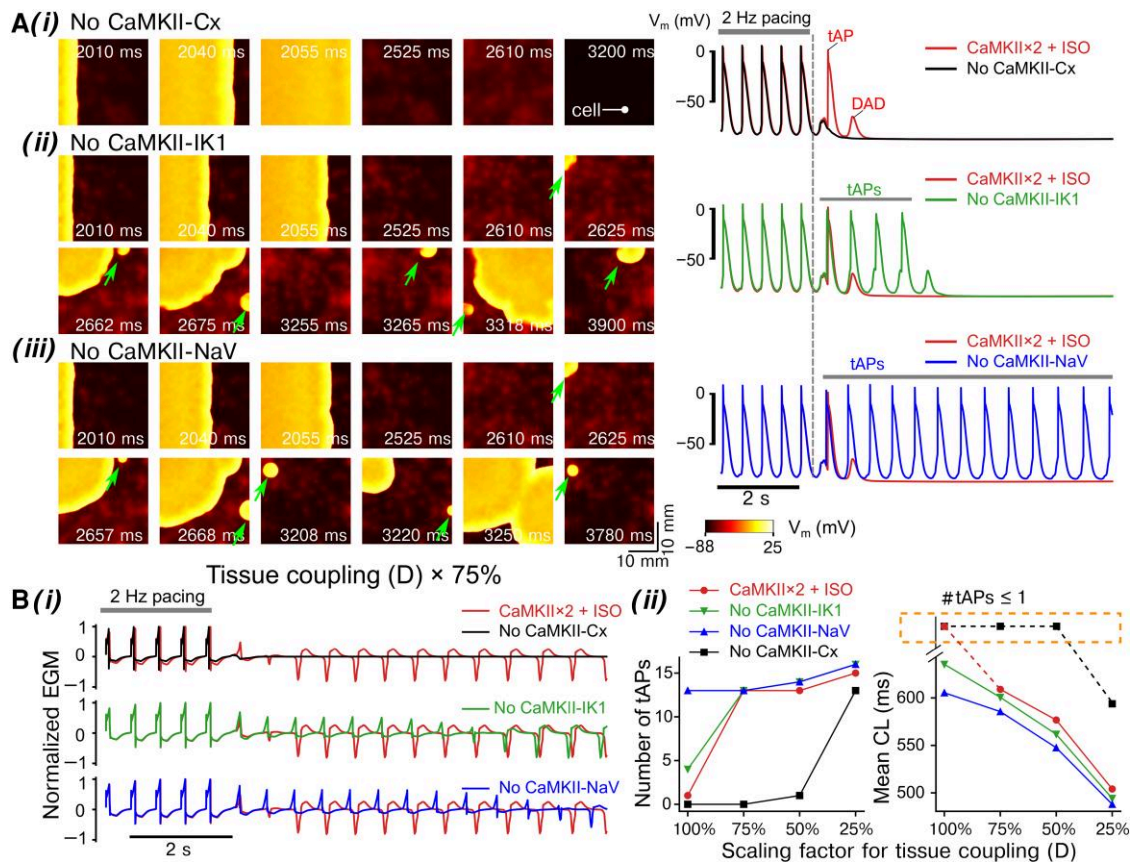


Figure 5 Dissecting the roles of CaMKII-dependent modulations of connexins (CaMKII-Cx), I_{K1} (CaMKII- I_{K1}), and Na^+ channel availability (CaMKII-NaV) in inducing tAPs in tissue. The 2D tissue slab was paced from the left side [site width indicated with a bar in top left of panel (i)] using a 2 Hz pacing (five beats) and pause protocol. The total simulated time was 10 s. (A, left) Time-stamped snapshots of tissue cell membrane voltage map featuring membrane voltage changes following the last pacing at $t = 2000$ ms. (A, right) Time courses of single cell AP extracted from the tissue. The location of the cell is marked in (A) (i), left. (i–iii) illustrate simulations after ISO application with two-fold CaMKII expression but removing CaMKII-dependent modulations of (i) gap junctions (No CaMKII-Cx), (ii) I_{K1} (No CaMKII- I_{K1}), and (iii) Na^+ channel availability (No CaMKII-NaV). The 2D tissue slab was paced from the left side using a 2 Hz pacing (five beats) and pause protocol. Triggered AP initiation is indicated by arrows. (B) (i) Simulated EGMs from the tissue simulations at reduced gap junction conductance (scaled to 75%, $D \times 75\%$) showing effects of removing the CaMKII-dependent modulations on gap junctions (Cx), I_{K1} , and Na^+ channel availability. (ii) Number of tAP propagation and CL for normal and reduced tissue conductivities (D scaled from 100 to 25%).

exacerbated the spontaneous triggered activity and created arrhythmogenic foci giving rise to a train of spontaneous APs that propagated throughout the tissue. Therefore, it is conceivable that in the atria, highly arrhythmogenic foci can be created by combining (i) cellular arrhythmogenic DADs, (ii) CaMKII-dependent functional electrotonic decoupling, and (iii) locally weakened electrical coupling due to fibrosis.

Our simulations uncover a protective role of the CaMKII actions on I_{Na} availability and I_{K1} activity. Indeed, removing CaMKII regulation on I_{Na} availability from our models resulted in substantially strengthened arrhythmogenic foci (Figure 5), without markedly modifying the CV of paced APs. These results can be explained by the timing of dynamic CaMKII activity during paced APs and Ca^{2+} -driven tAPs: in the paced APs, a delay between AP upstroke and CaT prevented peaking of CaMKII regulation of I_{Na} during AP upstroke, whereas during the early phase of Ca^{2+} -driven tAPs, strong CaMKII regulation of I_{Na} (by high $[Ca^{2+}]_i$) diminished cell excitability and the development of tAPs. The latter has additional implications on the pro-arrhythmic effects of sub-threshold DADs that failed to invoke tAPs. Sub-threshold DADs not only may promote tissue dispersion of cell excitability due to heterogeneous voltage-dependent I_{Na} availability,¹⁰² as shown in our analysis (Figure 3E) and working model (Figure 7), thereby causing conduction block that promotes re-entry,⁴⁴ but can also augment

this functional excitability heterogeneity via spatial dispersion of Ca^{2+} /CaMKII activities that modify I_{Na} , both creating vulnerable substrates for arrhythmia induction and maintenance. Thus, our simulations discovered novel insights into the potential effects of CaMKII on re-entrant arrhythmias. Likewise, CaMKII-dependent modulation of I_{K1} activity also produces protective effects against triggered activity (Figure 5). The increased I_{K1} upon CaMKII phosphorylation may help to counteract Ca^{2+} overload-induced depolarizing I_{NCX} to limit the arrhythmogenic propensity and thus reduce the current source, while it also may substantially increase the current sink of the surrounding cells in tissue, thereby contributing to alleviating the source–sink mismatch. However, the up-regulation of I_{K1} stabilizes rotors and may help to sustain AF-maintaining re-entry.¹⁰³ Combined, our simulations establish complex patterns of CaMKII actions in triggering atrial arrhythmogenesis across spatial scales, which allow to dissect (mal)adaptive from adaptive remodelling components.

PV is a major source of triggered activity in AF,⁹⁴ and thus, PV isolation is routinely performed in catheter ablation treatment of AF. Our simulation results underscore a prominent involvement of I_{K1} in shaping the higher propensity of PV to pro-arrhythmia. Consistent with clinical observations, our populations of PV-like cells and tissue display substantially increased

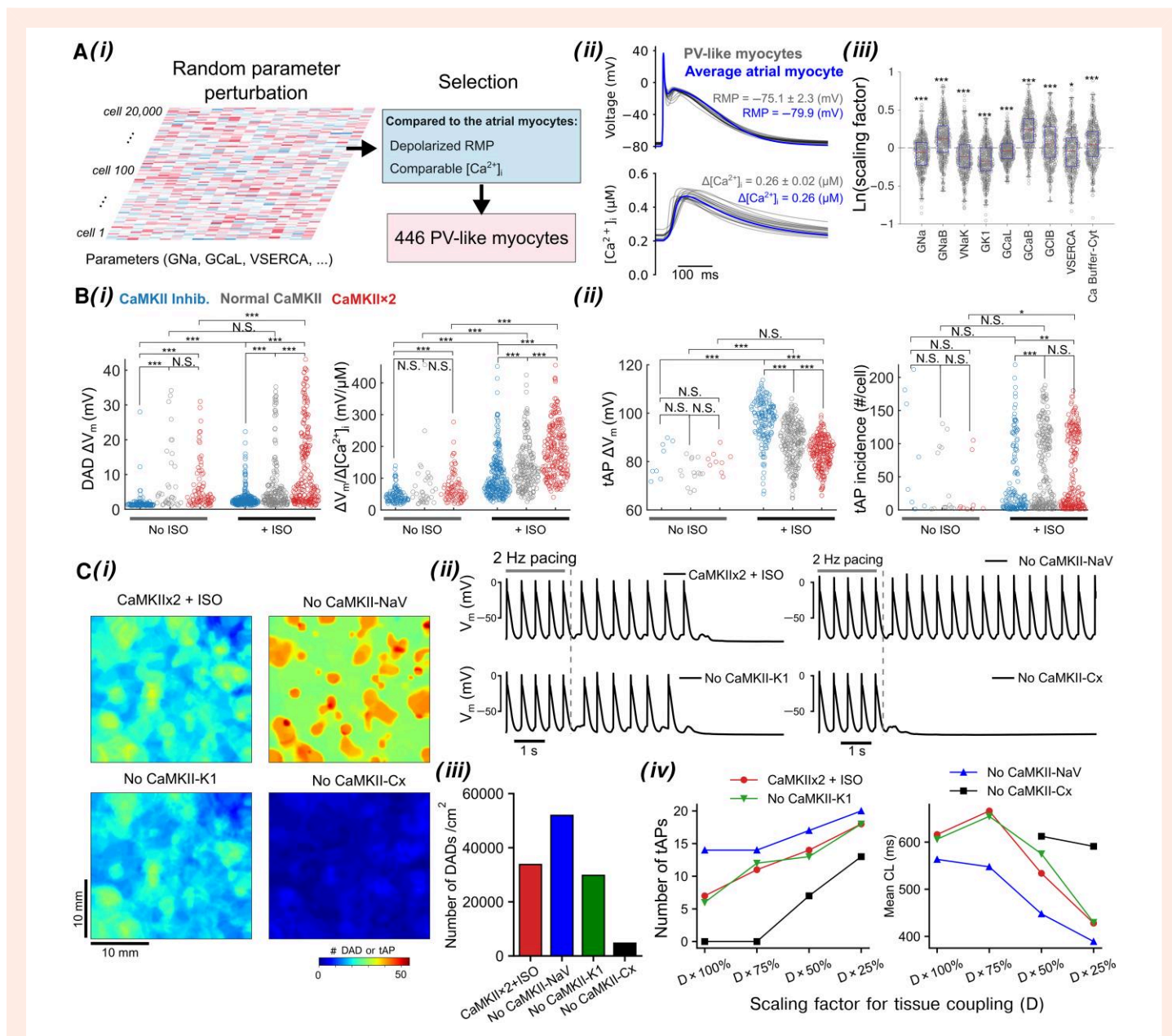


Figure 6 Populations of PV-like myocytes and tissues demonstrate critical roles of PKA and CaMKII signals in promoting V_m and Ca^{2+} instabilities in PVs. (A) (i) Illustration of generating PV-like cardiomyocytes models by introducing random parameter perturbation to the average atrial cardiomyocyte model; model calibration selected PV-like cardiomyocytes as having depolarized RMP compared to the average LA cardiomyocytes while the $[Ca^{2+}]_i$ being comparable. (A) (ii) Superimposed AP and $[Ca^{2+}]_i$ traces of PV-like cardiomyocyte population and the average LA cardiomyocyte model paced at 1 Hz. (A) (iii) Identification of subcellular parameters of the PV-like cardiomyocyte population that are significantly different from the average LA cardiomyocyte. Note that the y-axis plots the scaling factors for those subcellular parameters in logarithm, and the scaling factors of the average atrial cardiomyocyte are thus zero and indicated with a dashed line. Statistical test was performed using one-sample *t*-test with Bonferroni correction. (B) Characterization of (i, left) V_m amplitude and (i, right) $\Delta V_m/\Delta[Ca^{2+}]_i$ coupling strength for sub-threshold DADs, and (ii, left) mean amplitude and (ii, right) number of incidence for tAPs in each PV-like cardiomyocyte following a 2-Hz pacing-pause protocol. Statistical analysis was performed using Kruskal–Wallis test followed by planned comparisons with Wilcoxon rank sum test and Bonferroni correction. (C) Tissue simulations uncover precise contributions of CaMKII-dependent modulations of I_{Na} availability (CaMKII-NaV), I_{K1} activity (CaMKII-K1), and connexins (CaMKII-Cx). (i) Spatial distribution of sub-threshold DADs and tAPs in simulated tissue following 2 Hz pacing-pause protocol. (ii) APs of cardiomyocytes extracted from the tissue. (iii) Density of DAD and tAP incidence in tissue. (iv, left) incidence number and (iv, right) mean CL of tAPs in tissue with respect to gradual reduction of tissue coupling. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; N.S., not significant.

incidence of triggered activity compared to atrial cardiomyocytes. This may be explained by lower I_{K1} amplitude in PV myocytes compared to atrial cardiomyocytes, which could augment $\Delta V_m/\Delta[Ca^{2+}]_i$ gain in each myocyte and decrease the electrical sink, thereby exacerbating the source–sink mismatch in tissue.

5.3 Integrative framework to define (mal) adaptive remodelling associated with AF

It is well established that AF is associated with extensive remodelling of ion channels and Ca^{2+} handling proteins including their regulations by

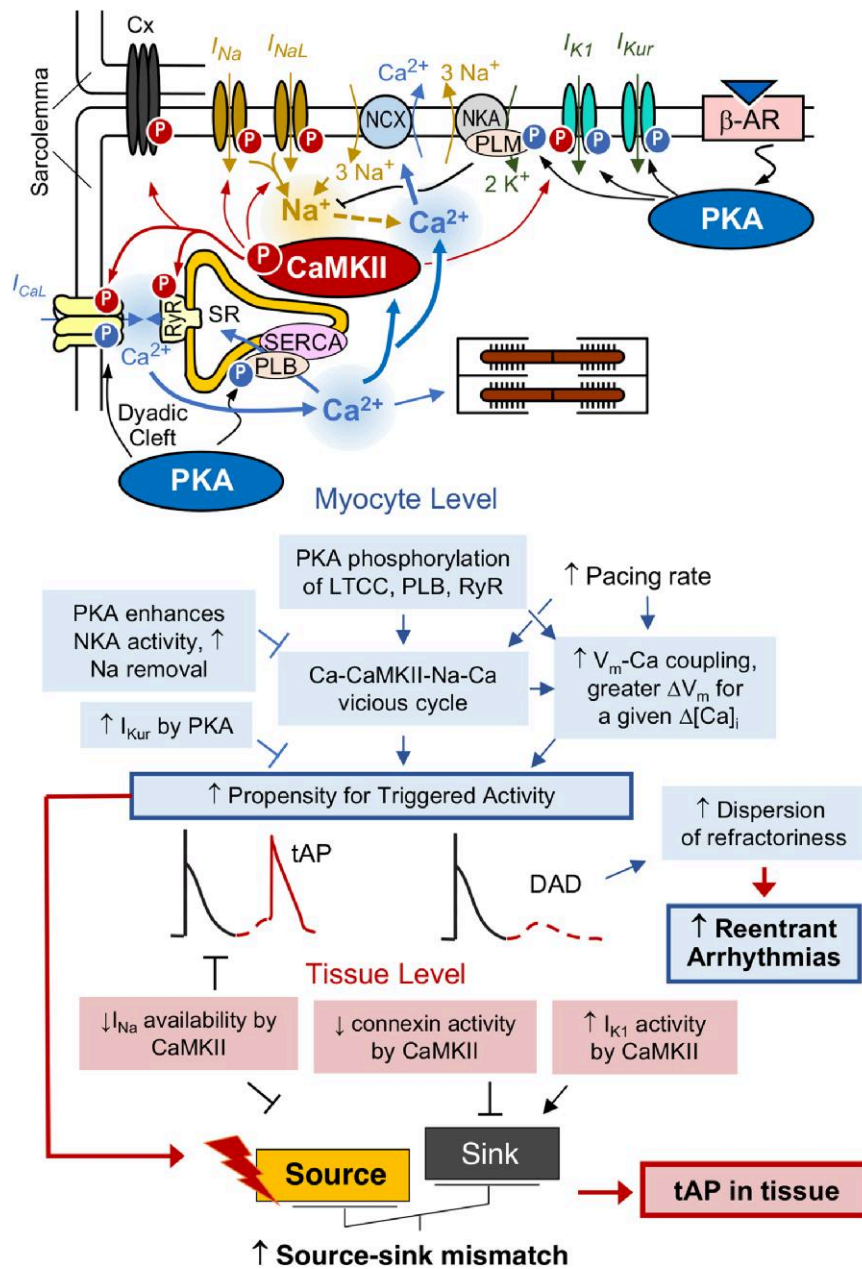


Figure 7 Working model schematic illustrates the mechanisms underlying PKA and CaMKII activations promoting DADs and tAPs in both atrial myocytes and tissues.

upstream signalling.^{3,10,93,104–107} Discerning the adaptive changes from maladaptive remodelling may provide a better understanding of AF pathophysiology and inform favourable anti-AF targets, but such effort requires a systems framework integrating the mechanistic contributions of AF-associated remodelling processes. To this end, our simulations reveal relative influences of each protein and its phosphorylation on the propensity to arrhythmia, thus providing mechanistic insights into determining the adaptive or maladaptive nature of the remodelling processes caused by AF. For example, our results show that the propensity to DADs is most sensitive to an increase in the maximum conductance of I_{CaL} (G_{CaL}) (Figure 1Di). Thus, I_{CaL} might contribute to DADs particularly in patients with paroxysmal AF wherein I_{CaL} is unchanged,¹⁰⁸ whereas previously documented reductions of I_{CaL} in chronic AF^{3,13,67,104,106,107,109} despite increased open probability¹¹⁰ could be an adaptive process to attenuate

arrhythmia propensity, but at the expense of causing re-entry-promoting APD abbreviation.^{10,111} Similarly, PKA- and CaMKII-dependent phosphorylation of LTCCs are positively correlated with pro-arrhythmia by causing cellular Ca²⁺ overload and triggered activity (Figure 1Dii). Conversely, increased activity of protein phosphatase 2A and PDE8-mediated LTCC dephosphorylation¹⁰⁷ that have been observed in atrial tissue from AF patients^{13,28} may constitute adaptive changes to reduce LTCC phosphorylation levels and the related cellular Ca²⁺ overload, but at the expense of causing re-entry-promoting APD abbreviation (Figure 1Diii). The augmented phosphorylation of PLB and RyR2 (e.g. due to abnormal local activity of PP1 within the multi-protein complexes) also promotes atrial arrhythmogenesis (Figure 1Dii), which is consistent with prior work showing that PP1 deficiency or stronger inhibition by endogenous inhibitor-1 within the RyR2^{86,112} and PLB^{28,113} complexes is associated with cardiac arrhythmogenesis in

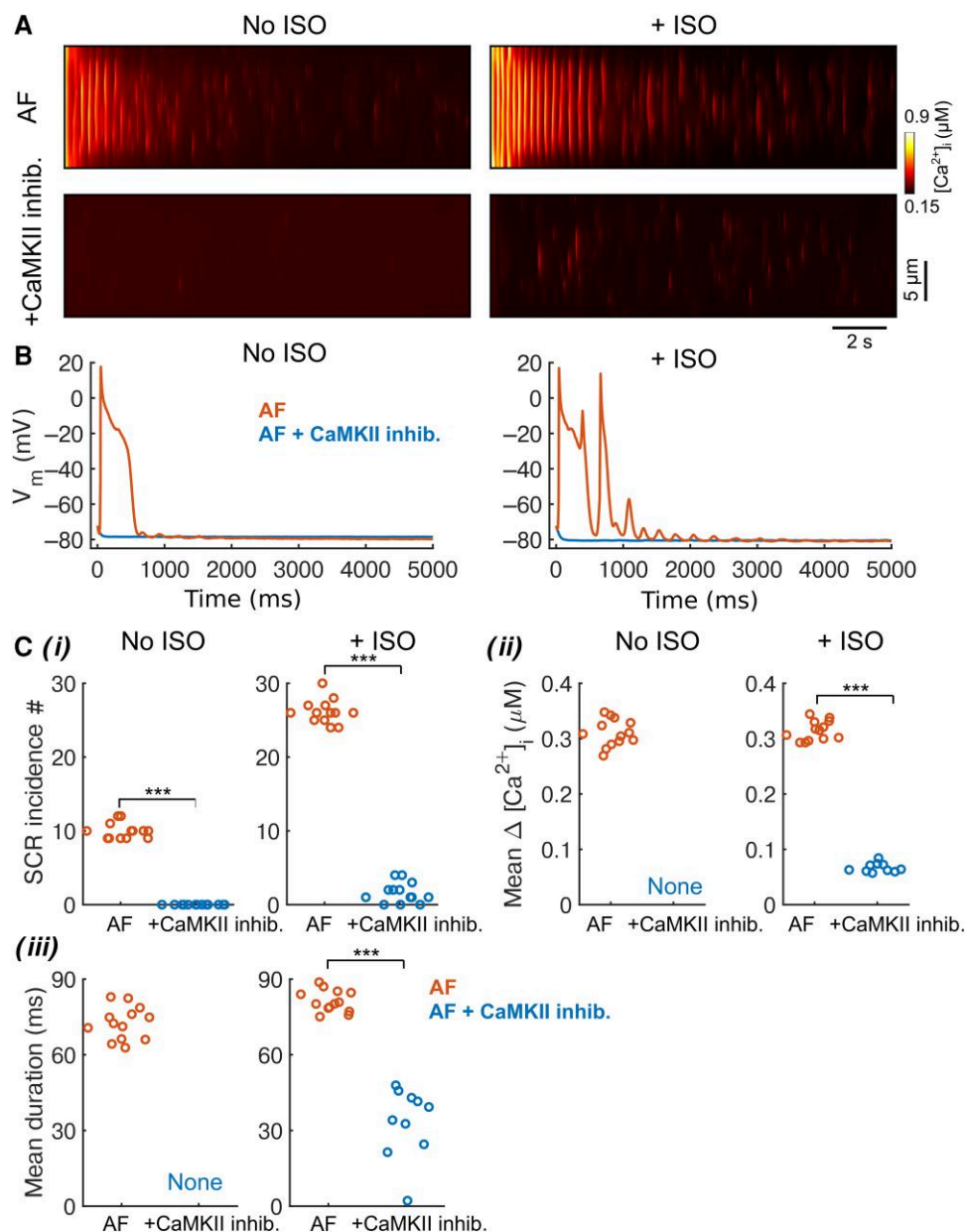


Figure 8 Three-dimensional spatial model of non-stimulated human atrial cardiomyocytes shows that AF-induced remodelling and ISO treatment promote tAPs and Ca²⁺ release events, which are ameliorated by CaMKII inhibition. The spatial cell model was pre-conditioned with same initial conditions (i.e. same ion channel states, local Ca²⁺ concentrations in each compartment, etc.) before switching on AF remodelling effects, and adding ISO or CaMKII inhibition treatment effects. (A) Simulated transversal line-scan of [Ca²⁺]_i in AF-remodelled human atrial cardiomyocytes showing effects of ISO treatment or CaMKII inhibition. (B) Membrane V_m of AF-remodelled cardiomyocytes and following treatments. (C) Characterization of SCR events in response to treatments measured with 13 transversal line scans spaced by 5.5 μm along the longitudinal axis. (i) Number of SCR incidence, (ii) mean [Ca²⁺]_i amplitude of SCR, and (iii) mean duration of SCR. Two-sample Student's t-test was applied to perform statistical test. ****P* < 0.001.

experimental models and patients. Preventing RyR2 phosphorylation in our model suppresses DADs and tAPs (see [Supplementary material online, Figure S16](#)). Of note, augmented CaMKII-induced target phosphorylation causes source–sink mismatch, which can be partially corrected by CaMKII-dependent modifications of I_{Na} availability and I_{K1}. Similarly, the enhanced I_{K1} in AF patients^{114–116} could also be interpreted as a compensatory mechanism against triggered activity, however, at the expense of shortening APD and promoting re-entry. Furthermore, increasing NCX expression diminishes propensity of DADs ([Figure 1Di](#)). Therefore, the

hallmark increase of NCX1 documented in AF^{10,18,22,28,72} may constitute an adaptive process to counteract the arrhythmogenic Ca²⁺ overload. On the other hand, NCX up-regulation increases ΔV_m/Δ[Ca²⁺]_i in chronic AF, and this could promote DADs during increased diastolic SR Ca²⁺ leak. These two apparently opposing effects of increased NCX may be both operative in AF, with the net effect depending on the study conditions and disease stages. Overall, our analysis unravels a complex landscape of AF-induced (mal)adaptive changes that we speculate may have pro-arrhythmic or anti-arrhythmic consequences depending on the

prevailing arrhythmogenic mechanism (triggered activity, re-entry, or both) in an individual patient. As such, effective anti-AF strategies should be selectively directed to correct maladaptively remodelled processes such as those we identified in present study, where effects of CaMKII and PKA could be either pro-arrhythmic or anti-arrhythmic. An attractive therapeutic strategy would therefore be the development of selective protein–protein interaction inhibitors, which could prevent CaMKII and PKA interactions with some of its targets while leaving other targets unaffected.

5.4 Limitations and future directions

We acknowledge that our study has limitations that may be addressed in future investigations. First, β -adrenoceptor stimulation can activate CaMKII through β AR-cAMP-Epac-dependent^{117,118} or nitric oxide-dependent¹¹⁹ pathways that were not included in our modelling framework. The direct crosstalk between β AR signalling and CaMKII might further favour CaMKII activity during β AR stimulation, thereby enhancing the synergistic crosstalk between PKA and CaMKII for promoting atrial arrhythmogenesis. Further, while the model captures the ability of CaMKII monomers to auto-phosphorylate neighbouring subunits thus prolonging the activated state of CaMKII, other post-translational modifications of CaMKII have been reported (oxidation,¹²⁰ O-GlcNAcylation,¹²¹ and S-nitrosylation¹²²) and are not yet included in the model. The model could be further extended to include the role of inhibitor-1 as a PKA-dependent regulator of CaMKII phosphorylation,^{123,124} whereas in the current formulation, PKA activation of inhibitor-1 is linked to CaMKII through the increase in Thr-17 phosphorylation of PLB and contributes to the synergy of CaMKII and PKA signalling. While there are limited kinetic data available detailing the characteristics of CaMKII activation and target phosphorylation, the predicted outcomes are generally consistent with available experimental measurements and functional readouts. Second, we focused on studying the crosstalk effects by investigating propensity to triggered activity. However, the CaMKII-dependent increase in I_{K1} , decrease in peak I_{Na} , and the Cx-mediated functional decoupling of tissue all could promote re-entry, another important arrhythmia mechanism.^{44,93,103} Indeed, the CaMKII-induced functional decoupling decreased CV in our simulations, which should reduce wavelength of excitation thereby promoting re-entry. However, we acknowledge that opposing effects of acute CaM/CaMKII inhibition on ventricular CV have been reported.^{52,53,125–127} Due to the extensive targets of CaMKII involved in determining CV, the exact action of CaMKII on Cx remains to be resolved. Nevertheless, our simulations show that CaMKII could induce sub-threshold DADs in tissue in the absence of its effect on Cx, and these sub-threshold DADs could create a substrate for re-entry by causing dispersion of excitability and conduction block. Of note, Cx can also be phosphorylated by other kinases including PKA and PKC, and the phosphorylation levels of Cx are high at basal conditions and are reduced with diseases including heart failure,^{128,129} whereas the exact effects of PKA on Cx are mixed from previous studies.^{130,131} Nevertheless, our additional simulations in which tissue connectivity was modestly (+10%) increased in the presence of ISO (see [Supplementary material online, Figure S19](#)) suggested that PKA effects on connexins only show minor impacts on the tAP properties in tissue simulations. Thus, our study provides novel mechanistic insights into PKA- and CaMKII-dependent arrhythmogenesis that should be experimentally demonstrated and validated in atrial tissue. Third, while our model incorporates detailed descriptions of protein phosphatase regulations for each substrate, it is important to acknowledge that the function of many of these phosphatase isoforms and targeting properties remains to be elucidated.^{132,133} The same applies to phosphodiesterase isoforms and regulations.¹⁰⁷ Our model assumes that the biophysical and biochemical properties of phosphorylation regulations are shared among many substrates. New experimental insights on the substrate-specific phosphorylation regulations, as they emerge, may be included to update our model to tease out contribution of individual substrate-specific protein phosphorylation in arrhythmogenesis and implications for therapy. Fourth, our model is deterministic and lacks descriptions of spatial subcellular details of Ca^{2+} diffusion. Merging the spatially detailed stochastic models^{27,134,135} with the

PKA and CaMKII signalling model presents several challenges and introduces uncertainties in model parameters, including spatial details of concentrations and activity of these kinases; in addition, to tease out the distinct effects of signalling on arrhythmogenesis, we did not explicitly include all AF-induced remodelling changes in our integrated signalling model. Nevertheless, we performed additional preliminary simulations using our recently developed 3D spatial model of human atrial cardiomyocytes incorporating stochastic subcellular Ca^{2+} signalling and AF-induced remodelling effects.¹³⁴ We found that AF-induced remodelling caused substantially increased Ca^{2+} release events and associated I_{NCX} that outweighed I_{K1} (see [Supplementary material online, Figure S20](#)) leading to tAPs that are amplified by ISO treatment; CaMKII inhibition suppressed these events in quiescent cells ([Figure 8A–C](#)) and attenuated the diastolic SCRs during steady-state pacing (see [Supplementary material online, Figure S21](#)). These results are consistent throughout our study using distinct types of models. Fifth, we simulated a heterogeneous tissue with small clusters of randomly varied electrophysiological properties. While the presence of electrophysiological heterogeneities in the heart is well known, the spatial arrangement of these heterogeneities is poorly elucidated. Also, the spatial distribution of cardiac innervation and expression of adrenoceptors as well as CaMKII in the atria and their AF-related changes have not been fully understood. Although CaMKII δ is considered the main cardiac isoform, CaMKII γ contributes importantly to cardiac remodelling.¹³⁶ Thus, future computational models should implement the contribution of different CaMKII isoforms. Our model framework can be readily modified to account for these critical spatial details, when available, to better understand the contribution of these heterogeneities in physiology and pathophysiology. Finally, as sex differences in cAMP/PKA pathway have been reported to contribute to sex differences in the Ca^{2+} -handling properties of cardiomyocytes,^{137,138} our novel integrative modelling framework provides a unique platform for studying sex differences in the regulation of upstream signalling on the cardiac EC coupling, which is likely to contribute to mechanistic discovery of sex-dependent differences in arrhythmogenesis.

We envision extending our integrative framework to incorporate emerging experimental insights of upstream regulation of cardiac EC coupling and to investigate a broad spectrum of arrhythmia mechanisms. As computational models are increasingly being applied in drug screening and therapeutic discovery,^{33,56,139,140} integrative models not only allow for studies under conditions where key signalling pathways are active or disrupted to faithfully represent arrhythmic hearts but also provide a framework to investigate non-ion channel anti-AF strategies (e.g. targeting upstream or downstream signalling). Thus, our integrative models are powerful and instrumental to assemble and reconcile existing knowledge into a coupled network, allowing for quantitatively dissecting the precise contributions of subcellular processes and their modifications by upstream regulatory pathways to initiation and maintenance of arrhythmia in the atria.

Supplementary material

[Supplementary material](#) is available at *Cardiovascular Research* online.

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Data availability

The data underlying this article are available in the article and in its online [supplementary material](#). Source codes, analysis scripts, and related parameter data are available for download at elegrandi.wixsite.com/grandilab/downloads and github.com/drgrandilab.

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Translational perspective

Despite significant advancement in our understanding of pathological mechanisms and alterations underlying atrial fibrillation (AF), a highly prevalent clinical arrhythmia causing substantial health and socio-economic burden, development of effective pharmacological therapeutics for AF remains an urgent unmet clinical need. We built a systems framework integrating key processes and their regulatory upstream signalling pathways that are involved in atrial electrophysiology and modified by AF. By simulating populations of single atrial cardiomyocyte models and heterogeneous tissues, our analysis demonstrated synergistic interactions between upstream signalling pathways that promote atrial arrhythmogenesis across spatial scales, added new insight into complex atrial arrhythmia mechanisms, and revealed adaptive and maladaptive alterations caused by AF, thus providing a powerful new tool for identifying innovative therapeutic approaches against AF.