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Real-time relationship between PKA biochemical signal network dynamics and increased action potential firing rate in heart pacemaker cells

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Abstract

cAMP-PKA protein kinase is a key nodal signaling pathway that regulates a wide range of heart pacemaker cell functions. These functions are predicted to be involved in regulation of spontaneous action potential (AP) generation of these cells. Here we investigate if the kinetics and stoichiometry of increase in PKA activity match the increase in AP firing rate in response to β -adrenergic receptor (β -AR) stimulation or phosphodiesterase (PDE) inhibition, that alter the AP firing rate of heart sinoatrial pacemaker cells. In cultured adult rabbit pacemaker cells infected with an adenovirus expressing the FRET sensor AKAR3, the EC₅₀ in response to graded increases in the intensity of β -AR stimulation (by Isoproterenol) the magnitude of the increases in PKA activity and the spontaneous AP firing rate were similar (0.4 ± 0.1 nM vs. 0.6 ± 0.15 nM, respectively). Moreover, the kinetics ($t_{1/2}$) of the increases in PKA activity and spontaneous AP firing rate in response to β -AR stimulation or PDE inhibition were tightly linked. We characterized

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Disclosures

None.

the system rate-limiting biochemical reactions by integrating these experimentally derived data into mechanistic-computational model. Model simulations predicted that phospholamban phosphorylation is a potent target of the increase in PKA activity that links to increase in spontaneous AP firing rate. In summary, the kinetics and stoichiometry of increases in PKA activity in response to a physiological (β -AR stimulation) or pharmacological (PDE inhibitor) stimuli match those of changes in the AP firing rate. Thus Ca^{2+} -cAMP/PKA-dependent phosphorylation limits the rate and magnitude of increase in spontaneous AP firing rate.

Keywords

Computational modeling; Coupled-clock system; Pacemaker; Phosphorylation

1. Introduction

Protein kinase A (PKA) is a key nodal signaling molecule that regulates a wide range of cellular functions [1]. However, its magnitude, dynamic and spatiotemporal regulation are not known in heart pacemaker cells. Recent live-imaging provides direct measurements of protein kinase dynamics in intact cells from different organs [2, 3], and specifically in the heart [4–6].

In heart pacemaker cells, integrated functions of molecular mechanisms on cell membrane surface and in the sarcoplasmic reticulum (SR) create a system of coupled biological clocks that determine the heart rate [7]. PKA plays an important role in regulating molecular function of variety of cellular locations: e.g. cell membrane (L-type [8], K-channels [9]) and Ca^{2+} storage in the SR (phospholamban (PLB) [10] and ryanodine receptors (RyR) [11]) (Fig. 1). Ca^{2+} -activated adenylyl cyclase (AC) expressed in pacemaker cells generates high cAMP activity [12, 13]. The high basal rate of cAMP production controls PKA-dependent phosphorylation, and is coupled to high basal phosphodiesterase (PDE) activity, which degrades cAMP and limits the extent to which basal cAMP levels increase, and the extent of pacemaker clock protein phosphorylation [14–16]. In response to β -adrenergic receptor (β -AR) stimulation or PDE inhibition, the coupled-clock intrinsic mechanisms, i.e., cAMP/PKA-dependent phosphorylation levels, become further activated [15], leading to increased action potential (AP) firing rate [17] [18]. Although PKA-dependent mechanisms are linked to AP firing rate, real-time measurements of PKA signaling in the context of heart pacemaker function are lacking. Specifically, it has never been demonstrated that the kinetics and stoichiometry of increases in PKA activity and protein phosphorylation in individual pacemaker cell are similar to and sufficient to account for changes in the kinetics and stoichiometry of increases in AP firing rate that occur in response to physiological (β -AR stimulation) or PDE inhibition. To address this question, we measured changes in kinetics and magnitude of PKA activity and of the spontaneous AP firing rate in response to β -AR stimulation or PDE inhibition. We characterized kinetics of rate-limiting biochemical reactions by integrating these and other experimental data into mechanistic computational model simulations. We found that the kinetics and stoichiometry of increases in PKA activity in response to a physiological (β -AR stimulation) or pharmacological (PDE inhibitor) match those of changes in the AP firing rate. Thus Ca^{2+} -cAMP/PKA-dependent

phosphorylation limits the rate and magnitude of changes in spontaneous AP firing rate in response to these stimuli.

2. Materials and Methods

Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The animal protocols have been approved by the Animal Care and Use Committee of the National Institutes of Health (protocol #034LCS2013). To test the hypothesis that the AP firing rate is regulated by PKA activity, we imaged PKA activity and measured AP firing rate of sinoatrial node pacemaker cells (SANC) in response to β -AR stimulation (isoproterenol, ISO), PDE inhibitor (IBMX), and PKA inhibitor (H-89). We characterized their kinetics by calculating the drug concentration that lead to 50% max response (EC_{50}) and the half time to reach steady state response ($t_{1/2}$). To measure PKA activity, we cultured adult rabbit pacemaker cells for 24 hours, infected them with a 3rd-generation of a genetically encoded A-Kinase activity receptor (AKAR3), and monitored PKA activity and its response to β -AR stimulation or PDE inhibition 24 hours later (see expended view for additional data on the probe properties). Prior experiments in cardiomyocytes [4] and in primary neurons [19] have demonstrated the ability and specificity of this probe to monitor PKA activity. We measured AP firing rate under the same experimental conditions in another subset of cultured cells.

To simulate the expected rate of cAMP production and the kinetics and magnitude of protein phosphorylation, we formulated a novel numerical model that integrated our previously experimentally measured levels of steady-state AC activity, cAMP and phospholamban phosphorylation in response to perturbations that influenced cAMP/PKA signaling and the changes in the kinetics and magnitude of PKA activity determined experimentally in the present study.

Data are presented as mean $\pm$ SEM. When the means of paired samples are to be compared, a paired t-test was employed. In cases where the samples are independent, a two-sample t-test was applied. $P < 0.05$ was taken to indicate statistical significance.

A detailed, expanded description of experimental and numerical methods is available in the on-line supplement.

3. Results

3.1 Imaging PKA FRET probe fluorescence and establishing its dynamic range for reporting changes in PKA activity in response to β -AR stimulation or PDE inhibition

The activation deactivation kinetics and the working range of the FRET probe

—To estimate the real-time kinetics and magnitude of change in PKA activity in live single SANC, we measured real-time PKA activity in 48-hour cultured pacemaker cells using a FRET-based A-kinase activity receptor 3 (AKAR3) probe [20] (Fig. 2A). To verify that introduction of the FRET probe did not affect pacemaker function, we determined whether the FRET probe infection alters the spontaneous AP firing rate of cultured SANC. The AP firing rate in the presence and absence of FRET probe did not change (104 ± 10 (n=15) and

101±9 (n=12) beats/min), respectively). Note that we used here only pacemaker cells that were spontaneously beating. For comparison of spontaneously beating to quiescent cells, see online supplement.

To characterize the PKA activation kinetics and its maximum response that could be elicited in these cells, we treated the cells with the PDE inhibitor IBMX (100 μM). PDE inhibition induced a marked and rapid significant increase in PKA activity ($R/R_0=1.4\pm0.02$, $t_{1/2}=116\pm27$ s n=7) (Fig. 2B, C). Application of an AC activator (forskolin, 50 μM) or β-AR activation (isoproterenol, 100 nM) in the presence of IBMX did not further increase PKA activity (Fig. S1). Hence, the average PKA activity elicited by 100 μM IBMX was taken as the maximum PKA activity in these cultured pacemaker cells. To determine the deactivation kinetics and the minimum response of PKA, we applied the PKA inhibitor, H-89 (10 μM). H-89 significantly reduced the IBMX-induced increase in PKA activity ($R/R_0=1.02\pm0.03$, $t_{1/2}=254\pm25$ s n=5). Direct application of 10 μM H-89, in the absence of PDE inhibition, significantly reduced the basal PKA activity to a level below baseline ($R/R_0=0.95\pm0.02$, n=4). Because higher concentrations of H-89 did not further decrease the PKA activity, we defined the average PKA activity in response to H-89 alone as the minimum PKA activity. Thus, the working range of the FRET probe in adult cultured rabbit SANC extends from an R/R_0 of 0.95 to 1.4.

Response to β-AR stimulation—The aforementioned characterization of the maximum and minimum bounds of PKA activity was achieved through pharmacological agents that inhibit PDE or PKA. Under physiological conditions, however, an increase in AP firing rate is achieved through β-AR stimulation. In order to quantify the kinetics and magnitudes of the increase in PKA activity in SANC in response to a physiologic stimulus, we applied graded increases in the concentration of ISO, a β-AR agonist to the cells. Graded increases in ISO concentrations were accompanied by graded increases in PKA activity (Fig. 2C). Specifically, in response to a low concentration of ISO (< 10 nM), there was a rapid increase in PKA activity, which subsequently decayed to a steady-state level. As the concentration of ISO increased, the initial overshoot in PKA activity was reduced, and in response to 100 nM ISO, the maximal initial increase in PKA activity was close to the steady-state level (Fig. 2C). These results of PKA dynamics have been observed in other systems [5]. The EC_{50} of the steady-state PKA activity increase in response to ISO was 0.4 ± 0.1 nM. Interestingly, adding IBMX in the presence of the maximal response to ISO alone only slightly increased PKA activity ($R/R_0=1.32\pm0.2$ in response to ISO only vs. 1.4 ± 0.02 in response to both ISO and IBMX, $p<0.05$). This suggests that in cultured SANC physiological stimulation increases the PKA activity almost to its maximal level.

3.2 The changes in kinetics and magnitude of PKA activity in response to graded increases in β-AR stimulation or PDE inhibition are tightly linked to concurrent changes in kinetics and magnitude of the spontaneous AP firing rate

We employed graded increases in the concentrations of ISO to quantify the relationship between changes in PKA activity and spontaneous AP firing rate. We found that these changes led to a progressive increase in the spontaneous AP firing rate (Fig. 2D, from 104 ± 14 to 173 ± 5 beats/min). Furthermore, over the entire range of [ISO] the graded

increases in PKA activity and AP firing rate are strikingly similar (Fig. 3A), yielding similar values of the EC₅₀ of the increase in PKA activity ($0.4 \pm 0.1 \text{ nM}$) and increase in spontaneous AP firing rate ($0.6 \pm 0.15 \text{ nM}$) in response to β -AR stimulation.

To compare the kinetics of the increases in PKA activity and spontaneous AP firing rate, only single concentrations of ISO were administered to each cell. Note that, the experiments to quantify PKA activity and spontaneous AP firing rate were not performed on the same cell (see limitation section below). In these cells, the increases in PKA activity and AP firing rate magnitudes were similar to those in response to ISO concentration achieved by cumulative increase of ISO (Fig. 3C). The kinetics of the increase in PKA activity and the increase in spontaneous AP firing rate over the entire range of single ISO concentrations (Fig. 3C) do not statistically differ (by ANCOVA). Moreover, statistically the $t_{1/2}$ of PKA activity and spontaneous AP firing over the entire range of single ISO concentrations are similar. Because PKA and AP firing dynamic do not statistically differ, one can claim that both are tightly linked.

To prove that neither time nor the presence of AKAR3 affects the changes in PKA activity and spontaneous AP firing rate in response to ISO we performed two types of experiments: (i) in response to drug vehicle application, neither PKA activity (Fig. S2A) nor spontaneous AP firing rate (Fig. S2B) significantly changed; (ii) furthermore, the presence of AKAR3 did not affect the response of AP firing rate to ISO (Fig. S3).

PKA activity and AP firing rate in response to PDE inhibition—To characterize the AP firing rate at the maximum PKA activity, we compared the responses of PKA activity and AP firing rate to the maximal PDE inhibition by IBMX ($100 \mu\text{M}$). The steady-state spontaneous AP firing rate increased from 103 ± 10 to 188 ± 10 beats/min. The $t_{1/2}$ of the increase in PKA activity in response to PDE inhibition ($116 \pm 27 \text{ s}$, $n=7$) was strikingly similar to the $t_{1/2}$ of the increase in spontaneous AP firing rate ($123 \pm 14 \text{ s}$, $n=5$). Interestingly, the kinetics of the increase in PKA activity and AP firing rate in response to IBMX are significantly slower than those in response to ISO ($p < 0.05$).

To determine the kinetics of PKA inactivation and reduction in AP firing rate in response to a reduction in PKA activity, we applied H-89 in the presence of IBMX. PKA activity was reduced from 1.4 ± 0.02 to 1.02 ± 0.02 . The spontaneous AP firing rate was reduced from 100 ± 5 to 26 ± 5 beats/min. The $t_{1/2}$ of the reduction in PKA activity in response to H-89 in the presence of IBMX was $254 \pm 25 \text{ s}$ ($n=5$), and that of the spontaneous AP firing rate was $175 \pm 22 \text{ s}$ ($n=5$). Note that the kinetics of the reduction of PKA activity and AP firing rate toward the baseline (by H-89) are slower than those of the increase in PKA activity in response to either maximal β -AR stimulation or to PDE inhibition (Fig. 2B). In summary, in response to PDE inhibition or β -adrenergic stimulation, the beating rate magnitude and kinetics are tightly linked to these changes in PKA activity.

3.3 Spatiotemporal synchronization of PKA activity in response to perturbations of cAMP

Spatio-temporal gradients of molecular dynamics enable a cell to expand its repertoire of functional outcomes [21]. In ventricular myocytes, compartmentalized cAMP/PKA signaling has been documented and studied through the use of AKAR variants and other

genetically encoded biosensors [6]. To assess changes in spatio-temporal gradients of PKA activity in response to graded β -AR stimulation, we measured the magnitudes of changes in PKA activity amplitude and its $t_{1/2}$ within different random regions of $1\mu\text{m}$ radius across the cell. In response to graded β -AR stimulation, changes in the spatio-temporal variability in PKA activity were quantified by calculating the coefficients of variance (CV) of changes in PKA activity amplitude and its $t_{1/2}$ measured at different regions within the cells. In order to gain a robust estimate of the spatial dynamics, only cells large enough to accommodate at least ten regions were analyzed.

In response to graded increases in ISO concentration (from 0.1 to 10nM), as the average PKA activity amplitude increased, local spatiotemporal (CV of PKA activity amplitude and its $t_{1/2}$) gradients in PKA activity become reduced (Fig 4 A–D). Specifically, graded increases in average PKA activity are accompanied by reductions in the coefficients of variance of PKA activity amplitude and $t_{1/2}$ gradients. Coefficients of variance of both changes in the amplitude of PKA activity and its $t_{1/2}$ gradients are linked (Fig. 4E).

Conversely application of H-89 in the presence of IBMX to lower the average PKA activity is associated with increase in spatiotemporal desynchronization of changes in PKA activity and $t_{1/2}$ (Fig. 5A–B). For comparison of spontaneously beating to quiescent cells, see online supplement (Fig. S4).

3.4 Elevating PKA activity in cultured sinoatrial node pacemaker cells to mimic the PKA activity in fresh cells

Prior observations indicate that after 48 hours in culture, the spontaneous AP firing rate of cultured pacemaker cells becomes reduced [22]; Our observations were similar. The beating rate was reduced from 152 ± 7 beats/min in fresh SANC to 101 ± 9 beats/min in cultured SANC ($p<0.05$). This reduction in AP firing rate is commonly accompanied by a reduction in phosphorylation of both phospholamban and RyR [22]. Furthermore, in response to a maximal β -AR stimulation, the spontaneous AP firing rate, and phospholamban and RyR phosphorylation levels in cultured SANC become similar to those in fresh isolated SANC [22]. We found that exposure of cultured SANC to 1nM of ISO increased the spontaneous AP firing rate in these cells to the value similar to the basal level AP firing rate of freshly isolated SANC (Fig. 3B). Therefore, we estimated that adding 1 nM of ISO to cultured cells mimics the basal phosphorylation level of freshly isolated SANC (Fig. 3B dashed line). We next assessed PKA activity and AP firing rate of cultured SANC responses to physiological stimulation (β -AR stimulation) or pharmacological intervention (PDE inhibitor) in the presence of 1.0 nM ISO to mimic basal PKA activity in freshly isolated SANC. β -AR stimulation (100 nM ISO) in the presence of 1 nM of ISO increased the PKA activity by 35% (Fig. 6A) with $t_{1/2}= 38\pm 8\text{s}$ ($n=4$). PDE inhibition (100 μM IBMX) in the presence of 1 nM of ISO (Fig. 6B) increased the PKA activity by 43% with $t_{1/2}= 43\pm 6\text{s}$ ($n=6$). We also directly measured the kinetics of the change in AP firing rate in response to drug stimuli in freshly isolated SANC. In response to β -AR stimulation or PDE inhibition the kinetics of the AP firing rate do not significantly differ from those kinetics of PKA activity: $t_{1/2}$ of the change in AP firing rate in response to ISO was $47\pm 15\text{s}$ ($n=7$) and $t_{1/2}$ of the change in AP firing rate in response to IBMX was $58\pm 14\text{s}$ ($n=5$).

3.5 Numerical model simulation of cAMP and PKA-mediated phosphorylation targets

Pacemaker function is regulated by a complex array of signaling molecules, including Ca^{2+} , cAMP and PKA, which interact with each other in a complicated non-linear fashion. Although our experiments quantified the magnitude and rate of change in PKA activity in response to physiological and drug interventions, and compared these to changes corresponding to AP firing rate, current experimental methods do not permit simultaneous quantification of changes in phosphorylation of target proteins and changes in AP firing rates. In the absence of such crucial dynamic experimental information, it is difficult to interpret the interplay between Ca^{2+} , cAMP, PKA activation and phosphorylation level in determining SANC AP firing rate. Therefore, we developed a computational model that integrated previously measured parameters of steady-state AC activity, cAMP and phospholamban phosphorylation levels in response to perturbations that influence cAMP/PKA signaling, and changes in the kinetics and magnitude of PKA activity measured in the present experiments (we used the experimental results of PKA activity under the conditions that mimics the PKA activity in fresh cells, see above). When intracellular Ca^{2+} changes, the model predicts changes in the spontaneous AP firing rate that result from the Ca^{2+} -dependent downstream changes in cAMP or the PKA-dependent phosphorylation of ion channels (f-channel, L-type channel and slow-activated potassium channels) and SR Ca^{2+} cycling kinetics (PLB and RyR phosphorylation) that are affected by changes in the ion channels. The model assumptions and equations are described in the expended view.

Numerical model simulation of the relative roles of M clock and Ca^{2+} clock protein phosphorylation in the maintenance of SANC automaticity—It is unclear which PKA target components of the clocks are crucial for maintaining an AP firing rate. We made use of our model to address this question. We estimated the relative contributions of changes in phosphorylation of surface membrane protein (L-type and funny current) and Ca^{2+} mechanisms (PLB and RyR) in maintenance of SANC automaticity at a constant cAMP, by calculating the changes in AP firing rate when only a single of the above mechanisms sensed the changes in cAMP (i.e., cAMP was “clamped” to basal level for the remaining other mechanisms) and comparing the change in AP firing rate to that when all mechanisms were functional (Fig. 7). As illustrated in Fig. 7, in the mid-range of cAMP (35–60 pmol/mg protein) L-type current, PLB phosphorylation together with HCN4 have substantial impact on the changes in spontaneous AP firing rate. When cAMP begins to drop below the basal level (16–18 pmol/ mg protein), however, PLB and HCN4 contribute equally to the changes in spontaneous AP firing rate. As cAMP level further drops, PLB phosphorylation dominates the changes in spontaneous AP firing rate. As cAMP progressively increases above the basal level, PLB phosphorylation progressively dominates and the contributions of both L-type current and HCN4 become progressively reduced. Thus, at cAMP levels higher or lower than basal conditions (i.e., 20 pmol/ mg proteins) the relative role of phosphorylated intracellular proteins (i.e., PLB) in maintaining SANC periodicity remains higher than the other targets. Therefore the model simulations predict that PLB is the most potent cAMP/PKA target over the entire range of [cAMP] (Fig. 7).

Numerical model simulations of cAMP and PKA-mediated phosphorylation targets in response to β -AR stimulation or PDE inhibition—Cardiac rhythm is a

dynamic process. Although steady state simulations of SANC automaticity as detailed above are instructive of the general mechanistic features, kinetic information is more crucial to an understanding of how changes in pacemaker function from the basal state are regulated. Specifically, the temporal changes in cAMP and PLB phosphorylation in response to perturbations that increase cAMP have not been quantified previously. We modeled the kinetics of changes in cAMP, PKA, PLB phosphorylation (the dominant phosphorylation target; see above) and AP firing rate in response to acute β -AR. Simulations of concurrent steady-state cAMP and PLB phosphorylation levels in response to β -AR stimulation or PDE inhibition (Fig. 8) employ values similar to the levels experimentally quantified previously [15, 23], and the PKA activity measured in the present study. The $t_{1/2}$ of the increase in PKA activity in response to maximal β -AR stimulation in the model simulation is 20 s (Fig. 8A), i.e. similar to the experimental results of the present study under conditions that mimic fresh cells (excluding direct and instant effect of the drug in the model). That PKA activity kinetics are similar to the experimental results presented above also validates the ability of the model to virtually approximate the kinetics of intracellular PKA signaling. The kinetics of the increase in the spontaneous AP firing rate in response to β -AR stimulation were similar to that of PLB phosphorylation, and lag behind the increases in cAMP and PKA activity (Fig. 8D). Therefore, the model simulation predicts that the kinetics of PKA activation are closely linked to the kinetics of the change in spontaneous AP firing rate in response to β -AR stimulation.

Simulated dynamic changes in cAMP, PKA, PLB phosphorylation and AP firing rate in response to PDE inhibition are illustrated in Fig. 8 E–H. PKA activity, cAMP and PLB phosphorylation increased in response to PDE inhibition. cAMP and PLB phosphorylation levels were similar to the steady levels quantified experimentally previously [14, 15], and the PKA activity kinetics were similar to the experimental results in the present study. In particular, $t_{1/2}$ of the increase in PKA activity in response to PDE inhibition in the model simulations is 30 s (Fig. 8E), i.e., similar to the presented experimental results under conditions that mimic fresh cells (excluding direct and instant effect of the drug in the model). Note that the simulated kinetics of changes in cAMP and PLB in response to PDE inhibition were slower than in response to β -AR stimulation. In contrast to β -AR stimulation, in response to PDE inhibition the kinetics of the change in spontaneous AP firing rate were more closely related to the kinetics of changes in PKA activity than the rate at which PLB phosphorylation increases (Fig. 8). Therefore, the model simulation predicts that in response to PDE inhibition phosphorylation states of cAMP/PKA-dependent targets other than PLB (i.e., If, L-type and ryanodine receptor phosphorylation) play more dominant role than in response to β -AR stimulation. The experiential PKA dynamics measured here were critical to understand by using numerical model how molecular clock mechanisms operate the AP firing rate.

4. Discussion

While it has been hypothesized that pacemaker function is tightly regulated by the overall phosphorylation levels of various signaling molecules that constitute the M and Ca^{2+} clocks, and that PKA is one of the important kinases that effect this phosphorylation process, experimental measurements of dynamics of PKA activity required to quantify cAMP-PKA

signaling in SANC to substantiate this hypothesis have been lacking. Here we present, for the first time, direct real-time measurements of PKA activity dynamics in single heart pacemaker cells using a technique similar to that used previously in ventricular myocytes [6, 24]. The most novel findings of our study are (1) that substantial PKA activity is present in SANC under basal conditions and (2) that, in response to perturbations that increase cAMP, the magnitude and kinetics of the increases in PKA activity and spontaneous AP firing rate are correlated. The significance of these findings is that changes in the kinetics and stoichiometry of PKA activity in response to drug interventions tightly regulates changes in SANC AP firing rate, validating the hypothesis that PKA-dependent phosphorylation regulates the coupled-clock system that drives the SANC AP firing rate [7]. We used cultured pacemaker cell model to explore the PKA activity dynamics. The use of this model has two limitations: 1) the number of cultured cell (and therefore proteins) is small and therefore it is particularly challenging to genetically to knock down or overexpress molecules of interest to test mechanistic hypotheses related to chronotropic stimuli. 2) After 48 hours in culture, the spontaneous AP firing rate of cultured and their electrophysiological characteristics pacemaker cells becomes reduced¹⁷, accompanied by a reduction in phosphorylation of both phospholamban and RyR [22]. In response to a maximal ISO concentration, however, the spontaneous AP firing rate, AP characteristics and phospholamban and RyR phosphorylation levels in cultured SANC are restored to those levels in fresh isolated SANC [22] making them a valid model of sinoatrial node pacemaker activity in the rabbit heart.

The use of the genetically encoded PKA activity sensor enabled us to detect changes in PKA activity dynamics in response to β -AR stimulation or PDE inhibition which would have been difficult to observe using traditional techniques. For example, we found that the kinetics of the increase in PKA activity and AP firing rate in response to PDE inhibition are significantly slower than those in response to the maximal β -AR stimulation. Moreover, the kinetics of the reduction of PKA activity and AP firing rate to baseline levels when PKA is inhibited are slower than those of the increase in PKA activity in response to either maximal β -AR stimulation or to PDE inhibition. Such temporal differences suggest that different mechanisms by which PKA is regulated may have a significant bearing on how PKA activity regulates cardiac rate in response to different biochemical signaling. Moreover, by normalizing the PKA activity to minimal (H-89) and maximal (IBMX) PKA activity, it becomes clear that the pacemaker cells bear an intrinsic ability to produce high rates of formation and degradation of cAMP. Similar results have been documented previously [14]. Although the baseline beating rate is similar in isolated, intact rabbit sinoatrial node tissue and isolated single SANC [25], the change in spontaneous AP firing rate in response to β -AR stimulation is lower in freshly isolated SANC than in intact sinoatrial node tissue [26]. Therefore, it is possible that during the isolation procedure of single SANC some mechanisms that regulate the response to ISO become altered. Moreover, in cultured rabbit pacemaker cells half maximal concentration to stimulate PKA activation and AP firing are by ISO are in the range of nM (Fig. 3) where in freshly isolated pacemaker cells half maximal stimulation concentration is higher in the range of μ M [23]. A similar trend was documented in cultured vs. fresh rat ventricular myocytes [27]. Therefore, it is possible that during culture some mechanisms that regulate receptor affinity, or mechanisms down stream

of receptor stimulation are altered. Additionally, in response to prolonged exposure to ISO, receptor desensitization can occur [28]. Therefore, during consecutive increases in ISO concentration, the higher concentration may have a desensitizing effect.

While spatial heterogeneity within the baseline PKA activity cannot be interpreted from heterogeneity in baseline PKA activity because the distribution of the FRET probe may not be equal in all cell loci. But, changes of fluorescence intensity (i.e., amplitude and $t_{1/2}$) at a given locus can be interpreted as related to the corresponding local changes in PKA activity, in a relative, dynamic sense, supporting the experimental analysis of local PKA kinetics presented here. Another novel finding of our study is that the local spatiotemporal gradients of changes in PKA activity occur in response to stimuli that activate PKA and that the integral of these gradients generates the average cell change in the magnitude and kinetics of the PKA activity, the averages of which in PKA activity are linked to the kinetics and magnitudes of the change in AP firing rate. As the intensity of β -AR stimulation increases, the variation in spatial gradients in PKA amplitude and the variation in temporal gradients of kinetics of PKA activity changes become reduced (Fig. 4). Conversely, when PKA is inhibited, spatiotemporal gradients of PKA activity within the cell increase (Fig. 5). Prior studies in SANC demonstrated that cAMP and PKA-dependent phosphorylation of SR Ca^{2+} cycling proteins in response to β -AR stimulation or PDE inhibition effect spatiotemporal synchronization of spontaneous local diastolic Ca^{2+} releases generated within the coupled clock system [29]. The synchronization of these local Ca^{2+} releases is linked to changes in the AP firing rate. Therefore it is tempting to speculate that local oscillatory in Ca^{2+} releases in SANC are associated with local oscillations in cAMP and PKA activity that have been documented in other cell types [2]. That the graded reductions in PKA spatial amplitude gradients and in the acceleration of $t_{1/2}$ gradients occur in parallel may suggest that PKA activity may oscillate **locally**, and that such local spatiotemporal heterogeneity oscillations are synchronized by β -AR stimulation. In the coupled-clock system, synchronization of local oscillations in PKA activity would lead to a larger average PKA signal that evolves rapidly and synchronizes local diastolic Ca^{2+} oscillations. Synchronization of local Ca^{2+} signals generates a larger ensemble Ca^{2+} signal, leading to increased diastolic depolarization current of the M clock, resulting in acceleration of the AP firing rate. Although we demonstrate here key spatiotemporal PKA activity using epifluorescence microscopy, future experiments using specific modalities that provide higher spatial resolution are required to provide more detailed insights. PKA signaling heterogeneity may originate from diffusion mechanisms, differential kinetics in PKA activation and/or inactivation at the level of protein complexes, e.g., compartmentalization of AKAPS which binds PKA. Indeed, such compartmentalized signaling may also be the source of differences in the kinetics of PKA activation between β -AR stimulation and PDE inhibition responses that have been observed in other cell types [5]. Our experiments, however, cannot link spatiotemporal heterogeneity of changes in PKA activity dynamics to specific subcellular compartments (e.g. cell membrane vs. nucleus vs. intracellular) that may occur in the context of compartmentalization of PKA activity, as in ventricular myocytes.³²

Employing a PKA biosensor in the present study also enabled us to extract precise quantitative information regarding the activation and deactivation kinetics of PKA. This

enabled us to construct a computational model that interfaces the coupled-clock system (i.e., ion channels and SR Ca^{2+} cycling kinetics) with a cAMP/PKA signaling module. An AC-cAMP/PKA module has been explored by computational models in prior studies in ventricular myocytes (e.g., Saucerman-McCulloch model [30], Heihman-Rudy model [31]) and in other cell types [2]. However, due to the unique properties of pacemaker cells, e.g., the fundamental importance of Ca^{2+} -activated AC, the ability to spontaneously generate APs, different mechanisms coupling ATP supply to ATP demand, a novel model design is required for pacemaker cells.

A previous mathematical model in SANC has integrated ionic mechanisms together with biochemical reactions that underlie the response of pacemaker cells to β -AR stimulation [32]. This model, however, lacks the ability to define the kinetics of signaling initiated by Ca^{2+} -activated AC [12, 13], and its coupling to other mechanisms that regulate changes in AP firing rate. In the present study, integration of our novel experimental data on PKA activity and kinetics into SANC models of ionic mechanisms enabled simulation of kinetics of cAMP accumulation, increases in PKA activation and PKA-mediated phosphorylation in response to drugs that increase AC-cAMP signaling.

Our novel integrated model of biochemical and biophysical mechanisms not only correctly simulated our experimental results, but also generated predictions providing an insight into the finer details of how pacemaker cells are regulated by PKA. The first prediction of our numerical model is that the level of PKA activity-dependent PLB phosphorylation in spontaneously beating SANC is higher than the numerically predicted level in ventricular myocytes beating at similar rates (~ 3 Hz) [33]. This prediction is in accord with high cAMP levels documented in pacemaker cells [34], and may be related to Ca^{2+} activation of AC [12, 13]. The second prediction of our numerical model simulations is that the changes of kinetics of PKA-dependent mechanisms are tightly linked to changes in AP firing rate. The third prediction of our numerical model is that PKA activity in response to β -AR stimulation is different than in response to PDE inhibition. This result is consistent with an interpretation of studies in ventricular cells suggesting that different phosphorylation targets have different sensitivities to PDE inhibition or β -AR stimulation, or that PKA in SANC is localized within microdomains and different stimuli may differently affect different compartments [5, 35]. Although our numerical model simulates the changes in cAMP kinetics and magnitude cAMP was not directly measured, novel insight generated by our model simulations is that kinetics of PKA-dependent phosphorylation are more closely linked to the control of the spontaneous AP firing rate than direct cAMP-dependent mechanisms. Our numerical model, however, is a “common-pool” type of model, and cannot delineate differences in cAMP and PKA signaling in different compartments (SR, cytosol, membranes), taking into account consideration of localization and co-localization of the various constituents. Future experiments that measure PKA and cAMP within different compartments are required for the development of next-generation models of local Ca^{2+} activation of cAMP/PKA signaling in pacemaker cells.

The delayed rectifier potassium current in ventricular myocytes is modulated by PKA-mediated phosphorylation [9] and is an essential component in primary cardiac pacemaker activity in the rabbit SAN. Sodium current that has a role on cardiac peacemaking in

“follower” cells is modulated by PKA-mediated phosphorylation [36]. Unfortunately, no experimental data exist on the PKA-mediated phosphorylation of these channels in pacemaker cells. Future experimental results on PKA-mediated phosphorylation of delayed rectifier potassium current and sodium current are required for the development of modeling of full description of cAMP/PKA signaling in pacemaker cells. Additionally, Ca^{2+} also activates Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) protein phosphorylation in parallel with Ca^{2+} -calmodulin-activated AC-cAMP/PKA signaling [37]. There is crosstalk in rabbit pacemaker cells between CaMKII and PKA activity and a reduction in CaMKII activity indirectly reduces steady-state cAMP levels [38]. Because of PKA and CaMKII crosstalk, our experimental and numerical model results reporting on PKA activity and AP firing rate indirectly include a CaMKII activity effect. The level of CaMKII and specific relative roles of CaMKII and PKA activation in pacemaker cells are not established. Future experiments, like those performed in ventricular myocytes [39] and employment of novel developed techniques to visualize CaMKII and calmodulin activities [40] may precisely define the relative role of CaMKII in protein phosphorylation cascades in regulation of SANC AP firing rate.

Limitations

In this paper, we measured PKA activity and spontaneous AP firing rate characteristics in a separate group of cells. Technical limitations prevented dual measurements in the same cell: (i) the equipment available to us limited the recording time of PKA activity to 10 sec; in contrast, to resolve spontaneous AP firing characteristics a recording time in the order of tens of msec is required. Therefore, we could not use the same camera to record both PKA and AP signals. (ii) The membrane of pacemaker cells in culture is deformed and it is difficult to implement a perforated patch recording technique. Because in each culture dish approximately 4–5 cells exhibited spontaneous beating and there was less than 10% chance to successfully patch the cell, measuring AP firing rate by patch recording simultaneously with PKA activity within same cell is very technically challenging. Future studies are required to overcome these limitations. Note that the high standard errors around PKA activity and spontaneous AP firing derived from beat-to-beat variability between cells. When EC_{50} or $t_{1/2}$ were each calculated within the same cell there was small standard error and similar values between the variables.

Prior to performing our experiments, we developed a numerical model based on previously integrated measured parameters of steady-state AC activity, cAMP and phospholamban phosphorylation levels in response to perturbations that influence cAMP/PKA signaling. We chose PKA activities that are close to 25, 50 and 75% response based on the model prediction, and therefore did not spread the ISO concentrations evenly. Thereafter, we trained the model with the new data to predict the changes in PKA activity in response to the entire physiological range of ISO concentrations. Therefore, although we did not directly measure PKA activity in response to the entire physiological range of ISO concentrations, we can predict it based on our novel mathematical model.

In pacemaker cells, in addition to PKA activation by β -AR stimulation and PDE inhibition, phosphatase (PPT) inhibition can increase the spontaneous AP firing rate [15]. Additional

information on cellular mechanisms affected by PPT and the identity of which dominate PPT isoforms exist in pacemaker cells are required to determine the potential effect of PPT inhibition on PKA dynamics.

5. Conclusions

Our experimental results show that changes in the kinetics and magnitude of the spontaneous AP firing rate in response to β -AR stimulation or PDE inhibition are attributable to changes in kinetics and amplitude of PKA activity. Changes in average PKA are attributable to changes in spatiotemporal synchronization of local gradients changes. We interpret our model simulations to indicate that basal PKA activity in SANC is higher than in ventricular myocytes cell, and that the rate of PKA-dependent phosphorylation can limit the rate and magnitude of changes in spontaneous AP firing rate in response to perturbations that change the cAMP level. Future experiments employing live-cell imaging of pacemaker cells with localized PKA probes and numerical modeling are required for a more precise definition of compartmentalization of intracellular PKA activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations and acronyms

AC	adenylyl cyclases
AKAR	A-kinase activity receptor 3
AP	action potential
β-AR	β -adrenergic receptor
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
ISO	isoproterenol
M	membrane
PDE	phosphodiesterase
PKA	protein kinase A
PLB	phospholamban
RyR	ryanodine receptors
SANC	sinoatrial node cells

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Figure 1. Schematic illustration of the coupled-clock pacemaker cell system

Basal Ca^{2+} -calmodulin activation of adenylyl cyclases (AC) initiates cAMP-PKA-dependent phosphorylation signaling, and in parallel to AC, activates calmodulin-dependent kinase II (CaMKII) phosphorylation signaling. cAMP positively shifts the f-channel activation curve. Phosphodiesterases (PDE) degrade cAMP production, while protein phosphatase (PPT) degrades phosphorylation. PKA and CaMKII phosphorylate: sarcoplasmic reticulum (SR) Ca^{2+} cycling proteins (ryanodine receptor (RyR), phospholamban (PLB), which bind to and inhibit the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA)); surface membrane ion channel proteins (L-type, K channel); and mitochondrial ATP production proteins to activate ATP production and sarcomeres to modulate contraction.

Figure 2. FRET based measurements of PKA activity

(A) Schematic of AKAR3. Phosphorylation of a PKA-specific substrate (LRRATLVD) by PKA leads to its association with the Forkhead association 1 (FHA1) domain bringing the cyan fluorescent protein (CFP) closer to yellow fluorescent protein (YFP) which results in increased FRET from YFP to CFP and hence an increase in the yellow (FRET)/cyan emission ratio. (B) Changes in PKA activity in response to the PDE inhibitor (IBMX) or to the PKA inhibitor (H-89) in a single cultured SANC. (C) Representative example of the PKA activity (AKAR3 emission ratio Y/C) in a single cultured SANC in response to cumulative increases in the ISO concentration. (D) Representative example of the increase in spontaneous AP firing in response to cumulative increases in the ISO concentration.

Figure 3. PKA activity in response to β -AR stimulation

(A) Average graded changes in PKA activity (emission ratio Y/C) and spontaneous AP firing rate in response to different ISO concentrations. (B) The relationship between the increase in PKA activity and the spontaneous AP firing rate. PKA activity was normalized to the minimum activity (the decrease in PKA in response to 10 μ M H-89) and to the maximal activity (the increase in PKA in response to 100 μ M IBMX). The dashed line represents the estimated basal level of PKA activity and spontaneous AP firing rate in freshly isolated SANC. (C) Half times ($t_{1/2}$) of the increase in PKA activity and spontaneous AP firing rate in response to different single concentrations of ISO.

Figure 4. Distribution of PKA amplitude and time response to β -adrenergic receptor stimulation (A) Distributions of PKA activity gradients amplitude among different spatial regimes within the same cell and (B) the relationship between average PKA activity amplitude and PKA coefficient of variation in response to graded increases in isoproterenol (ISO) concentration. (C) Distributions of the $t_{1/2}$ among different spatial regimes within the same cell and (D) the relationship between average $t_{1/2}$ and $t_{1/2}$ coefficient of variation in response to graded increases in isoproterenol (ISO) concentration. (E) Coefficient of variation of PKA activity and $t_{1/2}$ gradients at different ISO concentrations.

Figure 5. Distribution of PKA amplitude and time response to PDE inhibition or PKA inhibition
Distributions of the (A) PKA amplitudes and (B) the time response to achieve the activity in different spatial regimes within the same cell in response to PDE inhibition (IBMX) following PKA inhibition (H-89).

Figure 6. PKA activity in response to β -AR stimulation or PDE inhibition

Representative example of time course of change in PKA activity (AKAR3 emission ratio Y/C) in response to (A) β -adrenergic receptor stimulation (ISO 100 nM) in the presence of 1 nM ISO and (B) PDE inhibition (IBMX 100 μ M) in the presence of 1 nM ISO.*The estimated basal level of PKA activity (see text for details).

Figure 7. Model simulations of the synergism of different mechanisms in mediating SANC spontaneous AP firing rate

The relative role of each mechanism was tested at a constant cAMP, by determining the changes in AP firing rate when only the tested mechanism sensed the changes in cAMP (cAMP was “clamped” to basal level for the remaining other mechanisms) and comparing the resultant change in AP firing rate to that when all mechanisms (direct cAMP and PKA-dependent phosphorylation) were functional.

Figure 8. Kinetics and magnitude of cAMP/PKA-phospholamban phosphorylation and spontaneous AP firing rate in response to maximal β -adrenergic receptor stimulation or PDE inhibition

Model simulations in response to maximal β -adrenergic stimulation predict the rate and extent of changes in (A) PKA activity, (B) cAMP, and (C) PLB phosphorylation in response to maximal β -adrenergic receptor stimulation. (D) The simulations predict that the kinetics of the increase in PLB phosphorylation are similar to those of the increase in spontaneous AP firing rate. Model simulations in response to PDE inhibition predict the rate and extent of changes in (E) PKA activity, (F) cAMP, and (G) PLB phosphorylation in response to PDE inhibition. (H) The simulations predict that the kinetics of the increase in PKA are similar to those of the increase in spontaneous AP firing rate.