Constitutive Phosphodiesterase Activity Restricts Spontaneous Beating Rate of Cardiac Pacemaker Cells by Suppressing Local Ca\textsuperscript{2+} Releases

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Abstract—Spontaneous beating of rabbit sinoatrial node cells (SANCs) is controlled by cAMP-mediated, protein kinase A–dependent local subsarcolemmal ryanodine receptor Ca\textsuperscript{2+} releases (LCRs). LCRs activated an inward Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange current that increases the terminal diastolic depolarization rate and, therefore, the spontaneous SANC beating rate. Basal CAMP in SANCs is elevated, suggesting that cAMP degradation by phosphodiesterases (PDEs) may be low. Surprisingly, total suppression of PDE activity with a broad-spectrum PDE inhibitor, 3\'-isobutylmethylxanthine (IBMX), produced a 9-fold increase in the cAMP level, doubled cAMP-mediated, protein kinase A–dependent phospholamban phosphorylation, and increased SANC firing rate by 55%, indicating a high basal activity of PDEs in SANCs. A comparison of specific PDE1 to -5 inhibitors revealed that the specific PDE3 inhibitor, milrinone, accelerated spontaneous firing by 46% (effects of others were minor) and increased amplitude of L-type Ca\textsuperscript{2+} current (\(I_{\text{Ca,L}}\)) by 46%, indicating that PDE3 was the major constitutively active PDE in the basal state. PDE-dependent control of the spontaneous SANC firing was critically dependent on subsarcolemmal LCRs, i.e., PDE inhibition increased LCR amplitude and size and decreased LCR period, leading to earlier and amplified LCR Ca\textsuperscript{2+} release, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange current, and an increase in the firing rate. When ryanodine receptors were disabled by ryanodine, neither IBMX nor milrinone was able to amplify LCRs, accelerate diastolic depolarization rate, or increase the SANC firing rate, despite preserved PDE inhibition–induced augmentation of \(I_{\text{Ca,L}}\) amplitude. Thus, basal constitutive PDE activation provides a novel and powerful mechanism to decrease cAMP, limit cAMP-mediated, protein kinase A–dependent increase of diastolic ryanodine receptor Ca\textsuperscript{2+} release, and restrict the spontaneous SANC beating rate. (Circ Res. 2008;102:761-769.)

Key Words: sinoatrial node ■ phosphodiesterase ■ ryanodine receptors ■ local Ca\textsuperscript{2+} release
pholamban (PLB) phosphorylation, and controlled spontaneous SANC beating rate. Surprisingly, these effects markedly exceeded those induced by β-AR stimulation by isoproterenol (ISO). LCRs via RyR were critically involved in the PDE-dependent control of the spontaneous beating rate. When RyR were inhibited by ryanodine, PDE inhibition failed to amplify local Ca²⁺ release during late DD and failed to increase spontaneous firing of SANCs. Thus, high basal constitutive PDE activity in SANCs coexists with constitutively active ACs, providing a negative feedback on the latter to limit cAMP level. This leads to a suppression of basal LCRs during DD that acts as a brake to keep the basal spontaneous SANC firing under control.

Materials and Methods
An expanded Materials and Methods section is in the online data supplement, available at http://circres.ahajournals.org.

SA Node Cell Preparations and Electrophysiological Recordings
Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Single spindle-shaped spontaneously beating SANCs were isolated from rabbit hearts. Perforated or ruptured patch-clamp techniques were used to record APs or currents from spontaneously beating SANCs. The bath temperature was maintained at 35±0.5°C.

Confocal Imaging of LCRs
SANCs were loaded with fluo-3 acetoxymethyl ester (Molecular Probes, Eugene, Ore). All images were recorded in line scan mode, using confocal microscopy as previously described.⁴,⁵

Cell Permeabilization
A subset of SANCs was permeabilized with 0.01% saponin as previously described.⁴,⁵

Western Blotting
The detection of Ser16 PLB phosphorylation was performed in isolated SANCs using a phosphorylation P-Ser-16 PLB antibody (Badrilla) as previously described.⁵

cAMP Measurements
SA nodal or ventricular cells were homogenized, and cAMP was estimated using the Biotrak cAMP [¹²⁵I] assay system (RPA 509; Amersham Biosciences).

Statistical Analysis
Data are presented as means±SEM. The statistical significance of effects was evaluated by Student t test or ANOVA where appropriate. A value of P<0.05 was considered statistically significant.

Results
Effects of PDE Suppression on the Level of cAMP and Spontaneous Firing Rate of SANCs
To determine the extent of PDE-dependent control of basal cAMP level in isolated rabbit SANCs, we used a broad-spectrum PDE inhibitor, 3′-isobutylmethylxanthine (IBMX), which produced a 9-fold increase in cAMP, an effect larger than that of a saturating ISO concentration (Figure 1A). In spite of a high basal level of PDE activity, basal cAMP in isolated SANCs was substantially higher than in ventricular myocytes, confirming a prior study.⁵

Suppression of total PDE activity with IBMX dramatically accelerated SANC spontaneous beating rate (by 55%, Figure 1B), and this effect was reversible after drug washout. Analysis of AP parameters showed that IBMX-induced acceleration was accompanied by a marked increase in the DD rate (62.2±6.8 mV/sec in control versus 116.7±6.7 mV/sec with IBMX; n=7), whereas the maximum diastolic potential (−63.7±2.3 mV in control versus −64.1±2.8 mV with IBMX; n=7), AP amplitude (97.9±2.7 mV in control versus 96.8±3.4 mV with IBMX; n=7), and AP upstroke (6.6±0.7 V/sec in control versus 6.9±0.7 V/sec with IBMX; n=7) were unchanged. To verify the direct effect of cAMP on SANC spontaneous beating, we used a membrane-permeable cAMP analog, 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), which increased spontaneous beating rate by 36%, confirming a critical role of elevated cAMP to increase the spontaneous SANC beating rate (Figure 1C).

PDE3 Controls SANC Spontaneous Beating Rate in the Basal State
The PDE superfamily consists of 11 families, and PDE1 to -5 are present in the heart.¹⁰ To determine which PDE subtypes
mediate IBMX-induced increase in the spontaneous SANC beating rate, we used specific inhibitors of different PDE subtypes (see the online data supplement). Figure 2 shows that on average, the increase in the spontaneous beating rate produced by suppression of PDE1, -2, -4, or -5 was relatively small, whereas suppression of PDE3 by the specific PDE3 inhibitor milrinone substantially accelerated spontaneous firing, producing an effect almost equal to the effect of nonspecific PDE inhibitor IBMX. These data strongly suggest that constitutive activation of PDE3 is a major contributor to the total basal PDE activity. The positive chronotropic effect of IBMX markedly exceeded the effect of the saturating concentration of β-adrenergic agonist ISO (P<0.05). Although milrinone-induced acceleration of the beating rate was ∼12%, larger than that produced by ISO, it did not reach statistical significance (Figure 2).

**Positive Chronotropic Effects of PDE Inhibition Are Critically Dependent on Local RyR Ca2+ Releases**

Our prior work has demonstrated that normal spontaneous beating of SANCs is critically dependent on characteristics of LCRs, which are potently modulated by cAMP mediated PKA-dependent phosphorylation.3,5 To investigate how LCRs are affected by PDE inhibition, we used ryanodine, which locks RyRs in a subconductance open state and depletes the sarcoplasmic reticulum (SR) Ca2+ load. Consistent with our previous results,3 ryanodine inhibition of LCRs and reduction in the firing rate occurred concomitantly. Figure 3 shows that when RyRs were functionally disabled by ryanodine, a suppression of either PDE3 activity with milrinone or total PDE activity with IBMX induced only a minor increase in the spontaneous beating rate. Compared with control, acceleration of the beating rate by IBMX or milrinone in the presence of ryanodine was decreased by ∼14-fold. These results suggest that an essential feature of PDE inhibition–induced acceleration of the rabbit SANC firing rate is to affect Ca2+ release via RyR.

Ca2+ influx via L-type Ca2+ current (I_{CaL}) is a crucial component of Ca2+ cycling that sustains LCRs in SANCs. To verify whether the aforementioned effects of ryanodine to suppress the milrinone-induced increase in SANC spontaneous beating rate concomitantly suppress I_{CaL}, we recorded effects of milrinone on I_{CaL} in the presence of ryanodine. Consistent with our previous data,11 ryanodine pretreatment, per se, had no effect on the average I_{CaL} amplitude (Figure 4). PDE3 inhibition by milrinone increased I_{CaL} amplitude by ∼45% (Figure 4A). Moreover, the milrinone-induced increase in the I_{CaL} amplitude was not affected by ryanodine pretreatment (Figure 4). These data demonstrate that, even when suppression of PDE3 activity with milrinone markedly increased Ca2+ influx via increase of I_{CaL} amplitude, the positive chronotropic effect of milrinone was markedly suppressed if RyRs were disabled and LCRs were inhibited by ryanodine.
PDE Inhibition Modulates Spatiotemporal Characteristics of LCR

To determine how PDE inhibition specifically modifies spatiotemporal characteristics of LCRs to augment SANC firing, we used confocal microscopy to simultaneously measure LCR and APs in intact SANCs. Figure 5 shows that following PDE3 inhibition, there was a shift in the distribution of LCRs amplitudes (Figure 5B) and spatial widths (Figure 5C) to larger values. Milrinone increased the average LCR amplitude from 0.83±0.07 to 1.05±0.08 ΔF/F₀ (n=6, P<0.05) and width from 6.51±0.32 to 8.46±0.21 μm (full width at half maximum; n=6, P<0.01). Suppression of total PDE activity with IBMX also markedly increased average LCR amplitude from 0.78±0.03 to 0.89±0.04 ΔF/F₀ (n=4, P<0.05) and size from 5.66±0.56 to 8.32±0.73 μm (full width at half maximum; n=4, P<0.05). These data indicate that following PDE inhibition, LCRs are amplified, in part, by recruitment of additional RyRs to contribute to the local Ca²⁺ release.

Because the LCR period regulates the spontaneous cycle length and beating rate, we studied how LCR period is affected by PDE inhibition. During spontaneous beating, regularly occurring AP-induced SR Ca²⁺ releases depleted SR and inactivated RyR. When the SR content was replenished and RyR recovered from inactivation, LCR occurrence resumed. Therefore, the LCR period was estimated as an interval between the onset of AP-induced Ca²⁺ transient and the onset of subsequent spontaneous LCR (Figure 5A, inset). Both milrinone (Figure 5D) and IBMX (data not shown) induced a marked decrease in the LCR period, which was highly correlated with a concomitant decrease in the spontaneous cycle length.

Electrogenic Mechanisms of PDE Inhibition–Produced Acceleration of the SANC

Spontaneous Beating Rate

It is well recognized that an increase in cAMP in SANCs directly activates the inward hyperpolarization activated current (Iₕ), which controls the early phase of the DD. To directly probe the Iₕ contribution in the PDE inhibition–produced acceleration of spontaneous SANC firing, effects of IBMX and milrinone were compared in the absence and presence of 2 mmol/L Cs⁺, which effectively blocks Iₕ. The PDE inhibition accelerated the SANC spontaneous beating rate to almost the same extent in the presence and absence of

Figure 5. PDE3 inhibition increases the frequency and changes spatiotemporal properties of LCRs in intact SANCs. A, Simultaneous recordings of APs and confocal line scan images of a representative SANC before and following exposure to 50 μmol/L milrinone, LCRs are indicated by arrowheads. The inset below the bottom image illustrates how the LCR period is defined, ie, as the time from the prior AP-induced Ca²⁺ transient to the onset of LCR. B and C, Histograms of LCR size (full width at half maximum) and amplitude (ΔF/F₀) in control SANCs (n=6 cells; 62 LCRs) and after superfusion with milrinone (n=6 cells; 123 LCRs). D, The milrinone-induced decrease in the spontaneous cycle length is linked to its effect on the LCR period. Note that this relationship lies above the line of identity (dashed line), indicating that the period of LCRs is shorter than the spontaneous cycle length.
 Cs⁺ (Figure 6A and 6B). This result was consistent with the idea that İₛ plays a minor, if any, role in the PDE inhibition induced acceleration of the spontaneous SANC beating rate.

To elucidate additional ryanodine-sensitive electrogentic mechanisms involved in PDE inhibition–induced increase in DD rate and spontaneous beating rate, we investigated effects of the broad-spectrum PDE inhibitor IBMX on NCX current and delayed rectifier potassium (İₚₚ) current. Because LCRs activate an inward NCX current, IBMX-induced amplification of local Ca²⁺ releases would be expected to augment this current and increase DD rate. When LCRs are inhibited by ryanodine, PDE inhibition–induced increase of NCX current as well as increase in the DD rate would be suppressed. To test this idea, we simulated DD by a voltage ramp protocol and applied IBMX in the absence and presence of ryanodine. IBMX markedly increased the inward current developed during voltage ramp by 107%, from 0.73±0.08 to 1.51±0.20 pA/pF (n=3; Figure 6C and 6D). Ryanodine significantly decreased the inward current, from 0.80±0.10 to 0.52±0.10 pA/pF (P<0.01; n=6), and caused a more than 3-fold reduction in the IBMX-induced amplification of this current. In fact, the IBMX-induced increase in this inward current in the presence of ryanodine did not reach statistical significance (Figure 6C and 6D).

Iₖ current was measured as the amplitude of the outward tail current (Iₖₜₐᵢ), and effects of IBMX on Iₖₜₐᵢ were studied in the absence and presence of ryanodine. IBMX produced an ~5-mV shift of Iₖₜₐᵢ activation in the negative direction (Figure II in the online data supplement) and an ~12% increase in Iₖₜₐᵢ current amplitude (supplemental Figure III). However, both the shift of Iₖₜₐᵢ activation and increase in Iₖₜₐᵢ current amplitude were preserved in the presence of ryanodine (supplemental Figures II and III), suggesting that modulation of Iₖ by PDE inhibition is not affected by ryanodine pretreatment.

PDE Inhibition in SANCs Increases PLB Phosphorylation and Accelerates the Kinetics of SR Ca²⁺ Uptake

Although basal cAMP was high in SANCs, the link of cAMP to the spontaneous beating was not direct but required PKA-dependent phosphorylation of multiple proteins controlling Ca²⁺ homeostasis, including Iₖₜₐᵢ, and proteins controlling SR Ca cycling, ie, RyR and PLB. We used the phosphorylation status of PLB as a marker for PKA-dependent protein phosphorylation. Figure 7 shows that PLB phosphorylation at the PKA-dependent serine 16 site was markedly increased by both the broad-spectrum PDE inhibitor IBMX and the PDE3 inhibitor milrinone and was reversed by a specific peptide inhibitor of the PKA catalytic subunit 14-22 amide, PKI.

It is well known that markedly elevated PLB phosphorylation increases the SR Ca²⁺ pump rate and thus the rate at which Ca²⁺ is pumped back into SR. This effect results in a more rapid decay of the AP-induced Ca²⁺ transient. In intact

**Figure 6.** Contribution of İₛ and NCX currents in the positive chronotropic effect of PDE inhibition. A, Typical example of the increase in the spontaneous SANC beating rate produced by the broad-spectrum PDE inhibitor IBMX in the presence of complete Cs⁺ block by 2 mmol/L Cs⁺. Cs⁺ decreased beating rate in this cell from 165 to 140 bpm; 100 μmol/L IBMX increased the firing rate to 208 bpm in the presence of Cs⁺. B, Comparison of the relative increase in the beating rate by 50 μmol/L milrinone or 100 μmol/L IBMX in the presence or absence of 2 mmol/L Cs⁺. C, Original current recordings and voltage-clamp protocol for DD current. D, Relative increase in the average DD current amplitude (at −45 mV) by 100 μmol/L IBMX before and after 10 μmol/L ryanodine pretreatment. *P<0.05.

**Figure 7.** PDE inhibition increases PLB phosphorylation in SANCs. A, Representative Western blot of the basal level of phosphorylated at serine 16 and total PLB in SANCs and that following milrinone (50 μmol/L), milrinone and PKI (10 μmol/L), IBMX (100 μmol/L), or β-AR stimulation (1 μmol/L ISO). B, Relative values of phosphorylated PLB normalized to control (n=8).
SANCs, milrinone markedly decreased 90% decay time of AP-induced \(\text{Ca}^{2+}\)/\(\text{H}^{1}\) transient from 298.8±15.4 to 223.4±9.1 ms (\(P<0.001; n=6\)) and increased the average amplitude of AP-induced \(\text{Ca}^{2+}\)/\(\text{H}^{1}\) transient from 1.53±0.10 to 1.65±0.12 \(F/\text{F}_0\) (\(P<0.02; n=6\)), which was consistent with PLB phosphorylation–induced acceleration in SR \(\text{Ca}^{2+}\) pumping.

In Permeabilized SANCs, PDE3 Inhibition Modulates LCR Parameters and Increases SR \(\text{Ca}^{2+}\) Load

In SR-enriched microsomes of rabbit SA node, PDE3 activity is the major (≈75% of total) PDE activity, suggesting close association of PDE3 with SR.\(^{15}\) To verify direct effects of PDE3 inhibition on the LCR parameters in the absence of spontaneous APs, avoiding concomitant effects of sarcolemmal ionic currents, we permeabilized SANCs with saponin. Similar to its effect in intact SANCs, suppression of PDE3 activity by milrinone markedly increased the likelihood of LCR occurrence and partially synchronized their initiation by increasing number and size of LCRs in “skinned” SANCs (supplemental Figure IVA and IVB). To determine whether this PDE inhibition–induced augmentation of local \(\text{Ca}^{2+}\) releases in skinned SANCs was caused, at least in part, by stimulation of SR \(\text{Ca}^{2+}\) uptake and increase in the SR \(\text{Ca}^{2+}\) load, we applied a pulse of caffeine directly on the SANCs to rapidly empty the SR \(\text{Ca}^{2+}\) store. Representative images and average data (supplemental Figure VA and VB) showed that, indeed, following milrinone, there was a significant increase in the SR \(\text{Ca}^{2+}\) load by ≈12%.

PDE Inhibition Effects on PLB Phosphorylation and LCR Period Are Tightly Linked to Effects on the Spontaneous Beating Rate

The effects of cAMP PKA-dependent stimulation on the LCR period are likely mediated by an increase in phosphorylation of multiple proteins that regulate cell \(\text{Ca}^{2+}\) balance, including L-type \(\text{Ca}^{2+}\) channels, PLB phosphorylation, and probably that of other proteins not measured in the present study. Figure 8B shows that the increase of cAMP PKA-dependent protein phosphorylation produced by PDE inhibition or \(\beta\)-AR stimulation, and indexed by PLB phosphorylation–induced acceleration in SR \(\text{Ca}^{2+}\) pumping, was highly correlated with the concomitant decrease of the LCR period. Thus, interventions that use different pathways to increase the cAMP level (activation of ACs or inhibition of PDEs) are linked to a common mechanism, ie, an increase of cAMP PKA-dependent protein phosphorylation to decrease the LCR period and spontaneous cycle length (Figure 8C). The dependence of the spontaneous
cycle length on cAMP/PKA-dependent effects on the LCR period produced by β-AR stimulation or PDE inhibition form a single continuous function.

Discussion

PDEs Control cAMP Level and SANC Spontaneous Beating in the Basal State

The first novel finding of the present study was that intrinsic PDE activity potently controlled the basal cAMP level and spontaneous beating rate of SANCs. A suppression of the total PDE activity by the broad-spectrum PDE inhibitor IBMX led to a 9-fold increase in the level of cAMP (Figure 1A) and an ∼55% increase in the spontaneous beating rate of SANCs. Thus, high cAMP production by AC in SANCs coexists with high cAMP degradation by PDEs, demonstrating a unique type of balance between former and latter. Moreover, the system acting in this mode can rapidly react to stimuli that alter cAMP without involvement of receptor-dependent mechanisms. Remarkably, effects of total PDE inhibition on both cAMP and the spontaneous beating rate exceeded effects of the saturating concentration of β-AR agonist ISO (Figure 2), which may be attributable to the more efficient cAMP degradation by PDEs than cAMP production triggered by β-AR stimulation.

PDE3 Is the Major Constitutively Active PDE That Controls Spontaneous SANC Firing

The second discovery of the present study was that PDE3 was the major PDE subtype that controlled basal spontaneous SANC firing, whereas contribution of other PDEs was relatively small (Figure 2). It is known that PDE1 and PDE2 can hydrolyze both cAMP and cGMP and that PDE3 preferentially hydrolyzes cAMP, whereas PDE4 is specific for cAMP and PDE5 is specific for cGMP. Our data are consistent with the species-dependent variation in the activity of different PDE subtypes in the heart. For example, in the murine ventricular cells, PDE4 is the dominant PDE subtype, accounting for 60% of total cAMP hydrolyzing activity. In both rabbit SA node and ventricle, PDE3 is the most abundant subtype, accounting for ∼75% of total cAMP-hydrolyzing activity in microsomal fraction (PDEs attached to membranes) and ∼30% in cytosol. Importantly, an inhibition of an SR-associated PDE3 subtype is the key factor of PDE3 inhibition–induced augmentation of contractile responses in myocardium. Immunolabeling patterns of RyR clearly demonstrate a high density of RyR in SANCs beneath sarcolemma. The striking effects of milrinone on local subsarcolemmal Ca²⁺ releases both in intact and permeabilized SANCs (Figure 5 and supplemental Figure IV) could be explained by strategically positioned PDE3 in this region, which could control local cAMP level and PKA-dependent phosphorylation in the vicinity of sarcolemma and SR. Specifically, suppression of PDE3 activity markedly increased PKA-dependent phosphorylation of PLB (Figure 7), amplified SR Ca²⁺ ATPase pumping rate, and increased SR Ca²⁺ load (supplemental Figure V) in the subsarcolemmal SR.

PDE-Dependent Regulation of LCRs Provides Control Over Spontaneous Beating of SANCs

The third and probably the most important finding of the present study was the mechanism of the PDE-dependent control of the spontaneous firing of SANCs. LCRs in SANCs, as assessed by line scan or 2D images, are localized to the subsarcolemmal area. LCRs occur during terminal DD and begin as spark-like events; with time, they converge to become waves that propagate locally with a velocity of 156±14 μm/sec and to a distance up to 11.5±1.2 μm, which is followed by a steep AP-induced rise in global Ca²⁺. Whereas confocal images of the present study (Figures 5 and 8) provided only 1 line scan image and showed LCRs in a single location, 2D images show that multiple LCR occur simultaneously throughout SANCs. Numeric model simulations show that each LCR activates an inward NCX current equal to ∼0.27 pA that results in a membrane potential response of ∼0.17 mV. Experimental measurements of the present study showed that ryanodine-susceptible NCX current was equal to ∼0.28±0.03 pA/pF, which resulted in a total current equal to ∼9 pA (per 32 pF SANCs) and was in a good agreement with numeric simulations of ∼10 pA.

In the present study, PDE inhibition increased LCR amplitude and size, amplified subsarcolemmal Ca²⁺ release during DD, and shifted it to earlier times, which could be partially explained by an increased and earlier spontaneous RyR Ca²⁺ release flux attributable to an increase in PLB phosphorylation, increase in SR Ca²⁺ ATPase pumping rate, and increase in SR Ca²⁺ load (Figure 7 and supplemental Figure V). This amplification of subsarcolemmal Ca²⁺ release during DD led to a 2-fold increase of the inward NCX current (Figure 6D), which “boosted” DD rate and, as a result, increased the spontaneous beating rate (Figures 5 and 8). In the presence of ryanodine, this current was markedly suppressed, and PDE inhibition failed to substantially increase either NCX current (Figure 6D) or the spontaneous SANC beating rate (Figure 3).

Thus, intact RyR function was critically important for PDE inhibition–induced acceleration of SANC spontaneous beating. When RyR local subsarcolemmal Ca²⁺ release was inhibited by ryanodine, PDE inhibition was unable to increase LCRs, NCX current (Figure 6C and 6D), or spontaneous beating rate (Figure 3). In the presence of ryanodine, the positive chronotropic effect of PDE inhibition was suppressed in spite of preserved PDE inhibition–induced average increase of the Ic₅₀ amplitude (Figure 4) and I₈ amplitude (supplemental Figure III). Moreover, our data clearly demonstrate that in our experimental conditions, I₈ current likely had, at best, a minor role in IBMX or milrinone-induced acceleration of spontaneous SANC firing.

Different Signaling Pathways Use cAMP-Mediated, PKA-Dependent Modulation of LCRs to Control the Spontaneous SANC Beating Rate

The PDE-dependent control of LCRs and basal spontaneous beating rate uses the very same mechanism that is used by either β-AR stimulation or membrane-permeable cAMP analog to accelerate spontaneous SANC firing. All of these interventions directly or indirectly (1) elevate level of cAMP; (2) increase cAMP-mediated, PKA-dependent phosphorylation of Ca²⁺-cycling proteins; (3) augment LCRs; (4) amplify...
NCX current and increase terminal DD rate; and (5) accelerate spontaneous beating rate. Thus, regulation of local RyR Ca\(^{2+}\) release plays a central role in the control of spontaneous SANC firing, regardless of whether intracellular cAMP is increased via activation of AC, as during β-AR stimulation, or by suppression of cAMP degradation, as during PDE inhibition. Intact RyR function is essential for the chronotropic effect of all aforementioned interventions, because inhibition of RyR Ca\(^{2+}\) release by ryanodine prevents increase in the SANC spontaneous beating rate produced by β-AR stimulation,\(^{11,22,23}\) membrane permeable cAMP analog,\(^5\) or PDE inhibition (present study).

Previous studies in intact animals, isolated Langendorff heart preparations,\(^7−9\) and clinical trails have observed the positive chronotropic effect of PDE inhibition. However, complex effects of PDE inhibitors on both myocardium and blood vessels in vivo precluded a direct demonstration of PDE-dependent effects on the cardiac pacemaker function. Studies of the isolated SA node\(^24\) and of isolated SANCs\(^25\) have examined the effects of some PDE3 inhibitors on AP parameters\(^24\) and sarcolemmal ionic currents,\(^25\) respectively. In guinea pig SANCs, amrinone, another PDE3 inhibitor, increased the firing rate by \(\approx 12\% ,\)\(^25\) compared with \(\approx 47\% \) increase in the rabbit SANC beating rate by milrinone (this study). This difference could be explained, in part, by the different species used and, in part, by the fact that milrinone is regarded as a more potent PDE3 inhibitor.\(^7,8\) Moreover, only milrinone effectively inhibits PDE3 activity in SA node–enriched fraction,\(^26\) whereas other PDE3 inhibitors have minor effects, despite potent inhibition of PDE3 activity in ventricular tissue.\(^26\)

In summary, the present data show, for the first time, that basal level of cAMP-mediated, PKA-dependent phosphorylation and basal spontaneous beating rate of SANCs are under a tight restraint of constitutively active PDEs, with PDE3 as the major subtype. This PDE-dependent control was mostly executed through modulation of local RyR Ca\(^{2+}\) release. When PDEs were inhibited, the increase in cAMP activated cAMP-mediated, PKA-dependent phosphorylation of PLB and L-type Ca\(^{2+}\) channel. Acceleration of SR Ca\(^{2+}\) pumping and elevation of SR Ca\(^{2+}\) load increased LCR amplitude and size by partial synchronization and recruitment of additional RyR Ca\(^{2+}\) releases. The earlier occurring and amplified Ca\(^{2+}\) release beneath sarcolemma augmented the inward NCX current and accelerated DD rate and, as a result, spontaneous SANC beating rate. When RyR Ca\(^{2+}\) release was suppressed by ryanodine, efficient PDE-dependent control of the spontaneous beating rate was abolished, in spite of preserved PDE inhibition–induced augmentation of \(I_{\text{Ca,L}}\) and \(I_{\text{K}}\). Thus, constitutively active PDEs via reduction of cAMP PKA-dependent protein phosphorylation restrict local RyR Ca\(^{2+}\) release during DD to keep the basal spontaneous SANC firing under control. Suppression of PDE activity is a novel strategy to increase spontaneous beating rate without involvement of β-AR stimulation. It offers a new direction for using PDE-dependent control of SA node beating rate as a tool to adjust cardiac pacemaker function according to the requirements of aging or the diseased heart.

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Disclosures

None.

References


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SA node cell preparations and electrophysiological recordings: Single sinoatrial node cells (SANC) were isolated according to the modified method of Ito and Ono\textsuperscript{1}. New Zealand White rabbits (Charles River Laboratories, USA) weighing 1.8–2.5 kg were deeply anesthetized with sodium pentobarbital (50–90 mg/kg). The heart was removed quickly and placed in the Tyrode solution containing (in mmol/L): NaCl, 130; NaHCO\textsubscript{3}, 24; NaH\textsubscript{2}PO\textsubscript{4}, 1.2; MgCl\textsubscript{2}, 1.0; CaCl\textsubscript{2}, 1.8; KCl, 4.0; and glucose, 5.6; after continuous saturation with a mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2} the pH was maintained at 7.4; temperature was maintained at 36° C. The SA node region was cut into small strips (~1.0 mm wide) perpendicular to the crista terminalis (CT) and excised as shown in figure 1 (dashed line). The final SA node preparation consisted of SA node strips attached to the small portion of CT.

The SA node preparation was washed twice in Ca\textsuperscript{2+}-free Tyrode solution (34°C) containing (in mmol/L): NaCl, 140; KCl, 5.4; MgCl\textsubscript{2}, 0.5; NaH\textsubscript{2}PO\textsubscript{4}, 0.33; HEPES, 5; glucose, 5.5; pH= 6.9; then, incubated at 34°C for 30 min in Ca\textsuperscript{2+}- free Tyrode solution containing elastase type IIA (0.6 mg/ml; Sigma, Chemical Co.), collagenase type 2 (0.6 mg/ml; Worthington, NJ, USA) and 0.1% bovine serum albumin (Sigma, Chemical Co.). Thereafter, the SA node preparation was washed in modified Kraftbruhe (KB) solution, containing (in mmol/L): 70 potassium glutamate, 30 KCl, 10 KH\textsubscript{2}PO\textsubscript{4}, 1 MgCl\textsubscript{2}, 20 taurine, 10 glucose, 0.3 EGTA, and 10 HEPES (titrated to pH 7.4 with KOH), and kept at 4°C for 1h in KB solution containing 50 mg/ml polyvinylpyrrolidone (PVP 40, Sigma,
Chemical Co.). Finally, cells were dispersed from the SA node preparation by gentle pipetting in the KB solution and stored at 4°C.

**Electrophysiology recordings:** Perforated patch-clamp technique was employed to record action potentials (APs), and perforated or ruptured patch-clamp techniques were used to record currents using Axopatch -200D patch-clamp amplifier (Axon Instruments, Foster City, CA). Only spontaneous regularly beating spindle-shaped SANC were chosen for recordings of either APs or currents. Pipette solution for both perforated patch studies and current recordings (except L-type Ca\(^{2+}\) current) contained (in mmol/L): K-gluconate, 120; NaCl, 5; MgATP, 5; HEPES, 5; KCl, 20; pH, 7.2. For perforated patch-clamp experiments, β-escin (50 μmol/L, Sigma) was added to the pipette solution\(^2\). The bath temperature was maintained at 35±0.5°C.

Considering that NCX current is critically dependent upon rapidly changing level of Ca\(^{2+}\) produced by LCRs beneath sarcolemma and pharmacological or ionic interventions by themselves are able to eliminate LCRs and to alter NCX exchanger function, we measured NCX current under close to physiological conditions, i.e., used perforated patch to keep physiological ionic gradients close the intact and employed pre-conditioning pulses to ensure steady-state loading of the SR Ca\(^{2+}\), as previously described\(^3,4\). Recordings of NCX current were performed using a voltage ramp protocol from holding potential -60 to -45 mV, the upper level of the ramp was chosen based on our measurements of SANC take-off potential which is equal to -45 mV\(^5\).

Since IK,r and IK,s are the only currents contributing to the outward tail current\(^6\), we studied effects of IBMX on the outward tail currents in the absence and presence of Ry. For recordings of delayed rectifier potassium current (I\(K\)) (whole cell or perforated patch
clamp) depolarizing voltage clamp pulses (1000 ms) were applied from a holding potential of -50 mV; 4 μmol/L nifedipine was added to block interfering I_{Ca,L} current. Effects of IBMX (in the absence or presence of ryanodine) on the tail current steady-state activation were obtained by normalizing each tail current amplitude to the maximum I_{K,tail} current recorded under the specific experimental conditions.

For recordings of I_{Ca,L} (whole cell patch clamp), depolarizing voltage clamp pulses (300 ms) were applied from a holding potential of -50 mV; 10 μmol/L tetrodotoxin and 4 mmol/L 4-aminopyridine were added to block interfering currents. The bath solution contained the following (in mmol/L): NaCl, 117; TEA-Cl, 20; CsCl, 5.4; MgCl₂, 1; HEPES, 5; CaCl₂, 1.8; 4-AP, 4; pH = 7.4. The pipette solution contained the following (in mmol/L): NaCl, 10; TEA-Cl, 20; CsCl, 110; EGTA, 10; MgATP, 5; HEPES, 10; pH, 7.2 (as previously described⁴.

Effects of PDE inhibitors either on the beating rate (AP recordings) or ionic currents were studied in the absence or presence of ryanodine. In the latter case SANC were pretreated with ryanodine for 4 minutes before PDE inhibitors in the presence of Ry were added into the bath solution. The same time protocol was maintained either in the absence or in the presence of ryanodine. To minimize the interference from rundown, all currents were measured every 11 seconds and the effect of PDE inhibition was expressed as maximal current amplitude in the presence of PDE inhibitor normalized to the amplitude immediately before PDE inhibitor addition.

**Confocal imaging of local subsarcolemmal Ca^{2+} releases:** SANC were loaded with fluo-3 AM (Molecular Probes, Eugene, OR) and placed on the stage of a Zeiss LSM-410 inverted confocal microscope (Carl Zeiss, Inc., Germany). All images were
recorded in the linescan mode, with the scan line oriented along the long axis of the cell, close to sarcolemmal membrane and processed with IDL software (5.4, Research Systems, Boulder, CO). The amplitude of each LCR was expressed as a peak value (F) normalized to minimal fluorescence (F₀), its spatial size was indexed as the full width at half maximum amplitude (FWHM), and its duration characterized as the full duration at half maximum amplitude (FDHM). The number of LCR in permeabilized SANC was normalized per 100 μm of the linescan image and during a 1s time interval (as previously described⁷).

**Cell permeabilization:** A subset of cells was permeabilized with 0.01% saponin as previously described⁷ in a solution containing in mmol/L: K aspartate, 100; KCl, 25; NaCl, 10; MgATP, 3; MgCl₂, 0.81 (free [Mg²⁺] ~1 mM); HEPES, 20; EGTA, 0.5; phosphocreatine, 10 and creatine phosphokinase, 5U/ml; pH 7·2. The control experimental solution was, as above, with 0.03 mmol/L fluo-4 pentapotassium salt. The free [Ca²⁺], at a given total Ca²⁺, Mg²⁺, ATP and EGTA concentration was calculated, using a computer program (WinMAXC 2.50, Stanford University, CA). All experiments were done at the final Ca²⁺ concentration 100nmol/L. The bath temperature was maintained at 35±0.5°C.

**Western Blotting:** The detection of site specific PLB phosphorylation was performed in SANC as previously described⁸. Specifically, the SANC suspension was equally divided into 5 parts, and each part was individually treated: in the first group with 50 μmol/L milrinone, in the second group with 50 μmol/L milrinone + 15 μmol/L PKI, in the third group with 100 μmol/L IBMX or solvent control for 10 minutes; in the fifth group with 1 μmol/L isoproterenol for 5 minutes. All treatments were performed at
35±0.5°C. Then, cells were solubilized to dissociate fully PLB into its monomeric form (6.7 KDa). Proteins were resolved by 7.5% urea/SDS-PAGE gel and transferred (10 μg protein/lane) to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech). To detect PLB phosphorylation, the phosphorylation site specific P Ser\(^{16}\)PLB antibody (1:10000, Badrilla) and HRP conjugated secondary antibody (1:15000) (Bio Rad) were employed. PVDF membranes were exposed to the chemiluminescence (ECL, Amersham Pharmacia Biotech) reaction and quantified with a video documentation system Bio Rad.

**cAMP measurements:** Sinoatrial node cell suspensions were equally divided into 3 parts, and each part was individually treated: in the first group with 1 μmol/L isoproterenol, in the second group with 100 μmol/L IBMX or solvent control for 5 minutes at 35°C. Ventricular myocytes were treated with solvent control for 5 minutes at 35°C. After incubation, ice-cold ethanol was added to cell suspensions to have a final concentration of 65% ethanol. The extracts were centrifuged at 10000 g for 15 min at 4°C. The supernatants were saved in fresh tubes. The remaining precipitates were washed with ice cold 65% ethanol, carefully mixed and centrifuged as before. The supernatants were mixed with the previous ones and evaporated using a speed vacuum dryer. cAMP of the dried extracts are determined according to “The Biotrak™ cAMP \([^{125}\text{I}]\) assay system, Amersham Biosciences (RPA 509)” Briefly, samples were dissolved in the assay buffer prior to analysis. The solutions were mixed with a reconstituted antiserum from a lyophilized rabbit anti-succinyl cAMP and incubated for one hour at 4°C. The \([^{125}\text{I}]\) labeled cAMP was added to every tube and incubated for 3 more hours at 4°C. The standard kit secondary antibody were added to each tube and allowed to settle at room
temperature for ten minutes then centrifuged at 2600 rpm and 4°C. Supernatants were discarded and the tubes were inverted on absorbent pads for 5 min. The radioactivity of the pellets was counted in a γ counter. Total protein was determined with a BCA™ Protein Assay. The amount of the cAMP was expressed as pmoles/mg of protein.

**PDE inhibition:** To block total PDE activity we used, IBMX, a broad-spectrum PDE inhibitor, and for different PDE subtypes we used specific and selective PDE inhibitors: for PDE1 – MIMX; for PDE2 - EHNA; For PDE3 – milrinone; for PDE4 – rolipram and for PDE5 - zaprinast. The choice of concentrations of PDE inhibitors was based on the available information regarding the potency to increase the spontaneous beating rate either of the isolated heart or isolated right atrium preparation (including sinoatrial (SA) node). When this information were unavailable, we tested several concentrations of PDE inhibitor to determine the concentration that produced a maximum increase in the spontaneous SANC beating rate.

**Total PDE:** a concentration of 100 μmol/L IBMX was chosen for our experiments, since it was demonstrated that IBMX produced concentration-dependent increase in the spontaneous beating rate of the rabbit right atria (including SA node) with a maximum effect at this concentration.

**PDE2:** it was shown that the basal spontaneous beating rate of the rat right atrial preparation (including SA node) was not affected by a relatively high EHNA concentration, 20 μmol/L. In rabbit SANC we tested several EHNA concentrations in the range from 10 to 100 μmol/L, and a concentration of 10 μmol/L was chosen as the most potent.
**PDE3**: a concentration of 50 µmol/L milrinone was chosen for our experiments, since it was shown that a selective PDE3 inhibitor, milrinone, dose-dependently increased the spontaneous beating rate of the isolated guinea-pig heart with the maximal effect reached at this concentration\(^\text{13}\). Moreover, the effect of milrinone on the spontaneous heart beating rate was more potent than others PDE3 inhibitors\(^\text{13}\).

**PDE4**: rolipram, over a wide range of concentrations 0.1-100 µmol/L, did not have any positive chronotropic effect on the spontaneous beating rate of the guinea-pig right atrial preparation (including SA node)\(^\text{14}\). We studied effects of 2 -100 µmol/L rolipram on the spontaneous beating rate of rabbit SANC, and 2 µmol/L rolipram was the most potent.

**PDE5**: the basal spontaneous beating rate of guinea-pig right atrial preparation was not affected by 10 µmol/L zaprinast\(^\text{15}\). In our experiments zaprinast concentration was increased to 50 µmol/L, and effects of zaprinast on spontaneous beating rate of rabbit SANC was studied at this concentration.

**PDE1**: there is no information how specific PDE1 inhibitor, MIMX, affects the spontaneous heart beating rate, hence, we used information about MIMX’s inotropic effects. It was reported that 10 µmol/L MIMX was devoid of either a positive inotropic effect or an increase in cAMP level in rat ventricular myocytes\(^\text{16}\). We tested 10 µmol/L MIMX, which only slightly increased rabbit SANC beating rate; therefore, the MIMX concentration was increased to 30 µmol/L and effects of MIMX on SANC spontaneous beating rate was studied at this concentration.

**Drugs**: Drugs were added to the bath solution before the experiment. Broad spectrum PDE inhibitor 3-Isobutyl-1-methylxanthine, IBMX; specific PDE1 inhibitor 8-
Methoxymethyl-3-isobutyl-1-methylxanthine (MIMX), specific PDE2 inhibitor Erythro-9-(2-HYDROXY-3-NONYL)ADENINE (EHNA), specific PDE3 inhibitor 1,6-Dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile (milrinone), specific PDE4 inhibitor 4-[3-(Cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (rolipram), specific PDE5 inhibitor 1,4-Dihydro-5-[2-propoxyphenyl]-7H-1,2,3-triazolo[4,5-d]pyrimidin-7-one (zaprinast) and the membrane permeable cAMP analog 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (CPT-cAMP) were from Sigma. Isoproterenol and ryanodine were from Calbiochem.

**Online Data Supplement: Results**

**Effects of PDE inhibition on delayed rectifier potassium current \( I_K \).**

It is known that delayed rectifier potassium current (\( I_K \)) in rabbit SANC consists of two most important components: a rapidly activating component (\( I_{K,r} \)) and slowly activating component (\( I_{K,s} \)). It is well known that both currents are important for spontaneous beating of rabbit SANC: while \( I_{K,r} \) plays a major role in the pacemaker depolarization\(^{17}\), modulation of \( I_{K,s} \) is important for the positive chronotropic effect of \( \beta \)-AR stimulation\(^{18}\). A previous study also demonstrated that \( I_{K,r} \) and \( I_{K,s} \) are the only significant currents contributing into the outward tail current (\( I_{K,tail} \)) that occurred after a voltage clamp pulse\(^6\). Considering that both components of \( I_K \) might be affected by PDE inhibition, we studied effects of PDE inhibition on total \( I_{K,tail} \) either in the absence or in the presence of ryanodine.
I\textsubscript{K} was activated by 1-second step depolarizations from a holding potential -50 mV to a range of potentials between -40 and +40 mV and measured as an amplitude of the tail current following repolarization to the holding potential (ODS Fig 2A, bottom). In basal conditions broad spectrum PDE inhibitor, IBMX, shifted I\textsubscript{K,tail} activation in the negative direction for approximately 5 mV, however, this effect remained the same and was not affected by ryanodine pretreatment (ODS fig. 2C and 2D).

To study effects of PDE inhibition on I\textsubscript{K,tail} current amplitude, SANC were clamped from -50 mV to +40 mV for 1 second, voltage clamp pulses were given every 11 seconds, and the peak I\textsubscript{K,tail} current was measured and plotted against time. After application of 100 \textmu mol/L IBMX there was a significant (about 12%) increase in I\textsubscript{K,tail} current amplitude (ODS fig. 3A, B) and this effect was fully reversible upon washout (data not shown). Ryanodine (10 \textmu mol/L) did not affect I\textsubscript{K,tail} current amplitude, it remained the same 1.55 ± 0.31 pA/pF in control vs. 1.52 ± 0.31 pA/pF after 4 minute ryanodine application (n=5). After ryanodine pretreatment IBMX produced the same increase in I\textsubscript{K,tail} current amplitude as in control conditions (ODS fig. 3B, C). These data clearly indicate that ryanodine did not change the PDE inhibition-induced modulation of I\textsubscript{K}.

**Online Data Supplement: References.**


**Online Data Supplement: Figure legends.**

**ODS Figure 1:** Photograph of SAN preparation, CT indicates the crista terminalis; IVC indicates inferior vena cava; RA, right atrium and SVC, superior vena cava. The dashed line specifies the area from which SANC were isolated.

**ODS Figure 2:** Effect of IBMX on the I-V relationships and activation of the delayed rectifier current in SANC.

A, B, representative recordings of $I_K$ in the absence and presence of 100 μmol/L IBMX for 5 minutes. $I_K$ was measured as the tail current, $I_{K\text{,tail}}$, following repolarization to -50 mV from 1-sec step depolarizations between -40 and +40 mV. C, D, Steady state activation curves for $I_{K\text{,tail}}$ normalized to the maximum tail current in control (n=5, black squares), in the presence of 100 μmol/L IBMX (n=5, red squares) or 10 μmol/L Ry and 100 μmol/L IBMX (n=3, purple squares). Continuous curves were obtained by fitting of $I_K$ tail currents by a Boltzmann equation.
ODS Figure 3: Effects of IBMX on I\(_K\) current amplitude in the absence or presence of ryanodine.

A, B, Representative current traces demonstrating effects of 100 \(\mu\)mol/L IBMX on I\(_K\) current in the absence (A) or presence (B) of 10 \(\mu\)mol/L ryanodine. One-second step depolarizations were applied to +40 mV, from a holding potential of -50 mV. C, average data of the IBMX-induced increase in the I\(_{K,\text{tail}}\) current amplitude in the absence or presence of 10 \(\mu\)mol/L ryanodine. In control IBMX increased the I\(_{K,\text{tail}}\) current amplitude from 1.13 ± 0.17 pA/pF in control to 1.27 ± 0.17 pA/pF with IBMX (n=7). 4-minute ryanodine application itself did not change I\(_{K,\text{tail}}\) current amplitude, and IBMX application in the presence of 10 \(\mu\)mol/L ryanodine increased the I\(_{K,\text{tail}}\) current amplitude from 1.52 ± 0.31 pA/pF in control to 1.70 ± 0.33 pA/pF with IBMX (n=4).

ODS Figure 4: Suppression of PDE3 increases number and size of LCRs and SR Ca\(^{2+}\) load in ‘skinned’ SANC.

A, Representative confocal line-scan images recorded in saponin-permeabilized SANC bathed in 100 nmol/L free [Ca\(^{2+}\)] before (top) and after (bottom) superfusion with 30 \(\mu\)mol/L milrinone. B, average data of LCR number (normalized to 100 \(\mu\)m length and 1 sec time interval) and size (FWHM) in control (n=11 cells) and after superfusion with 30 \(\mu\)mol/L milrinone (n=14 cells). Error bars indicate standard error of mean. * P<0.05.

ODS Figure 5: Suppression of PDE3 increases number and size of LCRs and SR Ca\(^{2+}\) load in ‘skinned’ SANC.
A, effects of a rapid application of caffeine into the SANC cell in the absence (top) and presence (bottom) of 30 μmol/L milrinone. B, average data on the caffeine-induced increase in SR Ca^{2+} release of the initial rapid component indexed by F/F₀, in a control group of cells (n=25) and group of cells (n=9) subjected to 3 min superfusion with 30 μmol/L milrinone. Error bars indicate standard error of mean. * P<0.05.
Figure 2: Current-voltage (I-V) relationship of an ion channel under different conditions.

A) Control: The current-voltage response shows a smooth transition from depolarization to repolarization.

B) IBMX: The application of IBMX results in a change in the I-V response, indicating a modulation of the channel's activity.

C) Control and IBMX comparison: The graph compares the I-V response of the channel under control conditions and after IBMX treatment.

D) Ry + IBMX: The addition of Ry and IBMX further modifies the I-V curve, suggesting a synergistic effect.

ODS Fig 2: Data analysis and presentation.
A

Control

Milrinone

1 sec

B

Number of events per 1000 ms x 100 μm

Control Milrinone

LCR size, μm

Control Milrinone

ODS Fig. 4
Caffeine-induced Ca\textsuperscript{2+} release, $\Delta F/F_0$

Control: $1.5 \pm 0.2$

Milrinone: $2.0 \pm 0.3$

*Significantly different from Control