Sinoatrial Node Pacemaker Activity Requires Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase II Activation

Tatiana M. Vinogradova, Ying-Ying Zhou, Konstantin Y. Bogdanov, Dongmei Yang, Meike Kuschel, Heping Cheng, Rui-Ping Xiao

Abstract—Cardiac beating arises from the spontaneous rhythmic excitation of sinoatrial (SA) node cells. Here we report that SA node pacemaker activity is critically dependent on Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII). In freshly dissociated rabbit single SA node cells, inhibition of CaMKII by a specific peptide inhibitor, autacamtide-2 inhibitory peptide (AIP, 10 \(\mu\)mol/L), or by KN-93 (0.1 to 3.0 \(\mu\)mol/L), but not its inactive analog, KN-92, depressed the rate and amplitude of spontaneous action potentials (APs) in a dose-dependent manner. Strikingly, 10 \(\mu\)mol/L AIP and 3 \(\mu\)mol/L KN-93 completely arrested SA node cells, which indicates that basal CaMKII activation is obligatory to the genesis of pacemaker AP. To understand the ionic mechanisms of the CaMKII effects, we measured L-type Ca\(^{2+}\) current \((I_{\text{Ca,L}})\), which contributes both to AP upstroke and to pacemaker depolarization. KN-93 (1 \(\mu\)mol/L), but not its inactive analog, KN-92, decreased \(I_{\text{Ca,L}}\) amplitude from 12 ± 2 to 6 ± 1 \(\mu\)A/pF without altering the shape of the current-voltage relationship. Both AIP and KN-93 shifted the midpoint of the steady-state inactivation curve leftward and markedly slowed the recovery of \(I_{\text{Ca,L}}\) from inactivation. Similar results were observed using the fast Ca\(^{2+}\) chelator BAPTA, whereas the slow Ca\(^{2+}\) chelator EGTA had no significant effect, which suggests that CaMKII activity is preferentially regulated by local Ca\(^{2+}\) transients. Indeed, confocal immunocytochemical imaging showed that active CaMKII is highly localized beneath the surface membrane in the vicinity of L-type channels and that AIP and KN-93 significantly reduced CaMKII activity. Thus, we conclude that CaMKII plays a vital role in regulating cardiac pacemaker activity mainly via modulating \(I_{\text{Ca,L}}\) inactivation and reactivation, and local Ca\(^{2+}\) is critically involved in these processes. (Circ Res. 2000;87:760–767.)

Key Words: sinoatrial node ■ L-type Ca\(^{2+}\) channel ■ Ca\(^{2+}\)/calmodulin-dependent kinase II ■ local Ca\(^{2+}\) signaling

The human heart faithfully supplies blood to the body by beating more than 3 billion times in a lifetime. The sinoatrial (SA) node possesses automaticity and serves as the primary physiological pacemaker of the heart. The pacemaker action potential (AP) is initiated in a small group of primary pacemaker cells located in the center of the SA node and then propagates through transitional, peripheral regions to the atrial tissue.1,2 A number of ionic currents are involved in the SA node pacemaker activity, including 2 delayed rectifier potassium currents (mainly the rapidly activated component, \(I_{\text{Kr}}\)), L- and T-type Ca\(^{2+}\) currents (\(I_{\text{Ca,L}}\) and \(I_{\text{Ca,T}}\)), hyperpolarization-activated cation current (\(I_{h}\)), and others.3,4 In primary SA node cells, \(I_{\text{Ca,L}}\) plays an obligatory role in the generation of rhythmic spontaneous APs, because \(I_{\text{Ca,L}}\) is an important source of inward current for the AP upstroke and diastolic depolarization.5,6

Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), a ubiquitous and multifunctional enzyme, is widely involved in Ca\(^{2+}\)-dependent cellular processes. Signals that increase [Ca\(^{2+}\)], activate CaMKII.7 This kinase also retrospectively targets an array of molecules that affect Ca\(^{2+}\) levels. In the heart, CaMKII regulates the sarcoplasmic reticulum (SR) Ca\(^{2+}\) cycling by phosphorylating the Ca\(^{2+}\) release channels and the SR Ca\(^{2+}\)/ATPase regulator, phospholamban.8,9 In ventricular myocytes, activated CaMKII localizes closely to cardiac sarcolemmal membranes and mediates a frequency- and Ca\(^{2+}\)-dependent facilitation of \(I_{\text{Ca,L}}\), which counteracts the voltage- and Ca\(^{2+}\)-dependent inactivation of the channel.10–12 Furthermore, autophosphorylated (active) CaMKII retains its catalytic activity even in the absence of an increase in [Ca\(^{2+}\)].13–15; this biochemical property enables CaMKII to prolong the action of a transient Ca\(^{2+}\) signal or to function as a “frequency detector” of repetitive Ca\(^{2+}\) signals, which makes it ideally suited for the regulation of rhythmic activities such as heart beats.

Given the important role of \(I_{\text{Ca,L}}\) in initiating SA node pacemaker activity and the role of CaMKII in modulating \(I_{\text{Ca,L}}\), we hypothesize that CaMKII may be critically involved

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From the Laboratory of Cardiovascular Sciences (T.M.V., Y.-Y.Z., K.Y.B., D.Y., M.K., H.C., R.-P.X.), National Institute of Aging, Gerontology Research Center, Baltimore, Md, and National Laboratory of Biomembrane and Membrane Biotechnology (D.Y., H.C.), College of Life Sciences, Beijing University, Beijing, China.
Correspondence to Rui-Ping Xiao, MD, PhD, Laboratory of Cardiovascular Science, Gerontology Research Center, NIA, NIH, 5600 Nathan Shock Dr, Baltimore, MD 21224. E-mail xiaor@grc.nia.nih.gov
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in the regulation of SA node spontaneous excitations. Here we examined the effects of CaMKII on SA node spontaneous excitation and underlying ionic mechanisms, particularly modulation of $I_{\text{Ca,L}}$. The present results demonstrate that inhibition of CaMKII with a specific peptide inhibitor or a synthetic inhibitor, KN-93, can completely arrest SA node cells largely as a result of depressed $I_{\text{Ca,L}}$, amplitude, reduced window current, and slowed recovery of L-type Ca$^{2+}$ channels from inactivation. This finding shows, for the first time, a pivotal role of CaMKII in regulating cardiac pacemaker activity.

Materials and Methods

SA Node Cell Isolation

Single SA node cells were isolated according to the modified method of Ito and Ono. Albino rabbits weighing 1.8 to 2.2 kg were deeply anesthetized with sodium pentobarbital (50 to 90 mg/kg). The heart was rapidly excised, and the SA node region was excised and cut into small strips (0.5 to 1.0 mm wide) perpendicular to the crista terminals. The strips were first incubated in Ca$^{2+}$-free Tyrode solution containing (in mmol/L) NaCl 140, KCl 5.4, MgCl$_2$ 0.5, CaCl$_2$ 1.3, NaH$_2$PO$_4$ 0.33, HEPES 5, and glucose 5.5 (pH 6.9) at 33°C and then washed and dispersed in a modified Kraftbruhe solution by gentle pipetting and were stored at 4°C.

Electrophysiological Recordings

Perforated- or ruptured-patch-clamp techniques were used to record spontaneous APs or $I_{\text{Ca,L}}$, respectively, with Axopatch-1D patch-clamp amplifier (Axon Instruments). The bath temperature was maintained at 34±0.5°C. For perforated-patch experiments, amphotericin B (400 µg/mL, Sigma) or β-escin (40 to 50 µmol/L, Sigma) was added to the pipette solution. All potentials were corrected by subtracting the pipette-to-bath liquid junction potential, which was 13 mV for the perforated patch recordings of APs, as calculated using the Clampex 7 software package (Axon Instruments).

For $I_{\text{Ca,L}}$ recordings, depolarizing voltage-clamp pulses (300 ms) were applied from a holding potential of −50 mV; 10 to 30 µmol/L tetrodotoxin and 4 mmol/L 4-aminopyridine were added to block interfering currents. Activation-voltage relationships of $I_{\text{Ca,L}}$ were estimated from normalized conductance-voltage curves. Steady-state inactivation-voltage relationships of $I_{\text{Ca,L}}$ were measured using a 2-pulse protocol in which a variable-amplitude prepulse of 2000 ms was followed by a 200-ms test pulse to 0 mV from a holding potential of −70 mV. To measure the time required for recovery of Ca$^{2+}$ channels from voltage-dependent inactivation, the cell was depolarized to 0 mV for 300 ms to inactivate Ca$^{2+}$ channels, then repolarized to −50 mV for various durations to allow channel recovery (20 to 2300 ms), and finally depolarized to 0 mV for 300 ms. The percentage of $I_{\text{Ca,L}}$ restored in the test pulse was used to calculate the channel recovery.

Immunocytochemical Staining of CaMKII

The intracellular distribution of total and autophosphorylated (active) CaMKII was visualized in isolated SA node cells by confocal fluorescence microscopy. Cells were incubated overnight at 4°C with the monoclonal CaMKII antibodies (Affinity Bioreagents Inc), followed by an incubation for 4 hours with a Texas Red–conjugated anti-mouse antibody (Vector Laboratories Inc). Immunostaining of an autophosphorylated form of CaMKII was repeated after 30-minute pretreatment of cells with either of the CaMKII inhibitors KN-93 or autocamtide-2 inhibitory peptide (AIP). Immunostaining was then detected with a laser scanning confocal microscope (LSM-410, Zeiss).

Statistical Analysis

Data are given as mean±SEM. The difference of mean values were analyzed by Student t test; ANOVA was used to compare groups of data, and $P<0.05$ was considered statistically significant.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Inhibition of CaMKII Suppresses Spontaneous AP in SA Node Cells

We studied spontaneous excitation of freshly isolated, spindle-shaped SA node cells using the perforated patch-clamp technique (Figure 1). Under control conditions at 34°C, parameters of AP were as follows: amplitude, 84.2±2.9 mV; duration at 50% repolarization, 102.2±5.7 ms; upstroke velocity (dV/dt), 8.5±1.0 V/s; and cycle length, 357.1±17.0 ms (n = 24). The diastolic depolarization rate was 57.6±3.8 mVs, and the maximum diastolic potential (MDP) was −65.5±1.4 mV (n = 24). These values are highly comparable with those reported previously.

Figure 1 shows effects of a highly specific peptide inhibitor of CaMKII (AIP, membrane-permeable form, 10 µmol/L) on SA node pacemaking. This peptide, which corresponds to an
by superfusing cells with a specific CaMKII inhibitor, KN-93. \(^{20}\) Figures 2A through 2C show representative continuous recordings of APs in the absence (left panels) and presence (right panels) of KN-93, at different concentrations. KN-93 at a low concentration (0.3 \(\mu\)mol/L) reduced the rate of spontaneous excitations \((P<0.05)\) and absolute value of MDP (Figure 2A); these effects were reversible on washout of the inhibitor (data not shown). In the presence of 1 \(\mu\)mol/L KN-93, the negative chronotropic effect was more pronounced; excitations became irregular, and AP amplitudes became unstable. The disturbed pacemaker activity was associated with a \(\approx 10\)-mV reduction of MDP (Figure 2B).

More strikingly, application of a higher concentration of KN-93 (3 \(\mu\)mol/L) completely abolished spontaneous excitations (Figure 2C). In contrast, KN-92 (1 \(\mu\)mol/L; \(n=6\)), an inactive KN-93 analogue, had no significant effect on any examined AP parameter (Figure 2D), confirming the specificity of the CaMKII inhibitor. Figures 3A through 3D summarize the average effects of KN-93 on AP parameters that were similar to those induced by AIP (Figure 1B). The CaMKII inhibitor induced a robust change in all AP parameters in a dose-dependent manner. Spontaneous excitations were abolished in 2 of 6 cells and in 4 of 4 cells by KN-93 at concentrations of 1 and 3 \(\mu\)mol/L, respectively. Taken together, our data indicate that CaMKII plays an essential role in SA node pacemaking and that basal CaMKII activation is indispensable for the SA node cell to generate APs.

Effects of CaMKII on \(I_{\text{Ca}, L}\)

To delineate the ionic mechanisms underlying CaMKII-mediated modulation of SA node pacemaker activity, we measured \(I_{\text{Ca}, L}\) in the presence or absence of the CaMKII inhibitors, using a whole-cell patch-clamp configuration. KN-93 (1 \(\mu\)mol/L) induced a 50% decrease in \(I_{\text{Ca}, L}\) amplitude (from 12 \(\pm\)2 to 6 \(\pm\)1 pA/pF at 0 mV, \(P<0.01\)) without altering the current-voltage relationship (Figure 4A). The time-dependent inactivation of \(I_{\text{Ca}, L}\) fitted by a double-exponential function was not significantly altered by KN-93 (\(\tau_1=6.9\pm0.27\) ms and \(\tau_2=37.6\pm1.4\) ms \([n=19]\)) in control
cells; \( \tau_i = 7.7 \pm 0.31 \) ms and \( \tau_s = 33.9 \pm 1.4 \) [n = 10] in KN-93-treated cells). The inactive analog KN-92 (1 \( \mu \)mol/L) had no significant effect on \( I_{Ca,L} \) amplitude (13 \( \pm \) 1 pA/pF at 0 mV [n = 3, \( P > 0.05 \) versus control]) or other parameters examined (data not shown).

To define the specific mode of CaMKII-mediated modulation of \( I_{Ca,L} \), we examined the voltage-dependent activation and steady-state inactivation of \( I_{Ca,L} \). As shown in Figure 4B, the threshold for \( I_{Ca,L} \) activation was \( \approx -40 \) mV, and the saturation occurred at \( \approx +10 \) mV in the SA node cells. Inhibition of CaMKII (KN-93, 1 \( \mu \)mol/L) had virtually no effect on the slope factor (7.3 \( \pm \) 0.2 mV in controls versus 7.4 \( \pm \) 0.4 mV with KN-93), the midpoint voltage (\( V_0 = -16.1 \pm 1.3 \) mV in control versus \( -16.1 \pm 1.5 \) mV with KN-93), or the overall shape of the activation curve. In contrast, inhibition of CaMKII had a profound effect on the steady-state inactivation of the channel, as manifested by a parallel shift of the inactivation curve by 11.2 \( \pm \) 1.1 mV (\( P < 0.01 \)) toward more negative potentials (Figure 4B). In the presence of KN-93, the midpoint inactivation voltage was \( -45.2 \pm 1.3 \) mV, and the slope factor for inactivation was 6.1 \( \pm \) 0.2 mV, which indicates that a substantial number of L-type channels must be inactivated at the MDPs (\( -54.4 \pm 3.2 \) mV, n = 4) in the presence of CaMKII inhibitor (Figure 4B). Similarly, AIP (100 \( \mu \)mol/L in pipette solution, >30 minutes) shifted the steady-state inactivation curve of \( I_{Ca,L} \) leftward by 5.3 \( \pm \) 0.3 mV (\( P < 0.01 \)) without altering the activation curve (Figure 5A). Thus, CaMKII activity is required to alleviate the steady-state inactivation of L-type Ca\({\text{2+}}\) channels, maintaining the channel availability in the pacemaker cells.

In cells undergoing rhythmic excitations, L-type channel recovery from inactivation is another important determinant of channel availability. To gain further insight into the CaMKII-mediated modulation of pacemaker activity, we measured the time course for recovery of \( I_{Ca,L} \) from inactivation using a dual-pulse protocol (see Materials and Methods). The results are summarized in Figure 5B. At a holding potential of \( -50 \) mV, the recovery of \( I_{Ca,L} \) was fitted by 2 exponentials, with a fast component being predominant (\( \tau_r = 71 \) ms, \( A_1 = 70\% \); \( \tau_s = 612 \) ms, \( A_2 = 30\% \)).
CaMKII in L-type channel reactivation was confirmed by the fact that AIP markedly slowed the recovery of Ca\(^{2+}\) channels from inactivation (\(\tau_1 = 174\) ms, \(A_1 = 50\%\); \(\tau_2 = 1085\) ms, \(A_2 = 50\%)\) (Figure 5B). In the presence of KN-93 (1 \(\mu\)mol/L, \(n=6\)), both time constants were also markedly prolonged (\(\tau_1 = 120\) ms; \(\tau_2 = 2000\) ms), with the slow component being dominant (\(A_1 = 26\%; A_2 = 74\%\)). KN-92 (1 \(\mu\)mol/L, \(n=6\)), the inactive analogue of KN-93, had no significant effect on the recovery kinetics of the channel. These results reveal another mode of CaMKII action, ie, ensuring the reactivation of L-type channels during each excitation cycle. The markedly slowed recovery of the channel from inactivation contributes, at least in part, to KN-93–induced reduction of \(I_{\text{Ca, L}}\) amplitude depicted in Figure 4A. The effects of AIP and KN-93 on \(I_{\text{Ca, L}}\) indicate that some basal CaMKII activity exists in SA node cells dialyzed with 10 mmol/L EGTA, as is the case in rat ventricular myocytes.10

The results described above suggest that the inhibitory effects of CaMKII inhibitors on SA node pacemaker activity are largely mediated by suppressing \(I_{\text{Ca, L}}\) activation and reactivation. Next, we directly tested this idea using an L-type Ca\(^{2+}\) channel blocker, nifedipine (\(n=4\)). A representative example is shown in Figure 6. Similar to the CaMKII inhibitor, nifedipine (0.2 \(\mu\)mol/L) depressed all of the parameters of AP, including AP frequency, amplitude, rate of AP upstroke, and reduced MDP (Figure 6B). After a 5-minute perfusion, nifedipine completely arrested the pacemaker cell (Figure 6C). Thus, the effects of CaMKII inhibition on the pacemaker activity can be largely mimicked by a selective blockade of \(I_{\text{Ca, L}}\).

### Intracellular Localization of CaMKII

To determine intracellular distribution of autophosphorylated (active) as well as total CaMKII in the SA node cell, we used a site-specific antibody against CaMKII phosphorylated at Thr286 and another antibody that recognizes CaMKII regardless of its phosphorylation state. Confocal immunofluorescence imaging showed that active CaMKII was concentrated beneath the surface membrane (Figure 7B), whereas total CaMKII was present uniformly in the SA node cell (Figure 7A). Figure 7C shows the negative control image obtained in the absence of any primary antibody; the nonspecific staining was negligible. The restricted localization of active CaMKII to the surface membrane is consistent with the idea that CaMKII targets sarcosomal membrane–delimited substrates, particularly L-type Ca\(^{2+}\) channels, and that CaMKII activity is likely regulated by local Ca\(^{2+}\) gradients in the submembrane microdomains (see below). Pretreatment of cells with agents blocking CaMKII activity, KN-93 or AIP, significantly decreased the amount of the active form of CaMKII (Figure 7D), which substantiates the idea that both agents acted through inhibition of CaMKII.

### Role of Local Ca\(^{2+}\) in CaMKII-Mediated Modulation of Pacemaker Activity

Theoretically, Ca\(^{2+}\) influx through L-type channels as well as \(I_{\text{Ca, L}}\)-triggered Ca\(^{2+}\) release from the abutting SR may generate a local increase in \([\text{Ca}^{2+}]\), the activator of CaMKII. We therefore hypothesized that CaMKII is preferentially activated by local high Ca\(^{2+}\) transients in the microdomain of the surface membrane and that the active CaMKII, in turn,
mediates a positive feedback regulation of L-type channels, which contributes to the pacemaker activity. To test this hypothesis, we examined susceptibility of pacemaker APs and \( I_{Ca,L} \) to Ca\(^{2+} \) buffers with different kinetics, EGTA and BAPTA. Because the kinetics of BAPTA are \( \approx \)100-fold faster than those of EGTA,\(^2\) BAPTA is much more efficient in buffering local Ca\(^{2+} \) transients, even though both can effectively suppress global Ca\(^{2+} \) transients. Confocal imaging verified that global Ca\(^{2+} \) transients, as measured by the Ca\(^{2+} \) indicator fluo-4, nearly vanished after an exposure of the SA node cells to either BAPTA-AM (5 \( \mu \)mol/L) or EGTA-AM (30 \( \mu \)mol/L) for 10 minutes at 34°C (data not shown). However, only BAPTA significantly reduced the rate and amplitude of spontaneous AP by 54% and 14%, respectively, and reduced MDP by 8 mV (Figures 8B through 8D). It is noteworthy that BAPTA also led to an irregular beating pattern that was characterized by missing beats (Figure 8A). In contrast, the slow Ca\(^{2+} \) buffer, EGTA, only slightly reduced the rate of spontaneous excitations and did not change the amplitude of AP as well as MDP (Figure 8). In voltage-clamped cells, BAPTA hampered the \( I_{Ca,L} \) recovery from inactivation in a manner similar to that of the CaMKII inhibitor KN-93. Specifically, when EGTA (10 mmol/L) in the pipette solution was substituted by BAPTA (10 mmol/L), the \( I_{Ca,L} \) recovery time constants were increased to \( \tau_{1}=120 \) and \( \tau_{2}=3660 \) ms, respectively. Taken together, these results indicate that in SA node cells, the spontaneous AP and \( I_{Ca,L} \) are far more sensitive to BAPTA than to EGTA, which supports the idea that local Ca\(^{2+} \) transients are critically involved in CaMKII-dependent pacemaker activity.

### Discussion

#### CaMKII Activation Is Essential to Cardiac Pacemaker Activity

The spontaneous excitation of SA node pacemaker cells in the mammalian heart is under tight neuronal and hormonal control. For instance, β-adrenergic stimulation by the adrenal hormone epinephrine or the sympathetic neurotransmitter norepinephrine elicits a positive chronotropic effect through a cAMP/protein kinase A (PKA) signaling pathway.\(^4\)\(^,\)\(^2\) Conversely, parasympathetic stimulation exerts a potent negative chronotropic effect.\(^23\) In the present study, we demonstrated that in SA node cells basal CaMKII activation is essential for pacemaker activity. This conclusion is based on the following lines of evidence: (1) SA node cell pacemaker activity is depressed by the CaMKII inhibitor in a dose-dependent manner (Figures 1 through 3); (2) the abolition of spontaneous excitations by a high concentration of KN-93 (3 \( \mu \)mol/L) or inhibitory peptide AIP (10 \( \mu \)mol/L) suggests that a minimal level of CaMKII activation is obligatory to the genesis of the spontaneous excitations; and (3) an intermediate inhibition of CaMKII by KN-93 (0.3 to 1 \( \mu \)mol/L) or BAPTA (5 \( \mu \)mol/L) disrupts the rhythm and stability of pacemaker APs, which indicates that a certain amount of CaMKII activation is required to ensure optimal pacemaker function.

Compared with the well-characterized nervous and hormonal regulation, the CaMKII-mediated regulation of pacemaker activity is unique in several important aspects. First, CaMKII-dependent modulation is intrinsic to the SA node cells, operating tonically to maintain the excitability of the pacemaker cells. Second, in addition to its strong modulatory effects, CaMKII also plays an important permissive role in cardiac pacemaking, as demonstrated in the present study. In this regard, the effect of PKA is mostly modulatory because inhibition of basal PKA activity by 2 \( \mu \)mol/L H-89,\(^2\)\(^,\)\(^4\) which fully prevents the positive chronotropic effect induced by the β-adrenergic agonist isoproterenol, fails to abolish SA node pacemaker activity (data not shown). Third, CaMKII regulation of the pacemaker activity is a positive feedback by nature because CaMKII, which augments \( I_{Ca,L} \), is activated by local Ca\(^{2+} \) transients produced directly by \( I_{Ca,L} \) or indirectly by \( I_{Ca,L} \).
l-induced Ca\(^{2+}\) release from the SR. Finally, the CaMKII-mediated modulation could be self-adaptive. For example, an increase in heart rate would, on one hand, reduce the pacemaker \(I_{\text{Ca,L}}\) because of enhanced voltage-dependent inactivation and insufficient time for the channel recovery from inactivation. On the other hand, on the basis of its “memory” properties, CaMKII would serve as the “frequency detector” to integrate the local Ca\(^{2+}\) signals; the faster the heart beats, the more frequent the local Ca\(^{2+}\) transients, and thus the greater the CaMKII activity. The enhanced CaMKII activity alleviates steady-state inactivation and promotes channel recovery from inactivation, maintaining pacemaker activity at a higher set point. Thus, although negative feedback regulation, eg, \(I_{\text{Ca,L}}\) inactivation, stabilizes an established pacemaker frequency, the self-adaptive CaMKII activity permits the heart rate to change over a wider dynamic range.

**Ionic Mechanism of CaMKII Action**

The most prominent effects of CaMKII inhibition were the decrease of AP parameters (frequency, amplitude, and upstroke rate) or even an abolition of the spontaneous excitations (Figures 1 through 3). A decrease of MDP after CaMKII inhibition per se could not explain the changes in AP parameters, because in control cells membrane depolarization (\(\approx \text{mV}\)) induced an increase rather than a decrease in the rate of spontaneous excitations (data not shown). In KN-93–treated cells, AP parameters were only partially restored when MDP was restored by injection of a hyperpolarizing current (data not shown). To unravel the ionic mechanism underlying the modulatory effects of CaMKII on SA node pacemaker activity, we examined the possible involvement of \(I_{\text{Ca,L}}\) and found that suppression of CaMKII activity by KN-93 reduces \(I_{\text{Ca,L}}\) amplitude by 50% (Figure 4A), which is similar to previous observations in ventricular myocytes. The recovery of \(I_{\text{Ca,L}}\) from inactivation is also markedly slowed by AIP (Figure 5B), KN-93, or the fast Ca\(^{2+}\) buffer BAPTA. This is consistent with the leftward shift of the steady-state inactivation curve caused by the CaMKII inhibitors, which contributes to the decrease in L-type channel availability, particularly at the depolarized MDP.

In SA node cells, the leftward shift of steady-state inactivation curve of \(I_{\text{Ca,L}}\) is not associated with any shift of the voltage-dependent activation of the current, resulting in a markedly reduced “window” current (the overlap area of the steady-state inactivation and activation curves; Figures 4B and 5A). It has been shown that in rabbit SA node cells, the L-type window current contributes to the pacemaker potential. Thus, the decrease in the window current could, in part, explain the inhibitory effects of the CaMKII inhibitors on spontaneous excitations of SA node cells.

Because \(I_{\text{Ca,L}}\) constitutes one of the main ionic currents responsible for excitations of SA node cells, the suppression of \(I_{\text{Ca,L}}\) provides a straightforward explanation for inhibitory effects of the CaMKII inhibitor on AP. However, the paradox is that a reduction of the inward current was accompanied by a depolarization, instead of a hyperpolarization, of the membrane potential. One possible explanation is that the depolarization or inhibition of AP may secondarily reduce or preclude K\(^{+}\) conductance activated by an AP (eg, delayed rectifier K\(^{+}\) current) such that the net result is a reduction in the outward currents and thereby membrane depolarization. This interpretation is supported by a computer simulation of the SA node pacemaker using the OXSOFT HEART model. Direct evidence is given in Figure 6, which shows that the L-type channel antagonist nifedipine similarly reduces MDP, consistent with the previous reports. Taking these data together, we conclude that modulation of the voltage- and time-dependent properties of L-type channel inactivation by CaMKII is the primary mechanism underlying the CaMKII-mediated regulation of SA node pacemaker activity.

In addition to \(I_{\text{Ca,L}}\), several other ionic currents are involved in SA node pacemaker activity, including \(I_{\text{K_s}}\), \(I_{\text{f}}\), sustained current, T-type Ca\(^{2+}\) current, and the muscarinic K\(^{+}\) current \((I_{\text{K_ACh}})\). However, inhibition of \(I_{\text{f}}\) causes only minor changes in AP parameters. Recent studies in rabbit ventricular myocytes have demonstrated that CaMKII inhibition by peptide AC3-I has no significant effect on \(I_{\text{K_s}}\), which suggests that \(I_{\text{K_s}}\) and \(I_{\text{f}}\) are not critically involved in CaMKII-dependent regulation of spontaneous excitations. Further studies are required to determine possible contributions of the other pacemaker currents to CaMKII-dependent regulation of SA node pacemaker activity.

**Regulation of CaMKII Activation by Local Ca\(^{2+}\) Signaling**

CaMKII activity is regulated in a Ca\(^{2+}\)- and calmodulin-dependent manner. To delineate the contribution of local versus global Ca\(^{2+}\) transients in the temporal and spatial control of CaMKII activation, we directly visualized the intracellular distribution of the active versus the total CaMKII and found a uniform distribution of the total CaMKII but a highly localized distribution of the active CaMKII to the subsarcolemmal microdomain. This spatial pattern of active CaMKII in SA node cells fits nicely with the identified functional role of CaMKII in regulating the sarcoplasmic Ca\(^{2+}\) channels and supports the idea of a local control of CaMKII activation by local Ca\(^{2+}\) transients. Indeed, a fast Ca\(^{2+}\) buffer, BAPTA, significantly suppressed the spontaneous excitations of SA node cells and slowed \(I_{\text{Ca,L}}\) recovery from inactivation, which mimics the effects of direct inhibition of CaMKII. In contrast, the slow Ca\(^{2+}\) buffer EGTA has no significant effect on either AP or \(I_{\text{Ca,L}}\) in these SA node cells, probably because it cannot effectively buffer the local Ca\(^{2+}\). Thus results are in agreement with our previous observation in rat ventricular myocytes that BAPTA, but not EGTA, abolishes CaMKII-dependent facilitation during repetitive depolarizations. Thus, subsarcolemmal Ca\(^{2+}\) transients play a critical role in the local activation of CaMKII, which, in turn, mediates a positive feedback regulation of \(I_{\text{Ca,L}}\) in SA node cells.

In summary, whereas previous studies focused on the role of \(\beta\)-adrenergic and muscarinic stimulation in modulation of the heart rate, the present study demonstrates that SA node pacemaker activity is subject to an intrinsic regulation by CaMKII. The CaMKII-mediated regulation is unique as compared with the well-established hormonal or neuronal control because of its inherent positive feedback and self-adaptive properties. In addition, CaMKII may afford an important integrating mechanism for distinct Ca\(^{2+}\) and other...
signals\textsuperscript{32} to regulate heart rate. For example, \(\beta\)-adrenergic receptor stimulation may cross-talk with the CaMKII signaling pathway by enhancing \(I_{Ca,t}\) and SR Ca\(^{2+}\) cycling or by PKA-mediated, Ca\(^{2+}\)-independent phosphorylation of CaMKII at Thr286.\textsuperscript{33} Thus, in SA node cells under physiological conditions, CaMKII plays both permissive and modulatory roles in cardiac pacemaker activity via modulating L-type Ca\(^{2+}\) channels.

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