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Myristoylated peptides potentiate the funny current (I_f) in sinoatrial myocytes

Zhandi Liao,[1] Joshua R. St. Clair,[1] Eric D. Larson[1] and Catherine Proenza[1,2,*]

[1]Department of Physiology and Biophysics; [2]Department of Medicine, Division of Cardiology; University of Colorado; Aurora, CO USA

The funny current, I_f, in sinoatrial myocytes is thought to contribute to the sympathetic fight-or-flight increase in heart rate. I_f is produced by hyperpolarization-activated cyclic nucleotide sensitive-4 (HCN4) channels, and it is widely believed that sympathetic regulation of I_f occurs via direct binding of cAMP to HCN4, independent of phosphorylation. However, we have recently shown that Protein Kinase A (PKA) activity is required for sympathetic regulation of I_f, and that PKA can directly phosphorylate HCN4.[1] In the present study, we examined the effects of a myristoylated PKA inhibitory peptide (myr-PKI) on I_f in mouse sinoatrial myocytes. We found that myr-PKI and another myristoylated peptide potently and specifically potentiated I_f via a mechanism that did not involve PKA inhibition and that was independent of the peptide sequence, Protein Kinase C or phosphatidylinositol-4,5-bisphosphate. The off-target activation of I_f by myristoylated peptides limits their usefulness for studies of pacemaker mechanisms in sinoatrial myocytes.

Key words: sinoatrial node, hyperpolarization-activated cyclic nucleotide sensitive channel, HCN4, cAMP-dependent protein kinase, cardiac pacemaking, heart rate regulation

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*Correspondence to: Catherine Proenza; Email: catherine.proenza@ucdenver.edu

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Introduction

The sympathetic fight-or-flight increase in heart rate involves an increase in intracellular cAMP in pacemaker myocytes in the sinoatrial node; however, the molecular mechanisms for this signaling are not well understood. Hyperpolarization-activated, cyclic nucleotide sensitive-4 (HCN4) channels are among the downstream targets of cAMP in sinoatrial myocytes. HCN4 channels produce the "funny current" (I_f), a mixed Na^+/K^+ current with a reversal potential of ~-30 mV. Open HCN4 channels thus conduct a net inward current during diastole, and are thereby thought to contribute to the spontaneous "diastolic depolarization" that drives pacemaking. Sympathetic stimulation of β adrenergic receptors on sinoatrial myocytes shifts the voltage-dependence of activation of I_f to more depolarized potentials, and the ensuing increase in I_f during diastole can contribute to heart rate acceleration.

It is widely believed that β adrenergic activation of I_f occurs via direct binding of cAMP to HCN4 channel proteins, independent of phosphorylation. However, we recently demonstrated that the cAMP-dependent protein kinase (Protein Kinase A, PKA) is essential for sympathetic regulation of I_f in mouse sinoatrial myocytes.[1] Inhibition of PKA by intracellular perfusion with the PKA pseudosubstrate inhibitory peptide, PKI_{6-22} amide (PKI), almost abolished the ability of the β adrenergic agonist isoproterenol (ISO) to shift the voltage-dependence of I_f. Moreover, PKA caused a depolarizing shift in the voltage-dependence of activation of heterologously-expressed HCN4 channels. In vitro phosphorylation assays and mass spectrometry revealed that PKA can directly phosphorylate HCN4 at numerous sites in the intracellular amino and carboxyl terminals. A cluster of four serine/threonine residues within a single consensus PKA site in the distal carboxyl terminus was found to mediate the PKA-dependent shift in voltage-dependence of I_f.

We wanted to follow-up on two significant questions that were raised by our study: (1) does PKA phosphorylation

of HCN4 contribute to sympathetic regulation of heart rate? and (2) how do direct binding of cAMP and PKA phosphorylation interact in regulation of I_f in sinoatrial myocytes? In initial experiments directed at these questions, we discovered that a myristoylated version of PKI (myr-PKI) potentiated I_f, an effect opposite to that of the non-myristoylated PKI peptide. The present study is an aside that examines the basis for this unexpected effect. We report that myr-PKI and another myristoylated peptide specifically and potently activate I_f via a mechanism that does not involve inhibition of PKA, and that is independent of the peptide sequence, protein kinase C (PKC) activity or phosphatidylinositol-4,5-bisphosphate (PIP_2) synthesis. The off-target activation of I_f by myr-PKI precludes use of this reagent in studies of pacemaker mechanisms in sinoatrial myocytes.

Results

Myr-PKI increases I_f via a PKA-independent mechanism. As a follow-up to our studies of PKA regulation of I_f in sinoatrial myocytes, we attempted to do internally-controlled experiments in single cells by recording the current before and after wash-on of myristoylated PKI_{14-22} amide (myr-PKI, 2 μM), which is marketed as a cell permeable version of PKI. Surprisingly, myr-PKI rapidly and potently increased the amplitude of I_f in whole cell recordings from isolated sinoatrial myocytes from mice (n = 13; **Fig. 1A**). In most cells, the increase in I_f was followed quickly by a loss of voltage clamp control (e.g., **Fig. 1B and C**). Both the increase in I_f and loss of voltage control were generally reversible, however the loss of voltage clamp control prevented measurement of the voltage-dependence for I_f in the presence of myr-PKI, even at a lower concentration (200 nM; data not shown). These effects of myr-PKI appeared to result specifically from activation of I_f—rather than from non-specific effects on the membrane or activation of other ionic conductances—because myr-PKI had no effect on currents elicited by hyperpolarizing voltage steps when I_f was blocked with either Cs^+ (5 mM, n = 5, **Fig. 1A**) or ZD7288 (100 μM, n = 4, data not shown).

The potentiation of I_f by myr-PKI was surprising because it was opposite to effects we observed in our previous study[1] for the non-myristoylated PKI_{6-22} peptide, which was shown to (1) inhibit β adrenergic regulation of I_f in sinoatrial myocytes, (2) prevent PKA activation of heterologously-expressed HCN4 channels, (3) inhibit PKA phosphorylation of HCN4 in vitro and (4) have no effect on voltage clamp control. Thus, we next asked whether the myr-PKI potentiation of I_f actually involved inhibition of PKA. We addressed this question first by including the non-myristoylated PKI peptide (10 μM) in the patch pipette during whole-cell recordings, as we had done in our previous study. As shown in **Figure 1B**, myr-PKI still increased I_f even in the presence of a 5-fold excess of the non-myristoylated PKI peptide (n = 4), suggesting that PKI and myr-PKI affect I_f via different mechanisms.

In our earlier study, we demonstrated that PKA regulation of I_f was mediated by a site in the distal carboxyl terminus of HCN4; PKA had no effect on mutant HCN4-Cx4 channels, in which four serine/threonine residues within a single PKA site were neutralized to alanine (T1153A, S1154A, S1155A and S1157A).[1] As shown in **Figure 1C**, myr-PKI potentiated I_f produced by HCN4-Cx4 channels expressed in HEK cells (n = 4), demonstrating not only that myr-PKI acts via a mechanism distinct from that of PKA in its regulation of HCN4, but also that this mechanism is present in HEK and CHO (data not shown) cells as well as in mouse sinoatrial myocytes.

To determine whether the potentiation of I_f by myr-PKI depended on the PKI peptide sequence itself, we next examined the effects on I_f of another myristoylated peptide, myristoylated protein kinase C 19–31 inhibitory peptide (myr-PKC_{19-31}). We found that, like myr-PKI, myr-PKC_{19-31} dramatically increased I_f (n = 3; **Fig. 1D**). Thus, the mechanism by which myr-PKI activates HCN4 channels is independent of the exact peptide sequence, and is clearly independent of PKA inhibition. Given this, and the observation that non-myristoylated PKI or PKC_{19-31} peptides do not activate I_f (ref. 1, **Fig. 2B**), we next asked whether the myristoyl group itself was sufficient to mediate this effect. Accordingly, we applied to isolated sinoatrial myocytes myristic acid (2 μM, n = 5 or 100 μM, n = 3), myristamide (2 μM, n = 6), or methyl myristate (5 μM, n = 4 or 10 μM, n = 3). None of these compounds had any effect on I_f (data not shown), suggesting that both the myristoyl and peptide moieties of myristoylated peptides are necessary for their activation of HCN4 channels.

Myr-PKI potentiation of I_f is independent of PIP_2 and PKC. The membrane lipid PIP_2 causes a large depolarizing shift in the voltage dependence of activation of HCN channels.[2,3] We investigated whether myr-PKI increases I_f via an acute increase in PIP_2 levels by exposing sinoatrial myocytes to wortmannin, which blocks PIP_2 synthesis by inhibiting phosphatidylinositol 4-kinase. We found that pre-incubation of sinoatrial myocytes in wortmannin (15 μM, 30–60 min) had no effect on the ability of myr-PKI to potentiate I_f (n = 5; **Fig. 2A**). Acute wash-on of wortmannin (15 μM, 1–3 minutes) also did not attenuate the ability of myr-PKI to increase I_f, although it slightly decreased the amplitude of I_f, as expected for a modest reduction in PIP_2 levels (n = 4; data not shown). These data suggested that myristoylated peptides increase I_f via a mechanism that is independent of PIP_2 synthesis.

It has previously been shown that a variety of myristoylated peptides—including myr-PKI—can non-specifically inhibit PKC.[4] We therefore hypothesized that the mechanism for the potentiation of I_f by myr-PKI and myr-PKC_{19-31} could be via inhibition of PKC. To test this hypothesis, we inhibited PKC using either the non-myristoylated PKC_{19-31} inhibitory peptide in the patch pipette (10 μM; n = 10; **Fig. 2B**) or staurosporine, a broad-spectrum kinase inhibitor, which was applied acutely by perfusion for ~3 min (1 μM; n = 3; **Fig. 2C**) or by pre-incubation for 30–60 min (1 μM; n = 3; data not shown). Neither PKC_{19-31} nor staurosporine mimicked the potentiation of I_f caused by myristoylated peptides—indeed wash-on of staurosporine reduced the amplitude of I_f in some cells, perhaps via inhibition of PKA—and neither inhibitor altered the ability of myr-PKI to increase I_f (**Fig. 2B and C**). These data indicate that myristoylated peptides increase I_f

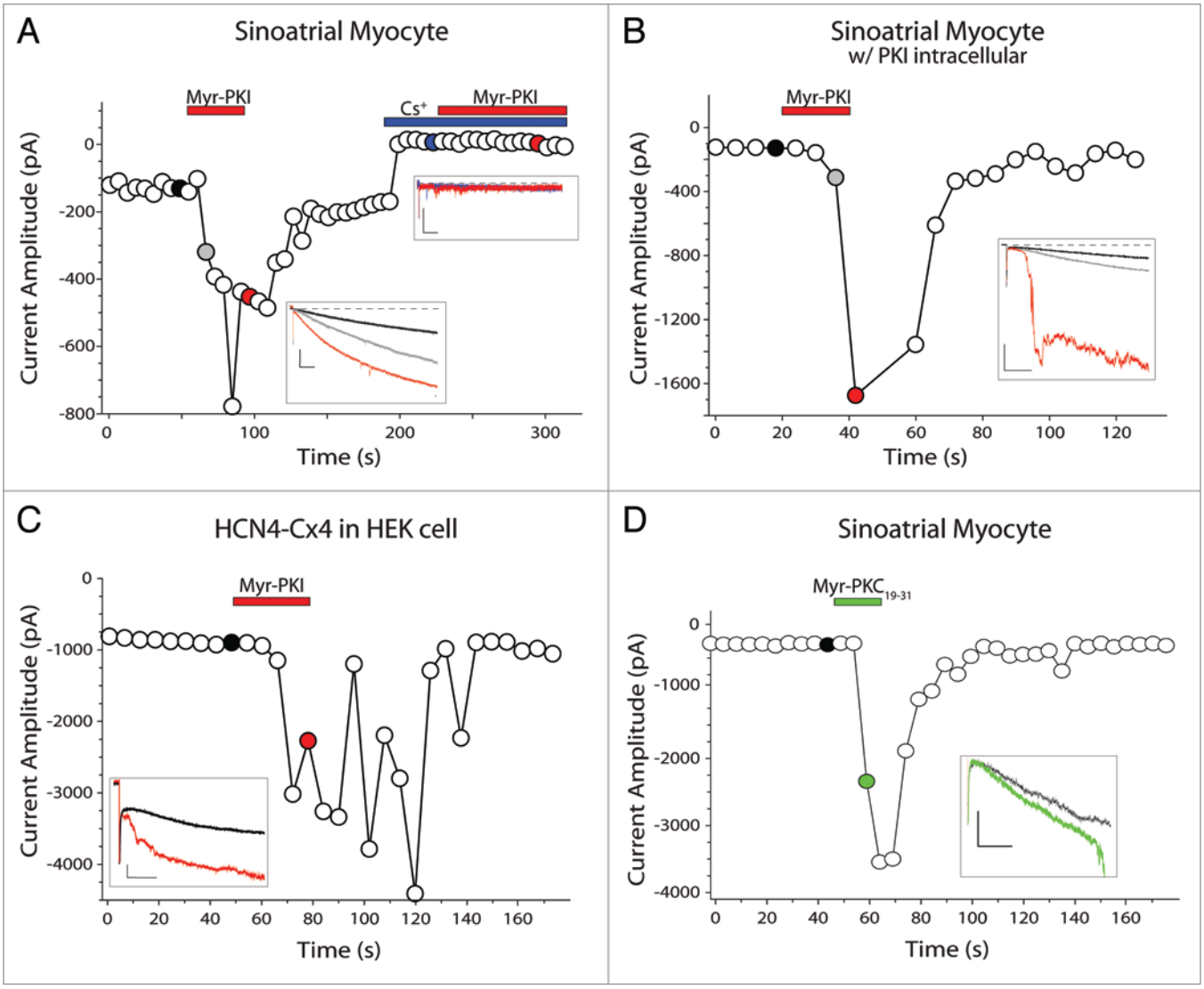

Figure 1. Myristoylated peptides increase I_f via a PKA-independent mechanism. I_f current amplitude as a function of time for sinoatrial myocytes exposed to 2 μM myr-PKI or 2 μM myr-PKC_{19-31}. Whole-cell currents were elicited by 2 s voltage steps to -120 mV from a holding potential of -50 mV every 6 s. Filled symbols indicate the traces shown in the insets. Dashed lines indicate zero current. (A) Myr-PKI effects on hyperpolarization-activated currents in the absence or presence of 5 mM Cs^+. Scale bars in insets, 100 pA/100 ms. (B) I_f amplitude for a myocyte perfused with myr-PKI in the presence of 10 μM non-myristoylated PKI in the patch pipette. Scale bars in inset, 400 pA/100 ms. (C) Current amplitude for HCN4-Cx4 channels expressed in HEK293 cells during perfusion of myr-PKI. Scale bars in inset, 500 pA/200 ms. (D) I_f amplitude for a myocyte perfused with 2 μM Myr-PKC_{19-31}. Scale bars in inset, 100 pA/100 ms.

via a PKC-independent mechanism. In addition, the data further support the conclusion that myr-PKI potentiation of I_f is independent of PKA inhibition, since staurosporine also blocks PKA (and a number of other kinases) with nanomolar affinity.[5]

In the course of these experiments, we also asked whether PKC activity can regulate I_f in sinoatrial myocytes, independent of the effects of myristoylated peptides. We found that phorbol 12-myristate 12-acetate (PMA, an activator of conventional and novel PKC isoforms) had no effect on I_f when it was perfused onto the cells for ~3 min (500 nM; **Fig. 2D**; n = 8). In addition, pre-incubation of the cells with PMA (500 nM, 30 min), or inclusion in the patch pipette of either the catalytic subunit of PKC (PKC_c; 30 nM) or the PKC_{19-31} inhibitory peptide (10 μM) had no significant effect on the voltage-dependence of activation for I_f ($V_{1/2}$ = -126.3 ± 3.1 mV, n = 6 in control intracellular; -123.9 ± 2.6 mV, n = 7 with PMA pre-incubation; -124.5 ± 5.0 mV, n = 10 with PKC_c intracellular; -120.1 ± 2.2 mV, n = 9 with PKC_{19-31} intracellular; $p > 0.05$ for all pairwise comparisons). Taken together, these data suggest that PKC is not a major modulator of I_f in sinoatrial myocytes.

Discussion

In this study we demonstrated that myristoylated peptides activate I_f in mouse sinoatrial myocytes via a mechanism that is independent of the peptide sequence, PKA, PKC or PIP_2. We also demonstrated that PKC has little effect on I_f in sinoatrial

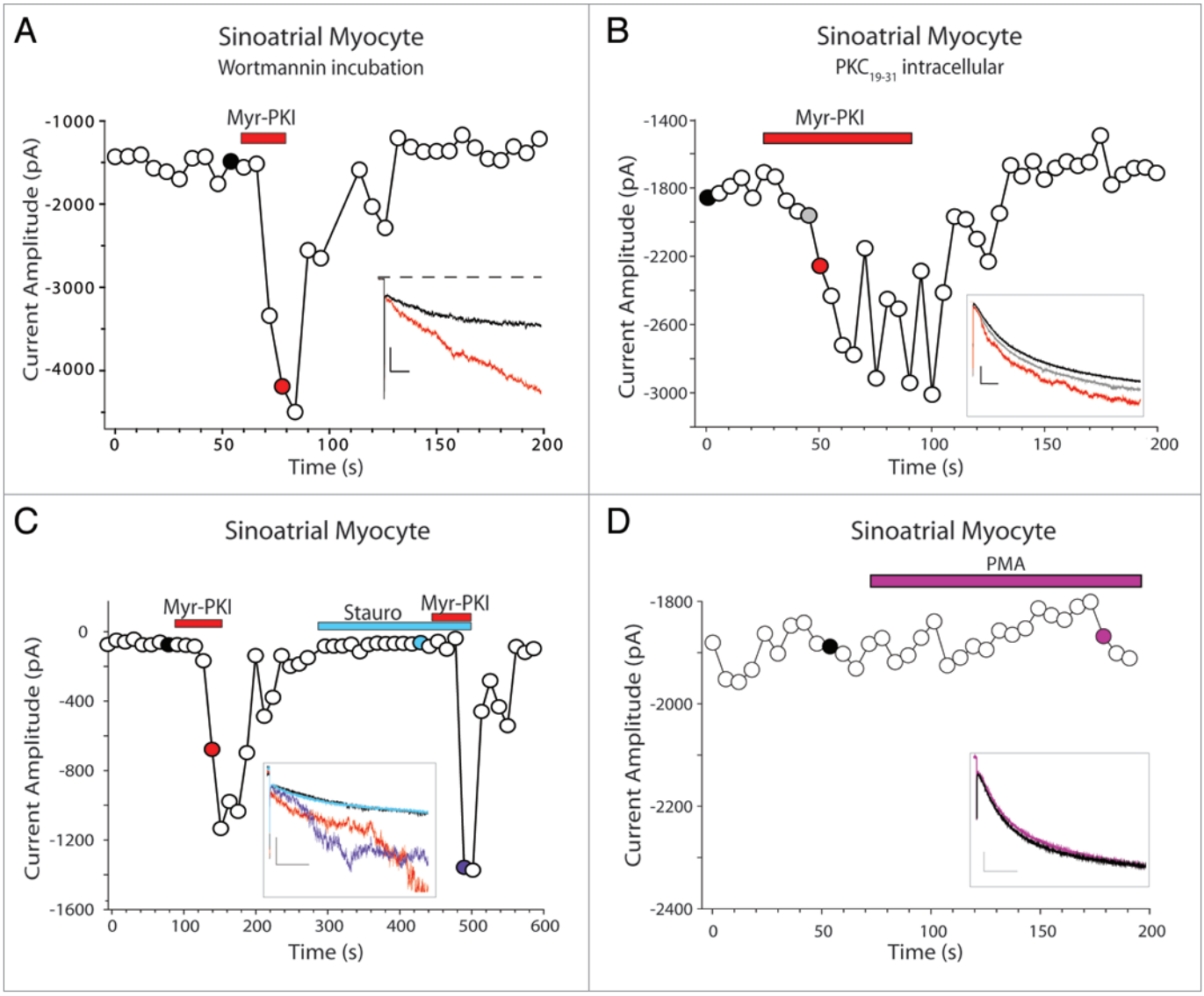

Figure 2. Potentiation of I_f by myristoylated peptides is independent of PIP_2 or PKC. I_f current amplitude as a function of time for sinoatrial myocytes exposed to 2 μM myr-PKI in the presence of (A) 15 μM wortmannin (scale bars in inset 1 nA/100 ms); (B) 10 μM PKC_{19-31} (scale bars in inset 400 pA/100 ms) or (C) 1 μM staurosporine (scale bars in inset 100 pA/200 ms). (D) I_f amplitude for a cell exposed to 500 nM PMA (scale bars in inset 400 pA/200 ms). Whole-cell currents were elicited by 2 s voltage steps to -120 mV from a holding potential of -50 mV every 6 s. Filled symbols indicate the traces shown in the insets.

myocytes, at least under the conditions used in this study.

Myristoylated peptides, including myr-PKI, myristoylated CaMKII inhibitory peptide and myristoylated control peptides have been previously shown to completely but reversibly halt spontaneous firing of isolated sinoatrial myocytes from rabbits or mice.[6,7] We have also observed rapid and reversible cessation of spontaneous activity in mouse sinoatrial myocytes exposed to 2 μM myr-PKI or 2 μM myr-PKC_{19-31} (data not shown). Our present study raises the possibility that potentiation of I_f is the mechanism by which myristoylated peptides stop spontaneous firing. Although modest activation of I_f would be expected to increase the spontaneous firing rate, excessive activation would simply clamp the cells near the I_f reversal potential (~-30 mV), thereby preventing compensation by other pacemaker mechanisms. This idea underscores the fundamental point that pacemaker activity in sinoatrial myocytes depends on a finely-tuned balance among many different ionic conductances working in concert, and that dysregulation of any part of the system can cause it to fail.

There are many possible mechanisms by which myristoylated peptides could activate I_f. For example, they could mimic, activate or interfere with an endogenous signaling pathway, or they could interact directly with HCN4 channels. Two physiological signaling molecules that are known to regulate I_f are cAMP and PIP_2. However, we consider it unlikely that myristoylated peptides act via either of these pathways. The effects of myristoylated peptides on I_f are quite different from any reported manipulations of cAMP in sinoatrial myocytes, including

acute wash-on of the membrane permeant cAMP analogue 8-Br-cAMP (data not shown). And, our present data argue against myristoylated peptide activation of PIP_2 synthesis (**Fig. 2A**). There are of course a multitude of other signaling pathways in sinoatrial cells that could regulate I_f. We have here ruled out PKC signaling as a mechanism for myr-PKI activation of I_f. Our experiments also suggested that I_f may be relatively unaffected by PKC activity in mouse sinoatrial cells. Investigation of other regulatory pathways that control HCN channel activity in the heart and brain is the focus of much ongoing work.

Overall, the major finding of this study is that myristoylated peptides have off-target effects that render them unsuitable for use in studies of pacemaker mechanisms in sinoatrial myocytes. The possibility that the dramatic activation of I_f by myristoylated peptides echoes a physiological regulatory mechanism awaits further study. However, the lack of requirement for a specific peptide sequence and the similar activation of HCN4 channels in sinoatrial myocytes and HEK cells indicate that any such mechanism does not involve myocyte-specific signaling pathways.

Materials and Methods

Materials. Wortmannin, staurosporine, PMA, PKC_c and myristic acid were obtained from Sigma-Aldrich; PKI_{6-22} amide, myr-PKI_{14-22} amide and PKC_{19-31} from EMD Chemicals; myr-PKC_{19-31} from AnaSpec; n-tetradecanamide and methyl tetradecanoate from Alfa Aesar; and ZD7288 from Tocris Bioscience.

Sinoatrial myocyte isolation and electrophysiology. Sinoatrial myocytes were isolated from adult (>7 weeks) male C57BL/6J mice as described previously in reference 1. Briefly, the sinoatrial node region was dissected at 35°C in a heparinized (10 U/ml) solution (in mM: 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 5 HEPES, 5.55 Glucose, 1 $MgCl_2$, 1.8 $CaCl_2$, pH adjusted to 7.4 with NaOH) and myocytes were isolated by digestion for 25–30 min in collagenase type II, protease type XIV and elastase.

Hyperpolarization-activated I_f currents were recorded from sinoatrial myocytes in the whole cell patch clamp configuration with pipettes that had resistances of ~1.5 to 3 MΩ when filled with an intracellular solution consisting of (in mM) 135 K-aspartate, 6.6 Na-phosphocreatine, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 0.1 Na-GTP, 4 Mg-ATP, pH adjusted to 7.2 with KOH. Cells were constantly perfused (~1–2 ml/min) with Tyrode's solution (140 NaCl, 5.4 KCl, 5 HEPES, 5.55 Glucose, 1 $MgCl_2$, 1.8 $CaCl_2$, pH adjusted to 7.4 with NaOH) containing 1 mM $BaCl_2$. All experiments were conducted at room temperature.

I_f was measured as the time-dependent component of current elicited by hyperpolarizing voltage steps. Conductance (G) for I_f was calculated as:

$$G = I/(V_m - V_r)$$

where I is the time-dependent component of inward current, V_m is the applied membrane voltage (corrected for a +14 mV junction potential error), and V_r is the reversal potential for I_f (-30 mV; ref. 8; Liao Z, Proenza C, unpublished). Conductance-voltage plots were fit with a Boltzmann equation to determine midpoint activation voltages ($V_{½}$).

HEK cell culture, transfection and electrophysiology. HEK293 cells were transiently transfected with cDNA encoding HCN4 or HCN4-CX4 plus CD8 using Fugene6 (Roche) according to the manufacturer's directions. Transfected cells were identified by anti-CD8 antibody coated beads (Invitrogen), and were used for electrophysiology 24–48 hours post-transfection. Cells were voltage-clamped in the whole cell configuration using pipettes with resistances of ~1.5 to 3 MΩ when filled with an intracellular solution that contained (in mM) 130 K-aspartate, 10 NaCl, 1 EGTA, 5 HEPES, 0.5 $MgCl_2$, 2 MgATP, pH adjusted to 7.2 with KOH. Cells were constantly perfused (~1–2 ml/min) with extracellular solution consisting of (in mM) 115 NaCl, 30 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 5 HEPES, pH adjusted to 7.4 with NaOH.

Statistics. All results are reported as mean ± SEM. Comparisons were performed using unpaired two-tailed t-tests.

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