**COMMUNICATION**

**Regulation of heart rate and the pacemaker current by phosphoinositide 3-kinase signaling**

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Heart rate in physiological conditions is set by the sinoatrial node (SN), the primary cardiac pacing tissue. Phosphoinositide 3-kinase (PI3K) signaling is a major regulatory pathway in all normal cells, and its dysregulation is prominent in diabetes, cancer, and heart failure. Here, we show that inhibition of PI3K slows the pacing rate of the SN in situ and in vitro and reduces the early slope of diastolic depolarization. Furthermore, inhibition of PI3K causes a negative shift in the voltage dependence of activation of the pacemaker current, I\(_F\), while addition of its second messenger, phosphatidylinositol 3,4,5-trisphosphate, induces a positive shift. These shifts in the activation of I\(_F\) are independent of, and larger than, those induced by the autonomic nervous system. These results suggest that PI3K is an important regulator of heart rate, and perturbations in this signaling pathway may contribute to the development of arrhythmias.

**Introduction**

The heartbeat normally originates in myocytes of the sinoatrial node (SN), the primary cardiac pacemaker. Evidence exists for both voltage-dependent and calcium-dependent “clocks” that determine the spontaneous rhythmicity of the SN (Eisner and Cerbai, 2009). A major component of the voltage-dependent mechanism is the hyperpolarization-activated pacemaker current, I\(_F\). The autonomic nervous system modulates both heart rate and I\(_F\) (DiFrancesco, 2006). Here we show that phosphoinositide 3-kinase (PI3K), an enzyme whose dysregulation underlies a number of pathological conditions including diabetes, long QT syndrome (a pathological increase in the interval between the Q and T waves on the electrocardiogram), and cancer, alters the endogenous rate of the SN and I\(_F\) independently of the autonomic nervous system. These findings are relevant to those disease states, as an increase in sympathetic tone required to compensate for the slowing induced by PI3K inhibition is itself arrhythmogenic.

**Materials and methods**

**Experimental animals**

All animal-related procedures were approved by the Stony Brook University Institutional Animal Care and Use Committee. Male C57BL/6j mice were purchased from The Jackson Laboratories. Adult male mongrel dogs (20–28 kg) were purchased from Covance Laboratories.

**Recording of mouse cardiac electrical activity ex vivo**

Isolated hearts were mounted on the IH-SR isolated heart perfusion system (Harvard Apparatus) and perfused with Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.52 mM CaCl\(_2\), 1.64 mM MgSO\(_4\), 24.88 mM NaHCO\(_3\), 1.18 mM KH\(_2\)PO\(_4\), 5.55 mM glucose, and 2 mM sodium pyruvate, aerated with 5% CO\(_2\) and 95% O\(_2\)) at 37°C. For electrocardiographic recording, one electrode was placed at the base of the heart next to the left atrium, and a second electrode was placed at the heart apex. Data were collected using the LabChart Pro 8.1.5 (ADInstruments) software system. When the heart rate reached a stable baseline, vehicle (dimethylsulfoxide) or PI-103 (Cayman Chemical) was added to the perfusate reservoir and circulated through the heart for 30 min before collecting the first set of data. Then isoproterenol (Iso; Sigma-Aldrich) was added to the perfusate reservoir and circulated through the heart for another 30 min before collecting the second set of data. The number of heartbeats in a 10-s portion of the recording was counted, and heart rate in beats per minute (bpm) was calculated.

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Recording of canine SN electrical activity ex vivo

Dogs were anesthetized with propofol (6–8 mg/kg intravenous) followed by inhalational isoflurane 2.5–3.5% and oxygen. Hearts were rapidly removed through a left thoracotomy and immersed in cold Tyrode’s solution (131 mM NaCl, 18 mM NaHCO3, 1.8 mM CaCl2, 0.5 mM MgCl2, 1.8 mM NaH2PO4, and 5.5 mM dextrose, aerated with 5% CO2 and 95% O2). For experiments with isolated SN preparations, the solution also contained 4.0 mM KCl. Recently we described an isolated superfused canine SN preparation that maintains a stable beating rate and maximum diastolic potential (MDP) for several hours (Sosunov and Anyukhovsky, 2012). Similar SN preparations were used in the present study. The preparations were placed in a tissue bath, superfused with Tyrode’s solution (37°C, pH 7.3–7.4), and allowed to beat spontaneously. Conventional microelectrode techniques were used to record transmembrane potentials. After 1 h in control Tyrode

potentially of ~30 mV. The isochronal (IC) activation curves of IF were fitted to the Boltzmann equation \( g_f(V) = g_f,max * \frac{1}{1 + \exp\left(\frac{V - V_{1/2}}{K}\right)} \), where \( V_{1/2} \) is the half-maximal voltage and \( K \) is the slope factor. When comparing different sets of data, statistical analysis was performed with either Student’s t tests or ANOVA; significance was set to \( P \leq 0.05 \). Results are given as mean ± SEM.

Steady-state (SS) estimates of current magnitude and activation curves were generated by fitting the IC data to SS assuming that the relaxation was exponential after the initial delay. The start of the fit was roughly 20 ms after the initiation of the voltage step to roughly 10 ms before its end (there were slight variations based on the experimental data). Fig. S1 shows the raw data for one experiment in black with the exponential fits in red, demonstrating the assumption of a single exponential decay is justified. The SS currents were generated from ~80 mV or ~90 mV to the most negative potentials for which experimental data were available. The tail currents were then multiplied by the ratio of the SS amplitude at a given voltage to the IC amplitude to generate the theoretical SS tails, which were then fit to the Boltzmann equation as described for the IC activation curves. Since the SS activation curves were all within a few percent of unity at ~110 mV, the SS calculation for that voltage was used to estimate the maximal IF density for comparison between experimental conditions.

Online supplemental material

Fig. S1 shows experimental IC current traces of IF activation overlaid with estimates generated by fitting the IC data to SS. The traces are indistinguishable. Fig. S2 shows IF activation curves generated by fitting IC data to SS. \( V_{1/2} \) is shifted to more negative potentials in cells treated with PI-103. This shift is reversed by infusion of PI(3,4,5)P3 through the patch pipette. Fig. S3 shows IF activation curves generated by fitting IC data to SS. \( V_{1/2} \) is shifted to more positive potentials in cells incubated with PI(3,4,5)P3 through the patch pipette. Fig. S4 shows IF activation curves generated by fitting IC data to SS. \( V_{1/2} \) in cells incubated with PI-103 is shifted to more positive potentials after treatment with ISO. Table S1 shows SS estimates of IF current density at ~110 mV. Exposure of rabbit SN myocytes to PI-103, ISO, or PI(3,4,5)P3 did not significantly alter current density.

Results

In a previous study on drug-induced long QT syndrome, we noticed that pharmacologic and genetic down-regulation of PI3K signaling appeared to reduce the spontaneous beating rate of isolated, perfused mouse hearts (Lu et al., 2012). To further investigate this observation, we mounted mouse hearts on a Langendorff apparatus, perfused them with or without a panisoform PI3K inhibitor (PI-103), and determined heart rate from recordings of cardiac electrical activity. Control mouse hearts perfused with vehicle had a spontaneous rate of 418 ± 17 bpm that increased to 559 ± 15 bpm after exposure to ISO (Fig. 1). Mouse hearts treated with PI-103 beat at only 90% of the rate of control hearts (377 ± 26 bpm) but still manifested a strong response to ISO stimulation (524 ± 23 bpm; Fig. 1). We also

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calculated mean heart rate from recordings of perfused wild-type \((n = 8)\) and PI3K p110\(\alpha\) knockout \((n = 4)\) hearts (Lu et al., 2012) and found that PI3K ablation caused a 9% decrease in rate \((418 \pm 7 \text{ bpm} \text{ [wild type] versus } 380 \pm 18 \text{ bpm [knockout]; } P < 0.05; \text{ t test})\). These results suggest that PI3K signaling modulates heart rate independently of sympathetic control.

The mouse heart beats much more rapidly than the human heart, so we next investigated the effects of PI3K inhibition on the cardiac rate of the adult dog, whose heart rate is closer to that of the human. We used ex vivo preparations of the SN in order to study heart rate independently of the autonomic nervous system. The left panel of Fig. 2 A shows a tracing of transmembrane potentials from a control SN that had stabilized to a constant rate after equilibrating for 1 h in the bath, and the adjacent panel shows the same control preparation 180 min later. There was no difference in rate. Rate increased from 108
bpm to 172 bpm in response to 5 min of superfusion with Iso, as shown in the third panel. Rate returned to the control range after a 15-min washout of the sympatetic agonist, as shown in the right panel of Fig. 2 A. Fig. 2 B shows data for a similar experiment in which the preparation was superfused with PI-103 after equilibrating to a constant rate at baseline. The rate declined from 100 bpm to 83 bpm after 180 min in the presence of the PI3K inhibitor. Exposure to Iso increased the rate to 166 bpm, and again this increase was completely reversed upon washout (right panel). Fig. 2 C plots the average rate in all of the experimental conditions above for the six preparations studied in each group. There was a significant decrease in pacing rate after the 180-min incubation with PI-103 as compared with control. Both control and PI-103–treated preparations responded well to Iso, although there was a trend for the rate to be lower in preparations superfused with PI-103. Fig. 2 D shows that the MDP was significantly more negative in the PI-103–treated preparations. Iso also caused a significant hyperpolarization of the MDP for both control and PI-103 conditions. This was reversed upon washout (Fig. 2 D). These results demonstrate that inhibition of PI3K leads to a reduction in beating rate in canine SN preparations without altering the robust effect of Iso.

We used myocytes isolated from the rabbit SN to study the effects of PI-103 on \( I_F \) because they are considered to be the most robust model to study this current (DiFrancesco et al., 1986). Rabbit SN myocytes were incubated for 2 h in the absence or presence of PI-103, and \( I_F \) was activated for 1 s by the voltage protocol shown in Fig. 3 A. The tail currents were then used to construct IC activation curves from which \( V_{1/2} \) was calculated (Fig. 3 B). The results indicate that PI-103 treatment caused a negative shift of 15.9 mV in \( V_{1/2} \) (−77.3 ± 0.7 mV [control] versus −93.2 ± 1.2 mV [PI-103]). If the shift in voltage dependence is due to inhibition of PI3K, this effect should be reversed by infusion of the PI3K second messenger, PI(3,4,5)P3, through the patch pipette. Indeed, PI(3,4,5)P3 infusion of cells incubated for 2 h with PI-103 resulted in an 11.7-mV positive shift in the midpoint voltage of the IC activation curve as compared with PI-103 treatment alone (\( V_{1/2} = −81.0 ± 0.9 \) mV [PI-103 + PI(3,4,5)P3]; Fig. 3 B). Shifts in voltage dependence should be accompanied by...
shifts in the time constant of activation ($\tau$). $\tau$ at $-110$ mV was more than doubled in PI-103–treated cells, and this alteration was completely reversed by PI(3,4,5)P3 (Fig. 3 C).

Because the 1-s pulses employed yield currents that are far from SS at some voltages, we used the well-known exponential decay of $I_F$ to generate theoretical SS activation curves for each of the voltage clamp conditions in this experiment. Fig. S1 shows that the fit to SS is indistinguishable from experimentally generated current traces for the first second. Fig. S2 shows the SS fits for activation curves under the control, PI-103, and PI-103 + PI(3,4,5)P3 conditions. The midpoints of activation were $-72.8$ mV, $-87.9$ mV, and $-76.8$ mV, respectively. Thus, each of the SS activation curves has a more positive activation than its IC counterpart. However, the differences between the experimental conditions are minimal. For control versus PI-103, the negative shifts were 15.9 mV or 15.1 mV for IC or SS, respectively. For PI-103 versus PI-103 + PI(3,4,5)P3, there were positive shifts of 12.2 mV or 11.1 mV for IC or SS, respectively.

Finally, we quantified the time dependence of PI-103–induced inhibition of $I_F$ by incubating SN myocytes for increasing times with the inhibitor and calculating current density at $-110$ mV. There was a progressive decrease in IC $I_F$ current density starting at 30 min. This became significant at 1 h and reached an almost maximal level by 2 h (Fig. 3 D). In cells exposed to PI-103 for 2 h, infusion of PI(3,4,5)P3 through the patch pipette caused the IC current density to return to near control levels (Fig. 3 D). However, substitution of either of two control phospholipids, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) or phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2), for PI(3,4,5)P3 in the pipette solution had no effect on the PI-103–induced reduction in $I_F$ current density at $-110$ mV in IC conditions, further indicating a specific role for PI3K inhibition.

Figure 3. Effects of PI-103 on $I_F$ in SN cells isolated from rabbit heart. (A) Representative traces of $I_F$ activation. Cells were untreated (control) or incubated for 2 h with 500 nM PI-103. Some cells treated with PI-103 were then infused with 1 µM PI(3,4,5)P3 in the pipette solution. Inset: The voltage-clamp protocol for $I_F$ activation was to apply 1-s hyperpolarizing voltage steps ranging from $-30$ mV to $-110$ mV in $-10$ mV increments from a holding potential of $-30$ mV followed by a depolarizing step to $+50$ mV for 0.5 s to record the tail currents, after which the preparation was stepped back to the holding potential. (B) Boltzmann fit of 1-s IC activation curves for $I_F$. $V_{1/2}$ and $K$ were derived from the curves. Control, $V_{1/2} = -77.3$ mV, $K = 11.2$ mV; PI-103, $V_{1/2} = -93.2$ mV, $K = 8.1$ mV; and PI-103 + PI(3,4,5)P3, $V_{1/2} = -81.0$ mV, $K = 11.6$ mV. (C) Mean time constants ($\tau$) from a single exponential fit of $I_F$ measured at $-110$ mV. **, $P < 0.01$ versus control. (D) Summary data of $I_F$ density measured at $-110$ mV from a holding potential of $-30$ mV. Cells were incubated with PI-103 for the times indicated before patching with or without infusion of 1 µM PI(3,4,5)P3 or the control lipids phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) or phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2). **, $P < 0.05$ versus control. Numbers above the bars indicate the number of cells examined in C and D. $n$ values for C also apply to B. Summarized data show mean ± SEM.
Current densities in Table S1 calculated from SS activation (see Materials and methods) show that PI-103 in the absence or presence of PI(3,4,5)P3 caused no significant change in \(I_F\) magnitude at \(-110\) mV, where the current is almost fully activated.

We next asked whether the control \(I_F\) activation curve reflects a maximal effect due to PI3K signaling. To answer this question, we added PI(3,4,5)P3 to the pipette solution. The results in Fig. 4, A and B, show the raw data in control conditions and with addition of PI(3,4,5)P3, respectively. Fig. 4 C shows the IC activation curves, which had midpoints of \(-83.6\) mV (control) and \(-75.0\) mV (PI(3,4,5)P3). Thus, the dynamic range of responsiveness to PI3K signaling is 24.5 mV (which is equal to the sum of the differences of the \(V_{1/2}\)s of PI-103 and PI(3,4,5)P3 from the control values) in IC conditions. Midpoints calculated from the theoretical SS curves in Fig. S3 were more positive than those calculated from the IC data (\(-73.7\) mV [control] and \(-63.1\) mV [PI(3,4,5)P3]). Again, the difference between the shifts in IC and SS activation curves was small (PI(3,4,5)P3) caused a positive shift of 8.6 mV or 10.6 mV for IC or SS, respectively, and \(I_F\) density at \(-110\) mV was not significantly different in the two conditions at SS (Table S1).

Although the SN rhythm is myogenic in origin, the autonomic nervous system modulates both \(I_F\) and sinus rate (DiFrancesco, 2006). Since sympathetic and parasympathetic stimulation alter \(I_F\) voltage dependence but not amplitude (DiFrancesco, 2006), the results in Fig. 1 (the in situ mouse heart) and Fig. 2 (the in vitro canine SN) suggested that the action of PI-103 is independent of autonomic regulation since although the inputs to the SN remain, there is no feedback control. We therefore investigated whether the shift in voltage dependence of activation we observed with exposure to PI-103 was dependent on the autonomic nervous system. SN myocytes were pretreated for 2 h with PI-103 and then exposed to CCh or Iso before measuring \(I_F\). Fig. 5, A and C, shows that the IC \(I_F\) current density at \(-110\) mV was further reduced upon exposure to CCh and increased in cells exposed to Iso. These differences in amplitude and current density are consistent with the difference in kinetics and open probability induced by the autonomic agonists. The IC activation curves in Fig. 5 B demonstrate a 6.9-mV negative shift in \(V_{1/2}\) induced by CCh (\(V_{1/2} = -90.2 \pm 1.2\) mV [PI-103] versus \(-97.1 \pm 1.9\) mV [PI-103 + CCh]), while Iso induced an 11.8-mV positive shift in IC midpoint voltage (\(V_{1/2} = -78.4 \pm 1.3\) mV [PI-103 + Iso]). The dynamic range of autonomic responsiveness in the presence of PI-103 is 18.7 mV ([\(V_{1/2} +\) Iso] - [\(V_{1/2} +\) CCh]) in IC conditions. This is very close to the value of 19.5 mV in control conditions reported by Zaza et al. (1996). We fit the data for the PI-103 and PI-103 + Iso conditions to SS and calculated midpoint voltages of \(-87.9\) mV and \(-74.3\) mV.
Figure 5. Effects of CCh or Iso on $I_F$ in isolated rabbit SN cells pretreated with PI-103. (A) Typical traces of $I_F$ activation in cells treated for 2 h with 500 nM PI-103 alone or treated with PI-103 and then exposed to 1 µM CCh or 10 µM Iso for 5 min. (B) Boltzmann fit of 1-s IC activation curves for $I_F$. $V_{1/2}$ and $K$ were derived from the curves. PI-103, $V_{1/2} = -90.2 \pm 1.2$ mV, $K = 9.2$ mV; PI-103 + CCh, $V_{1/2} = -97.1 \pm 1.9$ mV, $K = 9.3$ mV; PI-103 + Iso, $V_{1/2} = -78.4 \pm 1.3$ mV. (C) Summary data of $I_F$ density measured at -110 mV from a holding potential of -30 mV. Numbers above the bars indicate the number of cells examined. Summarized data show mean ± SEM.
are virtually fully activated. Our studies of spontaneous activity in the intact mouse heart and the isolated canine SN suggested that autonomic regulation was unaltered by PI3K inhibition. Since autonomic regulation is evidenced by a shift in the midpoint of the voltage dependence of activation, we examined the effects of both muscarinic stimulation and sympathetic stimulation on the $V_{1/2}$ of $I_F$. CCh caused a negative shift while Iso caused a positive shift in the midpoint of activation in IC conditions. The dynamic range (midpoint Iso–midpoint CCh) of 18.7 mV was entirely consistent with the previously reported value of 19.5 mV in control conditions (Zaza et al., 1996). SS calculations suggest there was no change in SS $I_F$ tail current density at –110 mV in the presence of Iso.

The novel action of PI3K inhibition on pacemaker activity is important for several reasons. First, inhibition of PI3K is a mechanism by which some drugs cause long QT syndrome (Lu et al., 2012; Yang et al., 2014). Second, reduced activity of cardiac PI3K is an expected outcome in type 2 diabetes. It is interesting to note that while basal heart rate is increased in diabetic people (Ewing et al., 1981; Hume et al., 1986), exposure to PI-103 caused rate slowing in the ex vivo mouse heart and the isolated canine SN where no sympathetic feedback is available. Presumably, this difference results from the well-known increase in sympathetic and decrease in parasympathetic tone that accompany diabetes. Our results raise the possibility that a myogenic reduction in heart rate caused by PI3K inhibition could be a factor mediating the hyperpolarizing-activated current (if) in cells isolated from the rabbit sino-atrial node. J. Physiol. 377:61–88. https://doi.org/10.1113/jphysiol.1986.sp016177

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Author contributions: R.Z. Lin conceived of the first experiment, analyzed the data, and cowrote the manuscript. Z. Lu designed, performed, and analyzed the patch-clamp data in Fig. 3 and Fig. 5. E.P. Anyukhovsky executed and analyzed the experimental data for Fig. 2. Y.P. Jiang executed the experiment in Fig. 1 and helped analyze the data. H.Z. Wang designed and analyzed the experiment in Fig. 4. J. Gao helped analyze the data in the supplemental figures and Table SI. M.R. Rosen helped design the studies and analyze the data in Fig. 2. L.M. Ballou helped analyze all the data and cowrote the manuscript. I.S. Cohen oversaw the execution of the patch-clamp experimental design and analysis, and cowrote the manuscript.

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