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COMMENTARY

Comparing pathways for long-term heart rate modulation by the funny current

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A recent report by Ira S. Cohen and collaborators proposes a novel mechanism for modulation of the pacemaker (funny) current involving the phosphoinositide 3-kinase (PI3K; Lin et al., 2019). Although when first described in 1979 (Brown et al., 1979) I_f was dubbed "funny" because of its peculiar features, based on verbal joking while running an experiment ("Wow. Look Hilary, isn't this funny? Yes Dario, it is funny, we should call it the funny current"), it soon became clear that no nickname could have been more fitting. It indeed had surprisingly odd properties, first of all that of being the first known inward current activated on hyperpolarization, but more oddities would become apparent with subsequent more detailed investigations, such as a mixed Na^+ and K^+ permeability (DiFrancesco, 1981) and the direct activation of channels by intracellular cAMP. This latter feature, described in 1991 (DiFrancesco and Tortora, 1991), was the first evidence that funny channels are unusually dually activated by both voltage (on hyperpolarization) and cAMP, but it was at the same time a hint, not immediately appreciated, that while atypical of voltage-dependent channels, this feature was typical of CNG channels such as the cGMP-activated channel of the retina. Nearly 20 yr were necessary after the discovery of I_f before the property of cAMP-dependent gating was confirmed with the cloning in the late 90s of the hyperpolarization-activated CNG (HCN) channels, the molecular components of native funny channels, shown to belong to the same superfamily of voltage-dependent Kv and CNG channels (Santoro et al., 1998).

Reductionism, based as it is on a limited knowledge of the real world, is apt to simplify the description of phenomena, but it is inevitably an approximation of reality requiring more generalized views whenever new knowledge is learnt. Thus, as soon as the bizarre (for a voltage-dependent current) direct cAMP-mediated activation of the funny current was discovered (DiFrancesco and Tortora, 1991), Ira Cohen's laboratory found that phosphorylation processes could also modulate I_f, requiring a more integrative view of the complexity of channel modulation (Chang et al., 1991).

Subsequent work confirmed the importance of phosphorylation-dependent processes in the modulation of funny channels, indicating the involvement of both serine-threonine and tyrosine kinases (Yu et al., 1993; Accili et al., 1997; Wu and Cohen, 1997; Arinsburg et al., 2006).

Data obtained in early experiments by modifying either phosphatase or kinase activity showed changes in the maximal current amplitude, as expected from alteration of channel trafficking, as well as changes in the voltage dependence of the current activation curve, which more likely reflect processes directly affecting channel gating and modulating the probability of channel opening.

The molecular counterparts of funny channels, the HCN channels, were also shown to be modulated by phosphorylation-dependent processes. Zong et al. (2005) provided evidence, in both heterologous expression experiments and in native cardiac myocytes and neurons, that inhibition of Src-induced tyrosine phosphorylation of HCN2 slows channel activation thus reducing their contribution to activity. In this study, Src inhibition slowed activation but did not shift the voltage dependence of current activation. Src was shown to phosphorylate a specific tyrosine residue in the C-linker (Tyr476 of mHCN2 or the homologous Tyr554 of hHCN4). A similar study investigating Src inhibition on HCN4 channels expressed in HEK293 cells and the interaction of Src with HCN4 channels in cardiac myocytes (Arinsburg et al., 2006) reported acceleration of current activation in the presence of constitutively activated Src, in agreement with Zong et al. (2005). In this case, however, a shift of the current activation curve was also reported.

PI3K is a member of a family of kinases involved in a variety of cellular functions including cell growth and differentiation, motility, apoptosis, intracellular trafficking, and others. In their recent work, Lin et al. (2019) show that inhibition of PI3K causes a negative shift in the voltage dependence of activation of the

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funny current, while addition of its second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3), induces a positive shift. A negative shift of I_f activation leads to a reduction of the current contribution to spontaneous activity and a decrease of the steepness of diastolic depolarization, resulting in a slowing of heart rate.

The authors propose that PI3K is an important regulator of heart rate and that dysfunctional PIK3 activity may lead to arrhythmias. The contribution of PI3K to heart rate modulation is particularly important in view of the involvement of PI3K dysregulation in pathologies such as diabetes, long QT syndrome (LQT), and heart failure.

Although the work of Lin et al. (2019) does not address the mechanistic link between PI3K inhibition and I_f inhibition, an obvious question arises about the mode of action of PI3K on funny channel function. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce PIP3, which acts as a second messenger of PI3K. Recruiting to the cell membrane of protein kinases by PIP3 then activates the Akt pathway, which in turn controls several downstream processes (Falkenburger et al., 2010). PI3K inhibition may therefore act via the Akt pathway or by effects on channel function of PIP3.

Phosphoinositides are known to act as lipid regulators of membrane proteins such as transporters and ion channels (Falkenburger et al., 2010), and previous work has already indicated phosphoinositide-dependent modulation of HCN function. In 2006, two groups (Pian et al., 2006; Zolles et al., 2006) investigated the effects of PIP2 and other phosphoinositides on HCN activation.

Pian et al. (2006) showed that increasing membrane concentration of PIP2 shifts the gating of HCN2 channels expressed in *Xenopus* oocytes and of the native I_f current in sinoatrial node (SAN) cells to more positive potentials, conferring to f/HCN channels activation ranges appropriate for their physiological function. The PIP2 dependence was shown to be responsible for the long known "run-down" effect originally described in 1986 with the first description of I_f in isolated SAN cells (DiFrancesco et al., 1986).

Similar observations were reported by Zolles et al. (2006), who showed that PIP2 acts as an f/h channel ligand whose binding shifts the voltage dependence of activation in the positive direction. They further showed in heterologous expression systems that while phosphatidylinositol-4-phosphate (PIP), PIP2, and PIP3 all shifted the activation curve of HCN1-2 and -4 isoforms (by >15 mV), phosphatidylinositol (PI) was ineffective. This suggests that the depolarizing action is due to the negatively charged headgroup present in all phospholipids except PI, where indeed the inositol ring is fully dephosphorylated and thus uncharged.

Based on these observations, it is tempting to speculate that the reduced phosphoinositide availability caused by inhibition of PI3K contributes to the negative shift of the I_f activation curve observed by Lin et al. (2019), like the one reported upon depletion of PIP2. A negative charge contribution of phospholipid headgroups would also explain the positive shift caused by addition of PIP3 (Fig. 3 B in Lin et al., 2019). In this respect, it is interesting to observe that according to early in vivo experiments, activation of PI3K increases not only the intracellular amount of PIP3, but also that of PIP2 (Franke et al., 1997).

These considerations, however, remain speculative until experimentally addressed. Furthermore, the comparison with the effects of PIP2 described earlier (Pian et al., 2006; Zolles et al., 2006) cannot be extended to the reduction of maximal I_f conductance caused by PIK3 inhibition. In this case, the data show that while intracellular perfusion with PIP3 is able to rescue the I_f density decrease following PI3K inhibition, the same does not occur with two other phosphatidylinositol bisphosphates (PI(4,5)P2) or PI(3,5)P2), indicating a specific role for PI3K inhibition (Fig. 3 D in Lin et al., 2019).

PI3K may act to control HCN4 channels through its downstream effector Akt, a serine-threonine protein kinase already reported to modulate the activity of other ion channels such as for example the L-type Ca channel (Viard et al., 2004; Lu et al., 2009) or the K channel Kv11.1 (Zhang et al., 2003). Modification of PI3K/Akt activity normally causes changes in the density of membrane (Ca^{2+}/K^+/Na^+) channel expression, either because of changes in channel trafficking or as a direct action on consensus Akt phosphorylation sites on channel proteins. In the work of Lin et al. (2019), however, while the large current density reduction caused by inhibition of PIK3 is clearly associated with a lower membrane expression reflecting reduced trafficking, the shift of the activation curve reflects, instead, a change in the open channel probability and points more favorably to a direct effect on the channel. PI3K activity thus represents a new mechanism adding to the multiple modulating factors already known to act on the same parameter, the voltage dependence of the funny current.

Why do we have multiple mechanisms? Blood flow is the product of cardiac rate times the stroke volume, so at any time cardiac rate turns out to be the most efficient and potentially rapid controlling factor of cardiac performance. The need to gear to the continuously variable internal and external conditions requiring metabolic adaptation, and the direct correlation between cardiac oxygen consumption and cardiac rate, fully justify that several processes fine-tuning heart rate to the desired level in all circumstances have been selectively developed, the most important of which is the autonomic nervous system. It is interesting to note in this respect that the action of PI3K is independent of autonomic regulation.

An obvious property that cAMP- and PI3K-dependent mechanisms do not share is speed. I_f modulation by cAMP is fast, to the extent that the moderate vagal activity responsible for I_f-mediated vagal tone (DiFrancesco et al., 1989) is able to adjust cardiac rate on a beat-to-beat basis (Warner and Cox, 1962). On the other hand, PI3K-induced changes, and as mentioned above other phosphorylation-dependent processes targeting the membrane expression of HCN channels and current density, are much slower (see, for example, Fig. 3 in Lin et al., 2019). Long-term mechanisms of heart rate adaptation are appropriate for processes such as training, ageing, circadian fluctuations, and similar processes.

Considering the long-term property of PI3K action, an intriguing possibility worth addressing is that PI3K acts by

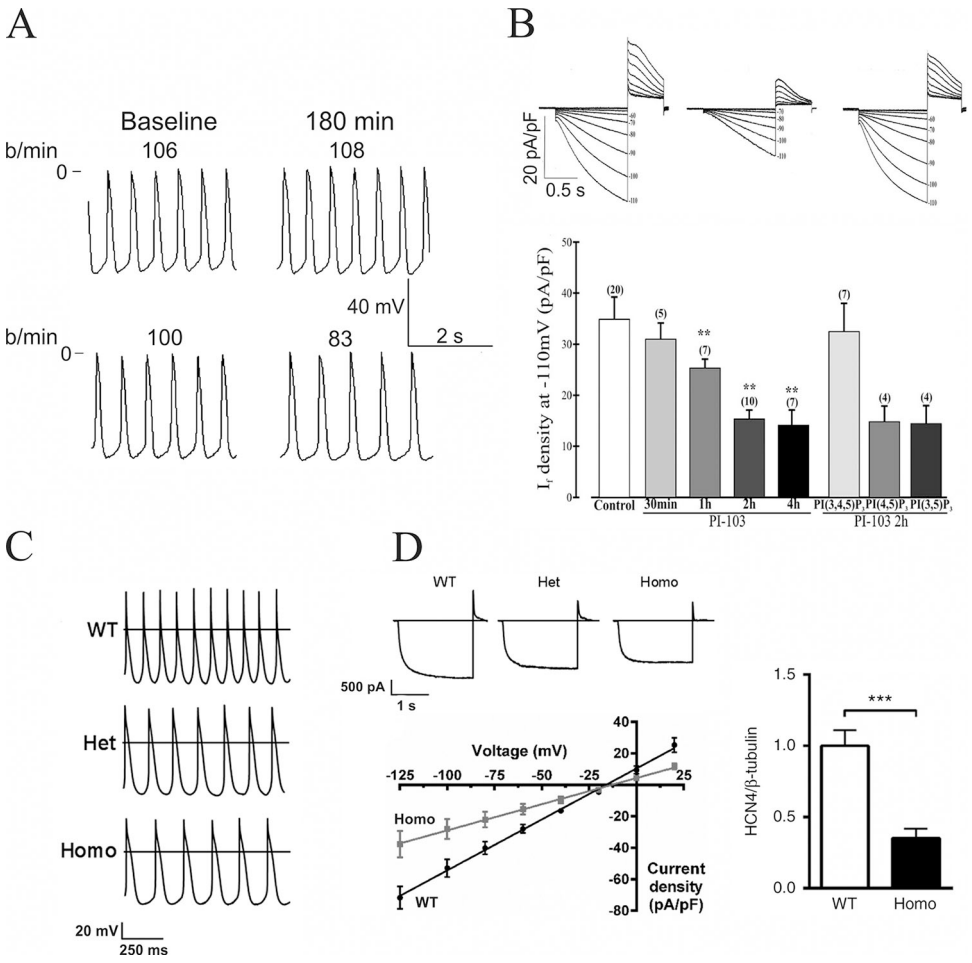

Figure 1. **Comparing effects on spontaneous rate and I_f of changes in PI3K and AMPK activity. (A and B)** Data from Lin et al. (2019). **(A)** Spontaneous rate of a dog SAN is reduced after 3h perfusion with 1 µM PI-103, a PI3K inhibitor (lower row), while no decline is seen in control conditions (upper row). **(B)** Top: I_f recorded from a rabbit SAN cell is reduced by external perfusion with PI-103 (middle), but returns to control levels if the cell is internally perfused with PIP3 (right). Bottom: I_f membrane density in SAN cells decreases with incubation time in the presence of PI-103. Cell infusion with PIP3, but not PI(4,5)P2 or PI(3,5)P2, rescues the I_f density to control values. **, P < 0.05 versus control. **(C and D)** Data from Yavari et al. (2017). SAN cells from mice carrying heterozygous (Het) or homozygous (Homo) activating mutation of γ2-AMPK (R299Q) beat at a slower rate (C) and express less funny channels (D, top) than wild-type cells. I_f membrane expression density (D, bottom left) and HCN4 expression level are ∼50% smaller in mutant that wild-type pacemaker cells (D, bottom right). ***, P < 0.001 versus WT.

regulating the activity of the AMP-dependent protein kinase (AMPK), a serine/threonine kinase activated by AMP under condition of energetic stress which is essential in the regulation of energy homeostasis of cardiac cells (Fig. 1).

It has been recently shown that γ2-AMPK, a subunit of AMPK enzyme, is highly expressed in the SAN and has a fundamental regulatory function in pacemaker cells by modulating heart rate via control of the I_f current (Yavari et al., 2017). γ2-AMPK function correlates inversely with I_f membrane expression and heart rate by a mechanism whose aim is to couple metabolic need and spontaneous rhythm: since heart rate correlates directly with cardiac oxygen consumption, the bradycardia induced by increased AMPK activity ensures direct and efficient adaptation of cardiac performance to the metabolic state of the cell.

Interestingly, a potential role of AMPK as a mediator of PI3K action is supported by data showing that in myoblasts, PI3K inhibition (by chemical inhibition, siRNA or expression of dominant-negative p110α, the catalytic subunit of class IA PI3K enzyme) activates AMPK, whereas expression of a constitutively active form of p110α reduces AMPK activity (Matheny et al., 2016).

These effects fit well the inverse correlation between PI3K activation and funny current availability. It will be interesting to investigate if there is a link between the PI3K and AMPK biochemical pathways converging on the I_f-dependent control of heart rate.

There are various reasons to justify the investigation of the cellular effects of PI3K inhibition. PI3K inhibitors have attracted strong interest as therapeutic targets since they have entered clinical use as anti-cancer agents. However, several adverse side effects including autoimmune dysfunction, skin toxicity, hypertension, hyperglycemia, and LQT seriously limit their use (Greenwell et al., 2017). In addition, pathological states such as diabetes, heart failure, and cancer are associated with dysregulation of PIK3 signaling. Knowledge of the PIK3 signaling

involved in the regulation of the funny current and heart rate highlights a new potential paradigm in the complex set of processes controlling cardiac pacemaker activity. In addition, it will contribute to a better understanding of the mechanisms underlying the relevance of PIK3 signaling to cellular homeostasis.

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