Cyclic AMP reverses the effects of aging on pacemaker activity and I_f in sinoatrial node myocytes

Emily J. Sharpe,[1] Eric D. Larson,[1] and Catherine Proenza[1,2]

[1]Department of Physiology and Biophysics and [2]Department of Medicine, Division of Cardiology, University of Colorado–Anschutz Medical Campus, Aurora, CO 80045

Aerobic capacity decreases with age, in part because of an age-dependent decline in maximum heart rate (mHR) and a reduction in the intrinsic pacemaker activity of the sinoatrial node of the heart. Isolated sinoatrial node myocytes (SAMs) from aged mice have slower spontaneous action potential (AP) firing rates and a hyperpolarizing shift in the voltage dependence of activation of the "funny current," I_f. Cyclic AMP (cAMP) is a critical modulator of both AP firing rate and I_f in SAMs. Here, we test the ability of endogenous and exogenous cAMP to overcome age-dependent changes in acutely isolated murine SAMs. We found that maximal stimulation of endogenous cAMP with 3-isobutyl-1-methylxanthine (IBMX) and forskolin significantly increased AP firing rate and depolarized the voltage dependence of activation of I_f in SAMs from both young and aged mice. However, these changes were insufficient to overcome the deficits in aged SAMs, and significant age-dependent differences in AP firing rate and I_f persisted in the presence of IBMX and forskolin. In contrast, the effects of aging on SAMs were completely abolished by a high concentration of exogenous cAMP, which restored AP firing rate and I_f activation to youthful levels in cells from aged animals. Interestingly, the age-dependent differences in AP firing rates and I_f were similar in whole-cell and perforated-patch recordings, and the hyperpolarizing shift in I_f persisted in excised inside-out patches, suggesting a limited role for cAMP in causing these changes. Collectively, the data indicate that aging does not impose an absolute limit on pacemaker activity and that it does not act by simply reducing the concentration of freely diffusible cAMP in SAMs.

The Journal of General Physiology

INTRODUCTION

Aerobic capacity decreases with advancing age, in part because of an age-dependent decline in maximum heart rate (mHR; Heath et al., 1981; Hagberg et al., 1985; Hawkins and Wiswell, 2003). This decline in mHR is considered to be an inherent property of aging because it occurs at approximately the same rate in all individuals, without regard for gender, lifestyle, or physical fitness (Robinson, 1938; Jose and Collison, 1970; Jose et al., 1970; Higginbotham et al., 1986; Ogawa et al., 1992; Fitzgerald et al., 1997; Wilson and Tanaka, 2000). The reduction in mHR results from a parallel, age-dependent decline in "intrinsic heart rate" (iHR; Jose and Collison, 1970; Christou and Seals, 2008; Larson et al., 2013). iHR is measured during autonomic blockade and thus reflects, at the whole-animal level, the intrinsic pacemaker activity of the sinoatrial node of the heart. The age-dependent decline in iHR indicates that sinoatrial node function is compromised with age. In contrast, the parallel declines in mHR and iHR indicate that the sympathetic "fight-or-flight" increase in heart rate is largely preserved in older individuals because sympathetic nervous system stimulation increases heart rate by approximately the same amount above the iHR baseline at all ages (Jose and Collison, 1970; Christou and Seals, 2008; Larson et al., 2013).

We previously reported that aging slows iHR and mHR in part by slowing the spontaneous action potential (AP) firing rate of individual sinoatrial node myocytes (SAMs; Larson et al., 2013). Spontaneous APs in SAMs initiate each heart beat by triggering a wave of depolarization that propagates through the myocardium to elicit contraction. Sinoatrial APs are characterized by a spontaneous depolarization during diastole that drives the membrane potential to threshold to trigger the subsequent AP. SAMs isolated from older animals have slower AP firing rates than SAMs from young animals because of changes in a limited set of AP waveform parameters, specifically a slower diastolic depolarization rate (DDR) and a hyperpolarization of the maximum diastolic potential (MDP; Larson et al., 2013).

The diastolic depolarization and spontaneous APs in SAMs arise as a result of the coordinated activity of a unique complement of ion channels that work in concert with intracellular Ca^{2+} signaling (Marionneau et al., 2005; Tellez et al., 2006; Mangoni and Nargeot, 2008; DiFrancesco, 2010; Lakatta et al., 2010). Most of the

Correspondence to Catherine Proenza: catherine.proenza@ucdenver.edu
E.D. Larson's present address is Dept. of Otolaryngology, University of Colorado–Anschutz Medical Campus, Aurora, CO 80045.

Abbreviations used: AP, action potential; βAR, β adrenergic receptor; DDR, diastolic depolarization rate; HCN, hyperpolarization activated, cyclic nucleotide sensitive; IBMX, 3-isobutyl-1-methylxanthine; I_f, funny current; iHR, intrinsic heart rate; ISO, isoproterenol; MDP, maximum diastolic potential; mHR, maximum heart rate; SAM, sinoatrial node myocyte; $V_{1/2}$, midpoint activation voltage.

The Rockefeller University Press $30.00
J. Gen. Physiol. 2017 Vol. 149 No. 2 237–247
https://doi.org/10.1085/jgp.201611674

proteins involved in the generation of spontaneous activity in SAMs are regulated directly or indirectly by cAMP. cAMP is a critical determinant of the AP firing rate in SAMs, and the basal concentration of cAMP is thought to be higher in SAMs than in other cardiac myocytes (Vinogradova et al., 2006). Activation of β adrenergic receptors (βARs) by the sympathetic nervous system accelerates the spontaneous AP firing rate in SAMs by stimulating additional cAMP production by adenylyl cyclases (Mattick et al., 2007). Such receptor-mediated cAMP signaling occurs within subcellular cAMP microdomains in many cell types (Dodge-Kafka et al., 2006; Warrier et al., 2007; Zaccolo, 2009; Xiang, 2011; Perera and Nikolaev, 2013).

The "funny current" (I_f) is a hallmark of SAMs and is among the cAMP-sensitive processes that determine the spontaneous AP firing rate. I_f is produced by hyperpolarization-activated, cyclic nucleotide–sensitive (HCN) channels, with HCN4 being the principal isoform in SAMs, where it is expressed at very high levels and is used as a immunohistochemical marker of the sinoatrial node (Moosmang et al., 2001; Marionneau et al., 2005; Huang et al., 2007; Liu et al., 2007; Viswanathan et al., 2007). HCN channels conduct both Na^+ and K^+ and have a net reversal potential of approximately −30 mV in physiological solutions. Thus, open HCN channels conduct a depolarizing inward current at diastolic potentials that contributes to the spontaneous diastolic depolarization. cAMP potentiates voltage-dependent activation of I_f either by binding directly to HCN4 channels (DiFrancesco and Tortora, 1991; Zagotta et al., 2003) or by protein kinase A (PKA)–mediated phosphorylation of HCN4 channels (Liao et al., 2010). In both cases, cAMP facilitates HCN4 channel opening by shifting the midpoint activation voltage ($V_{1/2}$) to more positive potentials. The importance of I_f for pacemaking is clearly illustrated by HCN4 mutations that cause sinoatrial node dysfunction in human patients and animal models (Herrmann et al., 2012; DiFrancesco, 2013; Baruscotti et al., 2015; Verkerk and Wilders, 2015) and by the ability of HCN channel blockers to slow heart rate (Borer, 2004; Stieber et al., 2006). I_f is thought to be a key determinant of the MDP and the DDR phases of the sinoatrial AP (Stieber et al., 2006; Bucchi et al., 2007; Severi et al., 2012), which are the main AP parameters responsible for the slower firing rate in SAMs from older animals (Larson et al., 2013). Accordingly, aging causes a hyperpolarizing shift in the voltage dependence of activation of I_f, which would be expected to contribute to the slower AP firing rate of aged SAMs (Larson et al., 2013).

In this study, we examined the ability of endogenous and exogenous cAMP to overcome age-dependent changes in AP firing rate and I_f in acutely isolated murine SAMs. We found that maximal stimulation of endogenous cAMP production in SAMs could not compensate for the age-dependent deficits in AP firing rate or I_f. In contrast, application of a high concentration of exogenous cAMP completely reversed the effects of aging in SAMs. These data establish that aging does not impose an absolute limit on the pacemaker activity of SAMs and suggest that the age-dependent shift in the voltage dependence of I_f contributes to slower heart rates in older individuals.

MATERIALS AND METHODS

All animal procedures were performed in accordance with protocols approved by the University of Colorado Denver–Anschutz Medical Campus Institutional Animal Care and Use Committee. Wild-type C57BL/6J male mice were obtained from the National Institute on Aging, Aged Rodent Colony. Young mice were 2–3 mo of age, and aged mice were 21–24 mo of age (corresponding to ∼17–20 and ∼65–69 human years, respectively; Fox et al., 2007; Nadon et al., 2008). All reagents were obtained from Sigma-Aldrich unless otherwise noted.

SAM isolation

SAMs were isolated as we have previously described (Liao et al., 2010, 2011; Larson et al., 2013; St Clair et al., 2013, 2015; Sharpe et al., 2016). Mice were anesthetized by isofluorane and euthanized by cervical dislocation. Hearts were removed and bathed in Tyrode's solution containing 10 U/ml heparin at 35°C (mM: 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 5 HEPES, 5.55 glucose, 1 $MgCl_2$, and 1.8 $CaCl_2$; pH adjusted to 7.4 with NaOH). The ventricles were removed, and the sinoatrial node was dissected from the atrial tissue, as defined by the borders of the crista terminalis, the interatrial septum, and the superior and inferior vena cavae. The sinoatrial node tissue was cut into three strips, which were digested for 10–15 min at 35°C in an enzyme solution containing 4.75 U elastase (Worthington Biochemical) and 3.75 mg Liberase (Roche) in a modified Tyrode's solution (mM: 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 5 HEPES, 18.5 glucose, 0.066 $CaCl_2$, 50 taurine, and 1 mg/ml BSA; pH adjusted to 6.9 with NaOH). After enzymatic digestion, SAMs were dissociated by mechanical trituration with a fire-polished glass pipette for 5–12 min at 35°C in a modified KB solution (mM: 100 potassium glutamate, 10 potassium aspartate, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 5 HEPES, and 0.1% BSA; pH adjusted to 7.2 with KOH). Calcium was gradually reintroduced to the cell suspension in five steps over the course of 22 min to a final concentration of 1.8 mM. Cells were stored in KB solution at room temperature for up to 6 h before electrophysiological recordings.

Electrophysiology

For patch clamp recordings, ∼100-µl aliquots of the isolated SAM cell suspension were transferred to a heated,

glass-bottomed recording chamber on the stage of an inverted microscope. SAMs were constantly perfused (1–2 ml/min) with Tyrode's solution at 35 ± 1°C during all experiments. Temperature was maintained with dual platform and in-line perfusion heaters (Warner Instruments). SAMs were identified based on their spontaneous contractions, distinctive morphology (Mangoni and Nargeot, 2001; St Clair et al., 2015), small size, lack of striations, characteristic spontaneous APs with a slow upstroke and a diastolic depolarization phase, and the presence of I_f with an amplitude ≥50 pA in response to a 1-s voltage step to −120 mV.

Patch clamp recordings used borosilicate glass pipettes with resistances of 1.5–3.0 MΩ. Data were acquired at 5–20 kHz and low-pass filtered at 1 kHz using an Axopatch 1D or 200B amplifier, Digidata 1322a or 1440a A/D converter, and Clampex software (Molecular Devices). The fast component of pipette capacitance was minimized in all recordings using the patch clamp amplifier. Membrane capacitance and access resistance (R_a) were estimated in whole-cell and perforated-patch recordings from responses to 10-mV test pulses using the membrane test function in Clampex. Data are reported only for cells with a stable R_a of <10 MΩ.

Current-clamp recordings. Spontaneous APs were recorded from isolated SAMs in current-clamp mode without current injection in either amphotericin B perforated-patch or whole-cell recording configurations. Whole-cell recordings were performed using an intracellular pipette solution composed of (mM) 110 K-aspartate, 20 KCl, 1 $MgCl_2$, 5 EGTA, 5 Mg-ATP, 5 creatine phosphate, and 5 HEPES; pH adjusted to 7.2 with KOH (Himeno et al., 2011), with or without 1 mM cAMP as indicated. Perforated-patch recordings were performed using a pipette solution composed of (mM) 135 KCl, 0.1 CaCl2, 1 MgCl2, 5 NaCl, 10 EGTA, 4 Mg-ATP, and 10 HEPES; pH adjusted to 7.2 with KOH, with amphotericin B (Thermo Fisher Scientific) added to a final concentration of 200 µg/ml. Amphotericin B was prepared fresh daily as a 20 mg/ml stock solution in DMSO, and the amphotericin B–containing pipette solution was made fresh hourly by diluting an aliquot of the stock solution into the intracellular solution and vortexing for at least 1 min. The final pipette solution containing amphotericin B was stored on ice and protected from light.

The extracellular Tyrode's solution for all current-clamp recordings was composed of (mM) 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 5.5 glucose, with pH adjusted to 7.4 with NaOH. Basal spontaneous AP firing rates were determined in the presence of 1 nM isoproterenol (ISO; EMD Millipore), which was used to promote stable AP firing as previously described (Clark et al., 2004; Liao et al., 2010, 2011; Larson et al., 2013; St Clair et al., 2013, 2015); 1 µM ISO or 100 µM 3-isobutyl-1-methylxanthine (IBMX) plus 10 µM forskolin (Tocris Bioscience, Minneapolis, MN) was added to the extracellular solution as indicated. APs were collected within the first 1 min after breaking into the cell (whole cell) or after achieving stable R_a <10 MΩ (perforated patch; usually within 2–3 min of initial seal formation). Instantaneous AP firing rates were determined offline based on AP events detected using the "template search" function of Clampfit software (Molecular Devices); mean firing rates are reported for 30-s recording windows (Larson et al., 2013; St Clair et al., 2015).

Voltage-clamp recordings of I_f. I_f was assayed in three different recording configurations. Amphotericin B perforated-patch recordings were performed as described above. Whole-cell recordings were performed as previously described (Liao et al., 2010, 2011; Larson et al., 2013; St Clair et al., 2013, 2015). In brief, cells were perfused with Tyrode's solution containing 1 mM $BaCl_2$ to block K^+ currents, and recording pipettes were filled with an intracellular solution consisting of (mM) 135 potassium aspartate, 6.6 sodium phosphocreatine, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, and 4 Mg-ATP; pH adjusted to 7.2 with KOH, with 1 mM cAMP added where indicated. Whole-cell recordings were performed >2 min after membrane rupture to allow for equilibration with the pipette solution.

Excised inside-out patch recordings of I_f were performed using an extracellular (pipette) solution consisting of (mM) 160 KCl, 1 $MgCl_2$, and 10 HEPES, with pH adjusted to 7.4 with KOH, and an intracellular (bath) solution consisting of (mM) 115 NaCl, 30 KCl, 1 $MgCl_2$, 5 HEPES, and 1 EGTA, with pH adjusted to 7.2 with NaOH. 100 µM cAMP was washed-on in the bath solution as indicated. After initial GΩ seal formation, excised membrane patches from SAMs were obtained either by rapidly pulling the pipette away from the cell while still in the bath or by lifting the cell out of the bath with the pipette and immediately resubmerging the pipette again. Patches were obtained from ∼70% of cells. Of those patches, ∼20% had I_f of >20 pA in response to a 2-s voltage step to −150 mV.

To evaluate the voltage dependence of activation of I_f, cells or patches were held at −50 mV, and I_f was elicited by 3-s test pulses from −60 to −160 mV in 10-mV increments. Conductance (G) was calculated from hyperpolarization-activated inward currents from Ohm's law:

$$G = \frac{I}{V - V_r},$$

where I is the time-dependent inward current, V is the test potential (corrected for a 14-mV liquid junction potential error in whole-cell and perforated-patch recordings), and V_r is the reversal potential for I_f (−30 mV; Liao et al., 2010). Conductances were plotted as a

function of test voltage (V) and fit with a Boltzmann equation to determine the $V_{1/2}$ and slope factor (k) for each cell:

$$f(V) = \frac{1}{1 + e^{(V - V_{1/2})/k}}.$$

Voltage-clamp recordings of Ca^{2+} currents. Whole-cell Ca^{2+} currents were recorded as we have previously described (Larson et al., 2013; St Clair et al., 2015) with an intracellular solution consisting of (mM) 130 CsCl, 1 $MgCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, and 0.1 Na-GTP, with pH adjusted to 7.2 with CsOH. Cells were constantly perfused at 35 ± 1°C with an extracellular solution containing (mM) 130 tetraethylammonium chloride, 2 $CaCl_2$, 1 $MgCl_2$, 10 4-aminopyridine, and 10 HEPES, with pH adjusted to 7.4 with CsOH. 8-Br-cAMP was included in the bath or patch pipette as indicated. Ca^{2+} currents were elicited by 200-ms depolarizing voltage steps between −70 and 60 mV in 10-mV increments from a holding potential of −90 mV. All voltages were corrected for a calculated −9-mV liquid junction potential. Mean current-voltage plots were fit with a modified Boltzmann equation:

$$I = \frac{G_{max} * (V - V_r)}{\left\{1 + exp\left[\frac{(V - V_{1/2})}{k_G}\right]\right\}},$$

where I is the peak current at a given test voltage (V), V_{rev} is the reversal potential, G_{max} is the maximum conductance, $V_{1/2}$ is the half-maximal activation potential, and k_G is the slope factor.

Statistics

All data are presented as mean ± SEM. Comparisons were made using unpaired Student's *t* tests, unless otherwise noted. Statistical significance was indicated by $P < 0.05$.

Online supplemental material

Fig. S1 shows that AP firing rates elicited by 100 µM IBMX and 10 µM forskolin are saturating because 300 µM IBMX plus 30 µM forskolin do not produce additional increases. Fig. S2 shows that extracellular 8-Br-cAMP does not increase either AP firing rate or Ca^{2+} current amplitude in SAMs.

RESULTS

Slower AP firing rates in aged SAMs persist during maximal stimulation of endogenous cAMP production

We previously reported that AP firing rates are slower in SAMs from aged mice than in SAMs from young mice, even in response to a saturating concentration of the βAR agonist ISO (Larson et al., 2013). Here we asked whether stimulation of downstream elements in the βAR-cAMP signaling pathway could be used to overcome the deficit in pacemaking in aged SAMs. To this end, we recorded spontaneous APs in the amphotericin B perforated-patch configuration from acutely isolated SAMs from young (2–3 mo) or aged (21–24 mo) mice. AP firing rates were determined in control conditions and in response to wash-on of saturating concentrations (Fig. S1) of the broad-spectrum phosphodiesterase inhibitor, IBMX (100 µM), plus the adenylate cyclase activator, forskolin (10 µM). Although IBMX plus forskolin significantly increased AP firing rates compared with control in both young and aged SAMs (young, 199.6 ± 31.4 AP/min, $n = 6$; aged, 151.7 ± 31.4, $n = 6$), the increase in aged cells was not sufficient to overcome the slower basal AP firing rate. Consequently, the age-dependent difference in AP firing rate persisted in the presence of IBMX and forskolin (Fig. 1).

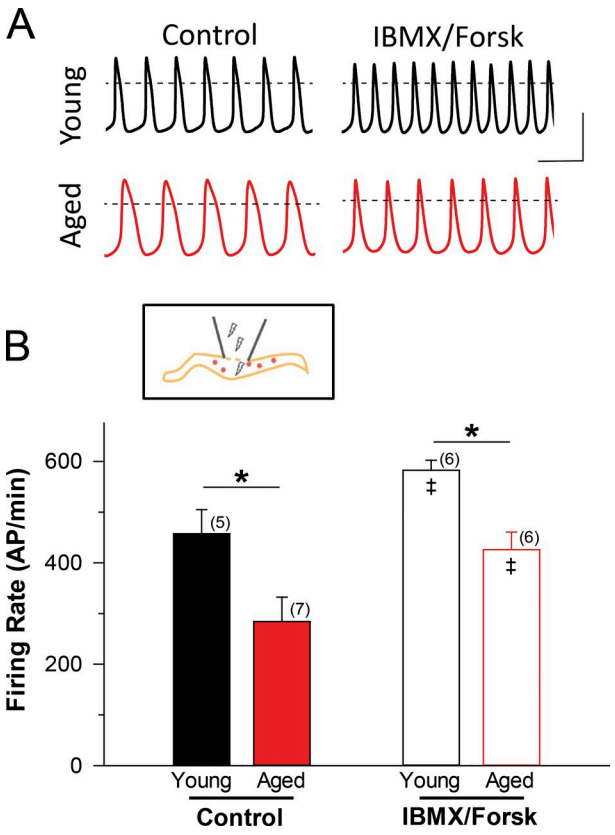

Figure 1. **Slower AP firing rates in aged SAMs persist during maximal stimulation of endogenous cAMP production.** (A) Representative spontaneous APs from perforated-patch recordings from SAMs isolated from young (black) or aged (red) mice under control conditions (left) or in 100 µM IBMX plus 10 µM forskolin (Forsk; right). Scale bar: 250 ms, 50 mV. Dashed lines indicate 0 mV. (B) Mean AP firing rates (±SEM) from young and aged SAMs in control or IBMX plus forskolin. Numbers in parentheses indicate numbers of cells. *, $P < 0.05$ versus corresponding condition in young cells (*t* tests); ‡, $P < 0.05$ versus control conditions for the same age before wash on of IBMX/forskolin (paired *t* tests). (inset) Schematic illustration of perforated-patch recording configuration. Lightning bolts represent amphotericin B, and red asterisks represent endogenous cAMP remaining in the cell during the recordings.

Exogenous cAMP rescues the AP firing rate of aged SAMs

We next asked whether stimulation of cAMP-responsive end effectors could be used to restore the maximum AP firing rate in aged SAMs, independent of the endogenous cAMP signaling pathways. We initially attempted to increase intracellular cAMP in SAMs by applying the "membrane-permeant" cAMP analogue, 8-Br-cAMP, in perforated-patch recordings. However, 8-Br-cAMP had little or no effect on AP firing rate or Ca^{2+} current amplitude (used as a control) across multiple lots, concentrations, and application times because of an apparent inability to cross the cell membrane in SAMs (Fig. S2).

As an alternative approach, we recorded spontaneous AP firing rates in whole-cell patch clamp recordings in the presence or absence of a saturating concentration of cAMP in the patch pipette (1 mM; St Clair et al., 2013). Remarkably, we found that this exogenous cAMP completely abolished the age-dependent difference in AP firing rate, producing a much larger increase in firing rate in aged SAMs than young SAMs (~258 vs. 95 AP/min, respectively; Fig. 2). These results establish that aging does not inherently limit the maximum AP firing rate of SAMs—SAMs from older animals can achieve the same firing rate as SAMs from younger animals if they are sufficiently stimulated.

Interestingly, the basal AP firing rates were not reduced in whole-cell compared with perforated-patch recordings for either young or aged SAMs (compare Fig. 1 B with Fig. 2 B; young perforated patch 382.9 ± 17.1 AP/min, n = 6 vs. young whole cell 457.4 ± 47.6, n = 5; aged perforated patch 274.4 ± 23.8, n = 6 vs. aged whole cell 284.1 ± 48.2 n = 7), despite the expectation that cAMP would be dialyzed in the whole-cell configuration (see below).

Different effects of endogenous and exogenous cAMP on the voltage dependence of I_f in SAMs

In addition to slowing the AP firing rate, aging also causes a hyperpolarizing shift in the voltage dependence of activation of I_f, both at rest and when βARs are maximally stimulated with ISO (Larson et al., 2013). We next evaluated the effects of endogenous and exogenous cAMP on I_f in whole-cell voltage-clamp recordings from SAMs from young and aged mice. I_f was elicited by 3-s hyperpolarizing voltage steps from −60 to −160 mV from a holding potential of −50 mV. Stimulation of endogenous cAMP by bath application of 100 µM IBMX plus 10 µM forskolin caused similar depolarizing shifts in the $V_{1/2}$ of I_f in aged and young SAMs (~9 and 11 mV, respectively; Fig. 3). Hence, the age-dependent difference in the $V_{1/2}$ of I_f remained in the presence of IBMX and forskolin (Fig. 3 C), mirroring the AP firing rate response. In contrast, addition of 1 mM cAMP in the patch pipette in whole-cell recordings produced a larger depolarizing shift in aged SAMs than in young

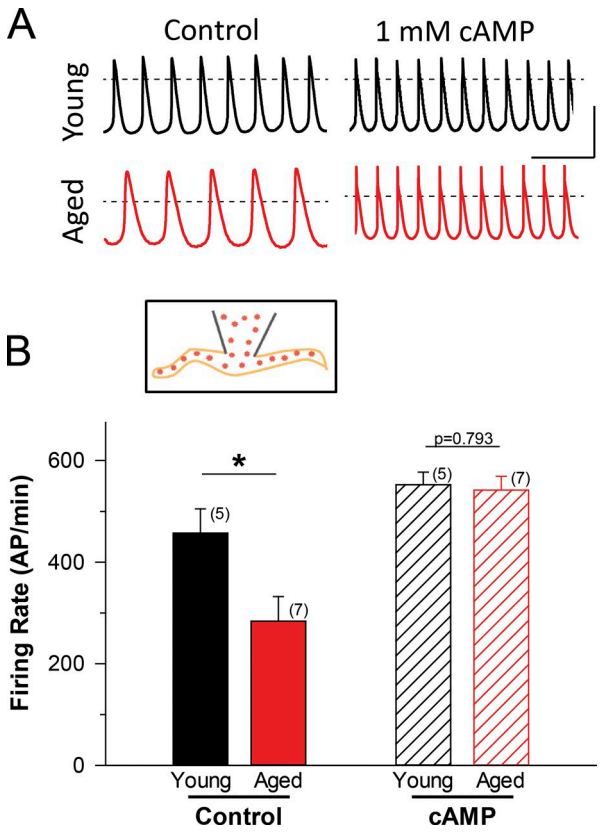

Figure 2. **cAMP rescues the slower AP firing rate in aged SAMs.** (A) Representative spontaneous APs from whole-cell recordings from SAMs isolated from young (black) or aged (red) mice under control conditions (left) or with 1 mM cAMP in the patch pipette (right). Scale bar: 250 ms, 50 mV. (B) Mean (±SEM) AP firing rates from whole-cell recordings from SAMs isolated from young (black) or aged (red) mice. Firing rates were recorded in control conditions (filled bars) or with 1 mM cAMP in the patch pipette (hatched bars). Numbers in parentheses indicate numbers of cells. P-values are from unpaired t tests. (inset) Schematic illustration of whole-cell recording configuration. Red asterisks represent cAMP introduced into the cell via the patch pipette in whole-cell recordings.

SAMs (~17 mV vs. 7 mV, respectively), such that the age-dependent difference in $V_{1/2}$ was abolished (Fig. 3).

Age-dependent changes in I_f persist in perforated-patch and excised inside-out patch recordings

The ability of exogenous cAMP to abolish the age-dependent differences in AP firing rate and $V_{1/2}$ of I_f suggested that these age-dependent differences could be caused by a lower concentration of cAMP in the cytoplasm of aged SAMs. However, such a mechanism would be surprising in whole-cell recordings (i.e., Figs. 2 and 3) because soluble cytoplasmic molecules like cAMP are thought to be diluted by the much larger volume of solution in the patch pipette. To further address this question in functional experiments, we next determined the voltage dependence of I_f in two opposing cAMP environments, a "cAMP-locked" environment,

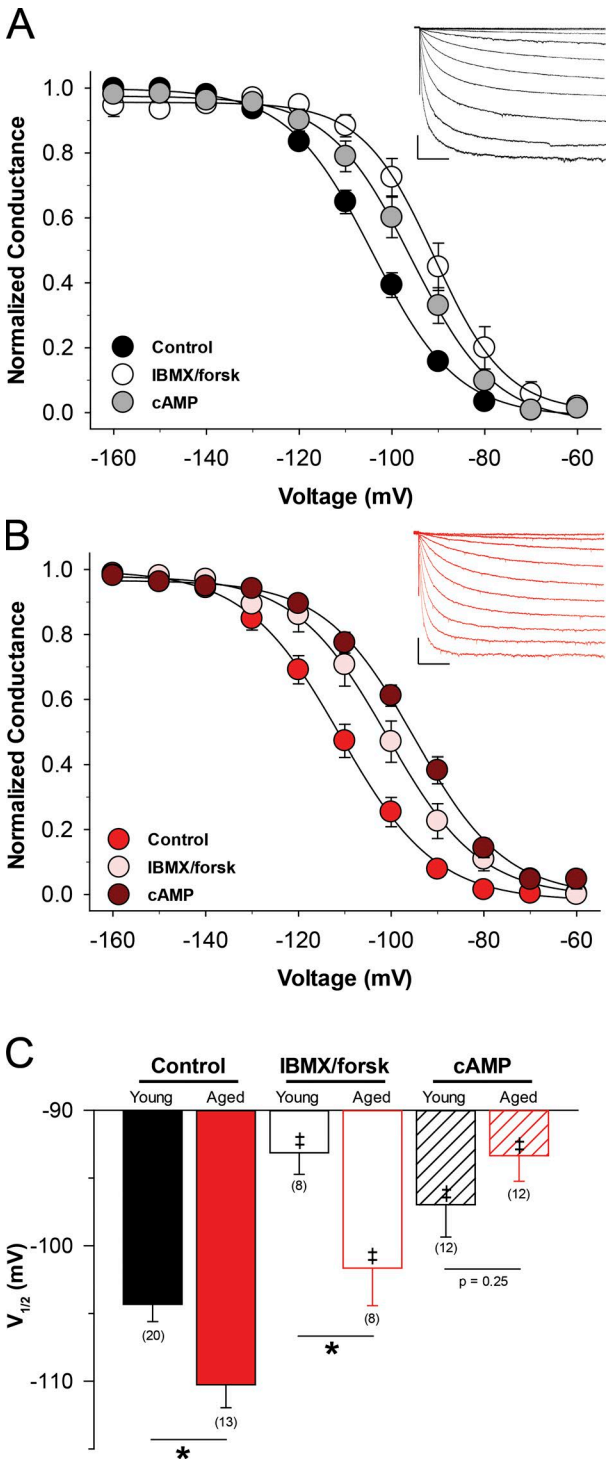

Figure 3. **Different effects of endogenous and exogenous cAMP on the voltage dependence of I_f in young and aged SAMs.** (A and B) Normalized mean (±SEM) conductance-voltage relationships for I_f in whole-cell recordings from young (A) and aged (B) SAMs in control conditions (black and red symbols), in the presence of IBMX plus forskolin (white and pink) or with 1 mM cAMP in the patch pipette (gray and dark red). (insets) Representative whole-cell I_f current families. Scale bars: 500 pA, 500 ms (A) and 200 pA, 500 ms (B). (C) Mean (±SEM) $V_{1/2}$ values for I_f in whole-cell recordings in control conditions (filled), with IBMX and forskolin in the bath (open), or with 1 mM cAMP in the patch pipette (hatched). *, $P < 0.05$ versus young SAMs in the same conditions (*t* tests); ‡, $P < 0.05$ versus control for the same age (ANOVAs with Holm-Šidák post-tests).

using the amphotericin B perforated-patch recording configuration, and a nominally "cAMP-free" environment, using the excised inside-out membrane patch recording configuration. If a lower concentration of cAMP were responsible for the hyperpolarized $V_{1/2}$ of I_f in aged SAMs, then $V_{1/2}$ values would be predicted to be more positive and the magnitude of the age-dependent shift in $V_{1/2}$ increased in perforated-patch relative to whole-cell recordings because wash-out of cAMP should be considerably reduced in the perforated-patch configuration. Meanwhile, age-dependent differences in I_f activation would be expected to be dramatically reduced or eliminated in excised-patch recordings in which cAMP is nominally absent.

As expected, the age-dependent difference in the $V_{1/2}$ of I_f was also observed in perforated-patch recordings (Fig. 4 A). However, there were no differences between perforated-patch and whole-cell recordings for either the absolute $V_{1/2}$ values (Fig. 4 B) or the age-dependent difference in $V_{1/2}$ (~6 mV in both configurations). In fact, the data trended, in both young and aged SAMs, toward more positive $V_{1/2}$ values in whole-cell recordings, opposite to the direction predicted if the cytoplasmic cAMP concentration were reduced by equilibration with the solution in the patch pipette (Fig. 4 B).

Remarkably, the age-dependent hyperpolarizing shift in $V_{1/2}$ of I_f also persisted in recordings from excised inside-out membrane patches in the absence of cAMP (Fig. 5). Wash-on of 100 µM cAMP in the perfusing solution produced significant depolarizing shifts in $V_{1/2}$ in patches from both young and aged SAMs. As in the case for whole-cell recordings, cAMP produced a larger shift in patches from aged SAMs (~15 mV vs. ~8 mV in young) and thereby abolished the age-dependent difference in the $V_{1/2}$ of I_f (Fig. 5).

DISCUSSION

In this study, we examined the effects of endogenous and exogenous cAMP on age-dependent changes in AP firing rate and I_f in acutely isolated SAMs from mice. The major findings are (a) the effects of aging in SAMs were completely reversed by a high concentration of exogenous cAMP, but not by maximal stimulation of endogenous cAMP, (b) both AP firing rates and the voltage dependence of I_f were not different in whole-cell versus perforated-patch recordings, and (c) the age-dependent hyperpolarizing shift in the voltage dependence of I_f persisted in excised inside-out membrane patches in the absence of cAMP. These results establish that aging does not impose an absolute limit on pacemaker activity

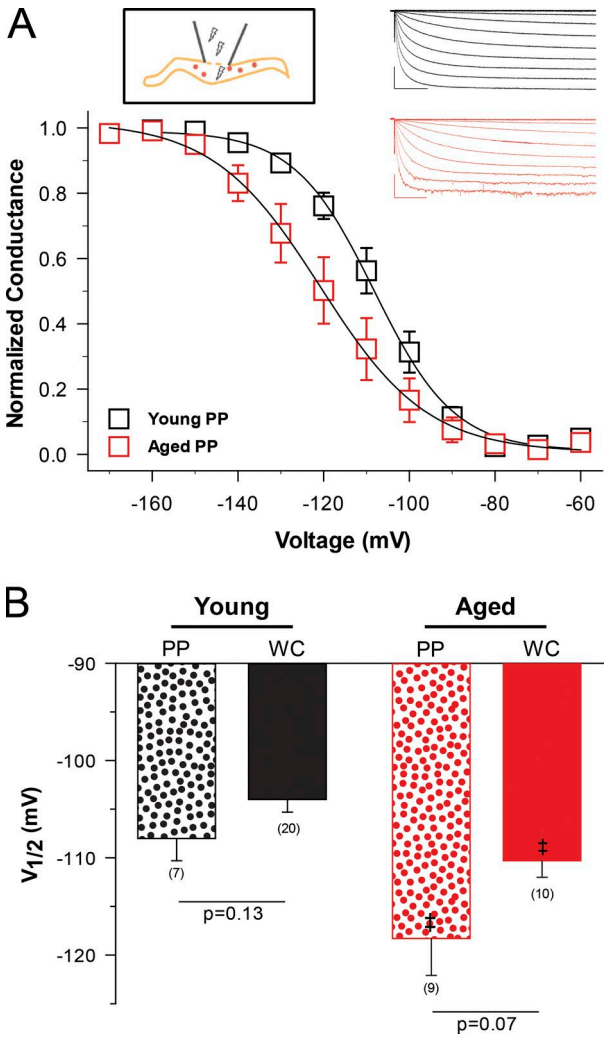

Figure 4. **Age-dependent changes in I_f in perforated-patch recordings.** (A) Normalized mean (±SEM) conductance-voltage relationships for I_f in SAMs isolated from young (black) or aged (red) mice in amphotericin B perforated-patch recordings. (insets) Schematic illustration of perforated-patch recording configuration and representative perforated-patch I_f current families in young (black) or aged (red) SAMs. Scale bars: 500 pA, 500 ms. (B) Mean (±SEM) $V_{1/2}$ values for I_f in young (black) and aged (red) SAMs in perforated-patch (PP, spotted bars) and whole-cell (WC, filled bars) recordings. P-values are comparisons between PP and WC for the same age (*t* tests); ‡, $P < 0.05$ compared with young in the same recording configuration (*t* tests).

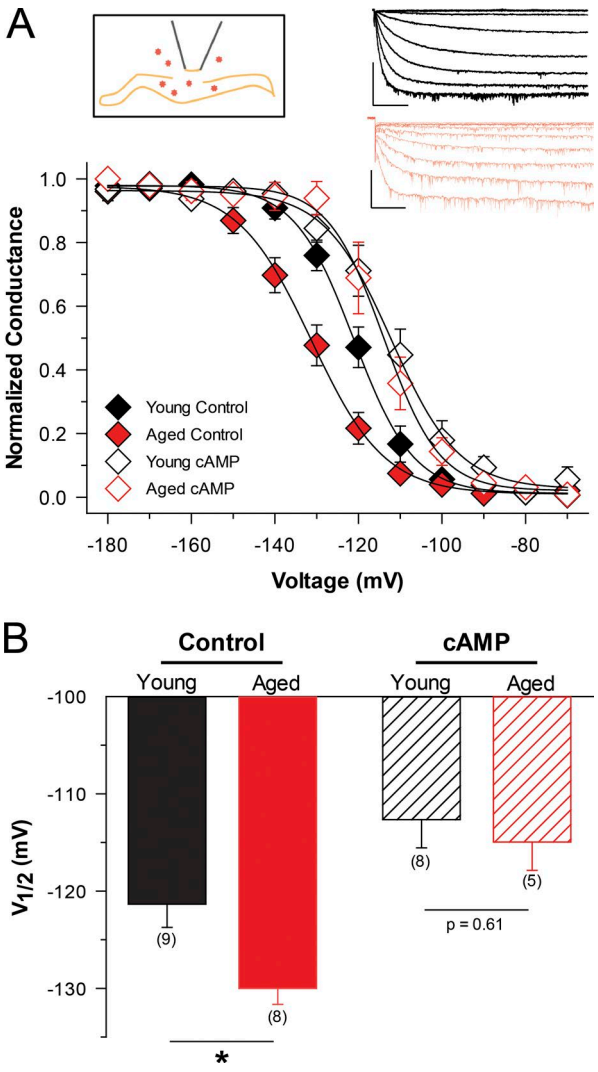

Figure 5. **Age-dependent hyperpolarizing shift in the voltage dependence of I_f persists in excised inside-out patches but is rescued by cAMP.** (A) Normalized mean (±SEM) conductance-voltage relationships for I_f from excised inside-out patches in SAMs isolated from young (black) and aged (red) mice in control conditions (filled) or upon wash-on of cAMP (open). (insets) Schematic illustration of excised patch recording configuration and representative I_f current families recorded from patches from young (black) and aged (red) SAMs. (B) Mean (±SEM) $V_{1/2}$ values for I_f in young (black) and aged (red) SAMs in control conditions (filled bars) and in the presence of 100 µM cAMP (hatched bars). *, $P < 0.05$ compared versus young control (*t* tests).

in SAMs, and they constrain the potential mechanisms by which aging exerts its effects on SAMs.

At face value, the ability of cAMP to rescue AP firing rate and I_f in aged SAMs could be taken to suggest that aging acts by reducing the cytoplasmic cAMP concentration. We did not directly measure cAMP in SAM extracts because the relevance of total cAMP concentration for cellular function is not clear, given that physiologically relevant cAMP signaling likely occurs in spatially and temporally restricted microdomains in SAMs as it does in other types of cardiac myocytes (e.g., Zaccolo, 2009; Mika et al., 2012; Perera and Nikolaev, 2013). However, even if total or local cAMP concentrations were reduced in aged SAMs, our results using different patch clamp recording configurations preclude a simple model for how changes in cAMP could cause the changes we observed. Specifically, we measured similar age-dependent changes in AP firing rate and I_f in whole-cell and amphotericin B perforated-patch recordings, despite the fact that small soluble cytoplasmic molecules like cAMP are

thought to be highly diluted in whole-cell (Hamill et al., 1981) but preserved in perforated-patch recordings (Lippiat, 2008). Although it is possible that restricted diffusion or longer-lasting effects of cAMP (e.g., mediated by PKA phosphorylation) could contribute to the similar whole-cell and perforated-patch results, a model in which age-dependent changes in SAMs arise solely because a lower cAMP concentration is difficult to reconcile with the observation that the age-dependent shift in $V_{1/2}$ of I_f persisted undiminished in excised inside-out membrane patches in which cAMP was absent from the perfusing solution and in which PKA phosphorylation would be expected to run-down rapidly (Ono and Fozzard, 1992; age-dependent shifts were −6, −9, and −9 mV in whole-cell, perforated-patch, and excised-patch recordings, respectively). Thus, the hyperpolarized voltage dependence of I_f in aged SAMs appears to result from a channel-associated factor, independent of known cAMP regulatory pathways.

These results raise two key questions: (1) what is the mechanism for the hyperpolarized $V_{1/2}$ of I_f that allows it to persist in excised patches, and (2) what is the link between the hyperpolarizing shift in I_f and the slower AP firing rate in aged SAMs? Possible mechanisms for a channel-associated shift in the $V_{1/2}$ of I_f include an age-dependent change in the expression of an HCN channel isoform, variant, or subunit. HCN4 is the predominant isoform in the sinoatrial node of all mammals. However, in the rat SAN, both HCN1 and HCN4 transcripts are down-regulated with age, with a larger relative decrease in HCN1 (Huang et al., 2007; Tellez et al., 2011). Because HCN1 is minimally sensitive to cAMP and has a more positive $V_{1/2}$ than HCN4 (Wainger et al., 2001; Altomare et al., 2003; Lolicato et al., 2011), it is conceivable that a relative decrease in HCN1 and increase in HCN4 expression in aged SAMs could contribute to the hyperpolarizing shift in $V_{1/2}$ and increased cAMP response. However, such isoform changes would be expected to contribute only minimally to our results, both because HCN1 is expressed at very low or undetectable levels in the mouse sinoatrial node (Moosmang et al., 2001; Liu et al., 2007) and because HCN1 activates ∼15-fold faster than HCN4, so a substantial decrease in HCN1 expression would be expected to be associated with slower I_f activation in aged cells, which we did not observe (unpublished data).

Could aging cause the hyperpolarizing shift in $V_{1/2}$ of I_f in SAMs by altering expression of an HCN channel–associated factor? Precedent for modulation of cAMP responsiveness of HCN channels by interacting proteins is provided by the neuronal HCN channel subunit, Trip8b, which allosterically regulates cAMP binding (Santoro et al., 2009; Zolles et al., 2009; Bankston et al., 2012; Saponaro et al., 2014). Although Trip8b is not expressed in the heart, we previously identified the existence of a distinct HCN4-specific, channel-associated factor that regulates the basal $V_{1/2}$ and cAMP sensitivity of I_f; we found that HCN4 channels are "preactivated" and insensitive to cAMP when expressed in CHO cells, owing to an as-yet-unidentified membrane-associated factor (Liao et al., 2012).

Regardless of the molecular mechanism, the age-dependent shift in $V_{1/2}$ of I_f almost certainly contributes to the slower AP firing rate of aged SAMs and to its rescue by cAMP. It is well established that reduction in I_f caused by knockout, mutation, or pharmacological blockade slows heart rate and SAM firing rate (Borer, 2004; Stieber et al., 2006; Herrmann et al., 2007, 2012; Boldt et al., 2010; Baruscotti et al., 2011; Verkerk and Wilders, 2015). It follows that the restoration of the $V_{1/2}$ of I_f with cAMP should also contribute to the restored AP firing rate. A direct link between the $V_{1/2}$ of I_f and AP firing rate is further suggested by their parallel changes in response to various manipulations of endogenous or exogenous cAMP across different recording conditions. Yet, our data do not establish a causal relationship between the $V_{1/2}$ of I_f and the AP firing rate. Indeed, it is nearly impossible to assess the independent contribution of I_f or any other process to AP firing rate in this highly interdependent system. In the case of I_f, alterations in the current affect membrane voltage not only directly but also indirectly, through resultant changes in other voltage-sensitive processes and downstream signaling cascades. We consider it probable that age-dependent slowing of AP firing rate and its rescue by cAMP result from changes in multiple processes within SAMs. Moreover, age-related slowing of iHR and mHR also involves tissue-level changes; for example, age-related fibrosis, reduction in the number of SAMs, and slowed sinoatrial node conduction are strongly correlated with slowed iHR (Kuga et al., 1993; Noujaim et al., 2004; Sanders et al., 2004; Hao et al., 2011; Akoum et al., 2012; Csepe et al., 2015).

We used mice in this study as a tractable experimental system to study aging; however, it is not clear whether results obtained in mouse SAMs can be directly extended to humans, given that there are three- to four-fold differences in iHR and mHR between humans and mice. Yet, aging causes similar relative decreases in iHR and mHR in mice and humans, and many of the fundamental mechanisms that drive pacemaking appear to be similar, as indicated by conserved protein expression and by similar heart rate responses to mutations and drugs. Although the relative contributions of different molecular processes to pacemaking may differ between mice and humans, our results suggest that new therapies could be developed for treatment of age-related sinoatrial node dysfunction by increasing the intrinsic AP firing rate of SAMs.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Heart Lung and Blood Institute (R01-HL088427) to C. Proenza. E.J. Sharpe was supported by grants from the National Institute on Aging (5T32-AG000279) and from the National Heart Lung and Blood Institute (F31 HL132408). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The authors declare no competing financial interests.

Author contributions: E.J. Sharpe, E.D. Larson, and C. Proenza conceived of the project. E.J. Sharpe and E.D. Larson conducted experiments. E.J. Sharpe, E.D. Larson, and C. Proenza analyzed the data. E.J. Sharpe and C. Proenza wrote the manuscript, which was edited by E.D. Larson.

Kenton J. Swartz served as editor.

Submitted: 25 July 2016
Accepted: 22 November 2016

REFERENCES

Akoum, N., C. McGann, G. Vergara, T. Badger, R. Ranjan, C. Mahnkopf, E. Kholmovski, R. Macleod, and N. Marrouche. 2012. Atrial fibrosis quantified using late gadolinium enhancement MRI is associated with sinus node dysfunction requiring pacemaker implant. *J. Cardiovasc. Electrophysiol.* 23:44–50. http://dx.doi.org/10.1111/j.1540-8167.2011.02140.x

Altomare, C., B. Terragni, C. Brioschi, R. Milanesi, C. Pagliuca, C. Viscomi, A. Moroni, M. Baruscotti, and D. DiFrancesco. 2003. Heteromeric HCN1-HCN4 channels: a comparison with native pacemaker channels from the rabbit sinoatrial node. *J. Physiol.* 549:347–359. http://dx.doi.org/10.1113/jphysiol.2002.027698

Bankston, J.R., S.S. Camp, F. DiMaio, A.S. Lewis, D.M. Chetkovich, and W.N. Zagotta. 2012. Structure and stoichiometry of an accessory subunit TRIP8b interaction with hyperpolarization-activated cyclic nucleotide-gated channels. *Proc. Natl. Acad. Sci. USA.* 109:7899–7904. http://dx.doi.org/10.1073/pnas.1201997109

Baruscotti, M., A. Bucchi, C. Viscomi, G. Mandelli, G. Consalez, T. Gnecchi-Rusconi, N. Montano, K.R. Casali, S. Micheloni, A. Barbuti, and D. DiFrancesco. 2011. Deep bradycardia and heart block caused by inducible cardiac-specific knockout of the pacemaker channel gene Hcn4. *Proc. Natl. Acad. Sci. USA.* 108:1705–1710. http://dx.doi.org/10.1073/pnas.1010122108

Baruscotti, M., A. Bucchi, R. Milanesi, M. Paina, A. Barbuti, T. Gnecchi-Ruscone, E. Bianco, L. Vitali-Serdoz, R. Cappato, and D. DiFrancesco. 2015. A gain-of-function mutation in the cardiac pacemaker HCN4 channel increasing cAMP sensitivity is associated with familial Inappropriate Sinus Tachycardia. *Eur. Heart J.* ehv582.

Boldt, A., U. Gergs, K. Pönicke, A. Simm, R.-E. Silber, and J. Neumann. 2010. Inotropic effects of ivabradine in the mammalian heart. *Pharmacology.* 86:249–258. http://dx.doi.org/10.1159/000320454

Borer, J.S. 2004. Drug insight: I_f inhibitors as specific heart-rate-reducing agents. *Nat. Clin. Pract. Cardiovasc. Med.* 1:103–109. http://dx.doi.org/10.1038/ncpcardio0052

Bucchi, A., M. Baruscotti, R.B. Robinson, and D. DiFrancesco. 2007. Modulation of rate by autonomic agonists in SAN cells involves changes in diastolic depolarization and the pacemaker current. *J. Mol. Cell. Cardiol.* 43:39–48. http://dx.doi.org/10.1016/j.yjmcc.2007.04.017

Christou, D.D., and D.R. Seals. 2008. Decreased maximal heart rate with aging is related to reduced β-adrenergic responsiveness but is largely explained by a reduction in intrinsic heart rate. *J. Appl. Physiol.* 105:24–29. http://dx.doi.org/10.1152/japplphysiol.90401.2008

Clark, R.B., M.E. Mangoni, A. Lueger, B. Couette, J. Nargeot, and W.R. Giles. 2004. A rapidly activating delayed rectifier K^+ current regulates pacemaker activity in adult mouse sinoatrial node cells. *Am. J. Physiol. Heart Circ. Physiol.* 286:H1757–H1766. http://dx.doi.org/10.1152/ajpheart.00753.2003

Csepe, T.A., A. Kalyanasundaram, B.J. Hansen, J. Zhao, and V.V. Fedorov. 2015. Fibrosis: a structural modulator of sinoatrial node physiology and dysfunction. *Front. Physiol.* 6:37. http://dx.doi.org/10.3389/fphys.2015.00037

DiFrancesco, D. 2010. The role of the funny current in pacemaker activity. *Circ. Res.* 106:434–446. http://dx.doi.org/10.1161/CIRCRESAHA.109.208041

DiFrancesco, D. 2013. Funny channel gene mutations associated with arrhythmias. *J. Physiol.* 591:4117–4124. http://dx.doi.org/10.1113/jphysiol.2013.253765

DiFrancesco, D., and P. Tortora. 1991. Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. *Nature.* 351:145–147. http://dx.doi.org/10.1038/351145a0

Dodge-Kafka, K.L., L. Langeberg, and J.D. Scott. 2006. Compartmentation of cyclic nucleotide signaling in the heart: the role of A-kinase anchoring proteins. *Circ. Res.* 98:993–1001. http://dx.doi.org/10.1161/01.RES.0000218273.91741.30

Fitzgerald, M.D., H. Tanaka, Z.V. Tran, and D.R. Seals. 1997. Age-related declines in maximal aerobic capacity in regularly exercising vs. sedentary women: a meta-analysis. *J. Appl. Physiol.* 83:160–165.

Fox, J.G., S.W. Barthold, M.T. Davisson, C.E. Newcomer, F.W. Quimby, and A.L. Smith, editors. 2007. The Mouse in Biomedical Research: Normative Biology, Husbandry, and Models. Vol. III. Second edition. Academic Press, Boston. 816 pp.

Hagberg, J.M., W.K. Allen, D.R. Seals, B.F. Hurley, A.A. Ehsani, and J.O. Holloszy. 1985. A hemodynamic comparison of young and older endurance athletes during exercise. *J. Appl. Physiol.* 58:2041–2046.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85–100. http://dx.doi.org/10.1007/BF00656997

Hao, X., Y. Zhang, X. Zhang, M. Nirmalan, L. Davies, D. Konstantinou, F. Yin, H. Dobrzynski, X. Wang, A. Grace, et al. 2011. TGF-β_1-mediated fibrosis and ion channel remodeling are key mechanisms in producing the sinus node dysfunction associated with SCN5A deficiency and aging. *Circ. Arrhythm. Electrophysiol.* 4:397–406. http://dx.doi.org/10.1161/CIRCEP.110.960807

Hawkins, S., and R. Wiswell. 2003. Rate and mechanism of maximal oxygen consumption decline with aging: implications for exercise training. *Sports Med.* 33:877–888. http://dx.doi.org/10.2165/00007256-200333120-00002

Heath, G.W., J.M. Hagberg, A.A. Ehsani, and J.O. Holloszy. 1981. A physiological comparison of young and older endurance athletes. *J. Appl. Physiol.* 51:634–640.

Herrmann, S., J. Stieber, G. Stöckl, F. Hofmann, and A. Ludwig. 2007. HCN4 provides a 'depolarization reserve' and is not required for heart rate acceleration in mice. *EMBO J.* 26:4423–4432. http://dx.doi.org/10.1038/sj.emboj.7601868

Herrmann, S., F. Hofmann, J. Stieber, and A. Ludwig. 2012. HCN channels in the heart: Lessons from mouse mutants. *Br. J. Pharmacol.* 166:501–509. http://dx.doi.org/10.1111/j.1476-5381.2011.01798.x

Higginbotham, M.B., K.G. Morris, R.S. Williams, R.E. Coleman, and F.R. Cobb. 1986. Physiologic basis for the age-related decline in

aerobic work capacity. *Am. J. Cardiol.* 57:1374–1379. http://dx.doi.org/10.1016/0002-9149(86)90221-3

Himeno, Y., F. Toyoda, H. Satoh, A. Amano, C.Y. Cha, H. Matsuura, and A. Noma. 2011. Minor contribution of cytosolic Ca^{2+} transients to the pacemaker rhythm in guinea pig sinoatrial node cells. *Am. J. Physiol. Heart Circ. Physiol.* 300:H251–H261. http://dx.doi.org/10.1152/ajpheart.00764.2010

Huang, X., P. Yang, Y. Du, J. Zhang, and A. Ma. 2007. Age-related down-regulation of HCN channels in rat sinoatrial node. *Basic Res. Cardiol.* 102:429–435. http://dx.doi.org/10.1007/s00395-007-0660-5

Jose, A.D., and D. Collison. 1970. The normal range and determinants of the intrinsic heart rate in man. *Cardiovasc. Res.* 4:160–167. http://dx.doi.org/10.1093/cvr/4.2.160

Jose, A.D., F. Stitt, and D. Collison. 1970. The effects of exercise and changes in body temperature on the intrinsic heart rate in man. *Am. Heart J.* 79:488–498. http://dx.doi.org/10.1016/0002-8703(70)90254-1

Kuga, K., I. Yamaguchi, and Y. Sugishita. 1993. Age-related changes of sinus node function and autonomic regulation in subjects without sinus node disease–assessment by pharmacologic autonomic blockade. *Jpn. Circ. J.* 57:760–768. http://dx.doi.org/10.1253/jcj.57.760

Lakatta, E.G., V.A. Maltsev, and T.M. Vinogradova. 2010. A coupled SYSTEM of intracellular Ca^{2+} clocks and surface membrane voltage clocks controls the timekeeping mechanism of the heart's pacemaker. *Circ. Res.* 106:659–673. http://dx.doi.org/10.1161/CIRCRESAHA.109.206078

Larson, E.D., J.R. St Clair, W.A. Sumner, R.A. Bannister, and C. Proenza. 2013. Depressed pacemaker activity of sinoatrial node myocytes contributes to the age-dependent decline in maximum heart rate. *Proc. Natl. Acad. Sci. USA.* 110:18011–18016. http://dx.doi.org/10.1073/pnas.1308477110

Liao, Z., D. Lockhead, E.D. Larson, and C. Proenza. 2010. Phosphorylation and modulation of hyperpolarization-activated HCN4 channels by protein kinase A in the mouse sinoatrial node. *J. Gen. Physiol.* 136:247–258. http://dx.doi.org/10.1085/jgp.201010488

Liao, Z., J.R. St Clair, E.D. Larson, and C. Proenza. 2011. Myristoylated peptides potentiate the funny current (I_f) in sinoatrial myocytes. *Channels (Austin).* 5:115–119. http://dx.doi.org/10.4161/chan.5.2.14195

Liao, Z., D. Lockhead, J.R. St Clair, E.D. Larson, C.E. Wilson, and C. Proenza. 2012. Cellular context and multiple channel domains determine cAMP sensitivity of HCN4 channels: Ligand-independent relief of autoinhibition in HCN4. *J. Gen. Physiol.* 140:557–566. http://dx.doi.org/10.1085/jgp.201210858

Lippiat, J.D. 2008. Whole-cell recording using the perforated patch clamp technique. *Methods Mol. Biol.* 491:141–149. http://dx.doi.org/10.1007/978-1-59745-526-8_11

Liu, J., H. Dobrzynski, J. Yanni, M.R. Boyett, and M. Lei. 2007. Organisation of the mouse sinoatrial node: structure and expression of HCN channels. *Cardiovasc. Res.* 73:729–738. http://dx.doi.org/10.1016/j.cardiores.2006.11.016

Lolicato, M., M. Nardini, S. Gazzarrini, S. Möller, D. Bertinetti, F.W. Herberg, M. Bolognesi, H. Martin, M. Fasolini, J.A. Bertrand, et al. 2011. Tetramerization dynamics of C-terminal domain underlies isoform-specific cAMP gating in hyperpolarization-activated cyclic nucleotide-gated channels. *J. Biol. Chem.* 286:44811–44820. http://dx.doi.org/10.1074/jbc.M111.297606

Mangoni, M.E., and J. Nargeot. 2001. Properties of the hyperpolarization-activated current (I_f) in isolated mouse sino-atrial cells. *Cardiovasc. Res.* 52:51–64. http://dx.doi.org/10.1016/S0008-6363(01)00370-4

Mangoni, M.E., and J. Nargeot. 2008. Genesis and regulation of the heart automaticity. *Physiol. Rev.* 88:919–982. http://dx.doi.org/10.1152/physrev.00018.2007

Marionneau, C., B. Couette, J. Liu, H. Li, M.E. Mangoni, J. Nargeot, M. Lei, D. Escande, and S. Demolombe. 2005. Specific pattern of ionic channel gene expression associated with pacemaker activity in the mouse heart. *J. Physiol.* 562:223–234. http://dx.doi.org/10.1113/jphysiol.2004.074047

Mattick, P., J. Parrington, E. Odia, A. Simpson, T. Collins, and D. Terrar. 2007. Ca^{2+}-stimulated adenylyl cyclase isoform AC1 is preferentially expressed in guinea-pig sino-atrial node cells and modulates the I_f pacemaker current. *J. Physiol.* 582:1195–1203. http://dx.doi.org/10.1113/jphysiol.2007.133439

Mika, D., J. Leroy, G. Vandecasteele, and R. Fischmeister. 2012. PDEs create local domains of cAMP signaling. *J. Mol. Cell. Cardiol.* 52:323–329. http://dx.doi.org/10.1016/j.yjmcc.2011.08.016

Moosmang, S., J. Stieber, X. Zong, M. Biel, F. Hofmann, and A. Ludwig. 2001. Cellular expression and functional characterization of four hyperpolarization-activated pacemaker channels in cardiac and neuronal tissues. *Eur. J. Biochem.* 268:1646–1652. http://dx.doi.org/10.1046/j.1432-1327.2001.02036.x

Nadon, N.L., R. Strong, R.A. Miller, J. Nelson, M. Javors, Z.D. Sharp, J.M. Peralba, and D.E. Harrison. 2008. Design of aging intervention studies: the NIA interventions testing program. *Age (Dordr.).* 30:187–199. http://dx.doi.org/10.1007/s11357-008-9048-1

Noujaim, S.F., E. Lucca, V. Muñoz, D. Persaud, O. Berenfeld, F.L. Meijler, and J. Jalife. 2004. From mouse to whale: a universal scaling relation for the PR Interval of the electrocardiogram of mammals. *Circulation.* 110:2802–2808. http://dx.doi.org/10.1161/01.CIR.0000146785.15995.67

Ogawa, T., R.J. Spina, W.H. Martin III, W.M. Kohrt, K.B. Schechtman, J.O. Holloszy, and A.A. Ehsani. 1992. Effects of aging, sex, and physical training on cardiovascular responses to exercise. *Circulation.* 86:494–503. http://dx.doi.org/10.1161/01.CIR.86.2.494

Ono, K., and H.A. Fozzard. 1992. Phosphorylation restores activity of L-type calcium channels after rundown in inside-out patches from rabbit cardiac cells. *J. Physiol.* 454:673–688. http://dx.doi.org/10.1113/jphysiol.1992.sp019286

Perera, R.K., and V.O. Nikolaev. 2013. Compartmentation of cAMP signalling in cardiomyocytes in health and disease. *Acta Physiol. (Oxf.).* 207:650–662. http://dx.doi.org/10.1111/apha.12077

Robinson, S. 1938. Experimental studies of physical fitness in relation to age. *Eur. J. Appl. Physiol.* 10:251–323. http://dx.doi.org/10.1007/BF02011412

Sanders, P., P.M. Kistler, J.B. Morton, S.J. Spence, and J.M. Kalman. 2004. Remodeling of sinus node function in patients with congestive heart failure: reduction in sinus node reserve. *Circulation.* 110:897–903. http://dx.doi.org/10.1161/01.CIR.0000139336.69955.AB

Santoro, B., R.A. Piskorowski, P. Pian, L. Hu, H. Liu, and S.A. Siegelbaum. 2009. TRIP8b splice variants form a family of auxiliary subunits that regulate gating and trafficking of HCN channels in the brain. *Neuron.* 62:802–813. http://dx.doi.org/10.1016/j.neuron.2009.05.009

Saponaro, A., S.R. Pauleta, F. Cantini, M. Matzapetakis, C. Hammann, C. Donadoni, L. Hu, G. Thiel, L. Banci, B. Santoro, and A. Moroni. 2014. Structural basis for the mutual antagonism of cAMP and TRIP8b in regulating HCN channel function. *Proc. Natl. Acad. Sci. USA.* 111:14577–14582. http://dx.doi.org/10.1073/pnas.1410389111

Severi, S., M. Fantini, L.A. Charawi, and D. DiFrancesco. 2012. An updated computational model of rabbit sinoatrial action potential to investigate the mechanisms of heart rate modulation.

J. Physiol. 590:4483–4499. http://dx.doi.org/10.1113/jphysiol.2012.229435

Sharpe, E.J., J.R. St Clair, and C. Proenza. 2016. Methods for the isolation, culture, and functional characterization of sinoatrial node myocytes from adult mice. *J. Vis. Exp.* (116):e54555.

St Clair, J.R., Z. Liao, E.D. Larson, and C. Proenza. 2013. PKA-independent activation of I_f by cAMP in mouse sinoatrial myocytes. *Channels (Austin).* 7:318–321. http://dx.doi.org/10.4161/chan.25293

St Clair, J.R., E.J. Sharpe, and C. Proenza. 2015. Culture and adenoviral infection of sinoatrial node myocytes from adult mice. *Am. J. Physiol. Heart Circ. Physiol.* 309:H490–H498. http://dx.doi.org/10.1152/ajpheart.00068.2015

Stieber, J., K. Wieland, G. Stöckl, A. Ludwig, and F. Hofmann. 2006. Bradycardic and proarrhythmic properties of sinus node inhibitors. *Mol. Pharmacol.* 69:1328–1337. http://dx.doi.org/10.1124/mol.105.020701

Tellez, J.O., H. Dobrzynski, I.D. Greener, G.M. Graham, E. Laing, H. Honjo, S.J. Hubbard, M.R. Boyett, and R. Billeter. 2006. Differential expression of ion channel transcripts in atrial muscle and sinoatrial node in rabbit. *Circ. Res.* 99:1384–1393. http://dx.doi.org/10.1161/01.RES.0000251717.98379.69

Tellez, J.O., M. Mczewski, J. Yanni, P. Sutyagin, U. Mackiewicz, A. Atkinson, S. Inada, A. Beresewicz, R. Billeter, H. Dobrzynski, and M.R. Boyett. 2011. Ageing-dependent remodelling of ion channel and Ca^{2+} clock genes underlying sino-atrial node pacemaking. *Exp. Physiol.* 96:1163–1178. http://dx.doi.org/10.1113/expphysiol.2011.057752

Verkerk, A.O., and R. Wilders. 2015. Pacemaker activity of the human sinoatrial node: an update on the effects of mutations in HCN4 on the hyperpolarization-activated current. *Int. J. Mol. Sci.* 16:3071–3094. http://dx.doi.org/10.3390/ijms16023071

Vinogradova, T.M., A.E. Lyashkov, W. Zhu, A.M. Ruknudin, S. Sirenko, D. Yang, S. Deo, M. Barlow, S. Johnson, J.L. Caffrey, et al. 2006. High basal protein kinase A-dependent phosphorylation drives rhythmic internal Ca^{2+} store oscillations and spontaneous beating of cardiac pacemaker cells. *Circ. Res.* 98:505–514. http://dx.doi.org/10.1161/01.RES.0000204575.94040.d1

Viswanathan, S., J.B.E. Burch, G.I. Fishman, I.P. Moskowitz, and D.W. Benson. 2007. Characterization of sinoatrial node in four conduction system marker mice. *J. Mol. Cell. Cardiol.* 42:946–953. http://dx.doi.org/10.1016/j.yjmcc.2007.02.008

Wainger, B.J., M. DeGennaro, B. Santoro, S.A. Siegelbaum, and G.R. Tibbs. 2001. Molecular mechanism of cAMP modulation of HCN pacemaker channels. *Nature.* 411:805–810. http://dx.doi.org/10.1038/35081088

Warrier, S., G. Ramamurthy, R.L. Eckert, V.O. Nikolaev, M.J. Lohse, and R.D. Harvey. 2007. cAMP microdomains and L-type Ca^{2+} channel regulation in guinea-pig ventricular myocytes. *J. Physiol.* 580:765–776. http://dx.doi.org/10.1113/jphysiol.2006.124891

Wilson, T.M., and H. Tanaka. 2000. Meta-analysis of the age-associated decline in maximal aerobic capacity in men: relation to training status. *Am. J. Physiol. Heart Circ. Physiol.* 278:H829–H834.

Xiang, Y.K. 2011. Compartmentalization of β-adrenergic signals in cardiomyocytes. *Circ. Res.* 109:231–244. http://dx.doi.org/10.1161/CIRCRESAHA.110.231340

Zaccolo, M. 2009. cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. *Br. J. Pharmacol.* 158:50–60. http://dx.doi.org/10.1111/j.1476-5381.2009.00185.x

Zagotta, W.N., N.B. Olivier, K.D. Black, E.C. Young, R. Olson, and E. Gouaux. 2003. Structural basis for modulation and agonist specificity of HCN pacemaker channels. *Nature.* 425:200–205. http://dx.doi.org/10.1038/nature01922

Zolles, G., D. Wenzel, W. Bildl, U. Schulte, A. Hofmann, C.S. Müller, J.O. Thumfart, A. Vlachos, T. Deller, A. Pfeifer, et al. 2009. Association with the auxiliary subunit PEX5R/Trip8b controls responsiveness of HCN channels to cAMP and adrenergic stimulation. *Neuron.* 62:814–825. http://dx.doi.org/10.1016/j.neuron.2009.05.008