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Age-associated abnormalities of intrinsic automaticity of sinoatrial nodal cells are linked to deficient cAMP-PKA-Ca\(^{2+}\) signaling

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Liu J, Sirenko S, Juhaszova M, Sollott SJ, Shukla S, Yaniv Y, Lakatta EG. Age-associated abnormalities of intrinsic automaticity of sinoatrial nodal cells are linked to deficient cAMP-PKA-Ca\(^{2+}\) signaling. Am J Physiol Heart Circ Physiol 306: H1385–H1397, 2014. First published March 14, 2014; doi:10.1152/ajpheart.00088.2014.—A reduced sinoatrial node (SAN) functional reserve underlies the age-associated decline in heart rate acceleration in response to stress. SAN cell function involves an oscillatory coupled-clock system: the sarcoplasmic reticulum (SR), a Ca\(^{2+}\) clock, and the electrogenic-sarcolemmal membrane clock. Ca\(^{2+}\)-activated-calmodulin-adenyl cyclase/CaMKII-cAMP/PKA-Ca\(^{2+}\) signaling regulated by phosphodiesterase activity drives SAN cells automatically. SR-generated local calcium releases (LCRs) activate Na\(^{+}\)/Ca\(^{2+}\) exchanger in the membrane clock, which initiates the action potential (AP). We hypothesize that SAN cell dysfunctions accumulate with age. We found a reduction in single SAN cell AP firing in aged (20–24 mo) vs. adult (3–4 mo) mice. The sensitivity of the SAN beating rate to a cAMP stress induced by both muscarinic and adrenergic receptor activation becomes decreased in advanced age. Additionally, age-associated coincident dysfunctions occur stemming from compromised clock functions, including a reduced SR Ca\(^{2+}\) load and a reduced size, number, and duration of spontaneous LCRs. Moreover, the sensitivity of SAN beating rate to a CAMP stress induced by phosphodiesterase inhibitor is reduced, as are the LCR size, amplitude, and number in SAN cells from aged vs. adult mice. These functional changes coincide with decreased expression of crucial SR Ca\(^{2+}\)-cycling proteins, including SR Ca\(^{2+}\)-ATPase pump, ryanodine receptors, and Na\(^{+}\)/Ca\(^{2+}\) exchanger. Thus a deterioration in intrinsic Ca\(^{2+}\) clock kinetics in aged SAN cells, due to deficits in intrinsic SR Ca\(^{2+}\) cycling and its response to a CAMP-dependent pathway activation, is involved in the age-associated reduction in intrinsic resting AP firing rate, and in the reduction in the acceleration of heart rate during exercise; aging; Ca\(^{2+}\) transient; intrinsic heart rate; pacemaker function; PKA signaling

AN AGE-ASSOCIATED REDUCTION in heart rate acceleration in response to stress is a major factor underlying the age-associated decline in cardiovascular reserve (29). Specifically, the heart rate increase in response to perturbations from the supine basal state, e.g., on assuming an upright posture or during exercise, is reduced in older vs. younger persons (43). That an exaggerated increase in plasma catecholamines accompanies the reduction in heart rate reserve in older persons suggests that the age-associated reduction in heart rate reserve, in part at least, is attributable to postsynaptic mechanisms that reside within the sinoatrial node (SAN) (14). Broadly, these postsynaptic mechanisms include intrinsic mechanisms that control the rate and rhythm of spontaneous action potential (AP) firing of SAN pacemaker cells, and autonomic receptor signaling that modulates these intrinsic pacemaker cell mechanisms (3, 22, 37). There is evidence that both mechanisms become altered with advancing age. 1) In humans the basal (i.e., in the absence of autonomic receptor activation) resting heart rate is not affected by age (13), but the basal intrinsic heart rate, i.e., in the presence of combined adrenergic and cholinergic receptor blockade, becomes reduced with advancing age (5, 7, 13, 26). This suggests that intrinsic function of SAN pacemaker cells becomes reduced with advancing age, and also that basal neurotransmitter signaling via β-adrenergic or cholinergic receptors in the SAN pacemaker cells changes with advancing age to compensate for the decline in intrinsic pacemaker cell function (i.e., to maintain basal resting heart rate). 2) The slope of the age-associated decline in maximal heart rate of humans during exercise in the presence of autonomic blockade is the same as that for the intrinsic heart rate at rest (27), suggesting that the reduction in the capacity to increase the heart rate in response to exercise is linked to changes in intrinsic mechanisms of the pacemaker cells. 3) The heart rate response to infusion of adrenergic (50, 54) or cholinergic agonists (9, 47) in humans at rest become reduced with advancing age. This suggests that age-associated deficiencies occur within postsynaptic signaling cascades. In other terms, it is possible that age-associated deficits in heart rate regulation within pacemaker cells involve deficits in both autonomic receptor-stimulated signaling to intrinsic clock mechanisms that regulate their normal automaticity, and to age-associated deficits in the response of intrinsic clock mechanisms to this receptor signaling.

A link between autonomic receptor-initiated signaling and intrinsic pacemaker cell mechanisms that are modulated by this receptor signaling has recently been demonstrated in adult mammals (30, 33, 52). Specifically, the basal pacemaker cell AP firing rate is regulated by intracellular Ca\(^{2+}\) cycling and Ca\(^{2+}\)-calmodulin activation of adenylyl cyclases (AC) and
calmodulin kinase II (CaMKII), leading to an increase in cAMP and to an increase in cAMP/PKA-dependent and CaMKII-dependent phosphorylation of sarcoplasmic reticulum (SR) Ca\(^{2+}\) cycling and surface membrane ion channel proteins (52, 55). The kinetics of this intrinsic basal signaling are controlled by basal phosphodiesterase (PDE) and phosphatase activities (33, 44, 51). The ticking speed of this intrinsic clock system becomes further activated, or dampened, respectively, by adrenergic or cholinergic neurotransmitter stimulation. Specifically, neurotransmitter activation of receptor and postsynaptic signaling cascades that activate and couple autonomic receptors to G proteins and to AC or guanylyl cyclase, lead to activation or suppression of protein kinases that modulate the extent of phosphorylation of intracellular Ca\(^{2+}\)-cycling proteins and surface membrane ion channel proteins that drives normal pacemaker clock activity.

In the present study, implemented in a mouse model, we hypothesized that both the intrinsic Ca\(^{2+}\)-AC-PKA, CaMKII-Ca\(^{2+}\) signaling cascade, and its modulation by neurotransmitter activation of autonomic receptors underlie age-associated deficits in SAN AP firing rate. In intact SAN isolated from adult and aged C57 black mice, we quantified changes in the expression and function of SAN pacemaker cell coupled-clock Ca\(^{2+}\)-cycling proteins under basal conditions, across a full range of concentrations of adrenergic or cholinergic receptor agonists and in response to a broad-spectrum PDE inhibitor. Furthermore, in spontaneously beating SAN pacemaker cells, we measured the initiator and an end effector of the cAMP/PKA/CaMKII signaling cascade, i.e., Ca\(^{2+}\) cycling initiating an AP. Finally, in isolated, single, permeabilized SAN pacemaker cells, we measured the characteristics of the SAN cells Ca\(^{2+}\) clock.

MATERIALS AND METHODS

The experiments were performed on adult (2–4 mo, n = 58) and aged (20–27 mo, n = 58) male C57Bl mice. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85–23, revised 1996). The experimental protocols have been approved by the Animal Care and Use Committee of the National Institutes of Health (protocol no. 034LCS2013).

SAN tissue isolation. The heart was quickly excised and placed into Tyrode solution (36 ± 0.5°C) of the following composition (in mM): 140 NaCl, 5.4 KCl, 1 MgCl\(_2\), 5 HEPES, 1.8 CaCl\(_2\), and 5.5 glucose, and titrated to pH 7.4 with NaOH. SAN tissue strips were identified by a rate of 4 ml/min. An insulated/Teflon-coated platinum electrode follows:

\[ M = \text{FWHM} \times \text{FDHM} \times 1/2\Delta F/F_0 \]

Confocal imaging of SAN pacemaker cells [Ca\(^{2+}\)] transient and Ca\(^{2+}\)-induced contractions. Cell shortening of unloaded and Ca\(^{2+}\) transient of loaded with fluo 4-AM SAN pacemaker cells isolated from adult (n = 12) and aged (n = 12) mice were recorded with a LSM 510 META confocal microscope (Carl Zeiss). Line (x) scan and frame (y) scan data were analyzed with Zeiss LSM image examiner (version 4.2, Carl Zeiss) or Imagel (NIH). SAN cells loaded (20 min, 23°C) with 5 µM fluo 4-AM (Molecular Probes, Eugene, OR) were superfused with normal Tyrode solution at 37°C (see above). Contraction and whole cell Ca\(^{2+}\) transients were recorded with line scan mode with the scanning speed of 1.93 ms/line with excitation wavelength of 488 nm. After stabilizing for 20 min, spontaneous transients were recorded either with Tyrode solution in control, or in the presence of drugs. Fluorescence data are presented as change in peak value (F) normalized to minimal fluorescence (F\(_0\)) (ΔF/F\(_0\)). Uprising the transient is expressed as the maximum positive derivative of δF/F\(_0\) (δΔF/F\(_0\)/δt max). The rate of recovery of intracellular calcium to diastolic levels was estimated from the duration of the Ca\(^{2+}\) transient at 85% Ca\(^{2+}\) transient decay time (CaR85).

Western blot analyses. Ten micrograms of total protein extracts from aged (24–27 mo, n = 6) and adult (3–6 mo, n = 6) mouse tissues were loaded per lane. Immunoblots were probed with mouse antibodies against sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 2 (SERCA2) (clone 2A7-A1; 1:5,000; Affinity BioReagents), Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) (clone R3F1; 1:2,000; Research Diagnostics), ryanodine receptor 2 (RyR2) (clone C3-33; 1:2,500; Affinity BioReagents), phospholamban (PLB) (Badrilla) or rabbit polyclonal anti-Ser-16-PLB (Badrilla), and reprobed for sarcomeric actin (clone 5C5; 1:10,000, Sigma). Horseradish peroxidase-conjugated secondary antibodies were visualized by ECL-Plus detection system (GE Healthcare).

Local Ca\(^{2+}\) Releases in permeabilized SAN pacemaker cells. SAN pacemaker cells, isolated from the SAN tissue of adult (n = 18) and aged (n = 17) mice, were permeabilized as previously described (34), using 0.01% saponin in a solution with the following composition (in mM): 100 Na\(_2\)H\(_4\)NO\(_3\) (t-aspatic acid potassium salt), 25 KCl, 10 NaCl, 3 MgATP, 0.81 MgCl\(_2\) (~1 mM free Mg\(^{2+}\)), 20 HEPES, 0.5 EGTA, 10 phosphocreatine, and creatine phosphokinase (5 U/mL), pH 7.2. After saponin washout, the solution was changed to the recording solution that contained 0.03 mM fluo 4 pentapotassium salt, 0.114 mM CaCl\(_2\) [free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) ~ 100 nM], 100 mM Na\(_2\)H\(_4\)NO\(_3\) (t-aspatic acid potassium salt), 25 mM KCl, 10 mM NaCl, 3 mM MgATP, 0.81 mM MgCl\(_2\) (~1 mM free Mg\(^{2+}\)), 20 mM HEPES, 0.5 mM EGTA, 10 mM phosphocreatine, and creatine phosphokinase (5 U/mL), pH 7.2. The cytosolic free Ca\(^{2+}\) at given total Ca\(^{2+}\), Mg\(^{2+}\), ATP, and EGTA concentrations was calculated using a computer program (WinMAXC 2.50, Stanford University). All images were recorded in the line-scan mode at 35 ± 0.5°C, with the scan line oriented along the long axis of the cell. Spontaneous Ca\(^{2+}\) releases were analyzed with IDL software (6.1, Research Systems, Boulder, CO). The amplitude of individual local calcium releases (LCRs) was expressed as a F/F\(_0\). LCR spatial size was indexed by FWHM, as the full width at half-maximum (FWHM) amplitude. LCR duration was characterized as the full duration at half-maximum (FDHM) amplitude. The number of LCRs was normalized per 100 μm of the line-scan image and during a 1-s time interval.

The integral of Ca\(^{2+}\) signal of an individual LCR was estimated as follows:

\[
\int \frac{dF\left(\tau\right)}{d\tau} = \int \frac{dF\left(\tau\right)}{d\tau} = \int \frac{d\left[F\left(\tau\right) - F_0\right]}{d\tau} \, d\tau = F_0 \cdot \Delta F_0.
\]

and presented in F/F\(_0\) (normalized Ca\(^{2+}\) signal) or absolute [Ca\(^{2+}\)]\(_i\) (ΔCa\(^{2+}\); nM). [Ca\(^{2+}\)]\(_i\), value in permeabilized SAN pacemaker cells was calculated as follows:

\[
[Ca^{2+}]_i = K_d(F/F_0)/(K_d/[Ca^{2+}]_i + 1 - F/F_0)
\]
\[
\Delta [Ca^{2+}] = [Ca^{2+}]_{i} - [Ca^{2+}]_{rest}
\]

where \( K_d \) is 864 nM, and free \([Ca^{2+}]\), at rest in the solution in permeabilized SAN pacemaker cells is known (for additional detail, see Ref. 38). The \( Ca^{2+} \) signal of the LCR ensemble was estimated by integrating the \( Ca^{2+} \) signal produced by each LCR and normalized per 100 \( \mu \)m of the line-scan image and during a 1-s time interval. The crude index that estimates the relative number of activated RyRs within an LCR has been estimated by dividing the \( Ca^{2+} \) signal of the LCR ensemble by the smallest \( Ca^{2+} \) signal of an individual LCR in permeabilized adult SAN pacemaker cells at 100 \( \text{nmol/l [Ca}^{2+}] \).

The \( Ca^{2+} \) load was indexed as \( Ca^{2+} \) release induced by a rapid (picrospritzer) caffeine (20 \( \text{mM} \)) application to the cell and was assessed as the amplitude (peak) of fluo 4 fluorescence (F/F0) transient, with the scan line oriented perpendicular to the cell membrane.

**Drugs.** Cyclopiazonic acid (CPA; 1–10 \( \mu M \)), isoproterenol (ISO; 1–1,000 \( \text{nM} \)), carbachol (CCh; 1–1,000 \( \text{nM} \)), ryanodine (10 \( \mu M \)), and 3’-isobutylmethylxanthine (IBMX; 1–100 \( \mu M \)) were obtained from Sigma Aldrich. All drugs dissolved in either DMSO or water as stock solution stored at \(-20^\circ\text{C}\) and diluted in Tyrode solution at working concentration before being applied to cells (33).

**Statistics.** Data are presented as means \( \pm \) SD (n = number of animal, SAN preparations, or SAN cells). P < 0.05 is considered as statistically significant. ANOVA with repeated measures for drug dose was employed to test for differences between adult and aged SAN tissue responses. A linear mixed-effects model was used to compare the SAN cells mean response among age or treatment groups for this repeated-measures data. These models accommodate the animals nested within treatment groups by including a random effect for animal in the model. Where necessary, a logarithmic transformation was used when the data were too skew and to make the variance more homogenous. To test drugs effects, the models first included an interaction was eliminated from the mixed-effects model. Student two-tailed t-test was used for analysis of Western blots in adult and aged SAN.

Only SAN cells that beat rhythmically were chosen to study, SAN cells that stopped beating or start to beat nonrhythmically during the control phase were excluded. Time controls were performed for each experiment to verify that the SAN cells response to drug effect was valid. One permeabilized cell was excluded from analyses due to occurrence of waves that unloaded the SR before caffeine application.

**RESULTS**

Age-associated reduction in SAN and SAN pacemaker cells intrinsic AP firing rate. The spontaneous beating rate of the intact, isolated SAN, i.e., the intrinsic beating rate (IBR) in the absence of neuronal input is lower in SAN from the aged (20 mo) hearts compared with SAN from adult (2 mo) hearts (318 \( \pm \) 17 beats/min, n = 11 vs. 405 \( \pm \) 14 beats/min, n = 12, P < 0.05) (Fig. 1A). The IBR of single cells isolated from the SAN of aged (24 mo, n = 4) mice is also reduced compared with that in cells isolated from adult (3 mo, n = 4) SAN (246 \( \pm \) 11 beats/min, n = 7 vs. 346 \( \pm \) 21 beats/min, n = 7, P < 0.01) (Fig. 1B).

**Age differences in response to neurotransmitter activation of autonomic receptor cascade signaling IBR in intact SAN.** The AP firing rate responses to \( \beta \)-adrenergic receptor (\( \beta \)-ARs) or cholinergic receptor stimulation by ISO or CCh, respectively, in adult (2 mo, n = 6) and aged (20 mo, n = 6) SAN are shown in Fig. 2. At high concentrations of ISO, the age-associated reduction in beating rate in control becomes reduced (and statistically is not significant between age groups) (Fig. 2A). The maximum beating rate increase in response to ISO averaged 204 \( \pm \) 20 beats/min in adult vs. 239 \( \pm \) 24 beats/min in aged SAN (nonsignificant). The sensitivity of the IBR of the aged SAN to \( \beta \)-AR stimulation by ISO is reduced compared with the adult SAN: EC50 (Fig. 2B) increases approximately.

![Fig. 1. Beating rate of sinoatrial node (SAN) tissue and isolated SAN pacemaker cells. A: average action potential (AP) firing rate of intact SAN tissue (adult n = 11; aged n = 12) and isolated SAN pacemaker cells (n = 7) from aged (n = 4) and adult (n = 4) mice. B and C: representative examples of extracellular signal recorded in SAN tissue (B) and contractions in single SAN pacemaker cells (C) from aged and adult mice. **P < 0.01, *P < 0.05 vs. adult, mixed-effects model for repeated-measures ANOVA. bpm, Beats per minute.](http://ajpheart.physiology.org/)
fourfold from 13 ± 6 nM in adult to 41 ± 4 nM in aged SAN (P < 0.05).

The maximum IBR was reduced in response to CCh 321 ± 27 beats/min in adult vs. 237 ± 27 beats/min in aged SAN (Fig. 2C). The threshold, EC50, and maximum beating rate responses to CCh were all reduced in aged vs. adult SAN (Fig. 2, C and D). EC50 increases approximately threefold from 41 ± 3 nM in adult to 128 ± 9 nM in aged SAN, P < 0.05 (Fig. 2D).

Blunted IBR responsiveness of aged SAN to PKA activation induced by PDE inhibition. Intrinsic mechanisms of SAN basal automaticity in numerous species (30), including mouse (33), are composed of constitutively active Ca2+-AC-cAMP-PKA-Ca2+ signaling, which leads to substantial basal phosphorylation of cell Ca2+ cycling proteins. PDE activity, by degrading cAMP, is a crucial regulator of this basal feed-forward signaling: in rabbit SAN pacemaker cells, a broad spectrum PDE inhibitor, IBMX, produced a ninefold increase in the cAMP level, and increased cAMP-mediated, protein kinase A-dependent PLB phosphorylation and the spontaneous AP firing rate to even a greater extent than did maximum β-AR stimulation (53).

The basal intrinsic adrenergic cascade signaling was probed in adult and aged SAN by examining the chronotropic effect of IBMX on IBR. Increasing IBMX from 1 μM to 100 μM dose-dependently increased IBR in both adult (2 mo) and aged (20 mo) SAN preparations (Fig. 3). The sensitivity to IBMX in aged SAN, however, is reduced compared with that in adult SAN; EC50 for IBMX to change in IBR is 5.4 ± 1 μM in adult and 22.8 ± 1 μM in aged SANs (n = 5 in each group, P < 0.01). At the saturating dose of IBMX (100 μM) (33), the IBR in the aged SAN becomes elevated to a comparable level to that in the adult SAN (similar to the effect of ISO in Fig. 2A). Thus a change in intrinsic SANpacemaker mechanisms within PDE-dependent Ca2+-AC-cAMP-PKA-Ca2+ signaling cascade accompanies advanced age.

Intrinsic AP firing rate and Ca2+ transients of single intact cells isolated from adult and aged SAN. The data in Fig. 2 suggest that the reduced sensitivity of the aged SAN to β-AR stimulation is, in part at least, attributable to an age-associated difference in the ability of the Ca2+-AC-cAMP-PKA signaling cascades to regulate Ca2+ cycling within aged SAN pacemaker cells.

To study the intracellular Ca2+ cycling kinetics, the end effector of this signaling cascade, SAN pacemaker cells isolated from adult (3 mo, n = 4) and aged (24 mo, n = 4) mice were loaded with the Ca2+ indicator fluo-4-AM and imaged by confocal microscopy. The spontaneous beating rate of the
isolated SAN pacemaker cells, like that of the isolated intact SAN, was reduced with age (Fig. 1). Representative Ca\(^{2+}\) transients in SAN pacemaker cells isolated from adult and aged SAN are illustrated in Fig. 4A. Ca\(^{2+}\) transient amplitude, indicated by maximal ΔF/ΔF\(_{0}\), was reduced by 23% in aged SAN pacemaker cells (4.11 ± 0.41, n = 6 adult cells vs. 3.1 ± 0.08, n = 7 aged cells, P < 0.05) The RyR Ca\(^{2+}\) release flux, indexed by dF/F\(_{0}\)/dt\(_{\text{max}}\) (Fig. 4B) was substantially reduced in aged SAN pacemaker cells by 38% (0.18 ± 0.02, n = 7 cells) vs. that in in adult cells (0.29 ± 0.01, n = 6 cells). Ca\(^{2+}\) transient duration (Fig. 4C) was increased in aged vs. adult SAN pacemaker cells by 57% (121 ± 6 ms, n = 7 aged cells vs. 77 ± 4 ms, n = 6 adult cells, P < 0.01). Increase in Ca\(^{2+}\) transient 85% decay time indicates that removal of Ca\(^{2+}\) from the cytosol was decreased, in part at least, due to decreased pumping into SR by SERCA.

These age-associated differences depicted in Figs. 1 and 4 can be interpreted to indicate that, under basal physiological conditions, compared with adult SAN pacemaker cells, spontaneous AP firing rate in aged SAN pacemaker cells is reduced and Ca\(^{2+}\) pumping kinetics and RyR Ca\(^{2+}\) release flux are both reduced.

**Response of SAN pacemaker cells AP firing rate to SR functional inhibition.** Given the aforementioned results, it might be expected that a stress on SERCA-2 function would lead to exaggerated Ca\(^{2+}\) cycling malfunction and to a greater reduction in the spontaneous AP firing rate in aged vs. adult SAN pacemaker cells. We employed two widely used methods to slow Ca\(^{2+}\) cycling: ryanodine, which targets Ca\(^{2+}\) release from RyR2, and CPA, which targets Ca\(^{2+}\) pumping into SR via SERCA. Ryanodine (10 μM) (Fig. 5A) decreased spontaneous IBR by 60% in adult (3 mo) SAN pacemaker cells and terminated spontaneous beating in four aged (24 mo) SAN pacemaker cells. CPA dose-dependently reduced the firing rate in SAN cells (Fig. 5B). In adult (3 mo) SAN pacemaker cells, 5 μM CPA reduced the beating rate by 38% with spontaneous regular activity preserved. However, in the presence of the same dose of CPA, aged (24 mo) SAN pacemaker cells showed abnormal or even no spontaneous activity, and overall IBR was reduced by 84% in aged SAN pacemaker cells (P < 0.05, Fig. 5D). Thus automaticity in aged pacemaker SAN cells is more vulnerable to inhibition of calcium cycling than their adult counterparts.

Intracellular Ca\(^{2+}\) transients were compared before and after CPA (5 μM) in SAN pacemaker cells from adult (n = 4) and aged (n = 4) mice to evaluate Ca\(^{2+}\) cycling. Figure 5C shows representative traces of Ca\(^{2+}\) transients recorded from adult and aged SAN pacemaker cells. Figure 5D shows summary data of CPA’s effects on Ca\(^{2+}\) cycling parameters. As expected, CPA decreased firing rate of SAN pacemaker cells and prolonged Ca\(^{2+}\) cycling duration in both adult and aged SAN pacemaker cells. However, Ca\(^{2+}\) transient duration and decay time constant in aged SAN pacemaker cells exhibited a significantly larger degree of prolongation after CPA (57 ± 11% in adult vs. 174 ± 57% in aged, P < 0.05). Inhibition of Ca\(^{2+}\) cycling with CPA caused larger inhibition of Ca\(^{2+}\) decay, concomitantly with a larger degree of inhibition of automaticity in aged SAN pacemaker cells.

**Spontaneous Ca\(^{2+}\) cycling studies in single permeabilized SAN cells.** A reduction in SR Ca\(^{2+}\) load of aged cells, due to a reduction in SERCA2 function, in part, could explain the age-associated differences in Ca\(^{2+}\) cycling in Fig. 4. We assessed the SR Ca\(^{2+}\) load by measuring the amplitude of the caffeine-induced releasable SR [Ca\(^{2+}\)]. Figure 6, A and B, shows representative examples of confocal images of Ca\(^{2+}\) transients in response to a rapid spritz application of 20 mM caffeine onto permeabilized SAN pacemaker cells bathed in physiological [Ca\(^{2+}\)].

In rabbit SAN, spontaneous LCRs occur during diastolic depolarization and activate an inward NCX current that accelerates the rate of diastolic depolarization to prompt the generation of the next AP (52). LCR characteristics are an important mechanism in determining the spontaneous AP firing rate. Prior studies showed that LCR characteristics are dependent on the SR Ca\(^{2+}\) load (44). That the SR Ca\(^{2+}\) load is reduced in single permeabilized SAN cells from aged heart (Fig. 6) suggests that LCR characteristics in aged SAN cells may be blunted. Indeed, Fig. 7 shows that the average size of the LCR and the integrated Ca\(^{2+}\) signal of the LCR ensemble in a given epoch were significantly reduced in aged (n = 10, cells from 5 mice) SAN cells vs. adult (n = 17, cells from 6 mice) permeabilized SAN cells bathed in 100 nM [Ca\(^{2+}\)]; the average amplitude of the LCR was not differ (Fig. 7A) and the average duration of the LCR was marginally significant in aged permeabilized SAN cells (Fig. 7C, P = 0.06; 29 ± 2.0 vs. 38 ± 1.8 ms in adult SAN cells), and number of LCR events showed a trend to decrease (Fig. 7D, P = 0.1) in aged vs. adult permeabilized SAN cells. A crude index that estimates the relative number of activated RyRs within an LCR (see MATERIALS AND METHODS) was significantly reduced by 2.8-fold in aged vs. adult permeabilized SAN cells (Fig. 7F).

That the sensitivity of IBR in intact SAN cells to PDE inhibition is reduced by age (Fig. 3) suggests that the increase
in LCR signal in response to PDE inhibition will also be reduced. Figure 8, A and B, illustrates representative LCRs in adult (n = 8, cells from 6 mice) and aged (n = 8, cells from 6 mice) permeabilized SAN cells in response to PDE inhibition (5 μM IBMX). On average, in adult SAN cells, IBMX induced a significant increase in LCR size (by 54%, Fig. 8C), duration (by 41%, Fig. 8D), integrated ensemble LCR Ca$^{2+}$ signal (by more than twofold, Fig. 8E), and a crude index of the relative number of activated RyRs within an LCR (more than twofold, Fig. 8F). In aged permeabilized SAN cells, however, IBMX was without significant effect (Fig. 8).

The reduced sensitivity of PLB phosphorylation in aged SAN cells to IBMX, the prolonged Ca$^{2+}$ transient duration in intact SAN cells the reduced SR Ca$^{2+}$ load and SR Ca$^{2+}$ cycling characteristics of permeabilized SAN cells from aged heart in control and in response to IBMX (Figs. 6–8) suggest that intrinsic SR Ca$^{2+}$ cycling and its response to PDE inhibition decline with age, and that these age-associated deficits, in part at least, underlie the age-associated reduction in IBR in intact SAN.

**Calcium cycling protein levels in adult and aged SAN.** SAN pacemaking function is dependent on quantitative change in SR Ca$^{2+}$ cycling protein expression and function. The relative quantity of these three proteins was evaluated by Western blots using specific antibodies against these calcium cycling proteins. Figures 9 and 10 show examples of Western blots with
samples of SAN and atrial and ventricular tissue proteins from the same pool of adult (2–4 mo) and aged (24–27 mo) mouse hearts. The expression levels of SERCA2, PLB, and NCX1 (Fig. 9) are relatively similar in all of the adult heart tissues. Although SERCA2 expression in the aged mouse ventricle and atrium is not significantly decreased compared with that in adult tissues, SAN SERCA2 expression is significantly lower in the aged tissues (Fig. 9A). Expression of the SERCA2...
inhibitory protein PLB, along with basal PLB-Ser16 phosphorylation, both remain unchanged compared with adult ones in all of the studied aged tissues (Fig. 9B). Note that the ratio of PLB to SERCA increased in aged tissue compared with adult tissue (Fig. 9C). A similar trend in mouse heart tissue has been documented previously (32). Similar to SERCA2, the expression of NCX1 is significantly decreased only in aged SAN tissue and remains unchanged with age in atrium and ventricle (Fig. 10A). In keeping with our findings in the rabbit SAN (36), the expression of RyR2 is lower in mouse SAN than in ventricular or atrial tissues (Fig. 10B). While RyR2 level remains unchanged in aged compared with adult ventricles, it is considerably lower in aged atria and is significantly diminished in aged SAN tissue, where longer exposure time on immunoblots is required to detect the low remaining RyR2 protein levels.

**DISCUSSION**

“Aging” is a rather nonspecific term that has been applied to a spectrum of life periods (e.g., neonatal, adult, and aged). While not solely a disease of the elderly, the risk of all types of heart disease, including atrial fibrillation (AF) increases with age (39). AF is the most common arrhythmia in older adults, with a prevalence of 9% in adults aged 80 yr or older (40). Similar trends for AF to increase with age have also been documented before in mice (24 compared with 2 mo) (35). It has been demonstrated previously that AF is associated with remodeling of intracellular Ca\(^{2+}\) homeostasis that plays a role in development of the contractile dysfunction and the changes in atrial electrophysiology (17). It is possible, therefore, that age-associated changes in mechanisms intrinsic to pacemaker cells may be involved in the increased incidence of AF that accompanies advancing age.

Alterations in some cardiovascular regulatory mechanisms may occur in early life span, but are not changed in adulthood and advanced age, and visa versa, or differ by sex. Furthermore, aged-associated changes may occur in different species or in different strains within species. Consequently, the effect of aging cannot be generalized, and it is important to strictly qualify results in aging studies with species, strain (23), sex (11, 42), and the specific age range examined (29). In the present study, we compared changes in heart rate mechanisms in adult (2–4 mo) and aged (20–27 mo) C57 black mice. Changes between the neonatal period and advanced age have been previously documented in rabbit (2 days vs. 5.6 yr) (1, 6) and cat (6 wk vs. 18 yr) SAN function (1, 6). Similarly, the present results demonstrate that IBR of the isolated mouse SAN and in cells isolated from the node are reduced in advanced age (Fig. 1). Numerous mechanisms involved in the initiation of the heartbeat, i.e., pacemaker normal automaticity,
change with advancing age. 1) The site of the leading pacemaker shifts toward the inferior vena cava in aged rats (3 vs. 24 mo) (62) and humans (24 vs. 66 yr) with SAN dysfunction (28), but the site of the leading pacemaker does not appear to be affected by age in rabbits (2 days vs. 5.6 yr) or cats (6 wk vs. 18 yr) (1). 2) SAN pacemaker cells from aged rats (24 mo) vs. 3-mo-old rats are more sensitive to blockade of neuronal Na+/H+ channel by TTX (62) or to blockade of funny current (If) by Cs2+ or ivabradine (62). Pacemaker cells from aged guinea pig (1 vs. 38 mo) are more sensitive to blockade of L-type blocker by nifedipine (24). Moreover, the L-type and If in mouse SAN cells decrease in advanced age (2 vs. 24 mo) (31). 3) There is an age-associated decrease in SAN RyR, Kv1.5, and HCN1 gene expression in rat (3 vs. 24 mo) and increase in Na1.5, Na1.5, and Ca1.2 gene expression (19, 21, 49, 62). Note, however, a reduction in If induced reduction in the heart rate, contributions of changes not only in If, but also of changes in Ca2+ cycling within SAN cells, because the membrane and Ca2+ clocks cross talk in response to any disturbance signal that directly perturbs either clock entrains the function of the other clock (60). 4) The maximal response to ISO in rat (3 vs. 24 mo) SAN pacemaker cells decreases in advanced age (45). 5) The number of cells in aged human (50 vs. 75 yr) SAN tissue decreases (8). 6) SAN Cx43 protein expression decreases with age in rat (1 vs. 38 mo); however, the expression of other cardiac connexins, Cx40 and Cx45, does not differ with age (25). 7) Sick sinus syndrome is associated with abnormal impulse initiation and propagation from the SAN and occurs most commonly in older patients (10). In mice exhibiting sick sinus syndrome, there is an increase in pacemaker cells apoptosis, a reduction in the number of cells, and CaMKII activity is increased (48).
Reduced response of the aged SAN to neurotransmitters. The results of the present study add an additional perspective on how the heart’s pacemaker function changes in advanced age.

Even under resting conditions, the normal heart beat-to-beat rhythms are neither strictly stationary nor completely random and continuously shift from one period to another, providing complexity to the beating interval variability (review in Ref. 59). Loss of this complexity in aging and cardiovascular disease is manifested as a reduction in heart rate variability (HRV) and this reduction correlates with an increase in both morbidity and mortality (46). Changes in HRV in vivo have mainly been attributed to the balance of sympathetic and parasympathetic neural impulses to the heart. Therefore, an imbalance of flux between the two arms of the autonomic system in the presence of cardiovascular disease has been thought to be the basis of changes in both the heartbeat interval and HRV. Changes in both the sympathetic (29, 41) and parasympathetic (12) system activity were documented in advanced age. However, because parasympathetic and sympathetic nerves discharge neurotransmitters that bind pacemaker cell receptors within the SAN tissue and modulate the heart rate and rhythm, the readout of HRV is a direct output of intrinsic pacemaker cell function. Preliminary evidence suggests that changes that occur in the properties of the pacemaker cells in the SAN likely contribute to age-associated reduction in HRV and heart rate complexity in vivo (57). Future experimental and theoretical data are needed to establish this perspective.

Our novel finding is that the sensitivity of the beating rate responses of the intact, isolated SAN to both muscarinic and adrenergic receptor activation becomes decreased in advanced age (Fig. 2). Thus the SAN in advanced age is “stuck in low gear” with respect to the heart rate response to neurotransmitters. Higher concentrations of the sympathetic adrenergic neurotransmitter, ISO, however, overcome the age-associated deficit, whereas the deficit to the cholinergic receptor agonist CCh persists even at high concentrations (Fig. 2). That the SAN intrinsic firing rate is reduced in the absence of exogenous neurotransmitters while the basal heart rate in vivo remains unchanged (26, 29) suggests that the latter is preserved by increased sympathetic nerve impulses (or epinephrine), or that vagal impulses become reduced with advancing age. Thus, although the sensitivity of the IBR to autonomic neurotransmitters is reduced in vivo (9, 50, 56), an age-associated shift in the balance of sympathetic and vagal nerve impulses preserve the basal heart rate in advanced age. In older humans, perturbations from the basal state elicit a reduced increase in heart rate and are associated with a greater neurotransmitter and epinephrine hormone spillover into the circulation (14). The heart rate response to β-adrenergic stimulation, however, declines with age (45). Thus, in humans as well as mice (present study), overcompensation of a diminished postsynaptic response to neurotransmitters by enhanced sympathetic nerve impulse and epinephrine occurs (14).

Age-associated changes in intracellular Ca\(^{2+}\)-AC-PKA signaling. The reduced beating rate sensitivity of the aged SAN to ISO or CCh (Fig. 2) may be attributable to changes in receptor number or affinity, or to signaling from the receptor to intracellular effectors that produce a change in beating rate. Recent studies in rabbit SAN pacemaker cells indicate that one intracellular effectors, the SR, spontaneously cycles Ca\(^{2+}\) in a roughly periodic manner; in other terms, the SR is a Ca\(^{2+}\) clock (30). The timekeeping by this clock and its interaction with surface membrane electrician molecules operate as a coupled-clock system that regulates the spontaneous pace-
maker cells’ AP firing rate, i.e., normal automaticity (60). The speed at which the SR cycles Ca\(^{2+}\) is a critical factor in determining how fast the coupled-clock system ticks, and thus the rate at which the pacemaker cells fire spontaneous APs. Phosphorylation of PLB and RyR in SAN by constitutive AC activation, i.e., in the absence of β-AR stimulation, which activates basal PKA activity, results in high basal levels of PKA-dependent protein phosphorylation of PLB and other cell proteins involved in Ca\(^{2+}\) homeostasis. Basal PDE and phosphoprotein phosphatase activities limit cAMP and phosphorylation levels to keep the basal set point of Ca\(^{2+}\)-AC-cAMP-PKA-Ca\(^{2+}\) signaling near the midpoint of its range (61). On stimulation of β-ARs, the cAMP-PKA signaling cascade that drives the basal beating rate becomes further activated, leading to increases in SR Ca\(^{2+}\) cycling and acceleration of the Ca\(^{2+}\) clock ticking speed.

Similarly, in the absence of β-AR stimulation, PDE inhibition increases cAMP, enhances local PKA-dependent phosphorylation of PLB in SAN, increases the Ca\(^{2+}\) ticking speed, and increases the spontaneous AP firing rate (53). The present results indicate that, with aging, the sensitivity of the IBR of the intact SAN to PDE inhibition becomes reduced (Fig. 3). Thus one mechanism that appears to be involved in the reduction in sensitivity of spontaneous SAN beating rate in advanced age is a reduced sensitivity of PLB phosphorylation to PDE inhibition. This mechanism may explain also the reduced sensitivity of the IBR to β-ARs stimulation (Fig. 2). However, because basal PLB phosphorylation before PDE inhibition does not differ with age, the latter per se cannot explain the reduced intrinsic SAN beating rate. Thus another mechanism distal to PLB phosphorylation must change with age.

Age-associated changes in Ca\(^{2+}\) cycling in sinoatrial nodal cells. Because PLB phosphorylation drives the kinetics of the Ca\(^{2+}\) clock, age-associated changes in the Ca\(^{2+}\) clock itself may occur. Indeed, the present results show that the age-associated reduction in basal AP firing rate in aged SAN pacemaker cells occurs in the context of sluggish Ca\(^{2+}\) cycling (Fig. 4), i.e., reduced Ca\(^{2+}\) flux rate in response to an AP, and reduced rate of Ca\(^{2+}\) removal from the cytosol, both compatible with a compromised SR Ca\(^{2+}\) function. Also, in response to pharmacological SR stress, the SR Ca\(^{2+}\) cycling becomes disabled to a substantially greater extent in aged vs. adult SAN cells (Fig. 5). Furthermore, in permeabilized aged SAN cells, in a fixed physiological Ca\(^{2+}\) milieu, the SR Ca\(^{2+}\) load is reduced compared with adult SAN cells (Fig. 6), and spontaneous, SR-generated LCRs that in intact cells promote spontaneous membrane potential depolarization are reduced in size, number, and duration in aged vs. adult SAN cells (Fig. 7). Finally, PDE inhibition markedly increases LCR size and amplitude in adult but not aged SAN cells (Fig. 8). Thus a reduction in Ca\(^{2+}\) clock kinetics in aged SAN cells appears to be involved in both age-associated reduction in the basal intrinsic AP firing rate (Fig. 1) and its reduced response to PDE inhibition (Fig. 3).

Age-associated changes in Ca\(^{2+}\) cycling protein expression. An important factor potentially contributing to age-associated changes in pacemaker activity (decreased beating rate and a distinctive decline in function) is the significant reduction in Ca\(^{2+}\) cycling protein expression in SAN. While in rat (23) the SERCA2 expression in the heart is reduced in advanced age, it remains unchanged with age in mouse (Fig. 9A). Age-related changes in Ca\(^{2+}\) cycling proteins in the heart have typically been studied only in whole heart samples without considering the distinctive features of the different regions of the heart. They have also often only been studied in adult and not aged tissues. This could account for discrepancies in the literature regarding age-related changes in cardiac Ca\(^{2+}\) cycling proteins (23, 58). The present study analyzed protein expression in the different dissected mouse heart tissues, thereby allowing identification of important regional differences. The most profound changes in the Ca\(^{2+}\) cycling protein levels were detected in SAN tissue compared with the other heart regions. While SERCA2 and RyR2 expression in C57B mouse SAN in the present study significantly decreased with age, these did not statistically differ in adult and aged ventricular tissue. Note, however, that these protein expressions do become reduced with aging over the same range in other species [e.g., rat Fisher 334 (6 vs. 24 mo), rat Wister (4 vs. 24 mo), and also in mouse FVB (3 vs. 28 mo)] (23). Atrial tissue reveals only a small decrease in NCX1, but a significant age-dependent loss of RyR2. These findings suggest that reduced SR Ca\(^{2+}\) load and blunted LCR in the aged SAN are potentially directly related to decreased levels of SERCA2 and RyR2. In addition, important age-associated changes in the mRNA profiles of different ion channel and exchanger genes have recently been described in the atrium and SAN (49). The ratio of PLB to SERCA in the present study increases in advanced age, suggesting the inhibition of SERCA by PLB could be higher in advanced age (44). In agreement with our study, mRNA levels of RyR2 were found to be significantly reduced in SAN tissue of rat aged 24 vs. 3 mo (62). That NCX protein levels (Fig. 10) also become reduced in advanced age suggests that reductions in proteins that pump Ca\(^{2+}\) into the SR and in RyR Ca\(^{2+}\) release to NCX are linked to the reduction in intrinsic SAN and SAN pacemaker cells beating rate (Fig. 1).

Age-associated changes in Ca\(^{2+}\) cycling in SAN are similar to those in ventricular myocytes. Coordinated actions of the same molecules in SAN and ventricular cells drive both chronotropy and inotropy. The gain of excitation-contraction coupling becomes reduced with aging in male Fisher 344 rats (3 vs. 24 mo). However, the excitation-contraction coupling gain was not affected by age in female myocytes (20). The rate of aging among components of the cardiovascular system varies among species and between sexes. Although rodents are one of the most popular aging models, age-associated changes in the expression of Ca\(^{2+}\)-transporting proteins differ in different rodent species (23). Both the age-associated reduction in kinetics of Ca\(^{2+}\) cycling and the reduction in cAMP-PKA-dependent stimulation of Ca\(^{2+}\) cycling observed in mouse SAN in the present study are strikingly similar observations compared with how aging affects rat ventricular myocytes. Specifically, in ventricular myocytes, Ca\(^{2+}\) removal from the cytosol following AP Ca\(^{2+}\) release is slowed (16), due in part to a reduced expression of SERCA2 (2, 29), and this results in a prolonged contraction (16, 29). The increase in SR protein phosphorylation in response to β-AR stimulation of ventricular cells becomes reduced with advancing age (15), and this is accompanied by a reduction in the β-AR-induced augmentation of the AP-induced SR Ca\(^{2+}\) release, acceleration of Ca\(^{2+}\) removal from the cytosol, and reduced augmentation of contraction amplitude and relaxation (18, 56). A coordinated diminution in the function of these molecules in SAN and
ventricular myocytes with aging would confer a protective adaptation: due to a slower Ca$^{2+}$ cycling in aged vs. adult ventricular cells in response to accelerated electrical impulses, aged ventricular myocytes become Ca$^{2+}$ overloaded (16) and generate spontaneous abnormal SR-generated Ca$^{2+}$ that can trigger abnormal APs (4). Slower SR Ca$^{2+}$ cycling in SAN pacemaker cells in response to β-AR stimulation results in a reduced rate of APs emanating from the SAN and conducted to ventricular cells. The reduction of the increase in heart rate during stress (e.g., β-AR stimulation during exercise) protects ventricular cells in the aged heart from this Ca$^{2+}$ overload, and this from arrhythmias. Other molecules of the coupled pacemaker clock system, e.g., PKA signaling and mitochondrial ATP production proteins, also may change with age and require further study.

In summary, results from both prior and the present studies appear to indicate that advancing age is accompanied by reduced function within and among the molecular functions that regulate the rate and strength of the heartbeat. In the present study, age-associated changes within the SAN lead to a reduction in the IBR. This reduction is accompanied by a reduced sensitivity of the beating rate responses to both muscarinic and adrenergic receptor activation. Moreover, the sensitivity of the IBR of the intact SAN to PDE inhibition becomes reduced in advanced age, indicating blunted intrinsic clock components responses to increase in cAMP/PKA activation. Furthermore, the age-associated reduction in basal AP firing rate of SAN pacemaker cells occurs in the context of “sluggish” Ca$^{2+}$ cycling, in part linked to a reductions in SR Ca$^{2+}$ content and SR Ca$^{2+}$ cycling proteins, and to a reduction in NCX proteins and an increased PLB-to-SERCA ratio. Thus a deterioration in intrinsic coupled-clock kinetics in aged SAN cells appears to be involved in the age-associated reduction in mouse intrinsic heart rate and is also likely implicated in the age-associated reduction in the acceleration of heart rate in response to stress.

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AUTHOR CONTRIBUTIONS


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