Cholinergic receptor signaling modulates spontaneous firing of sinoatrial nodal cells via integrated effects on PKA-dependent Ca\(^{2+}\) cycling and \(I_{\text{KACh}}\)

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Lyashkov AE, Vinogradova TM, Zahanich I, Li Y, Younes A, Nuss HB, Spurgeon HA, Maltsev VA, Lakatta EG. Cholinergic receptor signaling modulates spontaneous firing of sinoatrial nodal cells via integrated effects on PKA-dependent Ca\(^{2+}\) cycling and \(I_{\text{KACh}}\). Am J Physiol Heart Circ Physiol 297: H949–H959, 2009. First published June 19, 2009; doi:10.1152/ajpheart.01340.2008.—Prior studies indicate that cholinergic receptor (ChR) activation is linked to beating rate reduction (BRR) in sinoatrial nodal cells (SANC) via 1) a G\(_i\)-coupled reduction in adenylyl cyclase (AC) activity, leading to a reduction of cAMP or protein kinase A (PKA) modulation of hyperpolarization-activated current \((I_{f})\) or L-type Ca\(^{2+}\) currents \((I_{\text{Ca,L}})\), respectively; and 2) direct G\(_i\)-coupled activation of ACh-activated potassium current \((I_{\text{KACh}})\). More recent studies, however, have indicated that Ca\(^{2+}\) cycling by the sarcoplasmic reticulum within SANC (referred to as a Ca\(^{2+}\) clock) generates rhythmic, spontaneous local Ca\(^{2+}\) releases (LCRs) that become integrated to generate the net response to a given intensity of ChR stimulation in single, isolated rabbit SANC. The threshold CCh concentration \((CCh)\) for BRR was \(\sim 10\) nM, half maximal inhibition \((IC\(_{50}\)) was achieved at 100 nM, and 1,000 nM stopped spontaneous beating. \(G_i\) inhibition by pertussis toxin blocked all CCh effects on BRR. Using specific ion channel blockers, we established that \(I_{\text{KACh}}\) blockade by a specific inhibitor, tertiapin Q, mirrored that of CCh to reduced PLB phosphorylation. At IC\(_{50}\), CCh caused a time-dependent reduction in cAMP and reduced PKA-dependent Ca\(^{2+}\) cycling by the sarcoplasmic reticulum (SANC), manifested as rhythmic to hyperpolarization, first became apparent at [CCh] > 30 nM. At IC\(_{50}\), CCh reduced cAMP and reduced PKA-dependent phospholamban (PLB) phosphorylation by \(\sim 50\%\). The dose response of BRR to CCh in the presence of \(I_{\text{KACh}}\) blockade by a specific inhibitor, tertapin Q, mirrored that of CCh to reduced PLB phosphorylation. At IC\(_{50}\), Ca\(^{2+}\) caused a time-dependent reduction in the number and size of LCRs and a time dependent increase in LCR period that paralleled coincident BRR. The phosphatase inhibitor calyculin A reversed the effect of IC\(_{50}\) CCh on SANC LCRs and BRR. Numerical model simulations demonstrated that Ca\(^{2+}\) cycling is integrated into the cholinergic modulation of BRR via LCR-induced activation of NCX current, providing strong support for the experimental findings. Thus ChR stimulation-induced BRR is entirely dependent on \(G_i\) activation and the extent of \(G_i\) coupling to Ca\(^{2+}\) cycling via PKA signaling or to \(I_{\text{KACh}}\); at low [CCh], \(I_{\text{KACh}}\) activation is not evident and BRR is attributable to a suppression of cAMP-mediated, PKA-dependent Ca\(^{2+}\) signaling; as [CCh] increases beyond 30 nM, a tight coupling between suppression of PKA-dependent Ca\(^{2+}\) signaling and \(I_{\text{KACh}}\) activation underlies a more pronounced BRR.

NATURE HAS DEVISED A POTENT “brake” on the rate at which the heart’s pacemaker fires: signaling via cholinergic receptors (ChR) (6, 9). In situ, the ChR signaling cascade activated by vagal tone produces the most potent physiological suppression of the rate of spontaneous electrical impulses generated within the sinoatrial node, the heart’s primary pacemaker. Spontaneous beating rate reduction (BRR) of sinoatrial node (SAN) cells (SANC) via ChR signaling involves \(G_i\) protein coupling (15) to several downstream targets: signaling via \(G_{iR}\), activates ACh-activated potassium current \((I_{\text{KACh}})\), leading to membrane hyperpolarization (8, 9); signaling via \(G_{o}A\) inhibits adenylyl cyclase (AC) activity (10), which results in \(I_{f}\) in a reduction in cAMP, leading to a reduction of hyperpolarization-activated current \((I_{f})\) (via a shift of its voltage-dependent activation) and to changes in the early diastolic depolarization (DD) (11, 12, 14 and 2) a reduction of cAMP-mediated, PKA-dependent activity and phosphorylation of its downstream targets. Since L-type Ca\(^{2+}\) currents \((I_{\text{Ca,L}})\) activation is controlled by PKA-dependent signaling, e.g., during \(\beta\)-adrenoceptor \((\beta\text{-AR})\) stimulation (17, 20), or in its absence (34, 39), reduction of \(I_{\text{Ca,L}}\) has been suggested as an additional mechanism in BRR by ChR stimulation (39). Thus it is currently believed that ChR signaling slows heart rate entirely by effects on the surface membrane ion channels, including \(I_{\text{KACh}}\), \(I_{f}\), and \(I_{\text{Ca,L}}\) (9, 53).

On the other hand, recent studies demonstrate that PKA-dependent phosphorylation also controls Ca\(^{2+}\) cycling (47, 48) (referred as Ca\(^{2+}\) clock in SANC), manifested as rhythmic spontaneous subsarcolemmal Ca\(^{2+}\) releases (LCRs) (49) that affect the late DD via the electrogenic Na\(^{+}/\text{Ca}^{2+}\) exchanger (3, 4). A high basal PKA-dependent phosphorylation of Ca\(^{2+}\) cycling proteins [phospholamban (PLB), ryanodine receptors (RYR), and L-type Ca\(^{2+}\) channels] is a crucial component of LCR occurrence and is required for SANC spontaneous beating (47) and for the increase in the beating rate in response to \(\beta\)-AR stimulation. Although the concept of the crucial importance of changes in sarcoplasmic reticulum (SR) Ca\(^{2+}\) cycling in normal automaticity and its response to \(\beta\)-AR stimulation, based on observations of ryanodine effects, has been challenged (21), this challenge has been comprehensively rebutted (27, 29). Whereas inhibition of basal PKA activity by PKI, a specific peptide inhibitor, damps LCRs, resulting in BRR (47), there has been no prior investigation of how activation of ChR signaling impacts on basal AC-dependent cAMP production or

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PKA signaling and functioning of the Ca^{2+} clock within the physiological BRR mechanism.

The purpose of the present study is to determine how the aforementioned ChR-Gi-coupled signaling mechanisms (targeting both sarcolemmal ion channels and LCRs) become integrated to generate the net response to a given intensity of ChR stimulation by carbachol (CCh). Specifically, we examined the integrated effects of ChR stimulation on action potential (AP) characteristics; \(I_{\text{KACH}}, I_f, I_{\text{Ca,L}}; AC\) and guanylyl cyclase (GC) activities in the absence and presence of phosphodiesterase (PDE) inhibition; PKA-dependent PLB phosphorylation; SR Ca^{2+} cycling; and BRR. We also determined whether basal signaling via the ChR pathway in the absence of ChR-ligand activation is present within SANC, as in the case of high basal \(\beta\)-AR-independent cAMP/PKA-dependent signaling (47, 48). Finally, we employed numerical modeling to explore the coupling of SR Ca^{2+} cycling to membrane currents that affects BRR.

**METHODS**

Methods for SANC preparation, confocal Ca^{2+} imaging and electrophysiological recordings (48), Western blotting of PLB (24), and cAMP and cGMP measurements (52) have been described in detail previously, and additional details are provided in the Online Supplement.1 The animal protocols for this study were approved by the Animal Care and Use Committee of the Gerontology Research Center, National Institute on Aging (protocol no. 034LCS2010).

**Numerical modeling.** Our previous SANC model (3, 47), featuring individual stochastic LCRs, was modified to include CCh modulation of \(I_{\text{KACH}}, I_f, I_{\text{Ca,L}}\), and LCRs (see Online Supplement).

**Experimental protocols and drugs.** We applied a broad-spectrum cholinergic agonist, CCh (Calbiochem), to activate ChRs during spontaneous beating or during voltage-clamp experiments. A ChR antagonist, atropine (10 \(\mu M\), Sigma), was used in the presence of CCh to confirm that CCh-induced BRR occurred via ChR activation. To detect potential constitutive G_{\alpha}\_q protein activation in the absence of ChR activation, we pretreated a subset of SANC in Kraft-Bru¨he reagent (K-B), and during CCh experiments these cells were continuously superfused with Tyrode’s buffer containing PTX to detect a possible presence of constitutive G\_i activation (50). To disable \(I_{\text{KACH}}\), we incubated a subset of SANC for at least 1 h with 1 \(\mu M\) tertiapin-Q (TQ) (Sigma), a specific peptide inhibitor of \(I_{\text{KACH}}\) (23, 51). Cells were subsequently studied in current-clamp or voltage-clamp experiments in which SANC were continuously superfused with TQ vs. control cells (no TQ treatment). We employed 2 mM CsCl to block \(I_f\), cAMP or cGMP concentrations in SANC suspensions were measured in the absence and presence of IBMX, 100 \(\mu M\) (Sigma), to inhibit PDE activity. In some experiments the phosphatase inhibitor calyculin A (100 nM) was applied following CCh.

**Statistical analysis and nonlinear regression method.** Data are presented as means ± SE unless otherwise stated. Statistical significance of differences between means was evaluated by Student’s t-test or analysis of variance (ANOVA), when appropriate. A value of \(P < 0.05\) was considered statistically significant. To determine the IC_{50} for the average dose-response curves for a given pharmacological intervention, and to compare IC_{50} of different pharmacological interventions, a standard nonlinear regression method (GraphPad Software, Prism 5, 2007) was used. A detailed description of applied method is provided in the Online Supplement.

1 Supplemental data for this article are available online at the American Journal of Physiology Heart and Circulatory Physiology website.
rate in SANC pretreated with PTX in the absence of CCh. PTX pretreatment did not affect the basal beating rate in the absence of the ChR ligand (182 ± 5 bpm in control, n = 39, 177 ± 27 bpm in PTX-treated cells, n = 6), but it completely blocked CCh effects on BRR (Fig. 1B). Thus constitutive G_{i} activity is not present in the absence of ligand, and the effect of ChR stimulation to affect BRR requires ChR-G_{i} coupling.

Effect of CsCl or TQ on SANC membrane currents and responses to ChR stimulation with CCh. Figure 2A shows the average effect of CsCl on the total current-voltage relationship measured under the whole cell voltage-clamp configuration. Representative examples of raw currents measured across the voltage range in the presence or absence of Cs\(^{+}\) are illustrated in Supplemental Fig. S2A, i and ii. In main text Fig. 2A, note that CsCl markedly blocks the inward current at voltages negative to −60 mV. The inset in Fig. 2A shows the pre- and post-CsCl difference current-voltage relationship, i.e., the \(I_{\text{p}}\) IC50 does not have a major role in the average BRR by CCh.

Figure 4 illustrates the time course of the BRR response to CCh. Note also that even in the absence of CCh, which markedly blocks 2 mM CsCl in control cells (Supplemental Fig. S2B, i), total inward current is markedly diminished, similar to the effect of CsCl in control cells (Supplemental Fig. S2A, i vs. ii). In the absence of TQ CCh activates \(I_{\text{KAC}}\) (Supplemental Fig. S2A, iii), but in the presence of TQ CCh fails to do so (Supplemental Fig. S2B, iii). The average CCh effect in the presence of 2 mM CsCl on the steady-state \(I_{\text{KAC}}\) I-V relationship in response to CCh is shown in Fig. 2B. Note that TQ completely abolishes steady \(I_{\text{KAC}}\) current stimulated by CCh. The amplitude of the steady outward \(I_{\text{KAC}}\) current ∼3.2 pA/pF, measured in the presence of Cs\(^{+}\), within the voltage range relevant to diastolic depolarization (Fig. 2) is similar to the steady \(I_{\text{KAC}}\) current measured in the absence of CsCl in prior studies, i.e., ∼2.3 pA/pF (12) and 4 pA/pF (6).

Supplemental Fig. S4 shows the effect of CCh on \(I_{\text{CAL}}\) peak amplitude. On average, \(I_{\text{CAL}}\) was reduced by only 20% even in response to 10 μM CCh, i.e., a concentration tenfold higher than that to 1 μM, which stopped spontaneous beating (Figs. 1, 3, 4).

Roles of \(I_{\text{KAC}}\) activation or \(I_{\text{p}}\) suppression in ChR-induced suppression of BRR. Figure 3 shows the complete BRR and MDP dose response to CCh in the absence or presence of \(I_{\text{p}}\) blockade, or \(I_{\text{KAC}}\) blockade, or blockade of both \(I_{\text{p}}\) and \(I_{\text{KAC}}\) (by CsCl and TQ, together). Note in Fig. 3A that, on average, MDP hyperpolarization, which indirectly reflects the level of \(I_{\text{KAC}}\) activation in response to CCh, begins to occur at [CCh] > 30 nM. At [CCh] > 100 nM, the hyperpolarizing effects of \(I_{\text{KAC}}\) activation increased and parallel increased BRR. Importantly, TQ completely abolishes the hyperpolarization of MDP caused by any [CCh], either in the presence of \(I_{\text{p}}\) blockade (green circles vs. blue circles) or in the absence of \(I_{\text{p}}\) blockade (black circles vs. red circles in Fig. 3; see also Supplemental Fig. S3). Note in Fig. 3A the presence of an \(I_{\text{KAC}}\)-independent effect of CCh on BRR (red diamonds). Specifically, this \(I_{\text{KAC}}\)-independent BRR by CCh begins to occur at 10 nM and plateaus at 100 nM, accounting for 25–30% of the maximum BRR (Fig. 3). Note also that even in the absence of \(I_{\text{KAC}}\) blockade (Figs. 1A, red line, and 3A, black diamonds) BRR by CCh becomes evident at a [CCh] as low as 30 nM, at which no MDP hyperpolarization is evident (black circles). Figure 3B shows that IC50 \[\text{IC50} = \frac{\log(\text{IC50}_{\text{CCh}})}{M}\] estimated from the best fit of the average normalized dose-response curve for BRR to CCh shifts to lower (2.6-fold) [CCh] when the CCh effect was partially blocked by the \(I_{\text{KAC}}\) blocker TQ (Fig. 3B CCh+TQ, \[\text{IC50}_{\text{CCh}} = -7.36 M\]).

Figure 3A also shows that CsCl, which markedly blocks \(I_{\text{p}}\) (Fig. 2A), does not affect BRR at any [CCh] (green vs. black diamonds). Prior to CCh, CsCl caused a 15% BRR, as was reported earlier (6, 45). Furthermore, the CCh effect on BRR in the absence of hyperpolarization when both \(I_{\text{KAC}}\) and \(I_{\text{p}}\) are blocked (blue diamonds, Fig. 3A) is identical to that when \(I_{\text{KAC}}\) alone is blocked (red diamonds, Fig. 3A), indicating that \(I_{\text{p}}\) does not have a major role in the average BRR by CCh.

Figure 4 illustrates the time course of the BRR response to four different [CCh] in the presence or absence of \(I_{\text{p}}\) and/or
**Effects of ChR stimulation on cAMP, cGMP, and phospholamban phosphorylation.** Figure 5 shows the effects of [CCh] on total cAMP (Fig. 5A) and cGMP (Fig. 5B) in the absence (left bars) and presence of PDE inhibition by IBMX (right bars). In the absence of CCh or PDE inhibition total cAMP levels are 20 times higher than cGMP levels (Fig. 5, A and B, left bars). CCh, in the absence of PDE inhibition, had no detectable effect on either cAMP or cGMP (A and B, left bars). This lack of effect on total cAMP or cGMP is not surprising, as changes in functionally relevant local concentrations of nucleotides are often not apparent in the measurement of total nucleotides when PDE activity is present. PDE inhibition increased cAMP by sevenfold and doubled cGMP (Fig. 5, A and B, left bars). During a 20-min period of PDE inhibition by IBMX, CCh reduces total cAMP by 21 and 34% at 100 nM and 1 μM, respectively (Fig. 5A, right bars), but does not change cGMP (Fig. 5B, right bars). Although AC activity is not measured directly in this experiment, the reduction of cAMP over the 5-min period of CCh treatment in the presence of PDE inhibition indicates an effect of CCh to reduce net AC activity over that period.

**Effects of ChR stimulation on Ca$^{2+}$ cycling.** It has been shown previously that reduction of PLB phosphorylation reduces the kinetics of SR Ca$^{2+}$ cycling, which markedly affects the timing and amplitude of spontaneous LCRs (47). Figure 7, left, depicts line scan images of Ca$^{2+}$ beneath the cell surface membrane. The AP triggers a global SANC Ca$^{2+}$ transient. The AP is preceded by LCRs. At 100 nM, i.e., the IC$_{50}$ for BRR, CCh markedly affects both the AP-induced Ca$^{2+}$ transient and diastolic LCRs. The average effects of CCh on the AP-induced Ca$^{2+}$ transient and spontaneous LCRs are illustrated in Fig. 8. CCh decreases the maximum rate of rise of the AP-triggered Ca$^{2+}$ transient [d(F/F$_{0}$)/dt], an index of the SR Ca$^{2+}$ release flux (44) (Fig. 8A), reduces the amplitude of AP-triggered Ca$^{2+}$ transient and increases the times to 50 and 90% decay of cytosolic Ca$^{2+}$ transient. Figure 8B illustrates the average effects of CCh on LCR characteristics: LCR frequency of occurrence and size are reduced by 28 and 27%, respectively; the LCR period, i.e., the time between the maximum rate of the upstroke of the Ca$^{2+}$ transient triggered by the prior AP to the occurrence of maximum rate of upstroke of a subsequent LCR (as indicated in Fig. 8B inset), increased by ~43%. Figure 9 depicts the relationship of concom-
itantly measured cycle length to the LCR period during the evolution of the BRR, i.e., the prolongation of spontaneous AP cycle length with time (1–3 min) following CCh application. Note that the time-dependent prolongation of cycle length closely tracks the concomitant prolongation of LCR period.

The concomitant CCh induced inhibition of the spontaneous SANC beating rate, of Ca$^{2+}$/H11001 cycling characteristics and of PLB phosphorylation suggests that dephosphorylation mediates the effects of the IC$_{50}$CCh on Ca$^{2+}$ cycling and BRR. Thus we determined whether phosphatase inhibition could reverse the effects of CCh on LCR and BRR (Figs. 10 and 11). In Fig. 10 note that the phosphatase inhibitor calyculin A (100 nM) reverses the effects of IC$_{50}$ CCh on both cycle length and LCR characteristics. The relationship between cycle length and LCR period in control during exposure to CCh and following the addition of calyculin A in the continued presence of CCh is shown in Fig. 11. Note the close correlation between LCR period and cycle length, and, importantly, that the LCR period is always shorter than the cycle length.

Numerical modeling of CCh effect and continuity of the negative and positive chronotropic regulation by PKA. Ion channel-related mechanisms of BRR in SANC by ChR modulation, previously numerically modeled in 1999 by Demir et al. (9) and in 2002 by Zhang et al. (53), have formulated important ChR-induced changes of $I_{KACh}$, $I_f$, and $I_{Ca,L}$. Recent studies have shown that the signaling sequence, PKA $\rightarrow$ PLB $\rightarrow$ LCRs $\rightarrow$ NCX, underlies the late DD acceleration, providing a major contribution to the spontaneous beating rate in rabbit SANC (3, 47). The extent of PLB phosphorylation is high in the basal state (47, 48) but decreases under ChR stimulation with CCh (Fig. 6). This suggests that CCh also mediates BRR, at least in part, via the same signaling sequence.

In the present study, using our previous SANC model featuring individual stochastic LCRs in submembrane space (3, 47) (see Online Supplement for details), we explored the integration of classical BRR membrane mechanisms with the BRR produced by LCRs via NCX current ($I_{NCX}$) in response to the IC$_{50}$ CCh. Thus, in addition to the ion channel changes described by classical models, we also introduced experimentally measured changes of LCR characteristics (LCR period increase and LCR signal mass decrease). Our model simulations closely predict our experimental results for BRR produced by IC$_{50}$ CCh alone or combined with blockade of $I_f$ (Cs) and/or $I_{KACh}$ (TQ), or with phosphatase inhibition by calyculin A (Fig. 12). Our simulations show that the late diastolic $I_{NCX}$ (blue traces in Fig. 12A) mediates effects of LCRs in all perturbations and these LCR-induced ($I_{NCX}$-mediated) effects on the late DD substantially contribute to the BRR, which is in agreement with experimental results (Fig. 12).

When the emergence of LCRs is delayed, the diastolic $I_{NCX}$ is also delayed, resulting in a later DD acceleration and a longer cycle length (Fig. 12A, ii vs. i). When CCh-induced dephosphorylation is prevented by calyculin A, the diastolic $I_{NCX}$ also remains almost unchanged (Fig. 12A, iv), resulting in a rather limited BRR (12.9% in the model and 12.5% in experiment) produced by phosphorylation-independent $I_{KACh}$ and $I_f$. On the other hand, blockade of $I_{KACh}$ or $I_f$, or both $I_f$ and $I_{KACh}$, still accounts for less than half of the BRR compared with the BRR induced by 100 nM CCh alone in the model simulation, which is in agreement with the experimental results. These model simulations provide a theoretical basis for the LCR dependence of the BRR response to CCh.

DISCUSSION

The present study dissected the complex ChR-linked signaling cascade (Supplemental Fig. S5) to discover which mechanisms are recruited in the BRR in response to a given intensity of ChR stimulation in rabbit SANC. Graded ChR stimulation by CCh (10 to 1,000 nM) produced graded BRR: the threshold
[CCh] for BRR was /H11011 10 nM; the IC50 was 100 nM CCh; and in response to 1,000 nM CCh spontaneous APs ceased (Fig. 1, A and B). The entire BRR dose response to CCh requires Gi protein signaling, since pretreatment with PTX to prevent Gi activation abolished all effects of CCh (Fig. 1 B). PTX effects on If and ICa,L suppression by ChR activation have also been reported previously (14, 17). The present results show that, in the absence of CCh, PTX does not affect the basal beating rate, which indicates an absence of any significant constitutive Gi activity linked to spontaneous beating in rabbit SANC. The Gi protein activation responsible for the ChR induced BRR can be mediated by either Gαi subunit or Gβγ complex. The Gβγ complex directly activates IKACH, which affects BRR by hyperpolarizing the MDP (9, 12, 14). We observed that the CCh-induced MDP hyperpolarization only at a [CCh] /H11022 30 nM, and this effect continued to increase with increasing [CCh]. Beginning at 100 nM CCh, graded hyperpolarization of MDP paralleled BRR as the [CCh] increased. At 1,000 nM CCh, just prior to spontaneous AP cessation, the hyperpolarization resulted in the MDP shift to /H11002 /H11006 68.5 /H11006 1.2 mV (Supplemental Fig. S1, bottom). Thus IKACH is a highly effective mediator of BRR in response to ChR stimulation, but this effect occurs at higher intensities of ChR stimulation, as also observed in some prior studies that employed barium to block IKACH, or reported that the IC50 acetylcholine (ACh) to suppress If was 20 times less than the IC50 for IKACH activation (1, 6, 12).

Our results (Figs. 1, 3, and 4) also show that in response to lower [CCh] (up to 30 nM), whereas hyperpolarization of the MDP did not occur, BRR (15%) did occur; and, in the presence of full inhibition of IKACH, when no MDP hyperpolarization occurred at any [CCh] the BRR increased to

[Fig. 5. CCh effects on total cAMP (A; n = 5) and cGMP (B; n = 4) levels in spontaneously beating SANC suspensions in the absence or presence of phosphodiesterase (PDE) inhibition by IBMX. Each cell suspension from a given sinoatrial node was first divided into 2 major groups: no IBMX prior to Cch, or IBMX (100 μM, 20 min pretreatment before CCh). Each group was further subdivided into 3 groups: control, 100 nM CCh, and 1 μM CCh. Total incubation time in all cell groups was 25 min. In PDE-treated groups IBMX was added immediately at the beginning of incubation. In groups with CCh treatment, CCh was added at the 20th minute of incubation. In other words, the duration of exposure to CCh was 5 min. PDE inhibition in the absence of CCh increases both cAMP and cGMP (*P < 0.05). In the presence of PDE inhibition CCh significantly reduces cAMP (#P < 0.05) but does not affect cGMP.

[Fig. 6. A, top: representative Western blots of phosphorylated phospholamban (PLB) at the PKA-dependent phosphorylation site Ser-16 and total PLB immunolabeling in control SANC suspensions and cell suspensions exposed to a wide range of [CCh]. Bottom: average immunoblot data of phosphorylated PLB normalized to total PLB in response to CCh. The number of experiments at each [CCh] is indicated in parentheses. B: the average response at each [CCh] has been normalized to the maximum effect of CCh. The curves represent the best fit nonlinear regression model for the experimental data points. The curve for BRR in the presence of IKACH inhibition is the same as that was illustrated in Fig. 3B. The log[IC50] calculated from the nonlinear regression for each curve is shown in the inset. In 3 PLB phosphorylation experiments, cell suspensions received the entire range of [CCh]. Thus a final normalized dose-response curve could be calculated for each of these experiments, permitting direct calculation of the average IC50 and the variance around the average IC50. This is indicated by large solid gray point with ±SD on the PLB dose-response curve. Note that the IC50 for BRR by CCh in the presence of IKACH inhibition is within the SD of the IC50 for PLB dephosphorylation.

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activation by ChR stimulation inhibits AC activity (or reduces cAMP by activating PDE), which, in turn, reduces cAMP levels in SANC, shifting the $I_t$ current activation curve toward more negative membrane voltages, rendering $I_t$ less active (13). Earlier studies had extrapolated this effect of ACh (or CCh) in voltage-clamped SANC to spontaneously firing SANC to explain the ChR-induced BRR (12, 14). However, although ChR stimulation clearly affects $I_t$ activation under voltage clamp, it does not necessarily follow that such an effect need be involved in BRR, as has previously been contended (9) because of the attendant reduction in PKA-dependent signaling that is evoked by ChR stimulation. The present study, in fact, shows (Figs. 3 and 4) that the full effect of CCh on BRR persists at all [CCh] in the presence of $I_t$ blockade by 2 mM

25–30% at 100 nM [CCh] (Figs. 3 and 4). In addition to $I_{Kach}$ desensitization or fading (7), BRR induced by low [CCh] or ACh concentration ([ACh]) has previously been attributed to suppression of $I_t$ or $I_{Ca,L}$, which are modulated by cAMP or cAMP mediated PKA-dependent signaling, respectively (Supplemental Fig. S5).

Since our results demonstrate that Gi protein activation is required for the overall BRR by ChR stimulation, the hyperpolarization-independent BRR effect must be attributable to activation of the $G_{i\alpha}$ subunit, sometimes referred to as an indirect inhibitory signaling mediated through the cAMP-PKA pathway (9) (Supplemental Fig. S5): the active GTP-$G_{i\alpha}$ protein complex directly inhibits AC activity, and this is followed by a reduction in cAMP-mediated, PKA-dependent protein phosphorylation (8, 10, 22, 35). Indeed, in the present study, CCh reduced the AC activity (inferred from a 21 and 34% cAMP reduction over a 5-min period of CCh treatment in IBMX-pretreated cells, Fig. 6A, right bars) and was accompanied by 25–30% BRR when $I_{Kach}$ or $I_t$ or both are blocked (Fig. 3A, red and blue diamonds). Our experiments, however, cannot directly relate the cAMP reduction resulting from AC inhibition to the BRR in response to CCh, since the kinetics of former were not measured during 5 min of CCh incubation. Our results also show that in the absence of PDE inhibition total cGMP levels in SANC are only a fraction (1/20th) of cAMP levels, and whereas cGMP and cAMP both increase activation by ChR stimulation inhibits AC activity because $I_t$ channel subunits have a cAMP response element (Supplemental Fig. S5). The specific mechanism previously invoked for $I_t$ suppression is that Gi protein

* Figure 7. Confocal Ca$^{2+}$ images (left) and analog Ca$^{2+}$ transients (right), in a representative SANC prior to and during CCh exposure at the IC50 CCh. Both action potential (AP)-triggered Ca$^{2+}$ transients and local Ca$^{2+}$ release (LCR) characteristics are substantially reduced by CCh in spontaneously beating SANC. F, fluorescence during excitation; F0, background fluorescence.

* Figure 8. A: average ($n = 10$) changes in AP-induced Ca$^{2+}$ transient characteristics in the presence of CCh. d(F/F0)/dt, maximum rate of rise of the Ca$^{2+}$ transient measured as F/F0 fluo 3-AM fluorescence; T90, transient duration at 90% of transient amplitude; T50, transient duration at 50% of transient amplitude. In control: d(F/F0)/dt = 0.32 ± 0.002 s$^{-1}$; amplitude = 1.46 ± 0.08 F/F0; T90 = 264.4 ± 11 ms; T50 = 100.2 ± 3 ms. B: average ($n = 10$) change in LCR characteristics induced by CCh. In control: LCR amplitude = 1.1 ± 0.18 F/F0; LCR number per cycle = 1.02 ± 0.1; LCR size = 6.9 ± 0.7 μm; LCR period = 351 ± 22 ms ($P < 0.05$). The method used to define LCR period and LCR cycle length is illustrated in the inset of B. The cycle length is the time between 2 successive AP-induced Ca$^{2+}$ transients, and LCR period is measured as time from the preceding Ca$^{2+}$ transient upstroke to the maximum rate of rise of subsequent LCR ($*P < 0.05$).
CsCl (Fig. 2) and that the effects of CCh at IC50 on both BRR and Ca2+/H11001 cycling are reversed by phosphatase inhibition (Figs. 10 and 11), an effect distal to cAMP, i.e., on PKA-dependent phosphorylation (see below). We interpret our result to indicate that modulation of If activation by ChR stimulation does not participate in (i.e., is neither sufficient nor necessary for) the ChR-induced BRR. The results of prior studies also have been interpreted to indicate the absence of a substantial participation of If in ChR-induced BRR (6, 53).

Prior reports of ChR stimulation in spontaneously beating intact hearts differ in their interpretation of the role of If in the BRR. With the advent of TQ, two studies were performed on the isolated Langendorff preparations to estimate the participation of IKACh and If currents in the bradycardic response to CCh. Yamada (51) did find a marked contribution of If current to the negative chronotropic response of isolated rabbit heart to CCh. On the other hand, in isolated guinea-pig heart, Bolter and English (5) showed that If current played little or no role in vagal slowing or in the pacemaker response to ACh. The difference between these studies cannot be explained by a species difference, since a significant contribution of If current to pacemaker activity has been convincingly demonstrated in both rabbit and guinea pig isolated SAN (28). Moreover, both studies used 300 nM TQ to block IK,ACh, and 2 mM Cs to block If current. Thus results of our study are consistent with the findings of Bolter and English but not those of Yamada. Additional studies are required to define the contribution of the LCR mechanism to parasympathetic stimulation induced bradycardia of the intact, isolated heart.

We have previously demonstrated that, as a result of a high basal cAMP level, SANC have a high basal level of PKA-dependent phosphorylation of PLB and RyR (47). There is also indirect evidence for the importance of basal L-type Ca2+/H11001 channel phosphorylation (39). Graded dephosphorylation by a specific PKA inhibitor peptide, PKI, is highly correlated with graded reductions in the spontaneous basal beating rate (47). To determine whether ChR stimulation is indeed involved in reduction of basal PKA-dependent protein phosphorylation in SANC, we studied the effects of CCh on PLB phosphorylation as an index of PKA activity. Our results (Fig. 6A) show that at the IC50 CCh for BRR PLB phosphorylation is reduced by 50%, similar to the extent of estimated net reduction of AC activity [inferred from the CCh-induced cAMP reduction (Fig. 5A) over the 5 min of CCh treatment when PDEs are inhibited]. Furthermore, the IC50 for reduction in PLB phosphorylation by CCh mirrors that of BRR in the presence of IK,ACh blockade (Fig. 6B), and the IC50 CCh-mediated effects in BRR and Ca2+/H11001 cycling are reversed by phosphatase inhibition (Fig. 10, 11).

It is important to note although PDE inhibition was required to demonstrate a CCh-induced reduction in total cell cAMP levels, a local reduction in cAMP-mediated, PKA-dependent PLB phosphorylation and its effects on the kinetics of SR Ca2+/H11001 cycling are reversed by phosphatase inhibition (Fig. 10, 11).
cycling, manifested by the changes in cell Ca\(^{2+}\) signaling (see below), can be detected even in the absence of PDE inhibition. Thus evidence for a functionally significant reduction of cAMP-mediated-PKA-dependent signaling by CCh emerges even in the absence of PDE inhibition, even though the demonstration of a CCh effect on total cAMP requires the presence of PDE inhibition. Nonetheless, the requirement for PDE inhibition to observe an effect of CCh to reduce cAMP may be considered as a limitation of our method.

Numerous studies have documented that ChR stimulation reduces \(I_{Ca,L}\) (17, 18, 34, 38, 39), and this effect has been ascribed in some studies (38, 39) to a PKA-dependent reduction in ambient L-type Ca\(^{2+}\) channel phosphorylation. The effect of phosphatase inhibition to reverse the effect of CCh on Ca\(^{2+}\) cycling and BRR is consistent with this idea. There is substantial disagreement, however, as to whether ChR inhibitory effects on \(I_{Ca,L}\) do occur in the absence of concurrent β-AR stimulation. Our findings show that in the absence of β-AR stimulation CCh reduced the \(I_{Ca,L}\) amplitude by 20% (Supplemental Fig. S4), but at a tenfold higher [CCh] than that required to stop spontaneous beating. An effect of ChR stimulation to suppress \(I_{Ca,L}\) has previously been attributed to activation of soluble GC, leading to elevation of cGMP and to a subsequent increase in PDE2 activity (18, 19). This mechanism is controversial, since ChR do not appear to directly link to GC (Supplemental Fig. S5). It has also been suggested that NO mediates its effects through G protein coupling (41), a result consistent with our observations. Although we did not directly investigate a role for NO signaling in the BRR effect of ChR stimulation, our results do indicate that if such an effect is present, it must be a G\(_i\)-coupled effect, since G\(_i\) inhibition abolishes all effects of ChR stimulation on spontaneous AP rate without affecting the basal beating rate (Fig. 1B).

The phosphorylation of “Ca\(^{2+}\) clock” proteins, i.e., L-type Ca\(^{2+}\) channel, PLB, and RyRs, markedly affects the amplitude and kinetics of Ca\(^{2+}\) releases in cardiac cells, including SANC (32). The present results demonstrate that \(I_{Ca,L}\) in addition to reducing PLB phosphorylation, CCh, at the IC\(_{50}\) for BRR, reduces the amplitude and slows the kinetics of both cytosolic Ca\(^{2+}\) transients triggered by the AP and of rhythmic submembrane LCRs (Figs. 7 and 8) that occur spontaneously later in the cycle, i.e., during the late DD; and 2) these effects are reversed by phosphatase inhibition. Prior studies have demonstrated that LCRs activate \(I_{NCX}\) that drives in acceleration of the late DD toward the \(I_{Ca,L}\) activation threshold, thus igniting the subsequent AP firing (3, 4, 31). When the LCR period is prolonged and LCR net signal mass (i.e., the combination of...
LCR amplitude, spatial size, and frequency of occurrence) is
damped, as occurs in response to CCh (Fig. 8B), the diastolic
cycle length becomes prolonged. A hypothetical biophysical
mechanism for the prolongation could be that the LCR impact
on the DD via I_{NCX} is expected to be reduced and to occur later
in time following the prior AP, thereby resulting in a longer
time for the membrane potential to achieve the threshold for
I_{Ca,L}. Indeed, our results show that the time-dependent effect
of CCh to prolong the LCR period is highly correlated to the
concomitant time-dependent BRR (Fig. 9). The reduction in
beating rate by CCh, per se, reduces Ca^{2+} influx and the net
cell and SR Ca^{2+} load [via the Bowditch treppe effect (25)],
thus further damping LCRs. This “feed-forward” effect further
contributes to the extent of steady-state BRR by CCh.

We employed numerical modeling to provide theoretical
support for interpretation of our experimental results, i.e., that
a CCh-induced change in Ca^{2+} cycling and its specific
coupling role to I_{NCX} is crucial in physiologically relevant regime
of BRR. Our simulations at IC_{50} CCh for BRR clearly show
that LCRs, indeed, critically influence membrane function via
reducing and delaying (after prior AP) I_{LCR} (Fig. 12A). In
contrast, CCh induced BRR becomes substantially blunted
when I_{NCX} remains unchanged. Furthermore, when CCh-in-
duced dephosphorylation is largely prevented, phosphoryla-
tion-independent I_{KACH} and I_{r} produce a rather moderate BRR
(12.9% in the model simulation and 12.5% in our experiment
with calcycin A) (Fig. 12A, iv). Thus we conclude that sarcolemmal
electrogenic mechanisms and Ca^{2+} cycling [i.e., the system Ca^{2+}
clock as previously defined (30)] tightly cooperate to effect the strong and robust BRR at IC_{50} CCh.

It would be an oversimplification to assume that the intrinsic
spontaneous cycle length or AP characteristics of individual
rabbit SANC in isolation or their modulation by ChR stimula-
tion are identical to those of all SANC within the intact SAN.
The autonomic milieu is heterogeneous throughout the SAN,
with the central area having the greatest density of nerve
endings and autonomic receptors on SANC (2, 40). Further
shifts of the impulse origin from the initial, primary site in
SAN preparations occur in response to autonomic receptor
stimulation or to different ion manipulations (16, 36, 37, 43,
46). The complexity of the intact SAN notwithstanding, the
present findings, interpreted in the context of prior work (3, 4,
31, 32, 47, 48), support a novel general theoretical formulation
for the interplay of both cholinergic and adrenergic autonomic
regulation of spontaneous AP firing of isolated rabbit SANC
across the full physiological range of spontaneous beating rates
(Supplemental Fig. S5).

Since isolated SANC have no constitutive β-AR activity
(47), or ChR or G_{i} protein activation (Fig. 1B), the intrinsic AP
firing rate in SANC is determined by constitutive Ca^{2+}
activation of AC (33, 52) that results in cAMP-mediated PKA-
dependent phosphorylation of Ca^{2+} clock proteins (32, 47, 48,
52). A high constitutive PDE activity acts as a brake to control
the effects of intrinsic Ca^{2+}-activated AC activity by increas-
ing cAMP hydrolysis (48). ChR stimulation in vivo by ACH release
from the nerve endings “clamps” the basal PKA-
dependent Ca^{2+} signaling (Ca^{2+} clock) that is intrinsic to
SANC and that determines the AP firing rate in the absence of
any receptor stimulation. But ChR stimulation, rather than
increasing hydrolysis of cAMP to control the cAMP level, as
do PDEs, affects cAMP production, via a G_{i}α effect to reduce
AC activity. Still, concerted cAMP reduction by both constit-
tutively active PDEs and ChR stimulation regulates the AP
firing rate via a common downstream signaling point: PKA-
dependent phosphorylation of Ca^{2+} cycling proteins, and
resultant effects on Ca^{2+} cycling (Supplemental Fig. S5). As
the intensity of ChR increases, cycle length prolongation by AC-CAMP-PKA-LCR-NCX interactions is extended further by a
waxing I_{KACH} contribution, i.e., by an effect to reduce a tight
integration of both mechanisms. A marked alteration of ChR
signaling in AC Type 5 (a G_{i}-coupled AC) knockout mice in
which I_{KACH} is intact (35) is, in fact, consistent with this idea.

During the flight or fight reflex, i.e., in response to stress, as
persistent and more marked vagal tone withdrawal occurs,
the PKA-dependent Ca^{2+} cycling mechanisms become further
activated by β-AR stimulation, although the increase in cAMP
activation of PKA may be effected by different ACs (Ca^{2+}
inhibited) that link to β-ARs via G_{i} proteins (Supplemental
Fig. S5), rather than by ACs (Ca^{2+} activated) that control the
intrinsic AP firing rate (52).

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