Muscarinic Stimulation Facilitates Sarcoplasmic Reticulum Ca Release by Modulating Ryanodine Receptor 2 Phosphorylation Through Protein Kinase G and Ca/Calmodulin-Dependent Protein Kinase II

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Abstract—Although the effects and the underlying mechanism of sympathetic stimulation on cardiac Ca handling are relatively well established both in health and disease, the modes of action and mechanisms of parasympathetic modulation are poorly defined. Here, we demonstrate that parasympathetic stimulation initiates a novel mode of excitation–contraction coupling that enhances the efficiency of cardiac sarcoplasmic reticulum Ca store utilization. This efficient mode of excitation–contraction coupling involves reciprocal changes in the phosphorylation of ryanodine receptor 2 at Ser-2808 and Ser-2814. Specifically, Ser-2808 phosphorylation was mediated by muscarinic receptor subtype 2 and activation of PKG (protein kinase G), whereas dephosphorylation of Ser-2814 involved activation of muscarinic receptor subtype 3 and decreased reactive oxygen species–dependent activation of CaMKII (Ca/calmodulin-dependent protein kinase II). The overall effect of these changes in phosphorylation of ryanodine receptor 2 is an increase in systolic Ca release at the low sarcoplasmic reticulum Ca content and a paradoxical reduction in aberrant Ca leak. Accordingly, cholinergic stimulation of cardiomyocytes isolated from failing hearts improved Ca cycling efficiency by restoring altered ryanodine receptor 2 phosphorylation balance. (***Hypertension***. 2016;68:1171-1178. DOI: 10.1161/HYPERTENSIONAHA.116.07666.) ● Online Data Supplement

Key Words: Ca/calmodulin-dependent protein kinase II ■ calcium ■ carbachol ■ heart failure ■ protein kinase G ■ reactive oxygen species ■ ryanodine receptor calcium release channel

The function of the heart is regulated by 2 arms of the autonomic nervous system: the sympathetic and parasympathetic branches.[1,2] Whereas the sympathetic arm is responsible for boosting cardiac performance to support the fight or flight response, the parasympathetic stimulation adjusts cardiac pump activity to the reduced demands of the rest and digest state.[3] A pathological shift of autonomic balance to a dominant sympathetic state is a hallmark of heart failure (HF).[2,4–10] Hence, parasympathetic augmentation and particularly vagal stimulation have been effective means of restoring autonomic balance and alleviating HF.[2,6,8,10]

Ca-induced Ca release (CICR) from the sarcoplasmic reticulum (SR) via cardiac ryanodine receptors (RyR2s) plays a key role in cardiac excitation–contraction (EC) coupling. For this reason, impaired RyR2 function contributes to cardiac disease, including HF.[11–13] The sympathetic regulation of cardiac EC coupling and the resultant contractility has been extensively studied and is relatively well characterized.

Specifically, β-adrenergic receptor–mediated phosphorylation of key Ca-handling proteins (including L-type Ca channels [$Ca_v1.2$], RyR2, and phospholamban) results in increased SR Ca accumulation and enhanced CICR leading to improved contractility.[14–16] The dependence of CICR on the SR Ca content is highly nonlinear[17–19] such that a relatively small elevation in the SR Ca content during β-adrenergic stimulation results in a large increase of Ca release. This is particularly evident in HF where hyperphosphorylation of RyR2 increases RyR2-mediated SR Ca leak, thereby resulting in the depletion of the SR Ca store and weakened contractility.[11–13]

On the contrary, activation of the parasympathetic arm of the autonomic nervous system generally opposes the effects of sympathetic stimulation. However, the mechanism(s) responsible for physiological effects elicited by cholinergic stimulation are poorly defined. For example, the same exponential dependence of CICR on SR Ca content that is evidenced during adrenergic stimulation is expected to result in a drastic reduction

of Ca release during cholinergic stimulation, particularly when the SR Ca load is reduced. This raises an important question: in light of the reduced SR Ca content and the highly nonlinear dependence of CICR on SR Ca load, how is an efficient level of EC coupling maintained during rest when parasympathetic tone is elevated? Furthermore, even less is known about the molecular consequences of parasympathetic stimulation and their effects on cellular Ca handing in the failing heart. This knowledge is required for better understanding of the physiology of the heart in health and disease and is critical for optimization of HF therapies based on parasympathetic augmentation.

In the present study, we hypothesized that cholinergic stimulation enhances the efficacy of cardiac Ca cycling through changes in phosphorylation state of RyR2. We further hypothesized that this parasympathetic modulation of EC coupling is compromised in HF and that the beneficial effects of cholinergic stimulation involve improved utilization of intracellular Ca stores.

Methods

All animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; online-only Data Supplement).

Electrophysiological Recordings

Ca currents in mouse ventricular myocytes were recorded at 0 mV (from holding potential −50 mV) in the absence and presence of carbachol at room temperature in normal Tyrode solution. The pipette solution for voltage clamp experiments contained (mmol/L): 123 CsCl, 20 TEACl, 5 MgATP, 10 NaCl, 1 $MgCl_2$, 0.1 Tris GTP, 10 HEPES, and 0.2 Fluo-4 FF K5-salt (pH 7.2).

Biochemistry Assays

Protein analysis from cardiac samples were performed using Western blot (online-only Data Supplement).

Statistical Analysis

Data are presented as mean±SEM. The number of cells for Ca/reactive oxygen species (ROS) experiments (Figures 1 through 3, 5) or the number of hearts for Western blot experiments (Figures 2G, 4, and 5D) are shown as n in the figure legend. For each Ca/ROS imaging experiments, ≥3 animals per groups were used. Statistical analyses were performed using either *t* test, ANOVA with Tukey post hoc test, or paired *t* test. Values of $P<0.05$ were accepted as statistically significant.

Results

Cholinergic Stimulation Enhances SR Ca Store Mobilization

Despite its potentially important therapeutic significance, the impacts of cholinergic stimulation on myocyte Ca handling have not been sufficiently studied. Therefore, we investigated the effects of the cholinergic agonist, carbachol, on systolic and diastolic SR Ca release in wild-type mouse ventricular myocytes. Exposure of cardiomyocytes (paced at 0.5 Hz) to carbachol caused no change in the amplitude of the Ca transient; however, it resulted in a significant decrease in the SR Ca content (Figure 1A and 1B and 1D and 1E). Accordingly, the fraction of stored Ca that was released from the SR during a Ca transient (ie, fractional release [FR], the ratio of the Ca transient amplitude/total SR Ca content[20,21]) was increased by carbachol (Figure 1F).

FR is considered to be an index of efficacy of EC coupling during systolic Ca release.[17,19] FR is strongly influenced by both L-type Ca current and SR Ca load. To assess the role of potential changes in the L-type Ca current (I_{Ca}), we examined the effect of carbachol on I_{Ca}. Consistent with previous reports,[22,23] *I–V* curves for I_{Ca} were similar with and without carbachol (Figure 1C), thus revealing no changes in I_{Ca}. Next, we examined the load dependency of carbachol effects on EC coupling by measuring FR in carbachol-treated and carbachol-untreated (control) cells at matched SR Ca loads. To restore the reduced SR Ca content in carbachol-treated myocytes toward the baseline (normal) level, extracellular Ca was raised from 1 to 2.5 mmol/L. Then the SR Ca contents between the carbachol-treated and carbachol-untreated groups were adjusted by thapsigargin (60–150 nmol/L) at 2 different levels, that is, normal and low (60% of normal). Consistent with the previous report,[17] lowering SR Ca content to 60% of normal markedly depressed FR in the absence of carbachol. Strikingly, carbachol increased FR at low SR content (Figure 2A through 2C). Thus, carbachol facilitated systolic SR Ca release at low SR Ca content.

Next, we assessed the effects of carbachol on diastolic SR Ca leak by measuring Ca sparks in the load-matched myocyte groups. Whereas carbachol reduced Ca spark frequency under normal SR Ca load, it had no effect in cells with low SR Ca load (Figure 2D and 2E). Thus, cholinergic stimulation seems to enhance the efficacy of Ca store utilization by facilitating systolic Ca release at low SR Ca content without enhancing diastolic SR Ca leak.

To test the relevance of results obtained in mice to a large animal model, experiments were performed in canine ventricular myocytes. In agreement with the effects of carbachol on SR Ca release in mouse myocytes, carbachol did not promote diastolic Ca leak (Figure S1A and S1B in the
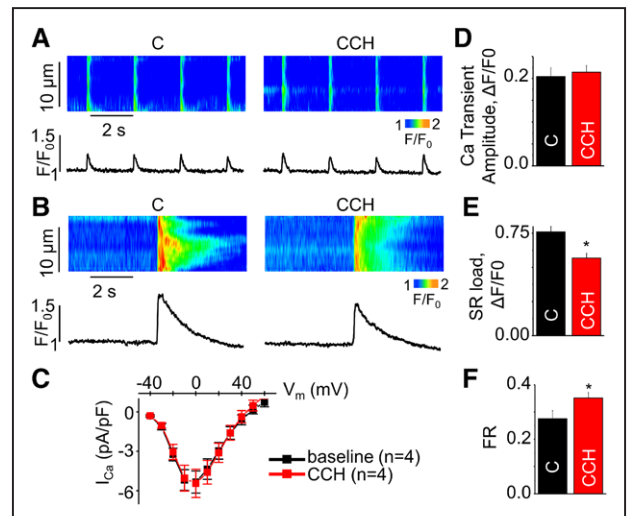
Figure 1. Effects of carbachol (CCH) on calcium (Ca) handling in wild-type mouse ventricular myocytes. Representative line-scan images for (**A**) Ca transients, (**B**) sarcoplasmic reticulum (SR) Ca with or without 10 μmol/L CCH. **C**, Voltage dependencies of I_{Ca} in baseline (black) and in the presence of CCH (red) (n=4). **D–F**, Summary data for Ca transient amplitude, caffeine-induced Ca transient amplitude (SR load), and fractional release (FR; ±SEM; n=11–16). *$P<0.05$ vs control (**C**). SR Ca content was obtained by application of 20 mmol/L caffeine.

online-only Data Supplement). Thus, cholinergic augmentation of SR Ca store utilization is observed in mouse and canine ventricular myocytes.

Cholinergic Stimulation Increases Phosphorylation of Ser-2808 While Decreasing That of Ser-2814

Cardiac EC coupling is modulated through changes in the phosphorylation status of the RyR2 at several sites including Ser-2808, Ser-2814, and Ser-2030. To examine the possible role of RyR2 phosphorylation in the observed effects of carbachol on Ca handling, we performed Western blot analysis using phosphospecific antibodies. Exposure of wild-type mouse cardiomyocytes to carbachol significantly increased RyR2 Ser-2808 phosphorylation while reducing Ser-2814 phosphorylation without affecting the status of Ser-2030 (Figure 2F and 2G; Figure S2A). Similar results were obtained in canine cardiac myocytes (Figure S1C and S1D). The concentration dependency of carbachol effects on RyR2 phosphorylation was also examined in the concentration range of 0.1 to 10 μmol/L (Figure S2B). These effects were prevented by pretreatment of the cells with the muscarinic receptor (MR) antagonist, atropine (Figure S2C), suggesting that phosphorylation changes were mediated by activation of muscarinic acetylcholine receptors.

Role of Changes in RyR2 Phosphorylation at Ser-2808 and Ser-2814 in Mediating Cholinergic Effects on SR Ca Release

RyR2 functional activity has consistently been demonstrated to be in direct relation with the level of Ser-2814 phosphorylation by Ca/calmodulin-dependent protein kinase II

(CaMKII).[24,25] Therefore, while in agreement with reduced Ca spark rate, the observed decrease in Ser-2814 phosphorylation cannot explain the increased fractional Ca release in myocytes exposed to carbachol. To investigate the involvement of the RyR2 phosphorylation site Ser-2808 in cholinergic-mediated regulation of SR Ca release, experiments were performed in mice genetically modified to render this site nonphosphorylatable by replacing serine at 2808 with alanine (RyR2-S2808A). Ablation of RyR2 phosphorylation at Ser-2808 prevented the increase in fractional Ca release at low SR Ca load observed in wild-type myocytes (Figure 3). Thus, RyR2 phosphorylation at Ser-2808 is indeed required for the observed load-dependent facilitation of SR Ca release by carbachol. At the same time, inhibiting CaMKII activity with KN-93 had no significant impact on FR (Figure S2D). This result supports the notion that muscarinic modulation of systolic Ca release is independent of CaMKII-dependent phosphorylation of Ser-2814. Taken together, these findings suggest that increased efficiency of Ca store utilization in cardiac cells (ie, facilitation of systolic release and inhibition of diastolic SR Ca leak) in response to muscarinic stimulation involves reciprocal changes in RyR2 phosphorylation at Ser-2808 and Ser-2814.

MR Subtype 2 and MR Subtype 3 Mediate 2 Divergent Roles of Cholinomimetics

Cardiac myocytes predominantly express the MR subtype 2 (M2R); however, the receptor subtype 3 (M3R) is also present.[26,27] Accordingly, selective inhibitors of the aforementioned MR subtypes AF-DX 116 (specific for M2R) and J 104129 fumarate (specific for M3R)[27,28] were used to examine the role of these receptor subtypes in mediating RyR2 phosphorylation in response to cholinergic stimulation. Inhibition of M2R (but not inhibition of M3R) prevented changes in RyR2 phosphorylation at Ser-2808 in mouse myocytes (Figure 4A and 4B). Moreover, to further confirm the involvement of M2R, we inhibited Gi protein with pertussis toxin. Application of pertussis toxin also prevented phosphorylation changes at Ser-2808 (Figure 4C and 4D). Notably, blocking M2R by AF-DX 116 had no effects on carbachol-dependent dephosphorylation on Ser-2814. Instead, the M3R antagonist, J 104129 fumarate, successfully inhibited Ser-2814 dephosphorylation by carbachol, indicating that the effects of carbachol exerted on RyR2 Ser-2808 and Ser-2814 are mediated via distinct MR subtypes.

Figure 2. Effects of carbachol (CCH) on calcium (Ca) handling at different sarcoplasmic reticulum (SR) Ca contents and ryanodine receptor (Ry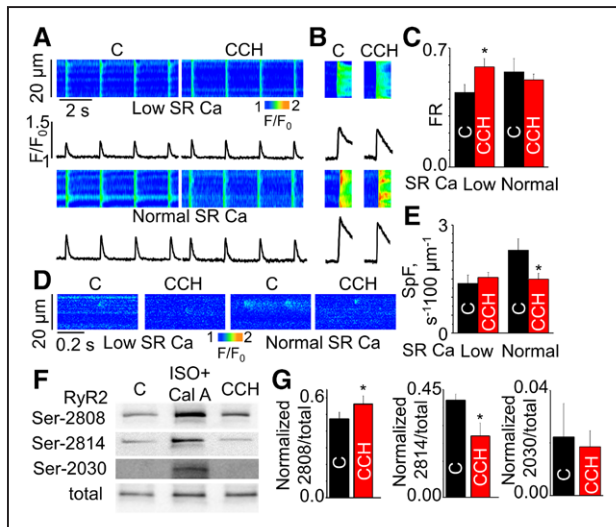R) 2 phosphorylation in wild-type mouse ventricular myocytes. Representative line-scan images for (**A**) Ca transients, (**B**) SR Ca content, and (**D**) Ca sparks at normal and low (60% of normal) SR Ca loads with or without 10 μmol/L CCH. **C** and **E**, Summary data for fractional release (FR) and spark frequency (SpF) at different SR Ca contents (±SEM, n=4–23). *P<0.05 vs control (**C**). **F** and **G**, Immunoblots and bar graphs for the effects of CCH on phosphorylation of RyR2 (±SEM, n=4–18). *P<0.05, paired *t* test. Calyculin A (Cal A)+isoproterenol (ISO) produces maximum phosphorylation. SR Ca content was obtained by application of 20 mmol/L caffeine.
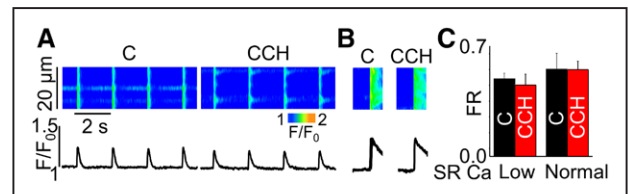
Figure 3. Ablation of the ryanodine receptor (RyR) 2 Ser-2808 phosphorylation site prevents a carbachol (CCH)–dependent increase in fractional release (FR). **A** and **B**, Representative line-scan images (**top**) and time-dependent profiles (**bottom**) for Ca transients and sarcoplasmic reticulum (SR) Ca content at low (60%) SR Ca loads under control conditions and in the presence of 10 μmol/L CCH. **C**, Summary data for FR in S2808A cells at normal and low SR Ca contents (±SEM; n=6–11). SR Ca content was obtained by application of 20 mmol/L caffeine.

Figure 4. Type 2 and 3 muscarinic receptor subtypes mediate carbachol (CCH)–dependent alterations in ryanodine receptor (RyR) 2 phosphorylation at Ser-2808 and Ser-2814. Representative Western blots (**A** and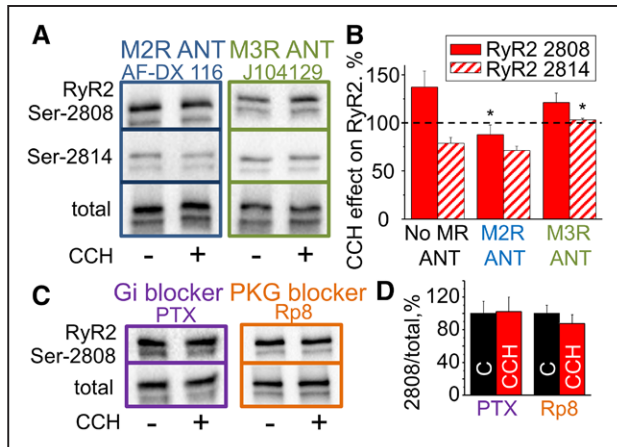 **C**) and the average effects of CCH on RyR2 Ser-2808 and Ser-2814 phosphorylation (**B** and **D**) in the presence of muscarinic receptor subtype 2 (M2R; AF-DX 116) and M3R (J104129) antagonists, pertussis toxin (PTX; inhibitor of Gi protein) and Rp8 (protein kinase G [PKG] inhibitor) (±SEM; n=4–7). *P<0.05 vs no MR antagonist (ANT).

Downstream Mediators of Cholinergically Promoted Modulation in SR Ca Release

Several different protein kinases have been shown to be able to phosphorylate RyR2 at Ser-2808 in vitro, including protein kinase A (PKA), protein kinase G (PKG), CaMKII, and protein kinase C (PKC).[29–34] Therefore, we applied specific pharmacological inhibitors of these kinases to test their roles on muscarinic-dependent phosphorylation of Ser-2808 (eg, H89, Rp-8-Br-PET-cGMPS [Rp8], KN-93, and bisindolylmaleimide I, respectively). Carbachol-dependent phosphorylation of Ser-2808 was prevented by inhibition of PKG (Figure 4C and 4D) but not by inhibition of PKA, PKC, or CaMKII (Figure S3A). Ser-2808 have been reported to exhibit a robust baseline phosphorylation independent of PKA and CaMKII.[29,35] Similarly, PKG and PKC inhibition failed to reduce Ser-2808 phosphorylation at baseline condition (Figure S3B). Collectively, these results suggest involvement of the M2R/Gi/PKG pathway in the cholinergic effects on Ser-2808. On the contrary, RyR2 Ser-2814 has been shown to be the primary CaMKII phosphorylation site. Therefore, we examined whether carbachol-dependent dephosphorylation of Ser-2814 is associated with changes in CaMKII activity. CaMKII activity was indeed decreased in the presence of carbachol suggesting that the decreased phosphorylation of Ser-2814 is attributable to reduced CaMKII activation (Figure S3C).

Cholinergic Modulation of Ca Cycling in Failing Canine Hearts

To examine the possible beneficial effects of cholinergic stimulation on Ca handling in myocytes from failing hearts, we measured spontaneous Ca release and RyR2 phosphorylation levels with or without carbachol in failing myocytes using a well-characterized canine model of chronic HF.[36–40] Consistent with our previous studies,[39,40] failing myocytes exhibited spontaneous Ca waves in the presence of 10 nmol/L isoproterenol. Carbachol decreased the frequency of these waves
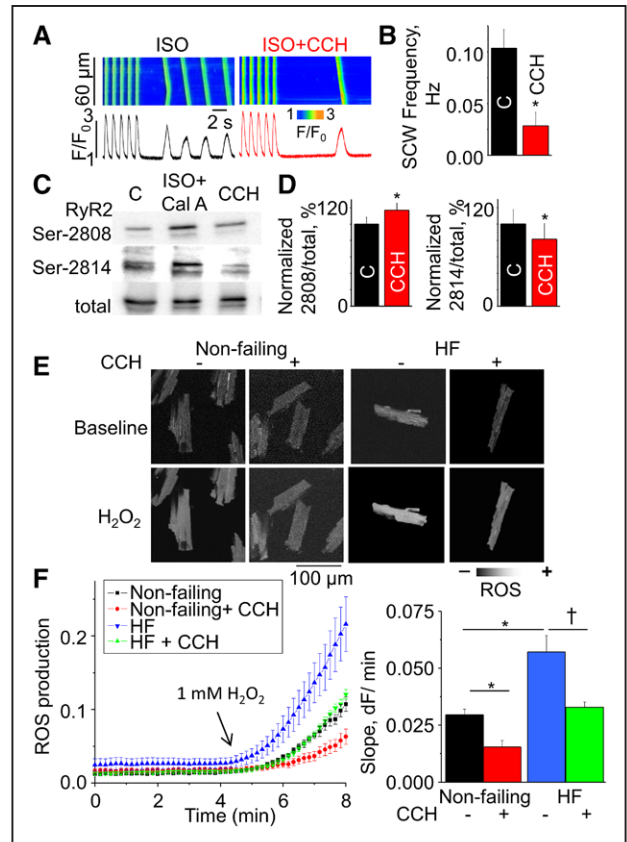

Figure 5. Effects of carbachol (CCH) on calcium (Ca) handling, ryanodine receptor (RyR) 2 phosphorylation and reactive oxygen species (ROS) production in heart failure (HF) canine myocytes. Confocal Ca recordings (**A**) before and after application of CCH in the presence of 10 nmol/L ISO in field-stimulated failing cells (2 Hz). **B**, Summary bar graph for spontaneous Ca wave frequency (SCW) (±SEM; n=21–25). *P<0.05 vs control. Immunoblots (**C**) and pooled data (**D**) showing effect of CCH on RyR2 phosphorylation (±SEM; n=3). *P<0.05, paired *t* test. **E**, Representative images of ROS generation measured using the ROS-sensitive dye, CM-H$_2$DCFDA, for nonfailing and failing myocytes under baseline condition and in the presence of 1 mmol/L H$_2$O$_2$. **F, Left**, Averaged traces of ROS production from each group. **F, Right**, Pooled data for ROS accumulation rates obtained from the slopes in the presence of 1 mmol/L H$_2$O$_2$ (±SEM; n=5–8). Data pointes were normalized to maximum fluorescence signal produced by 10 mmol/L H$_2$O$_2$. *P<0.05 vs nonfailing control. †P<0.05 vs failing control.

(Figure 5A and 5B). Also consistent with previous results,[39,40] RyR2 phosphorylation at Ser-2814 (but not at Ser-2808) was elevated in failing cardiomyocytes relative to nonfailing ones. Treatment with carbachol increased phosphorylation of RyR2 Ser-2808 but reversed hyperphosphorylation of RyR2 Ser-2814 (Figure 5C and 5D). Studies from our group and others have shown HF is associated with elevated ROS production and increased CaMKII activity.[38,39,41] Oxidative stress promotes constitutively active CaMKII through methionine oxidation.[42,43] To examine whether cholinergically medicated decreases in Ser-2814 phosphorylation are attributable to decreased ROS production, we measured the rate of ROS generation in response to H$_2$O$_2$ using the ROS-specific fluorescent indicator, CM-H$_2$DCFDA, in nonfailing and failing myocytes. Consistent with previous reports, failing myocytes showed a higher rate of ROS generation than nonfailing cells (Figure 5E

and 5F). Notably, carbachol reduced ROS in both the nonfailing and failing groups. Thus, the reduction of ROS generation by cholinergic modulation may contribute to the beneficial effects of vagal stimulation therapy by reducing CaMKII-dependent phosphorylation of RyR2 at Ser-2814.

Discussion

These data suggest a novel efficiency mode of cardiac EC coupling that enhances the utilization of intracellular Ca stores during the rest and digest state predominated by the parasympathetic tone. This efficient utilization of Ca store involves reciprocal changes in phosphorylation of RyR2 at Ser-2808 and Ser-2814, which in turn increases systolic release at low SR Ca content while inhibiting aberrant SR Ca leak. Such reciprocal alteration of RyR2 phosphorylation proved to be beneficial in failing hearts, where cholinergic/muscarinic stimulation improved the efficiency of Ca cycling by reducing pathological SR Ca leak while preserving enhanced systolic release.

Efficiency Mode in Myocyte Ca Cycling During Cholinergic Stimulation

Sympathetic modulation of the cardiac muscle has been extensively studied and is known to involve adrenergically mediated phosphorylation of key EC proteins, including phospholamban, $Ca_v1.2$, and RyR2.[14–16] This multiprotein response facilitates trans-sarcolemmal and SR Ca fluxes to increase the chronotropic and inotropic states of the heart (Figure 6A). In contrast, cholinergic stimulation is generally thought to act by slowing cardiac Ca cycling via activation of MRs; however, the specific mechanisms of the parasympathetic response remain to be defined. Here, we show that in ventricular myocytes, MR stimulation initiates a distinct mode of EC coupling that operates at reduced intra-SR Ca levels characteristic of resting state. However, the fraction of SR Ca release increases on MR stimulation without affecting L-type Ca current (Figures 1 and 2). Therefore, in contrast to the performance-oriented mode of EC coupling during sympathetic stimulation, the cholinergic efficiency mode is poised to attain maximal Ca release at minimal SR Ca load, thereby minimizing the energy costs of Ca cycling. Previously, it has been shown that the dependency of SR Ca release on the SR Ca content is highly nonlinear with release failing when SR Ca content falls below 60% of normal.[17,44] Our results show that SR Ca release is profoundly influenced by the cholinergic activation that makes Ca release more efficient at reduced SR Ca content.

Commonly, interventions that enhance RyR2 functional activity (such as CaMKII phosphorylation or caffeine) are expected to facilitate both systolic and diastolic release when the load is kept constant.[24,45] Notably, carbachol failed to increase Ca spark frequency at various SR Ca loads, despite improving systolic Ca release (Figure 2A through 2E; Figure S1A and S1B). Thus cholinergic stimulation facilitates systolic Ca release without promoting energetically wasteful diastolic Ca release, suggesting the increased utilization of intracellular Ca stores during parasympathetic dominance.

Reciprocal Modulation of RyR2 via Changes in Phosphorylation at 2808 and 2814

Increased phosphorylation of RyR2 Ser-2808 and Ser-2814 has been implicated in adrenergically induced elevation of systolic and diastolic SR Ca release.[24,46–48] However, little is known about the consequences of parasympathetic stimulation aside from the general notion that the effects oppose those of the sympathetic stimulation. Consistent with this antagonistic phenotype, cholinergic stimulation decreased RyR2 Ser-2814 phosphorylation along with diastolic Ca leak (Figures 2 and 5; Figure S1). However, Ser-2808 phosphorylation in both mouse and canine preparations was increased rather than decreased during cholinergic stimulation. Notably, increased Ser-2808 phosphorylation was associated with enhanced efficiency of EC coupling (increased fractional Ca release) at reduced SR Ca load (Figures 1 through 3). Thus, during cholinergic stimulation, reciprocal changes in phosphorylation at Ser-2808 and Ser-2814 confer a new mode of smart regulation of SR Ca release. Mainly, cholinergic stimulation results in an enhanced utilization of SR Ca stores through increased systolic Ca release at low SR Ca loads while reducing aberrant diastolic SR Ca leak at the same time.

We further delineated signaling cascades mediating the aforementioned effects of cholinergic stimulation (Figure 6B). Cardiac myocytes predominantly express the M2R and M3R.[26,27] Whereas M2R is coupled to Gi that inhibits adenylate cyclase and PKA but causes NO-dependent stimulation of PKG,[23,49] M3R interacts with Gq involved in activation of PKC.[26] Recently, M3R stimulation has been also shown to decrease ROS-mediated CaMKII activation.[50] Here, we found that during cholinergic stimulation, these 2 muscarinic pathways (ie, M2R and M3R) converged to increase SR Ca release efficiency through reciprocal changes in RyR2 phosphorylation. Specifically, our results demonstrated that RyR2 Ser-2808 phosphorylation is mediated by
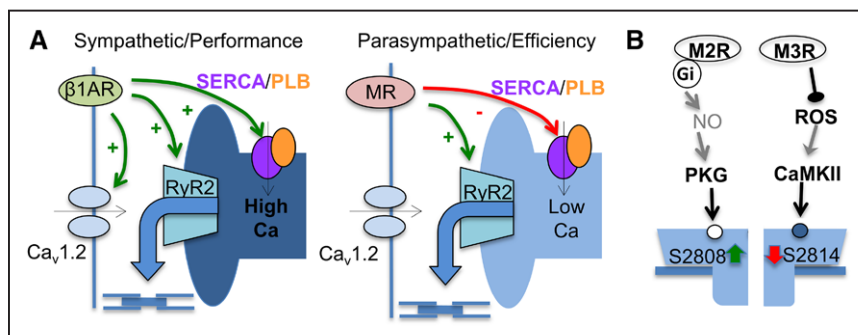
Figure 6. Calcium (Ca) cycling during sympathetic and parasympathetic dominance (**A**) and molecular mechanisms and pathways (**B**) involved in the regulation of reciprocal phosphorylation/dephosphorylation of ryanodine receptor (RyR) 2 at Ser-2808 and Ser-2814. M2R indicates muscarinic receptor subtype 2; M2R, MR subtype 3; PLB, phospholamban; ROS, reactive oxygen species; and SERCA, sarcoplasmic reticulum calcium transport ATPase.

M2R through activation of PKG, whereas dephosphorylation of RyR2 Ser-2814 seemed to be caused by the activation of M3R and the resultant reduction in ROS-dependent CaMKII activity. The precise mechanisms of this composite RyR2 modulation at the subcellular and molecular level remain to be defined, however. One possibility is that this modulatory response is an inherent property of an individual RyR2 channel. Consistent with this possibility, PKA-phosphorylation has been shown to increase the peak transient response while accelerating the rate of decay to a steady level (ie, adaptation) of single RyR2s during rapid and sustained elevations of Ca.[51] This in turn suggests that transient and steady phases of RyR2 activity can be differentially regulated in individual RyR2 channels. Alternatively, the effects could be mediated by the 2 phosphorylation pathways acting on 2 distinct pools of RyR2 channels in cardiomyocytes. Consistent with this possibility, cardiomyocytes have been shown to contain 2 sets of release sites with different response rate and predisposition to CaMKII phosphorylation (ie, fast, CaMKII dependent and slow, CaMKII independent).[52] As to the increase in fractional SR Ca release in response to carbachol, it could be attributed to increased sensitivity of RyR2 to luminal Ca and reduced threshold for Ca release termination. Consistent with this possibility, Ullrich et al[53] previously demonstrated that ablation of the Ser-2808 phosphorylation site blunts the increase in RyR2 activity observed in PKA phosphorylated RyR2 channels in response to an increase in luminal Ca. Further studies are required to establish the role(s) of these mechanisms in physiological modulation of cardiac EC coupling.

About the influence of cholinergic agonists on myocyte Ca handling and contractility, previous studies have yielded controversial results demonstrating stimulatory, biphasic, or inhibitory effects.[54–57] This variability in results could be attributed to the complex nature of muscarinic regulation of SR Ca release demonstrated here, including reciprocal changes in RyR2 phosphorylation mediated by 2 different MR subtypes.

Restoration of Compromised Ca Cycling as a Basis for Vagal Stimulation Therapy for HF

HF is characterized by altered autonomic balance, impaired functional performance, and a compromised energetic state.[4] Specifically, patients with HF exhibit sympathetic dominance,[2,5,7,58] a result of sympathetic overdrive and attendant reductions of parasympathetic tone. Reduced vagal tone in HF seems to be mainly because of abnormal presynaptic (ie, ganglionic) mechanisms.[59] Changes in MR subtypes have been also reported although the specific alterations remain controversial.[28,60] One of the main consequences of the sympathetic dominance that is characteristic of HF is increased RyR2-mediated diastolic Ca leak.[37,46,61] This leak has been variably attributed to RyR2 hyperphosphorylation at either Ser-2808 or Ser-2814. Given the intrinsic antagonistic relationships between sympathetic and parasympathetic influences on the heart, it is reasonable to suggest that cholinergic stimulation would oppose the detrimental effects of increased sympathetic stimulation on Ca handling. Indeed, a body of experimental evidence suggests that vagal nerve stimulation (VNS) may be a viable option for management of HF.[2,4,6,62,63] However, surprisingly, only limited information is available on the effects of cholinergic stimulation on HF-dependent changes in Ca handling. Here, we determined that cholinergic stimulation in myocytes reduced SR Ca leak while increasing systolic SR Ca release by decreasing RyR2 phosphorylation at Ser-2814 and increasing it at Ser-2808, respectively. These findings are consistent with a role for CaMKII phosphorylation in HF pathology and implicate dephosphorylation as a potential mechanism underlying the beneficial effects of parasympathetic stimulation on SR Ca cycling and contractility.[64] Notably, the improved Ca handling in failing myocytes was associated with a reduction in ROS coupled with reduced CaMKII activity. Based on previous reports, the reduction of ROS during cholinergic modulation could be a result of increased reducing-enzyme(s) activity or inhibition of ROS generation through NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, xanthine oxidase, and mitochondria.[65–67]

Perspectives

It is to be noted that VNS in cardiac disease is usually applied chronically (ie, days-months) rather than in an acute manner[68,69] although, recently, even brief stimulation of the vagal nerve has been reported to exert beneficial effects on cardiac performance in a rat model of ischemic HF[70] and has been shown to prevent cardiac remodeling in post MI rabbits.[71] Our study demonstrating the mode of action of muscarinic activation on myocyte Ca handling is consistent with, and provides insights into, the beneficial effects of short-term vagal stimulation. In particular, our results suggest that the therapeutic impact of cholinergic stimulation may involve enhanced efficiency of the SR Ca store utilization and inhibition of arrhythmogenic diastolic Ca release in failing cardiomyocytes. Because HF is associated with chronic alteration in the autonomic balance,[2,4,5,7] it is reasonable to assume that long-term VNS will provide more substantial and lasting therapeutic effects than brief stimulation. Although our study, which uses direct application of cholinomimetics to isolated myocytes, mimics the cellular effects of VNS that is often used in vivo, it is important to acknowledge that pleiotropic effects[68] may also contribute to the therapeutic benefits of VNS. Therefore, additional studies are necessary to define the systemic, cellular and molecular mechanisms of salutary effects of VNS with an ultimate goal of optimization of this potentially promising therapeutic modality.

Disclosures

None.

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Novelty and Significance

What Is New?

- Here, we demonstrate a previously unrecognized mechanism by which parasympathetic stimulation enhances the utilization of SR Ca stores through reciprocal changes in the phosphorylation of RyR2 via distinct muscarinic receptor subtypes. By showing improved Ca handling in failing myocytes after acute parasympathetic stimulation, we reveal a potential mechanism underlying the beneficial effects of vagal stimulation therapy.

What Is Relevant?

- The beating heart is regulated by the 2 branches of the autonomic nervous system: the sympathetic and parasympathetic branches. Certain pathologies, such as heart failure, are marked by an imbalance between the 2 branches, where the sympathetic overdrive is coupled to reduced parasympathetic tone. Interestingly, vagal nerve stimulation has become a promising therapeutic option for heart failure; however, there is little mechanistic insight for the success of this therapeutic modality. Thus, our study shows a new mode of cardiac regulation and lays a foundation for optimization of therapeutic vagal nerve stimulation.

Summary

The beneficial effect of parasympathetic augmentation on cardiac Ca handling in HF seems to involve restoration of normal phosphorylation balance and improved functional performance of RyR2, which in part may be a result of activation of PKG and decreased CaMKII activity.