Cardiac Resynchronization Therapy Restores Sympathovagal Balance in the Failing Heart by Differential Remodeling of Cholinergic Signaling

Deeptankar DeMazumder, David A. Kass, Brian O’Rourke, Gordon F. Tomaselli

**Rationale:** Cardiac resynchronization therapy (CRT) is the only heart failure (HF) therapy documented to improve left ventricular function and reduce mortality. The underlying mechanisms are incompletely understood. Although β-adrenergic signaling has been studied extensively, the effect of CRT on cholinergic signaling is unexplored.

**Objective:** We hypothesized that remodeling of cholinergic signaling plays an important role in the aberrant calcium signaling and depressed contractile and β-adrenergic responsiveness in dyssynchronous HF that are restored by CRT.

**Methods and Results:** Canine tachypaced dyssynchronous HF and CRT models were generated to interrogate responses specific to dyssynchronous versus resynchronized ventricular contraction during hemodynamic decompensation. Echocardiographic, electrocardiographic, and invasive hemodynamic data were collected from normal controls, dyssynchronous HF and CRT models. Left ventricular tissue was used for biochemical analyses and functional measurements (calcium transient, sarcomere shortening) from isolated myocytes (n=42–104 myocytes per model; 6–9 hearts per model). Human left ventricular myocardium was obtained for biochemical analyses from explanted failing (n=18) and nonfailing (n=7) hearts. The M2 subtype of muscarinic acetylcholine receptors was upregulated in human and canine HF compared with nonfailing controls. CRT attenuated the increased M2 subtype of muscarinic acetylcholine receptor expression and Gαi coupling and enhanced M3 subtype of muscarinic acetylcholine receptor expression in association with enhanced calcium cycling, sarcomere shortening, and β-adrenergic responsiveness. Despite model-dependent remodeling, cholinergic stimulation completely abolished isoproterenol-induced triggered activity in both dyssynchronous HF and CRT myocytes.

**Conclusions:** Remodeling of cholinergic signaling is a critical pathological component of human and canine HF. Differential remodeling of cholinergic signaling represents a novel mechanism for enhancing sympathovagal balance with CRT and may identify new targets for treatment of systolic HF. (Circ Res. 2015;116:1691-1699. DOI: 10.1161/CIRCRESAHA.116.305268.)

Key Words: acetylcholine ■ autonomic nervous system ■ cardiac resynchronization therapy ■ heart failure ■ receptors, muscarinic ■ vagal nerve stimulation

Heart failure (HF) is a leading cause of death worldwide. Despite contemporary medical advances, about half of patients with HF die within 5 years of diagnosis. Pharmacological approaches improve HF symptoms and delay mortality but do not reverse disease progression. Cardiac resynchronization therapy (CRT) is the only approach documented to improve left ventricular (LV) function and reduce mortality, albeit by mechanisms that are incompletely understood. Improving this understanding may help us improve on the response to CRT and identify new therapeutic targets that extend the benefits of CRT to a wider HF population.

CRT has salutary effects beyond restoration of electromechanical synchrony that involve remodeling of β-adrenergic signaling pathways and restoring sympathovagal balance.

Although mechanisms leading to depressed β-adrenergic signaling have been studied extensively, far less is known about concurrent functional alterations in cholinergic (parasympathetic/muscarinic) signaling or its role in the HF phenotype. Evidence from animal models and ongoing clinical trials suggests modulating cholinergic activity and restoring sympathovagal balance has salutary effects in HF, but the underlying molecular mechanisms have not been established. The effect of CRT on cholinergic signaling is unexplored.
Because β-adrenergic and cholinergic signaling pathways are intimately coupled, we hypothesized that remodeling of cholinergic signaling plays an important role in the aberrant calcium signaling and depressed contractile responses to β-adrenergic stimulation in dysynchronous HF (DHF) that are restored by CRT.

Methods
We studied 3 canine models: (1) normal controls (n=8); (2) DHF (n=10), which were first subjected to ablation of the left bundle branch and then, 6 weeks of right atrial tachypacing at 200 bpm; (3) CRT (n=10), which was developed as DHF for the first 3 weeks followed by biventricular tachypacing (LV lateral and right ventricular anterograde epicardium) for the next 3 weeks. Echocardiography, electrocardiography, and tissue Doppler (longitudinal strain speckle tracking with 4-chamber views) were performed at 3 and 6 weeks to assess dysynchrony (variance of peak systolic strain timing) as previously described.1,2 Calcium recordings and SS were assessed at 0.5 and 2 Hz, and with 2 mmol/L extracellular calcium, and yielded concordant findings. Isoproterenol, carbamylcholine, pirenzapine (M1-mAChR antagonist), 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP; M3-mAChR antagonist), atropine, and pertussis toxin (PTX) were purchased from Sigma-Aldrich Inc (St. Louis, MO). J 104129 fumarate, an antagonist with higher specificity than 4-DAMP for M3-mAChR, was purchased from Tocris Bioscience (Bristol, United Kingdom) to confirm findings in some experiments with 4-DAMP.

Myocardium was homogenized in lysis buffer (Cell Signaling Technology), and 50 to 100 µg of total protein was loaded for gel electrophoresis as previously described.4 mRNA expression was assessed by Taqman real-time polymerase chain reaction using the Path-ID Multiplex One Step RT-PCR kit (Applied Biosystems) as previously described.8 The canine-specific primer and probe sequences for M2-mAChR were as follows: cCHRM2-F (GGACAATTGGTTATTGGCTTTGTA), cCHRM2-R (GTTGGCTTACAAGTGTCAGACC), cCHRM2-T (ATCAACAGGCACCATACTCCGCC).

The antibodies used for Western blots were M2-mAChR (Sigma M9558 1:1500 for canine samples and Millipore Abs156 1:1500 for human samples), M3-mAChR Santa Cruz sc-9108 1:1200, Guo11 (Santa Cruz sc-392 1:250 dilution), and connexin-43 (Cx43) (Chemicon mab3068 1:1000). The same antibodies for M2 and Cx43 were used in immunostaining at dilutions of 1:100 and 1:50, respectively. Western blot analysis from LV myocardial samples has been previously reported.4,5 LV tissue was obtained from explanted failing and nonfailing hearts deemed unsuitable for transplantation after approval from the Institutional Review Boards at the Johns Hopkins University and University of Munich, Germany. Immunohistochemical analysis was performed and representative sections were selected by a senior pathologist not involved in the study and blinded to the canine model groups.

Figure 1. Echocardiographic, hemodynamic, and electrophysiological characteristics of normal, dysynchronous heart failure (DHF), and cardiac resynchronization therapy (CRT) animals at 6 weeks. Invasive pressure measurements revealed left ventricular end-systolic pressures (LVESP) (A) were similar between DHF (n=10) and CRT (n=10), but both were reduced compared with normal controls (n=8). Regional LV longitudinal strain (B) derived from echocardiographic speckle tracking analysis revealed similar, simultaneous strains in all regions for normal controls, but in DHF, septal shortening preceded the lateral wall with reciprocal septal stretch when the lateral wall contracted; restoration of synchrony was observed with CRT. Echocardiography-derived ejection fractions (G) and stroke volumes (D) were decreased in DHF and improved by CRT, but both remained lower than normal controls. Invasive pressure measurements revealed higher LV end-diastolic pressures (LVEDP) (E) and lower dp/dt (normalized to instantaneous developed pressure [IP]) (F) in DHF compared with CRT and normal controls. Despite a similar increase in heart rate (G) in DHF and CRT, the heart rate variability (H) was significantly lower in DHF compared with normal and CRT. All panels, *P<0.01 vs all other groups. SDNN indicates SD of NN intervals.
Fractional changes are presented as mean±SEM and compared using a paired t test. Categorical comparisons were performed using a χ 2 test. Absolute values are reported in box and whisker plots (mean, median, interquartile range, minimum, and maximum). Comparisons between multiple experimental groups were performed by ANOVA and a Tukey test. In vivo data at multiple time points were analyzed by repeated-measures ANOVA.

**Results**

The canine DHF model 6–8 was generated by left bundle branch ablation to disrupt synchronous activation combined with rapid right atrial pacing for 6 weeks. CRT was produced by switching to biventricular tachypacing from weeks 4 to 6. Because both models were tachypaced, they developed similar global...
LV dysfunction (Figure 1A) and were designed to interrogate responses specific to dysynchronous versus resynchronized ventricular contraction during hemodynamic decompensation. Compared with non-HF controls, DHF animals demonstrated significant regional dyssynchrony of LV shortening and depressed ejection fraction, stroke volume, dP/dt_{max}, and heart rate variability (Figure 1B–1H). These hemodynamic and electrophysiological changes were significantly improved by CRT.

We hypothesized that during tonic β-adrenergic stimulation, acute cholinergic stimulation suppresses CaT and SS in DHF more than in normal or CRT. In the continued presence of isoproterenol, cholinergic stimulation with carbamylcholine markedly depressed peak CaT and SS amplitudes in DHF myocytes by 59% and 74%, respectively. These responses were only modestly diminished in normal controls and even less so in CRT (Figure 2A and 2B; Online Figure IIA). Reversal of depression by atropine indicated an mAChR-specific effect. Some DHF cells were so sensitive to cholinergic stimulation that contraction was arrested despite continued isoproterenol exposure, and full restoration of the CaT and contraction required atropine (Online Movie I). Cholinergic stimulation also prolonged the CaT and SS more in DHF than in normal or CRT myocytes (Figure 2A and 2C; Online Figure IIB).

To identify the molecular basis of the model-dependent cholinergic responses, we performed immunohistochemistry, Western blot, and real-time polymerase chain reaction analyses. We observed an increase in M2-muscarinic acetylcholine receptor (M2-mAChR) mRNA and protein expression with dyssynchrony that was reversed by resynchronization (Figure 2D). The canine DHF model exhibits similar changes in β-adrenergic signaling as human cardiomyopathy. Thus, we observed similar experiments on LV myocardium from human hearts failing of ischemic and nonischemic pathogenesis. We observed similar increases in immunoreactive protein expression and subcellular localization of M2-mAChR (Figure 1E), suggesting that M2-mAChR remodeling is a generalizable pathophysiological feature of HF.

Does tonic cholinergic stimulation alter acute β-adrenergic responses? This question is germane to the effect of vagal nerve stimulation in HF. Under normal resting conditions, cholinergic signaling is the predominant autonomic influence on the heart. In the continued presence of cholinergic stimulation, CaT and SS responses to β-adrenergic stimulation were markedly depressed in DHF (by 42% and 56%) and less inhibited in normal and CRT myocytes (Figure 3A–3C; Online Figure IIIA and IIIB).

With cholinergic stimulation alone, peak CaT and SS amplitudes were decreased in DHF (by 18% and 27%), but remained unchanged in normal and CRT myocytes (Figure 4A and 4B; Online Figure IVA). Cholinergic stimulation prolonged CaT and SS in DHF but shortened them in normal and CRT myocytes (Figure 4C; Online Figure IVB). Reversal of cholinergic responses by atropine was not recapitulated by M1- or M3-mAChR inhibition (data not shown), suggesting that these effects are mediated via M2-mAChR, consistent with changes in subtype functional expression (Figure 1D).

Figure 3. Response to β-adrenergic stimulation in the setting of tonic cholinergic stimulation. A, Representative calcium transient (CaT) and sarcomere shortening (SS) from normal control, dysynchronous heart failure (DHF) and cardiac resynchronization therapy (CRT) myocytes are plotted in a format similar to Figure 2A. The myocytes were sequentially exposed to: carbamylcholine (CCh; [C], left column); isoproterenol (C+I, middle column); and atropine (+Atr; C+I+A, right column). B, The ratio of the peak responses for C+I:C and C+I+A:C+I for CaT (top) and SS (bottom) in normal control (empty bar), DHF (filled), and CRT (striped) myocytes. C, The ratio of the 80% duration of CaT and SS is plotted in a format similar to Figure 2B (n=19–32 myocytes per bar; n=6–9 hearts per bar). The individual data points are plotted in Online Figure IIIA. β-Adrenergic stimulation markedly increased the respective peak CaT and SS by 176±26% and 525±57% in normal control; and by 119±20% and 620±111% in CRT myocytes; but by only 65±9% and 165±29% in DHF. Addition of atropine caused an additional increase in respective peak CaT and SS by 93±24% and 228±58% in DHF myocytes, whereas normal and CRT myocytes showed little response (22%–63%). C. The ratio of the 80% duration of CaT and SS is plotted in a format similar to B. The individual data points are plotted in Online Figure IIIB. In the continued presence of CCh, isoproterenol shortened the respective durations of CaT and SS by 15±3% and 21±4% in DHF; by 21±4 and 30±4% in normal controls; and by 29±3% and 33±5% in CRT myocytes. Atropine further shortened the durations by 35±3% and 37±4% in DHF; by 25±5% and 18±4% in normal control; and by 10±3% and 14±5% in CRT myocytes. All panels, *P<0.05 vs all other groups.
Does M2-mAChR remodeling have functional effects independent of receptor activation? Exposure of DHF myocytes to atropine alone reversibly increased peak CaT and SS amplitudes (by 26% and 33%; Figure 4A), and SS (bottom) in normal control (empty bar), DHF (filled) and CRT (striped) myocytes is plotted in a format similar to Figure 3B (n=20–30 myocytes per bar; n=6–9 hearts per bar). The individual data points are plotted in Online Figure IVA. Cholinergic stimulation decreased the respective peak CaT and SS by 18±5% and 27±7% in DHF but had little or no effect in normal and CRT myocytes. These effects were reversed by subsequent addition of atropine, C. The ratio of the 80% duration of CaT and SS is plotted in a format similar to B. The individual data points are plotted in Online Figure IVB. Cholinergic stimulation prolonged the CaT and SS durations by 14±3% and 5±3% in DHF, whereas in normal and CRT myocytes, the CaT and SS durations were either shortened or unchanged. D, The ratio of the peak responses to atropine (A:E) and washout for CaT and SS in normal control (empty bar), DHF (filled), and CRT (striped) myocytes is plotted in a format similar to Figure 3B (n=20–30 myocytes per bar; n=3 hearts per bar). Atropine increased the peak CaT (left) and SS (right) by 26±5% and 33±11% in DHF and by 9±4% and 10±3% in normal controls, but had no effect in CRT myocytes. These effects were reversed by washing off atropine. All panels, *P<0.05 vs all other groups.

Figure 4. Response to cholinergic stimulation alone. A, Representative calcium transient (CaT) and sarcomere shortening (SS) are shown from normal controls, dysynchronous heart failure (DHF), and cardiac resynchronization therapy (CRT) myocytes sequentially exposed to standard Tyrode's extracellular solution (ECSS; E, thin gray line) followed by carbamylcholine (CCh [C], thick black line). B, The ratio of the peak responses for C:E and after the addition of atropine to CCh compared with CCh alone (C+A:C) for CaT (top) and SS (bottom) in normal control (empty bar), DHF (filled) and CRT (striped) myocytes is plotted in a format similar to Figure 3B (n=20–30 myocytes per bar; n=6–9 hearts per bar). The individual data points are plotted in Online Figure IVA. Cholinergic stimulation decreased the respective peak CaT and SS by 18±5% and 27±7% in DHF but had little or no effect in normal and CRT myocytes. These effects were reversed by subsequent addition of atropine, C. The ratio of the 80% duration of CaT and SS is plotted in a format similar to B. The individual data points are plotted in Online Figure IVB. Cholinergic stimulation prolonged the CaT and SS durations by 14±3% and 5±3% in DHF, whereas in normal and CRT myocytes, the CaT and SS durations were either shortened or unchanged. D, The ratio of the peak responses to atropine (A:E) and washout for CaT and SS in normal control (empty bar), DHF (filled), and CRT (striped) myocytes is plotted in a format similar to Figure 3B (n=20–30 myocytes per bar; n=3 hearts per bar). Atropine increased the peak CaT (left) and SS (right) by 26±5% and 33±11% in DHF and by 9±4% and 10±3% in normal controls, but had no effect in CRT myocytes. These effects were reversed by washing off atropine. All panels, *P<0.05 vs all other groups.

Does M2-mAChR remodeling have functional effects independent of receptor activation? Exposure of DHF myocytes to atropine alone reversibly increased peak CaT and SS amplitudes (by 26% and 33%; Figure 4A). These atropine-induced effects were infrequently observed in normal cells, suggesting that DHF hearts are biased toward M2-mAChR-Gαi–coupled signaling both in the absence and in the presence of cholinergic stimulation.

How does M2-mAChR-Gαi remodeling alter arrhythmic risk? In 30% to 50% of myocytes from all models, isoproterenol alone induced after-transients and after-contractions (Figure 5A), consistent with findings from a recent study on normal canine myocytes.20 Cholinergic stimulation completely abolished these disturbances in DHF and CRT, with little effect on peak CaT and SS in CRT myocytes. In the presence of tonic cholinergic stimulation, isoproterenol did not induce after-transients and after-contractions until exposure to atropine (Figure 5B). Compared with DHF, early after-transients and after-contractions were more frequently seen in normal and CRT myocytes. The atropine effect was not recapitulated by M1- or M3-mAChR inhibition (data not shown).

In myocytes from all models pretreated with PTX, isoproterenol promptly induced after-transients and after-contractions that were not affected by cholinergic stimulation (Figure 5C).

To characterize the role of M3-mAChR-Gαi signaling, we pretreated myocytes with PTX to inhibit Gαi signaling. With tonic β-adrenergic stimulation, PTX completely abolished the negative inotropic effects from cholinergic stimulation in all models (Figure 6A; Online Figure VA). In the presence of PTX and pirenzepine, an M1-mAChR–specific inhibitor, cholinergic stimulation increased peak CaT and SS in CRT but not in DHF cells (Figure 6B); this effect was suppressed by atropine (Online Figure VB) or M3-mAChR inhibition (Figure 6B; Online Figure VC). Consistent with these findings, immunohistochemical analyses demonstrated increased M3-mAChR expression, prominently at the intercalated discs in CRT (Figure 6C). Western blot analyses revealed increased M3-mAChR protein without any apparent effect on Gαq/11 expression (Figure 6D).

Discussion

We have identified upregulated M2-mAChR18,19 expression and function in human and canine HF compared with non-failing controls. CRT attenuated M2-mAChR expression and Gαi17,21,22-coupling and enhanced M3-mAChR23,24 expression in association with enhanced calcium cycling and SS. These changes in cholinergic signaling represent a novel mechanism for enhancing sympathovagal balance in CRT and may identify new targets for treatment of systolic HF.

Tonic β-adrenergic stimulation and increased Gαi expression are a hallmark of DHF. CRT reverses this phenotype by upregulating regulator of G-protein signal 2 (RGS2) and inhibiting Gαi signaling without decreasing Gαi expression, thereby improving calcium handling and sarcomere contraction.67 The M2-mAChR subtype18,19 in the heart is selectively coupled to Gαi and acts via well-characterized second messenger pathways. Coordinated increases in M2-mAChR and Gαi expression and coupling have been noted in the LV from synchronized failing hearts21,22 but
the functional significance was not known. Whether this coordinated remodeling is also a feature of dyssynchronized and resynchronized HF had not been explored.

Our results indicate that DHF hearts are biased toward M2-mAChR-\(G_{\alpha i}\)-coupled signaling, even in the absence of cholinergic stimulation. Extensive in vitro and ex vivo evidence indicates that G\(\alpha i\)-coupled cardiac M2-mAChRs are activated in proportion to G\(\alpha i\) expression\(^{25}\) and in this setting, atropine (I+I+C+A). The percent of normal, DHF, and CRT myocytes that demonstrated triggered activity (after-transients and after-contractions) in response to the corresponding solution exposures (n=42–104 myocytes per group; n=6–9 hearts per group). Myocytes that demonstrated isoproterenol-induced triggered activity were used only for analysis in this section and excluded from the analyses as shown in Figures 2 to 4 and 6. B, Representative CaT and SS from CRT myocytes are plotted (top) in a format similar to Figure 3A. In the presence of CCh (C), addition of isoproterenol (C-I) did not induce after-transients and after-contractions even after addition of atropine (C+I+C+A). The bar graph (bottom) shows the percent of normal, DHF, and CRT myocytes demonstrating triggered activity (after-transients and after-contractions) in response to the corresponding solution exposures (n=25–63 myocytes per group; n=6–9 hearts per group). Myocytes that demonstrated isoproterenol-induced triggered activity were used only for analysis in this section and excluded from the analyses as shown in Figures 2 to 4 and 6. C, Representative CaT and SS from normal control, DHF, and CRT myocytes pretreated with pertussis toxin (PTX) to inhibit G\(\alpha i\) are plotted in a format similar to A. In all myocytes from all models, sustained after-transients and after-contractions were noted with isoproterenol regardless of exposure to CCh. All panels, *\(P<0.05\) vs all other groups.

It is plausible that M2-mAChR remodeling occurs early on in HF as a compensatory mechanism to heightened sympathetic tone that, in the long-term, contributes to the pathology of HF, perhaps by depressing myocyte function, calcium handling, and \(\beta\)-adrenergic responsiveness. Increased cholinergic tone has been noted early in HF development\(^{29}\) and cholinergic transdifferentiation of cardiac sympathetic neurons has been observed in some HF models.\(^{30}\) By decreasing M2-mAChR-G\(\alpha i\)–mediated signaling, CRT improves \(\beta\)-adrenergic responsiveness. This,
Figure 6. Cholinergic stimulation mediates positive and negative inotropic effects via distinct muscarinic receptor subtypes. 

A. The peak sarcomere shortening (SS) responses (mean±SEM) corresponding to the indicated solution exchange protocol are compared in the absence (empty bars) or presence (filled) of pertussis toxin (PTX) for normal control (Nor; gray), dyssynchronous heart failure (DHF; red bars) and cardiac resynchronization therapy (CRT; blue) myocytes (n=30–52 myocytes from n=6–9 hearts for each bar). The individual data points are plotted in Online Figure VA. PTX increased the peak SS response to isoproterenol (left column) in DHF myocytes, but had no effect in normal and CRT myocytes. This is consistent with enhanced baseline G\(\alpha_i\) activity in DHF. In the continued presence of isoproterenol, pretreatment with PTX abolished the negative inotropic effects of cholinergic stimulation in all groups (middle column). The peak SS after addition of atropine was not significantly different with and without PTX for normal (\(P=0.43\)), DHF (\(P=0.13\)), and CRT (\(P=0.32\)) myocytes (right column). These data suggest that in the presence of saturating \(\beta\)-adrenergic stimulation, the negative inotropic effect from cholinergic stimulation is mediated via M2-muscarinic acetylcholine receptor (mAChR)-G\(\alpha_i\) signaling.

B. The ratio of the peak SS responses to carbamylcholine (CCh) alone compared with Tyrode's extracellular solution (ECS) using the same protocol as in Figure 4B is plotted in the absence and presence of PTX and an M3-mAChR–specific inhibitor (M3i; n=8–30 myocytes per bar; n=3–9 hearts per bar). All myocytes were continuously perfused with pirenzapine to block M1-mAChR–specific effects. The individual data points are plotted in Online Figure VB and VC. Compared with the absence of PTX, cholinergic stimulation in the presence of PTX increased the peak SS by 45±8% in CRT myocytes, but this effect was abolished with M3i. In DHF myocytes, PTX abolished the negative inotropic effect from cholinergic stimulation, but M3i had no significant effect. These data suggest that CRT myocytes are biased toward M3-mAChR–mediated positive inotropic effect, whereas normal and DHF myocytes are not.

C. Representative immunohistochemical staining sections of canine midmyocardial tissue from the left ventricular (LV) lateral wall (top) revealed increased M3-mAChR protein expression in CRT myocytes at the intercalated discs. Western blots of tissue lysates (5 hearts per group) revealed CRT increased M3-mAChR protein expression without any change in G\(\alpha_q\)/11 protein expression (bottom). Proposed mechanism for autonomic remodeling in DHF and with CRT. Cholinergic stimulation of LV myocytes by acetylcholine (ACh) released from parasympathetic vagus nerve branches can produce both inhibitory and stimulatory calcium and contractile responses in the heart via well-characterized M2-mAChR–G\(\alpha_i\) and M3-mAChR–G\(\alpha_q\) coupled signaling, respectively. DHF (red arrows and tracings) is associated with downregulation of \(\beta_1\)-adrenergic receptors (\(\beta\)-AR) and inhibition of adenylate cyclase (AC) from direct interactions with the \(\alpha\)-subunit of the PTX-sensitive inhibitory G protein (G\(\alpha_i\)) selectively coupled to M2-mAChRs. Furthermore, coordinated increases in M2-mAChR-G\(\alpha_i\)-coupled expression and signaling chronically inhibit basal AC-mediated downstream signaling and markedly impairs the efficiency of \(\beta\)-adrenergic responsiveness, resulting in smaller amplitudes and prolonged relaxation of CaT and SS. CRT (blue arrows and tracings) reverses this phenotype by differentially remodeling cholinergic mAChR signaling. By concurrently decreasing M2-mAChR and increasing RGS2 expression, CRT decreases the negative inotropic effects of G\(\alpha_i\) signaling. In addition, CRT increases M3-mAChR-G\(\alpha_q\)-mediated signaling associated with positive inotropic responses and putative cardioprotective effects. All panels, *\(P<0.05\) vs all other groups.
along with functional inhibition of Gtqi by RGS2, results in positive inotropic effects because of improved calcium handling and sarcomere response to β-adrenergic stimulation.

How does M2-mAChR-Gtqi remodeling alter arrhythmic risk? Ventricular arrhythmias are a major cause of death in patients with HF. Since the first report in 1859, extensive evidence from animal and clinical studies indicates that β-adrenergic signaling increases arrhythmic risk and cholinergic stimulation protects the heart from lethal arrhythmias. Despite model-dependent remodeling, there seems to be a large margin of safety at the cellular level for M2-AChR-Gtqi–coupled signaling to protect and rescue normal, DHF, and CRT hearts from arrhythmias. These results provide a mechanistic basis for prior observations, that is, increased arrhythmias with β-adrenergic stimulation and PTX, antiarhythmic effects of cholinergic stimulation, and may have important implications for vaso nerve stimulation and development of new antiarrhythmic therapies.

Whereas the highly prevalent M2-mAChR subtype is selectively coupled to Gtqi, the relatively scarce M3-mAChR is highly specific for stimulatory Gq with putative cardioprotective effects. Recent insights into the molecular structure, function, pharmacology, and fundamental physiological role of M3-mAChRs have identified them as a major target for drug development. Our results indicate that CRT may exert beneficial effects via M3-mAChR-Gq signaling, including enhanced calcium handling, sarcomere responsiveness, and positive inotropy. Notably, CRT increased M3-mAChR expression at the intercalated discs. In cardiomyocytes, M3-mAChR activation during ischemia preserves the phosphorylated levels of sarcolemmal connexin 43 to provide delayed cardioprotection. Furthermore, M3-mAChR-Gq signaling augments inositol 1,4,5-triphosphate (IP3)/diacylglycerol (DAG)-mediated calcium release, protein kinase C (PKC)-mediated phosphorylation, phosphoinositide-3-kinase/Akt-mediated reduced apoptosis, and RGS2 expression. CRT has similar effects, including increasing RGS2 expression particularly in clinical responders that may be exerted via M3-mAChR-Gq signaling. We could not specifically address this here because an in vitro model of CRT does not currently exist.

The notion that cholinergic signaling has a relatively limited effect on LV function belies much evidence. Our results indicate that remodeling of cholinergic signaling is paramount for restoration of autonomic balance by CRT (Figure 4E). The novel mechanisms identified herein offers an opportunity to apply targeted downregulation of M2-mAChR and upregulation of M3-mAChR in patients with HF who are not CRT responders or candidates. Moreover, the beneficial effects of CRT might be enhanced by vaso nerve stimulation and remodeling of the key signaling components reported herein may represent mechanistic pathways engaged by vaso nerve stimulation and open new avenues for pharmacological or pacing treatments for HF.

Acknowledgments
We gratefully acknowledge Dr Charles Steenbergen for blinded analysis of immunohistochemistry slides and selection of representative samples, Deborah DiSilvestre and Dr Swati Dey for technical assistance with some of the experimental protocols, Drs Federica Farinelli and Khalid Chakir for myocyte isolation and procurement, and Rick Tunin for preparation of the animal models.

Sources of Funding
This work was supported by P01 HL 77180 (D.A. Kass, B. O’Rourke, and G.F. Tomaselli).

Disclosures
None.

References


31. Zhao J, Su Y, Zhang Y, Pan Z, Yang L, Chen X, Liu Y, Lu Y, Du Z, Yang B. Activation of cardiac muscarinic M3 receptors induces delayed cardiac arrhythmias. The development of new and improved HF therapies remains a clinical, research and public health priority. CRT is the only HF therapy to decrease long-term mortality, restore autonomic balance, and improve both acute and chronic left ventricular function. The underlying mechanisms are largely unknown. Autonomic imbalance is associated with worsening HF and increased mortality risk, independent of left ventricular function and ventricular arrhythmias. The present study demonstrates a critical role of parasympathetic M3ARs in HF, arrhythmic risk, and CRT. It suggests that the beneficial effects of CRT involve differential remodeling of mACHRs. Further understanding these mechanisms can lead to the design and development of new, more effective HF therapies.
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Circ Res. 2015;116:1691-1699; originally published online March 2, 2015; doi: 10.1161/CIRCRESAHA.116.305268

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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DeMazumder et al., Cardiac resynchronization therapy restores sympathovagal balance in the failing heart by differential remodeling of cholinergic signaling. Circulation Research (2015)
Each panel shows the schematic of a cardiomyocyte cell membrane containing β-adrenergic (β₁-AR) and/or muscarinic (M₁, M₂, M₃ mAChR) receptors coupled to intracellular second messengers [Gα, Gαq, or adenylate cyclase (AC)]; the specific stimulators and/or inhibitors corresponding to the receptors that were employed in the solution exchange protocol are on top; the measured steady-state responses (calcium transient, sarcomere shortening) are at the bottom.

a. Each myocyte was exposed first to isoproterenol (Iso) to stimulate β₁-AR. Then, carbamylcholine (CCh) was added to stimulate mAChRs in the continued presence of Iso. Finally, atropine (Atr) was added to assess reversal of mAChR-specific effects. The data from this protocol are shown in Fig. 2a-c and Online Fig. 2a-b.

b. Similar to panel a, each myocyte was exposed first to CCh, then Iso in the continued presence of CCh, and finally Atr (data shown in Fig. 3a-c and Online Fig. 3a-b).

c. The myocyte was first exposed to CCh alone followed by Atr (Fig. 4a-c and Online Fig. 4a-b).

d. The protocol was same as in panel a, except that the experiments were performed in the presence of pertussis toxin (PTX; Gαi inhibitor) (Fig. 5c, Fig. 6a and Online Fig. 5a).

e. The protocol was same as in panel c, except for the presence of PTX and M₁-mAChR-specific inhibitor (M₁i) to assess for mAChR-stimulated effects not mediated by M₂-Gαi and M₁-Gαq (Fig. 6b and Online Fig. 5b).

f. The protocol was same as in panel e, except for the presence of M₃-mAChR-specific inhibitor (M₃i). Comparison of these data with those from panel e were used to assess for effects specific to the M₃-mAChR (Fig. 6b and Online Fig. 5c).
ONLINE FIGURE II: Response to cholinergic stimulation in the setting of tonic β-adrenergic stimulation

a. Peak response

For each individual LV cardiomyocyte (grey filled circles) from normal controls (left column), DHF (middle) and CRT (right) animals (N=6-9 hearts/group), the peak CaT (top row) and SS (bottom) amplitudes are plotted for sequential exposures to ECS (E), Iso alone (I), Iso+CCh (I+C), and Iso+CCh+atropine (I+C+A). For each LV cardiomyocyte within groups, the inset (log2 scale; grey empty circles) plots the ratio of the responses to CCh added to Iso compared to Iso alone (I+C:I) as well as the ratio of I+C+A:I+C. The corresponding mean±SEM values are indicated by the colored filled markers.

The I+C+A:I+C ratio was larger than the I+C:I ratio within each inset for all groups (p<0.001); these ratios were also distinct between groups (p<0.001).
b. Time to 80% duration

The 80% durations of the CaT (top row) and SS (bottom) are plotted in a format similar to that in Online Figure 2a.

The I+C+A:I+C ratio was larger than the I+C:I ratio within each inset for all groups (p<0.01). The SS I+C:I ratio in DHF was larger than those in normal or CRT (p<0.01).
ONLINE FIGURE III: Response to β-adrenergic stimulation in the presence of tonic cholinergic stimulation

a. Peak response

For each individual LV cardiomyocyte (grey filled circles) from normal controls (left column), DHF (middle) and CRT (right) animals (N=6-9 hearts/group), the peak CaT (top row) and SS (bottom) amplitudes are plotted for sequential exposures to ECS (E), CCh alone (C), CCh+Iso (C+I), and CCh+Iso+atropine (C+I+A). For each LV cardiomyocyte within groups, the inset (log2 scale; grey empty circles) plots the ratio of the responses to Iso added to CCh compared to CCh alone (C+I:C) as well as the ratio of C+I+A:C+I. The corresponding mean±SEM values are indicated by the colored filled markers.

The I+C+A:I+C ratio was larger than the I+C:I ratio within each inset in normal and CRT (p<0.001) but not in DHF. Whereas the C+I:C ratios for the CaT and SS in DHF were smaller than those in normal or CRT (p<0.01), the C+I+A:C+I ratios in DHF were larger (p<0.05).
b. Time to 80% duration

The 80% durations of the CaT (top row) and SS (bottom) are plotted in a format similar to that in Online Figure 3a.

The C+I:A+C ratio and the C+I:C ratio were significantly different within each inset in DHF and CRT (p<0.05), but these ratios were similar in normal controls. Whereas the C+I:A+C ratio was smaller than the C+I:C ratio in DHF, the converse was seen in CRT (p<0.01).
ONLINE FIGURE IV: Cholinergic stimulation alone in normal, DHF and CRT

a. Peak response

For each individual LV cardiomyocyte (grey filled circles) from normal controls (left column), DHF (middle) and CRT (right) animals (N=6-9 hearts/group), the peak CaT (top row) and SS (bottom) amplitudes are plotted for sequential exposures to ECS (E), CCh alone (C), and CCh+atropine (C+A). For each LV cardiomyocyte within groups, the inset (log2 scale; grey empty circles) plots the ratio of the responses to CCh compared to ECS alone (C:E) as well as the ratio of C+A:C. The corresponding mean±SEM values are indicated by the colored filled markers.

The C+A:C ratio was larger than the C:E ratio within each inset in normal and DHF (p<0.05) but these ratios were similar in CRT.
ONLINE FIGURE IV (continued):

b. Time to 80% duration

The 80% durations of the CaT (top row) and SS (bottom) are plotted in a format similar to that in Online Figure 4a.

For all groups, the C+A:C ratio was significantly different from the C:E ratio within each inset (p<0.05). Whereas the C+A:C ratio was larger than the C:E ratio in DHF, the converse was seen in normal and CRT (p<0.05).
ONLINE FIGURE V: Distinct effects of M2- and M3-muscarinic receptor signaling

a. Peak response to cholinergic stimulation in the presence of tonic β-adrenergic stimulation and PTX (Gαi inhibitor)

The 80% durations of the CaT (top row) and SS (bottom) are plotted in a format similar to that in Online Figure 2a.

Compared to the data in the absence of PTX (Online Fig. 2a), the corresponding I+C+A:I+C ratios and the I+C:I ratios were significantly diminished for all groups (p<0.0001).
b. Peak response to cholinergic stimulation alone in the presence of PTX (Gαi inhibitor)

The peak CaT (top row) and SS (bottom) amplitudes in the presence of PTX and an M1-mAChR-specific inhibitor are plotted in a format similar to that in Online Figure 4a.

The C:E ratios for the CaT and SS in CRT were larger than those in normal and DHF (p<0.05), and the C+A:C ratios in CRT were smaller than those in normal and DHF (p<0.05).
c. Peak response to cholinergic stimulation alone in the presence of PTX (Gαi inhibitor) and M3-receptor inhibitor

The peak CaT (top row) and SS (bottom) amplitudes in the presence of PTX, an M1-mACHR-specific inhibitor and an M3-mACHR-specific inhibitor are plotted in a format similar to that in Online Figure 4a.

For all groups, the C:A:C ratio was similar to the C:E ratio within each inset. Compared to the absence of an M3-mACHR inhibitor (Online Fig. 5b), the corresponding C:A:C ratios for the CaT and SS were significantly different in CRT (p<0.05) but they were the same in DHF and normal.