Muscarinic-dependent phosphorylation of the cardiac ryanodine receptor by protein kinase G is mediated by PI3K–AKT–nNOS signaling

Post-translational modifications of proteins involved in calcium handling in myocytes, such as the cardiac ryanodine receptor (RyR2), critically regulate cardiac contractility. Recent studies have suggested that phosphorylation of RyR2 by protein kinase G (PKG) might contribute to the cardioprotective effects of cholinergic stimulation. However, the specific mechanisms underlying these effects remain unclear. Here, using murine ventricular myocytes, immunoblotting, proximity ligation as-says, and nitric oxide imaging, we report that phosphorylation of Ser-2808 in RyR2 induced by the muscarinic receptor agonist carbachol is mediated by a signaling axis comprising phosphoinositide 3-phosphate kinase, Akt Ser/Thr kinase, nitric oxide synthase 1, nitric oxide, soluble guanylate cyclase, cyclic GMP (cGMP), and PKG. We found that this signaling pathway is compartmentalized in myocytes, as it was distinct from atrial natriuretic peptide receptor–cGMP–PKG–RyR2 Ser-2808 signaling and independent of muscarinic-induced phosphorylation of Ser-239 in vasodilator-stimulated phosphoprotein. These results provide detailed insights into muscarinic-induced PKG signaling and the mediators that regulate cardiac RyR2 phosphorylation critical for cardiovascular function.

Post-translational modifications of proteins involved in myocyte calcium (Ca) handling, including the cardiac ryanodine receptor (RyR2) (1), play a critical role in regulation of cardiac contractility as part of autonomic nervous system integration. Substantial effort has been devoted to investigating modulation of myocyte Ca handling by the sympathetic nervous system (2). However, detailed regulation of Ca handling by the parasympathetic system remains poorly understood. At the same time, reduced and/or altered parasympathetic tone is a common feature of heart disease (3), whereas parasympathetic augmentation is reportedly cardio-protective (4).

RyR2 is phosphorylated at three known sites, including serine 2808 (5). Initially thought to be fundamental to the adrenergic “flight or fight” response and contribute to altered Ca handling in heart failure (HF) (6), the mechanisms and role of RyR2 Ser-2808 phosphorylation in both physiology and disease is not fully defined (7, 8). Recently, it was shown that RyR2 Ser-2808 is phosphorylated by protein kinase G (PKG) in a nitric oxide (NO)-dependent manner to facilitate sarcoplasmic reticulum (SR) Ca release during conditions such as osmotic stress/swelling (9). Moreover, our group has shown a role for cholinergic-induced RyR2 Ser-2808 phosphorylation in improving efficiency of calcium handling (10). However, the specific signaling components of this novel RyR2 regulatory pathway and its relation to muscarinic and PKG signaling are unknown.

Muscarinic stimulation and PKG signaling have been recognized for their cardio-protective roles in various heart disease settings (11). Whereas Ca signaling is characterized by spatially restricted subdomains (12), the subcellular organization of muscarinic and PKG signaling and integration of Ca signaling within cardiac myocytes is not fully defined. It has been previously reported that specificity of PKG signaling in cardiomyocytes is maintained via the involvement of receptor-mediated particulate guanylate cyclase (pGC) and soluble guanylate cyclase (sGC) (13). In addition, there is evidence of cyclic guanylate monophosphate (cGMP) compartmentalization in cardiomyocytes whereby β3AR–eNOS–NO–sGC–NO–PKG (β2-adrenergic receptor–endothelial nitric oxide synthase–nitric oxide–sGC–cGMP–PKG) signaling occurs separately from a pGC-cGMP pool mediated via atrial natriuretic peptide receptor (ANPR)–pGC–cGMP–PKG signaling (14). Although extensively characterized in other cell types (15, 16), the specific molecular events linking muscarinic stimulation and cGMP–PKG-dependent phosphorylation of target effectors, including components of Ca handling, remain to be elucidated in cardiomyocytes.

The goal of the present study was to examine the specific molecular components and subcellular compartmentalization of this novel muscarinic PKG signaling cascade. Identifying molecular components in this pathway is critical to understanding fundamental properties of muscarinic signaling under physiological conditions to improve potential therapies that augment parasympathetic output to improve and/or preserve cardiovascular function.

Results

Previous data from our group suggested muscarinic acetylcholine receptor 2 (M2R) mediates carbachol (CCh)-dependent
PKG phosphorylation of RyR2 via PI3K/AKT/nNOS

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phosphorylation of RyR2 Ser-2808 to facilitate utilization of SR Ca stores to improve excitation contraction coupling (10). To further specify the role of M2R in mediating RyR2 phosphorylation, we employed M2R knockout mice (M2R KO) and measured RyR2 Ser-2808 phosphorylation using Western blot analyses. Isolated cardiomyocytes from M2R KO mice failed to exhibit increased RyR2 2808 phosphorylation in response to CCh as observed in WT myocytes (Fig. 1A) and displayed no differences in baseline RyR2 Ser-2808 phosphorylation (Fig. S1). Further, we used the M2R/M4R allosteric activator LY2119620 to investigate the role of M2R in regulating RyR2 Ser-2808 phosphorylation. LY2119620 significantly increased phosphorylation of RyR2 Ser-2808 in the presence of CCh (Fig. 1C). These results establish the specific role of M2R in CCh-mediated phosphorylation of RyR2 Ser-2808.

The downstream signaling systems linking M2R stimulation and RyR2 phosphorylation are unknown. Because nitric oxide (NO) has been associated with cholinergic stimulation (17) and PKG signaling (18) in various cell types, we examined the role of NO as a potential mediator of M2R-dependent RyR2 Ser-2808 phosphorylation. To this end, we measured NO release in response to CCh using fluorescent NO indicator DAF-FM in ventricular cardiomyocytes. Fig. 2 (A and B) shows that isolated cardiomyocytes treated with CCh display a significant increase in NO release as measured by 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM Diacetate) fluorescence. Additionally, we measured the NO byproducts nitrate and nitrite as an index for total NO generation in ventricular myocytes. Fig. S2 demonstrates that allosteric potentiation of M2R with LY2119620 and CCh significantly increases total nitrate/nitrite production in ventricular myocytes. This effect was mediated via nitric oxide synthase (NOS) as nonspecific inhibition curtailed total NO generation (Fig. S2).

In cardiac myocytes, NO is generated by two NOS isoforms: neuronal (NOS1, nNOS) and endothelial (NOS3, eNOS) nitric oxide synthase enzymes. Treatment of cardiomyocytes with a specific NOS1 inhibitor, Nω-propyl-L-arginine hydrochloride (NP), prevented NO release in response to CCh (Fig. 2, D–F). In addition, we employed pharmacological inhibitors of NOS1 or NOS3 and measured phosphorylation of RyR2 Ser-2808 using Western blotting analyses. Consistent with the results of the experiments with NO measurement, myocytes treated with the specific NOS1 inhibitor NP abrogated CCh-mediated increase in RyR2 Ser-2808 phosphorylation (Fig. 3A). In contrast, inhibition of NOS3 with Nω-(1-iminoethyl)-L-ornithine dihydrochloride (LN) did not prevent CCh-mediated increase in RyR2 Ser-2808 phosphorylation (Fig. 3A). Additionally, genetic ablation of NOS1 but
PKG phosphorylation of RyR2 via PI3K/AKT/nNOS

Figure 3. Nitric oxide synthase 1 mediates CCh-induced RyR2 Ser-2808 phosphorylation. A, representative Western blot showing that 10 \( \mu \text{M} \) CCh increases RyR Ser-2808 phosphorylation. Inhibition of NOS1 with specific inhibitor 1 \( \mu \text{M} \) NP abolishes CCh-induced RyR Ser-2808 phosphorylation. Inhibition of NOS3 with 1 \( \mu \text{M} \) LN failed to inhibit CCh-mediated RyR Ser-2808 phosphorylation (**, \( p < 0.05 \) versus CTL; †, \( p < 0.05 \) versus CCh + NP). Student’s t test. B, plot of OD normalized for phosphorylated RyR Ser-2808/total RyR levels. Data shown are median ± S.D. n = 3-4 WT mice, 3-4 experiments/group. C, representative Western blot showing that 10 \( \mu \text{M} \) NP abolishes CCh-induced RyR Ser-2808 phosphorylation (**, \( p < 0.05 \) versus CTL). Student’s t test. D, plot of OD normalized for phosphorylated RyR Ser-2808/total RyR levels. Data shown are median ± S.D. n = 3 NOS1 KO mice, 3-4 experiments/group. E, representative Western blot showing that 10 \( \mu \text{M} \) WM (100 nM) abrogates Akt Ser-473 phosphorylation (**, \( p < 0.05 \) versus CTL). Student’s t test. F, plot of OD normalized for phosphorylated Akt Ser-473/total Akt levels. Data shown are median ± S.D. n = 3-5 WT, n = 3-5 NOS3 KO mice, minimum of 3 experiments/group.

not NOS3 prevented RyR2 Ser-2808 phosphorylation induced by CCh (Fig. 3, C and E). It has been shown previously that ablation of NOS1 does not affect NOS3 protein expression (19). Similarly, NOS3 KO mice displayed no significant alteration in NOS1 protein compared with WT controls (Fig. S3). Additionally, we observed no significant changes in levels of total or RyR2 Ser-2808 phosphorylation in these samples (Fig. S3). Collectively, these results suggest that cholinergic-induced RyR2 Ser-2808 phosphorylation is mediated specifically by NOS1.

Endogenous NOS1 in various cell types has been linked to several upstream kinase systems including Akt (20, 21). We examined the role of this system in mediating M2R-dependent activation of NOS1 and RyR2 Ser-2808 phosphorylation. Strikingly, and for the first time in adult mouse ventricular cardiomyocytes, we show that application of CCh increases phosphorylation of Akt serine 473 (Fig. 4A). Thus, our data indicate that CCh activates Akt Ser-473 phosphorylation in ventricular myocytes. Because phosphoinositide-3 kinase (PI3K) is a known upstream effector of Akt, we investigated the role of PI3K to initiate Akt signaling under cholinergic stimulation. Cardiomyocytes treated with specific PI3K inhibitor wortmannin abrogated increased Akt Ser-473 phosphorylation in the presence of CCh (Fig. 4A). Moreover, wortmannin decreased RyR Ser-2808 phosphorylation in the presence of CCh (Fig. 4C). These results indicate that CCh initiates parasympathetic signaling via the PI3K–Akt–NO axis to increase RyR2 Ser-2808 phosphorylation in ventricular cardiomyocytes.

Our previous work using kinase inhibitors linked PKG with CCh-induced RyR2 Ser-2808 phosphorylation (10). To further
establish the role of PKG as the kinase responsible for cholinergic-induced RyR2 Ser-2808 phosphorylation, we examined the role of sGC as the putative mediator of NOS–PKG activation and PKG activation via 8-Br-cGMP. As shown in Fig. 5 (A and C), exposure of myocytes to both the specific sGC stimulator (BAY 58-2667) and 8-Br-cGMP enhanced phosphorylation of RyR2 Ser-2808. These results indicate that PKG mediates phosphorylation of RyR2 Ser-2808 during cholinergic stimulation.

Intracellular Ca is an important secondary messenger that could support alternative or complementary pathways downstream of muscarinic receptors. To assess the role of Ca in muscarinic-dependent RyR2 phosphorylation, we used the cell-permeable Ca chelator EGTA-AM to buffer intracellular calcium levels. EGTA-AM had no significant effect on RyR2 Ser-2808 phosphorylation in response to CCh-treated or control levels. EGTA-AM had no significant effect on RyR2 Ser-2808 phosphorylation. We recently demonstrated that acute muscarinic stimulation results in PKG-dependent phosphorylation of the cardiac RyR2 at serine 239 (22). As shown in Fig. 6A, CCh increased phosphorylation of VASP Ser-239. Interestingly, VASP Ser-239 phosphorylation was independent of NOS1, suggesting subcellular specificity/compartmentalization (Fig. 6C). In cardiac myocytes, activation of the atrial natriuretic peptide (ANP) receptor is known to activate PKG via pGC (23). To gain further insights into PKG signaling associated with RyR2 phosphorylation, we assessed the ability of this canonic PKG pathway to phosphorylate RyR2 Ser-2808. As demonstrated in Fig. 6F, exposure to ANP resulted in phosphorylation of RyR2 Ser-2808. These results suggest global and more specific PKG-dependent subcellular pathways mediating phosphorylation of RyR2 Ser-2808 and VASP Ser-239 during ANP and muscarinic stimulation, respectively.

Subcellular compartmentalization of cholinergic-induced phosphorylation of RyR2 Ser-2808 relative to VASP Ser-239 was assessed using a combination of immunofluorescence and proximity ligation assay (PLA). Whereas distribution of total RyR2 and Akt showed striated staining at Z-lines, immunoreactivity for total VASP exhibited more diffuse staining with no substantial overlap with RyR2 (Fig. S5). Differential distribution of RyR2 and VASP immunoreactivity is consistent with NOS1-dependent PKG signaling revealed in the RyR2 and VASP phosphorylation experiments above.

PLA generates a punctate fluorescent signal at locations where two co-labeled proteins are within 40 nm of each other (24). This approach was used to identify the spatial localization and distribution of phosphorylated RyR2, Akt, and VASP with a high degree of specificity. Similar to global phosphorylation seen in Western blotting experiments, CCh significantly increased spatial densities (puncta/mm2) of phosphorylated RyR2 Ser-2808 (Fig. 7A), phosphorylatedVASP F7 Ser-239 (Fig. 7C), and phosphorylated Akt Ser-473 (Fig. 7E) puncta/μm². At the same time, phosphorylated RyR2 was detected with markedly higher spatial density than phosphorylated VASP (Fig. 7, A and C). This suggests that M2R-mediated RyR2 phosphorylation utilizes a more spatially extensive signaling network than VASP. Consistent with this notion, the spatial density of PLA signals corresponding to phosphorylated Akt was comparable with that of RyR2 and markedly higher than VASP (Fig. 7E). Taken together, these results suggest distinct sGC-dependent subcellular pathways mediating phosphorylation of RyR2 Ser-2808 and VASP Ser-239.

Discussion

We recently demonstrated that acute muscarinic stimulation results in PKG-dependent phosphorylation of the cardiac RyR2 at serine 239 to enhance utilization of the SR Ca store for EC coupling (10). The goal of the present study was to examine the specific molecular components and subcellular compartmentalization of this novel muscarinic PKG signaling mechanism. Our experiments revealed that muscarinic agonist–induced PKG phosphorylation of RyR2 Ser-2808 involves activation of the M2R–PI3K–Akt signaling pathway with subsequent stimulation of NOS1 and generation of NO. The resultant cGMP-dependent stimulation of PKG enhances phosphorylation of RyR2 Ser-2808. Of note, CCh-dependent phosphorylation of RyR2 was independent of myocyte Ca signaling, further underscoring the distinctive nature of this mechanism. Moreover, we report that this novel muscarinic PKG activation pathway is compartmentalized and operates in cardiomyocytes independently from muscarinic PKG–dependent phosphorylation of VASP at Ser-239. Stimulation of the ANP receptor (ANPR) resulted in concurrent PKG-dependent phosphorylation of both target proteins.
proteins, RyR2 Ser-2808 and VASP Ser-239. These results suggest that RyR2 is subject to phosphorylation by PKG both locally and globally through muscarinic and ANP stimulation, respectively. Of note, the impacts of both interventions on RyR2 phosphorylation are specifically mediated by NOS1 (9), suggesting conversion of the two input types on the same RyR2 modulatory mechanism. Moreover, our present study demonstrated that in addition to this NO-dependent mechanism, PKG phosphorylates RyR2 Ser-2808 in a NO-independent manner through the canonical ANPR-pGC pathway. Thus, PKG phosphorylation of RyR2 Ser-2808 via NOS1 or ANPR pathways may be linked to cardio-protective signaling activated during osmotic stress and muscarinic or ANP stimulation. These results provide new insights into the relationship between intracellular Ca handling and cardio-protective cGMP–PKG signaling in the heart.

It has been previously reported that specificity of PKG signaling in cardiomyocytes is maintained via the involvement of pGC versus sGC (14). Consistent with this notion, our study demonstrated separate patterns of RyR2 PKG phosphorylation via ANPR and M2R, globally versus locally, respectively. Moreover, our results revealed mechanistically distinct NOS–PKG pathways targeting RyR2 and VASP. Distinct PKG signaling domains were further supported by different patterns of total RyR2 and VASP subcellular distributions (i.e., striated staining at Z-lines versus more homogeneous staining, respectively) and different spatial densities of PLA puncta for phosphorylated RyR2 and VASP. These results are also in agreement with the existence of two distinct NO pools in cardiac myocytes associated with the localization of NOS1 and NOS3 to the junctional space and caveolae, respectively (25). Additionally, compartmentalization of phosphodiesterase (PDE) isozymes that are differentially modulated by PKG-mediated feedback loops (26) could contribute to the observed selectivity of the PKG–RyR2 phosphorylation pathway.

Previous studies have suggested that in settings of β1AR (β1-adrenergic receptor) stimulation, Akt modulates RyR2 phosphorylation in a NO-dependent manner (27). It has been demonstrated that cAMP-dependent activation of EPAC stimulates the PI3K–Akt–NOS1 pathway to facilitate NO-dependent activation of Ca/calmodulin-dependent protein kinase II (CaMKII) and enhance RyR2 phosphorylation at the CaMKII phosphorylation site RyR2 Ser-2814 (28). In our experiments, CCh stimulation of the Akt-NOS1 pathway was not associated with an increase in RyR2 phosphorylation at Ser-2814 (not shown) (10) but enhanced RyR2 phosphorylation at Ser-2808. These effects could be attributed to the fact that CaMKII activity is influenced by multiple factors, including Ca and reactive oxygen species, whose levels differ under conditions of muscarinic and adrenergic stimulation (29). Moreover, Bers and co-workers (30) recently demonstrated that NO can activate or inhibit CaMKII, depending on whether exposure to NO occurs before or after association of CaMKII with Ca/calmodulin. The complex interplay between NOS1 and
RyR2 phosphorylation will require further examination in the setting of both adrenergic and cholinergic stimulation. It has been suggested that NO generated by NOS1 can modulate RyR2 directly via S-nitrosylation. However, the functional consequences and dissection of this mechanism in modulation of RyR2 has proved challenging. Our demonstration of muscarinic-mediated NO generation by NOS1 raises the possibility that muscarinic regulation of RyR2 may involve modification via S-nitrosylation. This possibility and its functional implications remain to be determined (1, 31).

Muscarinic and NO signaling are known to be impaired in HF (32–35). Moreover, augmentation of both muscarinic and NO–cGMP–PKG signaling has been demonstrated to alleviate HF (11). Specifically, parasympathetic augmentation via vagal nerve stimulation and PDE5 inhibitors has been shown to improve outcomes in HF animal models and human patients (36–39). Although the underlying mechanisms of muscarinic signaling impairment are unknown, derangement of NO signaling has been attributed to loss of NOS1 from the dyadic subdomain (40–42). This is consistent with the observed functional specificity of the M2R–NOS1–PKG–RyR2 pathway. Based on our demonstrated link between muscarinic and dyadic NOS1–PKG–RyR2 signaling, it is possible that impairment of the latter also contributes to compromised muscarinic regulation in HF. Thus, restoration of NOS1–PKG–RyR2 signaling could be considered as an avenue for rational therapy to alleviate HF.

**Experimental procedures**

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 National Institute of Health (NIH Publication 85-23, revised 2011).

**Isolation of ventricular cardiomyocytes**

3–6-Month-old C57BL/6J, NOS1$$^{-/-}$$, and NOS3$$^{-/-}$$ mice (Jackson Laboratory stock nos. 000664, 002986, and 002684) of either sex were used in this study. Intact ventricular myocytes were obtained by enzymatic digestion as described previously (43). Briefly, mice were anesthetized with 5% isoflurane in 95% oxygen until a deep plane of anesthesia was achieved. Hearts were rapidly excised and cannulated through the aorta for perfusion with ice-cold calcium-free Tyrode solution containing 140 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl2, 10 mM HEPES, and 5.5 mM glucose, pH 7.4. Cannulated hearts were then switched to a gravity flow Langendorff apparatus containing calcium-free Tyrode’s solution with a temperature of 37°C. Hearts were

Figure 7. Muscarinic-induced phosphorylation targets revealed via proximity ligation assay. A, PLA demonstrates that 10 μM CCh significantly increases phospho-RyR2 2808 puncta in WT ventricular myocytes ($^*$, p < 0.05 versus CTL). Student’s t-test. B, plot of punctae/μm². Data shown are median ± S.D. C, PLA analysis reveals a significant increase in phosphorylated VASP Ser-239 puncta in myocytes treated with 10 μM CCh ($^*$, p < 0.05 versus CTL). Student’s t-test. F, plot of punctae/μm². Data shown are median ± S.D. WT mice (labeled CTL), n = 3–4 mice, 3–4 experiments/group.
PKG phosphorylation of RyR2 via PI3K/AKT/nNOS

perfused for 5 min before switching to a perfusate solution containing Liberase TH (0.24 units; Roche Applied Science) for digestion of connective tissue. Following enzymatic digestion, hearts were minced and triturated in perfusion solution containing BSA (20 mg/ml).

Western blot analyses

Isolated ventricular myocytes were digested with radio-immune precipitation assay buffer (Sigma) with protease inhibitors (Sigma) and phosphatase inhibitors (Sigma). Protein concentrations were determined by a Bradford assay. Cardiac homogenates (25-50 μg) were subjected to 4–15% SDS-PAGE (Bio-Rad) and blotted onto nitrocellulose membranes (Bio-Rad). Phosphorylation status of proteins was detected using phospho-specific and total protein antibodies, including RyR2 Ser-2808 (Badrilla, catalog no. A010-30AP), RyRTotal (Thermo, catalog no. MA3-925), p-VASP Ser-239 (Cell Signaling, catalog no. 3114), VASP total (Cell Signaling, catalog no. 3132), p-Akt Ser-473 (Cell Signaling, catalog no. 4060), total Akt (Cell Signaling, catalog no. 4691), and GAPDH (Fitzgerald, catalog no. G109a). The ratio between phosphorylated and total protein was obtained for all values. GAPDH was used as a loading control. Images were processed with ImageJ software (National Institutes of Health).

Proximity ligation assay

For cellular imaging studies, freshly isolated cardiomyocytes were plated on laminin-coated coverslips and fixed with 2% paraformaldehyde. Fixed samples were washed with PBS followed by blocking/permeabilization with 3% fetal bovine serum + 0.2% Triton X-100 in PBS. Samples were then incubated with primary antibody overnight at 4°C. Following primary antibody incubation, samples were washed with PBS (five times for 5 min each at room temperature), and the PLA reactions were carried out using appropriate Duolink secondary antibodies (Sigma). For confocal microscopy and sDCI, samples were mounted (Invitrogen Prolong Gold) and cured prior to imaging performed using an A1R-HD laser-scanning confocal microscope (Nikon). sDCI was performed using an A1R-HD confocal microscope (Nikon) using a pinhole of 0.4 Airy units with spatial oversampling (pixel size of optical resolution/4.6, z-step size of optical sectioning/4), and three-dimensional deconvolution (NIS Elements software, Nikon). Primary antibodies used are listed under “Western blotting analyses.” Analysis was performed using custom MATLAB code (44, 45).

Nitric oxide imaging

Isolated ventricular myocytes were plated on laminin-coated coverslips and loaded with 10 μM DAF-FM (4-aminomethylamino-2′,7′-dihydrofluorescein) diacetate (Thermo Fisher Scientific) for 30 min in the dark at room temperature. Myocytes were allowed 30 min for de-esterification. Following de-esterification, cells were switched to 1.8 mM calcium Tyrode solution for imaging using a Nikon A1R-HD laser-scanning confocal microscope. Data are represented as rate of fluorescence/time or slope. Spermine NONOate was used as a positive control to determine maximal NO release.

Data analysis

Statistical analyses were completed using Origin and/or Microsoft Excel. Unpaired one-tailed Student’s t test or analysis of variance with post-hoc Fisher’s test was used to test statistical significance. Paired data were analyzed with the appropriate Student’s t test, following application of the F-test to evaluate equality of variances. Outlier data points were excluded by using the Grubbs outlier test with significance level of Alpha 0.05.

Data availability

All data are contained within the article.

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