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Phosphodiesterase 5 Restricts NOS3/Soluble Guanylate Cyclase signaling to L-type Ca^{2+} current in Cardiac Myocytes

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Abstract

Endothelial nitric oxide synthase (NOS3) regulates the functional response to β -adrenergic (β -AR) stimulation via modulation of the L-type Ca^{2+} current (I_{Ca}). However, the NOS3 signaling pathway modulating I_{Ca} is unknown. This study investigated the contribution of soluble guanylate cyclase (sGC) and phosphodiesterase type 5 (PDE5), a cGMP-specific PDE, in the NOS3-mediated regulation of I_{Ca} . Myocytes were isolated from NOS3 knockout (NOS3^{-/-}) and wildtype (WT) mice. We measured I_{Ca} (whole-cell voltage-clamp), and simultaneously measured Ca^{2+} transients (Fluo-4 AM) and cell shortening (edge detection). Zaprinast (selective inhibitor of PDE5), decreased β -AR stimulated (isoproterenol, ISO)- I_{Ca} , and Ca^{2+} transient and cell shortening amplitudes in WT myocytes. However, YC-1 (NO-independent activator of sGC) only reduced ISO-stimulated I_{Ca} , but not cardiac contraction. We further investigated the NOS3/sGC/PDE5 pathway in NOS3^{-/-} myocytes. PDE5 is mislocalized in these myocytes and we observed dissimilar effects of PDE5 inhibition and sGC activation compared to WT. That is, zaprinast had no effect on ISO-stimulated I_{Ca} , or Ca^{2+} transient and cell shortening amplitudes. Conversely, YC-1 significantly decreased both ISO-stimulated I_{Ca} , and cardiac contraction. Further confirming that PDE5 localizes NOS3/cGMP signaling to I_{Ca} ; YC-1, in the presence of zaprinast, now significantly decreased ISO-stimulated Ca^{2+} transient and cell shortening amplitudes in WT myocytes. The effects of YC-1 on I_{Ca} and cardiac contraction were blocked by KT5823 (a selective inhibitor of the cGMP-dependent protein kinase, PKG). Our data suggests a novel physiological role for PDE5 in restricting the effects of NOS3/sGC/PKG signaling pathway to modulating β -AR stimulated I_{Ca} , while limiting effects on cardiac contraction.

INTRODUCTION

Nitric oxide (NO), produced by NO synthase (NOS), is an important modulator of cardiac contractility [1]. Endothelial NOS (NOS3) is constitutively expressed within cardiac myocytes and is an important regulator of β -adrenergic (β -AR) signaling. For example, *in vivo* investigations examining cardiac performance (left ventricle pressure, pressure/volume loops) demonstrated that mice with genetic deletion of NOS3 had a greater contractile response to β -AR stimulation [2–4]. Conversely, mice with cardiac myocyte specific overexpression of NOS3 exhibited a blunted functional response to β -AR stimulation [5,6]. We have previously found that myocytes isolated from NOS3 knockout (NOS3^{-/-}) mice also had an enhanced

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contractile response (i.e., augmented shortening and Ca^{2+} transient amplitudes) to β -AR stimulation [7]. We further demonstrated that this effect was due to an increase in the L-type Ca^{2+} current (I_{Ca}).

Cyclic guanosine 3', 5'-monophosphate (cGMP) is an important second messenger and is hypothesized to be the signaling molecule of NOS3 [8]. Yet this pathway has not been completely characterized in cardiac myocytes. Previous studies have shown that exogenous cGMP or cGMP analogs are able to decrease the β -AR stimulated I_{Ca} [9,10]. In mammalian cardiac myocytes, it appears that activation of the cGMP-dependent protein kinase (PKG) is the main transducer of cGMP signaling [11]. As with cGMP, exogenously applied PKG can also inhibit β -AR stimulated I_{Ca} [12,13]. However, it is unknown whether the regulation of the L-type Ca^{2+} channel by NOS3 is via the cGMP/PKG pathway in cardiac myocytes.

An important characteristic of signaling pathways, such as the β -AR pathway, is their spatial localization [14]. Phosphodiesterases, which degrade cyclic nucleotides, are key proteins for compartmentalization by restricting the spread of the second messengers [15,16]. Phosphodiesterase type 5 (PDE5) is the main isozyme for compartmentation of cGMP produced via soluble guanylate cyclase (sGC) [17]. We have previously shown that inhibition of PDE5 is able to decrease β -AR stimulated I_{Ca} via stimulation of PKG [18]. Interestingly, a study demonstrated that the anti-adrenergic effect of PDE5 inhibition was absent in $\text{NOS3}^{-/-}$ mice [19]. These data suggest a functional interaction exists between NOS3 and PDE5. In addition, PDE5 inhibitors may also be utilized as a potential therapy for cardiovascular patients, but limited data exists on the mechanism for this protective effect [20]. Thus, the purpose of our study is to further examine if PDE5 and PKG are involved in the NOS3-mediated regulation of I_{Ca} . We hypothesize that the regulation of I_{Ca} by NOS3 signaling involves a tightly regulated signaling microdomain involving sGC/PKG/PDE5.

MATERIALS AND METHODS

Isolation of ventricular myocytes

Ventricular myocytes were isolated from $\text{NOS3}^{-/-}$ and their corresponding wildtype (C57BL/6J) mice (Jackson Laboratories, Bar Harbor, Maine), as previously described [7]. Briefly, the heart was mounted on a Langendorff apparatus and perfused with Ca^{2+} free normal Tyrode solution. Blendzyme Type II (0.077 mg/ml) (Roche Applied Science, Indianapolis, IN) was then added to the perfusate. After 5–10 minutes, the heart was taken down, the ventricles minced, and myocytes dissociated by trituration. Subsequently the myocytes were filtered, centrifuged, and resuspended in normal Tyrode solution containing 200 $\mu\text{mol/L}$ Ca^{2+} . Myocytes were used within 6 hours of isolation. All the animal protocols and procedures were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Measurement of L-type Ca^{2+} current (I_{Ca})

I_{Ca} was measured using the whole cell voltage-clamp technique with an Axopatch-200B amplifier and pClamp 8.1 software (Axon Instrument, Foster City, CA.), as described previously [7,18]. Electrodes (borosilicate glass tubing), with a resistance of 1.5–3 $\text{M}\Omega$, were filled with (in mmol/L): 120 CsCl, 6 MgCl_2 , 10 EGTA, 10 HEPES, 2 MgATP , pH 7.2 adjusted with CsOH. The bath solution consisted of (in mmol/L): 120 NaCl, 4 CsCl, 1 MgCl_2 , 1 CaCl_2 , 10 glucose, 5 HEPES, 1 L-arginine, pH 7.4 adjusted with CsOH or HCl. I_{Ca} was elicited by 200 ms pulses to 0 mV from a holding potential of -80 mV (following a pre-pulse to -40 mV) at a frequency of 0.2 Hz. This procedure isolates I_{Ca} by inactivation of the Na^+ current with the pre-pulse, and replacement of K^+ with Cs^+ eliminates K^+ current. Measurements were performed at room temperature.

Simultaneous measurement of Ca^{2+} transients and cell shortening

Functional measurements were performed as previously described [21]. Briefly, myocytes were loaded at room temperature with Fluo-4 AM (10 $\mu\text{mol/L}$, Molecular Probes, Eugene, OR) for 30 min, and then 30 min were allowed for intracellular de-esterification. The instrumentation used for cell fluorescence measurements was a Cairn Research Limited (Faversham, UK) epifluorescence system. $[\text{Ca}^{2+}]_i$ was measured by Fluo-4 epifluorescence with excitation at 480 ± 20 nm and emission at 535 ± 25 nm. The illumination field was restricted to collect the emission of a single cell. Data were expressed as $\Delta F/F_0$, where F is the fluorescence intensity and F_0 is the intensity at rest. Simultaneous measurement of shortening was also performed using an edge detection system (Crescent Electronics, Sandy, UT). Data were expressed as % of resting cell length (%RCL). Measurements were performed at room temperature.

Solution and drugs

Normal Tyrode (NT) solution consisted of (in mmol/L): 140 NaCl, 4 KCl, 1 MgCl_2 , 1 CaCl_2 , 10 glucose, 5 HEPES, 1 L-arginine, pH 7.4 adjusted with NaOH or HCl. Isoproterenol (ISO, 1 $\mu\text{mol/L}$, a non-selective β -AR agonist); zaprinast (ZAP, 10 $\mu\text{mol/L}$, a selective inhibitor of PDE5) [18,22]; YC-1 (10 $\mu\text{mol/L}$, NO-independent activator of sGC) [23]; T-0156 (1 $\mu\text{mol/L}$, a selective inhibitor of PDE5 [24,25], Tocris Bioscience, Ellisville, MO, USA); L-NIO (10 $\mu\text{mol/L}$, selective NOS3 inhibitor [7,26], Calbiochem, La Jolla, CA, USA); KT 5823 (0.05 $\mu\text{mol/L}$, selective inhibitor of PKG [27] Calbiochem, La Jolla, CA, USA) were prepared fresh each experimental day. All chemicals were from Sigma except where indicated.

Statistics

Data were presented as mean $\pm$ SEM. Differences between groups were evaluated for statistical significance ($P < 0.05$) by paired or unpaired Student's t tests.

RESULTS

Inhibition of PDE5 Decreased β -AR Stimulated I_{Ca} and Cardiac Contraction in WT Myocytes

We tested the effects of zaprinast (ZAP, 10 $\mu\text{mol/L}$), a specific PDE5 inhibitor, in wildtype (WT) myocytes during β -AR stimulation. We first examined if ZAP was able to modulate I_{Ca} . Figure 1A shows representative current traces (left) and time plot (right) of the effects of β -AR stimulation (isoproterenol, a non-selective β -AR agonist, ISO, 1 $\mu\text{mol/L}$) and PDE5 inhibition (ZAP). Summary data are shown in Figure 1B. After the ISO response reached steady state, the solution was switched to ISO plus ZAP, resulting in a significant reduction in I_{Ca} (NT: 3.2 ± 0.5 -pA/pF; ISO: 4.0 ± 0.5 -pA/pF; ISO+ZAP: 3.5 ± 0.5 -pA/pF, $P < 0.05$ vs ISO). ZAP is a selective PDE5 inhibitor; however it can also inhibit other PDE isoforms. Therefore, we corroborated our results with another PDE5 inhibitor, T-0156. As shown in Figure 1C, T-0156 also significantly reduced the β -AR stimulated I_{Ca} (NT: 2.5 ± 0.3 -pA/pF; ISO: 4.3 ± 0.6 -pA/pF; ISO+T-0156: 3.2 ± 0.4 -pA/pF, $P < 0.05$ vs ISO). The inhibition of PDE5-induced decrease in current was not due to "run down" since I_{Ca} started to increase upon washout of the inhibitor (i.e., ISO alone) as shown in the time plot (Figure 1A, right). In addition, ZAP had no effect on basal I_{Ca} (9 ± 7 % Δ from control, $P = \text{NS}$, $n = 6$ myocytes/3 hearts). Thus, PDE5 inhibition is able to blunt β -AR stimulated I_{Ca} .

We then tested if ZAP was able to modulate myocyte contraction. We simultaneously measured Ca^{2+} transients and cell shortening. Figure 1D shows representative shortening (left) and Ca^{2+} transient (right) traces of the effects of β -AR stimulation (ISO) and PDE5 inhibition (ZAP). Summary data are shown in Figure 1E. Consistent with previous reports, PDE5 inhibition resulted in a significant reduction in the β -AR stimulated shortening amplitude (NT:

5.0±0.6 %RCL; ISO: 10.0±1.4 %RCL; ISO+ZAP: 8.5±1.2 %RCL, $P<0.05$ vs ISO). Not previously observed, our data shows that PDE5 inhibition also significantly blunted β -AR stimulated Ca^{2+} transient amplitude (NT: 0.8±0.1 F/F₀; ISO: 1.2±0.1 $\Delta\text{F}/\text{F}_0$; ISO+ZAP: 1.1±0.1 $\Delta\text{F}/\text{F}_0$, $P<0.05$ vs ISO). ZAP had no effect on basal shortening (16±12 % Δ from control, $P=\text{NS}$) and Ca^{2+} transient (4±4 % Δ from control, $P=\text{NS}$, $n=5$ myocytes/2 hearts) amplitudes. There was a trend that ZAP increased basal shortening, which is consistent with previous results [19]. Thus, besides blunted ISO-stimulated myocyte shortening, PDE5 inhibition also decreases Ca^{2+} transient amplitude, in part via decreased I_{Ca} in WT myocytes.

Activation of sGC Decreased β -AR Stimulated I_{Ca} but not Cardiac Contraction in WT Myocytes

We continued investigating the cGMP signaling pathway by using YC-1, (10 $\mu\text{mol/L}$), a NO independent stimulator of sGC. We first examined the effects of YC-1 on I_{Ca} . Shown in Figure 2A is a representative time plot of the effects of β -AR stimulation (ISO, 1 $\mu\text{mol/L}$) and sGC activation (YC-1). Summary data are shown in Figure 2B. After the ISO response reached steady state, the solution was switched to ISO plus YC-1, resulting in a significant reduction in I_{Ca} (NT: 1.8±0.1 -pA/pF; ISO: 4.8±0.6 -pA/pF; ISO+ZAP: 3.9±0.6 -pA/pF, $p<0.05$ vs ISO). YC-1 had no effect on basal I_{Ca} (0.4±3 % Δ from control, $P=\text{NS}$, $n=5$ myocytes/2 hearts). Thus, sGC activation is able to blunt β -AR stimulated I_{Ca} .

We then tested if YC-1 was able to modulate myocyte contraction. Summary data are shown in Figure 2C. Contrary to the effect observed with PDE5 inhibition, we observed no effect of YC-1 on β -AR stimulated myocyte contraction. That is, YC-1 had no effect on shortening (NT: 4.9±0.9 %RCL; ISO: 10.8±2.2 %RCL; ISO+ZAP: 10.9±2.2 %RCL, $P=\text{NS}$ vs ISO; left panel) or Ca^{2+} transient (NT: 0.6±0.1 $\Delta\text{F}/\text{F}_0$; ISO: 0.9±0.1 $\Delta\text{F}/\text{F}_0$; ISO+ZAP: 0.9±0.1 $\Delta\text{F}/\text{F}_0$, $P=\text{NS}$; right panel) amplitudes. YC-1 also had no effect on basal shortening (19±11 % Δ from control, $P=\text{NS}$) and Ca^{2+} transient (-7±4 % Δ from control, $P=\text{NS}$, $n=6$ myocytes/3 hearts) amplitudes. These data suggest that activation of sGC is only able to reduce β -AR stimulated I_{Ca} but not cardiac contraction in WT myocytes.

Inhibition of PDE5 had no effect on β -AR Stimulated I_{Ca} or Cardiac Contraction in NOS3^{-/-} Myocytes

Since we observed different functional effects with PDE5 inhibition and sGC activation in WT myocytes, we further investigated the cGMP signaling pathway in myocytes isolated from NOS3^{-/-} hearts. We chose this model because it is known that PDE5 localization is altered leading to a lack of an effect on myocyte contraction [19]. Consistent with our previous study [7], NOS3^{-/-} myocytes had a larger increase in I_{Ca} with β -AR stimulation compared to WT myocytes (72±16% vs 32±6% of control). We tested if ZAP (10 $\mu\text{mol/L}$) was able to modulate I_{Ca} in NOS3^{-/-} myocytes. Figure 3A (left) shows a time plot of the effects of β -AR stimulation (ISO) and PDE5 inhibition (ZAP) in a NOS3^{-/-} myocyte. Summary data are shown in Figure 3A (right). After the ISO response reached steady state, the solution was switched to ISO plus ZAP, resulting in no further change in I_{Ca} (NT: 2.3±0.5 -pA/pF; ISO: 3.9±0.4 -pA/pF; ISO+ZAP: 3.9±0.5 -pA/pF, $P=\text{NS}$ vs ISO). This is in contrast to what was observed in WT myocytes. Figure 3B shows that ZAP had a much larger effect in reducing the β -AR stimulated I_{Ca} in WT vs NOS3^{-/-} myocytes (-22±5 vs 1±7 % Δ from ISO, $P<0.05$). These data suggest that NOS3 is essential for PDE5 inhibition to be effective. We further tested this hypothesis by acutely inhibiting NOS3 with the specific inhibitor L-NIO in WT myocytes. In the presence of L-NIO, ZAP had no effect on β -AR stimulated I_{Ca} (NT: 2.1±0.3 -pA/pF; ISO: 3.5±0.4 -pA/pF; ISO+ZAP: 3.6±0.4 -pA/pF, $P=\text{NS}$ vs ISO, Fig 3B). Thus, the effect of PDE5 inhibition on I_{Ca} was also ineffective in WT myocytes with acute NOS3 inhibition (1±3 % Δ from ISO, Figure 3B). ZAP was also without effect on basal I_{Ca} (-4±6 % Δ from control, $P=\text{NS}$, $n=5$

myocytes/2 hearts). The absence of an anti-adrenergic effect of ZAP with NOS3 knockout or acute inhibition indicates that the blunted I_{Ca} caused by PDE5 inhibition is NOS3 dependent.

We then tested if ZAP was able to modulate myocyte contraction. Consistent with our previous study [7], NOS3^{-/-} myocytes had a larger increase in cardiac contraction with β -AR stimulation compared to WT myocytes (shortening: 196 $\pm$ 47% vs 108 $\pm$ 21% of control; Ca²⁺ transient: 63 $\pm$ 9% vs 51 $\pm$ 9% of control). We observed that ZAP had no effect on ISO-stimulated cell shortening (NT: 3.6 $\pm$ 0.6 %RCL; ISO: 10.8 $\pm$ 2.4 %RCL; ISO+ZAP: 9.8 $\pm$ 1.8 %RCL, P=NS vs ISO; Figure 3C, left) and Ca²⁺ transient (NT: 0.5 $\pm$ 0.05 Δ F/F₀; ISO: 0.8 $\pm$ 0.1 Δ F/F₀; ISO+ZAP: 0.8 $\pm$ 0.1 Δ F/F₀, P=NS vs ISO; Figure 3C, right) amplitudes in NOS3^{-/-} myocytes. This different effect of PDE5 inhibition between WT and NOS3^{-/-} myocytes can be more clearly seen in figure 3D. ZAP decreased WT myocyte shortening significantly more than in NOS3^{-/-} myocytes (-15 $\pm$ 3 vs 3 $\pm$ 6 % Δ from ISO, P<0.05). The same result was also observed in Ca²⁺ transient amplitude (-7 $\pm$ 2 vs -1 $\pm$ 1 % Δ from ISO, P<0.05). ZAP also had no effect on basal shortening (14 $\pm$ 7 % Δ from control, P=NS) and Ca²⁺ transient (3 $\pm$ 4 % Δ from control, P=NS, n=7 myocytes/2 hearts) amplitudes. Thus, similar to PDE5 inhibition having no effect on NOS3^{-/-} myocyte β -AR stimulated I_{Ca} , ZAP was also without effect on β -AR stimulated myocyte contraction. Thus, the anti-adrenergic effects of PDE5 inhibition are dependent on NOS3.

Activation of sGC Decreased both β -AR Stimulated I_{Ca} and Cardiac Contraction in NOS3^{-/-} Myocytes

We also tested the effects of YC-1 (10 μ mol/L) in NOS3^{-/-} myocytes. Shown in Figure 4A (left) is a time plot of the effects of β -AR stimulation (ISO) and sGC activation (YC-1) on I_{Ca} in a NOS3^{-/-} myocyte. Summary data is shown in Figure 4A (right). Activation of sGC with YC-1 dramatically decreased ISO-stimulated I_{Ca} in NOS3^{-/-} myocytes (NT: 2.1 $\pm$ 0.1 - pA/pF; ISO: 4.2 $\pm$ 0.1 - pA/pF; ISO+ZAP: 2.3 $\pm$ 2.2 - pA/pF, P<0.05 vs ISO). Additionally, Figure 4B shows that there was a much greater reduction of I_{Ca} with YC-1 in NOS3^{-/-} myocytes compared to WT myocytes (-47 $\pm$ 5 vs -18 $\pm$ 4 % Δ from ISO, P<0.05). YC-1 had no effect on basal I_{Ca} (-0.4 $\pm$ 4 % Δ from control, P=NS, n=5 myocytes/2 hearts).

Interestingly, knockout of NOS3 abolished the differential regulation of YC-1 between I_{Ca} and cardiac contraction. That is, in NOS3^{-/-} myocytes, YC-1 now significantly decreased ISO-stimulated cell shortening (NT: 3.9 $\pm$ 0.7 %RCL; ISO: 10.6 $\pm$ 1.5 %RCL; ISO+ZAP: 7.9 $\pm$ 1.4 % RCL, P<0.05 vs ISO) and Ca²⁺ transient (NT: 0.6 $\pm$.1 Δ F/F₀; ISO: 1.1 $\pm$ 0.1 Δ F/F₀; ISO+ZAP: 0.9 $\pm$ 0.1 Δ F/F₀, P<0.05 vs ISO) amplitudes, and these data are summarized in Figure 4C. The distinct effects of sGC activation on cardiac contraction between WT and NOS3^{-/-} myocytes can be seen in Figure 4D (cell shortening: WT 3 $\pm$ 4 vs NOS3^{-/-} -24 $\pm$ 6.0 % Δ from ISO, P<0.05; Ca²⁺ transient: WT -2 $\pm$ 1 vs NOS3^{-/-} -11 $\pm$ 2 % Δ from ISO, P<0.05). YC-1 also had no effect on basal shortening (66 $\pm$ 35 % Δ from control, P=NS) and Ca²⁺ transient amplitude (23 $\pm$ 14 % Δ from control, P=NS, n=8 myocytes/2 hearts). There was a trend that YC-1 increased basal contraction, consistent with previous results [28]. These data suggest that PDE5 restricts NOS3/cGMP signaling pathway to the L-type Ca²⁺ channel. When PDE5 is mislocalized, as in NOS3^{-/-} myocytes, this compartmentalized effect is lost and now cGMP is able to modulate cardiac contraction.

Inhibition of PDE5 released cGMP to Regulate Cardiac Contraction in WT myocytes

We further investigated the hypothesis that PDE5 restricts cGMP signaling to the L-type Ca²⁺ channel by repeating the YC-1 contraction experiments in WT myocytes with PDE5 inhibition. As hypothesized, in the presence of ZAP, YC-1 now significantly decreased shortening (NT: 3.9 $\pm$ 0.7 %RCL; ISO: 8.7 $\pm$ 1.1 %RCL; ISO+YC-1+ZAP: 6.2 $\pm$ 0.9 %RCL, P<0.05 vs ISO) and Ca²⁺ transient (NT: 0.9 $\pm$ 0.1 Δ F/F₀; ISO: 1.4 $\pm$ 0.1 Δ F/F₀; ISO+YC-1+ZAP:

1.2±0.1 $\Delta F/F_0$, $P<0.05$ vs ISO) amplitudes. This is in contrast to the effects of YC-1 alone in WT myocytes. As shown in Figure 5A, YC-1, in the presence of ZAP had a much greater effect in reducing ISO-stimulated cell shortening ($-31\pm3\%$ Δ from ISO, $P<0.05$ vs YC-1 alone) and Ca^{2+} transients ($-12\pm1\%$ Δ from ISO, $P<0.05$ vs YC-1 alone). Interestingly, these reductions of cardiac contraction in WT myocytes are similar to the levels observed in NOS3^{-/-} myocytes (Figure 5B). YC-1+ZAP had no effect on basal shortening ($20\pm19\%$ Δ from control, $P=NS$) and Ca^{2+} transient ($10\pm4\%$ Δ from control, $P=NS$, $n=4$ myocytes/1 heart) amplitudes. There was a trend that YC-1+ZAP increased basal myocyte contraction, consistent with our YC-1 and ZAP alone results. These data suggest that upon PDE5 inhibition (or mislocalization with NOS3 knockout) the NOS3/cGMP signaling pathway “spills over” and modulates cardiac contraction.

Since YC-1 had a greater inhibitory effect on β -AR stimulated I_{Ca} in NOS3^{-/-} vs WT myocytes, we also repeated the YC-1 experiments on I_{Ca} in the presence of ZAP. In WT myocytes, YC-1 in the presence of ZAP decreased ISO-stimulated I_{Ca} (NT: 2.6 ± 0.3 -pA/pF; ISO: 4.5 ± 0.5 -pA/pF; ISO+YC-1+ZAP: 2.9 ± 0.5 , $P<0.05$ vs ISO). As shown in Figure 5C (left panel), the co-infusion of YC-1 and ZAP resulted in a much larger reduction in ISO-stimulated I_{Ca} ($-36\pm8\%$ Δ from ISO, $P<0.05$ vs YC-1 alone). As with the effects on contraction, this reduction of I_{Ca} in WT myocytes is similar to the levels observed in NOS3^{-/-} myocytes (Figure 5C, right panel). YC-1+ZAP had no effect on basal I_{Ca} ($0.4\pm4\%$ Δ from control, $P=NS$, $n=5$ myocytes/3 hearts).

PKG is involved in the NOS3/sGC signaling pathway

We investigated if PKG was the downstream effector of the NOS3/sGC signaling pathway by investigating the effects of KT5823 (a specific PKG inhibitor, 0.05 μ mol/L) on the YC-1 induced changes in β -AR stimulated I_{Ca} and contraction. Figure 6A shows that the effect of YC-1 to reduce I_{Ca} was eliminated in the presence of KT5823 (WT: 3 ± 7 , NOS3^{-/-}: $-5\pm5\%$ Δ from ISO, both $P<0.05$ vs YC-1 alone). KT5823 had no effect on basal I_{Ca} in either WT (control: 2.1 ± 0.3 -pA/pF; KT5823: 2.1 ± 0.4 -pA/pF, $P=NS$, $n=6$ myocytes/4 hearts) or NOS3^{-/-} myocytes (control: 2.1 ± 0.2 -pA/pF; KT5823: 2.1 ± 0.4 -pA/pF, $P=NS$, $n=5$ myocytes/2 hearts). We observed the same phenomenon in NOS3^{-/-} myocyte contraction (Figure 6B). That is, in the presence of KT5823, the reduction of shortening ($-5\pm4\%$ Δ from ISO, $P<0.05$ vs YC-1 alone) and Ca^{2+} transient ($-5\pm1\%$ Δ from ISO, $P<0.05$ vs YC-1 alone) amplitudes by YC-1 was greatly blunted. KT5823 also had no effect on basal shortening (WT: $21\pm16\%$ Δ from control, $P=NS$; NOS3^{-/-}: $-3\pm9\%$ Δ from control, $P=NS$) and Ca^{2+} transient (WT: $10\pm5\%$ Δ from control, $P=NS$; NOS3^{-/-}: $0\pm3\%$ Δ from control, $P=NS$, $n=4-7$ myocytes/1-2 hearts) amplitudes. There was a trend that PKG increased basal shortening in WT myocytes. Thus, PKG is the main signaling molecule of the anti-adrenergic effects of the cGMP signaling pathway.

DISCUSSION

We have previously shown that NOS3 is an important modulator of I_{Ca} . However, the NOS3 signaling pathway has not been completely characterized. Our data demonstrates that in WT myocytes, activation of sGC via YC-1 only decreased β -AR stimulated I_{Ca} (but not cardiac contraction) and this effect was prevented by PKG inhibition with KT5823. When PDE5 was inhibited by ZAP, sGC activation with YC-1 now reduced β -AR stimulated cardiac contraction. These results reveal a novel physiological role for PDE5 in limiting the functional effects of NOS3/sGC signaling to modulate β -AR stimulated I_{Ca} . Further, sGC signaling (YC-1) was drastically altered in NOS3^{-/-} myocytes in that there was an anti-adrenergic effect on both I_{Ca} and cardiac contraction. These effects were prevented by PKG inhibition. In addition, PDE5 inhibition (ZAP) had no effect on I_{Ca} or cardiac contraction in NOS3^{-/-} myocytes. These

differing effects of YC-1 and ZAP in WT and NOS3^{-/-} myocytes may be due the mislocalization of PDE5 in NOS3^{-/-} myocytes [19]. With PDE5 inhibition (or NOS3 knockout) the spatially restricted cGMP now “spills over” and is able to modulate cardiac contraction. Thus, our data demonstrates that NOS3/sGC modulates β -AR stimulated I_{Ca} via activation of PKG, and this signaling pathway exists in a microdomain formed by PDE5.

NOS3-mediated regulation of I_{Ca}

Our data demonstrates that inhibition of the cGMP-specific PDE (PDE5) via ZAP decreases I_{Ca} in WT (Figure 1) but not in NOS3^{-/-} (Figure 3) myocytes. These data suggest that NOS3 must be present to lead to the production of cGMP which will blunt the β -AR stimulation of I_{Ca} . In addition to inhibiting PDE5, ZAP is also a PDE1 inhibitor. While PDE5 is specific for hydrolyzing cGMP, PDE1 hydrolyzes both cAMP and cGMP. Previous studies have shown that specific PDE1 inhibition in cardiac myocytes increased cAMP levels by ~20% [29]. In a previous study, we found that ZAP (at the same concentration used in the current study) increased cGMP levels, but had no effect on basal or ISO-stimulated cAMP levels in isolated myocytes [18]. In addition, a previous study has shown that specific PDE1 inhibition (MIMX) had no effect on basal I_{Ca} , but significantly increased ISO-stimulated I_{Ca} (the opposite effect of ZAP) [29]. Furthermore, we used another selective inhibitor of PDE5 (T-0156) [24,25], and observed similar results as with ZAP (Figure 1). Hence, at the concentration used, ZAP is a selective inhibitor of PDE5 in cardiac myocytes.

We further explored the cGMP pathway by using YC-1, a NO-independent stimulator of sGC. Similar to PDE5 inhibition, YC-1 decreased β -AR stimulated I_{Ca} in WT myocytes (Figure 2). Unlike PDE5 inhibition, YC-1 was able to blunt the β -AR stimulated I_{Ca} in NOS3^{-/-} myocytes (Figure 4). Thus, the cGMP pathway is still able to modulate I_{Ca} in NOS3^{-/-} myocytes. NOS3 is localized to caveolae, along with superoxide dismutase, a superoxide anion scavenger [30, 31]. Superoxide dismutase will prevent the superoxide anion from reacting with NO, making NOS3 signaling more likely to be via the cGMP-dependent pathway [8]. Thus, we may not have observed an effect of PDE5 inhibition in NOS3^{-/-} myocytes most likely due to the lack of sGC activation and resultant cGMP production.

We also tested if PKG was involved in the NOS3 signaling pathway since PKG mediates the majority of the cGMP-dependent effects [11]. We observed that PKG inhibition did indeed prevent the reduction in I_{Ca} caused by YC-1 in WT and NOS3^{-/-} myocytes (Figure 6). Previous studies have shown that PKG is able to phosphorylate the α_{1C} and β_{2a} subunits of the L-type Ca^{2+} channel [32,33]. We have also previously shown in guinea pig cardiac myocytes that PDE5 inhibition decreased I_{Ca} through a PKG-dependent mechanism [18]. However, the involvement of NOS3 was not investigated in these studies. Our data demonstrate that the NOS3-induced activation of sGC is crucial for the cGMP mediated regulation of I_{Ca} . Thus, increasing cGMP levels either by NOS3-induced sGC activation or by inhibiting hydrolysis with PDE5 inhibition (in the presence of NOS3) will decrease β -AR stimulated I_{Ca} via PKG. In addition, we do not believe that NOS3^{-/-} myocytes have reduced basal PKG phosphorylation. Many studies have shown that PKG phosphorylation leads to a reduction in I_{Ca} [12,13,27]. However, our previous [7] and current results demonstrate that there is no difference in basal I_{Ca} between WT and NOS3^{-/-} myocytes, and PKG inhibition (KT5823) had no effect on basal I_{Ca} . These data suggest no difference in basal I_{Ca} PKG phosphorylation levels.

A previous study found that there was no difference in PDE5 expression or basal levels of PKG activity in myocytes isolated from WT and NOS3^{-/-} mice [19]. However, PDE5 inhibition was not able to increase PKG activity in NOS3^{-/-}-myocytes, while it significantly increased PKG in WT myocytes [19]. The failure to increase PKG activity in NOS3^{-/-} myocytes was not due to changes in PKG expression [34]. Specifically, in the presence of acute NOS inhibition in

WT myocytes (thus PKG expression will not be altered), PDE5 inhibition was unable to increase PKG activity. This is consistent with our data. PDE5 inhibition with ZAP decreased β -AR stimulated I_{Ca} in WT myocytes through a PKG-dependent pathway (Figures 1 and 6). However, in the presence of acute, specific inhibition of NOS3 with L-NIO in WT myocytes, ZAP was now ineffective in blunting β -AR stimulated I_{Ca} (Figure 3). These data suggest that, in the presence of NOS3 inhibition, PDE5 inhibition is unable to lead to the activation of PKG, even though expression is unaltered. Further, activation of sGC with YC-1 had a much larger functional effect in NOS3^{-/-} myocytes compared to WT myocytes (Figure 4). This effect was via activation of PKG (Figure 6). These data indicate that PKG is functional in NOS3^{-/-} myocytes; however it is not able to be activated by PDE5 inhibition with ZAP. Thus, we believe that the difference in response to PDE5 inhibition in WT vs NOS3^{-/-} myocytes is not due altered PDE5 and/or PKG expression but a difference in the NOS3/sGC/PDE5/PKG signaling cascade.

These data could have important clinical implications as there is increasing evidence which suggests the potential utility of PDE5 inhibitors to treat a variety of cardiovascular diseases [20]. These beneficial effects have been observed in protecting ischemic myocardium [35, 36] and not only preventing, but also reversing cardiac hypertrophy [37]. The protective effects of PDE5 inhibition have also been shown to, in part, precondition cardiac myocytes against necrosis and apoptosis [38], reduce ventricular fibrillation [35], and blunt calcineurin, NFAT, and ERK1/2 activation [37]. As discussed, PDE5 inhibition also reduced β -AR stimulated I_{Ca} in WT myocytes (Figure 1). Our studies reveal another important end target of PDE5 inhibition, L-type Ca^{2+} channels. Modulation of I_{Ca} could be a key contributor to the protective mechanisms observed with PDE5 inhibition. Our previous data showed that NOS3 knockout or acute inhibition increased β -AR stimulated I_{Ca} resulting in higher incidences of spontaneous activity (early and delayed afterdepolarizations) in myocytes [7], supporting the antiarrhythmic effects of PDE5 inhibition. It is also known that Ca^{2+} influx via I_{Ca} activates calcineurin, leading to NFAT dephosphorylation and the activation of hypertrophic signaling. Exogenous NO-mediated inhibition of I_{Ca} can inhibit this calcineurin activation [39]. Excessive Ca^{2+} influx via I_{Ca} also leads to apoptosis [40]. Thus, increased Ca^{2+} influx via β -AR stimulated I_{Ca} can be detrimental to the myocyte. Hence, decreasing β -AR stimulated I_{Ca} by PDE5 inhibition should be beneficial for the treatment of heart disease.

PDE5 Restricts NOS3/sGC Signaling

Our data and others [19,41] demonstrate that the effects of PDE5 inhibition are dependent on NOS3 and sGC activation. That is, PDE5 inhibition decreased β -AR stimulated I_{Ca} , and Ca^{2+} transient and shortening amplitudes in WT myocytes (Figure 1), but not in NOS3^{-/-} myocytes (Figure 3). However, when we activated sGC with YC-1 in WT myocytes, we only observed a reduction in β -AR stimulated I_{Ca} , but not cardiac contraction (Figure 2). Taken together, our data indicates that PDE5 restricts NOS3/sGC/PKG signaling to modulate β -AR stimulated I_{Ca} . PDEs are key controllers in limiting the spread of cGMP, thus shaping intracellular signaling microdomains [16]. Castro *et al.* demonstrated that PDE5 controls the cGMP pool produced by sGC, whereas cGMP produced via the particulate isoform is under the exclusive control of PDE2 [17]. Thus, our data is consistent with this scheme, since we observed that PDE5 restricts NOS3/sGC/PKG signaling to modulate β -AR stimulated I_{Ca} . Further evidence supporting this signaling microdomain is that NOS3, along with L-type Ca^{2+} channels, are localized to the caveolae [31,42]. A previous publication has shown that there is a marked co-localization of sGC with NOS3 [43]. Since, NOS3 is localized to the caveolae along with the L-type Ca^{2+} channel [31,42], it appears that sGC is localized to the same region. In addition, activation of sGC, but not the particulate isoform, activates PKG [34]. PKG phosphorylation also increases cGMP binding affinity [44] and the activity of PDE5

[45]. Thus, this NOS3/sGC/PKG/PDE5 signaling regulates I_{Ca} activity in a spatially localized microdomain (Figure 7A).

However, this pathway is altered in NOS3^{-/-} myocytes. A study showed that PDE5 inhibition was without effect on cardiac contraction in NOS3^{-/-} myocytes [19]. Our data is consistent in that we also observed no effect of PDE5 inhibition on contraction in NOS3^{-/-} myocytes (Figure 3). We further showed that PDE5 inhibition also had no effect on I_{Ca} (Figure 3). These data suggest that NOS3 is essential for PDE5 to be effective. That is, NOS3-derived NO will activate sGC to produce cGMP, and this specific pool of cGMP would be hydrolyzed by PDE5. This pathway is also important for PDE5 localization. In NOS3^{-/-} myocytes, PDE5 is no longer localized to the z-bands. This is due to the knockout of NOS3 and not some compensatory adaptation since adenoviral mediated expression of NOS3 in these myocytes restores the localization of PDE5 [19]. This effect was also observed with chronic NOS inhibition [41]. With PDE5 mislocalization, NOS3^{-/-} myocytes had a markedly different response to sGC activation. Not only did YC-1 result in a greater reduction of β -AR stimulated I_{Ca} compared to WT myocytes, it also resulted in depressed β -AR stimulated cardiac contraction (Figure 4), which was prevented by PKG inhibition (Figure 6). Thus, the resultant effects of YC-1 on NOS3^{-/-} myocytes may be due to cGMP “spilling out” of the compartment to decrease myofilament Ca^{2+} sensitivity via PKG phosphorylation of troponin I [19,46]. Unlike previous studies, we also observed decreased Ca^{2+} transient amplitude, which could be due to the reduced I_{Ca} . We further tested if PDE5 is indeed responsible for restricting sGC signaling in WT myocytes. In the presence of PDE5 inhibition, superfusion with YC-1 now blunted β -AR stimulated cardiac contraction (Figure 5). In fact, the cumulative effect of YC-1 and ZAP in WT myocytes on cardiac contraction and I_{Ca} was similar to the effects of YC-1 alone in NOS3^{-/-} myocytes (Figure 5). Thus, with NOS3 knockout or PDE5 inhibition, cGMP will no longer be restricted and will “spill over” and affect cardiac contraction via troponin I and additional I_{Ca} phosphorylation (Figure 7B).

Previous studies have demonstrated that the L-type Ca^{2+} channel ($Ca_v1.2$) exists as a preassembled macromolecular complex. That is, many of the kinases and phosphatases that regulate $Ca_v1.2$ activity are found as a macromolecular signaling complex [47,48]. This has been shown for PKA (as well as related signaling components- β_2 -adrenergic receptor, $G_s\alpha$, $G_i\alpha$, adenylate cyclase, and A-kinase anchoring protein), protein phosphatase 2a, and PKC. Further, this PKA/ β_2 receptor complex has been found to exist in the caveolae of ventricular myocytes [42]. Thus, we speculate that there is also a macromolecular complex with PKG and its signaling components (sGC, PDE5 and NOS3), but this remains to be determined.

In conclusion, our results demonstrate a novel physiological role for PDE5 within cardiac myocytes. That is, PDE5 confines the NOS3/sGC/PKG signaling pathway in a microdomain that regulates the L-type Ca^{2+} channel, while limiting the effects on cardiac contraction.

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Figure 1.

PDE5 inhibition reduced the response to β -adrenergic (isoproterenol, ISO) stimulation in WT myocytes. A) Representative traces (left) and time plot (right) of I_{Ca} with ISO (1 μ mol/L) and zaprinast (ZAP, 10 μ mol/L) in a WT myocyte. B) Summary data (mean $\pm$ SEM) of the effect of ISO and ZAP on I_{Ca} . C) Summary data (mean $\pm$ SEM) of the effect of ISO and T-0156 on I_{Ca} . D) Representative traces of shortening and Ca^{2+} transient with ISO and ZAP in a WT myocyte. E) Summary data (mean $\pm$ SEM) of the effects of ISO and ZAP on shortening and Ca^{2+} transient amplitudes. * $P < 0.05$ vs ISO alone; $n = 6-16$ cells/4 hearts.

Figure 2.

Activation of sGC with YC-1 only decreased ISO-stimulated I_{Ca} , but not cardiac contraction in WT myocytes. A) Representative time plot of I_{Ca} with ISO (1 μ mol/L) and YC-1 (10 μ mol/L) in a WT myocyte. B) Summary data (mean $\pm$ SEM) of the effect of ISO and YC-1 on I_{Ca} . C) Summary data (mean $\pm$ SEM) of the effect of ISO and YC-1 on shortening and Ca^{2+} transient amplitudes. *P < 0.05 vs ISO alone; n = 7–11 cells/2–3 hearts.

Figure 3.

PDE5 inhibition with zaprinast (ZAP) had no effect in NOS3^{-/-} myocytes. A) Representative time plot (left) and summary data (mean±SEM) (right) of I_{Ca} with ISO (1 μ mol/L) and ZAP (10 μ mol/L) in NOS3^{-/-} myocytes. B) Summary data (mean±SEM) of the effect of ZAP on I_{Ca} in WT, NOS3^{-/-}, and WT with acute NOS3 inhibition (WT +L-NIO) myocytes. C) Summary data (mean±SEM) of the effect of ZAP on ISO-stimulated shortening and Ca²⁺ transient amplitudes in NOS3^{-/-} myocytes. D) Summary data (mean±SEM) of the effects of ZAP on shortening and Ca²⁺ transient amplitudes in WT and NOS3^{-/-} myocytes. *P<0.05 vs WT; n=7–13 cells/3–4 hearts.

Figure 4.

Activation of sGC with YC-1 significantly decreased ISO-stimulated I_{Ca} , shortening and Ca²⁺ transients in NOS3^{-/-} myocytes. A) Representative time plot (left) and summary data (mean±SEM) (right) of I_{Ca} with ISO (1 μ mol/L) and YC-1 (10 μ mol/L) in NOS3^{-/-} myocytes. B) Summary data (mean±SEM) of the effect of YC-1 on I_{Ca} in WT and NOS3^{-/-} myocytes. C) Summary data (mean±SEM) of the effect of YC-1 on ISO-stimulated shortening and Ca²⁺ transient amplitudes in NOS3^{-/-} myocytes. D) Summary data (mean±SEM) of the effects of YC-1 on shortening and Ca²⁺ transient amplitudes in WT and NOS3^{-/-} myocytes. *P<0.05 vs ISO alone, ** P<0.05 vs WT; n=7–14 cells/3–4 hearts.

Figure 5.

Inhibition of PDE5 released the restriction of sGC signaling on cardiac contraction. A) Summary data (mean $\pm$ SEM) of the effect of YC-1 (10 μ mol/L) in WT myocytes ($\pm$ ZAP) on shortening and Ca^{2+} transient amplitudes. B) Summary data (mean $\pm$ SEM) of the effects of YC-1 + ZAP in WT myocytes and YC-1 in NOS3^{-/-} myocytes on shortening and Ca^{2+} transient amplitudes. C) Left: Summary data (mean $\pm$ SEM) of the effect of YC-1 in WT myocytes ($\pm$ ZAP) on I_{Ca} . Right: Summary data (mean $\pm$ SEM) of the effects of YC-1 + ZAP in WT myocytes and YC-1 in NOS3^{-/-} myocytes on I_{Ca} . *P<0.05 vs -ZAP. n=5–17 cells/2–5 hearts.

Figure 6.

PKG is the primary protein target for the NOS3/sGC signaling pathway. A) Summary data (mean $\pm$ SEM) of the effect of KT5823 (0.05 μ mol/L) on the YC-1 response on I_{Ca} in WT and NOS3^{-/-} myocytes B) Summary data (mean $\pm$ SEM) of the effects of KT5823 on the YC-1 response on shortening and Ca²⁺ transient amplitudes in NOS3^{-/-} myocytes. *p<0.05 vs -KT5823. n= 7–16 cells/2–4 hearts.

Figure 7. NOS3/sGC signaling is compartmentalized via PDE5. A) In WT myocytes, the effect of NOS3/sGC signaling is localized to modulate I_{Ca} . B) With NOS3 knockout or PDE5 inhibition, compartmentation of NOS3/sGC signaling is lost allowing for modulation of cardiac contraction. ATP= SR Ca^{2+} ATPase, PLB= phospholamban, RyR= ryanodine receptor. See text for other abbreviations.