Published in final edited form as:
Nitric Oxide. 2012 December 1; 27(4): 242–247. doi:10.1016/j.niox.2012.08.077.

Effects of increased systolic Ca^{2+} and β-adrenergic stimulation on Ca^{2+} transient decline in NOS1 knockout cardiac myocytes

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Abstract

We have previously shown that the main factor responsible for the faster $[Ca^{2+}]_i$ decline rate with β-adrenergic (β-AR) stimulation is the phosphorylation of phospholamban (PLB) rather than the increase in systolic Ca^{2+} levels. The purpose of this study was to correlate the extent of augmentation of PLB Serine^{16} phosphorylation to the rate of $[Ca^{2+}]_i$ decline. Thus, ventricular myocytes were isolated from neuronal nitric oxide synthase knockout ($NOS1^{-/-}$) mice, which we observed had lower basal PLB Serine^{16} phosphorylation levels, but equal levels during β-AR stimulation. Ca^{2+} transients (Fluo-4) were measured in myocytes superfused with 3mM extracellular Ca^{2+} ($[Ca^{2+}]_o$) and a non-specific β-AR agonist isoproterenol (ISO, 1μM) with 1mM $[Ca^{2+}]_o$. This allowed us to get matched Ca^{2+} transient amplitudes in the same myocyte. Similar to our previous work, Ca^{2+} transient decline was significantly faster with ISO compared to 3mM $[Ca^{2+}]_o$, even with matched Ca^{2+} transient amplitudes. Interestingly, when we compared the effects of ISO on Ca^{2+} transient decline between $NOS1^{-/-}$ and WT myocytes, ISO had a larger effect in $NOS1^{-/-}$ myocytes, which resulted in a greater percent decrease in the Ca^{2+} transient RT_{50}. We believe this is due to a greater augmentation of PLB Serine^{16} phosphorylation in these myocytes. Thus, our results suggest that not only the amount but the extent of augmentation of PLB Serine^{16} phosphorylation are the major determinants for the Ca^{2+} decline rate. Furthermore, our data suggest that the molecular mechanisms of Ca^{2+} transient decline is normal in $NOS1^{-/-}$ myocytes and that the slow basal Ca^{2+} transient decline is predominantly due to decreased PLB phosphorylation.

Keywords

Phospholamban; NOS1; calcium; β-adrenergic; myocyte

INTRODUCTION

An enhanced lusitropic response is a hallmark of β-adrenergic (β-AR) stimulation [1]. This is, in part, due to the faster $[Ca^{2+}]_i$ uptake into the sarcoplasmic reticulum (SR) by the SR Ca^{2+} ATPase (SERCA). SERCA's function is inhibited by its regulatory protein, phospholamban (PLB) [2]. The PLB-mediated inhibition of SERCA is relieved by its dissociation, which is

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caused by an increase in systolic Ca^{2+} levels and/or phosphorylation of PLB [3]. During β-AR stimulation, not only is PLB phosphorylated at Serine[16] by the cAMP-dependent protein kinase (PKA) but there are higher systolic Ca^{2+} levels as well. In our previous study [4], we set out to determine the major factor in the faster $[Ca^{2+}]_i$ decline during β-AR stimulation (i.e., the high systolic levels of $[Ca^{2+}]_i$ or PLB phosphorylation). We demonstrated that the faster $[Ca^{2+}]_i$ decline rate with β-AR stimulation was due to the phosphorylation of PLB, specifically at Serine[16], instead of the increase in systolic Ca^{2+} levels.

Cardiac relaxation is altered in many cardiomyopathies such as heart failure [5–7]. The slowed relaxation can be attributed to changes in myofilament kinetics and/or slowed $[Ca^{2+}]_i$ decline. Fully understanding each aspect of muscle relaxation will be critical for successful treatment of these diseases. Thus, the purpose of this study was to correlate the extent of augmentation of PLB Serine[16] phosphorylation to the rate of $[Ca^{2+}]_i$ decline. Previous studies have shown that neuronal nitric oxide synthase (NOS1) signaling is able to modulate basal PLB phosphorylation [8, 9]. Namely, basal PLB phosphorylation at Serine[16] is decreased in NOS1 knockout ($NOS1^{-/-}$) compared to wildtype (WT) myocytes [8, 9]. However, during β-AR stimulation, PLB Serine[16] phosphorylation levels are similar in $NOS1^{-/-}$ and WT myocytes [8, 9]. Since $NOS1^{-/-}$ myocytes have lower basal PLB Serine[16] phosphorylation but equal phosphorylation levels with β-AR stimulation, the extent of the increase of PLB Serine[16] phosphorylation is greater in $NOS1^{-/-}$ compared to WT myocytes. We hypothesize that not only the amount but the extent of augmentation of PLB Serine[16] phosphorylation are the key determinants of the Ca^{2+} transient decline rate. We expect a greater effect of ISO that should result in a more prominent effect on Ca^{2+} transient decline in $NOS1^{-/-}$ vs WT myocytes.

METHODS AND MATERIALS

Cardiomyocyte isolation

Ventricular myocytes were isolated from $NOS1^{-/-}$ and C57BL/6 (WT) (Jackson Laboratories, Bar Harbor, Maine) as previously described [8]. Briefly, the heart was cannulated and hung on a Langendorff apparatus. It was then perfused with Ca^{2+} free tyrode solution for 4 min. The solution was then switched to tyrode solution containing Liberase Blendzyme II (0.077 mg/ml) (Roche Applied Science, Indianapolis, IN). After 3–5 min, the heart was taken down, the ventricles minced, and myocytes were dissociated by trituration. Subsequently the myocytes were filtered through mesh, centrifuged, and resuspended in Tyrode solution containing 200 μmol/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 Myocyte Ca^{2+} transients

Ca^{2+} transient measurements were performed as previously described [10]. Briefly, myocytes were loaded at room temperature with Fluo-4 AM (10 μmol/L, Molecular Probes, Eugene, OR) for 30 min, and then another 30 min were allowed for intracellular de-esterification. The solution for de-esterification was Tyrode solution containing 200 μmol/L Ca^{2+}. The instrumentation used for cell fluorescence measurements was a Cairn Research Limited (Faversham, UK) epifluorescence system. $[Ca^{2+}]_i$ 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. Myocytes were stimulated at 1 Hz via platinum electrodes connected to a Grass Telefactor S48 stimulator (West Warwick, RI).

Western blot for PLB Serine^{16} phosphorylation

Whole hearts were perfused with the various experimental solutions (3mM $[Ca^{2+}]_o$ or ISO) for 3 min using a Langendorff apparatus, homogenized, and analyzed via western blot as previously described [11]. Membranes were probed using a custom antibody to PLB (Zymed, San Francisco, CA), Serine16 phosphospecific antibody (Badrilla, Leeds, UK) and normalized to calsequestrin (ABR, Golden, CO). Data expressed as Serine^{16} phosphorylation normalized to total PLB.

Solutions 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, pH 7.4 adjusted with NaOH or HCl. Isoproterenol (ISO, 1 μmol/L, a non-selective β-AR agonist) was prepared fresh each day. All chemicals were from Sigma (St. Louis, MO).

Experimental protocol

Our experimental protocol consisted of the myocyte first being superfused with control solution (NT with 1 mM $[Ca^{2+}]$) until steady-state was reached, the myocyte was then superfused with 3 mM $[Ca^{2+}]$ NT solution, which resulted in increased systolic Ca^{2+} levels. After reaching steady-state, the solution was then switched back to control solution (1mM $[Ca^{2+}]$ NT) resulting in the washout of 3 mM $[Ca^{2+}]_o$ and the Ca^{2+} transient amplitude returning back to basal levels (~ 3 minutes). The myocyte was then superfused with 1μM ISO (with 1mM $[Ca^{2+}]_o$) which increased myocyte systolic Ca^{2+} levels. Experiments were performed at room temperature.

Statistics

Data were presented as mean±SEM. Differences between groups were evaluated for statistical significance ($P < 0.05$) by ANOVA for multiple groups or paired/unpaired Student's *t* tests for two groups.

RESULTS

Ca^{2+} transient kinetics experimental protocol

Shown in Figure 1A is a representative experiment showing Ca^{2+} transients over time recorded in a NOS1$^{-/-}$ myocyte. The maximal Ca^{2+} transient amplitude for each treatment for NOS1$^{-/-}$ myocytes and adapted data for WT myocytes [4] is shown in Figure 1B. 3 mM $[Ca^{2+}]_o$ and ISO caused a significant increase in maximal Ca^{2+} transient amplitude compared to 1 mM $[Ca^{2+}]_o$ in WT and NOS1$^{-/-}$ myocytes. However, NOS1$^{-/-}$ myocytes had significantly lower maximal Ca^{2+} transient amplitudes at 1 mM $[Ca^{2+}]_o$, 3 mM $[Ca^{2+}]_o$, and ISO compared to WT myocytes. Shown in Figure 1C is the Ca^{2+} transient decline rate measured as time to 50% relaxation (RT_{50}) with 1 mM $[Ca^{2+}]_o$, 3 mM $[Ca^{2+}]_o$, and ISO. 3 mM $[Ca^{2+}]_o$ and ISO resulted in a faster RT_{50} compared to 1 mM $[Ca^{2+}]_o$ in WT and NOS1$^{-/-}$ myocytes. However, NOS1$^{-/-}$ myocytes had significantly slower RT_{50} at 1 mM and 3 mM $[Ca^{2+}]_o$. Interestingly, RT_{50} with ISO is similar between WT and NOS1$^{-/-}$ myocytes. Thus, Ca^{2+} transient decline was slower in NOS1$^{-/-}$ myocytes, but normalized with β-AR stimulation.

Since we observed differences in Ca^{2+} transient decline in NOS1$^{-/-}$ myocytes with 3mM $[Ca^{2+}]_o$ and ISO treatments, we further analyzed Ca^{2+} transient decline in NOS1$^{-/-}$ myocytes. We first examined PLB phosphorylation. We measured PLB Serine16 phosphorylation under similar experimental conditions (i.e., perfusion with 3 mM $[Ca^{2+}]_o$ or ISO for 3 min, which is a similar time point in which we matched the Ca^{2+} transient

amplitudes). Shown in Figure 2A, WT and NOS1$^{-/-}$ myocytes perfused with ISO had significantly increased Serine16 phosphorylation compared with myocytes perfused with 3 mM $[Ca^{2+}]_o$. However, NOS1$^{-/-}$ myocytes perfused with 3 mM $[Ca^{2+}]_o$, had decreased Serine16 phosphorylation levels but similar phosphorylation levels with ISO when compared to WT myocytes. Further analysis reveals that ISO produces a much larger increase in PLB Serine^{16} phosphorylation in NOS1$^{-/-}$ myocytes compared to WT myocytes (Figure 2B). Thus, the extent of the increase of PLB Serine^{16} phosphorylation with ISO was greater in NOS1$^{-/-}$ vs WT myocytes.

Although the maximum Ca^{2+} transient amplitudes with 3mM $[Ca^{2+}]_o$ and ISO were not statistically different, the faster Ca^{2+} transient decline rates with ISO may have resulted from the slightly higher peak systolic Ca^{2+} levels. Therefore, we further investigated the Ca^{2+} transient decline by grouping the NOS1$^{-/-}$ myocytes that had a higher maximal response to ISO together (n=18) and the myocytes that had a higher maximal response to 3mM $[Ca^{2+}]_o$ together (n=5). When grouping the data, the myocytes that had a higher maximum peak systolic Ca^{2+} with ISO also had a faster RT_{50} (Figure 2C). In this case, it is unknown if the faster Ca^{2+} transient decline with ISO is due to higher systolic Ca^{2+} levels or PLB Serine^{16} phosphorylation. However, when comparing the group that had a higher maximum peak systolic Ca^{2+} with 3mM $[Ca^{2+}]_o$ (Figure 2D), ISO still resulted in a faster Ca^{2+} transient decline RT_{50}. We observed the same phenomenon in WT myocytes [4]. We further analyzed this relationship between maximum peak systolic Ca^{2+} levels and RT_{50}. We plotted the maximum peak systolic Ca^{2+} levels with 1mM $[Ca^{2+}]_o$, 3mM $[Ca^{2+}]_o$, and ISO against their respective RT_{50} (Figure 2E). The higher peak systolic Ca^{2+} in the 1mM and 3mM $[Ca^{2+}]_o$ group correlated with a faster RT_{50} (slope of −41.3±4.3). However, in the ISO group, there was no correlation between peak systolic Ca^{2+} and RT_{50} (slope of −1.5±4.3). We observed the same phenomenon in WT myocytes [4]. These data suggest that with ISO at 1 mM $[Ca^{2+}]_o$, unlike in the absence of ISO, the rate of Ca^{2+} transient decline is not dependent on peak Ca^{2+} transient amplitude. These data suggest that during β-AR stimulation PLB Serine^{16} phosphorylation is the major factor responsible for the faster Ca^{2+} transient decline rate. Furthermore, these data suggest that the molecular mechanisms for Ca^{2+} transient decline are normal in NOS1$^{-/-}$ myocytes.

Ca^{2+} Transient kinetics with matched amplitudes

For accurate comparisons of Ca^{2+} transient decline rates, it has been shown that one must match the Ca^{2+} transient amplitudes [12]. By using our experimental protocol, we were able to match Ca^{2+} transient amplitude levels between the 3mM $[Ca^{2+}]_o$ and ISO groups and reduce the variability of Ca^{2+} handling between cells exposed to 3mM $[Ca^{2+}]_o$ and ISO by superfusing each myocyte with both solutions. Representative matched individual Ca^{2+} transient traces are shown in Figure 3A and the matched peak values of 3mM $[Ca^{2+}]_o$ and ISO are shown in Figure 3B.

Using the matched Ca^{2+} transient amplitude data, we examined the effects of 3mM $[Ca^{2+}]_o$ and ISO on Ca^{2+} transient decline by analyzing the time it takes the Ca^{2+} transient to decline by 25% (RT_{25}), 50% (RT_{50}), 75% (RT_{75}) and 90% (RT_{90}) from its peak amplitude. The Ca^{2+} decline with ISO at each time point was significantly faster compared to 3mM $[Ca^{2+}]_o$ (Figure 3C). Our previous study [4] showed that the faster effect of ISO occurred only in the first 50% of the decline in WT myocytes. This was determined by dividing the declining Ca^{2+} transient into intervals: RT_{50-25}, RT_{75-50}, and RT_{90-75}. For example, we subtracted the RT_{25} from the RT_{50} to get the RT_{50-25} interval, reflecting the time of the Ca^{2+} transient to decline from 25% from the peak amplitude to the 50% point. Data are shown in Figure 3D. The RT_{50-25} and RT_{75-50} intervals were significantly different between 3mM $[Ca^{2+}]_o$ and ISO. Thus, these data suggest that faster Ca^{2+} transient decline with ISO compared to 3mM $[Ca^{2+}]_o$ occurs in the first 75% in NOS1$^{-/-}$ myocytes.

Different effect of ISO on Ca^{2+} transient decline in $NOS1^{-/-}$ vs WT myocytes

The interval data observed in the $NOS1^{-/-}$ myocytes are somewhat different than what was observed in WT myocytes (i.e., first 75% vs 50% of the decline) [4]. Hence, we wanted to further investigate if there were any more differences in the effects of ISO on Ca^{2+} transient decline between WT and $NOS1^{-/-}$ myocytes. This was done by examining the effects of ISO on the decline rate (i.e., RT_{25}, RT_{50}, RT_{75}, and RT_{90}) of matched Ca^{2+} transients as a percent change of the decline with 3mM $[Ca^{2+}]_o$. For example, at the RT_{25}, ISO in WT myocytes resulted in 18±1% faster decline compared to 3mM $[Ca^{2+}]_o$, but in $NOS1^{-/-}$ myocytes, ISO had a 26±3% faster decline ($P<0.05$ vs WT). ISO also had a faster decline at the RT_{50} in $NOS1^{-/-}$ myocytes, but not at the RT_{75} or the RT_{90} (Figure 4A). We performed the same analysis investigating the effects of ISO in WT and $NOS1^{-/-}$ myocytes on our relaxation time intervals. Shown in Figure 4B, ISO had a significantly greater effect at the $RT_{50\text{-}25}$ interval in $NOS1^{-/-}$ vs WT myocytes. This pronounced effect of ISO on Ca^{2+} transient decline also resulted in a greater effect in reducing the Ca^{2+} transient RT_{50} when inspecting the maximal responses to ISO (Figure 4C). That is, ISO resulted in a 48±2% faster Ca^{2+} transient RT_{50} (compared to the RT_{50} with 1mM $[Ca^{2+}]_o$) in $NOS1^{-/-}$ myocytes, but only a 39±2% in WT myocytes ($P<0.05$ vs $NOS1^{-/-}$). There was no difference in the effects of 3mM $[Ca^{2+}]_o$ on Ca^{2+} decline between $NOS1^{-/-}$ and WT myocytes. Thus, these data suggest that ISO has a more prominent effect to augment Ca^{2+} transient decline in $NOS1^{-/-}$ myocytes.

DISCUSSION

The lowering of $[Ca^{2+}]_i$ is the initiating event that permits relaxation. The majority of the decline of $[Ca^{2+}]_i$ is due to Ca^{2+} resequestation into the SR via the SERCA/PLB complex and extrusion from the cell by the Na^+/Ca^{2+} exchanger (NCX). In murine myocytes, the bulk (>95%) of the $[Ca^{2+}]_i$ is resequestered back into the SR via SERCA [13], which is reversibly inhibited by PLB [3]. Ca^{2+} binding to SERCA results in the dissociation of PLB from SERCA to relieve this inhibition. PLB is also a key phosphoprotein in the heart, which can be phosphorylated on Serine[16] through PKA or on Threonine[17] through Ca^{2+}/calmodulin-dependent protein kinase. Phosphorylation at either site also results in the dissociation of PLB from SERCA. Thus, increasing $[Ca^{2+}]_i$ or PLB phosphorylation results in greater Ca^{2+} resequestation into the SR and faster Ca^{2+} decline.

Ca^{2+} decline during β-AR stimulation

Stimulation of the β-AR receptor results in increased myocyte contraction and faster relaxation [1]. In terms of Ca^{2+} handling, this will lead to an increase in peak systolic Ca^{2+} levels and faster decline. The higher systolic Ca^{2+} levels during β-AR stimulation should result in PLB dissociation and greater SR Ca^{2+} uptake. Furthermore, β-AR stimulation results in the phosphorylation of PLB at Serine[1614], which will also dissociate PLB from SERCA. We have previously shown that in WT myocytes the major factor for the faster Ca^{2+} decline during β-AR stimulation is PLB Serine[16] phosphorylation.

In addition to the greater Ca^{2+} uptake into the SR with β-AR stimulation, greater Ca^{2+} extrusion from the cell could also enhance the Ca^{2+} transient decline rate [15]. Thus, increased NCX, which removes Ca^{2+} from the cytosol throughout the action potential (besides phase 0), could result in faster Ca^{2+} transient decline. However, we believe that NCX does not play a role in the effects of β-AR stimulation on Ca^{2+} transient decline. Studies have found that β-AR stimulation does not affect NCX function [16]. In addition, NCX plays a minor role in the Ca^{2+} decline rates in mouse myocytes (<5%), so an effect, if any, would be very minor at best. Further, using the NCX knockout mouse, there was no difference in Ca^{2+} transient decline rates with β-AR stimulation in WT compared to knockout myocytes [17].

Besides greater Ca^{2+} uptake or extrusion, β-AR stimulation also decreases myofilament Ca^{2+} sensitivity via TnI phosphorylation. It is widely accepted that TnI phosphorylation during β-AR stimulation accelerates relaxation [18, 19]. A previous study [20] has shown that in unloaded myocyte experiments, there is little effect of TnI phosphorylation on myocyte relengthening. However, under loaded conditions (e.g. trabeculae), TnI phosphorylation does play a significant role in relaxation. Thus, under our experimental conditions, (i.e. unloaded myocyte) we believe that TnI does not play a role. Hence, with our previous work [4] showing that the major mechanism for the faster Ca^{2+} transient decline is PLB $Serine^{16}$ phosphorylation, we wanted to extend this observation and correlate the extent of augmentation of PLB $Serine^{16}$ phosphorylation to the rate of $[Ca^{2+}]_i$ decline.

NOS1 signaling in myocytes

Nitric oxide produced via NOS1 is an important cardiac signaling molecule [21]. Many studies have shown that NOS1 is a key modulator of cardiac myocyte function. By using cardiac myocyte NOS1 overexpressing mice [22], $NOS1^{-/-}$ mice [8, 23–26], and specific NOS1 inhibitors [8, 26], studies found that NOS1 signaling results in enhanced inotropy, lusitropy and augments the functional response to β-AR stimulation. In terms of Ca^{2+} handling, NOS1 signaling will accelerate Ca^{2+} transient decline under basal conditions and increase basal and β-AR stimulated systolic Ca^{2+} levels. Hence, $NOS1^{-/-}$ myocytes have slowed basal Ca^{2+} transient decline and depressed basal systolic Ca^{2+} levels. A molecular component of NOS1 regulation of myocyte contraction occurs via modulation of basal PLB $Serine^{16}$ phosphorylation levels. We[8] and others[9, 27] have shown that WT myocytes with acute NOS1 inhibition or $NOS1^{-/-}$ myocytes have reduced basal levels of PLB $Serine^{16}$ phosphorylation, while myocyte-specific NOS1 overexpression results in increased basal PLB $Serine^{16}$ phosphorylation. Interestingly, studies [8, 9] have shown that $NOS1^{-/-}$ myocytes have similar PLB $Serine^{16}$ phosphorylation levels and Ca^{2+} transient decline rates during β-AR stimulation. Since $NOS1^{-/-}$ myocytes have a lower basal PLB $Serine^{16}$ phosphorylation but equal phosphorylation levels with ISO, the extent of the increase of PLB $Serine^{16}$ phosphorylation is greater in $NOS1^{-/-}$ compared to WT myocytes. Our current results examining PLB $Serine^{16}$ phosphorylation (Fig 2A and 2B) are consistent with these previous studies, in which we show that ISO resulted in a 692±107% increase in PLB $Serine^{16}$ phosphorylation in $NOS1^{-/-}$ myocytes but only a 306±51% increase in WT myocytes. Thus, $NOS1^{-/-}$ myocytes represent a straightforward model system to investigate the role of PLB $Serine^{16}$ phosphorylation augmentation in modulating the Ca^{2+} transient decline.

Ca^{2+} transient decline in $NOS1^{-/-}$ and WT myocytes with high extracellular Ca^{2+} vs ISO

We investigated Ca^{2+} transient decline kinetics with ISO and 3mM $[Ca^{2+}]_o$ in $NOS1^{-/-}$ myocytes and compared them to WT data [4]. As shown in Figure 1C, WT had significantly faster Ca^{2+} transient RT_{50} during 1mM $[Ca^{2+}]_o$ and 3mM $[Ca^{2+}]_o$ compared to $NOS1^{-/-}$ myocytes. The slowed Ca^{2+} decline during 1mM $[Ca^{2+}]_o$ and 3mM $[Ca^{2+}]_o$ is consistent with lower basal PLB $Serine^{16}$ phosphorylation levels and agrees with previous work [8, 9]. Moreover, the Ca^{2+} transient RT_{50} with ISO is similar between WT and $NOS1^{-/-}$ myocytes, which suggest that β-AR stimulation normalizes the Ca^{2+} transient RT_{50} in $NOS1^{-/-}$ myocytes via similar PLB $Serine^{16}$ phosphorylation levels between WT and $NOS1^{-/-}$ myocytes and agrees with previous work [4]. Nevertheless, the faster RT50 with ISO (compared to 3 mM $[Ca^{2+}]_o$) in the $NOS1^{-/-}$ myocytes may be due to the higher (although not significant) systolic Ca^{2+} levels. However, if we group the $NOS1^{-/-}$ myocytes that had a larger Ca^{2+} transient amplitude with 3mM $[Ca^{2+}]_o$ (Figure 2D), the Ca^{2+} transient decline with ISO was still faster than 3mM $[Ca^{2+}]_o$. Thus, this highlights the importance of PLB $Serine^{16}$ phosphorylation in modulating the rate of Ca^{2+} decline.

In addition to PLB Serine^{16} phosphorylation, an increase in diastolic SR Ca^{2+} leak via increased ryanodine receptor (RyR2) activity could contribute to slowed basal Ca^{2+} transient decline. Indeed, Gonzalez et al [28] previously demonstrated that NOS1$^{-/-}$ myocytes have increased basal diastolic SR Ca^{2+} leak via increased RyR2 activity and slowed basal $[Ca^{2+}]_i$ decline. Thus, there is a balance shift towards increased SR Ca^{2+} release that slows the Ca^{2+} decline. However, we observed decreased RyR2 activity (and diastolic SR Ca^{2+} leak) [29] and decreased basal PLB Serine^{16} phosphorylation in NOS1$^{-/-}$ myocytes (8 and Figure 2A). Thus, our data suggests, that the balance is shifted towards a decrease in SR Ca^{2+} uptake that is responsible for the slowed basal $[Ca^{2+}]_i$ decline. During β-AR stimulation, RyR2 activity is increased [30, 31]. However, there is no difference in RT_{50} between WT and NOS1$^{-/-}$ myocytes (in which PLB Serine^{16} phosphorylation is the same). Furthermore, our previous work [4] showed that increased RyR activity with β-AR stimulation contributed to a faster rate of $[Ca^{2+}]_i$ rise, but had no effect on $[Ca^{2+}]_i$ decline. Therefore, during β-AR stimulation, the balance is shifted to greater SR Ca^{2+} uptake via PLB Serine^{16} phosphorylation that accelerates Ca^{2+} decline. Thus, if RyR2 activity was the major reason for the slowed Ca^{2+} transient decline, then we should still have observed slowed Ca^{2+} transient decline with ISO in the NOS1$^{-/-}$ myocytes vs WT.

We also examined the relationship between maximum systolic Ca^{2+} levels and the Ca^{2+} transient RT_{50} (Figure 2E). These data show that in the absence of ISO as systolic Ca^{2+} levels increased there was a direct relationship for a faster Ca^{2+} transient decline. However, this is not the case during β-AR stimulation. That is, the ISO group (at 1 mM $[Ca^{2+}]_o$) was not dependent upon systolic Ca^{2+} levels. We did not measure this relationship with ISO at 3 mM $[Ca^{2+}]_o$ and cannot establish that the same phenomenon will occur. The ISO at 1 mM $[Ca^{2+}]_o$ data are similar to what we observed in WT myocytes [4]. Thus, we suggest that the molecular mechanisms for the Ca^{2+} transient decline are not different between NOS1$^{-/-}$ and WT myocytes but just slower under basal conditions due to decreased PLB Serine^{16} phosphorylation.

As suggested in previous studies [4, 12], to properly analyze Ca^{2+} transient decline between groups, one should use matched Ca^{2+} transient amplitudes. Thus, we investigated the RT_{25}, RT_{50}, RT_{75}, RT_{90} and the time intervals ($RT_{50\text{-}25}$ and $RT_{75\text{-}50}$) of the Ca^{2+} transient decline with matched Ca^{2+} transient amplitudes (Figure 3). ISO resulted in a faster decline at all time points and a faster decline at the $RT_{50\text{-}25}$ and $RT_{75\text{-}50}$ intervals. This suggests that ISO has its greatest effect in increasing Ca^{2+} decline during the initial 75%. The effects of PLB Serine^{16} phosphorylation are observed in the initial 75% of the decline in NOS1$^{-/-}$ myocytes because, we believe that the increase in systolic Ca^{2+} with 3 mM $[Ca^{2+}]_o$ (or ISO) will not dissociate all the PLB from SERCA. However, phosphorylation of PLB Serine16 results in the dissociation of more PLB from SERCA (compared to 3 mM $[Ca^{2+}]_o$) resulting in a faster decline during the initial 50% in WT and 75% in NOS1$^{-/-}$ Ca^{2+} transient decline.

Ca^{2+} transient decline in NOS1$^{-/-}$ vs WT myocytes

Our data suggest that ISO has a more pronounced effect on Ca^{2+} transient decline in NOS1$^{-/-}$ myocytes compared to WT myocytes. This effect of ISO also resulted in a greater decline in RT_{50} in NOS1$^{-/-}$ vs WT myocytes (Figure 4C). We believe this greater effect of ISO in NOS1$^{-/-}$ myocytes is due to the lower basal PLB Serine^{16} phosphorylation. Thus, there is a greater quantitative increase in PLB Serine^{16} phosphorylation with ISO that results in a greater effect on Ca^{2+} transient decline. Furthermore, although not significant, there was a trend that 3mM $[Ca^{2+}]_o$ had a greater effect on RT_{50} in NOS1$^{-/-}$ myocytes (Figure 4C). NOS1$^{-/-}$ myocytes also had a steeper slope in the peak systolic Ca^{2+} levels with 1mM and 3mM $[Ca^{2+}]_o$ and their respective RT_{50} (Figure 2) compared to WT myocytes [4]. We believe that this may be due to the increased SERCA/PLB ratio in the NOS1$^{-/-}$ myocytes [24, 32]. These data highlight the major role of PLB phosphorylation in modulating Ca^{2+} transient

decline. Such that there is a compensatory change in SERCA/PLB ratio in NOS1$^{-/-}$ myocytes, but basal Ca^{2+} transient decline is still slower (compared to WT myocytes), which we believe is due to decreased basal PLB Serine^{16} phosphorylation.

In conclusion, our data suggest that the molecular mechanisms for the decline of $[Ca^{2+}]_i$ is similar between NOS1$^{-/-}$ and WT myocytes and emphasizes that not only the amount but the extent of augmentation of PLB Serine^{16} phosphorylation are key determinants for the Ca^{2+} transient decline rate.

Acknowledgments

GRANTS

This research was supported by the American Heart Association (Established Investigator Award 0740040N, PML Janssen) and the National Institutes of Health, R01JL091986, JP Davis; K02HL094692, R01HL079283, MT Ziolo).

References

1. Bers DM, Ziolo MT. When is cAMP not cAMP? Effects of compartmentalization. Circ Res. 2001; 89(5):373–375. [PubMed: 11532895]
2. Bers DM. Cardiac excitation-contraction coupling. Nature. 2002; 415(6868):198–205. [PubMed: 11805843]
3. MacLennan DH, Kranias EG. Phospholamban: a crucial regulator of cardiac contractility. Nat Rev Mol Cell Biol. 2003; 4(7):566–577. [PubMed: 12838339]
4. Roof SR, Shannon TR, Janssen PM, Ziolo MT. Effects of increased systolic Ca(2) and phospholamban phosphorylation during beta-adrenergic stimulation on Ca(2) transient kinetics in cardiac myocytes. Am J Physiol Heart Circ Physiol. 2011; 301(4):H1570–1578. [PubMed: 21765055]
5. Varian KD, Kijtawornrat A, Gupta SC, Torres CA, Monasky MM, Hiranandani N, Delfin DA, Rafael-Fortney JA, Periasamy M, Hamlin RL, Janssen PM. Impairment of diastolic function by lack of frequency-dependent myofilament desensitization rabbit right ventricular hypertrophy. Circ Heart Fail. Sep; 2009 2(5):472–481. [PubMed: 19808378]
6. Janssen PM, Periasamy M. Determinants of frequency-dependent contraction and relaxation of mammalian myocardium. J Mol Cell Cardiol. 2007; 43(5):523–531. [PubMed: 17919652]
7. Davis JP, Tikunova SB. Ca(2+) exchange with troponin C and cardiac muscle dynamics. Cardiovasc Res. Mar 1; 2008 77(4):619–626. [PubMed: 18079104]
8. Wang H, Kohr MJ, Traynham CJ, Wheeler DG, Janssen PM, Ziolo MT. Neuronal nitric oxide synthase signaling within cardiac myocytes targets phospholamban. Am J Physiol Cell Physiol. 2008; 294(6):C1566–1575. [PubMed: 18400986]
9. Zhang YH, Zhang MH, Sears CE, Emanuel K, Redwood C, El-Armouche A, Kranias EG, Casadei B. Reduced phospholamban phosphorylation is associated with impaired relaxation in left ventricular myocytes from neuronal NO synthase-deficient mice. Circ Res. 2008; 102(2):242–249. [PubMed: 18007024]
10. Kohr MJ, Wang H, Wheeler DG, Velayutham M, Zweier JL, Ziolo MT. Biphasic effect of SIN-1 is reliant upon cardiomyocyte contractile state. Free Radic Biol Med. 2008; 45(1):73–80. [PubMed: 18433725]
11. Kohr MJ, Wang H, Wheeler DG, Velayutham M, Zweier JL, Ziolo MT. Targeting of phospholamban by peroxynitrite decreases {beta}-adrenergic stimulation in cardiomyocytes. Cardiovasc Res. 2008; 77(2):353–361. [PubMed: 18006474]
12. Bers DM, Berlin JR. Kinetics of [Ca]i decline in cardiac myocytes depend on peak [Ca]i. Am J Physiol. 1995; 268(1 Pt 1):C271–277. [PubMed: 7840156]
13. Bers, DM. Excitation-Contraction Coupling and Cardiac Contractile Force. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2001.

14. Chu G, Lester JW, Young KB, Luo W, Zhai J, Kranias EG. A single site (Ser16) phosphorylation in phospholamban is sufficient in mediating its maximal cardiac responses to beta -agonists. J Biol Chem. Dec 8; 2000 275(49):38938–38943. [PubMed: 10988285]

15. Terracciano CM, Souza AI, Philipson KD, MacLeod KT. Na+-Ca2+ exchange and sarcoplasmic reticular Ca2+ regulation in ventricular myocytes from transgenic mice overexpressing the Na+-Ca2+ exchanger. J Physiol. Nov 1; 1998 512 (Pt 3):651–667. [PubMed: 9769411]

16. Ginsburg KS, Bers DM. Isoproterenol does not enhance Ca-dependent Na/Ca exchange current in intact rabbit ventricular myocytes. J Mol Cell Cardiol. Dec; 2005 39(6):972–981. [PubMed: 16242149]

17. Henderson SA, Goldhaber JI, So JM, Han T, Motter C, Ngo A, Chantawansri C, Ritter MR, Friedlander M, Nicoll DA, Frank JS, Jordan MC, Roos KP, Ross RS, Philipson KD. Functional adult myocardium in the absence of Na+-Ca2+ exchange: cardiac-specific knockout of NCX1. Circ Res. Sep 17; 2004 95(6):604–611. [PubMed: 15308581]

18. Layland J, Solaro RJ, Shah AM. Regulation of cardiac contractile function by troponin I phosphorylation. Cardiovasc Res. Apr 1; 2005 66(1):12–21. 21. [PubMed: 15769444]

19. Zhang R, Zhao J, Mandveno A, Potter JD. Cardiac troponin I phosphorylation increases the rate of cardiac muscle relaxation. Circ Res. Jun; 1995 76(6):1028–1035. [PubMed: 7758157]

20. Li L, Desantiago J, Chu G, Kranias EG, Bers DM. Phosphorylation of phospholamban and troponin I in beta-adrenergic-induced acceleration of cardiac relaxation. Am J Physiol Heart Circ Physiol. 2000; 278(3):H769–779. [PubMed: 10710345]

21. Ziolo MT, Kohr MJ, Wang H. Nitric oxide signaling and myocardial function. J Mol Cell Cardiol. 2008; 45(5):625–632. [PubMed: 18722380]

22. Loyer X, Gomez AM, Milliez P, Fernandez-Velasco M, Vangheluwe P, Vinet L, Charue D, Vaudin E, Zhang W, Sainte-Marie Y, Robidel E, Marty I, Mayer B, Jaisser F, Mercadier JJ, Richard S, Shah AM, Benitah JP, Samuel JL, Heymes C. Cardiomyocyte overexpression of neuronal nitric oxide synthase delays transition toward heart failure in response to pressure overload by preserving calcium cycling. Circulation. 2008; 117(25):3187–3198. [PubMed: 18541744]

23. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lemmon CA, Burnett AL, O'Rourke B, Rodriguez ER, Huang PL, Lima JA, Berkowitz DE, Hare JM. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature. 2002; 416(6878):337–339. [PubMed: 11907582]

24. Khan SA, Skaf MW, Harrison RW, Lee K, Minhas KM, Kumar A, Fradley M, Shoukas AA, Berkowitz DE, Hare JM. Nitric oxide regulation of myocardial contractility and calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. Circ Res. 2003; 92(12): 1322–1329. [PubMed: 12764022]

25. Khan SA, Lee K, Minhas KM, Gonzalez DR, Raju SV, Tejani AD, Li D, Berkowitz DE, Hare JM. Neuronal nitric oxide synthase negatively regulates xanthine oxidoreductase inhibition of cardiac excitation-contraction coupling. Proc Natl Acad Sci U S A. Nov 9; 2004 101(45):15944–15948. [PubMed: 15486091]

26. Wang H, Viatchenko-Karpinski S, Sun J, Gyorke I, Benkusky NA, Kohr MJ, Valdivia HH, Murphy E, Gyorke S, Ziolo MT. Regulation of myocyte contraction via neuronal nitric oxide synthase: role of ryanodine receptor S-nitrosylation. J Physiol. 2010; 588(Pt 15):2905–2917. [PubMed: 20530114]

27. Loyer X, Gomez AM, Milliez P, Fernandez-Velasco M, Vangheluwe P, Vinet L, Charue D, Vaudin E, Zhang W, Sainte-Marie Y, Robidel E, Marty I, Mayer B, Jaisser F, Mercadier JJ, Richard S, Shah AM, Benitah JP, Samuel JL, Heymes C. Cardiomyocyte overexpression of neuronal nitric oxide synthase delays transition toward heart failure in response to pressure overload by preserving calcium cycling. Circulation. Jun 24; 2008 117(25):3187–3198. [PubMed: 18541744]

28. Gonzalez DR, Beigi F, Treuer AV, Hare JM. Deficient ryanodine receptor S-nitrosylation increases sarcoplasmic reticulum calcium leak and arrhythmogenesis in cardiomyocytes. Proc Natl Acad Sci U S A. 2007; 104(51):20612–20617. [PubMed: 18077344]

29. Wang H, Viatchenko-Karpinski S, Sun J, Gyorke I, Benkusky NA, Kohr MJ, Valdivia HH, Murphy E, Gyorke S, Ziolo MT. Regulation of myocyte contraction via neuronal nitric oxide

synthase: role of ryanodine receptor S-nitrosylation. J Physiol. 2010; 588(Pt 15):2905–2917. [PubMed: 20530114]

30. Shan J, Kushnir A, Betzenhauser MJ, Reiken S, Li J, Lehnart SE, Lindegger N, Mongillo M, Mohler PJ, Marks AR. Phosphorylation of the ryanodine receptor mediates the cardiac fight or flight response in mice. J Clin Invest. Dec; 120(12):4388–4398. [PubMed: 21099118]
31. Curran J, Hinton MJ, Rios E, Bers DM, Shannon TR. Beta-adrenergic enhancement of sarcoplasmic reticulum calcium leak in cardiac myocytes is mediated by calcium/calmodulin-dependent protein kinase. Circ Res. Feb 16; 2007 100(3):391–398. [PubMed: 17234966]
32. Sears CE, Bryant SM, Ashley EA, Lygate CA, Rakovic S, Wallis HL, Neubauer S, Terrar DA, Casadei B. Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. Circ Res. 2003; 92(5):e52–59. 22. [PubMed: 12623875]

- Molecular mechanisms of $[Ca^{2+}]_i$ decline are similar in $NOS1^{-/-}$ and WT myocytes
- β-AR stimulation resulted in greater effect on $[Ca^{2+}]_i$ decline in $NOS1^{-/-}$ myocytes
- The amount of PLB phosphorylation is major determinant for the $[Ca^{2+}]_i$ decline rate
- Extent of augmentation of PLB phosphorylation also is a major determinant

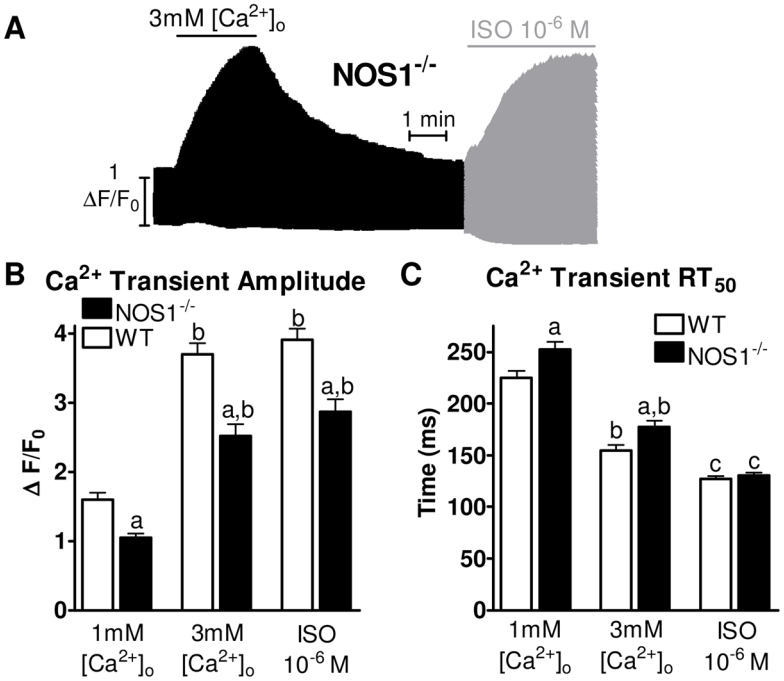

Figure 1.

Experimental Protocol. A) Representative time plot of the experimental protocol. B) Summary data (mean±sem) of maximum Ca^{2+} transient amplitude with 1 mM $[Ca^{2+}]_o$, 3mM $[Ca^{2+}]_o$, or ISO from WT (clear bar) and $NOS1^{-/-}$ (black bar) myocytes. C) Summary data (mean±sem) of Ca^{2+} transient decline measured as the RT_{50}. a $P<0.05$ vs corresponding WT, b $P<0.05$ vs 1 mM $[Ca^{2+}]_o$, c $P<0.05$ vs 1mM and 3mM $[Ca^{2+}]_o$.

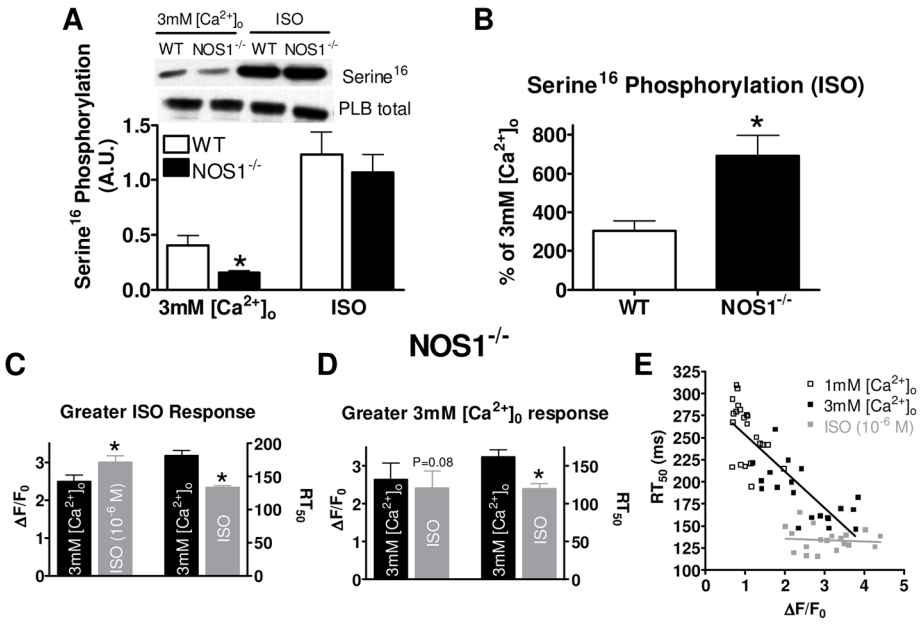

Figure 2.

Effects of ISO and 3mM $[Ca^{2+}]_o$ on WT and NOS1$^{-/-}$ PLB Serine^{16} phosphorylation and Ca^{2+} transient decline in NOS1$^{-/-}$ myocytes. A) Summary data (mean±sem) of PLB Serine^{16} phosphorylation with ISO or 3mM $[Ca^{2+}]_o$ (A.U.- arbitrary units). B) Summary data (mean±sem) of the ISO mediated increase in PLB Serine^{16} phosphorylation expressed as the % of 3mM $[Ca^{2+}]_o$. Summary data (mean±sem) of Ca^{2+} transient amplitude (left) and decline (right) in NOS1$^{-/-}$ myocytes which had a higher maximal Ca^{2+} transient amplitude to ISO (C) or 3 mM $[Ca^{2+}]_o$ (D). *P<0.05) vs corresponding WT or 3 mM $[Ca^{2+}]_o$. E) Individual values of peak $[Ca^{2+}]_i$ versus RT_{50} with Ca^{2+} (black, r^2=0.70) and ISO (gray, r^2=0.007).

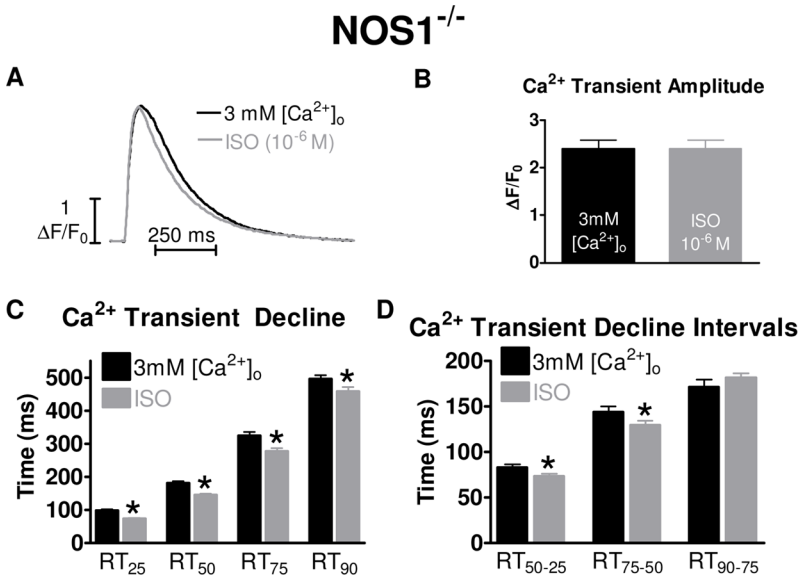

Figure 3.

Effects of 3 mM $[Ca^{2+}]_i$ and ISO on Ca^{2+} transient decline with matched Ca^{2+} transient amplitudes in NOS1$^{-/-}$ myocytes. A) Representative trace of matched $[Ca^{2+}]_i$ amplitudes with 3 mM $[Ca^{2+}]_o$ (black) or 10^{-6} M ISO (gray). B) Summary data (mean±sem) of matched Ca^{2+} transient amplitude with 3mM $[Ca^{2+}]_o$ (black) or ISO (gray). C) Summary data (mean±sem) of Ca^{2+} transient decline with 3mM $[Ca^{2+}]_o$ (black) or ISO (gray). (D) Summary data (mean±sem) of Ca^{2+} transient decline time intervals with 3mM $[Ca^{2+}]_o$ or ISO. * $P<0.05$ vs corresponding 3 mM $[Ca^{2+}]_o$.

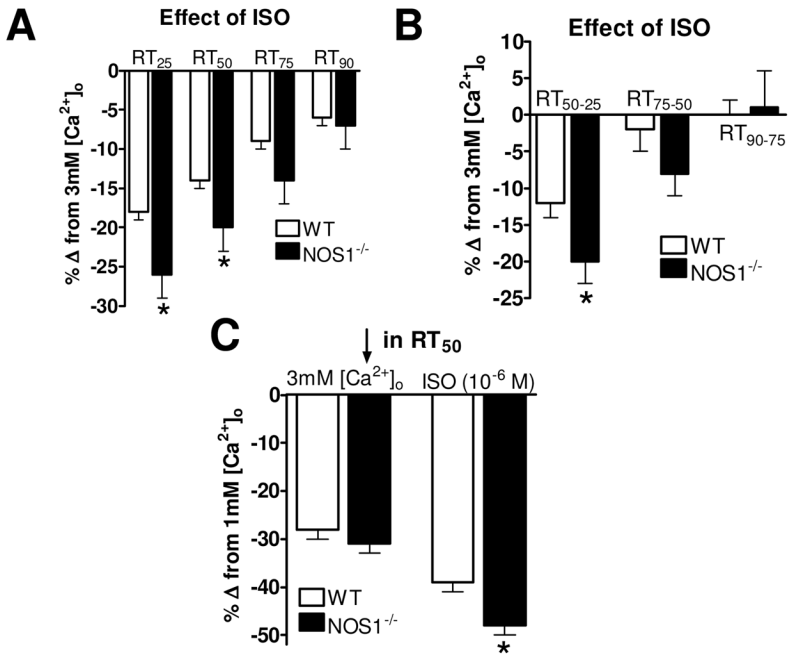

Figure 4.

Effect of ISO on Ca^{2+} transient decline in $NOS1^{-/-}$ vs WT myocytes. A) Summary data (mean±sem) of the effects of ISO on Ca^{2+} transient decline normalized to 3mM $[Ca^{2+}]_o$ in WT (clear bars) and $NOS1^{-/-}$ (black bars) myocytes. B) Summary data (mean±sem) of the effects of ISO on Ca^{2+} transient decline time intervals normalized to 3mM $[Ca^{2+}]_o$ in WT (clear bars) and $NOS1^{-/-}$ (black bars) myocytes. C) Summary data (mean±sem) of the effects of maximum 3mM $[Ca^{2+}]_o$ (left) and ISO (right) on Ca^{2+} transient RT_{50} normalized to 1mM $[Ca^{2+}]_o$ in WT (clear bars) and $NOS1^{-/-}$ (black bars) myocytes. * $P<0.05$ vs corresponding WT.