

Research Article

Prolonged Action Potential and after Depolarizations Are Not due to Changes in Potassium Currents in NOS3 Knockout Ventricular Myocytes

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Received 20 April 2012; Revised 13 July 2012; Accepted 16 July 2012

Academic Editor: Christopher Ahern

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Ventricular myocytes deficient in endothelial nitric oxide synthase (NOS3^{-/-}) exhibit prolonged action potential (AP) duration and enhanced spontaneous activity (early and delayed afterdepolarizations) during β -adrenergic (β -AR) stimulation. Studies have shown that nitric oxide is able to regulate various K⁺ channels. Our objective was to examine if NOS3^{-/-} myocytes had altered K⁺ currents. APs, transient outward (I_{to}), sustained (I_{Ksus}), and inward rectifier (I_{K1}) K⁺ currents were measured in NOS3^{-/-} and wild-type (WT) myocytes. During β -AR stimulation, AP duration (measured as 90% repolarization-APD₉₀) was prolonged in NOS3^{-/-} compared to WT myocytes. Nevertheless, we did not observe differences in I_{to} , I_{Ksus} , or I_{K1} between WT and NOS3^{-/-} myocytes. Our previous work showed that NOS3^{-/-} myocytes had a greater Ca²⁺ influx via L-type Ca²⁺ channels with β -AR stimulation. Thus, we measured β -AR-stimulated SR Ca²⁺ load and found a greater increase in NOS3^{-/-} versus WT myocytes. Hence, our data suggest that the prolonged AP in NOS3^{-/-} myocytes is not due to changes in I_{to} , I_{Ksus} , or I_{K1} . Furthermore, the increase in spontaneous activity in NOS3^{-/-} myocytes may be due to a greater increase in SR Ca²⁺ load. This may have important implications for heart failure patients, where arrhythmias are increased and NOS3 expression is decreased.

1. Introduction

Cardiac myocytes endogenously produce nitric oxide (NO) via two constitutively expressed NO synthase isoforms: endothelial NO synthase (NOS3) and neuronal NO synthase (NOS1). Both NOS1 and NOS3 play important roles in modulating cardiac function [1]. However, NOS1 and NOS3 signalings lead to different functional effects [2, 3]. NOS1 is localized to the sarcoplasmic reticulum (SR) and enhances cardiac contraction [2, 4], while NOS3 is localized to the caveolae and blunts the response to β -adrenergic (β -AR) stimulation due to a decreased L-type Ca²⁺ current (I_{Ca}) [5, 6]. We also observed that NOS3 knockout (NOS3^{-/-}) myocytes have prolonged action potential (AP) duration [5].

In addition to Ca²⁺ channels in the ventricular myocyte, the AP waveform is also determined by potassium (K⁺) channels, which are essential for proper electrical activity of the heart as they are responsible for the resting membrane potential (RMP), the plateau phase, and repolarization [7]. K⁺ channels are also the most variable channels with multiple components, such as transient outward K⁺ current (I_{to}), sustained outward K currents (I_{Ksus}), inward rectifier K⁺ current (I_{K1}), delayed rectifier K⁺ current (I_{Ks}), and so forth [7]. In addition, there are different expression patterns of the various K⁺ channels in different species [8].

A previous study has shown that sodium nitroprusside, a NO donor, can enhance I_{Ks} in guinea-pig cardiac myocytes [9]. Another study found that NOS3, but not NOS1, is

responsible for the Ca^{2+} -induced I_{Ks} enhancement [10]. I_{Ks} , which activates very slowly during depolarization and deactivates very slowly during repolarization, contributes to the repolarization during the late phase of the AP. However, I_{Ks} channels are not expressed in adult murine ventricular myocytes [8, 11]. Thus, our observed changes in AP waveform in $\text{NOS3}^{-/-}$ mouse myocytes may result from alterations of other K^+ channels, such as I_{Ksus} , I_{to} , and/or I_{K1} . In mouse hearts, I_{Ksus} is an important modulator of the plateau phase and repolarizing phase 3 of the AP. I_{to} contributes to the repolarizing phase 1 and the plateau phase of AP. Previous data [12] showed that NO and NO donors inhibited human atrial I_{to} . I_{K1} is responsible for setting the RMP and shaping the late repolarizing phase 3 of the AP. Gómez et al. [13] found that NO and NO donors can increase I_{K1} measured in human atrial cells. However, the role of NOS3 signaling on modulation of I_{to} , I_{Ksus} , and/or I_{K1} in ventricular myocytes is unclear. In addition to contributing to prolonged AP duration, altered K^+ channel function can contribute to the generation of arrhythmias [14, 15]. Indeed, there is an increase in the incidence of arrhythmias in $\text{NOS3}^{-/-}$ mice [16]. Furthermore, our and others' data have shown increased spontaneous activity (i.e., early and delayed afterdepolarizations) in $\text{NOS3}^{-/-}$ myocytes [5, 17]. Thus, the purpose of this study is to determine if NOS3 signaling modulates K^+ currents. In order to investigate the effects of NOS3 signaling on K^+ currents, we used ventricular myocytes isolated from $\text{NOS3}^{-/-}$ and corresponding WT mice to investigate if alterations in one or all of K^+ channels (I_{to} , I_{Ksus} , I_{K1}) play a role in the prolongation of the AP, an effect expected to increase the propensity for spontaneous activity in $\text{NOS3}^{-/-}$ myocytes.

2. Materials and Methods

2.1. Isolation of Ventricular Myocytes. Age-matched, male and female $\text{NOS3}^{-/-}$ mice [18] and their corresponding control (C57Bl/6J) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Ventricular myocytes were isolated as previously described [5]. Briefly, the heart was mounted on a Langendorff apparatus and perfused with Ca^{2+} free normal Tyrode solution for 4 min. Blendzyme Type IV (0.077 mg/mL) (Roche Applied Science, Indianapolis, IN, USA) was then added to the perfusate. After 2–5 minutes, the heart was taken down, the right and left 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+} . We randomly selected our myocytes and the region of the heart (epicardium, endocardium, midmyocardium, etc.) could not be determined. 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.

2.2. Measurement of Action Potential. Action potentials (AP) were recorded with an Axopatch-200B amplifier and pClamp 8.1 software (Axon Instrument, Foster City, CA, USA) using

the current-clamp mode. The pipette was filled with (in mM): NaCl (8), KCl (10), K-Aspartate (140), HEPES (5), MgATP (2), and pH 7.2 adjusted with KOH or HCl, with a resistance of 9–11 M Ω . Myocytes were perfused with normal Tyrode solution, which consisted of (in mM): NaCl (140), KCl (4), MgCl₂ (1), CaCl₂ (1), Glucose (10), HEPES (5), L-arginine (1), and pH 7.4 adjusted with NaOH or HCl. Isoproterenol (ISO, 1 μM , Sigma), a nonselective β -AR agonist, was prepared fresh each experimental day. A Grass S48 stimulator gated the amplifier for current injection to activate the AP, triggered by a 1.5 ms, 2 nA current injection. Measurements were performed at $37 \pm 1^\circ\text{C}$.

2.3. Measurement of Potassium Currents. The amphotericin-B-perforated patch clamp technique was used to measure the various K^+ currents. Myocytes were placed in a laminin-coated cell chamber (Cell Microcontrols, Norfolk, VA, USA) and superfused with bath solution containing (in mM): 135 NaCl, 5 MgCl₂, 5 KCl, 10 glucose, 1.8 CaCl₂, and 5 HEPES with pH adjusted to 7.4 with NaOH at temperature of $36 \pm 0.5^\circ\text{C}$. Nifedipine (2 μM) was added to the superfusate to block the L-type calcium current. Borosilicate glass micropipettes with tip resistance of 1.5–3 M Ω were filled with pipette solution containing the following (in mM): 100 K-aspartate, 40 KCl, 5 MgCl, 5 EGTA, 5 HEPES, and pH adjusted to 7.2 with KOH.

Inward rectifier K^+ current (I_{K1}) was elicited by voltage steps from -140 to $+40$ mV from a holding potential of -40 mV (which will inactivate the sodium current). The current was measured at the end of each 100-ms test pulse. I_{K1} inward conductance (mS/cm₂) was determined by calculating the slope of the linear portion of the current density-voltage relationship from -140 to -100 mV. Peak outward I_{K1} density was measured as the current at -60 mV (I_{-60}) [19]. All currents (in picoamperes (pA)) were normalized to the cell capacitance (measured in picofarads (pF)) and expressed as pA/pF.

Outward K^+ currents were elicited by a series of 300 ms test potentials from -50 to $+50$ mV from a holding potential of -60 mV (which will inactivate the sodium current). The sustained K^+ current (I_{Ksus}) was measured at the end of the 300 ms test pulse. The transient outward K^+ current (I_{to}) was determined by subtracting the sustained outward current from the peak outward current [19].

2.4. SR Ca^{2+} Load. SR Ca^{2+} load was measured at room temperature (22°C) by rapid application of 10 mmol/L caffeine for 10 sec. The amplitude of the caffeine-induced Ca^{2+} transient was used as an index of SR Ca^{2+} load [20].

2.5. Statistics. Myocyte data were presented as mean \pm SEM. Differences between multiple groups were evaluated for statistical significance using an ANOVA (followed by Neuman-Keuls test) or unpaired Student's *t*-test for two groups. Statistical significance was accepted at the level of $P < 0.05$.

3. Results

3.1. $\text{NOS3}^{-/-}$ Myocytes Have Prolonged β -AR Stimulated APD but No Change in RMP. We have previously shown that

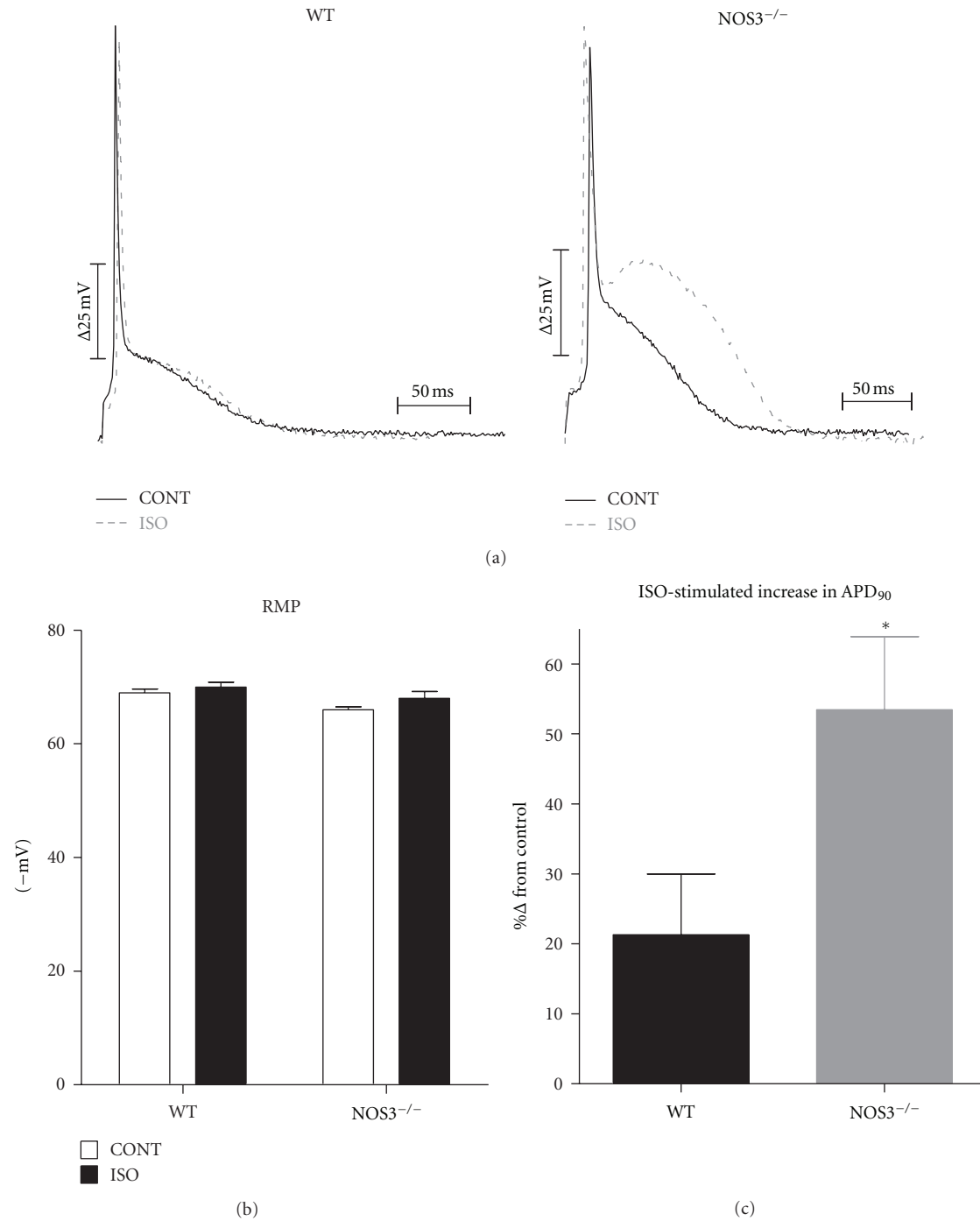


FIGURE 1: Prolonged APD₉₀ in NOS3^{-/-} myocytes after β -AR stimulation. (a) Representative AP traces from WT and NOS3^{-/-} myocytes (control-CONT; isoproterenol-ISO). (b) Summary data (mean \pm S.E.M.) of RMP in WT and NOS3^{-/-} myocytes. (c) Summary data (mean \pm S.E.M.) of β -AR stimulated increase of APD₉₀ in WT and NOS3^{-/-} myocytes. * $P < 0.05$, $n = 4, 7$ cells.

during β -AR stimulation, NOS3^{-/-} ventricular myocytes had a prolonged AP (measured as time to 90% repolarization-APD₉₀) when measured at room temperature [5]. We repeated these experiments to examine if the phenomenon occurred at body temperature. Thus, we measured AP waveform in control (wildtype, WT) and NOS3^{-/-} myocytes at 37°C. Representative AP traces measured in a WT and NOS3^{-/-} myocyte (\pm β -AR stimulation with ISO) are shown

in Figure 1(a). As shown in Figure 1(b), there was no difference between WT and NOS3^{-/-} resting membrane potential (basal or with β -AR stimulation). However, we found that during β -AR stimulation, NOS3^{-/-} myocytes (compared to WT) had a significant increase in APD₉₀, consistent with our previous finding (Basal, WT: 75 ± 17 ms, NOS3^{-/-}: 77 ± 10 ms; ISO, WT: 93 ± 24 ms, NOS3^{-/-}: 117 ± 17 ms). The increase in APD₉₀ with β -AR stimulation

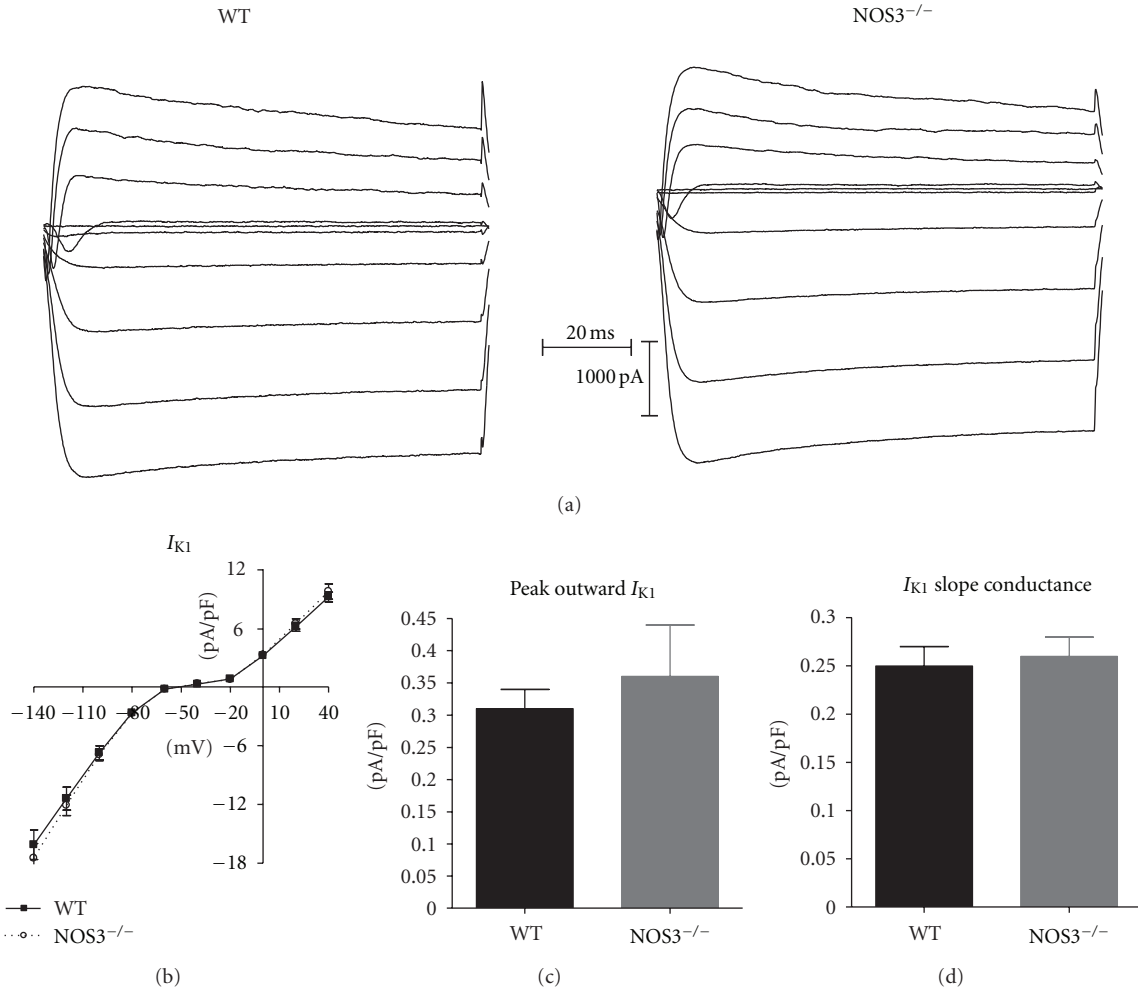


FIGURE 2: I_{K1} current was not changed in $NOS3^{-/-}$ myocytes. (a) Representative traces from WT and $NOS3^{-/-}$ myocytes. (b) I - V curves of I_{K1} in WT and $NOS3^{-/-}$ myocytes. (c) Summary data (mean \pm S.E.M.) of peak outward I_{K1} in WT and $NOS3^{-/-}$ myocytes. (d) Summary data (mean \pm S.E.M.) of I_{K1} slope conductance in WT and $NOS3^{-/-}$ myocytes. $n = 6, 15$ cells.

in $NOS3^{-/-}$ myocytes is shown in Figure 1(c). These data suggest that $NOS3^{-/-}$ myocytes have a prolonged AP with no change in the RMP during β -AR stimulation.

3.2. $NOS3^{-/-}$ Myocytes Do Not Have Altered Inward Rectifier K^+ Currents. An important determinant of RMP and APD_{90} is the inward rectifier K^+ current (I_{K1}). Since we had prolonged APD_{90} with β -AR stimulation in $NOS3^{-/-}$ myocytes, we investigated if these myocytes had altered I_{K1} . Representative I_{K1} currents measured in a WT and $NOS3^{-/-}$ myocyte are shown in Figure 2(a). As shown in Figures 2(b)–2(d), knockout of $NOS3$ did not alter the I_{K1} I - V relationship, the peak outward I_{K1} , or the I_{K1} slope conductance. These data suggest that $NOS3$ signaling does not modulate I_{K1} .

3.3. $NOS3^{-/-}$ Myocytes Do Not Have Altered Transient Outward or Sustained K^+ Currents. Since other K^+ currents are also involved in repolarization to determine the APD, we investigated if $NOS3^{-/-}$ myocytes had alterations in

other K^+ currents by measuring I_{to} and $I_{K_{sus}}$. Representative currents measured in a WT and $NOS3^{-/-}$ myocyte are shown in Figure 3(a). Our data show no difference in the I_{to} or $I_{K_{sus}}$ I - V relationship in $NOS3^{-/-}$ compared to WT myocytes (Figure 3). With our voltage protocol, $I_{K_{sus}}$ is composed of various K^+ currents, including $I_{K_{slow}}$. However, since we did not observe a difference in composite $I_{K_{sus}}$, we did not further investigate the contributing currents. These data indicate that $NOS3$ signaling does not modulate I_{to} or $I_{K_{sus}}$.

3.4. β -AR Stimulated SR- Ca^{2+} Load Was Higher in $NOS3^{-/-}$ Myocytes. Altered K^+ currents resulting in prolonged APD can contribute to the generation of afterdepolarizations [21, 22]. However, since we did not observe any differences in I_{to} , $I_{K_{sus}}$, or I_{K1} , we performed additional experiments to measure SR Ca^{2+} load, which is also known to contribute to afterdepolarizations [22, 23]. We observed no difference in basal SR Ca^{2+} loads between $NOS3^{-/-}$ and WT myocytes (1.8 ± 0.2 versus $1.9 \pm 0.2 \Delta F/F_0$). However, our data (Figure 4) show that $NOS3^{-/-}$ myocytes (compared to WT)

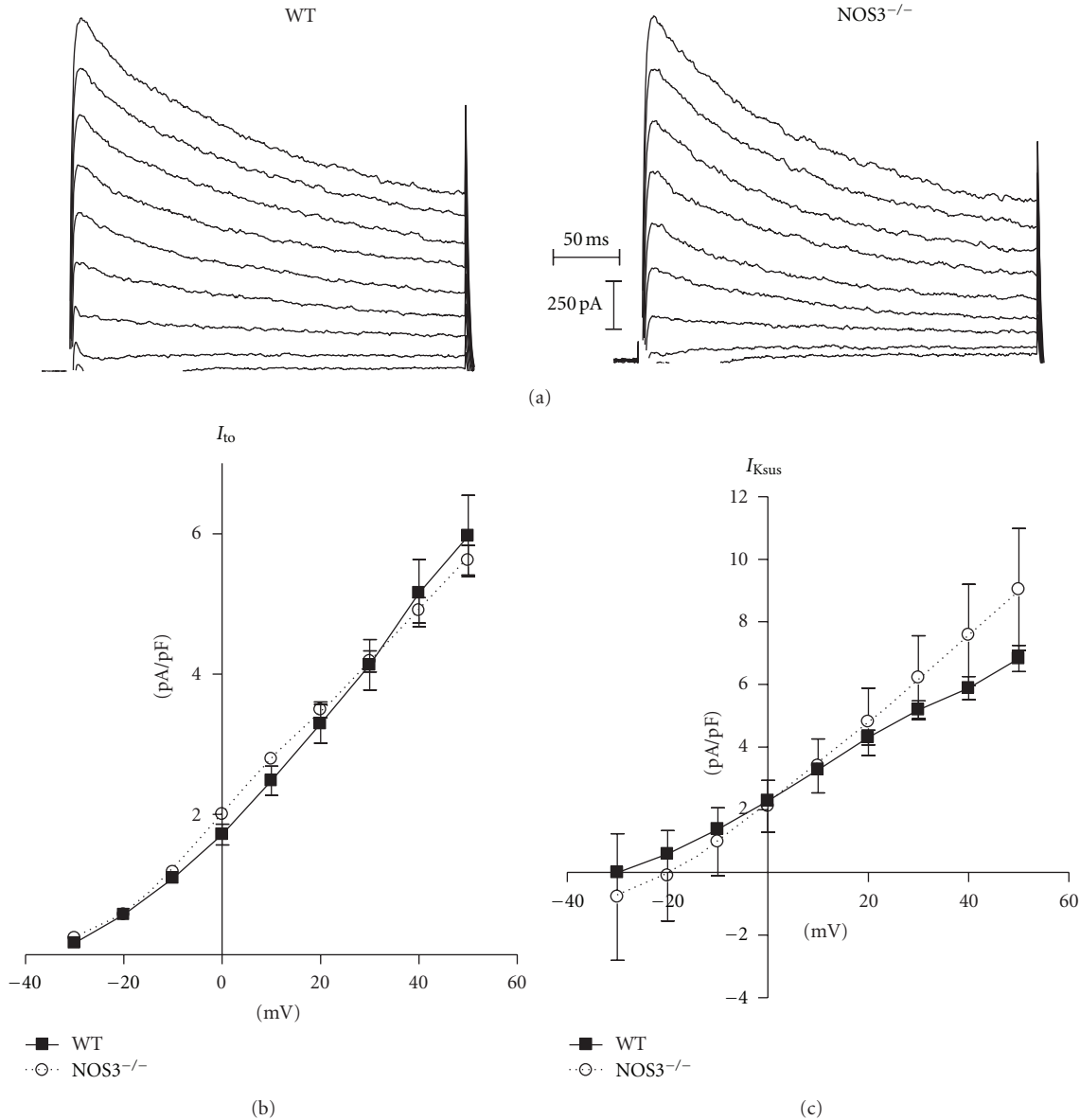


FIGURE 3: NOS3 knockout did not alter I_{to} or I_{Ksus} . (a) Representative current traces from WT and NOS3^{-/-} myocytes. (b) Summary data (mean \pm S.E.M.) of I_{to} I - V curves in WT and NOS3^{-/-} myocytes. (c) Summary data (mean \pm S.E.M.) of I_{Ksus} I - V curves in WT and NOS3^{-/-} myocytes. $n = 7, 13$ cells.

have a larger increase in their SR Ca^{2+} load with β -AR stimulation. These data suggest that with β -AR stimulation there is a larger increase in SR Ca^{2+} load in NOS3^{-/-} myocytes.

4. Discussion

The purpose of this study was to investigate the role of NOS3 signaling on the modulation of I_{to} , I_{Ksus} , and I_{K1} . Our data show that I_{to} , I_{Ksus} , and I_{K1} are not altered in NOS3^{-/-} ventricular myocytes, which suggests that NOS3 does not modulate I_{to} , I_{Ksus} , and I_{K1} . Furthermore, the increased afterdepolarizations with β -AR stimulation in NOS3^{-/-}

myocytes may be due to enhanced spontaneous Ca^{2+} waves resulting from increased β -AR stimulated SR Ca^{2+} load.

4.1. Nitric Oxide Modulation of the β -AR Pathway. Stimulation of β -AR pathway is an essential regulator of cardiac contractility, leading to positive inotropic and lusitropic effects [24]. Our and other previous studies showed that NOS3 signaling blunts the functional response to β -AR stimulation [2, 5, 25–28]. That is, NOS3^{-/-} myocytes have increased Ca^{2+} transient amplitudes and shortening amplitudes compared to WT, which is due to an enhanced β -AR stimulated I_{Ca} . Our data also demonstrated that NOS3^{-/-} myocytes had

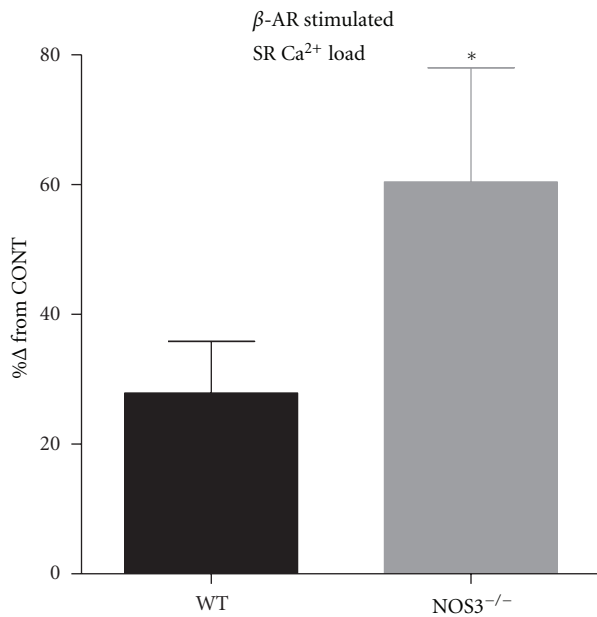


FIGURE 4: Larger increase in β -AR stimulated SR Ca^{2+} load in NOS3^{-/-} myocytes. Summary data (mean \pm S.E.M.) of the increase in SR Ca^{2+} load with β -AR stimulation in WT and NOS3^{-/-} myocytes (control- CONT). * $P < 0.05$ versus WT, $n = 11, 27$ cells.

prolonged APD during β -AR stimulation [5]. This current study examined if alterations in K^+ currents contributed to the prolonged APD in NOS3^{-/-} myocytes.

4.2. Prolonged AP in NOS3^{-/-} Myocytes Is Not due to Altered I_{to} , I_{Ksus} , and I_{K1} . Previous studies have shown that alterations in I_{K1} , which is an important K^+ channel responsible for setting the RMP and shaping the late phase 3 of AP, can contribute to prolonging the AP [14]. However, our data (Figure 2) shows that I_{K1} is unaffected with knockout of NOS3. Furthermore, we observed similar RMP between WT and NOS3^{-/-} myocytes (Figure 1), consistent with no difference in I_{K1} . Thus, I_{K1} is not the cause for prolonged APD in NOS3^{-/-} myocytes.

Besides I_{K1} , many other K^+ currents are key channels in shaping the AP waveform. I_{to} is a transient outward current that contributes to the plateau phase of AP. Inhibition of I_{to} has the ability to prolong the APD. In mouse myocytes, I_{Ksus} is the sustained outward component of the repolarizing K^+ current, and it plays an important role during the plateau and phase 3 of the AP. Interestingly, we also did not observe any differences in I_{to} and I_{Ksus} between NOS3^{-/-} and WT myocytes (Figure 3). These data indicate that I_{to} or I_{Ksus} is not involved in the prolonged APD in NOS3^{-/-} myocytes. Using our voltage protocol to elicit outward K^+ channels, our composite I_{Ksus} current will encompass $I_{\text{K,slow}}$ [29–32]. $I_{\text{K,slow}}$ is also an important K^+ current in mice responsible for $\sim 30\%$ of the repolarization [29]. Since we did not observe a difference in I_{Ksus} , these data suggest that $I_{\text{K,slow}}$ is also not modulated by NOS3 signaling. It should be noted that I_{to} and I_{Ksus} (including $I_{\text{K,slow}}$) are not the only operating currents during the plateau phase. I_{Ca} is an inward Ca^{2+} current that is counterbalanced by I_{to} and I_{Ksus} , which creates the

plateau phase of the AP. Hence, the duration of the plateau is determined by the balance between I_{Ca} and I_{to} and I_{Ksus} . We previously observed an increase in the β -AR stimulated I_{Ca} in NOS3^{-/-} myocytes compared to WT myocytes [5]. Thus, we suggest that in NOS3^{-/-} myocytes an increased I_{Ca} will overcome the unaltered I_{to} and I_{Ksus} , leading to more inward current resulting in prolonging the APD. This is consistent with previous studies which demonstrated that changes in I_{Ca} will affect APD [33–35].

Previous studies demonstrated that NO donors are able to modulate K^+ currents. The discrepancy between our findings and other groups may be due to the use of different species (human, guinea pig, and mouse). Another distinction between our current study and the previous studies is the use of endogenous NO (i.e., NOS3) or exogenous NO (i.e., NO donors). Our and others' work has shown the endogenous NO signaling via NOS1 and NOS3 is compartmentalized [1, 2]. However, when using NO donors, the entire myocyte will be exposed to NO, which is in contrast to the localized signaling observed with endogenous NO. In this study, we used isolated myocytes from NOS3^{-/-} and WT hearts to directly investigate the effects of NOS3 signaling on K^+ currents. While Gómez et al. [12] did observe effects of NO donors on I_{to} , they also did not observe any differences in I_{to} between WT and NOS3^{-/-} ventricular myocytes (consistent with our observations). Thus, we are confident in our findings that NOS3 signaling does not modulate I_{to} , I_{Ksus} , or I_{K1} .

4.3. Increased SR Ca^{2+} Load in NOS3^{-/-} Myocytes with β -AR Stimulation. β -AR stimulation can result in triggered arrhythmias [36], which are observed in NOS3^{-/-} mice [16]. These premature beats are analogous to afterdepolarizations in isolated myocytes, which we and others have demonstrated to occur in NOS3^{-/-} myocytes [5, 17]. While alterations in K^+ currents can contribute to the increased incidence of these triggered arrhythmias, we did not observe any difference in K^+ currents in NOS3^{-/-} myocytes. Another contributing factor to the generation of afterdepolarizations is an increase in the SR Ca^{2+} load [23, 37–39]. Studies have shown that early afterdepolarizations are due to an increase in I_{Ca} [40] and delayed afterdepolarizations are due to spontaneous release of Ca^{2+} from the SR (i.e., Ca^{2+} wave) [37]. However, recent data now suggests that early afterdepolarizations caused by β -AR stimulation may also be due to spontaneous SR Ca^{2+} release [41, 42]. Thus, an important finding in the present study is the increased SR Ca^{2+} load with β -AR stimulation in NOS3^{-/-} myocytes (Figure 4). It is known that enhanced Ca^{2+} influx via I_{Ca} will result in increased SR Ca^{2+} load [33, 43]. Thus, our higher SR Ca^{2+} load in NOS3^{-/-} myocytes is consistent with the observed greater Ca^{2+} influx via I_{Ca} . In fact, it has been suggested that Ca^{2+} waves and increased I_{Ca} act synergistically to produce early and delayed afterdepolarizations [42]. Thus, NOS3^{-/-} myocytes are tailored for afterdepolarizations since these myocytes exhibit an increased I_{Ca} and SR Ca^{2+} load during β -AR stimulation. In fact, our previous study showed that the vast majority of NOS3^{-/-} myocytes have afterpolarizations [5].

In addition to I_{Ca} and the various K^+ currents, it is known that other currents, most notably the sodium current [44], can not only affect APD but also contribute to the generation of afterdepolarizations. Since the purpose of this study was to examine if NOS3 signaling modulates K^+ currents, we did not measure sodium current. Thus, the effect of NOS3 on sodium current and its corresponding effect on APD and afterdepolarizations cannot be excluded. Future studies are needed to investigate if NOS3 signaling can modulate the sodium current.

In conclusion, in NOS3^{-/-} mouse ventricular myocytes, I_{to} , I_{Ksus} , and I_{K1} are normal and do not contribute to the prolonged APD and increased incidence of afterdepolarizations. We suggest that in NOS3^{-/-} myocytes the increase in β -AR stimulated I_{Ca} is the reason for the prolonged APD. Furthermore, the increased afterdepolarizations in NOS3^{-/-} myocytes during β -AR stimulation are caused by the increased I_{Ca} along with the increased SR Ca^{2+} load. This may have important implication for arrhythmias and sudden cardiac death in heart failure, where NOS3 expression is decreased [45, 46] and β -AR tone is increased [47].

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