Cardiac Neuronal Nitric Oxide Synthase Isoform Regulates Myocardial Contraction and Calcium Handling

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Abstract—A neuronal isoform of nitric oxide synthase (nNOS) has recently been located to the cardiac sarcoplasmic reticulum (SR). Subcellular localization of a constitutive NOS in the proximity of an activating source of Ca\(^{2+}\) suggests that cardiac nNOS-derived NO may regulate contraction by exerting a highly specific and localized action on ion channels/transporters involved in Ca\(^{2+}\) cycling. To test this hypothesis, we have investigated myocardial Ca\(^{2+}\) handling and contractility in nNOS knockout mice (nNOS\(^{-/-}\)) and in control mice (C) after acute nNOS inhibition with 100 \(\mu\)mol/L L-VNIO. nNOS gene disruption or L-VNIO increased basal contraction both in left ventricular (LV) myocytes (steady-state cell shortening 10.3±0.6% in nNOS\(^{-/-}\) versus 8.1±0.5% in C; \(P<0.05\)) and in vivo (LV ejection fraction 53.5±2.7% in nNOS\(^{-/-}\) versus 44.9±1.5% in C; \(P<0.05\)). nNOS disruption increased \(I_{Ca}\) density (in pA/pF, at 0 mV, \(-11.4±0.5\) in nNOS\(^{-/-}\) versus \(-9.1±0.5\) in C; \(P<0.05\)) and prolonged the slow time constant of inactivation of \(I_{Ca}\) by 38% (\(P<0.05\)), leading to an increased Ca\(^{2+}\) influx and a greater SR load in nNOS\(^{-/-}\) myocytes (in pC/pF, 0.78±0.04 in nNOS\(^{-/-}\) versus 0.64±0.03 in C; \(P<0.05\)). Consistent with these data, [Ca\(^{2+}\)], transient (indo-1) peak amplitude was greater in nNOS\(^{-/-}\) myocytes (410/495 ratio 0.34±0.01 in nNOS\(^{-/-}\) versus 0.31±0.01 in C; \(P<0.05\)). These findings have uncovered a novel mechanism by which intracellular Ca\(^{2+}\) is regulated in LV myocytes and indicate that nNOS is an important determinant of basal contractility in the mammalian myocardium. The full text of this article is available at http://www.circresaha.org.

Key Words: neuronal nitric oxide synthase • ventricular • contraction • calcium

It is now well-established that nitric oxide (NO) is constitutively generated within the heart, not only by the endothelium but also by the myocytes themselves.\(^1\) However, whether constitutive myocardial NO production regulates basal inotropy and calcium (Ca\(^{2+}\)) fluxes remains controversial (see reviews\(^2,3\)). In particular, some studies have shown that nonisoform specific inhibition of NO synthase (NOS) or targeted disruption of the endothelial NOS isoform (eNOS) has no effect on cell shortening or Ca\(^{2+}\) handling.\(^3,4\) Others, however, have indicated that endogenous NO production may tonically inhibit myocardial inotropy\(^5,6\) and the Ca\(^{2+}\) current.\(^7\) These studies assumed that eNOS was the only constitutive isoform involved in the autocrine control of myocardial function. In 1999, however, Xu and collaborators\(^8\) localized a neuronal-type NOS isoform (nNOS) to murine and human sarcoplasmic reticulum (SR). The subcellular localization of a constitutive NOS isoform in the proximity of an activating source of Ca\(^{2+}\) suggests that endogenous NO may exert a specific and localized action on ion channels/transporters involved in Ca\(^{2+}\) cycling. In the present study, we report that targeted disruption of the nNOS gene (nNOS\(^{-/-}\)) as well as acute pharmacological nNOS inhibition enhances basal left ventricular (LV) contraction and intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]) transients by increasing Ca\(^{2+}\) influx (via the Ca\(^{2+}\) current) and SR Ca\(^{2+}\) content. These findings have uncovered a novel mechanism by which [Ca\(^{2+}\)], is regulated in LV myocytes. We suggest that NOS may provide a negative feedback regulation of Ca\(^{2+}\) influx, because increases in [Ca\(^{2+}\)], stimulate NOS synthesis of NO, which in turn acts to inhibit Ca\(^{2+}\) influx. Such mechanisms would contribute to the maintenance of a tight control of [Ca\(^{2+}\)], in physiological conditions and may protect against Ca\(^{2+}\) overload in cardiac disease states.

Materials and Methods

Animals

Mice homozygous for targeted disruption of the nNOS gene (B6.129-NOS1\(^{tm1plh}\), nNOS\(^{-/-}\)) were purchased from Jackson Laboratories (Bar Harbor, Maine) and a colony was established at the John Radcliffe Hospital by backcrossing the nNOS\(^{-/-}\) on a C57BL/6 background. N3 littermate mice homozygous for the nNOS gene (nNOS\(^{+/-}\)) were used as controls in most protocols, in others...
age-matched C57BL/6 mice were used as wild-type controls as in previous studies. The treatment of all animals was in accordance with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act, 1986 (H.M.S.O.).

In Vivo Measurements of LV Function

Hemodynamic indices were measured in anesthetized mice (isoflurane) using a A 1.4F Millar Micro-tip catheter (SPR-671) inserted into the LV via the carotid artery. At the end of the experiment, the catheter was withdrawn from the LV to measure aortic pressures. Reported values represent an average of 20 consecutive cardiac cycles. Echocardiography was performed simultaneously using an Agilent Sonos5500 with a 7- to 15-MHz linear-array transducer. Parasternal short-axis images, 2-D and M-mode, were obtained at the level of the papillary muscles and stored digitally.

Myocyte Isolation and Techniques

Single LV myocytes were isolated using a standard enzymatic dispersion technique as described previously. For electrophysiological recordings, myocytes were superfused with a modified Tyrode solution (for composition, see expanded Materials and Methods, available in the online data supplement at http://www.circresaha.org). Membrane current was measured using the whole-cell configuration of the patch-clamp technique (Axopatch 200A, Axon Instruments). Cell length was concurrently monitored with a video-edge detection system (IonOptix Corp), with a temporal resolution of ~4.2 ms. Analog signals (current, voltage, and cell length) were digitized (Digidata 1200A, Axon Instruments) and stored on-line to computer for subsequent off-line analysis. Calcium load in nNOS

Assessment of SR Ca2+ Load

The SR Ca2+ content was quantified in voltage-clamped cells by discharging SR Ca2+ with a 10 second application of caffeine (10 mM/L, using a rapid solution-switching device) and integrating the resulting Na+/Ca2+ exchange (NCX) current, as described previously. In a cohort of myocytes from each group, the caffeine-induced calcium transient (indo-1) was recorded after exposure to 5 mM/L nickel for 5 minutes, and the time constant of decline of the caffeine-induced Ca2+ transient in the presence of Ni2+ was verified by the absence of an inward current in response to a 10 mM/L pulse of caffeine (data not shown).

Measurement of [Ca2+]i, Transients

Indo-1 fluorescence was monitored from cells preincubated with the acetoxymethyl ester of indo-1 (5 μM/L, Sigma) for 20 minutes at room temperature. Cells were field-stimulated to contract at 1 Hz at 35°C. In a cohort of cells from each group, calibration of the indo-1 signal was performed using the method of Terraciano and MacLeod. In some experiments, cells were imaged (Fluo-4, 100 μM/L loaded via the patch pipette) in line-scan mode (2.61 ms per line) using a Leica TCS-NT confocal microscopy system.

Immunoblotting

Western blots were performed on LV membrane subfractions using specific antibodies to the following: α1C subunit of the dihydropyridine calcium channel (Alomone); cardiac ryanodine receptor, calsequestrin, and SERCA2a (Affinity Bioreagents); phospholamban (Cyclacel); NCX (Abcam); and the plasmalemma Ca2+-ATPase (PMCA) (Laboratory Vision).

Acute Inhibition of Myocyte nNOS With N(4)-(1-imino-3-butenyl)-l-Ornithine (L-VNIO)

L-VNIO is a potent nNOS selective inhibitor. At the concentration used, its Kᵢ for nNOS inhibition is 120-fold lower than for eNOS inhibition. Cohorts of cells taken from the same isolates were either incubated with L-VNIO (100 μM/L) for 30 minutes (in addition, to ensure intracellular access for the drug, 100 μM/L L-VNIO was also added to the pipette solution) or were stored under normal conditions and used as control cells. Iₑᵣ, cell shortening, and SR load were assessed as outlined above.

Results

Contractile Parameters in nNOS+/− Mice

The role of nNOS in regulating cardiac function was first investigated in vivo in anesthetized mice with homoygous deletion of the nNOS gene (nNOS+/−) and their littermate controls. LV ejection fraction measured by transhoracic echocardiography was significantly greater in the nNOS−/− mice than in controls (53.5±2.7% in nNOS−/− versus 44.9±1.5% in controls; P<0.05; Figure 1A). There was no difference in resting heart rate, LV wall thickness, LV mass to body weight ratio, or aortic blood pressure (Table). Hemodynamic measurements showed a trend for a load-independent measure of LV contractility (maximal rate of rise in LV pressure normalized to instantaneous developed pressure, LV dP/dtmax/IP) to be higher in the nNOS−/− animals (P=0.059), and for the τ of LV isovolumetric relaxation to be slower (Table).

This finding of enhanced LV contraction in the nNOS−/− mice was confirmed as a single cell phenomenon in isolated myocyte studies. Cell shortening elicited by a step depolarization to 0 mV was significantly greater in nNOS−/− than in control myocytes over the physiological voltage range (Figures 2B and 2C) were larger in nNOS−/− myocytes. In both protocols, time to 50% relaxation was significantly greater in nNOS−/− myocytes than in controls. Myocyte length and capacitance did not differ between groups (data not shown).

nNOS-Dependent Regulation of [Ca2+]i

To investigate whether an increased SR load might contribute to the enhanced contraction in nNOS−/− myocytes, we measured the integral of the caffeine-induced NCX current (which reflects the amount of Ca2+ load in the SR). Both the amplitude (−1.59±0.6 versus −1.68±0.1 pA/pF; Figure 2A) and integral of the NCX current (0.64±0.03 pC/pF in controls versus 0.78±0.04 pC/pF in nNOS−/−; P<0.05; n=15 and 17, respectively; Figures 2B and 2C) were larger in nNOS−/− than in controls. The rate of decay of the caffeine-induced current was similar in the two groups (359.2±12.2 ms in controls versus 386.3±18.9 ms in nNOS−/−), suggesting similar NCX characteristics in both groups. In addition, the time constant of decline of the caffeine-induced Ca2+ transient in the presence of Ni2+ was similar in control and nNOS−/− myocytes (τ, 6.83±1.2 seconds in control versus 5.13±0.7 seconds in nNOS−/−, n=9 in each group; P=0.25), indicating that there are no significant differences in the slow mechanisms of Ca2+ extrusion between the two groups.

nNOS-mediated regulation of Ca2+ handling was confirmed in indo-1–loaded myocytes. [Ca2+]i transients had significantly greater amplitude in nNOS−/− myocytes com-
either method did not differ between nNOS activity by nNOS-derived NO, as suggested by Xu et al. 8 

the [Ca$^{2+}$], transient was significantly greater in the nNOS$^{-/-}$ myocytes (102±11 ms versus 148±11 ms; *P<0.05; Figure 3B), suggesting that additional mechanisms may be involved.

Calcium Current in nNOS$^{-/-}$ Myocytes

Thus, we investigated whether the increased SR load and contraction might result from modulation of another impor-
tant component of E-C coupling, the Ca\(^{2+}\) current. We found that \(I_{\text{Ca}}\) density was significantly greater in the nNOS\(^{-/-}\) myocytes at voltages from \(-30\) to \(+20\) mV (Figures 4A and 4B). At 0 mV, \(I_{\text{Ca}}\) density was \(-9.1 \pm 0.5\) pA/pF in control myocytes and \(-11.4 \pm 0.5\) pA/pF in nNOS\(^{-/-}\) myocytes \((P<0.01)\). Steady-state activation curves showed that the voltage at which \(I_{\text{Ca}}\) was half-maximally activated was \(-11.2 \pm 0.4\) mV in controls and \(-12.8 \pm 0.6\) mV in nNOS\(^{-/-}\) myocytes \((P=0.04;\) Figure 4C). The slope of activation was unaltered \((5.3 \pm 0.1\) mV in controls versus \(5.2 \pm 0.1\) mV in nNOS\(^{-/-}\)\). Similarly, the voltage at which \(I_{\text{Ca}}\) was half-inactivated was similar in both groups \((-28.5 \pm 0.6\) versus \(-28.7 \pm 0.6\) mV in nNOS\(^{-/-}\), slopes \(4.7 \pm 0.1\) and \(4.4 \pm 0.2\) mV, respectively; \(P=\)NS for both; Figure 4C).

An additional explanation for the increased SR Ca\(^{2+}\) load in nNOS\(^{-/-}\) myocytes can be found by looking at the decay characteristics of \(I_{\text{Ca}}\). The decay of steady-state \(I_{\text{Ca}}\) is best fitted by a double exponential function, yielding a fast and a slow time constant (Figure 4D). The fast time constant was not significantly different between the two groups \((6.08 \pm 0.3\) ms in controls versus \(6.83 \pm 0.5\) ms in nNOS\(^{-/-}\)), but the slow time constant was approximately 38% greater in the nNOS\(^{-/-}\) myocytes \((37.3 \pm 21.5\) versus \(26.9 \pm 1.6\) ms; Figure 4E). The inward current during slow decay of \(I_{\text{Ca}}\) is thought to contribute to Ca\(^{2+}\) loading of the SR,\(^1\) and thus its prolongation in nNOS\(^{-/-}\) myocytes may underlie their enhanced SR Ca\(^{2+}\) content. Moreover, we found that the steady-state current at the end of the pulse was more inward \((-0.32 \pm 0.07\) versus \(-1.02 \pm 0.04\) pA/pF; \(P<0.05\)) and the integral of \(I_{\text{Ca}}\) over the whole pulse (an overall measure of Ca\(^{2+}\) influx) was greater in nNOS\(^{-/-}\) myocytes \((-0.2 \pm 0.01\) versus \(-1.2 \pm 0.01\) pC/pF; \(P<0.05\)) for both. Both of these findings would contribute toward enhancing SR Ca\(^{2+}\) load.

We also examined whether nNOS disruption and the resulting increase in the [Ca\(^{2+}\)], transient were associated with changes in the expression of other Ca\(^{2+}\) cycling proteins in the LV myocardium. We found no differences in the protein level of the \(\alpha\)-subunit of the L-type Ca\(^{2+}\) channel (Figure 3C), NCX, SERCA, and PMCA (data not shown). However, expression of both calsequestrin and the ryanodine receptor Ca\(^{2+}\) release channel (RyR) were increased in the nNOS\(^{-/-}\) myocytes, whereas phospholamban levels were decreased (Figure 3C).

### Does the Increase in \(I_{\text{Ca}}\) in nNOS\(^{-/-}\) Myocytes Underlie the Increase in Cell Shortening?

To test whether the enhanced cell shortening in nNOS\(^{-/-}\) myocytes was predominantly a function of the increased Ca\(^{2+}\) influx via \(I_{\text{Ca}}\), we assessed steady state contraction and \(I_{\text{Ca}}\) after disabling the SR with thapsigargin. Cell shortening...
state activation and inactivation curves for 0.5% versus 2.9% H2O/H2O.
P in controls (*) did not differ between control (open triangles) and nNOS I steady-state.

Under these conditions, the increase in contraction in nNOS I myocytes may result from either a greater Ca2+ influx or an increase in the myofilament sensitivity to Ca2+. Activation curves, expressed as relative conductance, show a small negative shift in the voltage of half-maximal activation between nNOS I (filled squares) and control myocytes (open triangles). Inactivation curves, expressed as relative current, did not differ between control (open triangles) and nNOS I myocytes (filled circles). D, Example records from steady-state Ica recordings during a 200-ms depolarizing step from −40 to 0 mV. To illustrate that deactivation of Ica is slower in nNOS−/− myocytes than in controls, peak current amplitudes for each myocyte have been normalized to 1. E, Decay of Ica was best fitted by a double-exponential function. Average results show that the fast component of decay was not significantly different, whereas the slow time constant was significantly greater in nNOS−/− myocytes than in controls (*P<0.05).

remained larger in nNOS−/− myocytes than in controls (4.6±0.5% versus 2.9±0.3%; *P<0.05; Figure 5A) in the presence of thapsigargin, but the time to 50% relaxation was no longer prolonged in the nNOS−/− myocytes (174.8±5.7 ms in control versus 157.4±5.8 ms in nNOS−/−; *P=0.05). Under these conditions, the increase in contraction in nNOS−/− myocytes may result from either a greater Ca2+ influx or an increase in the myofilament sensitivity to Ca2+. We found that Ca2+ influx, measured by integrating the steady-state Ica over the whole pulse was larger in nNOS−/− than in controls (−0.22±0.02 versus −0.15±0.02 pC/pF; Figure 5B; *P<0.05). Moreover, the slow component of Ica decay was still prolonged in nNOS−/− myocytes after thapsigargin (42.5±2.2 ms in controls versus 36.5±5.1 ms in nNOS−/−; *P<0.05), indicating that the difference in Ica inactivation kinetics in nNOS−/− was not dependent on SR Ca2+. An index of myofilament sensitivity to Ca2+ was estimated by plotting the relationship between cell shortening and the integral of Ica in the presence of thapsigargin (Figure 5C). The slope of this relationship did not differ between nNOS−/− and controls, suggesting that under these conditions an increase in Ca2+ influx is mainly responsible for the increased contraction in nNOS−/− myocytes.

Figure 4. Calcium currents from nNOS−/− myocytes are larger and show slower inactivation. A, Example records of Ica (in pA/pF) elicited by a 200 ms depolarizing step from −40 to 0 mV in control and nNOS−/− myocytes. B, Current-voltage relationship shows Ica density is greater in nNOS−/− myocytes (filled squares) than in controls (open circles) over the voltage range −30 to +20 mV (*P<0.05, n=16 and 21, respectively). C, Steady-state activation and inactivation curves for Ica. Activation curves, expressed as relative conductance, show a small negative shift in the voltage of half-maximal activation between nNOS−/− (filled squares) and control myocytes (open triangles). Inactivation curves, expressed as relative current, did not differ between control (open triangles) and nNOS−/− myocytes (filled circles). D, Example records from steady-state Ica recordings during a 200-ms depolarizing step from −40 to 0 mV. To illustrate that deactivation of Ica is slower in nNOS−/− myocytes than in controls, peak current amplitudes for each myocyte have been normalized to 1. E, Decay of Ica was best fitted by a double-exponential function. Average results show that the fast component of decay was not significantly different, whereas the slow time constant was significantly greater in nNOS−/− myocytes than in controls (*P<0.05).

Effect of Acute nNOS Inhibition With L-VNIO on Cell Shortening, Ica, and SR Load
Our findings in nNOS−/− mice suggest that cardiac nNOS is an important determinant of basal contractility and Ca2+ in the mammalian myocardium. However, the potential contribution of compensatory mechanisms secondary to chronic and systemic disruption of nNOS is difficult to assess in this model. To circumvent this problem, we also tested the effect of acute pharmacological inhibition of nNOS with L-VNIO (100 μmol/L)5 in LV myocytes from control mice. Results were qualitatively similar to those seen in myocytes from nNOS−/− mice. Steady-state cell shortening was significantly increased in myocytes incubated with L-VNIO compared with control (10.3±0.7% versus 8.2±0.6%; *P<0.05; Figures 6A and 6B). The increase in cell shortening was associated with a 23% increase in the density of the steady-state Ica (Figures 6C and 6D). Fitting the decay of Ica with a double exponential showed an increase in the slow time constant (control 25.9±0.8 ms versus L-VNIO 30.5±1.6 ms; *P<0.05), whereas there was no difference in the fast time constant. In addition, the assessment of SR load was significantly greater in L-VNIO exposed cells than in control (Figures 6E and 6F).
Figure 5. Cell shortening is enhanced in nNOS−/− myocytes in the presence of thapsigargin. A, Example records of unloaded cell shortening (expressed as percent resting cell length) elicited by a 200-ms depolarizing step from −40 to 0 mV in control and nNOS−/− myocytes in the presence of thapsigargin. Cell shortening was significantly greater in nNOS−/− myocytes than in controls (n=8 and 9, respectively). B, Example records showing that ICa density recorded concurrently in the presence of thapsigargin was greater in nNOS−/− myocytes than in control myocytes. C, Relationship between cell shortening and the integral of ICa under these conditions shows no difference between nNOS−/− and control myocytes.

Discussion

Until recently, eNOS was thought to be the sole isoform constitutively expressed in ventricular myocytes and thus the source of NO involved in the autocrine regulation of myocardial contraction and Ca2+ homeostasis. Emerging evidence, however, indicates that nNOS is present in the cardiac SR, suggesting that this isoform may modulate ion channels/transporters involved in Ca2+ cycling and contraction. In the present study, we have demonstrated that nNOS gene disruption as well as pharmacological inhibition of nNOS significantly enhances contraction and [Ca2+] transient in isolated LV myocytes. In addition, we found that LV ejection fraction was significantly increased in nNOS−/− mice, and there was a trend for LV dp/dt max to be increased. These data indicate that cardiac nNOS plays a significant role in the autocrine control of cardiac contractility by regulating Ca2+ fluxes.

An increased basal LV dp/dt max/IP in nNOS−/− mice compared with C57Bl/6 mice has also been reported by Barouch et al., although they found no difference in an alternative index of LV systolic performance. These authors, however, did not see a significantly greater basal contraction or [Ca2+] transient in nNOS−/− LV myocytes in experiments presumably performed at room temperature. Conversely, both the present data and those of Ashley et al in field-stimulated LV cardiomyocytes show that at 35°C contraction is enhanced in nNOS−/−.

Several proteins involved in E-C coupling are potential targets for NO modulation. Xu et al suggested that nNOS-derived NO might inhibit the activity of the SR Ca2+-ATPase. The increased SR Ca2+ load in nNOS−/− myocytes and control myocytes after L-VNIO would be consistent with this hypothesis. However, if the main action of cardiac nNOS were to inhibit the SR Ca2+-ATPase, we would expect the speed of relaxation and the rate of decay of the [Ca2+] transient to be increased in nNOS−/− myocytes, as the re-uptake of Ca2+ into the SR would occur more quickly. In addition, a greater peak [Ca2+], transient is thought to result in faster kinetics of [Ca2+] decline. On the contrary, we found a slower time to 50% relaxation and a prolonged time-course of decay of the [Ca2+] transient in nNOS−/− myocytes, indicating that other mechanisms are likely to be involved.

A potential alternative target for NO is the cardiac ryanodine receptor Ca2+ release channel (RyR), which has been shown to be both activated and inhibited by NO. We found that the time to peak [Ca2+] was similar in nNOS−/− and in control LV myocytes, despite the increase in SR Ca2+ load and RyR protein expression in the nNOS−/− myocytes, both of which might be expected to result in a faster time to peak [Ca2+]. Given the inherent limitations of inferring the function of calcium transport/release systems from the kinetics of calcium transients of differing sizes, it is difficult to draw firm conclusions on this issue. However, our findings are in keeping with the notion that RyR activity could be inhibited in nNOS−/− myocytes, supporting the model put forward by Xu et al who proposed that NO may modulate E-C coupling via two discrete mechanisms: (1) a tonic inhibition of ICa and (2) an increase in RyR open probability. In basal conditions (ie, in the absence of agonists or mechanical stimuli) both effects appear to be dependent on nNOS. Our data also indicate that the nNOS-mediated changes in ICa have a greater influence on contraction, in agreement with recent findings by Trafford et al who demonstrated that sustained changes in the open probability of RyR only cause transient effects on [Ca2+] and cell shortening in rat LV myocytes.

Protein level and activity of the NCX (evaluated by the time course of decay of the caffeine-induced NCX current) did not differ between nNOS−/− and controls, nor were the protein levels of SERCA different between these two groups. However, we did find an increase in the Ca2+ store protein calsequestrin in nNOS−/− and a reduction in phospholamban. These changes are likely to be compensatory and of uncertain functional significance because acute NOS inhibition is sufficient to reproduce the nNOS−/− phenotype.

Because there is close spatial juxtaposition of L-type Ca2+ channels and junctional SR, nNOS-derived NO from the SR may also affect the L-type calcium channel. There are only a few reports on the effect of NOS inhibition on basal ICa in mammalian ventricular myocytes and the findings are inconsistent. In mouse ventricular myocytes at room temperature Vandecastelee et al found no effect of L-NMMA on basal ICa. However, Gallo et al saw a large stimulatory effect of L-NMMA and L-NA on guinea-pig ventricular ICa, at 35°C, suggesting that NO may exert a tonic inhibition of ICa. No difference in basal ICa has ever been reported after eNOS gene...
disruption. 4,26 We found an increased Ca density both in nNOS−/− and after L-VNIO, suggesting that an increase in Ca2+ influx may contribute to the enhanced myocyte contraction after nNOS disruption. Experiments showing that Ica and contraction remained greater in nNOS−/− myocytes after disabling the contribution of the SR with thapsigargin further support a predominant effect of nNOS on the Ca2+ channel. nNOS-derived NO could be acting via the guanylate cyclase/cyclic GMP second messenger pathway, as suggested by Gallo et al.25 In agreement with these findings, 8-BrcGMP has been shown to inhibit Ica in ferret right ventricular myocytes,27 and protein kinase G has been reported to cause a 40% inhibition of Ica in rat ventricular myocytes.28 However, it is also possible that the mechanism of action of nNOS-derived NO on the Ca2+ channel is unclear but it may involve a change in the phosphorylation state of the Ca2+ channel.32,33 Because the slow component of the time constant of decay is still prolonged in the nNOS−/− myocytes in the presence of thapsigargin, this effect does not appear to be dependent on SR load and it is likely to reflect NO-mediated regulation of the Ca2+ channel itself.

Stimulation of endothelial NO production induces earlier onset of myocardial relaxation with little or no effect on force of contraction in a variety of preparations.2 In rat ventricular myocytes, 8-Brcyclic GMP causes a small reduction in cell shortening and an earlier onset of relaxation in the absence of changes in the intracellular Ca2+ transient, suggesting that endogenous NO may reduce myofilament Ca2+ sensitivity via cyclic GMP.2 We explored this possibility by evaluating the relationship between cell shortening and the integral of Ica in the presence of thapsigargin (which disables the SR). Under these conditions, the relationship between Ca2+ entry and cell shortening is governed by myofilament sensitivity to Ca2+. Our finding that this relationship does not differ between nNOS−/− and control myocytes suggests that nNOS-derived NO has no significant effect on myofilament Ca2+ sensitivity. Disabling the SR also abolished the difference in time to 50%
relaxation between nNOS−/− and control myocytes, suggesting that slower relaxation and prolonged [Ca2+]i transients in nNOS−/− mice may result from inhibition of SR Ca2+ uptake, as has recently been demonstrated by Zhou et al.34

In conclusion, our results demonstrate that nNOS-derived NO regulates myocardial contraction and Ca2+ transients by controlling Ca2+ handling in LV myocytes. We have shown that gene-disruption or acute inhibition of nNOS increases Ca2+ influx both by increasing ICa, density and by slowing ICa, inactivation, leading to greater loading of the SR stores and hence greater Ca2+-induced Ca2+ release. This novel finding challenges the assumption that the only NOS isoform involved in the control of cardiac function is eNOS and as such may stimulate a re-examination of the data in this field. It is conceivable that nNOS plays a negative feedback role in preventing [Ca2+]i overload (particularly in the presence of submaximal β-adrenergic stimulation38), as increases in [Ca2+]i stimulate nNOS synthesis of NO, which in turn acts to inhibit Ca2+ fluxes. Such mechanisms may contribute to the maintenance of a tight control of intracellular Ca2+ in physiological conditions and may protect against the development of triggered arrhythmias in cardiovascular disease.

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In vivo measurements of LV function

Mice were anaesthetized at 5% and maintained with 1% isoflurane in oxygen, with body temperature regulated at 37°C. The right common carotid artery was dissected free and the distal portion occluded. Animals were allowed to equilibrate until hemodynamic indices were stable (up to 30 minutes); measurements were recorded on a Powerlab 4SP data recorder (AD Instruments) and stored digitally for later analysis. LV ejection fraction was calculated as: (end diastolic area) - (end systolic area) / (end diastolic area).

Myocyte isolation and techniques

To isolate LV myocytes the heart was initially perfused for 3 minutes with a nominally Ca\(^{2+}\)-free solution, followed by a further 7-8 minutes with an enzyme-containing solution (collagenase, 1 mg.ml\(^{-1}\), Worthington Biochemical Co., Ca\(^{2+}\) 0.05 mMol/L). The LV free wall was isolated and placed in a separate flask containing fresh enzyme solution. Myocytes were harvested following further five and ten minute digestion periods, washed twice and re-suspended in Dulbecco’s modified Eagle medium, supplemented with 2 mg/ml Ultraser G\(^{TM}\) (Gibco PRL, Paisley, Scotland). The myocyte suspension was stored at room temperature and cells were used within 8 hours of isolation.

In 86 LV myocytes the microelectrode resistance was 2.04±0.07 MΩ and series resistance was 5.34±0.2 MΩ (after membrane rupture); 70% series resistance correction and capacity compensation was routinely achieved. Peak inward calcium current (\(I_{ca}\)) was measured with respect to the current at the end of the step depolarization. Specific conductance (\(g\)) was calculated from \(g = I/(V_m - V_{rev})\) where \(I\) is the current amplitude, \(V_m\) is membrane voltage and \(V_{rev}\) the reversal potential. For each cell, the reversal potential
of $I_{Ca}$ was calculated from the current-voltage relationship (67.75±0.5 mV in controls, and 64.2±1.0 mV in nNOS $^{-/-}$, $P$=ns, n=16 and 21, respectively). Steady-state activation curves for each cell were constructed by plotting normalized conductance ($g/g_{max}$) as a function of membrane voltage. Data points were fitted to a Boltzmann function $g/g_{max} = 1/(1 + \exp((V_h - V_m)/k))$, where $g/g_{max}$ is the normalized conductance recorded at membrane voltage $V_m$, $V_h$ is the potential at which the current is half maximally activated and $k$ is the slope factor. Steady-state inactivation curves were constructed by plotting relative current amplitude obtained using a standard double-pulse protocol. The decay of $I_{Ca}$ was best fitted by a double exponential function, yielding a fast and a slow time constant.

The composition of the solution used during the measurements of $I_{Ca}$ and cell shortening contained in mMol/L: NaCl 134, KCl 5.4, MgCl2 1.2, CaCl2 1.8, glucose 11.1, HEPES 5, 4-AP 1, pH 7.4, NaOH. The patch electrode solution to measure $I_{Ca}$ contained (mMol/L): CsCl 110, TEA-Cl 20, HEPES 10, NaCl 10, MgCl2 1, MgATP 5, pH 7.2, CsOH.

**Measurement of LV myocyte contraction and calcium current**

$I_{Ca}$ and cell shortening were elicited by 200 ms step depolarizations from a holding potential of −40mV to test potentials ranging from −50 to +80 mV. 1 mMol/L 4-aminopyridine (4-AP) was used to block $I_o$. Steady-state $I_{Ca}$ and contraction parameters were also assessed during a step to 0 mV at a stimulation frequency of 1 Hertz (Hz). Measurements were taken from the average of 5 steady-state $I_{Ca}$ and contractions. Edge-detection algorithms allowed derivation of contractile parameters. Cell membrane capacitance ($C_m$) in all cells was measured by applying a −10 mV pulse, 50 ms in
duration, from a holding potential of –40 mV. The current transient was fitted to a mono-
exponential; the time course of decay of the current transient is related to the cell
capacitance. For each cell calcium current was normalized to capacitance and expressed
as current density in pA/pF.

**Assessment of SR Ca\(^{2+}\) load**

Steady-state conditions prior to the caffeine pulse were achieved by a train of 20
depolarizing pulses (200 ms depolarization from –45 mV to 0 mV).

**Measurement of calcium transients**

Excitation light (wavelength 355 nm) from a xenon arc lamp (75 W) was delivered to the
cell via a 100x oil immersion objective (Leica). Two photomultiplier tubes (Cairn)
collected emitted fluorescence at 410 and 495 nm. Background fluorescence (recorded in
the absence of a cell) was subtracted from the 410 and 495 signals, and the \([\text{Ca}^{2+}]_i\)
transient displayed as the 410/495 ratio.

**Immunoblotting**

A LV membrane subfraction was prepared as in Ashley et al. \(^1\) Following calibration to
confirm the linearity of the loading range for each protein/antibody combination, equal
amounts of protein samples (7.5-75 µg, Bradford Assay) were separated on
polyacrylamide-SDS gels and transferred to nitrocellulose membranes for Western
blotting with specific antibodies. Bands were visualized by chemiluminescence
(SuperSignal Substrate kit, Pierce Chemical) then quantified using the NIH Imaging
Program. Values were expressed in arbitrary units after subtraction of background and normalization to the GAPDH signal (Chemicon) where appropriate.

References