Supplementary Material and Methods

Myocyte isolation

Left ventricular (LV) myocytes were isolated using a standard enzymatic dispersion technique \(^1\). Briefly, the heart was initially perfused for 3 minutes with a nominally Ca\(^{2+}\)-free solution, followed by a further 8-9 minutes with an enzyme-containing solution (collagenase, 1 mg.ml\(^{-1}\), Worthington Biochemical Co., Ca\(^{2+}\) 0.05 mmol/L). Unless specified otherwise, myocytes were superfused with a modified Tyrode solution containing in mmol/L: NaCl 134, KCl 5.4, MgCl\(_2\) 1.2, CaCl\(_2\) 1.4, glucose 11.1, HEPES 5, pH 7.4, NaOH. 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 storage solution (in mmol/L; NaCl 120, KCl 5.4, MgSO\(_4\) 5, CaCl\(_2\) 0.2, Na-pyruvate 5, glucose 20, taurine 20, Hepes 10, pH 7.4, NaOH). The myocyte suspension was stored at room temperature and cells were used within 8 hours of isolation.

Measurement of LV myocyte contraction & [Ca\(^{2+}\)]\(_i\) transients

Cell length was measured in LV myocytes field-stimulated at 3 Hz by using a video-edge detection system (IonOptix Corp). Cell shortening and the time to 50% relaxation (TR\(_{50}\)) were compared in myocytes from nNOS\(^{-/-}\) mice and their wild type littermates. Contribution of SR on cell shortening and TR\(_{50}\) was assessed by using 10 µmol/L thapsigargin (TG) to irreversibly inhibit SERCA2a \(^1\).

The [Ca\(^{2+}\)]\(_i\) transient was evaluated in Fura-2-loaded (5 µmol/L, Molecular Probes) LV myocytes field-stimulated at 3Hz or exposed to caffeine (10 mmol/L for 10 s) after loading the SR by a train of more than 50 stimuli at 3 Hz. Fluorescence was excited at 365 and 380 nm and monitored at 510 nm (IonOptix Corp) and the amplitude of the [Ca\(^{2+}\)]\(_i\) transient was calculated as the difference between diastolic and peak Ca\(^{2+}\) fluorescence.

Calibration of the Fura-2 signal using the method of Chorvatova & Hussain \(^2\) showed that average diastolic and peak [Ca\(^{2+}\)]\(_i\), approximated to 117 nmol/L and 392 nmol/L in nNOS\(^{+/+}\) myocytes and 139 nmol/L and 602 nmol/L in nNOS\(^{-/-}\) myocytes.
The decay of the field-stimulated \([\text{Ca}^{2+}]_i\) transient was best fit by a double exponential \((\text{Clampfit, Axonpatch, 200A, Axon Instruments})\) and \(\tau_1\) was used for comparisons between genotypes. The decay of caffeine-induced \([\text{Ca}^{2+}]_i\) transient, which was fit by a single exponential using the IonOptix software, was used to investigate SR-independent mechanisms of \([\text{Ca}^{2+}]_i\) decay (mostly the \(\text{Na}^+\)-\(\text{Ca}^{2+}\) exchanger) \(^3\).

Since differences in action potential (AP) duration and \([\text{Ca}^{2+}]_i\) transient amplitude have been shown to influence \(\text{TR}_{50}\) \(^4\) and the rate of decay of the \([\text{Ca}^{2+}]_i\) transient (\(\tau\)) \(^5\), these parameters were compared after matching \([\text{Ca}^{2+}]_i\) transient amplitude in voltage-clamped myocytes (stimulated by a 25 ms depolarization pulse from -70 to +20 mV at 3 Hz) by lowering the extracellular concentration of \(\text{Ca}^{2+}\) to 0.9 mmol/L in nNOS\(^{-/-}\) myocytes or in nNOS\(^{+/+}\) myocytes after nNOS-specific inhibition with vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine, (L-VNIO, 100 \(\mu\)mol/L, Alexis). However, cell shortening or \([\text{Ca}^{2+}]_i\) transient amplitude data were compared between voltage-clamped myocytes exposed to the same extracellular Ca\(^{2+}\) concentration of 1.4 mmol/L. Pipette solution contained in mmol/L: 120 K aspartate, 20 KCl, 10 Hepes, 5 MgATP, 10 NaCl, pH adjusted to 7.2 using KOH.

It should be noted that acute nNOS inhibition with L-VNIO or SMTC (S-methyl-L-thiocitrulline) reproduce the phenotype of nNOS\(^{-/-}\) myocytes only if the inhibitor is added both in the perfusing solution and dialyzed into the cell through the patch pipette \(^1\). In our hands, 30 minutes incubation in a solution containing a nNOS inhibitor did not increase basal cell shortening but significantly prolonged relaxation \(^6\). Because we were predominantly interested in the latter, we compared TR\(_{50}\) in field-stimulated PLN\(^{+/+}\) myocytes after extracellular application of SMTC (100 nmol/L for 30 min).

**Immunoblotting**

Immunoblots were performed both in LV homogenates and isolated myocytes lysates \(^7\) from nNOS\(^{-/-}\) and nNOS\(^{+/+}\) mice using specific antibodies against SERCA2a, protein phosphatase 1 (PP1, Upstate) and 2A (PP2A, Santa Cruz) total PLN (Affinity Bioreagents) and its Ser\(^{16}\) (Upstate) or Thr\(^{17}\) (Cyclacel) phosphorylated fractions, total RyR (Affinity Bioreagents) and its Ser\(^{2809}\) phosphorylated fraction (Badrilla). Troponin I phosphorylation in
LV tissue homogenates was investigated using phosphorylation-independent and phosphorylation-specific monoclonal antibodies (mAbs 19 and 14, respectively, a kind gift from Prof. Ian Trayer, University of Birmingham, UK), as described previously. Evaluation of the phosphorylated fraction of PLB, RyR and TnI was carried out in the same membrane after stripping. PLB and RyR phosphorylation was also evaluated in LV homogenates from eNOS+/+ and eNOS−/− mice and nNOS+/+ mice incubated for 30 minutes with the nNOS-specific inhibitor S-methylthiocitrulline (SMTC, 100 nmol/L, Sigma). The lysis buffer contained (mmol/L) 50 Tris-HCl (pH 7.4), 100 NaCl, 1% Triton X-100, 50 NaF, 5 EDTA, 40 β-glycerophosphate, 0.2 ortho-vanadate, 0.001 aprotinin and 0.1 leupeptine.

The effect of isoproterenol on PLB phosphorylation was evaluated by immunoblotting the membrane fraction of LV myocytes that had been exposed to ISO (1 μmol/L, Sigma) for 5 minutes. In some experiments, PLB phosphorylation was evaluated in lysates of LV myocytes that had been incubated in a storage solution containing oxypurinol (100 μM, Sigma) or okadaic acid (OA; 2 μmol/L) for 30 min.

**Phosphatase assay**

LV myocytes were homogenised for 30 seconds using a Polytron PT1200 in a buffer solution containing (mmol/L): 50 Tris-HCl (pH 7.5), 10 MgCl₂, 50 KCl supplemented with protease inhibitors tosyl-L-arginine methyl ester (TAME, 10μg/ml), tosyl-l-lysine chloromethyl ketone (TLCK, 5 μg/ml) and phenyl methylsulfonyl fluoride (PMSF, 0.5 mmol/L). After centrifugation at 15800 × g for 5 minutes, the supernatant was removed and protein concentration was determined using the BCA assay (Pierce).

Purified recombinant human cardiac troponin I (TnI) was phosphorylated with γ³²P-ATP using the catalytic subunit of cAMP-dependent kinase (Sigma) to a specific activity of approximately 2000 cpm/pmol. Phosphatase activity was measured using 0.005 – 0.02 μg/ml extract in the presence of 2 μmol/L ³²P-TnI in phosphatase buffer at 37°C. Reactions were stopped and TnI precipitated by the addition of trichloracetic acid to 13.3%. The insoluble protein was removed by centrifugation at 15800 × g for 5 minutes and the liberated ³²P
phosphate determined by scintillation counting of the supernatant. TnI has been shown to be a good *in vitro* substrate for both PP1 and PP2a \(^{11,12}\).

**Protein phosphatase 1 inhibition assay**

Endogenous protein phosphatase inhibitor 1 (I-1) and 2 (I-2) were enriched from wild-type and nNOS\(^{-/-}\) hearts (n=5 each) using an extraction protocol described in detail previously \(^{13}\). The activity of endogenous protein phosphatase inhibitors was estimated by using a colorimetric type 1 phosphatase activity (PP1) inhibition assay (recombinant PP1-mediated dephosphorylation of the chromogenic substrate p-Nitrophenyl Phosphate, Sigma). The concentration of recombinant PP1 (0.12 µg) was adjusted to achieve ~80% dephosphorylation of 10 pmol p-Nitrophenyl Phosphate. Measurements were performed continuously over 30 min on a TECAN multi-well reader at 405 nm.

**Chemicals**

1H-[1,2,4]oxidazolo[4,3-a]quinoxalin-1-one (ODQ, 10µmol/L, Sigma) and Rp-8-Br-cGMP (100µmol/L, Merck Biosciences) were used to inhibit soluble guanylate cyclase and protein kinase G, respectively; cilostamide (1µmol/L,Sigma) was used to inhibit the cGMP-inhibited phosphodiesterase (PDE3). PKI (6-22 Amide, 1µmol/L and membrane permeable inhibitor amide 14-22, 1.7 µmol/L, Merck Biosciences) were used to inhibit PKA activity. Okadaic acid (OA, 10nmol/L, Sigma) and I-2 (500 nmol/L, Upstate) were used to evaluate the effects of PP2A and PP1 inhibition, respectively, in the regulation of myocyte relaxation in nNOS\(^{-/-}\) and nNOS\(^{+/+}\) mice.

**Statistics**

Data are expressed as mean ± s.e.m. and *n* indicates the number of LV myocytes used. For all comparisons, cells were obtained from a minimum of two hearts per genotype per protocol. Data were analyzed using *ANOVA* (Statview for Windows, SAS, version 5.0.1). A value of *P*<0.05 was considered to be statistically significant.
References


Supplementary Figures Legends

**Suppl. Figure I.**
Disabling the SR abolishes the differences in TR50 and tau between nNOS^{+/+} and nNOS^{-/-} myocytes. **A.** Representative raw traces of cell shortening in the presence of thapsigargin (TG) in nNOS^{-/-} and nNOS^{+/+} myocytes. Average data (lower panel) show that TR_{50} is no longer prolonged in the presence of TG. **B.** Representative caffeine-induced [Ca^{2+}]_{i} transients in nNOS^{-/-} and nNOS^{+/+} myocytes. Average data (lower panel) indicate that the tau of caffeine-induced [Ca^{2+}]_{i} transient is not different between nNOS^{+/+} and nNOS^{-/-}.

**Suppl. Figure II.**
**A.** Representative immunoblots and average Ser^{16}-phosphorylated and total PLN (blotted after stripping the membrane) in LV myocytes lysates from nNOS^{-/-} and nNOS^{+/+} mice (n=3 hearts in each group, * P=0.01). **B.** Ser^{16} or Thr^{17} phosphorylated PLN and total PLN in LV homogenates from eNOS^{-/-} and eNOS^{+/+} mice (n=3 hearts in each group). Averaged data show that PLN phosphorylation is not affected in LV homogenates from eNOS^{-/-} mice.

**Suppl. Figure III.**
**A.** Total and phosphorylated TnI in LV myocytes from nNOS^{-/-} and nNOS^{+/+} mice (n=3 hearts in each group). Average data show that TnI phosphorylated fraction is significantly reduced in nNOS^{-/-} mice (P=0.03). **B.** Total and Ser^{16} phosphorylated and total PLN in LV myocytes from nNOS^{+/+} incubated for 30 minutes in the presence or absence of SMTC (100 nmol/L) after exposure to isoproterenol 1 µmol/L for 3 minutes (n=3 hearts in each group). Average data show that PLN Ser^{16} phosphorylated fraction is not different after nNOS inhibition in the presence of β-adrenergic stimulation.
C. Incubation with SMTC significantly decreased RyR phosphorylation in nNOS\(^{+/+}\) myocyte lysates (n=3 hearts in each group, \(P=0.02\)). Similar results were obtained when nNOS\(^{-/-}\) myocytes were compared to wild type (not shown); however, as reported previously\(^1\), total RyR was significantly increased in nNOS\(^{-/-}\) myocytes compared with nNOS\(^{+/+}\).

**Suppl. Figure IV.**

Pretreatment with the XOR inhibitor, oxypurinol (100 \(\mu\)mol/L), did not affect PLN phosphorylation in nNOS\(^{-/-}\) (A) or nNOS\(^{+/+}\) (B) LV myocytes, (n=6 for each group and \(P>0.05\)).

C. Pretreatment of LV myocytes with oxypurinol (100 \(\mu\)mol/L) significantly prolonged TR\(_{50}\) in both groups, probably because of the Ca\(^{2+}\) sensitizing effect of this compound (nNOS\(^{-/-}\): n=19 myocytes in Ctr vs. n=12 myocytes incubated with OXY, \(P=0.01\); nNOS\(^{+/+}\): n=27 in Ctr vs. n=10 with OXY, \(P=0.02\); \(P=0.62\) for the interaction between the effect of oxypurinol and the genotype).

**Suppl. Figure V.**

Immunoblots using specific antibodies for PP1 and PP2A in nNOS\(^{+/+}\) and nNOS\(^{-/-}\) LV myocytes (from 3 hearts in each group). The expression of PP1 or PP2A is not affected by nNOS gene deletion.
Suppl. Fig. I

A

nNOS^{+/+}  nNOS^{-/-}

Thapsigargin

\[ \text{% cell shortening} \]

\[ \begin{array}{c}
0 \\
1 \\
2 \\
3 \\
4 \\
5 \\
\end{array} \]

\[ \begin{array}{c}
100 \text{ ms} \\
200 \text{ ms} \\
300 \text{ ms} \\
400 \text{ ms} \\
500 \text{ ms} \\
600 \text{ ms} \\
\end{array} \]

Thapsigargin 10 $\mu$mol/L

P=NS

nNOS^{-/-}  nNOS^{+/+}

Caffeine 10 mmol/L, 10s

P=NS

B

nNOS^{+/+}  nNOS^{-/-}

Amplitude of [Ca] transient

(F365/F380 ratio)

\[ \begin{array}{c}
0.0 \\
0.2 \\
0.4 \\
0.6 \\
0.8 \\
\end{array} \]

Caffeine

Caffeine

1 sec

nNOS^{-/-}  nNOS^{+/+}

Amplitude of [Ca] transient

(F365/F380 ratio)

\[ \begin{array}{c}
0.0 \\
0.2 \\
0.4 \\
0.6 \\
0.8 \\
1.0 \\
1.2 \\
\end{array} \]

P=NS
Suppl. Fig. II

A

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B

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Ser^{16}-PLN/PLN

Thr^{17}-PLN/PLN
Suppl. Fig. III

A

\[ \text{A} \]

<table>
<thead>
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B

\[ \text{B} \]

ISO 1\(\mu\text{mol/L}\)

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C

\[ \text{C} \]

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Suppl. Fig. IV

A

nNOS\(^{++/+}\)


c

Ser\(^{16}\) PLN

PLN

GAPDH


nNOS\(^{-/-}\)

Ctr OXY Ctr OXY Ctr OXY Ctr OXY


B

nNOS\(^{++/+}\)

\begin{align*}
\text{Ser}^{16}\text{PLN/PLN} & \\
\text{OXY} & \\
\end{align*}


nNOS\(^{-/-}\)

\begin{align*}
\text{Ser}^{16}\text{PLN/PLN} & \\
\text{OXY} & \\
\end{align*}


C

\begin{align*}
\text{nNOS}^{++/+} & \\
\text{Ctr} & \text{OXY} & \\
\text{nNOS}^{-/-} & \\
\text{Contrast} & \text{OXY} & \\
\end{align*}

TR\(_{50}\) (ms)
Suppl. Fig. V

nNOS\(^{+/+}\) nNOS\(^{-/-}\)

PP1

PP2A

GAPDH

\[\begin{array}{c}
\text{PP1} \\
\text{PP2A} \\
\text{GAPDH}
\end{array}\]

\[\begin{array}{c}
nNOS^{+/+} \\
nNOS^{-/-}
\end{array}\]

\[\begin{array}{c}
\text{PP2A/GAPDH} \\
\text{PP1/GAPDH}
\end{array}\]

\[\begin{array}{c}
0.8 \\
0.6 \\
0.4 \\
0.2 \\
0
\end{array}\]

\[\begin{array}{c}
nNOS^{+/+} \\
nNOS^{-/-}
\end{array}\]