Cardiac Nitric Oxide Synthase 1 Regulates Basal and β-Adrenergic Contractility in Murine Ventricular Myocytes

Euan A. Ashley, MRCP; Claire E. Sears, DPhil; Simon M. Bryant, PhD; Hugh C. Watkins, MD, PhD, FRCP; Barbara Casadei, MD, DPhil, FRCP

Background—Evidence indicates that myocardial NO production can modulate contractility, but the source of NO remains uncertain. Here, we investigated the role of a type 1 NO synthase isoform (NOS1), which has been recently localized to the cardiac sarcoplasmic reticulum, in the regulation of basal and β-adrenergic myocardial contraction.

Methods and Results—Contraction was assessed in left ventricular myocytes isolated from mice with NOS1 gene disruption (NOS1−/− mice) and their littermate controls (NOS1+/+ mice) at 3 stimulation frequencies (1, 3, and 6 Hz) in basal conditions and during β-adrenergic stimulation with isoproterenol (2 nmol/L). In addition, we examined the effects of acute specific inhibition of NOS1 with vinyl-L-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO, 500 μmol/L). NOS1−/− myocytes exhibited greater contraction at all frequencies (percent cell shortening at 6 Hz, 10.7±0.9% in NOS1−/− myocytes versus 7.21±0.8% in NOS1+/+ myocytes; P<0.05) with a flat frequency-contraction relationship. Time to 50% relaxation was increased in NOS1−/− myocytes at all frequencies (at 6 Hz, 26.53±1.4 ms in NOS1−/− myocytes versus 21.27±1.3 ms in NOS1+/+ myocytes; P<0.05). L-VNIO prolonged time to 50% relaxation at all frequencies (at 6 Hz, 21.28±1.7 ms in NOS1+/+ myocytes versus 26.45±1.4 ms in NOS1+/+ +L-VNIO myocytes; P<0.05) but did not significantly increase basal contraction. However, both NOS1−/− myocytes and NOS1+/+ myocytes treated with L-VNIO showed a greatly enhanced contraction in response to β-adrenergic stimulation (percent increase in contraction at 6 Hz, 25.2±10.8 in NOS1+/+ myocytes, 68.2±11.2 in NOS1−/− myocytes, and 65.1±13.2 in NOS1+/+ +L-VNIO myocytes; P<0.05).

Conclusions—NOS1 disruption enhances basal contraction and the inotropic response to β-adrenergic stimulation in murine ventricular myocytes. These findings indicate that cardiac NOS1-derived NO plays a significant role in the autocrine regulation of myocardial contractility. (Circulation. 2002;105:3011–3016.)

Key Words: nitric oxide synthase • myocytes • contractility • receptors, adrenergic, beta

Despite much investigation, the role of endogenous NO production in the regulation of cardiac contractility remains controversial. For example, application of the NO synthase (NOS) inhibitor nitro-L-arginine to rat left ventricular (LV) myocytes led to no change in the amplitude or cell shortening in one study,1 whereas in another study,2 contraction was increased. Similarly, NOS inhibition has been shown to enhance the inotropic response to β-adrenergic stimulation in isolated myocytes (see review)3. However, no difference in the response to isoproterenol was observed in papillary muscles from mice with selective disruption of the gene encoding the endothelial isoform of NOS (or NOS3).4 In humans, recent work using an intracoronary infusion of L-NAME-monomethyl-L-arginine suggests that endogenous (mostly endothelium-derived) NO may exert a small positive inotropic effect.5 Part of the explanation for these inconsistent results may be found in the differences between experimental preparations (eg, isolated myocytes versus whole-heart or in vivo studies), in the lack of isoform specificity of most NOS inhibitors,6 and in the recent discovery of subcellular localization of different NOS isoforms.7,8 Indeed, in addition to the well-established paracrine effects of endothelial or endocardial NOS3-derived NO on ventricular function,9 emerging evidence indicates that intracellular compartmentalization of NOS isoforms within cardiac myocytes may play a complex and previously unrecognized role in the autocrine regulation of myocardial function.7,8,10 In particular, the recent finding of a neuronal-type NOS1 isoform in the cardiac sarcoplasmic reticulum (SR)8 led us to hypothesize that NOS1-derived NO may be involved in the regulation of calcium fluxes and, thus, of myocardial inotropy. To test this idea, we investigated the effect of selective NOS1 gene disruption or NOS1 inhibition on basal and β-adrenergic contraction in murine LV myocytes.

Methods

NOS1 Knockout Mice

Selective disruption of the NOS1 gene was first achieved by Huang et al11 in 1993. We acquired mice with NOS1 gene disruption...
(NOS1+−/− mice) generated on a Bl6/129 background from the Jackson Laboratory, Bar Harbor, Me, and backcrossed them to BL6 (3 generations). F2 littermates, which were genotyped according to standard procedures, were used as controls (NOS1++/+ mice).

Isolation of Ventricular Myocytes

Treatment of all animals was in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (HMSO). Ventricular myocytes were prepared from 8- to 12-week-old mice by standard enzymatic techniques. Briefly, after cervical dislocation, the aorta was cannulated in situ, and the heart was excised and perfused with oxygenated, warmed, nominally Ca2+-free Tyrode’s solution (in mmol/L: NaCl 140, KCl 5.4, MgCl2 1.2, NaH2PO4 0.4, HEPES 5, taurine 20, and glucose 10), followed by switching to a solution containing 240 U/mL collagenase II (Worthington Biochemical Co), 0.075 mg/mL protease (Sigma Chemical Co), and 50 μmol calcium. The LV free wall was isolated and then incubated with gentle agitation at 37°C in a collagenase solution containing 50 μmol Ca2+. Dispersed cells were washed twice in BSA and then resuspended in a storage solution of DMEM supplemented with 2 mg/mL Ultraser G (GIBCO-BRL). Cells were stored at room temperature and used within 8 hours of isolation.

Field Stimulation

Single LV myocytes were suspended in a perfusion bath and then stimulated to contract (5 ms square pulse, voltage-adjusted to maximize capture) at 3 frequencies (1, 3 and 6 Hz). The perfusion solution contained (in mmol/L) NaCl 140, KCl 5.4, MgCl2 1.2, HEPES 5, glucose 10, and Ca2+ 1.4. All experiments were performed at 36±1.5°C. The steady-state contractions at each frequency were recorded and digitized at a rate of 256 Hz. Edge-detection algorithms allowed derivation of contractile parameters (Ionoptix). Measurements from 5 steady-state contractions were averaged for each cell at each frequency.

NOS1 Inhibition

Vinyl-L- N-[5-(1-imino-3-butenyl)]-L-ornithine (L-VNIO) is a potent, mechanism-based, highly selective inhibitor of NOS1.12 Myocytes were incubated in 500 μmol L-VNIO for 20 minutes.

β-Adrenergic Stimulation

After stable contractile parameters were recorded at 1, 3, and 6 Hz from NOS1+/− myocytes, NOS1+−/− myocytes, and NOS1+/+ myocytes incubated with L-VNIO, a solution switcher changed the perfusion solution to a solution containing 2 mmol/L isoproterenol. This concentration of isoproterenol has been shown to elicit an ~50% increase in the contraction amplitude of adult rat ventricular myocytes.1 After a stabilization period during which the cell was stimulated at 0.2 Hz, the frequency protocol was repeated.

Immunoblotting

Uprogulation of the NOS3 isoform has been demonstrated in pial vessels of NOS1+/− mice.13 To test the level of NOS3 protein in the myocardium of NOS1−/− mice, we carried out immunoblotting of LV homogenate from four 8- to 12-week NOS1−/− and NOS1+/− mice. In brief, freshly isolated tissue was frozen in liquid nitrogen and then crushed while frozen and homogenized on ice in a buffer containing 0.32 mol/L sucrose, 1 mmol/L EDTA, 50 mmol/L Tris, and a protease inhibitor cocktail (Boehringer-Mannheim). Crude membranes were prepared by centrifugation at 4°C and 1000g for 10 minutes to remove the debris and nuclei, followed by spinning of the supernatant at 100 000g for 1 hour. The final pellet was resuspended in buffer, and the protein concentration was determined by the Bradford assay. Protein samples (25 to 100 μg) were separated on 6% polyacrylamide-SDS gels and transferred to nitrocellulose membranes for Western blotting with mouse anti-NOS3 monoclonal antibody (No. 610296, 1:1000 dilution, Transduction Laboratories). Visualization of bands was by chemiluminescence (Supersignal Substrate kit, Pierce). Bands were quantified by using the National Institutes of Health (Scion) Imaging Program.

Statistical Analysis

Data are expressed as mean±SE. Statistical significance was determined by using either the Student t test for single-group comparisons or a general linear model ANOVA with Fisher post hoc testing (NCSS 2001). The null hypothesis was rejected at the P<0.05 level.

Results

Role of NOS1-Derived NO in Regulation of Myocyte Contraction

Contractile parameters of NOS1+/+ and NOS1−/− LV myocytes are illustrated in Figure 1. NOS1−/− myocytes exhibited a flat negative contraction-frequency response, which extended from 0.2 to 10 Hz (data not shown), consistent with observations from murine in vivo models14 and mathematical predictions.15 As shown, compared with NOS1+/+ myocytes, NOS1−/− myocytes exhibited similar shortening-phase dynamics but greater overall shortening at all frequencies (Figure 1A through 1C; percent cell shortening at 6 Hz, 10.67±0.9% for NOS1−/− myocytes versus 7.21±0.8% for NOS1+/+ myocytes [n=14 and 18, respectively]; P<0.05). Relaxation, evaluated by the time to 50% relaxation (TR50), was prolonged in the NOS1−/− myocytes (Figure 1D; at 6 Hz, 26.53±1.4 ms for NOS1−/− myocytes versus 21.27±1.3 ms for NOS1+/+ myocytes; P<0.05).

The effect of acute pharmacological inhibition of NOS1 was assessed in NOS1−/− myocytes incubated in Tyrode’s solution with or without L-VNIO. As illustrated in Table 1, TR50 was significantly increased after exposure to L-VNIO (at 6 Hz, 21.28±1.7 ms in NOS1−/− myocytes and 26.45±1.4 ms in NOS1+/+ +L-VNIO myocytes; P<0.05) to values similar to those seen in the NOS1−/− myocytes (Figure 1D). Although a trend toward increased cell shortening was observed in the presence of L-VNIO, it did not reach statistical significance (at 6 Hz, percent shortening was 7.80±0.75% in NOS1−/− myocytes versus 8.11±0.61% in L-VNIO myocytes; P=0.16). As in NOS1−/− myocytes, shortening-phase dynamics were not altered by acute NOS1 inhibition with L-VNIO (see Table 2).

Effect of NOS1-Derived NO on β-Adrenergic Responses

The increase in cell shortening after superfusion with 2 mmol/L isoproterenol was calculated for NOS1−/− myocytes, NOS1+/− myocytes, and NOS1+/+ myocytes incubated with L-VNIO. As shown in Figure 2, cell shortening in response to β-adrenergic stimulation was greatly enhanced in myocytes from NOS1−/− mice at stimulation frequencies of 3 and 6 Hz. The effect of acute inhibition of NOS1 was virtually identical to the effects of gene disruption, with significant potentiation of the response at 3 and 6 Hz. Shortening and relaxation parameters after isoproterenol did not differ between the groups (Table 3).

To test for a nonspecific effect of L-VNIO, NOS1−/− myocytes (n=12) were incubated with L-VNIO and tested at 6 Hz (Figure 2, right bar). There was no additional effect of L-VNIO in NOS1−/− myocytes on the magnitude or rate of cell shortening or relaxation.
Immunoblotting

To analyze whether the level of NOS3 protein is greater in the hearts of NOS1\(+/\) animals than in the hearts of their sibling controls, equal amounts of protein extracts from 4 NOS1\(+/\) and 4 NOS1\(-/-\) LVs were analyzed by Western blotting. As shown in Figure 3, the absence of NOS1 gene product was not associated with upregulation of NOS3 protein in LV homogenates. Further blots using protein derived from isolated LV myocytes also showed no difference in the level of NOS3 (n=2 hearts, data not shown).

Discussion

The present study shows that pharmacological inhibition or genetic disruption of NOS1 elicits (1) greater basal myocyte contraction, (2) slower basal relaxation, and (3) enhanced contractile responses to \(\beta\)-adrenergic stimulation. Taken together, these findings indicate that NOS1 exerts a previously unrecognized role in the autocrine regulation of myocardial function.

NOS1 Regulation of Basal Contraction and Relaxation

Studies until now have assumed that NO-mediated effects in cardiac myocytes isolated from normal hearts relate to NOS3-derived NO. After the discovery of a NOS1 gene product on the cardiac SR and the suggestion that NOS1-derived NO could inhibit sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a) function,\(^{8}\) we hypothesized that inhibition or disruption of NOS1 in cardiac myocytes would result in increased contraction and faster contraction and relaxation times secondary to a greater and more rapid uptake of calcium in the SR. By using 2 complementary approaches, we have demonstrated that myocyte contraction is indeed increased when NOS1 activity is suppressed. Contrary to our expectation, however, we found that TR50 is significantly

### TABLE 1. Contraction Characteristics of LV Myocytes After Specific Inhibition of NOS1 With L-VNIO

<table>
<thead>
<tr>
<th></th>
<th>1 Hz</th>
<th>3 Hz</th>
<th>6 Hz</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-VNIO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude, %</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>8.68±0.73 (20)</td>
<td>7.68±0.73 (20)</td>
<td>7.80±0.75 (19)</td>
</tr>
<tr>
<td></td>
<td>9.78±0.58 (32)</td>
<td>8.46±0.59 (31)</td>
<td>8.11±0.61 (29)</td>
</tr>
<tr>
<td>Time to peak, ms</td>
<td>97.13±3.85 (20)</td>
<td>67.69±3.85 (20)</td>
<td>55.41±3.95 (19)</td>
</tr>
<tr>
<td></td>
<td>105.12±3.05 (32)</td>
<td>77.45±3.10 (31)</td>
<td>62.47±3.20 (29)</td>
</tr>
<tr>
<td>TR50, ms</td>
<td>23.80±1.68 (20)</td>
<td>22.37±1.68 (20)</td>
<td>21.28±1.73 (19)</td>
</tr>
<tr>
<td></td>
<td>28.48±1.33* (32)</td>
<td>26.87±1.35* (31)</td>
<td>26.46±1.40* (29)</td>
</tr>
</tbody>
</table>

Values are mean±SE (n).  
*P < 0.05 vs control.
longer in NOS1−/− myocytes or after the administration of L-VNIO, whereas the time to peak contraction is unchanged. With a narrow focus on SERCA2a function, this is difficult to explain. Indeed, other work from our group16 has shown that the calcium current density is increased and that current inactivation time is slower in LV myocytes from NOS1−/− mice or after the administration of L-VNIO. These data suggest that the main effect of NOS1 in intact myocytes may not be at the level of the SERCA2a but at the L-type calcium channel. This is consistent with recent work indicating that myocardial NO production can tonically inhibit the L-type calcium current in frog17 and guinea pig18 cardiac myocytes and is consistent with experiments showing that NOS1-derived NO suppresses voltage-sensitive calcium entry in neurons.19 In addition, increasing consideration is being given to the notion that local concentrations of NO govern subcellular processes in microdomains.20 From this perspective, NOS1 would be well positioned to provide NO-mediated regulation of calcium fluxes.

Acute NOS1 inhibition with L-VNIO prolonged TR50 to values similar to those seen in myocytes from NOS1−/− mice, whereas the increase in cell shortening did not reach statistical significance. The reason for this difference in the effects of extracellularly applied L-VNIO is not clear and may depend on time or concentration differences in the action of L-VNIO on relaxation and contraction parameters. Indeed, in a separate set of experiments, we found that intracellular dialysis of L-VNIO through a patch-clamp pipette elicited a significant increase in cell shortening (data not shown).

NOS1-derived NO could affect relaxation in several ways. A tonic inhibition of SERCA2a remains possible, although such an effect should result in a faster relaxation in the presence of NOS1 inhibition/disruption. Stimulation of endothelial NO production induces an earlier onset of myocardial relaxation in a variety of preparations, with little or no effect on the force of contraction (see review). In isolated rat ventricular myocytes, 8-bromo-cGMP has been shown to cause a small reduction in cell shortening and an earlier onset of relaxation in the absence of changes in intracellular calcium.21 These findings suggest that NO may exert a lusitropic effect by causing a cGMP-mediated reduction in myofilament calcium sensitivity, possibly secondary to protein kinase G—mediated phosphorylation of troponin I.22 Consistent with our findings (Table 3), Shah et al21 also showed suppression of the lusitropic effect of 8-bromo-cGMP in the presence of β-adrenergic stimulation with isoproterenol, suggesting that the effects of protein kinase G and protein kinase A on myofilament calcium sensitivity may not be additive. However, data from our previous experiments16 show no differences in the relationship between calcium influx in the presence of thapsigargin and peak cell shortening in myocytes from NOS1−/− and control mice, indicating that NOS1-derived NO is unlikely to affect the contraction/relaxation cycle by desensitizing cardiac myofilaments to calcium.

**NOS1 and β-Adrenergic Inotropic Effect**

The role of NO in β-adrenergic modulation of cardiac contractility is an area of significant interest and debate.3 Early studies involving adult rat ventricular myocytes showed clear potentiation of the adrenergic response in the presence of NOS inhibition with nitro-L-arginine,1 a finding repro-

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**TABLE 2. Maximal Shortening Velocity of LV Myocytes**

<table>
<thead>
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<th></th>
<th>1 Hz</th>
<th>3 Hz</th>
<th>6 Hz</th>
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<tbody>
<tr>
<td>NOS1+/+</td>
<td>131±18 (13)</td>
<td>128±18 (13)</td>
<td>125±18 (13)</td>
</tr>
<tr>
<td>NOS1−/−</td>
<td>101±20 (11)</td>
<td>104±17 (14)</td>
<td>135±17 (14)</td>
</tr>
<tr>
<td>L-VNIO</td>
<td>136±19 (12)</td>
<td>158±19 (12)</td>
<td>150±19 (12)</td>
</tr>
</tbody>
</table>

Values are mean±SE (n) μm/s. No differences were significant.

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**Figure 2.** Percentage increase in cell shortening of LV myocytes after superfusion with 2 mmol/L isoproterenol. Response was enhanced at 3 and 6 Hz in NOS1−/− myocytes and in NOS1+/+ myocytes incubated with specific NOS1 inhibitor L-VNIO (500 μmol) compared with NOS1+/+ control myocytes (n=14, 12, and 13, respectively; P<0.05). Prior incubation of NOS1−/− myocytes with 500 μmol L-VNIO (right bar; n=12, 6 Hz only) did not lead to any additional effect, suggesting that effects of L-VNIO in blocking other NOS isoforms are minimal.

**Figure 3.** Mean±SE optical density measurements for 4 immunoblots of LV homogenate derived from NOS1+/+ and NOS1−/− myocytes. An equal amount of protein (25 μg) was loaded in each lane. Equal transfer was verified by using Ponceau S (Sigma) staining of the nitrocellulose membrane. Data show that there was no upregulation of NOS3 in NOS1−/− myocardium.
TABLE 3. TR50 in LV Myocytes Before and After Superfusion With 2 nmol/L Isoproterenol

<table>
<thead>
<tr>
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<th>1 Hz</th>
<th>3 Hz</th>
<th>6 Hz</th>
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<tr>
<td></td>
<td>NOS1+/+</td>
<td>NOS1−/−</td>
<td>L-VNIO</td>
</tr>
<tr>
<td>Before</td>
<td>22.7±0.6 (16)</td>
<td>27.1±0.9 (18)*</td>
<td>27.0±1.4 (11)</td>
</tr>
<tr>
<td>After</td>
<td>21.9±0.9 (16)</td>
<td>23.1±1.0 (18)</td>
<td>22.7±0.8 (11)</td>
</tr>
</tbody>
</table>

Values are mean±SE (n).

*P<0.05 vs NOS1+/+.

duced in studies using intracoronary infusions of Nω-nitro-L-arginine methyl ester in dogs. However, later studies in the NOS3−/− mouse produced inconsistent results. In particular, Gyurko et al. found an enhanced maximal LV dP/dT in response to β-adrenergic stimulation in NOS3−/− mice in both in vivo-perfused and Langendorff-perfused hearts, whereas others, using the same model, found no change either in calcium current or papillary muscle contractility. Godecke et al. using a differently generated NOS3−/− mouse, also found a greater maximal LV dP/dT in response to dobutamine in isolated hearts. Differences in experimental conditions and preparations might have been in part responsible for the reported inconsistency in experimental findings (see review). However, our data showing that cardiac NOS1-derived NO can contribute importantly to the autocrine regulation of β-adrenergic contractile responses may also help explain differences observed between nonspecific NO inhibition and selective NOS3 gene disruption. Indeed, inhibition of NOS1 by nitro-L-arginine (which inhibits NOS1 and NOS3 at similar Kᵦ) may have accounted, at least in part, for the observed enhancement of adrenergic responses in isolated rat myocytes. In the same way, selective disruption of NOS3 may not be sufficient to effect a reproducible potentiation of adrenergic responses in quiescent isolated ventricular myocytes in the absence of endothelium or of stimuli (such as stretch) that increase the myocyte NOS3-mediated production of NO.

Our aim was to test whether disruption of myocyte NOS1 activity enhanced the β-adrenergic inotropic response, and as such, we elected to use a physiological (submaximal) concentration of isoproterenol (2 nmol/L). Our findings support this hypothesis; however, they may not be applicable to all concentrations of isoproterenol, because the effect of NO has been shown to vary with the degree of β-adrenergic stimulation (see review). Indeed, Barouch et al. have recently reported that calcium transients and sarcomere length changes in response to higher doses of isoproterenol (0.1 to 1 μmol/L) are smaller in myocytes from NOS1−/− mice, suggesting that the maximal inotropic response may also be decreased.

Study Conclusions
In summary, our data indicate that myocardial NOS1-derived NO can tonically inhibit basal myocardial contractility and the response to β-adrenergic stimulation. Because both NOS1 gene transcription and biochemical activity are regulated by calcium, localization of this enzyme to the SR membrane is consistent with the idea that locally released NO modulates myocardial inotropy by exerting a negative feedback on intracellular calcium fluxes. An important question and the aim of our future work is to investigate the role of cardiac NOS1 in disease states, such as cardiac hypertrophy and failure, that are characterized by electromechanical dysfunction and calcium-handling abnormalities. Such involvement of NOS1 in myocardial dysfunction would be particularly interesting in light of the recent finding that cardiac-specific overexpression of cardiac-specific NOS2 does not result in a reduction in contractility.

Acknowledgments
This study was supported by The Wellcome Trust (Dr Ashley), the Royal Society (Dr Sears), and the British Heart Foundation (Dr Casadei, Bryant, and Watkins). Dr Ashley is a Wellcome Trust Research Training Fellow. Dr Casadei is a Senior Research Fellow of the British Heart Foundation, and Dr Sears is a Dorothy Hodgkin Fellow of the Royal Society. We also thank Dr Simon Golding for his expert technical help with immunoblotting.

References


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_Circulation_. 2002;105:3011-3016; originally published online May 20, 2002;
doi: 10.1161/01.CIR.000019516.31040.2D
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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