Elevated Afterload, Neuroendocrine Stimulation, and Human Heart Failure Increase BNP Levels and Inhibit Preload-Dependent SERCA Upregulation

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Background—In heart failure, brain-type natriuretic peptide (BNP) is elevated and the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) downregulated. We previously showed that preload-induced SERCA-upregulation is suppressed by exogenous BNP.

Methods and Results—Here we tested the hypothesis that afterload and neurohumoral activation would counterregulate preload-dependent SERCA upregulation through BNP, which finally results in decreased SERCA levels. We studied the effects of 6 hours preload, afterload, and isoproterenol stimulation on BNP and SERCA mRNA expression in rabbit and human failing muscles strips. Preload resulted in a pronounced upregulation of SERCA by 149\% (isotonic versus slack, \(P<0.01\)). This upregulation was largely suppressed in afterloaded muscles (isometric versus slack: +32\%; \(P<0.05\)). Similarly, presence of isoproterenol prevented SERCA upregulation in isotonic muscles. Afterload and isoproterenol resulted in a pronounced increase in BNP expression compared with slack by 225\% (\(P<0.05\)) and 198\% (\(P<0.01\)), respectively. Isoproterenol also increased expression of phospholamban by 84\% (\(P<0.01\)). SERCA upregulation in preloaded muscles is associated with frequency-dependent potentiation of contractile force, which is absent in afterloaded muscles. In failing human myocardium, BNP expression was upregulated compared with nonfailing (+631\%; \(P<0.05\)). Neither unloading nor preload or afterload induced a change in SERCA or BNP expression after 6 hours.

Conclusions—Afterload and neuroendocrine stimulation increase BNP expression thereby causing inhibition of preload-dependent SERCA upregulation. In failing human myocardium, high BNP expression may underlie the loss of preload-dependent upregulation of SERCA. BNP may thus contribute to adverse myocardial remodelling in heart failure. (Circ Heart Fail. 2008;1:265-271.)

Key Words: calcium ■ heart failure ■ mechanics ■ natriuretic peptides ■ sarcoplasmic reticulum

Hemodynamic load and neurohumoral stimulation are considered to play dominant roles in regulation of myocardial gene expression and to take part in the development of hypertrophy and the progression to heart failure. Heart failure shows the typical reactivation of a fetal gene expression program.\(^1\) Thereby the level of brain-type natriuretic peptide (BNP) is increased\(^2\) and the expression levels of calcium cycling proteins are changed. In particular, the expression of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) is reduced.\(^3\)

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In contrast to this general scheme of load- and neurohumoral-induced reactivation of the fetal gene program with SERCA downregulation, we previously showed that isolated preload increases SERCA expression whereas BNP is not regulated after 6 hours. Moreover, preload-dependent upregulation of SERCA could be abolished by addition of exogenous BNP. This effect was transmitted via the BNP receptor guanylyl cyclase A (GC-A), elevation of cGMP and activation of protein kinase G.\(^4\) Protein kinase G can inhibit calcineurin activation\(^5\) and the preload-dependent upregulation of SERCA-expression could indeed be inhibited by cyclosporine A.

Because SERCA is upregulated with preload but downregulated in heart failure with generally high preload, high afterload, and neurohumoral activation, quality of load and neurohumoral activation may determine the net effect on SERCA regulation.

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Accordingly, in the present study, we tested the hypothesis that afterload and neurohumoral activation counterregulate the preload-dependent upregulation of SERCA and that this is associated with an increase of BNP.

Methods

Muscle Preparation and Mechanical Stretch

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). Female chinchilla bastard rabbits (1.5 to 2 kg, Charles River, Kisslegg, Germany) were heparinized and anesthetized with thiopental sodium (50 mg/kg IV). Hearts were excised and retrogradely perfused with modified Krebs-Henseleit solution as described.6 Right ventricular trabeculae or thin papillary muscles were dissected and mounted in culture chambers (Scientific Instruments, Heidelberg, Germany) between a force transducer and a hook connected to a micrometer drive allowing for length adjustment. The system is equipped with a servomotor with force-feedback function and allows cultivation of functionally intact multicellular muscle preparations for up to 48 hours at 37°C with physiological protein turnover maintained.7 After [Ca\(^{2+}\)]c, was stepwise raised to 1.0 mmol/L, the Krebs-Henseleit solution was replaced with tissue culture medium at 1.25 mmol/L [Ca\(^{2+}\)]c (medium199 (M-199), Invitrogen, Karlsruhe, Germany) supplemented with 20 IU/L human insulin, 0.2% (wt/vol) BSA, 70 μmol/L streptomycin, and 100 IU/mL penicillin and equilibrated with 100% O\(_2\). Preparations were allowed to stabilize for 1 hour under continuous electric stimulation (1 Hz, 3 to 5 V) and subsequently assigned to the different experimental groups. In group 1, the isometric group, preparations were stretched progressively over 30 minutes to 3 mN/mm\(^2\) resting tension, corresponding to L\(_{\max}\), and stored at 4°C for up to 24 hours. In group 2, the isotonic group, preparations were also stretched to 3 mN/mm\(^2\) resting tension and allowed to shorten isotonically from this level of resting tension. Isotonic shortening means that afterload is 0. Results of this group have been published before. Here new experiments were performed, that confirmed our previous results. Group 3 was unloaded; ie, preparations remained slack under otherwise identical conditions. Isometric force development or isotonic shortening was recorded continuously over 6 hours at the designated loading conditions. At the end of the incubation period, muscle preparations were harvested from the culture chamber, rapidly frozen in liquid N\(_2\) and stored at −80°C. Freshly isolated RV papillary muscles not used for in vitro experiments were immediately frozen and served as a control group (fresh).

In a subset of preparations, after the 6-hour incubation period, the frequency dependence of force development was assessed by recording isometric force at stimulation rates of 1 to 4 Hz. Muscle strips in both stretched groups (isometric and isotonic) muscle strips were unloaded. In isotonic preparations, the force-feedback motor unit was switched off. Preparations of all groups were then stretched over 30 minutes as described above to 3 mN/mm\(^2\) resting tension. Force development was allowed to reach steady state before the frequency protocol was initiated.

Stimulation With Isoproterenol

Because oxidative stress degrades isoproterenol (isoprenaline), it was dissolved with vitamin C and culture medium was changed every 30 minutes. Isoproterenol (Sigma, Taufkirchen, Germany) in a 30 nM concentration was dissolved with vitamin C (Sigma) at a concentration of 300 nM in the culture medium. This isoproterenol concentration closely approximates the EC\(_{50}\) of the inotropic effect of isoproterenol in rabbit muscles strips,8 and in consideration of the higher potency of isoproterenol compared with epinephrine, this concentration is equivalent to epinephrine plasma levels measured in human heart failure.9 For control, vitamin C only was dissolved in culture medium. This isoproterenol concentration increases the shortening of the isotonic stretched muscles by 137% (P<0.05). This increase remained constant during the entire experiment (6 hours: +153%, P<0.05). The diastolic function was comparable to controls and was not influenced by isoproterenol.

Human Myocardium

Ventricular muscle strips were dissected from 7 end-stage heart failure patients undergoing cardiac transplantation as a result of ischemic or dilated cardiomyopathy (6 men, 1 woman, age 56.3±2.6 years). Detailed patient characteristics are provided in the online Data Supplement. For comparison, samples from 4 nonfailing donor hearts (3 men, 1 woman, age 21.2±5.1 years) that could not be used for cardiac transplantation for technical reasons, were used as controls. None of these patients had a history of cardiac disease. The study was approved by the institutional ethics committee, and all patients provided written informed consent with the use of tissue samples.

Right ventricular muscles strips were dissected from the hearts and placed in the system as described above. Calcium concentration and culture medium were identical to those used in the rabbit experiments described above. The only difference was that the muscle strips were stretched to human isometric L\(_{\max}\). This was the case at a resting tension of ~12 mN/mm\(^2\). The isotonic shortening in half of the muscles was activated after reaching L\(_{\max}\). Muscles were then treated as described above.

Quantitative mRNA Measurement in Rabbit and Human Myocardium

DNA-free total RNA was extracted from myocardial samples by a standard protocol with the RNeasy kit and RNase-free DNase Set (Qiagen, Hilden, Germany). First-strand cDNA synthesis was carried out with iScript cDNA synthesis kit (BioRad, Munich, Germany) according to manufacturer’s instructions. Real-time polymerase chain reactions were performed on a Biorad iQ-Cycler in a volume of 20 μL in a 96-well plate. The reaction mixture consisted of 1 μL cDNA with 19-μL SYBR GRN SUPERMIX (BioRad, Munich, Germany). After initial denaturation for 60 s at 95°C, the cycling program consisted of 40 cycles of 95°C for 15 s, 60°C (SERCA, phospholamban [PLB]), 58°C (BNP) or 62°C (glycerol-aldheydephosphate-3-dehydrogenase) for 10 s, and 72°C for 15 s. Emission at 530 nm was measured at the end of each cycle. Primer sequences used are provided in the online Data Supplement. For comparison, samples from 4 nonfailing donor hearts (3 men, 1 woman, age 21.2±5.1 years) that could not be used for cardiac transplantation for technical reasons, were used as controls. None of these patients had a history of cardiac disease. The study was approved by the institutional ethics committee, and all patients provided written informed consent with the use of tissue samples.

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Western Immunoblot Analysis

Frozen muscle strips were thawed on ice in 50 μL of homogenization buffer and homogenized. Protein concentrations of the suspensions were determined and 20 μg of samples subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis. Western blotting was carried out according to standard protocols, using antibodies against SERCA (monoclonal, Affinity Bioreagents) and glycerol-aldheydephosphate-3-dehydrogenase (monoclonal, Biotrend). For quantification an enhanced chemoluminescence detection system (Amersham) was used according to the manufacturer’s instructions. BNP secreted into the culture medium could not be detected because of dilution by a factor of 10,000.

Calculation and Statistical Analysis

Force values were transferred to tension by normalizing to the cross-sectional area of a preparation, which was calculated assuming an elliptical cross section using the formula cross-sectional area=\(D_1/D_2\times\pi\), with D1 and D2 representing width and thickness.

Gene- and protein-expression was analyzed using Student t test for unpaired samples and functional data (force-frequency relationship) using 2-way ANOVA, each test with a significance level of α=5%. Confidence intervals were calculated using a bootstrap procedure.10

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.
Results

Regulation of Calcium Cycling Proteins and BNP by Mechanical Load

We assessed the effect of different loading conditions on the expression of calcium cycling proteins and BNP. Muscle strips were either left at 0 load (slack) or stretched under isotonic (preload only) or isometric conditions (preload and afterload) for 6 hours.

After 6 hours of isotonic stretch, SERCA mRNA expression was upregulated by 149% (95% CI, 100% to 203%) compared with slack myocardium (Figure 1A, isotonic versus slack $P<0.001$). In contrast, isometric afterload greatly blunted upregulation of SERCA mRNA expression ($37\%$ [95% CI, 11\% to 67\%], isometric versus slack $P=0.028$).

Protein expression of SERCA was regulated likewise (Figure 2A). Compared with slack muscle strips, isotonic stretch led to an upregulation of SERCA protein expression by 51\% (95\% CI, 18\% to 92\%; isotonic versus slack $P=0.046$) and isometric stretch did not lead to a significant regulation of SERCA protein expression (Figure 2B).

In contrast to SERCA, BNP expression was significantly increased in the isometric group ($225\%$ [95\% CI, 54\% to 122\%]; control versus isoproterenol $P<0.001$; Figure 3).

Inhibition of Preload-Induced SERCA Upregulation by Isoproterenol

Addition of isoproterenol induced an upregulation of BNP-expression (Figure 5A). Compared with control muscles, isoproterenol increased BNP expression versus control by 84\% ($84\%$ [95\% CI, 54\% to 122\%], control versus isoproterenol $P<0.001$; Figure 5).

Analysis of Contractile Function

The force-frequency response largely depends on the activity of SERCA. The steeper force-frequency response in the isotonic stretched muscles compared with the other groups (Figure 4; ANOVA slack versus isotonic $P=0.013$, isometric versus isotonic, $P=0.025$) is consistent with the hypothesis that the increase in SERCA expression in the isotonic stretched muscles is of functional relevance.

Neuroendocrine Regulation of PLB

Stimulation with isoproterenol increased PLB expression versus slack control by $84\%$ ($84\%$ [95\% CI, 54\% to 122\%], control versus isoproterenol $P<0.001$; Figure 3).

Figure 1. Effect of 6-hour unloading (slack [ ], n=8), increased preload (isotonic [ ], n=8), increased preload and afterload (isometric [ ], n=8) on the mRNA expression of SERCA (A) and BNP (B). *$P<0.05$; $\&P<0.001$.

Figure 2. Effect of 6-hour unloading (slack [ ], n=4), increased preload (isotonic [ ], n=4), increased preload and afterload (isometric [ ], n=4) on the protein expression of SERCA and glycerol-aldehyophosphate-3-dehydrogenase. A, Example of a western blot; (B) statistic analysis of SERCA/glycerol-aldehyophosphate-3-dehydrogenase. *$P<0.05$.

Figure 3. Effect of 6-hour unloading (slack [ ], n=8), increased preload (isotonic [ ], n=8), increased preload and afterload (isometric [ ], n=8) and neuroendocrine stimulation (slack [ ], n=8; isoproterenol [ ], n=8) on the expression of PLB. *$P<0.05$; $\&P<0.001$. 

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isoproterenol increased BNP by 198% in slack (95% CI, 61% to 614%; control slack versus isoproterenol slack $P_{\text{H}11005}<0.006$) and by 131% in isotonic (95% CI, 59% to 400%; control isotonic versus isoproterenol isotonic $P_{\text{H}11005}<0.036$) muscles. Isoproterenol abolished the dependent SERCA upregulation (Figure 5B; control: $+71\%$ [95% CI, 19% to 105%], control slack versus control isotonic $P_{\text{H}11005}<0.015$; isoproterenol: $9\%$ [95% CI, 35% to 58%], isoproterenol slack versus isotonic $P_{\text{H}11005}<0.7545$).

**Discussion**

Our findings strongly suggest that the lack of mechanical load-dependent upregulation of SERCA under conditions of elevated afterload, neuro-humoral activation and in heart failure is causally related to BNP being upregulated and acting as a suppressor of load-dependent calcineurin signaling. Likewise, in failing human myocardium high BNP is associated with a loss of preload-dependent upregulation of SERCA. This is based on the following observations: 1)
Myocardial SERCA expression is increased by elevated preload (isotonic stretch). This increase in SERCA expression is associated with improved myocardial performance, underlining the functional relevance of the observed load-induced expression changes. 2) Increased afterload (isometric stretch) leads to an upregulation of BNP and suppresses preload-dependent upregulation of SERCA expression despite the same amount of preload. Lack of SERCA upregulation is associated with a lack of functional changes in isometrically contracting muscle strips. 3) Isoproterenol increases BNP expression and inhibits SERCA upregulation in preloaded muscles strips. 4) In failing human myocardium, BNP expression is high independent of short-term effects of biomechanical load, and preload does not upregulate SERCA expression.

Regulation of SERCA by BNP in the Human Failing Heart

Upregulation of myocardial BNP expression by afterload or neuroendocrine stimulation with its subsequent autocrine/paracrine actions on cardiac myocytes may contribute to maladaptive remodelling via downregulation of SERCA and thereby depress contractility. Indeed, BNP is negatively correlated with SERCA expression in human heart failure. A meta-analysis of clinical trials, evaluating the acute effects of recombinant BNP in decompensated heart failure, identified a tendency of increased mortality in BNP-treated subjects. In our previous work, we demonstrated that BNP regulates SERCA expression. Exogenous recombinant BNP reduced dose-dependently the expression of SERCA in unstimulated muscle strips, and also diminished the preload-dependent upregulation of SERCA.

In human failing myocardium, an increase in preload did not increase the expression of SERCA during 6 hours from the diminished levels compared with nonfailing myocardium. In contrast to this result, we could previously show this mechanism in human patients with a left ventricular assist device. Improvement of SERCA expression could only be observed in the subgroup of left ventricular assist device patients, where BNP levels were reduced. This shows, that in human myocardium an improvement is only possible, when BNP levels are normalized. The stimulus of elevated load cannot lead to a maintenance or increase of SERCA expression. This finally leads to a reduction SERCA expression and progression of heart failure. The differences in the present experiments with isolated human muscle strips compared with the left ventricular assist device study can be easily explained by the experimental time. After unloading, the BNP levels decrease much slower to become relevant within the 6-hour period studied here. The period for normalization of BNP in left ventricular assist device-treated patients is several months. Thus, the still high level of BNP and subsequent stimulation of GC-A can explain, why an increase in SERCA expression was not visible in the preloaded human muscle strips. Looking at the missing upregulation of BNP in the isometric stretched muscle strips it could be argued that the level of stretch was not sufficient to activate signal pathways like in the rabbit muscle strips. This, however, can be ruled out, because both—rabbit and human—muscle strips were stretched to their respective Lmax and an upregulation of BNP in the failing human heart can only be induced by stretching the muscles above Lmax.

Influence of Neuroendocrine Stimulation

Neuroendocrine stimulation can increase BNP and thereby suppress SERCA regulation. Isoproterenol increases BNP probably via the β2-receptor and involving Src, ras, and p42/p44 MAPK activation. Infusion of isoproterenol in rats also induced a downregulation of SERCA. BNP expression was not measured in these animals, but atrial natriuretic factor was highly upregulated by isoproterenol. Probably BNP is also upregulated in these animals and atrial natriuretic factor can also activate the GC-A, so that the downregulation of SERCA could be transmitted via BNP/atrial natriuretic factor and GC-A activation in these animals, too.

PLB showed a differential expression pattern between both stretched groups. A reduction of PLB expression in the isotonic group leads to a further increase in the SERCA/PLB ratio and would therefore further favor the calcium uptake into the sarcoplasmic reticulum. A load-dependent downregulation of PLB was also found in the overloaded right ventricle of the monocrotaline rat model. In single cells PLB was not regulated by stretch. Upregulation of PLB was also achieved by isoproterenol stimulation. The PLB promoter possesses an MCAT binding site that can be stimulated by isoproterenol.

Importance of the Type of Load

Load is an important factor for the expression of SERCA in the heart. Unloading the heart leads to a reduction of SERCA expression. The type of load (preversus afterload) is of particular importance and has different effects on hypertrophy, partially mediated via BNP. Here the different SERCA- and PLB-expression patterns between both stretched groups can also be explained by the influence of BNP. We previously showed that BNP—via the GC-A-cGMP-protein kinase G-pathway—has a negative effect on the preload-induced SERCA upregulation. In the isometric stretched muscles, the addition of afterload to the same preload as in isotonic stretched muscles reduced the SERCA upregulation markedly, but also increased BNP expression. BNP seems to be predominantly induced by afterload. Preload can as well induce BNP upregulation, but not so fast and not to the same extent. In vivo, an increase in afterload leads to a high and fast (hours) upregulation of BNP. In contrast, BNP upregulation in animal models of preload is slow and seen only after 3 days of large shunt or after 30 days of small shunt. In isolated paced cardiomyocytes, the activation of BNP was also higher when the stretch was performed during electric stimulation (in the presence of afterload) than when it was performed in the breaks between stimulation (presence of preload only). This is in line with our results of BNP being predominantly regulated by afterload.

The knowledge of how mechanical stress is sensed by the cardiomyocytes and transduced into intracellular signals is limited. Several mechanisms are currently being discussed. Some authors suggest pathways that involve auto- and/or paracrine factors released by mechanical stress such as...
endothelin-1 or angiotensin.24 Other authors have suggested pathways that are partially or completely independent from humoral or neuronal factors, but involve cellular mechanoreceptors like stretch activated channels,25 Na⁺/H⁺-exchanger,26 Z-disc proteins like MLP,27 or costamere proteins like melusin.28 MLP is thereby linked to the calcineurin signaling pathway.29

The Ca²⁺-dependent phosphatase calcineurin, via dephosphorylation of the transcription factor NFAT, has been implicated as an important mediator of hypertrophy.30,31 The SERCA promoter region possesses a consensus site for members of the GATA family of transcription factors,32 and NFAT is able to associate with GATA-4 to activate gene transcription. Transgenic mice overexpressing a constitutively active calcineurin indeed exhibited enhanced SERCA expression.33 We3 and others34 also demonstrated, that SERCA regulation was indeed calcineurin-dependent. Load-dependent differences in calcineurin activation could—because of the lack of rabbit NFAT antibodies—not be proven here. But in single cardiomyocytes a lower but longer calcium transient was found in isotonic contractions compared to isometric contractions.35 This profile of the calcium transient would favor an increase in calcineurin activity.36 Taken together, these observations make it likely that calcineurin activation is enhanced and thereby SERCA mRNA expression is increased via calcineurin by preload conditions.

Also other signal pathways can influence the regulation of SERCA in hypertrophy. Especially GSK3β, which controls the nuclear export of NFAT,37 has an influence on SERCA expression: GSK3β overexpression leads to a reduction of SERCA.38 Also JNK- and p38-MAPK diminish the nuclear localization of NFAT39,40 and could participate in a load-specific regulation of the calcineurin-NFAT pathway. An influence of these signaling pathways in our experiments cannot be ruled out.

In summary, we have demonstrated that preload induces a cardiomyocyte phenotype with increased sarcoplasmic calcium cycling by increased expression of SERCA and diminished expression of PLB. Afterload and neuroendocrine stimulation increase BNP expression that again leads to an inhibition of the preload-dependent SERCA regulation by blockade of calcineurin via the GC-A-cGMP-protein kinase G signal pathway. These findings suggest that blocking neuroendocrine stimulation by β-blockers and reduction of afterload in patients could diminish the BNP levels and thereby contribute to an improvement of the cardiac function through upregulation of SERCA.

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Disclosures
None.

References
One of the pathophysiological hallmarks of congestive heart failure (HF) is an impaired calcium homeostasis of the cardiac myocytes, resulting in contractile dysfunction. Reduced expression and function of the Ca\textsuperscript{2+}-ATPase of the sarcoplasmic reticulum (SERCA) is an important mechanism of defective calcium cycling. In HF, brain natriuretic peptide (BNP) is expressed and expression levels increase with the severity of HF. In a previous study we demonstrated that increased SERCA expression improves contractility. This beneficial adaptation is inhibited by BNP. In the present study, we report that in isolated rabbit myocardium, afterload or neuroendocrine stimulation with the mixed ET(A)/ET(B) and AT1 receptor antagonists in vivo and in vitro.

**Clinical Perspective**

One of the pathophysiological hallmarks of congestive heart failure (HF) is an impaired cardiac calcium homeostasis of the cardiac myocytes, resulting in contractile dysfunction. Reduced expression and function of the Ca\textsuperscript{2+}-ATPase of the sarcoplasmic reticulum (SERCA) is an important mechanism of defective calcium cycling. In HF, brain natriuretic peptide (BNP) is expressed and expression levels increase with the severity of HF. In a previous study we demonstrated that increased SERCA expression improves contractility. This beneficial adaptation is inhibited by BNP. In the present study, we report that in isolated rabbit myocardium, afterload or neuroendocrine stimulation with the β1-agonist isoproterenol lead to an upregulation of BNP. As a consequence of the higher BNP expression, SERCA upregulation, observed with preload only, was prevented. In human failing isolated myocardium the expression of BNP was markedly elevated and an increase in preload did not lead to an upregulation of SERCA. Our findings suggest that in patients with HF or in patients with high afterload and neuroendocrine stimulation, because of their endogenously expressed levels of BNP, the mechanism relevant for short-term compensation of elevated preload, i.e., upregulation of SERCA is not functional. We speculate that afterload is harmful partly because of increased BNP expression preventing SERCA upregulation. Similarly, neurohumoral activation by increased BNP expression independent from load may induce failure by preventing SERCA upregulation.

Anti-BNP strategies or careful titration of load may be effective in heart failure.
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