Endothelin ET_B Receptors in Arteries and Veins: Multiple Actions in the Vein

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ABSTRACT

Endothelin receptors (ET_A and ET_B) mediate responses to ET-1. ET_B receptor function seems to differ between a similarly sized arterial and venous pair, the rat vena cava (RVC) and rat thoracic aorta (RA). ET_B receptors mediate RVC contraction directly, but it is unclear whether ET_B receptors mediate contraction in RA. Because of these apparent differences in ET_B receptor-mediated vascular contraction, we hypothesize that relaxant ET_B-receptor mechanisms in RVC would be different from those in RA. RA and RVC rings were isolated from rats for measurement of isometric contraction. When contracted with prostaglandin F-2 α (PGF-2 α) (20 μ M), the ET_B receptor agonist sarafotoxin-6c (S6c) (100 nM) significantly relaxed RA and RVC. N^ω-Nitro-L-arginine (LNNa) (100 μ M) or endothelial denudation abolished relaxation to S6c in RA. By contrast, S6c-induced

relaxation of RVC was attenuated but not abolished by LNNa and endothelial denudation. RVC (PGF-2 α -contracted) relaxed to low concentrations of ET-1, whereas under the same conditions RA responded with contraction. ET-1-induced relaxation in RA was observed only with ET_A receptor blockade. Vessels from dopamine- β -hydroxylase-ET_B transgenic rats, which lack functional ET_B receptors in the vasculature, were also used. RVC (PGF-2 α -contracted) from these rats did not relax to ET-1. Thus, although both RA and RVC possess endothelial relaxant ET_B receptors, RA and RVC differ in that relaxant ET_B receptors may also be present in smooth muscle of RVC. Moreover, the mechanisms of endothelial cell ET_B receptor-mediated relaxation in RA and RVC are not the same.

Endothelin-1 (ET-1) is a 21-amino acid peptide that acts as a potent endogenous vasoconstrictor (Schiffrin, 1999). Although also made by other cell types, the dominant producers of ET-1 in the vasculature are endothelial cells. ET-1 has been implicated in the pathology of pulmonary arterial hypertension as well as systemic hypertension models, including deoxycorticosterone acetate-salt hypertension (Böhm and Pernow, 2007). ET-1 binds to and activates two G protein-coupled receptor subtypes: the ET_A and ET_B receptor (Davenerport, 2002). In both arteries and veins, ET_A receptor stimulation causes contraction (Zhang et al., 1999). In contrast, the role of ET_B receptors is much less clear and differs between vessel types.

Several reports characterize the ET_B receptor as a “clearance receptor,” the primary function of which is to sequester

and remove circulating ET-1 from the blood (Fukuroda et al., 1994). In this capacity, ET_B receptors decrease the amount of ET-1 available to interact with ET_A receptors and thus decrease vascular contraction because of ET_A receptor stimulation. Researchers have since discovered that ET_B receptor stimulation can directly modulate vascular tone in arteries and veins, albeit with different functions. In arteries, functional ET_B receptors exist in endothelial cells, in which receptor stimulation causes relaxation by increasing endothelial nitric oxide production (Tsukahara et al., 1994). In veins, ET_B receptors can mediate contraction (Sudjarwo et al., 1994) as well as relaxation (Zellers et al., 1994). There is evidence that both support and negate the involvement of the ET_B receptor in mediating contraction in arteries. For example, the ET_B receptor agonists sarafotoxin 6c (S6c) and IRL1620 do not cause arterial contraction directly in many arterial beds and isolated arteries (e.g., White et al., 1993; Caló et al., 1996; Thakali et al., 2004). However, as supported through antagonism studies, ET_B receptors mediate contraction in arteries from special circulations, including the coro-

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ABBREVIATIONS: ET, endothelin; S6c, sarafotoxin 6c; IRL1620, succinyl-(Glu⁹,Ala^{11,15})-ET-1-(8-21); RVC, rat vena cava; RA, rat thoracic aorta; (+/+), homozygous littermate to (sl/sl) rat; (sl/sl), homozygous spotting lethal ET_B receptor-deficient rat; PSS, physiological salt solution; PECAM, platelet/endothelial cell adhesion molecule; DAPI, 4,6-diamidino-2-phenylindole; NE, norepinephrine; PE, phenylephrine; PGF-2 α , prostaglandin F-2 α ; BQ-788, N-cis-2,6-dimethylpiperidinocarbonyl-L- γ -methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine; dH₂O, distilled water; LNNa, N^ω-nitro-L-arginine; ACh, acetylcholine.

nary artery (Bax et al., 1994; Sudjarwo et al., 1995), pulmonary artery (Teerlink et al., 1994; Montagnani et al., 2000), and skin (Warner et al., 1993). Finally, there are arteries in which ET_A and ET_B receptors mediate contraction (D'Orléans-Juste et al., 1993; Eguchi et al., 1997). Thus, the literature is mixed in terms of the role played by the ET_B receptor in arterial contractility. In the pair of vessels we use—the thoracic aorta and vena cava—S6c causes contraction in the vena cava but not the aorta.

It is unclear whether different arterial and venous responses to ET_B receptor stimulation are because endothelial ET_B receptors are linked to different mechanisms in arteries and veins, or whether veins have functional endothelial and smooth muscle ET_B receptors. Given the apparent differences in arterial versus venous contractile response to S6c, we hypothesize that relaxant ET_B receptor mechanisms in RVC would be different from those in RA. To test this hypothesis, we used pharmacological inhibition of ET receptors in normal rat tissues, as well as ET_B -deficient tissues from a novel strain of dopamine- β -hydroxylase (D β H)- ET_B receptor transgenic rats (*sl/sl*). These rats carry the spotting-lethal ET_B receptor mutation, which abrogates functional ET_B receptor expression because of a deletion of the first and second putative transmembrane domains. The D β H- ET_B transgene rescues these rats from a perinatally lethal gastrointestinal congenital abnormality (Garipey et al., 1996). The resulting adult rats do not express ET_B receptors in vascular tissues and provide confirmation of specific ET_B receptor functions.

Materials and Methods

Animal Care and Use. All procedures that involved animals were performed in accordance with the Institutional Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals at Michigan State University (East Lansing, MI). Normal male Sprague-Dawley rats, D β H- ET_B : ET_B (*sl/sl*) male rats (*sl/sl*), and their D β H- ET_B : ET_B (+/+) male littermates (+/+) (250–300 g) were used. All animals were genotyped before experimentation. Animals were euthanized with sodium pentobarbital (60 mg/kg i.p.).

Immunohistochemistry. Paraffin-embedded tissue sections were dewaxed, unmasked using unmasking reagent (Vector Laboratories, Burlingame, CA), and taken through a standard protocol. Primary antibody used was anti- ET_B antibody (rabbit polyclonal, 1:200; Alomone Labs, Jerusalem, Israel) in 1.5% blocking serum in phosphate-buffered saline or 1.5% blocking serum as a control. Development of slides proceeded according to the manufacturer's kit using 3,3'-diaminobenzidine as the developing substrate (Vector Laboratories), and slides were counterstained with hematoxylin (Vector Laboratories).

Immunofluorescence. Freshly dissected RVC were cleaned of adipose tissue in physiological salt solution (PSS) containing 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4 , 1.17 mM $MgSO_4 \cdot 7H_2O$, 14.8 mM $NaHCO_3$, 5.5 mM dextrose, 0.03 mM $Na_2EDTA \cdot 2H_2O$, and 1.6 mM $CaCl_2$, pH 7.2. The vessel was then cut lengthwise and pinned with lumen facing up. Tissues were fixed for 10 min in 4°C methanol and taken through a standard protocol. Primary antibodies used were as follows: anti- ET_B antibody (rabbit polyclonal, 1 μ g/ml; Alomone Labs) and anti-platelet/endothelial cell adhesion molecule (PECAM) antibody (goat polyclonal, 1 μ g/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 1.5% blocking serum, or both combined in 1.5% blocking serum. Secondary antibodies used were as follows: donkey anti-rabbit Cy-5 (1:400, Jackson ImmunoResearch Laboratories Inc., West Grove, PA), mouse anti-goat fluorescein isothiocyanate (1:800; Jackson ImmunoResearch Laboratories Inc.) in 1.5% blocking serum, or both combined in 1.5% blocking serum.

Tissues were then mounted on slides using Prolong Gold Medium with DAPI (Invitrogen, Carlsbad, CA) and viewed using fluorescence microscopy.

Isometric Contraction. Thoracic aortae (RA) and vena cavae (RVC) were dissected and cleaned of outer adipose tissue in PSS. Endothelium-intact tissue rings were then mounted in warmed, aerated PSS (37°C; 95% O_2 /5% CO_2) in isolated tissue baths (20 ml) for measurement of isometric contractile force using a 750 TOBS tissue organ bath system (Danish Myo Technology, Aarhus, Denmark) and PowerLab for Windows (ADInstruments, Colorado Springs, CO). In endothelium-denuded experiments, endothelial cells were mechanically removed before mounting, using a brush made from suture and an 18-gauge needle. The tissues were then placed under optimum resting tension (1 g for RVC and 4 g for RA, determined previously) and initially challenged with 10^{-5} M norepinephrine (NE) (RVC) or phenylephrine (PE) (RA) to test for tissue viability. Endothelium viability was confirmed by relaxation to 10^{-6} M acetylcholine after contraction by a submaximal concentration of PE (RA) or PGF-2 α (RVC). Tissues were washed every 15 min until they returned to resting tension. Cumulative concentration-response curves or responses to single concentrations of agonists were recorded. Antagonists, inhibitors, or their vehicles were incubated with the tissues for 1 h before addition of agonists. The specific agonists and antagonists (and corresponding solvents) were as follows: atrasentan (dimethyl sulfoxide), BQ-788 (dH_2O), indomethacin (ethanol), N^G -nitro-L-arginine (LNNA) (dH_2O), ET-1 (dH_2O), NE (dH_2O), PE (dH_2O), PGF-2 α (ethanol), and S6c (dH_2O).

Statistical Analysis. All data from contractility experiments were normalized to the maximal tissue contraction from PGF-2 α . From the normalized data, means, S.E.M., and variances were calculated. For samples of equal variance, statistical significance between groups was established using a two-tailed, unpaired Student's *t* distribution ($\alpha = 0.05$). For samples of unequal variance, the Mann-Whitney U test was used ($\alpha = 0.05$). All calculations were done using Excel (Microsoft, Redmond, WA).

Results

Localization of ET_A and ET_B Receptors. Both aorta and vena cava showed positive ET_B receptor staining in adventitial, medial, and intimal layers (Fig. 1A). Sections of aorta and vena cava untreated with primary antibody (Fig. 1B), or exposed to primary antibody and competing peptide (data not shown), showed no 3,3'-diaminobenzidine staining. This verifies that staining was specific for the primary ET_B antibody. To establish the presence of ET_B receptors in endothelial cells, freshly dissected vena cava were methanol-fixed and exposed to antibodies for both the ET_B receptor and PECAM-1. ET_B receptor and PECAM-1 localization was observed in vena cava (Fig. 2, A and B). DAPI was added to locate cell nuclei (Fig. 2C). An overlay of all three images showed localization of ET_B receptors within the endothelial cell cytoplasm (Fig. 2D). Parallel experiments using rat aorta were unsuccessful because of background autofluorescence, which made any specific staining indistinguishable from background staining.

Mechanism of ET_B Receptor-Mediated Relaxation in Aorta and Vena Cava. Thoracic aorta and vena cava from male Sprague-Dawley rats were used to establish the effects of ET_B receptor stimulation from basal tone. S6c (100 nM) did not cause contraction of aorta but caused contraction of short duration (<2 min) in vena cava (Fig. 3). A maximal concentration of S6c, as opposed to a cumulative concentration response curve, was used because of the desensitization that occurs with this agonist (Thakali et al., 2004). Next, vessels

Fig. 1. Representative immunohistochemical staining of ET_B receptor in paraffin-embedded, formalin-fixed rat aorta and vena cava. Arrows indicate the intimal, medial, and adventitial layers of each tissue. Dashed black line shows the separation between medial and adventitial layers. Positive staining for ET_B receptor antibody is shown in brown (A) compared with nonspecific staining in the absence of primary antibody (No Primary Ab) (B). Blue hematoxylin staining is used to locate cell nuclei in all pictures. Representative of four experiments.

were contracted with PGF-2 α (20 μ M) before being exposed to S6c (100 nM) (Fig. 4). S6c caused relaxation in both aorta and vena cava.

Aorta and vena cava were next incubated with LNNA (100 μ M) for 1 h or denuded of endothelium and then contracted with PGF-2 α (20 μ M) before being exposed to S6c (100 nM). Endothelial denudation and LNNA (100 μ M) abolished relaxation to ACh (1 μ M) and S6c (100 nM) in aorta (Fig. 5A). In vena cava, however, endothelial denudation and LNNA (100 μ M) abolished relaxation to ACh (1 μ M) but only attenuated relaxation to S6c (100 nM) (Fig. 5B). Inhibition of cyclooxygenase 1 and 2 by indomethacin (5 μ M) did not alter S6c- or ACh-induced relaxation in either the aorta or vena cava. PGF-2 α contraction, established before addition of S6c, was not significantly different ($p > 0.05$) from vehicle ($121.6 \pm 6.6\%$ PE contraction) in the presence of indomethacin ($134.9 \pm 19.1\%$ PE contraction) or endothelial denudation ($123.0 \pm 4.1\%$ PE contraction) but was significantly increased by LNNA ($163.0 \pm 10.5\%$ PE contraction; $p < 0.05$). Likewise, PGF-2 α contraction in vena cava was not significantly changed by indomethacin ($430.3 \pm 50.6\%$ NE contraction) or denudation ($239.5 \pm 33.5\%$ NE contraction) but was significantly increased by LNNA ($564.8 \pm 45.3\%$ NE contraction; $p < 0.05$) compared with vehicle ($309.5 \pm 31.9\%$ NE contraction).

Endothelin-1-Induced Relaxation in Aorta and Vena Cava. PGF-2 α (10 μ M)-contracted vessels were exposed to increasing concentrations of ET-1 (10 pM–100 nM). Vessels were incubated with vehicle, the ET_A receptor antagonist atrasentan (30 nM), or atrasentan (30 nM) and the ET_B receptor antagonist BQ-788 (100 nM), to distinguish between the effects of ET_A and ET_B receptor stimulation. ET-1 caused a concentration-dependent contraction in aorta treated with vehicle (Fig. 6A). Aorta incubated with atrasentan (30 nM)

Fig. 2. Representative immunofluorescent staining of methanol-fixed, en face mounted rat vena cava. A, rhodamine fluorescent staining of ET_B receptor antibody. B, fluorescein isothiocyanate fluorescent staining of PECAM-1 antibody. C, DAPI nuclear staining. An overlay of all three pictures shows the location of ET_B receptors, PECAM-1, and nuclei of endothelial cells (D). Representative of four experiments.

relaxed to ET-1, reaching a sustained minimum at a concentration of 10 nM. As the concentration of ET-1 was increased from 30 to 100 nM, aorta then contracted. Incubation with atrasentan (30 nM) and BQ-788 (100 nM) caused a rightward shift in the relaxant response of the aorta to ET-1, but contraction to ET-1 was not observed.

In contrast to the aorta, ET-1 caused concentration-dependent relaxation of vehicle-incubated vena cava with a maximal relaxation attained at 1 nM (Fig. 6B). As concentrations of ET-1 increased, the vena cava contracted in a concentration-dependent manner. When exposed to atrasentan (30 nM), the vena cava relaxed in a concentration-dependent manner up to 10 nM ET-1 before contracting as concentration increased. Incubation with both atrasentan (30 nM) and

Fig. 3. Representative tracings of aorta (top) and vena cava (bottom), showing contractions resulting from ET_B receptor stimulation with S6c (100 nM). Horizontal black bar represents 2 min of elapsed time. Representative of more than 50 experiments.

Fig. 4. Representative tracing of endothelium-intact aorta (top) and vena cava (bottom), showing relaxation by ET_B receptor stimulation with 100 nM S6c in vessels contracted with 20 μ M PGF-2 α . Representative of six experiments.

BQ-788 (100 nM) shifted the relaxation-response curve to the right, and contraction was not observed.

To further investigate the contribution of ET_B receptors to ET-1-induced relaxation, tissues from male ($D\beta H$)- $ET_B^{(sl/sl)}$ transgenic rats (sl/sl) and their $D\beta H$ - $ETB:ET_B^{(+/+)}$ male

Fig. 5. Measurement of relaxation to the agonists S6c and ACh in PGF-2 α (20 μ M)-contracted aorta (A) and vena cava (B), when exposed to different inhibitors for 1 h or endothelium-denuded (-endo). All bars represent mean $\pm$ S.E.M. for the number of animals indicated in parentheses. White bars represent S6c-induced relaxation. Black bars represent ACh-induced relaxation. Results are shown as percentages of initial PGF-2 α (20 μ M) contraction. Veh, vehicle treatment; Indo, indomethacin (5 μ M). LNNA was at 100 μ M. *, $p < 0.05$ versus vehicle.

littermates (+/+) were tested in the same protocol. Contractions to ET-1 in (sl/sl) aorta contracted with PGF-2 α (10 μ M) were not statistically different from those of (+/+) aorta, and aorta did not relax to ET-1. (Fig. 7A). ET-1 caused only concentration-dependent contraction in (sl/sl) vena cava contracted with PGF-2 α (10 μ M), whereas ET-1-stimulated relaxation was observed in (+/+) vena cava (Fig. 7B).

Discussion

Venous ET_B Receptors and Vascular Function. The main objective of this study was to test whether the mechanisms by which ET_B receptors modulate arterial relaxation are the same or different from those that mediate venous relaxation. We also address the idea of whether the ET_B receptor mediating relaxation could be activated by ET-1, the endogenous agonist for this receptor. The data we present here imply not only a different mechanism of endothelial ET_B receptor function between arteries and veins but also the possible existence of an ET_B receptor on venous smooth muscle cells that mediates relaxation. Understanding the mechanisms controlling venous and arterial responses to ET receptor stimulation may help define the roles of ET-1 and

Fig. 6. Measurement of endothelin-1-induced responses in PGF-2α (10 μM)-contracted aorta (A) and vena cava (B), exposed to vehicle, atrasentan (30 nM), or atrasentan (30 nM) with BQ-788 (100 nM). Vehicle or antagonists were incubated with tissue for 1 h before PGF-2α contraction and subsequent ET-1 exposure. Points represent mean ± S.E.M. for the number of animals indicated in parentheses. *, $p < 0.05$ versus vehicle.

veins in regulating blood pressure. Veins can respond differently than arteries when stimulated by the same agonist, including ET-1, in both physiological and pathological conditions (Thakali et al., 2004). This is important in light of the findings that ET_B receptor stimulation alone causes significant hypertension in rats that is unaffected by ET_A receptor blockade and may be largely mediated by the venous circulation (Fink et al., 2007).

ET_B Receptor-Mediated Relaxation in Venous and Arterial Endothelial and Smooth Muscle Cells. Endo-

Fig. 7. Measurement of endothelin-1-induced responses in PGF-2α (10 μM)-contracted aorta (A) and vena cava (B) from ET_B receptor-deficient rats (sl/sl) and their wild-type littermates (+/+). Points represent mean ± S.E.M. for the number of animals indicated in parentheses. *, $p < 0.05$ versus vehicle.

thelial cell ET_B receptors in most arterial beds stimulate NO release and relaxation, leading us to postulate that venous relaxation also depended on endothelial nitric-oxide synthase (Tsukahara et al., 1994). Even though immunohistochemical analysis confirmed that ET_B receptors are present in the endothelium of the vena cava, inhibition of endothelial nitric-oxide synthase did not abolish S6c-induced relaxation. The concentration of L-NAME (100 μM) used was adequate to abolish ET_B receptor-mediated relaxation in the aorta and acetylcholine-induced relaxation in both the aorta and vena

cava. This confirms that ET_B receptor-mediated relaxation in the aorta is wholly dependent on NO release but suggests that venous ET_B receptor-mediated relaxation is only partially dependent on NO release.

When we combine our findings from LNNA-exposed tissues with our findings in endothelium-denuded tissues, they suggest that there is a functional ET_B receptor in venous tissue, as least as revealed by S6c that can regulate relaxation.

Role of ET_B Receptors in Regulating Responses to ET-1 in Contracted Aorta and Vena Cava. Evidence strongly supports the existence of a contractile ET_B receptor in the vena cava. It is unclear, however, whether antagonism of both ET_A and ET_B receptors reveals the presence of a contractile ET_B receptor in the aorta. The addition of BQ-788 (100 nM) to atrasentan (30 nM) further reduced ET-1-induced contraction in the aorta, suggesting the presence of an aortic contractile ET_B receptor (Fig. 6). Other evidence, however, is contradictory to this idea. First, the ET_B agonist S6c (100 nM) does not directly contract isolated aorta but does contract vena cava (Fig. 3). Second, experiments with tissues from (*sl/sl*) and (+/+) rats revealed that contraction to ET-1 was neither rightward-shifted nor reduced in aorta that lack functional ET_B receptors (Fig. 7A). These collective results present a mixed conclusion as to the presence of an ET_B receptor mediating contraction in the aorta. There is, however, no question that a contractile ET_B receptor is present in the vena cava.

Use of ET-1 in testing the presence of relaxant receptors was important because ET-1 is the endogenous agonist for ET receptors. PGF-2 α -contracted vena cava demonstrated a functional ET_B receptor that mediates relaxation when activated by ET-1. This mechanism was not initially apparent in the aorta, because only contraction to ET-1 was observed in aorta controls. Aorta incubated with atrasentan, however, relaxed to ET-1. This implies that a functional and relaxant ET_B receptor exists in the aorta that is revealed by ET_A receptor blockade and requires a greater concentration of ET-1 than the vein does to activate it. ET-1-induced relaxation has been noted in vivo as well, in which injection of ET-1 results in a transient drop in blood pressure followed by a prolonged increase in arterial pressure (Pollock et al., 1995).

Limitations. There are limitations to some of the studies presented here. Making an association between ET receptor function in large conduit vessels and the role of the ET receptor in maintaining blood pressure is difficult. Large vessels make for more consistent experiments and more obvious differences between arteries and veins, but small-resistance vessels may respond differently than large vessels when taken through the same experimental protocol.

Whenever genetically modified animals are used in an experiment, unexpected phenotypical changes caused by the mutation can arise and should be noted. Although there are no outstanding phenotypical changes in (D β H)-ET_B:ET_B^(*sl/sl*) rats that separate them from their D β H-ET_B:ET_B(+/+) male littermates, blood pressure and circulating ET-1 concentrations are slightly elevated (Hochoer et al., 2001). We do not believe this changed our in vitro results.

With en face immunohistochemical staining, autofluorescence is problematic in thick tissues such as the aorta. We tried to locate ET_B receptors in the endothelium of aorta a

number of times, using varying concentrations of both primary and secondary antibodies as well as different blocking solutions. We were not able to separate the background fluorescence from the specific staining to provide a clear picture. In this study, we demonstrated a functional response to S6c that was endothelial cell-dependent in aorta (Fig. 5A). These data provide some confidence in the statement that ET_B receptors are present in the aortic endothelial cell. Moreover, literature reports have located PECAM-1 in en face mouse aorta (Dusserre et al., 2004) and ET_B receptors in rat aortic endothelial cells (Shichiri et al., 1998). We believe that this information, collectively, is sufficient to state that ET_B receptors are likely to be present in aortic endothelial cells.

Differing results from endothelium-denuded RVC are another limitation. Whereas treatment with S6c still caused relaxation in denuded RVC, the relaxation caused by ET-1 in denuded RVC was abolished. This does not call into question the existence of a functional ET_B receptor on venous smooth muscle cells but shows that ET-1 responses are more complex than S6c responses.

In conclusion, the mechanisms by which ET_B receptors cause relaxation in arteries are different from those in veins. We conclude that ET_B receptors in the vena cava regulate relaxation in a manner partially independent of either a functional endothelium or NO release. It is noteworthy that the endogenous ligand for the ET_B receptor (ET-1) causes relaxation in the vena cava that is not observed in the aorta. We have shown differences in ET_B receptor function in the aorta and vena cava, providing a new understanding of the function of venous ET receptors. These findings are important in understanding the different ways vascular tone is regulated in the venous and arterial circulation and may help to further understand how veins and arteries interact to maintain blood pressure.

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