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CFTR potentiation as a therapeutic strategy for pulmonary edema: a proof-of-concept study in pigs

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Abstract

OBJECTIVE—To determine the feasibility of using a CFTR potentiator, ivacaftor (VX-770, Kayledeco®), as a therapeutic strategy for treating pulmonary edema.

DESIGN—Prospective laboratory animal investigation.

SETTING—Animal research laboratory.

SUBJECTS—Newborn and 3-day to 1-week old pigs.

INTERVENTIONS—Hydrostatic pulmonary edema was induced in pigs by acute volume overload. Ivacaftor was nebulized into the lung immediately after volume overload. Grams of water per grams of dry lung tissue were determined in the lungs harvested 1 hour after volume overload.

MEASUREMENTS AND MAIN RESULTS—Ivacaftor significantly improved alveolar liquid clearance in isolated pig lung lobes *ex vivo* and reduced edema in a volume overload *in vivo* pig model of hydrostatic pulmonary edema. To model hydrostatic pressure-induced edema *in vitro*, we developed a method of applied pressure to the basolateral surface of alveolar epithelia. Elevated hydrostatic pressure resulted in decreased CFTR activity and liquid absorption, an effect which was partially reversed by CFTR potentiation with ivacaftor.

CONCLUSIONS—CFTR potentiation by ivacaftor is a novel therapeutic approach for pulmonary edema.

Keywords

Animal Models of Human Disease; Ion Channels; Membrane Transport; Pulmonary Biology; Treatment; CFTR; pulmonary edema; ion transport

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Introduction

Pulmonary edema, characterized by excessive liquid accumulation in the alveolar space, is commonly observed in many clinical scenarios such as acute respiratory distress syndrome, acute lung injury, heart failure and volume overload. Defective alveolar liquid clearance (ALC) plays a central role in the pathogenesis of pulmonary edema (1–3). ALC is an integrated process that requires basolateral $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity along with increased apical epithelial Na^+ channel (ENaC) and cystic fibrosis transmembrane conductance regulator (CFTR) activity (4–7). In several different experimental pulmonary edema models, it has been shown that dysregulation of each of these factors contributes to impaired liquid absorption (8–10).

Preclinical studies have suggested that liquid absorption can be enhanced by activation of β -adrenergic signaling that increases cAMP levels, which in turn stimulate ENaC, $\text{Na}^+\text{-K}^+\text{-ATPase}$, and CFTR activity (4–7). Despite this strong experimental evidence and promising results from the single-center BALTI study (11), results from the ALTA trial using inhaled β -agonists were disappointing and in some patients increased morbidity and mortality (12, 13). Several reasons for the lack of benefit have been postulated, including ineffective drug delivery to the injured lung, inability of the injured lung to clear liquid, and side effects of β -agonists on other organ systems such as development of cardiac arrhythmias. Thus, alternative strategies to enhance pulmonary liquid absorption are necessary.

We and others have identified CFTR as a key regulator of alveolar liquid absorption (9, 10, 14, 15). Adenoviral-mediated overexpression of CFTR *in vitro* increases liquid absorption (15), whereas CFTR-deficient animals or epithelia have decreased liquid absorption and ALC in response to cAMP elevation (10, 16). These data suggest that increasing CFTR activity is a potential therapeutic strategy to treat pulmonary edema. CFTR potentiators, which were originally designed as a treatment for people with cystic fibrosis (CF) who had certain CFTR gating mutations (17), also increase the open probability of wild-type CFTR (18). Recent work from our group established that the CFTR potentiator ivacaftor (VX-770/Kalydeco, Vertex Pharmaceuticals) augments pig and human, but not mouse, CFTR activity (19). Our objective in this study was to determine the impact of CFTR potentiation on liquid absorption and edema treatment using a porcine model.

Methods

Animals

All animal studies were reviewed and approved by the University of Iowa Animal Care and Use Committee. *CFTR*^{+/+} (denoted as “WT”) and *CFTR*^{-/-} (denoted as “CF”) (20) piglets were obtained from Exemplar Genetics (Exemplar Genetics, Sioux Center, IA).

Liquid clearance in *ex vivo* pig lung

ALC was measured in isolated pig lungs as described (10). Pigs were euthanized (Euthasol, Virbac, Fort Worth, TX) within 12 hours of birth or at 3 to 4 days of age for this study. Under basal conditions, pig lungs were isolated and were not pretreated with isoproterenol. Under stimulated conditions, isolated pig lungs were pretreated with isoproterenol (100

nmol/L perfused through pulmonary artery). Left and right lungs were paired for study: one lobe was treated with vehicle and the other with ivacaftor. As previously described (10), a bronchus of a piglet lung was cannulated with a catheter and instilled with 8 ml of BSA solution containing 0.1 mg/ml Evans blue dye (EBD, Sigma, St. Louis, MO) in the absence or presence of 10 μ mol/L ivacaftor. The lung was inflated with 100% oxygen through the catheter at 7 cm H₂O airway pressure, placed in a plastic bag, and then submerged in a water bath at 37°C. Alveolar liquid was aspirated at 10 min and 70 min after instillation. EBD concentration of the aspirate samples (provided in Online Table I) was assayed by measuring the absorbance at 620 nm in a Hitachi Model U2000 Spectrophotometer (Hitachi Inst., San Jose, CA) and ALC was calculated as previously described (21).

***In vivo* hydrostatic model of pulmonary edema**

Hydrostatic pulmonary edema was induced in 4 to 7 day-old pigs by acute volume overload using a modified protocol used in sheep (22, 23). Pigs were initially sedated with Ketamine (Ketaject, Phoenix Pharmaceuticals, Burlingame, CA; 20 mg/kg, i.m. injection) and Xylazine (AnaSed, Lloyd, Inc., Shenandoah, IA; 2 mg/kg, i.m. injection) and anesthetized using Propofol (Diprivan, Fresenius Kabi, Germany; 2 mg/kg, i.v. injection). A central line was placed to monitor central venous pressure (CVP). A peripheral i.v. route was established. Pigs were infused intravenously with saline at a constant rate to achieve an ~20% increase in body weight in 30–45 min. Immediately after volume overload, ivacaftor (10 μ mol/L in 1 ml of 10% DMSO/saline) or vehicle control (1 ml of 10% DMSO/saline) was nebulized into the lung. Pigs were euthanized (Euthasol) 1 hour after volume overload and grams of water per grams of dry lung tissue were determined as previously described (10).

In some experiments, inferior vena cava diameter was monitored during saline infusion using CT imaging to validate the model. Specifically, inferior vena cava minor diameter measurements were obtained from the transverse plane of inspiratory (25 cm H₂O) chest CT scans based on the contrast between the vessel and surrounding lung parenchyma. Measurements were made halfway between heart and diaphragm using ImageJ's tape measure feature.

In some experiments, lung compliance was monitored during saline infusion using a computer controlled respirator (flexiVent, SCIREQ, Inc., Montreal, Canada) as previous described (24). The pig was paralyzed using rocuronium (1 mg/kg) and mechanically ventilated. Lung quasi-static compliance was obtained using the maneuver pressure-volume ramp volume regulated.

Cell culture

Primary alveolar epithelial cells were isolated from lungs of piglets within 12 hours after birth and co-cultured in F media in the presence of 10 μ mol/L Y-27632 (Enzo Life Sciences, Switzerland), a ROCK inhibitor, and irradiated fibroblast feeder cells NIH-3T3-J2 and maintained at 37°C with 5% CO₂ using a previously reported method to expand human airway epithelia (25). After one week, amplified cells were then cultured at the air-liquid

interphase (ALI) as previously described (10) for 5–7 days in the absence of feeder cells and ROCK inhibitor.

***In vitro* model of elevated hydrostatic pressure**

Expanded porcine alveolar epithelial cells (5–7 days after seeding) were cultured on transwells at the ALI. Elevated hydrostatic pressure was modeled by applying pressure to the basolateral surface of cell culture inserts using a custom-made device that consists of a pressure column to apply the pressure by elevating a liquid column (filled with culture medium or buffer solution) to a height of 5–15 cm (Online Figure 2). Ussing chamber studies and liquid absorption measurements then were performed as described below.

Ussing chamber studies

Ussing chamber studies were performed using expanded pig alveolar epithelia 5–7 days after seeding. Cells were mounted in Ussing chambers and studied as previously described (26–28). Hydrostatic pressure (0–15 cm H₂O) was applied to the basolateral side. Na⁺ current was inhibited with 100 µmol/L amiloride on the apical side, an epithelial sodium channel blocker, followed by addition of 100 µmol/L DIDS (4,4'-diisothiocyanato-stilbene-2, 2'-disulfonic acid) apically to block the calcium activated Cl⁻ channel. Next, CFTR activity was increased by elevating cellular levels of cAMP with apical 10 µmol/L forskolin and 100 µmol/L IBMX (3-isobutyl-1-methylxanthine) (F&I). Finally, 100 µmol/L GlyH, a CFTR inhibitor, was added to the apical side. $I_{sc\ GlyH}$ is the decrease in current after apical addition of GlyH.

***In vitro* liquid absorption rate measurement**

The rate of liquid absorption by expanded pig alveolar epithelia (5–7 days after seeding) was measured as previously described (10). Briefly, the basolateral solution was replaced with fresh cell culture medium (500 µl) and saline (100 µl) was applied to the apical surface. In some experiments, hydrostatic pressure (5 cm H₂O) was applied to the basolateral side. Osmolality of the submucosal solution was adjusted to equal that of the mucosal solution. After incubation for 16 hours, the apical solution was collected and the volume was measured. In some experiments, 5 µmol/L forskolin & 50 µmol/L IBMX was added to the basolateral solution and 10 µmol/L ivacaftor to the apical solution.

Statistics

The statistical analysis was performed using different tests indicated in the Online Supplement and $P < 0.05$ was defined as statistically significant.

Results

Ivacaftor increases ALC *ex vivo*

We investigated the effect of ivacaftor on ALC *ex vivo* using an isolated non-perfused piglet lung model (10). Under basal conditions, instillation of ivacaftor had no effect on ALC in lungs from 3–4 day-old WT pigs (Fig. 1A). Next, pig lungs were stimulated with low-dose isoproterenol to induce CFTR phosphorylation (17) and then ivacaftor administered in the

instillate. As compared to control treatment, ivacaftor significantly increased ALC by 2-fold (Fig. 1B). To determine if CFTR expression is required for ivacaftor-mediated increase in ALC, we repeated studies using newborn CF pigs, since these CF pigs do not survive >48 hours due to meconium ileus. Following perfusion of isolated lung lobes with a low dose of isoproterenol, we found that ivacaftor did not potentiate ALC in CF pig lung lobes (Fig. 1C).

Ivacaftor potentiation attenuates pulmonary edema induced by hydrostatic pressure in vivo

One pathologic origin of cardiogenic pulmonary edema is increased hydrostatic pressure in pulmonary capillaries, which causes liquid filtration to flood the alveoli (29). Another important, yet underappreciated, contributing factor is impaired ALC from the alveolar space (1–3). To determine if ivacaftor can resolve edema *in vivo*, we modeled acute hydrostatic pulmonary edema via volume overload in pigs. Experiments were performed without pretreatment with isoproterenol since, *in vivo*, CFTR is thought to be dynamically phosphorylated in response to circulating catecholamine levels and protein kinase A (30). Pigs were infused intravenously with saline at a constant rate to achieve a 20% increase in body weight in 30 to 45 min (Fig. 2A). Edema formation in this model was confirmed by analysis of lung histology and changes in lung compliance (Online Figure 1). Next, ivacaftor was administered by nebulization because CFTR is primarily located on the apical surface of epithelial cells (10), and also to minimize the volume of distribution if given systemically. Ivacaftor treatment resulted in a significant decrease in the grams of water per grams of dry lung tissue (Fig. 2B), consistent with reduced edema.

Effect of elevated hydrostatic pressure on ion transport properties of alveolar epithelia in vitro

To understand the mechanisms by which ivacaftor promotes ALC *ex vivo* and reduced edema *in vivo*, we created an *in vitro* model of elevated hydrostatic pressure by applying 5 cm H₂O pressure to the basolateral surface of cultured alveolar epithelial cells (Online Figure 2A). To overcome technical challenges associated with culture of primary cells, we adapted a protocol to expand primary alveolar epithelial cells. This protocol is based on recent studies showing that expanded human alveolar epithelial cells maintain phenotypic features as non-expanded primary cells (31). We validated that expanded alveolar epithelia from pigs retain expression of several genes that are unique to native alveolar epithelia, including surfactant protein A, B and D (Online Figure 3).

We first investigated the effect of hydrostatic pressure on ion transport properties in alveolar epithelial cells. Elevated hydrostatic pressure was modeled by applying a range of pressures to the basolateral surface using a column elevated to a height of 5–15 cm. Based on Ussing chamber studies of electrochemical properties, 5 cm H₂O was chosen as the optimal pressure to obtain stable traces (Online Figure 2B). Representative Ussing chamber traces in response to 5 cm H₂O are shown in Fig. 3A. Surprisingly, elevated hydrostatic pressure increased amiloride-sensitive current, suggestive of higher ENaC activity (Fig. 3B). Moreover, CFTR activity, as shown by GlyH-inhibitable current, was decreased by 25% in response to 5 cm H₂O hydrostatic pressure (Fig. 3C, D). These data provide the first direct

evidence for the impact of elevated hydrostatic pressure on bioelectric properties of pig alveolar epithelia.

Ivacaftor rescues the impaired liquid absorption induced by elevated hydrostatic pressure *in vitro*

The observed decrease in GlyH-inhibitable current in response to elevated hydrostatic pressure (Fig. 3D) is consistent with the role of CFTR in liquid absorption. We therefore examined whether ivacaftor can increase liquid absorption under elevated hydrostatic pressure *in vitro*. In the absence of hydrostatic pressure, ivacaftor increased liquid absorption under basal conditions and when CFTR was stimulated using forskolin and IBMX (3-isobutyl-1-methylxanthine) (Fig. 4A, B). Next, we investigated the effect of elevated hydrostatic pressure on liquid absorption by applying 5 cm H₂O pressure to the basolateral surface of alveolar epithelial cells. We found a significant decrease in liquid absorption in response to 5 cm H₂O pressure compared to 0 cm H₂O, and a partial rescue by treatment with ivacaftor (Fig. 4C). These data are consistent with the ability of ivacaftor to increase ALC *ex vivo* and decrease edema *in vivo*.

Discussion

Pulmonary edema often manifests as acute respiratory failure requiring urgent therapies, including mechanical ventilation. Current treatment strategies for hydrostatic pulmonary edema rely on decreasing pulmonary capillary pressure, which include diuretics and vasodilators(32). These therapies are frequently ineffective and poorly tolerated for patients with common co-morbid conditions such as impaired renal function or hypotension(33–36). Moreover, these therapies do not target the dysregulated alveolar liquid transport processes, an important contributing factor for pathogenesis of pulmonary edema. Therapeutic approaches targeting clearance of alveolar fluid can rapidly relieve respiratory distress and prevent or reduce time on ventilatory support. Preclinical data identified β -adrenergic receptor agonists as a potential therapy for pulmonary edema because activation of β -adrenergic signaling accelerates ALC (14) by increasing activity of basolateral Na⁺-K⁺-ATPase and apical ENaC and CFTR (4–7). Importantly, *ex vivo* treatment with a β -agonist in resected human lungs improves ALC by 2.3-fold (37). These data served as one of the rationales for the BALTI trial, where β -agonist produced a significant 1.4-fold decrease in lower lung water that was associated with a lower Murray lung injury score (11). However, using β -agonists to treat acute lung injury did not improve clinical outcomes and showed a trend towards increase mortality (12, 13).

While numerous studies have focused on the active Na⁺ transport pathways and demonstrated central roles for ENaC and Na⁺-K⁺-ATPase in this process, more recently CFTR has been implicated in liquid absorption in alveolar epithelia in response to elevations in cAMP (9, 10, 14, 15). Increased transepithelial Cl⁻ transport results in osmolarity changes on the basolateral surface, promoting liquid absorption along the electrochemical gradient (38). Consistent with this concept, work from our group and others established that CFTR activity promotes liquid absorption *in vitro* and ALC *ex vivo* (9, 10, 14, 15). Therefore, we examined CFTR potentiation with ivacaftor as an alternative strategy for pulmonary edema

treatment. In *ex vivo* studies in isolated pig lungs, we demonstrated a 2-fold increase in ALC with ivacaftor. While ivacaftor was not compared head to head to β -agonist stimulation, the increase in ALC with ivacaftor is comparable to previously published findings from our group demonstrating that β -agonist increases ALC 2-fold (10). These data suggest that CFTR potentiation may provide a similar benefit as β -agonists without adverse cardiac effects as was observed in the two BALTI trials (11, 12).

In vivo and *ex vivo* studies demonstrate that pulmonary edema is caused by a pressure gradient of ~5–10 cm H₂O height between the pulmonary artery and left atrium (39). This pressure difference can be transmitted to alveolar epithelia through a change in the interstitial hydrostatic pressure. The change of interstitial hydrostatic pressure can be as high as 15 cm H₂O (from -11 to 3 cm H₂O), depending on the species (40). To our knowledge, no methods existed to evaluate the effect of elevated basolateral hydrostatic pressure on alveolar epithelia cells *in vitro*. Herein we report a system where pressure is applied to the basolateral surface of expanded alveolar epithelia, thus allowing analysis of bioelectric properties and liquid transport. We found that increased hydrostatic pressure (5 cm H₂O) resulted in a significant decrease in CFTR activity and impaired liquid transport. Importantly, ivacaftor rescued the impaired liquid transport. Our *in vitro* model of cultured alveolar epithelia lacks the capillary component; thus we do not know whether bioelectric and liquid transport properties would be similarly altered in the presence of an endothelial monolayer as *in vivo*.

In this study, we provide evidence that potentiation of CFTR activity using ivacaftor enhances liquid absorption and ALC to treat pulmonary edema. These results may appear to be contradictory to previous published data regarding the role of CFTR in pulmonary edema formation in mice (39). In that study, they used mice deficient in CFTR or a CFTR inhibitor to demonstrate that, in pulmonary edema, nitric oxide regulates ENaC activity, creating changes in the electrochemical gradient that lead to a switch in the directionality of Cl⁻ transport through CFTR (39). That results in cells switching from liquid absorption to secretion mode and suggests that CFTR plays a key role in edema formation by mediating Cl⁻ flux from the basolateral through the apical membrane. However, we speculate that CFTR only promotes liquid secretion when the apical surface is devoid of excess liquid (10) such that CFTR potentiation in the setting of existing lung edema would only result in liquid absorption without enhancing liquid secretion. It should be noted that CFTR has been suggested to potentiate edema formation in the setting of hydrosalpinx induced by chlamydia infection (41). In addition, CFTR potentiation with the CFTR activator CFTRact-16 had no additional benefit for LPS-induced lung injury in mice, though this study demonstrated a clear role for CFTR in ALC (42). These data indicate that the effectiveness of CFTR potentiation for edema treatment may be dependent upon the timing of drug administration and edema model system.

Since this study is a proof-of-concept pilot study, we focused on the experiments that would provide the most direct evidence that potentiation of CFTR by ivacaftor can treat pulmonary edema. We therefore chose a large animal preclinical model for these studies, though there are limitations associated with studies in larger animals. As compared to mice, pigs are not syngeneic and thus results are more variable. To minimize variability within experiments, we paired lung lobes from the same pig when possible. Studies in pigs are not high throughput;

thus, minimal animals were used and dose response studies were not performed. On the other hand, as compared to humans, studies in pigs have some advantages for research on pulmonary edema. In humans, pulmonary edema cannot be measured directly and is inferred by changes in chest x-ray, lung compliance, pulmonary capillary wedge pressure (PCWP), and arterial blood gases. In pigs, we can directly measure pulmonary edema by measuring the grams of water per grams of dry lung. We recognize that subsequent studies to establish the optimal dose, route, etc., are necessary to translate these findings into a treatment for patients.

In summary, this study provides the first proof-of-principle evidence that CFTR potentiation may be a therapeutic strategy for cardiogenic pulmonary edema. Further investigation is also necessary to determine if CFTR potentiation is effective in other models, such as pulmonary edema caused by chronic heart failure or acute lung injury. Finally, it will be necessary to identify the ideal route for drug administration since ivacaftor is formulated for oral intake rather than nebulization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

ALC	alveolar liquid clearance
ALI	air-liquid interface
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
ENaC	epithelial Na ⁺ channel
SPA/B/D	surfactant protein A/B/D

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Figure 1. Ivacaftor enhances ALC in isolated pig lungs, and CFTR is required for this effect (A) ALC was examined in 3–4 day-old WT pig lungs *ex vivo* following treatment with DMSO or ivacaftor (10 μ mol/L in instillate) for 1 hour (n=5 pigs). (B, C) WT or CF pig lungs were pretreated with isoproterenol (100 nmol/L perfused through pulmonary artery), and ALC determined *ex vivo* following treatment with DMSO or ivacaftor as in A. ALC was determined 1 hour later and compared to control without ivacaftor (%CTL) (n=7 for WT pigs and 7 CF pigs). Dashed lines: paired individual pigs with or without ivacaftor treatment;

solid lines: mean $\pm$ SE; filled squares: mean fold change with 95% confidence intervals relative to control. *See Online Table 1 for raw data.* * $P < 0.05$ by Paired t test on log.

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Figure 2. Ivacaftor alleviates hydrostatic pulmonary edema

(A) Hydrostatic pulmonary edema was induced *in vivo* by acute volume overload with saline infusion (indicated by red dashed lines). Changes in central venous pressure (CVP) were monitored during acute volume overload. Insets depict changes in the diameter of inferior vena cava in response to volume overload in different phases separated by red lines: a, pre-saline infusion phase; b, saline infusion phase; c, edema resolution phase (see Supplementary Methods for experimental details). (B) After completion of saline infusion, ivacaftor (10 μ mol/L in 1 ml of 10% DMSO/saline) or DMSO control (CTL) were

administered by nebulization and grams of water per grams of dry lung tissue determined 1 hour later. No saline infusion serves as baseline control. * $P < 0.05$ vs baseline; # $P < 0.05$ vs CTL treatment by ANOVA; n=5.

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Figure 3. Effect of elevated hydrostatic pressure on bioelectric properties of alveolar epithelia *in vitro*

(A) Representative Ussing chamber traces of expanded alveolar epithelial cells in response to elevated hydrostatic pressure and subsequent treatments as indicated by arrows. (B-D) Quantitation of amiloride-sensitive (B, $I_{sc\ Aml}$), cAMP-stimulated (C, $I_{sc\ F\&I}$), or GlyH-inhibitable current (D, $I_{sc\ GlyH}$) in WT pig alveolar cells exposed to 0 or 5 cm H₂O hydrostatic pressure. Dashed lines: paired individual pigs with or without hydrostatic pressure; solid lines: mean $\pm$ SE; red filled squares: mean change in Isc with 95% confidence intervals relative to control, expressed as percent of control. * $P < 0.05$; $n=6$ by paired t test on log.

Figure 4. Ivacaftor partially reverses the impaired hydrostatic pressured-induced liquid absorption by alveolar epithelia *in vitro*

(A, B) Liquid absorption (Jv) was assessed in expanded alveolar epithelial cells from WT pigs under basal conditions (A) or following treatment with forskolin+IBMX (5+50 $\mu\text{mol/L}$, B) in the absence or presence of 10 $\mu\text{mol/L}$ ivacaftor. n=10 transwells from 3 pigs. (C) Liquid absorption in expanded alveolar epithelia exposed to 0 or 5 cm H₂O in the absence (white bars) or presence of 10 $\mu\text{mol/L}$ ivacaftor (gray bars). Note that all groups were

exposed to low-dose forskolin+IBMX (5+50 $\mu\text{mol/L}$) prior to treatment with ivacaftor. * $P < 0.05$ vs. CTL; # $P < 0.05$ vs. 0 cmH_2O ; $n=3$ by ANOVA.

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