Allergic inflammation-induced neuropeptide production in rapidly adapting afferent nerves in guinea pig airways

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Myers, Allen C., Radhika Kajekar, and Bradley J. Undem. Allergic inflammation-induced neuropeptide production in rapidly adapting afferent nerves in guinea pig airways. Am J Physiol Lung Cell Mol Physiol 282: L775–L781, 2002; 10.1152/ajplung.00353.2001.—In the vagal-sensory system, neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) are synthesized nearly exclusively in small-diameter nociceptive type C-fiber neurons. By definition, these neurons are designed to respond to noxious or tissue-damaging stimuli. A common feature of visceral inflammation is the elevation in production of sensory neuropeptides. Little is known, however, about the physiological characteristics of vagal sensory neurons induced by inflammation to produce substance P. In the present study, we show that allergic inflammation of guinea pig airways leads to the induction of substance P and CGRP production in large-diameter vagal sensory neurons. Electrophysiological and anatomical evidence reveals that the peripheral terminals of these neurons are low-threshold Aδ mechanosensors that are insensitive to nociceptive stimuli such as capsaicin and bradykinin. Thus inflammation causes a qualitative change in chemical coding of vagal primary afferent neurons. The results support the hypothesis that during an inflammatory reaction, sensory neuropeptide release from primary afferent nerve endings in the periphery and central nervous system does not require noxious or nociceptive stimuli but may also occur simply as a result of stimulation of low-threshold mechanosensors. This may contribute to the heightened reflex physiology and pain that often accompany inflammatory diseases.

vagal neurons; nodose ganglion; jugular ganglion; capsaicin; mechanosensors; substance P

By contrast, the faster-conducting myelinated (A-fibers) sensory nerves in the vagus are relatively less sensitive to inflammatory mediators but are often exquisitely sensitive to mechanical stimulation (24). With respect to the vagal sensory innervation of guinea pig airways, substance P and related neuropeptides are localized nearly exclusively in the small-diameter (<25-μm-diameter) unmyelinated C-fiber class of sensory neurons (14, 24).

Activation of nociceptive C-fibers results in the local release of substance P and related neuropeptides at the site of inflammation, where they can contribute to the inflammatory reaction through a process referred to as neurogenic inflammation (5). Activation of nociceptors also causes the release of neuropeptides from their central terminals where they can augment synaptic neurotransmission and participate in “central sensitization” (17). In addition to leading to activation of C-fibers, a large literature reveals that inflammation also leads to an increase in the production of neuropeptides in sensory nerves. Increase in neuropeptide synthesis is associated with experimental inflammation of the joints (1), skin (22), eyes (15), teeth (9), lungs (26), gastrointestinal tract (28), and bladder (3). Increases in substance P content are found in human inflammatory diseases such as asthma (26) and are associated with heightened pain sensations in ulcerative colitis (13), appendicitis (6), and rheumatoid arthritis (20). Little is known, however, about the phenotype and physiology of the neurons that are induced by inflammation to produce neuropeptides.

In a study of inflammatory pain caused by injection of turpentine oil into rat paws, substance P production was noted to be induced in large-diameter neurons located in dorsal root ganglia (1). The specific responsiveness of the peripheral nerve endings of the large-diameter substance P-containing neurons was not directly studied, but the size of the neurons indirectly suggests that they likely belong to a nonnociceptive class of nerves. This is a highly relevant finding because it suggested to the authors a mechanism by...
which simply stimulating touch fibers may contribute to pain sensations, i.e., an allodynic response.

In the present study, we specifically address the hypothesis that substance P and calcitonin gene-related peptide (CGRP) are constitutively produced in visceral sensory nociceptive C-fibers, but production of these neuropeptides induced by inflammation occurs in large, fast-conducting, low-threshold mechanically sensitive fibers.

**MATERIALS AND METHODS**

**Immune sensitization and allergen challenge.** Adult male Hartley guinea pigs \((n = 4; 300–500 \text{ g})\) were passively sensitized to antigen by intraperitoneal injection with serum (20 ml/kg) containing IgG1 that was collected from guinea pigs actively sensitized to ovalbumin. Control guinea pigs were injected with serum from guinea pigs not sensitized to ovalbumin (27). After 24 h, control \((n = 6)\) guinea pigs were exposed to aerosolized antigen (0.01% ovalbumin) in a Plexiglas chamber \((\text{vol} = 1.1 \text{ m}^3)\). The guinea pigs were closely monitored for signs of an allergic response \((\text{gasping or rapid breathing})\) and then removed to breathe ambient air. This commonly employed animal model resulted in an eosinophilic bronchitis within 24 h of exposure (data not shown).

**Extracellular electrophysiology of airway nerve ending activity.** Twenty-four hours after antigen challenge, the animals were killed by CO2 inhalation and exsanguinated. The trachea/bronchus was prepared, as previously described, for extracellular recording of action potential discharge in nodose and jugular afferent nerve fibers that have defined receptive fields in the airway wall (24). The airways with intact right-side extrinsic vagal innervation \((\text{including nodose and jugular ganglia})\) were removed and placed in a dissection dish containing Krebs bicarbonate buffer solution gassed with 5% O2–5% CO2 and composed of \((\text{mM})\): 118 NaCl, 5.4 KCl, 1.0 NaH2PO4, 1.2 MgSO4, 1.9 CaCl2, 25.0 NaHCO3, and 11.1 dextrose. Connective tissue was trimmed away, leaving the trachea, larynx, and right mainstem bronchus with intact nerves \((\text{vagus, superior laryngeal, and recurrent laryngeal})\), including nodose and jugular ganglia. A longitudinal cut was made through the ventral surface of the larynx, trachea, and bronchi, and the airways were then pinned, mucosal side up, to a Sylgard-lined Perspex chamber. The right nodose and jugular ganglia, along with the rostral most vagus and superior laryngeal nerves, were gently pulled through a small hole into an adjacent compartment of the same chamber for recording of single fiber activity. Both compartments were superfused with Krebs solution. The temperature was maintained at 37°C with a flow rate of 6–8 ml/min. Extracellular recordings were performed by manipulating a fine aluminosilicate glass microelectrode filled with 3 M sodium chloride near neuronal cell bodies in the jugular or nodose ganglion.

Mechanically sensitive receptive fields were revealed when a burst of action potentials was elicited in response to von Frey filament stimulation. Conduction velocity and amplitude of the action potential were then compared with responses elicited by electrical stimulation of either the recurrent laryngeal or vagus nerve trunks to determine the trunk that supplied the fiber (24). Conduction velocities were calculated by electrically stimulating the receptive field and measuring the distance traveled along the nerve pathway divided by the time between the shock artifact and the recorded action potential.

Retrograde labeling of airway-specific neurons. Airway neurons in the nodose and jugular ganglia were labeled by retrograde tracing techniques 7–14 days before antigen challenge. Guinea pigs \((n = 32)\) were anesthetized with injection of ketamine \((50 \text{ mg/kg})\) with xylazine \((2.5 \text{ mg/kg im})\), the trachea was exposed, and 10 ml of rhodamine-dextran dye \((3% \text{ Fluororuby; Molecular Probes, Eugene, WA})\) was injected in five to six sites in the dorsal wall of the trachea. The guinea pigs were allowed to recover for 7–14 days. Jugular or nodose ganglia neurons that project axons to the trachea were identified by the accumulation of rhodamine-dextran in their cell bodies with a fluorescent microscope equipped with a filter set appropriate for visualizing rhodamine as previously described (2, 8). One ganglion and one neuron was recorded from each dye-injected animal (see below).

Twenty-four hours after antigen challenge, the animals were killed as above. Ganglia were isolated, fixed in 4% formaldehyde in phosphate-buffered saline \((\text{PBS})\) for 2 h at 4°C, rinsed in PBS, cryoprotected with 18% sucrose in PBS for 18–24 h, covered with optimum cutting temperature mounting medium, and frozen on dry ice. Serial sections of 12-μm-thickness \((\text{except for Fig. 1C, 16 μm})\) of each tissue type were cut on a cryostat, collected on silane-coated slides, and air-dried for 30 min. Sections were incubated with blocking solution containing 1% bovine serum albumin \((\text{BSA})\), 10% normal sheep serum, and 0.1% Tween 20 in PBS for 60 min. The tissue was incubated for 30 min at 37°C in a mixture of polyclonal rabbit antisemur to substance P \((\text{diluted 1:200; Peninsula Laboratories, Belmont, CA})\) and a mouse monoclonal antibody to neurofilament \((160 \text{ kDa; diluted 1:30; Boehringer Mannheim, Indianapolis, IN})\) in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin \((\text{PBS-TX}+\text{BSA})\).

In separate experiments, nodose and jugular ganglia from control and challenged animals were immunostained with a mixture of polyclonal rabbit antisemur to the vanilloid-1 receptor \((\text{VR-1; diluted 1:100; Chemicon International, Temecula, CA})\) and a rat monoclonal antibody to substance P \((\text{diluted 1:200; Chemicon})\). Slides were washed in PBS-TX+BSA and incubated for 1 h at 22°C with a mixture of either sheep anti-mouse antibody labeled with Alexa 594 \((\text{diluted 1:100; Molecular Probes})\) and sheep anti-rabbit antibody labeled with Alexa 488 \((\text{diluted 1:100; Molecular Probes})\) or, for VR-1 staining, sheep anti-rabbit antibody labeled with Alexa 488 \((\text{diluted 1:100; Molecular Probes})\) or sheep anti-rabbit antibody labeled with Alexa 594 \((\text{diluted 1:100; Molecular Probes})\) and sheep anti-rabbit antibody labeled with Alexa 488 \((\text{diluted 1:100; Molecular Probes})\) or, for VR-1 staining, sheep anti-rabbit antibody labeled with Alexa 488 or sheep anti-rat antibody labeled with Alexa 594 in PBS-TX+BSA. Separate sections were processed similarly, but the primary antibody was excluded to evaluate nonspecific staining. The slides were evaluated with the appropriate filter sets to allow separate visualization of Alexa 594 \((\text{rhodamine})\) or Alexa 488 \((\text{fluorescein})\). Sections were counted and photographed, and digital images were converted to black and white prints without additional image processing. For neurons that were used for intracellular recording, the ganglia were fixed, sectioned, and stained as above except rabbit anti-substance P primary antibody and sheep anti-rabbit antibody labeled with Alexa 350 \((\text{Molecular Probes})\) were viewed with an ultraviolet filter set, airway-labeled neurons were viewed with a rhodamine filter set, and the Lucifer yellow-injected neuron \((\text{see below})\) was viewed with a fluorescein filter set. The mean distance of the long axis and short axis of the neuronal soma was taken as the “neuronal diameter” throughout processing the data.

**Intracellular electrophysiology.** Twenty-four hours after antigen challenge, the vagus nerve and associated sensory ganglia were removed as above except the vagus nerve was held by a suction electrode for stimulation of vagal axons. Once a rhodamine-positive airway neuron was located with a fluorescent microscope, the fluorescent light was switched to...
bright-field illumination, and the neuron was impaled. Intracellular micropipettes were filled with 5% Lucifer yellow carboxyhydrate (Molecular Probes) in 0.1 M LiCl. The conduction velocity of the associated axon was determined, and capsaicin (1.0 μM) was superfused (2 min, 20 ml) over the ganglion, recording the corresponding change in the resting potential. Electronic voltage transients (see downward deflections in Fig. 3, A and B) were evoked by −100 pA, 1 Hz current injection, to monitor changes in membrane resistance. Only one neuron was exposed to capsaicin in each ganglion from each dye-injected animal. To conclusively demonstrate that the recorded neuron was the identified airway neuron, Lucifer yellow was ionophoresed into the neuron, and fluorescent light (excitation 450–480 nm, emission 515 nm) was used to observe the impaled cell. If both rhodamine and Lucifer yellow were colocalized, the preparation was fixed and prepared for immunostaining for substance P using an ultraviolet-labeled secondary antibody.

RESULTS

The 160-kDa neurofilament (NF) protein is a selective marker for large-diameter neurons in guinea pig vagal sensory ganglia (14, 24). As expected, in control animals (n = 6), all NF-negative neurons in vagal sensory ganglia had small diameters (diameter ~20 μm) and were uniformly positive for substance P immunoreactivity. Conversely, none of the large NF-positive neurons (mean diameter ~40 μm) expressed substance P immunoreactivity. Figure 1A shows several small-diameter substance P-positive neurons in a control guinea pig nodose ganglion. Consistent with our previous study (24), nodose neurons labeled retrogradely from the trachea/bronchus; however, almost all (713/727 from 6 animals) were large-diameter (>26 μm), NF-positive, substance P-negative neurons. The size distribution of nodose neurons retrogradely labeled from the airway was unimodal with a mean diameter of 43 ± 8 μm. The 14 nodose neurons labeled from the airway that were NF negative were all substance P positive, and all had an average diameter of 19 ± 5 μm. We therefore investigated whether allergic inflammation increased substance P in the large-diameter (>25 μm) NF-positive neurons. In ovalbumin-exposed, nonsensitized guinea pigs (n = 6), <1% of the large-diameter NF-positive neurons revealed substance P or CGRP immunoreactivity. By contrast, within 1 day of ovalbumin challenge in sensitized animals (n = 6), >30% of the large-diameter NF-positive neurons were substance P and CGRP immunoreactive (Fig. 1). Importantly, the substance P immunoreactivity was transported away from the cell soma and into both the peripheral and central processes (Fig. 1C).

When we repeated this experiment but evaluated only tracheal-specific nodose neurons (i.e., those that were previously retrogradely labeled by tracer dye and injected into a small region of the tracheal wall), we found similar results. As mentioned above, the retrogradely labeled neurons were large-diameter neurons (mean diameter >40 μm), and these were all negative for substance P immunoreactivity. Within 1 day of antigen challenge, an average of 32 ± 3% of the large-diameter, tracheal-specific neurons were immunoreactive for substance P (P < 0.01, n = 3 animals, data not shown).

The large-diameter nodose ganglion neurons project fast-conducting Aδ fibers to the airways (24). The terminals of these fibers are unresponsive to capsaicin and inflammatory mediators but are exquisitely sensitive to mechanical stimulation (24). We next addressed whether induction of substance P and CGRP in the large-diameter nodose neurons caused by allergen exposure was associated with a phenotypic switch in the
responsiveness of the nerve endings such that they became nociceptive in nature. Consistent with our previous findings (24), both C-fibers and A-fibers in the trachea/bronchus derived from cell bodies located in the jugular ganglia were responsive to capsaicin (Fig. 2D). By contrast, the nerve endings of nodose neurons (Aδ fibers, conduction velocity = 4–8 m/s) with receptive fields in the wall of the trachea or bronchus were uniformly negative in their response to capsaicin (1 μM) in both untreated and allergen-challenged ani-

Fig. 2. Tracheal nerve endings from nodose Aδ neurons remain unresponsive to capsaicin 24 h after antigen challenge. A: single unit recording from a nodose neuron with its nerve ending in the tracheal wall. Capsaicin (1 μM) applied to the mechanical receptive field in the airway had no effect, whereas mechanical stimulation (at bar) of the field with a von Frey fiber caused a burst of action potentials. The conduction velocity of this fiber was 5.1 m/s (horizontal bar = 10 s). B: by contrast, when capsaicin is applied to the nerve ending (1 μM at arrow) of a jugular ganglion Aδ neuron, action potentials are elicited (conduction velocity 5.2 m/s). A similar response is evoked when capsaicin is applied to the nerve endings of jugular C-fibers (not shown). C: capsaicin (arrows) has no effect on the nerve terminal of an Aδ neuron located in a nodose ganglion from an animal that was challenged with antigen 24 h earlier, whereas mechanical stimulation (at bar) of the field with a von Frey fiber caused a burst of action potentials. D: summary of responses to capsaicin applied to tracheal nerve ending in jugular Aδ fiber (n = 18) and C-fiber (n = 6) neurons and nodose Aδ fiber neurons (n = 12) in control guinea pig and from nodose Aδ fiber neurons (n = 12) from antigen-challenged guinea pigs.

Fig. 3. Intracellular recordings showing that capsaicin causes membrane depolarizations in airway-specific jugular neurons but has no effect on airway-specific nodose ganglion neuronal cell bodies from control or antigen-challenged guinea pigs. Rhodamine-dextran that was injected in the tracheal wall 7–14 days before recording collects in the cell soma of nodose neurons, allowing visualization of airway neurons for intracellular recordings from these cells in vitro. A: airway jugular neurons (identified by presence of rhodamine-dextran, not shown) with both large and small diameters depolarize in response to bath-applied capsaicin (1 μM). B: airway nodose Aδ neurons (identified by presence of rhodamine-dextran, somal size, and conduction velocity) from antigen-challenged guinea pig was unaffected by bath-applied capsaicin (1 μM). C: summary of the responses to capsaicin by airway jugular (Aδ and type C-fiber neurons) neurons from control (left, open bar; n = 12) and antigen-challenged (left, filled bar) guinea pigs (n = 8); capsaicin had no effect on nodose Aδ neurons from control (open bars; n = 6) or antigen-challenged (filled bars; n = 6) guinea pigs.
mals (Fig. 2). The peripheral endings of the nodose fibers in sensitized and allergen-challenged airways were also unresponsive to bradykinin (1 μM; n = 6, data not shown). Bradykinin (1 μM) vigorously stimulated the nociceptive endings in the guinea pig airways that are derived from jugular neurons (12). All the nodose nerve endings studied in the allergen-challenged airways were exquisitely sensitive to mechanical stimulation (von Frey threshold < 1 mN; Fig. 2). Thus although allergen challenge induced substance P synthesis in airway-specific nodose neurons, their peripheral terminals remained unresponsive to capsaicin and bradykinin and exquisitely sensitive to mechanical stimulation.

To further substantiate the hypothesis that at sites of inflammation a portion of the substance P is derived from capsaicin-insensitive neurons, we employed intracellular electrophysiological recording techniques combined with immunohistochemistry. The tracheal-specific neurons in the jugular and nodose ganglia were retrogradely labeled with dextran-conjugated rhodamine. One day after antigen inhalation, the nodose and jugular ganglia were removed, and the electrophysiological properties of the neurons were studied in situ using a fluorescent microscope. As expected, most tracheal-specific neurons in the nodose ganglia were large-diameter neurons (~40-μm-diameter). Capsaicin (1 μM) had no effect on the resting membrane potential in these neurons (Fig. 3). Tracheal-specific neurons in the jugular ganglia consistently responded to capsaicin with a 5- to 10-mV membrane depolarization (Fig. 3). This is in keeping with findings at the nerve endings in the airway wall in which ~90% of the fibers from the jugular ganglia are capsaicin-sensitive nociceptors (24). As shown above, not every large-diameter nodose neuron is induced to produce substance P following allergen inhalation. To confirm that at least some of the nodose neurons that failed to depolarize to capsaicin exposure were induced to produce substance P, we labeled neurons with Lucifer yellow, delivered through the microelectrode, and subsequently sectioned the tissue and evaluated the Lucifer yellow-filled cell for substance P immunoreactivity. The recorded trace in Fig. 3B is from a nodose neuron studied 1 day after allergen-induced airway inflammation. This cell was unresponsive to capsaicin but was subsequently shown to be positive for substance P immunoreactivity.

Capsaicin stimulates sensory neurons via interaction with the VR-1 (4). To provide additional support to the hypothesis that visceral inflammation induces substance P in capsaicin-insensitive sensory neurons, we evaluated the expression of VR-1 on airway-specific sensory neurons. In control animals, VR-1 immunoreactivity was found on airway-specific substance P-positive neurons in jugular ganglia but not in airway-specific substance P-negative neurons in nodose ganglia (Fig. 4). VR-1 staining was noted in small-diameter neurons in the nodose, but as mentioned above, only a small percentage of these neurons project fibers to the airways. In animals that were subjected to allergic inflammation, the induction of substance P in large-diameter nodose neurons was not associated with induction of VR-1 expression. Thus in airway-inflamed animals, there was a population of substance P-positive, VR-1-negative neurons, a finding consistent with the electrophysiological studies of the nerve endings and soma.

**DISCUSSION**

Allergic inflammation of guinea pig airways has been shown to cause an elevation in sensory neuropeptide content of pulmonary tissues and an induction of the preprotachykinin gene in nodose ganglion neurons (8). The present results support these findings and extend them by demonstrating that allergic inflammation of the airways leads to substance P and CGRP production in airway-specific, large-diameter, NF-pos-
itative neurons. More importantly, the results from electrophysiological studies at both the nerve ending in the airway wall and soma, together with results from histological studies, support the hypothesis that a population of the sensory neurons induced by inflammation to produce substance P and CGRP belong to the non-nociceptive, capsaicin-insensitive, low-threshold mechanosensitive Aβ class. On the basis of response characteristics and conduction velocity, these fibers are analogous to the rapidly adapting receptors characterized in in vivo studies.

Physiological studies on guinea pig airway afferent nerves support the hypothesis that the nodose fibers in the trachea/bronchus wall are likely activated by mechanical forces caused during normal respiration, whereas the jugular fibers in the airway provide nociceptive input, activated only during tissue damage and inflammation (24). In the present study, we found that allergic inflammation of the airways led to the induction of neurokinin synthesis in large-diameter nodose neurons but did not change their physiological characteristics. Thus after neuropeptide induction, the fibers remained highly sensitive to mechanical force but insensitive to capsaicin and bradykinin. Indeed, in other studies we have provided evidence that the sensitivity of nodose afferent fibers to mechanical stimulation may actually be increased by allergic inflammation (25). This means that at the site of airway inflammation, nociceptive fibers may represent a significant percentage of the neurokinin-containing afferent fiber population.

The function of neuropeptide-containing afferent nerve fibers has been extensively studied using selective nociceptor stimulants such as capsaicin (10). Indeed, capsaicin has proven to be an extraordinarily valuable and useful tool in the study of the constituent population of neuropeptide-containing afferent nerves. A practical implication of the present findings is that capsaicin may not be so useful in the study of a potentially important subset of neuropeptide-containing nerves, i.e., the afferent nerve fibers induced by inflammation to produce neuropeptides. Capsaicin stimulates sensory neurons via interaction with the VR-1 (10). To provide additional support to the hypothesis that visceral inflammation induces substance P in capsaicin-insensitive sensory neurons, we evaluated the expression of VR-1 on airway-specific sensory neurons. In control animals, VR-1 immunoreactivity was found on airway-specific substance P-positive neurons in the jugular ganglia but not in airway-specific neuron nodose ganglia (Fig. 4). VR-1 staining was noted in small-diameter neurons in the nodose, but as mentioned above, these neurons do not project fibers to the airways. In animals that were subjected to allergic inflammation, the induction of substance P in large-diameter nodose neurons was not associated with induction of VR-1 expression. Thus in airway-inflamed animals, there was a population of substance P-positive, VR-1-negative neurons, a finding consistent with the electrophysiological-physiological studies of the nerve endings and soma.

The molecular mechanisms responsible for the phenotypic change in sensory neuropeptide innervation cannot be discerned from the data presented. It is tempting to speculate, however, that nerve growth factor (NGF) may be a signaling molecule in this response. NGF is elevated by allergic inflammation (16), and we have previously noted that injection of NGF into the trachea induces substance P production in nodose neurons (11). In that study, the physiological phenotype of the neurons was not determined, but it is noteworthy that they belonged to the NF-positive, large-diameter subset of neurons, similar to those observed here to be affected by airway inflammation.

The physiological consequence of phenotypic changes in neuropeptide content in the somatosensory system has been recently demonstrated. For example, injection of turpentine oil into rat paws leads to substance P production in large-diameter Aβ neurons located in dorsal root ganglia and provides a potential mechanism by which simply stimulating touch fibers may lead to painful sensations, i.e., an allodynic response (23). Thus it was argued that substance P may be released in the central nervous system as a consequence of activation of low-threshold mechanosensors in the skin, therein amplifying synaptic transmission to the extent that it leads to sensations of pain. Furthermore, substance P and related neuropeptides are known to augment synaptic transmission in the nucleus of the solitary tract (18, 21). The results presented here in the visceral-sensory system are in accord with this same concept. Under normal conditions, the release of sensory neuropeptides peripherally and in the brain stem from vagal afferent nerves may be limited to occasions where the innervated organ is subjected to noxious-type stimuli. At sites of inflammation, however, the phenotypic switch in neuropeptide-containing fibers sets up the condition where simple mechanical deformation of the tissue occurs physiologically (i.e., during normal respiration, digestion, heart contractions, etc.) results in neuropeptide release in the brainstem. Therefore, an increase in neuropeptide released into this region from nonnociceptive afferent fibers may help explain some of the altered sensations and reflex physiology that often accompany inflammatory diseases of visceral tissues.

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