Neurogenic Inflammation in Rat Trachea Is Accompanied by Increased Negativity of Interstitial Fluid Pressure

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The present experiments were performed to investigate whether neurogenic inflammation in rat trachea (with edema formation and protein extravasation when the circulation is intact) induced by electrical field stimulation of neuropeptide-containing C fibers in the vagal nerve is accompanied by increased negativity of interstitial fluid pressure ($P_f$). Increased negativity of $P_f$ in the trachea occurs in dextran anaphylaxis and mast cell degranulation and facilitates edema formation under these circumstances. Experiments were performed after circulatory arrest had been induced in pentobarbital anesthesia to prevent edema formation, which will raise $P_f$ and potentially cause underestimation of an increased negativity of $P_f$. After induction of circulatory arrest, the vagal nerve was isolated and placed in a stimulating electrode. The trachea was then exposed and covered with mineral oil, and measurement of $P_f$ was started as soon as possible thereafter. $P_f$ was measured with sharpened glass capillaries (tip diameter, 3 to 7 μm) connected to a servo-controlled counterpressure system. $P_f$ in the control group ($n=12$) did not change throughout the observation period. Electrical stimulation of the left vagal nerve caused $P_f$ to fall in all experiments, from $-1.1 \pm 1.1$ mm Hg in the control condition to an average of $-10.6 \pm 3.4$ mm Hg ($n=9$, $P<.01$). In some experiments, a continuous recording of $P_f$ was obtained, showing that the reduction of $P_f$ started within 30 seconds after onset of stimulation to reach and later remain at a stable level within a few minutes. The experimental protocol was repeated after the C fibers had been nearly depleted of neuropeptides with capsaicin. In this group ($n=7$), vagal nerve stimulation had no effect on $P_f$, which averaged $-0.8 \pm 0.9$ and $-0.08 \pm 0.17$ mm Hg before and after stimulation, respectively ($P>.05$). Mast cell degranulation with C48/80 ($n=5$) and polymyxin B ($n=6$) resulted in $P_f$ of $-5$ to $-6$ mm Hg, and subsequent vagal nerve stimulation was without effect on $P_f$. This could suggest that the increased negativity of $P_f$ occurring in the initial phase of neurogenic inflammation in rat trachea involves mast cell degranulation. Finally, the presence and localization of immunoreactive calcitonin gene–related peptide (CGRP) and substance P were studied in transverse sections of trachea. Normally, the neuropeptides were typically located right under the mucosa, adjacent to mast cells and blood vessels. Capsaicin resulted in near depletion of immunoreactive calcitonin gene–related peptide and substance P. In conclusion, electrical field stimulation of the vagal C fibers in rat trachea for the first time provides experimental evidence for a functional innervation of loose connective tissue, causing increased negativity of $P_f$ ($Circ. Res. 1993;73:839-845$).

**Key Words** • interstitial fluid pressure • inflammation • electrical field stimulation • sensory fibers • C fibers • polymyxin B • C48/80 • mast cells • substance P • calcitonin gene–related peptide • immunohistochemistry • edema

We have recently reported that tracheal edema formation in dextran anaphylaxis is accompanied by increased negativity of interstitial fluid pressure ($P_f$). The increased negativity of $P_f$ seems to be a major driving force for the fluid filtration required to rapidly form the edema, which in this situation causes interstitial fluid volume to nearly double in 10 minutes, implying a dramatic increase in capillary filtration. The same phenomenon with increased negativity of $P_f$ in the rat trachea occurs after administration of the mast cell–degranulating substances C48/80 and polymyxin B. Increased negativity of $P_f$ has also been observed in rat skin in xylene-induced inflammation, burn injury, and dextran anaphylaxis. These data are the basis for stating that the loose connective tissues “actively” participate in transcapillary fluid balance by promoting fluid filtration through an increased negativity of $P_f$.

In the present experiments, we have investigated whether electrical field stimulation of sensory C fibers to the trachea induces an increased negativity of $P_f$. Electrical stimulation of the C fibers releases neuropeptides and rapidly causes edema and plasma protein extravasation. This neurally elicited reaction is called “neurogenic inflammation,” since it has the main characteristics of an inflammatory reaction with vasodilatation, edema formation, and protein extravasation.

In our previous experiments, the full extent of the increased negativity of $P_f$ could not be detected if...
edema was allowed to develop. The increase in interstitial fluid volume will increase $P_i$, potentially causing an underestimation of the increased negativity of $P_e$. Therefore, the present experiments were carried out after circulatory arrest had been induced with an intracardiac injection of saturated potassium chloride under pentobarbital anesthesia. This procedure prevents the capillary fluid filtration and edema development associated with inflammatory reactions.

Briefly, electrical field stimulation of the vagal nerve caused $P_e$ to fall from $-1$ to about $-10$ mm Hg. Denervation of the nerve and the peptide-containing fibers with capsaicin abolished this reaction. The chemical denervation was verified with immunohistochemistry. Thus, the present experiments for the first time provide experimental evidence for a functional innervation of the loose connective tissues, mediated through C fibers and their neuropeptides, which causes increased negativity of $P_e$.

Materials and Methods

Wistar Møller rats weighing 200 to 250 g were used in the experiments. The animals were fed an ordinary diet and were not fasted before experiments. Circulatory arrest was induced with an intracardiac injection of saturated KCl in pentobarbital anesthesia (50 mg/kg body wt IP). The rats were kept on a servocontrolled heating pad during the experiments.

Measurements

Interstitial fluid pressure. $P_e$ was measured with sharpened glass capillaries (tip diameter, 3 to 7 μm) connected to a servocontrolled counterpressure system.11 Measurement of $P_e$ was performed without introducing or removing fluid from the tissue, and the small size of the pipette tip allowed a virtually atraumatic measurement. The glass pipettes were filled with 0.5 mol/L NaCl colored with Evans blue. Punctures were performed under visual guidance with a microscope (Wild M3C, Leitz, Germany) and without applying stretch or compression at the site of the measurement.12 Pressure measurements were accepted when the following criteria were fulfilled: (1). Feedback gain could be altered without changing the recorded pressure. (2). After fulfillment of criterion 1, fluid was moved into the pipette by applying suction to the servocontrolled pump, visualized as increased electrical resistance in the pipette due to the lower toxicity in the fluid entering the pipette. (3). Zero measurement was unaltered from the prepuncture value after measurement had been performed. Zero measurement was performed in a plastic cup at the level of the puncture site.

Measurement of $P_e$ was started as soon as possible after the left vagal nerve had been isolated and placed in a stimulating electrode and the trachea had been exposed and covered with mineral oil. The surgical procedures required approximately 10 minutes. In agreement with our previous studies of the trachea, the measurements were grouped in the following time periods: 0 to 15, 16 to 30, 31 to 45, and 46 to 60 minutes after induction of cardiac arrest. The above criteria for accepting a measurement could sometimes not be met at all registration periods in each experimental animal. The counterpressure created by the pump was recorded with a venous pressure transducer (model 1280C, Hewlett-Packard Co, Calif) connected to a Hewlett-Packard amplifier (model 8805B) and recorder (model 7414A).

Electrical stimulation. Electrical stimulation was performed with an S48 stimulator (Grass Instrument Co, Quincy, Mass) at 20 V, 20 Hz, and 0.5 millisecond for a total of 15 minutes.

Immunohistochemistry. The tissue specimens were placed in 4% paraformaldehyde and 0.2% picric acid in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4, for 48 hours and thereafter saturated in 30% sucrose in 0.1 mol/L PBS, pH 7.4, for 24 hours. Frozen horizontal serial sections (50 μm thick) were cut on a freezing microtome. Immunoreactions for substance P (SP) and calcitonin gene–related peptide (CGRP) were performed on free-floating sections in tissue-culture wells. The sections were pretreated with 0.3% H2O2 in methanol to block endogenous peroxidase activity. Before incubation with primary antibody, the sections were soaked in 2% normal goat serum (Vector Laboratories, Inc, Burlingame, Calif) at room temperature for 30 minutes. Several rinses in PBS were performed between each step. The sections were incubated for 72 hours at 4°C with rat CGRP antibody (1:6000 dilution) and SP antibody (1:4000 dilution). Both antibodies were raised in rabbit (Cambridge Research Biochemicals, Cambridge, UK). To localize the bound antibody, a commercially available ABC kit (Vector Laboratories) was used for the avidin-biotin peroxidase reaction, in which 3′,3′-diaminobenzidine (Sigma Chemical Co, St Louis, Mo) served as the chromogen. The sections were mounted on gelatin-coated slides, air-dried and counterstained in methylene blue/azure II, and mounted in Eukitt (Kindler, Freiburg, Germany). The specificity of the immunoreaction was routinely controlled by preabsorption of the primary antibody with synthetic rat CGRP or SP (Cambridge Research Biochemicals) before incubation or omission of primary or secondary antibody. The immunocounters did not show immunolabeling for CGRP or SP.

Experimental Groups

Interstitial fluid pressure. The effect of electrical stimulation of the vagal nerve was studied in four experimental groups: (1) In group 1 (control group), the animals (n=12) were operated on as described above. No vagal dissection was performed, and no electrical field stimulation was applied. (2) In group 2 (control group with vagal nerve stimulation), the animals (n=9) were operated on as described above. After measurement of a control $P_e$, electrical field stimulation was applied as described above. Attempts were made to record $P_e$ continuously during the electrical stimulation. (3) In group 3 (capsaicin group), capsaicin (90 mg/kg, Sigma) was administered to seven rats in increasing doses of 20, 30, and 40 mg/kg on 3 consecutive days.10 The capsaicin was injected subcutaneously as a 1% solution dissolved in 10% ethanol and 10% Tween 80 in 0.9% NaCl. Experiments were performed 10 days later, and $P_e$ was measured as described for group 2 above. After measurements were completed (1 hour after circulatory arrest), the tracheas were fixed and processed for immunohistochemistry as described above. (4) In group 4 (polymyxin B or C48/80 followed by vagal nerve stimulation), six rats were given 1 mg polymyxin B (Sigma) in 1 ml 0.9% NaCl, and five rats were given 50
\( \mu \)g C48/80 (Sigma) in 1 mL of 0.9% NaCl IV via a PE-50 catheter in the femoral vein. One minute later, the rats were killed with an intravenous injection of 0.5 mL saturated potassium chloride. The vagal nerve and trachea were then prepared as described above, and measurement of \( P_d \) was started as soon as possible. Electrical stimulation of the vagus was performed as described above. If possible, \( P_d \) was recorded continuously before and during the nerve stimulation and then again at the end of the observation period.

**Histochemistry.** The localization of immunoreactive CGRP and SP was studied in two groups: In the control group, five rats received saline without capsaicin as a control injection and had no vagal stimulation. In the capsaicin group, three rats received capsaicin as described above. On the day when the tissue specimens were obtained (10 days after the first capsaicin injection), circulatory arrest was induced, and the trachea was exposed and covered with mineral oil. All biopsies were obtained 1 hour after induction of circulatory arrest in order to simulate the conditions for the experimental groups used for measurement of \( P_d \). The samples were taken from the area used for micropuncture and as far caudally from the trachea as possible without having to split the sternum, i.e., 2 to 5 mm above the carina. The samples were immediately transferred into fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 mol/L PBS, pH 7.4) and processed as described above.

The following parameters were evaluated in the histochemical sections: First, the number of peptide-containing nerves was quantified per visual field (objective, \( \times 40 \); eyepiece, \( \times 10 \)) in the epithelial layer and the remaining part of the trachea. The evaluation was performed in as many visual fields as possible, provided that the visual fields were free of cartilage and not overlapping. Second, the fraction of mast cells that had a peptide-containing nerve crossing the cell or in which the nerves were within one cell diameter from the cell body was assessed. Five to 12 sections were prepared from each rat for evaluation of the immunohistochemistry.

**Statistical Methods**

Data are presented as mean\( \pm \)1 SD unless otherwise specified. Differences within groups were tested with paired \( t \) tests and Wilcoxon tests. Differences between groups were tested with \( t \) tests and Mann-Whitney tests. A value of \( P<.05 \) was considered statistically significant.

**Results**

**Interstitial Fluid Pressure**

In the control group (\( n=12 \) experiments), \( P_d \) averaged -1.4\( \pm \)0.4 mm Hg and did not change throughout the experimental period. In the control group with vagal nerve stimulation (\( n=9 \) experiments), \( P_d \) before stimulation averaged -1.1\( \pm \)1.1 mm Hg. Electrical stimulation of the vagal nerve caused \( P_d \) to fall to -10.6\( \pm \)3.4 mm Hg (\( P<.01 \)) (Fig 1). In five of the experiments, we obtained a continuous recording of \( P_d \) showing that increased negativity of \( P_d \) occurred within 30 seconds after the onset of stimulation, leveled out within 3 to 5 minutes, and thereafter remained stable throughout the experimental period. One such recording is shown in Fig 2.

In the capsaicin group (\( n=7 \) experiments), vagal nerve stimulation after capsaicin denervation had no effect on \( P_d \), which averaged -0.8\( \pm \)0.9 and -0.1\( \pm \)1.7 mm Hg before and after stimulation, respectively (\( P>.05 \)) (Fig 1).

In group 4 (polymyxin B or C48/80 followed by vagal nerve stimulation), after the administration of polymyxin B, \( P_d \) averaged -5.2\( \pm \)1.2 mm Hg, and \( P_d \) measured during vagal nerve stimulation did not change (\( n=6 \) experiments). After obtaining a new zero recording, measurement was successfully repeated in four of the six experiments, giving an average \( P_d \) of -5.8\( \pm \)1.0 mm Hg (\( P>.05 \) compared with the measurement before stimulation). Administration of C48/80 resulted in \( P_d \) of -4.3\( \pm \)1.2 mm Hg (Fig 3), and electrical field stimulation of the vagal nerve did not change \( P_d \), being, on average, -4.2\( \pm \)0.9 mm Hg (\( P>.05 \)). After obtaining a new zero recording, measurement was successfully repeated in all five experiments and gave an average \( P_d \) of -5.2\( \pm \)0.9 mm Hg (\( P>.05 \) comparing the measurement before and after stimulation).

**Histochemistry**

In the control animals, immunoreactive CGRP and SP were most abundant in the loose connective tissue closely adjacent to the underlying epithelium and blood vessels. The content of SP was substantially higher than...
Fig 3. Plot shows interstitial fluid pressure after intravenous injection of C48/80 (group 4). Pre denotes control measurement; Stim, measurement during stimulation; and Post, measurement after stimulation. Open circles with bars represent mean±1 SD. Solid lines connect measurements in the same experiment.

that of CGRP (compare Figs 4 and 5). When quantifying the content of SP, a total of 68 visual fields met the criteria in the five normal rats (ie, no cartilage in the visual field), whereas 46 visual fields met the criteria in the capsaicin group. When quantifying CGRP, 36 visual fields (three rats) met the criteria in the capsaicin-treated rats, whereas 72 fields were quantified in the five normal rats. The numbers obtained from each rat were averaged, and the number used in the subsequent statistical analysis is the number of rats. The normal rats had, on average, 3.9±0.6 nerves (n=5) immunoreactive to SP in the epithelial layer and 4.4±0.7 nerves (n=5) in the corresponding cross section of the trachea. Capsaicin denervation reduced the number of nerves significantly to 1.5±0.9 and 1.0±0.9 in the epithelial layer and the remaining part of the cross section, respectively (P<.01 compared with the control group). The fraction of mast cells with SP-containing nerves in close proximity averaged 0.8±0.1 in control rats (n=5) and 0.2±0.2 in capsaicin-treated rats (n=3) (Figs 4 and 5). The number of CGRP-immunoreactive nerves was normally 2.0±0.2 (n=5) in the epithelial layer and 2.5±0.8 (n=5) in the remaining part of the cross section and fell to 0.7±0.2 (n=3) and 1.0±0.2 (n=3), respectively, after capsaicin treatment (P<.05 and P<.01 compared with the control value, respectively).

Discussion

The present study has shown that electrical field stimulation of C fibers to the trachea rapidly induced increased negativity of $P_d$ from −1 mm Hg in the control condition to about −10 mm Hg. This phenomenon was abolished if neuropeptides were depleted from the C fibers with capsaicin or when the mast cells were degranulated with polymyxin B or C48/80 before nerve stimulation. Thus, the experiments for the first time provide evidence for a functional innervation of the loose connective tissues in the trachea mediated via the neuropeptide-containing C fibers that causes increased negativity of $P_d$.

The parasympathetic, sympathetic, and sensory nerves innervating the trachea all contain peptides that are specific for the different nerve types: The sympathetic nerves contain neuropeptide Y, which constricts airway and vascular smooth muscle. The parasympathetic nerves carry vasoactive intestinal peptide and peptide histidine leucine, which are vasodilators and potent relaxants of bronchial smooth muscle. Finally, the sensory nerves contain CGRP and SP, which have vasodilatory effects. In addition, SP and neurokinins will increase capillary permeability in the trachea. Electrical stimulation of the C fibers in the vagal nerve releases the peptides in these fibers and induces a neurogenic inflammation consisting of vasodilatation, protein extravasation, and edema formation.7-9

Few studies have addressed the changes in transcapillary pressures and permeability that are required to increase the capillary fluid filtration to such an extent that the edema accompanying the neurogenic inflammation can be created in a few minutes. Based on measurements of normal albumin turnover in trachea, it can be calculated that approximately 150 minutes is required to generate a capillary filtrate that equals the interstitial fluid volume.1 The transcapillary fluid flux (Jf) is the product of the capillary filtration coefficient (CFC), the hydrostatic pressure (P), and the colloid osmotic pressure (COP) acting across the capillary wall. These parameters are interrelated in the following equation: $J_f=CFC[P_f−P−σ(COP−COP_{pl})]=CFCAP$, where subscripts c and if denote capillary and interstitial fluid, respectively, $σ$ is the capillary reflection coefficient for proteins, and ΔP is the net filtration pressure across the capillary wall (the imbalance between the transcapillary hydrostatic and colloid osmotic pressures). The increase in permeability in neurogenic inflammation is larger in the trachea than in the larger bronchi13 and is confined to the small venules.14 The normal ΔP, CFC, or σ in the trachea are not known, nor have the changes in these parameters been quantified in the trachea in inflammatory reactions. However, data from skin could allow some comparison: Histamine injection increases CFC two to three times in the canine hindpaw15,16 and reduces $σ$ from 0.9 to 0.7.17 Similar changes are observed in CFC and $σ$ after burn injury.18,19 The combined effect of increased CFC and decreased $σ$ as described above would result in a threefold to fivefold increase in the transcapillary filtration, which is not enough to explain an increase in $J_f$, of more than 15 times above control in dextran anaphylaxis and mast cell degranulation.12,20 Thus, an increase in ΔP is also required.

Increased negativity of $P_d$ accompanies mast cell degranulation in the trachea with C48/80 or polymyxin B.5 To investigate whether mast cells could also be involved in the increased negativity of $P_d$, secondary to the vagal nerve stimulation, two additional series of experiments were performed in which mast cell degranulation was induced by polymyxin B or C48/80 before electrical field stimulation of the vagal nerve. Mast cell degranulation with these substances reduced $P_d$ from −1 to −5 to −6 mm Hg, in agreement with our previous observations.2 In both groups, subsequent stimulation of the vagal nerve did not cause further increased negativity of $P_d$. There may be two explanations for this observation: (1) The mast cells are necessary to induce the increased negativity of $P_d$ after stimulation of the vagal C fibers. (2) The mast cells induce maximal negativity of $P_d$ and subsequent stimulation of the C fibers cannot add to this effect. The definitive experiment necessary to answer this question cannot be
performed since this would mean stimulating the sensory C fibers before administering the mast cell degranulators via the intravenous route. However, this would necessitate intact circulation, in which case the C fiber stimulation would cause edema and obscure the effect of the mast cell degranulators.

The immunohistochemistry was performed to verify that capsaicin caused denervation of neuropeptide-containing nerves, which was verified through a significant reduction in the number of immunoreactive SP and CGRP nerves in the animals given capsaicin. The dose of capsaicin was selected on the basis of the study of Buck and Burks, who quantified the effect of denervation by histochemistry 10 days after the capsaicin had been administered. To make the quantitative verification of the immunoreactive nerves more conclusive, the sections were 50 μm thick. However, the thick sections at the same time do not allow firm conclusions with respect to the detailed relation between the neuropeptide-containing nerves and other anatomic entities. The immunohistochemistry (Figs 4 and 5) showed that immunoreactive SP and CGRP are normally distributed in a circumferential manner in the loose connective tissue underlying the mucosa. Finer ramifications of the nerve fibers were seen within the epithelium. Furthermore, the amount of SP was considerably higher than that of CGRP.

Another interesting observation from the histochemistry was the close relation commonly observed between the CGRP- and SP-immunoreactive nerve fibers and the mast cells of the loose connective tissue in the trachea. A close proximity between mast cells and sensory nerves has previously been observed in the meningeal membranes and the intestine (for further references, see Reference 21) but, to our knowledge, not in the trachea. A functional relation has been suggested to exist between the peptide-containing nerves and mast cells, with SP as a main modulatory and possibly transmitter substance between the nervous system and the mast cells. In vitro experiments have shown that SP and acetylcholine may induce the release of mast cell granules. Furthermore, stimulation
of the vagal nerves to the mesentery will cause swelling of the mast cells.\textsuperscript{25} Even more in line with the present experiments, stimulation of the vagal nerve results in mast cell degranulation in the trachea,\textsuperscript{26} an effect that disappeared after capsaicin denervation with a dose and protocol similar to that used in the present study. However, despite the in vitro observations of nerves and/or transmitters being able to degranulate mast cells, the functional or physiological in vivo effect of mast cell degranulation after C fiber stimulation has, to our knowledge, previously not been demonstrated in loose connective tissues. The anatomic proximity between the mast cells and nerve fibers in the trachea has been observed previously.\textsuperscript{27} Similarly, it has been reported that, in the intestine, approximately two thirds of the mast cells are less than 2 \(\mu\)m from the neuropeptide-containing nerves.\textsuperscript{28} The somewhat higher number reported in the present study is likely due to the larger distance allowed by us to fulfill the criterion of “close proximity.” The reduction in mast cells with close proximity to the nerves after capsaicin denervation is of the same magnitude as the reduction of immunoreactive nerve fibers per visual field. The close proximity between mast cells and nerves, together with the observations that tracheal mast cells degranulate after vagal nerve stimulation\textsuperscript{26} and that mast cell degranulation and C fiber stimulation are not additive phenomena, makes it tempting to suggest the following functional relation: Mast cell degranulation would be induced by the stimulation of C fibers, subsequently causing an increased negativity of \(P_i\).

The precise mechanism that causes increased negativity of \(P_i\) in inflammation is not fully clarified, but involvement of the structural components of the tissue (ie, collagen and glycosaminoglycans) seems likely. It should be noted that in skin the amount of positive charge carried by histamine equals the amount of negative charge on hyaluronan.\textsuperscript{6} The mast cell degranulation could provide the amount of histamine required to interact with the highly charged glycosaminoglycans of the tissue and result in increased negativity of \(P_i\). Finally, perturbation of \(\beta_1\)-integrin function in skin was recently reported to cause development of edema concomitant with increased negativity of \(P_i\) to the level observed in the present study.\textsuperscript{29} This could suggest that \(\beta_1\)-integrin function is perturbed in neurogenic inflammation and is the final step in a chain of events resulting in release of a constrained collagen network,\textsuperscript{30} in turn causing increased negativity of \(P_i\).

Since increased negativity of \(P_i\) occurs concomitant with the stimulation of neuropeptide-containing C fi-
bers in the vagal nerve, the present study for the first time provides evidence for functional innervation of the loose connective tissue in rat trachea. Furthermore, the experiments suggest that the mast cells in the tissue are involved such that the neuropeptides initially cause degranulation of mast cells, which in turn induce increased negativity of $P_r$.

Acknowledgments
This study was supported by the Norwegian Council for Science and the Humanities, the Norwegian Heart Association, and the Lars and Gerd Volders Foundation for Allergy and Asthma Research. The technical assistance of Eli Kjorlaug, Gerd Salvesen, and Åse R. Eriksen is appreciated.

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_Circ Res._ 1993;73:839-845
doi: 10.1161/01.RES.73.5.839
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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