Sensitization of isolated rat vagal pulmonary sensory neurons by eosinophil-derived cationic proteins

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Asthma is characterized by chronic airway inflammation as well as eosinophil-derived cationic proteins. Eosinophils are known to secrete a number of cationic proteins, including major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) (16). Previous studies have shown that intratracheal instillation of eosinophil-derived cationic proteins such as MBP induces bronchoconstriction and bronchial hyperresponsiveness in a number of animal species (9, 17, 21). Similar effects can also be induced by intratracheal administration of synthetic cationic proteins such as poly-L-lysine (PLL) (10, 24, 46).

Most of the sensory inputs arising from airways and lungs are conducted in vagus nerves and their branches. Cell bodies of these sensory nerves reside in two adjacent but distinct anatomic structures, the nodose and intracranial jugular ganglia. It is known that >75% of vagal bronchopulmonary afferents are nonmyelinated (C) fibers. Stimulation of bronchopulmonary C-fiber afferents can cause bronchoconstriction, hypersecretion of mucus, and other pronounced effects on airway functions, which are mediated through cholinergic reflexes and endogenous release of tachykinins (8, 35). Recent studies from our laboratory (19, 20, 33) have demonstrated that airway exposure of eosinophil-derived cationic proteins (such as MBP and ECP) or synthetic cationic proteins (such as PLL and poly-L-arginine) induced stimulatory and sensitizing effects on vagal pulmonary C fibers. However, whether the effects resulted from a direct action of these proteins on the sensory nerves was not known. The present study was therefore carried out to determine the effects of these proteins on isolated rat vagal pulmonary sensory neurons. Our results obtained from perforated whole cell patch-clamp recordings showed that pretreatment with eosinophil major basic protein (MBP; 2 μM, 60 s) significantly increased the capsaicin-evoked inward current in these neurons; this effect peaked ~10 min after MBP and lasted for ~60 min; in current-clamp mode, MBP substantially increased the number of action potentials evoked by both capsaicin and electrical stimulation. Pretreatment with MBP did not significantly alter the input resistance of these sensory neurons. In addition, the sensitizing effect of MBP was completely abolished when its cationic charge was neutralized by mixing with a polyanion, such as low-molecular-weight heparin or poly-L-glutamic acid, before its delivery to the neurons. Moreover, a similar sensitizing effect was also generated by other eosinophil granule-derived proteins (e.g., eosinophil peroxidase). These results demonstrate a direct, charge-dependent, and long-lasting sensitizing effect of cationic proteins on pulmonary sensory neurons, which may contribute to the airway hyperresponsiveness associated with airway infiltration of eosinophils under pathophysiological conditions.

Major basic protein; eosinophil cationic protein; eosinophil peroxidase; airway inflammation; airway hyperresponsiveness

Asthma is characterized by chronic airway inflammation associated with airway infiltration of various inflammatory cells, particularly the eosinophils (7). Activated eosinophils are known to secrete a number of cationic proteins, including major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) (16). Previous studies have shown that intratracheal instillation of eosinophil-derived cationic proteins such as MBP induces bronchoconstriction and bronchial hyperresponsiveness in a number of animal species (9, 17, 21). Similar effects can also be induced by intratracheal administration of synthetic cationic proteins such as poly-L-lysine (PLL) (10, 24, 46).

Most of the sensory inputs arising from airways and lungs are conducted in vagus nerves and their branches. Cell bodies of these sensory nerves reside in two adjacent but distinct anatomic structures, the nodose and intracranial jugular ganglia. It is known that >75% of vagal bronchopulmonary afferents are nonmyelinated (C) fibers. Stimulation of bronchopulmonary C-fiber afferents can cause bronchoconstriction, hypersecretion of mucus, and other pronounced effects on airway functions, which are mediated through cholinergic reflexes and endogenous release of tachykinins (8, 35). Recent studies from our laboratory (19, 20, 33) have demonstrated that airway exposure of eosinophil-derived cationic proteins (such as MBP and ECP) or synthetic cationic proteins (such as PLL and poly-L-arginine) induced stimulatory and sensitizing effects on vagal pulmonary C fibers. However, whether the effects resulted from a direct action of these proteins on the sensory nerves was not known. To answer these questions, the purposes of this study were therefore to determine 1) whether the eosinophil-derived cationic proteins directly sensitize isolated pulmonary sensory neurons and enhance their responses to chemical and electrical stimulations and 2) whether the effect of these proteins is dependent on their positive charges. The excitability of the pulmonary sensory neurons isolated from rat nodose and jugular ganglia was determined by using the whole cell perforated patch-clamp recording technique.

MATERIALS AND METHODS

The experimental procedures were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, and were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

Identification of vagal pulmonary sensory neurons. Cell bodies of vagal sensory nerves arising from airways and lungs reside in nodose and intracranial jugular ganglia. These sensory neurons were identified by retrograde labeling from the lungs by using the fluorescent tracer 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI), as described previously (31). Briefly, young adult Sprague-Dawley rats (~150 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) and intubated with a polyethylene catheter (PE-150), with its tip positioned in the trachea above the thoracic inlet. DiI was initially sonicated and dissolved in ethanol, diluted in saline (1% ethanol vol/vol), and then instilled into the lungs (0.2 mg/ml; 0.2 ml × 2) with the animal’s head tilted upward at ~30°.

Primary culture of nodose and jugular ganglion neurons. After 7–10 days, an interval previously determined to be sufficient for DiI to diffuse to the cell body, the rats were anesthetized with halothane inhalation and decapitated. The head was immediately immersed in ice-cold HBSS. Nodose and jugular ganglia were extracted under a
dissecting microscope and placed in ice-cold DMEM-F12 solution. Each ganglion was desheathed, cut into ~10 pieces, placed in 0.125% type IV collagenase, and incubated for 1 h in 5% CO2 in air at 37°C. The ganglion suspension was centrifuged (150 g, 5 min), and supernatant was aspirated. The cell pellet was resuspended in 0.05% trypsin in HBSS for 5 min and centrifuged (150 g, 5 min); the pellet was then resuspended in a modified DMEM-F12 solution [DMEM-F12 supplemented with 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μM MEM nonessential amino acids] and gently triturated with a small-bore fire-polished Pasteur pipette. The dispersed cell suspension was centrifuged (500 g, 8 min) through a layer of 15% BSA to separate the cells from the myelin debris. The pellets were resuspended in the modified DMEM-F12 solution supplemented with 50 ng/ml 2.5S nerve growth factor, plated onto poly-L-lysine-coated glass coverslips, and incubated at 37°C in 5% CO2. Neurons were used within 24–48 h of culture.

Whole cell perforated patch-clamp recording. Coverslips with neurons isolated from nodose and jugular ganglia were transferred to a recording chamber (0.2 ml) that was superfused (2 ml/min) continuously by gravity feed (WGT-6 perfusion valve controller; Warner Instruments, Hamden, CT) with extracellular solution (ECS) containing the following chemicals (in mM): 136 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, 0.33 NaH2PO4, 10 glucose, and 10 HEPES, pH 7.4. Whole cell perforated patch clamping (50 μg/ml ganciclovir) was performed by using Axopatch 200B/pCLAMP 9.0 (Molecular Devices, Palo Alto, CA). The chemical stimulants were applied by a pressure-driven drug delivery system (ALA-VM8; ALA Scientific Instruments, Westbury, NY), with its tip positioned to ensure that the cell was fully within the stream of the injectate. The tip resistances of fire-polished micropipettes were 2–4 MΩ when filled with an internal solution consisting of the following (in mM): 92 potassium gluconate, 40 KCl, 8 NaCl, 1 CaCl2, 0.5 MgCl2, 10 EGTA, and 10 HEPES, pH 7.2. The whole cell perforated patch-clamping (50 μg/ml ganciclovir) was performed by using Axopatch 200B/pCLAMP 9.0 (Molecular Devices, Palo Alto, CA). The chemical stimulants were applied by a pressure-driven drug delivery system (ALA-VM8; ALA Scientific Instruments, Westbury, NY), with its tip positioned to ensure that the cell was fully within the stream of the injectate. The tip resistances of fire-polished micropipettes were 2–4 MΩ when filled with an internal solution consisting of the following (in mM): 92 potassium gluconate, 40 KCl, 8 NaCl, 1 CaCl2, 0.5 MgCl2, 10 EGTA, and 10 HEPES, pH 7.2. The series resistance was generally approximately ≈10 MΩ and was not compensated. Data were acquired at 5 kHz and filtered at 2 kHz. The experiments were performed at room temperature (~22°C).

Eosinophil-derived cationic protein purification. Eosinophils from patients with marked blood eosinophilia were collected by cytopheresis, and eosinophil granules and granule proteins were purified as previously described (40). Briefly, after cell lysis and granule isolation, granules were solubilized in 0.01 M HCl (pH 2.0) by vigorous suspension with a Pasteur pipette. After centrifugation (13,600 g, 5 min), the supernatant was fractionated on a Sephadex G-50 column equilibrated with 0.025 M sodium acetate (pH 4.3) containing 0.15 M NaCl. Acetate buffer was harvested for vehicle control conditions. Individual protein peaks were pooled, and protein concentrations were determined by absorbance at 280 nm. All eosinophil granule protein preparations were pure as assessed by Coomassie blue staining after SDS-PAGE.

Experimental protocols. Whole cell patch-clamp recordings were made in pulmonary sensory neurons selected based on the following criteria: 1) labeled with DiI as indicated by fluorescence intensity, 2) smooth surface and spherical shape without visible processes, 3) whole-cell capacitance <30 pF, and 4) responding to 1 μM capsaicin. These neurons presumably give rise to pulmonary C-fiber afferents (31). Neurons from nodose and jugular ganglia were cultured and studied separately. In our preliminary studies, we find no difference between neurons from these two different ganglion origins in their resting membrane potential and responses to capsaicin, electrical stimulation, and cationic proteins treatment. The data from the neurons of these two types of ganglia were therefore pooled for group analysis in this study. Five series of experiments were carried out.

In study series 1, we aimed to determine whether human eosinophil-derived MBP modulates the sensitivity of isolated pulmonary sensory neurons to chemical and electrical stimuli. Responses of neurons to capsaicin (0.3–1 μM, 2–5 s), a potent and selective activator of transient receptor potential vanilloid receptor subtype-1 (TRPV1), were tested before and at different time points after MBP pretreatment (2 μM, 60 s). The effect of MBP on the neuron responses to depolarizing current injections (10–400 pA, 485 ms; the magnitude of injected current was chosen in each individual neuron to evoke above firing-threshold membrane depolarization at control) was tested in current-clamp recording mode. At least 10 min and 5 min elapsed between two consecutive capsaicin and electrical stimulations, respectively.

In study series 2, we aimed to determine the effect of MBP pretreatment (2 μM, 60 s) on the input resistance of pulmonary sensory neurons. Ten hyperpolarizing current pulses (−10 to −100 pA, increment = 10 pA; 485 ms) were applied; the resulting membrane potential was plotted as a function of current, and the input resistance was determined by the slope.

In study series 3, we aimed to determine whether the effect of MBP was dependent on its positive charge. Three different polyanions, low-molecular-weight heparin (LMWH), poly-l-lysine, and poly-l-aspartic acid (PLAA), were used to test their effectiveness in blocking the effect of MBP.

In study series 4, we aimed to determine the effects of ECP and EPO, two other eosinophil-derived cationic proteins, on the sensitivity of pulmonary sensory neurons. In two separate groups of neurons, responses to capsaicin and current injection were determined in current-clamp mode, before and at different time points after pretreatments with these two proteins (2 μM, 60 s).

It has been suggested that MBP may function as an endogenous allosteric inhibitor of agonist binding to the muscarinic M2 receptor (28). Expression of M2 receptors in rat dorsal root ganglion neurons has recently been reported (5), but whether they are also expressed in vagal pulmonary sensory neurons is not known. Study series 5 was designed to determine whether pretreatment with methoctramine (2 and 20 nM), a selective M2-receptor antagonist, mimics the effect of MBP. Pulmonary sensory neurons responses to capsaicin (voltage-clamp mode) and injected current (current-clamp mode) were determined before and at different time points after the methoctramine pretreatment (60 s).

Chemicals. DiI was purchased from Molecular Probes (Eugene, OR). DMEM-F12 and trypsin were obtained from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma (St. Louis, MO). A stock solution of methoctramine (10 mM) was prepared in DMSO and that of capsaicin (1 mM) in 1% Tween 80, 1% ethanol, and 98% ECS. The solutions of chemicals at desired concentrations were prepared daily by dilution with ECS.

Statistical analysis. Data were analyzed by a one-way ANOVA. When the ANOVA showed a significant interaction, pair-wise comparisons were made with a post hoc analysis (Fisher’s least significant difference). Results were considered significantly different when P < 0.05. Data are means ± SE.

RESULTS

MBP potentiated capsaicin-evoked whole cell responses. In voltage-clamp mode, pretreatment with MBP (2 μM, 60 s) by itself did not induce any detectable current in isolated rat vagal pulmonary sensory neurons (n = 39). However, the MBP pretreatment dramatically potentiated the whole cell inward current evoked by capsaicin (Fig. 1A). The potentiation was found when capsaicin challenge was administered immediately (<1 s) after the completion of MBP pretreatment, which peaked −10 min later when the capsaicin (0.3–1 μM, 2–4 s)-evoked current was increased 4.8-fold; the response then gradually returned toward control but remained significantly elevated when tested at 60 min after MBP (Fig. 1B; P < 0.05, n = 6).

In current-clamp mode, pretreatment with MBP (2 μM, 60 s) substantially enhanced capsaicin-evoked membrane depolarization and action potential firing in these pulmonary sensory neurons (Fig. 2). The peak membrane potential depolarized by
capsaicin (0.3–1 μM, 2–5 s) was increased from −40.5 ± 3.7 mV at control to −21.4 ± 2.8 mV at 10 min after MBP (Fig. 2F; P < 0.05, n = 6). The duration of the capsaicin-evoked depolarization was also increased after MBP (e.g., Fig. 2; A–E); which was 13.5 ± 4.6 s at control and 19.4 ± 4.7 s at 10 min after the pretreatment (P < 0.05, n = 6). In addition, the number of action potentials evoked by capsaicin in these neurons was significantly enhanced from 2.3 ± 0.6 at control to 20.2 ± 6.2, 43.7 ± 12.4, 28.7 ± 8.1, and 21.3 ± 6.7 at 0, 10, 30, and 60 min, respectively, after MBP pretreatment (Fig. 2G; P < 0.05, n = 6). Pretreatment with the vehicle of MBP (acetate buffer) did not generate any effect on capsaicin-evoked inward current in voltage-clamp mode, and this pretreatment did not affect the capsaicin-evoked depolarization/action potential firing in current-clamp recordings (n = 5; data not shown).

MBP enhanced action potential generation evoked by electrical stimulation. In current clamp, pretreatment with MBP (2 μM, 60 s) alone evoked action potentials only in 2 of 23 neurons tested, the firing of which started during the last 15 s of the pretreatment and stopped within 10 min after the termination of MBP (not shown). MBP induced small but detectable changes in resting membrane potential in most of these neurons, but the change varied among them. For exam-

Fig. 1. Major basic protein (MBP) potentiated the capsaicin-evoked whole cell inward current in isolated rat vagal pulmonary sensory neurons. A: representative records in voltage-clamp mode illustrating the capsaicin (0.3 μM, 3 s)-evoked inward currents before and at different time points after pretreatment with MBP (2 μM, 60 s) in a jugular neuron (capacitance: 12.1 pF). Note the different current scale at 10 min after MBP. B: group data showing the potentiating effect of MBP (2 μM, 60 s) on the capsaicin (0.3–1 μM, 2–4 s)-evoked inward current. The responses at time 0 refer to the capsaicin-induced responses immediately (<1 s) after the termination of MBP pretreatment. Values are means ± SE; n = 6. *Significantly different from the control response (Con) before MBP (P < 0.05).

Fig. 2. MBP enhances the number of capsaicin-evoked action potentials (APs) in rat vagal pulmonary sensory neurons. A–E: representative records in current-clamp mode illustrating the responses to capsaicin (Cap; 0.3 μM, 2 s) before and at different time points after pretreatment with MBP (2 μM, 60 s) in a jugular neuron (21.2 pF). Insets: APs triggered by capsaicin shown on a large time scale. F: group data showing the effect of MBP on the capsaicin (0.3–1 μM, 2–5 s)-evoked peak depolarizing membrane potential (V_m). G: group data showing that MBP significantly increased the number of APs evoked by capsaicin (0.3–1 μM, 2–5 s). Values are means ± SE; n = 6. *Significantly different from the corresponding control before MBP (P < 0.05).
ple, at 5 min after MBP, depolarization was shown in 12 neurons (average: 3.3 mV), hyperpolarization was shown in 6 neurons (average: 2.6 mV), and no change was shown in the remaining 5 neurons. Thus the group data showed that pretreatment with MBP did not significantly alter the resting membrane potential in these neurons (Fig. 3). In sharp contrast, the number of action potentials evoked by an electrical stimulation (10–400 pA, 485 ms) was dramatically increased within 2–10 min after MBP in all 23 neurons tested; the potentiation lasted for >65 min (Fig. 3). Pretreatment with the vehicle of MBP did not cause any detectable change in the sensitivity of these neurons’ responses to current injection (n = 5).

Effect of MBP on the input resistance of pulmonary sensory neurons. It has been well documented that eosinophil-derived cationic proteins at high dose can induce injury and damage of airway mucosa (17, 38). We assume that, if MBP causes a similar membrane damage of the pulmonary sensory neurons, the input resistance of these neurons will be reduced. In seven pulmonary sensory neurons, input resistance was determined before and at different time points after MBP pretreatment (2 μM, 60 s) (Fig. 4). Although the input resistance slightly increased after pretreatment with MBP in some pulmonary sensory neurons (e.g., Fig. 4C), the group data were not significantly different from those at control (Fig. 4D; 320.1 ± 30.5 Ω at control and 334.7 ± 38.5, 327.7 ± 33.4, and 339.3 ± 34.1 Ω at 5, 10, and 30 min after MBP, respectively; P > 0.05, n = 7).

Role of polyanions in the MBP-induced sensitization of pulmonary sensory neurons. The sensitizing effect of MBP (2 μM, 60 s) was completely abolished when its cationic charge was neutralized by mixing with a polyanion such as LMWH (Fig. 5, A and B), PLGA (Fig. 5, C and D), or PLAA (not shown), before the delivery to the neurons. However, pretreatment with these polyanions (3 μM, 60 s) within 2 min before MBP did not prevent the neurons from being sensitized, nor could these polyanions reverse the sensitizing effect of MBP when they were applied within 2–10 min after administration of MBP (n = 3–5 in each of the conditions; data not shown).

Sensitization of pulmonary sensory neurons by EPO and ECP. Pretreatment with EPO (2 μM, 60 s) mimicked the effect of MBP on the pulmonary sensory neurons. The sensitizing effect of EPO on the neuron responses to capsaicin (0.3–1 μM, 1–6 s; n = 7; Fig. 6, A and C) and current injection (20–400 pA, 485 ms; n = 9; Fig. 6, B and D) lasted at least for 30 and 50 min, respectively.

Pretreatment with ECP (2 μM, 60 s) also increased pulmonary sensory neuron responses to both capsaicin (0.3 μM, 3–5 s; n = 5; Fig. 6E) and current injection (20–300 pA, 485 ms; n = 7; Fig. 6F). Compared with that of MBP or EPO, the effect of the same molar concentration of ECP was less intense and lasted a shorter duration; although a significant enhancement of the action potential firing evoked by current injection lasted for more than 35 min, a significant increase in the number of capsaicin-evoked action potentials was only found immediately after ECP pretreatment. Pretreatment with either EPO or ECP alone did not evoke any action potential or significantly alter the resting membrane potential of these sensory neurons.

Effect of M2-receptor blockade on the sensitivity of pulmonary sensory neurons. Pretreatment with either concentration (2 or 20 nM, 60 s) of methoctramine did not induce any detectable current or depolarization/action potentials in vagal pulmonary sensory neurons (n = 14). Surprisingly, methoctramine at both concentrations significantly and reversibly inhibited capsaicin-evoked inward current. The whole cell current evoked by capsaicin (0.3–1 μM, 1–8 s) was inhibited to 69.4 ± 10.1% (P < 0.05, n = 7) and 76.7 ± 8.9% (P < 0.05, n = 6) immediately after pretreatments with 2 and 20 nM methoctramine, respectively; the responses to capsaicin returned to control when tested at 10 min after the pretreatments. In contrast, pretreatment with either concentration of methoctramine did not induce any significant change (P > 0.05, n = 9 for both the 2 and 20 nM methoctramine groups) in the number of action potentials evoked by current injection (40–400 pA, 485 ms). These two concentrations of methoctramine were chosen based on the reported pKi values of methoctramine for the five muscarinic receptor subtypes (7.5, 8.7, 7.0, 7.6, and 7.0 for M1, M2, M3, M4, and M5 receptors, respect-

Fig. 3. MBP sensitizes rat vagal pulmonary sensory neurons to electrical stimulation. A: representative records in current-clamp mode illustrating the responses to current injection (200 pA, 485 ms) before and at different time points after pretreatment with MBP (2 μM, 60 s) in a nodose neuron (18.6 pF). B: group data showing that MBP pretreatment did not significantly alter the resting Vm of these sensory neurons. C: group data showing that the number of APs evoked by injected current (10–400 pA, 485 ms) was significantly increased after MBP pretreatment. Values are means ± SE; n = 23. *Significantly different from the control response before MBP (P < 0.05).
methoctramine at 2 nM is expected to occupy about one-half of M2 receptors and very few other muscarinic receptors, whereas 20 nM methoctramine occupies most of M2 receptors but also nearly one-half of M1 and M4 receptors.

DISCUSSION

Our results show that pretreatment with eosinophil MBP significantly increased the capsaicin-evoked inward current in rat vagal pulmonary sensory neurons (Fig. 1), and it substantially increased the number of action potentials evoked by either capsaicin or current injection (Figs. 2 and 3). The sensitizing effect of MBP peaked within 10 min and lasted >60 min after the pretreatment. In addition, similar potentiating effects were also induced by EPO and ECP, two other eosinophil-derived cationic proteins. These results are consistent with previous reports from our laboratory (19, 20, 33) that intratracheal delivery of either human eosinophil-derived or synthetic cationic proteins induced hypersensitivity of vagal pulmonary C-fiber afferents. Furthermore, this study provides the first evidence that pulmonary sensory neurons can be directly sensitized by these cationic proteins. Our data also show that the sensitizing effect of MBP was completely abolished when its cationic charge was neutralized by mixing with a polyanion, such as LMWH, PLGA, or PLAA, before its delivery to the neurons (Fig. 5). However, the polyanions were ineffective when they were delivered shortly (within 2–10 min) either before or after MBP. These results suggest that the cationic charge of these proteins plays an important role in their sensitizing effect on pulmonary sensory neurons.

Fig. 4. Effect of MBP on input resistance of rat vagal pulmonary sensory neurons. A: representative records in current-clamp mode illustrating the voltage responses of a nodose neuron (15.5 pF) to hyperpolarizing current pulses as shown in B (−10 to −100 pA, 485 ms) before and at different time points after pretreatment with MBP (2 μM, 60 s). C: data in A plotted as a current (I)-V_m relationship. Input resistance was measured by the slope of each I-V_m plot. D: group data showing that the input resistance of the sensory neurons was not significantly altered after MBP pretreatment (P < 0.05, n = 7).

Fig. 5. Role of polyanions in the MBP-induced sensitization of rat vagal pulmonary sensory neurons. Recordings in A and B were made in current-clamp mode from a single pulmonary jugular neuron (13.1 pF). A: responses to capsaicin (0.3 μM, 3 s) at control, at 10 min after 60-s pretreatment with a mixture of MBP (2 μM) and low-molecular-weight heparin (LMWH; 3 μM) and 30 min later, and at 10 min after pretreatment with MBP (2 μM; 60 s) alone. B: responses to current injection (0.6 nA, 240 ms) were tested 5 min after the capsaicin challenges under each of the pretreatment conditions (control, MBP + LMWH; MBP alone). Recordings in C and D were made in voltage- and current-clamp mode, respectively, from a single pulmonary nodose neuron (27.6 pF). C: responses to capsaicin (1 μM, 3 s) at control, at 10 min after 60-s pretreatment with a mixture of MBP (2 μM) and poly-L-glutamic acid (PLGA; 3 μM) and 30 min later, and at 10 min after pretreatment with MBP (2 μM, 60 s) alone and another 10 min later; pretreatment with PLGA (3 μM, 60 s) alone did not reverse the effect of MBP. D: responses to current injection (20 pA, 485 ms) were tested 5 min after the capsaicin challenges under each of the pretreatment conditions (control, MBP + PLGA, MBP alone, PLGA after MBP).
The present study shows a dramatic increase in the responses of firing rate to capsaicin and current injection after the MBP pretreatment in all of the neurons tested, despite only small and variable changes in resting membrane potential. This would suggest that the change, if any, in resting membrane potentials cannot account for the neuronal hypersensitivity induced by eosinophil-derived cationic proteins. The mechanism underlying the direct sensitizing effect of these proteins is not fully understood. Interestingly, it has been demonstrated recently that extracellular cations such as Mg$^{2+}$ and Ca$^{2+}$ can sensitize and gate TRPV1 (1). A similar effect can also be generated by certain polyamines (such as spermine, spermidine, and putrescine), the organic polycations that are known to modulate inflammation and nociception (2). In addition to their interactions with TRPV1, the endogenous cationic charged polyamines have been reported to modulate a number of ion channels, including inward rectifier K$^{+}$ channels (14, 30), NMDA (3, 51) and non-NMDA glutamate receptors (29), and calcium-sensing receptor (23). Whether eosinophil-derived cationic proteins have similar effects on these ion channels and consequently contribute to the sensitization of pulmonary sensory neurons observed in our study remains to be determined.

It has been previously proposed that eosinophil-derived cationic proteins may lead to airway hyperresponsiveness via an interaction of these proteins and airway epithelium (11, 24). Synthetic cationic proteins such as PLL have been reported to lower the electrical resistance of cultured airway epithelial cell layers, which presumably reflects the disruption of physical integrity of the epithelial membrane or the tight junctions between epithelial cells (47, 52). Indeed, it has been well documented that eosinophil MBP and other cationic proteins can cause injury or damage of airway epithelium in various species, including humans (17, 26, 38, 47, 52). The disruption and desquamation of the airway epithelial layer induced by these proteins may cause a reduction in the barrier function of the epithelium and therefore allow increased access of various irritants to underlying airway nerve endings and smooth muscles (24, 47). In the present study, we tested whether eosinophil-derived cationic proteins, when delivered briefly (60 s) at a low concentration (2 μM), caused a similar membrane damage in the pulmonary sensory neurons. Our data show that the input resistance of these neurons after MBP pretreatment was not significantly different from that of control (Fig. 4) and therefore do not reveal any evidence of membrane damage. This assumption is further supported by our observation that the sensitization of these sensory neurons appeared reversible after 60 min washout of the cationic proteins (e.g., Figs. 1–3).

It has been suggested recently that blockade of the inhibitory M$_2$ receptors on the parasympathetic nerves plays an important role in eosinophil-derived cationic protein-induced airway hyperreactivity (13, 27, 48). However, it is unlikely that blockade of M$_2$ receptor is responsible for the cationic protein-induced...
hypersensitivity of isolated pulmonary sensory neurons demonstrated in our present study because pretreatment with methoctramine (2 or 20 nM, 60 s), a selective M2-receptor antagonist, significantly and reversibly inhibited capsaicin-evoked whole cell inward current in these sensory neurons. A similar inhibitory effect of methoctramine on TRPV1-mediated responses has also been reported in rat dorsal root ganglion neurons (36), although a much higher concentration of methoctramine (1–100 μM) was used in that study.

Although our study shows that eosinophil-derived cationic proteins can directly sensitize pulmonary sensory neurons, our data do not exclude other possible mechanisms that may also contribute to the hypersensitivity of pulmonary C-fiber afferents when eosinophils infiltrate in the airways. Indeed, eosinophil-derived cationic proteins such as MBP have been reported to release prostaglandins from epithelial cells (50) and histamine from mast cells (43, 53) and basophils (45). These mediators are known to induce pronounced sensitizing effects on pulmonary C-fiber afferents, elevating their sensitivities to various chemical and mechanical stimulations (15, 22, 34). The importance of pulmonary C fibers in the regulation of airway functions in both physiological and pathophysiological conditions is well documented (8, 35). Stimulation of these sensory afferents is known to evoke dyspnecic sensation, airway irritation, and cough and to elicit reflex responses such as bronchoconstriction and hypersecretion of mucus, which are mediated through the central nervous system and cholinergic pathway (8, 35). Moreover, sensory neuropeptides such as tachykinins (e.g., substance P, neurokinin A) and calcitonin gene-related peptide released locally from these nerve endings on activation can produce additional local effects such as airway constriction, protein extravasation, mucosal edema, and inflammatory cell chemotaxis (4, 44). Therefore, it is reasonable to propose that when the airway infiltration of eosinophils occurs under certain pathophysiological conditions such as asthma, the hyperexcitability of pulmonary C-fiber nerves induced by these cationic proteins may play a part in the manifestation and development of the bronchial hyperreactivity, dyspneic sensation, and cough. Furthermore, in addition to eosinophil granule-derived proteins, a number of other cationic proteins have been identified in a variety of cell types, e.g., cathepsin G and neutrophil cationic proteins from neutrophil and platelet factor 4 from platelet. Thus it is very probable that these cationic proteins collectively play a significant part in the pathogenesis of airway hyperresponsiveness associated with airway inflammatory diseases (10, 41).

This study shows that the sensitizing effect of MBP on isolated pulmonary sensory neurons lasted for >60 min even after the cells had been continuously perfused with the control ECS (e.g., Figs. 1–3). This observation is interesting and may have significant physiological relevance, but its underlying mechanism is not understood. A previous study in our laboratory (32) has demonstrated that synthetic cationic protein PLL administered by intratracheal instillation induced a sustaining effect on vagal pulmonary C fibers in anesthetized rats, with a strikingly long duration (>120 min). In that study, our group postulated that the long sustaining effect may have involved a slow release of chemical mediators (e.g., PGE2 or bradykinin) from various cells in the airway mucosa on action of cationic proteins, as discussed above. Although a possible involvement of endogenous autacoids cannot be completely ruled out in the present study because certain potent mediators (e.g., PGE2 or PG12) may still be released from isolated neurons or from nonneuronal satellite cells present in the culture (25, 39, 49), other potential contributing factors should also be considered. For example, it is possible that the sustaining action of MBP is related to cascades of intracellular signaling events triggered by the cationic protein in these neurons (37). In addition, a recent study has further revealed that the binding of MBP with certain specific cell-surface receptors plays an important role in its cytostimulatory and cytotoxic properties (18), which may explain, perhaps in part, why the sensitizing effect on the pulmonary neurons in the present study persisted even after MBP was completely washed out of the recording chamber. It is also possible that the longer duration of the sensitizing effect of MBP than that of ECP found in the present study (Figs. 2, 3, and 6) is related to the fact that MBP may form a disulfide bond with a target molecule on these neurons because of the reactivity of the sulphydryl groups on MBP; in comparison, it is unlikely for ECP to form a disulfide bond because its cysteine residues form four disulfide bonds (6). Obviously, further investigations are required to bring a better understanding of the interaction between MBP and these neurons and the mechanism underlying the sustaining nature of its effect on these cells.

We have previously demonstrated that intratracheal instillation of eosinophil-derived cationic proteins can stimulate pulmonary C-fiber afferents (33). However, our data from the present study showed that pretreatment with MBP alone induced action potential firing only in a small portion (2 of 23) of the isolated pulmonary sensory neurons. Although different doses of the proteins applied and/or different sensitivities of pulmonary neurons in two distinct preparations (afferent nerve endings vs. the neuronal soma) may also be involved, the discrepancy of the stimulatory effect of the cationic proteins between our previous in vivo and present in vitro studies could also result from the possible release of various endogenous mediators in the in vivo experiments, as discussed above. In addition, although full-range dose responses were not established in this study, our data indicated that the order of the sensitizing potency of three different eosinophil-derived cationic proteins in the same molar concentration was MBP > EPO > ECP. It has been recognized that these proteins have marked differences in tertiary conformation and in the proportion of arginine and amino acid residues, which may influence their cationic charge capacity and interactions with cell environment (41). However, whether the different amount of cationic charge carried by these proteins is solely responsible for their different potency in sensitizing pulmonary sensory nerves is not known.

In conclusion, our results show that pretreatment with eosinophil-derived cationic proteins induces a pronounced sensitizing effect on isolated rat vagal pulmonary sensory neurons. Our data also indicate that the effects of these proteins are dependent on their cationic charge but are not likely due to the damage or injury of neuronal membrane. These results further suggest that the direct and long-lasting sensitizing effect of cationic proteins on pulmonary sensory nerves may play an important part in the manifestation of airway hyperresponsiveness associated with eosinophil infiltration in the airways.
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