Residual oil fly ash and charged polymers activate epithelial cells and nociceptive sensory neurons

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Oortgiesen, Marga, Bellina Veronesi, Gary Eichenbaum, Patrick F. Kiser, and Sidney A. Simon. Residual oil fly ash and charged polymers activate epithelial cells and nociceptive sensory neurons. Am J Physiol Lung Cell Mol Physiol 278: L683–L695, 2000.—Residual oil fly ash (ROFA) is an industrial pollutant that contains metals, acids, and unknown materials complexed to a particulate core. The heterogeneous composition of ROFA hampers finding the mechanism(s) by which it and other particulate pollutants cause airway toxicity. To distinguish culpable factors contributing to the effects of ROFA, synthetic polymer microsphere (SPM) analogs were synthesized that resembled ROFA in particle size (2 and 6 µm in diameter) and zeta potential (~29 mV). BEAS-2B human bronchial epithelial cells and dorsal root ganglion neurons responded to both ROFA and charged SPMs with an increase in intracellular Ca2+ concentration ([Ca2+]i) and the release of the proinflammatory cytokine interleukin-6, whereas neutral SPMs bound with polyethylene glycol (0-mV zeta potential) were relatively ineffective. In dorsal root ganglion neurons, the SPM-induced increases in [Ca2+]i were correlated with the presence of acid- and/or capsaicin-sensitive pathways. We hypothesized that the acidic microenvironment associated with negatively charged colloids like ROFA and SPMs activate irritant receptors in airway target cells. This causes subsequent cytokine release, which mediates the pathophysiology of neurogenic airway inflammation.

partial matter; capsaicin receptors; acid-sensitive receptors; zeta potential

EXPOSURE TO PARTICULATE MATTER (PM) air pollutants is associated epidemiologically with adverse health effects in the human population (10). The reported increases in morbidity and mortality are marked in individuals with preexisting pathological conditions such as respiratory or cardiac disorders. PM pollutants come from various sources (e.g., urban, natural, or industrial) and consist of complex mixtures of different organic and inorganic components. The heterogeneous composition of PM pollutants has complicated identifying the particulate component(s) that underlie the observed symptoms. Explanations for the mechanisms of action have focused on the soluble metal components (e.g., iron, vanadium, nickel) that cause the formation of oxidative radicals (11, 14, 29), on biological components (i.e., endotoxins) that cause activation of macrophages (2), and on the acidic nature of these particles (8, 10, 24). Residual oil fly ash (ROFA), an industrial particulate pollutant generated from the burning of low-sulfur residual oil (15), has been used in numerous studies to investigate the mechanisms of action of PM. Like other PM, ROFA contains various metals, sulfates, acids, and unknown materials complexed to its particulate core (11, 15). Tracheal instillation of ROFA in experimental animals produces acute lung injury and airway hyperresponsiveness (11, 37). In human and rodent macrophages, ROFA exposure results in the release of inflammatory cytokines, oxidative burst, and apoptosis (2, 16). Cultured airway epithelial cells and the human bronchial epithelial cell line BEAS-2B secrete inflammatory cytokines and mediators after exposure to ROFA (6, 32). Recent data from our laboratories (13, 40) suggest that ROFA initiates inflammatory cytokine release by activation of sensory irritant receptors. In ROFA-exposed BEAS-2B cells, antagonists of capsaicin- and acid-sensitive receptors inhibit the increase in the intracellular Ca2+ concentration ([Ca2+]i) and reduce the release of the proinflammatory cytokines interleukin (IL)-6 and IL-8 and their transcripts (40). In BEAS-2B cells and primary airway epithelial cells, ROFA-induced IL-6 release was most pronounced (6, 30). Therefore, the present studies concentrated on particle-mediated IL-6 release because this cytokine is secreted by various immune and nonimmune cell types and plays a prominent role in airway and neurogenic inflammation (3).

A variety of environmental pollutants (e.g., ozone, sulfuric acid, toluene 2,4-diisocyanate, sodium metabisulfite, cigarette smoke) cause airway disorders that have been associated with irritation, sensory nerve activation, and neurogenic inflammation (18, 19, 28, 35). The symptoms of airway inflammation and hyperresponsiveness caused by these pollutants are reduced in animals in which sensory input is impaired by capsaicin-induced denervation of nociceptors or by pretreatment with capsaicin- or neuropeptide-receptor antagonists (27). Recently, airway hyperresponsiveness

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and inflammatory cell influx were reduced in capsaicin-treated BALB/c mice when exposed to ROFA (13). These studies suggest that ROFA and other PM initiate airway inflammation by triggering sensory irritant receptors. Such receptors (e.g., capsaicin and acid sensitive) found on the cell bodies and nerve terminals of nociceptors are sensitive to a wide range of noxious stimuli such as heat, cold, and mechanical and chemical irritants (e.g., capsaicin, the pungent component in hot peppers and acids; see Refs. 7, 42). Sensory nerves originating from trigeminal, nodose, and dorsal root ganglion neurons (DRGs) extend their terminals into the nasal and/or pulmonary epithelium. Activation of irritant receptors on their nerve terminals by noxious stimuli results in the release of neuropeptides that mediate airway function through vasodilation, bronchoconstriction, and mucus secretion (1, 26). In addition to nociceptor nerve terminals, functional capsaicin receptors have been identified on nonneuronal cells (e.g., mast cells and glia cells) (4, 5). Human epithelial BEAS-2B cells also express capsaicin receptors, the activation of which causes the release of inflammatory cytokines (39). Together, these observations lead to the hypothesis that particulate pollutants trigger irritant receptors on a variety of airway target cells. The resulting neurogenic inflammatory pathways could contribute to PM morbidity and mortality.

Because PM pollutants are complex mixtures, analysis of their physicochemical properties is necessary to help identify the culpable component(s) and understand the mechanisms by which particulate pollutants cause inflammation. In the present study, we examined the effects of synthetic polymer microspheres (SPMs) synthesized to resemble ROFA particles in size and surface potential but lacking confounding factors such as metals or biologics. We compared the effects of ROFA and SPMs on the human bronchial epithelial cell line BEAS-2B and on mouse sensory DRGs. The data indicate that, at least in part, the inflammatory effects of ROFA particles on airway target cells may be explained by their negative surface charge, which appears to be sufficient to activate irritant receptors.

**MATERIALS AND METHODS**

Cells. BEAS-2B cells, derived from a human bronchial epithelial tumor cell transformed with an Ad12-SV40 construct (31), were obtained from the American Type Culture Collection (Manassas, VA). This cell line has been featured in numerous experiments (6, 36) to investigate the mechanisms of airway pollutant toxicity. In the present studies, cells were used from passages 60 to 85 and maintained in keratinocyte growth medium (KGM) that was supplemented with bovine pituitary extract (0.22-1.54 mg/ml of total lipid), human epidermal growth factor (5 ng/ml), hydrocortisone (0.5 µg/ml), insulin (5 µg/ml), and insulin (5 µg/ml). Cells were refreshed with KGM every 3-4 days after being washed once with warm Hank's balanced salt solution. When confluent, BEAS-2B cells were removed from their culture flasks by incubating them for 6 min in 0.25% trypsin and 1 mM EDTA (GIBCO BRL, Life Technologies, Grand Island, NY) and were neutralized with soybean trypsin inhibitor at 37°C. Cells were replated at a density of 1 × 10^5 cells/ml. For [Ca^{2+}]_i measurements, BEAS-2B cells were plated at a density of 1 × 10^5 cells/ml on fibronectin-coated glass (22 μm in diameter, no. 0 thickness) coverslips (Carolina Biological Supply, Burlington, NC). For cytokine measurements, BEAS-2B cells were cultured in 96-well culture plates (Costar, Cambridge, MA) at a density of 1 × 10^5 cells/ml. Experiments were performed when cells were 80-90% confluent, at which time their density was 5-7 × 10^5 cells/ml.

Under these conditions, the cells morphologically appeared as an epithelial monolayer and gave the most reproducible responses.

DRG explants were cultured from either fetal (18-20 days gestation) or newborn BALB/c mice (postnatal days 3-16). DRGs were taken from the C2-8 and T1-11 regions because neurons from these ganglia specifically innervate the upper and lower pulmonary airways. DRGs were isolated, and their neurons were dissociated after 1 h of treatment with 1.5 mg/ml of collagenase and 10 min of treatment with 50 µg/ml of DNase I (25).

For [Ca^{2+}]_i measurements, DRG neurons from newborn mice were plated on fibronectin-coated glass coverslips. For cytokine measurements, DRG neurons from fetal mice were cultured at a density of 1 × 10^5 cells/ml in fibronectin-coated 96-well culture plates. DRGs were maintained in culture in DMEM-F-12 medium (1:1) supplemented with 10% FCS and nerve growth factor (100 ng/ml of 7S-NGF) and refreshed with DMEM-F-12 medium every 2-3 days. Cells were used after 2-6 days for [Ca^{2+}]_i measurements and after 7-10 days in culture for cytokine release. Fibroblast and glia growth were inhibited by the addition of 10 µM cytosine arabinoside for 24 h after 2-3 days in culture.

ROFA and SPMs. Field ROFA was originally collected on a Teflon glass filter that was positioned downstream from the cyclone of an industrial power plant (Southern Research Institute, Birmingham, AL). Previous chemical analysis of ROFA indicated that it consisted of various metals (e.g., vanadium, iron, magnesium, nickel, copper), sulfates, acids, and other materials associated with an insoluble carbon core (11, 15). When suspended in aqueous solutions and vortexed, ROFA dissociates into soluble and particulate fractions. For samples of the particulate core, ROFA (50 µg/ml) was suspended in 1 ml of ultraviolet-distilled water, vortexed for 20 s, and centrifuged for 20 min at 21,000 rpm (49,000 g). The supernatant was removed, and the pellet was resuspended in 1 ml of water, vortexed, and centrifuged again. The washed ROFA particulate pellet used in these studies was obtained after five such washing cycles. ROFA filtrate was obtained after the suspension of ROFA was filtered through a 0.22-µm Millipore filter.

SPMs [poly(methacrylic acid) co-acrylic acid microspheres] were synthesized as described previously (12). Monomers (4-nitrophenyl methacrylate monomer) and the cross-linker N,N’-methylene-bis-acrylamide (recrystallized from methanol and cooled to −60°C) were mixed in a methanol-ethanol solution under argon. The reaction was degassed at room temperature for 20 min by freezing and thawing under argon. The polymer reaction was initiated by the addition of 2,2′-azobisisobutyronitrile, which resulted in a cloudy dispersion of particles after 10 min. The reaction was run for 4 h, after which time it was quenched by exposure to oxygen. The dispersed particles were washed three times with ethanol (EtOH), incubated with 1 N NaOH to hydrolyze the nitrophenol, and washed again five times with water. The SPMs were stored in deionized water at 4°C until use. We used SPMs with diameters of 2 and 6 µm at a 5:1 ratio.

The neutral SPM_PEG were synthesized by covalent binding of polyethylene glycol (PEG-5000) to the SPMs. The charged SPMs containing unhydrolyzed 4-nitrophenyl methacrylate
monomer groups from the above synthesis (700 mg) were transferred from pure EtOH into pure chloroform (CHCl₃) in four steps. The SPMs (500 mg) in EtOH were pelleted (300 rpm, 30 min), the supernatant was carefully removed, and 3 ml of CHCl₃-EtOH (20:80 vol/vol) were added. The SPMs were suspended by vortexing, agitated on a rotating table for 15 min, and then repelleted. This process was repeated with solutions of CHCl₃-EtOH (50:50 vol/vol, 3 ml and 80:20 vol/vol, 3 ml). The latter solution was near neutral density for the SPMs and thus did not pellet. To this solution, CHCl₃ (3.0 ml) was added, and the SPMs were isolated by flotation (1,000 rpm, 45 min). The reactive SPMs were finally washed two times in CHCl₃. The final floating pellet was suspended in CHCl₃ (2.0 ml). The SPM suspension (350 µl, 65 mg of SPM) was transferred into a reaction vessel to which CHCl₃ (2,150 µl) and triethylamine (250 µl, 1.8 mmol) were added. The SPMs were resuspended, and α-amino-ω-methoxy-polyethylene glycol (molecular weight 5,000; Shearwater Polymers) was added as a solid (147 mg, ~30 µmol). The mixture was vortexed for 5 min, sonicated for 5 min, and placed on a rotating table for 48 h. The reaction was washed twice with CHCl₃, after which the SPMPEG were transferred from pure EtOH into pure chloroform (CHCl₃) in 380 nm, and their fluorescent emission was detected at 510 nm with a PTI imaging system (Photon Technology International 710, South Brunswick, NJ). Ratio data of 340- to 380-nm fluorescence intensity were analyzed and recorded with ImageMaster software (Photon Technology International). In other experiments, the fluorescent dye fluo 3-AM (Molecular Probes) was used with the same loading protocol. In these studies, the cells were illuminated at 485 nm, and fluorescent emission was detected at 525 nm with Scion (Frederick, MD) imaging hardware and software. In all experiments, full images of small populations of fluorescence-labeled cells were recorded and stored on hard disk at the rate of 1 image/s. The ratio or intensity measurements for the individual cells were analyzed off-line with ImageMaster and Scion software. BEAS-2B and DRGs showed no significant difference in the [Ca²⁺] data between the two procedures (i.e., fluo 3-AM or fura 2-AM), as shown by the responses to prototype test agents (e.g., ionomycin). Experiments were carried out at 25–26°C.

Cells were initially exposed to the HEPES buffer alone to obtain baseline values. A constant flow of medium was applied to the cells by continuous superfusion (15 ml/min) of HEPES buffer through a 1-mm-diameter Teflon tube attached to a solenoid-driven valve. Superfusion of the cells with control HEPES buffer did not change the [Ca²⁺] responses of the cells during that period of time. Nevertheless, the data show (see Figs. 7 and 8) that in responding cells multiple responses could be induced on sequential exposures to SPMs. Switching between control buffers had no effect on [Ca²⁺], which indicated that the changes in [Ca²⁺] were not mechanical artifacts caused by switching the superfusion solution. The effects of receptor antagonists on SPM-mediated [Ca²⁺] responses were tested by preincubation of the cells with the specific antagonists for 5–10 min before test compounds (i.e., ROFA or SPMs) were applied in the presence of the antagonist. In experiments testing the effects of pH 6.5 or pH 5.0, 10 mM MES was added to buffer the acidic exposure solutions. At the end of each experiment, 2 µM ionomycin was added to the cells to compare the [Ca²⁺] changes. Only cells that showed a strong response with ionomycin were used for data analysis. Traces of cells were selected (i.e., those cells with strong ionomycin responses that did not show marked bleaching), and averaged data were obtained from three to five cells per exposure and three to five different experiments.

Cytokine measurement. Test compounds suspended in either KGM or DMEM-F-12 medium were exposed to the cells for 4 h following procedures previously described (6, 40). Media from the exposed cells were removed and analyzed by ELISA for IL-6 (R&D Systems, Minneapolis, MN). The effects of receptor antagonists on IL-6 release were tested by a 15-min preexposure to the antagonist, after which the medium was replaced by medium containing the test compound and the agonists for an additional 3.75 h. Plates were read on a Molecular Devices (Sunnyvale, CA) plate reader, and the data were analyzed with SoftMax Pro 1.2
software. Data were calculated in picogram per milliliter concentrations after comparison to an in-plate standard quadratic curve. Control values of IL-6 release obtained from cells exposed to culture medium alone amounted to 5−30 pg/mL. Each value represents at least six or seven individual samples from three or four different experiments.

Exposure. Cells were exposed to ROFA at 50 µg/ml. A previous study (40) showed that at this concentration the ROFA-mediated IL-6 release by BEAS-2B cells amounted to 70−80% of the maximal value. When suspended, the concentration of ROFA particles (50 µg/mL) and SPMs (18.8 µg/mL) was 2 × 10⁴ particles/mL as counted with a Neubauer counting chamber (light microscope, ×63).

In the [Ca²⁺]i measurements, the ROFA and SPMs were suspended in the HEPES buffer described in [Ca²⁺]i measurement. Cells were first exposed to the HEPES buffer alone to obtain the baseline or control values. For antagonist studies, separate trials examined the effects of the antagonists alone. For IL-6 release measurement, ROFA and SPMs were suspended in the culture media (i.e., DMEM-F-12 medium for DRGs and KGM for BEAS-2B cells). Separate measurements of the baseline values of media and of the effects of capsaicin (CPZ) and amiloride alone are presented.

Suspended ROFA acidified unbuffered salt solutions, indicating that protons were released. This property was retained by the washed particles because ROFA particulate pellets, obtained after several cycles of washing, still acidified nonbuffered saline. The pH of ROFA suspension (100 µg/mL) in a 10 mM NaCl solution (pH 6.9) was 4.0, and after five washes, the pH was 5.3. ROFA (100 µg/mL) suspended in 10 mM HEPES-buffered salt solution lowered the pH from 7.4 to 7.1 but did not alter the pH of KGM or DMEM-F-12 medium. The washed ROFA particles did not significantly change these cell culture solutions. Similarly, SPMs suspended at a density of 18.8 µg/mL lowered the pH of the HEPES buffer from 7.4 to 7.25 and also failed to change the pH of KGM or DMEM-F-12 medium. The pH changes of the solutions alone were insufficient to cause any of the observed effects. That is, BEAS-2B cells or DRGs did not respond when the buffer solution was changed from pH 7.4 to 7.1. In addition, exposure of the cells to ROFA or SPMs in buffers adjusted to pH 7.4 induced similar increases in [Ca²⁺]i.

Cell viability. Neutral red (NR) was prepared as a 0.4% aqueous solution. After exposure to the test compound, the cultures were removed from the incubator and washed with Hanks’ balanced salt solution. KGM (200 µl) containing 50 µg/mL of NR was added to each well and incubated at 37°C for 3 h. Confluent wells of cells exposed to a 0.1% saponin solution for 15 min provided a background reading of 100% cell death. After incubation, the dye medium was aspirated, and the cells were washed with 200 µl of formal-calcium solution for 2−3 min. After removal of this fixative, 200 µl of 1% acetic acid-50% EtOH were added to each well at room temperature for 15 min to extract the extraneous dye. Plates were read at 540-nm absorbance.

Chemicals. KGM and supplements were purchased from Clonetics (San Diego, CA). 7S-NGF, collagenase XI, DNase I, trypsin, soy broth trypsin inhibitor, and fibronectin were purchased from Sigma (St. Louis, MO). Capsaicin, CPZ, and amiloride were purchased from RBI Chemicals (Natick, MA). ROFA was generously supplied by Dr. R. B. Devlin (Human Studies Division, US Environmental Protection Agency, Chapel Hill, NC).

ROFA and SPMs were suspended immediately before use. Other chemicals were dissolved in their respective vehicles to yield 100−to 1,000-fold stock solutions, kept at −20°C, and diluted to their final concentrations immediately before use.

Statistics. Data were obtained from multiple samples from three to five separate experiments (i.e., trials). A one-way ANOVA with the Newman-Keuls postcomparison test was used to examine the significant (P < 0.05) effects of the different treatments compared with control trials (medium only) and the statistical differences among the different treatment groups. Instat 2 software from Graph Pad (San Diego, CA) was used for the statistical analysis. For IL-6 experiments, n is the number of samples. For [Ca²⁺]i measurements, n is the number of responding cells unless otherwise indicated. Data are expressed as means ± SE, and differences between groups are significant at P < 0.05.

RESULTS

ROFA exposure. Exposure of BEAS-2B cells to ROFA (50 µg/ml) elicited a transient increase in [Ca²⁺]i. (Fig. 1A). When the cells were washed with HEPES buffer, the [Ca²⁺]i returned to baseline values. Similarly, exposure of cultured DRGs to ROFA (50 µg/ml) caused a rapid increase in [Ca²⁺]i (Fig. 1B). This increase was seen in 18 out of 37 neurons (i.e., 49 ± 12% of the cells in 8 experiments). The amplitudes and time courses of the [Ca²⁺]i responses varied among individual cells. In both BEAS-2B cells and DRGs, the peak amplitude of responding neurons was 25−100% compared with that of the maximal [Ca²⁺]i increase elicited by 2 µM ionomycin. Exposure of BEAS-2B to ROFA produced a concentration-dependent release of IL-6, with submaximal values at 50−100 µg/ml (40). In the present study, both BEAS-2B cells and DRGs released IL-6 on exposure to 50 µg/ml of ROFA (Fig. 1C). The higher amount of IL-6 produced by BEAS-2B cells relative to DRGs may be contributed by the washed ROFA particulate pellets, ROFA filtrate, and the washed particulate pellet.

ROFA consists of a particulate core to which soluble and insuble components are attached. When vortexed for 20 s, ROFA dissociates into soluble and particulate fractions. To compare the differential effects of soluble and insoluble particulate components, DRGs were exposed to 50 µg/ml of suspended ROFA, the ROFA filtrate, and the washed particulate pellet. Exposure of all three fractions of ROFA (i.e., suspended, filtrate, and particulate) to DRGs evoked the release of similar amounts of IL-6 (P = 0.39 and 0.70 for ROFA suspension vs. filtrate and washed particulate fraction, respectively; Fig. 2). These data indicate that the washed particulate core as well as the filtrate, which contains the soluble ROFA fraction and possibly particulates in the ultrafine range, contribute to the IL-6 release by the total suspended ROFA.

We further examined the role of the physicochemical characteristics of ROFA. ROFA particles as measured by light microscopy (×63) ranged between 1 and 10 µm at their longest geometric dimension. Previously, Dreher et al. (11) reported that ROFA particles had an average aerodynamic diameter of 1.95 ± 0.18 µm.
With microelectrophoresis, ROFA particles were shown to carry a negative surface charge. The zeta potential of ROFA particles examined in a 30 mM sucrose-10 mM NaCl solution (adjusted to pH 7.4) was determined to be $-22.8 \pm 1.3$ mV (range between $-25$ and $-35$ mV; $n = 7$ measurements). This zeta potential, which approximates the surface potential of the particle, indicated that the proton concentration around ROFA particles would be higher than in the bulk solution. This relationship creates an acidic microenvironment associated with the individual particles. As described in MATERIALS AND METHODS, ROFA also acidified the solution depending on the buffer capacity of the vehicle. However, the pH changes in the exposure solutions (HEPES buffer or cell culture medium) were insufficient to cause any of the observed effects of ROFA.

Effects of SPMs. Based on the physicochemical data collected on ROFA, SPMs were synthesized with geometric diameters of $2.6 \pm 0.1$ and $6.6 \pm 0.3 \mu m$ (average $6 \pm SE$) (12), which were used as a suspension of 6- and 2-µm SPMs at a 5:1 ratio to resemble the average size range of ROFA. The zeta potential of SPMs was measured to be $-29 \pm 0.9$ mV ($n = 8$ measurements). The SPMs differed from ROFA because they are spherical with distinct diameters (2 and 6 µm in a 5:1 ratio) relative to the heterogeneously shaped and sized ROFA. However, SPMs acted as synthetic analogs of ROFA particles with respect to their average size and zeta potential, but SPMs lacked other confounding elements of ROFA such as heavy metals or possible biological (i.e., endotoxin) contaminants.

Epithelial BEAS-2B cells or DRGs were exposed to 18.8 µg/ml of SPMs, which amounted to $2 \times 10^4$ particles/ml. This was similar in application to the number of ROFA particles (between 1 and 10 µm in diameter) at 50 µg/ml. In both cell types, SPMs elicited a rapid increase in $[Ca^{2+}]_i$ (Fig. 3, A and B). The cells responded to SPMs with variable amplitudes and time courses that ranged from 25 to 95% of the maximal $[Ca^{2+}]_i$ increase evoked by 2 µM ionomycin. SPMs (18.8 µg/ml, $2 \times 10^4$ particles/ml) also induced the release of IL-6 in BEAS-2B cells and DRGs (Fig. 3C). A twofold higher concentration of SPMs only marginally increased the IL-6 release and the $[Ca^{2+}]_i$ response, indicating that SPMs had a (sub)maximal effect at this concentration (data not shown).

To determine the contribution of surface charges as a factor in particulate-mediated effects, the effects of the
charged SPMs were compared with those of neutral SPM\textsubscript{PEG}. The SPM\textsubscript{PEG} had a 0-mV zeta potential as measured by electrophoresis. Figure 4A shows that in BEAS-2B cells and DRGs, neutral SPM\textsubscript{PEG} caused no significant IL-6 release compared with that induced by the charged SPMs ($P < 0.001$). The IL-6 release caused by SPM\textsubscript{PEG} was slightly higher compared with control value only in BEAS-2B cells. In addition, neutral SPM\textsubscript{PEG} were markedly less effective then their charged counterparts in increasing [Ca\textsuperscript{2+}]\textsubscript{i} in DRGs. The cells were superfused in alternating order between separate experiments with SPM\textsubscript{PEG} and charged SPMs. Neutral SPM\textsubscript{PEG} caused an occasional [Ca\textsuperscript{2+}]\textsubscript{i} response in the cells ($2.5 \pm 1.3\%$ responding cells in 6 experiments, 82 DRGs), whereas the charged SPMs activated $27 \pm 4\%$ (percent responding cells in 6 experiments, 82 DRGs) of the cells.

Previous data (40) on the blocking effects of CPZ, a competitive antagonist of capsaicin receptors, and amiloride, a nonselective antagonist of acid-sensitive receptors, on ROFA effects in BEAS-2B cells suggested that activation of capsaicin- and/or acid-sensitive receptors initiated ROFA-induced cytokine release. We further explored the possibility that SPMs also activated sen-

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**Fig. 3.** Effects of synthetic polymer microspheres (SPMs). Recordings of [Ca\textsuperscript{2+}]\textsubscript{i} in BEAS-2B cells (A) and DRG neurons (B) loaded with fluo 3-AM and fura 2-AM, respectively, are shown. Superfusion with SPMs (18.8 µg/ml, $2 \times 10^4$ particles/ml) caused an immediate increase in [Ca\textsuperscript{2+}]\textsubscript{i}, that declined during SPM exposure. Maximal increase in [Ca\textsuperscript{2+}]\textsubscript{i} is shown after exposure to 2 µM IO. C: release of IL-6 by BEAS-2B cells and cultured DRGs after 4 h of exposure to SPMs (18.8 µg/ml). Medium values indicate baseline IL-6 release. Exposure to SPMs induced significant IL-6 release compared with medium control values ($P < 0.001$; $n = 10$ samples).

**Fig. 4.** Effects of charged SPMs and neutral SPMs bound with polyethylene glycol (SPM\textsubscript{PEG}) were compared ($n = 9$ experiments for all exposures). A: in BEAS-2B cells and DRGs, SPM\textsubscript{PEG} ($2 \times 10^4$ particles/ml) caused a much smaller IL-6 release compared with that induced by charged SPMs ($2 \times 10^4$ particles/ml; $P < 0.001$). IL-6 release caused by SPM\textsubscript{PEG} was significantly higher compared with control value in BEAS-2B cells but not in DRGs ($P < 0.001$). IL-6 release caused by charged SPMs was significantly higher then baseline medium values in both cell types ($P < 0.001$). B: changes in [Ca\textsuperscript{2+}]\textsubscript{i} were measured in 82 DRG neurons exposed to SPM\textsubscript{PEG} and charged SPMs. Data are from 3 experiments in which cells were superfused with SPM\textsubscript{PEG} and after a 5-min wash, the same cells were exposed to charged SPMs. In 3 other experiments, order of SPM superfusion was reversed. SPM\textsubscript{PEG} caused an [Ca\textsuperscript{2+}]\textsubscript{i} increase in $25 \pm 13\%$ of cells ($n = 82$ in 6 experiments), whereas charged SPMs activated $27 \pm 4\%$ of cells ($n = 82$ in 6 experiments).
sory irritant receptors. A subset of DRG nociceptors contains capsaicin- and acid-sensitive receptors (42). Increases in [Ca$^{2+}$]$_i$ were recorded in DRGs stimulated sequentially with pH 6.5, capsaicin (0.5 µM), and charged SPMs (18.8 µg/ml, $2 \times 10^4$ particles/ml; Fig. 5A). The neurons were washed between exposures with control buffer solution for 5–10 min to allow recovery. Although not all acid-sensitive receptor subtypes are activated by pH 6.5 (41), this relatively high pH value was chosen for comparison with the relatively small changes in acidic microenvironment associated with SPMs or ROFA particles. DRG sensitivity to SPMs correlated with their sensitivity to acid pH or capsaicin. Cells that responded to SPMs were always sensitive to either pH 6.5 or capsaicin. Of the 39 cells tested, 31% responded to all three challenges (i.e., pH 6.5, capsaicin, and SPMs; Fig. 4A), 8% responded to pH 6.5 and SPMs but not to capsaicin, 12% responded to capsaicin and SPMs but not to pH 6.5, 15% responded to SPMs and pH 6.5 but not to capsaicin, and 33% of the cells did not respond to any of the test compounds. This suggested that the SPM sensitivity was found in nociceptors, that is, in those sensory DRGs that express capsaicin- and acid-sensitive irritant receptors. Exposure to pH 5.0 or 6.5 and to 1 or 10 µM capsaicin also caused the release of IL-6 by DRGs, similar to that during SPM exposure (Fig. 5B).

The expression of capsaicin receptors is not confined to sensory neurons; they are also found on mast cells and glial cells (4, 5). Preliminary data indicate that BEAS-2B epithelial cells also contain capsaicin- and/or acid-sensitive pathways (39). Capsaicin (3 µM) and pH 6.5–5.0 increased [Ca$^{2+}$]$_i$ in BEAS-2B cells (Fig. 6, A and B). Most cells (90–100%) responded to pH 5.0, whereas the sensitivity to capsaicin or SPMs was found in 30–60% of the BEAS-2B cells. Amiloride (100 µM), which blocks many acid-sensitive receptors, inhibited the [Ca$^{2+}$]$_i$ increase induced by pH 5.0 in BEAS-2B cells by 24 ± 5% ($n = 7$ responding cells; Fig. 6C). The capsaicin-receptor antagonist CPZ at the relatively high concentration of 20 µM totally blocked the capsaicin-induced [Ca$^{2+}$]$_i$ increase. Exposure of BEAS-2B cells to pH 5.0 for 15 min or to capsaicin (10 µM) for 4 h caused a moderate release of IL-6, which was inhibited by 69 ± 9% ($n = 3$ samples) in the presence of amiloride (200 µM; Fig. 6C). Capsaicin (10 µM) caused a small release of IL-6 in BEAS-2B cells, which was almost

Fig. 5. Responses to SPMs, capsaicin (CAPS), and acid pH in DRGs. A: recording of [Ca$^{2+}$]$_i$ in a single DRG loaded with fluo 3-AM in response to pH 6.5, CAPS (0.5 µM), and charged SPMs. Between exposures to different test compounds, cells were washed with control buffer for 5 min. Maximal response was induced by 2 µM IO. B: release of IL-6 by DRG cultures after exposure to acid pH and CAPS. Cells were exposed to pH 5.0 or 6.5 for 15 min before their return to control culture medium (pH 7.4). IL-6 release was measured after 4 h. Cells were exposed to CAPS for 4 h, after which IL-6 release was measured ($n = 8$ samples for all exposures). Medium values indicate baseline IL-6 release. Exposure to acid pH or CAPS induced significant IL-6 release compared with medium control values ($P < 0.001$). Exposure to pH 5.0 caused significantly higher IL-6 release compared with pH 6.5 ($P < 0.05$), although different CAPS concentrations were not significantly different.
completely blocked (83 ± 2%; n = 3) by CPZ (20 µM; Fig. 6C).

The involvement of capsaicin- and/or acid-sensitive receptors in the cellular effects of charged SPMs was further examined by testing the effects of antagonists for these receptors on SPM-induced [Ca^{2+}] response and IL-6 release. In the presence of amiloride (100 µM), the [Ca^{2+}] response to SPMs in BEAS-2B cells was reduced by 35 ± 4% (n = 5 responding cells) compared with that by SPMs alone (Fig. 7A). It should be noted that this inhibition could be partially affected by other factors not related to amiloride (e.g., receptor desensitization). However, the amplitude of [Ca^{2+}] response did not further decline after removal of amiloride, which would be the case if these factors caused the inhibition observed in the presence of amiloride. To further clarify this point, BEAS-2B cells were preexposed to amiloride and stimulated with SPMs in the presence of amiloride and then again after 5–7 min of washing with control HEPES solution. In this case, the [Ca^{2+}] responses to SPMs were slightly increased after washout of amiloride (n = 7 responding cells; Fig. 7B). Although this does not exclude some decline of the [Ca^{2+}] over time, this suggested that amiloride only marginally inhibited
the SPM-induced response. In contrast, CPZ (5 µM) totally inhibited the [Ca\(^{2+}\)]\(_i\) responses to SPMs in BEAS-2B cells in a reversible manner (Fig. 7C).

In DRGs, amiloride (100 µM) totally blocked the [Ca\(^{2+}\)]\(_i\) response to charged SPMs (Fig. 8A). In contrast, CPZ (5 µM) reduced the SPM-induced increase in [Ca\(^{2+}\)]\(_i\) by only 28 ± 12% (n = 8 responding cells) compared with that induced by SPMs alone (Fig. 8B).

Exposure of BEAS-2B cultures to charged SPMs caused the release of IL-6, which was reduced by CPZ (10 µM) by 63 ± 11% (n = 10 samples) and by amiloride (200 µM) by 36 ± 7% (n = 13 samples; Fig. 9A). In DRGs, the SPM-induced IL-6 release was inhibited 32 ± 8% (n = 3 samples) by CPZ (10 µM) and 28 ± 6% (n = 4 samples) by amiloride (200 µM; Fig. 9B). In both BEAS-2B cells and DRGs, the inhibition by CPZ and amiloride was significant compared with the IL-6 release obtained by SPMs alone (P < 0.001). CPZ or amiloride did not cause significant IL-6 release by themselves above the baseline. Treatment of the cells with SPMs and/or the antagonists caused no significant cytotoxicity with the NR assay (data not shown).

DISCUSSION

An understanding of the pathways by which airborne particulate pollutants cause airway disorders is complicated by the heterogeneous composition of such pollutants. The present data indicate that physicochemical properties of ROFA are, in part, responsible for triggering inflammatory events in key target cells, namely bronchial epithelial cells and sensory neurons. Synthetic analogs of ROFA that resemble its size (2–6 µm) and zeta potential (−29 mV) also activate BEAS-2B epithelial cells and sensory DRGs by increasing the [Ca\(^{2+}\)]\(_i\), and causing release of the proinflammatory cytokine IL-6 (Figs. 1 and 3). The surface charge associated with colloidal particles like ROFA and SPMs appears to be a key factor in the stimulating effects because neutral particles were distinctly less effective (Fig. 4). SPMs specifically activated capsaicin- and acid-sensitive neurons (i.e., nociceptors), which respond in vivo by releasing neuropeptides and initiating neurogenic inflammation (42). The data suggest that SPMs and ROFA particles activate irritant receptors and trigger the subsequent release of inflammatory cytokines and neuropeptides from the respiratory epithelium and sensory terminals through the process of neurogenic inflammation. By this pathway, ROFA and other acidic PM may initiate and/or exacerbate symptoms of airway inflammation.

ROFA consists of an organic core to which metals, sulfates, biologics, and other components are associated. Soluble metals (e.g., iron and vanadium) may induce part of the ROFA-induced effects by the formation of oxidative radicals (11, 29). However, the washed insoluble particulate fraction of ROFA, which is largely devoid of inflammatory heavy metals, also caused IL-6 release equivalent to field ROFA (Fig. 2), suggesting...
that the supposedly inert particulate core itself has inflammatory potential. Exposure of the cells to SPMs further demonstrated that in the absence of metals, these analogs of ROFA (i.e., synthetic polymers resembling the average particle size and charge density of ROFA) could by themselves initiate the inflammatory events associated with ROFA exposure in culture.

SPMs activated neurons that were also sensitive to capsaicin and/or protons (Fig. 4). This suggests that the SPM-sensitive DRGs are nociceptors, similar to those involved in chemical irritation and neurogenic inflammation. Their irritant receptors are sensitive to a wide range of stimuli, including heat, cold, mechanical stimulation, chemical irritants, and acids. Presently, the mechanism by which PM and other airborne pollutants could directly activate these receptors or other subtypes of this receptor class is not established. We propose that the acidic microenvironment associated with negatively charged PM is, in part, responsible for the activation of acid-sensitive irritant receptors. ROFA particles and charged SPMs have negative zeta potentials, which can reduce the pH of the microenvironment of these particles (34). The negative surface charge of SPMs appears to play a role in the mechanism of action of the particles because data obtained with neutral SPMPEG (i.e., synthetic microspheres with 0-mV zeta potential) caused minimal IL-6 release and \([\text{Ca}^{2+}]_i\) responses compared with those of the charged SPMs (Fig. 4). In addition, we note that the PEG coating, which extends from the SPMs by a maximum of 70 Å (20), likely prohibits contact of the SPM particle core with the cell membrane, which should inhibit cellular activation by SPMPEG. Therefore, the surface chemistry of the particles determines whether cells are activated (i.e., \([\text{Ca}^{2+}]_i\) increases or IL-6 release). In physiological solutions, the Debye length, which defines the effective length of the counterion atmosphere and characterizes the surrounding double layer, is only 10 Å. This, together with the results of SPMPEG, implies that direct contact of the particle with the target cells and their receptors is necessary for SPMs or ROFA particles to evoke a response. Various subtypes of acid-sensitive receptor types have been cloned that express variable

Fig. 8. Effects of antagonists on SPM-mediated \([\text{Ca}^{2+}]_i\) response in DRGs. A: recordings of \([\text{Ca}^{2+}]_i\) in DRGs loaded with fura 2-AM in response to SPMs (18.8 µg/ml, 2 \times 10^4 particles/ml). Amiloride (100 µM) totally blocked response to SPMs. Amiloride block was reversible on washing with control solution. B: in presence of CPZ (5 µM), SPM-induced \([\text{Ca}^{2+}]_i\) was reduced by 11%. Maximal increases in \([\text{Ca}^{2+}]_i\) are shown after exposure to 2 µM IO.

Fig. 9. Effects of antagonists on IL-6 release induced by SPMs. A: release of IL-6 by BEAS-2B cells after 6 h of exposure to SPMs (18.8 µg/ml, 2 \times 10^4 particles/ml). In presence of CPZ (10 µM), IL-6 release was inhibited by 63 ± 11% (n = 10 samples). Amiloride (AM; 200 µM) inhibited ROFA-induced IL-6 release by 36 ± 7% (n = 13 samples). B: release of IL-6 by DRG cultures after 6 h of exposure to SPMs (18.8 µg/ml, 2 \times 10^4 particles/ml). In presence of CPZ (10 µM), IL-6 release was partially blocked by 32 ± 8% (n = 6 samples). AM (200 µM) inhibited ROFA-induced IL-6 release by 28 ± 6% (n = 6 samples). Medium values indicate baseline IL-6 release. Exposure to charged SPMs induced significant IL-6 release compared with medium control values and with exposures to CPZ and AM alone (P < 0.001). CPZ or AM exposure alone did not cause a significant IL-6 release compared with medium control values in either cell type. * Significant inhibition by CPZ and amiloride compared with exposure to SPMs alone, P < 0.001.
responses and sensitivities to acidic pH < 6.8 (41). In addition, the recently cloned VR1 vanilloid receptor is activated by various stimuli including capsaicin, capsaicin analogs, heat, and acidic pH (38). However, the acidic pH associated with SPMs is not likely to activate VR1 receptors because the acid sensitivity of this capsaicin receptor subtype is beyond that of pH 6.0.

Sensory irritation has previously been implicated in animal models of airway inflammation by ROFA (13) and other air pollutants (18, 28, 35) in vivo. Particularly, acidic components of PM appear to play a role in the observed symptoms (10, 24). However, the pulmonary effects of acid exposure appear variable. In rats, tracheal instillation with sulfuric acid solutions did not produce pulmonary inflammation (11), although humans, guinea pigs, rabbits, and rats exposed to sulfuric acid aerosols express signs of airway inflammation and hyperresponsiveness (21, 43). In addition, the effects of other airway toxicants depend on their acidity (33). There appears to be variability among different species and strains in the inflammatory response to airborne pollutants, with guinea pigs being more responsive to respiratory toxicants than mice and rats (22, 43). The level of airway inflammation resulting from exposure to acidic compounds may further depend on the physicochemical characteristics (e.g., organic vs. inorganic molecules, aerosol droplet size) of the acidic (macro)molecules. Acidic pH or capsaicin by themselves increased [Ca²⁺], and caused cytokine release in BEAS-2B cells and DRGs, although at a reduced magnitude compared with ROFA. In BEAS-2B cells, the acid (pH 5.0)-evoked IL-6 release is approximately half of that induced by SPMs, suggesting that acidic pH alone is not sufficient to explain the observed effects of SPMs. However, it should be noted that the cells were exposed to acidic pH (pH 6.5 or 5.0) for only 15 min because longer exposures were cytotoxic. The acidic nature of PM appears to play an important role in its underlying mechanism(s) of inflammation. Several reports (8, 17) describe synergistic effects of particles coated with sulfuric acid compared with the effects of separate exposure to the particles or to sulfuric acid alone. These reports suggest that the combined effects of the particles themselves and their acidic surroundings (i.e., the physicochemical complex of the particle core and its acidic microenvironment) contribute to the inflammatory effects of PM.

The data indicate that SPMs activate DRGs and epithelial BEAS-2B cells through different pathways. The receptor antagonists CPZ and amiloride inhibited the effects of SPMs in BEAS-2B cells and DRGs to variable extents. In BEAS-2B cells, amiloride had almost no effect on the SPM-mediated [Ca²⁺] response (Fig. 6), eliminating the involvement of amiloride-sensitive, acid-mediated pathways. This is consistent with the observation that amiloride only marginally inhibited the [Ca²⁺] increase during exposure to acidic pH. CPZ totally blocked [Ca²⁺], suggesting the involvement of capsaicin-sensitive receptors in BEAS-2B cells. In contrast, CPZ caused no significant inhibition of the SPM-induced [Ca²⁺] increase in DRGs, suggesting that their capsaicin receptors are not directly activated by SPMs. The complete block by amiloride indicates that acid-sensitive receptors or other amiloride-sensitive pathways mediate the SPM-induced responses in DRGs. The observation that the SPM sensitivity was found in capsaicin- and acid-sensitive nociceptors, while not all DRGs are sensitive to SPMs, indicates that SPMs may activate different irritant receptors.

An earlier study (40) reported that CPZ totally blocked the ROFA-induced IL-6 release in BEAS-2B cells. CPZ and amiloride only partly inhibited the SPM-mediated IL-6 release in DRGs and BEAS-2B cells. The discrepancies in the pharmacology between the [Ca²⁺] responses and IL-6 release can be viewed in terms of the different particle exposure and the different time scales of these measurements. First, because IL-6 release was measured after SPM exposure in cell culture medium, SPMs may be protein coated in these experiments. Second, calcium increases indicate an immediate receptor-mediated activation. However, it is likely that particles act on a variety of target sites within the 4-h exposure period that elapses between exposure and the cytokine measurements. Also, alternative pathways that mediate the cytokine release after the initial particle exposure may be differentially affected by the antagonists. We note that CPZ also inhibits calcium and potassium channels at the relatively high CPZ concentrations used (9, 23), which may have additional effects on IL-6 release. The initial effects of the particles appear to involve irritant receptors that mediate the elevation of [Ca²⁺]. In the continued presence of ROFA, other mediators such as oxidative radicals (11, 29) may be generated that can sustain IL-6 release in a CPZ-sensitive and/or -insensitive manner.

These data support the hypothesis that sensory irritation plays an initiating role in ROFA toxicity. The process of neurogenic inflammation in the airways involves complex interactions between neuronal and nonneuronal cells. The data show that both epithelial cells, which are the first to encounter xenobiotics, and sensory neurons, in which the terminals may be directly exposed under pathophysiological conditions, are activated by ROFA. Their differential sensitivities and cell-to-cell interactions will affect the onset and persistence of symptoms of PM-related airway disorders in both healthy individuals and those with preexisting pathological conditions.

In summary, the data demonstrate that ROFA and SPM particles cause immediate activation of BEAS-2B cells and DRGs and subsequent release of inflammatory cytokines. These cellular effects may initiate and sustain the symptoms of inflammation and hyperresponsiveness associated with PM. The effects of synthetic analogs that resemble ROFA in certain physicochemical properties suggest that the negatively charged particles themselves and their associated acidic microenvironment are inflammatory and may explain, at least in part, the mechanism(s) by which PM pollutants initiate airway toxicity.
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