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Effects of Recycled Paper Dust Extracts on Isolated Guinea Pig Trachea

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Abstract. The effect of paper dust collected at two different locations in a paper recycling plant (PD1 and PD2) on isolated nonsensitized guinea pig tracheal smooth muscle was studied in vitro. Dust extracts were prepared as a 1:10 w/v aqueous solution. Dose-related contractions of guinea pig tracheal rings were elicited with both PD1 and PD2. Pharmacologic studies were performed with atropine (10^{-6} M), indometacin (10^{-6} M), pyrilamine (10^{-6} M), LY171883 (10^{-5} M), nordihydroguaiaretic acid (10^{-5} M), and TMB8 (10^{-5} M). The possible role of endogenous neuropeptides in this constrictor process was studied by depleting neural mediators with capsaicin (5×10^{-6} M) before challenge with dust extracts. Constrictor effects were partially inhibited by a wide variety of the mediator blocking agents. The effects of both extracts were almost totally inhibited by the anticholinergic agent atropine, suggesting that a principal pathway mediating this response may involve the parasympathetic nervous system. The intracellular calcium-blocking agent TMB8 also induced a reduction of the contractile responses to PD1 and PD2 concsistent with the well established role of intracellular calcium in smooth muscle constriction. Pretreatment with capsaicin significantly increased the contractile activity of paper dust extracts but only at the higher doses of these extracts. This suggests that the effect of paper dust is not initiated by the release of mediators stored in sensory nerves but that the prerelease of these mediators may enhance the constrictor effects of these dusts. We suggest that paper dust extracts cause dose-related airway smooth muscle constriction possibly associated with the release of cholinergic as well as other mediators. The constrictor effect does not require tissue presensitization or the release of neuropeptides from sensory nerves.

Key words: Isolated guinea pig trachea—Paper dust extract—Airway reactivity.

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Introduction

Substances of plant origin have been identified as a cause of airway disease in industrial workers [2, 3]. Rylander [22] has described that aerosols of animal, vegetable, and microbial products (organic dusts) induce a wide variety of pulmonary diseases accompanied by subjective symptoms.

A number of clinical studies have associated exposure to paper dust with respiratory disease [6, 7, 9, 14, 31]. Thoren et al. [31, 32], Toren et al. [33, 34], and Deprez et al. [8] reported that employment in paper mills was associated with an increased risk of bronchial asthma and chronic obstructive pulmonary disease. Ericsson et al. [9] found a dose-dependent increase in upper respiratory symptoms among paper workers. Thoren et al. [32] described an increased prevalence of respiratory symptoms as well as asthma among workers processing soft paper products. This group did not, however, find impaired lung function. Sigsgaard et al. [28], by contrast, reported across-shift decreases in FEV_1 among paper workes. Heederik et al. [13] reported abnormalities in lung function associated with positive immediate intradermal reactions in workers exposed to soft-paper dust. Jarvholm et al. [15] studied workers exposed to heavy concentrations of paper dust and found increased lung elastic recoil pressure, incresaed residual volume, and a significant frequency of lower respiratory tract symptoms. Recently, Sigsgaard et al. [27, 28] described respiratory and mucosal symptoms (particularly chest tightness and itching of the nose, throat, and eyes) among garbage workers handling and recycling paper products.

To characterize possible mechanisms by which paper dust acts on the respiratory system in workers occupationally employed in recycling paper mills we investigated the effect of extracts of paper dust on isolated guinea pig tracheal rings. We suggest that paper dust is an inflammatory agent acting on the airway to induce directly both smooth muscle constriction (bronchospasm) and airway inflammation. We propose that these responses are mediated by the release of mediators from airway-associated cells (e.g. mast cells and epithelial cells) which can be characterized by our model.

Methods

Paper Processing

Paper dust was collected at a paper recycling plant whose workers were studied previously. The paper industry manufactures many types and quality of paper. For economic and environmental reasons it has become desirable to recycle the large quantities of paper now being discarded. The paper recycling process creates the potential for exposure to organic dusts, particularly paper dust. There are, however, other respiratory irritants used in the recycling process (chlorine gas, sulfur dioxide, chlorine dioxide, ammonia, caustic soda).

Recycling begins with the sorting of waste paper, which is separated by hand into bulky (e.g. cardboard) and fine (e.g. newspaper, magazine paper, paper towels) materials. The sorted paper is then treated mechanically in hot water under alkaline conditions. Most of the recycled material requires a special chemical cleaning process. Chemicals (e.g. sodium hydroxide, alkaline soda, neutral sulfite, sodium sulfite, sodium sulfide, hydrogen sulfide, chlorine gas, sulfur dioxide) and air are mixed with the paper in a custom-designed flotation cell. By using chlorine dioxide, paper pulps can be bleached in several stages to a high degree of brightness. Additional harmful exposures of the workers may involve mercaptan, talc dust,

and caustic lime dust. After bleaching, the paper is sprayed on paper machines forming a sheetlike material. The paper sheets are then transferred to a huge heated cylinder and then scraped off when dry. This process, as well as the initial hand sorting, is particularly dusty. The paper is then rolled and processed by the cylinders and subsequently divided and folded in special rerolling machines after which it is cut. In between the mechanical processes and the fully cooked pulps varying amounts of different chemicals are added. Paper dust was collected at the initial sorting stage of the recycling process and again at the later processing stage when bleaching and dyeing occur.

Clinical Findings in the Paper Recycling Industry

We studied a total of 101 male workers in a paper recycling plant in Zagreb, Croatia. The prevalence of chronic respiratory symptoms in the group of exposed workers studied was compared with those elicited among a population of nonexposed controls. Significant differences were found for the prevalences of chronic cough, chronic phlegm, chronic bronchitis, dyspnea, and chest tightness ($p < 0.05$). High prevalences were also noted for work shift-related symptoms including cough and irritation of the eyes, throat, and nose, as well as headache. Measurement of ventilatory capacities among these workers showed significantly decreased lung function for FEF_{50} and FEF_{25} compared with predicted ($p < 0.01$).

Paper Dust Extract Preparation

Paper dust extracts were prepared from the paper dust collected from the machines in a recycling plant investigated in the above epidemiologic study. Samples were collected from the initial (PD1) and the later (PD2) stages of the recycling process in this paper recycling plant. The paper dust extract was prepared as an aqueous solution in a weight to volume ratio of 1:10 by the standard method of Sheldon et al. [26] at the Institute of Immunology in Zagreb.

Protein and Endotoxin Determination

The protein content of the paper dust extract was determined by the Lowry method [16]. The amount of endotoxin (EU/mg) in paper dust extracts (PD1 and PD2) was determined by using the limulus amebocyte lysate assay [19].

Guinea Pig Trachea Preparation

We used the tracheas of young albino Hartley male guinea pigs (300–390 g) purchased from Charles River Laboratories, Wilmington, MA. The animals were sacrificed by asphyxiation, exposing them to 100% CO_2 for 5 min. The tracheas were removed within 3 min of sacrifice. The animal tissues were trimmed manually to removed connective and other tissues. Four segments (rings, each 4–6 mm wide) were cut from the trachea of each animal. Each ring was suspended between two L-shaped stainless steel hooks mounted in a 20-ml organ chamber containing Krebs-Hanseliet buffer of the following composition (μM): NaCl, 110.0; KCl, 4.80; $CaCl_2$, 2.35; $MgSO_4$, 1.20; $KHPO_4$, 1.20; $NaHCO_3$, 25.0; and dextrose, 110.0, in glass-distilled water. Organ chambers were maintained at 36.5 ± 0.5°C and were aerated continuously with 95% O_2 and 5% CO_2 to maintain pH = 7.5 ± 0.1. The tissue segments were initially set to 2 g of tension and were allowed to stabilize for approximately 1.5 h before the experiments began. During that period the tissue was washed at 15-min intervals. After this stabilization period, the tension in each tissue segment was readjusted to a baseline of 2 g for all subsequent assays. Isometric contractions were recorded using a Grass FTO3C force displacement transducer attached to a Grass polygraph recorder. Before the contraction-response assay with paper dust extract (PD1 or PD2) was performed, a challenge with carbachol (10^{-4} M) was run to test the viability of the individual rings and to establish maximal tissue contraction to carbachol.

Steady-State Characterization of the PD Extract Dose-Response Curve

A dose-response curve with paper dust extract was obtained by adding increasing volumes of extract or Krebs (used as a control) into the tissue bath in progressive ailquots of 10, 30, 100, 300, and 1,000 μl. The potency of the extract was determiend, comparing its biologic activity with the maximal contraction induced by carbachol (10^{-4} M) on the same tissue. In each experiment the responsiveness to maximal carbachol stimulation was established initially. This was followed by washing, reestablishment of the baseline, and a dose-response challenge with PD extract. The data were expressed as a percentage of the initial maximal (10^{-4} M carbachol) contraction. Concentration-response curves were plotted using the Kaleidagraph software (version 3.04) for the Power Macintosh 8200 (Cupertino, CA). Data points were fit by iteration to the logistic function

$$E = E_{max}/(1 + (EC_{50}/[A]^n)$$

Where E = observed muscle tension (g above baseline)
$[A]$ = the concentrations of the agonist
EC_{50} = the $[A]$ eliciting one half of the maximal response
n = slope of the curve.

Drug Treatment Protocol

In a typical drug experiment the tissue was washed and baseline reestablished after an initial contraction with carbachol (10^{-4} M) used to demonstrate tissue viability and to establish maximal (carbachol) contractile tension. A specific blocking agent (or a control solution) was then added to the organ bath and incubated with the tissue for 20 min. Paper dust extract (PD1 or PD2) dose response was then performed in the presence (or absence) of the blocking agent. After the dose response the tissue was washed again, and carbachol (10^{-4} M) was added to verify the viability of the tissue. In the drug experiments, the following specific blocking agents were selected for addition to the organ bath: atropine (10^{-6} M) (anticholinergic), pyrilamine (10^{-6} M) (antihistamine, H1 blocking agent), indomethacin (10^{-6} M) (prostaglandin synthesis inhibitor), TMB8 (10^{-5} M) [3,4,5-trimethoxybenzoic acid-8-(diethylamino)acetyl ester] (inhibitor of intracellular calcium mobilization), NDGA (10^{-5} M) (nordihydroguaiaretic acid) (arachidonic acid pathway inhibitor), and LY171883 (10^{-5} M) (leukotriene synthesis inhibitor). Four rings were obtained from each animal so that multiple drugs could be tested in one animal. In general, we used rings from six separate animals to test the effect of a blocking agent on one of the dust extracts.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) has the property of releasing stored mediators from sensory nerves in the airway [18]. Since the release of neuropeptides from sensory nerves is a potential mechanism for nonspecific airway inflammation in occupational airway disease [30], we postulated that depletion of irritant nerve mediators by capsaicin could abort the response of a dust or its extract which acted through such a mechanism. In a series of studies we explored this possible mechanism. We pretreated tracheal rings with capsaicin and then performed a challenge study with PD1 or PD2 as detailed above. We compared this with a sham pretreatment with Krebs solution. In additional rings from the same guinea pig we pretreated these tissues twice with capsaicin. Finally we also performed an experiment in which capsaicin was administered after paper dust extract challenge to study whether paper dust extract deplete sensory nerve mediators sufficiently to abort the effect of capsaicin.

Statistical Methods

Mean values of the tissue response at a given dose were compared by the paired *t*-test, matching control and drug-treated tissues. The Statview software (Brain Power Inc, Calabasas, CA) for Macintosh was used to perform the analysis.

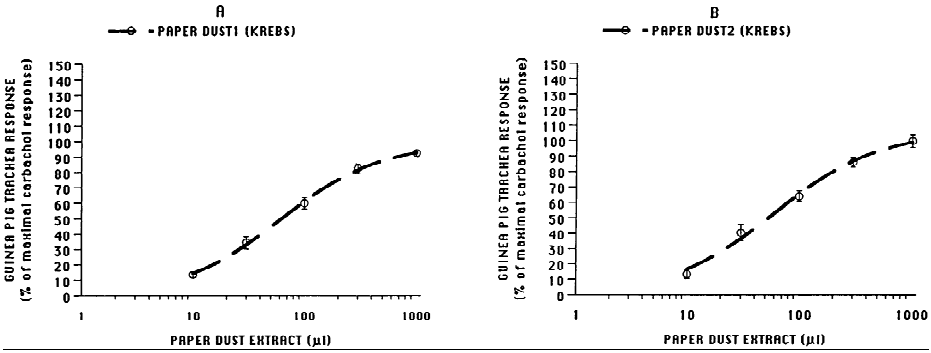

Fig. 1. Contractile activity of paper dust extracts (PD). *A,* PD1, and *B,* PD2 on isolated guinea pig tracheal smooth muscle as percentage of carbachol (10^{-4} M) contraction (mean ± S.E.).

Results

Dose-response curves to paper dust extract were measured in 36 guinea pig tracheal rings (each obtained from a separate animal) and expressed as a percentage of maximal carbachol (10^{-4} M) contraction. The dose responses to PD1 ($n = 19$) and to PD2 ($N = 18$) are shown in Figure 1-*A* and 1*B.* These extracts elicited similar dose-response characteristics; PD1 ($E_{max} = 98.4\%$, $EC_{50} = 60.397$ µl), and PD2 ($E_{max} = 105.77\%$, $EC_{50} = 59.474$ µl).

The dose-response curves to PD1 following pretreatment with atropine (10^{-6} M), indomethacin (10^{-6} M), and pyrilamine (10^{-6} M) are shown in Figure 2*A* and following TMB8 (10^{-5} M), NDGA (10^{-5} M), and LY171883 (10^{-5} M) in Figure 2*B.* Atropine abolished completely the constrictor response to PD1 in the range of PD1 concentrations tested. TMB8, NDGA, indomethacin, pyrilamine, and LY171883 also significantly reduced the constricting effect. The protective effects of these agents were only partial compared to those of atropine. For LY171883 the effects were seen primarily at the highest doses.

The effects of pretreatment on the PD2 dose response with atropine (10^{-6} M), indomethacin (10^{-6} M), and pyrilamine (10^{-6} M) are shown in Figure 3*A.* The effects of pretreatment with TMB8 (10^{-5} M), NDGA (10^{-5} M), and LY171883 (10^{-6} M) are shown in Figure 3*B.* Once again, atropine completely blocked the PD2 dose response. TMB8 consistently reduced the constricting reaction to PD2 at the dose of 300 and 1,000 µl, whereas NDGA and LY171883 demonstrated an inhibiting effect only at the highest dose. Pyrilamine and indomethacin had no effect on this response.

The effect of capsaicin (5×10^{-6} M) administered once (or twice) before the PD1 dose response is shown in Figure 4*A* and before the PD2 dose response in Figure 4*B.* Capsaicin pretreatment did not block the PD1 or PD2 effect; on the contrary, it increased significantly the constrictor effect of PD1 at the higest dose (1,000 µl) when adminstered before the dust extract ($p < 0.01$). The effect on PD2 was similar.

The effect of the two dust extracts on the ability of capsaicin to release neuropeptides was tested by our protocol involving the administration of capsaicin both before

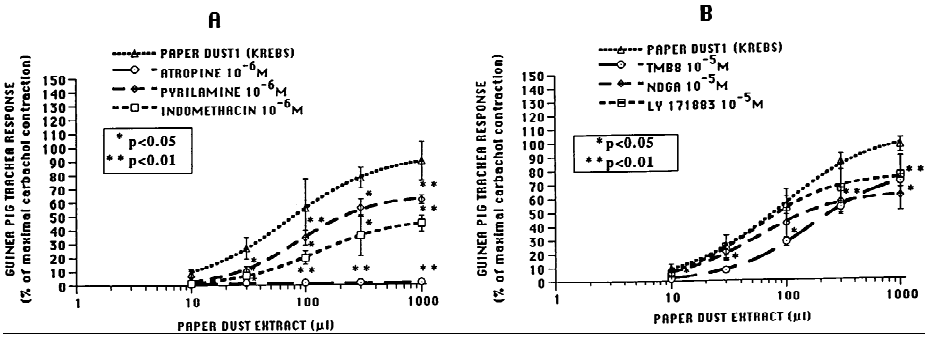

Fig. 2. Constrictor activity of paper dust extract (PD1) on isolated guinea pig tracheal smooth muscle *A*, following pretreatment with Krebs, atropine (10^{-6} M), pyrilamine (10^{-6} M), and indometacin (10^{-6} M) (mean ± S.E.). *B*, Krebs, TMB8 (10^{-5} M), NDGA (10^{-5} M), and LY177883 (10^{-5} M) (mean ± S.E.).

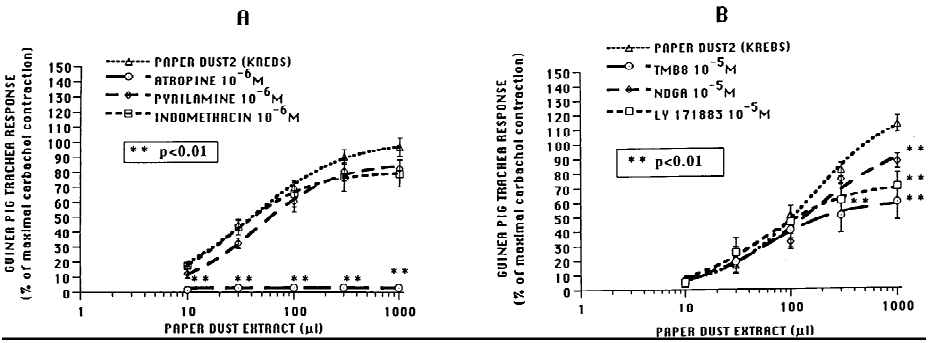

Fig. 3. Constrictor activity of paper dust extract (PD2) on isolated guinea pig tracheal smooth muscle. *A*, following pretreatment with Krebs, atropine (10^{-6} M), indomethacin (10^{-6} M), and pyrilamine (10^{-6} M) (mean ± S.E.). *B*, Krebs, TMB8 (10^{-5} M), NDGA (10^{-5} M), and LY171883 (10^{-5} M) (mean ± S.E.).

and after challenge with the dust extract. Our hypothesis was that if the extract acted by releasing neuropeptides, the challenge with capsaicin *after* PD treatment would lead to less constriction than the capsaicin challenge *before* PD treatment (as is the case with two sequential challenges with capsaicin). Since there was no significant difference between the effect of PD1 and PD2 on the constricting ability of capsaicin (data not shown), the data of the PD1 and PD2 experiments were pooled together. The constrictor effect of capsaicin (5×10^{-6} M) administered before PD (PDE1 or PD2) and after PD (PD1 or PD2) the dose response is shown in Figure 5*A.* There was no significant difference in the constricting effect of capsaicin before or after PD, suggesting that PD does not act by depleting airway nerve fibers of their peptide mediators. Sequential challenge with capsaicin (Fig. 5*B*) shows the expected reduction in capsaicin contraction with the second challenge.

The constricting effect of two doses of capsaicin (5×10^{-6} M) administered consecutively (i.e. second treatment with capsaicin 30 min after the first treatment with capsaicin) is shown in Figure 5*B.* As anticipated, the constrictor effect of capsaicin was

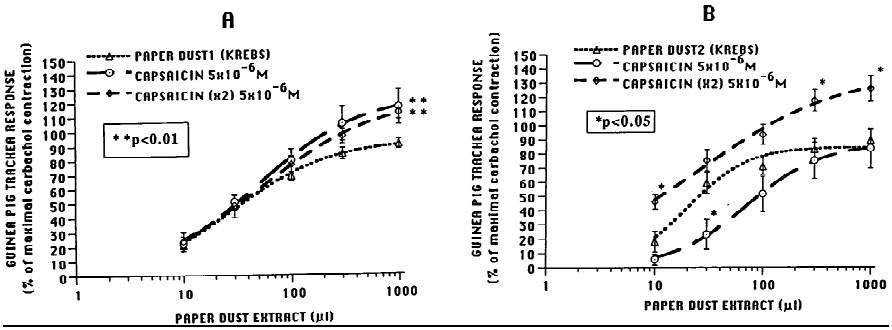

Fig. 4. Effect of capsaicin (5×10^{-6} M) administered once or twice (at 30-min interval) on subsequent paper dust extract dose response on isolated guinea pig tracheal smooth muscle. *A*, PD1; *B*, PD2 (mean ± S.E.).

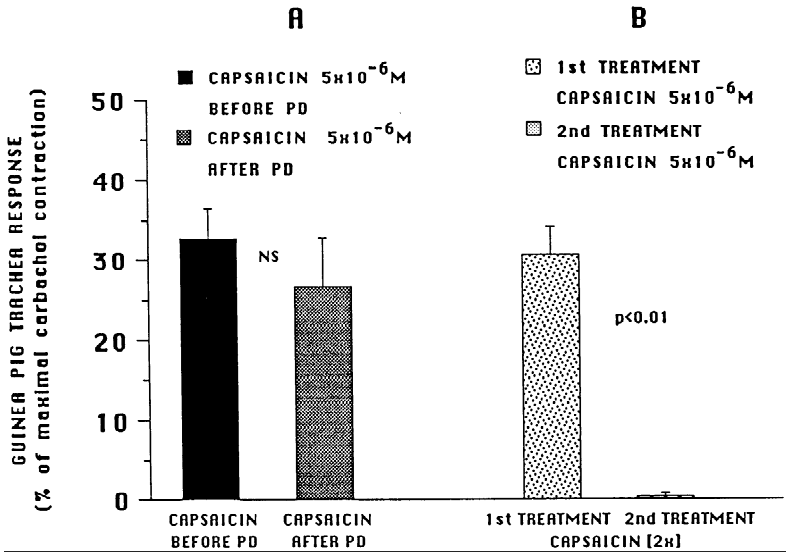

Fig. 5. *A*, contractile activity of capsaicin (5×10^{-6} M) on isolated guinea pig smooth muscle administered before and after paper dust extract (PD1 or PD2). There was no significant differnce between these two responses. *B*, contractile activity of capsaicin administered twice (with a 30-min interval between administraitons). The difference in the response between the first and second treatment with capsaicin was statistically significant ($p < 0.01$) (mean ± S.E.).

virtually abolished by the second (30 min later) treatment with capsaicin ($p < 0.01$), suggesting depletion of nerve fiber mediators.

The protein content measured by the Lowry method of the PD1 extract was found to be 12.5 µg/ml, and for PD2 it was 11.6 µg/ml. Standard assay curves for endotoxin were constructed both with and without extract to test the presence of substances in the extract which would have an inhibitory effect on the assay. No negative interference was noted. The endotoxin content of PD1 was 85.58 EU/ml (40.94 EU/mg of dust). The endotoxin content of PD2 was 543.75 EU/ml (164.77 EU/mg of dust). The assays were

run on the same extracts. However, the results did not yield parallel results. The protein contents of the two extracts were similar, whereas the endotoxin content of PD2 was four times that of PD1 (on an EU/mg dust basis).

Discussion

Our present investigation demonstrates that the paper dust extracts (PD1 an PD2) cause a dose-dependent airway constriction of isolated guinea pig trachea. These are complex organic dusts that we have not characterized in any biochemical detail. The advantage of studying whole extracts of this material rather than components (at this stage) is that the whole dust is more representative of worker exposure and thus more likely to reflect the clinical effect.

The data illustrated by these studies are similar to those of our previous studies with different organic dust extracts such as coffee, soy, spices, animal food, poultry and swine confinement building dust, brewery dust (hops, barley, yeast), as well as textile dust (cotton, hemp and wool) [23–25, 35–42]. The pharamcologic studies of paper dust extract on isoalted guinea pig tracheal smooth muscle suggest a complex effect of this airway irritant. We propose that several mediators may be involved in promoting airway constriction. The greatest protective effect against PD airway constriciton was noted following pretreatment with the muscarinic blocking agent atropine, suggesting a central role for the cholinergic nervous system in initiating these effects. This protective effect of atropine has been described with other organic dust extracts such as wool, brewery, and poultry confinement extracts [23, 24, 42].

In the present study as in previous evaluations of organic dust extracts (poultry, brewery, wool, soy) [23–25, 42], the suppression of constrictor effects caused by paper dust extract using the intracellular calcium channel blocker TMB8 may simply reflect the reliance of this response on intracellular calcium mobilization. An increase in intracellular Ca^{2+} occurs in many smooth muscle preparations induced to constrict by receptor and nonreceptor mechanisms [1, 12]. Calcium mobilization for the contractile mechanism may originate from intra- or extracellular stores. Since elevation of cytosolic calcium is involved in the sequence leading to smooth muscle constriction, the role for intra- and extracellular calcium-blocking agents in the treatment of dust-related airway obstruction remains to be explored.

Regoli [21] reported that isolated guinea pig trachea may be activated to constrict by a number of different peptide receptors. There are many neuropeptides with potent effects on airways. It has been shown that capsaicin acts largely through activation of a nonselective cation channel [20]. Fuller and co-workers [10, 11] demonstrated that capsaicin is an agent that stimulates airway nerves directly, initiating reflex effects. capsaicin acts specifically by depleting stores of substance P from sensory neurons and blocking further systnesis of this neuropeptide [5]. In animals, capsaicin stimulates C-fibers, causing the release of substance P [4, 29]. This response is subjects to tachyphylaxis [17]. The absence of a suppressor effect on paper dust extract contraction following capsaicin pretreatment suggests that sensory neuromediators are not primarily involved. It appears further that pretreatment of guinea pig trachea by paper dust extracts does not deplete neuromediators released by capsaicin since the latter remains

active after paper dust extract challenge. However, our study suggests that pretreatment with capsaicin augments the effect of PD1 and PD2. This suggests that preexisting airway irritation may enhance the effect of exposure to organic dusts such as paper dust. By contrast, repeated exposures to capsaicin in our study significantly decreased the constrictor reaction of guinea pig airway smooth muscle to capsaicin. Similar results have been shown by O'Neil [20], who demonstrated that prolonged exposure to capsaicin produces a subsequent densensitization or neuroinhibition.

The present study presents further evidence that extracts of the organic industrial products cause a nonspecific release of airway mediators. The source of these mediators does not appear to be sensory nerves. On the basis of these data we can speculate that the clinical effects of paper dust in workers employed in the paper processing and recycling industries may in part be related to nonimmunologic (non-IgE) mechanisms since the animals were not presensitized to the dust extracts. The role of individual mediators involved in these occupational airway diseases appears to vary with different organic dusts. The origin of these mediators is, as yet, not well defined.

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