PHARMACOLOGIC EFFECTS OF COCOA AND RYE FLOUR EXTRACTS ON ISOLATED GUINEA PIG TRACHEA

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Confectionery workers are exposed to a wide variety of organic dusts and aerosols. Previous studies with workers in a confectionery plant working with cocoa and rye flour indicate that these workers are at risk of developing adverse respiratory symptoms and lung function impairment. The effects of cocoa and rye flour extract on isolated guinea pig tracheal smooth muscle were studied using water-soluble extracts from cocoa and rye flour obtained from the studied confectionery plant. Dose-related contractions of non-sensitized guinea pig tracheal rings were demonstrated using both cocoa and rye flour extracts. Pharmacologic studies were performed by pretreating guinea pig tracheal tissue with drugs known to modulate smooth muscle contraction: atropine, indomethacin, pyrilamine, nordihydroguaiaretic acid (NDGA), acivicin, bromophenacyl bromide (BPB), 3,4,5-trimethoxybenzoate 8-(N,N-diethylamino)octyl ester (TMB8), captopril, and capsaicin. Constrictor effects of the dust extracts were inhibited by these agents, the pattern of which depended on the dust extract. Atropine consistently and significantly reduced the contractile effects of both extracts. These observations suggest a release of parasympathetic mediators by these extracts or more directly an interaction with muscarinic receptors. In addition, the constrictor effect of cocoa and rye flour extracts was significantly, but only partially, reduced by indomethacin, pyrilamine, BPB, and TMB8. Acivicin also partially decreased the constrictor effect of cocoa extract. Pretreatment of tracheal tissue with capsaicin also decreased the constrictor effects of high concentrations of cocoa and rye flour extracts. Data suggest that cocoa and rye flour extracts cause a dose-related constriction of airway smooth muscle by non immunological mechanisms involving cholinergic pathways and airway mediators such as histamine and the products of the arachadonic acid cascade. This effect is not dependent on the presensitization of guinea pigs.

The respiratory adverse effects of wheat flour and other grain products, including occupational asthma and allergic airway disease, have frequently been described in bakers (Wutrich & Baur, 1990; Valdivieso et al., 1994; Bohadana et al., 1994; DeZotti et al., 1994; Cullinan et al., 1997). Occupational asthma and respiratory hypersensitivity in bakers

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specifically caused by rye flour have been reported by several authors (Valero-Santiago et al., 1988; Garcia-Casado et al., 1995, 1996; Bataille et al., 1995; Anton et al., 1995; Baur & Czuppon, 1995). Our previous epidemiological studies of confectionery workers exposed to cocoa and flour dust in the workplace demonstrated a high prevalence of acute and chronic respiratory symptoms accompanied by lung function changes (Zuskin et al., 1994, 1998). A certain number of these confectionery workers also demonstrated immunological changes as indicated by positive skin tests to cocoa and flour antigens and increased total serum immunoglobulin E (IgE); however, most of these findings were nonspecific, and unassociated with allergic disease. Blaski et al. (1996) suggested that atopy may play, at most, a minor role in the development of grain dust-induced airway disease.

Matsumura et al. (1994) reported bronchial asthma and other symptoms in cooks who processed confectionery products. Gimenez et al. (1995) reported chronic respiratory findings as well as acute ventilatory effects induced by exposure to flour dust in grain mill workers. Respiratory symptoms and airway hyperresponsiveness were significantly greater among flour-exposed workers than among controls, despite exposure to very low concentrations of inspirable flour dust. Fries (1966, 1978) described airway and other hypersensitivity effects of chocolate, particularly among persons with allergic disease. Interestingly, Grob et al. (1993) suggested that the effects of cocoa or chocolate on workers occupationally exposed might be the result of dust contamination from jute and sisal bags in which cocoa beans are packed.

In order to characterize possible mechanisms by which cocoa and rye flour dusts act on the respiratory system of workers occupationally exposed to these dusts in bakeries and confectioneries, the effects of extracts of cocoa and rye flour were investigated on guinea pig isolated tracheal rings. Data suggest that cocoa and rye flour contain agents that act directly on the airway to induce smooth muscle constriction (bronchospasm). It is postulated that these responses are mediated by the release of mediators from airway-associated cells (e.g., mast cells, epithelial cells, nerves), which can be characterized in our model.

METHODS

Cocoa and Rye Flour Extract Preparation

Dust extracts were prepared from cocoa and rye flour collected from machines in a confectionery plant located in Zagreb, Croatia. In this plant the workers were part of an epidemiological study that investigated the effects of cocoa and flour dust exposure on respiratory functions (Zuskin et al., 1998). Cocoa and, rye flour extract were prepared as an aqueous solution in a weight to volume ratio of 1:10 at the Institute of Immunology of Zagreb. The extract was prepared by defatting the raw allergen material

with diethyl ether (boiling point 34°C). A 1:5 w/v extract was prepared by stirring the defatted material in phosphate-buffered saline (PBS) for 72 h at 4°C. The extract was then centrifuged and the supernatant was dialyzed for 48 h against PBS and after that for 24 h against distilled water. Subsequently the supernatant was filtered under sterile conditions. The filtered extract was divided into 7-ml aliquots in glass vials, and freeze-dried immediately. The vials were then stored at –20°C (Sheldon et al., 1967). This procedure provided a standardized, sterile extract. The protein content of the cocoa and rye flour extracts was determined by the method of Lowry et al. (1951).

Guinea Pig Trachea Preparation

The tracheas of 36 albino Hartley male guinea pigs (300–390 g) purchased from Charles River Labs (Wilmington, MA) were used. The animals were scarified by asphyxiation, exposing them to 100% CO_2 for 5 min. The tracheas were removed within 3 min of sacrifice. The tissues were manually trimmed to remove connective and other tissues. Four segments ("rings," each 4–6 mm wide) were cut from each trachea. Each ring was suspended between two L-shaped stainless steel hooks mounted in a 20-ml organ chamber containing Krebs–Henseliet buffer of the following composition (m*M*): NaCl, 110; KCl, 4.8; $CaCl_2$, 2.35; $MgSO_4$, 1.2; $KHPO_4$, 1.2; $NaHCO_3$, 25; and dextrose, 110, in glass-distilled water. Organ chambers were maintained at 36.5 ± 0.5°C, and were continuously aerated with 95% O_2 and 5% CO_2 to maintain pH 7.5 ± 0.1. The tissue segments were initially set to a level of 2 g 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 the baseline level 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 cocoa and rye flour dust extract 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 Cocoa and Rye Flour Extract Dose-Response Curve

Dose-response curves with cocoa and rye flour extracts were obtained by adding increasing volumes of cocoa or rye flour extract or Krebs (used as a control) into the tissue bath in progressive aliquots of 10, 30, 100, 300, and 1000 µl. The potency of the extract was determined, comparing its biological 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 initially established. This was followed by washing, reestablishment of the baseline, and a dose-response

challenge with cocoa or flour extract. The data were expressed as a percentage of the initial maximal (10^{-4} *M* carbachol) contraction. Concentration-response curves were plotted using Kaleidagraph software (version 3.04) for the Power Macintosh (8100, Cupertino, CA). Data points were fit by iteration to the logistic function:

$$E = E_{max}/(1 + (EC50/[A]^n)$$

where E is the observed muscle tension (grams above baseline), [A] the concentration of the agonist, EC50 the [A] eliciting one-half of the maximal response, and n the 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*). A specific blocking agent (or a control solution) was then added to the organ bath and incubated with the tissue for 30 min. Cocoa and rye flour extract dose response was then measured in the presence (or absence) of the blocking agent. After the dose response the tissue was again washed 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, $n = 6$), pyrilamine (10^{-6} *M*, antihistamine, H1 blocking agent, $n = 6$), indomethacin (10^{-6} *M*, prostaglandin synthesis inhibitor, $n = 6$), NDGA (10^{-5} *M*, nordihydroguaiaretic acid, arachidonic acid pathway inhibitor, $n = 6$), acivicin (10^{-5} *M*, leukotriene synthesis inhibitor, $n = 6$), BPB (10^{-5} *M*, (bromophenacyl bromide) phospholipase (PLA2) blocking agent), $n = 6$), TMB8 (10^{-5} *M*, 3,4,5-trimethoxybenzoic acid-8-(diethylamino) octyl ester, inhibitor of intracellular calcium mobilization, $n = 6$), captopril (10^{-5} *M*) [angiotensin converting enzyme (ACE) inhibitor], and capsaicin (5×10^{-6} *M*) (8-methyl-*n*-vanillyl-6-nonenamide, an agent that stimulates the release of neuropeptides).

Capsaicin has the property of releasing stored mediators from sensory nerves in the airway (Orawski et al., 1989; O'Neil, 1991). Since stimulation of irritant nerve receptors is a potential mechanism for nonspecific airway inflammation in occupational airway disease (Alexandre et al., 1993). It was postulated that depletion of irritant nerve mediators by capsaicin could abort the response of a dust or its extract that acted through such a mechanism. In a series of studies this mechanism was explored. Tracheal rings were pretreated with capsaicin and then performed a challenge study with cocoa or flour extracts as detailed earlier. This was compared to a sham pretreatment with Krebs solution.

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 paired *t*-test

was used because with each trachea divided into four segments we were able to compare drug-treated tissue (three segments) to control (one segment) in the same animal. The Statview version 4.1 software (Brain Power, Inc., Calabasas, CA) for Macintosh performed the statistical analysis. Similarly, response parameters (E_{max} and EC50) were characterized for individual tissues and compared between treatment protocols by the paired *t*-test. The confidence limit for statistical significance was taken to be $p < .05$.

RESULTS

The dose-response relationship between cocoa and rye flour extracts and smooth muscle constriction was 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 effects of cocoa extract ($n = 18$) and of rye flour extract ($n = 18$) are shown in Figure 1. These extracts elicited significant response characteristics (mean ± SE): cocoa ($E_{max} = 125.3 \pm 7.9\%$; EC50 = 30.09 ± 6.7 µl) and rye flour ($E_{max} = 104.0 \pm 2.6\%$; EC50 = 54.7 ± 13.6 µl).

The dose-response relationship with cocoa extract following pretreatment with atropine (10^{-6} *M*), pyrilamine (10^{-6} *M*), and indomethacin (10^{-6} *M*) is shown in Figure 2A. The responses following pretreatment with NDGA (10^{-5} *M*), BPB (10^{-5} *M*), and acivicin (10^{-5} *M*) are shown in Figure 2B. The results of pretreatment with captopril (10^{-5} *M*), TMBA (10^{-5} *M*), and capsaicin (5×10^{-6} *M*) are shown in Figure 2C. Atropine significantly and almost completely blocked the constrictor response to cocoa extract in the range of cocoa concentrations tested. The blocking effects of other mediator-modifying drugs were also significant, being quantitatively least for pyrilamine. TMB8 and capsaicin significantly diminished the constrictive effect of cocoa extract. The effect of captopril was only significant at doses of 300 µl and 1000 µl.

The effects of pretreatment with atropine (10^{-6} *M*), pyrilamine (10^{-6} *M*), and indomethacin (10^{-6} *M*) on rye flour extract constriction are shown in Figure 3A. The effects of NDGA (10^{-5} *M*), BPB (10^{-5} *M*), and acivicin (10^{-5} *M*) on rye flour extract constriction are seen in Figure 3B. The effects of captopril (10^{-5} *M*), TMB8 (10^{-5} *M*), and capsaicin (5×10^{-6} *M*) are illustrated in Figure 3C. Again atropine completely blocked the constrictor effect of rye flour dust extract. The effects of pyrilamine and indomethacin on the constrictor response were less marked except at a dose of 1000 µl. Acivicin did not significantly affect the constrictor reaction to rye flour at any of the doses administered. BPB, on the other hand, demonstrated a significant effect at doses from 30 µl to 1000 µl, while the effect of NDGA was seen only at doses of 300 or 1000 µl. TMB8 almost completely blocked the constrictive effects, while captopril and capsaicin were effective only at higher doses (captopril, 300 µl and 1000 µl; capsaicin, 1000 µl).

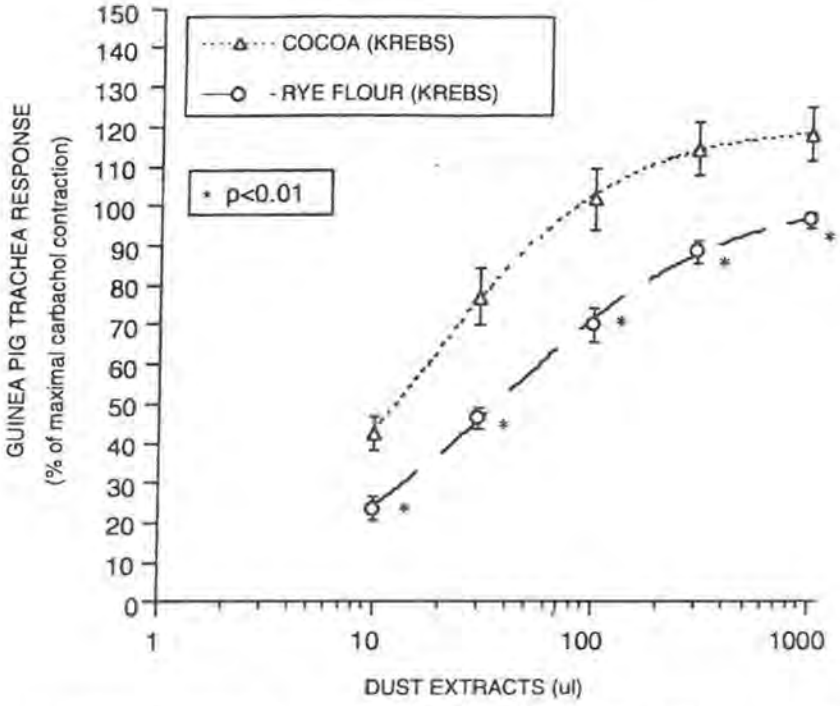

FIGURE 1. Contractile response of isolated guinea pig tracheal smooth muscle to cocoa and rye flour dust extracts as a percentage of maximal carbachol (10^{-4} *M*) contraction (mean ± SE). Asterisk indicates significantly different from control, $p < .01$.

The values for E_{max} (as a percent of control) and EC50 (μl) for cocoa and rye flour extract, following pretreatment with pharmacologic agents, are presented in Table 1. Data are shown as mean values for six guinea pigs calculated from each tissue tested per treatment. Most of the significant differences in tracheal smooth muscle response between control (Krebs solution) and drug-treated tissue were seen for E_{max}. EC50 was significantly different from control for atropine, pyrilamine, and TMB8 in cocoa extract. EC50 was significantly different from control for atropine, captopril, and capsaicin in rye flour extract. The analysis of the protein content in the cocoa extract showed a content of 45 μg/ml. Not unexpectedly, this was considerably less than the protein content in rye flour dust extract, which was 1.6 mg/ml.

DISCUSSION

Cocoa and rye flour extracts produce dose-dependent contractions of isolated unsensitized guinea pig tracheal smooth muscle similar to those seen with other organic dust extracts such as those of soy (Zuskin et al., 1991b), spices (Zuskin et al., 1988), animal food (Zuskin et al., 1992), wool dust (Schachter et al., 1995a), and paper recycling dust (Schachter et al., 1998).

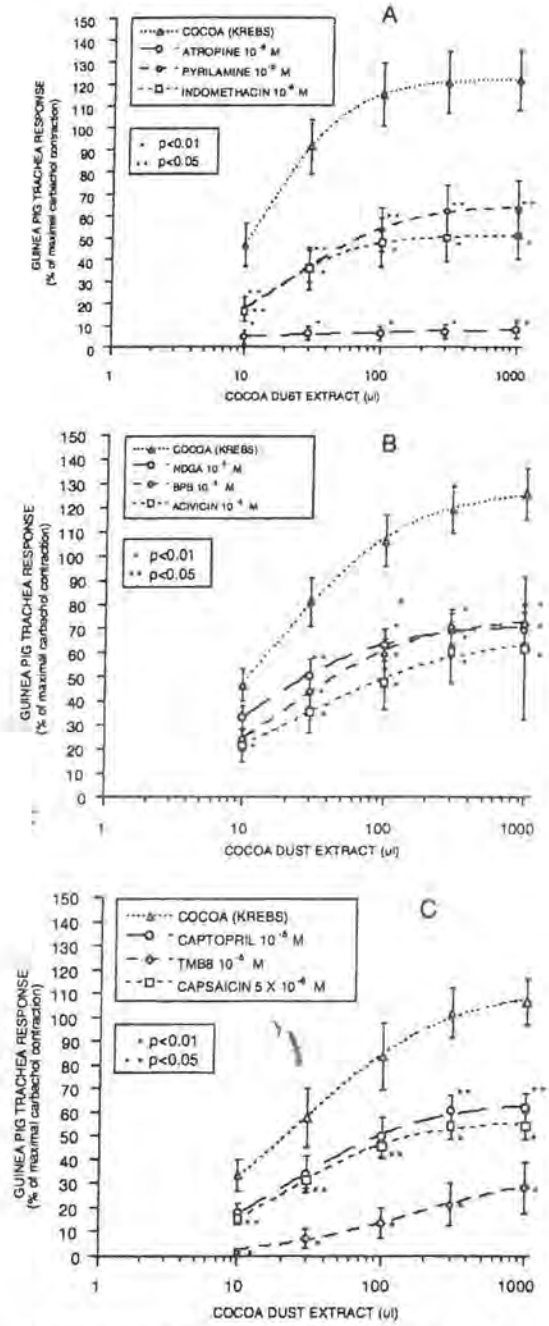

FIGURE 2. Modulation of constrictor activity of cocoa extract on isolated guinea pig tracheal smooth muscle by (A) pretreatment with Krebs, atropine (10^{-6} *M*), pyrilamine (10^{-6} *M*), and indomethacin, (B) pretreatment with Krebs, NDGA (10^{-5} *M*), TMB8 (10^{-5} *M*), and acivicin, and (C) pretreatment with Krebs, captopril (10^{-5} *M*), TMB8 (10^{-5} *M*), and capsaicin (5×10^{-6} *M*) (mean ± SE). Asterisks indicate significant differences from control.

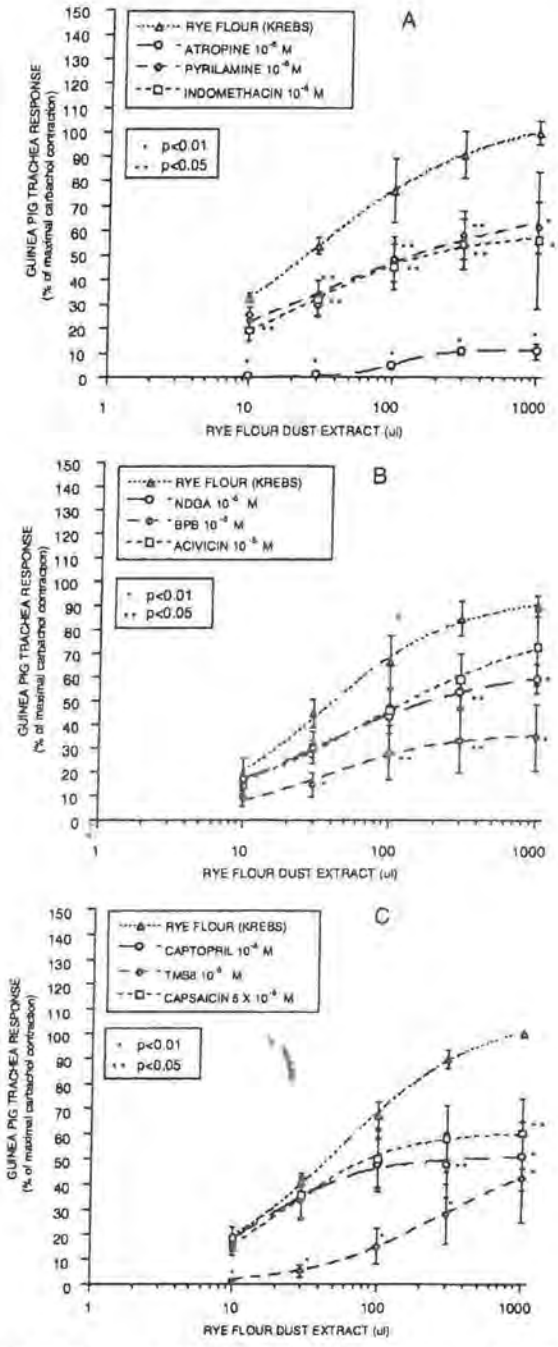

FIGURE 3. The modulation of constrictor activity of rye flour extract on isolated guinea pig tracheal smooth muscle by (A) pretreatment with Krebs, atropine (10^{-6} *M*), pyrilamine (10^{-6} *M*), and indomethacin (10^{-6} *M*), (B) pretreatment with Krebs, NDGA (10^{-5} *M*), BPB (10^{-5} *M*), and acivicin (10^{-5} *M*), and (C) pretreatment with Krebs, captopril (10^{-5} *M*), TMB8 (10^{-5} *M*), and capsaicin (5×10^{-6} *M*) (mean ± SE). Asterisks indicate significant differences from control.

TABLE 1. E_{max} and EC50 Values for Cocoa and Rye Flour Extracts

	Cocoa		Rye flour	
Pharmacologic agents	E_{max} (% of control)	EC50 (μl)	E_{max} (% of control)	EC50 (μl)
Krebs	123.7 ± 14.2	14.9 ± 2.0	103.3 ± 2.9	23.5 ± 2.6
Atropine	17.5 ± 15.5[b]	226.7 ± 209[b]	11.0 ± 1.4[a]	100.8 ± 19.0[b]
Pyrilamine	76.7 ± 7.8[a]	24.8 ± 3.4[b]	62.6 ± 13.0[b]	22.5 ± 5.5
Indomethacin	53.0 ± 10.4[a]	30.5 ± 13.3	59.5 ± 12.4	36.2 ± 15.8
Krebs	138.3 ± 17.1	31.3 ± 10.6	95.8 ± 3.6	65.8 ± 35.7
NDGA	72.4 ± 6.7[b]	15.9 ± 4.2	63.5 ± 8.0[a]	32.3 ± 2.2
BPB	75.0 ± 9.0[a]	25.3 ± 6.9	44.7 ± 15.6[b]	33.9 ± 6.8
Acivicin	65.8 ± 13.3[a]	71.4 ± 46.0	70.8 ± 18.2	45.7 ± 9.0
Krebs	115.1 ± 8.6	44.1 ± 16.0	112.4 ± 3.6	69.5 ± 13.1
Captopril	64.9 ± 5.8[a]	40.1 ± 11.8	58.8 ± 16.5[b]	28.3 ± 5.9[b]
TMB8	36.4 ± 16.2[a]	133.2 ± 37.8[b]	84.8 ± 29.7	324.9 ± 106.3
Capsaicin	56.9 ± 6.2[a]	25.5 ± 2.4	59.4 ± 17.0[b]	27.9 ± 1.1[b]

[a]Difference between krebs and pharmacologic agent statistically significant, $p < .05$.
[b]Difference between krebs and pharmacologic agent statistically significant, $p < .01$.

By analyzing the patterns of response to different drugs used in our experiments with cocoa and rye flour extracts, it was concluded that blockading of specific receptors has a modifying effect on the smooth muscle constrictor effect induced by both studied extracts. In particular, the muscarinic blocking agent atropine had a marked effect on the maximal (plateau) response to dust extract (E_{max}) and the dose-response curves for these extracts. The other agents tested only partially suppressed the effects of cocoa and rye flour dust extracts. For agents affecting the arachidonic acid pathway (e.g., indomethacin) the blocking effects were noted primarily for cocoa extract. These investigations suggest that in addition to cholinergic mechanisms there are probably other mediators involved in these events.

Modulation of the autonomic nervous system has been shown to affect bronchoconstriction resulting from organic aerosols. For example, Peters et al. (1989) have shown that *n*-formyl-methionyl-leucyl-phenylalanine (FMLP) causes bronchoconstriction in humans, while Fedan et al. (1994) reported that FMLP induced constriction in excised guinea pig tracheal smooth muscle. In these models, the bronchoconstrictor response in vivo was in part prevented by the anticholinergic agent ipratropium bromide. Similar findings were noted by Fuller et al. (1987) in clinical challenge studies using bradykinin.

Captopril, an angiotensin converting enzyme (ACE) inhibitor, has been associated with the enhancement of kinin-induced (e.g., bradykinin) contraction of airway smooth muscle (Dusser et al., 1988; Orawski et al., 1989). In our system these enzyme inhibitors paradoxically reduced the contractile effect of the cocoa and rye flour extracts but only at doses of 300 μl and 1000 μl. This could be explained by the hypothesis that the extracts

may contain peptides that mediate relaxation and are sensitive to captopril and other endopeptidase inhibitors. Alternatively, it may be possible that some inactive dust ingredient is normally cleaved to an active constrictor by these enzymes and is therefore not formed in the presence of their inhibitors.

TMB8, an inhibitor of calcium mobilization, suppressed cocoa and rye flour extract effects. This agent limits free intracellular calcium levels. An increase in intracellular calcium occurs in many smooth muscle preparations induced to constrict by receptor and nonreceptor mechanisms (Alexandre et al., 1993; Gustavson & Nilsson, 1993). Calcium mobilization for the contractile mechanism may originate from intra- or extracellullar stores. Since elevation of cytosolic calcium is involved in the sequence leading to smooth muscle constriction, the relative roles of intra- and extra-cellular calcium blocking agents in the prevention of dust-related airway obstruction remains to be explored.

Pretreatment with capsaicin (which depletes stored neuropeptides) significantly blocked the constrictor effect of cocoa extract but only minimally affected that of rye flour extract. This suggests that cocoa extract is a more potent irritant of sensory nerve receptors than rye flour extract.

These pharmacological studies of cocoa and rye flour extract on guinea pig tracheal smooth muscle suggest a complex interaction between these airway irritants and guinea pig tracheal tissue. Our experimental findings suggest, moreover, that the clinical effects of cocoa and rye flour dust in workers are probably related to nonimmunologic (non-IgE) mechanisms similar to those seen with other organic dusts extracts, such as green coffee, spices, swine confinement agents, animal food, poultry (Zuskin et al., 1983, 1988, 1991a, 1992, 1995), and soy and brewery dust extracts (Schachter et al., 1988a, 1995b). The striking effect of atropine in this model suggests that cholinergic mediators in particular play a role in this form of airway constriction, but other receptor mediators may also be involved; in particular, neuropeptides released from sensory nerves have been implicated. The role of these other mediators appears to vary with the extract. Such findings may have clinical and therapeutic implications for the acute and/or chronic respiratory symptoms and lung function changes seen to occur in workers exposed to organic dusts, such as those associated with cocoa and flour.

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