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Ruthenium red, but not capsazepine reduces plasma extravasation by cigarette smoke in rat airways

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1 Cigarette smoke increases vascular permeability in rat airways by activating release of tachykinin from capsaicin-sensitive sensory nerves. However, the mechanism by which cigarette smoke induces secretion of sensory neuropeptides is unknown. Here we hypothesized that cigarette smoke activates sensory nerve endings via a mechanism similar to that of capsaicin.

2 We studied the effects of ruthenium red, an inorganic dye which blocks the cation influx promoted by capsaicin and of the capsaicin antagonist capsazepine on the increase in vascular permeability produced by cigarette smoke, capsaicin, hypertonic saline and substance P in the trachea of pentobarbitone anaesthetized rats. We also investigated the ability of cigarette smoke to desensitize sensory nerve fibres.

3 Ruthenium red (10 mM) by aerosol blocked the increase in vascular permeability induced by capsaicin (0.5 μM) and reduced the response to cigarette smoke (5 puffs) but did not affect responses evoked by hypertonic saline (7.2%) or by substance P (10 μM) (all given by aerosol). Aerosols of capsazepine (0.1 mM) prevented extravasation by capsaicin, but did not inhibit response to cigarette smoke, hypertonic saline or substance P. Finally, pre-exposure to a high dose of cigarette smoke (10 puffs) prevented the extravasation caused by cigarette smoke (5 puffs) itself and by intravenous capsaicin (150 $\mu g\,kg^{-1}$), but not that by intravenous substance P (10 $nmol\,kg^{-1}$).

4 The present results show that cigarette smoke: (a) increases vascular permeability in the rat airways by a mechanism that is not antagonized by capsazepine, and is partially sensitive to rutheniun red; (b) produces desensitization of capsaicin-sensitive sensory nerves. We propose that chemical(s) contained in or agent(s) produced by cigarette smoke in the airways share partially a common pathway with capsaicin to activate peptide release from capsaicin-sensitive sensory nerves, but do not bind to the putative 'capsaicin receptor'.

Keywords: Cigarette smoke; ruthenium red; capsazepine; capsaicin; tachykinins; substance P; plasma extravasation; trachea; sensory nerves; hypertonic saline

Introduction

Inhalation of cigarette smoke produced irritation of the airways and an inflammatory response which includes vascular extravasation. Because capsaicin desensitization (Lundberg & Saria, 1983) or pretreatment with different NK_1 receptor antagonists (Delay-Goyet & Lundberg, 1991; Piedimonte *et al.*, 1992) inhibits plasma exudation by cigarette smoke completely, it is concluded that this response is due to tachykinin release from capsaicin-sensitive nerve endings. Cigarette smoke includes particulate and vapour phases and contains a variety of irritants (Stedman, 1968). The ability of cigarette smoke to increase vascular permeability is apparently due to the constituents of the vapour phase, and not to particulate materials or nicotine (Lundberg *et al.*, 1983). There is indirect evidence that irritants contained in the vapour phase of cigarette smoke affect capsaicin-sensitive sensory neurones. For instance, the inhalation of acrolein reduces the content of substance P (SP) and calcitonin gene-related peptide in the sensory nerves of rat airways (Springall *et al.*, 1990). However, it is not known by which mechanism cigarette smoke promotes the release of neuropeptides from the peripheral endings of capsaicin-sensitive sensory neurones, and thus produces inflammatory responses in the airways.

Capsaicin exerts two selective actions on sensory nerves. First, it produces excitation coupled to Ca^{2+}-dependent release of neuropeptides by the opening of a channel which allows Ca^{2+} and Na^{+} to enter the nerve (Marsh *et al.*, 1987; Wood *et al.*, 1988). Second, exposure to high concentrations of capsaicin makes sensory nerves no longer excitable by capsaicin itself and by several other stimuli (capsaicin desensitization) (Holzer, 1991). Ruthenium red, an inorganic dye with Ca^{2+} entry blocking properties, inhibits the action of capsaicin on sensory nerves by blocking the cation channel opened by this drug (Wood *et al.*, 1988; Maggi *et al.*, 1989; Dray *et al.*, 1990; Amann & Maggi, 1991). Capsazepine, a newly developed analogue of capsaicin, inhibits responses to capsaicin without exhibiting any excitatory activity on sensory nerves (Bevan *et al.*, 1991; Dickenson & Dray, 1991; Urban & Dray, 1991). Capsazepine in the guinea-pig airways *in vitro* has been shown to behave as a non-competitive antagonist of capsaicin (Belvisi *et al.*, 1992).

Here we have investigated whether cigarette smoke promotes release of sensory neuropeptides by sharing the pathway activated by capsaicin. To test this hypothesis, we have studied the effect of ruthenium red and capsazepine on the plasma extravasation evoked by cigarette smoke, and have compared responses obtained with cigarette smoke with the effects resulting from administration of capsaicin. The effect of cigarette smoke was also compared with effects produced by hypertonic saline and SP. Hypertonic saline was chosen because it increases vascular permeability in rat airways by releasing tachykinins from sensory nerves (Umeno *et al.*, 1990) and because neuropeptide release by hypertonic saline is independent of the activation of the cation channel operated by capsaicin (Tramontana *et al.*, 1991; Del Bianco *et al.*,

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1992). SP was chosen because it increases vascular permeability by acting directly at the vascular level without the involvment of a neuronal mechanism. Finally we have tested the possibility that cigarette smoke produces desensitization of the nerve fibres.

Methods

General

We used pathogen-free F344 rats (10–12 wk of age) from Simonsen Laboratories, Inc., Gilroy, CA, U.S.A., weighing 200–300 g. On the day of the experiments, the rats were anaesthetized with sodium pentobarbitone (65 mg kg^{-1}, i.p.; Anatomy products Co., Arcadia, CA, U.S.A.). Vascular permeability was quantified by injection of the albumin tracer, Evans blue dye (3% (w/v) solution in 0.9% NaCl; Polysciences Inc., Warrington, PA, U.S.A.; 30 mg kg^{-1}, i.v. for 5 s) at the beginning of each challenge. Five min later, the chest was opened, the aorta cannulated through the left ventricle, and the circulation was perfused for 2 min with PBS at pH 5. In experiments in which 10 puffs of cigarette smoke were used, the vascular perfusion was performed at the end of the 10 min exposure. The trachea was dissected and opened along the ventral midline, blotted, weighed, and then incubated overnight at 60°C in 3 ml of formamide (Fisher Scientific, Santa Clara, CA, U.S.A.). The extravasation of the dye-labelled macromolecules was assessed by measuring the optical density of the formamide extracts at a wavelength of 620 nm with a spectrophotometer (UV160U, Shimatzu, Columbia, MD, U.S.A.). The amount of Evans blue dye extravasated in the tissue, expressed in ng mg^{-1} of wet weight, was interpolated from a standard curve of Evans blue dye concentrations.

Aerosol administration

Rats were ventilated artificially at a frequency of 65 breaths min^{-1} and at a tidal volume of 3 ml, using a constant-volume ventilator (Harvard Apparatus, Millis, MA, U.S.A.) connected to a tracheal cannula (Umeno *et al.*, 1990). The cannula having a conical tip with an inner diameter of 1 mm, was inserted for 1–3 mm between the cricoid cartilage and the first tracheal ring. Aerosols of drugs were generated by ultrasonic nebulizers (Pulmo-Sonic 25, De Vilbiss, Somerset, PA, U.S.A.) and delivered to the airways through the inspiratory limb of the ventilator. In the experiments with cigarette smoke, rats were exposed to the smoke of a reference cigarette (2R1 cigarettes prepared by the University of Kentucky Tobacco and Health Research) containing 32.9 mg tar and 2.19 mg nicotine. Each puff of smoke was collected into a syringe (1 puff = 30 ml) and then was expelled over 30 s into the tracheal cannula, followed by a 30 s interval of ventilation with room air (Dusser *et al.*, 1989).

To test the effect of ruthenium red on the extravasation produced by the different agents, the compound or its vehicle (0.9% saline) was given over 2 min, 20 min before the delivery of each stimulus. The aerosol of capsazepine or its vehicle (1% (v/v) ethanol in 0.9% saline) was administered over 2 min, 5 min before the challenge with the different stimuli. Aerosols of capsaicin (dissolved in ethanol to 1 mM concentration and then diluted in 0.9% saline), its vehicle (0.2% ethanol (v/v)), 7.2% NaCl or SP were delivered over 2 min.

Desensitization procedure

To study desensitization produced by cigarette smoke on the extravasation evoked by subsequently administered stimuli, rats received air (5 puffs) or cigarette smoke (5 or 10 puffs). Twenty min later the different stimuli were delivered. These consisted of cigarette smoke (10 puffs), capsaicin (150 μg kg^{-1}, i.v., dissolved in 0.9% saline containing 10% (v/v) ethanol, 10% (v/v) Tween 80 and further diluted 10 times in 0.9% saline) or SP (10 nmol kg^{-1}, i.v. diluted in 0.9% saline). It may be presumed that capsaicin and SP by i.v. administration allowed to obtain a more uniform drug distribution in the trachea than the aerosol administration. Therefore, this experimental condition was chosen to test the ability of cigarette smoke to desensitize sensory nerves completely in this tissue.

Drugs

All of the drugs used in this study were injected in a volume of 1 mg kg^{-1} of body weight. Substance P was purchased from Peninsula Lab. (Belmont, CA, U.S.A.). Ruthenium red and capsaicin were purchased from Sigma (St. Louis, MO, U.S.A.). Capsazepine was a kind gift of Dr S. Bevan (Sandoz Institute for Medical Research, London, U.K.).

Statistical analysis

Data are expressed as ng of Evans blue dye per mg of tissue and are the mean ± standard error of the mean (s.e.mean). Mean values of spectrophotometric measurements of Evans blue dye extravasation were analysed by one-way analysis of variance (ANOVA). Comparisons between means were performed by the Dunnett's multiple range test. In the case of comparisons between two groups, the statistical analysis was performed by means of the Student's t test. Differences having a P value <0.05 were considered significant.

Results

General

Exposure of the rat airways to room air for 5 min produced an extravasation of Evans blue dye that was 13 ± 2.5 ng mg^{-1} ($n = 4$). Exposure to cigarette smoke produced a dose-related increase in tracheal vascular extravasation which reached a maximum after 10 puffs (195 ± 15 ng mg^{-1}, $n = 5$). For the following experiments a dose of 5 puffs was chosen because this dose resulted in a submaximal effect (Figure 1).

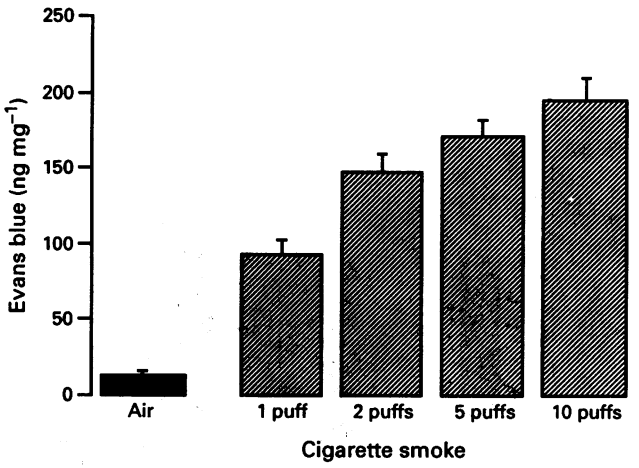

Figure 1 Effect of exposure to different numbers of puffs of cigarette smoke on the extravasation of Evans blue dye in the trachea of pentobarbitone anaesthetized rats. 2R1 cigarettes prepared by the University of Kentucky Tobacco and Health Research containing 32.9 mg tar and 2.19 mg nicotine were used. Each puff of smoke was collected into a syringe (1 puff = 30 ml) and then was expelled over 30 s into the tracheal cannula, followed by a 30 s interval of ventilation with room air. Each column is the mean ± s.e.mean (vertical bar) of at least 5 experiments.

Then, doses of capsaicin, hypertonic saline and SP that gave amounts of Evans blue dye extravasation comparable to that obtained with 5 puffs of cigarette smoke were selected. Ruthenium red aerosol given over 2 min, 20 min before the stimulus, reduced the dye extravasation evoked by aerosolized capsaicin (0.5 μM, for 2 min) in a dose-dependent manner, with almost complete inhibition at 10 mM (29 ± 5 ng mg^{-1}, $n = 5$) (Figure 2). Pretreatment with ruthenium red vehicle (0.9% saline, for 2 min, 20 min before) and challenge with 0.9% saline (for 2 min) gave an amount of Evans blue in the tissue that was 15.5 ± 3 ng mg^{-1} ($n = 5$).

Effect of ruthenium red and capsazepine

Next we compared the effects of 10 mM ruthenium red on the increase in vascular extravasation evoked by cigarette smoke, hypertonic saline and SP. Ruthenium red reduced extravasation evoked by cigarette smoke (5 puffs for 5 min) by 45%, and it did not affect the response to hypertonic saline (7.2% for 2 min) and slightly but significantly enhanced by 29% the effect of SP (10 μM for 2 min) (Figure 3).

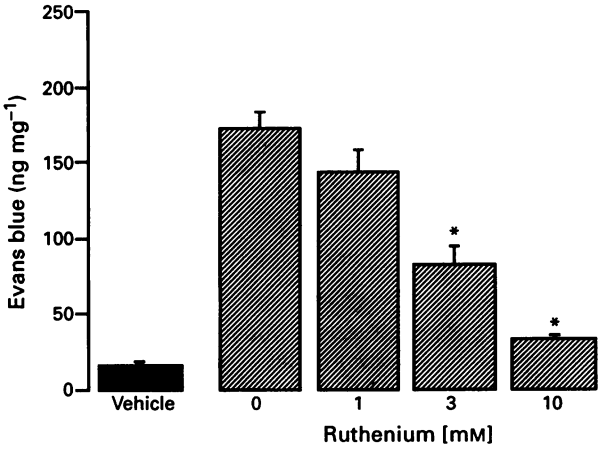

Figure 2 Effect of different concentrations of aerosolized ruthenium red (for 2 min, 20 min before capsaicin challenge) or 0.9% saline (0) on the Evans blue dye extravasation evoked by capsaicin aerosol (0.5 μM, for 2 min) in the trachea of pentobarbitone anaesthetized rats. Solid column (vehicle) indicates the extravasation produced by capsaicin vehicle delivered 20 min after 2 min pretreatment with 0.9% saline. Each column is the mean ± s.e.mean (vertical bars) of at least 5 experiments. $^*P < 0.01$.

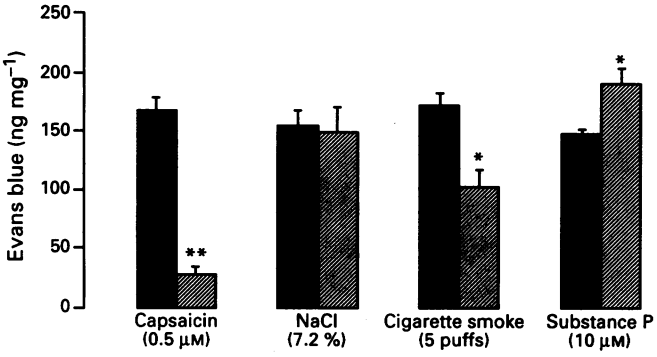

Figure 3 Effect of pretreatment with aerosolized ruthenium red (10 nM, for 2 min, 20 min before) (hatched columns) on the Evans blue dye extravasation evoked by aerosol administration of hypertonic saline (2 min) or substance P (2 min) and by delivery of cigarette smoke (5 puffs over 5 min) in the trachea of pentobarbitone anaesthetized rats. Solid columns = responses in absence of ruthenium red. Each column is the mean ± s.e.mean (vertical bars) of at least 5 experiments. $^*P < 0.01$, $^{**}P < 0.001$.

In another series of experiments, we found that capsazepine by aerosol (administered for 2 min, 5 min before the stimulus) inhibited the Evans blue dye extravasation evoked by aerosolized capsaicin (0.5 μM for 2 min) in a dose-dependent manner. The maximal inhibition was obtained with a concentration of 100 μM (Figure 4). Capsazepine aerosol (100 μM for 2 min) produced a slight increase in vascular extravasation (27 ± 5 ng mg^{-1}, $n = 4$) that was not significantly different as compared to the effect evoked by its vehicle (1% ethanol in 0.9% saline, administered for 2 min, 5 min before; 25 ± 4 ng mg^{-1}, $n = 4$). Then we tested the effect of this concentration of capsazepine on the responses produced by cigarette smoke and hypertonic saline: capsazepine did not affect the plasma extravasation induced by 5 puffs of cigarette smoke or by 7.2% NaCl (Figure 4).

Desensitization to cigarette smoke

Finally, we studied the effect of pre-exposure to cigarette smoke on the response to cigarette smoke itself and to intravenous capsaicin or SP. The response to cigarette smoke (5 puffs) was reduced by pre-exposure to 5 puffs of cigarette smoke (20 min before) by 58%. After pre-exposure to 10 puffs, the response to cigarette smoke was virtually abolished (2% of control). The response to capsaicin (150 μg kg^{-1}) was inhibited by pre-exposure to 5 puffs of cigarette smoke to 57% of that found in controls, and the response was markedly reduced by pre-exposure to 10 puffs of cigarette smoke (15% of control). The increase in Evans blue dye by SP (10 nmol kg^{-1}, i.v.) was not altered by pre-exposure to either 5 or 10 puffs of cigarette smoke (Figure 5).

Discussion

The present studies show that cigarette smoke-induced plasma extravasation is not sensitive to capsazepine, but it is partly reduced by ruthenium red. In the same series of experiments we found that ruthenium red or capsazepine did not affect Evans blue dye extravasation by hypertonic saline and ruthenium red had no influence on the response to SP. Finally, pre-exposure to cigarette smoke prevented the vascular extravasation evoked by capsaicin, but not that induced by SP.

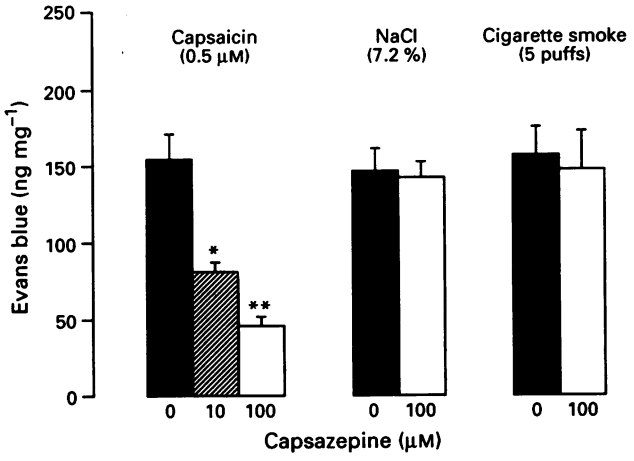

Figure 4 Effect of pretreatment with capsazepine aerosol (100 μM, open columns; 10 μM, hatched columns, for 2 min, 5 min before the challenge) or its vehicle (0.1% ethanol, for 2 min, 5 min before the challenge, solid columns) on the Evans blue dye extravasation evoked by aerosols of capsaicin (for 2 min) or hypertonic saline (for 2 min) and delivery of cigarette smoke (for 5 min) in the trachea of pentobarbitone anaesthetized rats. Each column is the mean ± s.e. mean (vertical bars) of at least 5 experiments. $^*P < 0.01$, $^{**}P < 0.001$.

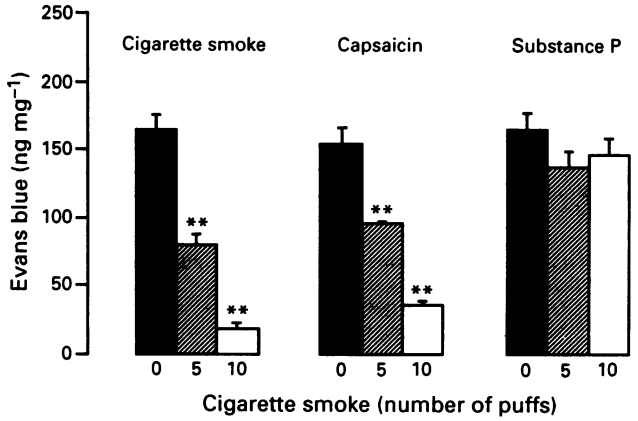

Figure 5 Effect of pretreatment with room air (over 5 min, solid columns), 5 puffs (over 5 min, hatched columns) or 10 puffs (over 10 min, open columns) of cigarette smoke on the Evans blue dye extravasation evoked by cigarette smoke (5 puffs over 5 min), capsaicin (150 $\mu g\,kg^{-1}$, i.v.) or substance P (10 $nmol\,kg^{-1}$, i.v.) in the trachea of pentobarbitone anaesthetized rats. Each column is the mean ± s.e.mean (vertical bars) of 6 experiments. $^{**}P<0.001$.

Ruthenium red is an effective functional antagonist of the action exerted by capsaicin on sensory nerves (Amann & Maggi, 1991). Ruthenium red prevents the release of sensory neuropeptides evoked by capsaicin but not by other stimuli such as high K^+ or bradykinin (Geppetti *et al.*, 1990; 1991). However, the use of ruthenium red for *in vivo* studies has been hampered by its toxicity (Amann *et al.*, 1990). We circumvented this problem by delivering the drug by aerosol. This mode of administration gave an effective local concentration in the airway without any apparent systemic toxic action; no rat died after the administration of this drug. Aerosol administration should minimize possible cardiovascular effects of ruthenium red, that theoretically could alter the response to the subsequent stimuli. However, cardiovascular effects by aerosolized ruthenium red, if any, does not seem to have a relevant action on subsequent plasma extravasation, because ruthenium red pretreatment selectively abolished dye extravasation by capsaicin, but not those by hypertonic saline or SP. These same considerations, discussed for ruthenium red, apply also to capsazepine (see below).

The observation that ruthenium red actually increases the response to SP shows that despite the high concentration of ruthenium red in the aerosol the amount of drug delivered over 2 min did not reduce the ability of postcapillary venules to respond to SP. Potentiation of bronchoconstriction by SP or by neurokinin A has been observed recently following subcutaneous administration of ruthenium red (Ballati *et al.*, 1992). These authors proposed that blockade of adrenergic outflow by ruthenium red could account for the observed potentiation. However, in the present study the reason why ruthenium red increases the response to SP remains to be defined.

Another drawback in the use of ruthenium red is that its selectivity for the capsaicin-activated channel is restricted to a narrow range of concentrations (Maggi *et al.*, 1988; 1989; Amann & Maggi, 1991). For this reason we compared the action of ruthenium red on the extravasation evoked by capsaicin with that exerted on the extravasation evoked by hypertonic saline. Hypertonic saline and capsaicin were both delivered by aerosol; both cause vascular extravasation by releasing tachykinins from sensory nerves (Umeno *et al.*, 1990; Piedimonte *et al.*, 1992). However, we found previously that hypertonic saline releases sensory neuropeptides by a mechanism that is insensitive to ruthenium red (Del Bianco *et al.*, 1992), in contrast with capsaicin. Because ruthenium red did not affect the dye extravasation induced by hypertonic saline but completely blocked the increase in vascular permeability evoked by capsaicin, it may be inferred that in the present experimental conditions its action is selectively directed at blocking the capsaicin-operated channel. As a consequence, the partial reduction produced by ruthenium red on cigarette smoke-evoked vascular extravasation suggests that cigarette smoke activates a mechanism, at least in part, similar to that stimulated by capsaicin.

Chemical(s) contained in cigarette smoke or agent(s) produced by contact of cigarette smoke with airway tissue could activate the capsaicin-operated mechanism by binding to the 'putative' receptor for capsaicin (Szallasi & Blunberg, 1990). To test this hypothesis, we used capsazepine, a recently developed compound which is devoid of any excitatory property and has been shown to block the action of capsaicin in different models, most probably by preventing the binding of capsaicin to its receptor (Bevan *et al.*, 1991; Dickenson & Dray, 1991; Urban & Dray, 1991). The finding that capsazepine blocked the increase in plasma extravasation evoked by capsaicin and did not affect the action of hypertonic saline indicates that capsazepine selectively antagonized the action of capsaicin. Because capsazepine did not reduce the response to cigarette smoke, it may be concluded that compound(s) contained in or produced by cigarette smoke, and which evokes release of sensory neuropeptides, exert this action without binding to the putative capsaicin receptor.

Exposure to a high dose of cigarette smoke prevented the action of a subsequent administration of cigarette smoke, thus indicating desensitization. This was not apparently due to an action of cigarette smoke at the vascular level because the response to intravenous SP was completely unaffected by such a treatment. However, protracted exposure to cigarette smoke impaired the ability of intravenous capsaicin to cause vascular extravasation in the airways, suggesting that the target of the desensitizing action of cigarette smoke is the sensory nerve. This finding is consistent with the observation that exposure to acrolein, an aldheyde contained in cigarette smoke, depleted the tissue content of sensory neuropeptides in the rat airways (Springall *et al.*, 1990). Several hypotheses on the modality by which capsaicin induces desensitization have been proposed: capsaicin may deplete the pool of releasable peptides from the sensory nerve endings (Hakanson *et al.*, 1987). It may, at low doses, exert selective desensitization. Selectivity is suggested by the observation that the nerve fibre no longer responds to capsaicin but is still excitable by other stimuli (Dray *et al.*, 1989). Finally, at higher doses, capsaicin exerts neurotoxic effects that lead to irreversible neuronal damage (Bevan & Szolcsanyi, 1990). Even though the present study clearly shows that cigarette smoke, like capsaicin, provokes sensory nerve desensitization, it does not provide information to indicate which of these different mechanisms proposed for explaining the desensitizing action of capsaicin is exerted by cigarette smoke.

Various endogenous agents have been reported to activate the 'efferent' function of capsaicin-sensitive sensory nerves by a ruthenium red-sensitive mechanism (Geppetti *et al.*, 1991; Mapp *et al.*, 1991). However, further studies are necessary to establish whether the compound(s) responsible for the ruthenium red-sensitive component of cigarette smoke action on sensory nerves is a chemical contained in cigarette smoke or an agent produced by cigarette smoke after contact with the airway tissue.

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