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INHIBITION OF LOW PH EVOKED ACTIVATION OF AIRWAY SENSORY NERVES BY CAPSAZEPINE, A NOVEL CAPSAICIN-RECEPTOR ANTAGONIST

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Summary: Low pH is a well known sensory irritant in pathological conditions such as inflammation. The mechanisms underlying this low pH effect were therefore studied in the guinea pig. Acid exposure caused marked nasal irritation via a specific subset of sensory nerves sensitive to capsaicin. Futhermore, acid caused bronchoconstriction via release of neuropeptides from capsaicin sensitive afferents. Interestingly, capsazepine, a recently developed competitive capsaicin receptor antagonist, selectively inhibited these responses to low pH. Ruthenium red, which blocks the cation channel associated with the capsaicin receptor, had effects similar to those of capsazepine. Therefore, acid irritation of the airway mucosa may involve capsaicin-receptor mechanisms and capsazepine represents a novel protective agent.

Sensory neurons which are also sensitive to the pungent agent in red hot peppers, capsaicin, respond to low pH with an increase in cation permeability (1). Stimulation of capsaicin-sensitive C-fibre afferents leads to generation of a sensory impulse and central perception of irritation/pain, but also to local inflammation via release of several neuropeptides such as substance P, neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) (2,3). Both indirect functional evidence (4) and subsequent direct biochemical measurements (5) suggest that low pH media promotes local peptide release from sensory nerves. Neuropeptide release from peripheral endings of capsaicin-sensitive afferents leads to a variety of responses including vasodilatation, plasma protein extravasation via substance P acting on NK1 receptors and bronchoconstriction via NKA acting on NK2 receptors (3,6,7). Based on binding experiments, it has been suggested that the effects of capsaicin on sensory neurons are mediated through an interaction with a specific membrane receptor (8). Capsaicin is known to activate an ion channel similar to that activated by protons (1), causing influx of cations, a response which

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Abbreviations: CGRP, calcitonin gene-related peptide; -LI, -like immunoreactivity; NKA, neurokinin A; R_L, lung resistance.

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can be inhibited by the dye ruthenium red (RR). Furthermore, since RR has been reported to inhibit citric acid induced cough (9) and sulfur dioxide induced bronchoconstriction (10), activation of sensory nerves by low pH and activation via the capsaicin receptor may have some common mechanism. Recently a selective, competitive capsaicin receptor antagonist, capsazepine (2-[2-(4-chlorophenyl)ethylamino-thiocarbonyl]-7,8-dihydroxy-2,3,4,5-tetrahydro-1H-2 benzazepine) has been developed (11). In the present experiments we have used capsazapine to test the hypothesis that low pH can activate sensory nerves by interacting with the capsaicin receptor. We used pH 5 solution as standard stimulus since acid solutions with pH < 6.2 evoked a sustained inward current due to increased cation conductance which is restricted largely to capsaicin-sensitive sensory neurons but solution with pH >6.2 did not (12). The airway mucosa can be exposed to such stimuli upon inhalation of acidic air pollutants or aspiration of gastric juice (4).

MATERIALS AND METHODS

Isolated perfused guinea-pig lung preparation. We have used isolated perfused guinea-pig lung model, where the peptide release and functional response can be measured in parallel (9). Briefly, adult Dunkin-Hartley guinea-pigs (bwt 250-350 g) of either sex were anaesthetized with Mebumal (pentobarbital, 60 mg/kg, i.p.). The lungs were perfused through the pulmonary artery with Krebs-Ringer solution. Then, the lungs together with the intubated trachea, cannulated heart and vagal nerves were dissected free and placed in thoracic chamber at 37°C and alternating negative pressures (-1 to -11 cm H_2O) were produced by a respirator (Harward, mod. 665) and a vacuum source connected to the chamber. The chamber pressure, the tracheal airflow and the tidal volume were recorded on a Grass Polygraph. The lung resistance (R_L) was calculated by an on-line computer. Alternatively the time to total lung closure was recorded to obtain a functional parameter for very strong bronchoconstriction responses. The vagal nerves were placed on two individual platinum electrodes connected to an electrical stimulator (bipolar current generator, Hässle, Sweden). In all experiments the ganglionic blocking agent chlorisondamine (10^{-6} M) was added to inhibit parasympathetic postganglionic nerve activation and terbutaline (10^{-7} M) was present in order to obtain stable basal performance and graded functional effects. The guinea-pig lung was perfused with capsaicin (10^{-8} M or 10^{-6} M), bradykinin ($5x10^{-6}$ M) or nicotine (10^{-4} M) for 3 min in separate preparations. Vagal stimulations were performed at 1 Hz for 1 min with a duration of 5 ms. For low pH experiments, tissues were perfused for 9 min at pH 5 with a buffer of the following composition (mM); NaCl 140, $CaCl_2$ 2.5, $MgSO_4$ 1.5, Na_2HPO_4 0.06, KH_2PO_4 6.6 and glucose 11.0. Capsazepine (10^{-6} or $5x10^{-6}$ M), RR ($5x10^{-6}$ M) or indomethacin (10^{-5} M) were given 10, 15 and 40 min, respectively, before and during provocation with various stimulants.

CGRP-LI and NKA-LI release. Tachykinins and CGRP are co-stored in the same large dense cored vesicles and since it is easier to detect CGRP than NKA release, overflow of CGRP like immunoreactivity (LI) was used as a biochemical marker for pulmonary sensory nerve activation in most experiments (9). Perfusate fractions (3 min) were collected through a right atrial catheter in a beaker placed on ice. After pH determination, acetic acid was added to give a final concentration of 0.2 M. The perfusate samples were desalted using SEP-PAK C_{18} cartridges, lyophilized and redissolved in buffer before determination of CGRP-LI by radioimmunoassay with an antiserum raised against human CGRP-alpha (13). In some experiments NKA-LI was measured using antiserum NKA-5 (14).

Nasal irritation. The nasal irritation response in concious guinea-pigs were recorded as nose wipings with the forepaws during 3 min (15) after local unilateral intranasal application of 25 µl saline containing capsaicin ($5x10^{-7}$ M), citric acid (0.4 M, pH 1.7) or nicotine ($3.5x10^{-3}$ M). The influence of combining capsazepine (10^{-4} M) or RR ($3x10^{-4}$ M) with these agents were also tested.

Capsaicin pretreatment. Capsaicin pretreatment was made under ketamine (50 mg/kg, i.m.) anesthesia by multiple s.c. injections during two days giving a total dose of 50 mg/kg of capsaicin. Bronchodilators, terbutaline (50 μg/kg, s.c.) and theophyllamine (10 mg/kg, i.m.) were given 10 min before each capsaicin injection (15). The capsaicin treated animals were used in some isolated lung and nasal irritation experiments three weeks later.

Drugs and statistics. Drugs from the following sources were used: capsazepine (Sandoz, London, England), capsaicin and ruthenium red (Fluka Chemie AG, Buchs, Switzerland), indomethacin (Sigma, MO, USA), chlorisondamine (Ciba-Geigy, AG, Basel, Switzerland), human CGRPα and NKA (Peninsula, Belmount, CA, USA), human [^{125}I] CGRPα and [^{125}I] NKA (Amersham Int., Amersham, England), terbutaline (Draco, Lund, Sweden). Capsazepine was dissolved in DMSO to give a stock solution of 10^{-1} M and further diluted (1:10) in saline containing 10 % Tween 80 and 10 % ethanol. Indomethacin was dissolved in absolute ethanol to a stock solution of $2x10^{-2}$ M and further diluted in the Kreb's solution.

Values are expressed as means ± S.E.M. For statistical analysis the Kruskal Wallis analysis of variance was used. Probablity $p<0.05$ was considered significant.

RESULTS

Inhibitory effect of capsazepine on bronchoconstriction and peptide release induced by capsaicin and low pH in perfused guinea-pig lung

Capsaicin (10^{-8} M) caused bronchoconstriction (as revealed by decreased tidal volume) (Fig 1), increased lung resistance, R_L (Fig 2A) and increased overflow of CGRP-like immunoreactivity (LI) in the perfusate (Fig 2B). Capsazepine did not cause any significant effect by itself. A threshold inhibitory effect of capsazepine on the capsaicin response was observed at 10^{-6} M (not shown). The effects of capsaicin (10^{-8} M) were virtually abolished by capsazepine ($5x10^{-6}$ M) (Figs 1, 2A,B) or RR ($5x10^{-6}$ M) (Figs 1, 2A,B). Capsaicin at high concentration

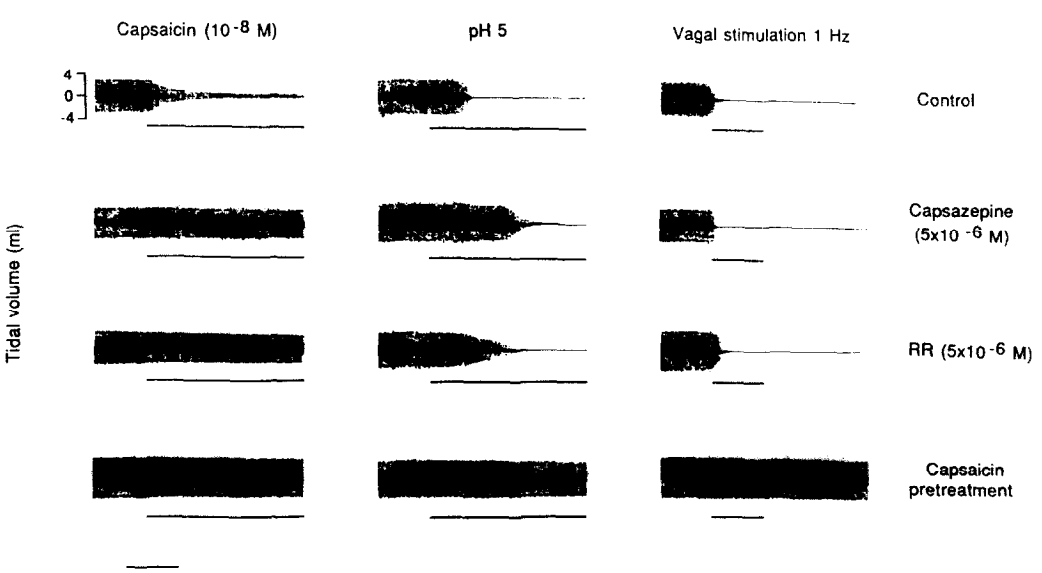

Fig 1. Original recordings showing the effects of capsaicin (10^{-8} M), pH 5 buffer or vagal stimulation (1 Hz for 1 min) on the tidal volume (ml) in the isolated perfused guinea-pig lung in control experiments (top panel), after pretreatment with capsazepine ($5x10^{-6}$ M, 2nd panel), RR ($5x10^{-6}$ M, 3rd panel) or capsaicin pretreatment (50 mg s.c., bottom panel). Reduction of tidal volume represents bronchoconstriction.

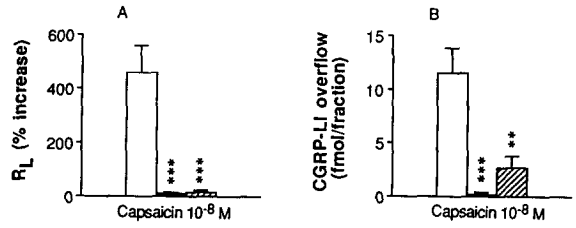

Fig 2. Effects of capsaicin (10^{-8} M) on (A) airway resistance (R_L) and (B) CGRP-LI overflow (fmol/fraction) in control experiments (open bars) as well as after pretreatment with capsazepine ($5x10^{-6}$ M) (filled bars) or ruthenium red ($5x10^{-6}$ M) (hatched bars). Peptide overflow represents the first fraction after exposure to stimuli. Values are expressed as mean ± SEM, n=4-6, ** p<0.01,*** p<0.001.

(10^{-6} M) caused marked CGRP-LI overflow and rapid total lung closure. The time to total closure (an indication of bronchoconstriction) was delayed and the CGRP-LI release reduced by 70-80 % by capsazepine ($5x10^{-6}$ M) and RR ($5x10^{-6}$ M) (Figs 3A,B). After systemic capsaicin pretreatment, which destroys sensory C-fibers, the CGRP-LI overflow and bronchoconstriction induced by capsaicin (10^{-8} M, or 10^{-6} M) were completely abolished (Fig. 1 or not shown).

When the acidic medium (pH = 5.06 ± 0.02) was introduced into the isolated lung preparation, the pH of the effluent was higher (pH = 6.04 ± 0.06). Capsazepine or RR per se did not significantly influence pH in the perfusion medium or in effluent (not shown). Low pH caused a progressive decrease in tidal volume (Fig 1) and increase in R_L leading to total airway closure (Figs 1, 3C) and a marked outflow of CGRP-LI and NKA-LI into the perfusate effluent (Fig 3D,E). The CGRP outflow evoked by low pH was about tenfold higher than that evoked

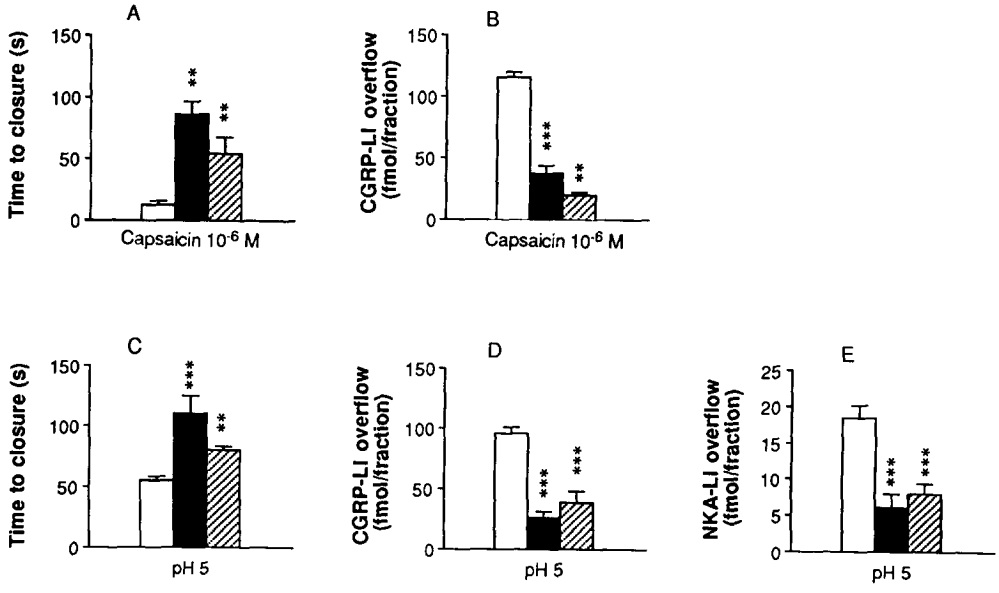

Fig 3. Effects of capsazepine ($5x10^{-6}$ M) (filled bars) or ruthenium red ($5x10^{-6}$ M) (hatched bars) on the bronchoconstriction (expressed as time to total closure) (A,C), or overflow of CGRP-LI (fmol/fraction) (B,D) evoked by exposure to capsaicin 10^{-6} M (A,B) or pH 5 buffer (C,D) and NKA-LI (fmol/fraction) overflow induced by pH 5 (E) (control responses, open bars). Peptide overflow represents the first fraction after exposure to stimuli. Values are given as mean ± SEM, n=4-7, ** p<0.01, *** p<0.001.

by 10^{-8} M capsaicin and similar in magnitude to that by 10^{-6} M capsaicin (Fig 3B, D). The low pH effect on peptide release could be reproduced at least once with only a slight reduction in the same preparation (not shown). The time to total closure of the ventilatory system upon exposure to pH 5 solution was significantly prolonged after pretreatment with capsazepine (Figs 1, 3C) and the CGRP and NKA overflows were significantly reduced (Fig 3D, E). Neither the functional response nor the peptide release evoked by pH 5 solution was influenced by the solvent for capsazepine (0.005% DMSO, 0.005% Tween 80, 0.005% ethanol, v/v) (n=3). After exposure to RR ($5x10^{-6}$ M), the peptide release evoked by low pH was significantly attenuated and the time to total lung closure was prolonged to a similar extent as for capsazepine (Figs 1, 3C,D). In controls the CGRP overflow was 96 ± 5.2, 152 ± 10.1 and 103 ± 10.1 fmol/3 successive 3 min fractions, and the NKA overflow was 18.4 ± 1.8, 30.9 ± 5.2, 20.1 ± 1.9 fmol/fraction (n=5). RR ($5x10^{-6}$ M) or capsazepine ($5x10^{-6}$ M) significantly inhibited CGRP and NKA overflow in all 3 fractions. Low pH after pretreatment with indomethacin caused CGRP and NKA overflow which was 72 ± 12 and 14.3 ± 1.8 fmol, respectively, in the first fraction. This slight reduction (25%, and 22%) of peptide overflow was not significantly different from the control effect. The time to total lung closure upon low pH was inconsistently increased in the presence of indomethacin (99 ± 34 sec to total closure compared to 55 ± 4 s in the control). After systemic capsaicin pretreatment, low pH medium perfusion for up to 9 min did not cause any bronchoconstriction (Fig 1) or release of sensory neuropeptides (not shown). The bronchoconstriction evoked by bolus injection of histamine ($5.4x10^{-9}$ mol) was not influenced by capsaicin pretreatment (not shown) suggesting that bronchial smooth muscle still responded to other stimuli.

In contrast to low pH and capsaicin effects, the bronchoconstrictor responses or peptide overflow evoked by electrical stimulation of the vagal nerves (Fig 1), bradykinin or nicotine were not significantly influenced by capsazepine. Thus, CGRP-LI release evoked by vagal stimulation, bradykinin or nicotine were 117 ± 11 %, 84 ± 11 % and 92 ± 6 % of control, respectively, after capsazepine ($5x10^{-6}$ M) pretreatment.

Inhibition of nasal irritation by capsazepine in conscious guinea-pigs

The nasal irritation index in conscious guinea-pigs, manifested as nose wiping with forepaws, was recorded during 3 min period. Intranasal application of 0.9% NaCl or the solvent for capsazepine evoked only marginal effects (0.71 ± 0.29, n=7 or 0.63 ± 0.38 n=8 nose wipings, respectively). Local application of capsaicin ($5x10^{-7}$ M), citric acid (0.4 M, pH 1.7) or nicotine ($3.5x10^{-3}$ M) caused clear-cut irritation (about 15 nose wipings), mainly during the initial one minute of the 3 min observation period (Fig 4). When capsaicin was combined with capsazepine (10^{-4} M) the nasal irritation response was reduced by 70±5 % (Fig 4). Furthermore, the citric acid effect was attenuated by 60±9 % (Fig 4). RR ($5x10^{-4}$ M) also inhibited the nasal irritation responses both to capsaicin and citric acid (by 75±11 % and 75±3 %, respectively) (Fig 4). The nose wiping response to nicotine, however, was not influenced by capsazepine or RR (Fig 4). Systemic capsaicin pretreatment abolished the nasal irritation induced by capsaicin or citric acid and markedly reduced the response induced by nicotine (by 86 ± 2%).

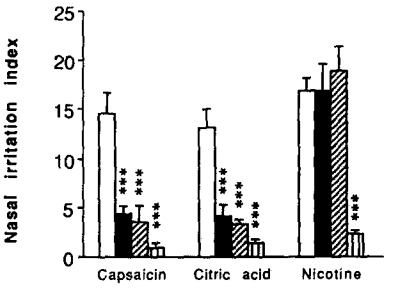

Fig 4. Nasal irritation index (number of nose wipings during 3 min) upon local application of capsaicin ($5x10^{-7}$ M), citric acid (0.4 M, pH 1.7) or nicotine ($3.5x10^{-3}$ M) in concious guinea-pigs in the absence (open bars) or presence of local capsazepine (10^{-4} M, filled bars), ruthenium red ($5x10^{-4}$ M, hatched bars) or systemic capsaicin pretreatment (vertical lined bars). Values are expressed as mean ± SEM, n=7-14, *** $p<0.001$.

Nicotine also evoked 3.6 ± 0.5 (n=8) sneezes and capsaicin pretreatment only partly attenuated this effect (by 34%, $p<0.05$, n=10).

DISCUSSION

The present data show some striking results. Low pH medium caused bronchoconstriction in the guinea-pig lung, most likely due to NKA release from capsaicin-sensitive sensory nerves (7). Surprisingly, the competitive capsaicin receptor antagonist capsazepine inhibited the bronchoconstriction response and, especially, the sensory neuropeptide releasing effect of low pH medium in the perfused lung. The increased acidity evoked a remarkable increase in CGRP-LI outflow, which was much higher than the response to vagal nerve stimulation and in the same range as capsaicin at a high concentration (10^{-6} M) (9). Capsazepine and RR inhibited the peptide release evoked by low pH medium or 10^{-6} M capsaicin to a strikingly similar extent. The remaining peptide overflow upon low pH after capsazepin or RR is in the same range as upon antidromic stimulation of vagal sensory nerves under control conditions i.e. probably still sufficient to evoke total lung closure (9). That the bronchoconstriction, and CGRP-LI and NKA-LI overflow induced by low pH were abolished after capsaicin pretreatment, further suggested the involvement of capsaicin sensitive sensory nerves in these responses. Therefore, occupancy of the capsaicin receptor (8) may thus influence permeability of the RR sensitive cation channel involved in the activation of sensory nerves by both protons and capsaicin (12). Alternatively, one can postulate that low pH medium causes the formation of an endogenous ligand for the capsaicin receptor on sensory nerves, although the existence of such an agent has to be biochemically and verified. The recent data that capsazepine (10^{-5} M) did not inhibit [H^+] induced $^{86}Rb^+$ efflux through cation channels in cultured dosal root ganglion cells of rat (16) further support the idea that some intermediate step is involved in the low pH evoked activation of sensory nerves in the guinea-pig airways, possibly involving non-neuronal elements. Many agents including prostaglandins may be formed in the lung at low pH medium and prostaglandins can release sensory neuropeptides via a RR sensitive pathway (17). However, the CGRP and NKA release and functional response upon low pH were not significantly modified in the presence of indomethacin, suggesting that cyclooxygenase products were not

involved to major extent (18). That capsazepine is a selective capsaicin receptor antagonist at the concentration used (11), was supported by the lack of influence of capsazepine on the bronchoconstriction or sensory neuropeptide release evoked by antidromic vagal nerve stimulation, bradykinin, and nicotine, stimuli which are not influenced by RR (9).

The nasal irritation experiments further show that local application of acid to the nasal mucosa was associated with capsazepine and RR sensitive irritant behavior in concious guinea-pigs. As a further sign of specificity, the nasal irritation evoked by nicotine was not changed by capsazepine despite the fact that the response to nicotine was absent after systemic capsaicin pretreatment, suggesting that nicotine acted on the same nerves as low pH. In this respect it was also of interest to note that although the nose wiping response to nicotine was entirely dependent on capsaicin sensitive nerves in the nasal mucosa, the accompanying sneezing was more resistant to capsaicin pretreatment as earlier reported which indicates that additional afferent mechanisms may be involved (19).

In this study, acid solution caused both reflex nasal rritation and bronchoconstriction via activation of capsaicin sensitive nerves. Patients with vasomotor rhinitis have hyperreactive nasal reflexes to capsaicin provocation (20) and the nasal secretory discharge and mucosal congestion in these patients are markedly ameliorated after local capsaicin densensitization (21).

The present data showing that the sensory nerve activation by low pH can be inhibited by the capsaicin receptor antagonist capsazepine may open up a new therapeutic principle both regarding inflammation and airway hyperreactivity. Furthermore, the existence of novel endogenous ligands for the capsaicin receptor may be proposed.

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