Mechanistic Studies of Acid Evoked Coughing in Anesthetized Guinea Pigs.

Brendan J. Canning, David G. Farmer and Nanako Mori

The Johns Hopkins Asthma and Allergy Center
5501 Hopkins Bayview Circle
Baltimore, MD 21224
USA

Correspondence:
Brendan J. Canning
The Johns Hopkins Asthma and Allergy Center
5501 Hopkins Bayview Circle
Baltimore, MD 21224
USA

Phone: 410-550-2156
Fax: 410-550-2130
e-mail: bjc@jhmi.edu

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Abstract

Experiments carried out in conscious guinea pigs suggest that citric acid evoked coughing is partly mediated by TRPV1 receptor-dependent activation of tachykinin-containing, capsaicin-sensitive C-fibers. In vitro electrophysiological analyses indicate, however, that acid also activates capsaicin-sensitive and –insensitive vagal afferent nerves by a TRPV1-independent mechanism, and studies in anesthetized guinea pigs show that coughing evoked by acid is mediated by activation of capsaicin-insensitive vagal afferent nerves. In the present study we have characterized the mechanisms of citric acid evoked coughing in anesthetized guinea pigs. Drugs were administered directly to the Krebs buffer perfusing the extrathoracic trachea. Citric acid was applied topically to the tracheal mucosa, directly into the tracheal perfusate in increasing concentrations and at 1 minute intervals. Citric acid dose-dependently evoked coughing in anesthetized guinea pigs. This was mimicked by hydrochloric acid but not by sodium citrate. The coughing evoked by acid was nearly or completely abolished by tetrodotoxin or by cutting the recurrent laryngeal nerves. Perfusing the trachea with a low chloride buffer potentiated the acid-induced cough reflex. In contrast, prior capsaicin desensitization, 10µM capsazepine, Ca++-free perfusate, 0.1µM iberiotoxin, 1µM atropine, 10µM isoproterenol, 10µM albuterol, 3µM indomethacin, 0.1µM HOE 140, a combination of neurokinin1 (NK1; CP99994), NK2 (SR48968) and NK3 (SB223412) receptor antagonists (0.1µM each), a combination of histamine H1 (3µM pyrilamine) and cysLT1 (1µM ICI198615) receptor antagonists, superior laryngeal nerve transection or epithelium removal did not inhibit citric acid evoked coughing. These and other data indicate that citric acid evoked coughing in anesthetized guinea pigs is mediated by direct activation of capsaicin insensitive vagal afferent nerves perhaps through sequential activation of acid sensing ion channels and chloride channels.
Citric acid and tartaric acid are used routinely in clinical and preclinical studies of the cough reflex (15, 27, 35). In conscious guinea pigs, citric acid evoked coughing is inhibited by the capsaicin receptor (TRPV1) antagonists capsazepine and iodo-resiniferotoxin (i-RTX) or the TRPV1 channel blocker ruthenium red, and can also be inhibited by neurokinin1 (NK₁), NK₂ and/or NK₃ receptor antagonists (1, 2, 7, 18, 34, 54, 55). The acid evoked coughing is mimicked by inhalation of the TRPV1 receptor agonists capsaicin, resiniferotoxin and anandamide in both animals and human subjects, and prior capsaicin desensitization abolishes citric acid evoked coughing in awake guinea pigs (10, 25, 35, 50). As the only tachykinin-containing nerves innervating the airways of normal, uninflamed guinea pigs are capsaicin-sensitive C-fibers (21, 32, 49, 56), these data provide overwhelming evidence that acid evoked coughing in conscious guinea pigs is dependent upon activation of capsaicin-sensitive, tachykinin-containing vagal afferent C-fibers innervating the airways.

In contrast to conscious guinea pigs, anesthetized cats, dogs and guinea pigs do not cough upon airway C-fiber activation (4, 29, 52, 53). Both capsaicin and bradykinin, selective stimulants of airway C-fibers, are consistently ineffective at evoking cough in anesthetized animals. Rather, bradykinin and capsaicin challenge may be acutely inhibitory to the cough reflex. Similarly, superior laryngeal nerve transection in guinea pigs, which primarily carry capsaicin-sensitive C-fibers innervating the larynx and trachea, potentiates coughing evoked by citric acid in conscious guinea pigs (4, 28, 51). Acid consistently elicits coughing and other C-fiber mediated reflexes are routinely evoked following anesthesia (3, 4, 29, 36, 40, 52, 53), so anesthesia does not prevent coughing, C-fiber activation or all C-fiber mediated reflexes. Thus, the C-fiber dependent component of acid induced coughing is sensitive to general anesthesia,
while a C-fiber independent component of the cough reflex is likely essential to acid evoked coughing.

Using in vitro electrophysiological analyses and functional studies of coughing in anesthetized guinea pigs, we recently described the cough receptors (4). Cough receptor activation results directly in coughing. These vagal afferents are capsaicin-insensitive but exquisitely sensitive to acid and punctuate mechanical stimuli. They are easily differentiated from C-fibers and rapidly (RARs) and slowly (SARs) adapting stretch receptors innervating the airways and lungs based on their action potential conduction velocity and their insensitivity to capsaicin, bradykinin, airway smooth muscle contraction, airway distension, and negative luminal pressures. Distributed primarily in the extrapulmonary airways and sensitive to both acid and punctuate mechanical stimuli, these vagal afferents are ideally situated and responsive to initiate coughing upon aspiration.

Our previous studies show that cough receptor activation is both sufficient and necessary for evoking cough from the trachea or larynx of anesthetized guinea pigs. Selectively activating the capsaicin-sensitive nerves innervating the trachea and larynx is consistently ineffective at evoking cough in anesthetized guinea pigs. We have also shown, however, that C-fiber activation may sensitize airway reflexes including cough, and coincident C-fiber activation by acid may regulate citric acid evoked cough in guinea pigs (42). We hypothesize that acid applied topically to the airway mucosa acts directly on the cough receptors to initiate coughing. In this study we further characterize the mechanisms of acid evoked coughing in anesthetized guinea pigs.
METHODS

All of the experiments described in this study were carried out using anesthetized male Hartley guinea pigs (300-500g, pathogen-free, Harlan) and were first approved by the Johns Hopkins Animal Care and Use Committee. Guinea pigs were anesthetized with 1.5g/ kg urethane (ip). The dose of urethane used produces a deep anesthesia lasting up to 9 hours, although experiments rarely lasted more than an hour. We confirmed adequate anesthesia throughout the experiment by monitoring withdrawal responses to a sharp pinch of a hindlimb or responses during surgical preparation. Additional anesthetic would have been provided if any responses to these stimuli were noted (no animals required additional anesthetic during the course of these experiments). When the experiments were completed, animals were killed by asphyxiation in a vessel filled with carbon dioxide followed by exsanguination. The American Veterinary Medical Association (AVMA) recommends this method of euthanasia.

We monitored coughing in anesthetized guinea pigs as described previously (4). Guinea-pigs were anaesthetised and secured supine on a warming pad. A midline incision in the neck exposed the trachea, which was cannulated at its caudal most end with a bent leur stub adaptor. The cannula, placed approximately equidistant from both the larynx and carina (1-2cm), was attached to a length of tubing that terminated inside a water-jacketed organ bath continuously filled with humidified and warmed air (The tubing and organ bath approximate the warming and humidifying functions and the dimensions and resistance of the nose). The innervation and vasculature of the trachea were carefully preserved throughout the dissection. A pressure transducer attached to a side port in the tracheal cannula monitored respiratory efforts, which were recorded digitally (Biopac data acquisition system, Santa Barbara, California).
Once the trachea was cannulated, the remaining rostral segment of the extrathoracic trachea was opened lengthwise with an incision along its ventral most aspect. Polyethylene (PE) tubing was threaded through the larynx and nasal cavity and out of a nostril. Warmed, oxygenated Krebs buffer (composition (mM) NaCl (118), KCl (5.4), NaHPO₄ (1), MgSO₄ (1.2), CaCl₂ (1.9), NaHCO₃ (25) and dextrose (11.1), pH=7.4) was superfused (3ml/ min) over the tracheal mucosa. The buffer was introduced into the tracheal lumen from the caudal most exposed segment of the trachea, and removed at the rostral end of the trachea by attaching the PE tubing threaded through the upper airways to a gentle suction source. Unless otherwise stated, the buffer contained 3µM indomethacin to block formation of neuromodulatory prostanoids.

Upon completion of the dissection, animals were allowed to breathe spontaneously and without any further manipulations for 10 minutes. Thereafter, we attempted to evoke cough by applying citric acid topically to the tracheal mucosa. Citric acid (0.001-2M) was dissolved in water and applied in 100µL aliquots, directly into the Krebs buffer perfusing the trachea. Concentration response curves were constructed in an ascending fashion, with each dose administered at 1 or 5 minute intervals. Cough was defined based on visual confirmation of a cough-like respiratory effort, and based on a change in tracheal pressure that produced a ≥500% increase in peak expiratory pressure preceded by an enhanced inspiratory effort all in <1 second.

We tried to modulate coughing evoked by topically applied citric acid with drugs added directly to the tracheal perfusate (thereby having a direct and selective effect in the trachea and no systemic effects) or by disrupting the extrinsic innervation of the trachea or by removing the tracheal epithelium. Interventions were carried out 15 minutes prior to constructing the citric acid concentration response curves, and unless otherwise stated, were maintained throughout the citric acid challenge. The superior (SLNs) or recurrent (RLNs) laryngeal nerves were transected
bilaterally. The epithelium was removed by rubbing the tracheal mucosa with a cotton swab and confirmed by microscopy and staining at the end of the experiments. Drugs evaluated for their effects on citric acid induced cough included 1µM tetrodotoxin (voltage sensitive Na+ channel blocker), 10µM capsazepine (TRPV1 antagonist), 0.1µM iberiotoxin (KCa channel blocker), 1mM 4-aminopyridine (Kv channel blocker), 10µM albuterol (β2 adrenoceptor agonist), 10µM isoproterenol (nonselective β-adrenoceptor agonist), 1µM atropine (muscarinic receptor antagonist), 3µM indomethacin (cyclooxygenase inhibitor), 0.1mM meclofenamic acid (studied in the presence of indomethacin. At this concentration, this cyclooxygenase inhibitor is reported to block the acid-sensitive ASIC3 channels; 59), 0.1µM FR 173657 (bradykinin B2 receptor antagonist), 0.1mM amiloride (Na+ and ASIC channel blocker), a combination of 3µM pyrilamine (histamine H1 receptor antagonist) and 3µM IC198615 (cysLT1 receptor antagonist – added in combination to block mast cell mediated effects), and a combination of CP99994, SR48968 and SB223412 (0.1µM each, neurokinin1 (NK1), NK2 and NK3 receptor antagonists, respectively). The appropriateness of these concentrations was either confirmed in previous studies using a similar preparation or were assumed based on in vitro studies (5, 6, 12, 14, 31, 41, 43).

We also determined the effects of altering the ionic composition of the Krebs buffer perfusing the trachea or prior capsaicin desensitization on citric acid evoked coughing in anesthetized guinea pigs. In some experiments, Ca++ was removed from the tracheal perfusate by omitting CaCl2 in the Krebs and adding EDTA (57). Replacing NaCl and KCl with Na citrate and KH2PO4, respectively, was used to assess the role of chloride in regulating the responses to acid (37, 45). Capsaicin desensitization was carried out by adding 10µM capsaicin to the tracheal perfusate (4). This evoked a transient slowing of respiration that gradually recovered,
after which the concentration response curve to citric acid was constructed (also see ref. 4).

Capsaicin was left in the perfusate throughout the citric acid challenges.

Vehicle control and negative control experiments were carried out in parallel. Vehicle control experiments were carried out for every intervention studied. Negative control experiments included studies of the effects of water on the cough response (the vehicle for citric acid), the effects of sodium citrate, and the effects of hydrochloric acid.

Statistical Analyses

A nonpaired experimental design had to be employed. Results are presented as a mean±sem of n experiments where n is a single animal. Differences amongst group means were assessed by one-way ANOVA and Sheffe’s f-test for unplanned comparisons. P-values of <0.05 was considered statistically significant. Rarely (<10% animals), guinea pigs did not cough during surgery or in response to citric acid or had basal respiratory rates of ≤45 breaths/minute. These animals were excluded from subsequent analyses.

Reagents

Tetrodotoxin, albuterol, isoproterenol, atropine, capsaicin, amiloride, pyrilamine, meclofenamic acid and indomethacin were purchased from Sigma. Capsazepine was purchased from Tocris. Iberiotoxin, SR48968 and SB223412 were gifts from GlaxoSmithkline. Schering Plough Research Institute and Fujisawa Pharmaceuticals provided CP99994 and FR173657, respectively. Zeneca provided ICI198615.
RESULTS

Citric acid applied topically to the tracheal mucosa of anesthetized guinea pigs evoked coughing (figure 1). The threshold concentration of citric acid for evoking cough was ~0.03M (64% of control animals coughed in response to ≥0.03M citric acid (range: 0-4 cumulative coughs) whereas only 14% of control animals coughed in response to ≤0.01M citric acid; n=83). All concentrations of citric acid ≥0.1M evoked 1-2 coughs even though the slowing of respiration evoked by the acid became progressively greater in magnitude as the concentration of acid increased (figure 2). At a single suprathreshold concentration (0.1M), increasing the volume of citric acid applied topically to >50µL failed to evoke more coughs even though the resulting effects on respiration became increasingly pronounced (figures 2 and 3). The coughing evoked by citric acid always occurred during the initial 5-10 seconds of bolus application onto the tracheal mucosa and was mimicked by hydrochloric acid but not by sodium citrate (figure 2). The vehicle for citric acid in these experiments, water, evoked no coughing when applied in volumes ≤1mL and evoked only modest effects on respiration (figures 2 and 3).

Increasing the time between citric acid challenges from 1 to 5 minutes failed to shift the acid concentration response curve. By contrast, constructing a second concentration response curve 10 minutes after completing the first curve revealed a marked desensitization of the subsequent response to acid (figure 4). Tetrodotoxin administered to the tracheal perfusate markedly inhibited citric acid evoked coughing. Severing the recurrent laryngeal nerves bilaterally abolished citric acid evoked coughing whereas bilateral cuts of the superior laryngeal nerves were without effect (figure 5).
Role of the Epithelium, Airway Smooth Muscle and Autacoids in Citric Acid Evoked Coughing.

Removing the tracheal epithelium by gently rubbing the mucosa for 20-30 seconds with a cotton swab evoked 4.5±0.7 coughs (n=8) and exposed the peripheral terminals of the cough receptors as revealed by intravital labeling with the styryl dye FM2-10. As expected, this had no effect on subsequently evoked coughing induced by citric acid applied topically (figure 6). Similarly, indomethacin, atropine, methacholine, isoproterenol, albuterol, FR173657 and a combination of pyrilamine and ICI198615, all of which were administered directly to the tracheal perfusate, did not modulate citric acid induced coughing, suggesting that locally produced prostanoids or bradykinin, airway smooth muscle contraction, mucus secretion, changes in mucosal blood flow/clearance or mast cell mediator release have no acute effects on topically applied citric acid evoked coughing in anesthetized guinea pigs (Table 1).

Role of Capsaicin-Sensitive C-Fibers in Citric Acid Evoked Coughing.

Prior capsaicin desensitization did not inhibit citric acid evoked cough in anesthetized guinea pigs. Similarly neither capsazepine nor a combination of NK1, NK2 and NK3 receptor antagonists applied topically to the tracheal mucosa inhibited citric acid induced coughing in anesthetized guinea pigs (Table 1).

Ionic Mechanisms of Citric Acid Evoked Coughing.

Citric acid evoked coughing was unaffected by iberiotoxin or removing Ca++ from the tracheal perfusate (Table 1). By contrast, lowering the chloride concentration in the tracheal perfusate markedly potentiated citric acid evoked coughing. Whereas 0 out of 207 animals in the various control and other treatment groups studied coughed in response to 0.001M citric acid, 5/6
animals coughed in response to 0.001M citric acid when the concentration of chloride in the Krebs perfusing the trachea was lowered (2.2±0.9 coughs; Figure 7). We also attempted to assess the effects of Kv channel blockade on citric acid evoked coughing. We found, however, that Kv channel blockade with 1mM 4-aminopyridine evoked spontaneous coughing in 4/5 animals (see below). Thereafter, the guinea pigs were generally unresponsive to acid or to mechanical and electrical stimuli used to evoke coughing.

The rapid adaptation to citric acid seen in this study of the cough reflex is suggestive of a role for Acid Sensing Ion Channels (ASICs; 31). Consistent with this notion, 100µM amiloride partially inhibited citric acid induced coughing (Table 1, Figure 7). We could not study a higher concentration of amiloride as this compound is reported to block TTX-sensitive sodium channels at concentrations >100µM (6). Therefore, we studied the effects of the cyclooxygenase inhibitor meclofenamic acid (59), which, along with several other compounds of this class, can block ASIC currents in patch clamp studies in rat DRG neurons and in COS cells transfected with ASIC3 (indomethacin, which does not inhibit ASIC currents, was added to the tracheal perfusate at a concentration of 3µM in all of these studies; see methods). At a concentration (1mM) that was reported to inhibit acid induced inward currents in patch clamp analysis by ~90%, meclofenamic acid had no effect on citric acid induced coughing in the anesthetized guinea pig (figure 7).

Spontaneous Coughing and Augmented Breaths in Anesthetized Guinea Pigs.

Nearly all animals coughed when surgically preparing the trachea for these experiments. Subsequently, spontaneous coughs were observed only rarely. Only 5 out of 50 representative control animals coughed spontaneously during the equilibration period or in between citric acid doses, with 3 of the 6 coughs not evoked by citric acid attributed to a mechanical event (e.g.
adjustment of the perfusion system or tissue restraints, turbulence in the tracheal perfusate).

Amongst 60 animals undergoing many of the interventions described above (e.g. nerve transection, various drugs added to the tracheal perfusate), only 5 animals coughed independent of the citric acid challenge (one animal had 2 spontaneous coughing bouts). Most (7 of 11) of these spontaneous coughing bouts consisted of a single cough. Repetitive coughing could be clearly attributed to adding 1mM 4-aminopyridine to the tracheal perfusate (4 of 5 animals coughed 4.8±2.6 times) or to lowering the Cl- concentration in the tracheal perfusate (1 of 6 animals studied coughed 12 times in rapid succession within 10 seconds of lowering the Cl-concentration in the buffer). We had reported previously that no animals (n=25) coughed after adding 3-10µM capsaicin to the tracheal perfusate (4, 42). In the present study, adding 10µM capsaicin to the tracheal perfusate for 10 minutes did evoke sporadic coughing in 5 of 9 animals (3±1 coughs in the 5 responders, 1.7±0.8 coughs overall; n=9), with the coughing beginning on average 96±56 seconds after initiating the challenge. Removing Ca++ from the tracheal perfusate and epithelium removal were associated with spontaneous coughing in 2 of 8 animals in each treatment group, but cause and effect was not obvious.

Augmented breaths (sighs) were also rarely observed during the equilibration period in any of the treatment groups. Just 10 of 50 representative control animals and 26 of 84 animals undergoing any of the interventions described in this study produced 2 or more augmented breaths during the 10 minute equilibration period. Only capsaicin (8 of 9 animals studied) appeared to consistently increase the frequency of augmented breaths. Citric acid challenges did, however, initiate many augmented breaths in nearly all of the treatment groups, either instead of coughing (particularly at lower concentrations of the acid) or subsequent to the coughing and the slowing of respiration evoked by the acid (see figure 1). All of 50 representative control animals
and 69 of 84 animals undergoing any of the interventions described above had augmented
breaths during the citric acid challenge. The majority (>80%) of animals studied produced
multiple (2-14) augmented breaths during the citric acid challenges. Severing either the RLNs or
SLNs, or TTX pretreatment decreased the number of augmented breaths induced by citric acid
challenge (8 of the 11 animals in these treatment groups sighed ≤1 time and 6 of these 11 did not
sigh at all during the citric acid challenges).

*Respiratory Slowing and Apnea Evoked by Citric Acid.*

As seen in Figures 1-3 and discussed above, subsequent to the evoked cough, citric acid
at concentrations ≥0.01M produced respiratory slowing and apneas that became progressively
larger as the concentration of citric acid increased. Following challenge with citric acid at
concentrations >0.1M, basal respiratory rate became depressed and never returned to that
measured during the equilibration period. These effects of acid on respiratory rate, like cough,
were largely unaffected by most of the interventions used in this study (figure 8). The slowing of
respiration induced by acid was, however, potentiated by removing Ca++ or lowering the Cl-
concentration in the tracheal perfusate. RLN or SLN transection, TTX or amiloride reduced the
effects of acid on respiration. The effects of citric acid on respiratory rate and apnea were
mimicked by capsaicin. Unlike citric acid induced coughing, which almost completely
desensitized in successive concentration response curves, the effects of acid on respiratory rate
were much less affected. Other than capsaicin and lowering the Cl- concentration in the
perfusate, none of the interventions consistently altered basal respiratory rate, which averaged
between 55-70 breaths/ minute in all treatment groups.
DISCUSSION

The data indicate that citric acid induced coughing in anesthetized guinea pigs is dependent upon activation of capsaicin insensitive vagal afferent nerves innervating the trachea and larynx. Capsaicin sensitive nerve activation is not required for initiating the cough reflex to acid in these anesthetized animals. Acid appears to act directly on the cough receptors. Indirect effects such as smooth muscle contraction, mucus secretion, inflammatory mediator release, axonal reflexes, vascular engorgement or edema within the airways do not appear to contribute to the cough response to acid in the anesthetized guinea pigs. Finally, the data also provide circumstantial evidence for a limited role for Acid Sensing Ion Channels (ASICs) but a prominent role for chloride in regulating the acid induced effects reported in this study.

The Role of Cough Receptor Activation in Citric Acid Induced Coughing.

We have shown previously that severing the recurrent laryngeal nerves prevents coughing evoked by electrical or mechanical stimulation of the tracheal or laryngeal mucosa of anesthetized guinea pigs whereas cutting the superior laryngeal nerves had no effect on coughing (4). Subsequent electrophysiological analyses revealed that the RLNs primarily carry the axons of afferents innervating the rostral (extrathoracic) trachea and larynx arising from the nodose ganglia whereas the capsaicin sensitive afferents innervating the rostral trachea and larynx project to these airways primarily via the SLNs. The laryngeal and tracheal afferents arising from both the jugular and nodose ganglia are activated by acid, but the afferents arising from the nodose ganglia are insensitive to capsaicin (31, 49). As shown here and previously, acid but not capsaicin applied topically to the tracheal mucosa consistently evokes coughing in the anesthetized guinea pigs. These data provide conclusive evidence that acid induced coughing in
anesthetized guinea pigs is mediated by the capsaicin-insensitive tracheal and laryngeal afferents arising from the nodose ganglia.

Coughing evoked by inhalation of citric acid in conscious guinea pigs is mediated in large part by activation of capsaicin-sensitive, tachykinin-containing C-fibers (1, 2, 7-10, 18, 25, 34, 35, 54, 55). In anesthetized guinea pigs, capsaicin-sensitive nerve activation can also sensitize subsequently evoked coughing reflexes and reflex bronchospasm initiated by activation of the cough receptors or airway mechanoreceptors, respectively (40, 42). However, the results of the present study indicate that capsaicin sensitive nerve activation by citric acid applied topically to the tracheal mucosa is neither necessary nor sufficient for initiating cough in anesthetized guinea pigs. This assertion is based on 5 points: 1) Neither capsaicin nor bradykinin reliably induce coughing in anesthetized guinea pigs no matter how they are delivered (4, 42), 2) Prior capsaicin desensitization does not inhibit citric acid induced coughing (4, 42, 50; present study), 3) Capsaicin and citric acid induced coughing in conscious guinea pigs is inhibited by peripherally acting and/or inhaled neurokinin receptor antagonists (3, 18, 55). We have shown that a combination of NK₁, NK₂ and NK₃ receptor antagonists added either to the tracheal perfusate or administered systemically do not inhibit citric acid induced coughing in anesthetized guinea pigs (present study; 42), 4) The majority of the capsaicin sensitive nerves innervating the larynx and rostral trachea project to these airways via the superior laryngeal nerves (SLNs) and terminate in the epithelium (4, 21). Neither severing the SLNs nor epithelium removal inhibited the acid–induced coughing described in the present study, and 5) Acid-induced activation of capsaicin sensitive nerves innervating the trachea and larynx is inhibited by capsazepine (14, 31). Capsazepine did not inhibit acid-induced coughing in the present study.
Some data presented here and published previously could be interpreted as evidence that capsaicin-sensitive nerve activation may be acutely inhibitory to the cough reflex in anesthetized animals (4, 29, 52, 53). This may be a unique feature of coughing studied during unconsciousness, but it may also reveal an additional important regulatory mechanism for the cough reflex. We found that consecutive citric acid concentration response curves were markedly different, with the second curve producing little or no coughing. This was not due simply to the duration of anesthesia, as the number of coughs evoked cumulatively by citric acid did not differ if the time interval between doses was increased from 1 to 5 minutes. It is possible that this adaptation is due to a desensitization of the airway afferent nerves to acid, but previous studies indicate that the response of airway vagal afferents to acid is stable over time (31) and in the present study the effects of acid on respiration were reduced but not abolished in successive citric acid concentration response curves. Capsazepine, which inhibits sustained activation of airway C-fibers by acid (31), actually potentiated citric acid-induced coughing in this study in anesthetized guinea pigs. Perhaps capsaicin sensitive nerve activation produces an acute, centrally mediated inhibition of cough. Higher concentrations of citric acid do evoke a marked slowing (depression or inhibition) of respiration and apnea, and it is certain that some brainstem pathways regulate both respiration and cough. These acid-induced effects on respiration were mimicked by capsaicin and attenuated by RLN or SLN transection. The SLNs project primarily capsaicin sensitive C-fibers to the trachea and larynx and as reported here and previously, SLN transection if anything potentiates coughing evoked in guinea pigs (4, 29, 52, 53).

Sustained activation of capsaicin-sensitive nerves evoked by acid is mediated by TRPV1 activation, while acute effects of bolus acid challenges of capsaicin sensitive nerves has been attributed primarily to TRPV1-independent mechanisms, perhaps involving ASICs (31). We
speculate that acid induced activation of capsaicin sensitive nerves through TRPV1 independent mechanisms accounts for the slowing of respiration evoked by acid observed in the present study. The effects of acid on respiration were enhanced by removing Ca++ from the tracheal perfusate (which greatly enhances the excitability of C-fibers while having no effect on cough receptor excitability; 57) and mimicked by capsaicin. Moreover, the ASIC channel blocker amiloride attenuated the effects of acid on respiratory rate while capsazepine had little if any measureable effects on the respiratory responses to acid. But our capsaicin desensitization protocol failed to prevent the acid induced effects on respiration. It is thus possible that desensitization to capsaicin does not prevent acid induced activation of capsaicin sensitive nerves, or that cough receptor activation or coughing itself may modulate respiration in these anesthetized guinea pigs.

Unlike in our previous studies in anesthetized guinea pigs and studies by other investigators in cats and dogs, we found that capsaicin evoked sporadic coughing in about half of the anesthetized guinea pigs used in this study. The coughing evoked was modest at best, on average evoking less than 2 coughs/guinea pig during a 10 minute continuous challenge with a supramaximally effective concentration of the vanilloid. It is interesting that the coughing occurred well after (about 100 seconds) initiating the capsaicin challenge, when afferent discharge would have slowed considerably due to desensitization. We speculate that the coughing evoked by capsaicin in this study occurred secondary to reflex effects (e.g. mucus secretion) induced in the lower airways resulting in a mechanical activation of the cough receptors innervating the intrathoracic trachea and extrapulmonary bronchi. It is unclear why in our previous experiments with anesthetized guinea pigs such coughing was not observed.
Mechanism of Cough Receptor Activation.

Previous studies of acid-induced responses in the airways have implicated a role for the axon reflex precipitated by C-fiber activation, de novo bradykinin formation, mast cell activation and/or airway parasympathetic reflexes (9, 33, 48). Cough receptors are not activated by smooth muscle contraction or stretch or a variety of transmitters and autacoids (4). Not surprisingly, therefore, citric acid induced coughing was unaffected by pretreatment with atropine, the β-agonists albuterol and isoproterenol, the cyclooxygenase inhibitor indomethacin, the bradykinin B2 receptor antagonist FR173657, the combination of pyrilamine and ICI198615 (histamine and cysteinyl-leukotriene receptor antagonists, respectively) or depletion of extracellular Ca++. This reveals an additional distinction between airway C-fibers and the cough receptors, as airway C-fibers can be directly activated by some autacoids (e.g. 5-HT, adenosine, bradykinin, ATP, anandamide, 15-HETE; 23, 25, 37, 56) and sensitized by others (e.g. bradykinin, histamine, PGE2, adrenaline; 8, 13, 17, 19, 30, 36). Rather, the effects of acid on the cough receptors appeared to be direct. This along with the effects of anesthesia on coughing highlights an important difference between our model for studying cough and cough evoked by aerosolized irritants in the conscious setting. With aerosol challenge, the coughing evoked by acid will be proportional to the concentration of acid, the amount of acid deposited in the large airways, the rate of acidification adjacent to the airway afferent nerve terminals, the mechanical effects (e.g. mucus secretion, vascular engorgement) evoked reflexively by the acid, and the duration of the acidification and mechanical effects evoked. It is likely, therefore, that smooth muscle contraction, vascular leakage, mucosal blood flow and mucus secretion and the cells and mediators that modulate these processes play a role in regulating responsiveness to inhaled citric acid in conscious animals.
The data presented suggest that the response to acid in the capsaicin insensitive cough receptors may be mediated in part by ASICs. Amiloride attenuated acid induced coughing and like ASICs, the response to acid rapidly adapted regardless of acid concentration or volume. The evidence is far from conclusive, however, as the effects of amiloride were subtle at best (higher doses of amiloride could not be evaluated due to their inhibitory effects on voltage-sensitive Na+ channels; 6). Also, meclofenamic acid, which has been reported to block the amiloride sensitive ASIC3 channel of rats in patch clamp analysis (59), had no effect on the citric acid evoked cough. The lack of effect of removing Ca++ from the tracheal perfusate also argues against ASICs in mediating the coughing initiated by acid (24). Perhaps other acid sensitive ion channels (e.g. ENaC, TASK, Kv, ClC) contribute to the cough receptor dependent cough initiated by citric acid (20, 39, 47, 61).

We also found that reducing the chloride (Cl-) in the buffer perfusing the trachea markedly potentiated citric acid induced coughing and induced spontaneous coughing in one animal. Although the results have been variable, furosemide, the Na+-K+-2Cl- transport inhibitor has been reported to have some inhibitory effects on coughing evoked in human subjects and in guinea pigs (26, 44, 58). A preliminary report suggests that both furosemide and niflumic acid, a Cl- channel blocker, can inhibit citric acid induced coughing in anesthetized guinea pigs using a design identical to the one used in this study (SB Mazzone, unpublished observations). In vitro studies of tracheal afferent nerve excitability have also shown inhibitory effects of the diuretic furosemide on nerve activation, although acid evoked activation was not studied (11). Perhaps Cl- channels regulate acid induced activation of the cough receptors. Precisely how Cl- channels are activated by acid and which Cl- channels are activated is unclear. The Cl- channel blocker niflumic acid was without effect on acid evoked responses of capsaicin
sensitive nerves innervating the guinea pig trachea in vitro, but markedly inhibited bradykinin- 
evoked activation of these neurons (37). The effect of niflumic acid on the responses evoked by 
bradykinin is thought to be due to effects on Ca++ activated Cl- channels (45). Since we found 
that removing Ca++ from the tracheal perfusate had no effect on acid-evoked coughing, there 
may either be sufficient extracellular Ca++ in the tissue to initiate Cl- channel activation or that 
the effects of removing Cl- from the perfusate are unrelated to effects on Ca++ activated Cl- 
channels.

Voltage-sensitive (Kv) potassium channels also regulate cough receptor excitability (43). 
The Kv channel blocker 4-AP evoked spontaneous coughing in anesthetized guinea pigs. By 
contrast, maxiK (or BK or Kca) channels do not appear to play a significant role in regulating 
cough receptor responsiveness to acid, as iberiotoxin had no effect on the acid induced coughing 
at concentrations which are known to modulate nerve mediated responses in guinea pigs (12). 
Moreover, it has been reported that meclofenamic acid can activate BK channels (and modulate 
activity of other potassium channels independent of its actions on cyclooxygenase; 16, 38, 46, 
60), and it also had no effect on acid induced coughing in this study. Removing Ca++ from the 
tracheal perfusate, which is known to sensitize airway C-fibers (57), was also without effect on 
the response to acid. This provides further evidence that neither extracellular Ca++ nor Ca++ 
influx modulates cough receptor excitability.

The data summarized here and elsewhere reveal potential therapeutic strategies for 
treating cough. The coughing initiated by aspiration of gastric fluid or refluxate or accumulated 
secretions is likely mediated by direct activation of the cough receptors either by acid or by 
mechanical effects. Failure to cough in response to such stimuli can have dire and potentially 
fatal acute consequences, so targeting the cough receptors for chronic therapy should be avoided.
In contrast, the data here and elsewhere suggest a modifying and amplifying role for capsaicin-sensitive nerves in regulating cough and other defensive respiratory reflexes. These afferents may be ideally targeted in chronic, poorly controlled cough, as they are sensitive to acid and a variety of inflammatory mediators in the airways including bradykinin, 15-HETE (a primary product of arachidonic acid formed by airway epithelial cells and an agonist for TRPV1; 22, 23, 40), 5-HT, adenosine and ATP (11-14, 49, 56). They may also express unique ion channels that can be targeted in chronic cough, including TRPV1 and TTX-insensitive Na+ channels. The preparation described here may prove useful in evaluating the local, systemic and central effects of potential therapies for cough.
REFERENCES


13. **Fox AJ, Laloo UG, Belvisi MG, Bernareggi M, Chung KF, and Barnes PJ.**


42. **Mazzone SB, Mori N, and Canning BJ.** Synergistic interactions between airway afferent nerve subtypes regulating the cough reflex in guinea-pigs. *J Physiol* 569(Pt 2): 559-573, 2005.


46. **Peretz A, Degani N, Nachman R, Uziyel Y, Gibor G, Shabat D, and Attali B.**


FIGURE LEGENDS.

Figure 1. Representative traces of citric acid evoked coughing in anesthetized guinea pigs.
Citric acid (0.001-2M) was applied topically in 100µL aliquots directly to the tracheal mucosa of anesthetized guinea pigs. The extrathoracic trachea was continuously perfused with Krebs bicarbonate buffer throughout the experiments. Concentrations of citric acid ≥0.03M consistently evoked 1-2 coughs within seconds of application to the tracheal mucosa. A) Lower concentrations of acid had little if any effect on respiratory rate, B) but higher concentrations of the acid initiated a slowing of respiration and apnea after initiating cough. Respiratory rate gradually increased after augmented breaths, which occurred following the acid challenges.

Figure 2: The effects of citric acid on coughing and respiratory rate in anesthetized guinea pigs are mimicked by HCl but not by sodium citrate or water. A) Cumulative coughs evoked by citric acid (n=83), sodium citrate (n=3) and HCl (n=3). Concentration response curves were created cumulatively, with increasing concentrations added in 100µL aliquots at 1 minute intervals. B) Effects of citric acid on respiratory rate. The data presented are the mean±sem of 19 representative control experiments. C) The number of coughs evoked by each dose of citric acid, applied in 100µL aliquots. These data are taken from 33 representative control experiments. D) The number of coughs evoked by a single, suprathreshold (0.1M) concentration of citric acid, applied in increasing volumes at 1 minute intervals (n=4). The effects of water (the vehicle for citric acid) applied in similar volumes are also presented (n=4).
Figure 3. Citric acid but not water applied topically to the tracheal mucosa evokes profound and immediate effects on respiratory rate in anesthetized guinea pigs. A) 0.1M citric acid was applied topically at 1 minute intervals and in progressively increasing volumes. Citric acid applied in any volume evoked coughing (see figure 2), and coincidentally, respiratory slowing was evoked (n=4). B) Water applied topically to the tracheal mucosa in volumes up to 1 mL evoked no coughing and produced only modest, slowly developing decreases in respiratory rate (n=4).

Figure 4: Citric acid induced coughing in anesthetized guinea pigs is desensitized following repeated challenges. A) Coughing was evoked by citric acid (0.001-2M) applied topically in 100µL aliquots in 1 (n=19) or 5 (n=26) minute intervals. The cumulative number of coughs evoked by citric acid is depicted in the graph. There was no effect on the potency or efficacy of citric acid for evoking cough when the time between challenges was altered. B) Repeating a citric acid induced cough response curve 10 minutes after constructing a similar curve results in little or no coughing in anesthetized guinea pigs (n=5).

Figure 5: Coughing evoked by citric acid applied topically to the tracheal mucosa in anesthetized guinea pigs is abolished by recurrent laryngeal nerve transection and by tetrodotoxin. Citric acid was applied topically in 100 µL aliquots at increasing concentrations and at 1 minute intervals. The cumulative number of coughs evoked by citric acid is depicted in the graph. Prior to citric acid challenge, the recurrent (RLN; n=4) or superior (SLN; n=3) laryngeal nerves were transected or tetrodotoxin (n=6) was added to the tracheal perfusate. Control experiments (n=19) were carried out in parallel. RLN transection and tetrodotoxin
abolished or nearly abolished (*) citric acid induced coughing whereas SLN transection had no inhibitory effect on the coughing evoked by citric acid.

**Figure 6: Epithelium removal does not prevent citric acid induced coughing evoked from the trachea of anesthetized guinea pigs.** A) Representative tracing showing the coughing evoked during epithelial removal prior to citric acid challenge. The epithelium was removed from the trachea by rubbing the mucosa with cotton swab. B) Intravital labeling of the tracheal mucosa with intact epithelium with FM2-10 reveals intense fluorescent labeling of the epithelium. C) Following epithelium removal, FM2-10 brilliantly and selectively labels afferent nerve terminals in the mucosa. Previous studies have shown that these labeled terminals correspond to the peripheral terminations of the cough receptors. Calibration bar: 50µm. D) Epithelium removal was without effect on citric acid evoked coughing in anesthetized guinea pigs. The results are the mean±sem of 8-14 experiments.

**Figure 7: Lowering the chloride concentration in the tracheal perfusate potentiates citric acid evoked coughing whereas amiloride partially attenuates citric acid induced coughing in anesthetized guinea pigs.** A) Citric acid was applied topically in 100 µL aliquots at increasing concentrations and at 1 minute intervals. The cumulative number of coughs evoked by citric acid is depicted in the graph. Prior to constructing the citric acid concentration response curve in some animals, the tracheal perfusate was changed to a modified Krebs buffer containing no Ca++ and EDTA (n=6), or to buffer containing low chloride content (NaCl and KCl were replaced with Na citrate, and KH2PO4, respectively; n=6). Control experiments (n=19) were carried out in parallel. Neither modified buffer altered significantly the number of coughs evoked cumulatively by citric acid. However, lowering the chloride concentration did increase
(*) the potency of citric acid for evoking cough and both modified buffers enhanced the effects of citric acid on respiratory rate (see figure 8). C) Pharmacologic attempts at blocking Acid Sensing Ion Channels (ASICs) with a high concentration of meclofenamic acid (n=4) and amiloride (n=8) were also without effect on the total number of coughs evoked cumulatively by citric acid. In all of these experiments, indomethacin was added to the tracheal perfusate, thereby rendering the actions of meclofenamic acid on cyclooxygenase redundant. D) Amiloride (*) but not meclofenamic acid partially attenuated citric acid evoked coughing evoked by threshold concentrations of the acid. See text for further details.

Figure 8: Modulation of basal respiratory rate and the citric acid induced slowing of respiration in anesthetized guinea pigs. A) Adding capsaicin to the tracheal perfusate (n=7) or lowering the chloride concentration in the perfusate (n=6) abrultly and significantly slowed respiratory rate in anesthetized guinea pigs. B) Lowering the chloride concentration in the perfusate also potentiated the effects of citric acid on respiration (the response to 0.001M citric acid is shown. Comparable effects were seen with all concentrations of citric acid). C) By contrast, recurrent (RLN; n=4) or superior (SLN; n=3) laryngeal nerve transection or TTX pretreatment (not shown; n=6) attenuated the effects of citric acid on respiration, both the acute effects of the acid (the responses to 1M citric acid are shown and are representative) and the sustained slowing of respiration evoked by the repeated acid challenges (note the differences in basal respiratory rates prior to the acid challenge). D) Removing Ca++ from the tracheal perfusate (n=6) increased the magnitude and duration of the effects of citric acid on respiration whereas amiloride (n=8) attenuated the effects of citric acid on respiration. Upon repeated exposures to the acid, the effects on respiration were reduced but not abolished (n=8). Control experiments were carried out in parallel for each treatment studied (n≥6).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>0.001-0.03M</th>
<th>0.001-0.1M</th>
<th>0.001-2M</th>
<th>n</th>
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<tr>
<td>Control</td>
<td>1.2±0.03</td>
<td>4.7±0.3</td>
<td>11.8±0.6</td>
<td>83</td>
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<td>-RLN</td>
<td>0±0</td>
<td>0±0*</td>
<td>0.3±0.3*</td>
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<tr>
<td>-SLN</td>
<td>1.3±0.3</td>
<td>5±1</td>
<td>13±1</td>
<td>3</td>
</tr>
<tr>
<td>1µM Tetrodotoxin</td>
<td>0±0</td>
<td>0.3±0.3*</td>
<td>3±2*</td>
<td>4</td>
</tr>
<tr>
<td>-Epithelium</td>
<td>1.9±0.6</td>
<td>5±1</td>
<td>12±2</td>
<td>8</td>
</tr>
<tr>
<td>No Indomethacin</td>
<td>1±0.4</td>
<td>4±1</td>
<td>12±2</td>
<td>3</td>
</tr>
<tr>
<td>Capsaicin Desensitization</td>
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<td>4±1</td>
<td>13±1</td>
<td>5</td>
</tr>
<tr>
<td>10µM Capsazepine</td>
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<td>20±4</td>
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<tr>
<td>0.1µM FR173567</td>
<td>1.1±0.4</td>
<td>4±1</td>
<td>11±2</td>
<td>13</td>
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<tr>
<td>Pyrilamine, ICI198615 (1µM each)</td>
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<td>8±3</td>
<td>14±5</td>
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<tr>
<td>CP99994, SR48968, SB223412 (0.1µM each)</td>
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<td>13±4</td>
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<tr>
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<td>8</td>
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<tr>
<td>0 Ca++/ EDTA</td>
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<td>4±1</td>
<td>13±2</td>
<td>6</td>
</tr>
<tr>
<td>Low Cl- Buffer</td>
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<td>7±1</td>
<td>14±2</td>
<td>6</td>
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<tr>
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<td>6</td>
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<td>7±1</td>
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<td>4</td>
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<tr>
<td>10µM Albuterol</td>
<td>1.0±0.7</td>
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<td>13±2</td>
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</table>

Table 1. Effects of chemical and physical interventions on citric acid evoked cough in anesthetized guinea pigs. Citric acid was applied topically in 100 µL aliquots at increasing concentrations and at 1 minute intervals. The cumulative number of coughs evoked by several concentration ranges of citric acid is tabulated. All drugs were added directly to the tracheal perfusate 15 minutes prior to citric acid challenge. Unless otherwise stated, 3µM indomethacin was added to the tracheal perfusate in all of these experiments. An asterisk (*) indicates a statistically significant decrease in the number of coughs evoked by citric acid relative matched controls. The mean data amongst the various control groups did not differ and were pooled for presentation purposes. See text for further details.
A 0.03M Citric Acid

B 0.3M Citric Acid

cough

augmented breath
A. Cumulative number of coughs against concentration (M) for different substances:
- Citric Acid
- HCl
- Na Citrate

B. Respiratory rate (breaths/min) over time (sec) for different concentrations of citric acid:
- 0M
- 0.001M
- 0.01M
- 0.1M
- 2M

C. Number of coughs against concentration (M) for citric acid:

D. Number of coughs against volume (µL) for 0.1M citric acid and water.
- 0.1M Citric Acid
- Water
A. 0.1M Citric Acid

B. H$_2$O

Respiratory Rate (Breaths/Min)

- 5µL
- 50µL
- 500µL
- 1000µL

Time (sec)
A

Cumulative Number of Coughs

[Citric Acid] (M)

5min interval

1min interval

B

Cumulative Number of Coughs

[Citric Acid] (M)

1st challenge

2nd challenge
Cumulative Number of Coughs

[Citric Acid] (M)

Control
RLN transection
SLN transection
1µM Tetrodotoxin
Figure A shows a recording of Epithelium Removal over 10 seconds. Figures B and C are images of epithelial tissues with and without removal, respectively. Figure D presents a graph of Cumulative Number of Coughs against [Citric Acid] (M), with two lines indicating Epithelium Intact and Epithelium Removed conditions.