Acid-sensing pathways of rat duodenum

YASUTADA AKIBA,1,2 PAUL H. GUTH,3 ELI ENGEL,4 IGOR NASTASKIN,1 AND JONATHAN D. KAUNITZ2,3

1Department of Medicine, School of Medicine, and CURE: Digestive Diseases Research Center, University of California, Los Angeles, California 90073

Acid-sensing pathways of rat duodenum. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 4): G268–G274, 1999.—We tested the hypothesis that the duodenal hyperemic response to acid occurs through activation of capsaicin-sensitive afferent nerves with subsequent release of vasodilatory substances such as calcitonin gene-related peptide (CGRP) and nitric oxide (NO). Laser-Doppler flowmetry was used to measure duodenal blood flow in urethan-anesthetized rats. Duodenal mucosa was superfused with pH 7.0 buffer with capsaicin or bradykinin or was acid challenged with pH 2.2 solution, with or without vanilloid receptor antagonists, a CGRP receptor antagonist, an NO synthase (NOS) inhibitor, or a cyclooxygenase inhibitor. The selective vanilloid receptor antagonist capsazepine (CPZ) dose dependently inhibited the hyperemic response to acid and capsaicin but did not affect bradykinin-induced hyperemia. Ruthenium red was less inhibitory than capsazepine. Selective ablation of capsaicin-sensitive nerves, CGRP-(8—37), and Nω-nitro-L-arginine methyl ester inhibited acid-induced hyperemia, but indomethacin did not. We conclude that luminal acid, but not bradykinin, stimulates CPZ-sensitive receptors on capsaicin-sensitive afferent nerves of rat duodenum. Activation of these receptors produces vasodilation via the CGRP-NO pathway but not via the cyclooxygenase pathway. Acid appears to be the endogenous ligand for duodenal vanilloid receptors.

laser-Doppler flowmetry; vanilloid (capsaicin) receptor; capsazepine; bradykinin; indomethacin

DUODENAL EPITHELIAL CELLS and underlying structures resist injury from frequent pulses of gastric acid. This resistance arises from fundamental mucosal host defense mechanisms, which are augmented in the presence of luminal acid. In the stomach, increased mucosal blood flow in response to an acid challenge is a well-accepted defense mechanism (19). The protective role of this hyperemic response to acid in duodenum is less well studied, possibly because of the close coupling between blood flow and the more-accepted duodenal defense mechanism bicarbonate secretion, which also increases after luminal acid superfusion (45). Several studies suggest that capsaicin-sensitive mucosal afferent nerves play an important role in sensing luminal acid and in mediating protective host responses to an acid challenge (27, 39). In one study, acid-induced augmentation of bicarbonate secretion was inhibited by indomethacin and ablation of capsaicin-sensitive afferent nerves (40), suggesting that the cyclooxygenase (COX) pathway is involved in the genesis of the protective response.

In the urinary bladder, airway, and heart, acid and capsaicin specifically activate afferent nerves, which release calcitonin gene-related peptide (CGRP) (12, 14, 21). This response is inhibited by the competitive capsaicin-receptor antagonist capsazepine (CPZ) but generally not by the nonselective COX inhibitor indomethacin (13, 14, 21). Nevertheless, bradykinin (BK)-induced CGRP release is inhibited by COX inhibitor (16). Comparative studies of the role of specific capsaicin receptors and COX on acid-induced hyperemia have not been performed in duodenum. Using measurement of duodenal blood flow as a convenient and reproducible means of studying the mucosal response to acid, we thus studied the roles of acid, BK, capsaicin, capsaicin-receptor antagonists, capsaicin-sensitive afferent nerves, and COX inhibition on duodenal blood flow.

Recently, a vanilloid receptor (VR1) was identified in dorsal root ganglion cells as a target molecule of capsaicin. Blot-hybridization analysis indicated that receptor mRNA was not present in intestine (6). Protons were thought to play a facilitative but not primary role in receptor activation, although a recent study documented that protons alone could activate VR1 transfected into a cell line, with half-maximal response (K0.5) at pH 5.4 and maximal response at pH 4.4 (41). This acid response curve raises the additional question as to how proton concentrations of this magnitude are achieved in body tissues, in which lactic acid is believed to take part in the modest local acidification during ischemia (37). In the upper gastrointestinal tract, where luminal acid is present at a concentration of 100 mM in the stomach and 10 mM in the duodenum (33), it is easier to imagine tissue acidification of this magnitude occurring. We previously demonstrated, for example, that superfusion with pH 2.2 solution, a non-damaging pH (29), acidified surface epithelial cells to pH ~6.2 (2), suggesting that the pH of the intercellular interstitium reaches an even lower level during luminal acid exposure. Of all organs, the upper gastrointestinal tract is the one area likely to regularly achieve proton concentrations high enough to activate capsaicin-sensitive afferent nerves in the absence of pathological conditions such as ischemia.

On the basis of the above considerations, we hypothesized that luminal protons pass through the mucosa and stimulate VR1 or a related vanilloid receptor on capsaicin-sensitive afferent nerves, producing a hyperemic response by the CGRP-nitric oxide (NO) pathway but independently of the COX and BK pathways.
MATERIALS AND METHODS

Chemicals

Capsazepine (CPZ) and ruthenium red (RR) were purchased from Research Biochemicals International (Natick, MA). Capsaicin, indomethacin, the CGRP receptor antagonist CGRP-(8–37), N G-nitro-L-arginine methyl ester (L-NAME), L-arginine (L-Arg), BK, HEPES, and other chemicals were obtained from Sigma Chemical (St. Louis, MO). Krebs solution contained (in mM) 136 NaCl, 2.6 KCl, 1.8 CaCl2, and 10 HEPES at pH 7.0. For acid superfusion, Krebs solution was titrated to pH 2.2 with 1 N HCl and adjusted to isotonicity (300 mM). Each solution was prewarmed to 37°C using a water bath, and temperature was maintained with a heating pad during the experiment. To make stock solutions, capsaicin was dissolved with 10% Tween 80, 10% ethanol, and 80% saline. CPZ was initially dissolved in DMSO. This solution was then mixed with other solvents for final concentration of 10% DMSO, 10% Tween 80, 10% ethanol and 70% saline. These solutions were diluted with Krebs buffer so that final concentrations of DMSO, Tween 80, and ethanol for superfusion were less than 0.01%. Krebs solution with 0.01% DMSO, Tween 80, and ethanol was used for vehicle superfusion.

Animal Preparation and Measurement of Duodenal Blood Flow

Male Sprague-Dawley rats weighing 225–275 g (Harlan Laboratories, San Diego, CA) were fasted overnight but had access to water. All studies were approved by the Animal Use Committee of the West Los Angeles Veterans Affairs Medical Center. Duodenal blood flow was measured with laser-Doppler flowmetry by the modified method as previously described (2). In brief, after urethane (1.25 g/kg) anesthesia, a tracheal cannula was inserted, and warmed saline was continuously infused through the left femoral vein at a rate of 1.08 ml/h with a Harvard infusion pump. Body temperature was maintained at 36–37°C by a heating pad. The abdomen was opened via a 3-cm midline incision, and the duodenum was exposed. The pylorus was tightly ligated, and the duodenum was filled with 0.5 ml saline prewarmed at 37°C. The anterior wall of the duodenum was incised between the pylorus and the papilla of Vater using a miniature electrocautery. A concave stainless steel disk (16-mm diameter and 1–2 mm deep) with a 3-mm central aperture was fixed water-tight on the mucosal surface with a silicone plastic adherent (Silly Putty, Binney & Smith, Easton, PA). The serosal surface of the duodenum was supported with the laser-Doppler flow probe (described below). A thin plastic coverslip was fixed to the disk with the silicone adherent to permit closed superfusion with solutions at a rate of 0.25 ml/min by means of a Harvard infusion pump.

For the measurement of duodenal blood flow, a right-angle probe (R-type, Transonic, Ithaca, NY) was surrounded with a silicone plastic adherent and attached on the duodenal serosa just below the chambered mucosa. Blood flow was measured as the voltage output of the laser-Doppler instrument (model BLF21, Transonic) and was expressed relative to the stable level (the basal level) 30 min after the superfusion started. Blood flow was recorded on the flow chart and determined every 5 min and expressed as a percentage of basal. We have demonstrated that a serosally placed probe can measure mucosal and submucosal blood flow in rat duodenum compared with a mucosally placed probe (2).

Experimental Protocol

After blood flow was stabilized with continuous superfusion of pH 7.0 Krebs buffer, the time was set as t = 0. The duodenal mucosa was superfused with pH 7.0 Krebs buffer from t = 0 min until t = 10 min and then either remained at pH 7.0 or was changed to pH 2.2 from t = 10 min until t = 20 min (acid challenge period), with or without the antagonists or inhibitors described below. The superfusate was changed to pH 7.0 from t = 20 min until t = 35 min (recovery period). The effects of these superfused compounds or of acid on blood flow were assessed at t = 15 min. Effects of all compounds (e.g., CPZ, L-NAME) on basal blood flow were assessed at t = 5 or t = 10 min.

Effect of intraluminal capsaicin with or without CPZ. To examine the effect of intraluminal capsaicin on duodenal blood flow, the duodenal mucosa was superfused with different concentrations of capsaicin (1 µM–1 mM) dissolved in pH 7.0 Krebs buffer. To confirm the inhibitory effects of CPZ on capsaicin-induced hyperemia in duodenum, CPZ (1 µM–1 mM) dissolved in pH 7.0 Krebs buffer was superfused for 10 min, followed by superfusion of CPZ combined with an ED 50 of capsaicin (300 µM) for an additional 10 min. Effects of capsaicin antagonists on acid- or BK-induced hyperemia. To determine the effects of capsaicin antagonists on baseline blood flow and on acid-induced hyperemia, CPZ (1 µM–1 mM), RR (0.1 mM–10 mM), or vehicle was superfused with pH 7.0 Krebs (presuperfusion) for 10 min, followed by superfusion with pH 2.2 buffer for 10 min. To test the effect of CPZ on BK-induced hyperemia, BK (0.5 mM) was superfused with pH 7.0 Krebs solution with or without CPZ (0.5 mM).

Effect of ablation of capsaicin-sensitive afferent nerves on acid-induced hyperemia. Ablation of capsaicin-sensitive afferent nerves was performed with high-dose capsaicin (125 mg/kg sc) as described previously (23). Capsaicin-treated rats were used ~10–14 days after the injections. Completeness of deafferentation was assessed by the 0.1% NH 4OH eye drop test as described previously (23). Duodenal mucosa of capsaicin-treated rat was superfused with pH 2.2 solution as described above.

Effect of CGRP-(8–37), L-NAME, and indomethacin on acid-induced hyperemia. To inhibit CGRP receptors, CGRP-(8–37) (0.1 mg/kg) was bolus-injected intravenously at t = ~10 min, as described previously (23), and L-NAME (0.1 mM) was topically superfused, as described by Holm and co-workers (18). The mucosa was superfused with pH 7.0 Krebs from t = ~10 min until t = 10 min and with pH 2.2 solution from t = 10 min until t = 20 min. L-NAME (0.1 mM) with or without L-Arg (50 mM) was superfused with pH 7.0 Krebs from t = 0 min until t = 10 min and with pH 2.2 buffer from t = 10 min until t = 20 min. To abolish the endogenous COX activity, rats were pretreated with indomethacin (5 mg/kg ip) 1 h before anesthesia (23).

Statistics

All data are expressed as means ± SE. Comparisons between groups were made by one-way ANOVA followed by Fisher’s least significant difference test. P < 0.05 was considered significant.

RESULTS

Hyperemic Effect of Intraluminal Capsaicin and Effect of CPZ on Capsaicin-Induced Hyperemia

Figure 1A depicts the effect of different concentrations of intraduodenal capsaicin on duodenal blood
flow. An approximate $K_{0.5}$ increase of duodenal blood flow was achieved by superfusion of 300 µM capsaicin, which increased duodenal blood flow 149%. This ED$_{50}$ of capsaicin was then used in combination with the specific capsaicin-receptor antagonist CPZ (Fig. 1B). A stoichiometric concentration of CPZ (300 µM) completely abolished the hyperemic effect of capsaicin, suggesting that affinity of CPZ to capsaicin receptor is 1:1.

Effect of Capsaicin Antagonists on Acid-Induced Hyperemia

The inhibitory effects of CPZ and RR on acid-induced hyperemia are depicted in Fig. 2. Superfusion of pH 2.2 solution increased duodenal blood flow 141% ± 10% ($P < 0.01$ vs. pH 7.0 superfusion). This hyperemia was dose-dependently inhibited by CPZ (Fig. 2A) and RR (Fig. 2B), although RR was less inhibitory than CPZ at concentrations greater than 0.1 mM. Neither inhibitor affected blood flow in the absence of acid superfusion. The approximate $K_{0.5}$ of CPZ for pH 2.2 superfusion was 300 µM.

Effect of CPZ on the Time Course of Acid- or BK-Induced Hyperemia

Figure 3 depicts the time course of the effect of CPZ (0.5 mM) on pH 2.2-induced hyperemia. The pH 2.2 plus vehicle solution rapidly increased duodenal blood flow, whereas CPZ abolished the acid-induced hyperemia without affecting baseline blood flow (pH 7.0 plus CPZ). Figure 4 depicts BK-induced hyperemia in duodenum. BK (0.5 mM) increased duodenal blood flow 151%; however, in contrast to acid, CPZ had no effect on BK-induced hyperemia.

Effects of Antagonists and Inhibitors on Acid-Induced Hyperemia

Figure 5 depicts the effects of several compounds on the hyperemic response to acid, measured at $t = 15$
min. Vehicle (0.01% DMSO, Tween 80, and ethanol in pH 7.0 Krebs) did not affect acid-induced hyperemia. CPZ (0.5 mM) abolished acid-induced hyperemia, whereas RR (10 mM) partially attenuated the hyperemic response (see Figs. 2–3). Rats deafferented with high-dose capsaicin (125 mg/kg sc) had no hyperemic response to luminal acid, whereas indomethacin pretreatment (Indo-treated, 5 mg/kg ip) did not affect the hyperemic response. Figure 6 depicts additional inhibitor experiments: L-NAME (0.1 mM) and CGRP-(8–37) (0.1 mg/kg iv) did not affect baseline blood flow but abolished acid-induced hyperemia. The effect of L-NAME was reversed by the addition of L-Arg (50 mM; Fig. 6).

DISCUSSION

We demonstrated that the hyperemic response to superfused acid is inhibited by specific and nonspecific capsaicin-receptor antagonists (CPZ and RR, respectively) and that the duodenal hyperemic response to acid involves capsaicin-sensitive afferent nerves and the CGRP-NO pathway but not the COX system. CPZ antagonizes the hyperemic response to acid and capsaicin but not to BK. This is the first study in which CPZ was used topically in the gastrointestinal tract to specifically assess the role of capsaicin receptors in the duodenal hyperemic response to capsaicin, BK, and acid.

Most studies have data that have been consistent with the hypothesis that protons are ligands for the capsaicin receptor in vitro and in vivo (4). Extracellular acidification activates capsaicin receptors of cultured dorsal root ganglion cells (5) and also stimulates tracheal C-fibers in guinea pigs via capsaicin receptors (11) and the relaxation of urinary bladder (31). In other studies, protons may modify the effect of capsaicin on...
VR1 in cultured dorsal root ganglion cells (25) and rabbit trigeminal ganglion neurons (32). Our hypothesis is supported by the most recent report by Tominaga et al. (41), in which it was demonstrated that protons directly and dose-dependently activate VR1 expressed in cultured cells at physiological temperature (37°C) and that CPZ inhibited this proton-evoked activation.

The effect of CPZ has not been extensively studied in the gastrointestinal tract. CPZ was used in in vitro binding assays using colon-derived membranes (17) and in a recent study by Leung and co-workers (35). In the latter study, capsaicin and HCl increased mesenteric arterial blood flow in rats, but systemic CPZ (100 µmol/kg sc) failed to inhibit this hyperemic response, although luminal RR (0.1%) was inhibitory (the latter result being consistent with our data). Their study, however, differs from ours in that topical CPZ was not used, arterial and not predominantly mucosal blood flow was measured, and the acid-induced increase in blood flow was relatively modest (15.5% vs. 40% in this study). The less-inhibitory effect of RR compared with CPZ can be accounted for by its molecular size (mol wt = 786 vs. 377), positive molecular charge, differing hydrophobicity, and possibly different molecular targets (22, 31). The use of inhibitors such as RR in vivo is also hindered by lack of control over inhibitor concentration at the receptor site; both previous studies of the use of RR in the gastrointestinal tract in vivo (36, 43) reported inhibition with intragastric concentrations of 1.3 mM, in contrast to the reported concentration that selectively inhibits capsaicin receptors of <20 µM (3).

The paradigm of CGRP release from afferent nerves in response to acid or capsaicin stimulation has been demonstrated in several organs and model systems. The best-studied model in the gastrointestinal tract is the stomach, in which superfusion of acid over mucosa-free slices augments CGRP release (15). Acid-induced, CGRP-mediated endothelial vasodilatory NO release is a well-accepted mucosal protective mechanism (for review, see Ref. 19). Several other systems are remark-

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**Fig. 5.** Effect of inhibitors and receptor antagonists on acid-induced hyperemia. CPZ abolishes and RR attenuates pH 2.2-induced hyperemia in control rats. Pretreatment with high-dose capsaicin (Cap-treated) also abolishes acid-induced hyperemia, whereas indomethacin (Indo-treated) does not. *P < 0.05 vs. pH 7.0 Krebs superfusion in control rats; †P < 0.05 vs. pH 2.2 + vehicle superfusion in control rats. Data are means ± SE from 6 rats.

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**Fig. 6.** Effect of calcitonin gene-related peptide (CGRP) antagonist CGRP-(8—37) and N\(^-\)nitro-L-arginine methyl ester (L-NAME) on acid-induced hyperemia. CGRP-(8—37) and L-NAME abolish pH 2.2-induced hyperemia, whereas neither affects baseline blood flow. L-Arginine (L-Arg) reverses the effect of L-NAME. *P < 0.05 vs. pH 7.0 Krebs superfusion in control rats; †P < 0.05 vs. pH 2.2 buffer superfusion in control rats. Data are means ± SE from 6 rats. SF, superfusion.
ably similar. Acid and capsaicin stimulate afferent nerves in excised trigeminal ganglia (32), isolated dorsal root ganglia (1, 25, 34), urinary bladder muscle (14), airway (21, 26, 30), and heart (12). Similar to our proposed mechanism, acid- and capsaicin-induced CGRP release are competitively antagonized by CPZ in guinea pig heart (12), guinea pig urinary bladder (14), and rat trachea (21) and guinea pig airway (30). Also in keeping with our proposed mechanism, indomethacin failed to inhibit acid-induced CGRP release in the latter three studies (14, 21, 30). Indomethacin, however, does inhibit CGRP release caused by mediators such as nicotine and bradykinin, but CPZ does not (12, 16, 21), in agreement with our findings. The mechanism underlying these differential effects remains uncertain.

The actual receptor subtype involved with mediating the duodenal hyperemic response to acid is unknown. VR1 has not yet been detected in the small or large intestine (6), although it is expressed in dorsal root ganglion cells, which contain CGRP-containing splanchnic afferent neurons, as well as the nodose ganglion, which contains vagal afferents (41). Because luminal CPZ inhibited the hyperemic effects of capsaicin and acid by a CGRP-dependent pathway, we postulate that a non-VR1 subtype of capsaicin receptor is present on splanchnic afferent neurons. Binding and pharmacokinetic studies with the ultrapotent capsaicin analog resiniferatoxin and CPZ suggest that there may be central and peripheral types of vanilloid receptors, with the peripheral types further subdivided into a urinary bladder type and an airway-colon type (1, 38, 44). The apparent Kᵣ for CPZ in duodenum is difficult to interpret because we did not measure CPZ concentrations at the receptor site. Nevertheless, our findings are consistent with the presence of the colon-airway type of receptor in duodenum, based on the CGRP-dependent mechanism and antagonism by CPZ but not by indomethacin. Another possibility is that CPZ could inhibit acid-induced hyperemia by nonspecific interactions with related ion channels and receptors, as suggested previously (10, 28). In the absence of definitive identification of a VR subtype from rat duodenum, these conclusions must remain somewhat speculative.

The mechanism by which CGRP released from capsaicin-sensitive afferent nerves stimulates NO production followed by vascular dilatation has been reported in stomach (8, 20). The inhibitory effect of L-NAME on acid-induced hyperemia confirmed that this mechanism was present in the duodenum. The report of Kao et al. (24), who found that inhibition of endogenous NO production reduced basal but not intraduodenal HCl-induced mesenteric blood flow in rat duodenum, is not confirmed by this study and is also disputed by the study of Holm et al. (18). The reversal of the inhibitory effect of L-NAME by high-dose L-Arg is consistent with a specific effect of L-NAME on NOS inhibition.

In recent studies, several groups have reported that amiloride-sensitive proton-gated Na⁺ channels (ASIC family) are present on sensory afferent neurons (7, 9, 42). The existence of a channel of this type on duodenal capsaicin-sensitive afferent nerves might present an alternative mechanism by which acid stimulates afferent nerve terminals. Nevertheless, the absence of immunoreactivity for any of the three known ASIC family members in intestine or enteric ganglia suggests that it is unlikely that this channel is involved with the duodenal hyperemic response (7).

In conclusion, we demonstrated that capsaicin-sensitive afferent nerves and capsaicin receptors are an integral component of the hyperemic response of rat duodenum to superfused acid. Furthermore, the CGRP-NO system, but not the COX system, is involved in this response. These data, combined with our prior observations of rat duodenum (2), help confirm the postulated sequence of events that take place after acid is introduced into the duodenal lumen: acid enters the epithelial cells, exiting via basolateral Na⁺-H⁺ exchange, and activates capsaicin receptors on afferent nerves, which releases CGRP, stimulating NOS and NO release in the vascular endothelium, relaxing vascular smooth muscle, and increasing duodenal mucosal blood flow. Protons are an endogenous ligand for the capsaicin receptor of rat duodenum.

We thank Dipti Shah for technical assistance. Address for reprint requests and other correspondence: J. D. Kaunitz, Bldg. 114, Ste. 217, West Los Angeles VA Medical Center, 11301 Wilshire Blvd., Los Angeles, CA 90073 (E-mail: jake@ucla.edu).

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