Mechanisms Underlying Capsaicin-Stimulated HCO_3^- Secretion in The Stomach - Comparison with Mucosal Acidification -

EITARO AIHARA, MASAMUNE HAYASHI, YOKO SASAKI,

ATSUSHI KOBATA AND KOJI TAKEUCHI

Department of Pharmacology and Experimental Therapeutics,

Kyoto Pharmaceutical University

Misasagi, Yamashina, Kyoto, Japan

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Running title: Capsaicin-Induced Gastric HCO_3^- Secretion

Address correspondence to: Dr. Koji Takeuchi
Department of Pharmacology and Experimental Therapeutics
Kyoto Pharmaceutical University
Misasagi, Yamashina, Kyoto 607, Japan
Tel (Japan) 075-595-4679; Fax (Japan) 075-595-4774

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Abbreviations:

Vanilloid receptor type 1	VR1
Transient receptor potential vanilloid type 1	TRPV1
Prostaglandin	PG
prostaglandin E_2	PGE_2
prostacyclin	PGI_2
nitric oxide	NO
PGE receptor	EP receptor
PGI_2 receptor	IP receptor
N^G-nitro-L-arginine methylester	L-NAME
calcitonin gene-related peptide	CGRP

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Abstract

The effects of capsaicin and mucosal acidification on gastric HCO_3^- secretion were compared in wild-type and prostacyclin (PGI_2) IP receptor or prostaglandin E receptor EP1 or EP3 knockout C57BL/6 mice as well as rats. Under urethane anesthesia, the stomach was mounted on an *ex-vivo* chamber, perfused with saline, and the secretion of HCO_3^- was measured at pH 7.0 using the pH-stat method. Capsaicin or 200 mM HCl was applied to the chamber for 10 min. Capsaicin increased the secretion of HCO_3^- in rats and wild-type mice, the response at 0.3 mg/ml being equivalent to that induced by acidification. This effect of capsaicin in rats was abolished by ablation of capsaicin-sensitive afferent neurons and attenuated by indomethacin, L-NAME and capsazepine (TRPV1 antagonist) but not FR172357 (bradykinin B2 antagonist) or the EP1 antagonist. The acid-induced HCO_3^- secretion was attenuated by indomethacin, L-NAME, the EP1 antagonist, and sensory deafferentation, but not affected by capsazepine or FR172357. PGE_2, NOR-3 (NO donor) and bradykinin stimulated the secretion of HCO_3^-, and the effect of bradykinin was blocked by indomethacin and L-NAME as well as FR172357. The stimulatory effect of capsaicin disappeared in IP (-/-) mice while that of acidification disappeared in EP1 (-/-) mice. Intragastric application of capsaicin increased mucosal PGI_2 but not PGE_2 levels in the rat stomach. These results suggested that both capsaicin and acid increase gastric HCO_3^- secretion via a common pathway, involving PG and NO as well as capsaicin-sensitive afferent neurons, yet their responses differ concerning TRPV1 or prostanoid receptor dependency.

Introduction

The gastric mucosa is kept intact by multiple protective mechanisms including humoral and neuronal factors, despite exposure to acid and other chemical hazards (Flemstrom & Garner, 1982). Capsaicin-sensitive afferent neurons play a central role in the neuronal mechanism of the stomach (Holzer, 1998). These afferent neurons regulate various gastric functions such as secretion, mucosal blood flow (GMBF) and motility, and modulate the mucosal integrity of the stomach (Holzer & Sametz; 1986 Holzer, 1998; Takeuchi et al., 1991; 1992). Vanilloid type 1 receptor (VR1), a nonselective cationic channel, has been recently cloned as the binding site of capsaicin (Caterina et al., 1997), and more recently, has been shown to be one of the transient receptor potential (TRP) family of ion channels (Clapham et al., 2001). Although the TRP family is activated by a diverse range of stimuli, including depletion of intracellular Ca^{2+} stores (Caterina et al., 1997), the VR1 receptor remains the only channel activated by vanilloids such as capsaicin and is now known as TRPV1 (Gunthorpe et al., 2002). Capsaicin stimulates these afferent neurons via TRPV1, resulting in the release of calcitonin gene-related peptide (CGRP), the predominant neurotransmitter of spinal afferents in the rat stomach, and by so doing exerts a gastroprotective action (Merchant et al., 1994). CGRP acts on endothelial cells to release nitric oxide (NO), and this molecule is also known to mediate, in large part, the action of CGRP (Holzer, 1998). Recent studies also showed that the activation of the bradykinin B2 receptor leads to the opening of TRPV1 and modifies the action of capsaicin (Ferreira et al., 2002; Shin et al., 2004).

The secretion of HCO_3^- from surface epithelial cells is a protective mechanism in the stomach, the HCO_3^- working in collaboration with mucus gel that adheres to the surface of mucosa (Flemstrom & Garner, 1982). We previously reported that capsaicin increased duodenal HCO_3^- secretion mediated by endogenous prostaglandins (PGs) and

NO as well as capsaicin-sensitive afferent neurons (Sugamoto et al., 2002; Kagawa et al., 2003). We also reported that PGE_2 stimulates HCO_3^- secretion through EP1 receptors in the stomach and EP3/EP4 receptors in the duodenum (Takeuchi et al., 1997; Aoi et al., 2004), while the action of capsaicin in the duodenum requires the presence of prostacyclin (PGI_2) IP receptors (Nakashima et al., 2004). However, few studies have examined the mechanisms involved in gastric HCO_3^- secretion in response to capsaicin.

In the present study, we investigated the regulatory mechanism of capsaicin-induced gastric HCO_3^- secretion, in relation to sensory neurons, TRPV1, PGs, NO and bradykinin B2 receptors, and compared it to that of the acid-induced response. In addition, since we have found that the responses to capsaicin and acid in the duodenum differ concerning PGI_2/IP dependency (Nakashima et al., 2004), we also examined these responses in the stomach using mice lacking EP1-, EP3- or IP-receptors.

Materials and Methods

Animals

Male Sprague Dawley rats (220-260 g, Nippon Charles River, Shizuoka, Japan) and male C57/BL6 mice (25-30 g) were used. Mice lacking the EP1, EP3 or IP receptors were generated as described previously (Oida et al., 1995; Ushikubi et al., 1998). No abnormality was detected in general body appearance or in the morphological feature of the gastroduodenal mucosa. The distribution of the EP1, EP3 and IP receptor genes was verified by Northern blot hybridization, which failed to detect mRNAs encoding the respective receptors in EP1 (-/-), EP3 (-/-) and IP (-/-) mice. These rats and knockout mice were deprived of food but allowed free access to tap water for 18 hr before the experiments. Studies were performed under urethane anesthesia (1.25 g/kg, i.p.) using 4~8 animals pre group. All experimental procedures were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Determination of gastric HCO_3^- secretion

The secretion of HCO_3^- was measured in the chambered stomach as described previously (Takeuchi et al., 1992). The abdomen was incised and the stomach was exposed, mounted on a chamber (exposed area, rat: 3.1 cm^2, mouse: 0.7 cm^2), and superfused with saline that was gassed with 100% O_2 and kept in a reservoir. The secretion of HCO_3^- was measured at pH 7.0 using a pH-stat method (Hiranuma Comtite-8, Mito, Japan) and by adding 2 mM HCl to the reservoir. To unmask HCO_3^- in the stomach, the acid secretion was completely inhibited by omeprazole given i.p. at a dose of 60 mg/kg. After basal HCO_3^- secretion had well stabilized, the animals were subjected to the following treatment. Capsaicin (0.03~0.3 mg/ml) or NOR-3 (a NO donor: 3 mg/ml) was topically applied to the chamber for 10 min, while PGE_2 (1 mg/kg) or bradykinin (30 μg/kg) was given i.v. as a

single injection. The doses of PGE_2, capsaicin and NOR-3 were chosen to effectively stimulate duodenal HCO_3^- secretion (Takeuchi et al., 1997; Sugamoto et al., 2001) while that of bradykinin was chosen to induce vasodilation, inflammation and glucose uptake via B2 receptors (Shiuchi et al., 2002). The secretion of HCO_3^- was also stimulated by exposure of the mucosa to 50~200 mM HCl (rat) or 50 mM HCl (mouse) for 10 min. In addition, the effects of indomethacin, N^G-nitro L-arginine methyl ester (L-NAME), ONO-8711 (EP1 antagonist)(Aoi et al., 2004), FR172357 (a bradykinin B2 antagonist)(Asano et al., 1997), capsazepine (a TRPV1 antagonist) or chemical ablation of capsaicin-sensitive afferent neurons were examined on the secretion of HCO_3^- induced by the above agents or the mucosal acidification. Indomethacin (5 mg/kg), ONO-8711 (10 mg/kg) or FR172357 (1 mg/kg) was given s.c. 30 min or i.v. 15 min before each treatment, while L-NAME (20 mg/kg) was given s.c. 3 hr before, because this agent acutely increased HCO_3^- secretion through a neural reflex due to an increase of blood pressure (Takeuchi et al., 1993; Aihara et al., 2005). Capsazepine (2.5 mg/ml) was applied to the chamber for 20 min starting from 10 min before capsaicin or acid treatment (Kagawa et al., 2003) or applied for 20 min followed by i.v. injection of bradykinin 10 min later. Chemical ablation of capsaicin-sensitive afferent neurons was achieved with repeated s.c. injections of capsaicin (total dose; 100 mg/kg) once daily for 3 days 2 weeks before the experiment (Holzer & Sametz, 1986; Takeuchi et al., 1992). All the injections were performed under ether anesthesia, and the rats were pretreated with terbutaline (0.1 mg/kg, i.m.) and aminophylline (10 mg/kg, i.m.) to counteract the respiratory impairment associated with capsaicin. To check for the effectiveness of the treatment, a drop of capsaicin solution (0.1 mg/ml) was instilled onto one eye of each rat, and wiping movements were counted as previously reported. In most of the above experiments, blood pressure was concomitantly measured via the femoral artery using a pressure transducer and amplifier system

(TP-200TL, AP-100F, RTA-1100A Nihon Koden).

Measurement of Mucosal PGE_2 and PGI_2 Levels

The mucosal PGE_2 and PGI_2 (6-keto $PGF_{1\alpha}$) levels in the stomach were measured after application of capsaicin (0.3 mg/ml) for 10 min. Thirty minutes later, the stomach was removed, weighed, and put in a tube containing 100% methanol plus 0.1 M indomethacin (Futaki et al., 1994). Then, the samples were minced with scissors, homogenized, and centrifuged at 12000 r.p.m. for 10 min at 4˚C. The supernatant of each sample was used for measuring levels of PGE_2 and 6-keto $PGF_{1\alpha}$ by EIA with PGE_2- and 6-keto $PGF_{1\alpha}$-kits (Cayman Chemical Co., Ann Arbor, MI), respectively.

Preparation of Drugs

Drugs used were urethane (Tokyo kasei, Tokyo, Japan), capsaicin and bradykinin (Nacalai Tesque, Kyoto, Japan), prostaglandin E_2 (PGE_2: Funakoshi, Tokyo, Japan), capsazepine, N^G-nitro L-arginine methyl ester (L-NAME) and indomethacin (Sigma Chemicals, St. Louis, Mo, USA), ONO-8711 (Ono Pharmaceutical Co., Osaka, Japan), NOR-3 [(±)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamine](Dojindo, Kumamoto, Japan), omeprazole (Astra Zeneca, Möndal, Sweden) and FR172357, terbutaline (Buricanyl^R, Fujisawa, Osaka, Japan) and aminophylline (Neophylline^R, Eizai, Tokyo, Japan). Capsaicin was dissolved in a Tween 80-ethanol solution (10% ethanol, 10% Tween and 80% saline, w/w: Wako, Osaka, Japan) for s.c. injection while it was suspended in a 0.5% carboxymethylcellulose solution (CMC: Nacalai Tesque) for topical application. PGE_2 or NOR-3 was first dissolved in absolute ethanol or dimethyl sulfoxide (DMSO), respectively, and diluted with saline to the desired concentrations. Omeprazole was suspended in a 0.5% CMC solution. Other agents were dissolved in saline. Each agent was prepared immediately before use and given in a volume of 0.5 ml per 100 g body weight in the case of i.p. or s.c. administration or in a volume of 0.1 ml per 100 g body weight in the

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case of i.v. administration, or applied topically to the chamber in a volume of 2 ml per rat or 0.7ml per mice. Control animals received saline or CMC in place of active agents.

Statistics

Data are presented as the mean±SE from 4~8 rats or mice per group. Statistical analyses were performed using a two-tailed Dunnett's multiple comparison test, and values of $p<0.05$ were regarded as significant.

Results

Effect of Capsaicin on Gastric HCO_3^- Secretion

Under urethane anesthesia, the rat stomach spontaneously secreted HCO_3^- at a steady rate of 0.2~0.4 μEq/10 min during a test period. Capsaicin (0.03~0.3 mg/ml) applied to the chamber for 10 min increased the secretion of HCO_3^- in a dose-dependent manner, and this effect was significant at the dose of 0.1 mg/ml or greater; the $\bullet HCO_3^-$ output at 0.1 and 0.3 mg/ml was 0.9±0.1 and 1.4±0.3 μEq/hr, respectively **(Figure 1)**. In the subsequent experiments, we used capsaicin at 0.3 mg/ml.

The stimulatory effect of capsaicin on HCO_3^- was significantly attenuated by the chemical ablation of capsaicin-sensitive afferent neurons as well as prior administration of indomethacin (5 mg/kg, s.c.) or L-NAME (20 mg/kg, s.c.)**(Figure 2)**. The $\bullet HCO_3^-$ output was 0.2±0.1, 0.1±0.2, and 0.1±0.1 μEq/hr, respectively, in the animals pretreated with indomethacin, L-NAME or capsaicin, all of which were significantly less than that (1.4±0.3 μEq/hr) of the vehicle-treated animals. Likewise, the stimulatory action of capsaicin was potently inhibited by co-application of capsazepine, the TRPV1 antagonist, the $\bullet HCO_3^-$ output being 0.3±0.2 μEq/hr, equivalent to that of the control group without capsaicin treatment **(Figure 3)**. By contrast, both the EP1 antagonist ONO-8711 (10 m/kg, s.c.) and the bradykinin B2 antagonist FR172357 (1 mg/kg, i.v.) did not significantly affect gastric HCO_3^- secretion in response to capsaicin **(Figure 2)**.

Effect of Mucosal Acidification on Gastric HCO_3^- Secretion

The secretion of HCO_3^- in the stomach was increased in a concentration-dependent manner, when the mucosa was acidified by exposure to 50~200 mM HCl for 10 min; the $\bullet HCO_3^-$ output induced by acidification at 200 mM HCl was

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1.7±0.2 μEq/hr **(Figure 4)**. The response to 200 mM HCl was significantly prevented by indomethacin, L-NAME and capsaicin pretreatment, but not FR172357, the degree of inhibition being 61.6%, 61.6% and 49.9%, respectively **(Figure 5)**. The EP1 antagonist ONO-8711 also significantly inhibited the gastric response induced by mucosal acidification. However, the acid-induced HCO_3^- secretion in the stomach was not significantly affected by co-application of capsazepine, and the $\bullet HCO_3^-$ output was 1.9±0.6 μEq/hr, almost equivalent to that (1.8±0.5 μEq/hr) of the vehicle-treated group **(Figure 6)**.

Effects of Capsaicin on Mucosal PGE_2 and 6-keto $PGF_{1\alpha}$ Levels in Rat Stomach

Mucosal PGE_2 and 6-keto $PGF_{1\alpha}$ levels in the normal rat stomach were 4.9±0.7 and 5.8±0.9 ng/g tissue, respectively. Intragastric application of capsaicin (0.3 mg/ml) for 10 min did not affect the amount of PGE_2 but significantly increased that of 6-keto $PGF_{1\alpha}$ to about 2.8-fold the control level, the value being 16.9±2.3 ng/g tissue **(Figure 7)**.

Effect of PGE_2, NOR-3 and Bradykinin on Gastric HCO_3^- Secretion

Since the response of HCO_3^- to capsaicin or acid in the stomach was attenuated by either indomethacin or L-NAME but not FR172357, it is assumed that both PGs and NO but not bradykinin are involved in the stimulatory mechanism of these agents. To further investigate the interactive role of PGs and NO in the mechanism of HCO_3^- secretion in the stomach, we examined the effects of PGE_2, NOR-3 (NO donor) and bradykinin on the secretion, in the absence or presence of indomethacin, L-NAME or FR172357.

Intravenous administration of PGE_2 (1 mg/kg) increased the secretion of HCO_3^- in the stomach, and the $\bullet HCO_3^-$ output was 1.5±0.5 μEq/hr, the value being equivalent to that induced by capsaicin at 0.3 mg/ml **(Figure 8)**. Likewise, the NO donor NOR-3 (3

mg/ml), applied topically to the mucosa for 10 min, also increased HCO_3^- secretion, the • HCO_3^- output being 1.0±0.2 μEq/hr. Neither indomethacin, L-NAME nor FR172357 significantly affected the increase of HCO_3^- secretion in response to PGE_2 (data not shown). Likewise, gastric HCO_3^- secretion was also stimulated by i.v. administration of bradykinin (30 μg/kg), reaching a maximal value of 160% of the basal level, though this effect was less potent than that of capsaicin or acidification and completely disappeared 1 hr **(Figure 9)**. The stimulatory effect of bradykinin on HCO_3^- was significantly antagonized by FR172357 and also attenuated by prior administration of indomethacin or L-NAME later. In addition, the stimulatory effect of bradykinin was almost totally blocked by chemical ablation of capsaicin-sensitive afferent neurons but not significantly affected by pretreatment with capsazepine.

Effect of Capsaicin on Gastric HCO_3^- Secretion in Wild-type and IP-Receptor Knockout Mice

We previously reported the importance of PGI_2/IP receptors in the HCO_3^- stimulatory action of capsaicin in the duodenum (Nakashima et al., 2004). Since capsaicin also stimulated HCO_3^- secretion in the stomach, in an indomethacin-inhibitable manner, we investigated which type of prostanoid receptor is involved in the capsaicin-induced response in the stomach, using EP1-, EP3- and IP-receptor knockout mice, in comparison with that induced by mucosal acidification.

The mouse stomach spontaneously secreted HCO_3^- at a rate of 0.1~0.3 μEq/10 min. No difference was found in the rate of basal HCO_3^- secretion between wild-type mice and mice lacking EP1-, EP3- or IP-receptors. Capsaicin (0.3 mg/ml) applied to the chamber for 10 min increased gastric HCO_3^- secretion in wild-type mice, and this effect was similarly observed in EP1- or EP3-receptor knockout mice but disappeared in mice lacking

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IP receptors **(Figure 10)**. The HCO_3^- secretion was also increased in wild-type mice by exposure of the mucosa for 10 min to 50 mM HCl, and this response was similarly observed in EP3- and IP- but not EP1-receptor knockout animals **(Figure 11)**. In wild-type animals, the responses induced by both capsaicin and acidification were significantly attenuated by prior administration of indomethacin (5 mg/kg, s.c.).

Discussion

The gastroduodenal mucosa responds to acidification with a significant rise in the secretion of HCO_3^- which, in collaboration with mucus, contributes to the mucosal tolerance of luminal acid (Flemstrom & Garner, 1982). We have previously reported that intraluminal application of capsaicin also stimulates the secretion of HCO_3^- in these tissues through the activation of capsaicin-sensitive afferent neurons (Takeuchi et al., 1991; 1992; Aoi et al., 2004; Nakashima et al., 2004). The present study confirmed that both acid and capsaicin produced an increase of HCO_3^- secretion in the stomach mediated by these afferent neurons, and clearly showed the difference in their modes of action in terms of sensitivity to TRPV1 and prostanoid receptors. Furthermore, we observed the involvement of endogenous PGs and NO in the stimulatory action of capsaicin in the stomach, essentially similar to the findings in the duodenum.

TRPV1 is a nonselective cation channel responsive to proton as well as capsaicin (Caterina et al., 1997). The binding sites of capsaicin are located at the intracellular site of the receptor protein (Jung et al., 1999) whereas the target of protons is thought to be located on the extracellular surface of the receptor protein (Jordt et al., 2000). In the present study, when the TRPV1 antagonist capsazepine was applied to the mucosa together with capsaicin or acid, it was found that this agent completely blocked the increase in gastric HCO_3^- secretion induced by capsaicin but not acid, despite that both responses are mediated by capsaicin-sensitive afferent neurons. These results are consistent with our previous findings in the duodenum, showing that capsazepine significantly mitigated the response induced by capsaicin but not mucosal acidification (Kagawa et al., 2003). Several investigators examined the effect of capsazepine on various events both in vivo and in vitro induced by acidification, but the results were controversial (Seno et al., 1998;

Akiba et al., 1999; McIntyre et al., 2001). Akiba et al (1999) reported that acid in the lumen induced a mucosal hyperemic response in the rat duodenum in a capsazepine-sensitive way and suggested luminal acid as the endogenous ligand for duodenal TRPV1. McIntyre et al (2001) reported pharmacological differences between the human and rat TRPV1 and demonstrated that capsazepine blocked the human but not rat TRPV1's response to low pH. At present, the reason for the different results between these studies remains unclear. The present results do not exclude the involvement of TRPV1 in the acid-induced secretion of HCO_3^-, yet it is likely that the target site of acid is different from that of capsaicin, ie., the binding site inhibitable by capsazepine. Alternatively, acid might activate these afferent neurons through acid-sensing ionic channels.

Endogenous PGs are particularly important in the local regulation of HCO_3^- secretion in the gastroduodenal mucosa. We previously found, using subtype-specific EP agonists and antagonists, that PGE_2 stimulates the secretion of HCO_3^- in the duodenum through EP3/EP4 receptors and in the stomach through EP1 receptors (Takeuchi et al., 1999; Aoi et al., 2004; Nakashima et al., 2004). Many investigators reported that mucosal acidification increases HCO_3^- secretion in these tissues, with a concomitant rise in mucosal PGE_2 levels (Sugamoto et al., 2001; Kagawa et al., 2003; Nakashima et al., 2004). Capsaicin also stimulated the secretion of HCO_3^- in the stomach in an indomethacin-inhibitable manner, suggesting the involvement of endogenous PGs in this action. However, capsaicin was reported to increase PGE_2 production in the duodenum but not in the stomach (Kagawa et al., 2003; Takeuchi et al., 2003). Notwithstanding, this agent has a variety of actions in the stomach, such as mucosal protection and hyperemia, mediated by capsaicin-sensitive afferent neurons and also depending on endogenous PGs (Takeuchi et al., 1991; 1992; Boku et al., 2001). The present study confirmed that intragastric application of capsaicin did not increase levels of PGE_2 but significantly enhanced levels of

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6-keto $PGF_{1\alpha}$, the PGI_2 metabolite, consistent with the findings in the mouse stomach (Boku et al., 2001). We previously reported that the gastroprotective action of capsaicin against HCl/ethanol was significantly attenuated by indomethacin in wild-type mice but totally disappeared in animals lacking IP receptors (Takeuchi et al., 2003). In the present study, capsaicin increased gastric HCO_3^- secretion in EP1- and EP3-receptor knockout mice, similar to wild-type mice, but not in the animals lacking IP receptors. Consistent with previous findings (Boku et al., 2001; Takeuchi et al., 2003), these results strongly suggest that endogenous PGI_2 plays a supportive role in the action of capsaicin in the stomach, probably by sensitizing the sensory neurons through IP receptors.

It should be, however, noted that the HCO_3^- secretion induced by acidification was unaltered in IP receptor knockout mice and disappeared in the animals lacking EP1-receptors. These results further support the idea that the response of HCO_3^- induced in the stomach by acidification and capsaicin, though in both cases depending on the sensory neurons, is mediated by different mechanisms concerning PG dependency; the former is mainly mediated by PGE_2 through EP1 receptors, while the latter depends on PGI_2/IP receptors. Similar results were obtained for the gastric hyperemic response induced by acid or capsaicin (Takeuchi et al., 2003). Although gastric hyperemic responses to these treatments were mitigated by capsaicin pretreatment (Holzer, 1998; Mimaki et al., 2002), the response induced by acid required the presence of EP1 receptors (Takeuchi et al., 2002) while that evoked by capsaicin required the presence of IP receptors (Takeuchi et al., 2003). Thus, it is not unreasonable that the presence of different prostanoid receptors is required for gastric HCO_3^- secretion in response to acid or capsaicin.

We observed that capsaicin had no effect on PGE_2 production but significantly increased PGI_2 generation in the stomach. Capsaicin-sensitive afferent neurons are known to distribute abundantly at peri-vascular sites, and the stimulation by capsaicin

releases CGRP / NO, resulting in increase of mucosal blood flow [Holzer, 1998]. Harada et al. (2002) reported that the activation of these afferent neurons ameliorated ischemia / re-perfusion-induced liver injury by increasing hepatic blood flow and by limiting inflammatory response through enhancement of endothelial PGI_2 production and suggested that the CGRP-induced activation of both endothelial NO synthase and cyclooxygenase-1 is involved in this mechanism. It is thus possible that capsaicin increases endothelial PGI_2 production locally in the stomach when applied topically in the mucosa. At present, the reason why capsaicin showed different effects on the production of PGE_2 and PGI_2 in the stomach remains unknown, yet there may be species or tissue differences in this action.

The present study also showed that the capsaicin-induced HCO_3^- secretion in the stomach was significantly attenuated by L-NAME, suggesting the involvement of endogenous NO in this process, in addition to PGs. Several studies showed that CGRP, the dominant neurotransmitter of spinal afferents, had various pharmacological actions, such as vasodilation, mediated by endogenous NO (Holzer & Sametz, 1986; Holzer, 1998; Lambrecht et al., 1993). We demonstrated in this study that the NO donor NOR-3 stimulated gastric HCO_3^- secretion, in agreement with our previous findings in the duodenum (Sugamoto et al., 2001). Nishihara et al (2002) reported that capsaicin increased the release of CGRP and NO in the rat stomach. Although we did not measure NO release in the stomach following capsaicin treatment, it is assumed that capsaicin activates primary afferent neurons, with the assistance of PGI_2, to liberate CGRP, which in turn stimulates NO release, resulting in an increase in gastric HCO_3^- secretion.

Bradykinin, a product of the kinin-kallikrein system often associated with inflammation, is also known to activate nociceptive-like afferent neurons through metabotropic G protein-coupled bradykinin B2 receptors (McGuirk et al., 1992; Maubach et

al., 1999). Recent study showed that the binding to B2 receptors activates an intracellular signaling cascade leading to the opening of TRPV1 channels (Chuang et al., 2001). We observed in the present study that bradykinin itself stimulated the secretion of HCO_3^- in the stomach. Furthermore, this response was attenuated not only by FR172357, the B2 antagonist, but also by indomethacin and L-NAME as well, suggesting the involvement of both PGs and NO in the response of HCO_3^- to bradykinin. The stimulatory effect of bradykinin was also significantly mitigated by the chemical ablation of capsaicin-sensitive afferent neurons but not affected by capsazepine, a TRPV1 antagonist. It is assumed that the stimulatory effect of bradykinin on HCO_3^- secretion is partly mediated by sensory neurons via B2 receptors but not through the interaction with TRPV1, in addition to endogenous PGs. Since bradykinin also potentiates the activation of TRPV1 by capsaicin through hydrolysis of endogenous posphatidylinositol-4,5-bisphosphate in a pospholipase C-dependent manner (Premkumar and Ahern, 2000; Chuang et al., 2001), it is possible that capsaicin-induced gastric HCO_3^- secretion is affected by the B2 receptor antagonist. However, the present study showed that the responses induced by capsaicin and acidification were not significantly affected by FR172357, suggesting no role for endogenous bradykinin in these responses. The reason for these results remains unexplained at present, and this point is currently under investigation in our laboratory.

Given the findings in the present study, we concluded that capsaicin stimulates the secretion of HCO_3^- in the stomach mediated by endogenous PGs and NO as well as capsaicin-sensitive afferent neurons, but not bradykinin B2 receptors. Mucosal acidification also increases gastric HCO_3^- secretion through sensory neurons mediated by both PGs and NO, similar to capsaicin, yet their modes of action differ in terms of capsazepine-sensitivity and prostanoid receptor-dependency. Although the luminal H^+ plays a modulator-type role in the physiological response mediated by capsaicin-sensitive afferent neurons in the

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stomach, it is unlikely that this action results from the interaction of H^+ with the capsazepine-sensitive site of TRPV1. The exact mechanism by which acid activates capsaicin-sensitive afferent neurons via TRPV1 or other sites on these neurons awaits further study.

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Footnotes

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Figure Legends

Figure 1 Effect of capsaicin on gastric HCO_3^- secretion in anesthetized rats. Capsaicin (0.03~0.3 mg/ml) was applied to the chamber for 10 min. In Fig. A, the data are presented as % of basal values and represent the mean±SE of values determined every 10 minutes from 4~7 rats. Fig. B shows total net HCO_3^- output for 1 hr after the capsaicin treatment, and the data are presented as the mean±SE for 4~7 rats. ＊Significant difference from control, at $P<0.05$.

Figure 2. Effects of pretreatment with various agents and capsaicin on gastric HCO_3^- secretion induced by capsaicin in anesthetized rats. Capsaicin (0.3 mg/ml) was applied to the chamber for 10 min. Indomethacin (5 mg/kg), ONO-8711 (10 mg/kg) or L-NAME (10 mg/kg) was given s.c. 1 or 3 hr before capsaicin, respectively. FR172357 (1 mg/kg) was given i.v. 15 min before the mucosal application of capsaicin. Chemical ablation of sensory neurons (capsaicin pretreatment) was achieved with 3 consecutive s.c. injections of capsaicin (total dose: 100 mg/kg) 2 weeks before the experiment. Figure shows total net HCO_3^- output for 1 hr after capsaicin treatment, and the data are presented as the mean±SE from 4~6 rats. Significant difference at $P<0.05$; ＊from control; # from saline.

Figure 3. Effect of capsazepine on gastric HCO_3^- secretion induced by capsaicin in anesthetized rats. Capsaicin (0.3 mg/ml) was applied to the chamber for 10 min. Capsazepine (2.5 mg/ml) was applied to the chamber for 20 min, starting 10 min before the capsaicin treatment. In Fig. A, the data are presented as % of basal values and represent the mean±SE of values determined every 10 minutes from 5~6 rats. Fig. B shows total net HCO_3^- output for 1 hr after the capsaicin treatment, and the data are

presented as the mean±SE for 5~6 rats. Significant difference at $P<0.05$; ＊from control; # from saline.

Figure 4. Effect of mucosal acidification on gastric HCO_3^- secretion in anesthetized rats. The acidification was performed by exposing the mucosa to 50~200 mM HCl for 10 min. In Fig. A, the data are presented as % of basal values and represent the mean±SE of values determined every 10 minutes from 4~6 rats. Fig. B shows total net HCO_3^- output for 1 hr after the acidification, and the data are presented as the mean±SE for 4~6 rats. ＊ Significant difference from control, at $P<0.05$.

Figure 5. Effects of pretreatment with various agents and capsaicin on gastric HCO_3^- secretion induced by mucosal acidification in anesthetized rats. The acidification was performed by exposing the mucosa to 200 mM HCl for 10 min. Indomethacin (5 mg/kg), ONO-8711 (10 mg/kg) or L-NAME (10 mg/kg) was given s.c. 1 or 3 hr before the acidification, respectively. FR172357 (1 mg/kg) was given i.v. 15 min before the mucosal acidification. Chemical ablation of afferent neurons (capsaicin pretreatment) was achieved with 3 consecutive s.c. injections of capsaicin (total dose: 100 mg/kg) 2 weeks before the experiment. Figure shows total net HCO_3^- output for 1 hr after the capsaicin treatment, and the data are presented as the mean±SE for 4~6 rats. Significant difference at $P<0.05$; ＊from control; # from saline.

Figure 6. Effect of capsazepine on gastric HCO_3^- secretion induced by mucosal acidification in anesthetized rats. The acidification was performed by exposing the mucosa to 200 mM HCl for 10 min. Capsazepine (2.5 mg/ml) was applied to the chamber for 20 min, starting 10 min before the acidification. In Fig. A, the data are presented as % of basal

values and represent the mean±SE of values determined every 10 minutes from 4~5 rats. Fig. B shows total net HCO_3^- output for 1 hr after the capsaicin treatment, and the data are presented as the mean±SE for 4~5 rats. Significant difference at $P<0.05$; ＊from control; # from saline.

Figure 7. PGE_2 and 6-keto-$PGF_{1\alpha}$ levels in the rat stomach after mucosal application of capsaicin under urethane anesthesia. Capsaicin (0.3 mg/ml) was applied to the chamber for 10 min, the mucosa was excised 30 min later, and then both PGE_2 and 6-keto-$PGF_{1\alpha}$ levels were measured by EIA. Data are presented as the mean±SE for 4~6 rats. ＊ Significant difference from the corresponding control, at $P<0.05$.

Figure 8. Effects of PGE_2 and NOR-3 on gastric HCO_3^- secretion in anesthetized rats. PGE_2 (1 mg/kg) was given i.v., while NOR-3 (3 mg/ml) was applied to the chamber for 10 min. Data represent total net HCO_3^- output for 1 hr after the administration of PGE_2 or NOR-3 and are presented as the mean±SE for 4-5 rats. ＊Significant difference from control, at $P<0.05$.

Figure 9. Effect of bradykinin on gastric HCO_3^- secretion in anesthetized rats. Bradykinin (30 μg/kg) was given i.v. after basal HCO_3^- secretion had been stabilized. Indomethacin (5 mg/kg) was given s.c. 1 hr before bradykinin while FR172357 (1 mg/kg) was given i.v. 15 min before. L-NAME (20 mg/kg) was given s.c. 3 hr before bradykinin. Capsazepine (2.5 mg/ml) was applied for 20 min to the chamber 10 min before the administration of bradykinin. Chemical ablation of sensory neurons (capsaicin pretreatment) was achieved with 3 consecutive s.c. injections of capsaicin (total dose: 100 mg/kg) 2 weeks before the experiment. In Fig. A, the data are presented as % of basal

values and represent the mean±SE of values determined every 10 minutes from 4~5 rats. Fig. B shows total net HCO_3^- output for 1 hr after the capsaicin treatment, and the data are presented as the mean±SE for 4~5 rats. Significant difference at $P<0.05$; ＊from control; # from bradykinin +saline.

Figure 10. Effect of capsaicin on gastric HCO_3^- secretion in wild-type, and EP1-, EP3- and IP-receptor knockout mice under urethane anesthesia. Capsaicin (0.3 mg/ml) was applied to the chamber for 10 min. In some wild-type mice, indomethacin (5 mg/kg) was given s.c. 1 hr before the capsaicin treatment. In Fig. A, the data are presented as % of basal values and represent the mean±SE of values determined every 10 minutes from 4~7 rats. Fig. B shows total net HCO_3^- output for 1 hr after the capsaicin treatment, and the data are presented as the mean±SE for 4~7 rats. Significant difference at $P<0.05$; ＊from control wild-type mice; # from wild-type mice treated with capsaicin+saline.

Figure 11. Effect of mucosal acidification on gastric HCO_3^- secretion in wild-type, and EP1-, EP3- and IP-receptor knockout mice under urethane anesthesia. The acidification was performed by exposing the mucosa to 50 mM HCl for 10 min. In some wild-type mice, indomethacin (5 mg/kg) was given s.c. 1 hr before the capsaicin treatment. In Fig. A, the data are presented as % of basal values and represent the mean±SE of values determined every 10 minutes from 4~8 rats. Fig. B shows total net HCO_3^- output for 1 hr after the capsaicin treatment, and the data are presented as the mean±SE for 4~8 rats. Significant difference at $P<0.05$; ＊from control wild-type mice; # from wild-type mice treated with 50 mM HCl+saline.

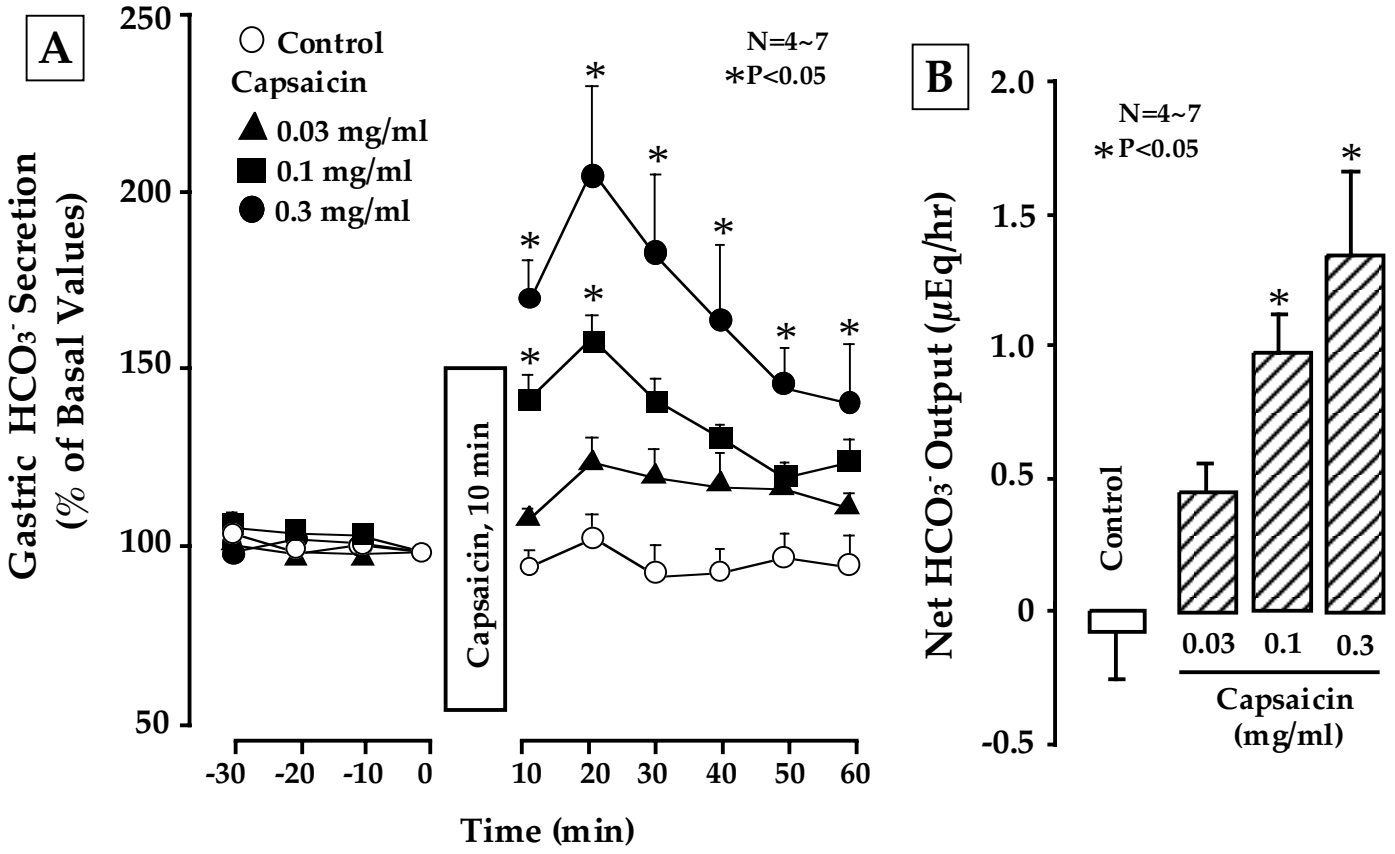
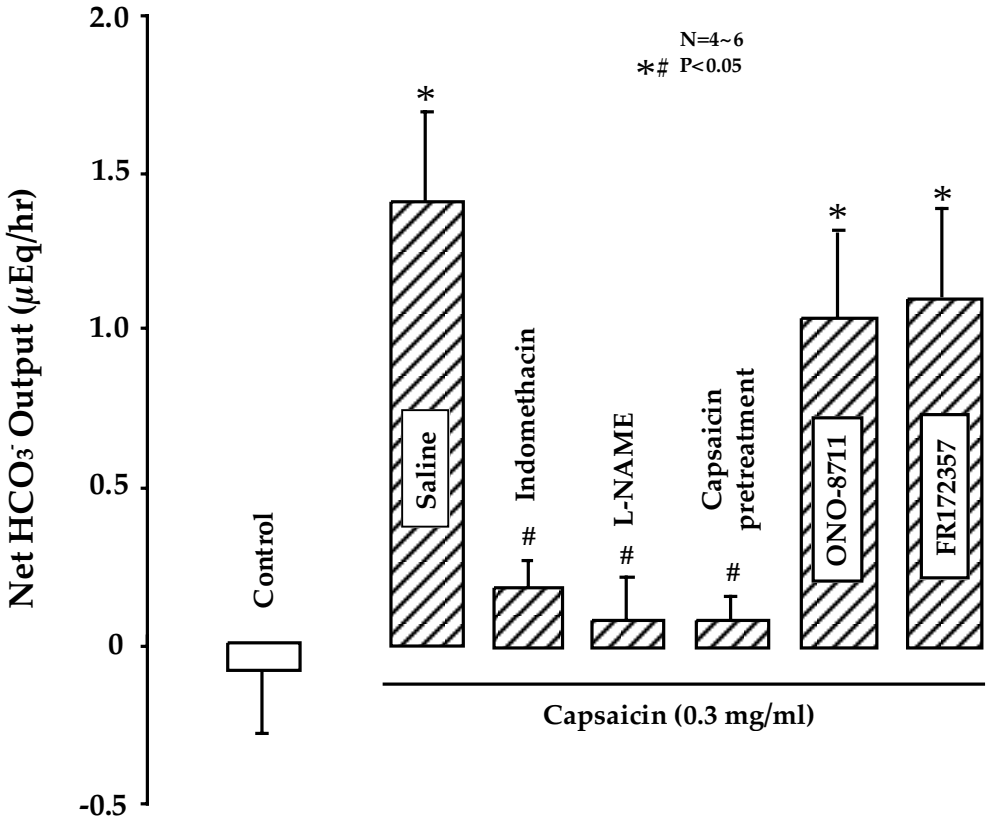


Figure 1



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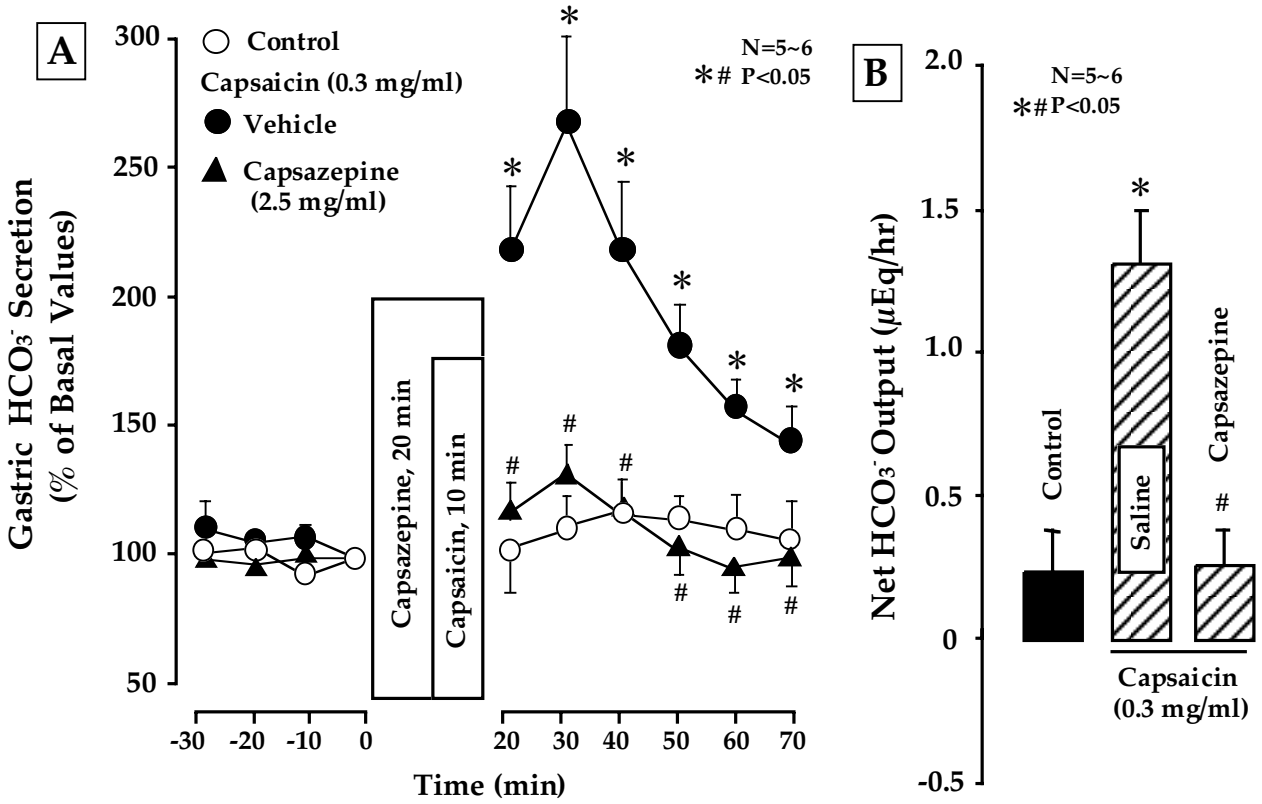

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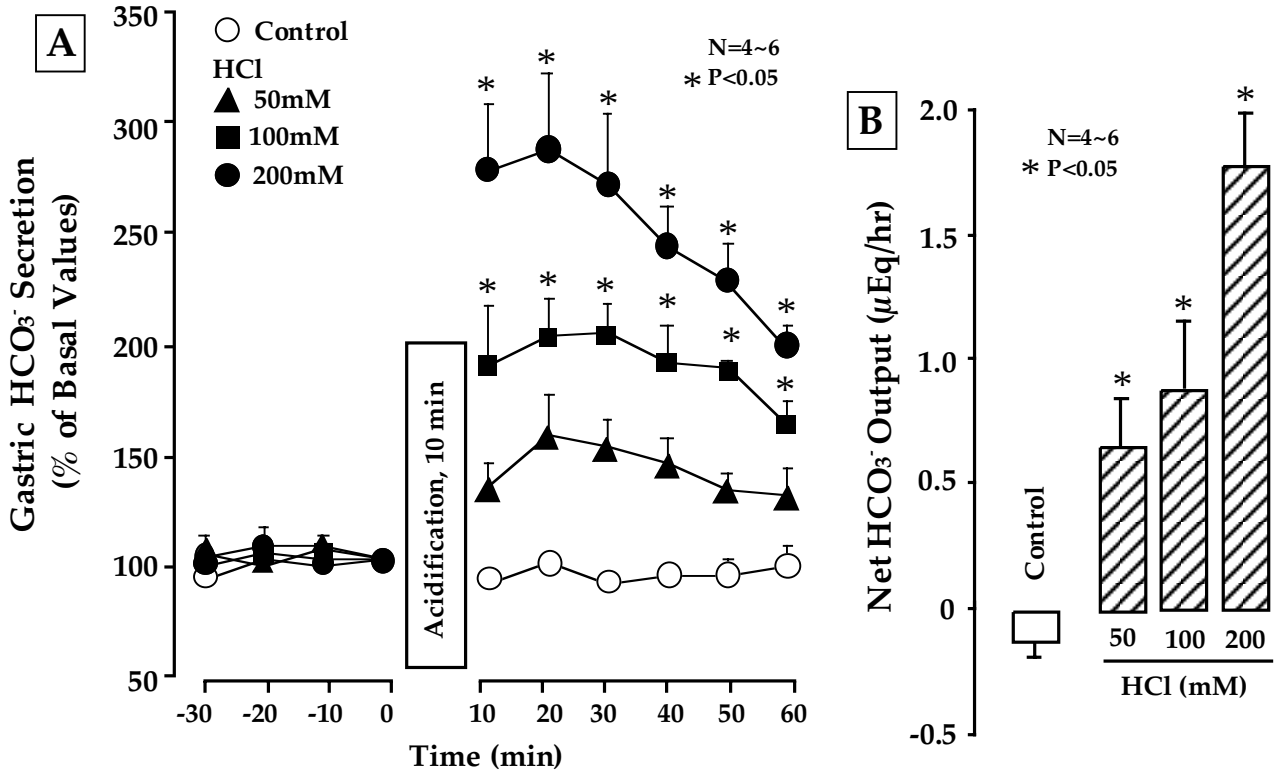

Figure 4

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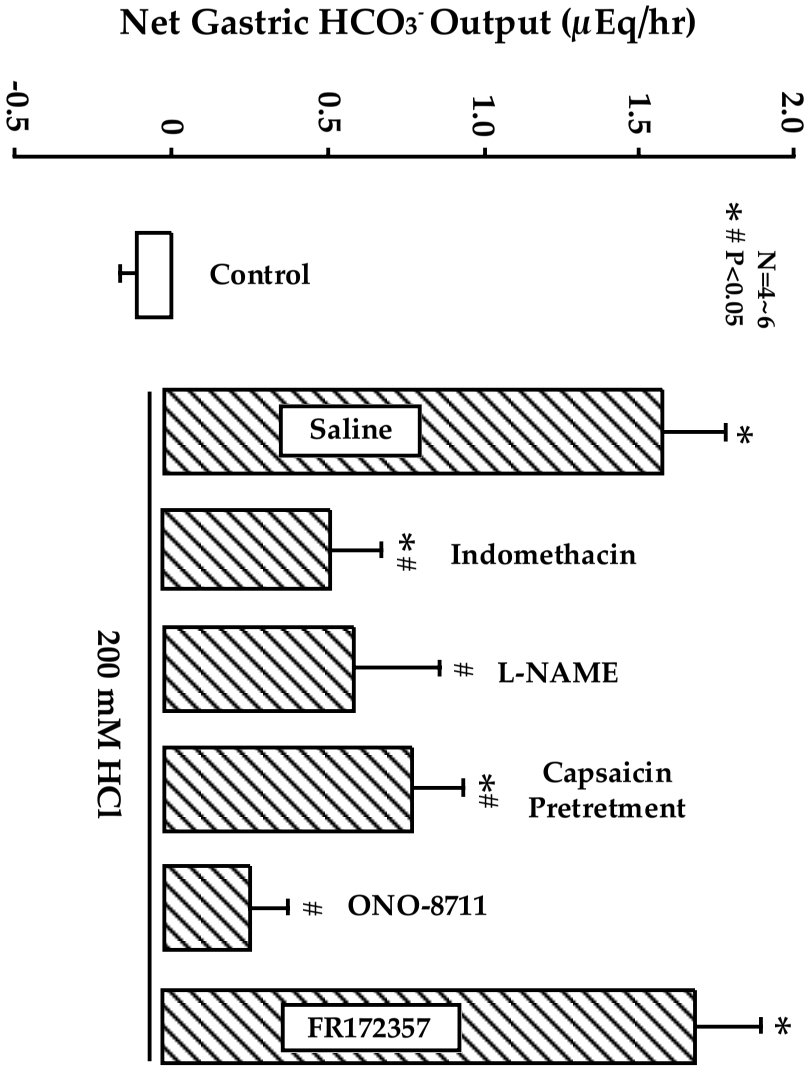

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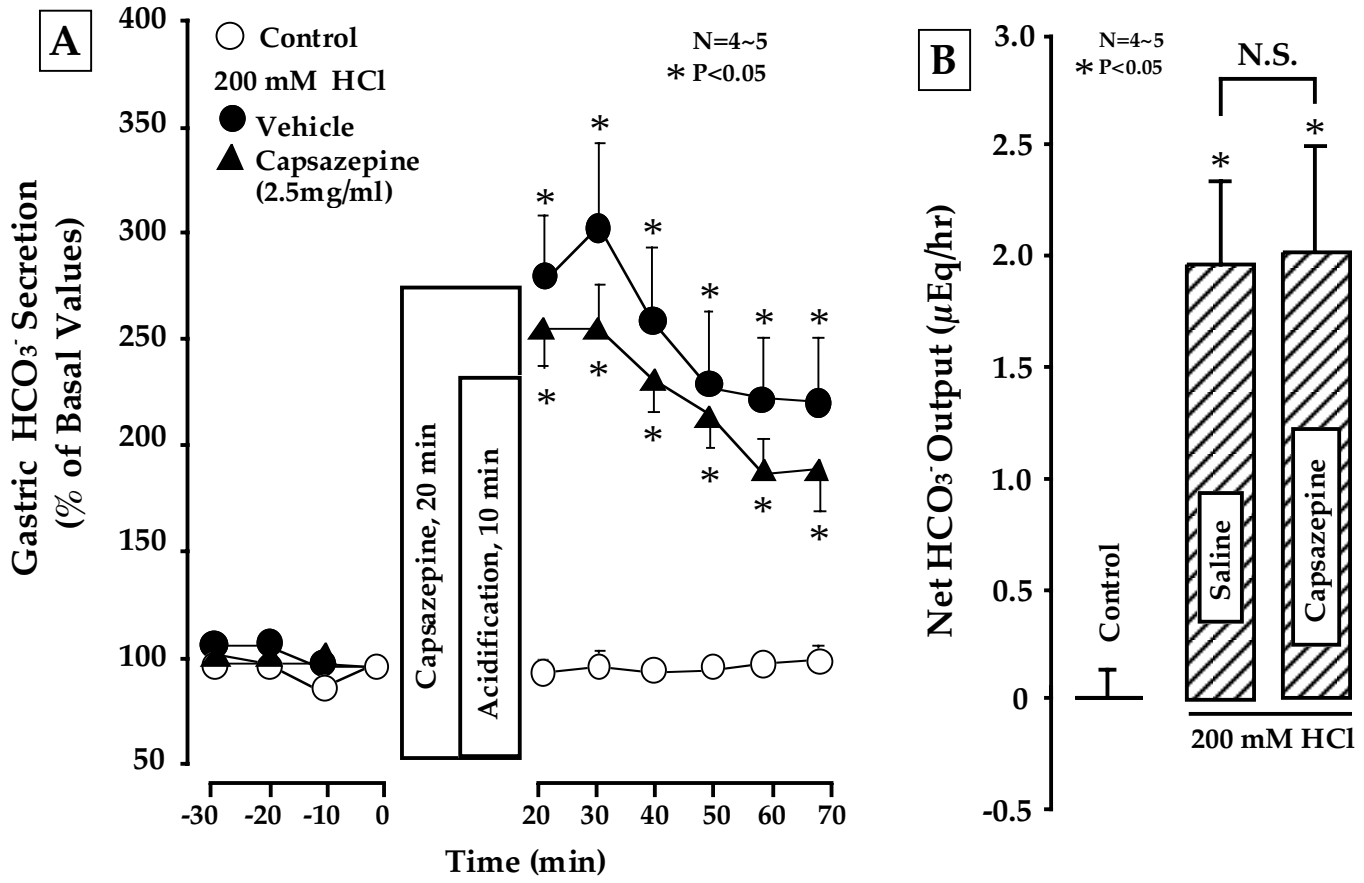

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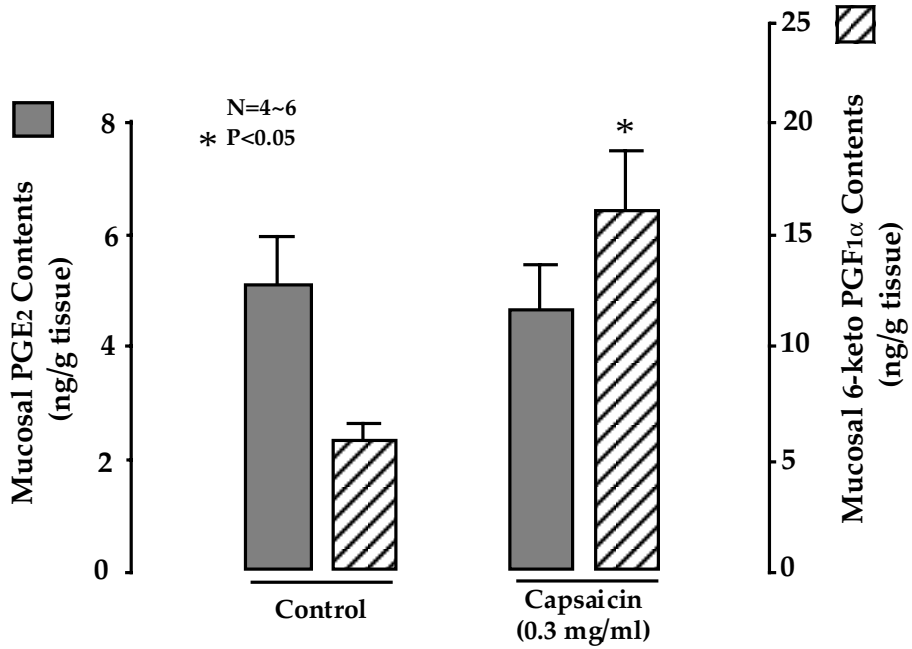


Figure 7

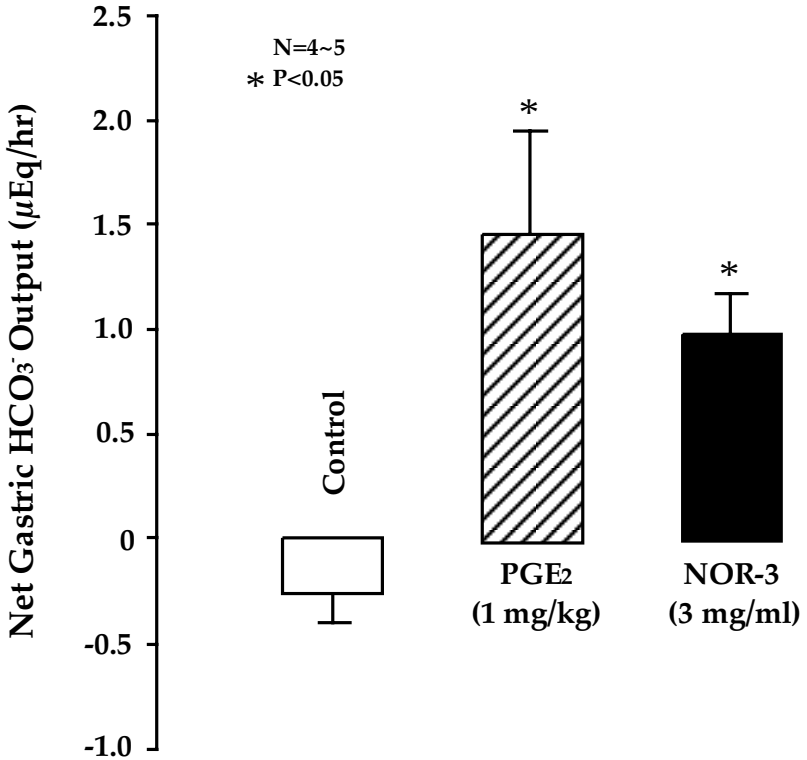


Figure 8

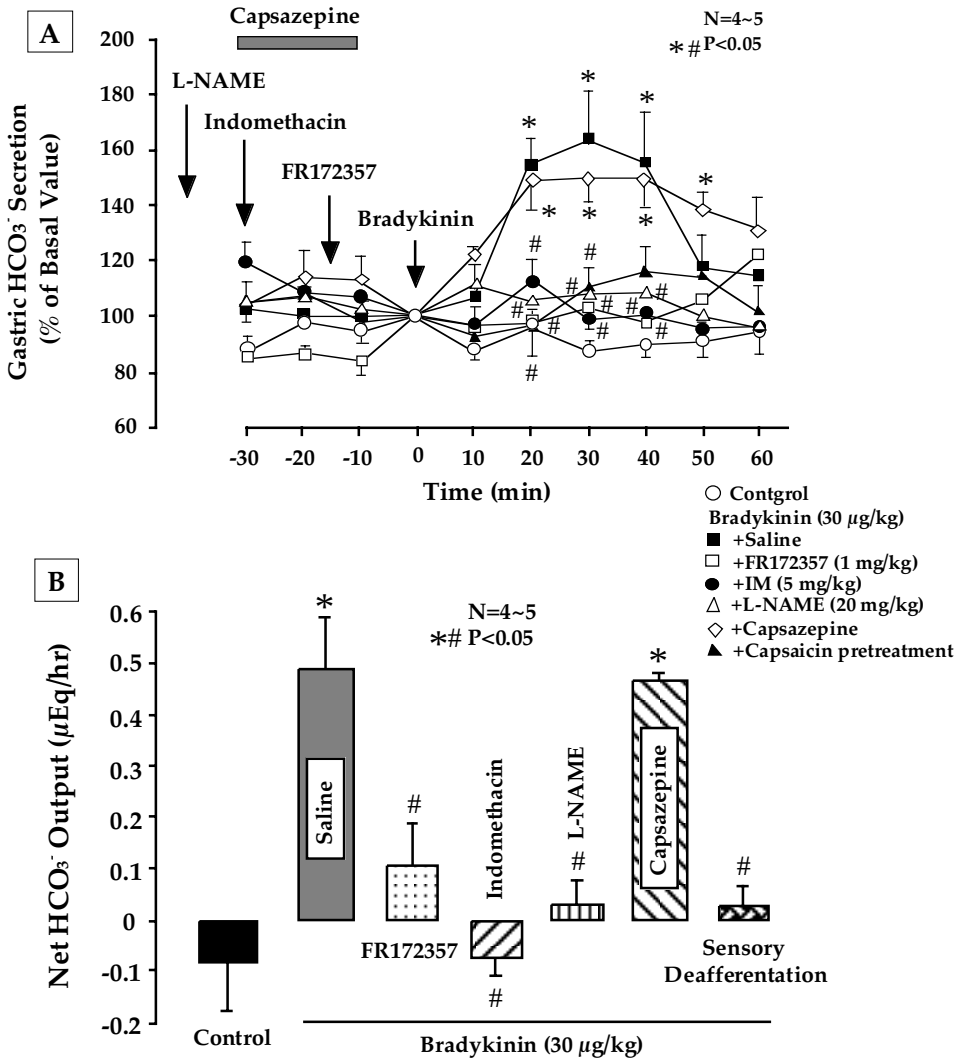
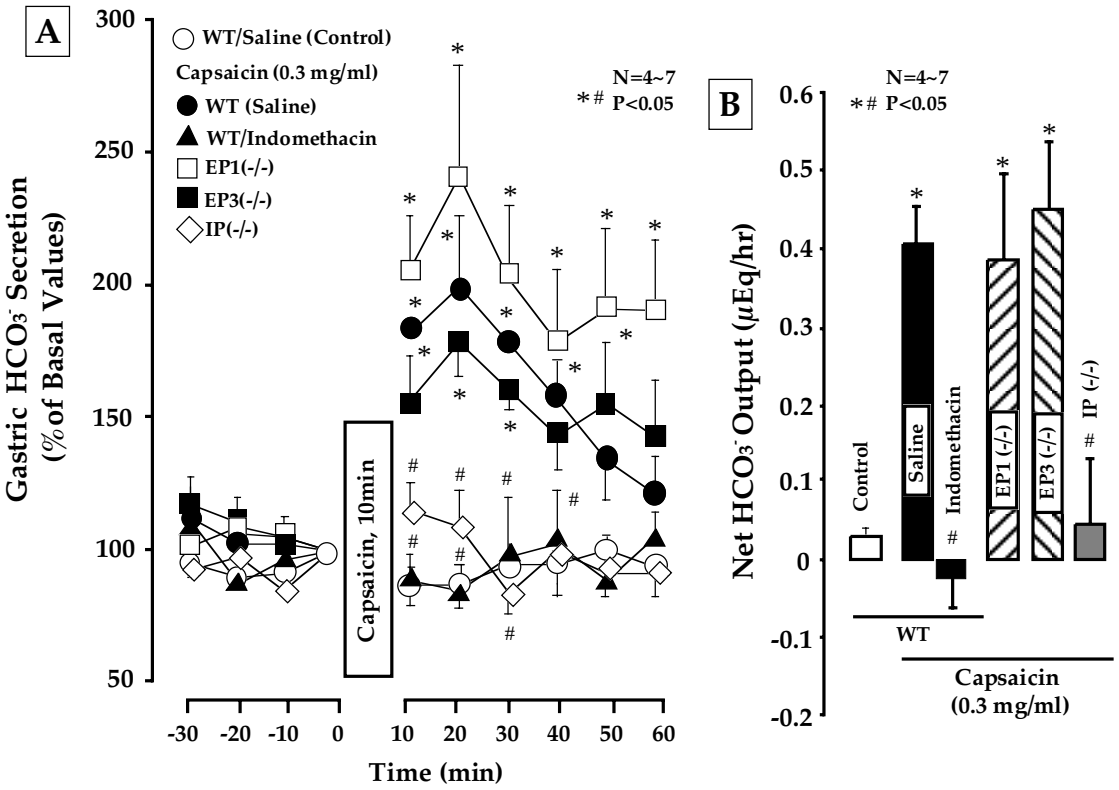


Figure 9



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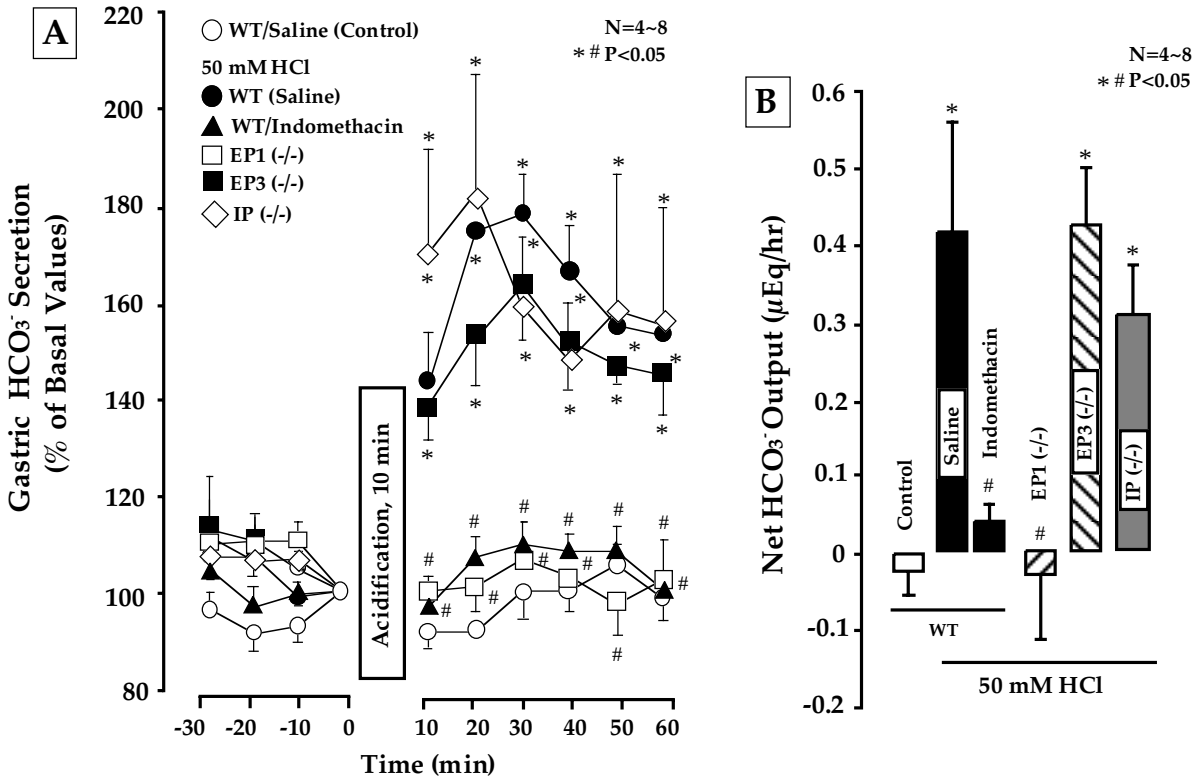

Figure 11