Participation of prostaglandin E receptor EP4 subtype in duodenal bicarbonate secretion in rats

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Aoi, Masako, Eitaro Aihara, Masato Nakashima, and Koji Takeuchi. Participation of prostaglandin E receptor EP4 subtype in duodenal bicarbonate secretion in rats. Am J Physiol Gastrointest Liver Physiol 287: G96–G103, 2004; 10.1152/ajpgi.00038.2004.—We examined, by using a selective PGE receptor subtype EP4 agonist and antagonist, the involvement of EP4 receptors in duodenal HCO$_3^-$ secretion induced by PGE$_2$ and mucosal acidification in rats. Mucosal acidification was achieved by exposing a duodenal loop to 10 mM HCl for 10 min, and various EP agonists were given intravenously 10 min before the acidification. Secretion of HCO$_3^-$ was dose-dependently stimulated by AE1-329 (EP4 agonist), the maximal response being equivalent to that induced by PGE$_2$, whereas that through EP3 receptors is regulated by both cAMP and Ca$^{2+}$; yet there is cooperation between the actions mediated by these two receptors. The neuronal reflex pathway is not involved in stimulatory actions of these prostanoids.

SECRETION OF HCO$_3^-$ from surface epithelial cells is one of the mucosal defensive mechanisms and plays an important role in protecting the duodenal mucosa against acid injury (6, 4, 18). Although the physiological regulation of this secretion involves several factors such as PGs, peptides, and neuronal factors (4, 10, 19, 20), endogenous PGs are particularly important in the local control of the process. We (23) previously investigated the relationship between PGE receptor (EP) subtypes and HCO$_3^-$ secretion, using specific EP1, EP2, and EP3 agonists, and found that the secretion was stimulated by EP agonists having a potent affinity for EP3 receptors. Using EP3 receptor knockout mice, we (21) also demonstrated the involvement of EP3 receptors in the acid-induced secretion of HCO$_3^-$ in the duodenum. At that time, however, the possibility remained that the stimulatory action of PGE$_2$ is mediated by EP4 receptors, because specific EP4 agonists and antagonists were not available. It is now known that the increase in duodenal HCO$_3^-$ secretion is mediated by the stimulation of adenylate cyclase (AC) activity and an elevation in intracellular cAMP levels (8, 16, 20) and that the activation of EP4 receptors results in an increase of intracellular cAMP via Gs protein (3). Thus it is possible that EP4 receptors play a role in the regulation of duodenal HCO$_3^-$ secretion. Recently, a highly specific EP4 agonist (ONO-AE1-329) and antagonist (ONO-AE3-208) developed in Japan have become available (1, 11).

The present study was designed using the selective EP4 agonist and antagonist to clarify the involvement of EP4 receptors in duodenal HCO$_3^-$ secretion induced by PGE$_2$ or mucosal acidification in rats in addition to the role of EP3 receptors. We also investigated the cooperative effect of stimuli sent through EP3 and EP4 receptors on this secretion, including the intracellular signaling pathway of HCO$_3^-$ secretion mediated by these receptors.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 180–200 g (Charles River, Shizuoka, Japan) were used in all experiments. The animals were kept in individual cages with raised mesh bottoms and were deprived of food but allowed free access to tap water for 18 h before the experiments. Studies were carried out by using 4–7 rats under urethane anesthesia (1.25 g/kg ip). All experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Determination of HCO$_3^-$ secretion. HCO$_3^-$ secretion was determined in a duodenal loop or a chambered stomach, according to previously published methods (18, 23). In brief, the abdomen was incised, and a duodenal loop (1.5 cm) was made between the pyloric ring and the area just above the outlet of the common bile duct to exclude the influences of bile and pancreatic juice. The loop was then perfused with saline, which was gassed with 100% O$_2$ and kept in a reservoir. Duodenal HCO$_3^-$ secretion was measured at pH 7.0 by using a pH-stat method (Hiranuma Comtite-8, Mito, Japan) and by adding 2 mM HCl to the reservoir. After basal HCO$_3^-$ secretion was well stabilized, the following prostanoids were administered intravenously as a single bolus injection, and the HCO$_3^-$ secretion was measured for 1 h thereafter: PGE$_2$ (0.1–3 mg/kg), sulprostone (EP1/EP3 agonist: 0.3–3 mg/kg), and ONO-AE1-329 (0.3–3 µg/kg). These prostanoids are considered to be EP receptor subtype-specific agonists (1–3, 11, 16). In some animals, the effect of ONO-AE3-208 (EP4 antagonist: 1 mg/kg) on duodenal HCO$_3^-$ secretion induced by PGE$_2$, sulprostone, or ONO-AE1-329 was examined. This agent was given intravenously 10 min before the above prostanoids. In some cases, the effect of ONO-AE1-329 (3 µg/kg) and sulprostone (0.03 mg/kg), either alone or in combination, on the HCO$_3^-$ secretion was examined. The secretion of HCO$_3^-$ was also stimulated by perfusing the loop with 10 mM HCl for 10 min (mucosal acidification). In another experiment, effects of the Ca$^{2+}$ antagonist verapamil (0.2 mg/kg) and the phos-
phodiesterase inhibitor IBMX (10 mg/kg) on the stimulatory action of sulprostone or ONO-AE1-329 were examined. Verapamil was given intravenously 5 min before the administration of these agents, whereas IBMX was given subcutaneously 1 h before. We also examined the stimulatory action of PGE₂, sulprostone, and ONO-AE1-329 in the rat duodenum after chemical ablation of capsaicin-sensitive afferent neurons (chemical deafferentation). Chemical deafferentation was induced with subcutaneous injections of capsaicin once daily for 3 consecutive days (total dose: 100 mg/kg) 2 wk before the experiment. All capsaicin injections were performed under ether anesthesia, and the rats were pretreated with terbutaline (0.1 mg/kg im) and aminophylline (10 mg/kg im) to counteract the respiratory impairment associated with capsaicin injection. To check for the effectiveness of the treatment, a drop of capsaicin solution (0.1 mg/ml) was instilled into one eye of each rat, and wiping movements were counted as previously reported (19).

In a separate experiment, the role of EP4 receptors in the stimulatory action of PGE₂ in the stomach was investigated. The stomach was exposed, mounted on a chamber (exposed area: 3.1 cm²), and perfused with saline, and the secretion of HCO₃⁻ was measured as described for the duodenal preparation. To unmask HCO₃⁻ in the stomach, acid secretion was completely inhibited by omeprazole given intraperitoneally at a dose of 60 mg/kg. Omeprazole at this dose has been shown to have no influence on gastric HCO₃⁻ secretion in rats (7). PGE₂ (1 mg/kg) or ONO-AE1-329 (3 µg/kg) was given intravenously after basal secretion had stabilized. In some cases, ONO-8711 (EP1 antagonist, 10 mg/kg) or ONO-AE3-208 (1 mg/kg) was administered subcutaneously 30 min or intravenously 10 min before the administration of PGE₂, respectively.

Preparation of drugs. Drugs used were urethane (Tokyo Kasei, Tokyo, Japan); PGE₂ (Funakoshi, Tokyo, Japan); sulprostone, ONO-AE1-329, ONO-AE3-208, and ONO-8711 (Ono, Osaka, Japan); indomethacin and verapamil (Sigma, St. Louis, MO); IBMX (Aldrich, Milwaukee, WI); capsaicin (Nacalai Tesque, Kyoto, Japan); aminophylline (Neophylline, Eizai, Tokyo, Japan); terbutaline (Buricanyl, Fujisawa, Osaka, Japan); and omeprazole (Astra Zeneca, Mönal, Sweden). Indomethacin and IBMX were suspended in saline with a trace of Tween 80 (Wako, Osaka, Japan), whereas verapamil was dissolved in saline. Omeprazole was suspended in a 0.5% carboxymethylcellulose solution. PGE₂ and other EP receptor ligands were first dissolved in absolute ethanol and then diluted with saline to a desired concentration. Capsaicin was dissolved in a Tween 80-ethanol solution (10% ethanol, 10% Tween, and 80% saline, wt/wt). Each agent was prepared immediately before use and given in a volume of 0.5 ml/100 g body wt in the case of intraperitoneal and subcutaneous administration or in a volume of 0.1 ml/100 g body wt in the case of intravenous and intramuscular administration. Control animals received saline in place of the active agent.

Statistics. Data are presented as the means ± SE from 4–7 rats per group. Statistical analyses were performed by using a two-tailed Dunnett’s multiple comparison test, and values of P < 0.05 were regarded as significant.

RESULTS

Effect of ONO-AE1-329, a specific EP4 agonist, on duodenal HCO₃⁻ secretion. Under the present experimental conditions, the rat duodenum spontaneously secreted HCO₃⁻ at a steady rate of 0.7–1.4 µeq/10 min during a 90-min test period. Intravenous administration of ONO-AE1-329 (0.3–3 µg/kg) caused a dose-dependent increase of HCO₃⁻ secretion in the duodenum (Fig. 1A). After administration of this agent at 3 µg/kg, the levels of secretion increased to a maximal value that was 282.1% of the basal level, and remained elevated for at least 50 min, the ΔHCO₃⁻ output being 3.4 ± 0.5 µeq/h. The stimulatory action of ONO-AE1-329 (3 µg/kg) was completely attenuated by prior administration of ONO-AE3-208, a specific antagonist of EP4 receptors, and the ΔHCO₃⁻ output was −0.5 ± 0.4 µeq/h, which is significantly lower than that obtained in the animals given saline instead of the EP4 antagonist (Fig. 1B).

Effect of ONO-AE3-208 on stimulatory action of PGE₂ and sulprostone. It was previously found that PGE₂ stimulates duodenal HCO₃⁻ secretion through the activation of EP3 receptors (23), and this study showed that the EP4 agonist also increases the secretion. To confirm the involvement of EP4 receptors in the stimulatory action of PGE₂ and the ineffectiveness of ONO-AE3-208 (EP4 antagonist) against the HCO₃⁻ response mediated by the activation of EP3 receptors, we examined the effect of ONO-AE3-208 on the responses induced by PGE₂ as well as sulprostone.

Intravenous administration of PGE₂ (1 mg/kg) increased duodenal HCO₃⁻ secretion over control levels, the maximal value being 2.6 ± 0.2 µeq/10 min. The ΔHCO₃⁻ output was 3.6 ± 0.8 µeq/h, which is significantly greater than that (0.1 ± 0.4 µeq/h) observed in the animals given saline alone. As evident in Fig. 2, ONO-AE3-208 (1 mg/kg iv) significantly antagonized the stimulatory action of PGE₂ (1 mg/kg) in the duodenum, the inhibition being 80%. Likewise, duodenal
HCO₃⁻ secretion was also significantly stimulated by intravenous administration of sulprostone (3 mg/kg), the ΔHCO₃⁻ output being 2.1 ± 0.2 μeq/h (Fig. 3). In contrast to the effect of PGE₂, the stimulatory action of sulprostone was not affected by the EP4 antagonist ONO-AE3-208 (1 mg/kg), and the ΔHCO₃⁻ output was 2.9 ± 0.6 μeq/h, which is not significantly different from that observed in the rats given saline instead of the EP4 antagonist. As has been previously reported (23), sulprostone stimulates duodenal HCO₃⁻ secretion mediated by the activation of EP3 receptors. Thus these results showed that ONO-AE3-208 mitigates the stimulatory action of PGE₂ by interacting with PGE₂ at EP4 receptors.

**Effect of ONO-AE3-208 on acid-induced duodenal HCO₃⁻ secretion.** The secretion of HCO₃⁻ in the duodenum increased when the mucosa was acidified by perfusing the duodenal loop with 10 mM HCl for 10 min. After the acidification, the HCO₃⁻ secretion reached a maximal value of 2.3 ± 0.2 μeq/10 min 20 min later and remained elevated for >60 min thereafter (Fig. 4). This response was significantly inhibited by prior administration of indomethacin (5 mg/kg), and the ΔHCO₃⁻ output caused by the acidification decreased from 3.8 ± 0.8 to 0.7 ± 0.2 μeq/h, the inhibition being 76.3%. The acid-induced HCO₃⁻ secretion in the duodenum was also significantly attenuated by pretreatment of the animals with ONO-AE3-208 (1 mg/kg), the ΔHCO₃⁻ output being 0.9 ± 0.3 μeq/h, which is ~23.7% of the response obtained in the rat given saline.

**Effect of coadministration of ONO-AE1-329 plus sulprostone on duodenal HCO₃⁻ secretion.** It was found that both sulprostone and ONO-AE1-329 stimulate duodenal HCO₃⁻ secretion, and this action is mediated by the activation of EP3 and EP4 receptors, respectively. Furthermore, the acid-induced secretion in the duodenum is mediated by endogenous PGs through both EP3 and EP4 receptors. Thus it is possible that the stimuli mediated by these different receptors cooperate to induce HCO₃⁻ secretion in the duodenum. To clarify whether there is any positive interaction between these stimuli, we examined the effect of a low dose of sulprostone or ONO-AE1-329, either alone or in combination, on the secretion of HCO₃⁻.

Neither sulprostone (0.03 mg/kg) nor ONO-AE1-329 (0.3 μg/kg) by itself produced a significant increase in duodenal HCO₃⁻ secretion, although the secretion tended to increase temporarily after the administration; the ΔHCO₃⁻ output was 0.8 ± 0.2 and 0.5 ± 0.1 μeq/h, respectively (Fig. 5). However, when sulprostone was given with ONO-AE1-329 at the same doses, the treatment caused a marked increase in HCO₃⁻ secretion, reaching a maximal value of 260 ± 48.5% of the basal level, and the ΔHCO₃⁻ output was 3.0 ± 0.2 μeq/h, which is highly significant compared with that induced by either of these agents alone.
Ep4 receptors and duodenal HCO₃⁻ secretion

Effects of verapamil and IBMX on duodenal HCO₃⁻ secretion induced by sulprostone or ONO-AE1-329. It was found that sulprostone and ONO-AE1-329 cooperated to potentiate the secretory response in the duodenum. To further investigate the intracellular mediator coupled with the EP3 and EP4 receptors in the stimulation of secretion, we examined the effects of verapamil and IBMX on the stimulatory action of sulprostone or ONO-AE1-329. Sulprostone (1 mg/kg) and ONO-AE1-329 (1 µg/kg) administered intravenously in submaximal doses each produced an increase in duodenal HCO₃⁻ secretion; the ΔHCO₃⁻ output was 2.3 ± 0.2 and 1.6 ± 0.3 µeq/h, respectively. Intravenous administration of verapamil (0.2 mg/kg) did not cause any significant alteration in the response to ONO-AE1-329. However, this agent significantly mitigated the stimulatory action of sulprostone, the ΔHCO₃⁻ output being 0.8 ± 0.3 µeq/h, which is ∼34.7% of that obtained in control rats given saline instead of verapamil (Fig. 6). On the other hand, the stimulatory action of both sulprostone and ONO-AE1-329 was significantly enhanced by prior administration of IBMX (10 mg/kg sc). In the animals pre-treated with IBMX, the ΔHCO₃⁻ output induced by sulprostone or ONO-AE1-329 was 4.2 ± 0.4 or 3.5 ± 0.4 µeq/h, respectively, both values being significantly greater than those observed in control rats given either agent alone. Neither verapamil nor IBMX alone had any influence on basal HCO₃⁻ secretion in the duodenum, and the ΔHCO₃⁻ output was not significantly different compared with that in control animals given saline alone.

Effect of capsaicin pretreatment on responses induced by PGE₂, sulprostone, and ONO-AE1-329. It is believed that the acid-induced HCO₃⁻ secretion is mediated via an axonal reflex pathway in addition to endogenous PGs (10, 19). Because this response is substantially inhibited by indomethacin, it is assumed that endogenous PGE₂ also stimulates the reflex pathway on the afferent side, in addition to directly stimulating the epithelial cells, both resulting in an increase in HCO₃⁻ secretion. Thus we examined the effect of chemical ablation of capsaicin-sensitive afferent neurons on the stimulatory action of PGE₂, sulprostone, and ONO-AE1-329.

Intravenous administration of PGE₂ (1 mg/kg), sulprostone (1 mg/kg), and ONO-AE1-329 (3 µg/kg) produced a significant increase in duodenal HCO₃⁻ secretion in control rats, the ΔHCO₃⁻ output being 3.6 ± 0.7, 2.4 ± 0.6 and 3.4 ± 0.8 µeq/h, respectively (Fig. 7). These agents similarly increased the secretion of HCO₃⁻ in the rats pretreated with capsaicin (1 mg/kg) and either sulprostone or ONO-AE1-329. In these rats, the ΔHCO₃⁻ output was stimulated by exposure of the duodenum to 10 mM HCl for 10 min, the ΔHCO₃⁻ output induced by sulprostone or ONO-AE1-329 being 0.8 ± 0.3 eq/h, whereas ONO-AE1-329 (10 mg/kg) was given intravenously 10 min before exposure to 10 mM HCl, whereas ONO-AE1-329 (10 mg/kg) was given intravenously immediately followed by intravenous administration of ONO-AE1-329. A: data are presented as the means ± SE of values determined every 10 min from 5 rats. B: net HCO₃⁻ secretion is mediated via an axonal reflex pathway on the afferent side, in addition to directly stimulating the epithelial cells, both resulting in an increase in HCO₃⁻ secretion. Thus we examined the effect of chemical ablation of capsaicin-sensitive afferent neurons on the stimulatory action of PGE₂, sulprostone, and ONO-AE1-329.

Intravenous administration of PGE₂ (1 mg/kg), sulprostone (1 mg/kg), and ONO-AE1-329 (3 µg/kg) produced a significant increase in duodenal HCO₃⁻ secretion in control rats, the ΔHCO₃⁻ output being 3.6 ± 0.7, 2.4 ± 0.6 and 3.4 ± 0.8 µeq/h, respectively (Fig. 7). These agents similarly increased the secretion of HCO₃⁻ in the rats pretreated with capsaicin (1 mg/kg) and either sulprostone or ONO-AE1-329. In these rats, the ΔHCO₃⁻ output was stimulated by exposure of the duodenum to 10 mM HCl for 10 min, the ΔHCO₃⁻ output induced by sulprostone or ONO-AE1-329 being 0.8 ± 0.3 eq/h, whereas ONO-AE1-329 (10 mg/kg) was given intravenously immediately followed by intravenous administration of ONO-AE1-329. A: data are presented as the means ± SE of values determined every 10 min from 5 rats. B: net HCO₃⁻ secretion is mediated via an axonal reflex pathway on the afferent side, in addition to directly stimulating the epithelial cells, both resulting in an increase in HCO₃⁻ secretion. Thus we examined the effect of chemical ablation of capsaicin-sensitive afferent neurons on the stimulatory action of PGE₂, sulprostone, and ONO-AE1-329.

Intravenous administration of PGE₂ (1 mg/kg), sulprostone (1 mg/kg), and ONO-AE1-329 (3 µg/kg) produced a significant increase in duodenal HCO₃⁻ secretion in control rats, the ΔHCO₃⁻ output being 3.6 ± 0.7, 2.4 ± 0.6 and 3.4 ± 0.8 µeq/h, respectively (Fig. 7). These agents similarly increased the secretion of HCO₃⁻ in the rats pretreated with capsaicin (1 mg/kg) and either sulprostone or ONO-AE1-329. In these rats, the ΔHCO₃⁻ output was stimulated by exposure of the duodenum to 10 mM HCl for 10 min, the ΔHCO₃⁻ output induced by sulprostone or ONO-AE1-329 being 0.8 ± 0.3 eq/h, whereas ONO-AE1-329 (10 mg/kg) was given intravenously immediately followed by intravenous administration of ONO-AE1-329.
with capsaicin 2 wk before the experiment, and the \( \Delta \text{HCO}_3^- \) output in these animals was 3.0 ± 0.6, 3.0 ± 0.8 and 4.6 ± 0.7 \( \mu \text{eq/h} \), respectively, neither of which is significantly different from that in the corresponding control group.

Effect of an EP4 agonist and antagonist on gastric HCO\(_3^-\) secretion. To further investigate the profile of ONO-AE1-329, an EP4 agonist, we examined the effect of this agent on gastric HCO\(_3^-\) secretion. In addition, we examined the effect of ONO-AE3-208, an EP4 antagonist, on the stimulatory action of PGE\(_2\) in the stomach. The rat stomach secreted HCO\(_3^-\) at a rate of 0.3–0.6 \( \mu \text{eq/10 min} \) in the animals given omeprazole (60 mg/kg) to inhibit the secretion of acid. Intravenous administration of ONO-AE1-309 (3 \( \mu \)g/kg) did not have any effect on the secretion of HCO\(_3^-\) in the stomach. On the other hand, PGE\(_2\) (1 mg/kg) produced a significant increase in gastric HCO\(_3^-\) secretion, similarly in the duodenum, and the \( \Delta \text{HCO}_3^- \) output at 1 mg/kg was 1.6 ± 0.4 \( \mu \text{eq/h} \), ~45% of that obtained in the duodenum at the same dose (Fig. 8). This response induced by PGE\(_2\) was significantly inhibited by prior administration of ONO-8711 (10 mg/kg), the EP1 antagonist, but not ONO-AE3-208 (1 mg/kg), the EP4 antagonist. The \( \Delta \text{HCO}_3^- \) output in the presence of ONO-8711 was 0.3 ± 0.1 \( \mu \text{eq/h} \), which is only 18.7% of that observed in control rats given saline.

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**Fig. 6.** Effect of verapamil and IBMX on the stimulatory action of sulprostone (A) or ONO-AE1-329 (B) in anesthetized rats. HCO\(_3^-\) secretion was stimulated by intravenous administration of sulprostone (0.03 mg/kg) or ONO-AE1-329 (1 \( \mu \)g/kg). IBMX (10 mg/kg) was given subcutaneously 30 min before the administration of these agents, whereas verapamil (2 mg/kg) was given intravenously 5 min before. Data are presented as the total net HCO\(_3^-\) secretion (HCO\(_3^-\) output) for 1 h after administration of sulprostone or ONO-AE1-329 and represented as the means ± SE for 4–7 rats. Significant difference at \( P < 0.05 \); *from saline; #from control.

**Fig. 7.** Effect of capsaicin pretreatment on the stimulatory action of PGE\(_2\), ONO-AE1-329 or sulprostone in anesthetized rats. Left: HCO\(_3^-\) secretion was stimulated by intravenous administration of PGE\(_2\) (1 mg/kg), ONO-AE1-329 (3 \( \mu \)g/kg), or sulprostone (1 mg/kg). Capsaicin pretreatment (total 100 mg/kg sc) was performed 2 wk before the experiment. Data are presented as the means ± SE of values determined every 10 min from 5 rats. Right: net HCO\(_3^-\) output for 1 h after administration of these agents. The data are presented as the means ± SE for 5 rats. *Significant difference at \( P < 0.05 \) from control. Note no difference in the response to any prostanoids tested between normal and capsaicin-pretreatment groups.

**Fig. 8.** Effects of ONO-AE1-329 and PGE\(_2\) on gastric HCO\(_3^-\) secretion in anesthetized rats. PGE\(_2\) (1 mg/kg) or ONO-AE1-329 (3 \( \mu \)g/kg) was given intravenously. In some animals, ONO-AE3-208 (1 mg/kg) or ONO-8711 (10 mg/kg) was given intravenously 10 min or subcutaneously 30 min before the administration of PGE\(_2\), respectively. A: data are presented as the means ± SE of values determined every 10 min from 4–6 rats. B: net HCO\(_3^-\) output for 1 h after administration of the prostanoids. Data are presented as the means ± SE for 4–6 rats. *Significant difference from control at \( P < 0.05 \).
DISCUSSION

Previous studies well demonstrated the importance of PGE$_2$ in the local regulation of duodenal HCO$_3^-$ secretion (6, 9, 18, 21). Endogenous PGE$_2$ indeed mediates the acid-induced HCO$_3^-$ response, the process being very important in the mucosal defense against acid injury (4, 18). The present study confirmed our previous findings that duodenal responses to PGE$_2$ as well as mucosal acidification are mediated through the activation of EP3 receptors (21, 23) and further demonstrated the involvement of EP4 receptors in the stimulatory process. It is assumed that the presence of both EP3 and EP4 is essential for maintaining the mucosal integrity of the duodenum.

Development of specific agonists and antagonists for PGE receptor subtypes has contributed much to our understanding of which EP receptor subtype is involved in the various actions of PGE$_2$ in the gastrointestinal tract. We have used several EP receptor subtype-specific agonists and reported that the stimulatory action of PGE$_2$ toward secretion of HCO$_3^-$ in the stomach and duodenum is mediated by the activation of EP1 and EP3 receptors, respectively (23). These results were confirmed in experiments using EP1- or EP3-receptor knockout mice (22). In addition, we also demonstrated the importance of EP3 receptors in the duodenal response induced by mucosal acidification (21). However, because EP4 agonists and antagonists as well as EP4 knockout mice were not available, we could not totally exclude the possibility that EP4 receptors participate in the HCO$_3^-$ response in the duodenum. Recently, ONO-AE1-329 and ONO-AE3-208 have become available as a specific EP4 agonist and antagonist, respectively (1, 11), allowing one to examine the participation of EP4 receptors in the regulation of duodenal HCO$_3^-$ secretion.

We confirmed in the present study the participation of EP3 receptors in duodenal HCO$_3^-$ secretion induced by PGE$_2$ or mucosal acidification. As expected, the secretion in the duodenum was significantly stimulated by ONO-AE1-329, a specific EP4 agonist, the effect being dose-dependently mitigated by ONO-AE3-208, the EP4 antagonist. In addition, this agent significantly attenuated the secretion of HCO$_3^-$ induced by both PGE$_2$ and acidification but not the EP1/EP3 agonist sulprostone. These results showed that ONO-AE3-208 is a selective antagonist of EP4 receptors and demonstrated that EP4 receptors are involved in the stimulatory response to endogenous PGs, along with EP3 receptors. The question then arises as to why the response to PGE$_2$ is substantially reduced when one of these receptors is inhibited. One possible explanation is that PGE$_2$ stimulates the secretion of HCO$_3^-$ by activating either receptor subtype EP3 or EP4, but the activation of both subtypes is required for full stimulation. This idea was supported by the present finding of a marked potentiation in the secretory response when sulprostone (EP1/EP3 agonist) and ONO-AE1-329 (EP4 agonist) were administered together at a dose at which either agent alone did not significantly increase the secretion.

Duodenal HCO$_3^-$ secretion results from an elevation of intracellular cAMP levels and can be elicited also by a receptor-independent AC activator like forskolin (8, 16, 17, 20). Ca$^{2+}$ also functions as an intracellular mediator in HCO$_3^-$ secretion, because the secretion was stimulated by A$_{23187}$ and inhibited by the removal of Ca$^{2+}$ from the serosal solution (5, 17). Consistent with previous findings (23), we observed that the stimulatory action of the EP1 and EP3 agonist sulprostone in the rat duodenum was significantly mitigated by verapamil and potentiated by pretreatment with IBMX, an inhibitor of phosphodiesterase, suggesting that PGE$_2$-stimulated HCO$_3^-$ secretion is mediated by both Ca$^{2+}$ and cAMP. EP receptor subtypes are coupled with different signal transduction systems; activation of EP1 receptors causes an elevation of intracellular Ca$^{2+}$ levels via Ca$^{2+}$ channels independent of phosphoinositol turnover, whereas that of EP2 and EP4 receptors results in an elevation of intracellular cAMP levels (3). The EP3 receptor has four splicing variants coupled to different signaling pathways (15). The EP3A receptor is linked to the activation of G$_i$ protein, whereas EP3B and EP3C are coupled with the activation of G$_o$, protein, resulting in stimulation of AC activity. In addition, the activation of EP3D causes an elevation of intracellular Ca$^{2+}$ by stimulating phosphoinositol turnover. In the present study, the duodenal response to ONO-AE1-329 was significantly augmented by pretreatment with IBMX but not affected by verapamil, confirming the mediation by cAMP of the action of the EP4 agonist. In general, a synergetic response to pharmacological actions is produced by the activation of two different signaling pathways. For example, stimulation of acid secretion involves an initial elevation of intracellular Ca$^{2+}$ and/or cAMP followed by activation of a cAMP-dependent protein kinase cascade that triggers the translocation and insertion of the proton pump enzyme into the apical membrane of parietal cells (24). Full response of acid secretion requires both cAMP and Ca$^{2+}$, and the lack of either factor results in substantial decrease in the response. The same is observed in regulation of HCO$_3^-$ secretion, although the exact mechanism for a synergetic response or the effectors that are activated by Ca$^{2+}$ and cAMP remain to be fully identified. Furthermore, at present, it remains unknown whether EP3 agonists activate the Ca$^{2+}$ and cAMP pathways at a similar time or dose, despite the activation of EP3 receptors being coupled with these two pathways. Notwithstanding, it seems that costimulation of these pathways by both EP3 and EP4 agonists produces a synergetic increase in duodenal HCO$_3^-$ secretion. This idea may also apply to the secretion of HCO$_3^-$ induced by acidification of the mucosa, and a malfunction of either the EP3 or EP4 receptor system results in a substantial loss of this response. Certainly, one would predict partial agonist and antagonist effects of these subtype-specific EP agonists. However, this possibility may be excluded, because ONO-AE1-329 or ONO-AE3-218 is a highly specific and full EP4 agonist or antagonist, respectively (1, 11).

Morimoto et al. (13) demonstrated by Northern blot analysis the significant expression of EP3 and EP4 receptors in the gastroduodenal mucosal layer containing epithelial cells and also in the neurons of the myenteric ganglia throughout the gastrointestinal tract. These results are compatible with the present observation that HCO$_3^-$ secretion, an epithelial function, is mediated by EP3 and EP4 receptors in the duodenum. However, we could not identify by immunohistochemical staining the exact locations of these receptors in the duodenum, because an antiserum for the receptor proteins has yet to be made available.

It is believed that the acid-induced secretion of HCO$_3^-$ is mediated via an axonal reflex pathway, in addition to endogenous PGs (10). Because this response is substantially inhibited by indomethacin, it is speculated that the afferent
side of this reflex pathway is influenced by PGs, probably by facilitating the neuronal excitation in response to H⁺. Indeed, we have previously reported that acid-induced HCO₃⁻ secretion was significantly attenuated by chemical ablation of capsaicin-sensitive afferent neurons and that the stimulatory action of capsaicin was also suppressed by indomethacin (12, 19). It is assumed that local PGE₂ release stimulates the reflex pathway on the afferent side and may also directly stimulate the epithelial cells, both resulting in an increase in the secretion of HCO₃⁻. The EP3 receptors, which are a prerequisite for the acid-induced duodenal HCO₃⁻ secretion, might be on cells at the afferent side of the reflex pathway. Thus it is assumed that local PGE₂ release would stimulate the reflex pathway on the afferent side and may also directly stimulate the epithelial cells, both resulting in an increase in HCO₃⁻ secretion. However, the stimulatory action of PGE₂, sulprostone, or ONO-AE1-329 was not significantly affected by capsaicin pretreatment, suggesting a direct action of these prostanoids on the duodenal epithelial cells to stimulate HCO₃⁻ secretion. Certainly, we cannot exclude the possibility that endogenous PGs affect the secretory response mediated by these afferent neurons.

Recently, we found that the duodenal response to capsaicin was absent in prostacyclin receptor (IP receptor) knockout mice (14). Because capsaicin and acid stimulate these afferent neurons via different mechanisms, the former action is blocked by capsaazepine, a competitive antagonist of vanilloid type 1 receptors, whereas the latter is not (12). The possibility still remains that the EP3 and EP4 receptors, which are required for the acid-induced duodenal HCO₃⁻ secretion, might be on cells at the afferent side of the reflex pathway and sensitize the neuronal responses to acid through these receptors.

We (22, 23) previously reported that PGE₂ stimulates HCO₃⁻ secretion in the stomach mediated by EP1 receptors. In the present study, both PGE₂ and sulprostone but not ONO-AE1-329 increased gastric HCO₃⁻ secretion. Furthermore, the stimulatory action of PGE₂ in the stomach was significantly attenuated by ONO-8711, the EP1 antagonist, but not ONO-AE3-208, the EP4 antagonist. These results confirmed the involvement of EP1 receptors in the gastric response to PGE₂ and clearly demonstrated that EP4 receptors do not play any role in the stimulation of HCO₃⁻ secretion in the stomach.

Taking the present and previous findings together, it is concluded that EP4 receptors are involved in the duodenal HCO₃⁻ response induced by PGE₂ or acidification in addition to EP3 receptors. The process by which HCO₃⁻ is secreted through these receptors differs with regard to second-messenger coupling; the stimulation through EP4 receptors is mediated by cAMP, whereas through EP3 receptors is mediated by both cAMP and Ca²⁺, yet there is cooperation between the actions mediated by these two receptors. Sensory neurons have no interaction with the stimulatory action of these prostanoids.

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