GASTROESOPHAGEAL REFUX (GER) is a common clinical disorder associated with a variety of respiratory symptoms, including chronic cough and the exacerbation of asthma (15, 16, 37). Although GER causes pulmonary symptoms by microaspiration of the gastric content, it is not possible to document this pathogenesis consistently. Therefore, other mechanisms must be considered. The vagally mediated reflex has been suggested as the cause of the esophageal-tracheobronchial reflex (28), because acidification of the esophagus has been shown to increase total respiratory resistance in dogs and humans (27, 28). Although the cholinergic nerve has been reported to be involved in the pathway (2), the involvement of C fibers contained in the vagal nerve has not been shown.

Tachykinins such as substance P and neurokinin A exist in the C fibers, which are distributed in the epithelium, smooth muscle, and blood vessels of airway tissues in many mammals, including humans (14, 24). Tachykinins affect the airway tissues in many ways and induce neurogenic inflammation: for example, they contract airway smooth muscle (31, 39), increase bronchial secretion (3), increase vascular permeability (26), and induce cough (19). It is known that tachykinins are released from the lung by capsaicin (pungent principal of red pepper), histamine, bradykinin, prostaglandin F₂α (8), allergic response (21), and antidromic electrical stimulation of the vagus nerve (34).

Tachykinins are effectively degraded by peptidase, especially neutral endopeptidase (NEP), which exists in the airways (17) and lungs (23, 32). The inhibition of NEP potentiates sensitivity and reactivity to tachykinins in the airways (7, 19, 36), including human airways (12). Furthermore, recombinant NEP inhibits cough induced by substance P and capsaicin (20). These reports suggest that NEP degrades tachykinins, thereby reducing their physiological effects in the airways.

Nerves containing tachykinins and tachykinin-binding sites exist in the esophagus as well as in the airways (18, 40). Because the esophageal and pulmonary systems share a similar embryological origin, the foregut, it would not be surprising if they also shared a common nerve pathway. Thus we postulated that a nonadrenergic noncholinergic (NANC) excitatory nerve communicating between airways and esophagus might exist. If so, stimulation of the esophagus with gastric acid would cause tachykinin release in the airways through the pathway. To test this hypothesis, we examined the airway plasma extravasation by stimulating the esophagus by using HCl in the presence or absence of NEP inhibitor, in the presence or absence of NK₁ receptor antagonist, and in normal or tachykinin-depleted animals.

METHODS

We used 65 male Hartley guinea pigs weighing 250–350 g. These were treated according to the guidelines of Animal Care at Kumamoto University and the Guidelines in the Care and Use of Animals approved by the Council of the American Physiological Society.

Capsaicin Pretreatment

To deplete tachykinins, the guinea pigs were treated with capsaicin as follows (14). First, they were given ketamine (50 mg/kg ip), aminophylline (10 mg/kg ip), and salbutamol (0.2 mg/kg sc) to reduce the pain and bronchoconstriction induced by the capsaicin injection. Then capsaicin (40 mg/kg sc) or its vehicle was injected. The same procedure was repeated 5 days later (10 guinea pigs for capsaicin pretreatment, 40 guinea pigs for vehicle pretreatment). One week after the second capsaicin injection, the animals were prepared for data collection.
Animal Preparation

Animals were anesthetized intraperitoneally with pentobarbital sodium (25 mg/kg). Additional injections were given as necessary. The carotid artery was cannulated to monitor systemic blood pressure (Recticorder, Nikon Kohden Kogyo, Tokyo, Japan) and to sample arterial blood for gas analysis. The jugular vein was cannulated to inject drugs. One hundred percent oxygen was supplied with a loosely fitted 100-ml polyethylene mask. All animals were pretreated intravenously 30 min before experimentation with atropine (1 mg/kg) and propranolol (1 mg/kg) to block muscarinic and ß-adrenergic receptors. The doses of atropine and propranolol were determined according to previous studies (5).

The esophageal wall was partly sectioned at the level of the fifth tracheal cartilage ring, and a catheter (3-Fr Indwelling Feeding Tube for Infant, ATOM, Tokyo, Japan) was placed in the midesophageus. We ligated the esophagus at the upper portion to inhibit HCl leakage. We then exposed the lower end of esophagus from the abdomen and ligated it to block communication between the esophagus and the stomach. Thus there was no leakage of fluid from the esophagus.

Experimental Procedure

To study the involvement of tachykinins in airway plasma extravasation by esophageal stimulation, we stimulated the esophagus by intraesophageal 1 N HCl (0.4 ml) or saline (0.9%, pH 6.4) infusion and measured the leakage of Evans blue dye in the airways. The esophagus was stimulated for 1 min, and HCl was collected. This procedure did not cause leakage of HCl into the mediastinum because the pH-indicator paper (pH-Box, Merck, Darmstadt, Germany) showed that the pH of the adventitia of the esophagus was 7.0 in all animals. The trachea and bronchi were also examined to investigate the effect of HCl leakage on the airways. After they were fixed in 4% paraformaldehyde and embedded in paraffin, hematoxylin and eosin staining was performed. No histological degeneration such as necrosis was seen in the airway adventitia, muscularis, lamina propria, or epithelium.

Effect of phosphoramidon on airway plasma extravasation induced by esophageal stimulation with HCl. Five minutes after injecting phosphoramidon (2.5 mg/kg) or its vehicle (0.9% saline, pH 6.4) intravenously, we injected Evans blue dye (30 mg/kg) intravenously. Then we infused 1 N HCl (0.4 ml) into the esophagus for 1 min. Ten minutes later, we measured the Evans blue dye leakage. The dose of phosphoramidon was determined according to the previous study in which it significantly increased antigen-induced airway extravasation associated with tachykinin release (6).

Effect of specific NK₁-receptor antagonist FK-888 on the HCl-induced airway plasma extravasation in the presence of phosphoramidon. Three minutes after injecting phosphoramidon, we injected each animal with FK-888 (0.1 mg/kg and 0.4 mg/kg) or its vehicle (4% propylene glycol, 1% ethanol, 95% distilled water). Two minutes later we injected Evans blue dye (30 mg/kg) intravenously, infused 1 N HCl (0.4 ml) for 1 min, and measured the Evans blue dye leakage.

Effect of systemic capsaicin treatment on HCl-induced airway plasma extravasation in the presence of phosphoramidon. Five minutes after giving phosphoramidon (2.5 mg/kg) intravenously to all animals, we injected Evans blue dye intravenously (30 mg/kg). Then we infused 1 N HCl (0.4 ml) or 0.9% saline into the esophagus of the capsaicin-treated or nontreated animals for 1 min and measured the Evans blue dye leakage.

Effect of bilateral vagotomy on HCl-induced airway plasma extravasation in the presence of phosphoramidon. All animals were pretreated with phosphoramidon (2.5 mg/kg) intravenously. We cut the bilateral vagal nerve at the fourth tracheal cartilage in five guinea pigs and left five intact. Five minutes later, we injected Evans blue dye (30 mg/kg) intravenously, infused 1 N HCl (0.4 ml) into the esophagus for 1 min, and measured the Evans blue dye leakage 10 min later.

Effect of bilateral vagotomy on substance P-induced airway plasma extravasation in the presence of phosphoramidon. To exclude the effect of bilateral vagotomy on local hemodynamics, we studied the airway plasma extravasation induced by substance P in the vagotomized guinea pigs. All animals were pretreated with phosphoramidon (2.5 mg/kg) intravenously. We cut the bilateral vagal nerve at the fourth tracheal cartilage in five guinea pigs and left five intact. Five minutes later, we injected substance P (0.6 µg/kg) for 1 min and Evans blue dye (30 mg/kg) intravenously, and we measured the Evans blue dye leakage 10 min later. In the preliminary experiment, we found that the dose of substance P used in the study caused almost the same Evans blue dye leakage as intraesophageal HCl did in the presence of phosphoramidon (data not shown). During the experiment, we monitored heart rate, arterial blood pressure, and arterial blood-gas analysis.

Measurement of airway microvascular leakage. Vascular permeability was quantified by using a modified method of Saria and Lundberg (33).

The tissue content of Evans blue dye after experimental intervention was determined by perfusing the systemic circulation with 0.9% saline to remove intravascular dye. After the induction of leakage (10 min after completion of the intraesophageal HCl infusion), the thorax was opened and a blunt-ended 14-gauge needle was passed through a left ventriculotomy into the aorta. The ventricle was cross clamped, and blood was expelled through an incision into the right atrium at 100-mmHg pressure with ~100 ml of 0.9% saline. The lungs were then removed. The connective tissue, vasculature, and parenchyma were gently scraped, and the airways were divided into two components: the lower part of the trachea and the main bronchi. The tissues were blotted dry and weighed, and their dye content was extracted in formamide at 37°C for 24 h. Dye concentration was quantified from light absorbance at 620 nm, and its tissue content (ng dye/mg wet tissue) was calculated from a standard curve of dye concentration in the range of 0.5–10 µg/ml.

Drugs

The following drugs were used: atropine sulfate, propranolol, capsaicin, formamide (Sigma Chemical, St. Louis, MO); Tween 80 (Nacalai Tesque, Tokyo, Japan); Evans blue dye, propylene glycol (Wako Pure Chemical, Osaka, Japan); ketamine, salbutamol (Sankyo, Tokyo, Japan); aminophylline (Eisai, Tokyo), phosphoramidon, substance P (Peptide Institute, Osaka, Japan); pentobarbital sodium (Dainippon Pharmaceutical, Osaka, Japan); and 1 N HCl (Katayama Chemical, Osaka). FK-888 was a generous gift from Fujisawa Pharmaceutical (Osaka, Japan).

Capsaicin (5 mg) was suspended in 80% normal saline, 10% Tween 80, and 10% ethanol. Evans blue dye, phosphoramidon, substance P, salbutamol, aminophylline, atropine sulfate, and propranolol were dissolved in 0.9% NaCl. FK-888 was dissolved in 4% propylene glycol, 1% ethanol, and 95% distilled water.
The data are expressed as means ± SE. The data were analyzed for significance by using repeated-measures one-way analysis of variance. Post-hoc analysis was performed by using Fisher’s protected least-significant difference test. The Mann-Whitney test was used to test the effect of bilateral vagotomy on airway plasma extravasation, heart rate, and arterial blood pressure in the substance P-treated animals. Significance was defined as $P < 0.05$.

### RESULTS

We monitored the mean blood pressure and collected arterial blood for gas analysis just before the injection of phosphoramidon. We studied seven groups. There was no difference among the seven groups (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Arterial Blood Pressure, mmHg</th>
<th>$\text{P}_2CO_2$, Torr</th>
<th>$\text{P}_2O_2$, Torr</th>
<th>Base Excess, meq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>46.7 ± 5.1</td>
<td>7.35 ± 0.03</td>
<td>51.0 ± 3.5</td>
<td>375.1 ± 25.4</td>
</tr>
<tr>
<td>Group 2</td>
<td>49.6 ± 6.1</td>
<td>7.36 ± 0.02</td>
<td>44.1 ± 5.3</td>
<td>370.7 ± 20.8</td>
</tr>
<tr>
<td>Group 3</td>
<td>49.5 ± 1.8</td>
<td>7.29 ± 0.02</td>
<td>48.8 ± 8.7</td>
<td>381.8 ± 32.9</td>
</tr>
<tr>
<td>Group 4</td>
<td>47.3 ± 2.8</td>
<td>7.32 ± 0.03</td>
<td>48.5 ± 4.7</td>
<td>377.1 ± 18.5</td>
</tr>
<tr>
<td>Group 5</td>
<td>50.1 ± 6.1</td>
<td>7.33 ± 0.03</td>
<td>47.3 ± 4.4</td>
<td>369.2 ± 31.2</td>
</tr>
<tr>
<td>Group 6</td>
<td>43.6 ± 3.7</td>
<td>7.29 ± 0.03</td>
<td>53.1 ± 3.8</td>
<td>358.2 ± 29.3</td>
</tr>
<tr>
<td>Group 7</td>
<td>43.3 ± 4.2</td>
<td>7.23 ± 0.04</td>
<td>51.3 ± 4.3</td>
<td>397.3 ± 25.2</td>
</tr>
</tbody>
</table>

Values are means ± SE for 5 guinea pigs in each group. $\text{P}_2CO_2$, arterial $\text{P}_2CO_2$; $\text{P}_2O_2$, arterial $\text{P}_2O_2$; group 1, pretreated with phosphoramidon intravenously and intraesophageal saline infusion; group 2, pretreated with phosphoramidon intravenously and intraesophageal HCl infusion; group 3, pretreated with phosphoramidon and FK-888 (0.4 mg/kg) intravenously and intraesophageal HCl infusion; group 4, capsaicin-treated animals pretreated with phosphoramidon intravenously and intraesophageal HCl infusion; group 5, vagotomized animals pretreated with phosphoramidon intravenously and intraesophageal HCl infusion; group 6, vagotomized animals pretreated with phosphoramidon intravenously and intravenous substance P injection; group 7, nonvagotomized animals pretreated with phosphoramidon intravenously and intravenous substance P injection.

Statistical Analysis

The data are expressed as means ± SE. The data were analyzed for significance by using repeated-measures one-way analysis of variance. Post-hoc analysis was performed by using Fisher’s protected least-significant difference test. The Mann-Whitney test was used to test the effect of bilateral vagotomy on airway plasma extravasation, heart rate, and arterial blood pressure in the substance P-treated animals. Significance was defined as $P < 0.05$.

Effect of Specific NK$_1$-Receptor Antagonist FK-888 on the HCl-Induced Airway Plasma Extravasation in the Presence of Phosphoramidon (Fig. 2)

The NK$_1$-receptor antagonist FK-888 significantly and in a dose-related manner inhibited the HCl-induced plasma extravasation potentiated by phosphoramidon. The inhibition was greater in the trachea than in the main bronchi.

Effect of Systemic Capsaicin Treatment on HCl-Induced Airway Plasma Extravasation in the Presence of Phosphoramidon (Fig. 3)

The plasma extravasation potentiated by phosphoramidon was significantly inhibited in the capsaicin-treated animals.
The effect of bilateral vagotomy on HCl-induced airway plasma extravasation in the presence of phosphoramidon (Fig. 4)

The plasma extravasation potentiated by phosphoramidon was significantly inhibited in the trachea of the vagotomized animals.

Effect of Bilateral Vagotomy on Substance P-Induced Airway Plasma Extravasation in the Presence of Phosphoramidon

The plasma extravasation was not different between the vagotomized and sham-operated animals (Fig. 5). The heart rate and arterial blood pressure were not different (Fig. 6) and arterial blood-gas analysis was not different between the animals (groups 6 and 7 in Table 1).

**DISCUSSION**

In guinea pigs in which cholinergic and adrenergic nerves were pharmacologically blocked, we found that 1) esophageal stimulation by HCl caused airway plasma extravasation, 2) the extravasation was potentiated by inhibiting NEP, and 3) the potentiation was inhibited by the tachykinin antagonist FK-888 (NK1-receptor antagonist). We also found that the HCl-induced plasma extravasation was completely inhibited in the capsaicin-treated guinea pig. The pH of the adventitia of the esophagus was 7.0 in all groups 10 min after HCl.

**Fig. 2.** Effect of specific NK1-receptor antagonist FK-888 on HCl-induced airway plasma extravasation in presence of phosphoramidon. A: trachea. B: main bronchi. Evans blue dye leakage induced by intraesophageal 1 N HCl infusion in presence of phosphoramidon was significantly inhibited by FK-888 in a dose-related manner in guinea pig trachea and main bronchi. All values are means ± SE; n = 5 guinea pigs for each experiment. *P < 0.05. **P < 0.01.

**Fig. 3.** Effect of systemic capsaicin treatment on HCl-induced airway plasma extravasation in presence of phosphoramidon. A: trachea. B: main bronchi. In guinea pigs pretreated with systemic capsaicin injection, Evans blue dye leakage induced by intraesophageal 1 N HCl infusion in presence of phosphoramidon was significantly inhibited in trachea and main bronchi. All values are means ± SE; n = 5 guinea pigs for each experiment. *P < 0.05. **P < 0.01.
infusion, and no histological change was seen in the airway adventitia, muscularis, lamina propria, or epithelium. So the plasma extravasation was not associated with the leakage from the intraesophageal HCl.

There are four types of neural pathways in the guinea pig airways and lungs: adrenergic, cholinergic, NANC inhibitory nerve, and NANC excitatory nerve. In the present study, we pretreated the animals with atropine and propranolol to avoid both adrenergic and cholinergic nerve effects (5), leaving active only the NANC inhibitory and NANC excitatory nerves. The NANC excitatory nerve in the airways contains neuropeptides, tachykinins (substance P and neurokinin A), and calcitonin gene-related peptide (CGRP). Substance P and CGRP mainly cause airway plasma extravasation, and neurokinin A causes airway smooth muscle contractions. It is known that NEP exists in the lung and airways in many animals, including humans (17, 35), and that NEP cleaves substance P and neurokinin A at the acting site of the peptides (13, 29). Previous studies suggest that the cleavage of substance P and/or neurokinin A is blocked by inhibiting the NEP activity (7, 19, 36). In the present study, esophageal stimulation by HCl causes airway plasma extravasation in the trachea, and the HCl-induced extravasation was potentiated in the trachea and main bronchi by NEP inhibitor phosphoramidon. This suggests that the airway plasma extravasation was induced by the release of tachykinin-like substances in the airways.

There are three types of tachykinin receptors: NK₁, NK₂, and NK₃. The NK₁ receptor exhibits the greatest affinity for substance P and exists on the bronchial smooth muscle and bronchial blood vessels. The NK₂ receptor exhibits the greatest affinity for neurokinin A and is found on the bronchial smooth muscle (1). The NK₃ receptor exhibits the greatest affinity for neurokinin B and appears to be localized at the bronchial parasympathetic ganglia (30). Thus, in the airway plasma extravasation, endogenously released tachykinins may act on NK₁ receptors. Therefore, we used the NK₁-receptor antagonist FK-888 (9) to study the effect of endogenously released tachykinins to cause airway plasma extravasation. In the present study, the potentiation of HCl-induced airway plasma extravasation in the presence of phosphoramidon was inhibited by tachykinin antagonist FK-888 in a dose-related manner.

Fig. 4. Effect of bilateral vagotomy on HCl-induced airway plasma extravasation in presence of phosphoramidon. A: trachea. B: main bronchi. In vagotomized guinea pigs, Evans blue dye leakage induced by intraesophageal 1 N HCl infusion in presence of phosphoramidon was significantly inhibited in trachea. All values are means ± SE; n = 5 guinea pigs for each experiment. NS, not significant. **P < 0.01.

Fig. 5. Effect of bilateral vagotomy on substance P-induced airway plasma extravasation in presence of phosphoramidon. Plasma extravasation was not different between vagotomized and sham-operated animals (control). All values are means ± SE; n = 5 guinea pigs for each experiment.
Furthermore, our study showed that, in the systemic capsaicin-treated guinea pigs, phosphoramidon failed to potentiate airway plasma extravasation induced by stimulation of esophagus with HCl. Systemic capsaicin treatment causes the release of tachykinins and finally depletes tachykinins in the peripheral nerves (14, 16a, 25). From these results, we suggest that esophageal stimulation by HCl causes the release of tachykinin-like substances, resulting in plasma extravasation in the airways.

There may be several pathways involved in the release of tachykinin-like substances in the airways when the esophagus is stimulated by HCl. Because we focused on the neural pathways, we blocked communication between the esophagus and airways by ligating the upper and lower parts of the esophagus. Thus we can ignore the effect of the aspiration of HCl into the main bronchi or that the vagus nerve; and/or NANC inhibitory and/or NANC excitatory nerves may modulate the release of tachykinins. However, in the vagotomized guinea pigs, the tracheal plasma extravasation induced by esophageal stimulation with HCl in the presence of phosphoramidon was significantly higher than that by sham stimulation in nonvagotomized guinea pigs (Figs. 1 and 4; P < 0.05). This suggests that there are other nerve pathways or mechanisms communicating between the esophagus and airways. Two possible pathways are 1) a local axon reflex pathway (4) and 2) a spinal reflex pathway. Because peptide-containing sensory nerve fibers in the airways originate from vagal and dorsal root ganglia (24, 38), the vagus nerve is not the only nerve supplying the airways and/or lungs with tachykinins. But a previous study argues against a significant spinal contribution to the tachykinergic innervation of guinea pig trachea (22). Therefore, we could suggest alternative possible mechanisms. 1) Because of the intimate association between esophagus and trachea, it is possible that the tachykinins released in the esophagus diffuse to the trachea or are carried to the trachea via local circulation. 2) Similarly, the local circulation may also lead to changes in the pH of the tracheal interstitium. In the present study, the plasma extravasation potentiated by phosphoramidon in the trachea was higher than in the main bronchi, although it was not statistically significant. Because substance P containing nerves are distributed highest at the level of large intrapulmonary bronchi (22), we speculate that 1) higher activity of NEP may exist in the trachea than in the main bronchi or that 2) the neural and/or vascular communications may be more intimate between esophagus to trachea than esophagus to main bronchi.

In summary, our results suggest that 1) tachykinin-like substances are released to cause plasma extravasation in the airways by intraesophageal HCl stimulation; and 2) there are neural pathways communicating between the esophagus and airways, including the vagus nerve; and/or 3) there are vascular pathways communicating between the esophagus and airways via local circulation modulating the plasma extravasation.

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