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The effects of calcitonin gene-related peptide on tracheal smooth muscle of guinea-pigs *in vitro*

[1]Hiroki Ninomiya, Yoshiyuki Uchida, Takeo Endo, Masahito Ohtsuka, Akihiro Nomura, Mikio Saotome & Shizuo Hasegawa

Department of Pulmonary Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

1 The effect of calcitonin gene-related peptide (CGRP) on airway smooth muscle is controversial. The aim of this study was to determine whether the action of CGRP on tracheal strips of guinea-pigs is modulated by epithelium and whether this peptide-induced action involves other mediators including nitric oxide (NO) and endothelin (ET)-1.

2 CGRP produced a weak dose-dependent increase in guinea-pig tracheal tension *in vitro* ($-\log EC_{50}$ = 8.5 ± 0.1, maximum contraction = $8.3 \pm 1.2\%$ of 50 mM KCl-induced contraction, $n=6$). In epithelium-depleted preparations, CGRP (10^{-7} M)-induced contraction was significantly potentiated from $9.0 \pm 1.9\%$ to $41.1 \pm 6.0\%$ ($n=6$).

3 L-N^G-nitro-arginine methyl ester (L-NAME, 10^{-4} M), which inhibits NO synthesis, enhanced the contractile response to CGRP from $9.0 \pm 1.9\%$ to $31.2 \pm 1.1\%$ ($n=6$). Indomethacin (10^{-5} M) also enhanced the response to CGRP, although the effect was weak ($13.4 \pm 3.2\%$, $n=6$).

4 Anti-ET-1 serum changed the CGRP-induced contraction into a relaxation. After incubation of the trachea with ET-1 (10^{-7} M) to attenuate ET-1-induced responses, the CGRP-induced contraction also changed into a relaxation. BQ-123 (an ET_A receptor antagonist) and BQ-788 (an ET_B receptor antagonist) caused the same conversion of the CGRP response, from contraction to relaxation, although the relaxing effect elicited by BQ-788 was more potent than that by BQ-123. Maximum inhibitory responses were $-31.0 \pm 3.3\%$ and $-13.0 \pm 2.3\%$ of 50 mM KCl-induced contraction, respectively ($n=6$).

5 In primary culture, guinea-pig tracheal epithelial cells released ET-1, and CGRP (10^{-5} M) significantly increased the release of ET-1.

6 These data suggest that the action of CGRP is modulated by airway epithelium and this mechanism involves the release of NO and ET-1. Especially, the majority of contractile action elicited by CGRP consists of an action of ET-1 via the predominant ET_B receptor.

Keywords: Calcitonin gene-related peptide (CGRP); airway epithelium; bronchoconstriction; nitric oxide; endothelin-1; L-N^G-nitro-arginine methyl ester (L-NAME); BQ-123; BQ-788

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide formed as an alternative product of the calcitonin gene (Amara *et al.*, 1982). It has a diverse spectrum of pharmacological activities, which include activity as a potent vasodilator (Brain *et al.*, 1985). In vascular smooth muscle, CGRP causes vasodilatation via two separate mechanisms; endothelium-dependent and independent (Gray & Marshall, 1992; Holzer *et al.*, 1993). When CGRP-induced vasodilatation is dependent on the presence of intact endothelium, it is usually inhibited by L-N^G-nitro-arginine methyl ester (L-NAME), suggesting that this relaxation is caused via the release of nitric oxide (NO).

In contrast to vascular smooth muscle, the activity of CGRP on airway smooth muscle is controversial. Early studies have indicated that CGRP constricts airway smooth muscle in man and guinea-pigs (Palmer *et al.*, 1987; Hamel & Ford-Hutchinson, 1988). Recent studies, however, have shown that CGRP has a weak relaxant effect on precontracted bronchial smooth muscle (Martling *et al.*, 1990; Kannan & Johnson, 1992).

Airway epithelium can release several substances which can relax or constrict airway smooth muscle. Epithelium-derived relaxing factors (EpDRF) including prostanoids and NO, cause airway relaxation (Barnett *et al.*, 1988; Filep *et al.*, 1993). On the other hand, airway epithelium is capable of releasing endothelin-1 (ET-1), which is a potent bronchoconstrictor (Uchida *et al.*, 1988; Ninomiya *et al.*, 1992). The condition of airway epithelium is critical in *in vitro* experiments; differences in the state of the epithelium can lead to differences in the response to CGRP.

We hypothesized that the effect of CGRP on airway smooth muscle might be modulated by airway epithelium as a consequence of the release of relaxing and/or constricting factors. This may be one of reasons that several investigators have demonstrated different results. To test this hypothesis, we evaluated (1) the effect of epithelium removal, (2) the effects of inhibition of relaxing factors or constricting factors, which are known to be released from epithelium, on the response of tracheal smooth muscle to CGRP. Furthermore, we observed the effect of CGRP on ET-1 synthesis from airway epithelial cells in culture.

Methods

Pharmacological experiments

Male Hartley-strain guinea-pigs (SLC farm, Japan) weighing 300-400 g were anaesthetized with sodium pentobarbitone 50 mg kg^{-1}, i.p. and killed by cutting the carotid artery. The trachea was removed and placed in Krebs-bicarbonate solution. The composition of Krebs-bicarbonate solution was (mM): NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 25 and glucose 5.5. Following the removal of adherent fats and connective tissues, the trachea was cut open along its longitudinal axis opposite to the smooth muscle, and strips consisting of two adjacent cartilage rings were prepared. The three tracheal rings were connected to each other via

[1] Author for correspondence.

cotton sutures, then placed in 5 ml water-jacketed organ baths containing Krebs-bicarbonate solution, which was gassed with 95% O_2/5% CO_2 and kept at 37°C. The tracheal strips were attached to Grass FT034 force-displacement transducers (Nihon Kohden, Japan). Mechanical responses were isometrically recorded by multi-channel polygraphs (WI-681G; Nihon Kohden). Tissue preparations were equilibrated under 2 g resting load for at least 1 h and washed every 15 min with fresh Krebs-bicarbonate solution before the start of each experiment. Concentration-response curves for CGRP were obtained by its cumulative addition to the bath in 3 fold increments. To prevent the effect of endogenous CGRP, 0.3 mM clonidine was added before the experiment. Clonidine has an inhibitory effect on the release of neuropeptides including CGRP (Grundström & Andersson, 1985). In experiments carried out to examine the effects of inhibitors and antagonists, tracheal preparations were exposed to each agent for 30 min before the addition of CGRP. The following agents were used: indomethacin (10^{-5} M) as a cyclo-oxygenase inhibitor, L-NAME (10^{-4} M) as an inhibitor of NO synthesis, BQ-123 ($10^{-9}-10^{-5}$ M) as an ET_A receptor selective antagonist and BQ-788 ($10^{-9}-10^{-5}$ M) as an ET_B receptor selective antagonist. To reduce the activity of ET-1, anti-ET-1 serum was added before another experiment. Preincubation with ET-1 (10^{-7} M) for 1 h was done for the same purpose. To examine the effect of epithelium, the epithelium was removed mechanically by gently rubbing the luminal surface with a cotton-tipped applicator. We previously found that this method can remove the majority of epithelial cells (more than 85%) without producing obvious damage to underlying mucosal and smooth muscle layers (Ninomiya *et al.*, 1991). All responses were standardized to the degree of contraction induced by 50 mM KCl, which was measured for each tracheal preparation before the experiments.

Epithelial cells in culture

Tracheal epithelial cells were prepared as described previously (Endo *et al.*, 1992). Hartley-strain guinea-pigs were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Tracheae were removed and trimmed of connective tissues. The tracheae were then incubated at 4°C overnight in 0.1% pronase dissolved in sterile 50% Dulbecco's modified Eagle's medium (DMEM) and 50% Ham's nutrient F12 medium (GIBCO, U.S.A.) containing 5% foetal calf serum, penicillin (5 u l^{-1}), streptomycin (100 mg l^{-1}), gentamicin (50 mg l^{-1}), and fungizone (2.5 mg l^{-1}). This medium was referred to as DMEM/F12. After overnight incubation, tracheal lumens were washed with 10 ml DMEM/F12 in order to detach the epithelial cells, which were filtered through 100 μm nytex mesh (Tetko, U.S.A.). The collected cells were centrifuged at 100 × *g* at 4°C for 10 min and resuspended twice in DMEM/F12. The total cell count was calculated with a standard haemocytometer, and the viability of collected cells was more than 95%, which was determined with trypan-blue dye exclusion. Tracheal epithelial cells were plated at a density of 5×10^5 cells/well on 15-mm, 24-well cluster tissue culture plates (Corning, U.S.A.) and incubated at 37°C in 5% CO_2/95% air. The medium was changed every day, and the cells grew to confluent monolayers in 5–6 days. The study was performed with these confluent cells. The medium was then changed to DMEM/F12 without serum, and cells were cultured for another 3 days to obtain a steady basal level of ET-1 synthesis. Cells were incubated in DMEM/F12, without serum, containing 10^{-6} M CGRP and 10^{-5} M phosphoramidon for 24 h, and the supernatant was taken and centrifuged at 1200 × *g* at 4°C for 10 min. The concentration of ET-1 in the supernatant was measured with a sandwich-enzyme immunoassay kit (Wako, Japan), which was designed in accordance with a previous study (Suzuki *et al.*, 1989).

Drugs

Drugs used were: human α-CGRP (Bachem, U.S.A.), L-NAME (Sigma, U.S.A.), ET-1 (Peptide Institute, Japan), indomethacin (Sigma), clonidine (Sigma), pronase (type XIV; Sigma), foetal calf serum (Nakarai Chemical, Japan, penicillin (Sigma), streptomycin (Sigma), gentamicin (Sigma), fungizone (Sigma), BQ-123 (cycro(-D-Trp-D-Asp(ONa)-Pro-D-Val-Leu)) and BQ-788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp (COOMe)-D-Nle-Ona) (gifts from Dr M. Yano of Banyu Pharmaceutical Co., Japan). Stock solutions of CGRP were prepared in 20 mM acetic acid and dilutions made in saline. Stock solutions of ET-1 were prepared in 0.1 mM acetic acid and dilutions made in saline. For administration, the stock solutions were diluted with Krebs-bicarbonate solution. Rabbit anti-human ET-1 serum was a gift from Mitsubishi Life Science, Japan.

Statistical analyses

Agonist-induced responses for each tissue are expressed as a percentage of the reference contraction obtained by 50 mM KCl. All data are given as the mean ± one s.e.mean. Statistical comparisons were performed by one-way ANOVA. If a significant variance was detected, individual group differences were determined by use of Dunnett's test. A probability less than 0.01 was regarded as significant to reject the null hypothesis.

Results

Effects of epithelium

CGRP produced only weak contraction of tracheal strips in a dose-dependent fashion under basal tension (Figure 1). The maximal contractile response was 8.3 ± 1.2% of 50 mM KCl-induced contraction and $-\log EC_{50}$ was 8.5 ± 0.1 ($n = 6$). We used 10^{-7} M CGRP in the following experiments to obtain maximal contraction. Epithelial removal enhanced the contractile response to CGRP (10^{-7} M) from 9.0 ± 1.9% to 41.1 ± 6.0%, while KCl-induced contraction did not change (Figure 2a). CGRP-induced contraction was not affected in the presence or absence of phosphoramidon (10^{-5} M). These observations suggest that the effect of epithelial removal is not just due to decrease in activity by degradation, but also to loss of EpDRF, which is induced by CGRP. To confirm the influence (existence) of relaxing factors, the effects of indomethacin and L-NAME were examined. Both indomethacin (10^{-5} M) and L-NAME (10^{-4} M) increased the contractile response to CGRP (13.4 ± 3.2% and 31.2 ± 1.1%, respectively),

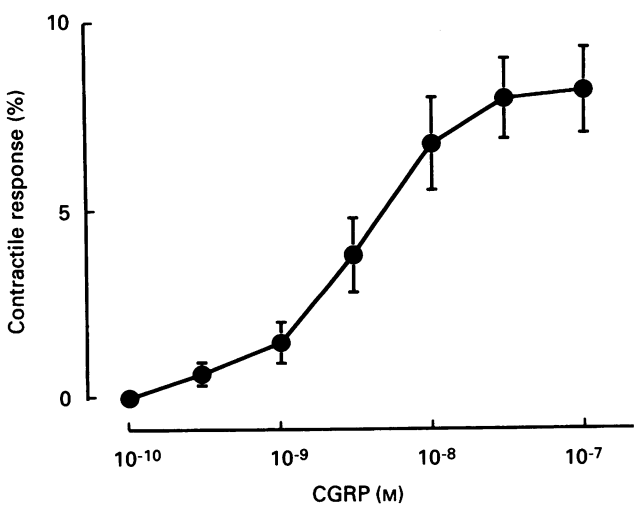

Figure 1 Concentration-response curve for the effect of CGRP on guinea-pig trachea. Increases in tension are expressed as a percentage of the response (reference contraction) to KCl (50 mM). Each point is the mean of 6 observations of separate animals and vertical lines indicate s.e.mean.

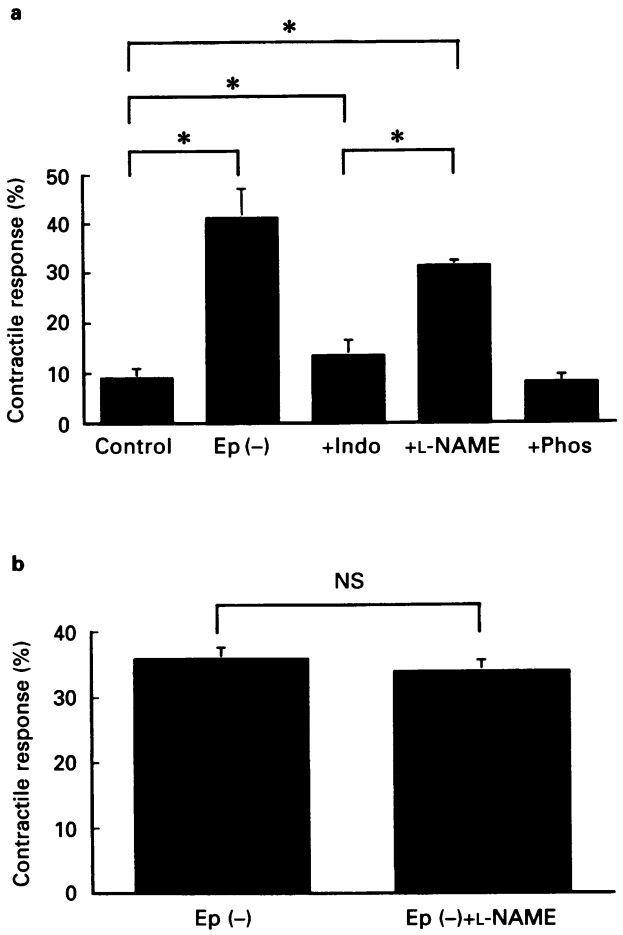

Figure 2 (a) Contractile responses of guinea-pig trachea to CGRP (10^{-7} M). Epithelium-intact preparations (control), pretreatment with 10^{-5} M phosphoramidon (+Phos), 10^{-5} M indomethacin (+Indo), 10^{-4} M L-NAME (+L-NAME), and epithelium-denuded preparations (Ep (−)) are shown. (b) Effect of L-NAME (10^{-4} M) on CGRP (10^{-7} M)-induced contraction of epithelium-denuded trachea. *Values significantly different in the presence of drug ($P<0.01$). Responses are expressed as a percentage of the response to KCl (50 mM). Data represent the mean±s.e.mean ($n=6$).

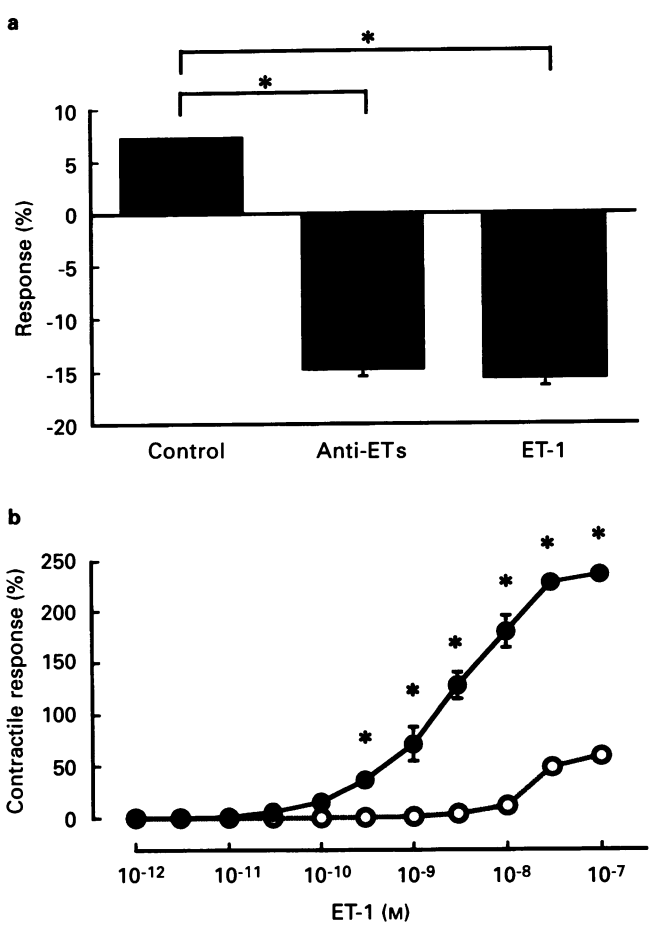

Figure 3 (a) Effects of inhibition of endothelin-1 (ET-1) on guinea-pig tracheal responses to CGRP (10^{-7} M). Vehicle control (Control), pretreatment with 10^{-7} M ET-1 (ET-1), and anti-ET-1 serum (+Anti ETs.) are shown. (b) Concentration-response curves to ET-1 after preincubation with ET-1 (○) and control (●). *Values significantly different in the presence and absence of the treatment ($P<0.01$). Responses are expressed as a percentage of the response to KCl (50 mM). Data represents the mean+s.e.mean ($n=6$).

although the effect of indomethacin was smaller than that of L-NAME. L-NAME had no effect on the contractile response to CGRP without epithelial (Figure 2b). These observations suggest that CGRP stimulates the release of NO and arachidonic acid metabolites, although NO is the more important relaxing factor.

Effects of inhibition of ET-1

The CGRP-response was observed as a relaxation from 7.2±0.8% to −14.7±4.1%, when preincubated with 1/500 anti-ET-1 serum, while rabbit serum appeared to have no effect (Figure 3a). This anti-ET-1 serum (1/500) caused a 2.5 fold shift to the right of the ET-1 concentration-response curve (data not shown). The CGRP-response also changed into a relaxation (−15.6±3.5%), when the tissue was preincubated with ET-1 to inhibit the response to ET-1 (Figure 3a). The concentration-response curve to ET-1 was significantly shifted to the right with a reduction in the maximal contractile effect after a preincubation with ET-1 (control; 235±7.4%, preincubation; 59±9.8%) (Figure 3b). To elucidate which subtype of endothelin receptor mediates the CGRP-response, we used a selective ET_A receptor antagonist, BQ-123 and a selective ET_B receptor antagonist, BQ-788. The effect of BQ-788 was more potent than that of BQ-123, although both of the antagonists changed the CGRP-induced contraction into a relaxation (Figure 4a). Maximal responses to BQ-123 10^{-5} M and BQ-788 10^{-5} M were −13.0±2.3% and −31.0±3.3%, respectively. When the epithelium was removed, the response to CGRP in the presence of BQ-788 was partly inhibited (Figure 4b), suggesting that ET-1 is released by CGRP from both epithelium and other tissues.

Release of ET-1 from cultured epithelial cells

Tracheal epithelial cells in culture were incubated for 24 h in the presence of CGRP (10^{-6} M). Phosphoramidon (10^{-5} M) did not affect the basal release of ET-1. The content of ET-1 in the supernatant incubated with CGRP significantly increased approximately 4 times compared with that with phosphoramidon (Figure 5).

Discussion

In this study, we have demonstrated that the effects of CGRP on tracheal smooth muscle are modulated by the release of both constricting and relaxing factors. Our evidence suggests that ET-1 is the contractile factor, and NO and arachidonic acid metabolites are the relaxing factors released by CGRP.

ET-1 has a potent constrictor activity on airway smooth muscle (Uchida *et al.*, 1988). Lung tissue has the highest levels of ET-1 as determined in a comparative assay of ET-1 mes-

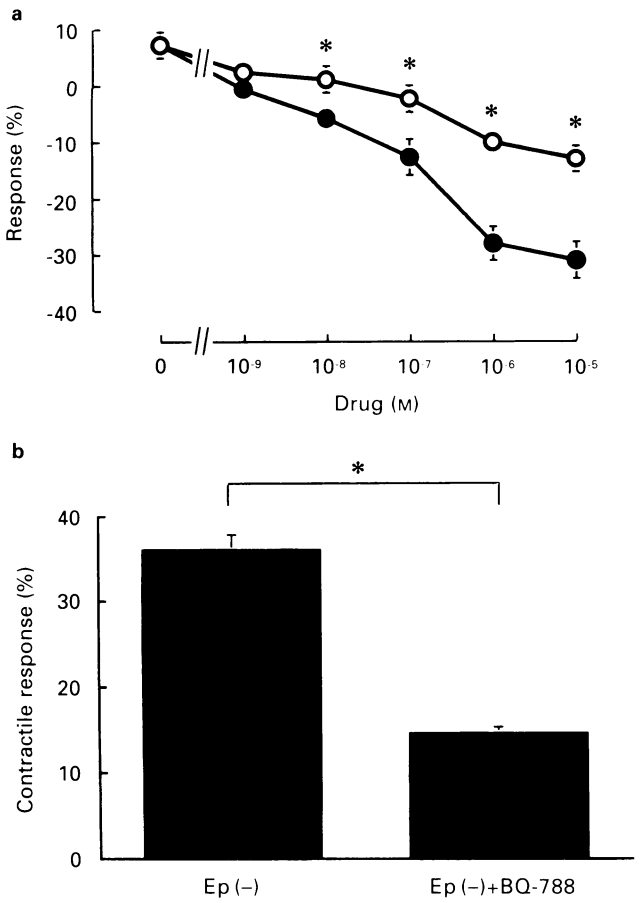

Figure 4 (a) Concentration-response curves for the inhibitory effect of BQ-123 (○) and BQ-788 (●) on the CGRP (10^{-7} M)-induced response. *Values significantly different between BQ-123 and BQ-788 ($P<0.01$). (b) Effect of BQ-788 (10^{-6} M) on CGRP-induced contraction of epithelium denuded trachea (Ep (−)). *Values significantly different in the presence of BQ-788 ($P<0.01$). Responses are expressed as a percentage of the response to KCl (50 mM). Data represent the mean ± s.e.mean ($n=6$).

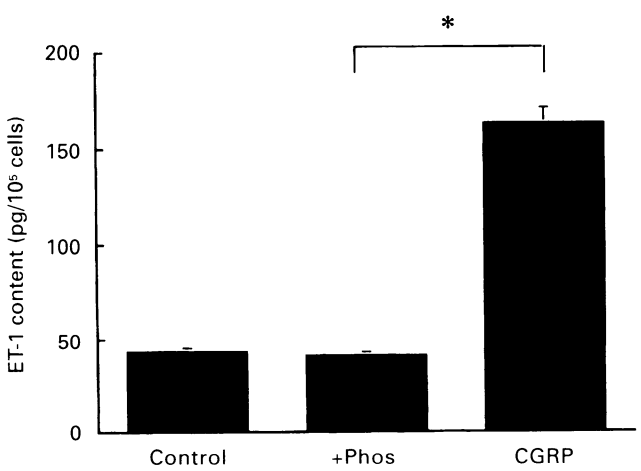

Figure 5 Effect of CGRP on synthesis of endothelin-1 (ET-1) in guinea-pig cultured, tracheal epithelial cells. Epithelial cells were cultured in serum free DMEM/F12 (1/1, v/v) medium for 3 days, and CGRP and phosphoramidon were added to 6 wells each at a final concentration of 10^{-6} M and 10^{-5} M, respectively. After 24 hours of culture, a medium was separated and ET-1 content was measured as described in the text. *Values significantly different in the presence of phosphoramidon with CGRP (CGRP) and without CGRP (+Phos) ($P<0.01$). Data represent the mean ± s.e.mean.

senger RNA levels in human organs, suggesting that the lung is a principal organ of ET-1 production (Bloch *et al.*, 1989). In airways, vascular endothelial cells and airway epithelial cells are the major sites of ET-1 synthesis (Rozengurt *et al.*, 1990), although alveolar macrophages, neutrophils and smooth muscle also release ET-1 (Gaston 1994). The present study has shown that CGRP provokes the contractile response of guinea-pig trachea via the release of ET-1. The precise cells which release ET-1 have not been determined. BQ-788 incompletely inhibited the contractile response of epithelium denuded preparations, suggesting that the epithelium is one of the releasing sites, but other tissues could also release ET-1.

Two different ET receptors, i.e., ET_A and ET_B receptors, have recently been cloned from cDNA library in bovine and rat lungs, respectively (Arai *et al.*, 1990; Sakurai *et al.*, 1990). The ET_A receptor is ET-1 selective whereas the ET_B receptor is equisensitive to all endothelin isopeptides. BQ-123 is a selective ET receptor antagonist; IC_{50} values for ET_A and ET_B receptors are 7.3 nM and 18000 nM, respectively (Ihara *et al.*, 1992). BQ-788 is a selective ET_B receptor antagonist; IC_{50} values for ET_A and ET_B receptors are 1300 nM and 1.2 nM, respectively (Ishikawa *et al.*, 1994). Nagase *et al.* (1995) have shown that both BQ-123 and BQ-788 reduced the pulmonary responses to ET-1 in guinea-pig *in vivo*. They have demonstrated that the inhibitory effect of BQ-788 on ET-1-induced bronchoconstriction is more potent than that of BQ-123, suggesting that ET_B is the predominant receptor on airway smooth muscle. In human peripheral lung, the majority of endothelin receptors in airway smooth muscle are the ET_B subtype (Knott *et al.*, 1995). These data are consistent with the present observation that the inhibitory effect of BQ-788 on the contractile response to CGRP was significantly greater than that of BQ-123. It is suggested that CGRP-induced contraction may be mediated via the release of ET-1, and ET-1 constricts airway smooth muscle predominantly via the ET_B receptor.

We have demonstrated that removal of the epithelium enhances the contractile response to CGRP. Several investigators have shown that epithelial damage may contribute to bronchial reactivity in a number of ways, including degradation of mediators and release of EpDRF (Barnes *et al.*, 1985; Cuss & Barnes, 1987). Airway epithelial cells are rich in the enzyme neutral metalloendopeptidase (enkephalinase) that degrades peptides, such as tachykinins and endothelins (Borson *et al.*, 1989; Candenas *et al.*, 1992). This is unlikely to be explained by degradation of CGRP since phosphoramidon, as an inhibitor of enkephalinase, did not change the response to CGRP in this study. Another possibility is that the airway epithelium releases a relaxing factor which may account for the effect of epithelium removal. One proposed EpDRF is prostaglandin E_2 (PGE_2) which is produced by epithelial cells and has a potent relaxing effect on airway smooth muscle (Barnett *et al.*, 1988). On the other hand, NO may be another EpDRF, since NO is also released from airway epithelium and relaxes airway smooth muscle (Dupuy *et al.*, 1992; Filep *et al.*, 1993). CGRP relaxes thoracic aorta and gastric microvasculatures by releasing NO (Gray & Marshall, 1992; Holzer *et al.*, 1993). NO relaxes airway smooth muscle *in vitro* and *in vivo* (Buga *et al.*, 1989; Dupuy *et al.*, 1992). Several lung tissues, including epithelial cells possess nitric oxide synthase (NOS) and release NO (Kobzik *et al.*, 1993). These observations support the view that CGRP relaxes airway smooth muscle via the release of NO. In the present study, the source of NO was not determined. The CGRP-induced contraction with L-NAME showed no difference in the presence and absence of airway epithelium, suggesting that the epithelium is a major source of NO.

In airways, CGRP is released from terminals of sensory neurones in tracheo-bronchial tissues and neuroendocrine cells in the airway epithelium (Shimosegawa & Said, 1991). Capsaicin causes the release of various mediators including substance P and CGRP from nerve endings and neuroendocrine cells, and induces neurogenic inflammation (Barnes, 1989). Capsaicin-induced tracheal contraction is partly mediated by

CGRP (Tchirhart *et al.*, 1990). When neurogenic inflammation occurs in pathophysiological status such as asthma, CGRP may be released and enhance some reactions, including vasodilatation and bronchoconstriction. These reactions may lead to a deterioration in the patient's condition (Barnes *et al.*, 1992). It is speculated that the effects of CGRP are potentiated in airways in conditions of inflammation, since the induction of ET-1 and NOS in airway epithelium have been observed in asthmatics (Vittoli *et al.*, 1992; Hamid *et al.*, 1993). Further studies are necessary to determine the role of CGRP in pathophysiological conditions.

In conclusion, the results of the present study demonstrate that CGRP has effects on the guinea-pig trachea via the release of ET-1 and NO. Airway epithelium is partly involved in this mechanism and modulates the responsiveness to CGRP.

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