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Sensitization of pulmonary chemosensitive neurons by bombesin-like peptides in rats

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Abstract

Small cell lung cancer (SCLC) patients suffer from pulmonary stresses such as dyspnea and chest pain, and the pathogenic mechanisms are not known. SCLC cells secrete a variety of bioactive neuropeptides including bombesin-like peptides. We hypothesize that these peptides may enhance the sensitivity of the pulmonary chemosensitive nerve endings, contributing to the development of these pulmonary stresses in SCLC patients. This study was therefore carried out to determine the effects of bombesin and gastrin-releasing peptide (GRP), a major bombesin-like peptide, on the sensitivities of pulmonary chemoreflex and isolated pulmonary vagal chemosensitive neurons. In anesthetized, spontaneously breathing rats, intravenous infusion of bombesin or GRP significantly amplified the pulmonary chemoreflex responses to chemical stimulants such as capsaicin and ATP. The enhanced responses were completely abolished by perineural capsaicin treatment of both cervical vagi, suggesting the involvement of pulmonary C-fiber afferents. In isolated pulmonary vagal chemosensitive neurons, pretreatment with bombesin or GRP potentiated the capsaicin-induced Ca^{2+} transient. This sensitizing effect was further demonstrated in patch-clamp recording studies; the sensitivities of these neurons to both chemical (capsaicin and ATP) and electrical stimuli were significantly enhanced by the presence of either bombesin or GRP. In summary, our results have demonstrated that bombesin and GRP up-regulate the pulmonary chemoreflex sensitivity in vivo and the excitability of isolated pulmonary chemosensitive neurons in vitro.

Keywords

bombesin; gastrin-releasing peptide; pulmonary stress; small cell lung cancer; vagal sensory neuron

INTRODUCTION

Small cell lung cancer (SCLC) constitutes ~25% of all pulmonary malignances, follows an aggressive clinical course, and is characterized by early metastases (30,31). It is well documented that patients with advanced SCLC suffer from persistent cough, dyspnea, chest pain and pulmonary inflammation (27,30). These pulmonary stresses are debilitating and severely impair the quality of life of these patients. More importantly, the pathogenic mechanism of these symptoms in SCLC patients is not fully understood. Human SCLC cells originate from neuroendocrine cells of the lung and retain some of their characteristics; one of the most prominent features of SCLC cells is the presence of small dense-core neurosecretory granules and the ability to secrete a variety of neuropeptides, particularly bombesin-like peptides (BLPs) such as gastrin-releasing peptide (GRP) and neuromedin B (11,15,31). These BLPs are

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GRANTS

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known to act as autocrine growth factors in several SCLC culture model systems and have potent mitogenic action on these cells (8,11,15,19).

Most sensory inputs arising from lung structures are conducted in vagus nerves and their branches (10,22). Cell bodies of these sensory nerves reside in nodose and intracranial jugular ganglia. Morphological evidence has shown that approximately 75% of vagal pulmonary afferent nerves are non-myelinated (C-) fibers (2). Activation of these C-fiber sensory endings by chemical irritants is known to elicit pulmonary chemoreflex (apnea, bradycardia and hypotension), cough, chest pain, bronchospasm, and hypersecretion of mucus (10,22). Furthermore, a number of neuropeptides, such as tachykinins and calcitonin gene-related peptide (CGRP), are synthesized in the cell bodies of these C-fiber afferents and released from nerve-endings in the airways upon activation. Tachykinins and CGRP are known to have potent effects on a number of target cells in the airways (e.g., airway smooth muscle, mast cells, etc.). Thus, sustained stimulation of these sensory endings can also induce neurogenic inflammation in the airways (5,34). More importantly, recent studies have clearly demonstrated that pulmonary epithelial neuroendocrine cells, the precursor cells of SCLC, are extensively innervated by branches of these sensory endings expressing high levels of tachykinins (1,7). However, the potential effects of these peptides on the vagal pulmonary C-fiber endings have not been investigated.

In light of the existing knowledge described above and the information that is currently lacking, the present study was carried out to investigate: 1) whether intravenous (i.v.) infusion of bombesin or GRP enhances the pulmonary chemoreflex sensitivity in intact rats; if so, 2) whether the effect of these peptides is caused by a direct sensitization of the vagal pulmonary C-fiber afferents. The excitability of these isolated sensory neurons was determined by using the fura-2 based Ca^{2+} imaging and the whole-cell patch-clamp recording techniques.

MATERIALS AND METHODS

The procedures described below were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and also were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Measurements of cardiorespiratory responses

Sprague-Dawley rats (360–420 g) were initially anesthetized with an intraperitoneal injection of α -chloralose (100 mg/kg) and urethane (500 mg/kg) dissolved in a 2% borax solution; supplemental doses of α -chloralose (~ 10 mg/kg/h) and urethane (~ 50 mg/kg/h) were injected (i.v.) to maintain abolition of pain reflexes elicited by paw-pinch. The right femoral artery, the left jugular vein, and the right femoral vein were cannulated for recording arterial blood pressure (ABP), injection and infusion of various chemical agents, respectively. The jugular venous catheter was advanced until its tip was positioned just above the right atrium. The volume of each bolus injection was 0.1 ml, which was first injected into the catheter (~ 0.2 ml) and then flushed into the circulation by an injection of 0.3 ml saline. A short tracheal cannula was inserted after a tracheotomy, and tracheal pressure (P_t) was measured (Validyne MP 45-28) via a side-port of the tracheal cannula. Body temperature was maintained at $\sim 36^\circ\text{C}$ by means of a heating pad placed under the animal lying in a supine position. At the end of the experiment, the animal was killed by an injection of KCl (200 mg/kg; i.v.).

Rats breathed spontaneously via the tracheal cannula. Respiratory flow was measured with a heated pneumotachograph and a differential pressure transducer (Validyne MP45-14), and integrated to give tidal volume (V_T). Respiratory frequency (f), expiratory duration (T_E), V_T ,

minute ventilation ($\dot{V}_E$), ABP and heart rate were analyzed (Biocybernetics TS-100) on a breath-by-breath basis by an on-line computer as described previously (23).

Identification and culture of vagal pulmonary neurons

Sensory neurons innervating the lungs and airways were identified by retrograde labeling from the lungs by using the fluorescent tracer, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) as described previously (20). Young adult Sprague-Dawley rats (150–200 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and intubated with a polyethylene catheter (PE 150) with its tip positioned in the trachea above the thoracic inlet. DiI was initially sonicated and dissolved in ethanol, diluted in saline (1% ethanol v/v), and then instilled into the lungs (0.2 mg/ml; 0.2 ml $\times$ 2) with the animal's head tilted up at $\sim 30^\circ$.

After 7–11 days, an interval previously determined to be sufficient for the dye to diffuse to the cell body, the animals were anesthetized with halothane inhalation and decapitated. The head was immediately immersed in ice-cold Hank's balanced salt solution. Nodose and jugular ganglia were extracted and digested as described previously (20). Briefly, each ganglion was desheathed, cut into ~ 10 pieces, placed in 0.125% type IV collagenase, and incubated for 1 h in 5% CO_2 in air at 37°C . The ganglion suspension was centrifuged ($150 \times g$, 5 min) and supernatant-aspirated. The cell pellet was resuspended in 0.05% trypsin and 0.53 mM EDTA in Hanks' balanced salt solution for 5 min and centrifuged ($150 \times g$, 5 min); the pellet was then resuspended in a modified DMEM/F-12 solution (DMEM/F-12 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 μM MEM nonessential amino acids) and gently triturated with a small bore fire-polished Pasteur pipette. The dispersed cell suspension was centrifuged ($500 \times g$, 8 min) through a layer of 15% bovine serum albumin to separate the cells from the myelin debris. The pellets were resuspended in the modified DMEM/F-12 solution supplemented with 50 ng/ml 2.5S nerve growth factor, plated onto poly-L-lysine-coated glass coverslips, and then incubated overnight (5% CO_2 in air at 37°C).

Calcium imaging

Intracellular Ca^{2+} was monitored using the fluorescent Ca^{2+} indicator fura-2 AM (14). Cells were loaded with 5 μM fura-2 AM for 30 min at 37°C , then rinsed ($\times 3$) with extracellular solution (ECS; containing in mM: 136 NaCl, 5.4 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 0.33 NaH_2PO_4 , 10 glucose, 10 Hepes, pH at 7.4), and allowed to de-esterify for at least 30 min before use. Ratiometric Ca^{2+} imaging was performed using a Zeiss fluorescence inverted microscope equipped with a variable filter wheel (Sutter Instruments; Novato, CA) and digital CCD camera (Princeton Instruments; Trenton, NJ). Dual images (340 and 380 nm excitation, 510 nm emission) were collected and pseudocolor ratiometric images monitored during the experiments by using the software Axon Imaging Workbench (Axon Instruments; Union City, CA). The imaging system was standardized with a two-point calibration, using a Ca^{2+} -free standard (–) and a Ca^{2+} -saturated standard (+). Both standards contained 11 μM fura-2 [44 μl of 10 mM fura-2 Penta K^+ salt, 8 ml of 20 mM Hepes-Na (pH 7.4), 32 ml H_2O] and were prepared as follows: (– standard) 18 ml fura-2, 1.98 ml of 10 mM EGTA-Na (pH 7.6); (+ standard) 18 ml fura-2, 1.98 ml of 10 mM CaCl_2 . The parameters used for the two-point calibration include: the dissociation constant of fura-2 (K_d ; 225), the ratio values for the (–) and (+) concentration standards ($R_{\min}$ and $R_{\max}$), and the fluorescence intensity at 380 nm excitation for the (–) and (+) concentration standards ($\text{Den}_{\min}$ and $\text{Den}_{\max}$). $[\text{Ca}^{2+}]_i$ (in nM) was calculated according to the following equation: $[\text{Ca}^{2+}]_i = K_d (R - R_{\min}) / (R_{\max} - R) (\text{Den}_{\min} / \text{Den}_{\max})$. Typical $R_{\min}$, $R_{\max}$ and $\text{Den}_{\min} / \text{Den}_{\max}$ values were 0.59, 2.54 and 2.27, respectively.

Electrophysiology

Whole-cell perforated patch-clamp recording was performed as previously described (20). In short, the coverslip containing the attached cells was placed in the center of a small (0.2 ml) recording chamber that was perfused by gravity feed (VC-6 perfusion valve controller; Warner Instruments, Hamden, CT) with ECS, and ECS containing bombesin or GRP at 2 ml/min. The chemical stimulants (capsaicin and ATP) were applied by a pressure-driven drug delivery system (ALA-VM8; ALA Scientific Instruments, Westbury, NY), with its tip positioned to ensure that the cell was fully within the stream of the injectate. The intracellular solution contained (in mM) 92 potassium gluconate, 40 KCl, 8 NaCl, 1 CaCl₂, 0.5 MgCl₂, 10 EGTA, 10 Hepes, pH at 7.2. Recordings were made in the whole-cell perforated patch configuration (50 µg/ml gramicidin) using Axopatch 200B/pCLAMP 9.0 (Axon Instruments). The experiments were performed at room temperature (~22 °C). The data were acquired at 5 kHz and filtered at 2 kHz. Series resistance was compensated at ~80%. The resting membrane potential was held at -70 mV.

Experimental protocols

Four series of experiments were carried out. *Study series 1.* The effects of bombesin and GRP on pulmonary chemoreflex sensitivity were determined in anesthetized, spontaneously breathing rats. Response to right atrial bolus injection of capsaicin (0.25 or 0.5 µg/kg), a potent and selective stimulant of C fibers, was tested before, during and 15 min after a 2-min constant infusion of bombesin (1 µg/kg/min; i.v.) or GRP (3 µg/kg/min; i.v.). The effects of bombesin and GRP on the response to ATP (0.2 or 0.25 mg/kg; i.v.), another selective stimulant of C-fiber afferents, were also determined in a separate group of animals. *Study series 2.* The role of vagal C-fiber afferents in the effects of bombesin and GRP on the pulmonary chemoreflex sensitivity was evaluated after perineural capsaicin treatment (PNCT) of both vagi, which produced a selective and reversible blockade of the C-fiber conduction in the vagus nerve. PNCT was applied as previously described (23). Briefly, cotton strips soaked in capsaicin solution (250 µg/ml) were wrapped around a 2–3 mm segment of the isolated cervical vagi for 20 min and then removed. The success of PNCT was verified in each animal by the abolition of capsaicin (1 µg/kg; i.v.) -induced reflex responses. *Study series 3* was carried out to determine whether the capsaicin-induced Ca²⁺ transient in pulmonary chemosensitive neurons was altered by the presence of bombesin or GRP. The Ca²⁺ transient evoked by capsaicin (30 or 50 nM; 15 s) was determined before, during and ~15 min after a pretreatment with bombesin (1 nM; 5 min) or GRP (3 nM; 5 min). *Study series 4* was carried out to investigate the effects of bombesin and GRP on the sensitivity of isolated pulmonary chemosensitive neurons. Capsaicin (0.3 or 1 µM; 1–4 s) -induced whole-cell inward current was determined before, during and 15–60 min after a pretreatment with bombesin (10 nM; 5 min) or GRP (30 nM; 5 min). The effect of bombesin on pulmonary sensory neurons responses to ATP (0.3 or 1 µM; 2–4 s) and current injection (10 pA increments from 0 to 100 pA; 230-s duration) were also determined in separate groups of neurons.

Chemicals

Fura-2 AM and DiI were purchased from Molecular Probes (Eugene, OR). DMEM/F12, trypsin-EDTA and 2.5S-nerve growth factor were obtained from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO). Stock solution of capsaicin (1 mM) was prepared in a vehicle of 1% Tween80, 1% ethanol, and 98% saline and that of ATP (10 mM) was prepared in saline. Stock solutions of GRP (0.1 mM) and bombesin (1 mM) were both prepared in distilled H₂O and kept in small aliquots at -80 °C. The solutions of these chemicals at desired concentrations were prepared daily by dilution with saline on the basis of the animal's body weight in *study series 1* and 2, or with ECS in *study series 3* and

4. No detectable effect of the vehicles of bombesin and GRP was found in our preliminary experiments.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA). When the ANOVA showed a significant interaction, pair-wise comparisons were made with a *post hoc* analysis (Fisher's least significant difference). Results were considered significant when $P < 0.05$. Data are means $\pm$ SE.

RESULTS

Intravenous infusion of bombesin and GRP enhanced pulmonary chemoreflex sensitivity in anesthetized rats

Infusion of either bombesin (1 $\mu\text{g/kg/min}$, 2 min; i.v.) or GRP (3 $\mu\text{g/kg/min}$, 2 min; i.v.) caused an irregular, rapid and shallow breathing, and mild decreases in ABP and heart rate in the majority (24/29) of the animals studied (e.g., Fig. 1, *B* and *E*). During bombesin infusion, the respiratory depressor response to the same near-threshold dose of capsaicin (0.25 or 0.5 $\mu\text{g/kg}$) injected into right atrium as a bolus was markedly augmented (Fig. 1, upper panel; Fig. 2, left panel); for example, the apneic ratio (apneic duration/average baseline expiratory duration) was increased significantly from 1.69 ± 0.23 at control to 3.88 ± 0.37 during the bombesin infusion ($P < 0.05$, $n = 17$) (Fig. 2B). The potentiating effect of bombesin was reversible in 15 min (apneic ratio: 1.47 ± 0.18). Similarly, infusion of GRP also significantly enhanced the response to the same dose of capsaicin (apneic ratio: 1.60 ± 0.23 at control; 3.41 ± 0.49 during GRP; $P < 0.05$, $n = 7$) (Fig. 1, lower panel; Fig. 2, right panel).

A bolus injection of ATP at a dose just above the threshold (0.2 or 0.25 mg/kg; i.v.) elicited an inhibitory effect on breathing after two- to four-breath delay in anesthetized rats (e.g., Fig. 3, *A* and *D*). The respiratory depressor effect of the same dose of ATP was markedly augmented and prolonged by either bombesin or GRP infusion (Fig. 3). Bombesin (0.95 ± 0.05 at control; 7.72 ± 1.87 during bombesin; $P < 0.05$, $n = 5$) induced slightly more potent enhancing effect than GRP (apneic ratio: 0.92 ± 0.04 at control; 5.61 ± 1.67 during GRP; $P < 0.05$, $n = 5$). The potentiating effects of both bombesin and GRP were reversible in 15 min.

PNCT abolished the effects of bombesin and GRP in anesthetized rats

Perineural capsaicin treatment (250 $\mu\text{g/ml}$; 20 min) of both cervical vagi caused only slight but not significant increases in baseline f , V_T , ABP and heart rate. However, it almost completely abolished the effect of a 2-min infusion of bombesin ($n = 8$) or GRP ($n = 7$) on the baseline breathing pattern (e.g., Fig. 4, *B* and *E*). In addition, the treatment completely blocked the potentiation of the pulmonary chemoreflex response to capsaicin (0.5 $\mu\text{g/kg}$; i.v.) induced by infusion of either bombesin or GRP (Fig. 4).

Bombesin and GRP potentiated capsaicin-induced Ca^{2+} transient in isolated pulmonary chemosensitive neurons

To identify the sensory neurons that give rise to vagal pulmonary C fibers mediating the pulmonary chemoreflexes, neurons isolated from nodose and jugular ganglia were selected based upon the following criteria for this and next study series: 1) labeled with DiI; 2) diameter $< 30 \mu\text{m}$; and 3) responding to capsaicin (20). Data obtained from nodose and jugular neurons were pooled because no detectable difference was found between them. Application of capsaicin (30 or 50 nM; 15 s) evoked a reversible Ca^{2+} transient in these small- to medium-size pulmonary sensory neurons. A 5-min treatment with neither bombesin (1 nM) nor GRP (3 nM) caused any detectable Ca^{2+} transient in these neurons (e.g., Fig. 5, *A* and *C*). However, during bombesin treatment, the peak response of the capsaicin-evoked Ca^{2+} transient was

significantly increased from 65.6 ± 11.4 nM at control to 109.3 ± 21.7 nM during bombesin ($P < 0.05$, $n = 13$) (Fig. 5, A and B). This augmented response to capsaicin gradually declined, but remained higher than control after washout for ~15 min (81.6 ± 12.3 nM). Similarly, pretreatment with GRP also significantly enhanced the capsaicin-evoked Ca^{2+} transient (55.7 ± 6.0 nM at control; 147.1 ± 50.7 nM during GRP; 120.1 ± 39.5 nM after washout; $P < 0.05$, $n = 7$) (Fig. 5, C and D).

Bombesin and GRP sensitize isolated pulmonary chemosensitive neurons to chemical and electrical stimuli

In voltage-clamp mode, capsaicin (0.3 or 1 μM ; 1–4 s) evoked a mild whole-cell inward current in small- to medium-size (11.5–29.2 pF) pulmonary sensory neurons (e.g., Fig. 6A). Perfusion of bombesin (10 nM; 5 min) or GRP (30 nM; 5 min) alone failed to stimulate these sensory neurons. However, during the bombesin treatment, the response to the same concentration of capsaicin was markedly enhanced (Fig. 6, A and B); e.g., the capsaicin-evoked whole-cell inward current density increased significantly from 10.4 ± 2.4 pA/pF at control to 20.8 ± 5.3 pA/pF during bombesin ($P < 0.05$, $n = 9$), which gradually declined after washout for 15–60 min (14.1 ± 3.2 pA/pF). In a separate group of pulmonary sensory neurons, pretreatment with GRP also significantly potentiated the inward current induced by capsaicin (15.2 ± 2.9 pA/pF at control to 22.2 ± 5.3 pA/pF during GRP; $P < 0.05$, $n = 6$) (Fig. 6, C and D).

The sensitizing effect of bombesin/GRP on pulmonary sensory neurons was not limited to capsaicin as the chemical stimulant. Pretreatment with bombesin (10 nM; 5 min) significantly enhanced the whole-cell inward current evoked by ATP ($n = 8$) (Fig. 7, A and B). Furthermore, in current-clamp mode, pretreatment with the same dose of bombesin also significantly increased the number of action potentials evoked by current injection ($n = 7$) (Fig. 7, C and D), suggesting the involvement of voltage-gated ion channels.

DISCUSSION

Our results showed that, in anesthetized, spontaneously breathing rats, intravenous infusion of bombesin or GRP induced rapid shallow breathing, and slightly reduced ABP and heart rate. Infusion of these two peptides also significantly potentiated the pulmonary chemoreflex responses to chemical stimulants such as capsaicin and ATP. These effects of bombesin and GRP were completely abolished when the conduction of pulmonary C fibers was blocked by PNCT of both vagi, suggesting the involvement of these afferents. Bombesin is also known to induce airway smooth muscle contraction (6) which may in turn activate bronchopulmonary C-fiber afferents (10,22). To void this possible effect, we studied the direct actions of bombesin and GRP on isolated pulmonary C neurons. Our results clearly indicated that pretreatment with bombesin or GRP potentiated the capsaicin-induced Ca^{2+} transient in pulmonary chemosensitive neurons. The sensitizing effect was further confirmed by patch-clamp recording which showed that the whole-cell responses to both chemical and electrical stimulations were significantly potentiated by these two peptides.

Bombesin is a 14-amino acid bioactive peptide first isolated from frog skin. One of its major mammalian homologues, namely BLPs, is GRP, a 27-amino acid peptide which is most closely related to bombesin, sharing all but 1 of last 10 amino acid at the COOH-terminal end (13). BLPs have been shown to contribute to diverse biological functions in the central nervous system and peripheral tissues, including regulation of homeostasis, body temperature, metabolism and behavior, and secretion of gastrointestinal, adrenal, and pituitary hormones (15,19). These peptides also have important developmental and growth effects. They are potent mitogens for SCLC cells as well as normal bronchial epithelial cells (3,11,19,33). On the other hand, SCLC cells are known to synthesize and secrete GRP and other BLPs (11,31,36), and GRP has been suggested as a good serum marker of human SCLC (25). Indeed, BLPs have

been considered as the prototypical autocrine growth factors in SCLC (15,24). We have now, for the first time, demonstrated that GRP and bombesin enhance the sensitivity of pulmonary chemoreflex in vivo and the excitability of isolated pulmonary chemosensitive neurons in vitro. These sensory endings are known to release tachykinins upon stimulation. Substance P, the major type of tachykinin in human lung, has been shown to induce rapid mobilization of Ca^{2+} from internal stores of SCLC cell lines and promote clonal growth of these cells (12,21, 35). On the other hand, (D-Arg¹,D-Trp^{5,7,9},Leu¹¹)substance P, a potent antagonist of the tachykinin receptor, can exert a pronounced inhibitory action on both tumor growth in vivo and signal transduction pathways of SCLC cells in vitro (21,32,35). Taken together, the information described above suggests that the effects of BLPs on these sensory endings may be involved in the deteriorating process of this aggressive lung cancer, though direct evidence has not been established.

Immunohistochemical studies have shown that vagal C-fiber afferents innervate all levels of the respiratory tract from trachea to alveoli in various mammalian species (1,4,7). When these C-fiber endings are activated, action potentials conducted through the vagus nerves to the central nervous system elicit the pulmonary chemoreflex response, and other cardiorespiratory reflex responses, such as bronchoconstriction, mucus hypersecretion, bronchial vasodilation, plasma extravasation, dyspneic sensation and cough (10,22). Although we did not attempt to measure these airway responses in the present study, they are known to be mediated through both the cholinergic reflex pathways and local axon reflex; the latter involves a release of tachykinins and CGRP from these sensory endings (5,10,22,34). It is well documented that bronchopulmonary C fibers can be stimulated by various endogenous substances such as H^+ , anandamide, and serotonin in normal lungs (9,22); some of these substances are known to be secreted at high concentrations during airway inflammation. Furthermore, these C-fiber afferents can be activated by various inhaled irritants such as cigarette smoke, SO_2 , ozone, acid aerosol, and also by lung expansion (10,22); expansion of the lung is a normal stimulus and can occur in many physiological conditions (e.g., increased tidal volume during exercise, sigh, etc). Under normal conditions, these stimuli (endogenous chemicals, inhaled irritants and lung expansion) may not generate a significant stimulatory effect on bronchopulmonary C fibers. However, when the excitability of these afferent endings is enhanced by BLPs released locally from SCLC cells in the lungs, their stimulation thresholds will be lowered and they can, therefore, be activated by these stimuli, which may consequently contribute, at least in part, to the pathogenesis of pulmonary stresses in SCLC patients.

Pulmonary C fibers are generally known to possess polymodal sensitivities (9,10,22). The two chemical stimulants applied in this study, capsaicin and ATP, are known to activate two different ligand-gated ion channels, transient receptor potential vanilloid 1 (TRPV1) and P2X₃ purinoceptor, respectively (9,14). Current injections stimulate these sensory neurons due to the activation of various voltage-dependent ion channels (9,20). The possible mechanism underlying the enhanced excitability of pulmonary C neurons after bombesin and GRP pretreatment were not determined in the present study. However, it is known that the effect of bombesin and related peptides is mediated by three major subtypes of the mammalian bombesin receptors designated BB₁ (NMB preferring), BB₂ (GRP preferring), and BB₃ (orphan bombesin receptor subtype-3, BRS-3) (13,19). Although the expression of these bombesin receptors on the soma or terminals of pulmonary C-fiber afferents has not been documented, significant levels of GRP specific binding, as well as mRNA as determined by RT-PCR for all three mammalian bombesin receptors were detected in normal human lung tissue (17,26). All bombesin receptors are known to be G-protein-coupled receptors that are coupled via Gq/11 to activation of phospholipase C, and subsequently activate protein kinase C. The latter has been demonstrated to enhance the neuronal excitability by increasing the phosphorylation of various ion channels such as the TRPV1 (28,37) and TTX-resistance Na^+ channel (18,29). In addition, stimulation of bombesin receptors is believed to be linked to the activation of cytosolic

phospholipase A₂, release of arachidonic acid and production of prostaglandin E₂ (19,31). It is well documented that prostaglandin E₂ enhances the excitabilities of pulmonary C-fiber afferents in vivo and isolated pulmonary vagal sensory neurons in vitro through the cyclic AMP/protein kinase A transduction cascade (14,16,20).

Two different experimental approaches were employed in this study: pulmonary C-fiber chemoreflexes in vivo and isolated pulmonary chemosensitive neurons in vitro. Our results showed that intravenous infusion of bombesin or GRP elicited a rapid, shallow and irregular breathing, accompanied by mild decreases in ABP and heart rate in the majority of the animals studied (e.g., Fig. 1, *B* and *E*), indicating the stimulation of pulmonary C fibers. However, pretreatment with bombesin or GRP alone did not cause any detectable inward current in patch-clamp recording or any Ca²⁺ transient in the imaging study (e.g., Fig. 5, *A* and *C*). The discrepancy of the stimulatory effect of these two peptides between the in vivo and in vitro experiments was probably related to the different sensitivities of these two preparations (afferent nerve endings vs the neuronal soma) and/or the different doses of bombesin/GRP applied in these two approaches. Nevertheless, the ratio of the effective doses between bombesin and GRP (1 : 3) was consistent in all the study series, suggesting that the same subtype(s) of bombesin receptors was probably involved.

In summary, our results demonstrate that bombesin and GRP up-regulate the pulmonary chemoreflex sensitivity in vivo and the excitability of isolated pulmonary capsaicin-sensitive neurons in vitro. These results indicate that BLPs, one of the major autocrine factors in SCLC, may enhance the sensitivity of pulmonary chemosensitive nerve endings and contribute to the development of the pulmonary stresses in SCLC patients.

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Fig. 1.

Representative experimental records illustrating the potentiating effects of bombesin (BN) and GRP on the pulmonary chemoreflex responses to capsaicin in anesthetized rats. *A*, *B* and *C*: responses to right atrial injection of capsaicin (0.5 µg/kg) before, during and 15 min after, respectively, intravenous infusion of bombesin (1 µg/kg/min; 2 min; rat body weight 390 g). *D*, *E* and *F*: responses to capsaicin (0.5 µg/kg) before, during and 15 min after, respectively, intravenous infusion of GRP (3 µg/kg/min; 2 min; rat body weight 375 g). Injectate (0.1 ml) of capsaicin (Cap) was first slowly injected into the catheter (dead space volume 0.2 ml) and then flushed (at arrow), as a bolus with saline (0.3 ml). V_T , tidal volume; ABP, arterial blood pressure.

Fig. 2.

Effects of bombesin (BN, 1 $\mu\text{g/kg/min}$; 2 min) and GRP (3 $\mu\text{g/kg/min}$; 2 min) on respiratory responses to a bolus injection of capsaicin (0.25 or 0.5 $\mu\text{g/kg}$) in anesthetized rats. *A* and *C*: ventilatory responses to capsaicin before (open circle) and during (filled circle) intravenous infusion of bombesin ($n = 17$) and GRP ($n = 7$), respectively. V_T , tidal volume; f , respiratory frequency; $\dot{V}_E$, minute ventilation. The vertical dashed line depicts the time of injection. *B* and *D*: apneic responses to capsaicin before (control), during and 15 min after (recovery) intravenous infusion of bombesin and GRP, respectively. Apneic duration, longest expiratory duration within 3 s after the injection. Baseline T_E , average expiratory duration over 10 control breaths. Data represent means $\pm$ SE. * $P < 0.05$ as compared with the corresponding control.

Fig. 3.

Effects of bombesin (BN, 1 $\mu\text{g/kg/min}$; 2 min) and GRP (3 $\mu\text{g/kg/min}$; 2 min) on pulmonary chemoreflex responses to ATP in anesthetized rats. *A* and *B*: responses to right atrial injection of ATP (0.25 mg/kg) before and during, respectively, intravenous infusion of bombesin (rat body weight 410 g). *D* and *E*: responses to ATP (0.2 mg/kg) before and during, respectively, intravenous infusion of GRP (rat body weight 410 g). *C* and *F*: apneic responses to capsaicin before (control), during and 15 min after (recovery) intravenous infusions of bombesin ($n = 5$) and GRP ($n = 5$), respectively. Apneic duration, longest expiratory duration within 10 s after the injection. Baseline T_E , average expiratory duration over 10 control breaths. Data represent means $\pm$ SE. * $P < 0.05$ as compared with the corresponding control.

Fig. 4.

Perineural capsaicin treatment of both cervical vagi abolished the potentiating effects of bombesin (BN, 1 $\mu\text{g/kg/min}$; 2 min) and GRP (3 $\mu\text{g/kg/min}$; 2 min) on pulmonary chemoreflex responses to capsaicin in anesthetized rats. *A* and *B*: responses to right atrial injection of capsaicin (0.5 $\mu\text{g/kg}$) before and during, respectively, intravenous infusion of bombesin (rat body weight 405 g). *D* and *E*: responses to capsaicin (0.5 $\mu\text{g/kg}$) before and during, respectively, intravenous infusion of GRP (rat body weight 400 g). *C* and *F*: apneic responses to capsaicin before (control), during and 15 min after (recovery) intravenous infusion of bombesin ($n = 8$) and GRP ($n = 7$), respectively. The apneic ratio at 100% indicates that there was no apnea. Data represent means $\pm$ SE.

Fig. 5.

Potentiating effects of bombesin (BN, 1 nM; 5 min) and GRP (3 nM; 5 min) on capsaicin-evoked Ca^{2+} transient in isolated pulmonary vagal sensory neurons. *A* and *C*: experimental records illustrating the potentiating effect of bombesin in a nodose neuron (diameter 22 μm) and GRP in a jugular neuron (18 μm), respectively, on the Ca^{2+} transient evoked by capsaicin (Cap, 50 nM; 15 s). *B* and *D*: group data showing the potentiating effects of bombesin ($n = 13$) and GRP ($n = 7$), respectively, on capsaicin (30 or 50 nM; 15 s) -evoked Ca^{2+} transient. Data represent means $\pm$ SE. * $P < 0.05$ as compared with the corresponding control.

Fig. 6.

Pretreatments with bombesin (BN, 10 nM; 5 min) and GRP (30 nM; 5 min) potentiated the whole-cell inward current evoked by capsaicin (Cap) in isolated pulmonary vagal sensory neurons. *A*: experimental records illustrating the effect of bombesin pretreatment on inward current evoked by capsaicin (0.3 μ M, 2 s) in a jugular neuron (20.8 pF). *B*: group data showing the effect of bombesin on capsaicin (0.3 or 1 μ M; 1–4 s) -elicited inward current ($n = 9$). *C*: experimental records illustrating the effect of GRP pretreatment on inward current evoked by capsaicin (1 μ M, 1 s) in a nodose neuron (15.8 pF). *D*: group data showing the effect of GRP on capsaicin (0.3 or 1 μ M; 1–4 s) -elicited inward current ($n = 6$). Data represent means $\pm$ SE.

* $P < 0.05$ as compared with the corresponding control.

Fig. 7.

Pretreatment with bombesin (BN, 10 nM; 5 min) sensitizes isolated pulmonary vagal sensory neurons to ATP and electrical stimulation. *A*: experimental records illustrating the effect of bombesin on inward current evoked by ATP (1 μ M, 2 s) in a nodose neuron (21.2 pF). *B*: group data showing the effect of bombesin on ATP (0.3 or 1 μ M, 2–4 s; $n = 8$) -evoked inward current. *C*: experimental records illustrating that during bombesin treatment, the identical stimulus evoked a greater number of action potentials compared with that of control. *D*: averaged data of 7 pulmonary sensory neurons. Data represent means $\pm$ SE. * $P < 0.05$ as compared with the corresponding control.