Br. J. Pharmac. (1987), **91**, 95–101

Calcitonin gene-related peptide is localised to human airway nerves and potently constricts human airway smooth muscle

[1]*James B.D. Palmer, *Francis M.C. Cuss, **Peter K. Mulderry, **Mohamed A. Ghatei, †David R. Springall, †Alain Cadieux, **Stephen R. Bloom, †Julia M. Polak & *Peter J. Barnes

Departments of Medicine (*Respiratory and **Endrocrinology Divisions) and †Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0HS

1 In human airways synthetic human sequence calcitonin gene-related peptide (hCGRP), a novel peptide produced by alternative processing of mRNA from the calcitonin gene, caused concentration-dependent contraction of human bronchi (EC_{50} 4.9×10^{-9} M) and was significantly more potent than substance P or carbachol.

2 The contractile response was unaffected by atropine (2×10^{-6} M), propranolol (10^{-6} M), indomethacin (10^{-5} M), tetrodotoxin (3×10^{-6} M), chlorpheniramine (10^{-4} M), cimetidine (10^{-5} M), or FPL55712 (10^{-4} M) suggesting a direct effect of CGRP on airways smooth muscle.

3 CGRP was detected in human airways by radioimmunoassay with highest concentrations in cartilaginous airways.

4 CGRP was localised by immunocytochemistry to both nerves and ganglia in human airways.

5 CGRP, is a potent constrictor of human airways and may have important effects on airway function and be implicated in the pathogenesis of bronchial hyper-responsiveness and asthma.

Introduction

Alternative processing of RNA from the calcitonin gene can occur (Amara *et al.*, 1982). In thyroid C cells, mRNA codes for calcitonin but in neural tissue an alternative mRNA is produced; the latter codes for a high molecular weight protein, which by proteolytic processing results in a 37 amino acid peptide, termed calcitonin gene-related peptide (CGRP) (Rosenfeld *et al.*, 1983). In man, immunoreactivity to CGRP has been identified in the central nervous system (Gibson *et al.*, 1984) and in the eye (Terenghi *et al.*, 1985). In animals, immunoreactivity to CGRP has been identified in the central nervous system (Rosenfeld *et al.*, 1983; Gibson *et al.*, 1984) and in nerves supplying many tissues including the heart and systemic blood vessels (Mulderry *et al.*, 1985), skin (Nicholl *et al.*, 1985), and lung (Springall *et al.*, 1984). CGRP is a potent vasodilator in rabbit aorta *in vitro* (Brain *et al.*, 1985) and in human infusion studies (Struthers *et al.*, 1985). In the lung, CGRP is probably localised to afferent nerve fibres and may coexist with another neuropeptide substance P (SP) (Lundberg *et al.*, 1985; Cadieux *et al.*, 1986), which contracts human (Lundberg *et al.*, 1983; Finney *et al.*, 1985) and animal (Karlsson *et al.*, 1984; Webber *et al.*, 1984) airways *in vitro* and is also a potent vasodilator (Pernow *et al.*, 1975). We have now studied the effect of human CGRP (hCGRP) and potential antagonists on human airway smooth muscle *in vitro* and compared this to SP and carbachol. We have also measured the content of CGRP in human airway smooth muscle by radioimmunoassay and its distribution in human airways by immunocytochemistry.

[1]Correspondence to Dr J. Palmer, Department Clinical Pharmacology, Cardiothoracic Institute, Brompton Hospital, Fulham Road, London SW3 6HP.

Methods

Tissue preparation

Human bronchi were obtained from 6 patients (mean age 61 years, range 52–72) undergoing thoracotomy

for bronchial carcinoma. All were smokers, none was asthmatic. None of the patients was receiving bronchodilator drugs prior to surgery. Tissue was placed in ice cold Krebs solution within 5–10 min of surgical removal. Small cartilaginous bronchi approximately 3–4 mm in diameter were dissected from tissue distal to the tumour and mounted in a ring preparation in a 10 ml organ bath containing oxygenated Krebs solution (pH 7.4, temp. 37°C) continuously gassed with 5% CO_2 and 95% O_2. In all experiments tissue was studied within 6–12 h of surgical removal.

Smooth muscle responses

Changes in isometric tension were measured with Grass FT.03 force-displacement transducers (Grass Instruments, Quincy, Mass. U.S.A.) and recorded on a Grass model 7D polygraph. The bronchial rings were mounted under a tension of 2 g, which in preliminary length tension experiments was found to be optimal. The tissues were then equilibrated for 90 min during which they were washed four times. Cumulative concentration-response curves to human synthetic CGRP, SP and carbachol were then constructed.

Radioimmunoassay

For radioimmunoassay, in addition to lung tissue obtained at thoractomy human tracheae obtained at post mortem within 6–8 h of death were used. CGRP was assayed in human airways using an antiserum raised in New Zealand White rabbits immunised with synthetic human sequence CGRP carbodiimide conjugated to bovine serum albumin in a final dilution of 1:400,000. Radiolabelled hCGRP was prepared by conjugation labelling of hCGRP with N-succininimidyl 3-(4-hydroxy 5-[^{125}I]iodophenyl) propionate (Bolton-Hunter reagent, Amersham Radiochemicals) and subsequent purification on reverse-phase high performance liquid chromotography (h.p.l.c.). Standard curves were constructed using synthetic hCGRP, and 1.5 fmol was detectable within 95% confidence limits. Peptides were extracted from airway tissue by boiling in 0.5 M acetic acid (Bryant & Bloom 1982). Acid tissue extracts were centrifuged and the identity of hCGRP-like immunoreactivity was verified by gel-filtration. The supernatant was applied directly to Sephadex G50 (0.9 × 60 cm) superfine columns eluted with 0.06 mol l^{-1} phosphate buffered saline (pH 7.4) containing 0.3% bovine serum albumin at a flow rate of 3.6 ml h^{-1} and precalibrated with synthetic hCGRP, blue dextran as a marker of void volume (V_0) and Na^{125}I as a marker of total volume (V_t). Fractions were collected at 10 min intervals and assayed directly for hCGRP.

Immunocytochemistry

Fresh human lung samples obtained as described previously were fixed by immersion in 0.4% solution of *p*-benzoquinone in phosphate buffered saline (PBS: 0.01 M phosphate, pH 7.4, 0.15 M saline) for 2 h at 4°C (Bishop *et al.*, 1978). After fixation the tissues were washed overnight in PBS containing 15% sucrose and 0.01% sodium azide. They were then snap-frozen and made into cryostat blocks, which were sectioned at a thickness of 10 μm at −20°C. Tissue sections were stained by the modified indirect immunofluorescence method (Gu *et al.*, 1983), using antiserum raised against synthetic hCGRP conjugated to bovine serum albumin by the glutaraldehyde method. Sections were soaked for 30 min in PBS containing 0.2% Triton X-100 prior to applying the first layer antiserum. The sections were then incubated with anti-CGRP (dilution 1:200) for 20 h at 4°C. After three rinses in PBS the CGRP antiserum was reapplied for 5 h followed by three more rinses. After incubating with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit immunoglobulin (Cappel, diluted 1:100), slides were then washed again in PBS and mounted in PBS-glycerol pH 7.4 (1:1 by volume) and examined by means of a Leitz fluorescence microscope fitted with epi-illumination. Preabsorbtion of the antiserum with synthetic rat and hCGRP at a concentration of 1 mmol ml^{-1} of diluted antibody completely abolished immunostaining. Negative controls included sections incubated with normal rabbit serum and FITC-conjugate alone.

Drugs and solutions

The Krebs solution was of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4.7H_2O$ 1.2, $CaCl_2.6H_2O$ 2.5, $NaH_2PO_4H_2O$ 1.2, $NaHCO_3$ 26 and glucose 11.

The following drugs were used:- atropine (Sigma), chlorpheniramine (May and Baker), cimetidine (Smith Kline and French), synthetic human sequence CGRP (Peninsula Laboratories), FPL 55712 (sodium 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate, Fisons), indomethacin, isoprenaline, (±)-propranolol, substance P, tetrodotoxin (Sigma). The atropine, propranolol, substance P, tetrodotoxin, chlorpheniramine and FPL55712 were made up daily in deionized water. Aliquots of CGRP were freeze dried with 1% heat-treated serum albumin to reduce adherence to glass and stored at −80°C until use. Cimetidine was dissolved in 1 N HCl and neutralized with 0.1 N NaOH prior to use, the final volume being made up in deionized water. The isoprenaline was made up in ascorbate (0.1 mg ml^{-1}) as a stock solution and stored at −20°C. The indomethacin was

dissolved in sodium bicarbonate solution (100 mM) and kept at −20°C.

Statistical analysis of results

Data are expressed as the mean ± standard error of the mean (s.e.mean). The EC_{50} values are expressed as geometric means. Differences between EC_{50} concentrations and the maximum contractions of the individual preparations are analysed by Student's t test for unpaired data a level of $P < 0.05$ being taken as statistically significant.

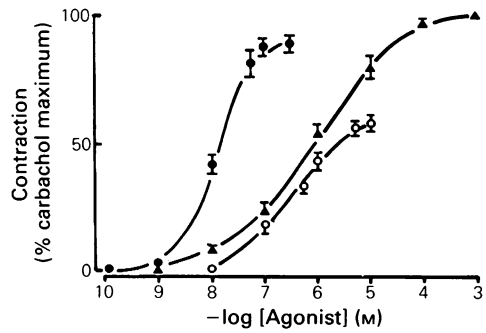

Figure 1 Cumulative concentration-response curves of human bronchial smooth muscle to hCGRP (●), substance P (○), and carbachol (▲). Abscissa scale: concentration of agonist (M), logarithmic scale. Ordinate scale: response to CGRP and substance P expressed as a percentage of the maximum contractile response to carbachol.

Results

Contractile responses

CGRP, SP and carbachol caused concentration-dependent contraction of human bronchi (Figure 1). Apparent geometric mean EC_{50} (the concentration of drug required to produce 50% of the maximum response to that agonist) values were: CGRP 2.3×10^{-8} M (range 4.0×10^{-9}–1.4×10^{-8} M), SP 2×10^{-7} M (range 1.1×10^{-7}–6.3×10^{-7} M), carbachol 9×10^{-7} M (range 3.3×10^{-7}–3.5×10^{-6} M); the EC_{50} value for CGRP was significantly lower than those for carbachol ($P < 0.01$) and SP ($P < 0.05$) ($n = 8$). CGRP produced 88% ± 6 (NS) and SP 55% ± 5 ($P < 0.01$) of the maximum contractions produced by carbachol (10^{-3} M). The contractile effects of CGRP were slow in onset, and persisted for up to 4 h despite repeated washing; however, they were readily counteracted with isoprenaline (10^{-5} M) (Figure 2). This was in contrast to the contractile effects of both SP and carbachol which were reversible by approximately 80% with frequent washing. Drugs tested which could potentially modify the contractile response to CGRP, SP and carbachol were atropine (2×10^{-6} M), propranolol (10^{-6} M), indomethacin (10^{-5} M), tetrodotoxin (3×10^{-6} M), chlorpheniramine (10^{-4} M), cimetidine (10^{-5} M), and the leukotriene antagonist, FPL 55712 (10^{-4} M). None of these produced any significant change in either the potency or maximum response to CGRP or substance P. Atropine 2×10^{-6} M reduced the contractile response to carbachol 10^{-3} M by 86.2% (± 9.3) mean ± s.e.mean. CGRP and SP were assayed in the bath fluid by radioimmunoassay to correct for drug losses, giving a corrected EC_{50} value of 4.9×10^{-9} M for CGRP and 3.1×10^{-8} M for SP based on actual bath concentrations of the drugs. Despite correcting for bath losses, CGRP remained significantly more potent than SP ($P < 0.05$).

Radioimmunoassay

The gel permeation profile showed two peaks of immunoreactivity, one of which eluted in the position corresponding to the hCGRP standard. The nature of the material eluted in the second peak is unknown but its low molecular weight suggests the possibility of a cross-reacting CGRP fragment on non-specific interfering material. CGRP-like immunoreactivity was found in the following concentrations: trachea 1.5 ± 0.3, bronchus 1.4 ± 0.3, peripheral lung 0.9 ± 0.1 (all pmol g^{-1} tissue, mean ± s.e.mean, $n = 8$).

Immunocytochemistry

CGRP-like immunostaining was localised exclusively to nerves (Figure 3a). No mucosal endocrine cells were

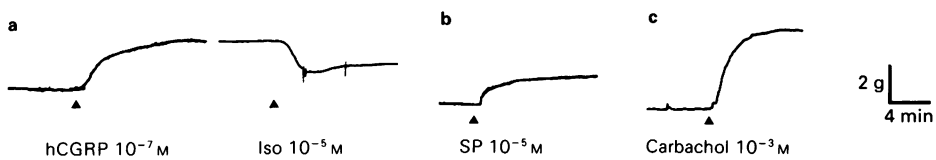

Figure 2 Individual traces of contractile responses of human bronchial smooth muscle to (a) hCGRP showing also relaxation to isoprenaline (Iso); (b) subtance P (SP) and (c) carbachol.

stained. CGRP-immunoreactive nerve fibres were scattered and associated with intra pulmonary vessels and airways rather than lung parenchyma. The fibres were associated principally with vascular (Figure 3b) and airway (Figure 3c) smooth muscle. However, th were also seen in the connective tissue (Figure 3 below the epithelium and in the adventitia of airwa sometimes in small bundles. Very occasionall

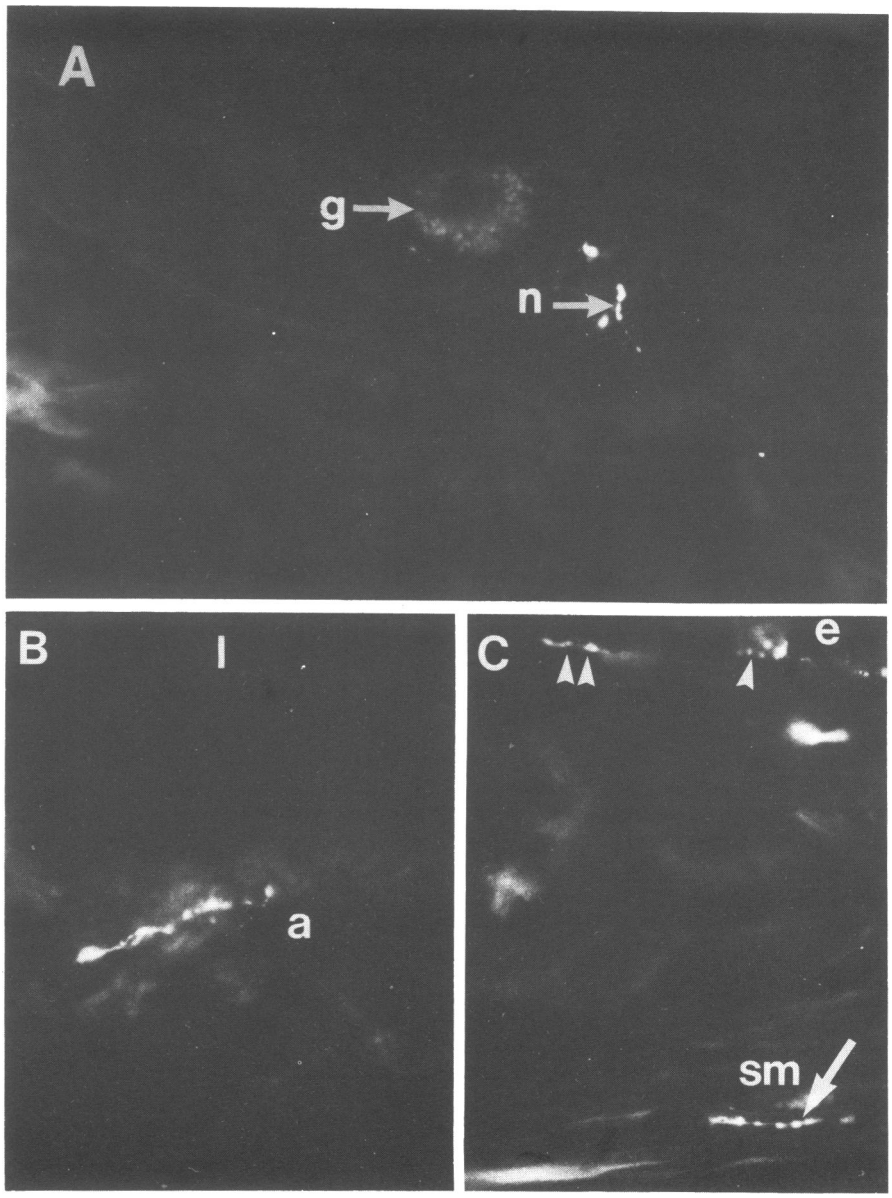

Figure 3 (a) hCGRP-immunoreactive nerve fibre in a nerve bundle (n) in the adventitia of an intrapulmonary bronchus. A weakly immunostained ganglion cell (g) is also seen (immunoflourescence method; magnification × 420). (b) Nerve fibre immunoreactive for hCGRP in the adventitia (a) of an arteriole (l = lumen) near to an intrapulmonary bronchus (magnification × 950). (c) Transverse section of a bronchiole showing hCGRP-immunoreactive nerve fibres (arrow) in the smooth muscle (sm), just below (arrowhead) the epitheium (e) and in the sub-epithelial connective tissue (double arrowhead) (magnification × 610).

immunoreactive nerves were detected just beneath the airway epithelium (Figure 3c). The density of immunoreactive nerves was less in the smaller airways, such as terminal bronchioles than in the larger airways. Numerous slender, varicose immunoreactive nerve fibres were also observed around ganglion cells (Figure 4); occasional ganglion cells within airways also displayed CGRP-like immunoreactivity (Figure 3a).

Discussion

CGRP is known to be a potent vasodilator both *in vitro* and *in vivo*, but is effects on human isolated bronchi have not previously been studied. We have shown that CGRP contracts human bronchi *in vitro*, and is apparently much more potent than either SP or carbachol. The contractions induced by CGRP are slow in onset, sustained, and functionally antagonized with the β-adrenoceptor agonist, isoprenaline, indicating an active contractile process.

In rat aorta the vasodilator response is endothelium-dependent and reduced by indomethacin, implying that some of the effect may be dependent on cyclo-oxygenase products (Brain *et al.*, 1985), but in airways indomethacin had no effect on contractile responses. Contractile responses were similarly unaffected by muscarinic and β-adrenoceptor blockade, also by antagonists to histamine or leukotrienes. These findings indicate that CGRP had a direct effect on airway smooth muscle, rather than indirect effect resulting from the release of other known pharmacological agents with local effects on airway smooth muscle. This suggests that in airway smooth muscle CGRP may act by activation of a specific receptor. A similar conclusion has been reached in studies on pancreatic acinar cells (Zhou *et al.*, 1985), and recently specific receptors for CGRP have been characterized in brain homogenates by direct binding with ^{125}I-labelled CGRP (Tschopp *et al.*, 1985).

Immunocytochemically in guinea-pigs CGRP is localised to the same nerves as SP (Lundberg *et al.*, 1985) and it is possible that CGRP functions as a co-transmitter with SP in the airways. CGRP is released by rat trigeminal ganglion cells *in vitro* (Mason *et al.*, 1984) and it seems likely that CGRP may act as a neurotransmitter or neuromodulator. The distribution of CGRP in the spinal cord is similar to that of SP and suggests a sensory role for this peptide (Gibson *et al.*, 1984). Neonatal administration of capsaicin to rats causes a permanent degeneration of unmyelinated sensory nerve fibres and concomitant reduction in SP-immunoreactive fibres in airways (Lundberg *et al.*, 1983). A similar reduction in CGRP-immunoreactivity in airways has also been found (Lundberg *et al.*, 1985; Cadieux *et al.*, 1986), suggesting that CGRP may be distributed in nerves similar to SP.

Both CGRP and SP produce a wheal and flare response in human skin when injected intradermally

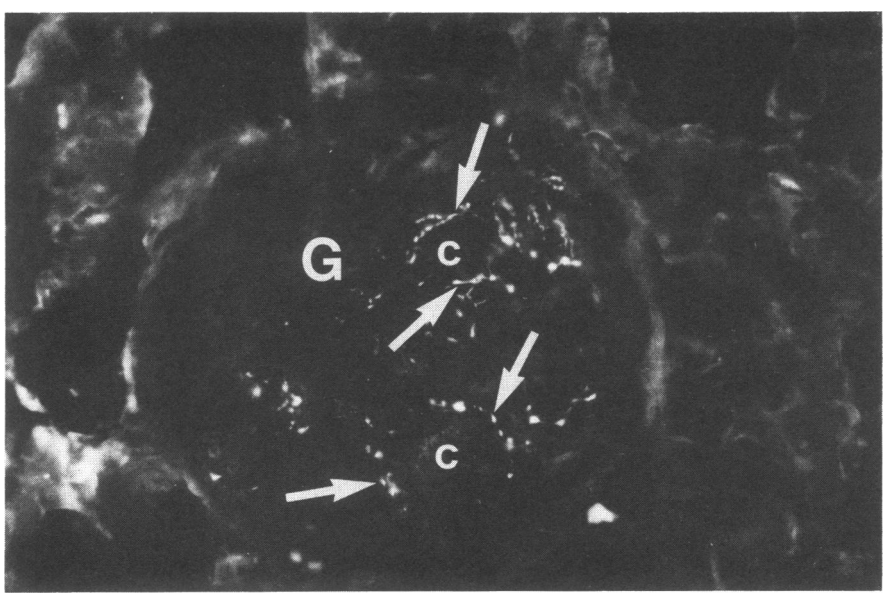

Figure 4 hCGRP-immunoreactive nerve fibres in a ganglion (G) close to an intrapulmonary bronchus. The nerve fibres (arrows) are seen surrounding both immunoreactive ganglion cells (c) (magnification × 420).

(Brain *et al.*, 1985; Foreman *et al.*, 1983; Barnes *et al.*, 1986), the flare response to CGRP having a very long duration. SP has also been implicated in the pathogenesis of bronchial oedema and the response to irritants (Lundberg & Saria, 1983). For the following reasons it is likely that CGRP may also play a role in the pathogenesis of airway oedema. Both sensory neuropeptides may have a role in the airway inflammatory response in asthma, possibly being released by an axon reflex (Barnes, 1986). If CGRP were released from airway nerves by an axon reflex then it might have potent effects on airway smooth muscle and on bronchial microvasculature, leading to airway oedema and plasma extravasation, so contributing to the pathology of asthma. Our findings suggest that CGRP may have a more important function than SP in the control of human airway smooth muscle and may play a role in the pathology of asthma and bronchial hyperresponsiveness.

J.P. and F.C. are supported by the Medical Research Council of Great Britain. We are grateful to the thoracic surgeons at Brompton, Colindale, Hammersmith and Harefield Hospital for providing lung tissue for these studies.

References

AMARA, S.G., JONAS V., ROSEFELD, M.G., ONG, E.S. & EVANS, R.M. (1982). Alternative RNA processing in calcitonin gene expression generates mRNA's encoding different polypeptide products. *Nature*, **198**, 240–244.

BARNES, P.J. (1986). Asthma as an axon reflex. *Lancet*, **i**, 242–245.

BARNES, P.J., CONRADSON, T-B.G., DIXON, C.M.S. & FULLER, R.W. (1986). A comparison of the cutaneous actions of substance P, neurokinin A and calcitonin gene-related peptide in man. *J. Physiol.*, (in press).

BISHOP, A.E., POLAK, J.M., BLOOM, S.R. & PEARSE, A.G.E. (1978). A new technique for immunocytochemical localisation of peptidergic innervation. *J. Endocrinol.*, **71**, 25–26.

BRAIN, S.D., WILLIAMS, T.J., TIPPINS, J.R., MORRIS, H.R. & McINTYRE, I. (1985). Calcitonin gene related peptide is a potent vasodilator. *Nature*, **313**, 54–56.

BRYANT, M.G. & BLOOM, S.R. (1982). Measurement in tissue. In *Radioimmunoassay of Gut Regulatory Peptides*. ed. Bloom, S.R. & Long, K.D. pp. 36–41. London: W.B. Saunders.

CADIEUX, A., SPRINGALL, D.R., MULDERRY, P.K., RODRIGO, J., GHATEI, M.A., TERENGHI, G., BLOOM, S.R. & POLAK, J.M. (1986). Occurrence, distribution and ontogeny of CGRP immunoreactivity in the rat lung: Effect of capscaicin treatment and surgical denervations. *Neurosci.*, (in press).

FINNEY, M.J.B., KARLSSON, J-A. & PERSSON, C.G.A. (1985). Effects of bronchoconstrictors and bronchodilators on a novel human small airway preparation. *Br. J. Pharmac.*, **85**, 29–37.

FOREMAN, S.P., JORDAN, C.C., OEHEME, P. & RENNER, H. (1983). Structure activity relationships for some substance P-related peptides that cause wheal and flare reaction in human skin. *J. Physiol.*, **335**, 449–465.

GIBSON, S.J., POLAK, J.M., BLOOM, S.R., SABATE, I.M., MULDERRY, P.K., GHATEI, M.A., McGREGOR, G.P., MORRISON, J.F.B., KELLY, J.S. & ROSENFELD, M.G. (1984). Calcitonin gene-related peptide immunoreactivity on the spinal cord of man and of eight other species. *J. Neuroscience*, **4**, 3101–3111.

GU, J., ISLAM, N. & POLAK, J.M. (1983). Repeated application of first layer antiserum provides immunofloures-cence staining: a modification of the indirect immunofloresecence staining procedure. *Histochem. J.*, **15**, 375–482.

KARLSSON, J-A., FINNEY, M.J.B., PERSSON, C.G.A. & POST, C. (1984). Substance P antagonists and the role of tachykinins in non-cholinergic bronchocontriction. *Life Sci.*, **35**, 2681–2691.

LUNDBERG, J.M., ANDERS, F-C., HUA, X., HOKFELT, T. & FISCHER, J.A. (1985).Co-existence of substance P and calcitonin gene related peptide like immunoreactivity in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. *Eur. J. Pharmac.*, **108**, 315–319.

LUNDBERG, J.M., MARTLING, C.R. & SARIA, A. (1983). Substance P and capscain - induced contraction of human bronchi. *Acta physiol. scand.*, **119**, 243–252.

LUNDBERG, J.M., & SARIA, A. (1983). Capscain induced desensitization of airway mucosa to cigarette smoke mechanical and chemical irritants. *Nature*, **302**, 251–253.

MASON, R.T., PETERFREUND, R.A., SAWCHENKO, P.O., CORRIGAN, A.Z., RIVIER, J.E. & VALE, W.N. (1984). Release of the predicted calcitonin gene-related peptide from cultured rat trigeminal ganglion cells. *Nature*, **308**, 653–655.

MULDERRY, P.K., GHATEI, M.A., RODRIGO, J., ALLEN, J.M., ROSENFELD, M.G., POLAK, J.M. & BLOOM, S.R. (1985). Calcitonin gene-related peptide in cardiovascular tissues of the rat. *Neuroscience*, **14**, 947–954.

NICHOLL, C.G., SPRINGALL, D.R., MULDERRY, P.K., GHATEI, M.A., BLOOM, S.R. & POLAK, J.M. (1985). Distribution and ontogeny of CGRP, a major skin neuropeptide. *Clin Sci.*, **68**, 71P.

PERNOW, B., & ROSELL, S. (1975). The effect of substance P on blood flow in canine adipose tissue and skeletal muscle. *Acta physiol. scand.*, **93**, 139–141.

ROSENFELD, M.G., MERMOD, J.J., AMARA, S.G., SWANDSON, L.H., SAWCHENKO, P.E., RIVIER, J., VALE, N.N. & EVANS, R.M. (1983). Production of a novel neuropeptide encoded by the calcitonin gene via tissue specific RNA processing. *Nature*, **304**, 129–135.

SPRINGALL, D.R., POLAK, J.M., GHATEI, M.A., LACKIE, P. & BLOOM, S.R. (1984). Calcitonin gene-related peptide (CGRP), a new regulatory peptide widely distributed in lung. *J. Path.*, **143**, 306–307.

STRUTHERS, A.D., BROWN, J., STEVENSON, J.C., BEA-

CHAM, J.L., MORRIS, H.R. & MACINTYRE, I. (1985). The cardiovascular and endocrine effects of human calcitonin gene-related peptide (CGRP) in man. *Clin. Sci.*, **68**, 18P.

TERENCHI, G., POLAK, J.M., CHATEI, M.A., MULDERRY, P.K., BUTLER, J.M., UNGER, W.G. & BLOOM, S.R. (1985). Distribution and origin of calcitonin gene related peptide (CGRP), immunoreactivity in the sensery innervation of the mammalian eye. *J. comp. Neurol.*, **233**, 506–516.

TSCHOPP, F.A., HENKE, H., PETERMANN, J.B., TOBLER, P.H., JANZER, R., HOKFELT, T., LUNDBERG, J.M., CUELLO, C. & FISHER, J.A. (1985). Calcitonin gene-related peptide and its binding sites in the central nervous system and pituitary. *Proc. natn. Acad. Sci. U.S.A.*, **82**, 248–252.

WEBBER, S.E., & FOREMAN, J.C. (1984). The effect of substance P and related peptides on the guinea pig lung strip. *Agents & Actions*, **14**, 425–428.

ZHOU, Z-C., VILLANUEVA, M.L., GARDNER, J.D., & JENSEN, R.T. (1985) Pancreatic acini possess a distinct receptor for calcitonin gene-related peptide (CGRP). *Gastroenterology*, **88**, 1643.

(Received February 12, 1986.
Revised November 21, 1986.
Accepted January 2, 1987.)