Morphine, but not sodium cromoglycate, modulates the release of substance P from capsaicin-sensitive neurones in the rat trachea *in vitro*

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1 Opioids have been shown to inhibit substance P (SP) release from primary afferent neurones (PAN). In addition, opioid receptors have been identified on PAN of the vagus nerves. Sodium cromoglycate (SCG) decreases the excitability of C-fibres in the lung of the dog *in vivo*. We have utilised a multi-superfusion system to investigate the effect of opioids and SCG on the release of SP from the rat trachea *in vitro*.

2 Pretreatment of newborn rats with capsaicin (50 mg kg⁻¹ s.c. at day 1 and 2 of life) resulted in a $93.2 \pm 6.3\%$ reduction in tracheal substance P-like immunoreactivity (SP-LI) content when determined by radioimmunoassay in the adult.

3 Exposure to isotonicity elevated potassium concentrations (37–90 mM), capsaicin (100 nM–10 μ M), and bradykinin (BK; 10 nM–1 μ M) but not des-Arg⁹-BK (1 μ M) stimulated SP-LI release by a calcium-dependent mechanism.

4 SCG (1 μ M and 100 μ M) did not affect spontaneous, potassium (60 mM)- or BK (1 μ M)-stimulated SP-LI release.

5 Morphine (0.1–100 μ M) caused dose-related inhibition of potassium (60 mM)-stimulated SP-LI release with the greatest inhibition of $60.4 \pm 13.7\%$ at 100 μ M. The effect of morphine was not mimicked by the κ -opioid receptor agonist, U50,488H (10 μ M) or the δ -opioid receptor agonist, Tyr-(D-Pen)-Gly-Phe-(D-Pen) (DPDPE).

6 The effect of morphine was totally abolished by prior and concomitant exposure to naloxone (100 nM) which had no effect on control release values.

7 We conclude that opioid receptors, predominantly of the μ -opioid receptor subtype, inhibit SP-LI release from PAN in the rat trachea and suggest that centrally inactive μ -opioid receptor agonists may have therapeutic potential in the treatment of asthma.

Keywords: Morphine; sodium cromoglycate; trachea substance P; primary afferent neurone

Introduction

In airways of mammals, including the rat, immuno-histochemical and morphological studies have localised the undecapeptide substance P (SP), possibly coexisting with calcitonin gene-related peptide (CGRP), and the related tachykinins, neurokinin A (NKA) and neurokinin B (NKB), to capsaicin-sensitive vagal and non-vagal primary afferent neurones (PANs). These sensory fibres innervate both bronchial and vascular smooth muscle, submucosal glands and the respiratory epithelium (Lundberg *et al.*, 1984; Hua *et al.*, 1985; McDonald *et al.*, 1988).

Recent evidence suggests that airway irritation causes local defence reactions including bronchoconstriction, vasodilatation and an increase in vascular permeability (Lundberg & Saria, 1982a; 1983; Lundberg *et al.*, 1983a). Activation of local axon reflexes, with release of mediators including SP from peripheral endings of sensory fibres, contributes to these reactions (Lundberg *et al.*, 1985). Indeed it has been postulated that these axon reflexes may have relevance to the pathogenesis of asthma (Barnes, 1986).

Opioid receptors exist on small-diameter PANs in the spinal cord (LaMotte *et al.*, 1976). Autoradiographic studies have demonstrated opioid receptors on vagal PANs (Atweh *et al.*, 1978). Several studies have shown that opioids inhibit the release of SP from neurones *in vitro* (Jessell & Iversen, 1977; Lembeck & Donnerer, 1985) and *in vivo* (Yaksh *et al.*, 1980). Opioids have also been shown to modulate non-adrenergic, non-cholinergic (NANC) bronchoconstriction and plasma extravasation induced by antidromic stimulation of the vagus

nerves in the guinea-pig *in vivo* (Belvisi *et al.*, 1988; 1989). These authors concluded that this effect may be mediated by presynaptic inhibition of SP release from the peripheral termini of vagal afferents.

Sodium cromoglycate (SCG) has been reported to depress the excitability of C-fibres in the canine lung *in vivo* (Dixon *et al.*, 1980). In addition, bradykinin, a known stimulant of vagal afferent C-fibres (Kaufman *et al.*, 1980), causes SCG-sensitive bronchoconstriction in man *in vivo* (Fuller *et al.*, 1987).

We have developed a multi-superfusion system to study the control of the release of SP-like immunoreactivity (LI) from rat trachea *in vitro* and have investigated the effect of morphine on potassium- and capsaicin-stimulated SP-LI release. In addition, the effect of SCG on potassium- and bradykinin-stimulated SP-LI release was studied.

Preliminary data have been presented to the British Pharmacological Society (Ray *et al.*, 1989).

Methods

Experimental protocol

Male albino rats (Wistar 350–400 g) were obtained from the departmental colony in Bristol. Animals were killed by a terminal injection of sodium pentobarbitone (100 mg kg⁻¹, i.p.). Eight or ten spirally cut tracheae (Constantine, 1965) were mounted in parallel surfasil (Pierce Chemical Co.)-coated glass chambers and simultaneously superfused (Watson-Marlow 202S/AA) at 37°C with oxygenated (95% O₂/5% CO₂) physiological salt solution (composition (mM): NaCl 120, KCl 4.7, CaCl₂ 2.6, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11.1) at 5 ml min⁻¹ to allow equilibration. After at least 60 min

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bovine serum albumin (BSA; 1 mg ml⁻¹) and three peptidase inhibitors were added to the physiological salt solution and the flow rate adjusted to 1 ml min⁻¹. Peptidase inhibitors were phosphoramidon (1 µM), captopril (100 µM) and bacitracin (20 mg ml⁻¹) (Turner *et al.*, 1985). After a further 15 min four fractions of 5 min from each trachea were simultaneously collected in vials containing a final concentration of 0.1% trifluoroacetic acid (TFA), ethylenediaminetetracetic acid, disodium salt, dihydrate (EDTA, 1 mM), and dithiothreitol (DTT, 1 mM). Drugs were added to the superfusion fluid reservoir for 4 min during the third collection period. Where the KCl concentration was raised the NaCl concentration was correspondingly reduced to maintain the isotonicity of the superfusing medium. Some experiments were performed on trachea from rats which had been pretreated neonatally with capsaicin. Rats received capsaicin (50 mg kg⁻¹) subcutaneously 24 h and 48 h after birth. Control littermates received equal volumes of the vehicle which consisted of 10% ethanol: 10% Tween 80 (v/v) in 0.9% (w/v) saline.

Substance P-like immunoreactivity extraction and radioimmunoassay

Superfusates were applied to Sep-Pak C₁₈ cartridges (Waters Associates) and washed with 0.1% TFA. Peptides were eluted with acetonitrile-0.1% TFA (80:20 v/v) and evaporated to 0.5 ml under nitrogen. The final SP-LI content of the trachea was extracted by plunging each trachea into a microcentrifuge tube containing 400 µl of boiling homogenizing medium (0.1% TFA, 1 mM EDTA, 1 mM DTT) for 10 min. The tubes were then transferred to ice and the samples homogenised in the tubes with a motor-driven Teflon pestle. A further 200 µl of homogenizing medium was added to wash the pestle. The samples were then centrifuged for 15 min at 8000 g and the supernatants decanted and retained for radioimmunoassay (RIA). Samples were then lyophilised and stored at -70°C. RIA of SP-LI was performed with an antiserum directed against the C-terminus of the peptide, as previously described (Harmar & Keen, 1986). None of the agents employed interfered with the RIA at the concentrations used.

Calculation of substance P-like immunoreactivity release

Changes in SP-LI release were calculated as the difference in fractional release constants (FRC) between the mean of fractions 1 and 2 and the mean of fractions 3 and 4 based on the recovery of exogenous SP (40 pg ml⁻¹) determined as 62.2 ± 3.2% (*n* = 12).

Statistical analysis

Results are expressed as means ± s.e.mean. Differences between means were analysed by Student's *t* test for unpaired data (two-tailed) or analysis of variance when appropriate. A *P* value of less than 0.05 was considered to be significant.

Drugs

Drugs and chemicals were obtained from the following sources: phosphoramidon, bacitracin, bovine serum albumin (BSA), DTT, capsaicin, SP, BK, des-Arg⁹ BK, *trans*-3,4-dichloro-N-methyl-N(2-(1-pyrrolidinyl)cyclohexyl)-benzene-

acetamine (U50,488H), Tyr-(D-Pen)-Gly-Phe-(D-Pen) (DPDPE), naloxone hydrochloride, cromolyn sodium, (Sigma Chemical Co., Poole, Dorset); EDTA, ethylene glycol *bis*-(2-aminoethyl) tetraacetic acid (EGTA; Fisons Ltd., Loughborough); captopril (E.R. Squibb & Sons, Inc., Princeton, U.S.A.), morphine hydrochloride (McFarlan Smith Ltd., Edinburgh) and sodium pentobarbitone (May & Baker Ltd., Dagenham).

Results

Effect of neonatal pretreatment with capsaicin

Neonatal administration of capsaicin caused a significant reduction in tracheal SP-LI content when extracted and assayed after the rats had attained 350 g body weight. SP-LI content of the trachea was reduced from 2125 ± 135 pg (*n* = 8) in vehicle-treated rats to 144 ± 17 pg (*n* = 8) in capsaicin-treated littermates representing a 93.2 ± 6.5% reduction.

Stimulation of SP-LI release

Capsaicin (100 nM–10 µM), KCl (37–90 mM), and BK (10 nM–1 µM), caused dose-related increases in SP-LI release above spontaneous values. Des-Arg⁹ BK (1 µM) did not increase SP-LI release. Replacement of calcium ions by magnesium with addition of 1 mM EGTA did not affect spontaneous SP-LI release but caused a total abolition of the increase in release stimulated by 60 mM KCl, a 97.8 ± 21.0% reduction of that by 1 µM capsaicin and a 97.6 ± 23.7% reduction of that by 1 µM BK (see Table 1).

Effect of sodium cromoglycate on substance P-like immunoreactivity release

Tracheae were preincubated in SCG (1 µM and 100 µM) for 60 min prior to and during the equilibration period. SCG (1 µM and 100 µM) did not significantly affect spontaneous, 60 mM KCl or 1 µM BK stimulated SP-LI release when compared to control values (Table 2).

Effect of opioid agonists on KCl-stimulated substance P-like immunoreactivity release

Morphine (0.1–100 µM), when present 2 min prior to and during exposure to 60 mM KCl, produced dose-dependent inhibition of SP-LI release: at a dose of 3 µM the inhibition was 33.6 ± 11.2% (*n* = 10, *P* < 0.01), at 10 µM 49.3 ± 16.7% (*n* = 10, *P* < 0.01) and at 100 µM 60.4 ± 13.7% (*n* = 10, *P* < 0.001; Figure 1). Morphine (100 µM) did not inhibit SP-LI release by capsaicin (1 µM). If naloxone (100 nM) were present during the equilibration and stimulation periods then the inhibitory effect of morphine (30 µM) on KCl-stimulated SP-LI release was abolished (Figure 2). Naloxone (100 nM) alone did not affect SP-LI release. The κ-receptor agonist, U50,488H (10 µM) did not significantly alter 60 mM KCl-stimulated release. Results were: KCl-stimulated control (60 mM): 100.0 ± 9.0% (*n* = 14); U50,488H (10 µM): 111.2 ± 9.6% (*n* = 14; *P* > 0.05). In a series of experiments we examined the effect of the δ-receptor agonist DPDPE. The maximum dose which did not increase recovery of exogenous SP was 1 µM. This effect may reflect inhibition of neutral endopeptidase. At

Table 1 Effect of calcium-free medium on substance P-like immunoreactivity (SP-LI) release by (a) 60 mM KCl, (b) 1 µM capsaicin and (c) 1 µM bradykinin (BK)

	Calcium control	Calcium-free	% inhibition
(a) KCl 60 mM	100.0 ± 16.7 (8)	-3.9 ± 14.8 (8)	≥ 100.0***
(b) Capsaicin 1 µM	100.0 ± 20.9 (10)	2.2 ± 1.6 (10)	97.8 ± 21.0***
(c) BK 1 µM	100.0 ± 22.3 (10)	2.4 ± 8.0 (10)	97.6 ± 23.7***

Release is expressed as a percentage of control for (*n*) preparations.

*** *P* < 0.001

Table 2 Effect of sodium cromoglycate (SCG) on (a) spontaneous, (b) 60 mM KCl and (c) 1 μ M bradykinin (BK)-stimulated substance P-like immunoreactivity (SP-LI) release

	Spontaneous	KCl	BK
(1) Control	100.0 $\pm$ 6.6 (30)	100.0 $\pm$ 10.3 (15)	100.0 $\pm$ 12.0 (15)
SCG 1 μ M	107.2 $\pm$ 7.5 (30)	97.5 $\pm$ 11.0 (15)	106.0 $\pm$ 16.2 (15)
(2) Control	100.0 $\pm$ 7.6 (30)	100.0 $\pm$ 10.9 (15)	100.0 $\pm$ 10.8 (15)
SCG 100 μ M	104.8 $\pm$ 5.0 (30)	98.8 $\pm$ 14.6 (15)	95.9 $\pm$ 18.3 (15)

Release is expressed as a percentage of control.
Values are means $\pm$ s.e.mean of *n* animals.

this dose DPDPE did not affect SP-LI release by KCl. Results were: KCl-stimulated control (60 mM): 100.0 $\pm$ 10.3% (*n* = 10); DPDPE (1 μ M): 99.3 $\pm$ 7.0% (*n* = 10; *P* > 0.05).

Discussion

We have investigated the release of SP-LI by a variety of neuronal stimulants in the superfused rat trachea. Treatment of newborn rats with capsaicin caused a greater than 90% reduction in tracheal SP-LI content. Capsaicin causes the selective destruction of chemosensitive C-fibre PANs under these conditions (Jancso *et al.*, 1977). This suggests that the

great majority of tracheal SP-LI is localized to PANs, a finding that is consistent with those of Lundberg and associates who found that treatment of newborn rats with capsaicin abolishes vagally mediated neurogenic plasma extravasation in the trachea (Lundberg & Saria, 1982b) and causes an almost total loss of SP-immunoreactive fibres from the airways (Lundberg *et al.*, 1984).

A markedly increased outflow of SP-LI was observed during exposure to KCl, capsaicin and BK. Removal of calcium ions from the superfusion medium abolished release to KCl and significantly reduced it to capsaicin and BK. The effect of BK was not mimicked by des-Arg⁹-BK. BK receptors have two distinct populations, classified as B₁ and B₂ (Regoli & Barabé, 1980). BK is a potent activator of C-fibres (Kaufman *et al.*, 1980; Dray *et al.*, 1988) and stimulates SP-LI release from capsaicin-sensitive neurones in guinea-pig airways (Saria *et al.*, 1988). In our studies the BK B₁ receptor agonist, des-Arg⁹-BK (Regoli & Barabé, 1980) was inactive suggesting that the BK receptor stimulating calcium-dependent SP-LI release is not of the B₁ subtype. This result agrees with the report that C-fibre activation by BK is mediated by the BK B₂ receptor (Dray *et al.*, 1988).

SCG has been reported to suppress C-fibre excitability in the canine lung *in vivo* (Dixon *et al.*, 1980) and to inhibit BK-stimulated bronchoconstriction in man (Fuller *et al.*, 1987). In both studies, subjects were exposed to SCG 10 min prior to challenge by capsaicin and BK respectively. Since BK is a known stimulant of vagal afferents (Kaufman *et al.*, 1980) we postulated that SCG might inhibit SP-LI release by BK from PANs in the rat trachea. However, we found no apparent changes in spontaneous, KCl- or BK-stimulated release of SP-LI after a prior exposure to SCG of 2 h. We conclude that it is possible that SCG can inhibit unmyelinated afferent pathways but that this effect does not manifest itself as a suppression of SP-LI release.

The neurotransmitter(s) mediating NANC neurogenic inflammatory reactions in the airways are currently unknown. Several neuropeptides, including SP, have been proposed as mediators of NANC neurogenic plasma extravasation, bronchoconstriction, vasodilatation and mucus secretion (Joos *et al.*, 1988). With regard to plasma extravasation, the most promising candidate is SP. SP is a powerful stimulant of plasma extravasation in rat airways *in vivo* (Lundberg & Saria, 1982b). Furthermore, vagally mediated NANC neurogenic plasma extravasation is abolished by capsaicin-pretreatment (Lundberg & Saria, 1982b) and inhibited by a SP receptor antagonist (Lundberg *et al.*, 1983b) although the latter observation is balanced by the finding that the SP receptor antagonist possesses potent local anaesthetic activity (Post *et al.*, 1985). In the present study, morphine inhibited 60 mM KCl-stimulated SP-LI release by a dose-related, naloxone-sensitive mechanism. Opioid receptors are known to be present on PANs in the spinal cord (LaMotte *et al.*, 1976) and vagus nerve (Atweh *et al.*, 1978). Opioids have been demonstrated to inhibit SP-LI release *in vitro* (Jessell & Iversen, 1977; Lembeck & Donnerer, 1985) and *in vivo* (Yaksh *et al.*, 1980). Furthermore, in guinea-pig airways, morphine inhibits NANC neurogenic plasma extravasation *in vivo* (Belvisi *et al.*, 1989). The receptor subtype mediating inhibition of SP-LI release is not known. Morphine is relatively selective for μ -opioid receptors but also stimulates δ -opioid

Figure 1 Effect of morphine on substance P-like immunoreactivity (SP-LI) release evoked by KCl 60 mM. Data are mean differences for 10 animals; vertical bars show s.e.mean. *** *P* < 0.001; ** *P* < 0.01.

Figure 2 Effect of naloxone (100 nM) on morphine (30 μ M)-sensitive substance P-like immunoreactivity (SP-LI) release evoked by KCl 60 mM. Data are means expressed as a percentage of 60 mM KCl-stimulated control; vertical bars show s.e.mean. ** *P* < 0.01 compared to control. *n* = 9 for control; *n* = 7 for naloxone; *n* = 8 for morphine; *n* = 6 for morphine (morph) plus naloxone (nal).

receptors at high doses (Paterson *et al.*, 1983). In our study, the effect of morphine was highly sensitive to antagonism by the selective μ -opioid receptor antagonist, naloxone (Paterson *et al.*, 1983). This finding, together with the fact that the κ -opioid receptor agonist, U50,488H, and the δ -opioid receptor agonist, DPDPE, did not mimic the action of morphine suggests a predominant role for the μ -opioid receptor. Precise classification requires a highly selective full antagonist at the δ -opioid receptor. The usefulness of the best available, ICI 174,864, has been questioned because it exhibits agonist activity (Dray & Nunan, 1984).

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