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Effects of levodropropizine on vagal afferent C-fibres in the cat

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1 Levodropropizine (LVDP) is an effective antitussive drug. Its effects on single-unit discharge of vagal afferent C-fibres were tested in anaesthetized cats to assess whether an inhibition of vagal C-fibres is involved in its antitussive properties. Vagal C-fibres, identified by their response to phenylbiguanide (PBG), were recorded via suction electrodes from the distal part of the cut vagus. Based on their response to lung inflation, C-fibres were classified as pulmonary (19 fibres) or non-pulmonary (6 fibres).

2 PBG increased the discharge rate of both C-fibre types and activated a respiratory reflex causing apnoea. This reflex was abolished when the second vagus nerve was cut as well, while PBG-mediated stimulation of the C-fibres was not affected by vagotomy.

3 LVDP was administered intravenously and the C-fibre response to PBG was compared with that before administration of the drug. LVDP reduced both the duration of apnoea and the response of the C-fibre to PBG.

4 Comparison of the C-fibre responses to PBG and to a mixture of PBG and LVDP revealed that the period of apnoea was shortened and the discharge rate of the C-fibre reduced when LVDP was present.

5 The LVDP-induced inhibition of the C-fibre response to PBG was on average 50% in pulmonary and 25% in non-pulmonary fibres.

6 These results suggest that LVDP significantly reduces the response of vagal C-fibres to chemical stimuli. It is, thus, likely that the antitussive effect of LVDP is mediated through its inhibitory action on C-fibres.

Keywords: Levodropropizine; antitussive effect; pulmonary C-fibre; vagal afferent fibres; coughing

Introduction

Levodropropizine (S(−)-3-(4-phenyl-piperazin-1-yl)-propane -1,2-diol; LVDP), is a non-opioid compound with antitussive effects, which is used as an effective and well-tolerated antitussive drug in clinical practice (Guffanti, 1989). Experimentally, LVDP has been shown to reduce the mechanically or electrically induced coughing in rabbits and guinea-pigs (Malandrino *et al.*, 1988). This antitussive effect of LVDP was also shown against coughing induced by irritant aerosols and by capsaicin (Lavezzo *et al.*, 1992). It has been suggested that LVDP exerts its antitussive effect by a peripheral mechanism, its central sedative effect was reported to be very small (Malandrino *et al.*, 1988; Melillo *et al.*, 1988). In this regard, LVDP was suggested to reduce cough (i) by interfering with stimulus activation of peripheral endings of sensory nerves and (ii) by modulation of neuropeptides involved in the cough reflex (Kohrogi *et al.*, 1988; Lavezzo *et al.*, 1992; Daffonchio *et al.*, 1993; Yamawaki *et al.*, 1993). The involvement of sensory neuropeptides in the cough reflex is well documented by the fact that exogenous administration of substance P evokes coughing (Forsberg & Karlsson, 1986; Karlsson *et al.*, 1988) most likely by a direct stimulation of the sensory nerve endings (Kohrogi *et al.*, 1988). The effect of LVDP in preventing coughing was markedly reduced by depletion of neuropeptides (Gamse *et al.*, 1981) which supports the involvement of neuropeptides in the peripheral action of LVDP. Thus, LVDP has been suggested to inhibit both the direct stimulation of sensory nerve endings by irritant stimuli and the release or activation of neuropeptides acting on these nerve endings.

Coughing is known to be the most dramatic reflex that results from stimulation of the afferent vagal fibres that innervate the respiratory tract. Although there appears to be some support for the notion that no single category of afferent is solely responsible, there is good evidence that certain types of afferent are critically involved in the induction of coughing from particular parts of the airways. However present evidence suggests that myelinated rapidly adapting receptors (RAR) and nonmyelinated C-fibres supply the critical afferent input when cough and sensations are evoked by chemical stimulation within the lung (Widdicombe, 1954; 1989; Coleridge & Coleridge. 1984). For example capsaicin, a specific stimulant of C-fibres, has been shown to induce coughing in human subjects (Midgren *et al.*, 1992). However, large doses of capsaicin deplete nonmyelinated fibres of their neuropeptides (Lundberg & Saria, 1983) and they abolish the cough reflex to some irritant stimuli (Forsberg & Karlsson, 1987). Thus, capsaicin exerts its effect either directly by stimulation of C-fibres or indirectly through release of neuropeptides. Both bronchial and pulmonary C-fibres are stimulated by capsaicin, but activation of the cough reflex has been suggested to result from stimulation of bronchial C-fibres (Tatar *et al.*, 1988; Widdicombe 1989). LVDP has been demonstrated to counteract this capsaicin-induced cough in animals and human subjects (Lavezzo *et al.*, 1992; Mistretta *et al.*, 1992). Moreover, LVDP has been shown to reduce acetic acid-induced writhing reflex, a visceral pain reflex in which C-fibre activation is involved (Melillo *et al.*, 1988). Therefore, it is likely that LVDP reduces the response of C-fibres to chemical stimulants irrespective of the origin of C-fibres. Thus, we tested the effects of LVDP on PBG-evoked stimulation of two groups of C-fibres (pulmonary and non-pulmonary) to assess whether their inhibition is involved in the antitussive effect of LVDP.

Methods

Experiments were conducted in 10 cats of either sex (average body mass 4.05 ± 1.05 kg). The right femoral vein was cannulated (PE-90) under halothane anaesthesia. General anaesthesia was maintained by slowly injecting a heated chloralose-

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urethane solution (40 mg kg^{-1} glucochloralose, 200 mg kg^{-1} urethane). A second PE-90 catheter, advanced through the left femoral vein into the inferior vena cava, was used for later infusion of phenylbiguanide (PBG, Aldrich-Chemie, Steinheim, Germany) or a mixture of PBG and levodropropizine (S(−)-3-(4-phenyl-piperazin-1-yl)-propane-1,2-diol; LVDP, Dompe' famaceutici, SpA, Italy). The right ventricle was cannulated via the right jugular vein by a curved-tip catheter with side holes (PE-90) for later infusion of LVDP. Arterial blood pressure (Pa) was measured through a catheter in the right femoral artery. Rectal temperature was measured with a thermistor, and body temperature was maintained near 38°C with an infrared lamp. The trachea was cannulated (7 mm i.d.) in the midcervical region, and a pneumotachometer was attached to the tracheal cannula. A pressure transducer was connected to the tracheal cannula through a side hole to measure airway pressure. Respiratory parameters, tidal volume (V_T) and respiratory frequency (f_R) was measured with a pneumotachometer (Fleich no. 0.8) and differential pressure transducer/signal conditioner (Gould Godart, model BV 17212, Bilthhoven, Netherlands) and ventilation ($\dot{V}$) was calculated as $V_T \cdot f_R$. Tracheal concentrations of O_2 and CO_2 were monitored by a respiratory mass spectrometer. Mean systemic arterial ($\bar{P}a$) and airway (Paw) pressures were continuously recorded with pressure transducers (P 23 ID, Statham).

Vagus nerve preparation and recording techniques

Both vagus nerves were exposed in the cervical region and carefully dissected free from surrounding connective tissue. One nerve was cut and its nerve sheath was retracted from its peripheral end for about 2–3 mm. This small piece of nerve sheath was then sucked into a glass pipette (1 mm o.d.) by use of a syringe. The glass pipette was attached to a micro-manipulator which allowed the free end of the nerve to be moved and its mechanical stabilization to be secured. The nerve was held in 0.9% NaCl solution, and a small volume of saline or a diluted Pronase enzyme (Pronase E, Merck, Darmstadt, Germany) was injected into its peripheral end to separate individual fibres. Fine nerve strands were sucked from the cut end into a borosilicate glass microelectrode, with a syringe attached to the microelectrode. An Ag/AgCl wire, introduced into the electrode close to its tip, was connected to the head stage buffer amplifier of a WP Micro Probe System (A-773, New Haven, U.S.A.). The reference electrode was placed in the bath solution. The nerve signal was further amplified (Tektronix,5A 22N), displayed on a storage oscilloscope (Tektronix, model 5113) and stored on an FM tape (Store 7 DS, Racal). For single-unit analysis, the tape was played back at a slower speed into a window discriminator (Frederic Haer) and also to a storage oscilloscope (Tektronix, model 5113), which allowed storage of one representative action potential during any experimental test as reference standard for comparison with the signals played back from the tape. Only action potentials of the same amplitude and shape as the reference standard were counted in order to ensure separation of individual units in a given experimental test.

Identification of pulmonary C-fibres

Twenty-five fibres with irregular spontaneous activity with no respiratory modulation which responded by an increased firing rate to an infusion of phenylbiguanide (PBG, 20 $\mu g\ kg^{-1}$ into the inferior vena cava) were classified as C-fibres. Of these, 19

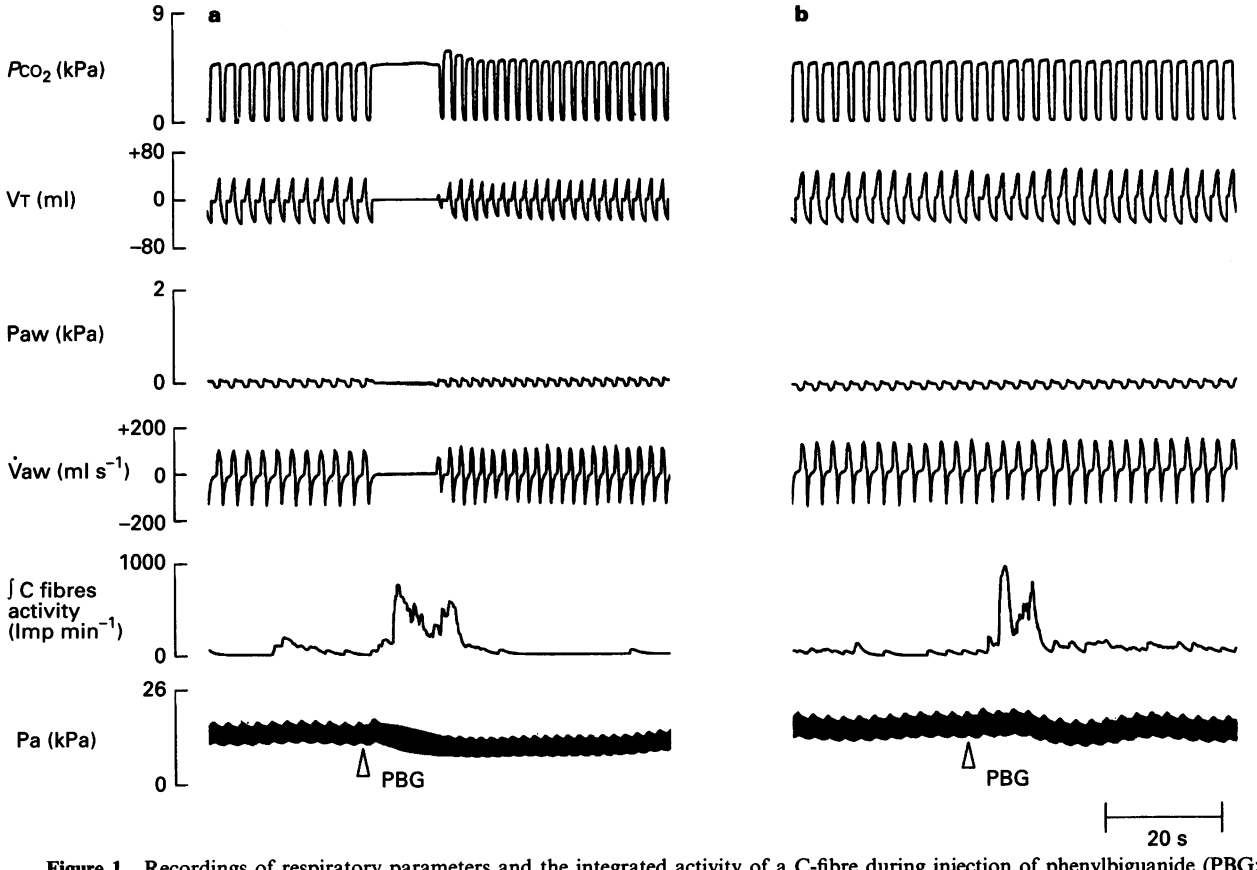

Figure 1 Recordings of respiratory parameters and the integrated activity of a C-fibre during injection of phenylbiguanide (PBG; at arrow head) in unilaterally (a) or bilaterally (b) vagotomized cat. Traces from top to bottom; P_{CO_2}, tracheal P_{CO_2}; V_T tidal volume (+: inspiratory, −: expiratory volume); Paw, airway pressure; $\dot{V}aw$, airway flow (+: inspiratory, −: expiratory flow); integrated C-fibre activity; and $\bar{P}a$, arterial blood pressure. Note that bilateral vagotomy abolished the respiratory response to PBG while C-fibre response remained intact.

which also responded to lung inflation, were identified as pulmonary C-fibres. For lung inflation, the tracheal tube was connected to a pressure source of 15–20 cmH_2O for 7 s. C-fibres not responding to lung inflation were found within fine nerve strands in which pulmonary C-fibres were recorded (multiple unit filament). Although the precise origin of these fibres could not be determined, the close vicinity of these and pulmonary C-fibres within the vagus nerve suggests that they were arising from the lung. Therefore, these fibres reported here as non-pulmonary C-fibres are most likely bronchial C-fibres.

Repeated application of capsaicin has often been shown to produce a functional desensitization of C-fibres as well as depletion of neuropeptides from nerve terminals which in turn can reduce indirectly the effects of chemical stimulants on nerve endings. Thus, although capsaicin is a more specific stimulant of C-fibres, we avoided its application and used PBG instead. Before running our experimental protocol, we tested the response of each individual C-fibre to repeated infusions of PBG and found that no reduction in their responses occurred when PBG infusions were at least 20–30 s apart. Any two successive infusions of PBG were at least 10 or 5 min apart in the two protocols of our experiments (see below). Therefore, any reduction in the response of C-fibres to PBG was avoided in these experiments.

Experimental protocol

In the unilaterally vagotomized cat, each vagal strand was tested for single-unit or multiple-unit activity by use of a window discriminator and a storage oscilloscope (see Methods). After classifying a given unit as a C-fibre and recording its control activity for 3–5 min, its response to PBG was tested; 1.5 ml freshly dissolved PBG at a concentration of 50 $\mu g\ ml^{-1}$ was infused within 10 s into the inferior vena cava by use of a roller pump (Ismatec, MV-GE, Zürich, Switzerland). Thereafter, LVDP was infused for 6 min into the right ventricle at a concentration of 20 mg ml^{-1} and a rate of 0.5 ml min^{-1}, to a total dose of 15 mg kg^{-1}. At the end of this infusion, fibre activity was recorded for 2–3 min for control after LVDP. PBG infusion was then repeated to test for LVDP-mediated changes in the response of C-fibres to PBG. This protocol was applied on both pulmonary C-fibres (19 fibres) and non-pulmonary C-fibres (6 fibres).

Two of the C-fibres were tested after bilateral vagotomy. Since changes caused by PBG infusion were virtually independent of vagotomy, the data for all fibres are presented and discussed together.

Another group of 6 C-fibres was tested for their response to a mixture of PBG and LVDP. Both PBG and LVDP were mixed (*in vitro*), giving a solution with concentrations of 50 $\mu g\ ml^{-1}$ PBG and 10 mg ml^{-1} LVDP. After testing the response of the C-fibre to PBG as in above protocol, 1.5 ml of this mixture (PBG = 20 $\mu g\ kg^{-1}$, LVDP = 4 mg kg^{-1}) was infused within 10 s through the same catheter into the inferior vena cava. This infusion of PBG plus LVDP was 5 min apart from that of PBG alone.

Data analysis

Paired *t* tests were performed to determine if a variable or its response to PBG changed significantly from control after infusion of LVDP or of a mixture of LVDP and PBG. Significance was accepted at the 5% level, unless stated otherwise.

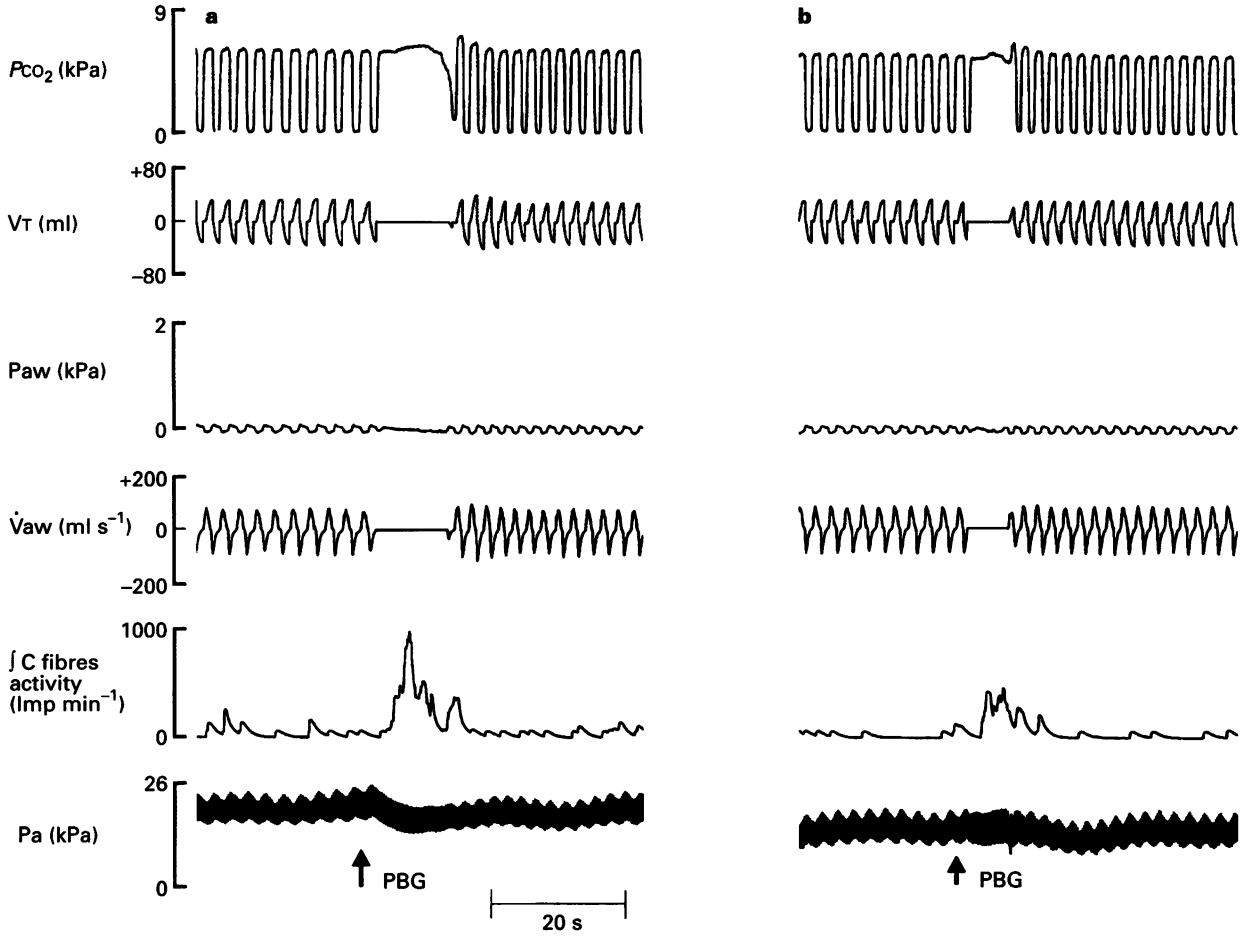

Figure 2 C-fibre and respiratory responses to comparable doses of PBG during control (a) and after infusion of levodropropizine (b) in a unilaterally vagotomized cat. Note that the responses are attenuated after administration of levodropropizine.

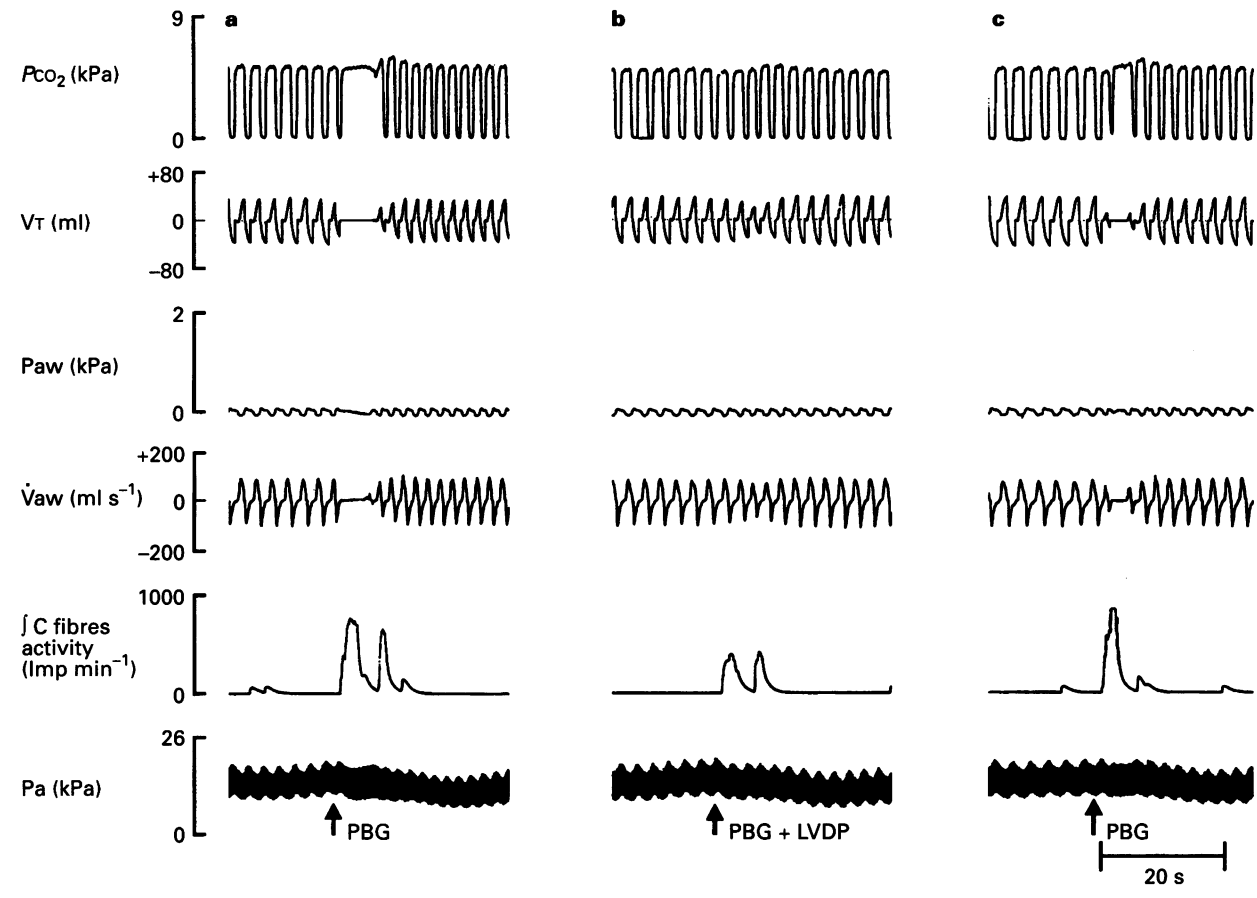

Figure 3 Respiratory parameters and integrated C-fibre activity during injections of PBG (a), a mixture of PBG and LVDP (b) and PBG again (c). The order of injections was from (a) to (c). Note that the inhibitory effects of LVDP were reversible.

Results

The original recording in Figure 1 shows the integrated activities of two C-fibres (one after unilateral (a), the other after bilateral vagotomy (b)) with systemic blood pressure ($\bar{P}a$) and respiratory parameters: airway flow ($\dot{V}aw$), airway pressure (Paw), tidal volume (V_T) and tracheal P_{CO_2}. As PBG was infused (indicated by the arrow heads) into the unilaterally vagotomized cat (a), the firing rate of the C-fibre increased and a respiratory reflex causing apnoea was activated. Bilateral vagotomy (b) abolished the respiratory reflex while the C-fibre response (increased activity) to PBG remained intact. In both conditions PBG infusion slowed the heart rate and caused the arterial blood pressure, $\bar{P}a$, to fall. The effects of PBG on the activity of C-fibres and length of apnoea were repeatedly reproducible (not shown here).

Figure 2 shows that the increase in firing rate of a C-fibre in response to PBG infusion during control (a) was significantly reduced after administration of LVDP (b). LVDP also shortened the period of apnoea by PBG infusion.

The impulse rate of the C-fibre shown in Figure 3 markedly increased during infusion of PBG (a). However, this increase in activity of the fibre was less when the same dose of PBG was infused together with LVDP (b). Apnoea elicited by PBG infusion (a) was abolished after combination of PBG with LVDP (b). However, both the stimulation of C-fibre and the respiratory reflex evoked by PBG were restored as PBG was infused alone some minutes later (c). This suggests that the effects of LVDP on C-fibre stimulation were reversible.

Mean values (± s.e.) for discharge rate of 19 pulmonary C-fibres and 6 non-pulmonary C-fibres which were examined for their response to PBG are compiled in Figure 4. As shown in this figure, both groups of C-fibre exhibited a pronounced increase in firing rate during the infusion of PBG. Infusion of LVPD significantly attenuated the increase in firing rate evoked by the infusion of PBG, but did not significantly affect control activity of the fibres. This LVDP-mediated reduction in the response of fibres to PBG was 50 ± 9% in pulmonary C-fibres and 25 ± 9% in non-pulmonary C-fibres.

Data from the 6 C-fibres which were examined for their responses to infusion of the mixture of PBG and LVDP revealed also that the increase in impulse rate of fibres was significantly smaller during infusion of PBG and LVDP than during infusion of PBG alone. The average reduction of responses was 40 ± 5%.

Mean values (± s.e.) for the duration time of apnoea occurring during infusion of PBG before ($n=10$) and after administration of LVDP ($n=10$) and during infusion of the mixture of PBG and LVDP ($n=9$; Figure 5), indicate that apnoea was significantly shorter after administration of LVDP and was almost abolished during infusion of PBG and LVDP. However, PBG did not evoke apnoea after bilateral vagotomy (not shown here).

In Table 1 are compiled data for cardiorespiratory parameters of unilaterally (**A**) or bilaterally (**B**) vagotomized cats, obtained during control or during infusion of LVDP. LVDP induced a slight increase in V_T and f_R and, thus, caused a decrease in endtidal P_{CO_2} (PE'_{CO_2}). Mean arterial blood pressure was decreased during infusion of LVDP. However, these cardiorespiratory changes were independent of bilateral vagotomy (compare Table 1, **A** with **B**).

Discussion

In the present study we searched exclusively for C-fibres, and preferentially for those originating from the lungs, which were sensitive to lung inflation (pulmonary C-fibres). However, we recorded about 250 fibres among which we were able to identify 19 as pulmonary C-fibres which we tested together

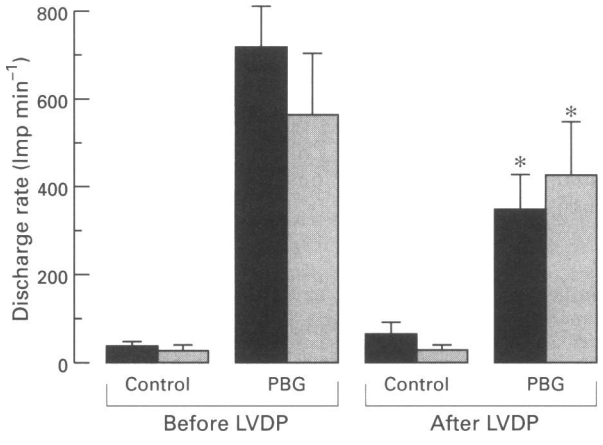

Figure 4 Average discharge rates of 19 pulmonary C-fibres (solid columns) and 6 non-pulmonary C-fibres (stippled columns) during control and during infusion of PBG before and after administration of LVDP. *Significantly different from corresponding values before LVDP.

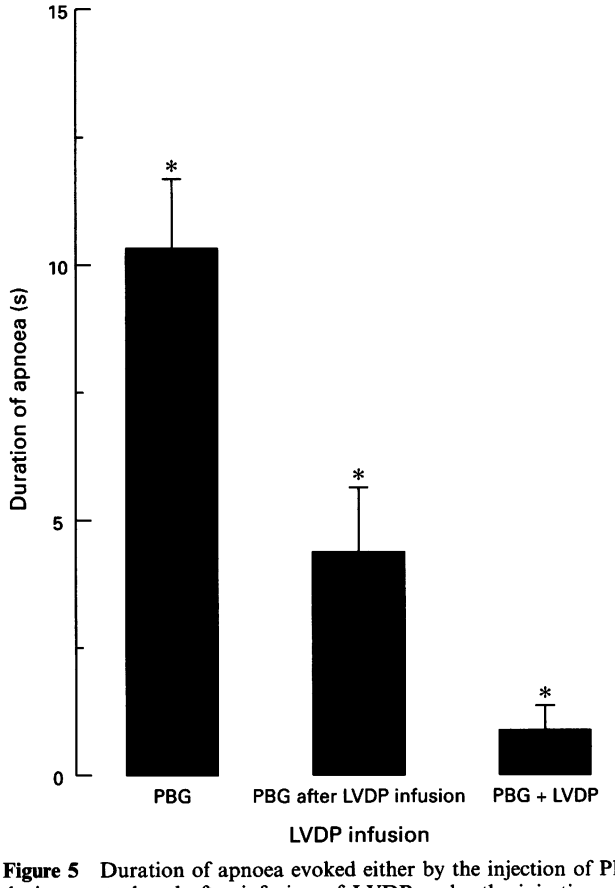

Figure 5 Duration of apnoea evoked either by the injection of PBG during control and after infusion of LVDP or by the injection of a mixture of PBG and LVDP. *Significantly different from control at 1% level.

with 6 non-pulmonary C-fibres for their responses to LVDP. Since LVDP reduced the response of all C-fibres to PBG (see Figure 4), it was extremely difficult to identify a second or third C-fibre in one cat once LVDP had been administered to test the first C-fibre. Thus, the number of fibres was not representative of the fibre composition of the whole nerve or of the ease with which they were found.

Since C-fibres fire at a very low rate, action potentials should be counted over a long recording period to determine a

Table 1 Cardio-respiratory parameters before, during and 2–3 min after the end of infusion of levodropropizine (LVDP) into the right ventricle

A Unilaterally vagotomized (10 observations in 6 cats)	*Control*	*During LVDP*	*After LVDP*
VT	33.8	38.7*	33.7
(ml)	3.3	3.8	3.4
fR	18.3	19.4	19.6
(min^{-1})	1.5	1.6	1.9
$\dot{V}E$	621	755*	664
(ml min^{-1})	76	92	89
$PE'CO_2$	38.2	34.6*	36.8
(Torr)	2.1	1.9	1.9
Pa	128	97*	102*
(mmHg)	5	5	6
B Bilaterally vagotomized (5 observations in 5 cats)	*Control*	*During LVDP*	*After LVDP*
VT	41.0	47.0*	42.2
(ml)	5.6	5.6	5.0
fR	15.9	17.3	17.4
(min^{-1})	0.8	1.4	1.6
$\dot{V}E$	650	816*	737
(ml min^{-1})	85	110	108
$PE'CO_2$	38.1	34.0*	34.7*
(Torr)	2.8	2.7	2.2
Pa	124	88*	91*
(mmHg)	11	9	9

Values are mean ± s.e.
*Significantly different from control at 1% level.

reliable firing rate for their control activity. In our experiments the viability of fine vagal filaments was relatively short and the time required for completion of our protocol did not allow us to record these fibres for periods longer than 3–5 min for their control activity. Therefore, we avoided drawing conclusions about the effects of LVDP on control activity of C-fibres. Since coughing is induced by the chemical stimulation of C-fibres, the effects of LVDP on stimulated C-fibres are discussed in relation to its antitussive properties.

The effects of LVDP lasted longer than the usual recording life of a C-fibre. This restriction did not allow us to determine either a dose-related response curve for LVDP effects on C-fibres or to test the reversibility of C-fibre responses to PBG after administration of LVDP. However, in two fibres which were recorded over 1 h we found that the LVDP-mediated decrease in their response to PBG lasted about 40 min.

C-fibres and levodropropizine

In the present study we tested the effects of LVDP on single C-fibre units as well as on a respiratory reflex activated by overall stimulation of all C-fibres. We have previously shown that activation of this respiratory reflex caused rapid shallow breathing or apnoea (Shams & Scheid, 1990; Karla *et al.*, 1992; Orr *et al.*, 1993). Our present results clearly demonstrate that LVDP not only reduced the activity of a single fibre in response to PBG but also diminished or even abolished the overall stimulation of C-fibres causing apnoea. We chose two different protocols to examine the effects of LVDP on C-fibres. LVDP was either administered over a period of 6 min by infusion or it was immediately injected as a mixture with PBG. The results obtained with either protocol did not differ. Irrespective of the form of application, LVDP diminished the response of fibres to PBG. Thus, our results suggest that LVDP may inhibit the activity of C-fibres once they are stimulated with endogenous stimulants. This effect of LVDP may contribute to its antitussive properties demonstrated in experimental animals and human subjects.

In animals, coughing has been elicited either by electrical

stimulation (Cavanaghi *et al.*, 1976) or mechanical and chemical irritation (Malandrino *et al.*, 1988; Lavezzo *et al.*, 1992) of the tracheal mucosa or by electrical stimulation of the vagus nerve (Pickering & James, 1979). Malandrino *et al.* (1988) used all these methods to test the effects of LVDP on experimentally induced coughing in the guinea-pig and rabbit. They found a significant inhibition of coughing after administration of LVDP irrespective of the method used to elicit coughing. The antitussive effect of LVDP was abolished after 30 min in their experiments with electrical stimulation of the vagus nerves. In fact, LVDP-mediated reduction in the response of C-fibres to PBG was abolished after 40 min in the present experiments. Thus comparable doses of LVDP exert antitussive properties in accordance with the course of inhibition of the C-fibre activity. This inhibitory effect of LVDP appears not to be confined to C-fibres of a certain location as indicated by the fact that LVDP inhibited both pulmonary C-fibres and non-pulmonary (bronchial) C-fibres in our experiments. Since C-fibres are suggested to contribute to the critical afferent input for cough sensations (Coleridge & Coleridge, 1984), it is likely that the inhibitory effect of LVDP on these fibres accounts for its antitussive effects.

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