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Does naloxone induce relaxation of guinea-pig airway smooth muscle?

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The effects of the opiate receptor antagonist naloxone were investigated on isolated preparations of guinea-pig trachealis contracted with either histamine, methacholine or KCl. The commercially available solution of naloxone (Narcan) induced concentration-dependent relaxation of the contracted airway preparations. In stark contrast, aqueous solutions of naloxone were without any significant relaxant effect. Aqueous solutions of the preservatives (methyl and propyl hydroxybenzoate) present in the vehicle used in the commercial formulation of naloxone mimicked exactly the relaxant effects induced by Narcan. Thus, naloxone does not directly induce relaxation of airway smooth muscle. The effects of Narcan can be solely attributed to the activity of the preservatives present in the vehicles. The mechanism underlying the bronchodilator activity of methyl and propyl hydroxybenzoate is unknown but is not related to receptor blockade or to alterations in the intracellular levels of cyclic AMP.

Opioids administered intravenously to experimental animals and man induce increases in airways resistance, laryngospasm and respiratory stimulation (Foldes et al 1966; Jennett et al 1968; Willette et al 1983). Furthermore, asthmatic attacks induced by a combination of chlorpropamide and ethanol in diabetic patients are mimicked by intravenous administration of enkephalin analogues and inhibited by the opiate receptor antagonist naloxone (Leslie et al 1980). It has been proposed, therefore, that in such diabetic patients the asthmatic episodes are mediated via endogenous peptides with opiate-like activity (Leslie et al 1980). Recently, however, it has been reported that the preservatives (methyl and propyl hydroxybenzoate) present in commercially available solutions of naloxone possess vasodilator activity (Brandt et al 1983; Crockard et al 1983). In the study of Leslie et al (1980) it is not clear whether the patients received the appropriate vehicle, in which commercial preparations of naloxone are formulated, as a control. Thus, in view of the reported smooth muscle relaxant activity of the preservatives, it is conceivable that the anti-asthmatic effect observed by Leslie et al (1980) was not entirely attributable to the opiate receptor-blocking activity of the naloxone.

The object of this study, therefore, was to investigate whether naloxone possesses any direct relaxant activity in isolated preparations of guinea-pig airway smooth muscle. A preliminary account of these findings has been reported to the British Pharmacological Society (Soulioti et al 1986).

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Methods

Male Dunkin-Hartley guinea pigs were killed by stunning and bleeding. Tracheae were rapidly excised from the animals, dissected free of extraneous connective tissue and then prepared as follows.

Tension studies. Spirally cut preparations of tracheae were suspended in Krebs-Henseleit solution (KHS) at 37 °C and bubbled with 95% O_2 and 5% CO_2. An initial stretching tension of 20 mN was applied to the tissues which were left for 60 min to equilibrate, during which time the bathing medium was changed three times. Changes in tension were recorded using isometric force-displacement transducers (FT03C; Grass Instruments, Quincy, Mass.) coupled to a Grass (model 7) curvilinear, ink-writing polygraph. The composition of the KHS used in these studies was as follows (in mM); NaCl 118; KCl 4·7; $MgSO_4$ 1·2; KH_2PO_4 1·2; $CaCl_2$ 2·5; $NaHCO_3$ 25 and glucose 11·7.

Following equilibration, the tissues were contracted with histamine (1×10^{-4} M) to gauge the normality and viability of the tissue. Following washout of the histamine and return to baseline tension, an interval of 30 min was allowed before addition of the same concentration of histamine. This time at the peak of the tonic (sustained) component of the contraction a cumulative concentration-effect curve was constructed using the relaxant solution under test (Narcan, naloxone hydrochloride or the vehicle present in Narcan). Control preparations which received saline in place of drug were run in parallel with those in which test solutions were studied. Drug-induced relaxations, corrected for any fall-off in tension recorded in the control preparations, were calculated as a percentage of the maximum relaxation achieved. Most control preparations exhibited little loss of tension over the 60 min test period. In several experiments KCl (9×10^{-2} M) or methacholine (1×10^{-6} M) was used as the contracting agent in place of histamine. In experiments using these two latter

agonists the protocol adopted was identical to that described above.

Cyclic (c) AMP studies. Tracheal preparations were rapidly removed from the tissue bath at the desired time points, blotted dry on absorbant filter paper and frozen in liquid nitrogen (time elapsed less than 10 s). The frozen preparations were then weighed before being placed in 2 ml capacity Teflon vials (pre-cooled in liquid nitrogen) containing 1 ml of 5% trichloroacetic acid (TCA) and a 0·9 mm stainless steel grinding ball. Tissues were then microdismembranated for 30 s (Braun Mikro-dismembrator II). The pulverized material was transferred to a 5 ml polypropylene centrifugation tube, residual material being removed from the Teflon vial with a further 1 ml of TCA. Each tube was left on ice for 10 min before centrifugation at 8000*g* for 15 min at 4 °C. The supernatant was decanted off into a large Pyrex tube and extracted six times with five volumes of water-saturated diethyl ether to ensure removal of the TCA and lipids present in the extract. Residual traces of ether were evaporated by heat (60 °C for 5 min) and the samples stored at −20 °C until required for assay. Storage at −20 °C is sufficient to prevent breakdown of cAMP after deproteinization and ether washing.

Before assaying for cAMP content, 450 µl aliquots of each extract were freeze-dried (Edwards EF4 Modulyo). The freeze-dried material was reconstituted in adequate buffer to allow duplicate measurements of cAMP to be made. cAMP content was determined using the commercially available protein-binding assay kit (Radiochemical International, TRK 432). For each assay a standard curve was constructed and tritium radioactivity was counted in a liquid scintillation spectrometer (Packard tri-carb, 460CD) after addition of 1 ml of scintillation cocktail (Picofluor 30, Packard).

In the text, the absolute levels of cAMP are expressed as pmol mg^{-1} wet weight of the tissue. Recovery of 40 pmol unlabelled cAMP added to 5% TCA before microdismembranation of the tissue was observed. Mean recovery value was 97 ± 4% (mean ± s.e.m.; n = 10).

Drugs

The drugs used were: histamine acid phosphate, methacholine chloride (BDH), Narcan (DuPont), methyl and propyl hydroxybenzoate (methyl and propyl parabens, Nipa Laboratories), naloxone hydrochloride (Sigma), propranolol hydrochloride (ICI) and flurbiprofen (Boots). Solutions of drugs were freshly prepared in 0·9% w/v NaCl solution (saline).

Results

Tension studies. Cumulative additions of Narcan (1×10^{-6} to 1×10^{-4} M) produced apparently concentration-related relaxations of tracheal preparations in which tone had been induced using either histamine, methacholine or KCl. Fig. 1a illustrates a typical tension recording from an experiment in which histamine was used as the contracting agent. The relaxant responses induced by Narcan solution were readily reversed by washing out the tissue bath with fresh KHS and

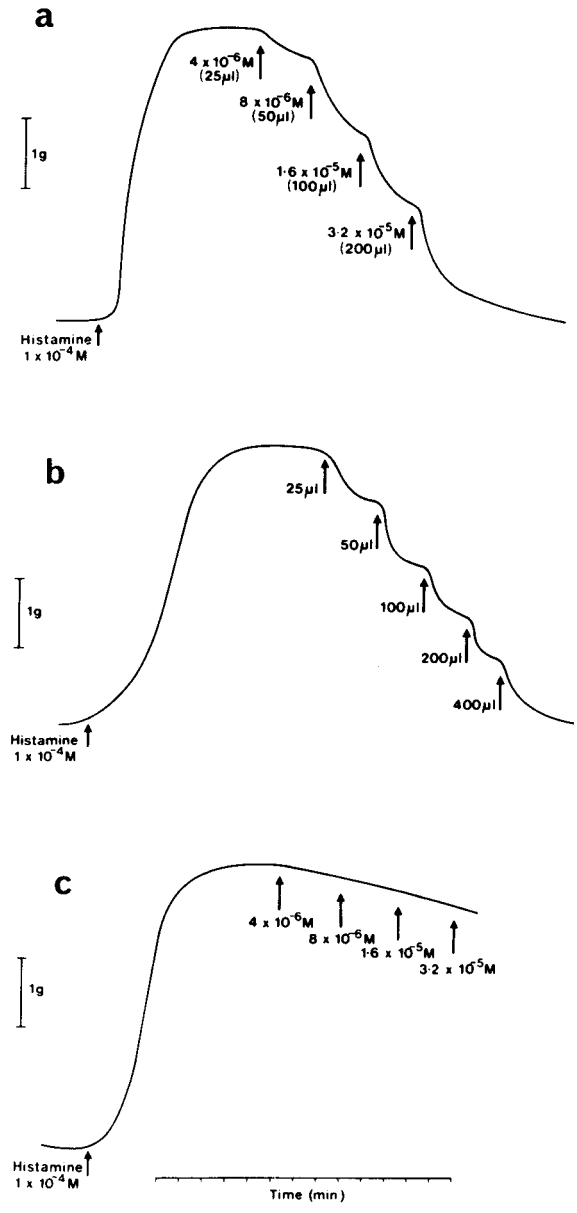

FIG. 1. Typical recordings of tension changes induced by Narcan (panel a), vehicle present in Narcan solutions (panel b) and aqueous solutions of naloxone hydrochloride powder (panel c) in spirally cut preparations of guinea-pig isolated trachea. In the examples shown tone was induced in the preparations by addition of histamine (1×10^{-4} M) before construction of a cumulative concentration-effect curve. The time bar is calibrated in min.

re-addition of histamine (1×10^{-4} M) or simply by addition of more histamine at the peak of the relaxation induced by Narcan.

Fig. 1b illustrates a typical tension recording from an experiment identical to that shown in Fig. 1a except that in place of Narcan solutions the appropriate vehicle was used—a mixture of methyl and propyl hydroxybenzoates made up according to the manufacturer's instructions. Equivalent volumes of vehicle to those employed in the construction of the concentration-effect curve to Narcan produced an almost identical relaxation profile (Fig. 1b). As was the case using Narcan, the relaxant effects of the vehicle were readily reversed by washing the tracheal preparations with fresh KHS and addition of further histamine. Both the magnitude and time course of relaxations produced by the vehicle were similar to those induced by Narcan.

To test whether naloxone itself possessed any smooth muscle relaxant activity, aqueous solutions of pure substance were tested in a similar manner. A typical recording from one such experiment is illustrated in Fig. 1c. Naloxone, in the concentration range 1×10^{-6} to 1×10^{-4} M was without significant relaxant effect when compared with time-matched control preparations run in parallel. Similar results to those illustrated in Fig. 1 were obtained when either methacholine or KCl was substituted for histamine.

Fig. 2 illustrates mean cumulative concentration-effect curves from six experiments comparing the relaxant activity of Narcan and its vehicle. There exists no significant difference between the curves. These relaxant effects of both Narcan and its vehicle were completely unaffected by pretreatment of the airway preparations with propranolol (1×10^{-6} M) or flurbiprofen (1×10^{-6} M).

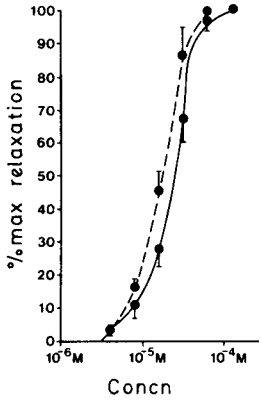

FIG. 2. Mean concentration-effect curves for Narcan (●——●) and vehicle (methyl and propyl parabens; ●- - -●) for relaxation of histamine-contracted guinea-pig isolated tracheal preparations. Abscissa: concentration of Narcan (M) or equivalent volume of vehicle. Ordinate: % maximum relaxation of preparation. Each point represents the mean of six different experiments. Standard errors of the mean are denoted by the vertical bars.

cAMP studies. cAMP levels were measured at the peak of contraction induced by either histamine or methacholine, immediately before addition of either Narcan or its vehicle (control levels) and at the peak of relaxation induced by a single, maximally effective concentration of Narcan or equivalent volume of its vehicle. No measurements of cAMP were made at intermediate relaxant levels since it was argued that if changes in cAMP did occur, they would be most marked after drug concentrations producing maximum relaxation. The results are summarized in Table 1.

Table 1. The effects of Narcan and its vehicle (methyl and propyl hydroxybenzoate) on cAMP levels in guinea-pig isolated trachealis.

Treatment	cAMP (pmol mg^{-1})
Control	0·29 ± 0·04 (n = 6)
Narcan	0·24 ± 0·05 (n = 5)
Vehicle	0·22 ± 0·03 (n = 5)

Each value is the mean ± s.e.m. The number of experiments is given in parentheses.

Neither Narcan nor its vehicle produced any significant alteration in the levels of cAMP in the tissues despite relaxing the tracheal preparations maximally. By way of comparison, equivalent relaxations of tracheal preparations induced by isoprenaline are accompanied by an approximate 150% increase in the levels of cAMP measured in the tissues (Giembycz & Rodger, unpublished observation).

Discussion

The experiments described in this paper clearly establish that naloxone does not possess any intrinsic ability to relax isolated preparations of airway smooth muscle. Since aqueous and/or saline solutions of naloxone prepared from pure substance were devoid of bronchodilator activity, the observed relaxation (of contracted trachealis) induced by the commercially available solution of naloxone (Narcan) can be wholly attributed to the vehicle used in the formulation. This conclusion is sustained by the observation (Fig. 1b) that the vehicle alone (a saline solution of methyl and propyl hydroxybenzoate made up according to the manufacturer's instructions) induced almost identical relaxations of the tracheal preparations, with respect to both time course and magnitude, as did Narcan itself. These effects of methyl and propyl parabens in airway smooth muscle, therefore, are in accord with the relaxant activity reported for these substances in other smooth muscles (Brandt et al 1983; Crockard et al 1983). Thus it is entirely conceivable that the anti-asthmatic effect observed by Leslie et al (1980) in-vivo was not entirely attributable to the opiate receptor blocking activity of naloxone. Indeed, we have recently shown that methyl and propyl hydroxybenzoate can provide protection

against exercise-induced asthma in certain individuals (Soulioti et al 1986) and some benzoates, in the form of Balsam of Peru or Flowers of Benzoin, were commonly used in the treatment of asthma in the eighteenth and nineteenth centuries.

The mechanism underlying the bronchodilator effect of methyl and propyl hydroxybenzoate remains obscure. It does not appear to involve cAMP-dependent processes (for example those activated via β-adrenoceptor agonists, phosphodiesterase inhibitors or relaxant prostanoids) since the level of this cyclic nucleotide (which is implicated in the relaxation process of smooth muscle, Rodger 1986) was not significantly altered by concentrations of the preservatives that produced maximum relaxation of the isolated airway preparations. This conclusion is confirmed by the finding that neither β-adrenoceptor blockade nor inhibition of cyclo-oxygenase modified the relaxant activity of the preservatives. In two recent experiments in which the levels of cGMP were also measured, there occurred no increase in the concentration of this nucleotide after full relaxation with the vehicle. It is unlikely that the preservatives acted as receptor antagonists at both histamine and methacholine receptors since, at similar concentrations, they also reversed contractions induced by KCl, which exerts its effect by directly depolarizing the smooth muscle cell membrane. The relaxant effect of the preservatives was readily reversed by addition of higher concentrations of spasmogen or by washing the tissues with fresh Krebs-Henseleit solution. These latter observations argue against a poisoning effect on cellular metabolism.

Methyl and propyl hydroxybenzoate are chemically similar to the local anaesthetics benzocaine, procaine and amethocaine, all of which are derived from *p*-amino benzoic acid. Indeed, methyl hydroxybenzoate has been reported to possess local anaesthetic activity (Nathan & Sears 1961; Mostow et al 1979). It is possible, therefore, that the bronchodilator activity of methyl and propyl hydroxybenzoate is attributable to a local anaesthetic/membrane stabilizing effect which could act to inhibit the transmembrane influx of Ca^{2+} ions that is essential to maintain contraction. This possibility remains to be tested.

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