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Inhibitory effect of capsaicin on cholinergic transmission in ovine airways: evidence for non-cholinergic contractions

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Abstract

Electrical stimulation of ovine trachealis smooth muscle and bronchial ring segments induced neurogenic and monophasic atropine-sensitive contractions. Pretreatment of the tissues with capsaicin (100 μM) significantly reduced these contractions indicating a possible contribution of a peptidergic neurotransmitter to the contractions. The effect of capsaicin on electrically induced contractions was significantly inhibited by capsazepin indicating an action on vanilloid receptors. In both preparations, electrically induced contractions were not modified by tachykinin NK_1- and NK_2-receptor antagonists singly and in combination. It was therefore concluded that a component of the atropine-sensitive electrically induced contractions of ovine airways smooth muscles involved the release of a peptide neurotransmitter which is probably not a tachykinin. However, an action of capsaicin on prejunctional vanilloid receptors located on cholinergic nerves cannot be ruled out.

Keywords: Tachykinin receptor; Ovine airway; Neurotransmission; Vanilloid receptor; Capsaicin

1. Introduction

Previous studies have shown that excitatory responses to electrical-field stimulation (EFS) in airway smooth muscles are predominantly cholinergic in origin. (Coburn and Tomita, 1973; Davis et al., 1982; Taylor et al., 1984). In some species however, there is evidence for non-adrenergic non-cholinergic (NANC) contractile responses (Lundberg and Saria, 1982; Lundberg et al., 1983; Matera et al., 1997). These excitatory NANC responses are possibly mediated via the release of substance P (SP) and tachykinins from airway sensory nerves, since these responses could be abolished by pretreatment with capsaicin to deplete neuropeptides from unmyelinated sensory nerves (Jancso et al., 1967) and also by tachykinin antagonists (Lundberg et al., 1983). We, and others (Sheller and Brigham, 1982; Mustafa et al., 1999), have shown that EFS of ovine airways smooth muscles produced atropine-sensitive contractions confirming cholinergic origin of these responses. When the tissues were precontracted with 5-hydroxytryptamine (5-HT) in the presence of atropine, EFS induced frequency-dependent tetrodotoxin-sensitive relaxation. In the trachea, this relaxation response involved a combination of peptidergic and adrenergic components (Mustafa et al., 1999). The neurotransmitter mediating relaxation in the bronchioles is yet to be identified. In all cases, the relaxation responses to electrical stimulation were always preceded by initial contractions (Mustafa, 1996; Mustafa et al., 1999) which were frequency-dependent. We concluded that these contractile responses, which were obtained in the presence of atropine, probably involved a neuronal release of NANC excitatory neurotransmitter(s). Since these NANC contractions were only demonstrable in the presence of other spasmogens, we hypothesized that a component of the cholinergically mediated electrically induced contractions could be due to an excitatory NANC neurotransmitter co-released during stimulation. The present studies were therefore designed to test this hypothesis.

2. Materials and methods

The trachea together with the lung of Merino sheep were obtained from the slaughter house, placed in Krebs'

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solution and transported to the laboratory within 30 min. The experimental procedure for setting up the tissue was similar to that used in previous studies (Mustafa et al., 1994). A piece of the trachea was cleaned of adhering adipose and connective tissue and opened longitudinally through the cartilage rings diametrically opposite the trachealis muscle. Thereafter, it was pinned flat on a corkboard and strips of smooth muscle 10 mm in length and 5 mm in width were dissected free from the underlying cartilage. An incision was made in the parenchyma and small bronchioles (3–4 mm in diameter) were dissected out without damage to the epithelium and cut into 5-mm ring segments. Each muscle preparation was suspended in 20 ml organ baths containing Krebs' (NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2, KH_2PO_4 1.2 $NaHCO_3$ 26, glucose 11.1 mM: pH = 7.4) solution (37°C) gassed with a 95% O_2 and 5% CO_2 mixture. Isometric tension was recorded through Dynamometer, UFI transducers on a two-channel lectromed recorder MX216. The tracheal strips were stretched to predetermined optimal tension (2 g) and bronchiolar rings to 1 g and were allowed to equilibrate for 60 min, during which time they were washed twice before adding agonists. Agonists were added cumulatively to the bath and allowed to equilibrate with the tissue until a steady response was obtained.

2.1. Electrical stimulation

For electrical stimulation, tissues were suspended between two platinum plate electrodes (bronchial ring segments) or passed through a pair of platinum ring electrodes (tracheal strips). The electrodes were connected to a Grass S8800 stimulator, delivering square wave pulses. The tissues were stimulated using optimum parameters, (50 V, 0.5 ms) previously determined by us (Mustafa et al., 1999). The tissues were stimulated for 10 s at frequencies ranging from 0.1 to 40 Hz.

In some experiments, the preparations were contracted with 5-HT (10 μM) before stimulation. This concentration of 5-HT produced approximately 50% of the maximal response of the preparations to this agonist. In this series of experiments, atropine (1 μM) was added to the Krebs' solution to block cholinergically mediated responses.

2.2. Effect of capsaicin on electrically induced contractions

In order to determine whether a capsaicin-sensitive peptide contributed to the electrically evoked contractions, the following experiments were performed. After establishing control responses to electrical stimulation, capsaicin (100 μM) was added to the bath and allowed to be in contact with the tissues for 20 min after which the tissues were washed and allowed to equilibrate for 20 min before stimulation.

2.3. Effect of tachykinin antagonists on electrically induced contractions

In order to determine whether tachykinins contribute to the electrically induced contractions, the effects of selective tachykinin receptor antagonists on the contractile responses were examined. Specifically, electrically induced contractions were obtained in the absence and also in the presence of the appropriate concentrations of the antagonists. In all cases, the antagonists were allowed to equilibrate with the tissues for 30 min before stimulation.

2.4. Drugs

These included atropine sulphate, 5-HT creatinine sulphate, (−)-propranolol hydrochloride, capsaicin, carbamoylcholine chloride and SP, all purchased from Sigma (St. Louis, MO, USA). Phentolamine mesylate was obtained from Ciba. Capsazepin, L-659,877 (a peptide), L-703,606 (*cis*-2-(diphenylmethyl)-*N*-[(2-iodophenyl)methyl]-1-azabicyclo[2,2,2]octan-3-amine) oxalate were obtained from RBI. Capsaicin and L-703,606 were dissolved in ethanol while capsazepin was dissolved in methanol. All other drugs were dissolved in distilled water.

2.5. Analysis of results

Data are presented as mean ± S.E. of (n) experiments. Where necessary, differences between mean values were compared using paired or unpaired Student's t-test as appropriate. Where multiple comparisons were necessary one-way analysis of variance (ANOVA) was used followed by Student–Newman–Keuls test. The difference was assumed to be significant where $P < 0.05$.

3. Results

3.1. EFS-induced contraction

EFS (0.1–40 Hz) of the tracheal and bronchiolar preparations elicited frequency-dependent contractions. The contractions were rapid in onset and stopped immediately when stimulation ceased, Fig. 1. In agreement with our previous observations (Mustafa et al., 1999), these contractions were abolished by tetrodotoxin (1 μM, $n = 8$, data not shown) confirming that they were neurogenically mediated. EFS-induced contractions were also abolished by atropine (1 μM) in the two preparations (Fig. 1), also confirming that the excitatory innervation of ovine airway smooth muscle was cholinergic in origin.

In preparations contracted with 5-HT in the presence of atropine (1 μM), EFS-induced frequency-dependent relaxation (Fig. 2). These relaxation responses were preceded by initial contractions that were frequency-dependent.

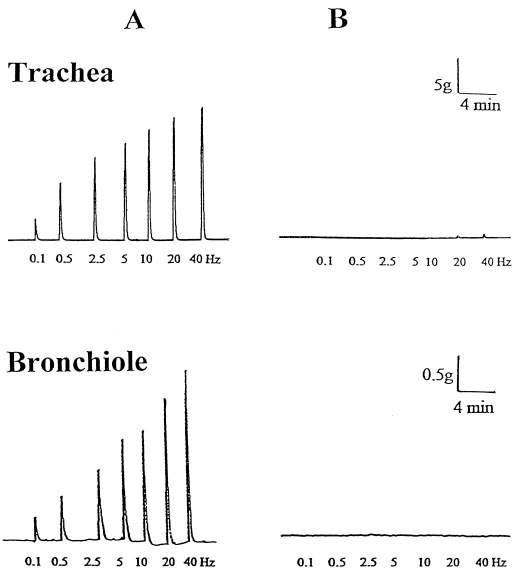

Fig. 1. A typical trace showing frequency-dependent contractions of ovine tracheal strip (A) and bronchial rings (B) to electrical stimulation. The tissues were stimulated at various frequencies for 10 s using square wave pulses (50 V, 0.5 ms).

3.2. Effect of treatment with capsaicin on EFS-induced contractions

Capsaicin treatment alone had no effect on the resting tone of the preparations. However, it significantly reduced electrically induced contractions in both tracheal strips and bronchiolar segments (Fig. 3). Capsaicin treatment reduced the responsiveness to electrical stimulation at all frequencies and, in tissues precontracted with 5-HT, abolished the initial contractions preceding the relaxation phase. In another series of experiments, the preparations were stimulated in the presence of capsaicin. The results showed that there was a greater reduction in electrically induced contractions, especially at the higher frequencies of stimulation, when the tissues were stimulated in the presence of capsaicin than when capsaicin treatment was followed by washing (Fig. 3). Capsaicin (100 μM) had no effect on carbachol-induced contractions indicating a prejunctional site of action.

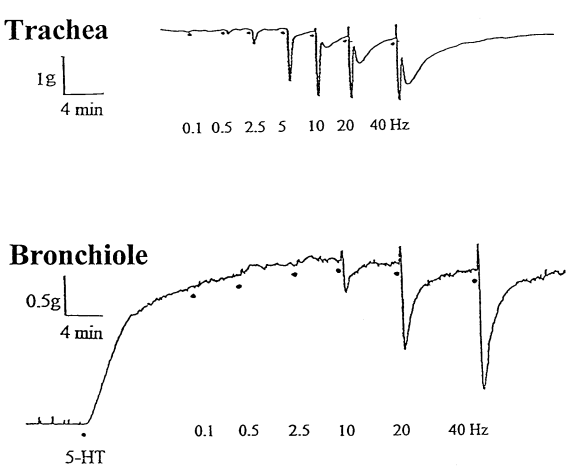

Fig. 2. A typical trace showing dual response of 5-HT (10 μM)-precontracted ovine tracheal strips and bronchial ring segments to electrical stimulation. Note that the relaxation was preceded by an initial contraction. Atropine (1 μM) was present in the Krebs' solution throughout the experiment.

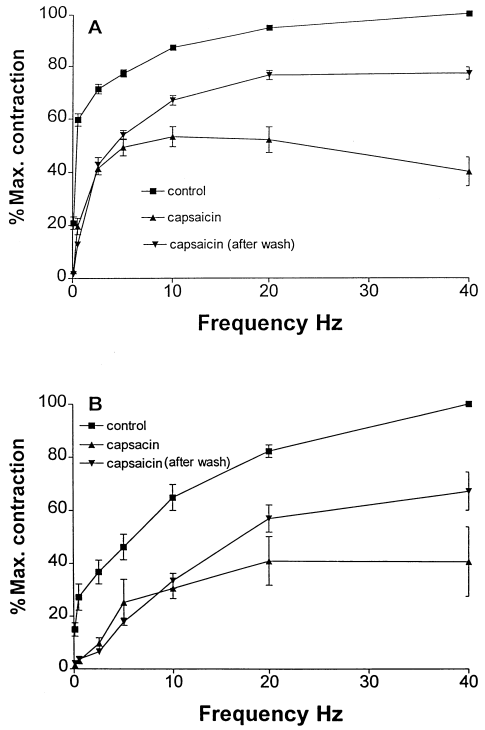

Fig. 3. Effect of pretreatment with capsaicin on cholinergic neurotransmission in ovine tracheal strip (A) and bronchial ring segments (B). The stimulation parameters are as shown in the legend to Fig. 1.

3.3. Effect of SP on basal tone and its effect on EFS-induced contractions

SP produced concentration-dependent contractions in the ovine trachea and bronchiole. The threshold concentration of SP required to evoke a contraction was 1 nM. SP (100 nM) did not increase the contractile responses to EFS (Fig. 4). Increasing the concentration of SP to 1 μM also failed to enhance EFS-induced contractions (data not shown).

3.4. Effect of neurokinin antagonists on EFS-induced contractions

L-659,877, a selective tachykinin NK_2 receptor antagonist, (1 μM) did not affect the contractile responses in the trachea or the bronchiole. It also did not affect the NANC contractions preceding the relaxation phase (Fig. 5). L-

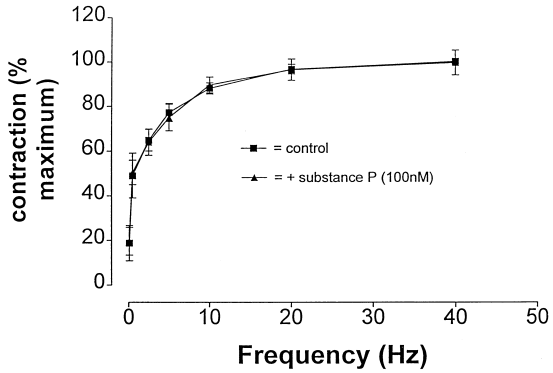

Fig. 4. Effect of SP (100 nM) on electrically induced contractions of the ovine trachealis smooth muscle. Each point is the mean ± S.E. of four experiments.

703,606, a selective tachykinin NK_1 receptor antagonist (1 μM), similarly did not affect the contractile responses in the trachea or the bronchiole, and also did not affect the initial contractions preceding the relaxation phase (data not shown). A combination of these two antagonists had no effect on EFS-induced contractions, either at basal tension or after precontraction with 5-HT (i.e., no effect on the initial contractions preceding relaxation).

3.5. Effect of capsazepin on the inhibitory effect of capsaicin

Capsazepin, a vanilloid receptor antagonist, at concentrations up to 30 μM had no effect on electrically induced contractions of the trachea strips and bronchial segments.

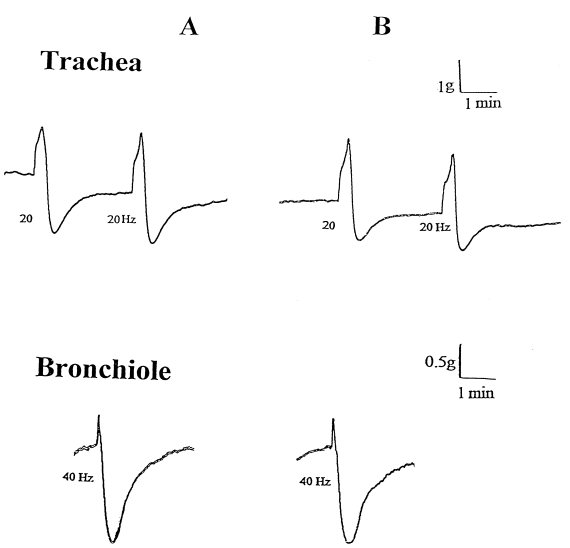

Fig. 5. A typical trace showing non-cholinergic contractile responses to electrical stimulation in the trachea strips and bronchial rings in the absence (A) and presence (B) of L-659,877 (1 μM), a selective tachykinin NK_2 receptor antagonist. The tissues were precontracted with 5-HT (10 μM) in the presence of atropine (1 μM) to block cholinergic contractions.

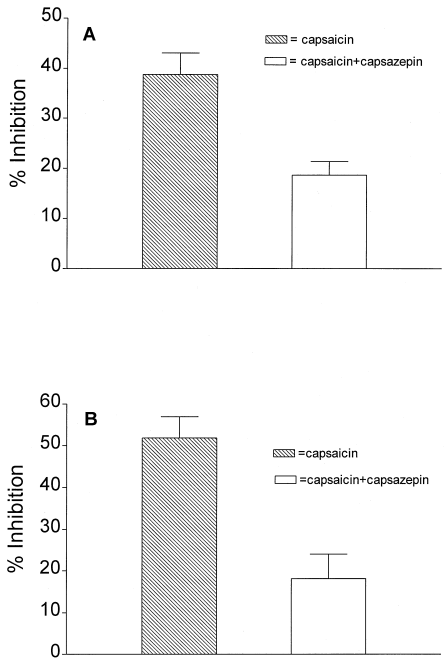

Fig. 6. Effect of capsazepine (30 μM) on capsaicin-induced inhibition of cholinergic contractions in ovine trachea strips (A) and bronchial (B) ring segments ($n = 4$).

However, as shown in Fig. 6, capsazepin (30 μM) significantly ($P < 0.05$) reduced the inhibitory effect of capsaicin on electrically induced contractions.

4. Discussions

In this study, we have confirmed that electrically induced neurogenic contractions of ovine airway smooth muscles are cholinergic in origin since they were abolished by atropine (Sheller and Brigham, 1982; Mustafa et al., 1999). We have also confirmed that precontraction of the preparations with 5-HT in the presence of atropine unmasked a non-cholinergic contraction. Our intention in this study was to determine if a component of the electrically induced contractions involved activation of this NANC contractile mechanism. Since we have shown that capsaicin abolished the NANC contractions (Mustafa, 1996; Mustafa et al., 1999), indicating the involvement of a peptide as the neurotransmitter, our approach was to examine the effect of peptide depletion with capsaicin on electrically induced contractions of the airways smooth muscle preparations. The rationale was that pretreatment of the tissues with capsaicin would reduce the response to electrical stimulation if a component of the response was due to activation of excitatory NANC nerves.

Our results showed that capsaicin significantly reduced the contractile responses to electrical stimulation in both preparations, indicating the involvement of capsaicin-sensitive excitatory NANC nerves in these responses. A similar observation has been made in guinea pig trachea by Stret-

ton et al. (1989) who showed that capsaicin pretreatment, to deplete sensory nerves of tachykinins, significantly reduced cholinergic responses in vivo and in vitro. It is now known that capsaicin exerts its pharmacological actions by activating specific receptors called vanilloid receptors. Upon binding to this receptor, capsaicin opens a cation conductance which is permeable to both divalent and monovalent cations. The resulting cation influx leads to impulse generation and to a release of neuromediators (Szolcsanyi, 1990). These receptors are selectively and competitively antagonized by capsazepine (Bevan et al., 1992; Szallasi, 1994). Therefore, our observation in this study that capsazepin attenuated the inhibitory effect of capsaicin would confirm that capsaicin produced its effects by activating vanilloid receptors.

It is well-known that capsaicin-sensitive primary afferent neurones synthesize, store and release a variety of neuropeptides including tachykinins SP, neurokinins A and B. Sensory neurones containing these neuropeptides have been localized to the airways of several species including man (Lundberg et al., 1984; Martling 1987). Subthreshold concentrations of the tachykinins have been shown to potentiate cholinergic neurotransmission in the rabbit (Armour et al., 1991) and guinea pig (Tanaka and Grunstein, 1986; Hall et al., 1989; Belvisi et al., 1994) leading to the suggestion that these neuropeptides could play a neuromodulatory role in the airways. Pharmacological actions of the tachykinins are mediated via tachykinin NK_1-, NK_2- and NK_3-recptors. There seems to be a species variation in tachykinin receptor subtype mediating contractions in airways' smooth muscles. For example, tachykinin NK_1 subtype mediates contractions in the rat (Joos et al., 1994) and rabbit (John et al., 1993) while in the guinea pig, tachykinin-induced contractions are mediated via tachykinin NK_1 and NK_2 receptors. However, only tachykinin NK_2 receptors are involved in the non-cholinergic response to electrical stimulation (Hall et al., 1989; Kamikawa and Shimo, 1993; Girard et al., 1997; Heavey et al., 1997). Tachykinin NK_3 receptors do not appear to be involved in tachykinin-induced contractions in any species. Recent studies, using rank order of agonist potencies as the criterion, have shown that tachykinin NK_2 receptors mediate tachykinin-induced contractions in ovine trachea (Reynolds et al., 1998). We therefore performed experiments to determine if tachykinins could account for the capsaicin-sensitive component of the neurogenic cholinergic responses of the ovine airway smooth muscles to electrical stimulation. Our results showed that SP produced concentration-dependent contractions of the tracheal strips and bronchial rings. The range of SP concentrations used in this study was similar to those used by Reynolds et al. (1998) confirming the presence of tachykinin receptors in these preparations. However, contrary to the observation that SP enhanced cholinergic responses in guinea pig (Hall et al., 1989; Belvisi et al., 1994) and rabbit airways (John et al., 1993), a subthreshold concentration of SP did not enhance electrically induced responses in either the tracheal strips or bronchial ring segments. Since no enhancement was observed even when the concentration of SP was increased to 100 nM, it seems unlikely that SP or a related tachykinin could modulate cholinergic responses in these preparations. This is further supported by results obtained using selective tachykinin receptor antagonists. Our results showed that neither the selective tachykinin NK_1 (L-703,606) nor tachykinin NK_2 (L-659,877) receptor antagonists singly and in combination reduced electrically induced contractions in both the tracheal strip and bronchial ring segments. Also, none of these tachykinin NK_1 and NK_2 receptor antagonists affected the non-cholinergic contractions preceding relaxation in these preparations suggesting that activation of these receptors did not contribute to the cholinergic contractions. It is unlikely that inclusion of a neutral peptidase inhibitor to the physiological solution would alter the outcome of this investigation. If anything, the endopeptidase inhibitor, by preventing the breakdown of the peptides, would allow the peptides to accumulate and thus reduce the efficacy of the antagonists. Belvisi et al. (1994) had previously shown that neurokinin receptor antagonists, at concentrations that prevented enhancement of cholinergic contractions induced by SP, did not affect cholinergic neurotransmission in the guinea pig bronchial rings suggesting that endogenous tachykinins do not modulate cholinergic neurotransmission in the guinea pig trachea.

In conclusion, these data confirm the existence of NANC excitatory nerves in ovine trachea and bronchioles. The fact that SP did not enhance electrically induced contractions, coupled with the failure of tachykinin receptor antagonists to modify the contractions would indicate that the neurotransmitter elaborated by these nerves is not likely to be SP or a related peptide. We cannot, however, rule out the possibility that the inhibitory effect of capsaicin on cholinergic contractions could be due to an irreversible inhibition of neurotransmitter release mediated via prejunctional vanilloid receptors located on cholinergic nerves in these preparations.

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