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Endothelin-induced contractions of tracheal smooth muscle and identification of specific endothelin binding sites in the trachea of the rat

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1 The presence of specific binding sites and the contractile activity of the novel peptide, endothelin have been investigated in rat trachea.

2 Endothelin (10^{-8}–10^{-5} M) induced long-lasting contraction of rat tracheal rings superfused with Krebs solution (EC_{50} 5.4×10^{-6} M). Contractions of the tissue to 10^{-6} M endothelin were attenuated in Ca^{2+}-free medium containing 0.1 mM EGTA but unaffected by nicardipine (10^{-7} M).

3 After equilibration in Ca^{2+}-free medium (without EGTA) a return to normal Ca^{2+} concentrations (2.5 mM), 30 min or 60 min following endothelin (10^{-6} M), produced a sustained contraction of the tissue.

4 Specific binding sites for endothelin were identified on rat tracheal smooth muscle (K_D 1.34×10^{-10} M, maximal binding 1.2 fmol mm^{-2}). Specific binding sites were also identified on nerve trunks. Endothelin binding was unaffected by co-incubation with nicardipine (10^{-7} M) or verapamil (10^{-7} M).

5 The discrepancy between the apparent K_D for endothelin binding and the EC_{50} for endothelin-induced contraction suggests that the endothelin binding sites identified in this study may not be associated with the receptors mediating contraction.

6 These results indicate that endothelin binding sites are present on tracheal smooth muscle. The mechanism of endothelin-induced contraction, whilst being dependent on extracellular calcium, does not appear to involve binding to the dihydropyridine- or verapamil-sensitive sites on the voltage-dependent Ca^{2+} channel. Its long duration of action may be associated with a sustained increase in Ca^{2+} permeability.

Introduction

Yanagisawa *et al.* (1988) have recently described a potent vasoconstrictor peptide, endothelin, isolated from cultured endothelial cells that elicits sustained increases in arterial pressure. Endothelin is a 21 amino acid peptide that has a regional homology with some neurotoxins which act on membrane ion channels. The action of endothelin on isolated vasculature has been shown to be dependant on extracellular Ca^{2+}, unaffected by antagonists of other vasoconstrictors and to be attenuated by nicardipine (Yanagisawa *et al.*, 1988), which lead to the hypothesis that this compound might be an endogenous ligand of dihydropyridine-sensitive, voltage-dependent Ca^{2+} channels. Since the calcium agonist Bay K 8644 elicits contractions of airway smooth muscle (Advenier *et al.*, 1986), we have examined the possibility that endothelin may also be an endogenous agonist of Ca^{2+} channels in airway smooth muscle and elicit sustained increases in airway tone.

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Methods

Effects on smooth muscle

Male Wistar rats (150–350 g) were stunned by a blow to the head and killed by cervical dislocation. The

trachea was removed, placed in Krebs solution and adherent fat and connective tissue removed. Rings consisting of 3 adjacent cartilage plates were suspended under a resting tension of 1 g and superfused with Krebs solution (composition in mM: NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 5.7) at 2 ml min^{-1}. The solution was pre-gassed with 95% O_2, 5% CO_2 and superfused the tissues at 37°C. Two tissues were used from each animal and were pre-equilibrated for 60 min. The resting tension at the end of this period was 0.9 ± 0.03 g. Agonists were added in the superfusing buffer and were in contact with the tissue for 30 s (60 s in the case of KCl).

In all tissues contractile responses to methacholine were established before examination of endothelin responses. Responses to methacholine or KCl were determined to single additions of the drug and complete recovery to resting tensions was allowed between concentrations. Cumulative concentration-response curves to endothelin were obtained by incremental increasing concentrations at the plateau of the previous response. Contractions of tracheal smooth muscle by endothelin were compared to those of rings of rat aorta under the conditions described for the tracheal rings. In parallel experiments, where the effects of nicardipine (10^{-7} M) on endothelin responses were investigated, nicardipine was included in the superfusate and the tissues were equilibrated with the antagonist for 30 min before endothelin was introduced.

In another series of experiments, following the initial equilibration, responses to methacholine 10^{-6} M and 50 mM KCl were determined. The tissues were then incubated in Ca^{2+}-free Krebs containing 0.1 mM EGTA for 15 min. Responses to methacholine and KCl were repeated and the contractile responses to endothelin 10^{-6} M established. The results of these experiments were compared to those of time-matched controls superfused with normal Krebs solution. In another study following a 60 min preincubation in nominally Ca^{2+}-free medium (i.e. without EGTA), the effect of adding 2.5 mM Ca^{2+} back to the superfusing medium 30 or 60 min after addition of endothelin was determined.

In vitro *autoradiography*

Adult male Wistar rats ($n = 15$) were killed by decapitation, the trachea was rapidly removed, tissue blocks prepared by snap freezing in melting Arcton (dichlorodifluoromethane) and stored under liquid nitrogen until required. From each animal consecutive cryostat sections were cut at a thickness of 10 μm and mounted onto acid alcohol washed chrome alum scrubbed slides. All of the sections were pre-incubated in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 5 mM $MgCl_2$, 40 mg ml^{-1} Bacitracin and 0.1% bovine serum albumin (w/v) for 15 min at room temperature. Following this some sections ($n = 4$) were incubated, alone, in the same buffer with [^{125}I]-endothelin (25 pM–2.5 nM; specific activity 1145 Ci $mmol^{-1}$) for 15 min at room temperature. To assess non-specific binding, additional serial sections from each animal were co-incubated with radiolabelled peptide (200 pM) and an excess of unlabelled endothelin (10^{-6} M). For competition experiments serial sections from each animal were co-incubated with radiolabelled peptide (200 pM) and other vasoactive but unrelated peptides (CGRP, VIP, ANP, gastrin all 10^{-6} M), the calcium antagonists nicardipine (10^{-7} M) and verapamil (10^{-7} M), and the K^+ channel blocking agents 4-aminopyridine (5 mM) and tetraethylammonium (30 mM) for 30 min at room temperature. The incubation was terminated by washing (2 × 5 min) in cold buffer (4°C) followed by one rinse in ice-cold distilled water, after which all sections were dried rapidly under a stream of cold air. Autoradiographs were generated by exposing labelled tissue sections and the polymer based ^{125}I standards (10 μm thick) to Hyperfilm-^{3}H for 4 days at 4°C. From the subsequent autoradiograms, binding sites on smooth muscle were analysed by IBAS 2000 computer assisted image analysis. Grey values of the radioactive standards were measured and a standard curve produced and stored on disc. The grey values of the tissue images were then measured by means of this standard curve and converted to the amount of ^{125}I-bound per unit surface area of tissue component measured. Smooth muscle and nerve trunks were identified in serial sections counterstained with haemotoxylin and eosin.

Drugs

Endothelin (Scientific Marketing, London, U.K.); acetyl-β-methylcholine bromide, 4-aminopyridine, bovine serum albumin (BSA), ethylene glycol-tetraacetic acid (EGTA), nicardipine hydrochloride, tetraethylammonium chloride, verapamil hydrochloride, (Sigma, Poole, Dorset, U.K.); calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), atrial naturetic peptide (ANP), gastrin (Peninsula Laboratories, St Helens, U.K.); Hyperfilm-^{3}H (Amersham International, Aylesbury, U.K.).

Statistics

Results are expressed as mean ± s.e.mean. Values were compared by means of a two-tailed Mann-Whitney U test for unpaired observations. Log EC_{50}

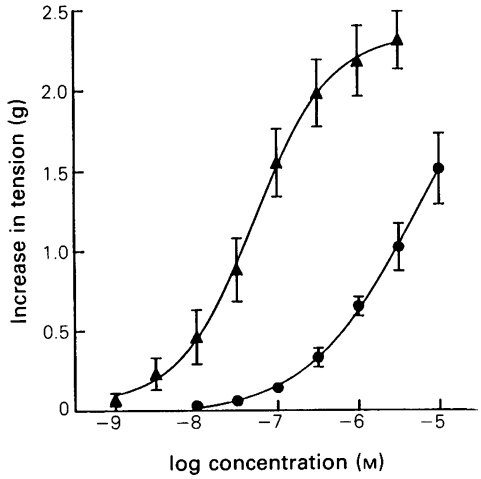

Figure 1 Concentration-response curves for endothelin on rat aorta (▲) or trachea (●) superfused with Krebs solution at $2\,ml\,min^{-1}$ and under a resting tension of $0.9 \pm 0.03\,g$. Results are expressed as the increase in tension (g) over resting and are a minimum of 6 observations. Vertical lines show s.e.mean.

values were calculated from the mean data following fitting to a sigmoid curve; K_D and maximal binding capacity were calculated by Scatchard analysis of the binding data. (GraphPad, ISI Software).

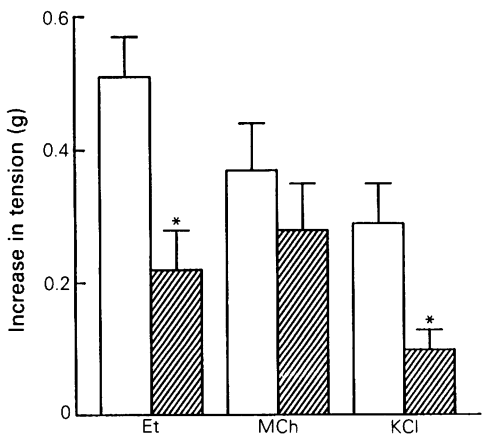

Figure 2 The response of rat trachea to 10^{-6} M endothelin (Et) after 15 min incubation in calcium free medium containing 0.1 mM EGTA compared to time matched controls. Also shown are the responses to methacholine (MCh 10^{-6} M) and KCl (50 mM) in normal medium and following 15 min incubation in calcium-free medium containing 0.1 mM EGTA. Open columns: responses in normal medium and hatched columns: responses in calcium-free medium containing 0.1 mM EGTA. $^*P < 0.05$ $n = 5$. Vertical bars show s.e.mean.

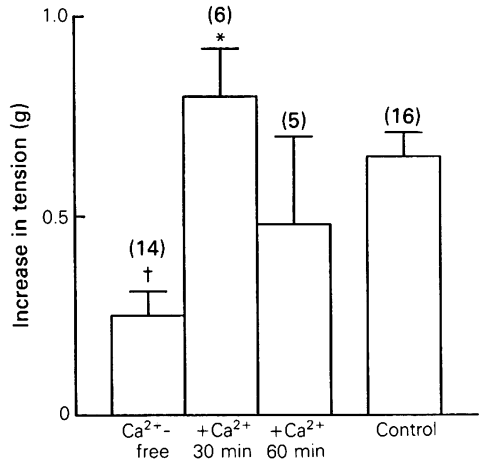

Figure 3 Endothelin (10^{-6} M) induced increases in tension of rat tracheal rings after 1 h incubation in calcium-free medium and the subsequent increases in tracheal tension following return to 2.5 mM calcium 30 or 60 min post endothelin. $^*P < 0.05$; ($\dagger P < 0.05$ compared to responses to endothelin in normal Krebs solution) (Mann-Whitney U test). Values in parentheses are the number of observations.

Results

In Krebs solution with normal Ca^{2+} concentrations, endothelin (10^{-8}–10^{-5} M) produced a concentration-dependent contraction of rat trachea and had a similar potency to methacholine (log EC_{50} − 5.27 and −5.89 respectively, $n \geqslant 5$). Concentrations of endothelin sufficient to produce a maximum contractile response were not achieved due to limited availability of the peptide. However, the maximal effect of endothelin 10^{-5} M (1.51 ± 0.22 g, $n = 6$) was not different from the maximum contraction to methacholine 3×10^{-5} M (1.24 ± 0.24, $n = 6$). Under the same conditions endothelin (10^{-9}–10^{-6} M) was 100 times more potent in producing contraction of rat aortic rings (log EC_{50} − 7.27 ($n \geqslant 6$) compared to −5.27 in trachea) (Figure 1). Endothelin-induced contraction of rat trachea was long lasting, responses to 10^{-6} M taking 5.3 ± 1.4 min to achieve a plateau and 57.0 ± 7.9 min to return to resting tensions ($n = 8$).

In Ca^{2+}-free solutions containing 0.1 mM EGTA responses to endothelin 10^{-6} M were reduced by 67% ($P < 0.03$) and those to 50 mM KCl by 76% ($P < 0.05$), the response to methacholine 10^{-6} M was not significantly affected ($n = 5$; Figure 2). Under these conditions the duration of action of endothelin was also reduced to 17 ± 6 min; at equivalent times,

contractions of tissues in Krebs with normal calcium concentrations were 75.6 ± 14% of their maximum levels ($n = 5$).

Thirty minutes after addition of endothelin (10^{-6} M) to tissues superfused with nominally Ca^{2+}-free medium for 60 min, changing the superfusate to Krebs containing 2.5 mM Ca^{2+} produced a sustained contraction of the tissue which was the same as the response to endothelin in normal medium but which was significantly greater than that following 60 min pre-incubation in Ca^{2+}-free medium, ($P < 0.05$, $n = 6$) (Figure 3). Return to normal Ca^{2+} concentrations 60 min after addition of endothelin (10^{-6} M) also elicited contraction of the tissues ($n = 5$) but the effect was smaller than those seen at 30 min post endothelin. In tissues that had not been exposed to endothelin return to normal Ca^{2+} concentrations had no effect on their resting tone. The contractile effects of endothelin (10^{-7}–3×10^{-6} M) were unaffected by nicardipine 10^{-7} M compared to time matched controls ($n = 4$; Figure 4).

In vitro *autoradiography*

Specific high affinity binding sites were identified in the smooth muscle of the trachea and on nerve trunks in the adventitia (Figure 5). These structures were identified in labelled tissue sections which were post-fixed in 10% formalin and counterstained with haematoxylin and eosin. Binding was rapid and reached equilibrium within 15 min ($n = 4$). The binding was specific; almost 100% of the total binding was displaced by coincubation with unlabelled endothelin (10^{-6} M). Scatchard analysis of the binding data suggested that there is a single class of binding site to smooth muscle (dissociation constant (K_D) 1.34×10^{-10} M, maximum binding 1.2 fmol mm^{-2}). However, in view of the scatter ($r = 0.89$) a second lower affinity site may be present. Endothelin binding was unaffected by co-incubation with CGRP, VIP, ANP or gastrin, the K^+ channel blocking agents tetraethylammonium and 4-aminopyridine or the Ca^{2+} antagonists nicardipine and verapamil. Non-displaceable binding to the respiratory epithelium was seen.

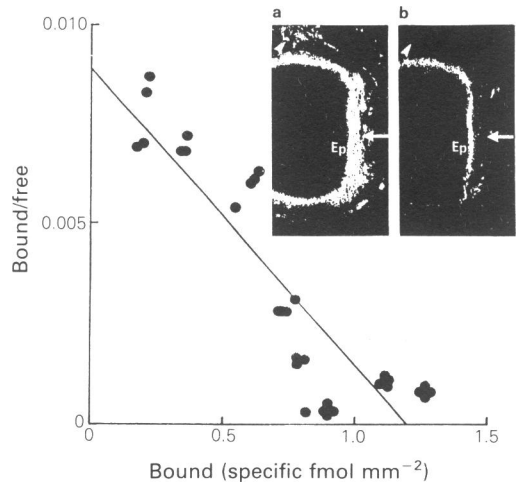

Figure 5 [^{125}I]-endothelin binding in the absence (a) and presence (b) of a 1000 fold excess of unlabelled endothelin. Specific binding is seen on the smooth muscle (arrow) and nerve trunks (arrowheads). Non-specific binding to the epithelium (Ep) was also seen. The scatchard plot of [^{125}I-endothelin (25–2500 pM) binding to 10 μm sections of rat trachea suggested a specific endothelin binding site with an apparent K_D of 1.34×10^{-10} M. Each point is the mean of 3 observations taken from serial sections from a total of 4 animals.

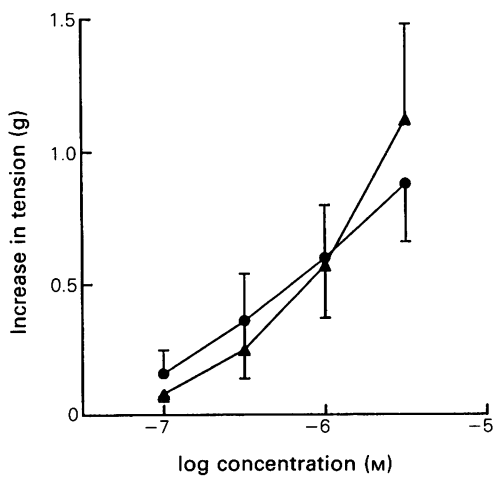

Figure 4 Effect of 10^{-7} M nicardipine (▲) on the increase in tension of rat tracheal rings elicited by endothelin. Nicardipine was present in the superfusate and the tissues were pre-incubated for 30 min before the addition of endothelin. (●) control responses to endothelin ($n = 4$). Vertical lines show s.e.mean.

Discussion

Calcium ions are essential for contraction of smooth muscle, calcium in the extracellular medium

gaining entry to the cell following the opening of specific ion channels. Of the two types of channel that have been proposed, voltage-dependent calcium channels have a calcium permeability that is linked to the transmembrane potential difference (Rodger, 1985; 1988; Daniel, 1988), and are specifically inhibited by dihydropyridine derivatives such as nifedipine and nicardipine (Advenier *et al.*, 1984). Endothelin, a potent vasoconstrictor (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988; Hirata *et al.*, 1988) has recently been suggested as a putative endogenous ligand for the dihydropyridine-sensitive calcium channels in vascular smooth muscle (Yanagisawa *et al.*, 1988).

Yanagisawa *et al.* (1988) have shown that preproendothelin mRNA is not expressed in porcine lung, suggesting that little endothelin is produced by the pulmonary microvasculature. We, however, have demonstrated that there are specific binding sites for this ligand in the rat trachea and that these are associated predominantly with the smooth muscle and nerve trunks. Furthermore, endothelin elicits contractions of tracheal smooth muscle, with a potency similar to that of substance P (on guinea-pig trachea), but 1–2 orders of magnitude less potent than neurokinins A or B (Devillier *et al.*, 1988). Contraction of the tissue to endothelin, however, is long lasting, persisting for up to 60 min at 10^{-6} M, even though the tissue was constantly washed by superfusion, which suggests that bound endothelin might be resistant to dissociation from its binding sites.

Scatchard analysis of the binding data gave an apparent dissociation constant in the order of 10^{-10} M. In contrast our observed EC_{50} for contraction of tracheal smooth muscle is 5.4×10^{-6} M. This disparity between the K_D for binding and the EC_{50} may in part be related to loss of the peptide during the superfusion, although in 3 experiments we have determined that losses of ^{125}I endothelin through the superfusion apparatus are negligible. Hirata *et al.* (1988), have, similarly demonstrated a discrepancy between the K_D for endothelin binding to rat aortic smooth muscle ($2–4 \times 10^{-10}$ M) and concentrations of the peptide that increase intracellular calcium levels ($10^{-9}–10^{-7}$ M). Although in their study the concentration of endothelin eliciting a maximum response was not obtained, this concentration range is consistent with our observed EC_{50} for contraction of rat aorta (5.4×10^{-8} M). Whilst the nature of the superfusion may not result in equilibrium conditions, these observations do suggest that in the rat trachea 'specific' endothelin binding over the concentrations studied ($25 \times 10^{-12}–25 \times 10^{-10}$ M) may not be related to the receptors mediating the contractile response.

It has been suggested that the mechanism of action of contractile agonists such as acetylcholine, histamine and leukotriene D_4 may be independent of Ca^{2+} entry through either voltage-dependent or receptor coupled channels, but is related to the release of activator Ca^{2+} from intracellular sites, whilst those to KCl are dependent on extracellular calcium (Farley & Miles 1978; Rodger 1988). In the present study endothelin-induced contractions were attenuated in Ca^{2+}-free medium containing 0.1 mM EGTA and its duration of action was shortened. In the same tissues the contactile response to KCl was also reduced but that to methacholine was not significantly affected. These observations, therefore, are consistent with a requirement for extracellular calcium in the expression of the contractile effects of endothelin.

Although endothelin-induced contraction of the trachea was attenuated in Ca^{2+}-free medium (residual contractile activity perhaps being due to the availability of Ca^{2+} bound to cartilage; Raeburn & Rodger, 1984) addition of Ca^{2+} to the medium superfusing the tissues elicited a contraction of the tissue, which was dependent or prior exposure to endothelin. The sustained contractions of the tissue, therefore, may be related to the tight association of endothelin to its binding site resulting in prolonged Ca^{2+} channel opening. Furthermore, since a contractile response to Ca^{2+} addition could still be achieved 60 min post-endothelin, the mechanism of recovery to resting tensions (60 min at 10^{-6} M) in the presence of a continued increase in Ca^{2+} permeability remains uncertain.

Under identical experimental conditions, endothelin was 100 times more potent in eliciting contractions of rat aorta. This greater affinity of endothelin on vascular smooth muscle may indicate that the endothelin binding sites in the rat trachea are different from those in the rat aorta. Furthermore, unlike porcine coronary artery (Yanagisawa *et al.*, (1988) or guinea-pig trachea (Uchida *et al.*, 1988), contraction of the rat trachea was unaffected by the calcium antagonist nicardipine. Whilst, Hirata *et al.* (1988) have recently shown that endothelin binding to rat aorta is not affected by calcium channel blockers, in their studies the endothelin-induced increase in intracellular calcium was. We have shown in the rat trachea that the binding of endothelin to airway structures is unaffected by either nicardipine or verapamil and this, coupled with the absence of effect of nicardipine on the contractile activity of endothelin, suggests that, at least in the rat airway, endothelin is unlikely to be interacting with the dihydropyridine binding site.

Finally potassium channel blocking agents such as tetraethylammonium elicit contractions of smooth muscle through opening of voltage-dependent calcium channels (Allen *et al.*, 1986; Daniel, 1988). Our binding studies have indicated that endothelin

binding to tracheal smooth muscle is unaffected by either tetraethylammonium or 4-aminopyridine, thus specific endothelin binding is unlikely to be related to an interaction with K^+ channels.

In conclusion, specific endothelin binding sites have been identified in the rat airway and endothelin has been shown to elicit long-lasting contractions of tracheal smooth muscle, albeit less potent than as an agonist on vascular smooth muscle. However, its molecular site of action does not appear to be at the dihydropyridine binding site or at the K^+ channel. Its long duration of action may be associated with a sustained increase in Ca^{2+} permeability.

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