Mechanical and biochemical responses to endothelin-1 and endothelin-3 in bovine bronchial smooth muscle

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1 In this study, mechanical responses to endothelin-1 and endothelin-3 were examined in bovine bronchial smooth muscle. In addition, the involvement of phosphatidylinositol 4,5-bisphosphate hydrolysis (PIP₂) in the responses to these peptides was assessed by measurement of inositol (1,4,5) trisphosphate (I(1,4,5)P₃) production using a specific mass assay.

2 ET-1 evoked contractions of bovine bronchi which were concentration-dependent and initiated at between 10⁻⁹ M and 10⁻⁸ M. ET-1-evoked responses were unaffected by slight elevation of tone with potassium chloride (3 × 10⁻² M), methacholine (10⁻⁶ M) or U46619 (10⁻⁷ M).

3 Contractions to ET-1 were not altered by pre-incubation with atropine (10⁻⁵ M), indomethacin (10⁻⁵ M), nifedipine (10⁻⁵ M), phosphoramidon (3.67 × 10⁻⁵ M) or by removal of the epithelium.

4 ET-3 evoked small contractions which were not concentration-dependent. In the presence of phosphoramidon (3.67 × 10⁻⁵ M) however, concentration-dependent contractions were obtained to ET-3 which were unaffected by atropine (10⁻⁵ M) or by removal of the epithelium, but were significantly attenuated by indomethacin (10⁻⁵ M). Nifedipine (10⁻⁵ M) virtually abolished this response.

5 Both ET-1 and ET-3 (in the presence of phosphoramidon)-evoked contractions were significantly enhanced by the presence of the phorbol ester phorbol 12,13-dibutyrate (10⁻⁸ M). Neither ET-1-, nor ET-3-mediated responses were antagonized by the protein kinase C (PKC) inhibitor, Ro 31-8220 (3 × 10⁻⁹–3 × 10⁻⁸ M).

6 ET-1 (3 × 10⁻⁷ M) evoked a biphasic rise in levels of I(1,4,5)P₃ which was unaltered by pre-incubation with atropine, whilst ET-3 (10⁻¹⁰–3 × 10⁻⁷ M) failed to alter levels of I(1,4,5)P₃ at any time point examined, even in the presence of phosphoramidon (3.67 × 10⁻⁵ M).

7 These results suggest that, in bovine bronchial smooth muscle, ET-1 does not evoke contraction via cyclo-oxygenase metabolites, does not evoke release of the neurotransmitter substance acetylcholine, or require calcium influx via dihydropyridine-sensitive channels. ET-1 evokes I(1,4,5)P₃ production, but stimulation of protein kinase C may not be critical for the associated contraction. In contrast, ET-3-evoked contractions are partly mediated by cyclo-oxygenase metabolites. ET-3 does not stimulate PIP₂ hydrolysis, nor activate PKC, but may, either directly or as a requirement of intermediates released in response to ET-3, rely upon extracellular calcium.

Keywords: Endothelin-1; endothelin-3; phosphatidylinositol hydrolysis; bovine bronchi; protein kinase C

Introduction

Endothelin-1 (ET-1) is a potent vasoactive peptide produced from endothelial cells (Yanigisawa *et al.*, 1988). Other cells however, including porcine and canine tracheal epithelial cells (Black *et al.*, 1989) and human bronchial cells (Mattoli *et al.*, 1990) also produce ET-1. Although less potent in bronchial compared to vascular tissues, it is nevertheless the most potent bronchoconstrictor substance yet isolated, producing prolonged contractions in airways, both *in vivo* and *in vitro* (McKay *et al.*, 1992). In addition, acute pulmonary alveolar hypoxia increases lung ET-1 levels in conscious rats (Shirakami *et al.*, 1991), while elevated endothelin levels have been detected in patients with acute severe asthma (Nomura *et al.*, 1989). Accordingly, a role may exist for ET-1 in airway diseases, in particular in asthma, a condition characterized by hyperreactivity of airway smooth muscle.

The mechanisms by which ET-1 acts are incompletely understood. One possible pathway is the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂). This biochemical pathway is thought to be an important signal-transduction mechanism coupling receptor occupation and contraction in response to a variety of spasmogens in airway smooth muscle (e.g. cholinceptor

agonists, Grandordy *et al.*, 1986; histamine, van Amsterdam *et al.*, 1990).

The products of this hydrolytic reaction are the second messengers inositol (1,4,5) trisphosphate (I(1,4,5)P₃), which releases intracellular stored calcium (Burgess *et al.*, 1983) and diacylglycerol (DAG), which stimulates protein kinase C (PKC, Nishizuka, 1983). It has been suggested (Rasmussen & Barrett, 1984) that the two second messengers produced from PIP₂ hydrolysis may exhibit differences in the time course of their action. The I(1,4,5)P₃/Ca²⁺ system may be responsible for an initial transient response while the DAG/protein kinase C system underlies the sustained phase; the sum of these responses being a combined integrated response. Abnormal increases in intracellular calcium levels (Barnes *et al.*, 1992) or inappropriate activation of protein kinase C (Obanime *et al.*, 1989) may be important in asthma.

ET-1-stimulated PIP₂ hydrolysis has been demonstrated in vascular smooth muscle cells (Marsden *et al.*, 1989) and in human bronchial smooth muscle cells (Mattoli *et al.*, 1991). In these studies, however, measurement of PIP₂ hydrolysis was by assessment of accumulation of a number of inositol phosphates after incubation with [³H]-inositol. Interpretation of such data is complicated and, in particular, it does not give any indication of the degree to which inositol monophosphate levels are derived from I(1,4,5)P₃ rather than from

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other inositol polyphosphates or, indeed, from possible hydrolysis of phosphatidylinositol. More accurate results are obtained using specific assays for $I(1,4,5)P_3$, which has the additional advantage of permitting the use of fresh, unradio-labelled tissue.

In this present study, to assess the importance of endothelins in airway disease, we examined several aspects of the mechanical and biochemical responses evoked by ET-1 and its isoform endothelin-3 (ET-3) in bovine bronchial smooth muscle.

We have attempted, using mechanical measurements, to establish the underlying reasons for potency differences between bronchial and vascular tissues and to provide some evidence for the type of endothelin receptor present in these bronchi.

The importance of PIP_2 hydrolysis in bovine bronchial smooth muscle has been examined by assessment of changes in levels of $I(1,4,5)P_3$ in response to ET-1 and ET-3. In addition, evidence for the importance to the contraction of PKC has been obtained by manipulation of kinase activity with phorbol esters, which mimic DAG and accordingly stimulate PKC (Castagna *et al.*, 1982) and Ro 31-8220, which inhibits this kinase (compound 3, Davis *et al.*, 1989).

Methods

Tissue collection and preparation

Bovine bronchial tissues were obtained from cattle within 30 min of slaughter. Tissues were dissected free of connective tissue and fat in oxygenated Krebs-Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1.

Measurement of contractile responses

Contractile responses were measured from rings of bronchial smooth muscle (3–5 mm external diameter) in vertical organ baths (10 ml) at $37 \pm 0.5^\circ C$ in oxygenated (95% O_2 , 5% CO_2) Krebs-Henseleit solution. Resting tension (2 g) was applied by inserting two platinum wires into the lumen. One wire was anchored and the other attached to a force displacement transducer (Grass FT03T). Concentration-response curves (CRCs) were constructed to ET-1 and ET-3. Drugs were added directly to the organ bath and antagonists were added 15 min before agonists. Phorbol 12,13-dibutyrate was added 5 min prior to addition of the endothelins. Where appropriate, removal of the epithelium was achieved by gentle rubbing of the intimal surface.

Preparation of samples for $I(1,4,5)P_3$ assay

The effects of ET-1 and ET-3 on levels of $I(1,4,5)P_3$ were examined in bovine bronchial smooth muscle by a modification of the method of Palmer & Wakelam (1990). Small ring segments (3–5 mm external diameter) of bronchial tissue were placed in 0.9 ml fresh Krebs-Henseleit solution at $37 \pm 0.5^\circ C$, gassed with 95% O_2 , 5% CO_2 , pH 7.4 in 3.5 ml test tubes. After equilibrating for 40 min, tissues were stimulated with ET-1 or ET-3 (each 10^{-10} – 3×10^{-7} M) for the required time (5, 10, 20, 30, 60, 120 and 300 s) and the reaction stopped by addition of 0.1 ml ice-cold perchloric acid (PCA) 20% ($4^\circ C$). Preliminary experiments indicated that recovery with this technique was 79–84% ($n = 4$). In addition, preliminary results demonstrated that statistically similar results were obtained when samples were prepared by terminating the incubation in liquid nitrogen ($n = 6$). Antagonists, where appropriate were added 15 min before agonists. Experiments were conducted in duplicate. Tissues were homogenized (hand held glass homogenizer) in 2% PCA ($4^\circ C$) and a 200 μ l sample of homogenate taken for estimation of protein by the method of Peterson (1977). The remainder of

the homogenate was treated for removal of perchloric acid by addition of potassium hydroxide (1.5 M) in HEPES (60 mM) and a minimal amount of universal indicator. Samples of the treated homogenate were taken for assay of $I(1,4,5)P_3$ levels by the method of Palmer & Wakelam (1990).

Materials

The following chemicals were used; atropine sulphate (Sigma), endothelin-1 (Novabiochem), endothelin-3 (Novabiochem), D-*myo*-inositol (1,4,5) trisphosphate potassium salt (Calbiochem), indomethacin (Sigma) D-*myo*-[3H]-inositol (1,4,5) trisphosphate potassium salt (20–60 Ci mmol $^{-1}$), methacholine chloride (Sigma), nifedipine (Sigma), phorbol 12,13-dibutyrate (Sigma), N-(α -rhamnopyranosyloxyhydroxy phosphinyl)-L-leucyl-L-tryptophan (phosphoramidon, Sigma), potassium chloride (Analar), Ro 31-8220 (a gift from Roche Products Ltd. (UK)), U46619 ((15S)-hydroxy-11 α , 9 α -(epoxymethane) prosta-5z,13E-dienoic acid, a gift from Upjohn). Concentration in the text refers to the salts, with the exception of nifedipine and phorbol 12,13-dibutyrate which are expressed as the base. Stock solutions of drugs were prepared in distilled water except for indomethacin, nifedipine, phorbol 12,13-dibutyrate and Ro 31-8220 which were dissolved in ethanol. In each case, subsequent dilutions were made in Krebs-Henseleit solution. Where appropriate, control CRCs included an equivalent concentration of ethanol.

Analysis of results

Unfortunately, due to financial restraints, a maximum response to ET-1 could not be obtained and a pD_2 value could not be calculated. In this present study therefore, results have been expressed in terms of the negative log concentration of the $EC_{200\text{ mg}}$ ($-\log EC_{200\text{ mg}}$; the concentration effecting a 200 mg wt. contraction). This system of expressing results is similar to that used by MacLean *et al.* (1992).

Results are expressed as mean $\pm$ s.e.mean. Statistical significance between data samples was tested by two-way analysis of variance. Significance between $EC_{200\text{ mg}}$ values was calculated by Student's *t* test. A probability level of $P < 0.05$ was considered significant. Number of observations (*n*) refers to the number of animals used.

Results

Endothelin-1

Contractile-responses ET-1 evoked a concentration-dependent contraction of bovine bronchi (control $-\log EC_{200\text{ mg}}$; 7.05 ± 0.13 , $n = 12$). This compares with an equivalent $-\log EC_{200\text{ mg}}$ of 6.44 ± 0.4 for methacholine in the same tissue). Contraction was initiated at between 10^{-9} M and 10^{-8} M.

The contraction evoked by ET-1 in bovine bronchi was unaffected by pre-incubation with KCl (Figure 1a, 3×10^{-2} M), U46619 (Figure 1b, 10^{-7} M), or methacholine (Figure 1c, 10^{-6} M). Each of these agents evoked small (10–20% of own maximum contractions at the concentrations used; e.g. 10^{-6} M methacholine evokes a contraction of 0.29 ± 0.04 g, cf. maximum response 2.4 ± 0.27 g, $n = 14$). The steady-state contraction evoked was considered to be the baseline for subsequent ET-1-evoked contractions. ET-1-evoked contractions were unaffected by atropine (Figure 1d, 10^{-5} M), indomethacin (Figure 2a, 10^{-5} M), nifedipine (Figure 2b, 10^{-5} M), phosphoramidon (Figure 2c, 3.67×10^{-5} M) or by removal of the epithelium (Figure 2d, $n = 6$ in each case). The phorbol ester, phorbol 12,13-dibutyrate (PDBu, 10^{-8} M), however evoked a significant leftward shift of the CRC ($P < 0.001$, $n = 6$), which was abolished by the presence of Ro 31-8220 (3×10^{-8} M, Figure 3a, $n = 6$), while Ro 31-8220 (Figure 3b, 3×10^{-8} M, $n = 6$) failed to alter significantly the response to ET-1.

Figure 1 Concentration-response curves to endothelin-1 (ET-1), alone (■) or in the presence of (a) potassium chloride (□, 3×10^{-2} M), (b) U46619 (□, 10^{-7} M), (c) methacholine (□, 10^{-6} M) or (d) atropine (□, 10^{-5} M), in bovine bronchial rings. None of these compounds evoked any significant changes in control responses to ET-1. Number of observations (n) = 6 in each case.

Figure 2 Concentration-response curves to endothelin-1 (ET-1), alone (■) or in the presence of (a) indomethacin (□, 10^{-5} M), (b) nifedipine (□, 10^{-5} M), (c) phosphoramidon (□, 3.57×10^{-5} M) or (d) after removal of the epithelium by gentle rubbing (□), in bovine bronchial rings. Indomethacin, nifedipine and phosphoramidon each failed to alter control responses to ET-1. Removal of the epithelium likewise failed to alter these responses to ET-1. Number of observations (n) = 6 in each case.

I(1,4,5)P₃ levels In bovine bronchial smooth muscle, ET-1 evoked a biphasic rise in I(1,4,5)P₃ levels (Figure 4). There was an initial rise, which was maximal at around 10 s, followed by a second rise peaking at 60 s. In response to 3×10^{-7} M ET-1, the mean maximum increase at 10 s was 306% ($P < 0.001$), while at 60 s the increase amounted to 242% ($P < 0.001$, $n = 8$). The initial rise preceded, while the later rise occurred approximately coincidentally with, the time for initiation of contraction. The pattern of changes in I(1,4,5)P₃ levels evoked by ET-1 resembled that seen in response to methacholine in this tissue (Nally *et al.*, 1992), where rises at 10 s and 60 s of 461% and 345% respectively were observed in response to methacholine 10^{-3} M. Nevertheless, neither the initial, nor the later rise in I(1,4,5)P₃ levels evoked by ET-1 was altered by pre-incubation with atropine (10^{-5} M, $n = 6$).

Endothelin-3

Contractile responses In untreated bovine tissues, endothelin-3 (ET-3, 10^{-10} – 3×10^{-7} M) evoked small (0.25–0.5 g wt.) contractions which were not concentration-dependent ($n = 6$). In the presence of phosphoramidon (3.67×10^{-5} M) however, the response to ET-3 became concentration-dependent ($-\log EC_{200 \text{ mg}}$; 8.28 ± 0.26) and similar or greater in magnitude and potency to that seen with ET-1 in this tissue (Figure 5). Subsequent experiments using ET-3 were therefore all con-

ducted in the presence of phosphoramidon. Under these conditions, the contractions evoked by ET-3 were unaffected by pre-incubation with atropine (10^{-5} M, $n = 6$) or by removal of the epithelium. PDBu (10^{-8} M) evoked a significant leftward shift of control (plus phosphoramidon) responses to ET-3 (Figure 6a, PDBu $-\log EC_{200 \text{ mg}}$, 9.7 ± 0.09 , $P < 0.001$), which was reversed by the additional presence of Ro 31-8220 (3×10^{-8} M, Figure 6a). Ro 31-8220 (Figure 6b, 3×10^{-9} – 3×10^{-8} M) did not alter responses to ET-3 in the presence of phosphoramidon. Indomethacin (Figure 7a, 10^{-5} M) significantly ($P < 0.001$) attenuated the responses evoked by ET-3 under these conditions. Three of six tissues did not evoke a response of 200 mg wt in the presence of phosphoramidon and indomethacin and accordingly the $-\log EC_{200 \text{ mg}}$ could not be calculated. Responses to ET-3, in the presence of phosphoramidon, were virtually abolished by pre-incubation with nifedipine (Figure 7b, 10^{-5} M), such that the $-\log EC_{200 \text{ mg}}$ could not be obtained.

I(1,4,5)P₃ levels ET-3 (10^{-10} – 3×10^{-7} M) failed to alter levels of I(1,4,5)P₃ in bovine bronchial smooth muscle, at any time point examined (5–300 s, $n = 8$), either in the presence (Figure 8, 3.67×10^{-5} M) or absence of phosphoramidon.

Figure 3 Concentration-response curves (CRCs) to endothelin-1 (ET-1) (■, $n = 12$), in the presence of (a) phorbol 12,13-dibutyrate, (PDBu, 10^{-8} M) alone (□, $n = 6$) or PDBu (10^{-8} M) and Ro 31-8220 (3×10^{-8} M) (●, $n = 6$) in combination and (b) to endothelin-1 (ET-1) (●, $n = 6$) in the presence of Ro 31-8220 (□, 3×10^{-8} M, $n = 6$) alone. PDBu evoked a significant leftward shift, which was abolished in the additional presence of Ro 31-8220, while Ro 31-8220 alone failed to evoke any significant alteration, of the CRC to ET-1.

Figure 4 Time-dependent changes in levels of inositol (1,4,5)-triphosphate (I(1,4,5)P₃) in response to endothelin-1 (3×10^{-7} M), measured as pmol mg⁻¹ protein. There was an initial rise, maximal at 10 s, followed by a return to baseline by 20 s and a second rise in I(1,4,5)P₃ levels at 60 s. Each of these rises was significant with respect to zero time control values. Number of observations (n) = 10 in each case.

Figure 5 Concentration-response curves (CRCs) to endothelin-3 (ET-3), in the presence (□) and absence (■) of phosphoramidon (3.67×10^{-5} M). In the presence of phosphoramidon, the response to ET-3 became concentration-dependent. Number of observations (n) = 6 in each case.

Figure 6 Concentration-response curves (CRCs) to endothelin-3 (ET-3, $\square$, $n = 12$), alone and in the presence of (a) phorbol 12,13-dibutyrate, (PDBu, 10^{-8} M, $\bullet$, $n = 6$) as well as ($\circ$) a combination of PDBu (10^{-8} M) and Ro 31-8220 (3×10^{-8} M) ($n = 6$) and (b) CRCs to ET-3 ($\square$, $n = 6$) in the presence of Ro 31-8220 ($\bullet$, 3×10^{-8} M, $n = 6$). PDBu evoked a significant leftward shift of the CRC to ET-3, which was attenuated by the additional presence of Ro 31-8220. Ro 31-8220 alone evoked no significant change in the contractions to ET-3. Each of these experiments involving ET-3 was conducted in the presence of phosphoramidon (3.67×10^{-5} M) throughout.

Discussion

The potency of ET-1 in stimulating contraction in bovine bronchial smooth muscle was lower than that observed in vascular smooth muscle (e.g. rabbit pulmonary artery, MacLean & McGrath, 1991). This low potency does not reflect a lack of tone *in vitro*, since pre-contraction with other spasmogens, such as KCl, MCh or U46619 failed to alter the CRC to ET-1. ET-1 does not act by the release of the neurotransmitter substance acetylcholine, since atropine did not alter the response, nor does it act by release of prostaglandins since indomethacin was also ineffective. It is unlikely that ET-1 acts by release of agents from the epithelium since the response to ET-1 was unaffected by epithelium removal.

It seems unlikely from the present study that the lower potency of ET-1 in bovine bronchi compared to vascular tissue is due to ET-1 acting via an ET_B-type receptor in this tissue. If this were the case then it would be expected that the response to ET-1 would resemble that of ET-3. This does not however appear to be the case.

Early evidence had shown that, in contrast to ET-1, no concentration-dependent response to ET-3 could be shown. However, many of the mechanical differences between ET-1 and ET-3 appeared to be due to the breakdown of ET-3

Figure 7 Concentration-response curves (CRCs) to endothelin-3 (ET-3), in the presence of phosphoramidon alone ($\square$) and in the presence of (a) phosphoramidon (3.67×10^{-5} M) and indomethacin ($\bullet$, 10^{-5} M) and (b) phosphoramidon (3.67×10^{-5} M) and nifedipine ($\bullet$, 10^{-5} M). Indomethacin and nifedipine each had a significant attenuating effect on responses to ET-3 in the presence of phosphoramidon. Number of observations (n) = 6 in each case.

Figure 8 Time-dependent changes in levels of inositol (1,4,5)-trisphosphate (I(1,4,5)P₃) in response to endothelin-3 (ET-3; 10^{-6} M) in the presence of phosphoramidon (3.67×10^{-5} M), measured as pmol mg⁻¹ protein. ET-3 failed to evoke any significant rise in I(1,4,5)P₃ levels at any time point measured. Number of observations (n) = 10 in each case.

under the influence of neutral endopeptidase since the presence of the endopeptidase inhibitor, phosphoramidon, resulted in a concentration-dependent response to ET-3 in bovine tissue. This is in keeping with the substantial enhancement of ET-3 responses recently demonstrated in both human and rabbit bronchus (McKay *et al.*, 1992).

Differences do however exist between the responses to ET-1 and ET-3. In bovine tissue, ET-3, but not ET-1 responses are inhibited by indomethacin and hence are partly evoked by the generation of cyclo-oxygenase products. The lack of effect of indomethacin on ET-1 responses is in contrast to, for example, the guinea-pig, where responses to this peptide in bronchi (Filep *et al.*, 1991), trachea (White *et al.*, 1991) and lung (Pons *et al.*, 1991) were to a greater or lesser extent mediated via the formation of cyclo-oxygenase metabolites. In human bronchi however, (Advenier *et al.*, 1990; McKay *et al.*, 1991) indomethacin is without effect on ET-1 stimulated responses and this may reflect a species difference.

ET-1 responses were also unaltered by nifedipine, a dihydropyridine which antagonizes voltage-dependent calcium channels. This is similar to results obtained in human bronchial tissue, (McKay *et al.*, 1991) where the contractions evoked by this agonist were unaffected by verapamil. In contrast, ET-3-stimulated responses were virtually abolished by nifedipine and contraction to ET-3 would therefore appear to rely on an influx of external calcium. In the present study we also examined the effect of stimulating or inhibiting PKC with, respectively, PDBu or Ro 31-8220. It was not possible during these or subsequent experiments to exceed 10^{-8} M PDBu since concentrations greater than this evoked a large contraction, masking the contractions evoked by the endothelins. The effects of the protein kinase C inhibitor, Ro 31-8220, suggest that contractions generated by ET-1 do not rely heavily upon PKC activation, since this inhibitor failed to attenuate the ET-1-evoked response in bovine bronchial smooth muscle. The leftward shift evoked by PDBu indicates an additive effect between PKC activation and the signals evoked by ET-1.

Contractions to ET-3 are also unlikely to utilise PKC activation, since, even in the presence of phosphoramidon, contractions were unaffected by Ro 31-8220 although they were enhanced by PDBu. The ability of PDBu to shift CRCs leftward is not evidence *per se* that ET-1 or ET-3 act via PKC activation since, in several tissues, it has been suggested that a maximum physiological response can result from the synergistic action of an increase in $[Ca^{2+}]_i$ concentration and the formation of diacylglycerol (Yamanishi *et al.*, 1983; Nishizuka, 1984), here mimicked by PDBu. Accordingly, the inability of Ro 31-8220 to inhibit these responses suggests rather that PKC activation is not critical to the mechanical response to either endothelin.

Evidence for ET-1 and ET-3 acting via different signal-transduction mechanisms and hence different receptors has been obtained from the measurement of $I(1,4,5)P_3$. ET-1 stimulated a substantial rise in $I(1,4,5)P_3$ levels in bovine tissue whilst, even in the presence of phosphoramidon, ET-3 evoked no such response. The substantial changes in $I(1,4,5)P_3$ levels evoked by ET-1 within the first few seconds following stimulation, precede increases in isometric force development. The rapid time course for production of $I(1,4,5)P_3$ is accordingly in keeping with a receptor-activated response initiating smooth muscle contraction.

We have therefore demonstrated that, while ET-1 elevates $I(1,4,5)P_3$ levels, PKC activation does not appear to be crucial to the associated contraction. This suggests that both

'limbs' of the PIP_2 hydrolysis pathway are not necessarily utilised as signals mediating the response to the activation of receptors by ET-1. A similar pattern is seen in rat pancreatic acinar cell line (Gallacher *et al.*, 1990) in response to another polypeptide, bombesin, suggesting that it is not possible to assume, from $I(1,4,5)P_3$ production, that PKC activation is important to a particular response in any given system.

The biphasic nature of the $I(1,4,5)P_3$ response to ET-1 suggests that this peptide did not fully desensitize and differs from the time course of formation of $I(1,4,5)P_3$ in response to ET-1 in other systems. In brain capillary endothelial cells for example (Vigne *et al.*, 1990), the response to ET-1 was transient, while a sustained elevation of $I(1,4,5)P_3$ levels was observed in Rat-1 fibroblasts in response to this peptide (Plevin *et al.*, 1991); this latter result demonstrating another system where desensitization does not appear to occur. It is important to recognise that a mass assay, as opposed to an accumulation assay, cannot take into account potential changes in the metabolism, rather than the generation of $I(1,4,5)P_3$. Indeed, the fall in $I(1,4,5)P_3$ levels observed at 20 and 30 s is probably due to increased activity of the 5-phosphatase and 3-kinase responsible for $I(1,4,5)P_3$ metabolism. Such changes in metabolism of $I(1,4,5)P_3$ have indeed been implicated in regulation of levels of this second messenger in bovine tracheal smooth muscle in response to carbachol (Chilvers *et al.*, 1991). Thus the biphasic $I(1,4,5)P_3$ response is due to a change in the equilibrium between $I(1,4,5)P_3$ generation and metabolism. It is probable that the major regulating aspect is, however, the phospholipase C. Although the pattern of the changes in IP_3 levels evoked by ET-1 resembles that observed for MCh in this tissue (Nally *et al.*, 1992), pre-incubation with atropine did not alter the IP_3 response to ET-1, confirming our earlier conclusion that ET-1 did not act via release of acetylcholine.

In conclusion, these results give further evidence of the possible importance of ET-1 in airway smooth muscle. It appears that, in this tissue, the actions of ET-1 are not via acetylcholine release or by generation of cyclo-oxygenase products and occur via a different receptor from that utilised by ET-3. This receptor, when activated by ET-1, stimulates hydrolysis of PIP_2 , with subsequent production of $I(1,4,5)P_3$. Neither PKC activation nor influx of extracellular calcium through dihydropyridine-sensitive calcium channels is a prerequisite for contraction to ET-1.

Addition of ET-3 does not rely on activation of protein kinase C, nor does stimulation of PIP_2 hydrolysis occur in response to this peptide. Instead ET-3 relies upon ingress of extracellular calcium through dihydropyridine-sensitive, voltage-operated channels, for production of contraction. In addition, a proportion of the contraction evoked by ET-3 appears to be mediated by cyclo-oxygenase metabolites. It is unclear if the stimulatory effect of ET-3 involves generation of arachidonic acid for eicosanoid production, perhaps through Ca^{2+} -stimulated phospholipase A_2 activation or if it is a stimulation of cyclooxygenase *per se*.

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