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Evidence for multiple endothelin receptors in the guinea-pig pulmonary artery and trachea

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1 The responses of the three peptides, endothelin 1 (ET-1), endothelin 2 (ET-2) and endothelin 3 (ET-3) were analysed on isolated circular segments of pulmonary arteries and trachea from the guinea-pig.

2 In the pulmonary artery, the vasomotor responses to the endothelins, expressed as the maximum contraction (E_{max}%), had the order ET-1 > ET-2 > ET-3 while the order of potency (pD_2) was ET-1 = ET-2 > ET-3. ET-1 and ET-2 caused cross-desensitization, but did not affect the responses to ET-3. ET-3 did not cause cross-desensitization to ET-1 or ET-2 although it induced homologous desensitization. Finally, the effects of ET-1 and ET-2 were additive to those of ET-3. The additive effect of ET-3 to those of ET-1 or ET-2 was more difficult to demonstrate, given the profound contraction produced by ET-1 and to a lesser extent by ET-2.

3 In the trachea, the rank order of potency, additivity and desensitization were different from the pulmonary artery. Basically, all three peptides were equipotent but less potent than ET-1 in the artery. There was no evidence for additivity and only a slight tendency to tachyphylaxis was seen.

4 The guinea-pig pulmonary artery appears to be endowed with one receptor type which is sensitive to ET-1/ET-2 and with another receptor type which responds preferentially to ET-3. In the trachea, neither of these receptors appears to be present since all three peptides apparently act on a homogeneous population of receptors with characteristics different from those of the two arterial receptors. This suggests a third non-isopeptide selective type of endothelin receptor.

Keywords: Endothelin; *in vitro* pharmacology; pulmonary blood vessels; tachyphylaxis; trachea; endothelin receptors

Introduction

Three endothelin genes with vasoactive products have been described in the human genome (Inoue *et al.*, 1989). The products expressed by these genes are distinct from each other but display a considerable homology. The 'classical' endothelin, endothelin 1 (ET-1), is the product originally isolated from porcine aortic endothelial cells. ET-3 differs from ET-1 in 6 out of 21 residues (Yanagisawa *et al.*, 1988a,b). ET-2 bears a close resemblance to ET-1, with Leu^6 and Met^7 substituted for Trp^6 and Leu^7, respectively (Inoue *et al.*, 1989). The three endothelins are potent vasoconstrictors as originally shown in porcine isolated aorta and coronary arteries (Inoue *et al.*, 1989). Different pharmacological profiles have led to the suggestion that there are endothelin receptor subtypes (Inoue *et al.*, 1989; Yanagisawa & Masaki, 1989). This assumption has gained further support from both ligand binding studies (Watanabe *et al.*, 1989; Masuda *et al.*, 1989) as well as recent work with cloned receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990). In the guinea-pig pulmonary vascular bed a remarkable degree of tachyphylaxis was noted for ET-1 (Cardell *et al.*, 1990). This is in agreement with findings in guinea-pig femoral artery (Wiklund *et al.*, 1988) and porcine aorta (Ishikawa *et al.*, 1988). In the present study we have examined endothelin-induced responses in the pulmonary artery and trachea of guinea-pig in an attempt to differentiate between different types of endothelin receptors. A preliminary account of some of this work was presented at the IUPHAR symposium on Chemistry and Biology of Endothelin (Cardell *et al.*, 1991).

Methods

Young male guinea-pigs (200–300 g) were killed by a blow on the neck. The lungs, including the heart and trachea, were quickly removed and immersed in a cold (+4°C) buffer solution (for composition, see below). Lobar arteries (second to third branches) and a distal portion of the trachea were dissected free of surrounding tissue. The vessels and the trachea were used in the experiments either immediately or, occasionally, following overnight storage in a cold buffer solution. Circular segments were mounted on two L-shaped metal prongs. One prong was connected to a force displacement transducer attached to a Grass polygraph for continuous registration of isometric tension and the other to a displacement device. The mounted segments were immersed in small (2.5 ml) temperature-controlled (37°C) tissue baths containing the buffer solution. The solution was equilibrated with 5% CO_2 in O_2, giving a pH of 7.4.

Initially, a tension of 1–2 mN was applied to the arterial segments and 2–3 mN was applied in the tracheal segments. The segments were subsequently allowed to stabilize at this level of tension for 90 min. The contractile ability of each segment was then examined by exposure to a potassium rich (60 mM) buffer solution (for composition, see below). Only when two reproducible contractions could be elicited was the individual segment used in further studies. The integrity of the vascular endothelium was assessed at the end of most experiments by obtaining a dilator response to acetylcholine (10^{-6} M) (Furchgott, 1984). The presence of tracheal epithelium was confirmed by staining with a 5% silver nitrate solution followed by light microscopy (Abrol *et al.*, 1984). There were no differences in the response to the endothelins when concentration-response curves obtained by cumulative application were compared to those obtained by a single dose procedure (Cardell *et al.*, 1990).

The log concentration-response relationship was approximated by linear regression analysis of the data within the 5% to 95% response interval. The pD_2 value (i.e. the negative logarithm of the concentration eliciting half the maximum response) and E_{max}% (the maximal contraction elicited by an agonist expressed as a percentage of the contraction induced by 60 mM K^+) was calculated for each experiment.

The strong tachyphylaxis caused by the endothelins was used as the basis of a series of desensitization tests (Miasiro & Paiva, 1990). Both the homologous and the cross-desensitization patterns for the three peptides were investigated. In the homologous desensitization tests the segments

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were first contracted by ET-1, ET-2 or ET-3. After repeated washes during a 90 min recovery period the initially contracted segment had returned to its 'resting' state (baseline). A single dose of the peptide was then reapplied to the segment. The cross desensitization test was performed according to the same principle, but at the point of reapplication a different endothelin was administered.

Solutions and drugs

The following solutions were used: (a) standard buffer solution (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11. (b) 60 mM K^+ buffer solution: as above, but substituting equimolar amounts of NaCl with KCl. Analytical-grade chemicals and twice-distilled water were used for preparing all solutions.

The following drugs were used: acetylcholine chloride (Sigma, U.S.A.), endothelin 1, endothelin 2, endothelin 3 (Peninsula, U.S.A.) 5-hydroxytryptamine creatine sulphate (Sigma, U.S.A.), histamine dihydrochloride (Sigma, U.S.A.), and prostaglandin $F_{2\alpha}$ (Astra, Sweden).

Endothelins were dissolved in and further diluted in saline containing bovine serum albumin (1%) and used in the experiments within 30 min to avoid possible degradation. The concentrations of the agents are expressed as the final molar concentration in the bath.

Statistics

Data were tested by analysis of variance (ANOVA) followed by Scheffe's method in order to test specific differences between groups (Wallenstein *et al.*, 1980).

Results

Contractile responses

ET-1, ET-2 and ET-3 elicited strong concentration-dependent contractions of the pulmonary lobar artery (Figure 1a) and the trachea (Figure 1b). The responses were slow in onset and long lasting.

In the pulmonary artery, ET-1 induced the greatest maximum constriction of the three peptides. The potencies of ET-1 and ET-2 were similar, while ET-3 was less active as compared to the other two peptides (Figure 1a and Table 1). Notably, ET-3 did not produce a contraction in vessels from approximately every sixth animal tested. These experiments were not included in the calculations. In the segments that did not respond to ET-3, histamine elicited strong reproducible contractions which could be reversed by acetylcholine (not illustrated).

Table 1 Guinea-pig pulmonary artery and trachea: maximum response (E_{max}%) to endothelin, expressed as a percentage of the contraction induced by 60 mM potassium, and sensitivity (pD_2) expressed as the negative logarithm of the concentration eliciting half maximum response

Pulmonary artery	n	E_{max}%	pD_2
ET-1	14	168 ± 21	8.65 ± 0.18
ET-2	9	121 ± 19 **	8.67 ± 0.16 NS
ET-3	9	100 ± 19 * **	7.79 ± 0.16 ** **
ANOVA		**	**
Trachea	n	E_{max}%	pD_2
ET-1	8	74 ± 13	7.81 ± 0.26
ET-2	5	75 ± 15	8.03 ± 0.39
ET-3	7	67 ± 15	7.94 ± 0.16
ANOVA		NS	NS

ET-1, endothelin 1; ET-2, endothelin 2; ET-3, endothelin 3.
The values represent the mean ± standard deviation.
Analysis of variance (ANOVA) was used followed by Scheffe's method in order to test specific differences between groups. * $P < 0.05$; ** $P < 0.01$; NS = no significant difference.

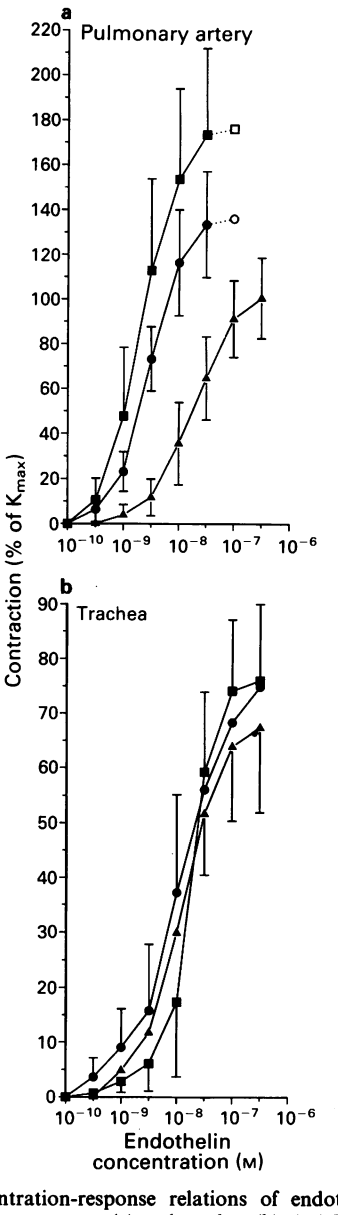


Figure 1 Concentration-response relations of endothelins (ET) in guinea-pig pulmonary artery (a) and trachea (b): (■) ET-1; (●) ET-2 and (▲) ET-3. Responses are expressed as a percentage of potassium (60 mM)-induced contraction and each point is the mean with s.d. shown by vertical bars. Pulmonary artery $n = 9$–14. Trachea: $n = 5$–8. In some arterial experiments a concentration of 10^{-7} M of ET-1 (□) and ET-2 (○) was also tested (172 ± 28%, $n = 7$ and 124 ± 13%, $n = 4$, respectively). Since there was no significant difference compared to 3×10^{-8} M for these peptides, the concentration 3×10^{-8} M was used for calculation of E_{max}%.

In the trachea, the maximum contractile responses to ET-1, ET-2 and ET-3 were smaller ($P < 0.01$) than the corresponding responses in the pulmonary arteries. The potencies of ET-1 and ET-2 were significantly less in the trachea than in the pulmonary artery. In contrast to the findings in the pulmonary arteries, there was no significant difference in the maximum contractile response or in potency between the peptides (Table 1).

Additivity experiments

In arterial segments maximally constricted by ET-3, application of ET-1 or ET-2 induced an additional contraction (Figure 2a). In segments maximally contracted by ET-1 or

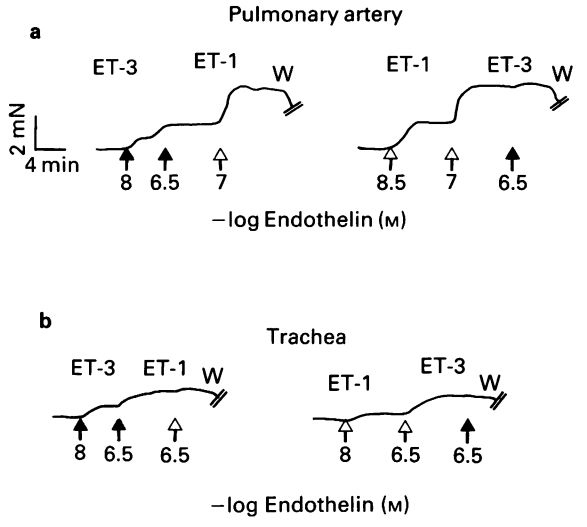


Figure 2 Typical examples of endothelin (ET)-induced contractions of guinea-pig pulmonary artery (a) and trachea (b). Cumulative application of ET-3 followed by ET-1 and cumulative application of ET-1 followed by ET-3, respectively. This type of experimentation was repeated four times in the trachea and six times in the pulmonary artery. At each arrow ET-1 (open) or ET-3 (filled) was added to the buffer solution. W, wash.

ET-2, only a small additive contraction was induced by ET-3. This contraction was more obvious in vessels precontracted by ET-2 than ET-1. None of the peptides induced an additional constriction of tracheal segments already maximally contracted by one of the other two peptides (Figure 2b).

The time course for the development of contraction of pulmonary arteries and trachea was equally long lasting and did not differ appreciably among the three peptides. However, the tracheal segments reached their maximum contraction somewhat more slowly than the vessel segments (Figure 2).

Homologous desensitization

After 2 h of repeated washing, arteries initially contracted by ET-1, ET-2 or ET-3 had returned to their 'resting' state (baseline). When ET-1 or ET-2 was reapplied, no or occasionally a small transient contraction (<7%) was seen (Figure 3a,b). Tachyphylaxis could also be demonstrated with ET-3; reapplication induced only about 40% of the initially induced contraction (Figure 3c). In the trachea, the tachyphylaxis seen after reapplication of the same peptide was rather weak for all three peptides. Furthermore, there were no major differences in the tachyphylaxis patterns for the three peptides. They all reached about 70% of the initial contraction (Figure 3d–f).

Cross desensitization

Vessel segments, initially contracted by ET-1, returned to their 'resting' contractile state after 2 h of repeated washes. When ET-2 was added to these segments, no or at most a weak transient contraction (<10%) was seen (Figure 4a). The same pattern was seen when ET-1 was given to segments previously exposed to ET-2 (Figure 4b). There was no apparent tachyphylaxis when applying ET-1/ET-2 after ET-3 (Figure 4c,d) or when applying ET-3 after ET-1/ET-2 (Figure 4e,f). Preparations that were tachyphylactic to ET-1/ET-2 still responded not only to ET-3 but also to histamine (10^{-6} M), 5-hydroxytryptamine (10^{-6} M), KCl (60 mM) and prostaglandin $F_{2\alpha}$ (10^{-6} M) (not illustrated).

In the trachea, the end tachyphylaxis seen after cross desensitization was identical for all three peptides and did not differ from the tachyphylaxis seen after homologous desensitization (Figure 4g–l).

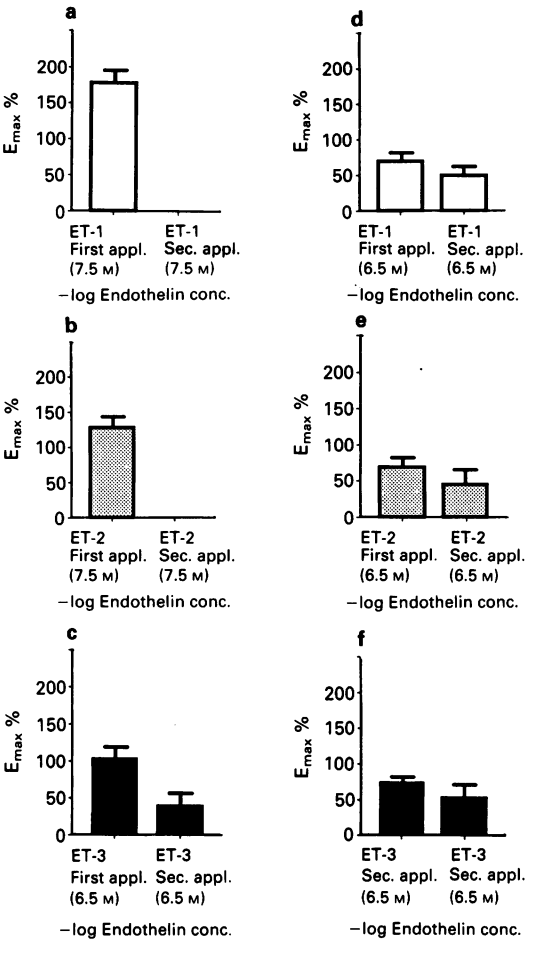
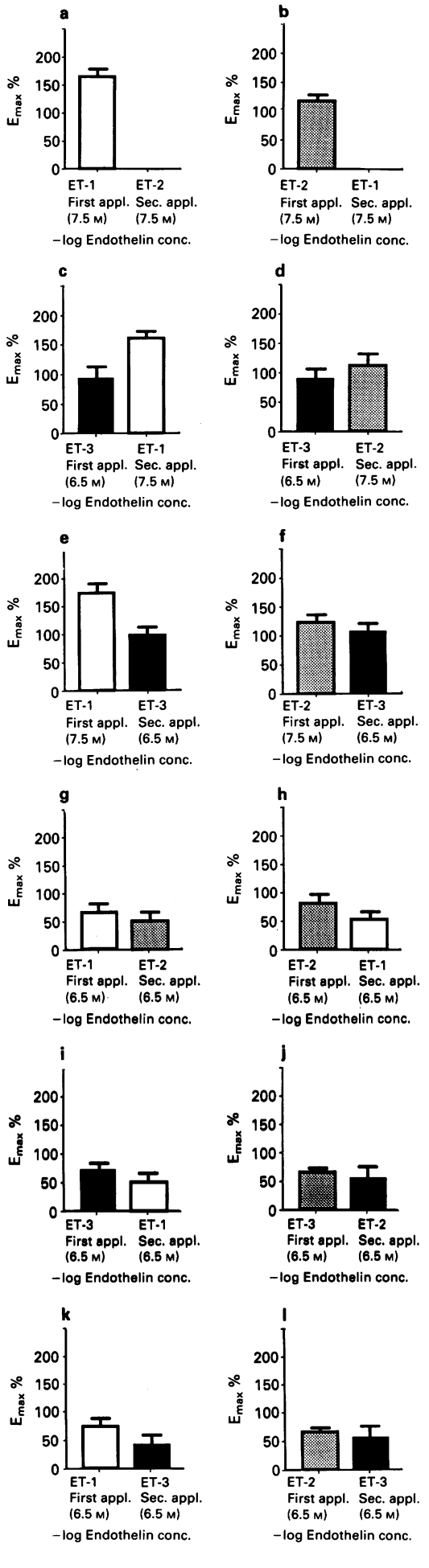


Figure 3 Homologous desensitization of endothelin 1 (ET-1), ET-2 and ET-3. Quantitative data for guinea-pig pulmonary arterial (a–c) and tracheal (d–f) segments initially contracted by ET-1, ET-2 or ET-3. After 2 h of repeated washings the segments had returned to their baseline. (a–b) When ET-1 or ET-2 was reapplied no persistent contraction was induced. (c) When ET-3 was reapplied a small contraction was seen. (d–f) After reapplication of the same peptide, ET-1, ET-2 or ET-3 to the tracheal segments a second contraction, slightly smaller than the first was seen. The responses are expressed as a percentage of potassium (60 mM)-induced contraction and the columns represent the mean with s.d. shown by vertical bars. n = 3–4 for each experiment.

Discussion

The present study demonstrates that ET-1, ET-2 and ET-3 induce a slowly developing, long lasting contraction of guinea-pig isolated tracheal and pulmonary arterial segments. Repeated washes for up to 2 h are required before the muscle tension returns to the control level (Ohlstein *et al.*, 1989). This may reflect slow dissociation of the endothelins from their receptors and/or long lasting intracellular effects (Gu *et al.*, 1988; Borges *et al.*, 1989). The vasoconstrictor activity in terms of maximum tension showed the order ET-1 > ET-2 > ET-3 and the rank order of potency was ET-1 = ET-2 > ET-3. This profile is in agreement with findings in rat pulmonary artery (Nakajima *et al.*, 1989) and porcine coronary artery (Inoue *et al.*, 1989). In the tracheal segments the order of maximum contractile activity was ET-1 = ET-2 = ET-3 and the order of potency was the same.

In vivo, a bolus injection of ET-1, ET-2 or ET-3 produces an initial depressor response, followed by an increase of the systemic blood pressure (Wright & Fozard, 1988; de Nucci *et al.*, 1988; Le Monnier de Gouville *et al.*, 1990). Although the initial relaxation is characteristic of the systemic response, such a response is seldom seen *in vitro* (Folta *et al.*, 1989). The tachyphylaxis pattern seen *in vitro* is also different from that



found under *in vivo* conditions since only the vasodepressor effects of the endothelins display tachyphylaxis *in vivo* (Le Monnier de Gouville *et al.*, 1990). Furthermore, regional and species-related differences in the endothelin responses are well known phenomena (Minkes & Kadowitz, 1989; Faraci, 1989; Lippton *et al.*, 1988). Endothelin is believed to exert its biological activity through interaction with specific receptors on the target cells (Miyazaki *et al.*, 1989; Watanabe *et al.*, 1989). The pharmacological profiles of the three structurally distinct endothelins suggest the existence of different receptor types (Inoue *et al.*, 1989) and two receptors for endothelin have been proposed (Randall *et al.*, 1989; Warner *et al.*, 1989; Fu *et al.*, 1989). In the present study, the three endothelins were used in an attempt to illustrate the existence of different types of endothelin receptors in the guinea-pig lung.

Treatment of pulmonary arteries with ET-1 led to desensitization towards ET-1. The desensitization seemed to be of the homologous type, since previous treatment with ET-1 did not alter the expected responses of either potassium or histamine. The same homologous desensitization pattern was seen for ET-2 and ET-3. The strong cross-tachyphylaxis reaction between ET-1 and ET-2 in the pulmonary artery implies that they act at the same functional receptor site or sites. However, it must be realized that tachyphylaxis does not have to involve receptor inactivation or desensitization, post receptor events may also desensitize. ET-3, on the other hand, produced tachyphylaxis upon reapplication, but there was no evidence of cross-tachyphylaxis towards ET-1 or ET-2.

ET-3 has been shown to be more easily dissociated from the relevant endothelin receptor than ET-1, probably due to a more polar nature of the N-terminal part of the molecule (Yanagisawa *et al.*, 1988a). This alone cannot explain the difference in the tachyphylaxis pattern. A more likely explanation is that ET-1 and ET-2 act on the same functional site while ET-3 acts on another site. In recent ligand binding studies on chicken cardiac membranes and rat lung membranes, two distinct types of endothelin binding sites have been found (Watanabe *et al.*, 1989; Masuda *et al.*, 1989). One site has a high affinity for ET-1 and ET-2 whereas the other site interacts preferentially with ET-3.

In the additivity experiments carried out in the present study the arterial segments which had reached the E_{max}-level for ET-3, could be further contracted by ET-1/ET-2. In the reverse situation, where ET-1 or ET-2 were used to maximally contract the arteries, ET-3 induced a small additive contraction. The latter situation was more difficult to demonstrate, given the profound contraction produced by ET-1 and to a lesser extent, ET-2. These findings give further support to the view that different receptors are involved in the vasoconstrictor responses to the endothelins.

In the trachea the pharmacological profile of the three endothelins differed from that in the pulmonary artery in that there was no difference between them in potency or intrinsic activity. Furthermore, ET-1, ET-2 or ET-3 did not further contract segments precontracted by either of the other two peptides. This is partly in agreement with the conclusions of Maggi *et al.* (1989) who postulated an endothelin receptor in the guinea-pig bronchi different from that in the guinea-pig aorta.

Figure 4 Cross desensitization of endothelin 1 (ET-1), ET-2 and ET-3. Quantitative data for guinea-pig pulmonary arterial (a–f) and tracheal (g–l) segments initially contracted by ET-1, ET-2 or ET-3. After repeated washing and a 2 h recovery period another peptide was applied in the same dose. (a–b) Strong cross tachyphylaxis reaction between ET-1 and ET-2. (c–d) No sign of tachyphylactic interference between ET-3 and ET-1/ET-2. (e–f) Application of ET-3 after contraction induced by ET-1 or ET-2 resulted in a normal contraction slightly smaller than control. (g–l) Only a slight tendency of tachyphylaxis is seen between the three peptides in the tracheal segment. The responses are expressed as a percentage of potassium (60 mM)-induced contraction and the columns represent the mean with s.d. shown by vertical bars. $n = 3$–4 for each experiment.

Recently, two separate endothelin receptors were cloned. Through ligand binding studies one was found to correspond to an ET-1 selective receptor type (Arai *et al.*, 1990), whereas the other was a non-isopeptide-selective type designated ET_B (Sakurai *et al.*, 1990). The ET_B receptor mRNA was not found to be present in vascular smooth muscle cells. This receptor could very well correspond to the tracheal endothelin receptor described in this paper.

According to Sakurai *et al.* (1990) the ET-1/ET-2 selective receptor found in the vascular smooth muscle cells should be referred to as an ET_A receptor. It is therefore tempting to propose that the contractile vascular endothelin receptor population in the pulmonary artery is dominated by the ET-1/ET-2 receptor site (most likely an ET_A-receptor) while the postulated ET-3 receptor site is of minor significance. In the trachea a third non-isopeptide-selective type of endothelin receptor is found, probably being of the ET_B-type.

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