Respiratory
Pharmacology and
Pharmacotherapy

Airways Smooth Muscle: Peptide Receptors, Ion Channels and Signal Transduction

Edited by
D. Raeburn
M. A. Giembycz

Birkhäuser Verlag
Basel · Boston · Berlin

Editors:

Dr. David Raeburn
Department Head
Discovery Biology
Rhône-Poulenc Rorer Ltd
Dagenham Research Centre
Dagenham
Essex RM10 7XS
England

Dr. Mark A. Giembycz
Lecturer
Department of Thoracic Medicine
Royal Brompton National Heart and Lung Institute
Dovehouse Street
London SW3 6LY
England

Library of Congress Cataloging-in-Publication Data

Airways smooth muscle : peptide receptors, ion channels and signal transduction / edited by D. Raeburn ; M. A. Giembycz.
p. cm. – (Respiratory pharmacology and pharmacotherapy)
Includes bibliographical references and index.
ISBN-13: 978-3-0348-7364-2 e-ISBN-13: 978-3-0348-7362-8
DOI: 10.1007/978-3-0348-7362-8
1. Respiratory Muscles – Cytochemistry. 2. Smooth muscle – Cytochemistry. I. Raeburn, D. (David), 1953– . II. Giembycz, M. A. (Mark A.), 1961– . III. Series.
[DNLM: 1. Respiratory Muscles – physiology. 2. Muscle, Smooth – – physiology. 3. Receptors, Peptide – physiology. 4. Ion Channels – – physiology. 5. Signal Transduction – physiology. WF 102 A29863 1995]
QP121.A425 1995
612.2 – dc20
DNLM/DLC
for Library of Congress

Die Deutsche Bibliothek - CIP - Einheitsaufnahme

Airways smooth muscle : Peptide receptors, ion channels and signal transduction. - 1995
/ ed. by D. Raeburn ; M. A. Giembycz.
Basel ; Boston ; Berlin : Birkhäuser, 1995
(Respiratory pharmacology and pharmacotherapy)
ISBN-13: 978-3-0348-7364-2

NE: Raeburn, David [Hrsg.]

Softcover reprint of the hardcover 1st edition 1995
P.O. Box 133
CH-4010 Basel/Switzerland

ISBN-13: 978-3-0348-7364-2

9 8 7 6 5 4 3 2 1

Contents

Contributors

Ratna Bose, Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada
Stephen G. Farmer, Pulmonary Pharmacology Section, Zeneca Pharmaceuticals Group, Wilmington, Delaware, USA
Susan J. Gunst, Department of Physiology and Biophysics, Indiana University School of Medicine, Indianapolis, Indiana, USA
Douglas W. P. Hay, Department of Pulmonary Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania, USA
Thomas R. Jones, Department of Pharmacology, Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Canada
Gregory J. Kaczorowski, Department of Membrane Biochemistry and Biophysics, Merck Research Laboratories, Rahway, New Jersey, USA
Jason Kelley, University of Vermont College of Medicine, Given, Burlington, Vermont, USA
Alan J. Knox, Respiratory Medicine Unit, City Hospital, Nottingham, UK
Carlo Alberto Maggi, Department of Pharmacology, A. Menarini Pharmaceuticals, Florence, Italy
David Raeburn, Department of Inflammation, Rhône-Poulenc Rorer, Inc., Collegeville, Philadelphia, USA
Ian W. Rodger, Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada
Sami I. Said, Department of Veterans Affairs, Medical Center at Northport, New York, and University Medical Center, Stony Brook, New York, USA
Neil C. Thomson, Department of Respiratory Medicine, Western Infirmary, Glasgow, Scotland, UK
Stephen L. Underwood, Rhône-Poulenc Rorer Ltd., Dagenham Research Centre, Dagenham, Essex, UK

Airways Smooth Muscle: Peptide Receptors, Ion Channels and Signal Transduction
ed. by D. Raeburn and M. A. Giembycz

CHAPTER 1
Endothelins

Douglas W. P. Hay

Department of Pulmonary Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania, USA.

1. Introduction

The endothelium is a critical cellular layer in the cardiovascular system which releases a variety of biologically active substances [1, 2]. In the

1980s considerable attention in the field of endothelial cell biology was directed towards the isolation and characterization of endothelium-derived modulators of the responsiveness of the underlying vascular smooth muscle. The focus of these studies was on relaxant substances released from the endothelium, such as prostacyclin and, in particular, an unknown material which was designated endothelium-derived relaxing factor or EDRF. Recent research has provided convincing evidence that EDRF is nitric oxide or a nitric oxide-containing moiety [3]. During this period there was limited research and information on endothelium-derived vasoconstrictor substances. However, evidence was provided that thromboxane and superoxide elicited contraction of vascular smooth muscle following their release from the endothelium [1]. Furthermore, two groups of researchers reported that a protease-sensitive material in the supernatant of cultured bovine aortic endothelial cells contracted vascular smooth muscle [4–6]. This suggested the presence of a vasoconstrictor peptide that was released from the endothelium.

The research area of endothelium-derived constrictor substances was irreversibly transformed in 1988 when Yanagisawa and co-workers published a landmark paper which described the isolation, purification, cloning and expression, and initial pharmacological characterization of a potent vasoconstrictor 21-amino acid peptide, named endothelin, which was released from porcine aortic endothelial cells [7]. It was demonstrated subsequently that this material was a member of a mammalian family of vasoconstrictor peptides, the endothelins (ETs), designated ET-1, ET-2 and ET-3, which are encoded by three similar but distinct ET-related genes [8]. ET-1 is the original porcine/human ET, ET-2 differs by two amino acid substitutions from ET-1, and ET-3 differs by six amino acids. The genes encoding ET-1, ET-2 and ET-3 are localized to chromosomes 6, 1 and 20, respectively [9–11]. Interestingly, the ETs possess a close structural and functional homology to a group of snake venom toxins, the sarafotoxins, which are found in the venom of the Middle Eastern burrowing asp, *Atractaspis engaddensis* [12–14].

An illustration of the massive amount of research conducted on the biology of the ETs and their putative physiological and pathophysiological roles is the realization that there have been over 3300 publications on this peptide family since the discovery of ET-1 nearly 6 years ago. Perhaps not surprisingly, in light of its recognized potent vasoconstrictor properties and the fact that it was originally isolated from endothelial cells, initial interest on the ETs focused on their activity and potential pathophysiological relevance in the cardiovascular system. However, research quickly revealed that the ETs possess a spectrum of diverse biological activities in a variety of other systems and tissues [12–14]. For example, there has been a significant increase in information in recent years on the actions of the ETs in the pulmonary system.

In this chapter a comprehensive review of our current knowledge of the influence of the ETs in various cells in the pulmonary system will be presented, with particular attention being paid to ET receptors. In addition, the evidence for a potential pathophysiological role of the ETs in respiratory tract disorders will be summarized and critiqued. Before the topics which are the major focus of this chapter are addressed, a brief synopsis of some of the general characteristics of the ETs and their relation to the pulmonary system, namely, distribution, synthesis, release, metabolism, uptake and clearance, will be given. Note, that most of the material relates to ET-1, which, compared to ET-2 and ET-3, has been the focus of research.

2. Distribution, Synthesis, Release, Metabolism, Uptake and Clearance

2.1. Distribution

Of the tissues and organs examined, ET levels in the lung are among the highest detected [15–18]. ET-1 mRNA was detected in human lung homogenates [19]. The cellular origins of ET have still to be clarified, although evidence indicates that they include the endothelium, epithelium, endocrine cells and some inflammatory cells. ET-like immunoreactivity (ir-ET) is present in most epithelial cells in the conducting airways of the rat and mouse [20]. In rabbit trachea, ET-1 immunoreactivity was detected in cells scattered throughout the epithelium, and was also observed in cultured tracheal epithelial cells [21]. Furthermore, in rat foetal lung significant quantities of ET mRNA are detected, localized in respiratory epithelial cells of bronchioles and also blood vessels [22]. In this study Northern blot analysis detected two forms of ET mRNA (2.5-kilobase and 3.7-kilobase forms), both of which were present in lung.

Immunocytochemical analysis and molecular biological techniques have also demonstrated ir-ET and mRNA, respectively, for the three ET isoforms in human airway epithelial and endocrine cells. Immunoreactivity was observed predominantly in pulmonary endocrine cells and was less evident in the airway epithelium (in about 50% of human adults) [23]. The amounts of immunoreactivity and mRNA present in vascular endothelial cells was highest in the developing lung, started to decrease before birth and was minimal in adults, and, accordingly, it was proposed that ET may play a role in growth regulation.

The expression and release of ET-1 has been demonstrated from human macrophages, a predominant inflammatory cell in the lung; the expression but not the release of ET-3 was noted [24]. Furthermore, ir-ET-1, but not ir-ET-3, is present in mouse primary bone marrow mast cells in large quantities [25].

2.2. *Synthesis*

It was proposed originally by Yanagisawa and co-workers that ET-1 was synthesized *via* an unusual, two-step, proteolytic process [7]. The initial stage involved the formation of a 39-amino acid residue intermediate, designated "big endothelin", from a 203-residue preproendothelin *via* the activity of an endopeptidase(s) which is specific for the paired dibasic amino acid residues [7]. Note, the human form of preproendothelin consists of 212-amino acid residues and big endothelin possesses 38 amino acids [8, 26]. Big endothelin then undergoes a previously unknown type of cleavage between Trp^{73} and Val^{74}, *via* the activity of a putative endopeptidase with chymotrypsin-like activity, which was called "endothelin-converting enzyme" or "ECE" [7]. The biosynthetic pathway for ET-1, in addition to the two potential molecular targets for therapeutic intervention, namely the ECE and also the ET receptors, are shown in Figure 1.

ET-1, Big ET-1 and also the carboxyterminal of Big ET-1 (big ET^{22-39}) are detected in the supernatant of cultured endothelial cells [27,

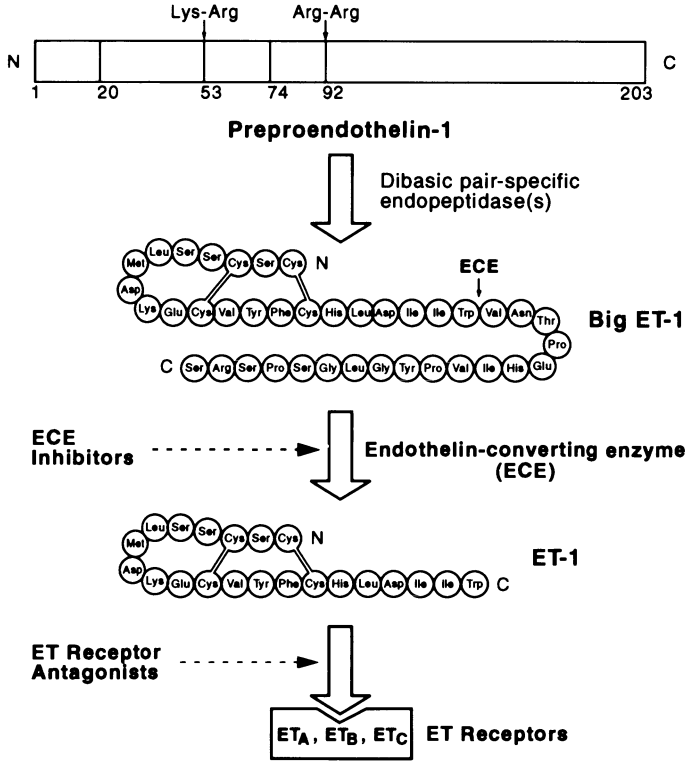

Figure 1. Endothelin-1 (ET-1) biosynthetic pathway and potential molecular targets for novel therapeutic agents (ET receptor antagonists and ECE inhibitors). Adapted from [7].

28] and in human plasma [29]. The vasoconstrictor potency of big ET-1 in isolated blood vessels is appreciably less than ET-1 [30, 31], suggesting that the formation of ET-1 is critical for optimal biological activity of the ET system. Furthermore, evidence indicates that ET is not stored preformed but is released constitutively by *de novo* synthesis, with the regulation of synthesis predominantly at the transcription level [12, 14, 32].

Until recently, there was considerable constroversy as to the identity, location and characteristics of the putative ECE, with evidence for the existence of at least three ECE-like enzymatic activities, two of which are cytosolic and the other membrane-associated. Most of the information favoured a membrane-bound, neutral metalloprotease, which is sensitive to phosphoramidon, as the most likely candidate as the physiologically relevant ECE [14, 32]. A phosphoramidon-sensitive (IC_{50} = 0.5 μM) neutral protease which was able to convert big ET-1 to ET-1 was identified in rat lung, with a relative abundance in the membrane versus the cytosol of 4:1 [33]. Furthermore, the partial solubilization and purification of ECE from porcine lung was reported recently [34] and successful purification to homogeneity of ECE from rat lung microsomes was demonstrated [35]. The purified enzyme had a molecular weight of 130 kD and specifically catalyzed the conversion of big ET-1 to ET-1 *via* a mechanism which was inhibited by phosphoramidon and metal chelators [35]. Sucrose-gradient ultracentrifugation analysis of rat lung microsomal fractions revealed that a major portion of the phosphoramidon-sensitive ECE activity appears to be associated with the Golgi apparatus, where it co-exists with endogenous ET-1 [36]. It was proposed that in rat lung most of the big ET-1 is converted to ET-1 during its passage *via* intracellular secretory pathways, although a smaller component of ECE may exist as an ecto-enzyme on the plasma membrane [36], accounting for the rapid one-pass conversion of intravenous big ET-1 to ET-1 in perfused lungs [37].

There is also evidence *in vivo*, and using perfused lungs, that the ECE responsible for the conversion of big ET-1 to ET-1 in airways is sensitive to phosphoramidon [38–40].

The presence of an aspartic protease with ECE activity in rat lung was reported [41]. Furthermore, evidence was presented for a novel, serine protease enzyme, which is present in the soluble fraction of porcine lung which degraded big ET-1 by cleavage at the bond between Val^{22} and Asn^{23}. This enzyme, which was sensitive to diisopropylfluorophosphate, was designated ET-Val-generating endopeptidase [42]. It was also suggested that lung mast cell-derived chymase produces extracellular processing of big ET-1 to ET-1 in the perfused rat lung [43]. The physiological significance of these putative ECEs in the pulmonary system has still to be ascertained and most of the evidence indicates that, as in other systems, the relevant ECE in the lung is a phosphoramidon-sensitive metalloprotease. In support of this assertion, and as

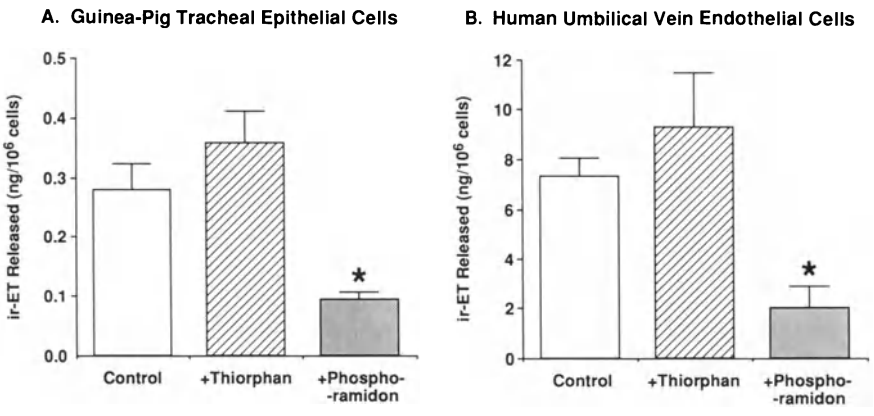

Figure 2. Effect of thiorphan (10 μM) or phosphoramidon (10 μM) on basal release of ir-ET-1, over a 24 hour period, from (A) cultured guinea-pig tracheal epithelial cells and (B) cultured human umbilical vein endothelial cells (HUVECs). Results are the Mean ± S.E.M. of 4 experiments. * indicates signficant compared to control, $P < 0.05$. The results indicate the ET release in both guinea-pig tracheal epithelial cells and HUVECs is sensitive to phosphoramidon (metalloprotease inhibitor; NEP inhibitor) but not thiorphan (NEP inhibitor) suggesting that the ECE responsible for the conversion of big ET-1 to ET-1 in both systems is a phosphoramidon-sensitive metalloprotease. Note the different scales for the y-axes in the two graphs indicating that the release of the ETs from HUVECs is much greater (ca. 30-fold) than from guinea-pig tracheal epithelial cells.

outlined in Figure 2, experiments performed in the author's laboratory indicate that basal release of ir-ET from guinea-pig cultured tracheal epithelial cells, and also human umbilical vein endothelial cells (HUVECs), is inhibited by phosphoramidon (100 μM) but not thiorphan (10 μM), another inhibitor of neutral endopeptidase (NEP; EC 3.4.24.11) [44]. The basal release of ir-ET from HUVECs was about 30-fold higher than from guinea-pig tracheal epithelial cells.

Considerably less is known about the processing of the precursors for ET-2 or ET-3 than for ET-1, and it is a controversial issue as to whether different ECEs exist which are specific for the individual ET peptides [45, 46].

2.3. Release

As indicated above, ET mRNA and ir-ET have been detected in airway epithelial cells and ECE is present in the lung. In support of these observations, ET-1 is released basally from porcine, canine [47] and human cultured bronchial epithelial cells [48] and guinea-pig [49, 50] and rabbit tracheal epithelial cells [51]. In addition, ET-3 was detected in supernatants from canine and porcine cultured tracheal epithelial cells [47]. ET-1 is also released from murine cultured bone marrow-derived mast cells following long-term incubation with IgE (10-fold

increase after 20 hr) [25]. The release of ET from cultured tracheal epithelial cells is increased by endotoxin, thrombin and various cytokines [49–51], whereas in perfused guinea pig lungs capsaicin, antidromic nerve stimulation or anoxia did not increase the outflow of ir-ET [52]. The regulatory role of cytokines on ET-1 synthesis and release may be important in inflammation and tissue repair associated with many pulmonary disorders, processes in which the cytokines are thought to play a key role.

2.4. Metabolism

The epithelium inhibits ET-1-induced contractile responses in guinea-pig trachea *via* a mechanism which is sensitive to phosphoramidon, an inhibitor of NEP [54]. The epithelium is a rich source of NEP [54] and it was hypothesized that this modulatory role was indicative of ET-1 being metabolized by epithelium-derived NEP [53]. The ETs were subsequently observed to be good substrates for NEP [55, 56]. Further functional data in support of a modulatory role of epithelium-derived NEP on ET-1-indiced contraction in guinea-pig trachea has been provided [57, 58], including the observation that recombinant human NEP decreased ET-1-induced contraction [58]. *In vivo* studies in guinea pigs have also demonstrated that phosphoramidon potentiates ET-1-induced bronchoconstriction [59]. In human bronchus, evidence indicated that NEP is involved in the local metabolism of ET-3 but not ET-1; similar findings were observed in rabbit bronchus [60]. However, other studies demonstrated that phosphoramidon potentiated contraction induced by ET-1 [61, 62], and also ET-2 and ET-3 [61], in human bronchus. It was concluded that the epithelium modulated ET-induced contraction in human bronchus, in part because of the removal of the metabolic influence of NEP, but also *via* other mechanisms [61]. In pig lung membrane fraction, NEP was identified as the principal metabolic pathway for ET-1 [63].

These and other data suggest strongly that two phosphoramidon-sensitive enzymes have opposing effects in the control of ET-1 levels in airways: thus, phosphoramidon inhibits the putative ECE responsible for the formation of ET-1 from big ET-1 and also NEP which appears to be involved in the breakdown of ET-1.

Although, NEP appears to be the major enzyme involved in ET-1 degradation in the airways, other pathways may also contribute. For example, soybean trypsin inhibitor essentially abolished the degradation of ET-1 induced by activated human polymorphonuclear neutrophils (PMNs), whereas phosphoramidon was without effect, suggesting that cathepsin G, rather than NEP, is involved in ET-1 metabolism by these cells [64].

2.5. Uptake and Clearance

Intravenously administered ET-1 is rapidly eliminated from the circulation in rats, with accumulation predominantly in the lungs and kidneys [65–67]; for example, it was calculated that 82% of [^{125}I]-ET-1 was taken up by the lungs [67]. Substantial removal of ET-1 by the pulmonary circulation ($>50\%$ in a single passage) also occurred in guinea-pig and rat isolated perfused lungs [68] and in rabbits significant uptake of ET-1 was demonstrated by the pulmonary but not the coronary circulation [69]. In contrast, during infusion of ET-1 in pig no evidence was observed for clearance in the lungs [17]. In two studies in humans, involving intravenous infusion of ET-1, it was estimated that the lung is the main organ responsible for its removal (approximately 50% of the elimination) [70, 71]. In contrast, another study concluded that ET was not extracted by the pulmonary system in humans [72]. Thus, marked species, in addition to regional, differences appear to exist in the contribution of the pulmonary circulation to the uptake and clearance of ET.

3. Receptors

3.1. Background

It quickly became apparent that quantitative and qualitative differences existed in the pharmacological profiles of the ET isoforms [12–14, 73]. For example, ET-1 is more potent than ET-2 in eliciting vasoconstriction whereas they have equivalent potencies as vasodilators [8, 74, 75]. In addition, it has been observed that administration of the ETs *in vivo* in animals elicits an initial transient depressor response followed by a sustained increase in blood pressure [7, 8, 74, 76]. This formed part of the initial indirect evidence to suggest that the ETs exert their diverse biological effects *via* multiple ET receptors which possess different tissue distributions [13, 14].

3.2. Biochemical and Molecular Biological Studies

Results of biochemical and molecular biological studies subsequently provided unequivocal evidence for the existence of distinct ET receptor subtypes. It is noteworthy that the lung, largely because of the abundance of ET receptors, has been used extensively in studies investigating ET receptor characterization, subtyping, isolation and purification.

For example, cross-linking, affinity labeling studies in rat lung membranes provided evidence for two ET receptors, a 44 kD type which had

higher affinity for ET-1 and ET-2 than ET-3 and another with a molecular weight of 32 kD which had a higher affinity for ET-3 [77]. Ligand binding and affinity labeling studies in porcine tissues, including lung, also suggested the presence of two distinct ET receptor subtypes, an ET-1-specific receptor and a receptor that was common to the ET/sarafotoxin family [78]. In this study, in both porcine and rat lung membranes three major bands, with molecular weights of about 120 000, 47 000 and 35 000, were labeled with [^{125}I]-ET-1 in cross-linking experiments. ET-1 prevented the labeling in all bands whereas ET-3 abolished only the labeling in the 35 000 molecular weight band. In another study photoaffinity labeling of the ET-1 receptor in bovine and rat lung membranes yielded only one labeled band with a molecular weight of 34 000 [79].

Using several systems, including rat and bovine lung, the cloning and expression of cDNA for a selective and also for a non-selective ET receptor, designated ET_A and ET_B, respectively, has been accomplished [14, 73, 74, 80–82]. The ET_A receptor has a higher affinity for ET-1 or ET-2 compared with ET-3, whereas, the ET_B receptor has equal affinity for the various ET isoforms. Evidence has also been provided in rat cultured anterior pituitary cells and rat PC12 phaeochromocytoma cells, for an ET receptor subtype, designated ET_C [14], which possesses selectivity for ET-3 [83, 84]. This receptor has recently been cloned from *Xenopus laevis* dermal melanophores [85]. All the ET receptors belong to the superfamily of G-protein-linked, seven transmembrane-spanning receptors [14, 73, 74, 80–82, 85]. A comparison of the characteristics of the three ET receptors is given in Table 1.

The human ET_A and the ET_B, but not ET_C, receptors have been cloned [82, 86–90]. For the ET_A or ET_B receptor there is considerable homology between species ($\geq 88\%$), although significant differences exist between the ET_A and ET_B receptors. The ET_A receptor was localized to chromosome 4 [87]. High or moderate distribution of mRNA for ET_A and ET_B receptors in human tissues has been detected in various tissues including lung (Table 1) [82, 88, 90]. There is no information on the distribution and function of ET_C receptors in humans.

The solubilization of ET_A and ET_B receptors from rat lung [91] and the purification of the ET_B receptor from bovine lung [92, 93] have been reported. In bovine lung, two forms of the ET_B receptor were identified, a 34 kDa species which was proposed to be a proteolytic compound of the native form (52 kDa molecular weight). Both forms exhibited nearly identical ligand affinities and specificities indicating that the binding activity of the receptor is located within the 34 kDa structure [92]. Cross-linking of [^{125}I]-ET-1 and [^{125}I]-ET-3 labeled solubilized ET receptors both produced a major band of 48 kDa and a minor band, 37 kDa, which was regarded as a proteolytic product [91].

Table 1. Properties of the ET_a, ET_B and ET_C receptor[1]

Parameter	ET_A	ET_B	ET_C	References
Relative agonist affinities	ET-1 ⩾ ET-2 ⩾ ET-3	ET-1 = ET-2 = ET-3	ET-3 ⩾ ET-1 = ET-2	14, 73, 74, 82, 85, 86, 88, 90
Seven transmembrane, G-protein linked receptor	yes	yes	yes	14, 73, 74, 80–82, 85, 88, 90
Homology	—	64% to ET_A	47% to ET_A 52% to ET_B	73, 82, 85, 86, 90
Amino acid number	427	442	424	82, 85, 86, 88–90
Potential glycosylation sites	2	1	2	82, 85, 86–90
Prominent distribution	heart, lung, colon, blood vessels, brain	endothelium, brain, lung, kidney, blood vessels, duodenum, adrenal, colon	unknown	82, 88, 90
Selective agonists	?	S6c, [Ala1,3,11,15]-ET-1 IRL 1620, BQ-3020	ET-3	85, 99, 110, 120, 121
Selective antagonists	BQ-123 FR 139 317	IRL 1038	None	100, 101, 122

[1] ET_A and ET_B are the human receptors whereas ET_C was cloned from *Xenopus laevis* dermal melanophores.

3.3. Functional Studies

There is accumulating data from functional studies in support of ET receptor subtypes in the pulmonary system. In the initial series of studies examining this phenomenon Maggi and co-workers comprehensively investigated the contractile effects of several members of the ET and sarafotoxin families and peptide analogs in isolated tissues including guinea-pig airways [94–96]. For example, they noted that the hexapeptide ET-(16-21) was a full agonist in guinea-pig bronchus but was without effect in rat aorta, whereas ET-1 effectively contracted both tissues. Based on these observations they provisionally designated two ET receptor subtypes as ET_A (representing the aorta) and ET_B (for bronchus) [95]. However, a subsequent study indicated that ET-(16-21) was a much less effective agonist in guinea-pig trachea and was without marked effect on [^{125}I]-ET-1 binding [97]. These data suggests that ET-(16-21) may be of limited usefulness for ET receptor classification. Following studies comparing the relative contractile activities of ET-1, ET-2 and ET-3, in addition to their ability to cause cross-sensitization, it was proposed that distinct ET receptors mediate contraction in guinea-pig pulmonary artery and trachea [98].

More direct evidence for ET receptor subtypes in the pulmonary system has been provided utilizing selective ligands, in particular, sarafotoxin S6c, the ET_B-selective agonist [99] and peptide ET_A-selective antagonists such as the cyclic pentapeptide BQ-123 [100] and FR 139317 [101]. Using these experimental tools, functional evidence was provided for distinct ET receptors mediating contraction in guinea-pig pulmonary artery and rat aorta (ET_A-subtype) compared with guinea-pig trachea and bronchus (non-ET_A, probably ET_B) [102, 103]. In human bronchus a non-ET_A (ET_B?) receptor population is the predominant one mediating ET-1-induced contraction whereas in human pulmonary artery contraction is meditated *via* the ET_A receptor [104] (Figure 3). In human bronchus ET_A receptors may be involved in smooth muscle proliferation [105].

In addition, regional differences in the relative distribution of ET_A and non-ET_A, probably ET_B, receptors occurs in guinea-pig airways. Thus, the contribution of ET_B receptors, assessed functionally using sarafotoxin S6c, predominated over ET_A receptors in guinea pig bronchus, and increased from the upper trachea (little contribution) to the lower trachea (marked contribution) [104]. In a more recent study utilizing various ligands, including antagonists, which interact with ET_A and ET_B receptors, it was concluded that ET_B receptors mediate contractions produced by ET-1 in guinea-pig trachea, bronchus and parenchyma and ET_A receptor activation also contributes to the response in guinea-pig trachea and parenchyma [106]. *In vivo* experiments indicate that ET-induced bronchoconstriction in guinea-pigs was not sensitive

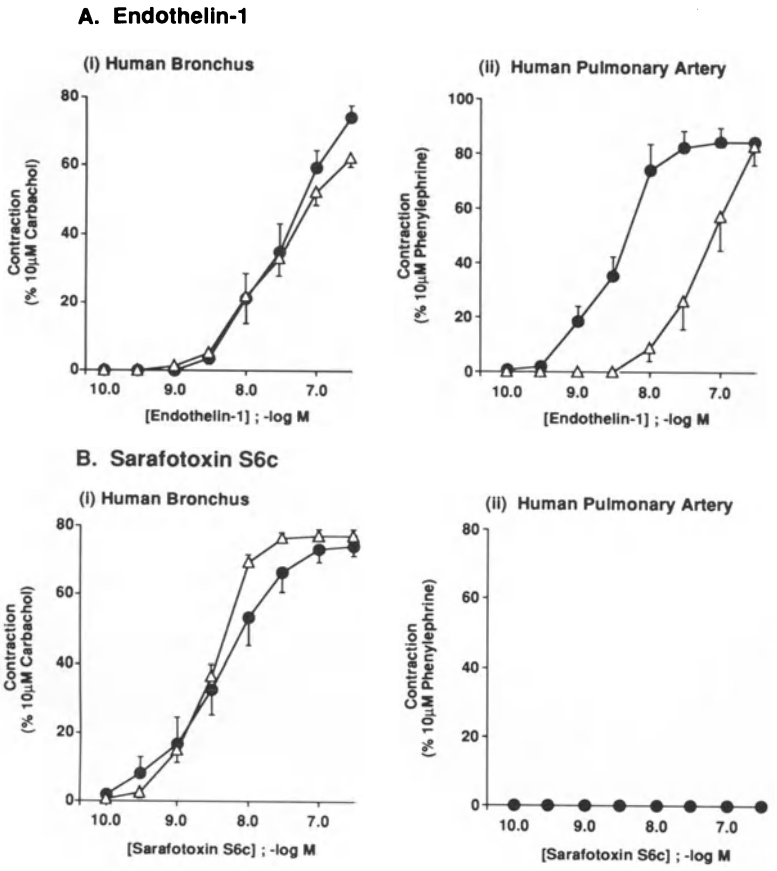

Figure 3. Effect of BQ-123 (10 μM), the ET_A receptor antagonist, on responses to (A) ET-1 and (B) sarafotoxin S6c (S6c), the ET_B-selective agonist, in (i) human bronchus and (ii) human pulmonary artery. Results are expressed as a percentage of the reference contraction and are the Mean ± S.E.M. of 5–7 experiments; ● = Control; △ = +10 μM BQ-123. BQ-123 antagonized ET-1-induced contraction in human pulmonary artery ($pK_B = 6.2$) but not in human bronchus. Furthermore, S6c did not contract human pulmonary artery. The results provide evidence that ET-1 induced contraction in human pulmonary artery is mediated *via* ET_A receptor activation whereas in human bronchus it is due to stimulation of a non-ET_A receptor, probably ET_B. Adapted from [104].

to BQ-123 and, therefore, does not appear to be mediated by ET_A receptor activation [107]. Interestingly, evidence was provided for different receptors mediating ET-1-induced contraction (non-ET_A) and prostanoid release in human bronchus (ET_A) [104]. In rat perfused lungs ET-1-induced PGI_2 release was antagonized by BQ-123 [108].

It was proposed initially that ET_A receptors on vascular smooth muscle elicit contraction whereas ET_B receptors located on endothelial cells produce vasodilatation [14, 73]. In support of this hypothesis, stimulation of the ET_A receptor subtype appears to mediate contraction

elicited by the ETs in most vascular smooth muscle [109–112]. ET_B-selective agonists have been shown to elicit endothelium-dependent relaxation in isolated vascular smooth muscles [113] and ET-1-induced relaxation in rat aorta is antagonized by the ET_B receptor antagonist, IRL 1038 [114]. However, ET_B-receptor activation mediates contraction in some vascular smooth muscles [113, 115–117]. It was proposed, based on binding studies in rat brain and atrium, that there are two subtypes of ET_B receptors, called ET_{B1}, which were designated as super-high affinity sites (in the pM range) and ET_{B2}, high affinity sites [118]. It was hypothesized that ET_{B1} receptors are involved in the vasodilator effects of the ETs, whereas, ET_{B2} receptors mediate the vasoconstriction.

There is *in vivo* functional evidence, obtained by exploring the responses and cross-tachyphylaxis induced by ET-1, ET-2 and ET-3, for the existence of ET_A-like and ET_C-like receptors in the cat pulmonary vascular bed [119].

Selective ligands for the ET_A, ET_B and ET_C receptor have been identified (Table 1). These include sarafotoxin S6c, [$Ala^{1,3,11,15}$]-ET-1 IRL 1620 and BQ-3020 (ET_B receptor agonists) [99, 110, 120, 121], BQ-123 and FR 139317 (peptide ET_A receptor antagonists) [100, 101] and IRL 1038 (peptide ET_B receptor antagonists) [122]. These compounds will continue to be invaluable experimental tools with which to decipher the receptors mediating the diverse biological effects of the ETs. In addition, the recently identified non-peptide receptor antagonists, such as Ro 46-20058 [111], SB 209670 [112] and CGS 27830 [123], may also be of therapeutic benefit in the various diseases in which the ETs have been implicated. Ro 46-20058 has about equivalent potency as an ET_A and ET_B receptor antagonist [111], whereas SB 209670 and CGS 27830 have higher affinity for the ET_A versus the ET_B receptor (ca. 20-fold) [112, 123]. A natural product isolated from the bayberry *Myria cenfera*, 27-0-caffeoyl myricerone (50-235) has been proposed to a selective ET_A receptor antagonist ($K_i = 51$ nM) with minimal affinity for the ET_B receptors [109, 124]. There are no known ET_C receptor antagonists.

It is likely that the present classicfiation of ET receptors is incomplete and there is increasing evidence from functional and binding studies for the existence of further subtypes of ET receptors [116, 125–128]. For example, evidence was presented for a novel subtype of isopeptide-nonselective ET_B receptor which mediates contraction to sarafotoxin S6c, ET-1, ET-2, ET-3 and IRL 1620 in swine pulmonary vein (not inhibited by the purported ET_B receptor antagonist, IRL 1038), and which is distinct from the ET_B receptor mediating ET-3-induced relaxation on precontracted swine pulmonary artery (antagonized by IRL 1038) [116].

3.4. Receptor Localization

Binding and autoradiographic studies indicate that significant quantities of a single class of high-affinity ET-1 binding sites are differentially located in various regions of the respiratory tract of several species including humans [97, 129–134]. In human bronchial tissues, labeling of [^{125}I]-ET-1 was localized largely to airways and vascular smooth muscle, with little or no binding on cartilage, connective tissues, the submucosal layer (including glandular cells) and the epithelium [129, 132]. Similar results were observed in mouse, rat and guinea-pig tracheal sections [132]. In contrast, recent studies in sheep trachea have revealed high densities of specific binding sites for ET-1 in cells associated with submucosal glands and in the submucosa immediately below the epithelium (lamina propria). In addition, in human, and also rat and guinea-pig airways, it was observed that there was significant binding associated with alveolar septae and parasympathetic ganglia, and also with paravascular nerves and nerves in the connective tissues [129, 130, 132, 134].

A single, specific high affinity binding site for [^{125}I]-ET-1 was detected on human cultured bronchial smooth muscle cells, with an apparent binding affinity (K_d) of 0.11 nM, and a maximum binding capacity (B_{max}) of 22.1 fmol/10^6 cells [48].

In contrast to some of the above studies, binding sites in airway epithelium have been reported. For example, some diffuse binding in the guinea-pig tracheal epithelium was noted, although the density of binding was much less (16%) than that detected in the smooth muscle and especially in the submucosal region (6%) [97]. Two binding sites for ET-1 were observed, with ET-3 able to interact with both sites. Similarly, significant labeling of [^{125}I]-ET has been detected in rat airway epithelium [65] and feline tracheal epithelial cells (two sites) [135]. A single class of binding sites for [^{125}I]-ET-1 ($K_d = 390$ pM and $B_{max} = 307$ fmol/mg protein) was detected in rat alveolar type II cells [136]. Furthermore, *in situ* hybridization techniques have revealed strongly positive staining for ET_B receptor probes in ciliated and non-ciliated rat and rabbit bronchial epithelial cells [137]. In addition, endothelium of large-calibre vessels, type II alveolar epithelial cells and visceral pleural mesothelium were stained. The presence of ET receptors on the same cells that are able to synthesize ET suggests that there may be autocrine regulation of its secretion [137].

Following *in vivo* labeling studies in rats the highest density of labeling of [^{125}I]-ET-1 was observed in the lung and kidney [65]. Furthermore, in a comparison of [^{125}I]-ET binding to various tissue membrane fractions, the highest density of binding was detected in trachea followed by lung parenchyma and *vas deferens* [138].

Both ET_A and ET_B receptors are abundant in mammalian lung. Using immunohistochemical and immunoprecipitation techniques with