On the molecular pharmacology of EndothelinA receptors : or how EndothelinA agonists can make a difference

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On the Molecular Pharmacology of Endothelin$_A$ Receptors

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Contents

Chapter 1

Introduction
Based on: ET_A Receptor Antagonists or Allosteric Modulators?

Jo GR De Mey, **Matthijs G Compeer**,
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The Endothelins

The existence of an endothelium-derived contractile factor (EDCF) was predicted in 1982 [1]. In 1988, Masashi Yanagisawa and colleagues reported on the identification of one of these EDCFs, which they named endothelin (ET) [2]. In mammals, this peptide belongs to a family of in total 3 isopeptides, ET-1, ET-2 and ET-3 [3]. Their amino acid sequences are shown in Fig. 1.1. Effects of these peptides are mediated via two types of receptors, Endothelin$_A$ (ET_A) and Endothelin$_B$ (ET_B) receptors [4], that display only 50 % sequence homology [5]. Both receptor subtypes are G-protein coupled receptors (GPCRs), subtype A [6, 7], but as this receptor family is known to signal also via systems other than G-proteins [8], the preferred term is 7 transmembrane domain receptors (7TMRs), as they cross the cellular membrane 7 times.

In the vasculature, mainly ET_A receptors mediate vasoconstriction and vasospasm and are therefore considered an interesting, large potential therapeutic target [9, 10]. The ET_B receptors on the other hand are involved in endothelium-dependent vasodilatations in the vasculature, counteracting the effects of ET_A receptors, as well as scavenging and clearance of circulating ET [11-13]. The endogenous agonists ET-1 and ET-2 bind and activate ET_A receptors with similar affinity and efficacy, (in the low or subnanomolar range), whereas ET-3 binds with much lower affinity to these ET_A receptors [14] (Fig. 1.1). All three ETs bind with similar affinity and activate with similar efficacy ET_B receptors. Therefore, ET-1 and ET-2 are considered non-selective ET-receptor agonists and ET-3 is a somewhat selective (30 fold), or ET_B preferring agonist [15, 16].

The most studied isoform, ET-1, is a 21 amino acid, bicyclic paracrine mediator [17, 18] and is the most abundant ET isoform in the vasculature. Via ET_A receptors, it can induce a variety of responses such as contractions and spasm in the vasculature, cellular proliferation, inflammation and oxidative stress [9, 10]. ET-2 is abundant in the urogenital and gastrointestinal systems [19-21] and differs from ET-1 in amino acid structure at positions 6 and 7 in the N-terminal loop [3] (Fig.1.1). Despite these structural differences, ET-2 is considered an ET_A agonist similar to ET-1, as these have similar affinity and efficacy [4, 10] (Fig.1.1). ET-3 is mainly known for its roles during embryonic development [22] and as a neurotransmitter in the adult central nervous system [23]. It differs in 6 amino acids from ET-1 and ET-2, which may explain its preference for ET_B over ET_A receptors [14] (Fig. 1.1).

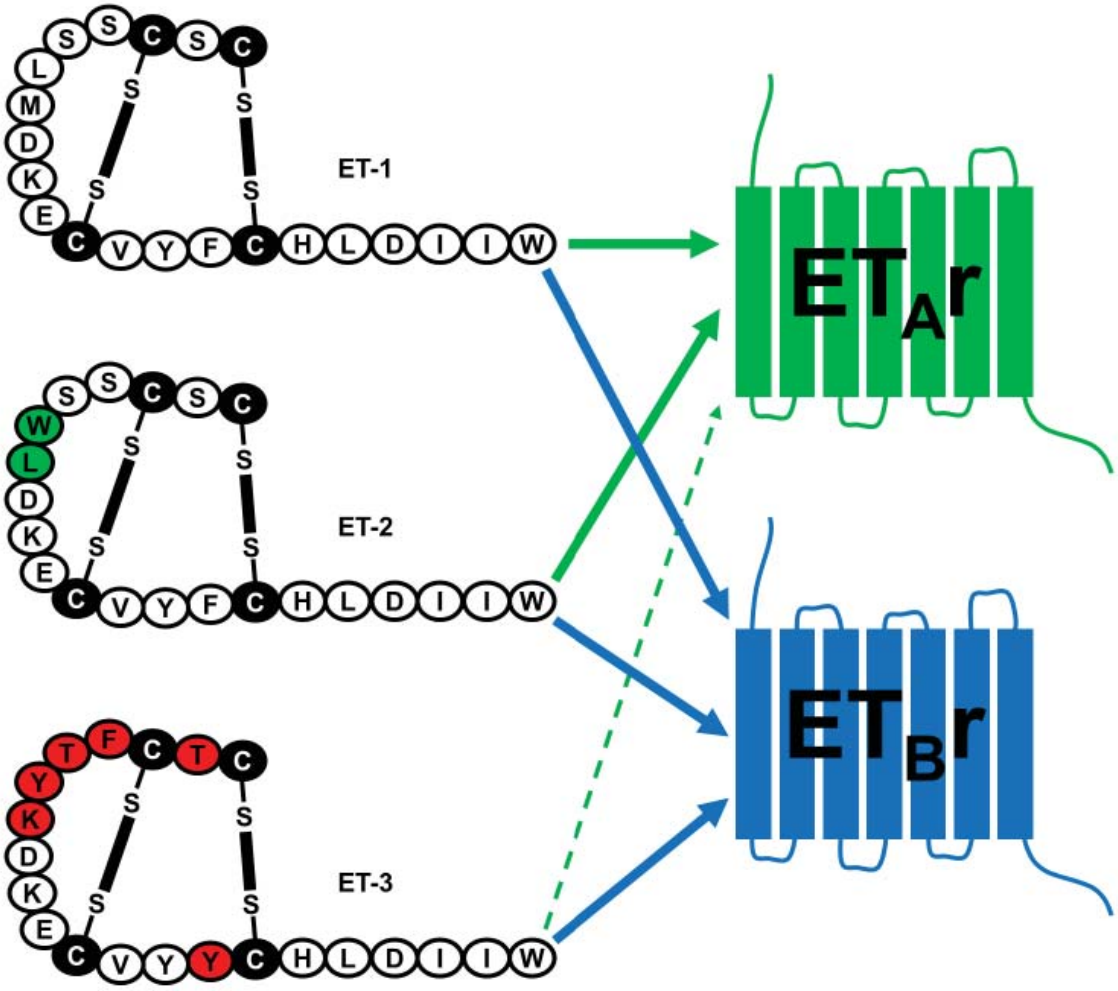

Figure 1.1. Amino acid structure of three ET isoforms and their preference for ET receptors.

Each ET isoform has its own gene, precursor peptides and enzymes to form the final effector 21 amino acid bicyclic peptide [3, 24], so (regulation of) expression of each isoform is distinct from the others.

The Endothelin receptors

An ET_A receptor is a flexible 7TMR protein that can isomerize between inactive and active conformations. Binding of an endogenous agonist to its orthosteric binding site on the receptor promotes transition of the receptor from an inactive to an active state that can interact with intracellular proteins involved in signal transduction [25]. The ET_A receptor interacts with several of these intracellular G-proteins, including $G_0\alpha$, $G_{i3}\alpha$, $G_{i1}\alpha$, $G_{i2}\alpha$ and $G_q\alpha/G_{11}\alpha$. It depends on the agonist used to activate the ET_A receptor which specific G-protein further mediates downstream signaling [26].

These downstream mechanisms can involve calcium influx and activation of phospholipase C-β (PLC-β), protein kinase C (PKC) and Rho-Kinase, and these intracellular mediators can in turn act as effectors of vasospasms, oxidative stress, cellular proliferation etc [10].

The ET_A receptor can be phosphorylated by G-receptors kinases (GRKs) and can subsequently bind arrestins [27-29]. Originally it was proposed that activated G proteins, GRK activity and arrestin binding cause desensitization, tachyphylaxis, internalization and tolerance of agonist-induced effects [30]. However, because ET_A mediated vasoconstrictor responses persist for a long time, chronically activated ET_A seems to be little affected by these negative feedback mechanisms. The long-lasting responses may involve tight binding of agonists to their receptors [31, 32], but this would not explain the molecular mechanisms of ongoing signaling by activated ET_A.

ET_A activation is promoted by binding of the endogenous peptidergic agonists to their orthosteric binding sites on the receptor. As previously mentioned, ET-3, which differs on 6 amino acid positions from ET-1 and ET-2 (Fig. 1.1), has much lower affinity for ET_A receptors compared to ET-1 and ET-2 [4]. Truncation, amidation or extension of the C-terminal Trp^{21} of ET-1 abolishes binding [33-35]. The linear analogue 4^{Ala}ET-1, in which both disulfide bonds are absent due to cysteine to alanine replacement, also does not bind to ET_A receptors [36, 37]. These observations indicate that the C-terminal tail, selected amino acids in the N-terminal loop and both disulfide bonds are all required for polyvalent binding of endogenous peptides to ET_A receptors [38, 39]. The orthosteric binding site of ET_A receptors would thus contain more than one functional domain. Studies using site-directed mutagenesis, chimeric receptors and photoaffinity labeling indicate that these orthosteric binding domains are located between transmembrane helices 1, 2, 3 and 7, and between transmembrane helices 4, 5 and 6 of ET_A receptors [40-43], (Fig. 1.2).

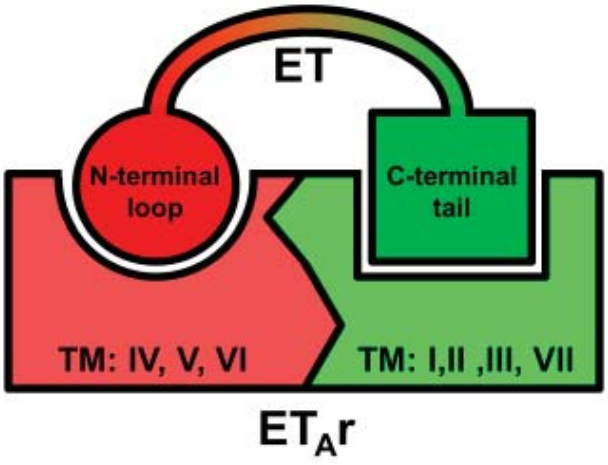

Figure 1.2. Schematic representation of the interaction between ET and ET_A. The initial interaction occurs when the ET C-terminal tail binds to the domain on ET_A consisting of TM I-III and VII. Consecutively the ET N-terminal loop can bind to another binding domain on ET_A consisting of TM IV-VI.

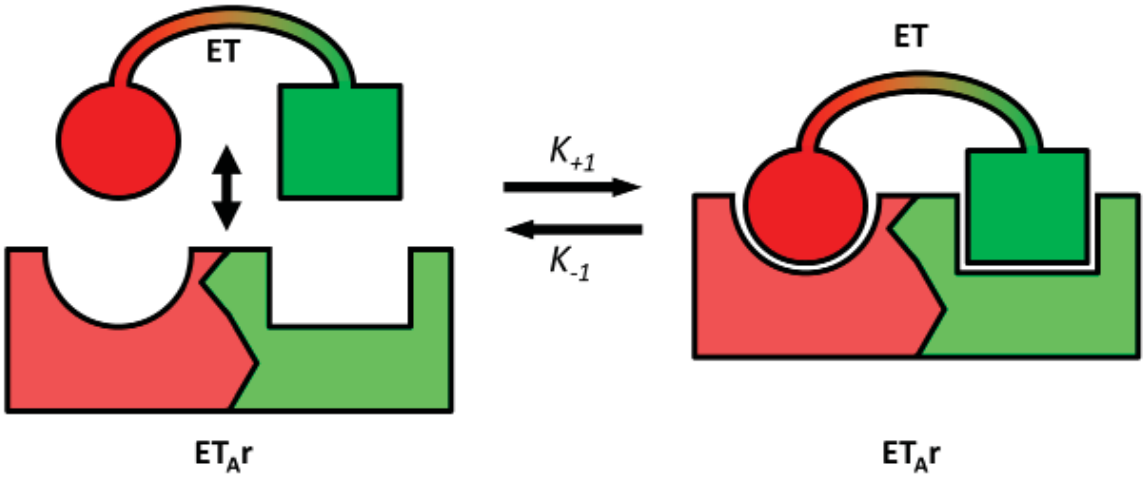

Figure 1.3. The 'dynamic' interaction of ET with ET_A receptors. When the dissociation rate constant (k_{-1}) is considerably less than the association rate constant (k_{+1}), a low concentration can locally act with high potency as a tight binding paracrine mediator.

Conditions of dynamic equilibrium govern most classical and modern theories of molecular pharmacology [44]. These conditions do not easily apply to ET_A in view of the slow dissociation of ET-1/ET_A receptor complexes [32, 45, 46]. Hence, reported affinity measures such as the 'equilibrium' dissociation rate constant K_d are mere approximations. When the dissociation rate constant of the agonist/receptor complex (k_{-1}) is considerably less than its association rate constant (k_{+1}), very low concentrations of agonist can act locally with high potency because $K_d = k_{-1}/k_{+1}$ (Fig. 1.3) [47, 48].

This is an effective mechanism for a paracrine mediator. However, conditions of dynamic equilibrium become hard to establish in routine experimental settings such as acute concentration-response studies. Not only is the agonist concentration a crucial factor, but the duration and history of agonist exposure (and therefore the patience of the scientist) also become determining factors that influence binding and effects. Theoretically, both tightness of agonist/receptor complexes and slow reversibility of receptor activation can contribute to long-lasting agonism. Both mechanisms complicate the potential of an antagonist to inhibit the agonist-induced responses. Additionally, physiology requires a counterbalancing system, likely to be located in the arterial wall itself, to functionally antagonize the persistent constrictor responses induced by ETs. Candidate functional antagonists include endothelium-derived nitric oxide (NO), the release of which can be stimulated by ET-1 [49], or vasodilator neurotransmitters from peri-arterial sensory-motor nerves, of which the release can also be stimulated by ET-1 [50, 51].

Receptor antagonists

An antagonist is still frequently seen as an agent that can occupy an orthosteric binding site on the receptor (i.e. where the endogenous ligand binds to, and activates, the receptor) without altering the activation of the receptor: a neutral competitive antagonist [25]. In general pharmacology, however, an antagonist is defined as a drug that reduces the action of another drug, generally an agonist [47]. An antagonist can therefore be a chemical antagonist, a functional antagonist, a physiological antagonist, a neutral competitive antagonist, an inverse agonist and a negative allosteric modulator.

An allosteric modulator is, as its name suggests, a compound that modulates a receptor by binding to a site on the receptor, other than the orthosteric site; an allosteric site (Fig. 1.4). These allosteric sites are an interesting drug target for several reasons. Evolution may pressure a receptor to accommodate endogenous ligands on an orthosteric site, but may spare an allosteric site from this pressure. An allosteric site might therefore be a drugable target unique to its receptor,which infers that targeting an allosteric site could yield drugs with greater receptor subtype selectivity, a more physiological signaling profile and a greater efficacy/safety profile [52].

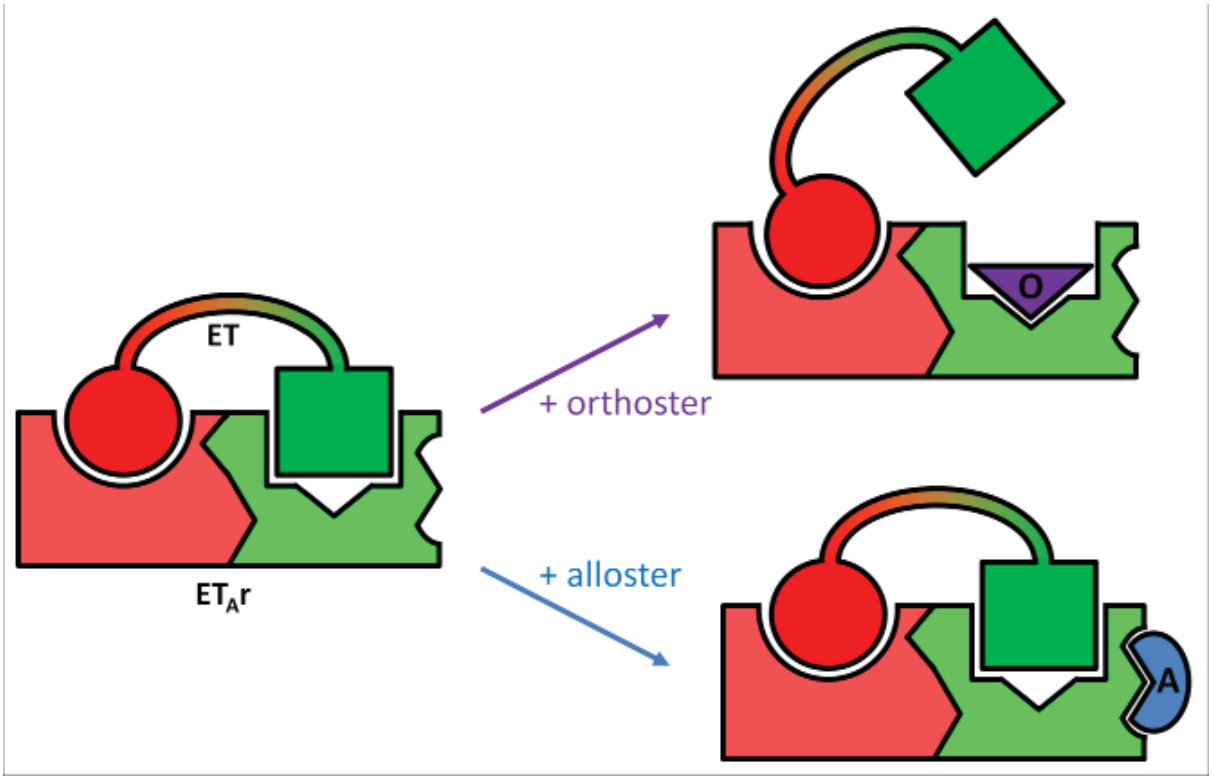

Figure 1.4. Mechanisms by which an antagonist can interact with an agonist/receptor complex. An orthosteric antagonist would bind to a site on the receptor shared with the agonist. An allosteric antagonist can bind to a distinct site on the receptor, away from the agonist binding site.

The concept of allosterism in itself describes one kind of interaction by a compound (with its receptor) (Fig. 1.4), but the nature of an alloster can necessitate detailed nomenclature [53]. Positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs), i.e. ligands that increase or decrease receptor activity, respectively, can be further specified into enhancers, enhancer-inhibitors and allo-competitors, amongst others.

To complicate the matter even further, labeling a compound an orthoster or alloster is all a matter of perspective [54]. An endogenous ligand of a 7TMR can be considered an orthoster as it increases receptor activation by binding to the extracellular site. However, an intracellular bound G-protein alters receptor activity and could therefore be considered an orthoster, which automatically makes the endogenous ligand on the extracellular site of the receptor an alloster.

So what about ET receptor antagonists (ERAs)? Soon after the discovery of ET-1 and the cloning of its ET_A and ET_B receptors, low molecular weight compounds were identified that can prevent the binding of radioactively labeled ET-1 [55, 56]. These ERAs initially resulted from screening efforts (e.g. BQ123 [57] and bosentan [58]) and later, rational drug design focused on the C-terminal hexapeptide that is common to all endogenous ET peptides, resulting in several classes of ERAs available nowadays [59], of which a few well-described ERAs are listed in Table 1.1.

Table 1.1. Characteristics of widely used ERAs.

Name	Class	MW	ET_A IC_{50} (nM)	ET_B IC_{50} (nM)	Selective
BQ123 [57]	peptide-based	611	22	18000	ET_A
FR139317 [60]	peptide-based	591	0.53	4050	ET_A
PD156707 [61]	butenolide	507	0.31	417	ET_A
BMS193884 [62]	biphenyl sulfonamide	395	1.4	1900	ET_A
Bosentan [58]	pyrimidine sulfonamide	552	4.7	95	-

Some of these discriminate between ET receptor subtypes, whereas others do not [63]. Literature suggests that all ERAs act as neutral competitive antagonists. However, ERAs can prevent agonist binding, but do not reverse established agonist-receptor complexes in membrane preparations [32, 46], adding evidence for the tight nature of the agonist/receptor complexes. As a result, certain ERAs prevent responses to ET-1 but do not influence responses that were initiated by the peptide [31]. As mentioned earlier, the endothelin system is (still) considered as a promising therapeutic target [9, 64]. Antagonizing the ET-receptors, either selectively against ET_A or non-selectively against both ET_A and ET_B, is currently the preferred approach,

rather than targeting ET-production [65] or ET breakdown [66]. In addition to its obvious potential in the cardiovascular system, in which currently the only clinically approved ET receptor antagonists, bosentan (Tracleer®) and ambrisentan (Volitris®) are used to treat pulmonary arterial hypertension [67, 68], targeting the ET system is considered in other pathophysiological conditions. Because ET-1 and its receptors have emerged as relevant players in tumor growth and metastasis, by e.g. regulating mitogenesis and cell survival [69], its therapeutic window in treatment of cancer, specifically ovarian cancer, is currently being studied [8]. Targeting ET_A receptors in cancer would not only benefit cancer treatment, but also cancer-related pain [70]. And in other types of pain, whether the pain is related to an inflammatory responses or an acute pain, ET-1 appears to induce nociception that could be treated, to some extent, by the use of ERAs [71]. And since ET-1 regulates cell proliferation and extracellular matrix turnover, patients that suffer from scleroderma is another patient group that could benefit from anti-ET drugs [72].

Relevance for anti-ET therapy

Some of the clinical trials with ERAs have been less successful than anticipated [13, 55, 56], perhaps as a result of underestimation of the complexity of the molecular pharmacology of ET_A receptors. To resolve this, it will be necessary to define the signaling mechanisms during the long-lasting effects resulting from ET_A stimulation. Testing of reversing or curative effects of putative antagonists, either functional or acting on ET_A, should take into consideration that ET-1 dissociates only slowly from ET_A receptors.

Functional antagonists of ET_A receptors are any vasodilator compound that (in) directly relaxes ET_A mediated vasoconstriction. In a recent study this is the case for not only NO and calcitonin gene-related peptide (CGRP), but also for forskolin (direct stimulus of adenylate cyclase), isoproterenol (β-adrenergic stimulus of adenylate cyclase) and pinacidil (K_{ATP}-channel opener) [73].

It is worth considering currently available, but also future ERAs, as negative allosteric modulators. Criteria of allosterism that could be addressed [54, 74, 75] include distinct effects on apparent agonist affinity and efficacy, probe/agonist-dependence and system dependence. These approaches could result in drugs that become more effective when the endogenous ET system is more activated, and in drugs that can discriminate between ET_A mediated effects of the distinct endogenous agonists ET-1, ET-2 and ET-3.

Aim of this thesis

ET_A receptors have a rather unique pharmacology regarding their interactions with their endogenous ligands. This is not compatible with homeostasis of the vascular system, unless an endogenous system exists that counterbalances effects of the tight agonism of the ET_A agonists. Therefore, firstly we aimed to find which physiological mechanism could effectively counterbalance the vascular responses to ET-1-induced, ET_A mediated responses (**Chapter 2**).

Because this a-typical receptor pharmacology complicates utilizing the therapeutic potential of targeting the ET_A receptors in various pathologies, we secondly aimed to characterize to which extent the amino acid structure of the endogenous agonists contributes to the tight binding to and activation of ET_A receptors (**Chapter 3**).

Thirdly, we aimed to determine which of the intracellular signaling mechanisms is responsible for the long-lasting, persistent responses to ET_A agonists (**Chapter 4**).

Fourthly, we aimed to define in more detail the interaction of ETs with ET_A receptors and to define a role for allosteric modulators rather than neutral competitive antagonists to interact with ET_A receptors (**Chapter 5**).

Finally, we will discuss the implications of our findings and how, in the future, our work can contribute to better, more effective compounds to (therapeutically) target ET_A receptors (**Chapter 6**).

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Chapter 2

Stimuli of Sensory-Motor Nerves Terminate Arterial Contractile Effects of Endothelin-1 by CGRP and Dissociation of ET-1/ET_A Receptor Complexes

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Abstract

Endothelin-1 (ET-1), a long-acting paracrine mediator, is implicated in cardiovascular diseases but clinical trials with ET-receptor antagonists were not successful in some areas. We tested whether the quasi-irreversible receptor-binding of ET-1 (i) limits reversing effects of the antagonists and (ii) can be selectively dissociated by an endogenous counterbalancing mechanism.

In isolated rat mesenteric resistance arteries, ET_A-antagonists, endothelium-derived relaxing factors and synthetic vasodilators transiently reduced contractile effects of ET-1 but did not prevent persistent effects of the peptide. Stimuli of peri-vascular vasodilator sensory-motor nerves such as capsaicin not only reduced but also terminated long-lasting effects of ET-1. This was prevented by CGRP-receptor antagonists and was mimicked by exogenous calcitonin gene-related peptide (CGRP). Using 2-photon laser scanning microscopy in vital intact arteries, capsaicin and CGRP, but not ET_A-antagonism, were observed to promote dissociation of pre-existing ET-1/ET_A-receptor complexes.

Irreversible binding and activation of ET_A-receptors by ET-1 (i) occur at an antagonist-insensitive site of the receptor and (ii) are selectively terminated by endogenously released CGRP. Hence, natural stimuli of sensory-motor nerves that stimulate release of endogenous CGRP can be considered for therapy of diseases involving ET-1.

Introduction

Prototypic G-protein coupled receptors (GPCR) are characterized by tight agonist concentration-response relationships on the short run and by tolerance on the long run. For instance, acute β_2-adrenoceptor stimulated cAMP production and the resulting smooth muscle relaxation are readily reversible as a result of rapid dissociation of the agonist-receptor complexes. This property underlies the therapeutic applicability of drugs that inhibit the synthesis or the receptor-binding of endogenous GPCR-agonists. During prolonged exposure to agonists, β_2-adrenergic effects fade as a result of phosphorylation, desensitization, uncoupling from the G-proteins and internalization of the receptors (for review see [1]).

In sharp contrast, the GPCR-agonist endothelin-1 (ET-1) causes long-lasting effects. Its in vitro arterial contractile effects persist after thorough washout of the agonist [2]. Its in vivo vasopressor effects are maintained long after clearance of the peptide from the circulation by the lungs and the kidneys [3]. The 21 amino acid bicyclic peptide, that is constitutively expressed by the endothelium and that can be induced in several other cell types [4, 5], is implicated in several cardiovascular diseases [4, 6, 7], cancers [8] and pain [9]. Its vasoconstrictor, pro-inflammatory, oxidative and mitogenic effects are mediated by ET_A receptors [4, 6, 7] while more beneficial effects such as endothelium-dependent vasodilatation and scavenging of circulating ET-1 are mediated by distantly related ET_B receptors [4-7, 10]. ET_B agonism can be mimicked by short C-terminal fragments of ET-1 [11-13] but high affinity ET_A agonism requires the full length, both disulfide bonds and distinct amino acids in the N-terminal loop of the peptide [12, 14-18]. This suggests that distinct parts of ET-1 have different functions in binding and activation of ET_A receptors. Several classes of low molecular weight ET_A selective or mixed ET-receptor antagonists have been developed primarily on the basis of prevention of the binding of ET-1 to its receptors [4-6, 19-21]. These compounds are thought to compete with the C-terminal tail of the agonist. They can prevent ET-1-induced effects in vitro (for review see [2]) and in animal studies [4, 6, 19]. They are, however, less effective in reversing the effects of ET-1 in vitro [2], in animal studies [22] and in clinical trials [6, 23]. This may be due to the atypical properties of ET_A receptors.

Irreversible agonism by ET-1 is incompatible with homeostasis unless counterbalancing systems exist. ET-1 can stimulate NO release from the endothelium [24]. NO reduces ET-1 synthesis [25] and counteracts vasoconstriction initiated by ET_A receptors on smooth muscle cells [4, 7, 26]. ET-1 can also promote activity of transient receptor potential (TRP) cation channels that stimulate release of

vasodilator neurotransmitters from peri-arterial sensory-motor nerves (SMN) [27, 28]. Hence, in cardiovascular diseases characterized by reduced bioavailability of endothelium-derived NO, ET-1 and ET_A effects are upregulated [4] and can be tempered by counterbalancing effects of SMN [29-31]. Whether the latter involves functional antagonism or a selective effect on ET_A receptors has not been addressed.

In this study, we hypothesized that polyvalent agonist-receptor binding by ET-1 limits reversing effects of ET-receptor antagonists and used physiological reasoning to search for a superior inhibitor. For these purposes we studied rat mesenteric arteries in which ET_A and ET_B receptors are expressed by several cell types [31-33]. We discovered that calcitonin-gene related peptide (CGRP) released from peri-arterial SMN terminates long-lasting vasoconstrictor effects of ET by promoting dissociation of ET-1/ET_A receptor complexes.

Results

Key role of smooth muscle ET_A receptors in long-lasting arterial contractile responses to ET-1

In isolated rat mesenteric resistance arteries, the ET_B selective agonist $Ala^{1,3,11,15}$ET-1 [10] (1 nM–1 μM) caused neither contraction (Table 1) nor relaxation (data not shown). In contrast, the non-selective agonist ET-1 [10] potently stimulated contractions (Fig. 2.1A, table 2.1). The concentration-response relationship was steep and the responses were quasi-irreversible ($T_{1/2}$ >20 min versus $T_{1/2}$ ≈30 sec for similarly strong contractile responses to norepinephrine (NE) (Fig. 2.1A/B). Contractile effects of ET-1 and their persistence were not modified by 1 μM BQ788 (ET_B antagonist) [10, 34], 100 μM L-NAME and 10 μM indomethacin (which reduce endothelial influences), nor by pre-treatment with capsaicin (1 μM during 20 min, which reduces effects of SMN) (Fig. 2.1A and table 2.1). Mechanical removal of the endothelium resulted in a small increase in the sensitivity for ET-1 (Table 2.1). The sensitivity to ET-1 was reduced in presence of the ET_A antagonists BQ123 [10], [35] (1 μM), SB234551 [10, 21] (10 nM) or bosentan [10, 19] (3 μM studied in presence of 1 μM BQ788 to focus on ET_A antagonism by bosentan, a mixed $ET_{A/B}$ receptor antagonist) (Fig. 2.1A, Suppl. Fig. 2.1.1A).

Partial and transient reversing effects of ET_A antagonists

Although BQ123, SB234551 and bosentan prevented contractile responses to up to

Table 2.1. Arterial effects of ET-1 and two analogues.

Agonist	Condition	EC_{50} (nM)	E_{max} (% NE_{max})	Tension (%NE_{max}; 8 min after agonist removal)
ET-1	-	4.9±0.8	88.5±4.0	80.9±3.5
	Denuded	2.1±0.2 *	100.1±6.2	82.54±5.4
	L-NAME + INDO	3.5±0.5	100.4±5.0	79.2±3.3
	CAPS + L-NAME + INDO	3.6±0.3	104.0±4.0	84.5±14.0
Rh-ET-1	CAPS + L-NAME + INDO	4.1±0.3	102.0±10.0	80.6±14.6
$Ala^{1,3,11,15}$-ET-1	CAPS + L-NAME + INDO	>1 µM	0	0

Potency, efficacy and persistence (response remaining at 8 min after agonist removal) are shown for arteries without and with pre-treatment with capsaicin (1 µM, 20 min; CAPS) and presence of L-NAME (100 µM) and indomethacin (10 µM; INDO) and for denuded arteries. n = 6–10. *: p<0.05 vs control.

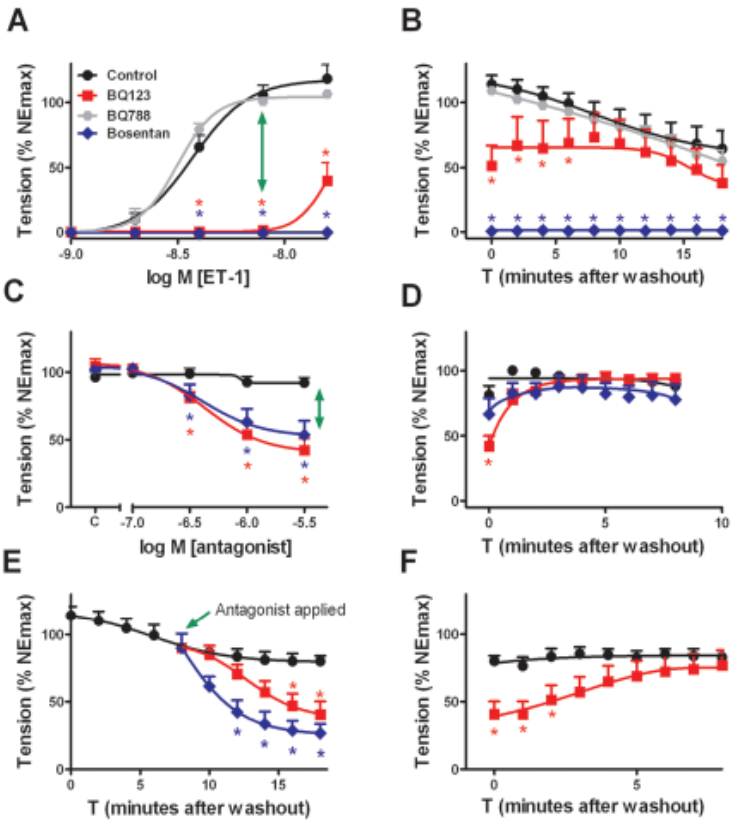

Figure 2.1. Partial and reversible reversing effect of ET-receptor antagonists on arterial contractile responses to ET-1 and their persistence. Isolated rat mesenteric resistance arteries were studied after treatment with capsaicin and in continuous presence of L-NAME (100 µM) and indomethacin (10 µM). A, responses to 0.25–16 nM ET-1 in absence (black) and presence of BQ123 (1 µM, red), BQ788 (1 µM, grey) or bosentan (3 µM in presence of 1 µM BQ788, blue). BQ123 and bosentan prevented responses to up to 8 nM ET-1. B, vasomotor tone after removal of free agonist and antagonist. C, effects of BQ123 and bosentan (0.1–3.0 µM) in the presence of 8 nM ET-1. D, vasomotor tone after removal of free agonist and antagonist. E, effect of BQ123 (1 µM) and bosentan (3 µM) on contractile responses initiated by 8 nM ET-1 that persisted in absence of the peptide. F, vasomotor tone after removal of free antagonist. n = 6–20. *, P<0.05 vs. control.

8 nM ET-1, the antagonists could only partly (≈50%) reverse contractile responses initiated by 8 nM ET-1 (Fig. 2.1C, Suppl. Fig. 2.1.1C). The relaxing effect of the antagonists was reversible, i.e. vasomotor tone rapidly recovered after washout of the ET-receptor ligands (Fig. 2.1D, Suppl. Fig. 2.1.1D). This indicates irreversible agonism and reversible antagonism. In addition, contractile effects of ET-1 that persisted in absence of free agonist were partly and transiently reduced by the antagonists (Fig. 2.1E/F, Suppl. Fig. 2.1.1E/F).

Transient reversing effects of endothelium-derived and exogenous vasodilators

In contrast to ET-antagonists, several vasodilator stimuli fully reversed contractile responses to ET-1 (Fig. 2.2A). This was the case for acetylcholine (endothelium-dependent vasodilator), forskolin (direct activator of adenylyl cyclase), isoproterenol

(beta-adrenergic stimulus of adenylyl cyclase), Na-nitroprusside (NO-donor) and pinacidil (activator of K_{ATP}-channels) (Fig. 2.2C). However, vasomotor tone rapidly recovered in absence of vasodilators and ET-1 (Fig. 2.2D). Moreover, contractions remaining after exposure to ET-1 could be relaxed by for instance acetylcholine (Fig. 2.3B) but again this inhibitory effect was reversible (Fig. 2.3C).

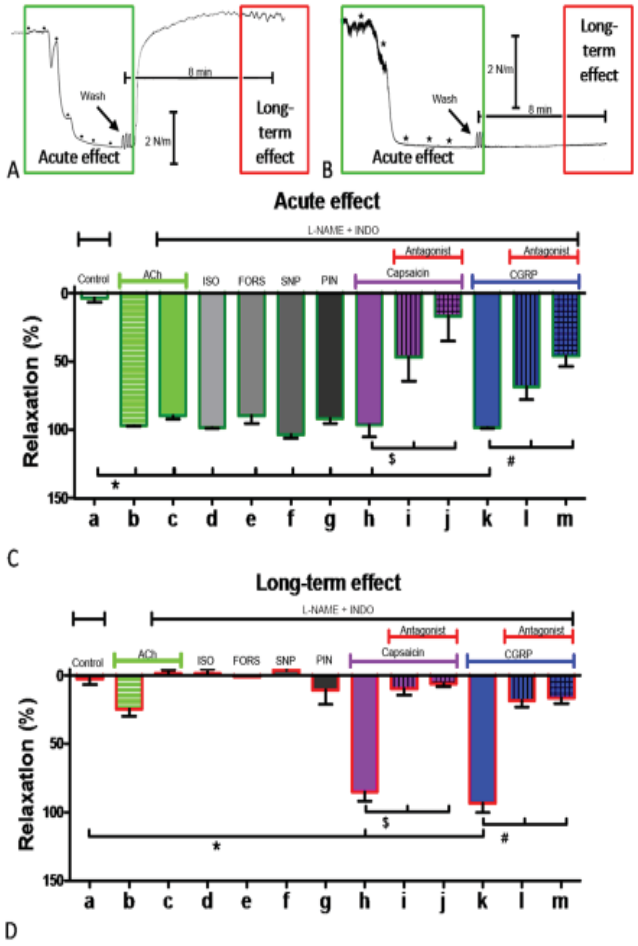

Figure 2.2. Capsaicin and CGRP relax endothelinergic arterial contraction and prevent the persistent contractile effect of ET-1.
Isolated rat mesenteric resistance arteries were studied in presence of L-NAME (100 µM) and indomethacin (10 µM) (as indicated) and were contracted with ET-1 (16 nM). Increasing vasodilator concentrations were administered until a maximal effect was observed. Thereafter vasoconstrictor and vasodilator stimuli were removed from the organ chamber while the recording of active wall tension continued for >10 min. A and B, typical tracings of active wall tension (WT) versus time (t) illustrating acute relaxing effects (green box) of acetylcholine (A; 0.01–10 µM) and capsaicin (B; 0.01–1 µM) and rapid recovery of contraction after removal of the vasodilator (long-term effect, red box) in the case of acetylcholine (A) but not capsaicin (B). C, maximal acute relaxing effects of various dilators. D, long-term effects of various dilators. a, time control; b and c, acetylcholine; d, isoproterenol; e, forskolin; f, Na-nitroprusside; g, pinacidil; h – j, capsaicin in the absence (h) and presence of $CGRP_{8-37}$ (i) or BIBN4096BS (j); k - m, CGRP in the absence (k) and presence of $CGRP_{8-37}$ (l) or BIBN4096BS (m). For concentrations of vasodilators see “Methods” section. n = 6–8. *, $ and #: P<0.05 vs. control, capsaicin or CGRP, respectively.

Effects of TRP-channel activators and CGRP

In contrast to these vasodilators, capsaicin relaxed ET-1-induced contractions (Fig. 2.2B/C) and prevented their recovery (Fig. 2.2B/D). This was also observed with rutaecarpine and with allyl isothiocyanate (Fig. 2.4A/B). In the case of rutaecarpine these effects were endothelium independent (Suppl. Fig. 2.1.2). Capsaicin, rutaecarpine and allyl isothiocyanate stimulate release of several neurotransmitters from SMN [27, 36-40]. The CGRP-receptor antagonists $CGRP_{8-37}$ [41] (1 µM) and BIBN4096BS [42] (20 nM)) reduced both the relaxation and the prevention of persistent effects of ET-1 by the SMN stimuli (Fig. 2.2C/D, Fig. 2.4A/B). Moreover, exogenous CGRP relaxed ET-1-induced contractions (Fig. 2.2C), prevented recovery of contractions initiated by ET-1 (Fig. 2.2D) and

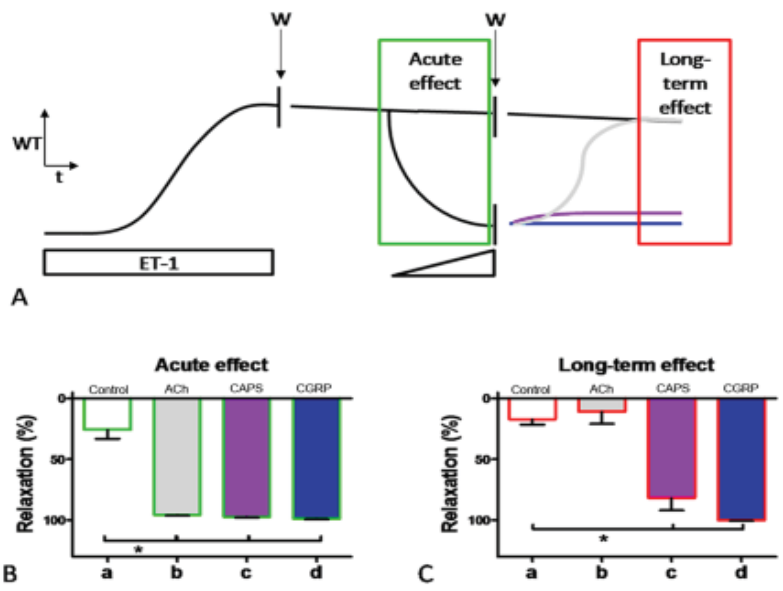

Figure 2.3. Capsaicin, CGRP and acetylcholine relax endothelinergic arterial contraction that remained after removal of ET-1 from its biophase but only capsaicin and CGRP prevent the persistent contractile effect of ET-1.
A, schematic tracings of active wall tension (WT) versus time (t) illustrating i) ET-1-induced contractions that are not reversed upon agonist removal (W), ii) acute effects of various dilators and iii) prevention of long-term ET-1 effects by capsaicin (purple) and CGRP (blue) but not acetylcholine (grey). B, maximal acute relaxing effects of acetylcholine (b), capsaicin (c) or CGRP (d). C, long-term effects of acetylcholine (b), capsaicin (c) or CGRP (d). For concentrations of vasodilators see "Methods" section. n = 6–8. *: P<0.05 vs. control.

caused long-lasting inhibition of the persistent effects initiated by ET-1 (Fig. 2.3A/B). These effects were endothelium independent (Suppl. Fig. 2.1.2) and were reduced by CGRP-receptor antagonists (Fig. 2.2C/D). The contraction that persisted after exposure to ET-1 and that was transiently inhibited by ET-receptor antagonists (Fig. 2.1E/F) or by acetylcholine (Fig. 2.3B/C), was terminated by capsaicin and by CGRP (Fig. 2.3B/C).

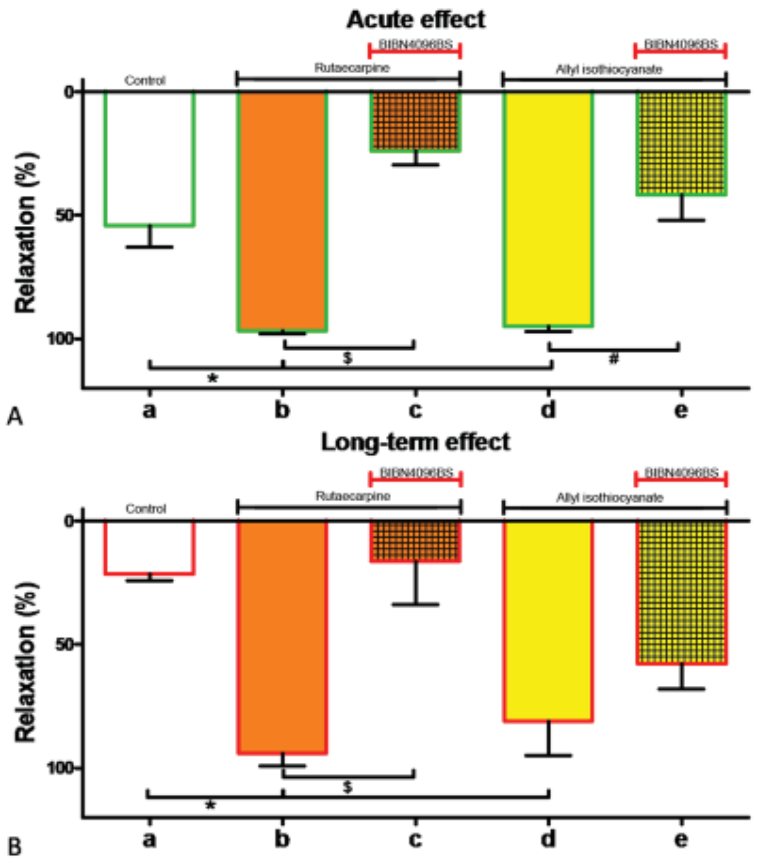

Figure 2.4. Stimuli of SMN, like rutaecarpine and allyl isothiocyanate, relax endothelinergic arterial contraction and prevent the persistent contractile effect of ET-1.
Isolated rat mesenteric resistance arteries were precontracted with 16 nM ET-1. Increasing concentrations of vasodilator compounds were administered until a maximal effect was observed. Thereafter vasoactive stimuli were removed from the organ chamber while the recording of active wall tension continued for >10 min. A, maximal acute relaxing effects of rutaecarpine and allyl isothiocyanate. B, long-term effects of rutaecarpine and allyl isothiocyanate. a, time control; b, rutaecarpine; c, rutaecarpine in presence of BIBN4096BS; d, allyl isothiocyanate; e, allyl isothiocyanate in presence of BIBN4096BS. For concentrations of vasodilators see "Methods" section. n = 6–8. *, $ and #: P<0.05 vs. control, rutaecarpine and allyl isothiocyanate, respectively.

When arteries were transiently exposed to a high concentration of CGRP (100 nM; Fig. 2.5A) or to ET-1 (16 nM) and then to CGRP (100 nM; Fig. 2.5B), exogenous ET-1 (1–16 nM; applied after removing other vasoactive compound from the organ bath) caused contractions with a potency and an efficacy that deviate only marginally from those observed in controls (Fig. 2.5C/D). This suggests that CGRP does not induce a long-lasting relaxing effect (Fig. 2.5C) but rather promotes dissociation of previously established ET-1/ET_A receptor complexes allowing re-application of ET-1 to again induced contractile responses (Fig. 2.5D).

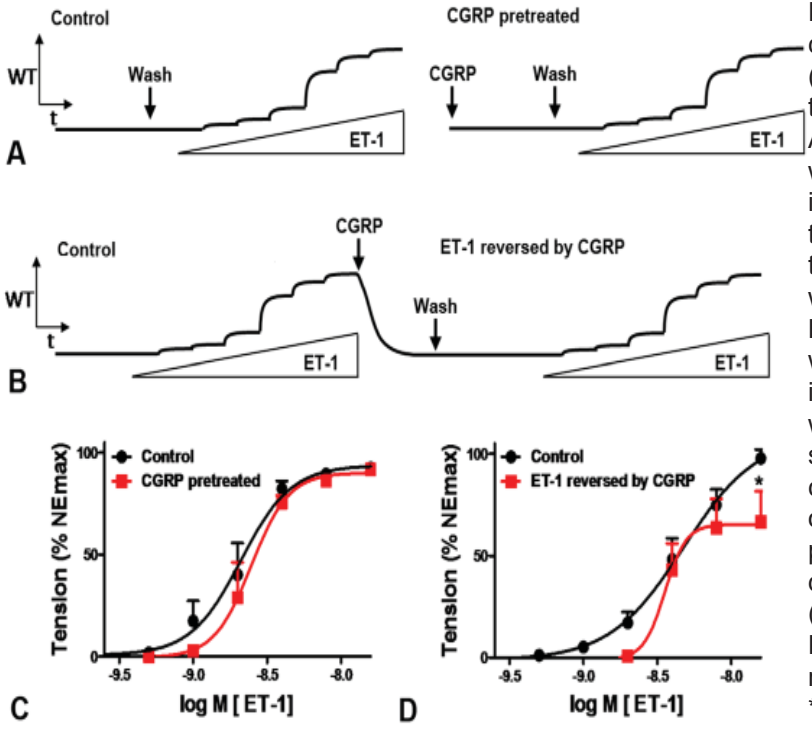

Figure 2.5. Unaltered arterial contractile responses to ET-1 (0.25–16 nM) following exposure to CGRP.
A, schematic tracings of active wall tension (WT) versus time (t) illustrating contractile reponses to ET-1 (0.25–16 nM) in arteries transiently treated with (right) or without (left) CGRP (100 nM). B, schematic tracing of active wall tension (WT) versus time (t) illustrating initial ET-1 effects which were reversed by CGRP before a second concentration response curve was generated. C: Effect of ET-1 (0.25–16 nM) in arteries pre-treated with CGRP (100 nM, during 16 min). D: Effect of ET-1 (0.25–16 nM) in arteries in which ET-1-induced contractions were reversed by CGRP (100 nM). n = 6. *: $p<0.05$ vs control.

Modulation of ET-1/ET_A receptor binding

We used rhodamine-labeled ET-1 (Rh-ET-1) and two-photon laser scanning microscopy (TPLSM) focusing on the tunica media, to visualize binding of ET-1 to the smooth muscle. Contractile properties did not differ between Rh-ET-1 and ET-1 (Table 2.1). Binding of Rh-ET-1 (16 nM) to smooth muscle (Fig. 2.6D) was reduced by BQ788 (1 µM; Fig. 2.6E) and was prevented by presence of either ET-1 (16 nM) or of both BQ788 (1 µM) and BQ123 (1 µM) [2] indicating selective binding to ET_A and ET_B receptors. Once established, binding of Rh-ET-1 persisted after washout of free Rh-ET-1 and was not reversed by BQ123 (1 µM; Fig. 2.6F) indicating quasi-irreversible receptor-binding of the agonist. In contrast, capsaicin (1 µM) and exogenous CGRP (100 nM; investigated in presence and absence (not shown) of 1 µM BQ788), reversed the binding of Rh-ET-1 to smooth muscle that remained after exposure to Rh-ET-1 (Fig. 2.6I/M). Thereafter, Rh-ET-1 could again label the arterial smooth muscle (Fig. 2.6J/N).

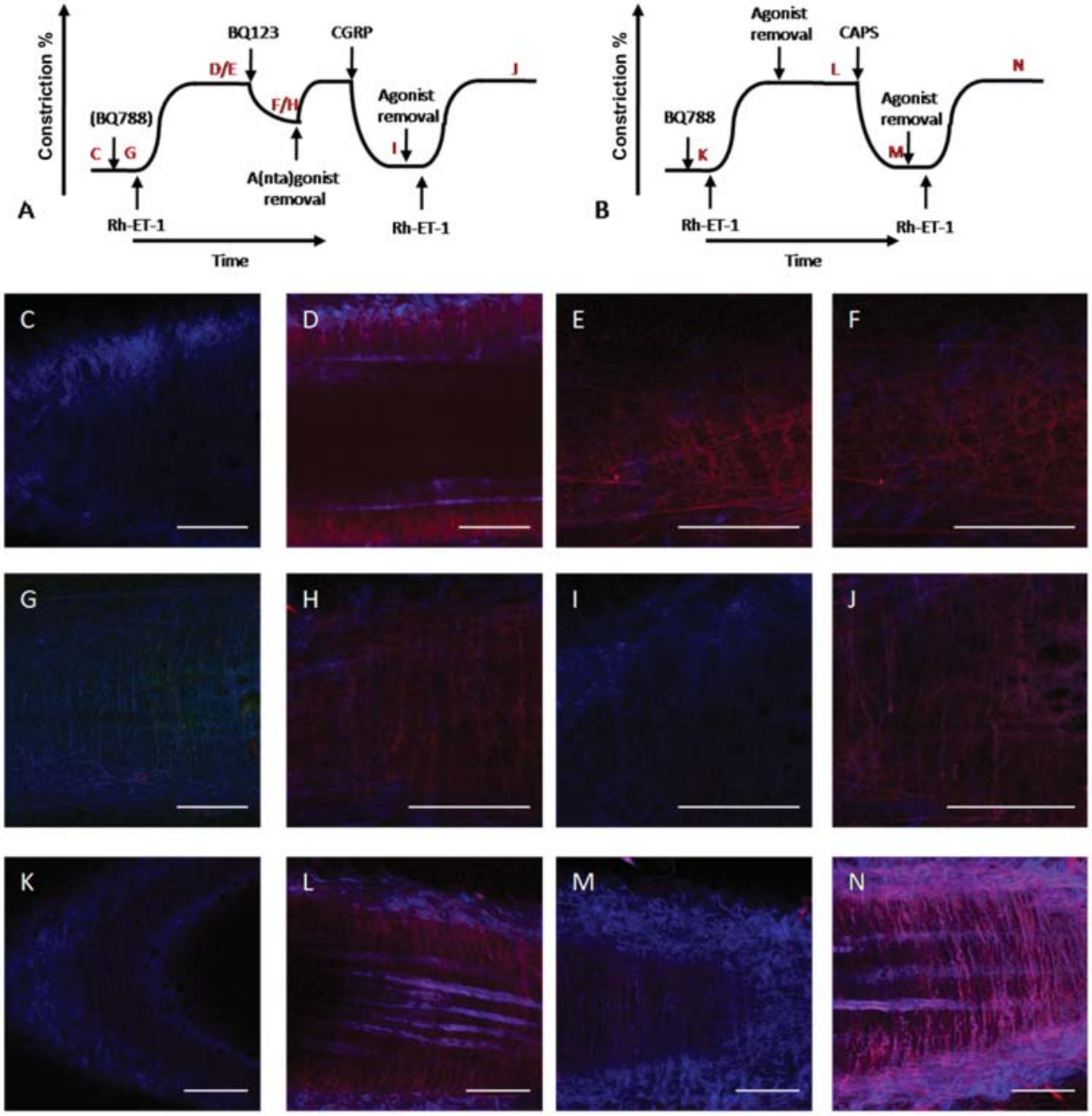

Figure 2.6. Binding of ET-1 is not reversed by ET-receptor antagonists (C-F) but can be reversed by CGRP (G-J) and by capsaicin (K-N).

Isolated rat mesenteric arteries were canullated, pressurized and mounted under a 2-photon laser scanning microscope. Analyses focussed on the smooth muscle layer (bordered by the autofluorescent (blue) internal and elastic laminae) (C, G, H)). Experiments were performed in continuous presence of BQ788 (1 μM) except panels C and D. A and B illustrate schematic tracings of active wall tension versus time illustrating the order of (i) administration of rhodamine-labeled ET-1 (Rh-ET-1, 16 nM), (ii) application of pharmacological agents and (iii) removal of agonists and antagonists. C, autofluoresence. D - F, labeling of vascular smooth muscle (D, red) observed in presence of Rh-ET-1 is not noticeably affected by administration of BQ788 (E, 1 μM) and BQ123 (F, 1 μM). G, autofluorescence. H – J, labeling induced by exposure to Rh-ET-1 (16 nM) persists in absence of free label and is resistant to ET-receptor antagonists (H) but is rapidly abolished (I) by exposure of the artery to CGRP (100 nM); thereafter labeling of smooth muscle can be re-established by exposure to Rh-ET-1 (16 nM) (J). K, autofluoresence. L – N, largely similar experiment using capsaicin (CAPS, 1 μM). Labeling induced by exposure to Rh-ET-1 (16 nM) that persists in absence of free label (L) is abolished (M) by exposure of the artery to CAPS (1 μM); thereafter labeling of smooth muscle can be re-established by exposure to Rh-ET-1 (16 nM) (N). Scale bars: 50 μm. Findings are representative for 4 arteries of 3 rats.

Discussion

The novel finding of our work is that while ET-receptor antagonists partly and transiently reduce endothelinergic vasoconstriction as a result of bitopic and irreversible agonist-receptor binding, stimuli of SMN can terminate effects initiated by ET-1 through CGRP-receptors that promote dissociation of ET-1/ET_A receptor complexes. This may lead to novel therapies of diseases involving ET-1.

We compared effects and mechanisms of action of competitive and physiological antagonists of ET-1 in isolated rat mesenteric resistance arteries. In these vessels, which influence local blood flow and total peripheral resistance and contribute to the development of hypertension [43], ET-receptor subtypes are expressed by several cell types [31-33]. However, a selective ET_B agonist did not modify vasomotor tone. Contractile effects of ET-1 were not modified by an ET_B antagonist, pre-treatment with capsaicin or inhibition of NO-synthases and cyclo-oxygenases. Thus, initiation and maintenance of contractile responses to ET-1 were dominated by smooth muscle ET_A receptors and were hardly affected by basal or endothelinergic influences of SMN or the endothelium.

Ligand-binding studies and analyses of structure-affinity and structure-selectivity relationships previously indicated quasi-irreversible and polyvalent binding of ET-1 to ET_A receptors [2, 5, 15, 17, 44, 45]. The high affinity of ET-1 for ET_A receptors is due to slow dissociation of the agonist-receptor complexes [2]. ET-1 requires the C-terminal Trp^{21}, both disulphide bonds and distinct amino acids in the N-terminal loop for high affinity binding to ET_A receptors [2, 5, 12-15, 18]. It has therefore been proposed that several parts of ET-1 interact with distinct sites on the ET_A receptor [15, 17]. To the best of our knowledge, the consequences of this polyvalent and irreversible binding of ET-1 to ET_A receptors for signaling have not been addressed before. We show that ET_A antagonists can prevent binding and contractile effects of ET-1 but that they are less effective in reversing effects induced by ET-1. This discrepancy has also been observed in vivo (e.g. [22]) and was even more marked in an in vitro study using another ET_A antagonist [46]. In addition, we report that ET-receptor antagonists reduce not only responses in presence of ET-1 but also responses that had been initiated by ET-1 and that persisted in absence of free agonist. These findings combined with earlier models of ET_A receptor function [15, 17] can be integrated into a model regarding ET-1/ET_A interactions and ET_A mediated signaling as depicted in Fig. 2.7. A part of ET-1, and the low molecular weight antagonists, binds with high affinity to one binding site on the receptor (site H). Thereafter another part of the ET-1 molecule binds to a second distinct binding-site

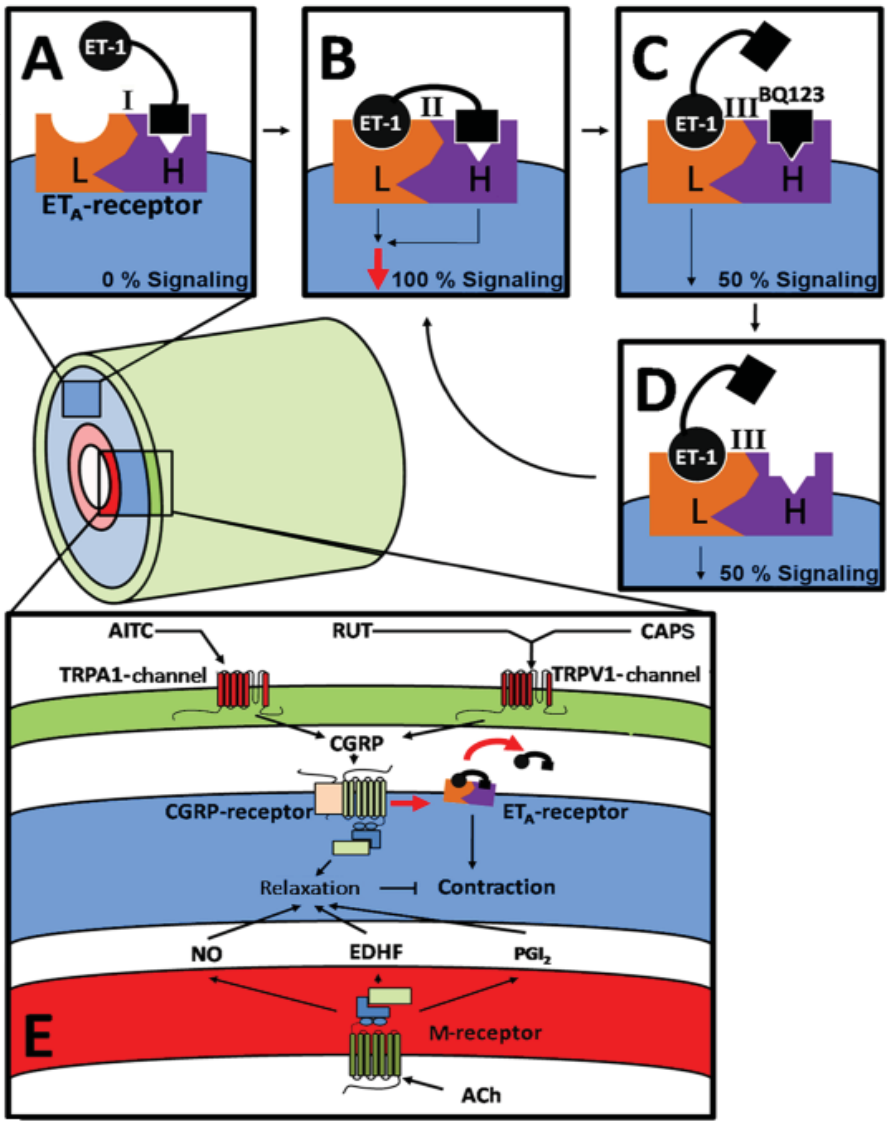

Figure 2.7. Schemes illustrating the interaction of ET-1 with arterial smooth muscle ET_A receptors (A – D) and the influences of vasodilators.
A, Initially, a part of ET-1 binds to a high affinity binding site (H) on an ET_A receptor. B, next, another part of ET-1 binds to a low affinity binding site (L) of the receptor. Signaling is triggered by the occupied site L and reinforced by the occupied site H. C, while binding of ET-1 to site L is quasi-irreversible, the binding of ET-1 to site H remains dynamic and can be competed off by low molecular weight antagonists such as BQ123. D, binding of antagonists is readily reversible after which bivalent binding of ET-1 to the ET_A receptor and cooperative signaling can be re-established. E, schematic representation of the endothelium (red), arterial smooth muscle (blue) and peri-arterial SMN (green). Endothelium-derived relaxing factors such as nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2) released upon stimulation of e.g. endothelial muscarinic receptors (M) by acetylcholine (ACh), counteract the ET-1/ET_A contractile effect by their relaxing effect (functional antagonism). Stimulation of TRPA1-channels by allyl isothiocyanate (AITC) or of TRPV1-channels by capsaicin (CAPS) or rutaecarpine (RUT) leads to release of CGRP. In addition to functional antagonism, stimulation of post junctional CGRP-receptors causes dissociation of ET-1/ET_A receptor complexes resulting in termination of thepersistent vasoconstrictor effect of ET-1.

on the receptor (site L). Binding of ET-1 at site H is dynamic and remains susceptible to competition by the low molecular weight antagonists. It precedes and is required for binding at site L which (i) is insensitive to antagonists, (ii) triggers signalling and (iii) binds the agonist quasi-irreversibly. This model explains the lower potency than affinity and the steepness of the concentration-effect relationships of ET-1 when signaling by ET_A receptors is enhanced by cooperativity between the two binding sites of ET-1. In addition, it takes into account the flexibility of ET-1 as indicated by X-ray crystallography and NMR spectroscopy studies [47, 48] and displays similarities to the “address and message domain model” proposed for other GPCR agonists [49, 50]. Because similar findings were obtained with BQ123, SB234551

and bosentan which represent i) hydrophilic and lipophilic antagonists and ii) ET_A selective and mixed antagonists, internalization and heterodimerization of receptors do not seem to be involved.

Our model predicts that compounds which accelerate dissociation of ET-1/ET_A receptor complexes have a larger and more long-lasting inhibitory effect on responses initiated by ET-1 compared to neutral competitive antagonists. Aspirin-like molecules were reported to display such an allosteric inhibitory effect at millimolar concentrations [45, 51]. We focused on the endothelium and on SMN, two structures that counterbalance ET-1/ET_A effects in vivo [4, 7, 25, 29-31], to identify a similar but more potent mechanism. The endothelium-dependent vasodilator acetylcholine and several directly acting vasodilators fully relaxed ET-1-induced contractions. However, these relaxations were transient and did not inhibit the persistent contractile effect initiated by ET-1. This indicates mere functional antagonism. In contrast, stimuli of SMN not only reversed ET-1-induced contractions but also prevented their recovery. This was observed with capsaicin (pungent vanilloid TRPV1-stimulus of Capsicum Spec [40]), rutaecarpine (an alkaloid TRPV1-agonist from the chinese traditional medicinal herb Evodia Rutaecarpa [39]) and with allyl isothiocyanate (an organosulfur TRPA1-channel activator of Allium and Brassica [37]). For rutaecarpine the effects were not modified by removal of the endothelium excluding a role for endothelial TRPV1-channels [52]. The effects of SMN-stimuli were reduced by CGRP-receptor antagonists and mimicked by exogenous CGRP, a neurotransmitter that can be released from peri-arterial SMN [27, 36, 40]. However, they could not be reproduced by agents that stimulate adenylyl cyclase (forskolin and isoproterenol), generate NO (Na-nitroprusside) or open K_{ATP}-channels (pinacidil) and thus activate components of the classical signal-transduction mechanism triggered by CGRP-receptors (for review see [53]). Furthermore, the contractile potency and efficacy of ET-1 were hardly modified by pre-exposure to capsaicin or CGRP or after “termination” of the persistent effect of ET-1 by CGRP. This suggests that the cAMP-independent effect of CGRP against ET-1 involves dissociation of ET-1/ET_A receptor complexes and not a long-lasting relaxing effect.

Clearly, this invites for further investigations into the molecular mechanism(s) induced by CGRP in this setting. These studies should focus on possibilities like i) heterodimers between ET_A and CGRP-receptors, ii) rapid phosphorylation followed by desensitization of ET_A receptors mediated by e.g. G protein receptor kinases [54], which can be activated by CGRP-receptor stimulation [55] and iii) possible interactions between the different subunits of CGRP receptors, most notably receptor activity modifying protein 1, and ET_A receptors.

We used imaging to study the effects of CGRP-receptor activation on binding of ET-1 to arterial smooth muscle ET_A receptors. In line with earlier findings, fluorescent labeling of ET-1 at Lys9 did not modify the pharmacology of the agonist [2], [56], [57]. We observed intense staining of intact vascular smooth muscle which could be prevented by ET-1 and by combined ET_A and ET_B antagonism ([2] and this study). Thus, we show that ET-1 agonist-receptor binding can be visualised in a vital tissue without the need for supra-physiological receptor densities. Compared to conventional radioligand binding experiments with microsomes or intact arteries (e.g. [58] it has the added value that (i) small tissue samples can be used efficiently without the need for large numbers of arteries and animals, (ii) dissociation of ET-1/ET_A complexes can be monitored in real-time and (iii) effects of second messengers and endogenously released mediators (e.g. neurotransmitters) can be registered. In line with the model that we propose, labeling of vascular smooth muscle persisted after removal of free label. In addition, labeling was not reversed by BQ123 in the presence of BQ788. In contrast, capsaicin and CGRP each abolished pre-existing labeling. Thereafter, Rh-ET-1 could again label the smooth muscle with comparable intensity. This strengthens the conclusion that CGRP-receptor stimulation promotes dissociation of the agonist ET-1 from contractile ET_A receptors.

In summary (Fig. 2.7), CGRP released from SMN promotes the dissociation of the ET-1/ET_A receptor complexes that are responsible for the long-lasting effects of the peptide. Hence CGRP can be more suited to inhibit vascular effects of ET-1 compared to functional antagonists and competitive antagonists. We could not demonstrate that this mechanism acts as a negative feedback under normal conditions because desensitization of SMN and presence of CGRP-receptor antagonists do not alter the sensitivity to ET-1 ([33] and this study). This is in line with observations that ET-1 does not directly stimulate but modulates effects of TRP channel activators [27, 28]. The negative feedback may become operative during ischemia and inflammation which stimulate SMN activity. Several other aspects remain to be addressed to validate CGRP-receptor agonism and SMN as valid targets for therapy of ET-1-related diseases. These include effects of SMN and CGRP against endogenously produced ET-1 in other vessels and other species. In the mean time it may be worthwhile to consider how widely available natural and orally active stimuli of SMN could be applied in diseases that involve ET-1 but in which clinical efficacy of ET-receptor antagonists has been hard to prove [6, 23].

Materials and Methods

Experimental protocols were approved by the Ethics Committee on Experimental Animal Welfare of Maastricht University.

Solutions and Drugs

Bosentan [19], BIBN4096BS [42] and SB-234551 [21] were obtained from Actelion Pharmaceuticals (Allschwill, CH), Boehringer Ingelheim Pharma KG (Biberach, D) and GlaxoSmithKline (Stevenage, UK) respectively, and dissolved in DMSO. Allyl isothiocyanate [37], capsaicin [38, 40], forskolin and indomethacin were purchased from Sigma Aldrich (Zwijndrecht, NL) and dissolved in ethanol. Acetylcholine, isoproterenol, L-NAME (Nω (G)-nitro-L- arginine methyl ester), Na-nitroprusside, norepinephrine, and isoproterenol were purchased from Sigma Aldrich (Zwijndrecht, NL) and dissolved in Krebs-Ringer bicarbonate (KRB) solution. Pinacidil was obtained from Sigma Aldrich (Zwijndrecht, NL) and dissolved in DMSO. BQ123 [20] and BQ788 [34] were obtained from Bachem (Weil am Rhein, D) and dissolved in DMSO. Human CGRP, $CGRP_{8-37}$ [41], ET-1 and $Ala^{1,3,11,15}ET\text{-}1$ [10] were obtained from Bachem (Weil am Rhein, D) and dissolved in KRB solution. Rutaecarpine [39] was a kind gift from Prof. Yu Huang (Chinese University of Hong Kong, China) and was dissolved in DMSO. The maximal concentrations of the solvents never exceeded 0.1% and did not alter arterial reactivity.

Tissue Preparation

16 weeks old male WKY rats (Charles River, Maastricht, NL) were euthanized by CO_2 inhalation. Second-order side branches of the superior mesenteric artery were isolated, and either mounted in a wire-myograph and stretched as previously described [33, 59] or mounted in a pressure-myograph and pressurized at 80 mm Hg [27, 60]. In some arteries, the endothelium was mechanically removed[27, 33, 59].

Vasomotor responses

At optimal diameter (340±6 μm) the contractile response to 10 μM NE averaged 4.1±0.2 N/m. The relaxing responses to acetylcholine (10 μM) during this precontraction averaged 93.7±0.7% and was absent in denuded arteries.

Effects of ET-receptor antagonists.

The effect of the ET_A antagonists BQ123 [20] (1 μM), bosentan [19] (3 μM, in presence of BQ788 (1 μM)) or SB234551 [21] (10 nM) was assessed when applied 20 min. before ET-1 induced contractions (0.25–16 nM). In addition, the effect of increasing concentrations (0.1–3 μM) of BQ123 and bosentan was assessed during contractions induced by 8 nM ET-1. Also, the effect of SB234551 (10 nM) during ET-1-induced contraction (8 nM) was determined. Finally, the effect of the antagonists was determined during contractions that remained after removal of ET-1 from its biophase. Before these experiments, peri-arterial SMN were desensitized [33, 38, 40]. In addition, L-NAME (100 μM) and indomethacin (10 μM) were continuously present.

Effects of candidate functional antagonists.

During ET-1-induced contractions (16 nM), and during contractions that remained after removal of ET-1 (16 nM) from its biophase, arterial relaxing responses to increasing concentrations acetylcholine (0.01–10 μM), capsaicin (0.01–1.0 μM), CGRP (0.1–100 nM), Na-nitroprusside (0.01–10 μM), rutaecarpine (0.1–10 μM), allyl isothiocyanate (0.01–10 μM), forskolin (0.1–3 μM), isoproterenol (0.01–3 μM) or pinacidil (0.01–10 μM) were assessed. These experiments were performed in absence of pharmacological inhibitors and were repeated in presence of L-NAME (100 μM) and indomethacin (10 μM) and in presence of CGRP-receptor antagonists (BIBN4096BS [42] (20 nM) or $\alpha CGRP_{8-37}$ [41] (1 μM)). Some of these experiments were repeated in denuded arteries.

Synthesis of fluorescently labeled ET-1

0.35 mg ET-1 (0.14 μmol) was dissolved in 50 μL dimethylformamide +1 μL N,N-diisopropylethylamine. 50 μL Rhodamine-succinimidyl ester (Rh-SE) stock solution (6.3 μmol/μl 33% acetonitril/67% methanol) was added and left overnight for coupling. After 16 hours HPLC and MALDI-TOF analyses showed that >90% of ET-1 was mono-labeled. Rh-ET-1 was purified using semi-preparative reversed-phase HPLC using a Vydac C-18 column (250×10 mm, 10 μm). A lineair gradient of acetonitrile in water/0.1% TFA (flow rate 5 ml/min; 0.5%B/min) was applied to elute peptides. Rh-ET-1 was lyophilized and stored at −20°C until use.

Two-photon laser scanning microscopy (TPLSM)

After isolating and pressurizing the arteries, TPLSM was performed as previously described [27, 60]. In short, tissue samples were excited with Tsunami Ti:sapphire laser (Spectra-Physics), which was pumped by a Millennia Vs 5 W pump laser (Spectra-Physics) and mode locked at 840 nm, with a 82.5 MHz repetition rate and 100 fs pulse width. Autofluoresence was visualized at 400 to 450 nm and focal planes were positioned within the tunica media. Arteries were incubated with Rh-ET-1 (16 nM) and labeling of structures in the vessel wall was assessed at 620 to 660 nM. Subsequently, the effect of preincubation with BQ123 (1 μM), BQ123 (1 μM) + BQ788 (1 μM) on labeling was determined. Labeling of arterial smooth muscle in the arterial wall by Rh-ET-1 (16 nM) can be prevented by ET-1 (16 nM) [2]. Finally, the effects of BQ123 (1 μM), removal of free label and antagonist and of administration of either CGRP (100 nM) or of capsaicin (1 μM) on labeling were determined. These experiments were performed in presence of BQ788 (1 μM) [2].

Data and Statistical Analysis

Contractile responses are expressed as percentage of the maximal contractile response to 10 μM NE in absence of pharmacological inhibitors (NEmax). Relaxing responses are expressed as percentage reduction of the level of pre-contraction. Concentration-response curves (CRC) were fitted to a non-linear sigmoid regression curve (Graphpad Prism 5.0). All data are shown as mean ± SEM. Statistical significance was assessed using either one-way ANOVA (comparison of EC_{50} and E_{max}) or two-way ANOVA (comparison of CRCs). Bonferroni's post-hoc test was used to compare multiple groups. A P value <0.05 was considered statistically significant.

Acknowledgments

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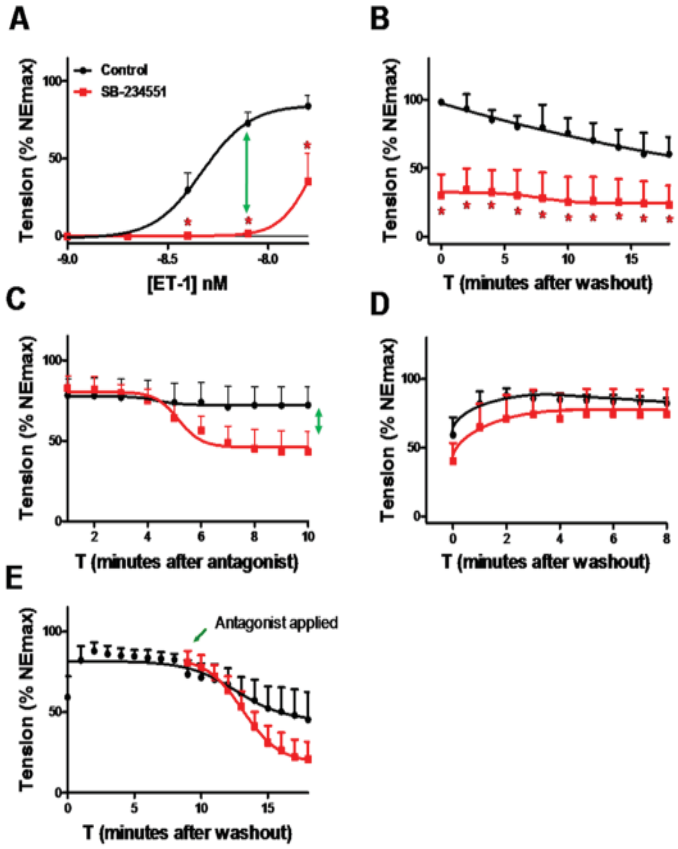
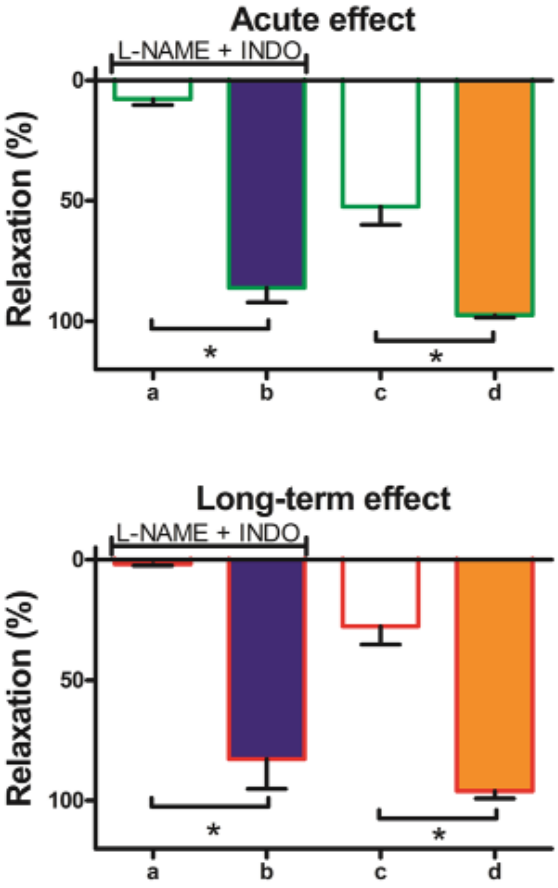
Chapter 2.1

Supplementary Data concerning Chapter 2



Supplementary Figure 2.1.1. Partial and reversible reversing effect of the ET_A receptor antagonist SB234551 on arterial contractile responses to ET-1 and their persistence. Isolated rat mesenteric resistance arteries were studied after treatment with capsaicin (1 μM during 20 min.) in the continuous presence of L-NAME (100 μM) and indomethacin (10 μM). A, responses to 0.25−16 nM ET-1 in the absence (black) and presence of SB234551 (10 nM, red). Note that SB234551 prevented responses to up to 8 nM ET-1. B, vasomotor tone after removal of free agonist and antagonist. C, effects of SB234552 (10 nM) on contractile responses to 8 nM ET-1. D, vasomotor tone after removal of free agonist and antagonist. E, effect of SB234551 (10 nM) on the contractile response initiated by 8 nM ET-1 and persisting in the absence of the peptide. F, vasomotor tone after removal of free antagonist. Data are expressed as % of the maximal response to norepinephrine (NEmax) prior to exposure to any drug, and are shown as mean ± SEM (n = 6). *, the difference from control is statistically significant (P<0.05).



Supplementary Figure 2.1.2. Effects of CGRP and rutaecarpine are endothelium-independent. Isolated, denuded rat arteries were studied in presence of L-NAME (100 μM) and indomethacin (10 μM) as indicated. Arteries were precontracted with 16 nM ET-1. Next, increasing concentrations of vasodilator compounds were administered until a maximal effect was observed. Thereafter vasoactive stimuli were removed from the organ chamber while the recording of active wall tension continued for >10 min. A, maximal acute relaxing effects of CGRP and rutaecarpine. B, long-term effects of CGRP and rutaecarpine. a, time control; b, CGRP; c, rutaecarpine.

Chapter 3

Endothelin-1 and -2: Two Amino Acids Matter

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Abstract

Endothelin-1 (ET-1) and endothelin-2 (ET-2; Trp^6Leu^7ET-1) are expressed by different cell types, but are considered to display identical pharmacological properties on endothelin receptors. We studied agonist-dependent aspects of endothelin$_A$ (ET_A) receptor function and the importance of amino acids 6 and 7 of ET-1 and ET-2 in this respect.

We used isolated rat mesenteric resistance arteries in wire myographs, in a setting that minimizes influences of endothelium and sensorimotor nerves, to study arterial smooth muscle ET_A receptor-mediated vasomotor responses, to ET-1, ET-2 and chimeras thereof (Trp^6ET-1 and Leu^7ET-1).

ET-1 and ET-2 cause arterial contractions with comparable sensitivities and maximal responses. BQ123 (ET_A antagonist) reduces sensitivity to ET-1 more potently than that to ET-2 (pK_B: 7.1 ± 0.2 versus 5.6 ± 0.4). However, 1 μM BQ123 relaxes maximal contractile responses to ET-2 more markedly than those to ET-1. Leu^7ET-1 is a contractile agonist with lower potency and similar maximal effect compared to ET-1 and greater sensitivity to BQ123 than ET-2. Up to 256 nM Trp^6ET-1 did not cause contraction and did not antagonize arterial responses to ET-1.

Arterial smooth muscle ET_A receptor function displays agonist-dependent aspects. This involves roles of amino acids on position 6 and 7 of the endothelin sequence. Agonist-dependent pathologies may benefit from the design of specific, agonist-selective ET receptor antagonists.

Introduction

Evolution left *homo sapiens* with three distinct potent vasoactive members of the endothelin family; endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) [1, 2]. The biological effects of these 21 amino acid bicyclic peptides are mediated by two distantly related 7 transmembrane domain receptors (7TMRs): endothelin$_A$(ET_A) and endothelin$_B$(ET_B) receptors [1-3]. Binding to ET_B receptors requires the 6 amino acid C-terminus, which is identical for all three ETs, making them equipotent at this receptor [4]. The 15 amino acid N-terminal loop, with disulfide bonds between Cys^1 and Cys^{15} and between Cys^3 and Cys^{11}, determines selectivity for the ET_A receptor [5]. Compared to ET-1 and ET-2, ET-3 differs in 6 amino acids within the N-terminal loop, distinguishing ET-3 as an ET_B selective ligand [3, 6].

ET-1 and ET-2 were reported to be non-selective ET receptor agonists. Their amino acid sequences differ only at positions 6 and 7 within the N-terminal loop. They bind to ET_A and ET_B with equal affinities and their pharmacological properties were proposed to be identical [2, 3]. However, ET-1 and ET-2 are expressed by different cell types, restricting their paracrine and autocrine function to distinct tissues [7]. ET-1 is mainly found in the cardiovascular system where it causes, amongst other effects, long-lasting vasoconstriction mediated by tight binding to ET_A receptors [8-11]. ET-2 is mainly found in the gastrointestinal tract and the urogenital system [7]. Via ET_A receptors, ET-2 can modulate immune cell function [12] and ovulation [13]. ET-1 and ET-2 are expressed at different developmental stages in the embryo and in different cell types and organ systems in the adult [1]. While ET-1 is intimately involved in the cardiovascular system [8], ET-2 seems to have selective functions in for instance the ovaries [13, 14]. The pharmacological properties of ET-1 and ET-2 have been considered to be identical [2]. This may be surprising because during the course of evolution other endothelin isoforms were lost [1]. We therefore compared both peptides beyond apparent affinities and efficacies.

Here we tested the hypothesis that ET-1 and ET-2 display distinct ET_A receptor mediated pharmacological properties. We studied inhibitory effects of an antagonist of ET_A receptors (BQ123, [15]) on arterial responses to the two endogenous agonists. In addition, we evaluated the arterial effects of two newly synthesized ET-1/ET-2 chimeras, one in which we substituted Leu^6 of ET-1 for Trp^6 of ET-2 (Trp^6ET-1 (or Met^7ET-2)) and another one in which we substituted Met^7 of ET-1 for Leu^7 of ET-2 (Leu^7ET-1 (or Leu^6ET-2)). Our observations indicate agonist-dependent modulation of ET_A receptor function and marked effects of amino acids 6 and 7 of the endothelin sequence in this respect.

Materials and methods

Experiments were performed in accordance with institutional guidelines and were approved by the Ethics Committee on Experimental Animal Welfare of the Maastricht University.

Solutions and compounds

BQ123 (Sigma Aldrich, Zwijndrecht, NL) and BQ788 (Peptides International, Louisville, USA) were dissolved in DMSO. Capsaicin (CAPS) and indomethacin (INDO) (Sigma Aldrich, Zwijndrecht, NL) were dissolved in ethanol. Human ET-1, human ET-2, 4AlaET-1, Sarafotoxin 6c (S6c) (Bachem, Weil am Rhein, D), noradrenaline (NA) and Nω(G)-nitro-L- arginine methyl ester (L-NAME) (Sigma Aldrich, Zwijndrecht, NL) were dissolved in Krebs Ringer bicarbonate buffer (KRB) containing (in mM): NaCl: 118.5; KCl: 4.7; $CaCl_2$: 2.5; $MgSO_4$: 1.2; KH_2PO_4: 1.2; $NaHCO_3$: 25.0; glucose: 5.5. The maximal solvent concentration never exceeded 0.1% and did not significantly modify arterial vasomotor responses.

De novo synthesis of Trp^6ET-1 and Leu^7ET-1

Single batches of Trp^6ET-1 and Leu^7ET-1 were synthesized by manual solid-phase peptide synthesis on 4-methylbenzhydrylamine (MBHA) resin using the in situ neutralization/activation procedure for tBoc-peptide synthesis as described [16], but using O-(6-Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HCTU) instead of O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) as a coupling reagent. To allow controlled Cys^3-Cys^{11} and Cys^1-Cys^{15} disulfide formation within ET peptides, two acetamidomethyl (Acm)-protected cysteines (1;15) were used that could selectively be deprotected during folding, ensuring the correct folding of the N-terminal loop of the peptide. The peptides were cleaved from the resin by treatment with anhydrous hydrofluoric acid for 1 h at 0 °C, using 4 *v*-% *p*-cresol as a scavenger. Following cleavage, the peptides were purified by preparative high-performance liquid chromatography (HPLC). Fractions containing the desired product were identified by electrospray ionization mass spectrometry, pooled and lyophilized.

Peptide Folding

The peptides were folded by a two-step protocol. The first disulfide bond was formed stirring the purified peptide in 0.05 M Tris buffer pH 8.0, 3 M Gn.HCl (0.2mg/ml) for

72 h at 4°C. For the second disulfide bond the solution was adjusted to 10% AcOH, purged with nitrogen, and Acm groups were removed by addition of 2 equivalents of iodine (0.12 M in methanol). Reaction progress was monitored by analytical HPLC and ESI-MS. Products were purified by semi-preparative HPLC and lyophilized. Presence of two disulfide bonds in the peptides in solution was checked by HPLC after completing the functional experiments.

Recording of vasomotor responses

Male, 16 weeks old Wistar Kyoto rats (Charles River, Maastricht, The Netherlands) were euthanized by CO_2-inhalation. Second-order branches of the superior mesenteric artery were isolated by dissection in KRB at room temperature. To record isometric tension development, freshly isolated 2mm long arterial segments were mounted in wire myographs (DMT, Aarhus, DK) in which 5mL KRB was maintained at 37°C and aerated with 95 % O_2/5 % CO_2. The arterial segments were stretched to the diameter at which the largest contractile response to 10 µM NA was obtained [9, 17]. The optimal internal diameter of the segments averaged 306 ± 8 µm and contractile responses to 10 µM NA averaged 3.9 ± 0.1 N/m.
Arterial segments were pre-treated with 1 µM CAPS for 20 min and were thereafter studied in the continuous presence of 100 µM L-NAME and 10 µM INDO. These interventions minimize the effects of sensorimotor nerves and of the endothelium, which we have previously shown to express immunoreactive ET_A and ET_B receptors [9, 17].

Pharmacological protocols

Increasing concentrations of an endothelin isopeptide (cumulative concentration-response curve, CCRC) were administered to resting arteries to record contractile effects. The effect of ET_B receptor activation was assessed using the ET_B agonists 4^{Ala}ET-1 [18] and S6c [19] and the ET_B antagonist BQ788 [20].

Competition Experiments. Using arterial segments in parallel, CCRCs for a putative agonist were constructed in the absence and in the presence of 1 µM of an antagonist. Effects of the antagonist on the position (ratio of EC_{50}, pK_B) and on the height of the agonist CCRC (E_{MAX}) were monitored.

Inhibition Experiments. In arteries made to contract with an ET, we acutely applied the same concentration of the antagonist as we used in the competition experiments. Thereafter, we assessed the effect of removal of the receptor ligands on contractility

of the arterial segments as a measure for the remaining receptor activation. Because endothelins cause long-lasting arterial contractile effects [9, 11], comparable inhibition experiments were performed on agonist-initiated contractions, where we removed the free agonist before assessing the inhibitory effects of the antagonist.

Only one set of experiments was performed in one set of arterial segments, i.e. distinct pharmacological protocols were not performed in series in the same set of arterial segments. Experiments comparing BQ123-induced inhibition of ET-1- and ET-2-induced contractile effects were analyzed in comparison to control curves within the same rat and not to curves obtained in rats used to compare the various endothelinergic peptides

Data analysis and statistics

Data are shown as mean ± SEM. Contractile responses are expressed as percentage of the maximal contractile response to NA observed prior to the administration of any pharmacological inhibitor (NA_{MAX}). Individual CCRC were fitted to a non-linear regression curve and ED_{50} and pK_B values were calculated using GraphPad Prism 5.02. Data were analyzed using one-way ANOVA (comparison of pD_2, pK_B and E_{max}) or two-way ANOVA (comparison of CCRC). Bonferroni's post-hoc test was used to compare multiple groups. $P < 0.05$ was considered to denote statistical significance.

Results

Vasomotor responses to ET-1 and ET-2

Nanomolar concentrations of ET-1 and ET-2 caused long-lasting contractions in isolated rat mesenteric resistance arteries. When compared in vessels from the same animals, the potency (pD_2: 8.4 ± 0.1 and 8.5 ± 0.1, respectively) and the maximal effect (E_{MAX}: 102 ± 5 % vs. 99 ± 10 %) did not differ significantly between both peptides (Fig. 3.1A), in line with earlier findings [21, 22] and in addition their effects were equally persistent (Fig. 3.1D and 3.1F). In contrast, the ET_B selective agonists S6c [19], found in snake venom, and 4^{Ala}ET-1 [18], a linear analogue of ET-1 where the four Cys residues are replaced by Ala, did not contract the isolated arteries at up to 1 µM (Fig. 3.1A) [23]. Furthermore, presence of 1 µM 4^{Ala}ET-1 or of 1 µM BQ788, an ET_B selective antagonist [20], tended to alter the sensitivity to ET-1 but this did not reach statistical significance (Fig. 3.1B). These findings indicate that ET-1 and ET-2 cause seemingly similar ET_A receptor mediated arterial smooth

muscle contractions, although sensitivity to ET-2 in these 2nd order mesenteric arterial side branches is rather variable in experiments performed in sets of rats within a few weeks interval (Fig. 3.1A and 3.1E).

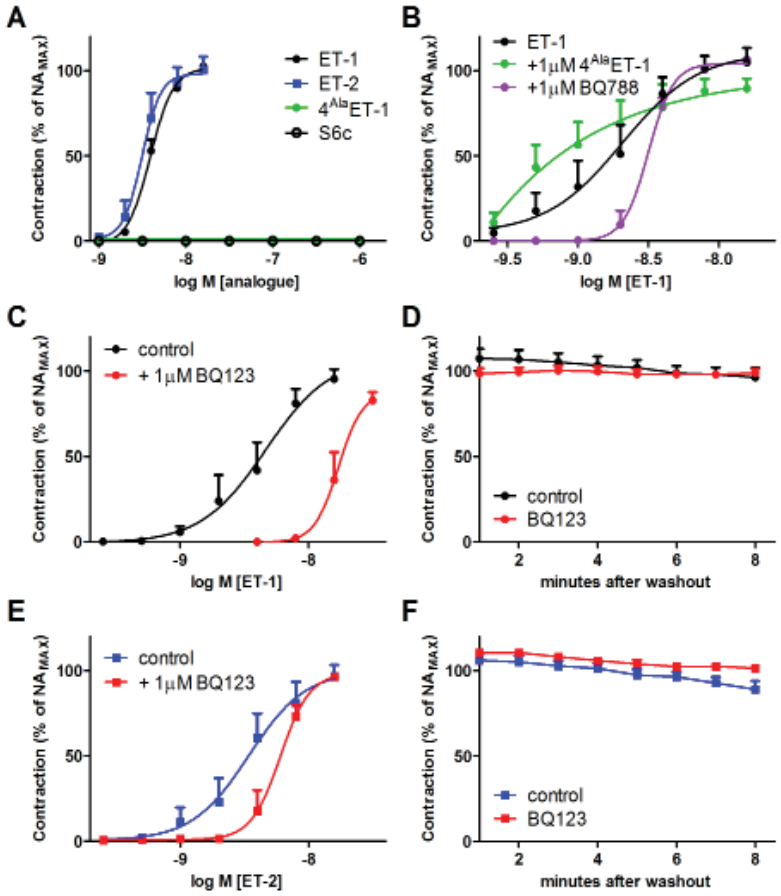

Figure 3.1. Vasomotor effects of isoforms and analogues of ET-1. ET-1 and ET-2 induce contractions with comparable affinity and efficacy, whereas the selective ET_B receptor agonists 4^{Ala}ET-1 and S6c do not induce responses (A). Neither 4^{Ala}ET-1 nor the selective ET_B receptor antagonist BQ788 significantly alter the ET-1-induced contractile responses (B). Sensitivity to ET-1-induced contractions (C) and ET-2-induced contractions (E) are reduced in the presence of selective ET_A antagonist 1 µM BQ123. Once full contractions are established, they are sustained following removal of BQ123 and ET-1 (D) or ET-2 (F).

Effects of an ET_A antagonist

Presence of 1 µM of the ET_A selective antagonist BQ123 [15] reduced the sensitivities to ET-1 and ET-2 without significant alteration of their maximal contractile effects (Fig. 3.1C and 3.1E). In contrast to earlier reports, where BQ123 inhibited ET-2-induced contractions more effective than ET-1-induced contractions in a preparation of either rings of the superior mesenteric artery or the perfused mesenteric arterial bed [24], the effect of the antagonist wasobserved to be more pronounced against ET-1 (11 fold reduction of sensitivity) than against ET-2 (2 fold) in our preparation using 2nd order mesenteric artery side branches and a different pharmacological study protocol. As a consequence, the apparent affinity of BQ123 was 30 times higher against ET-1 (pK_B: 7.1 ± 0.2) than against ET-2 (pK_B: 5.6 ± 0.4). Presence of BQ123 did on the other hand not prevent the development of persistent long-lasting contractile effects of ET-1 and ET-2 (Fig. 3.1D and 3.1F).

Application of 1 µM BQ123 during maximal contractile responses to ET-1 or ET-2 of comparable amplitude, resulted in significant relaxation (Fig. 3.2A and 3.2B). BQ123 relaxed ET-1-induced contractions by 43 ± 7 % but inhibited ET-2-induced effects to a significantly larger extent (92 ± 1 %). In both cases, the relaxing effect of BQ123 was rapidly reversible as tonic contractions redeveloped within 1 to 2 min after flushing both the ET and the antagonist from the organ chamber content (Fig. 3.2A and 3.2B). Similarly, when contractile responses were initiated by an ET and then allowed to proceed in the absence of free agonist, application of 1 µM BQ123 caused a reversible relaxation (Fig. 3.2C and 3.2D). Again this relaxing effect was significantly smaller for ET-1- (56 ± 1 %, Fig. 3.2C) than for ET-2-initiated contractions (90 ± 2 %; Fig. 3.2D).

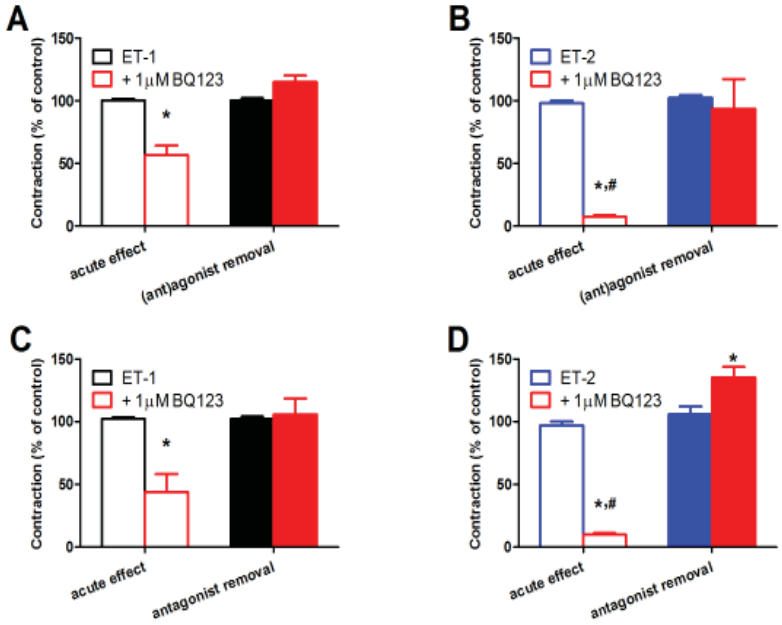

Figure 3.2. Inhibitory effects of BQ123 on sustained and maintained contractions. 1 µM BQ123 reduces ET-1-induced contractions by approximately 50% in presence (A) or following removal (C) of free agonist. The BQ123-induced inhibitions were rapidly reversed upon removal of antagonist and/or agonist. ET-2-induced contractions were reduced by 1 µM BQ123 by approximately 90% in presence (B) or following removal (D) of free agonist. Again, these BQ123-induced inhibitions were rapidly reversed upon removal of the ligand(s). * $P < 0.05$ vs control, # $P < 0.05$ vs BQ123 effect on ET-1.

These observations with BQ123 indicate agonist-dependent modulation of arterial smooth muscle ET_A receptor function. We next evaluated whether this could be attributed to one of the two amino acids that differ between the sequences of ET-1 and ET-2.

De novo chimera synthesis

Two chimeras of ET-1/ET-2 were synthesized and studied. The folded Leu^7ET-1 (CSCSS**L6L7**DKECVYFCHLDIIW) had an observed mass of 2472.2 Da, fitting well between the calculated monoisotopic mass (2472.1) and the average mass (2473.9) of the folded peptide. The folded Trp^6ET-1 (CSCSS**W6M7**DKECVYFCHLDIIW) peptide had an observed mass of 2563.3 Da, fitting well between the calculated monoisotopic mass (2563.0) and the average mass (2565.0) of the folded peptide. An observed mass difference of -144.2 Da between reduced and folded peptide

corresponded to the expected mass reduction caused by the loss of 2 protons and 2 Acm groups due to the formation of 2 disulfide bonds. The correct conformation of disulfide bonds (Cys^3-Cys^{11}; Cys^1-Cys^{15}) was ensured by applying a two step disulfide formation procedure (see Material and Methods), allowing the second disulfide bond between Cys^1 and Cys^{15} to form only after the first disulfide bond between Cys^3 and Cys^{11} was correctly formed.

Vasomotor effects of ET-1/ET-2 chimeras

Trp^6ET-1 (0.25 to 256 nM) did not induce contractile responses (Fig. 3.3A) and no contraction developed when the peptide was removed from the organ chamber (Fig. 3.3B). Also, presence of 256 nM Trp^6ET-1 did not significantly modify contractile responses to ET-1 (pD_2: 8.3 ± 0.3 and 8.4 ± 0.1; E_{MAX}: 102 ± 5 % and 97 ± 7 % in absence and presence of Trp^6ET-1, respectively). These indicate a particularly low affinity of Trp^6ET-1 for arterial smooth muscle ET_A receptors.

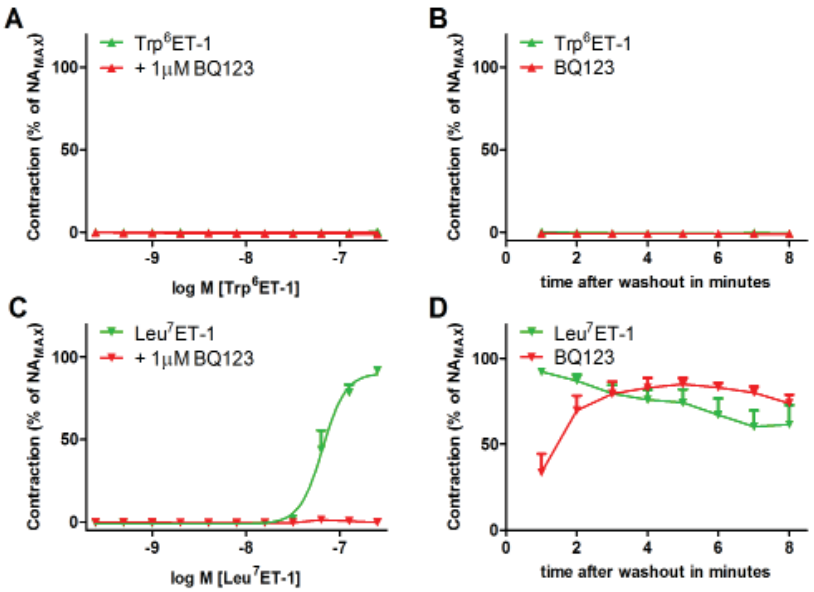

Figure 3.3. Vasomotor responses to Trp^6ET-1 and Leu^7ET-1. Trp^6ET-1 did not induce contractions up to 256 nM (A) and no contraction developed following removal of the putative vasoactive compounds (B). Leu^7ET-1 induced ET_A mediated contractions, which were, up to 256 nM, fully inhibited by 1 µM BQ123 (C). These contractions were sustained following removal of free agonist (D) and developed following removal of free vasoactive compounds (red lines).

Increasing concentrations of Leu^7ET-1 caused contractile responses in rat mesenteric resistance arteries with a potency (pD_2: 7.2 ± 0.1) that was 10 – 20 times smaller than that of ET-1 but with a similar maximum (Fig. 3.3C). As observed with ET-1 and ET-2, the arterial contractile effect of Leu^7ET-1 was long-lasting, i.e. it persisted after washout of the free unbound analogue (Fig. 3.3D). Presence of 1 µM BQ123 prevented contractile responses to up to 256 nM Leu^7ET-1 (Fig. 3.3C). Availability of only limited amounts of Leu^7ET-1 prevented estimation of the apparent affinity of the antagonist in this setting. However, comparison with Fig. 1 suggests that affinity of BQ123 versus Leu^7ET-1 is larger than versus ET-2 (Trp^6Leu^7ET-1). Also, it is noteworthy that while presence of BQ123 prevented Leu^7ET-1-induced contraction,

a strong response developed rapidly after washout of both ligands (Fig. 3.3D). This indicates tight binding of Leu^{7}ET-1 to ET_A receptors and inhibition of ET_A receptor activity by BQ123.

Discussion

The main findings of this work are that i) not only ET-1, but also ET-2 causes long-lasting arterial contractions, ii) arterial smooth muscle ET_A receptors display agonist-dependent function, iii) BQ123 acts as a negative allosteric modulator of ET_A receptors and iv) substitution of a single amino acid in the N-terminal loop of ET-1 can have profound pharmacological consequences. This may lead the way to the development of agonist-selective ET_A receptor antagonists.

To study ET_A receptor function we used rat mesenteric resistance arteries, which take part in the regulation of local and total peripheral vascular resistance and in the development of hypertension [25]. We performed all experiments after desensitization of peri-arterial sensorimotor nerves and during continuous inhibition of cyclo-oxygenases and NO-synthases. The selective ET_B receptor agonists 4^{Ala}ET-1 and S6c and the ET_B receptor antagonist BQ788 were without effects, the latter not only versus ET-1-induced contractions but also versus ET-2-induced contractions (data not shown). ET-1 and ET-2 caused contractions and the sensitivity to these non-selective ET receptor agonists was reduced by BQ123. These results indicate that the responses investigated are mediated by smooth muscle ET_A receptors [3] and are not modulated by endothelium, sensorimotor nerves or ET_B receptors as we have previously proposed [9].

Not surprisingly, sensitivity and maximal responses to ET-1 and ET-2 did not differ significantly in arteries from the same animals. The responses to both peptides were long-lasting. They persisted after removal of free unbound agonist, in line with earlier findings in different preparations [22], by a procedure that abolishes arterial responses to other contractile stimuli within less than 2 min [9], in line with earlier findings . For ET-1 this has been attributed to tight binding of the peptide to ET_A receptors [9, 26, 27]. To us it seems fair to propose that, also for ET-2, the rate of dissociation of the agonist/receptor complexes is particularly slow although estimates of this parameter have not been reported for this isopeptide yet. Our proposal is strengthened by the finding that BQ123 caused reversible relaxations of ET-2-induced and ET-2-initiated contractions as previously reported for ET-1 [9, 10].

The cyclic pentapeptide and ET_A selective antagonist BQ123 [15] reduced sensitivity and responses to both endothelins. The antagonist, however, reduced the sensitivity to ET-1 more markedly than that to ET-2. Conversely, the antagonist reduced responses to ET-2 more markedly than those to ET-1. This agonist-dependence and these different effects under resting and activating conditions are not compatible with neutral competitive antagonism but indicative of negative allosteric modulation of ET_A receptor function [26, 28, 29]. In this view, BQ123 binds to ET_A receptors at a site that is topographically distinct from the orthosteric binding sites of ET-1 and ET-2. This binding of the modulator changes the conformation of the receptors and thereby alters their affinities for the orthosteric ligands. In addition the bound modulator reduces the intrinsic activity of the agonist/receptor complexes. Activation resulting from tight binding of ET-2 to ET_A receptors was reduced to a larger extent than that resulting from ET-1. Whether also some of the low molecular weight non-peptidergic ET receptor antagonists are negative allosteric modulators rather than neutral competitive antagonists [26] largely remains to be established. For bosentan, however, it has been shown that its binding site does not fully coincide with that of ET-1 on ET_A receptors [30] and ABT-627 was shown to promote internalization of ET_A receptors [31]; an effect that is not compatible with neutral competitive antagonism.

To gain insight in the mechanism of agonist-dependence of ET_A receptor function, we synthesized chimeras of ET-1 and ET-2. Chemical analyses performed before and after the ex vivo experiments demonstrated that both compounds remained intact and were of the desired molecular weight. Leu^7ET-1 behaved as a full ET_A agonist but was less potent than ET-1 and ET-2. Presence of BQ123 reduced the sensitivity to Leu^7ET-1 more markedly than that to ET-2 (Trp^6Leu^7ET-1). However, BQ123 reduces the intrinsic activity of Leu^7ET-1 rather than its affinity for ET_A receptors. This suggestion is based on the observation that while BQ123 prevented contractile responses to the chimera, the antagonist did not prevent the development of a strong contractile response when both unbound ligands were removed. Apparently, Leu^7ET-1 bound tightly to ET_A receptors even in the presence of BQ123 and the antagonist inhibited the activity of the agonist/receptor complexes. The findings with Leu^7ET-1 thus strengthen the notions of agonist-dependence of ET_A receptor function and the allosteric properties of BQ123.

Trp^6ET-1, on the other hand, displayed neither agonistic nor antagonistic properties at concentrations at which even a low-affinity ET_A agonist like ET-3 induces contractile responses. It is unlikely that these observations result from a failure of synthesis, as methods of synthesis used were identical to those of the biologically active Leu^7ET-1. In earlier work, replacement in ET-1 of the

amino acids at positions 6 or 7 by alanine did not modify binding affinity to ET_A receptors in microsomes [32], nor did these substitutions alter constrictor activity [33]. However, in the current study, Leu^6 is replaced by a tryptophan, which will have a greater impact on structure-function of a small peptide. While endothelinergic peptides containing the combinations 'Leu^6 Met^7' for ET-1 and 'Trp^6 Leu^7' for ET-2 can bind and activate ET_A receptors, combination of tryptophan on position 6 and methionine on position 7 leads to a marked loss of binding affinity, despite that these substitutions would not alter backbone confirmation [34]. It remains to be determined what the consequences of this combination are for the conformation and flexibility of the agonist molecule [35] leading to the observed loss in biological activity.

Agonist-dependence (or probe-dependence) is one of the main properties of allosteric modulation of receptor function. Further exploration of this mechanism in the endothelin field may ultimately lead to the development of ET receptor antagonists that discriminate between endogenous endothelins (orthosteric agonists) acting in different organs or that become more efficacious with increasing activity of the endothelin axis [28, 29].

In conclusion, arterial smooth muscle ET_A receptors display agonist-dependent properties, involving the roles of amino acids on position 6 and 7 of the endothelin sequence. Agonist-dependent pathologies may benefit from the design of specific, agonist-selective ET receptor antagonists.

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Chapter 4

Endothelin-1 and Endothelin-2 Initiate and Maintain Contractile Responses by Different Mechanisms in Rat Mesenteric and Cerebral Arteries

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Submitted

Abstract

Endothelin-1 (ET-1) and endothelin-2 (ET-2) cause potent long-lasting vasoconstrictions by tight binding to smooth muscle ET_A receptors. We tested the hypotheses that different mechanisms mediate initiation and maintenance of arterial contractile responses to ET-1 and ET-2 and that this differs between vascular beds.

Segments of rat mesenteric resistance artery (MRA) and basilar artery (BA) were studied in wire myographs with and without functional antagonists.

The sensitivity and maximum of MRA contractile responses to ET-1 were not, or only moderately, reduced by stimuli of soluble guanylate cyclase, adenylate cyclase or K^+-channels and by an inhibitor of receptor-operated ion-channels. Yet, each of these reduced the maintenance of ET-1 effects and relaxed ET-1-induced contractions in MRA. A calcium-channel antagonist did not alter sensitivity, maximum and maintenance of ET-1 effects but relaxed ET-1-induced contractions in MRA. A phospholipase C inhibitor prevented contractile responses to ET-1 and ET-2 in MRA and BA, and relaxed ET-1- and ET-2-induced responses in MRA and ET-1 effects in BA. A Rho-kinase inhibitor did not modify sensitivity, maximum and maintenance of responses to both peptides in both arteries but relaxed ET-2, but not ET-1, effects in MRA and ET-1 effects in BA.

Phospholipase C plays a key role in arterial contractile responses to ETs but ET-1 and ET-2 initiate and maintain vasoconstriction by different mechanisms and these differ between rat mesenteric and basilar arteries. Selected functional antagonism may be considered for agonist- and vascular bed selective pharmacotherapy of ET-related diseases.

Introduction

Endothelins (ETs) are bicyclic 21 amino acid peptidergic paracrine mediators [1, 2] involved in the cardiovascular system [3], chronic pain [4] and cancers [5]. In mammalian species, the ET family consists of three members, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), the former being most prevalent in the cardiovascular system as a regulator of vascular tone [6]. After release from the endothelium, ET-1 causes contraction of vascular smooth muscle cells (VSMCs) via endothelin ET_A receptors [7]. In most ex vivo settings of freshly isolated arteries, there is no observable effect of endothelial or smooth muscle ET_B receptors on vasomotor tone [8, 9]. Activated ET_A receptors can stimulate several signal-transduction pathways including NADPH-oxidases, phospholipases, Rho-kinase and cellular-influx of calcium ions [6, 10, 11]. For several other vasoconstrictor agonists it has been established that transient stimulation of calcium-influx is followed by calcium-sensitization resulting from Rho-kinase mediated inhibition of myosin light-chain phosphatase illustrating different molecular mechanisms underlying the initiation and maintenance of vasoconstrictor responses [12-14].

ET-1 binds tightly to ET_A receptors [9, 15], causing arterial contractions followed by long-lasting vasospasms that are refractory to inhibition by ET-receptor antagonists [2, 9, 16-18]. This might explain why ET-receptor antagonists are rather ineffective in treating ET-related diseases in clinical trials [3, 19, 20]. In view of the tight agonist binding, functional antagonists may be better suited for therapeutic purposes than receptor antagonists.

The endogenous ET-2 (Trp^6Leu^7 ET-1) has binding affinities and efficacies at ET-receptors that are seemingly similar to those of ET-1 and has therefore been considered to display identical pharmacological properties [21]. Recently however we reported quantitative differences between the effects of ET_A antagonists on arterial responses to ET-1 and ET-2 [8, 22] and an elegant review of the literature identified several differences in the functions of ET-1 and ET-2 in the cardiovascular system, ovaries, immunology and cancer [23].

Here we tested the hypotheses that i) different mechanisms mediate ET-induced contractions and vasospasms and ii) that these mechanisms display agonist- and system dependence. For the latter we focused on differences between ET-1 and ET-2 and between mesenteric and cerebral arteries.

Methods

Experiments were performed in accordance with the institutional guidelines and were approved by the Ethics Committee on Experimental Animal Welfare of the Maastricht University.

Solutions and compounds

Bay412272 and Bay602770 were a kind gift from Dr. JP Stasch (Bayer Healthcare, Wuppertal, D) and were dissolved in DMSO. Felodipine, Staurosporine (Sigma Aldrich, Zwijndrecht, NL), Chelerythrine chloride, Pyr3, OH-fasudil, Ro318220 and U73122 (Tocris Bioscience, Bristol, UK), were also dissolved in DMSO, the latter by heating to 70°C for 2h. Indomethacin (INDO, cyclooxygenase-inhibitor) (Sigma Aldrich, Zwijndrecht, NL) was dissolved in 100% ethanol. Human ET-1 and human ET-2 (Bachem, Weil am Rhein, D), noradrenaline (NA), phenylephrine (PHE), acetylcholine (ACh), pinacidil, isoproterenol, forskolin and L-NAME (NO synthase-inhibitor) (Sigma Aldrich, Zwijndrecht, NL) were dissolved in Krebs Ringer bicarbonate buffer (KRB) containing, in mM: NaCl: 118.5; KCl: 4.7; $CaCl_2$: 2.5; $MgSO_4$: 1.2; KH_2PO_4: 1.2; $NaHCO_3$: 25.0; glucose: 5.5. K^+-KRB was KRB in which all NaCl was replaced by KCl. Buffers with intermediate K^+-concentration were prepared by mixing appropriate volumes of KRB and K^+-KRB. The maximal solvent concentrations did not exceed 0.1% and did not significantly modify vascular reactivity.

Recording of vasomotor responses

16 weeks old male Wistar Kyoto rats (Charles River, Maastricht, NL) were euthanized by CO_2-inhalation. The basilar artery and 2nd-order branches of the superior mesenteric artery were isolated by dissection in KRB at room temperature. To record isometric tension development, 2 mm long freshly isolated arterial segments were mounted in wire myographs (DMT, Aarhus, DK) in which the segments were kept in KRB at 37°C and aerated with 95% O_2/5% CO_2. The mesenteric resistance artery segments were progressively stretched to the diameter at which the largest contractile response to 10 μM NA was observed [24]. The optimal internal diameter of the segments averaged 311 ± 7 μm and contractile responses to 10 μM NA and to 40 mM K^+ averaged 4.7 ± 0.2 N/m and 4.1 ± 0.3 N/m, respectively. The basilar artery segments were distended to a diameter corresponding to 90% of the diameter at a transmural pressure of 100 mmHg (0.9 D_{100}). The internal diameter of these segments averaged 385 ± 13 μm and contractile responses to 40 mM K^+ averaged 2.5 ± 0.2 N/m.

The arterial segments were treated with 1 μM CAPS for 20 min during contraction induced by, 40 mM K^+, to desensitize peri-arterial sensory motor-nerves. The segments were thereafter studied in the continuous presence of INDO (10 μM) and L-NAME (100 μM) to inhibit prostaglandin- and NO-synthesis, respectively. This approach allowed focusing on vascular smooth muscle function, minimizing the effect of the endothelium and sensory motor-nerves [25].

Due to the quasi-irreversible nature of ET-induced contractions, a single set of experiments was performed in one set of arterial segments, i.e. distinct pharmacological protocols were performed in parallel rather than in series. Also, arteries from different rats were used to monitor anti-ET effects of different putative functional antagonists.

Pharmacological protocols

To determine workable concentrations of putative functional antagonists, we recorded contractile responses to 40 mM K^+ and 10 μM PHE in the presence of increasing concentrations of the compounds. The lowest concentration with the maximal inhibitory effect was then selected for further experiments. The reversibility of these effects was tested after 10 min. repeatedly rinsing the organ chamber with compound-free buffer. Stimuli of relaxing mechanisms and the calcium-antagonist were tested versus K^+-induced contractions while inhibitors of receptor-activated mechanisms were evaluated versus PHE-induced contractions. Effects of the putative functional antagonists on i) the apparent potency, ii) the maximal effect and iii) the maintenance of ET-induced arterial contractions were determined by constructing cumulative concentration-response curves (CCRC) to ET-1 or ET-2 in the absence (Fig. 4.1A) and in the continuous presence of the pre-determined concentration of the compound administered 15 min before the peptide (Fig. 4.1B). In addition, the relaxing effect of the antagonist was determined by first inducing contraction with increasing concentrations of the ET and subsequently administering the compound in the presence of the peptide (Fig. 4.1C). The three CCRCs for the ET were performed in arterial segments from the same rats. They started at 0.25 nM and the concentration of the peptide was doubled at 5 min interval until 16 nM was reached. This concentration was left in contact with the tissues for 15 min allowing the first segment, which was not exposed to antagonist (Fig. 1A), to be used as a time-control for the maintenance and relaxation parts of the protocol (Fig. 4.1B and 4.1C). Ultimately, the peptide and compound were rinsed from the organ chamber contents and tension was recorded for 8 more min to monitor reversibility of the ET-induced contraction and of the antagonist effects.

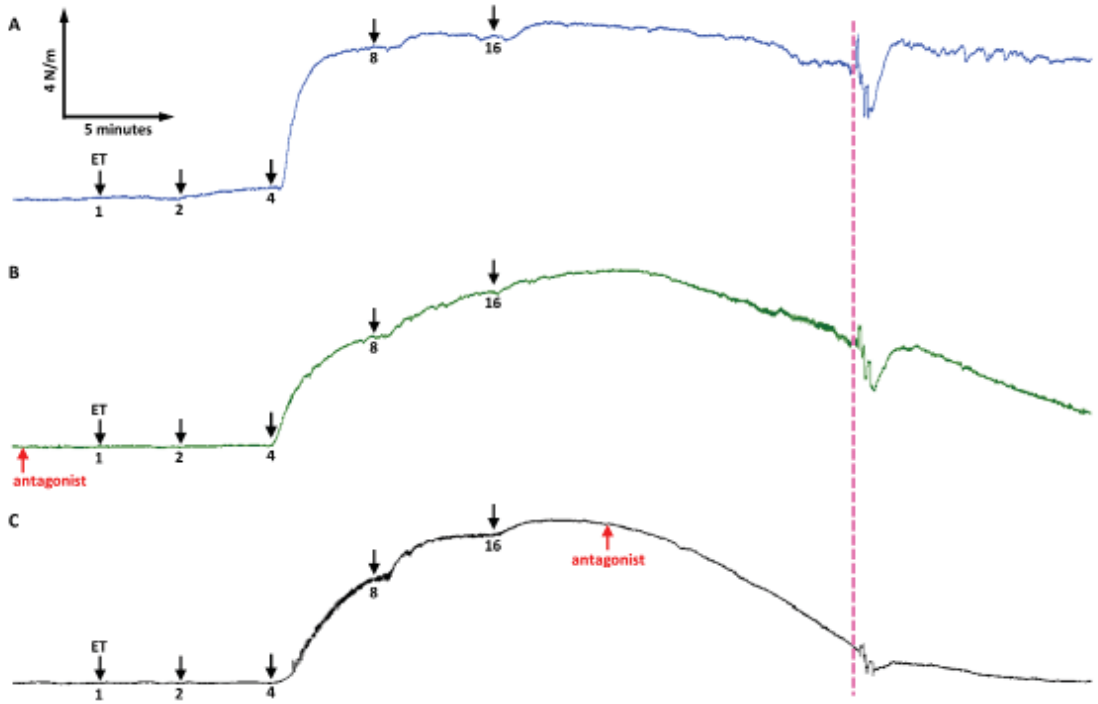

Fig. 4.1. Original tracings of active wall tension as a function of time, illustrating the pharmacological protocols. A. Contractile responses to increasing concentrations of ET-1 or ET-2 and the vasospasm and persistence thereof were recorded. The red dashed line indicates washout of compounds from the organ chamber content. B. Effects of a putative functional antagonist on the potency and maximal effect of the ET and its maintenance was recorded in another arterial segment in parallel. C. In a third arterial segment, the relaxing effect of the putative functional antagonist was recorded during ET-induced vasospasm.

Data analysis and statistics

Data are shown as mean ± SEM with n ≥ 6 for each observation. Contractile responses are expressed as percentage of the maximal contractile response to 10 µM NA (NA_{MAX}) or 40 mM K^+ observed prior to the administration of any pharmacological inhibitor for mesenteric and basilar artery preparations, respectively. Individual CCRC were fitted to a non-linear regression curve and ED_{50} values were calculated using GraphPad Prism 5.02. Data were analyzed using one-way ANOVA (comparison of EC_{50} and E_{MAX}) or two-way ANOVA (comparison of CCRC). Bonferroni's post-hoc test was used to compare multiple groups.

Results

General effects of functional antagonists

Fig. 4.2 summarizes mesenteric resistance artery contractile responses to 40 mM K^+ and to 10 µM PHE in the presence of functional antagonists. Maximal reduction of K^+-induced responses was observed with 1 nM felodipine

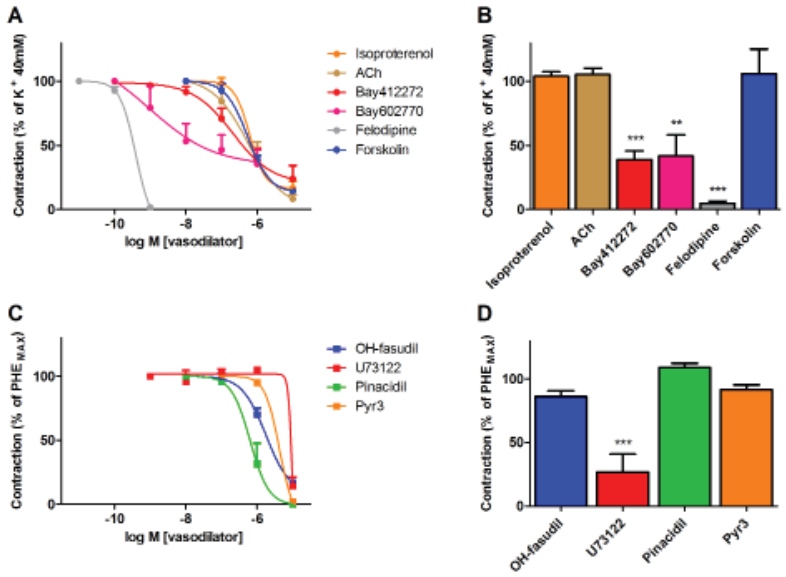

Fig. 4.2. Effects of functional antagonists on contractile responses to 40 mM K^+ (top) and 10 µM PHE (bottom). The reversibility of the effects of the vasodilator compounds (B and D) was evaluated at 10 minutes after flushing the antagonist from the organ chamber. Findings were expressed as % of the contractile response to 40 mM K^+ or to 10 µM PHE (PHE_{MAX}) and are shown as means ± SEM (n = 6 – 8). ** P < 0.01, *** P < 0.001.

(inhibitor of L-type voltage-operated Ca^{2+}-channels, L-VOCC [26]), 1 µM Bay602770 (haem-independent activator of sGC [27]) and with 10 µM of each acetylcholine (endothelium-dependent vasodilator [28]), Bay412272 (haem-dependent stimulator of sGC [29]), forskolin (direct stimulus of AC [30]), and isoproterenol (β-adrenoceptor agonist [31]) (Fig. 4.2A). Responses to PHE were reduced more than 80% in the presence of 10 µM of each pinacidil (opener of ATP-sensitive K^+-channels [32]), U73122 (inhibitor of PLC [33]), OH-fasudil (inhibitor of Rho-kinase [34]) and Pyr3 (inhibitor of receptor-operated Ca^{2+}-channels [35]) (Fig. 4.2B). 1 nM felodipine did not significantly modify contractile responses to PHE, and both 10 µM U73122 and 10 µM OH-fasudil did not significantly modify K+-induced contraction (data not shown), as expected. Conflicting results were obtained with candidate inhibitors of protein kinase C. They either had no effect on contractile responses at up to 10 µM (Ro318220) or they reduced responses to K^+ and PHE to the same extent (staurosporine and chelerythrine chloride; data not shown). Inhibitory effects of acetylcholine, forskolin, isoproterenol, OH-fasudil, pinacidil and Pyr3 were abolished within 10 min of washout of the compounds while inhibitory effects of Bay412272, Bay602770, U73122 and especially felodipine were only poorly reversible (Fig. 4.2C and 4.2D).

Mesenteric resistance artery response to ET-1

After sensorimotor nerve desensitization and in the presence of L-NAME and indomethacin, mesenteric resistance arteries responded to ET-1 with concentration-dependent contractions (Fig. 4.1, 4.3 and 4.4). The maximal response was well maintained in the presence of 16 nM ET-1 and long-lasting after washout of free unbound peptide (Fig. 4.1A, 4.3B and 4.4B).

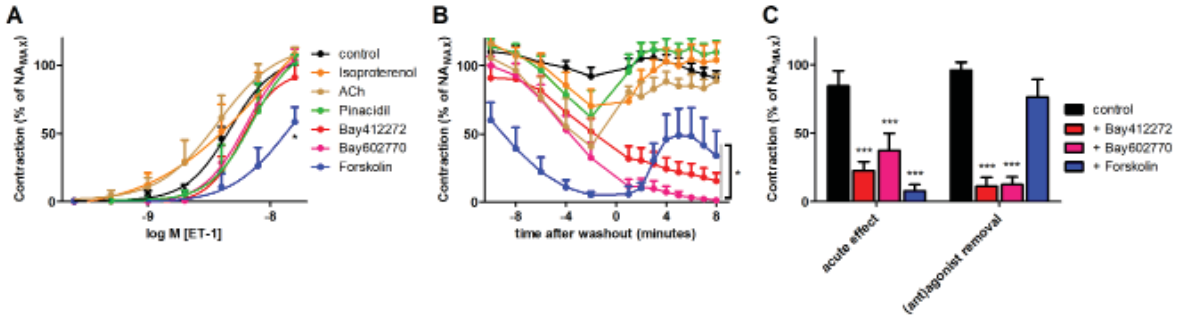

Fig. 4.3. Effects of the antagonists on sensitivity and maximal contractile responses to ET-1 (A) and their effects on the persistent ET-1-induced vasospasm before and after removal of free compounds from the tissue (washout) (B). C: The acute relaxing effect of the antagonists on ET-1-induced vasospasms and reversibility of the relaxation when the agonist and antagonist were removed. Findings were expressed as % of the contractile response to 10 µM noradrenaline (NA_{MAX}) and are shown as means ± SEM (n = 6 – 8).* P < 0.05, *** P < 0.001.

Presence of most of the functional antagonists at concentrations that inhibited K^+- or PHE-induced contraction, did not significantly modify sensitivity or maximal responses to ET-1. This was the case for acetylcholine, Bay412272, Bay602779, felodipine, isoproterenol, OH-fasudil, pinacidil and Pyr3 (Fig. 4.3A and 4.4A). Forskolin moderately reduced the sensitivity to ET-1 (Fig. 4.3A) and U73122 markedly attenuated the initiation of contraction by 0.25 to 16 nM ET-1 (Fig. 4.4A). Despite lack of effect on sensitivity and maximal responsiveness to ET-1, presence of several

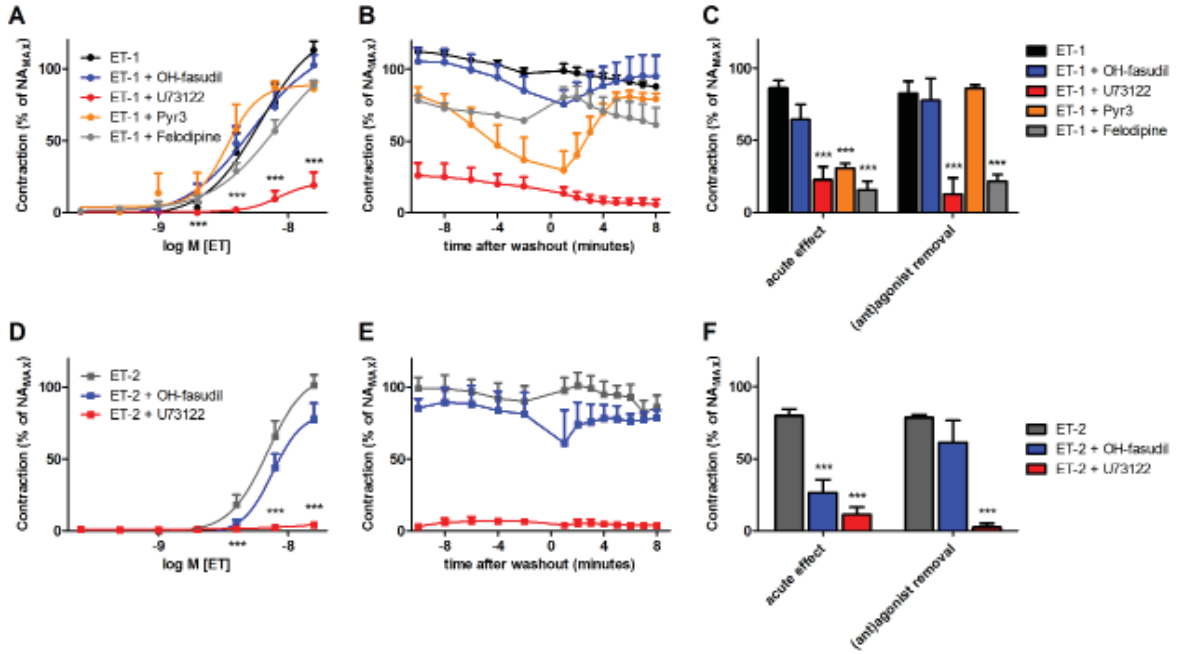

Fig. 4.4. Top: effects of felodipine, Pyr3, OH-fasudil and U73122 on ET-1-induced contractions (A) and persistence of vasospasms before and after washout (B), and the acute relaxing effect of the felodipine, Pyr3, OH-fasudil and U73122 on ET-1-induced vasospasms and reversibility of the relaxation when the agonist and antagonist were removed (C). Bottom: effects of OH-fasudil and U73122 on ET-2-induced contractions (D) and persistence of vasospasms (E), and the acute relaxing effect of OH-fasudil and U73122 on ET-2-induced vasospasms and reversibility of this effect (F). Findings were expressed as % of the contractile response to 10 µM noradrenaline (NA_{MAX}) and are shown as means ± SEM (n = 6).*** P < 0.001.

types of functional antagonists reduced the maintenance of the contractile response to the peptide (Fig. 4.3B and 4.4B). 10 min after reaching the maximal response to ET-1, the level of contraction was significantly lower than in the time control in preparations exposed to 10 μM isoproterenol, 10 μM acetylcholine, 10 μM pinacidil, 10 μM Bay412272, 1 μM Bay602770, 10 μM forskolin (Fig. 3B), or 10 μM Pyr3 (Fig. 4.4B). In contrast, presence of 1 nM felodipine and 10 μM OH-fasudil did not significantly alter the maintenance of mesenteric resistance artery responses to ET-1 (Fig. 4.4B).

In view of the different findings on initiation and maintenance of contraction we determined whether the functional antagonists could relax ET-1-induced contractions. In an earlier study we demonstrated this for isoproterenol, acetylcholine and pinacidil [9]. Here we observed marked relaxation with stimuli of soluble guanylate cyclase or adenylate cyclase which was long-lasting for both Bay412272 and Bay602770 and readily reversible for forskolin (Fig. 4.3C). Also U73122, Pyr3 and felodipine, but not OH-fasudil, markedly relaxed ET-1-induced contraction (Fig. 4.4C). While the effects of the PLC inhibitor and the L-VOCC blocker (felodipine) were long-lasting that of the inhibitor of receptor-operated calcium-channels (Pyr3) was readily reversible. It is noteworthy that felodipine which did not alter the potency, maximal effect and maintenance of ET-1-induced contracting did induce a marked relaxation of the response to peptide (Fig. 4.4).

Mesenteric resistance artery responses to ET-2

The potency and maximal contractile effects of ET-2 and their maintenance and persistence did not differ from those of ET-1 (Fig. 4.4). Presence of U73122 largely prevented contractile responses to ET-2 and the PLC inhibitor markedly relaxed ET-2-induced contraction. Presence of OH-fasudil did not significantly modify the sensitivity and the maximum and maintenance of responses to ET-2. These findings are very similar to those with ET-1 (Fig. 4.4). However, unlike ET-1-induced contractions (Fig. 4.4C), ET-2-induced contractions could be significantly relaxed by OH-fasudil (Fig. 4.4F).

Basilar artery responses to ET-1and ET-2

The cerebral arteries were somewhat more sensitive to ET-1 and ET-2 than the mesenteric resistance arteries and also in these vessels the contractile responses to the peptides were maintained and long-lasting (Fig. 4.4 and 4.5). As was the case for the mesenteric vessels, presence of U73122 largely suppressed basilar artery

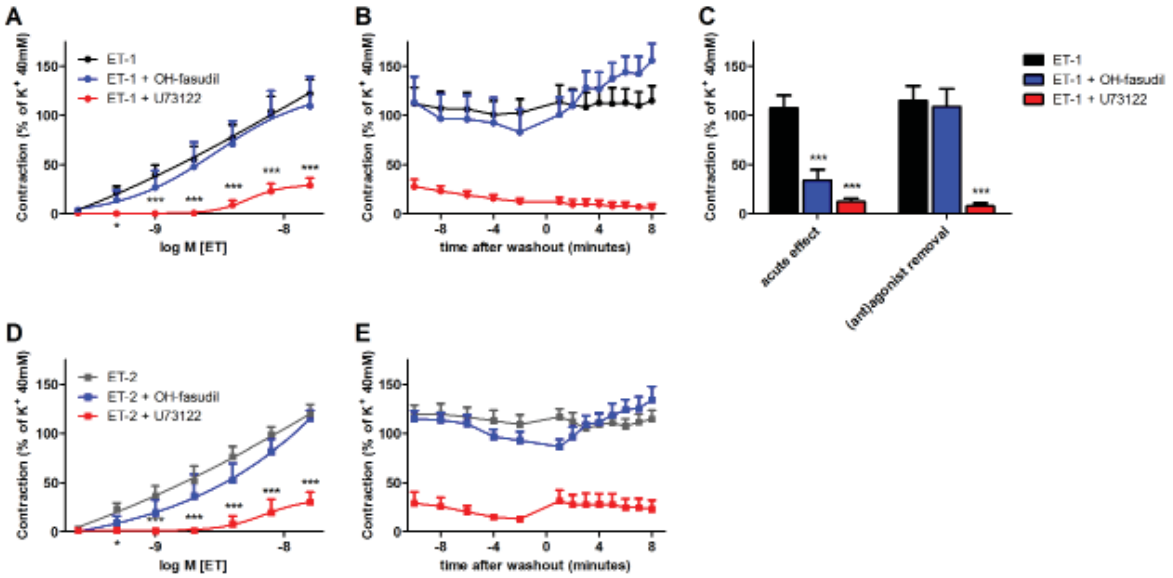

Fig. 4.5. Effects of U73122 and OH-fasudil, in the basilar artery, on sensitivity and maximal contractile responses to ET-1 (A) and ET-2 (D), on the persistence of the vasospasms before and after washout (B, E), and on the acute relaxing effect of U73122 and OH-fasudil on ET-1- induced vasospasms and reversibility of the relaxation (C). Findings were expressed as % of the contractile response to 40 mM K^+ and are shown as means ± SEM (n = 6).* P < 0.05, ** P < 0.01, *** P < 0.001.

contractile responses to ET-1 and ET-2 (Fig. 4.5A, 4.5D) and the PLC inhibitor markedly relaxed contractile responses to ET-1 (Fig. 4.5C). While presence of OH-fasudil did not modify sensitivity, maximum and maintenance of responses to ET-1, this RhoK-inhibitor markedly relaxed ET-1-induced contractions in rat basilar arteries (Fig. 4.5C). This is noteworthy because OH-fasudil had no statistically significant effects on responses to ET-1 in rat mesenteric resistance arteries (Fig. 4.4C).

Discussion

The main findings of this work are that several functional antagonists reduce the maintenance of ET-induced arterial contractions more markedly than their initiation and that this differs between ET-1 and ET-2 and between rat mesenteric resistance and basilar arteries.

Rat small artery vasomotor responses to ETs are mediated by smooth muscle ET_A receptors [7]. The peptides bind tightly to these receptors leading to long-lasting contractions that are refractory to reversal by ET_A antagonists [2, 9, 16-18]. This is even more marked in rat cerebral arteries than mesenteric resistance arteries [36] and invites to consider other types of inhibitors such as a “physiological” antagonist like CGRP [9, 37], negative allosteric modulators of receptor function [8, 15, 22] and functional antagonists. Here we concentrated on anti-endothelinergic effects of

inhibitors of contractile mechanisms and of stimuli of relaxing mechanisms. We verified the selectivity of these pharmacological tools by analyses of their effects on K^+-induced and α_1-adrenergic contractions.

The molecular mechanisms of vasoconstriction induced by agonists that activate 7 transmembrane domain receptors are still only partly understood. A widely held view suggests differences during initiation and maintenance of smooth muscle contraction. The agonists would stimulate PLC and cause an initial transient marked increase in cytoplasmic calcium-concentration that is followed by increased calcium-sensitivity of the contractile apparatus resulting from RhoK-mediated inhibition of myosin light chain phosphatase [12-14]. Most of the uncertainties remain in the origin of the calcium ions, in the nature of the channels involved in intracellular calcium-release and -influx over the sarcolemma and in the transition to RhoK-dependent mechanisms. In general, ET_A receptors have been shown to stimulate among others several G-proteins, PLC activity, a transient increase in intracellular calcium-concentration and RhoK activity [10, 11, 13, 38] and most of these were confirmed in smooth muscle cells from rat mesenteric resistance arteries [39, 40]. However, the distinct roles of these processes in the initiation and maintenance of ET-induced contractions are much less clear. Here, we observed that different mechanisms are involved in the initiation and maintenance of ET_A mediated arterial contractions but found no evidence for involvement of RhoK herein. We rather obtained the first indications that this displays agonist- and system-dependence.

The widely used PLC inhibitor U73122, at a concentration that did not reduce K^+-induced responses, largely prevented and reversed contractile effects of ET-1 and ET-2 in both mesenteric arteries and to ET-1 in basilar arteries. This suggests a major overall role for PLC in arterial ET_A mediated contractions. The precise isoenzymes were proposed to differ from PLC-δ1 involved in sustained α_1-adrenergic arterial contraction [39]. The use of U73122 as a selective general inhibitor of phospholipases has recently been challenged [41] but there is no valid pharmacological alternative available yet. A for this study potentially serious additional effect is potent PLC-independent inhibition of plasmalemmal calcium-channels reported in one study [42]. PLC synthesizes inositol trisphosphate that stimulates intracellular calcium-release and diacylglycerol that stimulates PKC which can among others promote calcium-influx. The former is in line with a strong but transient mesenteric resistance artery contractile response to ET-1 in the absence of extracellular calcium [43]. The roles of the latter could not be investigated because putative inhibitors were either ineffective or lacked the desired selectivity.

In contrast to U73122, the dihydropyridine inhibitor of L-VOCC felodipine and the RhoK-inhibitor OH-fasudil, at concentrations that inhibited K^+-induced and α_1-adrenergic responses, respectively, did not significantly modify sensitivity, maximum and maintenance of mesenteric artery responses to ET-1. This invited to consider involvement of receptor-operated calcium channels with the use of Pyr3. This pyrazole compound was recently reported to selectively inhibit transient receptor potential canonical channels (TRPC3) and Orai1, a component of store-operated calcium channels [35, 44]. Pyr3 did not alter initiation of mesenteric artery responses to ET-1 but reversibly inhibited their maintenance and reversibly relaxed ET-1-induced contractions in this tissue. Because TRPC3 and Orai1 functions are modulated by diacylglycerol, either directly or via PKC [45-47], these channels might be linked to ET-1 induced PLC activation and involved in the sustained mesenteric artery responses to the peptide.

Stimuli of relaxing mechanisms that act via hyperpolarization, AC or sGC did not alter sensitivity or maximal responses to ET-1 but could relax ET-1-induced vasospasms. These results indicate different intracellular mechanisms involved in initiation and maintenance of smooth muscle contractions. It is noteworthy that we did not observe a marked difference between the effects of a haem-dependent stimulator of sGC (Bay412272) and a haem-independent activator of sGC (Bay602770) [48] although ET-1 potently stimulates NADPH oxidase activity via ET_A receptors [49]. The stimuli that produced non-reversible relaxation of ET-1-induced vasospasms were those that were characterized, based on their effects on K^+-induced and α_1-adrenergic contractions, as poorly reversible, and likely did not alter binding of ET-1 to ET_A receptors. The effects of stimuli of relaxing mechanisms and of the inhibitors of contractile mechanisms are presented in Fig. 6, in which the proposed mechanisms for the initial contraction and subsequent vasospasms are summarized.

As previously described, effects of ET_A receptor antagonists are agonist-dependent, i.e. the extent of the modulation by these receptor antagonists depends on which agonist is used to activate ET_A receptors [8, 22]. We therefore aimed to determine if such agonist-dependence also applied to functional antagonists and we found that RhoK-inhibition, in contrast to ET-1-induced vasospasms, reversibly relaxed ET-2-induced vasospasms. Because the Rho/RhoK-mediated pathway is a signaling pathway parallel of PLC [13] and the effects of the PLC-inhibitor did not differ between ET-1- and ET-2-induced responses, ET-1 and ET-2 display a functional selectivity and could be considered as biased agonists [50]. ET-1 selectively activates PLC signaling, whereas ET-2 is more biased to a non-selective, parallel activation of PLC and Rho/RhoK pathways. This is worth some consideration when functionally

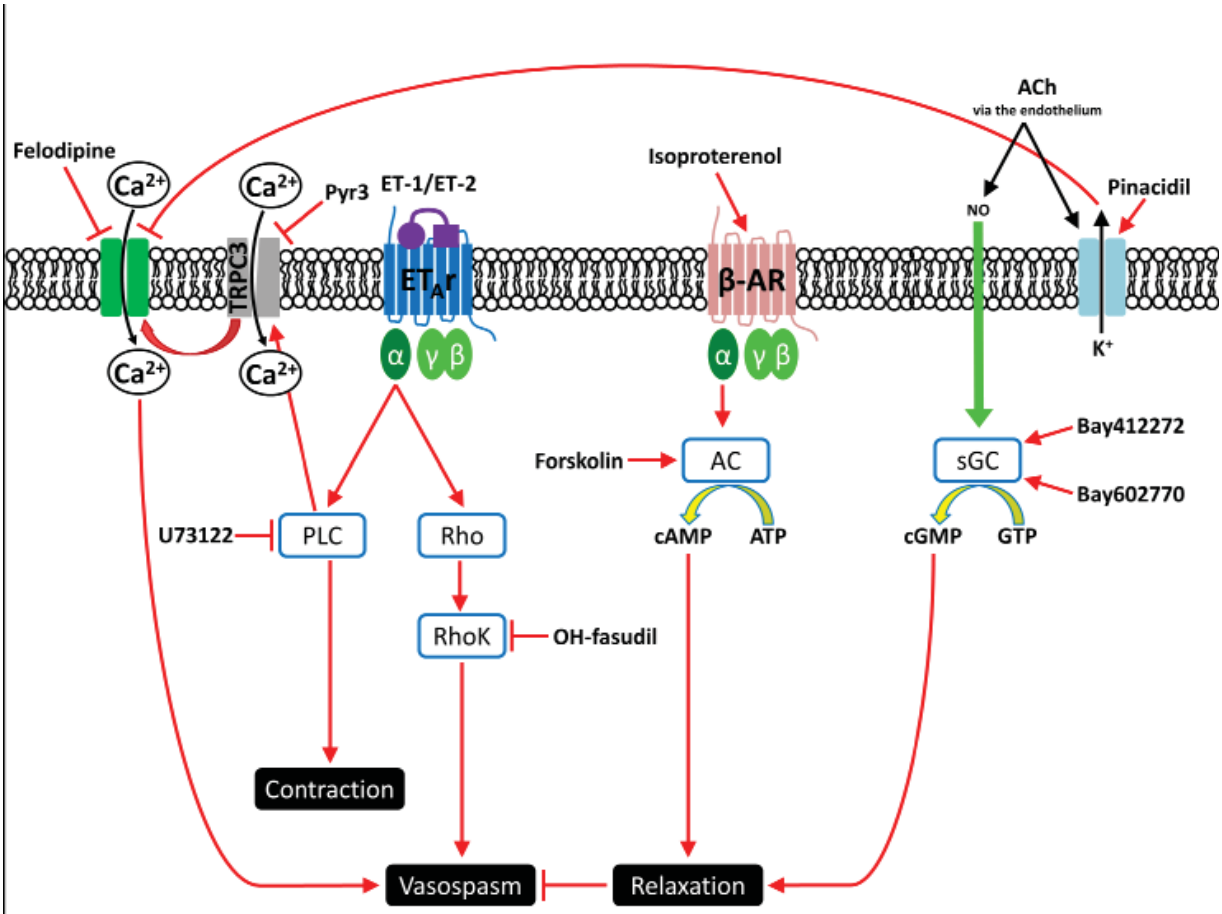

Fig. 6. The intracellular signaling pathway leading to ET_A mediated contractions and vasospasms and targets to inhibit these pathways. U73122, the PLC- inhibitor, is the only compound that inhibits both contractions and vasospasms and is therefore likely to be the main mediator of the initiating contraction. α: G-protein α subunit; β: G-protein β subunit; β-AR: β-adrenoceptor; γ: G-protein γ subunit; AC: adenylate cyclase; ACh: acetylcholine; ATP: adenosine triphosphate: Bay412272: sCG stimulator; Bay602770: sCG activator; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; ET-1: endothelin-1; ET-2: endothelin-2; ET_Ar: endothelin$_A$ receptor; felodipine: calcium channel blocker; forskolin; AC activator; GTP: guanosine triphosphate; isoproterenol: β-adrenoceptor agonist; NO: nitric oxide; OH-fasudil: RhoK-inhibitor; pinacidil: ATP-sensitive potassium channel opener; PLC: phospholipase C; Pyr3: TRPC3-blocker; RhoK: Rho-kinase; sGC: soluble guanylate cyclase; TRPC3: Transient receptor potential cation channel 3; U73122: PLC -inhibitor.

antagonizing ET_A mediated signaling, as agonist-dependence, one of the indicators of allosterism [50, 51] at a receptor level, is now also found as an intracellular occurrence.

To further explore allosteric modulation, we addressed another criterion; system-dependence [52]. To this end, we compared mesenteric arteries with basilar arteries, a system that is resistant to ET_A receptor antagonist and to the physiological antagonist CGRP [36]. Like in mesenteric arteries, the PLC-inhibitor U73122 largely prevented and reversed contractile effects of ET-1. But OH-fasudil, the RhoK-inhibitor, induced a reversible relaxation of ET-1-induced vasospasms in basilar arteries, in contrast to its (lack of) effect in mesenteric arteries. This suggests a functional selectivity of

ET-1 towards a biased, PLC-mediated response in mesenteric arteries and a non-selective, non-biased parallel PLC- and Rho/RhoK-mediated response.

In conclusion, the intracellular signaling mechanism of ET_A mediated contractile responses not only changes during sustained receptor activation but is also dependent on which agonist activates ET_A receptors. Unlike Rho/RhoK, PLC is involved in contractions as well as vasospasms induced by ET-1 or ET-2 in rat mesenteric arteries. The initiation and maintenance of ET-induced contractile responses are mediated by different mechanisms, downstream of PLC. Additionally, the intracellular signaling mechanism is not only agonist-dependent but also system-dependent, as Rho/RhoK signaling is involved in ET-1-induced responses in basilar arteries. When using functional antagonists as a method of pharmacologic intervention in the ET system, both system- and agonist-selectivity of the functional antagonist should be considered.

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Chapter 5

Agonist-Dependent Modulation of Arterial Endothelin$_A$-Receptor Function

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Abstract

Endothelin-1 (ET-1) causes long-lasting vasoconstrictions. These can be prevented by ET_A receptor antagonists but are only poorly reversed by these drugs. We tested the hypothesis that endothelin ET_A receptors are susceptible to allosteric modulation by endogenous agonists and exogenous ligands.

Isolated rat mesenteric resistance arteries were pretreated with capsaicin and studied in wire myographs, in the presence of L-NAME and indomethacin to concentrate on arterial smooth muscle responses.

Endothelins caused contractions with equal maximum but differing potency (ET-1 = ET-2 > ET-3). ET-1_{1-15} neither mimicked nor antagonized these effects in the absence and presence of ET_{16-21}. 4^{Ala}ET-1 (ET_B agonist) and BQ788 (ET_B antagonist) were without effects. BQ123 (peptidergic ET_A antagonist) reduced the sensitivity and relaxed the contractile responses to endothelins. Both effects depended on the agonist (pK_B: ET-3 = ET-1 > ET-2; % relaxation: ET-3 = ET-2 > ET-1). Also with PD156707 (non-peptidergic ET_A antagonist) agonist-dependence and a discrepancy between preventive and inhibitory effects were observed. The latter was even more marked with bulky analogues of BQ123 and PD156707.

These findings indicate allosteric modulation of arterial smooth muscle ET_A receptor function by endogenous agonists and by exogenous endothelin-receptor antagonists. This may have consequences for diagnosis and pharmacotherapy of diseases involving endothelins.

Introduction

The endogenous mammalian endothelins ET-1, ET-2 and ET-3 are bicyclic 21 amino acid paracrine mediators. They share a 6 amino acid C-terminal tail but differ in 2 to 6 amino acids in the N-terminal loop (Table 1). Their roles in physiology and in diseases are mediated by two subtypes of 7 transmembrane domain receptors (7TMR) [1-6]. Activation of ET_A receptors causes cell growth and proliferation, vasospasm, oxidative stress and, inflammation. Endothelial ET_B receptors scavenge endothelins from the circulation and are proposed to counterbalance the deleterious effects of endothelins [3, 7]. ET-1 and ET-2 bind with equal high affinity to ET_A and ET_B while ET-3 binds with considerably lower affinity to ET_A than ET_B [8-10]. During the past two decades, several classes of low molecular weight compounds were discovered that prevent binding of endothelins to ET_A and/or ET_B (for review see [8, 11]). These endothelin-receptor antagonists (ERAs) are regarded as neutral competitive antagonists although their binding-site does not necessarily coincide with the agonist-binding sites [12-16].

Structure-affinity, -selectivity and -activity relationships indicate a key role of the C-terminus of endothelins in ET_B binding and activation [14, 17]. ET_A receptor function seems more complex. We and others proposed that ET-1 binds polyvalently to ET_A receptors [14, 17-19]. For other peptidergic 7TMR-agonists such as calcitonin-gene related peptide (CGRP), corticotrophin-releasing factor (CRF) and parathyroid hormone (PTH), it has been reported that distinct parts of the agonist molecule and of the receptor govern binding-affinity (address domain) and signaling (message domain) [20, 21].

ET_A-mediated vasoconstrictor effects of ET-1 are potent, long-lasting and refractory to reversal by ERAs. In vitro and in vivo, they persist for long periods of time after washout or scavenging of the agonist [19, 22, 23]. While ERAs can prevent receptor-binding and effects of ET-1, they do not reverse established agonist-binding [24, 25] and have variable influences on ET-1-induced effects [19, 26, 27]. These unusual pharmacological properties may be due to tight binding of ET-1 to ET_A. The reported half-life of ET-1/ET_A complexes ranges from 7 to 77 hours (for review see [28]). Little is known about ET-2 and ET-3 in this respect. In contrast to ERAs, salicylates [24] and, more recently, the neuropeptide CGRP [19] were reported to promote dissociation of ET-1/ET_A complexes. This suggests that ET_A receptor function is susceptible to allosteric modulation [18].

Table 5.1. Amino acid sequence of ET isoforms and fragments.

Ligand	N				5				10				15				20	C
ET-1[a]	Cys	Ser	Cys	Ser	Ser	Leu	Met	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET-2[a]	Cys	Ser	Cys	Ser	Ser	Trp	Leu	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET-3[a]	Cys	Thr	Cys	Phe	Thr	Tyr	Lys	Asp	Lys	Glu	Cys	Val	Tyr	Tyr	Cys	His	Leu	Asp	Ile	Ile	Trp
4^{Ala}ET-1	Ala	Ser	Ala	Ser	Ser	Leu	Met	Asp	Lys	Glu	Ala	Val	Tyr	Phe	Ala	His	Leu	Asp	Ile	Ile	Trp
$ET\text{-}1_{1-15}$[a]	Cys	Ser	Cys	Ser	Ser	Leu	Met	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys						
$ET\text{-}1_{11-21}$[b]											Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET_{16-21}																His	Leu	Asp	Ile	Ile	Trp

[a] denotes presence of a disulphide bond between Cys^{1} and Cys^{15} and between Cys^{3} and Cys^{11}.

[b] denotes presence of a disulphide bond between Cys^{11} and Cys^{15}.

Here we tested the hypothesis that ET_A receptor pharmacology meets at least two criteria of allosteric modulation namely probe-dependence and differential modulation of affinity and efficacy by antagonists (for recent reviews see [29, 30]). For this purpose we used (i) isolated rat mesenteric resistance arteries, (ii) isoforms and fragments of ET-1 (Table 5.1), (iii) the peptidergic and the non-peptidergic ET_A selective antagonists BQ123 and PD156707, respectively and (iv) large analogues of these ERAs such as fluorescently labeled and homobivalent constructs (Fig. 5.1). The small muscular arteries that we used are involved in the regulation of local blood flow and blood pressure and in the development of hypertension [31]. ET receptors are expressed by their endothelial cells, smooth muscle cells and sensory-motor nerves [32], but we focused here on smooth muscle ET_A. We monitored effects of candidate ligands on the initiation, maintenance and persistence of arterial contractile responses and found that two prototypic ET_A receptor antagonists acted as allosteric inhibitors of the binding and activation of arterial smooth muscle ET_A receptors by endogenous ET-isoforms.

Methods

Experiments were performed in accordance with the institutional guidelines and were approved by the Ethics Committee on Experimental Animal Welfare of the Maastricht University.

Solutions and compounds

BQ123 (Sigma Aldrich, Zwijndrecht, NL) and BQ788 (Peptides International, Louisville, USA) were dissolved in DMSO. Capsaicin (CAPS) and indomethacin (INDO) (Sigma Aldrich, Zwijndrecht, NL) were dissolved in ethanol. Felodipine (Sigma Aldrich, Zwijndrecht, NL) was dissolved in polyethylene glycol 400. Human ET-1, human ET-2, ET-3, $Ala^{1,3,11,15}$ET-1 (4^{Ala}ET-1), $ET\text{-}1_{11\text{-}21}$, $ET_{16\text{-}21}$ (Table 5.1) (Bachem, Weil am Rhein, D), noradrenaline (NA) and L-NAME (Sigma Aldrich, Zwijndrecht, NL) were dissolved in Krebs Ringer bicarbonate buffer (KRB) containing (in mM): NaCl: 118.5; KCl: 4.7; $CaCl_2$: 2.5; $MgSO_4$: 1.2; KH_2PO_4: 1.2; $NaHCO_3$: 25.0; glucose: 5.5.

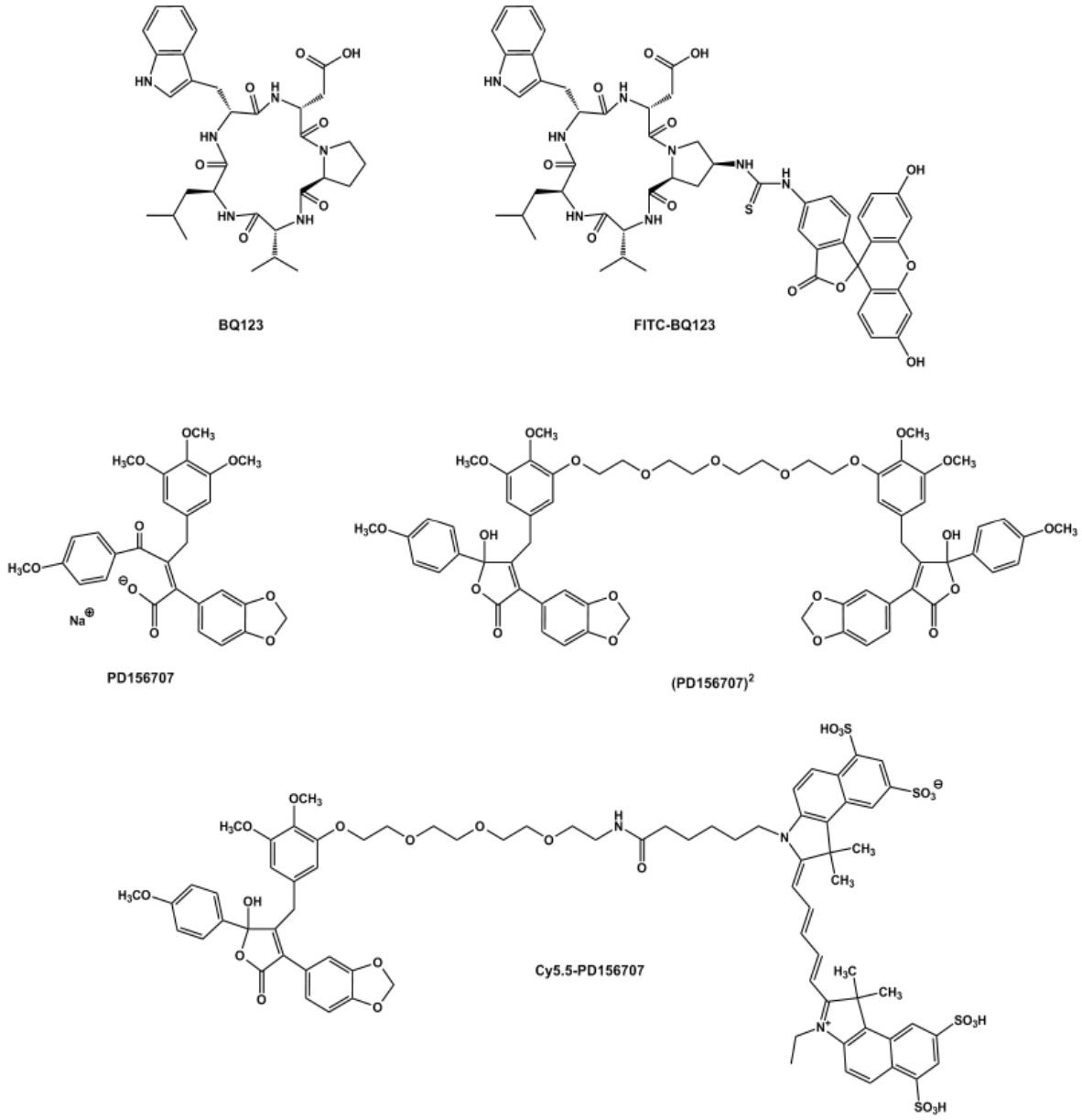


Figure 5.1. Structure of the low molecular weight ET receptor antagonists and their high molecular weight analogues used in this study.

PD156707, Cy5.5- PD156707 and FITC-BQ123 (Fig. 1) were synthesized as described [33, 34]. Synthesis of the intact bicyclic loop of ET-1 (ET-1_{1-15}) and synthesis of homobivalent PD156707 (Fig. 5.1), are detailed in the supplementary data. K^+-KRB was KRB in which all NaCl was replaced by KCl. Buffers with intermediate K^+-concentration were prepared by mixing appropriate volumes of KRB and K^+-KRB. The maximal solvent concentration never exceeded 0.1% and did not significantly modify vascular reactivity.

Recording of vasomotor responses

Male, 16 weeks old WKY rats (Charles River, Maastricht, The Netherlands) were killed by CO_2-inhalation. Second-order branches of the superior mesenteric artery were isolated by dissection in KRB at room temperature. To record isometric tension development, freshly isolated 2mm long arterial segments were mounted in wire myographs (DMT, Aarhus, DK) in which 5mL KRB was maintained at 37°C and aerated with 95% O_2/5% CO_2. The arterial segments were progressively stretched to the diameter at which the largest contractile response to 10 µM NA was observed [19, 32]. The optimal internal diameter of the segments averaged 306 ± 8 µm and contractile responses to 10 µM NA averaged 3.7 ± 0.1 N/m.

Except when specifically mentioned, arterial segments were pre-treated with 1 µM CAPS for 20 min and were thereafter studied in the continuous presence of 100 µM L-NAME and 10 µM INDO. These interventions desensitize peri-arterial sensory motor nerves and inhibit the synthesis of NO and prostaglandins, respectively [35]. They were used because in rat mesenteric resistance arteries not only arterial smooth muscle cells but also sensory- motor nerves and endothelial cells express immunoreactive ET_A and ET_B receptors [32, 36] and it was previously reported that ET-1 and ET-3 can induce the release of endothelium-derived relaxing factor (EDRF) [37].

Pharmacological protocols

We studied agonistic, antagonistic, competitive and inhibitory effects of putative ETA-receptor ligands and their reversibility as illustrated in Fig. 5.2.

Agonism. Increasing concentrations of an endothelin isopeptide or -fragment (cumulative concentration-response curve, CCRC) were administered to (i) resting arteries to record contractile effects and to (ii) arteries partly depolarized with 40 mM K^+ to record relaxing effects (Fig. 5.2A).

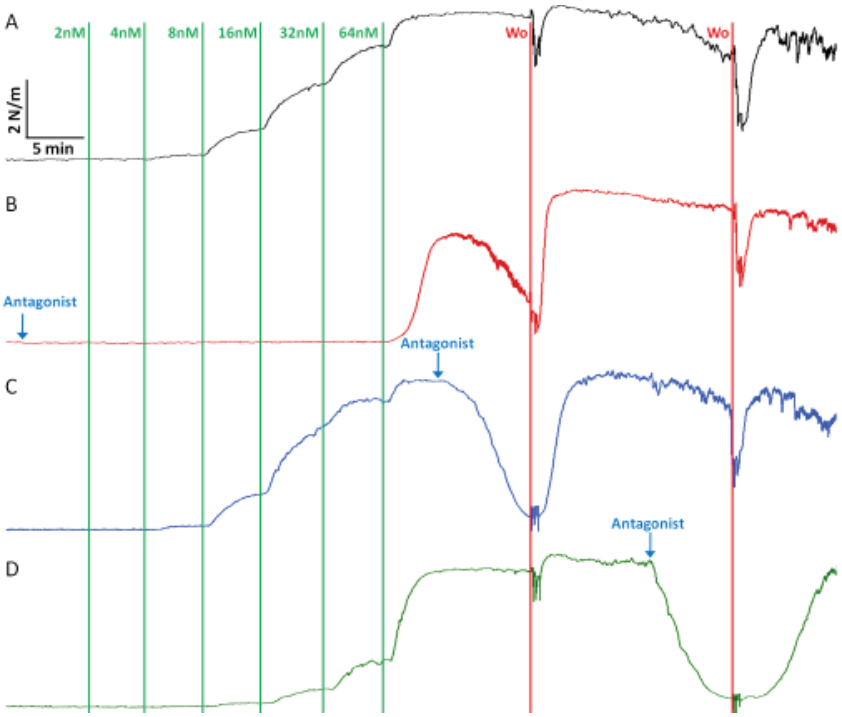


Figure 5.2. Typical tracings of arterial active wall tension versus time illustrating the study protocols. Contractile responses to increasing concentrations of an agonist (e.g. ET-2, vertical green lines, indicated concentrations are final concentrations) were recorded in absence (A) and presence (B) of an antagonist (e.g. 1 μM BQ123 (blue arrow)). Also, the inhibitory effect of this antagonist (blue arrow) was recorded during contractions observed in presence of the agonist (C) and during contractions that persisted after removal (washout, Wo) of free agonist (D).

Antagonism. For peptides that did not display agonism, a CCRC for an agonist (ET-1, ET-2 or ET-3) was constructed in the presence of 1 μM of the compound. The sensitivity (pD_2) and the maximal response (E_{MAX}) to the agonist were compared in parallel in presence and absence of the compound.

Competition Experiments. Using 4 arterial segments in parallel, CCRCs for an agonist were constructed in the absence and in the presence of a low, an intermediated and a high concentration of the putative antagonist (Fig. 5.2B). Effects of the compound on the position (ratio of EC_{50}, A'/A) and on the height of the agonist CCRC (ΔE_{MAX}) were monitored. Log (A'/A-1) was plotted as a function of the antagonist concentration ([B], Schild-plot).

Inhibition Experiments. Results from the competition experiments (contraction as a function of increasing agonist concentration ([A]) in absence and presence of 3 concentrations of putative antagonist ([B]) were plotted as a function of [B]. From this the inhibitory effect of a selected concentration of the antagonist ($[B]_y$) on the response to a selected concentration of an agonist ($[A]_x$) was calculated (predicted inhibition, PI). Then two arterial segments from the same rats were used. Both were exposed to $[A]_x$ and the responses were allowed to stabilize. Next, one preparation was also exposed to $[B]_y$ and the other served as a time-control (Fig. 5.2C). The effect of $[B]_y$ was allowed to stabilize and was compared to the predicted inhibition (PI).

Because endothelins can cause long-lasting effects, comparable inhibition experiments were performed on agonist-initiated contractions. Here, $[A]_x$ was applied and the effect was allowed to stabilize. $[A]_x$ was removed from the organ chamber and the influence of $[B]_y$ on the remaining effect was monitored 8 min later (Fig. 5.2D) and was compared to the predicted inhibition (PI).

Reversibility. Towards the end of each of the foregoing experiments, all putative ET_A receptor ligands were removed from the organ chambers (washout) and wall tension was recorded for > 20 min.

Only one set of experiments was performed in one set of arterial segments, i.e. distinct pharmacological protocols were not performed in series in the same set of arterial segments.

Data analysis and statistics

Data are shown as mean ± SEM. Contractile responses are expressed as percentage of the maximal contractile response to NA observed prior to the administration of any pharmacological inhibitor (NA_{MAX}). Individual CCRC were fitted to a non-linear regression curve and ED_{50}, pA_2 and pK_B values were calculated using GraphPad Prism 5.02. Data were analyzed using one-way ANOVA (comparison of pD_2, pA_2, pK_B and E_{MAX}) or two-way ANOVA (comparison of CCRC). Bonferroni's post-hoc test was used to compare multiple groups. Schild plots were constructed with linear regression analysis.

Results

Nanomolar concentrations of ET-1 ($ET\text{-}1_{1\text{-}21}$) and of ET-2 ($Trp^6\text{-}Leu^7\text{-}ET\text{-}1_{1\text{-}21}$) and micromolar concentrations of ET-3 ($Thr^2\text{-}Phe^4\text{-}Thr^5\text{-}Tyr^6\text{-}Lys^7\text{-}Tyr^{14}\text{-}ET\text{-}1_{1\text{-}21}$) caused contractions in isolated mesenteric resistance arteries (Fig. 5.3A). ET-1 and ET-2 were similarly potent and significantly ($p < 0.001$ and $p < 0.01$) more potent than ET-3 (pD_2: 8.4±0.1, 8.5±0.1 and 6.8±0.1, respectively). The maximal effects did not significantly differ between the peptides (E_{MAX}: 101.8±5.1%, 98.2±7.5% and 101.8±10.9%, respectively). They were sustained and faded only slowly after removal of the free agonist (Fig. 5.2 and 5.3B). Felodipine (1 nM), a dihydropyridine calcium channel blocker that inhibited tonic arterial contractile responses to 40 mM K^+, moderately reduced sentivity and maximal responses to ET-1 and ET-2; and this did not differ significantly between the two peptides (data not shown).

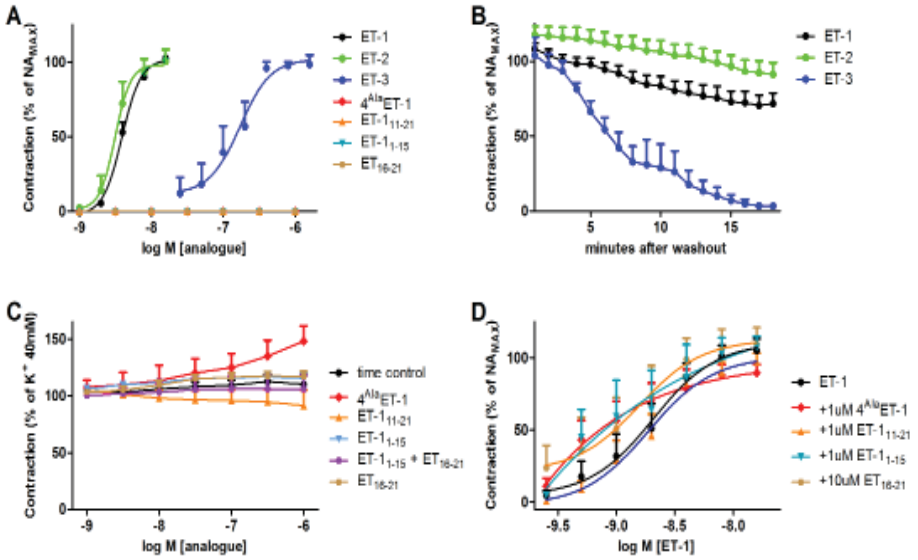


Figure 5.3. Vasomotor effects of isoforms, analogues and fragments of ET-1. A, only intact ET isoforms cause arterial contractile responses. B, these responses are only slowly reversible. C, 4^{Ala}ET-1 and fragments of ET-1 fail to induce relaxation in arteries contracted with 40 mM K^+. D, presence of 1 µM 4^{Ala}ET-1 or fragments of ET-1 does not modify contractile responses to ET-1. Findings were expressed as % of the contractile response to 10 µM noradrenaline (NA_{MAX}) or as % of the K^+-induced pre-contraction (C) and are shown as means ± SEM (n = 3 – 17).

For ET-3 (T½: ≈ 5 min) this was less pronounced than for ET-1 and ET-2 (T½; >18 min, Fig. 5.3B) but still more slowly than for equally strong contractile responses to for instance 10 µM NA T½: < 1 min, data not shown).

Presence of the ET_B selective antagonist BQ788 (1 µM, [38]) did not modify contractile effects of ET-1 (pD_2; 8.5±0.1 versus 8.4±0.1) and ET-3 (pD_2; 6.7±0.2 versus 6.8±0.1). 4^{Ala}ET-1, an ET_B selective linear analogue of ET-1 [39], did not cause contraction in resting arteries (Fig. 5.3A), did not cause relaxation in depolarized arteries (Fig. 5.3C) and did not modify contractile effects of ET-1 (Fig. 5.3D) at up to 1 µM. Likewise, the fragments $ET\text{-}1_{11\text{-}21}$, $ET\text{-}1_{1\text{-}15}$ and $ET_{16\text{-}21}$, and the combination of the N-terminal loop ($ET\text{-}1_{1\text{-}15}$) plus the C-terminal tail of ET-1 ($ET_{16\text{-}21}$) failed to stimulate contraction or relaxation and did not modify the contractile potency of intact $ET\text{-}1_{1\text{-}21}$ at up to 1 µM (Fig. 5.3A, C, D).

Presence of 1 µM BQ123, a peptidergic ET_A-selective antagonist [40], did not modify basal tension but reduced the contractile effects of all three endothelin isoforms (Fig. 5.4). This effect of BQ123 was more marked versus ET-3 than versus ET-1 and less marked versus ET-2 than versus ET-1 (Fig. 5.4). Presence of BQ123 did not prevent initiation of long-lasting contractile responses by ET-1 or ET-2, i.e. sustained responses persisting in the absence of free agonist (Fig. 5.4A-D). Moreover, presence of 1 µM BQ123 prevented contractile responses to 1 µM ET-3 (Fig. 5.4E) but a strong contraction developed within 1 min after washout of the free agonist and antagonist (Fig. 5.4F).

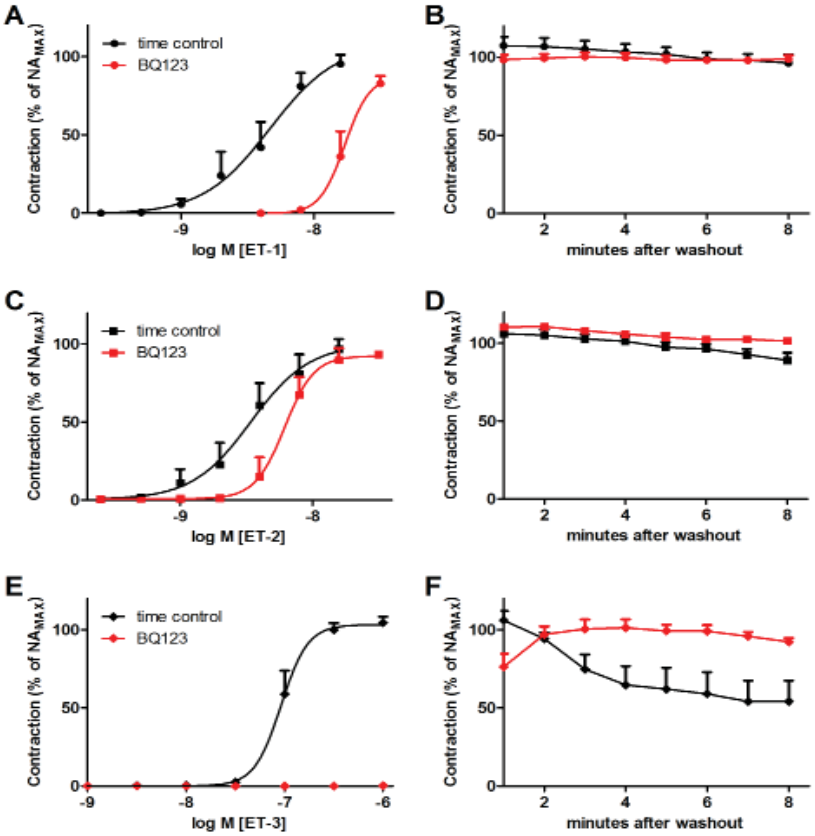


Figure 5.4 Contractile responses to ET-1 (A), ET-2 (C) and ET-3 (E) in absence (black) and presence of 1 µM BQ123 (red). B, D and F show changes in vasomotor tone after washout of free agonist (black) and after washout of both free agonist and antagonist (red). Findings were expressed as % of the contractile response to 10 µM noradrenaline (NA_{MAX}) and are shown as means ± SEM (n = 4 – 7).

Effects of ET_A selective antagonists were analyzed in more detail to gain insight in their agonist dependence. Using a range of concentrations, presence of BQ123 (3 nM – 3 µM) was observed to reduce the sensitivity but not the maximal responses to ET-1 and ET-3 (Fig. 5.5).

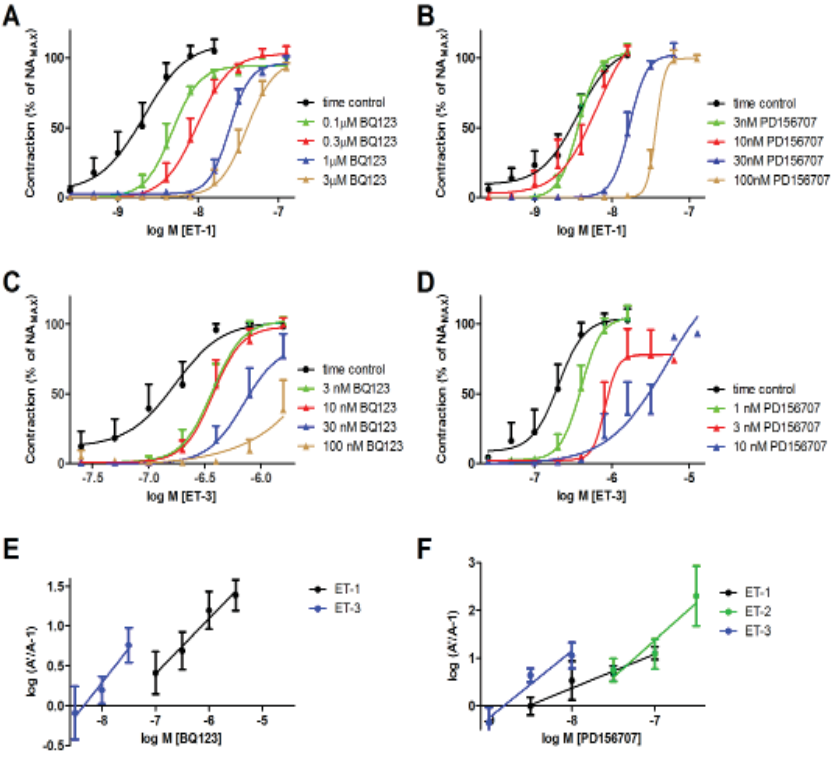


Figure 5.5. A-D, effects of presence of BQ123 (0.1 – 3.0 µM, A, C) or PD156707 (3 – 100 nM, B,D) on contractile responses to ET-1 (A, B) or ET-3 (C, D). Findings were expressed as % NA_{MAX} and are shown as means ± SEM (n = 4 – 7). E, F, Schild-plots for BQ123 (E) and PD156707 (F) versus ET-1, ET-2 and ET-3.

The slopes of the Schild-plots did not significantly deviate from unity (Table 5.2). The apparent affinity of BQ123 did not differ significantly versus ET-3 (pA_2; 8.3±0.4) and ET-1 (pA_2; 7.6±0.4) but both were significantly larger ($p < 0.01$) than the apparent affinity of BQ123 versus ET-2 (Fig. 5.4C, pK_B; 5.6±0.4). Also, presence of the non-peptidergic ET_A selective antagonist PD156707 (1 – 300 nM, [41]) did not modify basal tension and reduced the sensitivity to the contractile effects of the ET isopeptides (Fig. 5.5; Table 5.2). Again this was agonist-dependent; apparent affinity (pA_2) of PD156707 averaged 8.5±0.3, 7.9±0.3 and 8.8±0.2 versus ET-1, ET-2 and ET-3, respectively. This agonist-dependency of PD156707 (1 log unit) seems to be less marked than that of BQ123 (2.5 log units).

Table 5.2. Schild-analyses of contractile responses to endothelins in presence of ET_A antagonists.

Antagonist	Agonist	Slope	pA_2 or pK_B[a]
BQ123	ET-1	0.69 ± 0.08	7.6 ± 0.4
BQ123	ET-2	1.0 ± 0	5.6 ± 0.4[a]
BQ123	ET-3	0.86 ± 0.35	8.3 ± 0.4
PD156707	ET-1	0.71 ± 0.15	8.5 ± 0.3
PD156707	ET-2	1.55 ± 0.6	7.9 ± 0.3
PD156707	ET-3	1.4 ± 0.35	8.8 ± 0.2
FITC-BQ123	ET-1	0.65 ± 0.13	8.3 ± 0.7
Cy5.5-PD156707	ET-1	1.09 ± 0.15	8.2 ± 0.5
$(PD156707)^2$	ET-1	1.07 ± 0.35	7.7 ± 0.5

Data shown as mean ± SEM (n = 4-8).
[a] denotes calculation of a pKB value for BQ123 versus ET-2.

To evaluate whether ET_A receptor activation influences effects of ET_A selective antagonists, BQ123 and PD156707 were applied during contractions induced by ET-1, ET-2 or ET-3 (Fig. 5.2C) and the effects were compared to predictions from the "competition experiments" (Fig. 5.2B, Fig. 5.5). In view of the observed differences in apparent potency of the agonists and antagonists, we used different combinations of concentrations of the compounds. 1 µM BQ123 reduced the response to 8 nM ET-1 to a lesser extent than predicted and 100 nM PD156707 reduced the response to 16 nM ET-1 to a lesser extent than predicted (Table 5.3). In contrast, 1 µM BQ123 reduced the response to 64 nM ET-2 to a larger extent than predicted and 30 nM BQ123 reduced the response to 1.6 µM ET-3 to a larger extent than predicted (Table 5.3). Unlike the agonist effects of all three endothelins, the inhibitory effects of both antagonists were rapidly reversible. In all cases, contractile responses recovered within minutes after washout of both the agonist and the antagonist (e.g. Fig. 5.2C, Table 5.3).

To obtain additional evidence for topographically distinct agonist- and antagonist-binding sites underlying the observed effects of the antagonists we used bulky analogues of BQ123 and PD156707 (Fig. 5.1). Presence of 0.1-3 µM FITC-BQ123, 3-100 nM Cy5.5-PD156707 or 10-100 nM homobivalent PD156707 $((PD156707)^2$, two PD156707 molecules linked by a spacer, Fig. 5.1) reduced the sensitivity but not the maximal responses to ET-1 (Suppl Fig. 5.1.1).

Table 5.3. Predicted and observed inhibitory effects of ET_A antagonists on contractile responses in the presence of an ET and on contractile responses persisting after exposure to an ET.

Agonist	Antagonist	Predicted[a]	Observed in presence of agonist	Observed after agonist
8 nM ET-1	1 μM BQ123	−99 ± 1[b]	−43 ± 7[b,c]	−52 ± 1[b,c]
64 nM ET-2	1 μM BQ123	−29 ± 5[b]	−92 ± 1[b,c]	−96 ± 1[b,c]
1.6 μM ET-3	30 nM BQ123	−17 ± 17	−89 ± 4[b,c]	−90 ± 4[b,c]
16 nM ET-1	1 μM FITC-BQ123	−82 ± 11[b]	−26 ± 26[c]	−14 ± 12[c]
16 nM ET-1	0.1 μM PD156707	−99 ± 1[b]	−51 ± 8[b,c]	−20 ± 13[c]
16 nM ET-1	0.1 μM Cy5.5-PD156707	−99 ± 0[b]	+2 ± 7[c]	−12 ± 18[c]
16 nM ET-1	0.1 μM $(PD156707)^2$	−99 ± 1[b]	−4 ± 15[c]	−13 ± 5[c]

Data are expressed as percent change and are shown as means ± SEM (n = 4-8).
[a] Inhibitory effects were predicted from the results of competition-design experiments (e.g. Fig. 5.5) under concentration- and contractile amplitude-matched conditions.
[b] the effect is statistically significant ($p < 0.05$).
[c] the difference from the predicted value is statistically significant ($p < 0.05$).

The apparent affinity of these compounds did not differ significantly from that of the smaller BQ123 and PD156707 pharmacophores, respectively (Table 5.2). However, unlike their low molecular weight counterparts, administration of 1 μM FITC-BQ123, 100 nM Cy5.5-PD156707 or 100 nM $(PD156707)^2$ did not significantly reduce contractile responses observed in the presence of 16 nM ET-1 and neither did they reduce the contractile responses that had been initiated by 16 nM ET-1 and persisted after agonist removal (Table 5.3).

Discussion and Conclusions

The main findings of this work are: (i) ET_A mediated arterial contractile responses to not only ET-1 but also ET-2, and to a lesser extent ET-3, persist upon removal of free agonist, (ii) $ET\text{-}1_{1\text{-}15}$ does not cause ET_A agonism or antagonism in the absence or presence of $ET_{16\text{-}21}$ and (iii) effects of ET_A selective antagonists depend on the presence and type of ET_A agonist and on the size of the ET_A antagonist. These findings indicate that distinct ligand-binding domains are present on arterial smooth muscle ET_A receptors and that the antagonists used have distinct effects on these domains.

To unravel ET_A receptor function we used native rat mesenteric resistance arteries that take part in the regulation of local and total peripheral vascular resistance and in the development of hypertension [31]. Experiments were performed after desensitization of peri-arterial sensory-motor nerves and during continuous inhibition of NO-synthases and cyclo-oxygenases. We have previously shown that mechanical removal of the endothelium does not alter the contractile response to

ET-1 [19]. Contractile responses to ET-1 and ET-3 were not modified by the ET_B selective antagonist BQ788 [19] and the selective ET_B agonist 4^{Ala}ET-1 did not cause contraction, relaxation or altered responses to ET-1. Our results are thus not influenced by the endothelium, sensory-motor nerves and ET_B receptors.

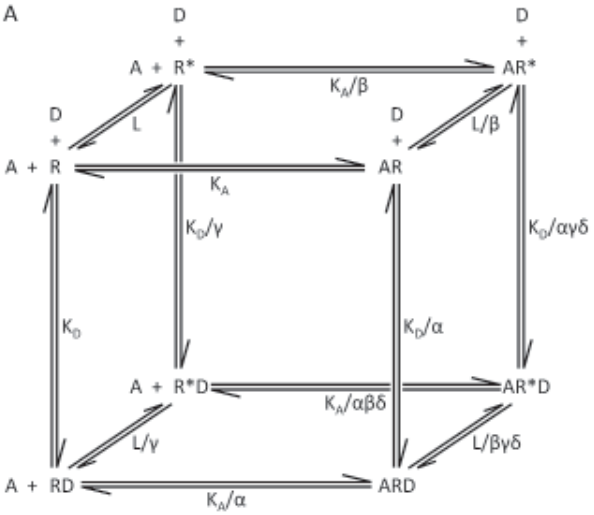
In view of the observed potency order (ET-1 = ET-2 >> ET-3) and sensitivity to two ET_A receptor selective antagonists, endothelin-induced contractions are mediated by arterial smooth muscle ET_A receptors [4, 8, 42, 43]. These receptors seem to function as monomers rather than as oligomeres [44]. This is suggested by the observation that the apparent affinity for homobivalent PD156707 is not larger but, if anything, smaller than that for PD156707 itself [45].

The arterial effects of ET-2, and to a lesser extent ET-3, are maintained and long-lasting after removal of the free agonist, in line with earlier findings with ET-1 [19, 23]. For all three endothelins, the long-lasting response can be reversibly reduced by an ERA. For ET-1, this has been attributed to tight, slowly reversible binding of the peptidergic agonist to ET_A receptors [19, 24, 25, 28]. This also seems to be case for ET-2/ET_A and for ET-3/ET_A complexes, as contractile responses to these peptides rapidly recovered after exposure to an ERA (Fig. 5.2 and Fig. 5.4F). Previous studies proposed the presence of multiple binding domains for ET-1 on ET_A receptors with distinct binding and signaling properties [14, 15, 17, 19]. For several other peptide-7TMR interactions, a clear functional distinction of these domains has been reported for the ligand and its receptor, with one domain mediating binding (address) and another one mediating signaling (message) [20, 21]. However, distinctive roles for the C-terminal tail and the N-terminal loop of ET-1 in dynamic high affinity binding, tight binding and activation of ET_A receptors [14], are not confirmed by the present study. 4^{Ala}ET-1, ET-1_{11-21} and ET_{16-21} did not display ET_A antagonist- or agonist-effects in line with earlier ligand-binding studies [39, 46]. Moreover, ET-1_{1-15}, the intact N-terminal loop segment of ET-1, did not display antagonism or agonism in the absence and in the presence of the C-terminal tail segment ET_{16-21}. Hence, the entire intact 21 amino acid structure of an endothelin seems to be required to bind and activate ET_A receptors [17, 47].

We next focused on differences between ET-1, ET-2 and ET-3, the endogenous ET receptor agonists that share the C-terminal tail and differ in amino acid sequence of the N-terminal loop (Table 5.1). While many earlier studies addressed effects of N-terminal loop amino acids on the affinity and selectivity for ET receptor subtypes [9, 17, 39, 48], our experiments aimed at their consequences for modulation of ET_A receptor function.

The cyclic pentapeptide BQ123 is one of the first selective inhibitors of ET-1/ET_A binding [40]. In line with competitive antagonism, it reduced the sensitivity and responses to ET-1, ET-2 and ET-3. Yet, preventive effects of BQ123 were more marked for ET-3 and ET-1 than for ET-2, in contrast to earlier reports where preventive effects were more marked for ET-3 and ET-2 than for ET-1 [49]. In addition, the relaxing effects of BQ123 were larger than predicted in the case of ET-2 and ET-3, but smaller than predicted for ET-1. An early review by Bax and Saxena reported on agonist-dependence of competitive antagonists in the endothelin system [50]. However, probe-dependence in combination with differential effects on affinity and efficacy, as our results show, indicate allosteric modulation rather than neutral competitive antagonism [29, 30]. Not only BQ123 but also the butenolide PD156707 reduced the sensitivity to ET-2 less markedly than that to ET-1 and ET-3 and relaxed ET-1-induced contractions to a lesser extent than predicted. The latter was previously reported for other non-peptidergic ERAs such as bosentan and SB-234551 [19]. In contrast, presence of a vasodilator such as the Ca^{2+}-channel blocker felodipine reduced the responses to 1-16nM ET-1 and ET-2 only moderately and this did not differ between the two peptides. This invites for a future detailed comparison of allosteric properties between the various classes of ERAs [11]. In order to evaluate saturability of antagonist effects, another criterion of allosterism [29, 30], this should include more antagonist concentrations and a thereby more powerful Schild analysis than used in the present study.

Fig. 6 illustrates ET_A receptor function along the lines of a recent model of allosteric modulation of 7TMRs [30]. Because ET_A receptors have not been observed to display constitutive activity, the receptor isomerization constant (L) is large. Endothelins i) bind to the orthosteric binding site according to their dissociation constant (K_A) that is considerably larger for ET-3 than for ET-1 and ET-2 and ii) promote receptor activation (agonist intrinsic efficacy $\beta > 1$). An antagonist (D) displaying negative allosteric modulation such as BQ123 i) binds to a topographically distinct site according to its dissociation constant (K_D) and ii) does not activate the receptor (antagonist intrinsic efficacy $\gamma \leq 1$). Binding of an orthosteric agonist and of an allosteric modulator changes the conformations of the receptors which may influence, besides receptor activation, also affinity and efficacy properties at the alternative sites. This is represented by cooperativity factors (α and δ). These are considered to be reciprocal, e.g. binding and efficacy of an endothelin influences binding and efficacy of BQ123 and vice versa [29, 30]. Combined with this scheme, our observations suggest that i) binding of BQ123 reduces the sensitivity to subsequently administered ET-2 less markedly than that to ET-1 or ET-3 (α: 1 < ET-2 << ET-1 ≤ ET-3) and that ii) receptor binding and activation by ET-2, compared to the other orthosteric agonists, more markedly



B

Allosteric model legend

R	Inactive receptor	
R*	Active receptor	
L	Receptor isomerization constant: R/R*	>> 1
A	Agonist (ET-1, ET-2, ET-3)	
K_A	Agonist K_{on}/K_{on}	ET-1 = ET-2 << ET-3
D	Antagonist (BQ123)	
K_D	Antagonist K_{off}/K_{on}	cte
α	Binding cooperativity	1 < ET-2 << ET-1 ≤ ET-3
β	Intrinsic efficacy of the agonist	1 < ET-1 ≈ ET-2 ≈ ET-3
γ	Intrinsic efficacy of the antagonist	≤ 1
δ	Activity cooperativity	ET-3 ≤ ET-2 << ET-1 < 1

Figure 5.6. Proposed model of allosteric modulation of ET_A receptors by an antagonist. A, interactions of ligand, receptor and antagonist such as BQ123. B, definition of dissociation constants, efficacies and cooperativity factors along with their rank orders.

promotes an inverse agonistic effect of BQ123 (δ: ET-3 ≤ ET-2 << ET-1 < 1). More quantitative analysis of allosteric mechanisms as previously described [51] proves difficult in our functional assay as we did not observe antagonist-induced reduction of maximal responses to the agonists.

Observations with large analogues of the ERAs provide additional support for an allosteric mechanism. Fluorescently labeled BQ123, fluorescently labeled PD156707 and homobivalent PD156707 reduced the sensitivity to ET-1 to the same extent as the low molecular weight pharmacophores. However, the large ERA failed to cause a statistically significant relaxation of ET-1-induced responses. That each FITC, Cy5.5 and an additional PD156707 moiety would impair the inverse agonistic property of the antagonist can not be excluded at present. It is, however, more likely that tight binding of ET-1 to ET_A limits the access of the antagonists to their allosteric binding sites and that this structural hindrance is more marked for large bulky antagonists than for their small molecular weight counterparts. Unfortunately this also complicates the potential use of molecular imaging techniques to directly prove the existence of distinct orthosteric and allosteric binding sites with the use of fluorescently labeled agonist and -antagonists.

Although ET-1 and ET-2 have been considered to display identical pharmacological properties [4, 8] we observed marked differences between these closely related peptides (summarized in Fig. 5.6). This points to pivotal roles of the amino acids at positions 6 and 7 in the orthosteric agonists. These residues do not interfere with the affinity of the peptide for ET_A receptors in the resting state [8]. They would rather result in a different conformation of the ET-1/ET_A and ET-2/ET_A complexes. Whether

this contributes to different physiological and pathological functions of ET-1 and ET-2, despite similarity of binding affinities, may become the subject of future studies. In a recent study, Millecamps et al. described that the effects of ET-1 and ET-2 are modified to a markedly different extent in an experimental model of chronic pain [52].

Our observations may have consequences for diagnosis and drug discovery. Because endothelins are paracrine mediators [53] that bind tightly to their receptors, circulating levels of free peptides may not be informative. As an alternative, effects of ERAs can be evaluated. BQ123 has been administered into the human forearm with the goal to monitor the contribution of ET-1 to basal peripheral vascular resistance in health and disease [54-58]. If allosteric modulation by BQ123 also applies for human vascular smooth muscle ET_A, reported findings for hypertensive, heart failure and diabetic patients must be regarded as an underestimation. Furthermore, the observation that not only affinity but also efficacy can be modulated by ET_A antagonists and that this displays agonist-dependence may redirect drug-discovery programs. Potent inhibitors of ET-1/ET_A binding have been observed to be only partly effective or even ineffective on agonist-occupied receptors [19, 26]. This may be remedied by shifting the attention from agonist-binding to allosteric modulation of receptor activation.

In summary, two prototypic ET_A receptor antagonists were observed to act as allosteric inhibitors of the binding and activation of arterial smooth muscle ET_A receptors by endogenous ET isoforms. This included differential effects on the sensitivity and on the responses to the endogenous endothelin isopeptides ET-1, ET-2 and ET-3. Ultimately this may be helpful for the design of diagnostics and drugs that discriminate between the roles of these closely related endogenous mediators in health and disease.

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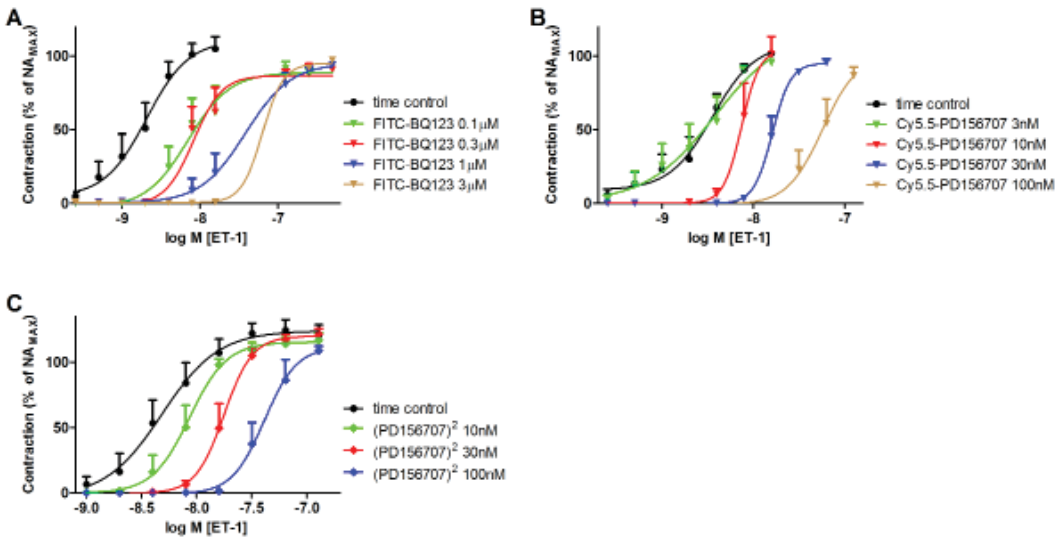
Chapter 5.1

Supplementary Data concerning Chapter 5



Supplementary Figure 5.1.1. Effects of presence of FITC-BQ123 (0.1 – 3.0 µM, A), Cy5.5-PD156707 (3 – 100 nM, B) or PD156707^2 (10 – 100 nM, C) on contractile responses to ET-1.

Synthesis of ET-1$_{1-15}$

Peptide H-CSC(Acm)SSLMDKEC(Acm)VYFC-NH$_2$ was synthesized on a MBHA-polystyrene resin (0.44mmol/g) by manual SPPS on a 0.3mmol scale using *in situ* neutralization/HCTU activation for Boc-chemistry as previously described [1]. After chain assembly N$^{\alpha}$-Boc-groups were removed and the resin-bound peptide was cleaved with anhydrous HF for 1h at 0ºC with 4% *p*-cresol as a scavenger. After cleavage the peptide was dissolved in a sodium acetate buffer (0.1M, pH 4), containing 6M Gn.HCl and purified on a Vydac C$_{18}$ RP-HPLC column (50mm x 250mm, 25ml/min) with a lineair gradient of acetonitrile in water/0.1% TFA, yielding 26.2mg. After HF cleavage Cys^3 and Cys^{11} stayed protected with acetamidomethyl-protecting groups (Acm), Cys^1 and Cys^{15} were deprotected. To generate the first disulfide bond (Cys^1-Cys^{15}), 5.6mg of the purified peptide (3.0µmol, 1eq) was dissolved in 28ml 0.1M ammonium bicarbonatebuffer (pH 7.8). The solution was incubated at room temperature and stirred during the reaction. The reaction progress was monitored by ESI-MS and analytical HPLC. After 3 hours the reaction was completed and the solution was acidified to pH 4 with acetic acid. After lyophilization the second disulfide bond (Cys^3-Cys^{11}) was formed by iodine treatment in methanol as previously described [2]. The lyophilized product was solved in 1.375ml of a 80:20 methanol:water mixture containing 5M HCl and purged with nitrogen. Then 247.5µl of iodine 0.24M in methanol (59 µmol, 20 eq) was added. The reaction mixture was purged with nitrogen and kept at room temperature for 40 minutes. To stop the reaction an excess of ascorbic acid 5% in a sodium acetate buffer (0.1M, pH 4) containing 6M Gn.HCl was added. After purification, product containing fractions were pooled and lyophilized to yield 1.1mg. ESI-MS showed a mass of 1712.54±0.50, fitting well between the calculated monoisotopic mass (1711.62) and average mass (1713.01).

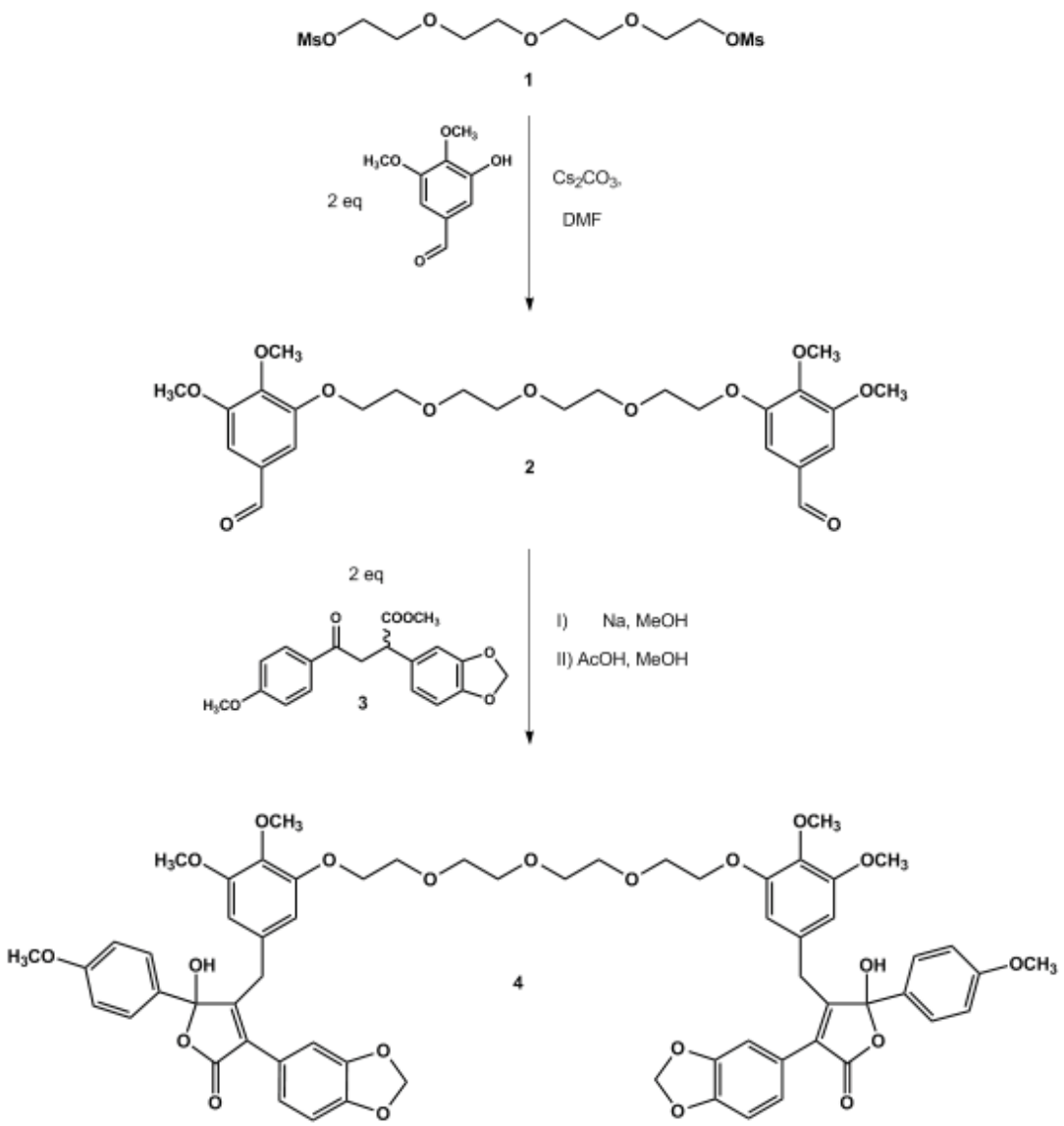
Synthesis of (PD156707)^2

The homobivalent ligand (PD156707)^2 has been synthesized by a two-step procedure starting from commercially available 3,4-dimethoxy-5-hydroxybenzaldehyde (Frinton Laboratories, Inc., Vineland, NJ) and tetraethyleneglycole dimethanesulfonate (1). The resulting bisaldehyde (2) was then converted to the final bifunctional ET_A antagonist (4) by reaction with two equivalents of ketoester (3) (Suppl. Fig. 5.1.2).
To a solution of 3.64 g (20.0 mmol) 3,4-dimethoxy-5-hydroxybenzaldehyde in 30 ml of DMF are added 3.47 g (9.9 mmol) tetraethyleneglycole dimethanesulfonate (1) and 7.17 g (22.0 mmol) Cs_2CO_3 and the mixture is heated at 100°C for 4 hours. The final solution is poured on ice and extracted with ethyl acetate. The organic layers are combined and washed with brine, dried over magnesium sulfate and filtered.

Evaporation of the solvent yields 4.97 g (9.5 mmol, 95%) of 3-(2-(2-(2-(2-(2,3-dimethoxy-5-formylphenyloxy)ethoxy)ethoxy)-ethoxy)ethoxy)-4,5-dimethoxybenzaldehyde (2) as a waxy light-yellow solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 9.83 (s, 2H), 7.13 (d, 2H, J = 1.8 Hz), 7.11 (d, 2H, J = 1.8 Hz), 4.24-4.20 (m, 4H), 3.93 (s, 6H), 3.91 (s, 6H), 3.90-3.86 (m, 4H), 3.74-3.70 (m, 4H), 3.69-3.65 (m, 4H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 191.0, 153.7, 152.8, 144.1, 131.5, 109.0, 106.4, 77.5, 77.2, 77.0, 76.6, 70.8, 70.7, 69.6, 68.9, 61.0, 56.3.

To a solution of 600 mg (26.1 mmol) sodium in 120 ml of methanol under argon are added 6.85 g (20.0 mmol) of methyl-2-(benzo[1,3]dioxol-5-yl)-4-(4-methoxyphenyl)-4-oxobutanoate (3) and the mixture is heated at 50°C for 3 h. Then an amount of 5.22 g (10.0 mmol) of the bisaldehyde (2) dissolved in 20 ml of methanol is added and the reaction mixture is heated at reflux for 48 h. Acetic acid (2 ml) is added and the mixture is further heated at reflux for 24 h. After evaporation of the solvent water and ethyl acetate are added and the organic layer is separated, washed with brine and dried over $MgSO_4$.

Filtration and evaporation of the solvent yields 9.07 g of a foamy, pale solid (7.94 mmol, 79%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.38 (d, 4H, J = 8.3 Hz), 6.94-6.73 (m, 10H),6.20 (s, 2H), 5.96 (s, 2H), 5.94 (s, 4H), 5.34 (br, 2H), 3.98-3.91 (m, 4H), 3.78 (s, 6H), 3.75-3.50 (m, 16H), 3.72 (s, 6H), 3.59 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 175.3, 160.6, 160.2, 152.8, 152.0, 148.1, 147.6, 136.9, 131.9, 128.8, 128.1, 127.4, 123.2, 123.0, 113.9, 109.5, 108.4, 108.1, 106.2, 106.0, 101.3, 70.7, 70.5, 69.7, 68.5, 60.8, 55.8, 55.3, 32.1. HRMS (ESI): m/z = 1165.3662 (M + Na+), calc. *m/z* = 1165.3676.



Supplementary Figure 5.1.2. Synthesis of PD156707[2].

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Chapter 6

Discussion and Future Directions

Since its discovery and characterization, the ET system was, and is, considered to be a therapeutic target with large potential. Involvement of ET_A receptors and/or ET_B receptors, in combination with one or more ET isoforms, in hypertension and other cardiovascular diseases, has become evident over the past years. Seizing the opportunity for anti ET drugs, the search was on for compounds that could bind to ET receptors and prevent binding of agonists. Leads were then 'rushed' into trials but, as only bosentan and more recently ambrisentan are currently used in clinical practice, for treatment of pulmonary arterial hypertension, pulmonary fibrosis and scleroderma, the full therapeutic potential of ERAs is far from reached. Therefore, opportunities to more effectively target the ET system for the treatment of hypertension should not be overlooked. In addition, this more effective targeting of the ET system may, in the near future, prove valuable for pathologies such as cancer and chronic pain, in which the role of the ET system has only started to become apparent over the past few years.

Due to the rather unique pharmacology of ETs however, a neutral competitive antagonist may not fit the need to displace an ET isoform and the focus to inhibit the ET system may, or should, shift to other mechanisms. These may include, but should not be limited to, compounds that promote dissociation of ETs from ET_A receptors, ERAs, that by tight binding can also be long-acting, and ERAs that can act ET isoform specifically and have an effect despite the tightly bound agonists. These latter two would benefit from an allosteric mechanism of action rather than a competitive antagonistic mechanism. Additionally, one of the mechanisms to displace ETs from ET_A receptors should be an endogenous system, as long-lasting ET agonism is not compatible with vascular homeostasis.

In **Chapter 2** of this thesis, we aimed to find which physiological mechanism could effectively counterbalance the vascular responses to ET-1-induced, ET_A mediated responses. We found that the long-lasting contractions induced by ET-1 were due to the quasi-irreversible nature of its binding to ET_A receptors. The effects of ET-1 could only be partially and transiently reversed by ERAs, indicative of irreversible agonism and reversible antagonism. In addition, endothelium-derived and exogenous vasodilators could only transiently reverse the effects of ET-1. Only CGRP, either exogenously applied or endogenously released from sensory motor-nerves via stimuli of TRP-channels, could effectively relax ET-1-induced contractions. Using a fluorescently-labeled ET-1 that, regarding affinity and efficacy for ET_A receptors did not differ from unlabeled ET-1, we could visualize the binding to smooth muscle ET_A receptors. We confirmed that this was due to tight binding by labeled ET-1 to ET_A receptors and that this was unaltered by ERAs. Yet, exogenous and endogenously

released CGRP could displace labeled ET-1 from ET_A receptors, after which labeled ET-1 could again label the arterial smooth muscle.

These findings indicate that ET-1 binding to ET_A receptors is a complex mechanism in which binding and signaling are mediated by different parts of the ET-1 molecule. Once ET-1 is bound to ET_A receptors, vasodilator mechanisms or ERAs do not displace ET-1, resulting in a fully signaling ET-1/ET_A complex upon removal of the vasodilators or ERAs. CGRP however, which is released endogenously when sensory-motor nerves are stimulated by e.g. compounds found in red pepper and garlic, does promote dissociation of ET-1 from ET_A receptors. This may explain, in part, the cardiovascular health benefits of the Mediterranean diet and should be an incentive for everyone to include garlic and red pepper in their diet, not just because it tastes great, but also because it might provide better cardiovascular health.

Subsequent experiments demonstrated that CGRP can act as a “physiological” antagonist of ET-1/ET_A-function not only in rat mesenteric resistance arteries but also of pressor responses and regional vasoconstrictor responses in intact rats. Also, we could identify that activated CGRP-receptors have arterial anti-endothelinergic effects via G-protein βγ subunits and not via cAMP, a second messenger that plays a major role in its general vasodilator effects.

In **Chapter 3**, we aimed to characterize to which extent the amino acid structure of the endogenous agonists contributes to the tight binding to and activation of ETA receptors. So, we dug deeper into the characteristic interactions of ET isoforms with ET_A receptors. We confirmed that ET_B agonists did not induce any effect and neither ET_B agonists nor ET_B antagonists altered sensitivity and maximal responses to ET-1. We also confirmed that ET-1 and ET-2 display similar potency and maximal effect and found that they induce similar vasospasms, as removal of the free ETs did not affect their induced contractions. An ET_A antagonist, however, inhibited ET-1-induced contractions to a larger extent than it did ET-2-induced contractions. And in contrast, it relaxed ET-2-induced vasospasms to a larger extent than it did ET-1-induced vasospasms. Interchanging the amino acids that differ between ET-1 and ET-2 makes the agonists lose affinity and highlights the agonist-selective extent of inhibition by an antagonist.

These findings indicate that, despite their initial, apparently similar pharmacology, ET-1 and ET-2 differently activate ET_A receptors as shown by the inhibitory effect of an antagonist, likely due to their amino acid sequence, as supported by the results of interchanging the amino acids that distinguish ET-1 and ET-2. This knowledge

should drive the smart design of an antagonist that can ET isoform-selectively target ET_A receptors. In addition, as an ET_A antagonist exerts greater inhibition of activated rather than inactive receptors, this could reduce antagonistic effects on part of the ET system that is not over-activated.

Because we now know that ET-1 and ET-2 induce vasoconstrictions and consecutive vasospasms by binding tightly to ET_A receptors, in **Chapter 4**, we aimed to determine which of the intracellular signaling mechanisms is responsible for the long-lasting, persistent responses to ET_A agonists. Using functional antagonists, i.e. compounds that inhibit contractile mechanisms or stimulate relaxing mechanisms, we studied which mechanisms are responsible for the vasoconstrictions and for the vasospasms, if this differed between ET-1 and ET-2 and if this knowledge could be useful in a system that does not potently respond to ERAs or CGRP: the basilar artery. We found that mainly PLC is responsible for the initial vasoconstrictor responses and for the consecutive vasospasm, but that these are mediated by different mechanisms downstream of PLC. Again we found agonist-dependent effects of ET_A activation, regarding intracellular signaling pathways, and that this pathway in the basilar arterial system differs from that in mesenteric arteries. Functional antagonists could effectively relax ET-induced vasospasms without affecting the initial contractile response.

Firstly, these results emphasize that taking into account which ET isoform activates ET_A receptors can help finding the best inhibitory approach and that it is worth considering in which arterial system one wishes to intervene. Secondly, since functional antagonists exert their effects against vasospasms rather than the initial contraction, a long-acting/slowly dissociating vasodilator could effectively counterbalance the long-lasting effects induced by ETs. This could be a preferable option in a system that does not respond well to ERAs or our endogenous functional antagonist CGRP.

Inhibiting a downstream mediator of ET_A induced effects may not fit the need of selectively inhibiting the ET system, as these mediators are generally involved in several intracellular 7TMR signaling mechanisms. However, a few mediators, such as TRPC3, may be rather specific for ET_A signaling and antagonizing this target may help counteracting ET-induced vasospasms.

Finally, in **Chapter 5**, we aimed to define in more detail the interaction of ETs with ET_A receptors and to define a role for allosteric modulators rather than neutral competitive antagonists to interact with ET_A receptors. So, we got into the mechanism of agonist-

dependent modulation of ETA receptors. In an attempt to relate the structure of the agonist to its functional effects, i.e. the initial contractile response and the consecutive vasospasm, we studied fragments of ETs but found that only the intact 21 amino acid peptide can bind to and activate the receptor. We found that, in addition to ET-1 and ET-2, ET-3 also induces vasospasms, although to a smaller extent. The inhibitory effect of an antagonist of this receptor activation was dependent on both presence and type of ETA agonist, and on the size of the antagonist. The agonist-dependence of the antagonist affinity differed between two distinct ETA antagonists, and these modulate receptor activity differently, depending on the agonist responsible for receptor activation.

To visualize antagonist-binding, preferably to an ETA receptor that was already labeled with fluorescently labeled ET-1, we characterized fluorescently labeled antagonists. The fluorescent label did not alter antagonist affinity for ETA receptors, but the labeled, and thereby bulky antagonists were unable to relax ET-1-induced vasospasms. The bulkiness of the antagonist was likely to sterically hinder the binding to ET¬A receptors with the agonist bound, and these bulky antagonists were therefore unable to induce relaxation. Unfortunately this complicated visualization of fluorescently labeled and bound agonist and antagonist, but it indicated that only small molecules would be fit to antagonize activated ETA receptors. This in turn complicates the use of fluorescently labeled antagonists as diagnostic tools, as they would only visualize receptors that are not bound to the endogenous agonists.

These findings support that the effects of at least some ETA antagonists are caused allosterically, rather than competitively. With this in mind, new or improved ETA antagonists could be able to more effectively target and inhibit ETA receptors, without having to displace the tightly bound agonist from the receptors, or to more effectively displace the agonist from the receptors (in a CGRP-like manner) without the antagonists having to inhibit the receptor activity in itself.

In figure 6.1, the mechanisms underlying the effects of ET_A activation by ETs, as well as the mechanisms that could antagonize activated ET_A receptors, are summarized. It becomes apparent that the choice of a particular antagonizing method with either allosteric ET_A receptor antagonists (yellow compound in the magnification), functional antagonists (inhibitors of contractile mechanisms or stimuli of relaxing mechanisms) or physiological antagonists (such as CGRP) will be based upon selectivity to the ET system, long-lasting relaxing effects and the ability to promote dissociation of ETs from ET_A receptors.

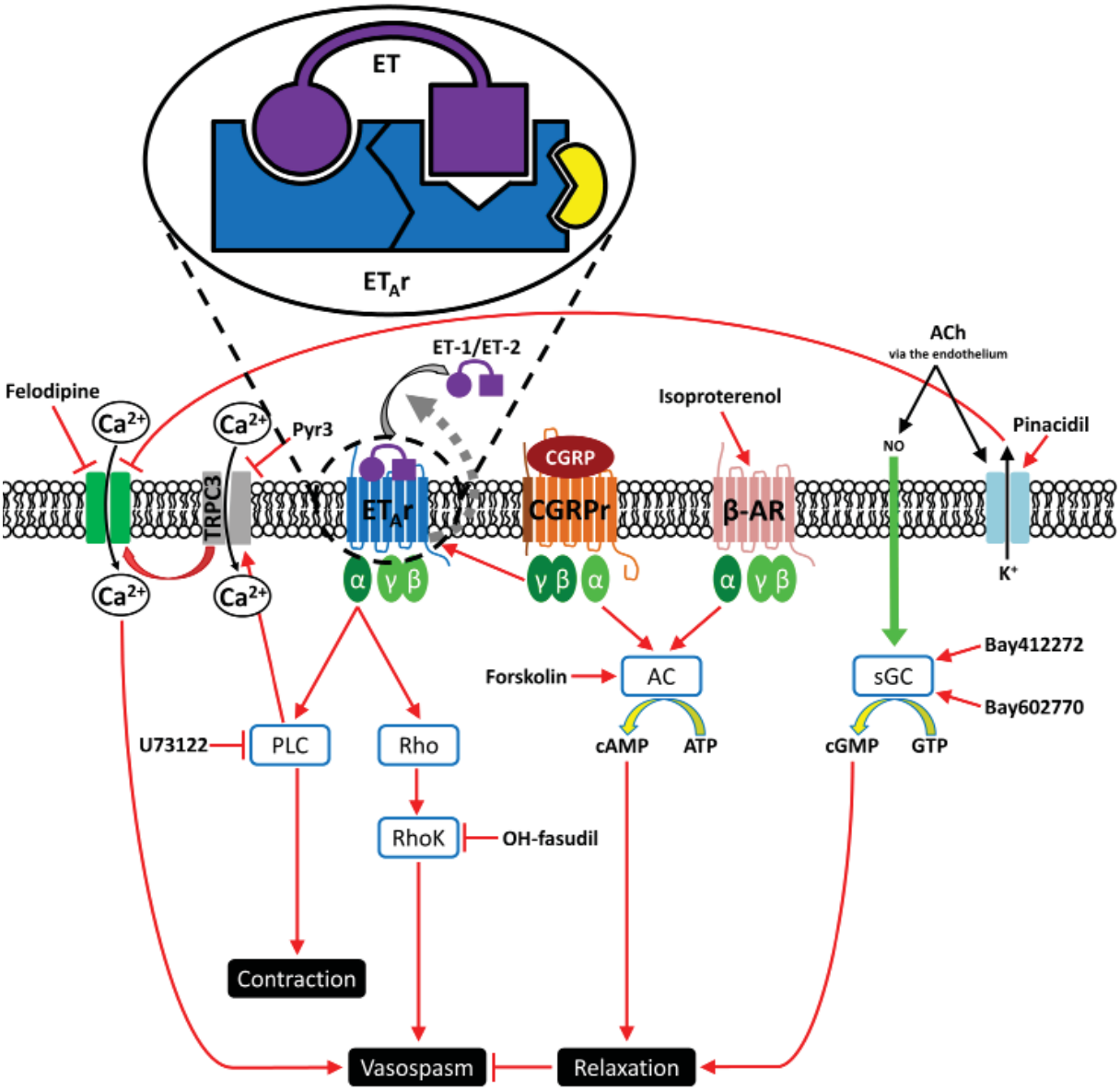

Fig. 6.1. The intracellular signaling pathway leading to ET_A mediated contractions and vasospasms and targets to inhibit these pathways. U73122, the PLC inhibitor, is the only compound that inhibits both contractions and vasospasms and is therefore likely to be the main mediator of the initiating contraction. Antagonizing the ET_A receptors can be done by using an allosteric modulator that can inhibit ET_A receptor activity (yellow compound in the magnification), using an endogenous physiological antagonist (CGRP, which promotes dissociation of ETs from ET_A receptors) or using functional antagonists to counterbalance ET_A receptor activity (inhibitors of contractile mechanisms or stimuli of relaxing mechanisms).

α: G-protein α subunit; β: G-protein β subunit; β-AR: β-adrenoceptor; γ: G-protein γ subunit; AC: adenylate cyclase; ACh: acetylcholine; ATP: adenosine triphosphate: Bay412272: sCG stimulator; Bay602770: sCG activator; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; ET-1: endothelin-1; ET-2: endothelin-2; ET_Ar: endothelin$_A$ receptor; felodipine: calcium channel blocker; forskolin; AC activator; GTP: guanosine triphosphate; isoproterenol: β-adrenoceptor agonist; NO: nitric oxide; OH-fasudil: RhoK-inhibitor; pinacidil: ATP-sensitive potassium channel opener; PLC: phospholipase C; Pyr3: TRPC3-blocker; RhoK: Rho-kinase; sGC: soluble guanylate cyclase; TRPC3: Transient receptor potential cation channel 3; U73122: PLC -inhibitor.

In conclusion, in this thesis on the molecular pharmacology of ET_A receptors, these receptors are, more than 20 years after their discovery, still considered as a therapeutic target with large potential. In the years to come, the focus should shift towards methods to more effectively target ET_A receptors, taking into consideration the system in which an antagonist should act, and taking into consideration the agonist that the antagonist should be designed against. New or improved antagonists should act as allosteric modulators rather than competitive antagonists, taking full advantage of this new window of opportunities. As an antagonist against a long-acting agonist, the allosteric modulator would ideally be a long-acting compound itself that can effectively displace ETs from ET_A receptors, thereby returning ET_A receptors back to the inactive state. This concept of allosteric modulation of ET_A receptors should be extrapolated to other pathologies in which the ET system is a promising target, and anti-ET drugs may (finally) live up to its promise.

Chapter 7

Summary

In this thesis on the molecular pharmacology of ET_A receptors, we started by highlighting the importance of this type of studies (**chapter 1**). Since its discovery it soon became apparent that the ET system plays a significant part in blood pressure regulation. Despite extensive efforts however, most ET-targeting drugs did not find their way into clinical practice. This may in part be due to the 'rush to the clinic' of ERAs, which may have hampered or attenuated the process of optimally designing drugs that best suited the need to inhibit the ET system, and rather just focused on binding to ET_A (and/or ET_B) receptors, thereby hoping to block the binding of ETs.

Unfortunately, the rather unique pharmacology of ET_A receptors complicates inhibition by neutral competitive antagonists. In **chapter 2**, we found that the persistent arterial contractile effects of ET-1 were caused by tight binding of ET-1 to ET_A receptors. ERAs were unable to continuously inhibit these active ET-1/ET_A receptor complexes, as the ERAs did not bind tightly to ET_A receptors, which results in continuously activated ET_A receptors, also after removal of the ERAs. In addition, ERAs did not promote dissociation of ET-1 from ET_A receptors. As tightly-bound, continuously active ET-1/ET_A receptor complexes are not compatible with vascular homeostasis, we identified a role for CGRP, released from sensory-motor nerves as a terminator of ET-1/ET_A receptor complexes, as CGRP promotes immediate dissociation of these complexes. This may explain to some extent the cardiovascular protection attributed to a diet high in peppers and garlic, as compounds from these plants trigger release of CGRP.

This atypical receptor pharmacology can complicate the use of potentially therapeutic drugs. To better understand the interaction of the agonists with ET_A receptors, we characterized the importance of the agonist amino acid structure for the binding to and activation of ET_A receptors in **chapter 3**. We found that ET_A receptors on arterial smooth muscle cells display agonist-dependent properties that depend on the amino acids on position 6 and 7 of the ET sequence. Although ET-1 and ET-2 display similar affinity to and maximal effect on ET_A receptors and that both agonists bind tightly to these receptors, their ET_A mediated activities are differently modulated by an ERA. Moreover, the substitution of a single amino acid in the agonist sequence drastically reduces affinity for ET_A receptors and therefore has profound pharmacological consequences.

As ERAs display agonist-dependent effects regarding ET_A receptor activity, we continued to find which intracellular signaling mechanisms were involved in the persistent ET_A agonist responses in **chapter 4**. We again found that the intracellular signaling mechanisms involved in contractile responses were dependent on which

agonist activated ET_A receptors, and that there were different mechanisms involved in the initial contractile response and the sequential vasospasm. The main facilitator of both the contractions and vasospasms was identified as PLC, downstream of which TRPC3 and L-VOCC were intermediate steps in the mechanism of vasospasm. The ET_A mediated activation of the intracellular signaling mechanism was not only agonist-dependent, but also system-dependent.

Then, in **chapter 5**, we present evidence that two ERAs act as allosteric modulators rather than as competitive antagonists. After establishing that ET_A receptor binding and activation requires the full 21 amino acid sequence of the agonists, we found that the effects of ERAs depend on the presence and type of ET_A agonist and on the size of the ERA. These effects can only be attributed to an allosteric mechanism rather than to a competitive antagonist mechanism.

In **chapter 6** we discuss these findings in light of our current understanding of anti-ET therapy and we suggest that in order to more effectively target ET_A receptors, we should take into consideration which of the ET_A agonists are involved and that when designing novel ERAs or improving on current ERAs, some attention should go out to possible long-acting effects of the ERA that would ideally dissociate ETs from ET_A receptors.

Chapter 8

Samenvatting

In dit proefschrift over de moleculaire farmacologie van Endotheline$_A$ (ET_A) receptoren zijn we begonnen met het benadrukken van het belang van dit soort studies (**hoofdstuk 1**). Sinds de ontdekking werd het snel duidelijk dat het ET systeem een belangrijke bijdrage levert aan reguleren van de bloeddruk. Ondanks intensieve pogingen zijn de meeste op ET gerichte medicijnen op weg naar klinische toepassing gesneuveld. Dit kan deels te wijten zijn aan de 'sprint naar de kliniek' van ET receptor antagonisten (ERAs), wat er voor gezorgd kan hebben dat het design proces van medicijnen, gericht op het remmen van het ET systeem, niet volledig zorgvuldig is doorlopen. Dit proces heeft zich misschien te veel gefocust op het binden van medicijnen aan ET_A (en/of ET_B) receptoren, waarmee in theorie het binden van ETs voorkomen zou worden.

Helaas is de unieke farmacologie van ET_A receptoren een complicerende factor met betrekking tot de inhibitie door neutrale competitieve antagonisten. In **hoofdstuk 2** hebben we gevonden dat de aanhoudende arteriële contracties van ET-1 worden veroorzaakt door een moeilijk omkeerbare binding van ET-1 aan ET_A receptoren. ERAs waren niet in staat om de geactiveerde ET-1/ET_A receptor complexen continu te remmen, omdat de ERAs wel dynamisch en gemakkelijk omkeerbaar binden aan ET_A receptoren. Dit resulteert in langdurige, aanhouden geactiveerde ET_A receptoren, ook na het verwijderen van ERAs. Daar komt bij dat ERAs niet zorgen voor een dissociatie van ET-1 van de ET_A receptoren af. Aangezien moeilijk omkeerbaar gebonden, continu actieve ET-1/ET_A receptor complexen niet compatibel zijn met vasculaire homeostase, hebben we een rol voor calcitonine-gen gerelateerd peptide (CGRP) kunnen identificeren. CGRP wordt vrijgemaakt uit sensorische motor zenuwen en werkt als een terminator van ET-1/ET_A receptor complexen, daar het een directe dissociatie van deze complexen induceert. Dit zou deels een verklaring kunnen zijn van de cardiovasculaire bescherming die wordt toebedeeld aan een dieet waarin veel peper en knoflook wordt gebruikt, omdat stoffen uit deze planten zorgen voor een vrijmaking van CGRP uit de sensorische motor zenuwen.

De ongebruikelijke, atypische receptor farmacologie van ET_A receptoren kan het gebruik van medicijnen met een potentieel therapeutische toepassing bemoeilijken. Ter vergroting van het begrip van de interacties tussen de agonisten en de ET_A receptoren hebben we in **hoofdstuk 3** het belang van de aminozuur structuur van de agonist voor het binden aan en activeren van ET_A receptoren gekarakteriseerd. We hebben gevonden dat ET_A receptoren op arteriële gladde spiercellen agonist-afhankelijke eigenschappen vertonen die afhankelijk zijn van de aminozuren op posities 6 en 7 van de ET sequentie. Ook al lijken ET-1 en ET-2 vergelijkbare affiniteit voor en maximaal effect op ET_A receptoren te hebben en lijken beide agonisten

moeilijk omkeerbaar te binden aan deze receptoren, hun ET_A gemedieerde activiteiten lijken verschillend te worden gemoduleerd door een ERA. Daarbij is de uitwisseling van een enkel aminozuur in de agonist sequentie verantwoordelijk voor een drastische afname van affiniteit voor ET_A receptoren en heeft daarom grote farmacologische gevolgen.

Daar ERAs agonist-afhankelijke effecten hebben met betrekking tot ET_A receptor activiteit, hebben we verder gezocht naar het intracellulaire signaleringsmechanisme dat betrokken is bij de persisterende ET_A agonist responses in **hoofdstuk 4**. We hebben gevonden dat ook welk intracellulaire signaleringsmechanisme betrokken wordt bij de contractiele response afhankelijk is van met welke agonist de ET_A receptoren worden geactiveerd. Daarbij zijn er verschillende mechanismes betrokken bij de initiële contractiele response en het daaropvolgende vasospasme. Het signaleringsmolecuul PLC was geïdentificeerd als de faciliterende factor van zowel de contracties als de vasospasmes, en downstream van PLC waren TRPC3 en L-VOCC betrokken bij het mechanisme van vasospasme. De ET_A gemedieerde activatie van het intracellulaire signaleringsmechanisme was daarbij niet alleen agonist-afhankelijk, maar ook systeem-afhankelijk.

Vervolgens tonen we in **hoofdstuk 5** aan dat twee ERAs als allosterische modulators in plaats van competitieve antagonisten functioneren. Nadat we hadden vastgesteld dat de volledige 21 aminozuur sequentie van de agonist noodzakelijk is om ET_A receptoren te kunnen binden en activeren, hebben we gevonden dat de effecten van ERAs afhankelijk zijn van het type en de aanwezigheid van de ET_A agonist en van het formaat van de ERA. Deze effecten kunnen enkel worden toegeschreven aan een allosterisch mechanisme en niet zozeer aan een competitief antagonist mechanisme.

In **hoofdstuk 6** bediscussiëren we de bevindingen tegen het licht van onze huidige kennis van anti-ET therapie. We stellen voor dat, om ET_A receptoren effectiever als therapeutisch doelwit te gebruiken, we rekening moeten houden met welk van de ET_A agonisten betrokken zijn bij de specifieke situatie. Daarnaast moet, wanneer er nieuwe ERAs worden ontworpen, of wanneer huidige ERAs worden verbeterd, er aandacht moet worden geschonken aan mogelijke langdurige effecten van de ERA zelf, die uiteindelijk idealiter de ETs moet dissociëren van ET_A receptoren.

Appendix

Dankwoord

Een thesis is nooit het werk van slechts een individu; een van de redenen dat in papers altijd wordt gesproken van de wetenschappelijke ‘wij’. Tot deze mensen wil ik me dan ook richten in dit dankwoord. Daarnaast zijn er ook enkele anderen die zeker nog een woord van dank verdienen.

Allereerst wil ik mijn promotor bedanken. Jo, je hebt mijn interesse voor de farmacologie optimaal aangewakkerd. Dit begon al bij het aanleren van onze voornaamste labtechniek, het myograafwerk en je hebt gelijk: iemand kan helemaal tot rust komen wanneer hij of zij bezig is met de isolatie en het monteren van de vaatjes. Daarnaast was je altijd plezierig betrokken vanaf het meedenken over (opzet van) experimenten tot het geduldig maar snel reviseren van manuscripten. Verder wil ik je ook bedanken voor de ruime mogelijkheden die je hebt geboden om mijn werk aan een zo breed mogelijk publiek te presenteren. Dit heeft mij meer dan alleen een betere wetenschapper gemaakt.

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Naast de bijdrages van mijn Farmacologie-collega’s heb ik veel samengewerkt met collega’s van de andere afdelingen van Maastricht University en van andere universiteiten. Naast de frisse kijk van anderen op het eigen werk nodigde de samenwerking ook uit tot het verbreden van de eigen interesse.

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List of Publications

Labruijere S, Van den Boogaerdt AJ, Maassen van den Brink A, **Compeer MG**, De Mey JG, Danser AH, Batenburg WW. Long-lasting physiological antagonism of calcitonin gene-related peptide towards endothelin-1 in rat and human blood vessels. *Submitted*.

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Matthijs Gerrit Compeer was born on February 8, 1985 in Vlissingen, where he attended and graduated from Scheldemond College in 2003 completing the Nature & Health profile and Latin.

He then moved to Maastricht to attend the transnationale Universiteit Limburg (tUL), a collaboration between Maastricht University and Universiteit Hasselt. In 2007, he completed his Bachelor in Molecular Life Sciences after an internship in Vascular Pharmacology with Prof. De Mey. Following this Bachelor Degree, in 2009 he completed his Master in Cardiovascular Biology & Medicine, a 2 year research master program organized by the Cardiovascular Research Institute Maastricht (CARIM). Again, his final internship was at the lab of Prof. De Mey.

In 2009, he continued his work in Vascular Pharmacology starting his PhD program on the molecular pharmacology of endothelin receptors at the department of Pharmacology within CARIM, at Maastricht University. His PhD program was funded by TI Pharma, a public-private partnership which in this case consisted of Maastricht University, Erasmus MC and Actelion. During his PhD program, he has published his data in peer-reviewed international scientific journals. Additionally, he has presented his work on (inter)national research conferences for which he was honored with, amongst others, the Best Poster Prize at the 10th International Symposium on Resistance Arteries in Rebild, Denmark, and the Promising Investigator Award at the New Investigator Symposium of the 24th Meeting of the International Society of Hypertension in Sydney, Australia.

Parallel with his scientific research, he has also been active as a teaching associate within the Bachelor program of Biomedical Sciences at Maastricht University and has supervised a number of Bachelor and Master students in the lab of Vascular Pharmacology.