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Endothelin-1 and -2: Two amino acids matter

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ABSTRACT

Aims: Endothelin-1 (ET-1) and endothelin-2 (ET-2; Trp^{6}Leu^{7}ET-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.
Main methods: 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^{6}ET-1 and Leu^{7}ET-1).
Key findings: 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^{7}ET-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^{6}ET-1 did not cause contraction and did not antagonize arterial responses to ET-1.
Significance: 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) (Braasch et al., 2009; Masaki, 2004). 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 (Braasch et al., 2009; Davenport, 2002; Masaki, 2004). 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 (Mihara and Fujimoto, 1992). 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 (Arai et al., 1990). 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 (Davenport, 2002; Sakurai et al., 1992).

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 (Davenport, 2002; Masaki, 2004). However, ET-1 and ET-2 are expressed by different cell types, restricting their paracrine and autocrine function to distinct tissues (Levin, 1995). 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 (Hynynen and Khalil, 2006; Meens et al., 2010, 2011; Yanagisawa et al., 1988). ET-2 is mainly found in the gastrointestinal tract and the urogenital system (Levin, 1995). Via ET$_A$-receptors, ET-2 can modulate immune cell function (Takizawa et al., 2005) and ovulation (Ko et al., 2006).

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 (Braasch et al., 2009). While ET-1 is intimately involved in the cardiovascular system (Hynynen and Khalil, 2006), ET-2 seems to have selective functions in for instance the ovaries (Ko et al., 2006; Meidan and Levy, 2007). The pharmacological properties of ET-1 and ET-2 have been considered to be identical (Masaki, 2004). This may be surprising because during the course of evolution other endothelin isoforms were lost (Braasch et al., 2009). 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, (Ihara et al., 1992)) on arterial responses to the two endogenous agonists.

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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, 4^{Ala}ET-1, Sarafotoxin 6c (S6c) (Bachem, Weil am Rhein, D), noradrenaline (NA) and N_ω(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 (Schnolzer et al., 1992), 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 (0.2 mg/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 2 mm long arterial segments were mounted in wire myographs (DMT, Aarhus, DK) in which 5 ml 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 (Meens et al., 2010, 2009). 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 (Meens et al., 2010, 2009).

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 (Saeki et al., 1991) and S6c (Deng et al., 1995) and the ET_B-antagonist BQ788 (Ishikawa et al., 1994).

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 (Meens et al., 2010; Yanagisawa et al., 1988), 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. 1A), in line with earlier findings (Bogoni et al., 1996; Saito et al., 1991) and in addition their effects were equally persistent (Fig. 1D and F). In contrast, the ET_B-selective agonists S6c (Deng et al., 1995), found in snake venom, and 4^{Ala}ET-1 (Saeki et al., 1991), 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. 1A) (Maguire and Davenport, 1995). Furthermore, presence of 1 μM 4^{Ala}ET-1 or of 1 μM BQ788, an ET_B-selective antagonist (Ishikawa et al., 1994), tended to alter the sensitivity to ET-1 but this did not reach statistical significance (Fig. 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. 1A and E).

Effects of an ET_A-antagonist

Presence of 1 μM of the ET_A-selective antagonist BQ123 (Ihara et al., 1992) reduced the sensitivities to ET-1 and ET-2 without significant alteration of their maximal contractile effects (Fig. 1C and E). In contrast to earlier reports, where BQ123 inhibited ET-2-induced contractions more effectively than ET-1-induced contractions in a preparation of either rings of the superior mesenteric artery or the perfused mesenteric arterial bed (Donoso et al., 1996), the effect of the antagonist was observed 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. 1D and F).

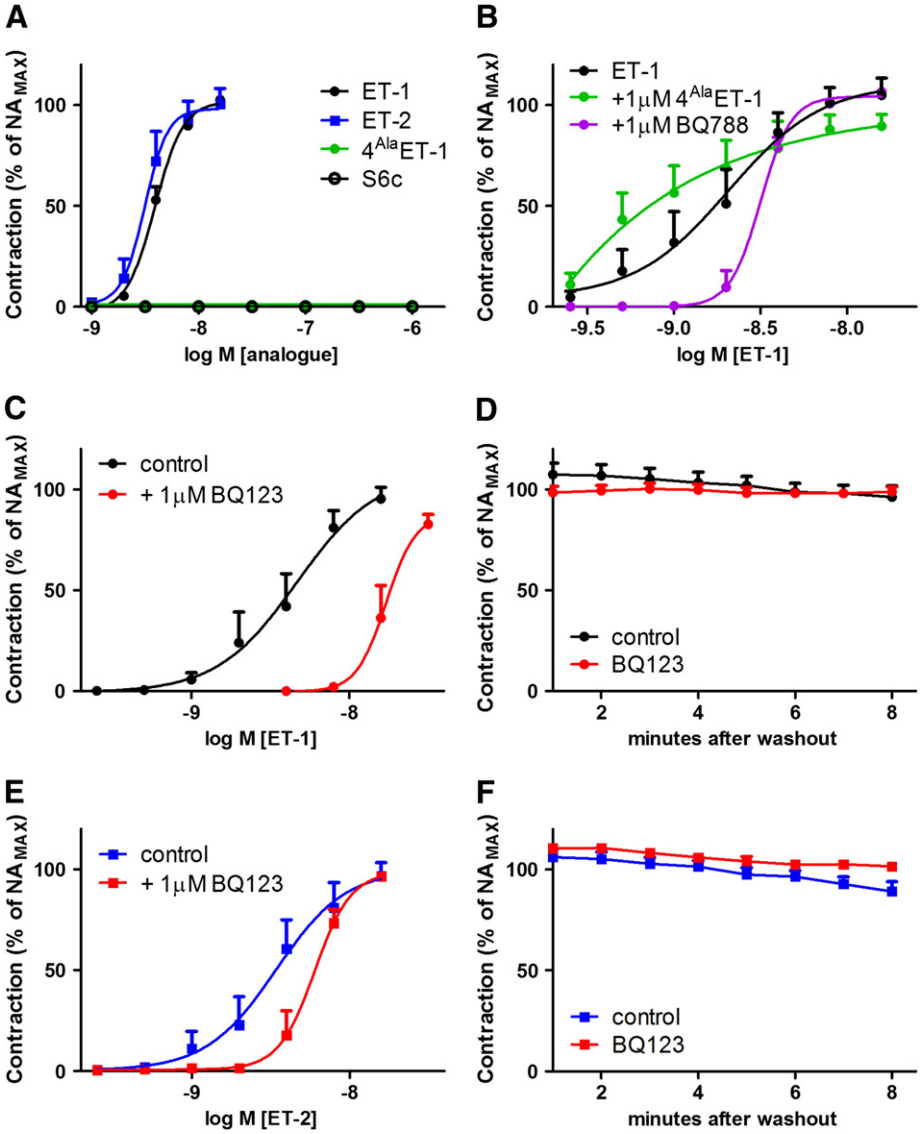

Fig. 1. Vasomotor effects of isoforms and analogues of ET-1. ET-1 and ET-2 induce contractions with comparable affinity and efficacy, whereas the selective ET_B-receptor agonists 4^{Ala}ET-1 and S6c do not induce responses (A).Neither 4^{Ala}ET-1 nor the selective ET_B-receptor antagonist BQ788 significantly 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).

Application of 1 μM BQ123 during maximal contractile responses to ET-1 or ET-2 of comparable amplitude, resulted in significant relaxation (Fig. 2A and B). 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. 2A and B). 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. 2C and D). Again this relaxing effect was significantly smaller for ET-1- (56 ± 1%, Fig. 2C) than for ET-2-initiated contractions (90 ± 2%; Fig. 2D).

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^{7}ET-1 (CSCSS**L^{6}L^7**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^{6}ET-1 (CSCSS**W^{6}M^7**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^{6}ET-1 (0.25 to 256 nM) did not induce contractile responses (Fig. 3A) and no contraction developed when the peptide was removed from the organ chamber (Fig. 3B). Also, presence of 256 nM Trp^{6}ET-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^{6}ET-1, respectively). These indicate a particularly low affinity of Trp^{6}ET-1 for arterial smooth muscle ET_A-receptors.

Increasing concentrations of Leu^{7}ET-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. 3C). As observed with ET-1 and ET-2, the arterial contractile effect of Leu^{7}ET-1 was long-lasting, i.e. it persisted after washout of the free unbound analogue (Fig. 3D). Presence of 1 μM BQ123 prevented contractile responses to up to 256 nM Leu^{7}ET-1 (Fig. 3C). Availability of only limited amounts of Leu^{7}ET-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^{7}ET-1 is larger than versus ET-2 (Trp^{6}Leu^{7}ET-1). Also, it is noteworthy that while presence of BQ123 prevented Leu^{7}ET-1-induced contraction, a strong response developed rapidly after washout of both ligands (Fig. 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

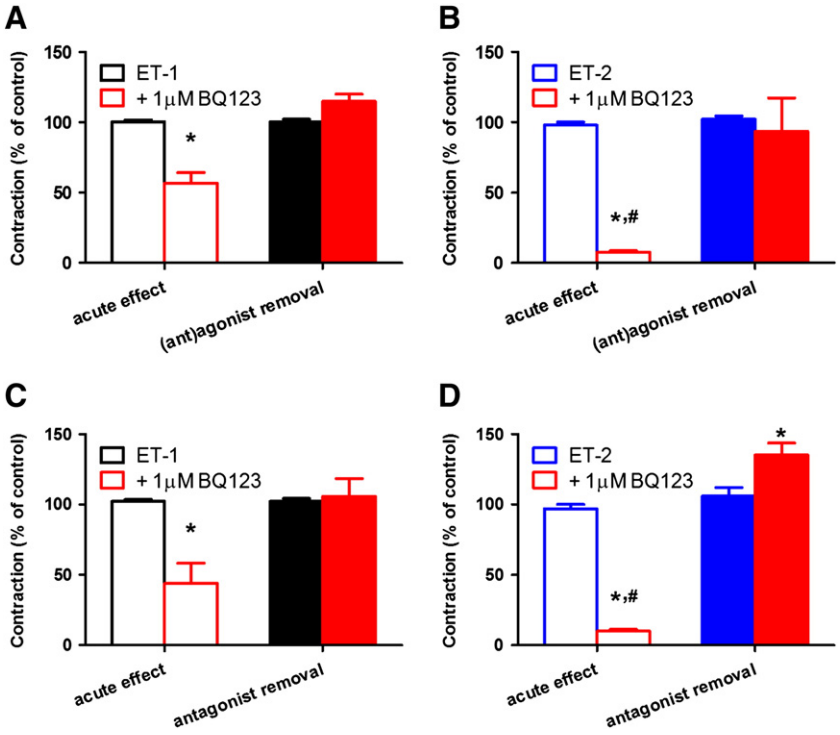

Fig. 2. Inhibitory effects of BQ123 on sustained and maintained contractions. 1 μM BQ123 reduces ET-1-induced contractions by approximately 50% in presence (A) or following removal (C) of free agonist. The BQ123-induced inhibitions were rapidly reversed upon removal of antagonist and/or agonist. ET-2-induced 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.

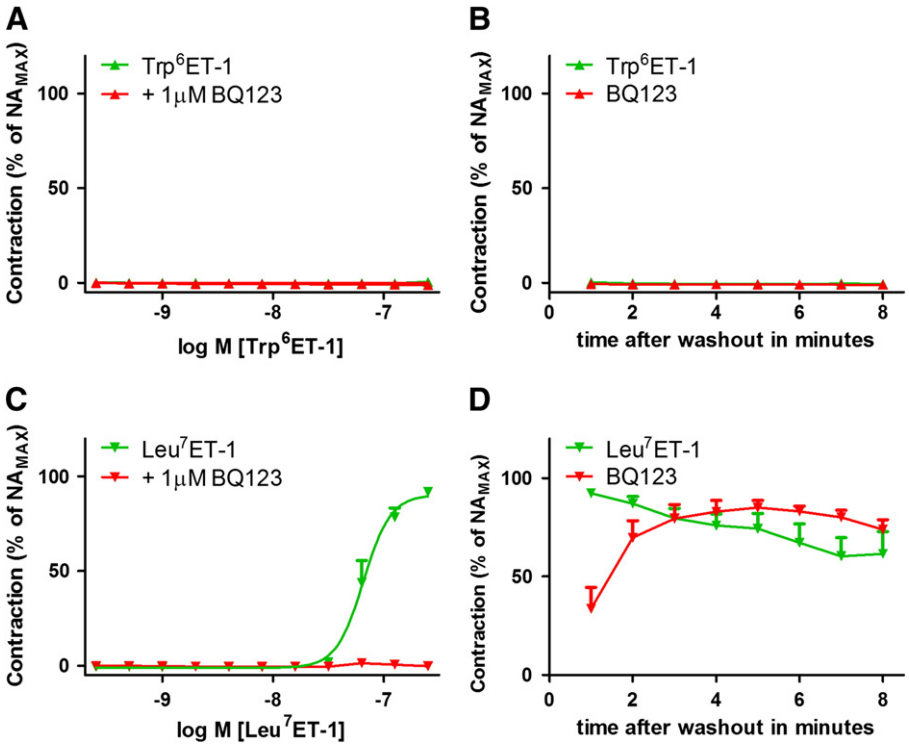

Fig. 3. Vasomotor responses to Trp^6ET-1 and Leu^7ET-1. Trp^6ET-1 did not induce contractions up to 256 nM (A) and no contraction developed following removal of the putative vasoactive compounds (B). Leu^7ET-1 induced ET_A-mediated contractions, which were, up to 256 nM, fully inhibited by 1 μM BQ123 (C). These contractions were sustained following removal of free agonist (D) and developed following the removal of free vasoactive compounds (red lines).

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 (Mulvany and Aalkjaer, 1990). 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 (Davenport, 2002) and are not modulated by endothelium, sensorimotor nerves or ET_B-receptors as we have previously proposed (Meens et al., 2010).

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 (Saito et al., 1991), by a procedure that abolishes arterial responses to other contractile stimuli within less than 2 min (Meens et al., 2010), in line with earlier findings. For ET-1 this has been attributed to tight binding of the peptide to ET_A-receptors (De Mey et al., 2011; Hilal-Dandan et al., 1997; Meens et al., 2010). 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 (Meens et al., 2010, 2011).

The cyclic pentapeptide and ET_A-selective antagonist BQ123 (Ihara et al., 1992) 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 (Christopoulos and Kenakin, 2002; De Mey et al., 2011; Keov et al., 2011). 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 (De Mey et al., 2011) 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 (Breu et al., 1995) and ABT-627 was shown to promote internalization of ET_A-receptors (Chiou et al., 2000); 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^{7}ET-1 more markedly than that to ET-2 (Trp^{6}Leu^{7}ET-1). However, BQ123 reduces the intrinsic activity of Leu^{7}ET-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^{7}ET-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^{7}ET-1 thus strengthen the notions of agonist-dependence of ET_A-receptor function and the allosteric properties of BQ123. Trp^{6}ET-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^{7}ET-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 (Tam et al., 1994), nor did these substitutions alter constrictor activity (Huggins et al., 1993). 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 conformation (Wallace and Janes, 1995). It remains to be determined what the consequences of this combination are for the conformation and flexibility of the agonist molecule (Lattig et al., 2009) 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 (Christopoulos and Kenakin, 2002; Keov et al., 2011).

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.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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