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RESEARCH PAPER

Agonist-dependent modulation of arterial endothelin$_A$ receptor function

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BACKGROUND AND PURPOSE
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.

EXPERIMENTAL APPROACH
Rat isolated 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.

KEY RESULTS
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 (peptide 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-peptide 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.

CONCLUSIONS AND IMPLICATIONS
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 the diagnosis and pharmacotherapy of diseases involving endothelins.

Abbreviations
4^{Ala}ET-1, H-Ala-Ser-Ala-Ser-Ser-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp-OH; 7TMR, 7 transmembrane domain receptor; BQ123, cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu); BQ788, N-cis-2,6-dimethylpiperidinocarbonyl-β-tBu-Ala-D-Trp(1-methoxycarbonyl)-D-Nle-OH; CAPS, capsaicin; CCRC, cumulative concentration-response curve; CGRP, calcitonin gene-related peptide; CRF, corticotrophin-releasing factor; Cy5.5, cyanine dye 5.5; DMSO, dimethyl sulfoxide; EDRF, endothelium-derived relaxing factor; ERA, endothelin receptor antagonist; ET-1, endothelin-1; ET-$1_{11–21}$, H-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-OH; ET-$1_{1–15}$, H-Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-OH; $ET_{16–21}$, H-His-Leu-Asp-Ile-Ile-Trp-OH; ET-2, endothelin-2; ET-3, endothelin-3; FITC, fluorescein isothiocyanate; INDO, indomethacin; KRB, Krebs Ringer bicarbonate buffer; L-NAME, N^G-nitro-L-arginine methyl ester; PD156707, (sodium 2-benzo(1,3)dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enoate); PTH, parathyroid hormone

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Introduction

The endogenous mammalian endothelins ET-1, ET-2 and ET-3 are bicyclic 21-amino acid paracrine mediators. They share a six-amino acid C-terminal tail but differ in two to six 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; Masaki *et al.*, 1991; Yanagisawa *et al.*, 1998; Masaki, 2004; Bagnato and Rosano, 2008; Hans *et al.*, 2008; Kirkby *et al.*, 2008). 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 (Hynynen and Khalil, 2006; Kirkby *et al.*, 2008). 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 (Inoue *et al.*, 1989; Sakurai *et al.*, 1992; Davenport, 2002). 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 Davenport, 2002; Palmer, 2009). These endothelin receptor antagonists (ERAs) are regarded as neutral competitive antagonists although their binding site does not necessarily coincide with the agonist binding sites (Sakamoto *et al.*, 1993; Sokolovsky, 1993; Lee *et al.*, 1994; Breu *et al.*, 1995; Webb *et al.*, 1996).

Structure-affinity, -selectivity and -activity relationships indicate a key role for the C-terminus of endothelins in ET_B-binding and -activation (Sakamoto *et al.*, 1993; Lattig *et al.*, 2009). ET_A receptor function seems more complex. We and others proposed that ET-1 binds polyvalently to ET_A receptors (Sakamoto *et al.*, 1993; Lattig *et al.*, 2009; Meens *et al.*, 2010; De Mey *et al.*, 2011). For other peptide 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 signalling (message domain; Conner *et al.*, 2007; Hoare, 2007).

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 (Yanagisawa *et al.*, 1988; Meens *et al.*, 2010; 2011). While ERAs can prevent receptor binding and effects of ET-1, they do not reverse established agonist binding (Hilal-Dandan *et al.*, 1997; Blandin *et al.*, 2000) and have variable influences on ET-1-induced effects (Pierre and Davenport, 1999; Adner *et al.*, 2001; Meens *et al.*, 2010). 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 h (for review, see De Mey *et al.*, 2009). Little is known about ET-2 and ET-3 in this respect. In contrast to ERAs, salicylates (Blandin *et al.*, 2000) and, more recently, the neuropeptide CGRP (Meens *et al.*, 2010) were reported to promote dissociation of ET-1/ET_A complexes. This suggests that ET_A receptor function is susceptible to allosteric modulation (De Mey *et al.*, 2011).

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

Table 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}.

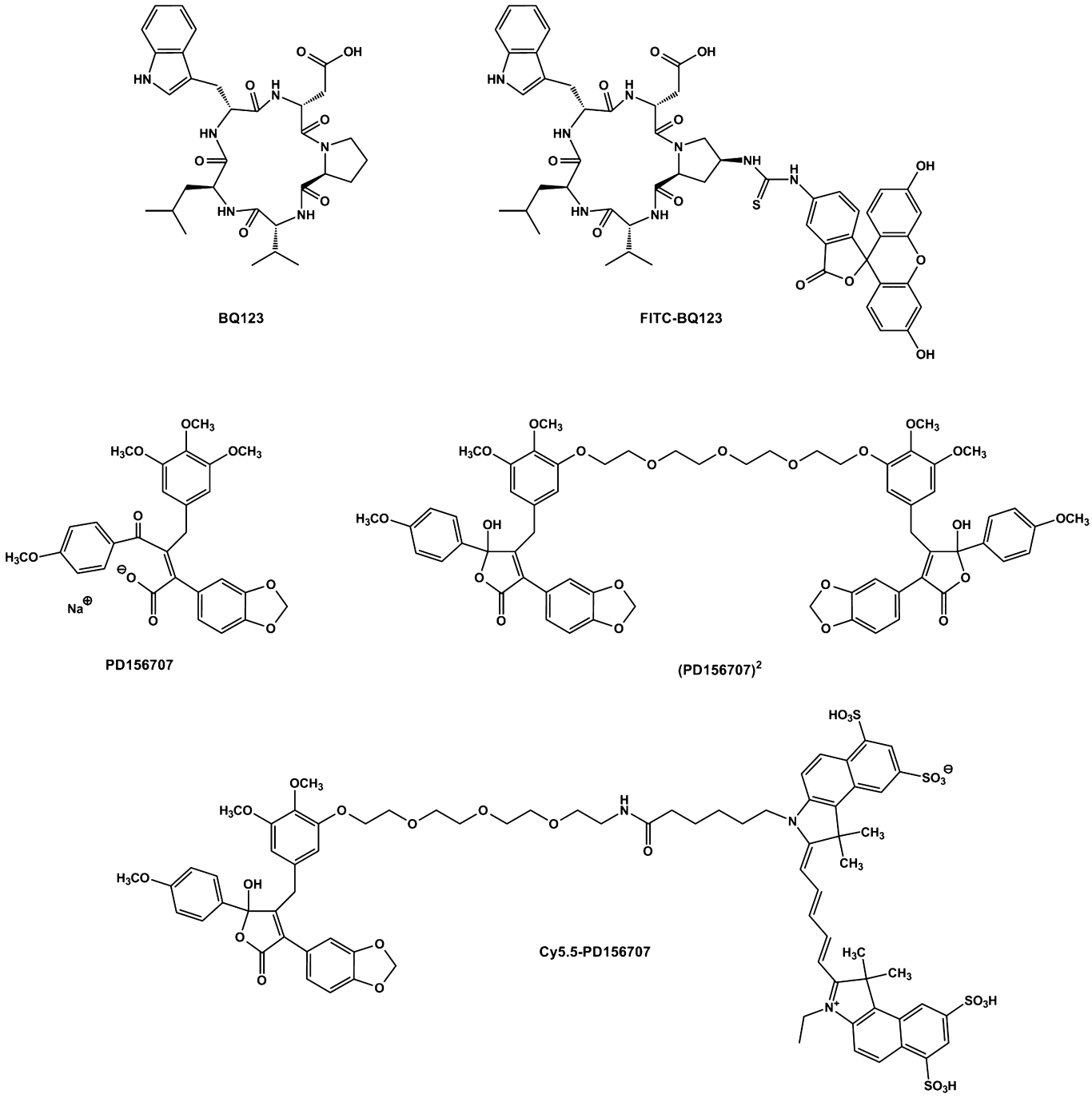


Figure 1

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

Kenakin and Miller, 2010; Keov *et al.*, 2011). For this purpose, we used (i) rat isolated mesenteric resistance arteries, (ii) isoforms and fragments of ET-1 (Table 1), (iii) the peptide and the non-peptide ET_A-selective antagonists BQ123 and PD156707, respectively and (iv) large analogues of these ERAs such as fluorescently labelled and homobivalent constructs (Figure 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 (Mulvany and Aalkjaer, 1990). ET receptors are expressed by endothelial cells, smooth muscle cells and sensory motor nerves (Meens *et al.*, 2009), but we focused here on smooth muscle ET_A. We monitored the 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, the Netherlands) and BQ788 (Peptides International, Louisville, KY, USA) were dissolved in dimethyl sulfoxide (DMSO). Capsaicin (CAPS) and indomethacin (INDO; Sigma Aldrich) were dissolved in ethanol. Felodipine (Sigma Aldrich) 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-$1_{11–21}$, $ET_{16–21}$ (Table 1; Bachem, Weil am Rhein, Germany), NA and L-NAME (Sigma Aldrich) 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 and glucose, 5.5. PD156707, cyanine dye 5.5 (Cy5.5)-PD156707 and fluorescein isothiocyanate (FITC)-BQ123 (Figure 1) were synthesized as described previously (Höltke *et al.*, 2007; Brosseau *et al.*, 2009). Synthesis of the intact bicyclic loop of ET-1 (ET-$1_{1–15}$) and synthesis of homobivalent PD156707 (Figure 1), are detailed in the online supplement (Supporting Information Materials S1 and S2). 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 week-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 2 mm long arterial segments were mounted in wire myographs (DMT, Aarhus, Denmark) in which 5 mL 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 (Meens *et al.*, 2009; 2010). 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^{-1}.

Except when specifically mentioned, arterial segments were pretreated 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 perivascular sensory motor nerves and inhibit the synthesis of NO and prostaglandins, respectively (De Mey *et al.*, 2008). 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 (Wang and Wang, 2004; Meens *et al.*, 2009) and it was previously reported that ET-1 and ET-3 can induce the release of endothelium-derived relaxing factor (EDRF) (Warner *et al.*, 1989).

Pharmacological protocols

We studied agonistic, antagonistic, competitive and inhibitory effects of putative ET_A receptor ligands and their reversibility as illustrated in Figure 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 (Figure 2A).

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 the presence and absence of the compound.

Competition experiments. Using four arterial segments in parallel, CCRCs for an agonist were constructed in the absence and presence of a low, an intermediate and a high concentration of the putative antagonist (Figure 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 three 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 (Figure 2C). The effect of $[B]_y$ was allowed to stabilize and was compared to the 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 (Figure 2D) and was compared to the 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, that is, 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 before the administration of any pharmaco-

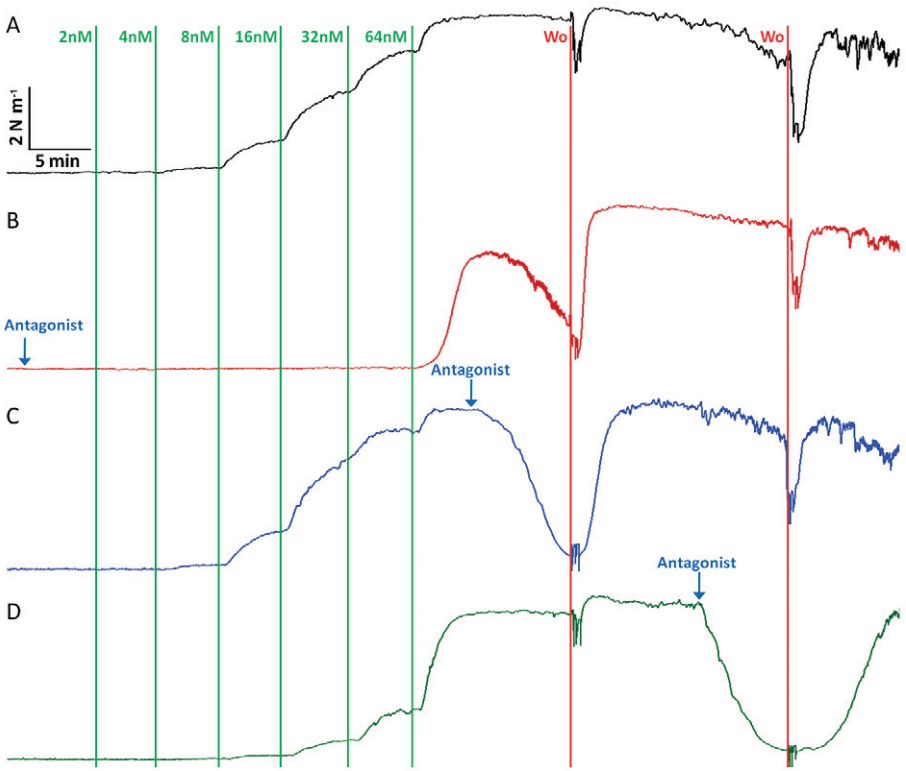


Figure 2

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 the 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).

logical 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 (GraphPad Software Inc., La Jolla, CA, USA). Data were analysed 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-$1_{1–21}$) and of ET-2 (Trp^6-Leu^7-ET-$1_{1–21}$) and μM concentrations of ET-3 (Thr^2-Phe^4-Thr^5-Tyr^6-Lys^7-Tyr^{14}-ET-$1_{1–21}$) caused contractions in isolated mesenteric resistance arteries (Figure 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 (Figures 2 and 3B). For ET-3 ($t_1/_2 \approx 5$ min), this was less pronounced than for ET-1 and ET-2 ($t_1/_2 > 18$ min, Figure 3B) but still slower than for equally strong contractile responses to, for instance, 10 μM NA ($t_1/_2 < 1$ min, data not shown).

Felodipine (1 nM), a dihydropyridine calcium channel blocker that inhibited tonic arterial contractile responses to 40 mM K^+, moderately reduced sensitivity and maximal responses to ET-1 and ET-2; and this did not differ significantly between the two peptides (data not shown).

The presence of the ET_B-selective antagonist BQ788 [1 μM (Ishikawa *et al.*, 1994)] 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 (Saeki *et al.*, 1991), did not cause contraction in resting arteries (Figure 3A), did not cause relaxation in depolarized arteries (Figure 3C) and did not modify contractile effects of ET-1 (Figure 3D) at up to 1 μM. Likewise, the fragments ET-$1_{11–21}$, ET-$1_{1–15}$ and $ET_{16–21}$, and the combination of the N-terminal loop (ET-$1_{1–15}$) plus the C-terminal tail of ET-1 ($ET_{16–21}$) failed to stimulate contraction or relaxation and did not modify the contractile potency of intact ET-$1_{1–21}$ at up to 1 μM (Figure 3A, C and D).

The presence of 1 μM BQ123, a peptide ET_A-selective antagonist (Ihara *et al.*, 1992), did not modify basal tension but reduced the contractile effects of all three endothelin isoforms (Figure 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 (Figure 4). The presence of BQ123 did not prevent initiation of long-lasting contractile responses by ET-1 or ET-2, that is, sustained responses persisting in the absence of

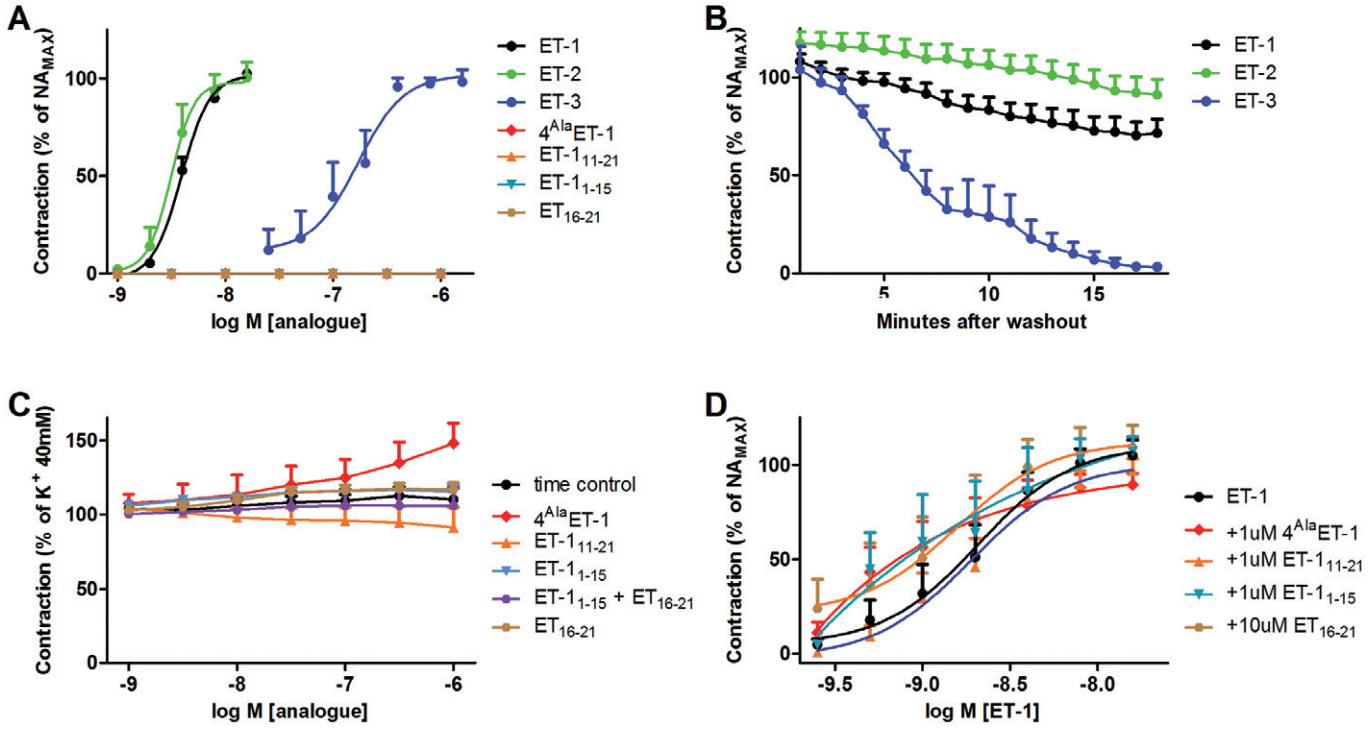


Figure 3

Vasomotor effects of isoforms, analogues and fragments of ET-1. (A) Only intact ET isoforms caused arterial contractile responses. (B) These responses were only slowly reversible. (C) 4^{Ala}ET-1 and fragments of ET-1 failed to induce relaxation in arteries contracted with 40 mM K^+. (D) The presence of 1 μM 4^{Ala}ET-1 or fragments of ET-1 did not modify contractile responses to ET-1. Findings are expressed as % of the contractile response to 10 μM NA (NA_{MAX}) or as % of the K^+-induced precontraction (C) and are shown as means ± SEM (n = 3–17).

free agonist (Figure 4A–D). Moreover, the presence of 1 μM BQ123 prevented contractile responses to 1 μM ET-3 (Figure 4E) but a strong contraction developed within 1 min after washout of the free agonist and antagonist (Figure 4F).

Effects of ET_A-selective antagonists were analysed in more detail to gain insight into their agonist-dependence. Using a range of concentrations, the presence of BQ123 (3 nM–3 μM) was observed to reduce the sensitivity but not the maximal responses to ET-1 and ET-3 (Figure 5). The slopes of the Schild plots did not significantly deviate from unity (Table 2). The pA_2 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 pA_2 of BQ123 versus ET-2 (Figure 4C, pK_B; 5.6 ± 0.4). Also, the presence of the non-peptide ET_A-selective antagonist PD156707 [1–300 nM (Maguire *et al.*, 1997)] did not modify basal tension but reduced the sensitivity of the tissue to the contractile effects of the ET-isopeptides (Figure 5; Table 2). Again, this was agonist-dependent; 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).

To evaluate whether ET_A receptor activation influences the effects of the ET_A-selective antagonists, BQ123 and PD156707 were applied during contractions induced by ET-1, ET-2 or ET-3 (Figure 2C) and the effects were compared to predictions from the 'competition experiments' (Figures 2B and 5). In view of the observed differences in apparent potency of the agonists and antagonists, we used different combinations of concentrations of the compounds. BQ123 1 μM 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 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 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. Figure 2C, Table 3).

In additional experiments, contractions were first initiated by ET-1, ET-2 or ET-3 and BQ123 or PD156707 was applied 8 min later during the response that persisted in the absence of free agonist (Figure 2D). BQ123 1 μM reduced the response initiated by 8 nM ET-1 to a lesser extent than predicted (Table 3) and this effect was reversible. The inhibitory effect of BQ123, albeit smaller than predicted, was comparable in the presence and absence of free ET-1 (Table 3). BQ123 1 μM markedly reduced the contraction initiated by 64 nM ET-2 (Table 2). This was rapidly reversible, as contractile tone redeveloped within minutes in the absence of BQ123 and ET-2 (Figure 2D, Table 3). Likewise, contractions initiated by 1.6 μM ET-3 were markedly relaxed by 30 nM BQ123 (Table 3).

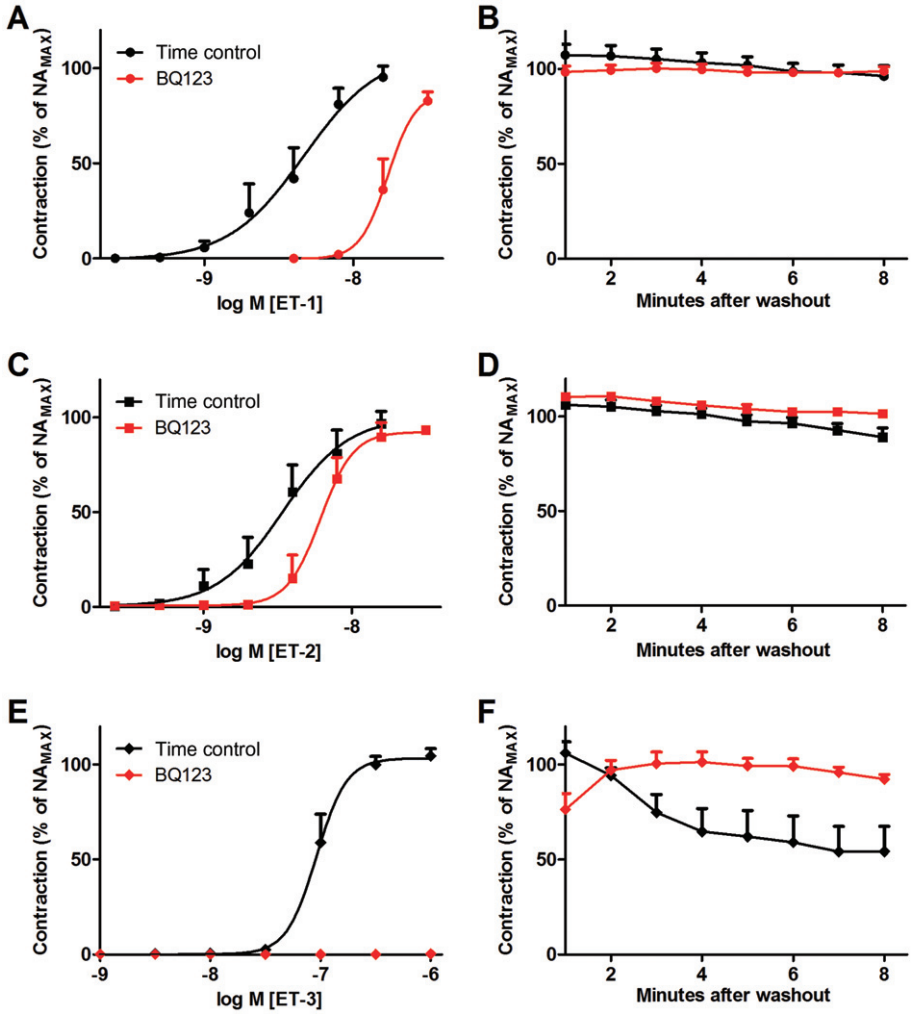


Figure 4

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

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 (Figure 1). The 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, Figure 1) reduced the sensitivity but not the maximal responses to ET-1 (Supporting Information Figure S1). The pA_2 of these compounds did not differ significantly from that of the smaller BQ123 and PD156707 pharmacophores, respectively (Table 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 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, persisted upon removal of free agonist; (ii) $ET\text{-}1_{1–15}$ did not cause ET_A agonism or antagonism in the absence or presence of $ET_{16–21}$; and (iii) the effects of the ET_A-selective antagonists depended 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 (Mulvany and Aalkjaer, 1990). Experiments were performed after desensitization of peri-

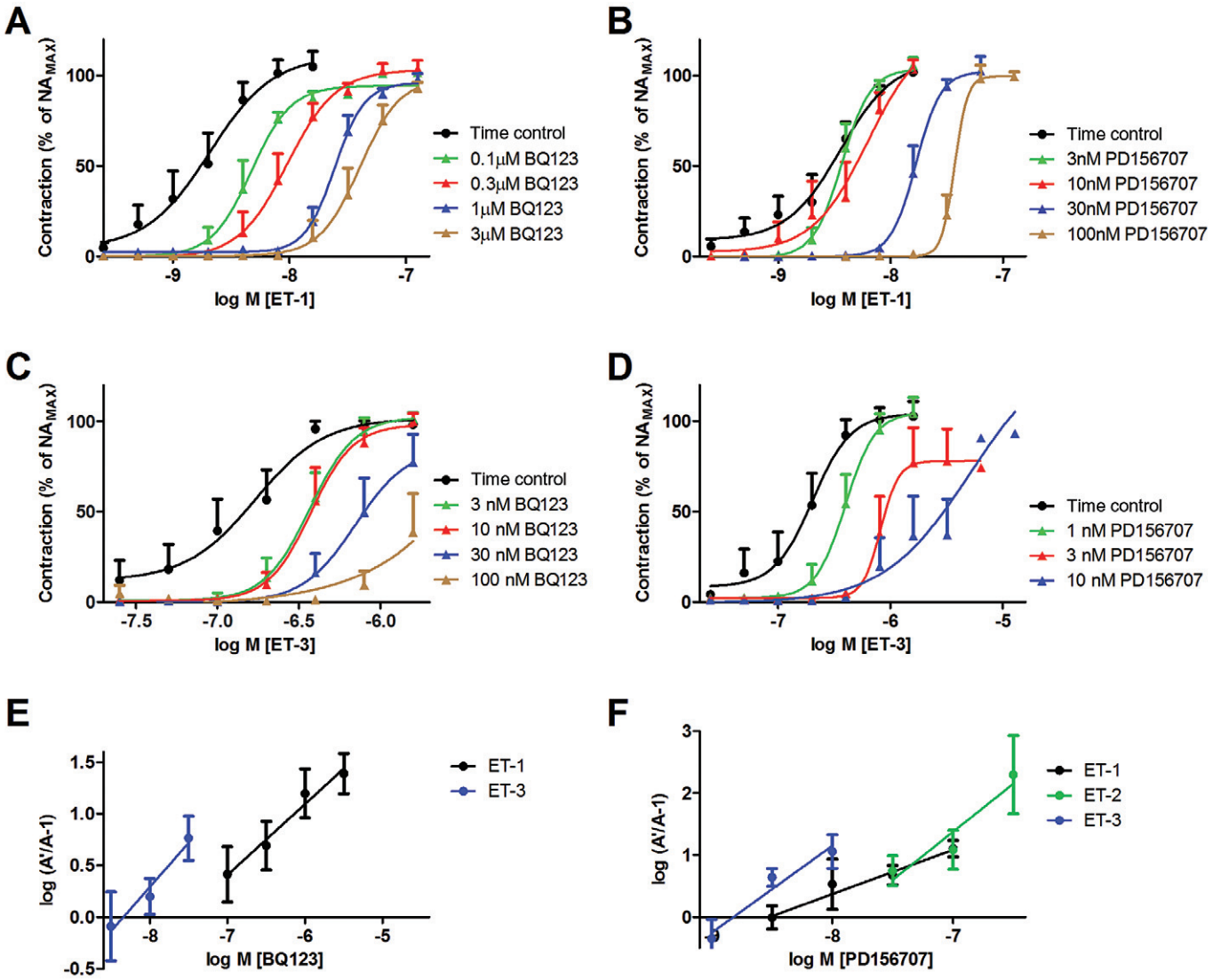


Figure 5

(A–D) Effects of the 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 are 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.

arterial sensory motor nerves and during continuous inhibition of NO synthases and COXs. We have previously shown that mechanical removal of the endothelium does not alter the contractile response to ET-1 (Meens *et al.*, 2010). Contractile responses to ET-1 and ET-3 were not modified by the ET_B-selective antagonist BQ788 (Meens *et al.*, 2010), and the selective ET_B agonist 4^{Ala}ET-1 did not induce a contraction, relaxation or alter the responses to ET-1. Our results are thus not influenced by either the endothelium, sensory motor nerves or 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 (Warner, 1990; D'Orleans-Juste *et al.*, 1993; Davenport, 2002; Masaki, 2004). These receptors seem to function as monomers rather than as oligomeres (Christopoulos and Kenakin, 2002). This is suggested by the observation that the pA_2 for homobivalent PD156707 was not larger, but if anything, smaller than that for PD156707 itself (Portoghese, 1989).

The arterial effects of ET-2, and to a lesser extent ET-3, were maintained and long-lasting after removal of the free agonist, in line with earlier findings with ET-1 (Yanagisawa *et al.*, 1988; Meens *et al.*, 2010). For all three endothelins, the long-lasting response could be reversibly reduced by an ERA. For ET-1, this has been attributed to tight, slowly reversible binding of the peptide agonist to ET_A receptors (Hilal-Dandan *et al.*, 1997; Blandin *et al.*, 2000; De Mey *et al.*, 2009; Meens *et al.*, 2010). 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 (Figures 2 and 4F). Previous studies proposed the presence of multiple

Table 2

Schild analyses of contractile responses to endothelins in the 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 pK_B value for BQ123 versus ET-2.

binding domains for ET-1 on ET_A receptors with distinct binding and signalling properties (Sakamoto *et al.*, 1993; Sokolovsky, 1993; Lattig *et al.*, 2009; Meens *et al.*, 2010). 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 signalling (message; Conner *et al.*, 2007; Hoare, 2007). 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 (Sakamoto *et al.*, 1993), were 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 (Saeki *et al.*, 1991; Doherty *et al.*, 1993). 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 (Randall *et al.*, 1989; Lattig *et al.*, 2009).

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 1). While many earlier studies addressed effects of N-terminal loop amino acids on the affinity and selectivity for ET receptor subtypes (Saeki *et al.*, 1991; Sakurai *et al.*, 1992; Tam *et al.*, 1994; Lattig *et al.*, 2009), 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 (Ihara *et al.*, 1992). 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 (Donoso *et al.*, 1996). 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 (Bax and Saxena, 1994). However, probe-dependence in combination with differential effects on affinity and efficacy, as our results showed, indicate allosteric modulation rather than neutral competitive antagonism (Kenakin and Miller, 2010; Keov *et al.*, 2011). 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-peptide ERAs such as bosentan and SB-234551 (Meens *et al.*, 2010). In contrast, the presence of a vasodilator such as the Ca^{2+}-channel blocker felodipine reduced the responses to 1–16 nM ET-1 and ET-2 only moderately and this did not differ between the two peptides. This suggests that a future detailed comparison of allosteric properties between the various classes of ERAs should be performed (Palmer, 2009). In order to evaluate saturability of antagonist effects, another criterion of allosterism (Kenakin and Miller, 2010; Keov *et al.*, 2011), this should include more antagonist concentrations and a thereby more powerful Schild analysis than used in the present study.

Figure 6 illustrates ET_A receptor function along the lines of a recent model of allosteric modulation of 7TMRs (Keov *et al.*, 2011). 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 co-operativity factors (α and δ). These are considered to be reciprocal, for example, binding and efficacy of an endothelin influences binding and efficacy of BQ123 and *vice versa* (Kenakin and Miller, 2010; Keov *et al.*, 2011). 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 promotes an inverse agonistic effect of BQ123 (δ: ET-3 ≤ ET-2 < < ET-1 < 1). More quantitative analysis of allosteric mechanisms as previously described (Ehlert, 2005) proved to be difficult in our functional assay, as we did not observe antagonist-induced reduction of maximal responses to the agonists.

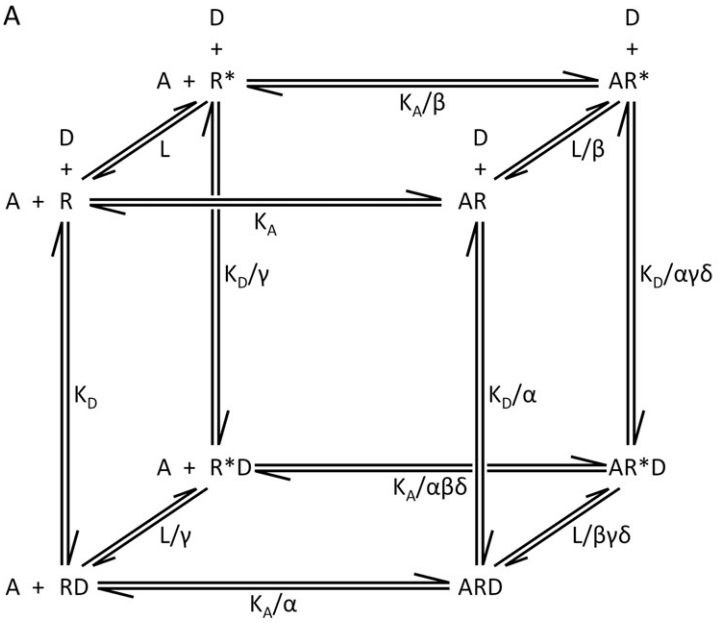
Observations with large analogues of the ERAs provide additional support for an allosteric mechanism. Fluorescently labelled BQ123, fluorescently labelled PD156707 and homobivalent PD156707 reduced the sensitivity to ET-1 to the same extent as the low molecular weight pharmacophores. However, the large ERA failed to cause a statistically significant relaxation of ET-1-induced responses. The possibility that an additional FITC, Cy5.5 and an additional PD156707 moiety would impair the inverse agonistic property of the

Table 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 % change and are shown as means ± SEM (n = 4–8).
[a]Inhibitory effects were predicted from the results of competition-design experiments (e.g. Figure 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 ($P < 0.05$).



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_{off}/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 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 co-operativity factors along with their rank orders.

antagonist cannot 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 labelled agonist and antagonists.

Although ET-1 and ET-2 have been considered to display identical pharmacological properties (Davenport, 2002; Masaki, 2004), we observed marked differences between these closely related peptides (summarized in Figure 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 (Davenport, 2002). 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*. demonstrated that the

effects of ET-1 and ET-2 are modified to a markedly different extent in an experimental model of chronic pain (Millecamps *et al.*, 2010).

Our observations may have consequences for diagnosis and drug discovery. Because endothelins are paracrine mediators (Wagner *et al.*, 1992) 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 aim of monitoring the contribution of ET-1 to basal peripheral vascular resistance in health and disease (Bohm *et al.*, 2002; Cardillo *et al.*, 2002; 2004; Nohria *et al.*, 2003; Stauffer *et al.*, 2010). If allosteric modulation by BQ123 also applies to 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 programmes. Potent inhibitors of ET-1/ET_A binding have been observed to be only partly effective or even ineffective on agonist-occupied receptors (Adner *et al.*, 2001; Meens *et al.*, 2010). 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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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Antagonism by bulky antagonists.
Figure S2 Two-step synthesis of $(PD156707)^2$.
Material S1 Synthesis of $ET\text{-}1_{1–15}$
Material S2 Synthesis of $(PD156707)^2$

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