BJP British Journal of Pharmacology

DOI:10.1111/bph.12332
www.brjpharmacol.org

RESEARCH PAPER

Endothelin-1 and endothelin-2 initiate and maintain contractile responses by different mechanisms in rat mesenteric and cerebral arteries

M G Compeer[1], G M J Janssen[1] and J G R De Mey[1,2]

[1]*Department of Pharmacology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands, and* [2]*Department of Cardiovascular and Renal Research, Institute of Molecular Medicine (IMM), University of Southern Denmark, Odense, Denmark*

Correspondence
Professor Jo G R De Mey, Department of Pharmacology, Cardiovascular Research Institute Maastricht, Maastricht University, PO Box 616, 6200MD Maastricht, The Netherlands. E-mail: j.demey@maastrichtuniversity.nl

Keywords
endothelin-1; endothelin-2; ET_A receptors; PLC; RhoK; vasoconstrictions; vasospasms; mesenteric resistance artery; basilar artery; vascular smooth muscle

Received
3 May 2013
Revised
29 July 2013
Accepted
1 August 2013

BACKGROUND AND PURPOSE
Endothelin (ET)-1 and 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 among vascular beds.

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

KEY RESULTS
Sensitivity and maximum of MRA contractile responses to ET-1 were not, or only moderately, reduced by stimulation of soluble GC, AC or K^+-channels and by an inhibitor of receptor-operated ion channels. However, each of these reduced 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 PLC 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.

CONCLUSIONS AND IMPLICATIONS
PLC played a key role in arterial contractile responses to ETs, but ET-1 and ET-2 initiated and maintained vasoconstriction through different mechanisms, and these differed between MRA and BA. Selective functional antagonism may be considered for agonist- and vascular bed selective pharmacotherapy of ET-related diseases.

Abbreviations
Bay412272, 3-(4-amino-5-cyclopropylpyrimidine-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo(3,4-b)pyridine; Bay602770, 4-((4-carboxybutyl) [2- (5-fluoro-2-([4′-(trifluoromethyl) biphenyl-4-yl]methoxy)phenyl)ethyl] amino}methyl)benzoic acid; CCRC, cumulative concentration–response curve; ET, endothelin; KRB, Krebs Ringer bicarbonate buffer;

L-VOCC, L-type voltage-operated calcium channel; PLC, phospholipase C; Pyr3, 1-[4-[(2,3,3-trichloro-1-oxo-2-propen-1-yl)amino]phenyl]-5-(trifluoromethyl)-1H-pyrazole-4-carboxylic acid; RhoK, Rho-kinase; Ro318220, 3-(3-(4-(1-methyl-1H-indol-3-yl)-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-1H-indol-1-yl)propyl carbamimidothioate; sGC, soluble guanylate cyclase; TRPC3, transient receptor potential cation channel 3; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione

Introduction

Endothelins (ETs) are bicyclic 21 amino acid peptides, acting as paracrine mediators (Yanagisawa *et al.*, 1988; Wagner *et al.*, 1992) in the cardiovascular system (Kirkby *et al.*, 2008), in chronic pain (Khodorova *et al.*, 2009) and in cancer (Buther *et al.*, 2012). In mammalian species, the ET family consists of three members, ET-1, ET-2 and ET-3, the first being most prevalent in the cardiovascular system as a regulator of vascular tone (Masaki, 2004). After release from the endothelium, ET-1 causes contraction of vascular smooth muscle cells via endothelin ET_A receptors (Rizzoni *et al.*, 1997; receptor nomenclature follows Alexander *et al.*, 2011). In most *ex vivo* preparations of freshly isolated arteries, there is no observable effect on vasomotor tone attributable to ET_B receptors in endothelium or smooth muscle (Meens *et al.*, 2010; Compeer *et al.*, 2012a). Activated ET_A receptors can stimulate several signal-transduction pathways including NADPH-oxidases, phospholipases, Rho-kinase (RhoK) and cellular influx of calcium ions (Badr *et al.*, 1989; Neylon, 1999; Masaki, 2004). For several other vasoconstrictor agonists, it has been established that transient stimulation of calcium influx is followed by calcium sensitization resulting from RhoK-mediated inhibition of myosin light-chain phosphatase, illustrating different molecular mechanisms underlying the initiation and maintenance of vasoconstrictor responses (Somlyo and Somlyo, 2000; Loirand *et al.*, 2006; Wynne *et al.*, 2009).

ET-1 binds tightly to ET_A receptors (Meens *et al.*, 2010; De Mey *et al.*, 2011), causing arterial contractions followed by long-lasting vasospasms that are resistant to inhibition by ET receptor antagonists (Yanagisawa *et al.*, 1988; Inoue *et al.*, 1989; Hilal-Dandan *et al.*, 1997; Adner *et al.*, 2001; Meens *et al.*, 2010). This might explain why ET receptor antagonists are rather ineffective in treating ET-related diseases in clinical trials (Schneider *et al.*, 2007; Kirkby *et al.*, 2008; Kohan *et al.*, 2012). 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 (Davenport, 2002). Recently however we reported quantitative differences between the effects of ET_A antagonists on arterial responses to ET-1 and ET-2 (Compeer *et al.*, 2012a,b), 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 (Ling *et al.*, 2013).

Here we tested the hypotheses that (i) different mechanisms mediate ET-induced contractions and vasospasm 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

Animals

All animal care and experimental procedures were in accordance with the institutional guidelines and were approved by the Ethics Committee on Experimental Animal Welfare of the Maastricht University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 66 animals were used in the experiments described here.

Recording of vasomotor responses

Sixteen week old male Wistar Kyoto rats (Charles River, Maastricht, The Netherlands) were killed by CO_2 inhalation. The basilar artery and second-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, Demark) 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 noradrenaline was observed (Meens *et al.*, 2009). The optimal internal diameter of the segments averaged 311 ± 7 μm and contractile responses to 10 μM noradrenaline and to 40 mM K^+ averaged 4.7 ± 0.2 and 4.1 ± 0.3 $N{\cdot}m^{-1}$, 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 $N{\cdot}m^{-1}$.

Following the stretch procedure, the arterial segments were treated with 1 μM capsaicin 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 indomethacin (10 μM) and L-NAME (100 μM) to inhibit prostaglandin and NO synthesis, respectively, except when we studied the effect of ACh-induced NO. This approach allowed us to focus on vascular smooth muscle function, minimizing the effect of the endothelium and sensory motor nerves (De Mey *et al.*, 2008), on which ETs can have complicating effects (Warner *et al.*, 1989; Wang and Wang, 2004).

Due to the quasi-irreversible nature of ET-induced contractions, a single set of experiments was performed in one set of arterial segments, that is 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 phenylephrine 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 channel antagonist were tested against K^+-induced contractions. Inhibitors of receptor-activated mechanisms were evaluated against phenylephrine-induced contractions and their selectivity was verified by testing their effects on 40 mM K^+-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 (Figure 1A) and in the continuous presence of the predetermined concentration of the compound administered 15 min before the peptide (Figure 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 (Figure 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 the antagonist (Figure 1A), to be used as a time control for the maintenance and relaxation parts of the protocol (Figure 1B and C). Finally, the peptide and compound were washed from the organ chamber and tension was recorded for a further 8 min to monitor the reversibility of the ET-induced contraction and of the antagonist effects.

Data analysis

Data are shown as mean ± SEM with $n \geq 6$ for each observation. Contractile responses are expressed as percentage of the maximal contractile response to 10 μM noradrenaline (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

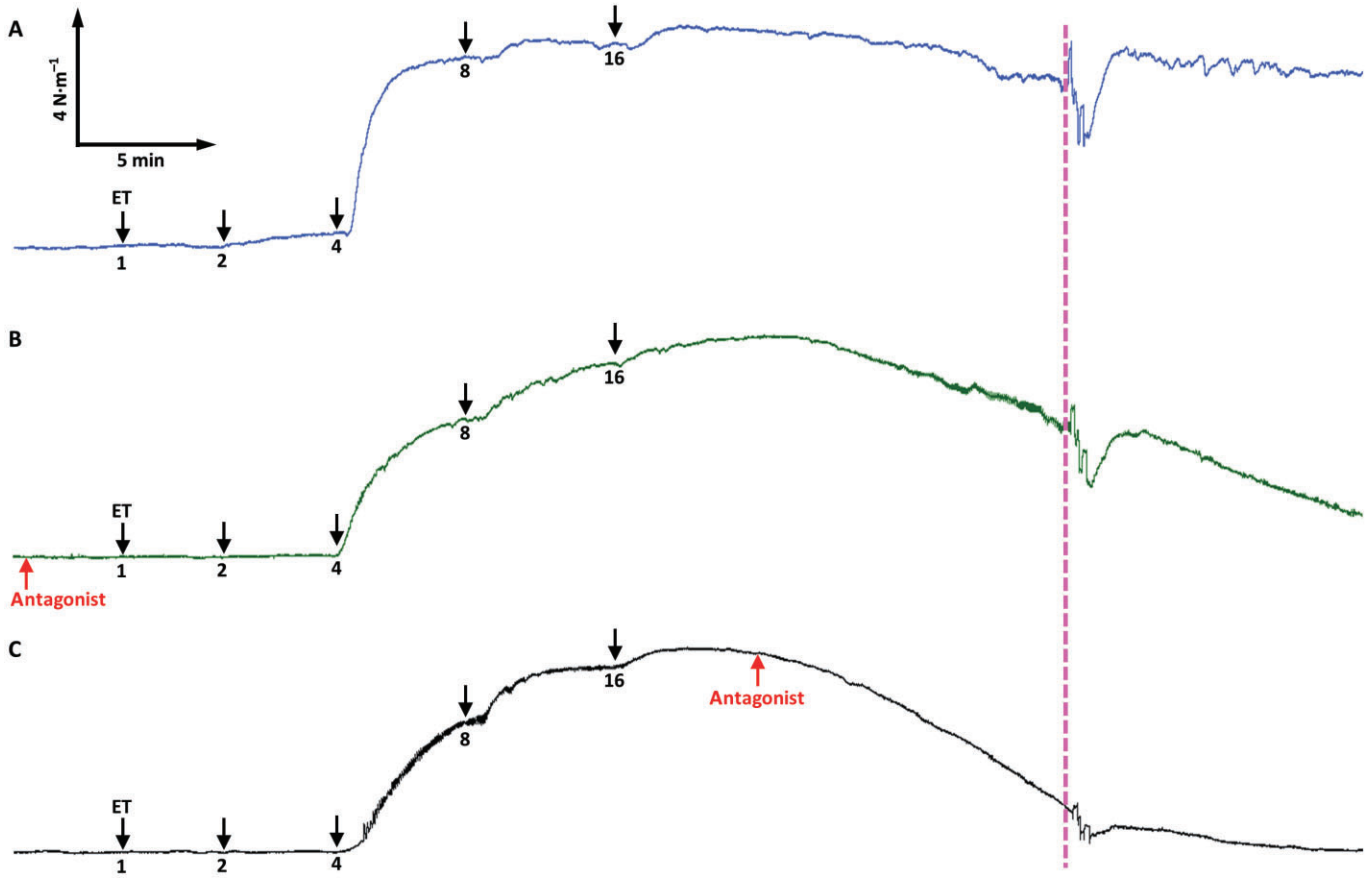

Figure 1

Original tracings of active wall tension in mesenteric artery segments, as a function of time, illustrating the pharmacological protocols. (A) Contractile responses to increasing concentrations of ET-1 or ET-2 and the vasospasm its and persistence were recorded. The red dashed line indicates washout of compounds from the organ chamber. (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.

to a non-linear regression curve and ED_{50} values were calculated using GraphPad Prism 5.02 (GraphPad Software, San Diego, Ca, USA). Data were analysed 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.

Materials

Bay412272 and Bay602770 were a kind gift from Dr JP Stasch (Bayer Healthcare, Wuppertal, Germany) and were dissolved in DMSO. Felodipine, staurosporine (Sigma Aldrich, Zwijndrecht, The Netherlands), 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 2 h as instructed by the supplier. Indomethacin (COX inhibitor) and capsaicin (TRPV1 cation channel activator; Sigma Aldrich) was dissolved in 100% ethanol. Human ET-1 and human ET-2 (Bachem, Weil am Rhein, D), noradrenaline, phenylephrine, ACh, pinacidil, isoprenaline, forskolin and L-NAME (NOS inhibitor; 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; glucose: 5.5. K^+-KRB was KRB in which all NaCl was replaced by KCl. Buffers with intermediate K^+-concentrations 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.

Results

General effects of functional antagonists

Figure 2 summarizes the contractile responses of segments of mesenteric resistance artery to 40 mM K^+ and to 10 μM phenylephrine in the presence of functional antagonists.

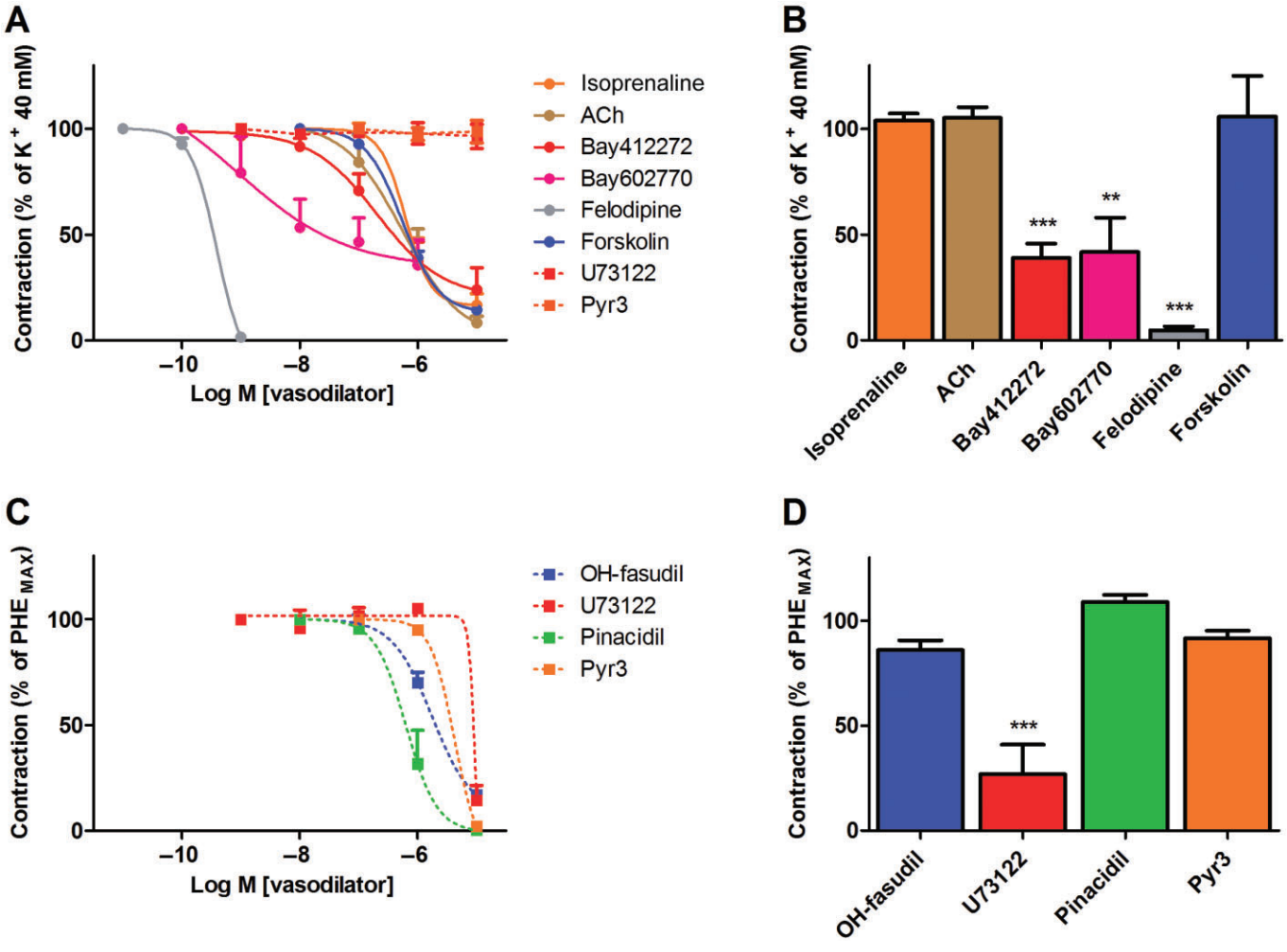

Figure 2

Mesenteric artery; effects of functional antagonists on contractile responses to 40 mM K^+ (upper) and 10 μM phenylephrine PHE (lower). The reversibility of the effects of the vasodilator compounds (B and D) was evaluated at 10 min after flushing the antagonist from the organ chamber. Results are expressed as % of the contractile response to 40 mM K^+ or to 10 μM phenylephrine (PHE_{MAX}) and are shown as means ± SEM (n = 6–8). $^{**}P < 0.01$, $^{***}P < 0.001$, significant effect of antagonist.

Maximal reduction of K^+-induced responses was observed with 1 nM felodipine [inhibitor of L-type voltage-operated Ca^{2+}-channels (L-VOCC; Herlitz *et al.*, 1983)], 1 μM Bay602770 [haem-independent activator of soluble GC (sGC; Pankey *et al.*, 2011)] and with 10 μM of each ACh [endothelium-dependent vasodilator (De Mey *et al.*, 1982)], Bay412272 [haem-dependent stimulator of sGC (Stasch *et al.*, 2001)], forskolin [direct stimulus of AC (Seamon *et al.*, 1981)] and isoprenaline [β-adrenoceptor agonist (Aviado *et al.*, 1958); Figure 2A]. Responses to phenylephrine were reduced more than 80% in the presence of 10 μM each of pinacidil [opener of ATP-sensitive K^+-channels (Carlsen *et al.*, 1983)], U73122 [inhibitor of PLC (Bleasdale *et al.*, 1990)], OH-fasudil [inhibitor of RhoK (Kandabashi *et al.*, 2002)] and Pyr3 [inhibitor of receptor-operated Ca^{2+}-channels (Kiyonaka *et al.*, 2009); Figure 2B]. 1 nM felodipine did not significantly modify contractile responses to phenylephrine, and both 10 μM U73122 and 10 μM Pyr3 did not significantly modify K^+-induced contraction (Figure 2A), as expected. Conflicting results were obtained with candidate inhibitors of PKC. They either had no effect on contractile responses at up to 10 μM (Ro318220) or they reduced responses to K^+ and phenylephrine to the same extent (staurosporine and chelerythrine chloride; data not shown). Inhibitory effects of ACh, forskolin, isoprenaline, 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 (Figure 2C and D).

Mesenteric resistance artery response to ET-1

After sensory motor nerve desensitization, and in the presence of L-NAME and indomethacin, mesenteric resistance arteries responded to ET-1 with concentration-dependent contractions (Figures 1, 3 and 4). The maximal response was well maintained in the presence of 16 nM ET-1 and long-lasting after washout of free unbound peptide (Figures 1A, 3B and 4B).

Presence of most of the functional antagonists at concentrations that inhibited K^+- or phenylephrine-induced contraction, did not significantly modify sensitivity or maximal responses to ET-1. This was the case for ACh, Bay412272, Bay602779, felodipine, isoprenaline, OH-fasudil, pinacidil and Pyr3 (Figures 3A and 4A and Table 1). Forskolin moderately reduced sensitivity to ET-1 (Figure 3A) and U73122 markedly attenuated the initiation of contraction by 0.25–16.0 nM ET-1 (Figure 4A). Despite lack of effect on sensitivity and maximal responsiveness to ET-1, presence of several types of functional antagonists reduced the maintenance of the contractile response to the peptide (Figures 3B and 4B). Ten minutes 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 isoprenaline, 10 μM ACh, 10 μM pinacidil, 10 μM Bay412272, 1 μM Bay602770, 10 μM forskolin (Figure 3B), or 10 μM Pyr3 (Figure 4B). In contrast, the presence of 1 nM felodipine or 10 μM OH-fasudil did not significantly alter the maintenance of mesenteric resistance artery responses to ET-1 (Figure 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 isoprenaline, ACh and pinacidil (Meens *et al.*, 2010). Here we observed marked relaxation with stimuli of sGC or AC, which was long-lasting for both Bay412272 and Bay602770 and readily reversible for forskolin (Figure 3C). Also U73122, Pyr3 and felodipine, but not OH-fasudil, markedly relaxed ET-1-induced contraction (Figure 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 contractions, did induce a marked reversal of the response to peptide (Figure 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 (Figure 4 and Table 1). The PLC inhibitor, U73122, largely

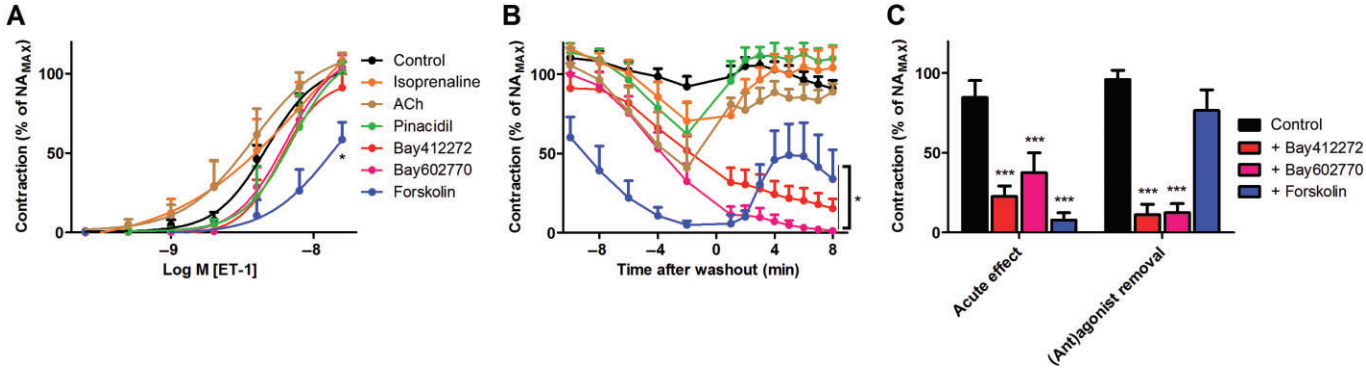

Figure 3

Mesenteric artery; 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 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. Results are 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, significant effect of antagonist.

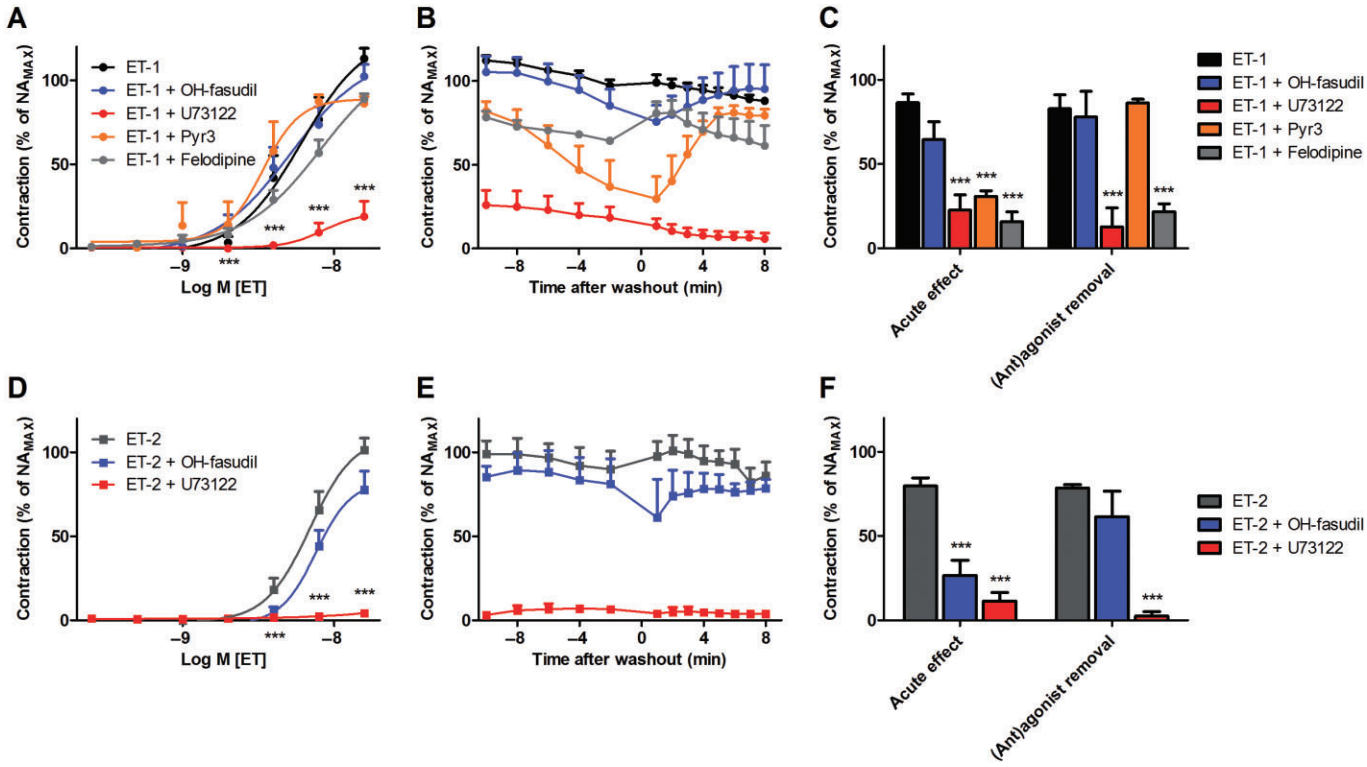

Figure 4

Upper set of results show responses to ET-1 in mesenteric artery; 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). Lower set of results show responses of mesenteric artery to ET-2; 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). Results are expressed as % of the contractile response to 10 μM NA_{MAX} and are shown as means ± SEM ($n = 6$). ***$P < 0.001$, significant effect of antagonist.

prevented contractile responses to ET-2 and markedly relaxed ET-2-induced contraction. The RhoK inhibitor, 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 (Figure 4). However, unlike ET-1-induced contractions (Figure 4C), ET-2-induced contractions were significantly relaxed by OH-fasudil (Figure 4F).

Basilar artery responses to ET-1 and ET-2

These 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 (Figures 4 and 5, Table 1). As observed with the mesenteric vessels, U73122 largely suppressed basilar artery contractile responses to ET-1 and ET-2 (Figure 5A, D) and the PLC inhibitor markedly relaxed contractile responses to ET-1 (Figure 5C). While the 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 (Figure 5C). This is noteworthy because OH-fasudil had no statistically significant effects on responses to ET-1 in rat mesenteric resistance arteries (Figure 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 (Rizzoni *et al.*, 1997). The peptides bind tightly to these receptors leading to long-lasting contractions that are refractory to reversal by ET_A antagonists (Yanagisawa *et al.*, 1988; Inoue *et al.*, 1989; Hilal-Dandan *et al.*, 1997; Adner *et al.*, 2001; Meens *et al.*, 2010). This is even more marked in rat cerebral arteries than mesenteric resistance arteries (Meens *et al.*, 2011) and suggests that inhibitors other than receptor antagonists, such as a 'physiological' antagonist CGRP (Meens *et al.*, 2010; 2012), negative allosteric modulators of receptor function (De Mey *et al.*, 2011; Compeer *et al.*, 2012a,b) and functional antagonists might be more effective in reversing ET-induced vasospasm . Here we have concentrated on the 'anti-endothelinergic' effects of some inhibitors of contractile mechanisms and of stimulants of relaxing mechanisms. We verified the selectivity of these pharmacological tools by

Table 1

EC_{50} values of ET-1 and ET-2 in absence and presence of functional antagonists, in segments of mesenteric resistance artery MRA and basilar artery BA

ET	Functional antagonist	EC_{50}
ET-1 (MRA)	–	4.7 ± 0.3 nM
	Isoprenaline	3.8 ± 0.4 nM
	ACh	3.7 ± 0.6 nM
	Pinacidil	6.8 ± 0.4 nM
	Bay412272	5.9 ± 0.3 nM
	Bay602770	6.3 ± 0.5 nM
	Forskolin	13.6 ± 0.3 nM
	U73122	–
	OH-fasudil	5.2 ± 0.2 nM
	Pyr3	3.3 ± 0.2 nM
	Felodipine	8.2 ± 0.3 nM
ET-2 (MRA)	–	6.3 ± 0.4 nM
	U73122	–
	OH-fasudil	7.6 ± 0.3 nM
ET-1 (BA)	–	2.9 ± 0.2 nM
	U73122	–
	OH-fasudil	3.0 ± 0.4 nM
ET-2 (BA)	–	2.8 ± 0.1 nM
	U73122	–
	OH-fasudil	5.1 ± 0.3 nM

analyses of their effects on contractions induced by K^+-and by α_1-adrenoceptor agonists. It remains to be established if NOS, COX and sensory motor nerves alter the effects of candidate functional antagonists, as these systems were inhibited in our experiments, in order to focus on smooth muscle ET_A receptors.

The molecular mechanisms of vasoconstriction induced by agonists that activate 7-TM receptors are still only partly understood. A widely held view suggests important differences between the mechanism(s) of initiation and those involved in maintenance, of smooth muscle contractions. 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 (Somlyo and Somlyo, 2000; Loirand *et al.*, 2006; Wynne *et al.*, 2009). 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 (Badr *et al.*, 1989; Neylon, 1999; Saleh *et al.*, 2009; Wynne *et al.*, 2009), and most of these were confirmed in smooth muscle cells from rat mesenteric resistance arteries (Clarke *et al.*, 2008; Morris *et al.*, 2010). 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. We did obtain the first indications that the mechanisms involved display 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 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 (Clarke *et al.*, 2008). The use of U73122 as a selective general inhibitor of phospholipases has recently been challenged (Klein *et al.*, 2011), but there is no valid pharmacological alternative available yet. A potentially serious additional effect for this study is the potent PLC-independent inhibition of plasmalemmal calcium channels (Pulcinelli *et al.*, 1998). PLC synthesizes inositol trisphosphate which stimulates intracellular calcium-release and diacylglycerol which stimulates PKC, and thus can promote calcium influx. The former is compatible with a strong, but transient mesenteric resistance artery contractile response to ET-1 in the absence of extracellular calcium (Boonen and De Mey, 1990). 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-adrenoceptor responses, respectively, did not significantly modify sensitivity, maximum and maintenance of mesenteric artery responses to ET-1. We therefore looked at the involvement of receptor-operated calcium channels with the use of Pyr3. This pyrazole compound was recently reported to selectively inhibit the cation channel, TRPC3, which is known to be involved in ET-1-induced vasospasms after subarachnoid haemorrhage (Xie *et al.*, 2007), and Orai1, a component of store-operated calcium channels (Kiyonaka *et al.*, 2009; Schleifer *et al.*, 2012). 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 the functions of TRPC3 and Orai1 are modulated by diacylglycerol, either directly or via PKC (Kawasaki *et al.*, 2010; Vazquez *et al.*, 2010; Harteneck and Gollasch, 2011), these channels might be linked to ET-1-induced PLC activation and involved in the sustained mesenteric artery responses to the peptide, but not to the initiation of the contractile response.

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

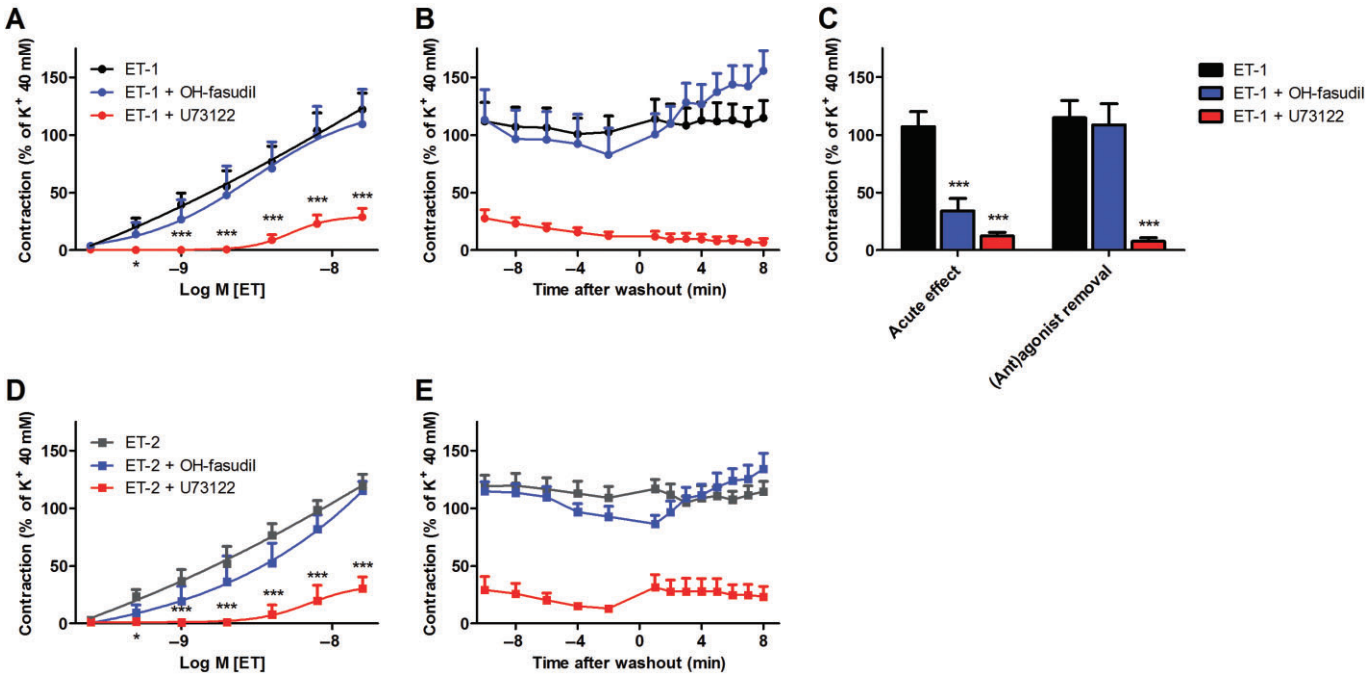

Figure 5

Basilar artery; effects of U73122 and OH-fasudil 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 ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant effect of antagonist.

activator of sGC (Bay602770; Evgenov *et al.*, 2006) although ET-1 potently stimulates NADPH oxidase activity via ET_A receptors (Elmarakby *et al.*, 2005). 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, possibly due to their hydrophobicity profiles, and probably did not alter binding of ET-1 to ET_A receptors.

In mesenteric resistance arteries, most functional antagonists reduced the maintenance of ET-1-induced contractions more markedly than the initiation of the responses. In addition, these functional antagonists could markedly reduce the contraction elicited by prior application of ET-1. For most functional antagonists, the amplitude of this acute relaxing effect did not differ significantly from the reduction of the maintenance of ET-1-induced responses. Felodipine, however, did not influence maintenance, but induced a marked relaxation. We have no clear explanation yet for this discrepancy. It will be of interest to verify whether it is shared by other dihydropyridine and non-dihydropyridine inhibitors of the L-VOCCs. The binding of these compounds to calcium channels is known to be influenced by membrane potential (Morel and Godfraind, 1988). Whether this or other aspects of the signal transduction of ET_A receptors modulate the pharmacology of L-VOCCs may be the subject of future investigations.

As previously described, effects of ET_A receptor antagonists are agonist-dependent, that is the extent of the modulation by these receptor antagonists depends on which agonist is used to activate ET_A receptors (Compeer *et al.*, 2012a,b). We therefore investigated if such agonist-dependence also applied to functional antagonists and we found that RhoK inhibition reversibly relaxed ET-2-induced vasospasm, in contrast to the lack of effect on ET-1-induced vasospasm. Because the Rho/RhoK-mediated pathway is a signalling pathway parallel to PLC (Wynne *et al.*, 2009) 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 (Kenakin and Miller, 2010). Thus, ET-1 selectively activated PLC signalling, whereas ET-2 was more biased to a non-selective, parallel activation of PLC and Rho/RhoK pathways, in mesenteric resistance arteries. In basilar arteries, ET-1 activated both PLC and Rho/RhoK pathways during vasospasm. This is worth some consideration when functionally antagonizing ET_A-mediated signalling, as agonist dependence, one of the indicators of allosterism (Kenakin and Miller, 2010; Keov *et al.*, 2011) at a receptor level, is now also found in intracellular signalling systems.

To further explore allosteric modulation, we addressed another criterion: system dependence (Christopoulos *et al.*, 2004). To this end, we compared mesenteric arteries with basilar arteries, a system that is resistant to ET_A receptor antagonism and to the physiological antagonist CGRP (Meens *et al.*, 2011). As observed with 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 basilar arteries.

It is unlikely that smooth muscle or endothelial ET_B receptors affected our current findings. We have not found a role of contractile ET_B receptors in freshly isolated arteries (Meens *et al.*, 2010; 2011; Compeer *et al.*, 2012a) and vasodilation induced via ET_B receptors is mediated by endothelial NO (Hirata *et al.*, 1993). We have used L-NAME to block NOS and thereby any NO-mediated relaxation.

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

Acknowledgements

This study was performed within the framework of Dutch Top Institute Pharma, project T2-301: Renin Angiotensin System Blockade beyond Angiotensin II.

Conflict of interest

None.

References

Adner M, Shankley N, Edvinsson L (2001). Evidence that ET-1, but not ET-3 and S6b, ET(A)-receptor mediated contractions in isolated rat mesenteric arteries are modulated by co-activation of ET(B) receptors. Br J Pharmacol 133: 927–935.

Alexander SP, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th edition. Br J Pharmacol 164 (Suppl. 1): S1–324.

Aviado DM, Jr, Wnuck AL, De Beer EJ (1958). Cardiovascular effects of sympathomimetic bronchodilators; epinephrine, ephedrine, pseudoephedrine, isoproterenol, methoxyphenamine and isoprophenamine. J Pharmacol Exp Ther 122: 406–417.

Badr KF, Murray JJ, Breyer MD, Takahashi K, Inagami T, Harris RC (1989). Mesangial cell, glomerular and renal vascular responses to endothelin in the rat kidney. Elucidation of signal transduction pathways. J Clin Invest 83: 336–342.

Bleasdale JE, Thakur NR, Gremban RS, Bundy GL, Fitzpatrick FA, Smith RJ *et al.* (1990). Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. J Pharmacol Exp Ther 255: 756–768.

Boonen HC, De Mey JG (1990). G-proteins are involved in contractile responses of isolated mesenteric resistance arteries to agonists. Naunyn Schmiedebergs Arch Pharmacol 342: 462–468.

Buther K, Compeer MG, De Mey JG, Schober O, Schafers M, Bremer C *et al.* (2012). Assessment of endothelin-A receptor expression in subcutaneous and orthotopic thyroid carcinoma xenografts *in vivo* employing optical imaging methods. Endocrinology 153: 2907–2918.

Carlsen JE, Kardel T, Jensen HA, Tango M, Trap-Jensen J (1983). Pinacidil, a new vasodilator: pharmacokinetics and pharmacodynamics of a new retarded release tablet in essential hypertension. Eur J Clin Pharmacol 25: 557–561.

Christopoulos A, May LT, Avlani VA, Sexton PM (2004). G-protein-coupled receptor allosterism: the promise and the problem(s). Biochem Soc Trans 32 (Pt 5): 873–877.

Clarke CJ, Forman S, Pritchett J, Ohanian V, Ohanian J (2008). Phospholipase C-delta1 modulates sustained contraction of rat mesenteric small arteries in response to noradrenaline, but not endothelin-1. Am J Physiol Heart Circ Physiol 295: H826–H834.

Compeer MG, Meens MJ, Hackeng TM, Neugebauer WA, Holtke C, De Mey JG (2012a). Agonist-dependent modulation of arterial endothelinA receptor function. Br J Pharmacol 166: 1833–1845.

Compeer MG, Suylen DP, Hackeng TM, De Mey JG (2012b). Endothelin-1 and -2: two amino acids matter. Life Sci 91: 607–612.

Davenport AP (2002). International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature. Pharmacol Rev 54: 219–226.

De Mey JG, Claeys M, Vanhoutte PM (1982). Endothelium-dependent inhibitory effects of acetylcholine, adenosine triphosphate, thrombin and arachidonic acid in the canine femoral artery. J Pharmacol Exp Ther 222: 166–173.

De Mey JG, Megens R, Fazzi GE (2008). Functional antagonism between endogenous neuropeptide Y and calcitonin gene-related peptide in mesenteric resistance arteries. J Pharmacol Exp Ther 324: 930–937.

De Mey JG, Compeer MG, Lemkens P, Meens MJ (2011). ETA-receptor antagonists or allosteric modulators? Trends Pharmacol Sci 32: 345–351.

Elmarakby AA, Loomis ED, Pollock JS, Pollock DM (2005). NADPH oxidase inhibition attenuates oxidative stress but not hypertension produced by chronic ET-1. Hypertension 45: 283–287.

Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HH, Stasch JP (2006). NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. Nat Rev Drug Discov 5: 755–768.

Harteneck C, Gollasch M (2011). Pharmacological modulation of diacylglycerol-sensitive TRPC3/6/7 channels. Curr Pharm Biotechnol 12: 35–41.

Herlitz H, Aurell M, Conradson T (1983). Felodipine, an arteriolar dilator with pronounced antihypertensive effect. Lancet 1: 409–410.

Hilal-Dandan R, Villegas S, Gonzalez A, Brunton LL (1997). The quasi-irreversible nature of endothelin binding and G protein-linked signaling in cardiac myocytes. J Pharmacol Exp Ther 281: 267–273.

Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K *et al.* (1993). Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. J Clin Invest 91: 1367–1373.

Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K *et al*. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A 86: 2863–2867.

Kandabashi T, Shimokawa H, Mukai Y, Matoba T, Kunihiro I, Morikawa K *et al*. (2002). Involvement of rho-kinase in agonists-induced contractions of arteriosclerotic human arteries. Arterioscler Thromb Vasc Biol 22: 243–248.

Kawasaki T, Ueyama T, Lange I, Feske S, Saito N (2010). Protein kinase C-induced phosphorylation of Orai1 regulates the intracellular Ca2+ level via the store-operated Ca2+ channel. J Biol Chem 285: 25720–25730.

Kenakin T, Miller LJ (2010). Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. Pharmacol Rev 62: 265–304.

Keov P, Sexton PM, Christopoulos A (2011). Allosteric modulation of G protein-coupled receptors: a pharmacological perspective. Neuropharmacology 60: 24–35.

Khodorova A, Montmayeur JP, Strichartz G (2009). Endothelin receptors and pain. J Pain 10: 4–28.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: Reporting *in vivo* experiments: The ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Kirkby NS, Hadoke PW, Bagnall AJ, Webb DJ (2008). The endothelin system as a therapeutic target in cardiovascular disease: great expectations or bleak house? Br J Pharmacol 153: 1105–1119.

Kiyonaka S, Kato K, Nishida M, Mio K, Numaga T, Sawaguchi Y *et al*. (2009). Selective and direct inhibition of TRPC3 channels underlies biological activities of a pyrazole compound. Proc Natl Acad Sci U S A 106: 5400–5405.

Klein RR, Bourdon DM, Costales CL, Wagner CD, White WL, Williams JD *et al*. (2011). Direct activation of human phospholipase C by its well known inhibitor u73122. J Biol Chem 286: 12407–12416.

Kohan DE, Cleland JG, Rubin LJ, Theodorescu D, Barton M (2012). Clinical trials with endothelin receptor antagonists: what went wrong and where can we improve? Life Sci 91: 528–539.

Ling L, Maguire JJ, Davenport AP (2013). Endothelin-2, the forgotten isoform: emerging role in the cardiovascular system, ovarian development, immunology and cancer. Br J Pharmacol 168: 283–295.

Loirand G, Guerin P, Pacaud P (2006). Rho kinases in cardiovascular physiology and pathophysiology. Circ Res 98: 322–334.

Masaki T (2004). Historical review: endothelin. Trends Pharmacol Sci 25: 219–224.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Meens MJ, Fazzi GE, van Zandvoort MA, De Mey JG (2009). Calcitonin gene-related peptide selectively relaxes contractile responses to endothelin-1 in rat mesenteric resistance arteries. J Pharmacol Exp Ther 331: 87–95.

Meens MJ, Compeer MG, Hackeng TM, van Zandvoort MA, Janssen BJ, De Mey JG (2010). Stimuli of sensory-motor nerves terminate arterial contractile effects of endothelin-1 by CGRP and dissociation of ET-1/ET(A)-receptor complexes. PLoS ONE 5: e10917.

Meens MJ, Mattheij NJ, Nelissen J, Lemkens P, Compeer MG, Janssen BJ *et al*. (2011). Calcitonin gene-related peptide terminates long-lasting vasopressor responses to endothelin 1 *in vivo*. Hypertension 58: 99–106.

Meens MJ, Mattheij NJ, van Loenen PB, Spijkers LJ, Lemkens P, Nelissen J *et al*. (2012). G-protein betagamma subunits in vasorelaxing and anti-endothelinergic effects of calcitonin gene-related peptide. Br J Pharmacol 166: 297–308.

Morel N, Godfraind T (1988). Selective modulation by membrane potential of the interaction of some calcium entry blockers with calcium channels in rat mesenteric artery. Br J Pharmacol 95: 252–258.

Morris GE, Nelson CP, Standen NB, Challiss RA, Willets JM (2010). Endothelin signalling in arterial smooth muscle is tightly regulated by G protein-coupled receptor kinase 2. Cardiovasc Res 85: 424–433.

Neylon CB (1999). Vascular biology of endothelin signal transduction. Clin Exp Pharmacol Physiol 26: 149–153.

Pankey EA, Bhartiya M, Badejo AM, Jr, Haider U, Stasch JP, Murthy SN *et al*. (2011). Pulmonary and systemic vasodilator responses to the soluble guanylyl cyclase activator, BAY 60-2770, are not dependent on endogenous nitric oxide or reduced heme. Am J Physiol Heart Circ Physiol 300: H792–H802.

Pulcinelli FM, Gresele P, Bonuglia M, Gazzaniga PP (1998). Evidence for separate effects of U73122 on phospholipase C and calcium channels in human platelets. Biochem Pharmacol 56: 1481–1484.

Rizzoni D, Porteri E, Piccoli A, Castellano M, Bettoni G, Pasini G *et al*. (1997). The vasoconstriction induced by endothelin-1 is mediated only by ET(A) receptors in mesenteric small resistance arteries of spontaneously hypertensive rats and Wistar Kyoto rats. J Hypertens 15 (12 Pt 2): 1653–1657.

Saleh SN, Albert AP, Large WA (2009). Activation of native TRPC1/C5/C6 channels by endothelin-1 is mediated by both PIP3 and PIP2 in rabbit coronary artery myocytes. J Physiol 587 (Pt 22): 5361–5375.

Schleifer H, Doleschal B, Lichtenegger M, Oppenrieder R, Derler I, Frischauf I *et al*. (2012). Novel pyrazole compounds for pharmacological discrimination between receptor-operated and store-operated Ca(2+) entry pathways. Br J Pharmacol 167: 1712–1722.

Schneider MP, Boesen EI, Pollock DM (2007). Contrasting actions of endothelin ET(A) and ET(B) receptors in cardiovascular disease. Annu Rev Pharmacol Toxicol 47: 731–759.

Seamon KB, Padgett W, Daly JW (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci U S A 78: 3363–3367.

Somlyo AP, Somlyo AV (2000). Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J Physiol 522 (Pt 2): 177–185.

Stasch JP, Becker EM, Alonso-Alija C, Apeler H, Dembowsky K, Feurer A *et al*. (2001). NO-independent regulatory site on soluble guanylate cyclase. Nature 410: 212–215.

Vazquez G, Tano JY, Smedlund K (2010). On the potential role of source and species of diacylglycerol in phospholipase-dependent regulation of TRPC3 channels. Channels (Austin) 4: 232–240.

Wagner OF, Vierhapper H, Gasic S, Nowotny P, Waldhausl W (1992). Regional effects and clearance of endothelin-1 across

pulmonary and splanchnic circulation. Eur J Clin Invest 22: 277–282.

Wang Y, Wang DH (2004). Prevention of endothelin-1-induced increases in blood pressure: role of endogenous CGRP. Am J Physiol Heart Circ Physiol 287: H1868–H1874.

Warner TD, Mitchell JA, de Nucci G, Vane JR (1989). Endothelin-1 and endothelin-3 release EDRF from isolated perfused arterial vessels of the rat and rabbit. J Cardiovasc Pharmacol 13 (Suppl. 5): S85–S88; discussion S102.

Wynne BM, Chiao CW, Webb RC (2009). Vascular smooth muscle cell signaling mechanisms for contraction to angiotensin II and endothelin-1. J Am Soc Hypertens 3: 84–95.

Xie A, Aihara Y, Bouryi VA, Nikitina E, Jahromi BS, Zhang ZD *et al*. (2007). Novel mechanism of endothelin-1-induced vasospasm after subarachnoid hemorrhage. J Cereb Blood Flow Metab 27: 1692–1701.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y *et al*. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332: 411–415.