0022-3565/09/3311-87–95$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 331:87–95, 2009

Vol. 331, No. 1
155143/3517524
Printed in U.S.A.

Calcitonin Gene-Related Peptide Selectively Relaxes Contractile Responses to Endothelin-1 in Rat Mesenteric Resistance Arteries[S]

Merlijn J. P. M. T. Meens, Gregorio E. Fazzi, Marc A. van Zandvoort, and Jo G. R. De Mey

Departments of Pharmacology and Toxicology (M.J.P.M.T.M., G.E.F., J.G.R.D.M.) and Biomedical Technology (M.A.v.Z.), Cardiovascular Research Institute Maastricht, Maastricht University, The Netherlands

Received April 15, 2009; accepted July 21, 2009

ABSTRACT

We tested the hypothesis that endothelin-1 (ET-1) modulates sensory-motor nervous arterial relaxation by prejunctional and postjunctional mechanisms. Isolated rat mesenteric resistance arteries were investigated with immunohistochemistry, wire-myography, and pharmacological tools. ET_A- and ET_B-receptors could be visualized on the endothelium and smooth muscle and on periarterial fibers containing calcitonin gene-related peptide (CGRP). Arterial contractile responses to ET-1 (0.25–16 nM) were not modified by blockade of ET_B-receptors, NO-synthase, and cyclooxygenase or desensitization of transient receptor potential cation channel, subfamily V, member 1 (TRPV1) with capsaicin. ET-1 reversed relaxing responses to CGRP in depolarized arteries. This effect was inhibited by ET_A-antagonists. It was not selective because ET-1 also reversed relaxing responses to Na-nitroprusside (SNP) and because phenylephrine (PHE; 0.25–16 μM) similarly reversed relaxing responses to CGRP or SNP. Conversely, contractile responses to ET-1 were, compared with PHE, hypersensitive to the relaxing effects of the TRPV1-agonist capsaicin and to exogenous CGRP, but not to acetylcholine, forskolin, pinacidil, or SNP. In conclusion, ET-1 does not stimulate sensory-motor nervous arterial relaxation, but ET_A-mediated arterial contractions are selectively sensitive to relaxation by the sensory neurotransmitter CGRP. This does not involve NO, cAMP, or ATP-sensitive K^+ channels.

The bicyclic 21-amino-acid peptide endothelin-1 (ET-1) is involved in pulmonary hypertension, heart failure, and cancer (Bagnato and Rosanò, 2008; Kirkby et al., 2008; Opitz et al., 2008). It binds with comparable high affinity to two distinct receptor subtypes, ET_A and ET_B, belonging to the G-protein-coupled receptor superfamiliy (Davenport, 2002; Masaki, 2004). In the vasculature, the detrimental effects of ET-1, (i) long-lasting vasoconstriction (Kawamata et al.), (ii) cell growth, proliferation, and migration, (iii) production of reactive oxygen species, and (iv) inflammation, are mediated by ET_A-receptors on vascular smooth muscle cells (VSMCs) (Masaki, 2004; Hynynen and Khalil, 2006; Schneider et al., 2007). Counterbalancing beneficial effects such as nitric oxide (NO) synthesis, vasodilatation, and scavenging of circulating ET-1 are mediated by endothelial ET_B-receptors (Nakashima and Vanhoutte, 1993; Woods et al., 1999; Johnström et al., 2005; Schneider et al., 2007). Yet, therapeutic effects in experimental animal models and patients do not differ profoundly between selective ET_A-antagonists and mixed ET_A/ET_B-antagonists (Masaki, 2004; Battistini et al., 2006; Dhaun et al., 2007; Schneider et al., 2007; Opitz et al., 2008). This may be due to effects of ET-1 on other cell types.

ET_A-receptors are not only expressed by VSMC but also by periarterial sensory-motor nerves (Wang and Wang, 2004; Plant et al., 2006). These nerves mediate nonadrenergic noncholinergic vasodilatation involving the neurotransmitter calcitonin gene-related peptide (CGRP) that can activate ad-

This study was performed within the framework of Top Institute Pharma Project T2–108-1: Metalloproteases and Novel Targets in Endothelial Dysfunction.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.109.155143.

[S] The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: ET-1, endothelin-1; BIBN4096BS, 1-piperidinecarboxamide, *N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl); bosentan, 4-*tert*-butyl-*N*-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide; BQ123, cyclo (D-Trp-D-Asp-Pro-D-Val-Leu); BQ788, *N*-*cis*-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl1-D-1methoxycarbonyl-tryptophanyl-D-norleucine; L-NAME, N^{ω}-nitro-L-arginine methyl ester; TRPV1, transient receptor potential cation channel, subfamily V, member 1; VSMC, vascular smooth muscle cell; CGRP, calcitonin gene-related peptide; AC, adenylate cyclase; CAPS, capsaicin; KRB, Krebs-Ringer bicarbonate-buffered physiological salt solution; INDO, indomethacin; ACh, acetylcholine; PHE, phenylephrine.

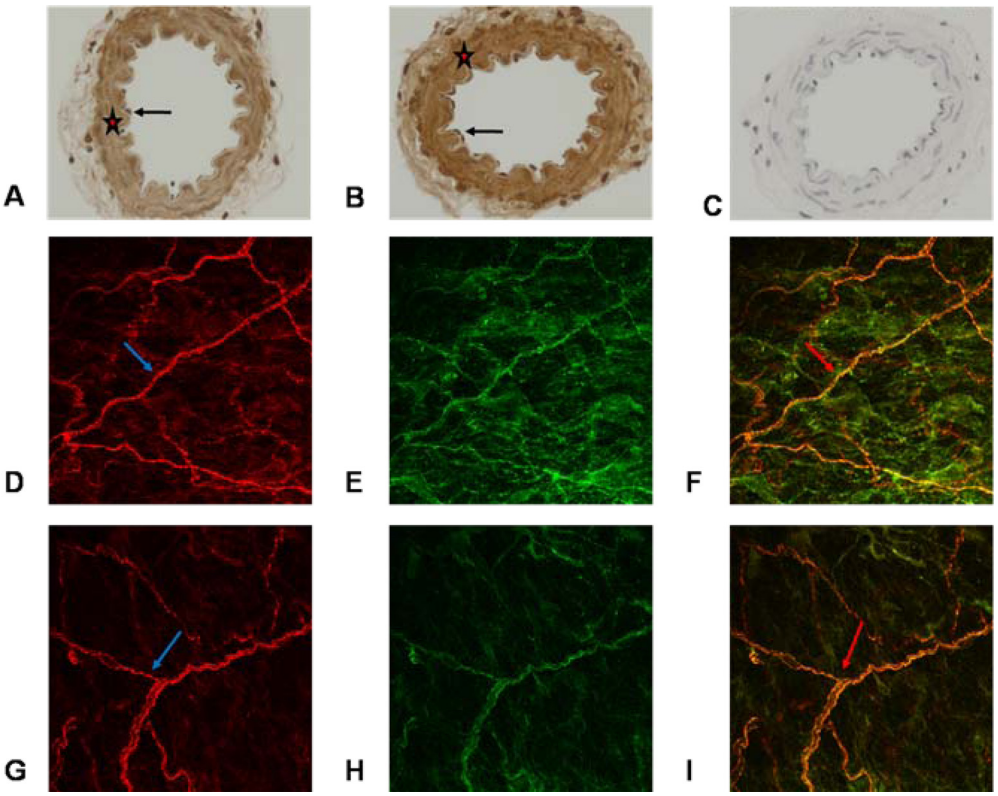

Fig. 1. Immunohistochemistry illustrating the distribution of ET_A- and ET_B-receptors in rat mesenteric arteries. A and B, distribution of ET_A- (A) and ET_B- (B) receptors on cross-sections of rat mesenteric arteries. Intense staining can be observed in the tunica media (stars) and endothelium (arrows). C, negative control, no primary antibody. D, localization of CGRP- (red) containing sensory-motor nerves in the adventitia of a rat mesenteric artery (arrow). E, distribution of ET_B-receptors (green) in the same optical sections. F, merger of D and E revealing ET_B-receptors on CGRP-containing sensory-motor nerves (arrow). G, localization of CGRP- (red) containing sensory-motor nerves at the media-adventitia border of a rat mesenteric artery (arrow). H, distribution of ET_A-receptors (green) in the same optical sections. I, merger of G and H revealing ET_A-receptors on CGRP-containing sensory-motor nerves (arrow).

enylate cyclase (AC) (Edvinsson et al., 1985; Brain and Grant, 2004; De Mey et al., 2008). ET_B-receptors are not only expressed by endothelial cells but also by VSMC (Adner et al., 2001; Masaki, 2004; Hynynen and Khalil, 2006; Schneider et al., 2007) where they can promote vasoconstriction through inhibition of the synthesis of cAMP. This was illustrated by the effects of ET_B-receptor selective agonists and antagonists on vasomotor effects of the direct AC-stimulus forskolin (Adner et al., 2001).

Here, we tested the hypothesis that the mixed ET-receptor agonist ET-1 modulates sensory-motor nervous arterial relaxation by prejunctional and postjunctional mechanisms. In isolated rat mesenteric resistance arteries, we determined the distribution of ET-receptor subtypes by immunohistochemistry and the vasomotor effects of acetylcholine (Caterina et al., 1997), an NO-donor, exogenous CGRP, and pharmacological stimulation and desensitization of sensory-motor nerves with capsaicin (CAPS) (Szallasi and Blumberg, 1999; De Mey et al., 2008). Functional experiments were performed in the presence of ET-1 and ET-receptor antagonists and, for comparison, also in the presence of the α_1-adrenergic agonist phenylephrine and depolarizing solution, which do not affect and stimulate periarterial sensory-motor nervous function, respectively (De Mey et al., 2008; Burnstock, 2009). We observed that ET-1 does not promote or inhibit sensory-motor nervous vasodilatation and that ET_A-mediated arterial contractions are selectively sensitive to relaxation by endogenously released and exogenous CGRP.

Materials and Methods

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

Tissue Preparation. Twenty-week-old male Sprague-Dawley rats (Charles River, Maastricht, The Netherlands) were euthanized by CO_2 inhalation. Second-order side branches of the superior mesenteric artery were isolated by dissection in Krebs-Ringer bicarbonate-buffered physiological salt solution (KRB) containing 118.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, and 5.5 mM glucose. Arterial samples were immersed in neutral buffered 4% formalin or used to record vasomotor responses immediately.

Immunohistochemistry. Arterial segments were fixed (24 h; room temperature), stored in 70% ethanol, and embedded in paraffin and transversely sectioned (4 μm thick) or used as whole mounts.

Cross-sections were used to visualize immunoreactive ET_A- and ET_B-receptors in the endothelium, media, and adventitia. Sections were deparaffinized and rehydrated; endogenous peroxidases were blocked (20 min. 0.3% H_2O_2; room temperature). Subsequently, sections were incubated with sheep polyclonal antibodies raised against a peptide fragment selective for an intracellular C-terminal fragment of ET_A- or ET_B-receptors, respectively (210-507A or 210-506A, 1:800; Alexis, Zandhoven, Belgium; see Supplemental Figs. 1–3 and Supplemental Data concerning antibody validation). Excess antibody was removed and sections were incubated with horseradish peroxidase-conjugated polyclonal rabbit anti-sheep antibodies (1:400; Dako Denmark A/S, Glostrup, Denmark). The localization of horseradish peroxidase was visualized with 3,3′-diaminobenzidine (Sigma-Aldrich, Zwijndrecht, The Netherlands). All sections were counterstained with hematoxylin. Negative controls were incubated with the secondary antibody only (Fig. 1).

For whole-mount immunohistochemistry, fixed arterial segments were rehydrated and incubated with primary antibodies directed against rat αCGRP (CA1137, 1:2000; BioTrend, Köln, Germany) and ET_A- or ET_B-receptors (210-507A, 1:2000, or 210-506A, 1:3000; Alexis). Secondary antibodies (Alexa Fluor 488- or Alexa Fluor 546-labeled donkey anti-sheep or goat anti-rabbit IgG; Invitrogen, Carlsbad, CA) and two-photon laser scanning microscopy were used to visualize immunoreactivity, as described previously (De Mey et al., 2008). Additional experiments were performed with an antibody

against an N-terminal fragment of ET_B-receptors (ab12980, 1:3000; Abcam, Cambridge, MA).

Vasomotor Responses. Arterial segments (2 mm long) were mounted and stretched as described previously (De Mey et al., 2008). At optimal diameter (425 ± 35 μm) the contractile response to 10 μM norepinephrine (NE) averaged 4.52 ± 0.75 N/m ($n = 4$, $N = 90$).

Contractile responses to increasing concentrations of K^+ (5.9–40 mM), phenylephrine (PHE, 0.25–16 μM), and ET-1 (0.25–16 nM) were recorded. Thereafter, vessels were discarded in view of the long-lasting nature of some of the effects of the peptide (Yanagisawa et al., 1988; Adner et al., 2001). The effects of persistent desensitization of sensory-motor nerves with CAPS (1 μM during 20 min; Szallasi and Blumberg, 1999; De Mey et al., 2008), competitive antagonism of CGRP-receptors with BIBN4096BS [20 nM; this concentration caused a significant rightward shift of CGRP-induced relaxations during ET-1-, PHE-, and K^+-induced contractions (not shown) (Doods et al., 2000)] and of blockade of the synthesis of NO and prostaglandins with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) and indomethacin (INDO, 10 μM) were analyzed.

To evaluate whether ET-1 could selectively reverse arterial relaxing responses, arteries were partly depolarized with 40 mM K^+ in the presence of L-NAME and INDO, relaxed by either CGRP (0.1–100 nM) or Na-nitroprusside (SNP, 0.01–10 μM) and then exposed to increasing concentrations of ET-1 or PHE. These experiments were performed before and after desensitization of sensory-motor nerves.

Arterial relaxing responses to increasing concentrations of acetylcholine (ACh, 0.01–10 μM), CAPS (0.01–1.0 μM), CGRP (0.1–100 nM), SNP (0.01–10 μM), forskolin (0.1–3 μM), isoproterenol (0.01–3 μM), and pinacidil (0.01–10 μM) were compared during contractions induced by 16 μM PHE or 16 nM ET-1, in the continuous presence of L-NAME and INDO. Some of these experiments were also performed in presence of a CGRP-antagonist and after desensitization of sensory-motor nerves.

A 20-min incubation with the ET_A-antagonist BQ123 (1 μM), the ET_B-antagonist BQ788 (1 μM), and the mixed ET-antagonist bosentan (10 μM) was used to evaluate the role of receptor subtypes in the arterial effects of ET-1 (Davenport, 2002).

Solutions and Drugs. Bosentan and BIBN4096BS were obtained from Actelion Pharmaceuticals (Allschwil, Switzerland) and Boehringer Ingelheim Pharma KG (Biberach, Germany), respectively, and were dissolved in dimethyl sulfoxide. CAPS, INDO, and forskolin were purchased from Sigma-Aldrich and dissolved in ethanol. ACh, PHE, L-NAME, NE, SNP, and isoproterenol were purchased from Sigma-Aldrich and dissolved in KRB solution. BQ123, BQ788, and pinacidil were obtained from Sigma-Aldrich and dissolved in dimethyl sulfoxide. Human α-CGRP and ET-1 were obtained from Bachem (Weil am Rhein, Germany) and dissolved in KRB solution. The maximal concentrations of the solvents never exceeded 0.1% and did not alter arterial reactivity.

Data and Statistical Analysis. Contractile responses are expressed as a percentage of the maximal contractile response to 10 μM NE before the administration of any pharmacological inhibitor. Relaxing responses are expressed as the percentage reduction of the level of precontraction. Individual concentration-response curves were fitted to a nonlinear sigmoid regression curve (Graphpad Prism 5.0; GraphPad Software Inc., San Diego, CA). Sensitivity (EC_{50}) and maximal effect (E_{max}) are shown as mean ± S.E.M. Statistical significance of effects and differences were analyzed using either one-way analysis of variance (comparison of EC_{50} and E_{max}) or two-way analysis of variance (comparison of concentration-response curves). Bonferroni's post hoc test was used to compare multiple groups. A p value of <0.05 was considered statistically significant.

Results

Histological Observations. Figure 1 illustrates the presence of immunoreactive ET-receptors in rat mesenteric resistance arteries. On cross-sections, both ET_A- (Fig. 1A) and ET_B-receptors (Fig. 1B) can be observed: i) on the endothelium, ii) throughout the tunica media, and iii) on adventitial cells. Two-photon laser scanning microscopy of whole-mount arterial preparations was used to visualize ET-receptors at the media-adventitial border. Immunoreactivity for not only ET_A- but also ET_B-receptors (Fig. 1E) is observed on fibers that have a density and a structure comparable with those of fibers containing CGRP (Fig. 1D). Figure 1, F–I, illustrate colocalization of CGRP with ET_A- and ET_B-receptors, respectively. Regarding ET_B-receptors, similar findings were obtained with an antibody raised against an N-terminal fragment, rather than a C-terminal fragment (Supplemental Fig. 4).

Contractile Responses to ET-1. In rat mesenteric resistance arteries, ET-1 (0.25–16 nM) caused potent (EC_{50} 3.3 ± 1.1 nM) concentration-dependent contractions (Fig. 2). Maximal ET-1-induced contraction (E_{max}) averaged 92 ± 5% of the response to 10 μM NE (NE_{max}) (Fig. 2). The peptide was considerably more potent than PHE (EC_{50} 1.2 ± 0.04 μM) or K^+, but its E_{max} was slightly smaller than that of the α_1-

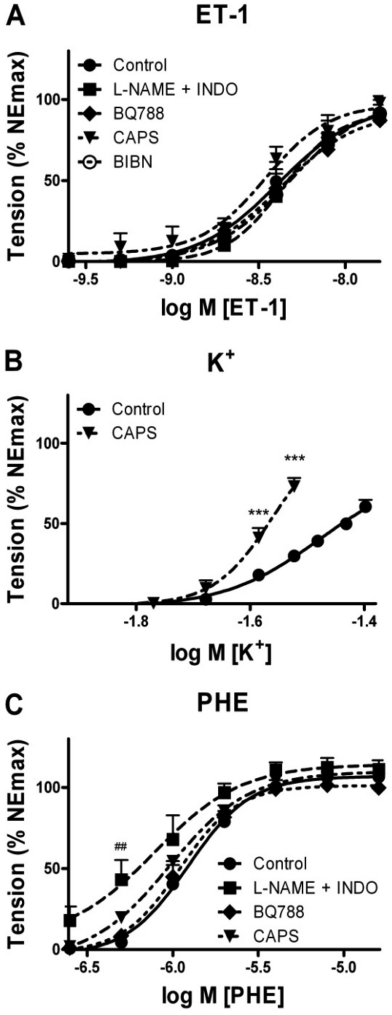

Fig. 2. Effects of contractile stimuli. A, effects of increasing concentrations of ET-1 in the absence (●) and presence of L-NAME + INDO (■), BQ788 (♦), BIBN4096BS (○), or pretreatment with CAPS (▼). B, contractions induced by increasing concentrations of K^+ in the continuous presence of L-NAME + INDO, without (●) and after pretreatment with CAPS (▼). C, effects of increasing concentrations of PHE in the absence (●) or presence of L-NAME + INDO (■), BQ788 (♦), or pretreatment with CAPS (▼). ##, $p < 0.001$, CAPS pretreated versus control.

adrenergic agonist (107 ± 4% of NE_{max}; $p < 0.05$ versus E_{max} ET-1) (Fig. 2).

Effects of L-NAME and Indomethacin. Presence of L-NAME (100 μM) and INDO (10 μM) increased the sensitivity to K^+ as described previously (Hilgers and De Mey, 2009) (Fig. 2). Blockade of NOS and COX did, however, not modify the sensitivity and maximal responses to ET-1 (EC_{50} 3.9 ± 1.3 nM; E_{max} 89 ± 10%) or PHE (EC_{50} 0.78 ± 0.1 μM; E_{max} 111 ± 6%) (Fig. 2A).

Effects of CAPS and CGRP-Antagonist. Pretreatment with CAPS (1 μM during 20 min) or presence of BIBN4096BS (20 nM) increased contractile responses to K^+ (Fig. 2B and De Mey et al., 2008). After desensitization of sensory-motor nerves or in the presence of the CGRP-antagonist, however, contractile effects of ET-1 were unaltered (EC_{50} 3.0 ± 0.7 nM and E_{max} 97 ± 6%, after desensitization; EC_{50} 3.9 ± 1.3 nM and E_{max} 89 ± 10%, in the presence of BIBN4096BS) (Fig. 2A).

Effects of ET-Receptor Antagonists. Presence of the ET_B-antagonist BQ788 (1 μM) did not modify sensitivity or maximal contractile responses to ET-1 (EC_{50} 2.9 ± 0.7 nM; E_{max} 87 ± 7%) (Fig. 2). This was also the case in the presence of L-NAME and INDO, and after desensitization of sensory-motor nerves (Supplemental Fig. 5). Presence of the ET_A-antagonist BQ123 (1 μM) or of the mixed ET-antagonist bosentan (10 μM), on the other hand, markedly reduced sensitivity to the contractile effects of ET-1 (not shown). Neither ET-receptor antagonist significantly affected contractile responses to 40 mM K^+ in the presence of L-NAME and INDO before or after desensitization of sensory-motor nerves with CAPS (Fig. 3).

ET-1 Reverses Relaxing Responses. To evaluate whether ET-1 selectively reverses arterial relaxing responses, arteries were made to contract by partial depolarization (40 mM K^+) in the presence of L-NAME (100 μM) and INDO (10 μM) and were then relaxed with either exogenous CGRP (0.1–100 nM) or SNP (0.01–10 μM). When a stable relaxation was established, increasing concentrations of ET-1 were administered (typical tracing, Fig. 3A). ET-1 reversed the relaxing effect of CGRP (Fig. 3C). The sensitivity and maximal effect of this ET-1-induced response (EC_{50} 2.9 ± 0.7 nM; E_{max} 87.1 ± 7%; Fig. 3C) were comparable with those of the direct contractile effect of ET-1 (Fig. 2A). Reversal of CGRP-induced relaxation by ET-1 was antagonized by BQ123 (1 μM) or bosentan (10 μM) (Fig. 3C). The combination of BQ123 (1 μM) and BQ788 (1 μM) did not antagonize to a larger extent than BQ123 (1 μM) alone (Fig. 3C). Similar results were obtained after desensitization of the sensory-motor nerves (Fig. 3.E), which markedly increased the sensitivity of depolarized arteries to the relaxing effects of exogenous CGRP (Fig. 3, B and D).

ET-1 not only reversed relaxing responses to CGRP, but also those to SNP (Fig. 3F). The potency of ET-1 was nonsignificantly smaller than the direct contractile potency of the peptide (EC_{50} 3.9 ± 0.7 nM; Fig. 3.G). During relaxations initiated by the NO donor, the effects of ET-receptor antagonists were comparable with those during CGRP-induced relaxations (Fig. 3G).

Not only ET-1, but also PHE reversed relaxing responses of depolarized arteries to either CGRP or SNP (Fig. 4). The potency of the α_1-agonist averaged 1.1 ± 0.5 μM and 3.8 ± 0.1 μM in the presence of the neuropeptide and the NO-donor, respectively (Fig. 4).

ET-1-Induced Contractions Are Hypersensitive to Relaxation by Endogenous and Exogenous CGRP. In arteries contracted by 16 nM ET-1, CAPS caused marked relaxations (E_{max} 87 ± 7%; Fig. 5C). These relaxations were reduced in the presence of BIBN4096BS (20 nM) (E_{max} 37 ± 26%, $p < 0.05$ versus control; Fig. 5D), were absent in arteries that had been pretreated with a high concentration of CAPS (1 μM during 20 min; not shown) and not modified by the presence of BQ788 (1 μM; Supplemental Fig. 6). In arteries contracted to the same extent by 16 μM PHE, CAPS-induced relaxations were significantly smaller than relaxations during ET-1-induced contraction (E_{max} 38 ± 12%, $p <$ 0.05 versus control) (Fig. 5C). The difference between CAPS-induced relaxations in ET-1- and PHE-precontracted arteries was diminished in the presence of BIBN4096BS. This suggests that ET-1-induced contractions are more sensitive to CGRP-receptor activation by endogenous CGRP released on activation of the TRPV1 channels on the sensory-motor nerves. In line with this, the sensitivity to the relaxing effect of exogenous CGRP was significantly larger during amplitude-matched contractions induced by 16 nM ET-1 compared with 16 μM PHE (EC_{50} 1.73 ± 0.1 nM versus 6.2 ± 0.04 nM, $p < 0.05$, ET-1 versus PHE; Fig. 5E). CGRP-induced relaxations of ET-1-induced contractions were not significantly modified by L-NAME + INDO, BQ788 (1 μM), or pretreatment with CAPS (Supplemental Fig. 7).

In contrast to CAPS and exogenous CGRP, ACh (endothelium-dependent vasodilator) in the absence and presence of L-NAME + INDO, forskolin (direct activator of adenylate cyclase), isoproterenol (β-adrenergic stimulus of adenylate cyclase), SNP (NO donor), and pinacidil (activator of ATP-sensitive K^+-channels) each caused comparable concentration-dependent relaxations of contractile responses to 16 nM ET-1 or 16 μM PHE (Fig. 5 and Supplemental Fig. 7).

Discussion

In rat mesenteric resistance arteries, ET_A- and ET_B-receptors are expressed by several cell types but contractile responses to exogenous ET-1 involve exclusively ET_A-receptors and are not modulated by ET_B-, endothelial, or sensory-motor nervous effects of the peptide. On the other hand, ET-1-induced arterial contractions are particularly sensitive to relaxation by endogenous and exogenous sensory-motor neurotransmitter CGRP.

We confirm the presence of ET_A- and ET_B-receptors on arterial smooth muscle and sensory-motor nerves and of ET_B-receptors on the endothelium (for review, see Davenport, 2002; Masaki, 2004; Johnström et al., 2005; Battistini et al., 2006; Hynynen and Khalil, 2006; Dhaun et al., 2007; Schneider et al., 2007; Kirkby et al., 2008; Opitz et al., 2008; Chichorro et al., 2009). We also document the presence of ET_A-receptors on peripheral arterial endothelium. Endothelial ET_A-receptors were previously observed in the central nervous system (Jesmin et al., 2007), but seem to be present in the peripheral circulation as well. These may be linked to aspects of ET-signaling beyond the scope of our investigations, e.g., permeability and/or inflammation. Presence of ET_A-receptors on sensory-motor nerves in rat mesenteric arteries and both ET_A- and ET_B-receptors on the trigeminal

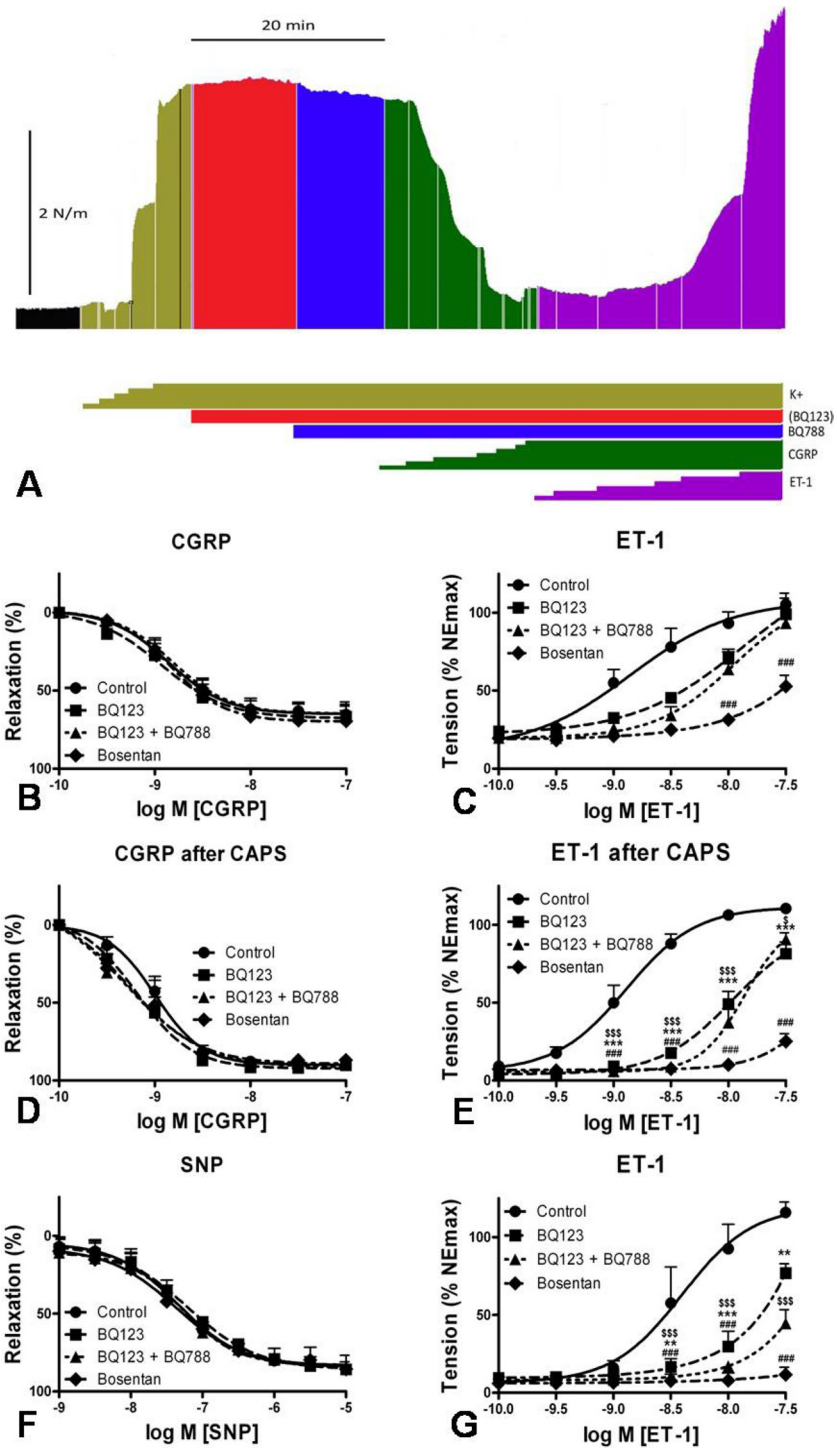

Fig. 3. Effect of CGRP and SNP on K^+-induced contraction, followed by reversal of the relaxations by ET-1. A, typical tracing of wall tension versus time illustrating consecutive effects of K^+ (yellow), BQ123 (red), BQ788 (blue), CGRP (green), and ET-1 (purple). B, effect of increasing concentrations of CGRP on K^+-induced contraction. C, reversal of CGRP-induced relaxations by increasing concentrations of ET-1 in absence (●) and presence of i) BQ123 (■), ii) BQ123 + BQ788 (▲), and iii) bosentan (♦). D, effect of increasing concentrations CGRP on K^+-induced contraction after CAPS pretreatment. E, reversal of CGRP-induced relaxations by increasing concentrations of ET-1 after CAPS pretreatment in absence (●) and presence of i) BQ123 (■), ii) BQ123 + BQ788 (▲), and iii) bosentan (♦). F, effects of increasing concentrations of SNP, during K^+ precontraction. G, reversal of SNP-induced relaxations by increasing concentrations ET-1 in absence (●) and presence of i) BQ123 (■), ii) BQ123 + BQ788 (▲) and iii) bosentan (♦). ** or ***, $p < 0.01$ or 0.001, BQ123 versus control; $ or $$$, $p < 0.05$ or 0.001, BQ123 + BQ788 versus control; ###, $p < 0.001$, bosentan versus control.

ganglion and on renal mechanosensitive nerves was reported previously (Wang and Wang, 2004; Kopp et al., 2006; Uddman et al., 2006; Chichorro et al., 2009). With the two anti-ET_B antibodies that we used, ET_B-receptors seem to be present on periarterial sensory-motor nerves as well. Although sympathetic ganglia express ET_B-receptors (Dai et al., 2004), it is unlikely that the nervous staining that we observed would be on sympathetic fibers. It localizes on fibers containing CGRP that run close to but do not colocalize with the more abundant neuropeptide Y-immunoreactive sympathetic fibers (De Mey et al., 2008). We conclude from our histological findings that the expression of ET-receptor subtypes by vascular cell types is more widespread than generally proposed.

In isolated arteries, nanomolar concentrations of ET-1 were required to induce contraction. This contrasts with the subnanomolar ligand-binding affinities of ET-1 for ET_A- and ET_B-receptors on cells and cell membranes (Davenport, 2002; Masaki, 2004; Johnström et al., 2005; Battistini et al., 2006; Hynynen and Khalil, 2006; Dhaun et al., 2007; Schneider et al., 2007; Kirkby et al., 2008; Opitz et al., 2008). Opposing

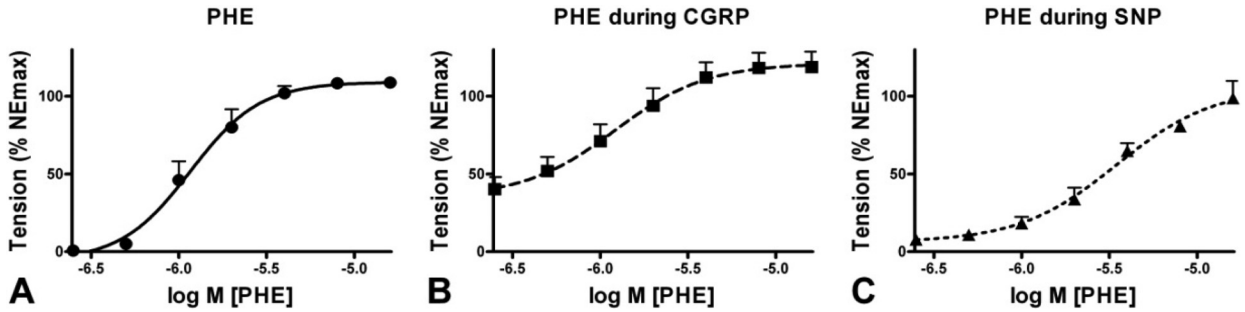

Fig. 4. Effects of PHE on basal tension (A) and during relaxation of depolarized arteries initiated by 100 nM CGRP (B) or 0.1 μM SNP (C).

stimulatory and inhibitory effects may contribute to the discrepancy between the binding affinities and the contractile potency of ET-1 (D'Orléans-Juste et al., 2002).

Pretreatment with CAPS and the presence of a CGRP-receptor antagonist or of L-NAME plus INDO, increases contractile responses to K^+ (De Mey et al., 2008; Hilgers and De Mey, 2009). These interventions and the ET_B-receptor antagonist BQ788 did, however, not modify sensitivity and maximal arterial contractile responses to ET-1. Thus, ET_B-mediated scavenging of agonist (Johnström et al., 2005), release of NO and prostaglandins (Tirapelli et al., 2005), and sensory-motor nervous vasodilatation do not contribute to the discrepancy between binding and contractile effects of ET-1. These findings do not exclude that ET-1 can stimulate the endothelium and the sensory-motor nerves. They suggest that local effects on the endothelium as seen, for instance, during intraluminal application of the peptide can be overruled by the strong ET_A-mediated arterial vasoconstriction.

Activation of ET_B-receptors on arterial smooth muscle can inhibit adenylate cyclase (Adner et al., 2001) which participates in the relaxing effects of CGRP (Brain and Grant, 2004). We verified this in conditions where endothelial relaxing effects were prevented by partial depolarization and by inhibition of NOS and COX. ET-1 reversed the relaxing effect of exogenous CGRP. The effect was antagonized by BQ123 (1 μM) and not modified to a larger extent by the presence of both BQ123 and BQ788 (1 μM). Bosentan (10 μM) resulted in a more marked inhibition. This may be due to a higher degree of ET_A-blockade or to access of the synthetic antagonist to both membrane-bound and intracellular receptors (Roux et al., 1999; Iglarz et al., 2008). Because depolarization can stimulate sensory-motor nerves (De Mey et al., 2008) to which ET-1 can bind, the experiment was repeated after pretreatment of the arteries with CAPS. As expected, the contractile effect of depolarization and the sensitivity to the relaxing effect of CGRP were increased. The capacity of ET-1 to reverse CGRP-induced relaxation was not modified, however. We conclude that stimulation of arterial smooth muscle ET_A-receptors can counteract arterial relaxing responses to CGRP. This turned out to be nonselective. Not only ET-1, but also the α_1-adrenergic agonist PHE caused concentration-dependent reversal of CGRP-induced relaxation. Furthermore, both ET-1 and PHE not only reversed relaxing effects of CGRP but also those of SNP that involve NO and cGMP rather than cAMP (Brain and Grant, 2004). Again, observations with antagonists indicate a role for smooth muscle ET_A-receptors rather than ET_B-receptors. We conclude that stimulation of arterial smooth muscle ET_A-receptors can functionally antagonize relaxing responses in partially depolarized arteries irrespective of their mechanism of action.

The lack of effect of L-NAME, INDO, pretreatment with CAPS and CGRP-receptor antagonism, combined with the effects of ET-1 on relaxing responses to exogenous CGRP and SNP, suggests that the strong smooth muscle effects of ET-1 overrule its weaker relaxing effects via endothelium and sensory-motor nerves. We therefore compared effects of indirectly and directly acting relaxing agents during contractions of similar amplitude induced by ET-1 and PHE. The α_1-adrenergic agonist is proposed to not directly affect the endothelium or sensory-motor nerves (Dora et al., 2000; Burnstock, 2009; Hilgers and De Mey, 2009). Relaxing effects of the endothelium-dependent agonist ACh were largely comparable in the presence of ET-1 and PHE under a broad variety of conditions. This indicates that the contractile effect of exogenous ET-1 is not refractory to the effects and is not noticeably accompanied by release of endothelium-derived NO or -hyperpolarizing factor. In contrast, acute relaxing effects of CAPS, a selective agonist of the TRPV1 channels that are expressed almost exclusively by C-type sensory-motor nerves (Caterina et al., 1997; Szallasi and Blumberg, 1999), were considerably larger in the presence of ET-1 than in the presence of PHE. Because ET-receptors can potentiate the function of TRPV1 channels (Plant et al., 2006, 2007; Kawamata et al., 2008) and modulate postjunctional arterial smooth muscle responses (Adner et al., 2001), we also assessed effects of exogenously supplied CGRP. Findings with CGRP-receptor antagonist and in depolarized arteries before and after pretreatment with CAPS indicate that this neuropeptide contributes to the relaxing effects of the vanilloid (De Mey et al., 2008). Arterial relaxing responses to exogenous CGRP were, in contrast to acetylcholine and Na-nitroprusside, found to be significantly more effective during ET-1-induced contraction than during PHE-induced contraction. This indicates a postjunctional mechanism resulting in hypersensitivity to sensory-motor nervous relaxation. Additional roles for the sensory-motor cotransmitters NO and substance P (Burnstock, 2009) seem unlikely in view of the observations with L-NAME and because exogenously administered tachykinin does not relax the arteries that we used (De Mey et al., 2008).

Arterial relaxing effects of endogenous and exogenous CGRP have been attributed to stimulation of AC in the VSMC and, to a lesser extent, to activation of ATP-sensitive K^+ channels and release of endothelium-derived NO (for review, see Brain and Grant, 2004). These mechanisms do not seem to be responsible for the hypersensitivity of ET-1-

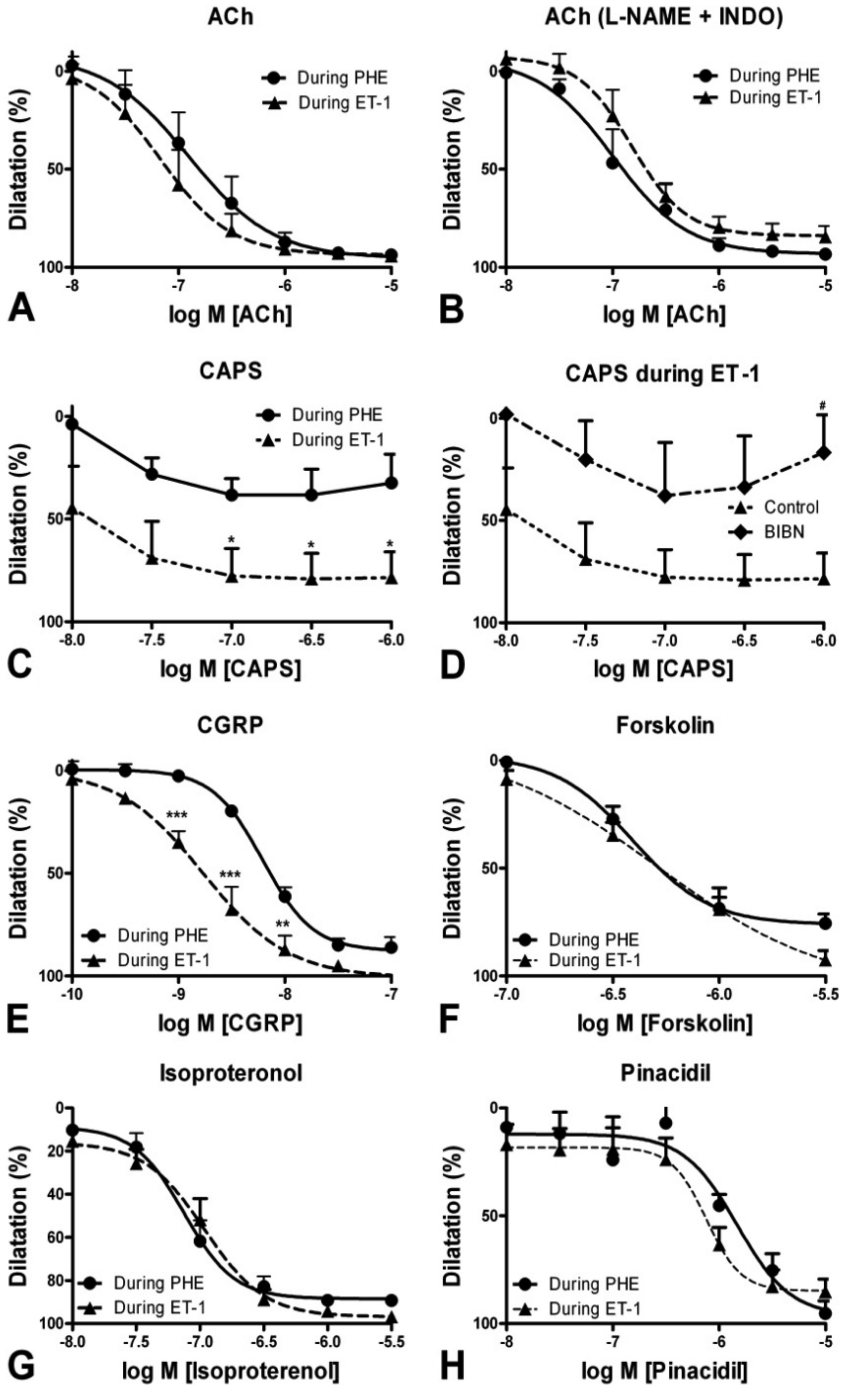

Fig. 5. Effect of relaxing agents during ET-1- (▲) or PHE-induced contraction (●). A, effect of increasing concentrations of ACh. B, effect of increasing concentrations of ACh in the presence of [scap]l-NAME + INDO. C, effect of increasing concentrations of CAPS. D, effect of increasing concentrations of CAPS in the presence (♦) and absence of BIBN4096BS. E, effect of increasing concentrations of CGRP. F, effect of increasing concentrations of forskolin. G, effect of increasing concentrations of isoproteronol. H, effect of increasing concentrations of pinacidil. *, **, or ***, $p < 0.05$, 0.01, or 0.001, relaxation in the presence of PHE versus ET-1. #, $p < 0.05$, control versus BIBN4096BS.

induced contractions to the relaxing effects of CGRP, because ACh, forskolin, isoproterenol, pinacidil, and SNP were equally effective during contractions elicited by ET-1 or the α_1-adrenergic agonist PHE.

Vasomotor effects of the endothelium involve relaxing and contractile factors (for review, see Yanagisawa et al., 1988; Masaki, 2004; Félétou and Vanhoutte, 2006a,b; Moncada and Higgs, 2006). Not only mechanical forces and blood-borne chemicals but also activation of the underlying VSMC can stimulate the endothelium through myo-endothelial gap junctions (Dora et al., 2000; Yashiro and Duling, 2000). To this we recently added sympathetic nervous stimulation of the VSMC (Hilgers and De Mey, 2009). With the present study, potential cross-talk between yet another arterial cell type and the endothelium is highlighted by the observation of selective functional antagonism between the sensory-motor neurotransmitter CGRP and the endothelium-derived peptide ET-1. This is summarized in Fig. 6.

The molecular mechanism that is responsible for the high, rather than low, potency of endogenous and exogenous CGRP to counteract the effects of exogenous ET-1 is unknown. In future experiments we will address whether CGRP-receptor stimulation interferes with agonist binding characteristics and activation of ET_A-receptors (Sakamoto et al., 1993; Bouallegue et al., 2007). In the mean time we suggest that increasing the bioavailability of CGRP in the blood vessel

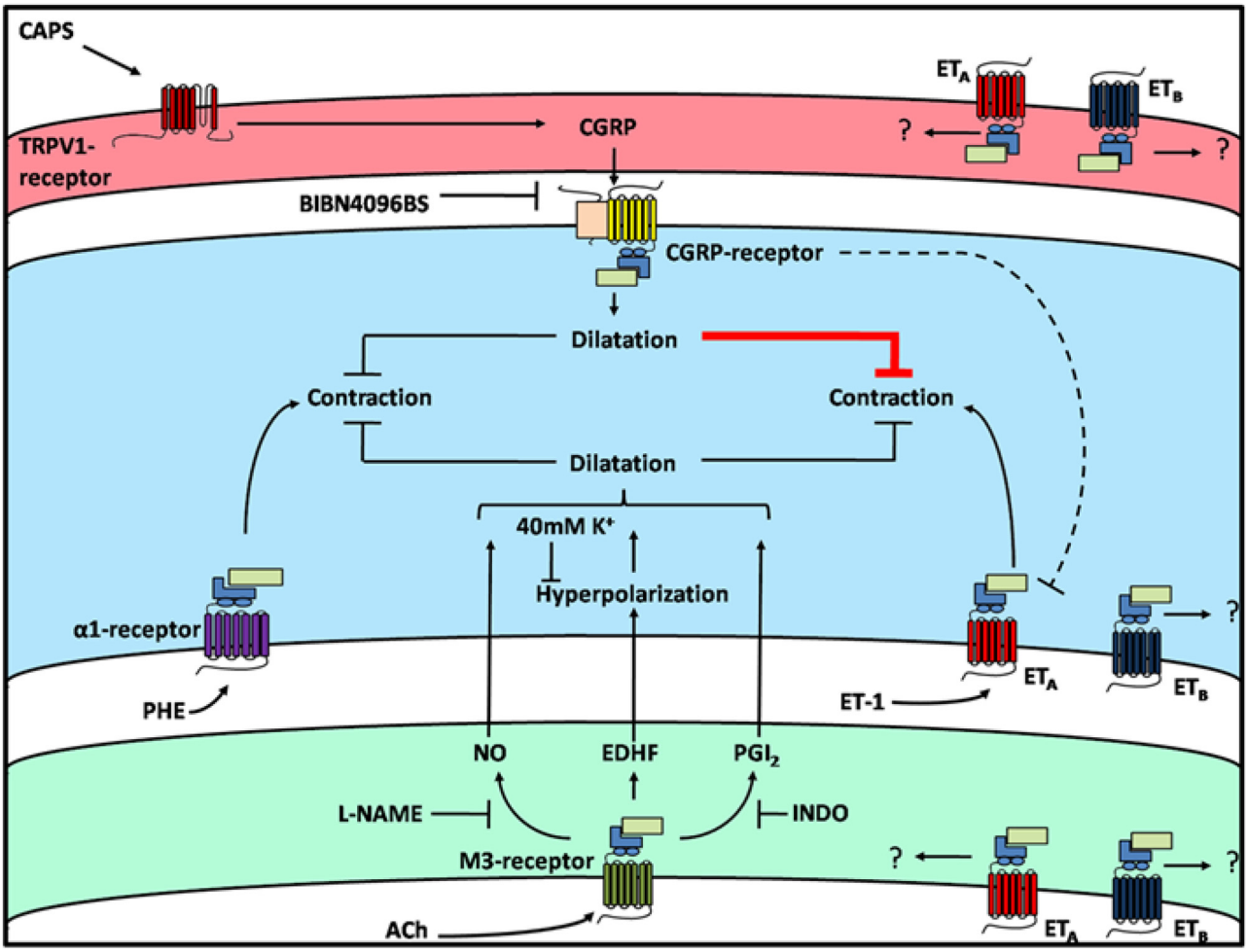

Fig. 6. Schematic overview illustrating presence of ET_A- and ET_B-receptors on sensory-motor nerves (pink), vascular smooth muscle cells (blue), and endothelial cells (green). ACh reverses both α_1-adrenergic contractions and ET-1-induced contractions with equal efficacy. CAPS (CGRP) also reverses both PHE- and ET-induced contractions, but ET-1-induced contractions are more sensitive to CAPS (CGRP) (red line). For clarity, sympathetic nerves are not shown. ?, presence of immunoreactive receptors that did not modify the vasomotor responses investigated.

wall and mimetics of the molecular mechanism of action of CGRP might be suited for therapy of diseases involving ET-1 (Hynynen and Khalil, 2006; Bagnato and Rosanò, 2008; Kirkby et al., 2008; Opitz et al., 2008).

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Address correspondence to: Dr. Jo G. R. De Mey, Department of Pharmacology and Toxicology, Cardiovascular Research Institute Maastricht, Maastricht University, PO box 616, 6200 MD, Maastricht, The Netherlands. E-mail: j.demey@farmaco.unimaas.nl