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RESEARCH PAPER

G-protein βγ subunits in vasorelaxing and anti-endothelinergic effects of calcitonin gene-related peptide

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BACKGROUND AND PURPOSE

Calcitonin gene-related peptide (CGRP) has been proposed to relax vascular smooth muscle cells (VSMC) via cAMP and can promote dissociation of endothelin-1 (ET-1) from ET_A receptors. The latter is not mimicked by other stimuli of adenylate cyclases. Therefore, we evaluated the involvement of G-protein βγ subunits (Gβγ) in the arterial effects of CGRP receptor stimulation.

EXPERIMENTAL APPROACH

To test the hypothesis that instead of α subunits of G-proteins (Gαs), Gβγ mediates the effects of CGRP receptor activation, we used (i) rat isolated mesenteric resistance arteries (MRA), (ii) pharmacological modulators of cyclic nucleotides; and (iii) low molecular weight inhibitors of the functions of Gβγ, gallein and M119. To validate these tools with respect to CGRP receptor function, we performed organ bath studies with rat isolated MRA, radioligand binding on membranes from CHO cells expressing human CGRP receptors and cAMP production assays in rat cultured VSMC.

KEY RESULTS

In isolated arteries contracted with K^+ or ET-1, IBMX (PDE inhibitor) increased sodium nitroprusside (SNP)- and isoprenaline (ISO)- but not CGRP-induced relaxations. While fluorescein (negative control) was without effects, gallein increased binding of [^{125}I]-CGRP in the absence and presence of GTPγS. Gallein also increased CGRP-induced cAMP production in VSMC. Despite these stimulating effects, gallein and M119 selectively inhibited the relaxing and anti-endothelinergic effects of CGRP in isolated arteries while not altering contractile responses to K^+ or ET-1 or relaxing responses to ISO or SNP.

CONCLUSION AND IMPLICATIONS

Activated CGRP receptors induce cyclic nucleotide-independent relaxation of VSMC and terminate arterial effects of ET-1 via Gβγ.

Abbreviations

2,5-DDA, 2′5′-dideoxyadenosine; AC, adenylyl cyclases; CAPS, capsaicin; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CRC, concentration–response curve; ET-1, endothelin-1; FORS, forskolin; Gβγ, G protein βγ subunits; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; ISO, isoprenaline; K_{ATP}, ATP-sensitive K^+-channels; KRB, Krebs–Ringer bicarbonate-buffered physiological salt solution; KT5720, (9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester; M119, 2-(3,4,5-trihydroxy-6-oxoxanthen-9-yl) cyclohexane-1-carboxylic acid'[Synonym] 274122[standardizedcid]; MRA, mesenteric resistance arteries; NA, noradrenaline; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PI3K, phosphatidylinositol-3-kinase; RAMP1, receptor activity modifying protein 1; SNP, sodium nitroprusside; SQ22536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine, 9-THF-Ade; VSMC, vascular smooth muscle cells

Introduction

Endothelin-1 (ET-1), a bicyclic 21 amino acid paracrine mediator, is implicated in cardiovascular diseases, cancer and chronic pain (Yanagisawa *et al.*, 1988; Masaki, 2004; Hynynen and Khalil, 2006; Bagnato *et al.*, 2008; Kirkby *et al.*, 2008; Khodorova *et al.*, 2009). It causes long-lasting potentially deleterious effects by tight binding to ET_A receptors (for review, see De Mey *et al.*, 2009; 2011). These unusual molecular pharmacological properties of the endothelinergic system can limit therapeutic effects of inhibitors of endothelin-converting enzymes and of ET receptor antagonists (Meens *et al.*, 2010; De Mey *et al.*, 2011). Drugs with a less classical mechanism of action (e.g. allosteric inhibitors of ET receptors) might be more efficacious. In 2000, Blandin *et al.* reported that high millimolar concentrations of aspirin-like compounds promote the dissociation of complexes between ET-1 and ET_A receptors (Talbodec *et al.*, 2000). We recently described a similar effect for submicromolar concentrations of exogenously supplied and endogenously released calcitonin-gene related peptide (CGRP) (Meens *et al.*, 2010).

CGRP, a neuropeptide released by perivascular sensory motor nerves that express ET receptors (Brain and Grant, 2004; Burnstock, 2009; Meens *et al.*, 2009), ranks among the most potent endogenous vasodilators (Brain and Grant, 2004). It has been proposed to play a beneficial counterbalancing role in several experimental models of hypertension characterized by an up-regulated ET system (Supowit *et al.*, 1997; Wang and Wang, 2004; Xie and Wang, 2009). In addition to these favourable CGRP-mediated effects, it is well established that CGRP receptor stimulation induces adverse events during pathologies, such as migraine and septic shock (Brain and Grant, 2004; Ho *et al.*, 2010). CGRP receptors activate adenylate cyclases (AC) through stimulating subunits of G-proteins (Gαs) (Brain and Grant, 2004). In turn, cAMP promotes vasodilatation through PKA, ATP-sensitive K^+-channels (K_{ATP}) and release of endothelium-derived NO (Brain and Grant, 2004). However, the anti-ET-1 effects of CGRP are not mimicked by vasodilators that cause release of endothelium-derived relaxing factors (e.g. ACh), nor by vasodilators that cause β-adrenoceptor-mediated (e.g. isoprenaline, ISO) or direct stimulation of AC (e.g. forskolin, FORS), open K_{ATP} (e.g. pinacidil) or act as an NO-donor (e.g. sodium nitroprusside, SNP) (Meens *et al.*, 2010).

Here we tested the hypothesis that instead of Gαs, βγ subunits of GTP-binding regulatory proteins (Gβγ) can mediate the effects of CGRP receptor activation. For this purpose, we used (i) isolated rat mesenteric resistance arteries (MRA); (ii) pharmacological modulators of cyclic nucleotides; and (iii) the recently discovered low molecular weight inhibitors of the functions of Gβγ, gallein and M119 (Bonacci *et al.*, 2006; Lehmann *et al.*, 2008). To validate these tools with respect to CGRP receptor function, we performed ligand-binding studies and measured the production of cAMP by cultured vascular smooth muscle cells (VSMC). Our results show that activated CGRP receptors relax VSMC and terminate the arterial effects of ET-1 via Gβγ.

Methods

All animal care and experimental procedures were approved by the Ethics Committee on Experimental Animal Welfare of Maastricht University.

Drugs and solutions

Krebs–Ringer bicarbonate-buffered physiological salt solution (KRB) contained (in mM) 118.5 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 5.5 glucose. Isoosmotic high K^+-KRB solution (125 mM K^+) was KRB in which all the NaCl was replaced by KCl. Buffers containing intermediate K^+ concentrations were prepared by mixing KRB and K^+-KRB. Capsaicin (CAPS) (Caterina *et al.*, 1997; Szallasi and Blumberg, 1999) and FORS were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands) and dissolved in ethanol. ACh, H-89, ISO, noradrenaline (NA), SNP and SQ22536 were purchased from Sigma Aldrich and dissolved in KRB solution. Human αCGRP and ET-1 were obtained from Bachem (Weil am Rhein, Germany) and dissolved in KRB solution. 2′5′-dideoxyadenosine (2′5′-DDA), fluorescein, IBMX, KT5720, ODQ and wortmannin were obtained from Sigma Aldrich and dissolved in dimethylsulfoxide (DMSO). Gallein was obtained from Tocris Bioscience (Bristol, UK) and dissolved in DMSO. M119 was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (USA) and dissolved in DMSO. The maximal concentrations of the solvents never exceeded 0.1% and did not alter arterial reactivity.

Wire myography

Tissue preparation. Sixteen-week-old male WKY rats (Charles River, Maastricht, the Netherlands) were killed by CO_2 inhalation. Intact MRA (second-order side branches of the superior mesenteric artery) were isolated and directly mounted in a wire myograph. Arterial segments (2 mm) were either distended to the diameter at which maximal contractile responses to 10 μM NA were observed (D_{opt}) (Hilgers and De Mey, 2009; Meens *et al.*, 2009; 2010) or until a diameter corresponding to 90% of the passive diameter at a transmural pressure of 100 mmHg ($0.9D_{100}$) (Mulvany and Halpern, 1977). The two different normalization protocols did not result in significant differences with regard to the initial wall tension between the different groups of arteries (Figure S1A). In addition, arteries tensioned using either method displayed similar contractile responses to K^+ (Figure S1B) or ET-1 (Figure S1C).

Contribution of cAMP and cGMP to CGRP-induced effects. MRA and pharmacological interventions were used to study the contribution of cAMP and cGMP to the arterial effects of CGRP. The pharmacological inhibitors included (i) ODQ (guanylyl cyclase inhibitor; 20 μM) (Garthwaite *et al.*, 1995); (ii) IBMX (non-selective PDE inhibitor, 30 μM) (Beavo, 1995); (iii) 2′5′-dideoxyadenosine (2,5-DDA; AC inhibitor, 10 μM) (Fain *et al.*, 1972); (iv) SQ22536 (AC inhibitor, 10 μM) (Weinryb and Michel, 1974); (v) H-89 (PKA inhibitor, 1 μM) (Chijiwa *et al.*, 1990); or (vi) KT5720 (PKA inhibitor, 1 μM) (Kase *et al.*, 1987). At D_{opt} (382 ± 21 μm), maximal

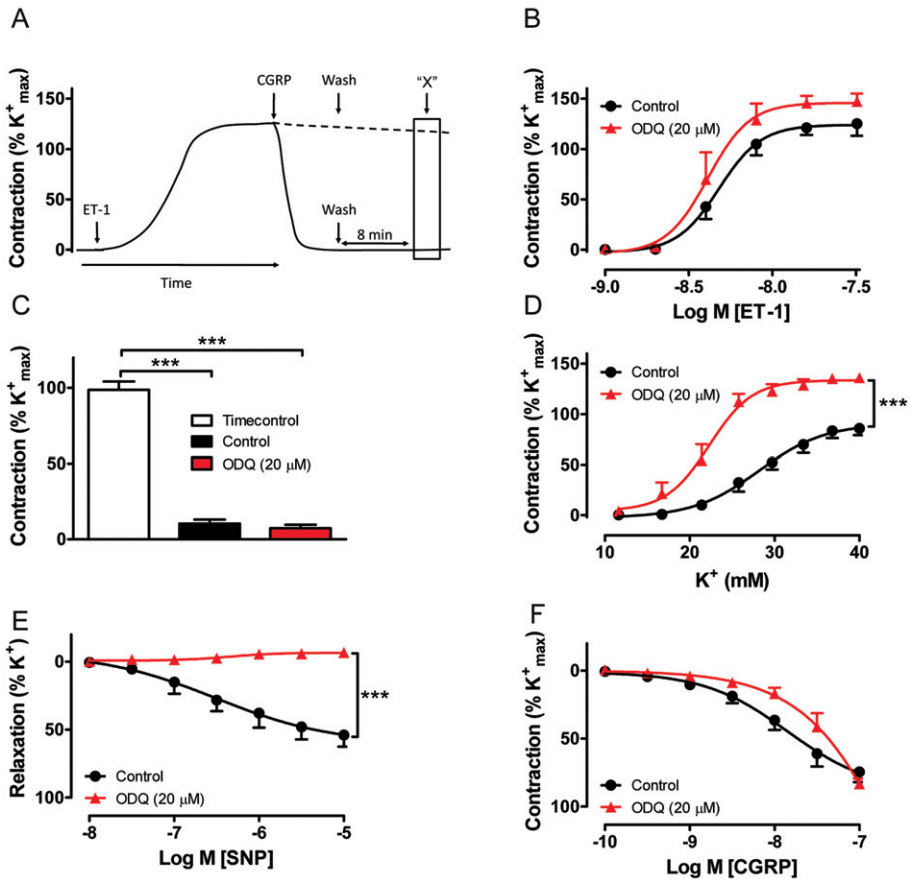

Figure 1

In rat MRA, inhibition of soluble guanylyl cyclase inhibits relaxations induced by SNP but does not inhibit the relaxing and anti-endothelinergic effects of CGRP. (A) Schematic trace illustrating a contraction induced by ET-1, which was terminated by CGRP. The anti-endothelinergic effect of CGRP was determined at 'X' 8 min after removal ('Wash') of all vasoactive compounds and was compared with a time control experiment conducted in parallel. (B) ET-1-induced contractions were not affected by the presence of ODQ. (C) Long-lasting contractile effect of ET-1 assessed 8 min after removal of all vasoactive compounds from the organ baths. Transient exposure to CGRP reduced the long-lasting ET-1-induced contractions (11 ± 3% vs. 99 ± 6% of K^+_{max}. $P < 0.001$). This anti-endothelinergic effect of CGRP was not altered in the presence of ODQ. (D) K^+-induced contractions were increased in the presence of ODQ (E_{max} 136 ± 5 vs. 86 ± 7% of K^+_{max}. $P < 0.001$). (E) SNP-induced relaxations during 40 mM K^+-induced contractions were abolished in the presence of ODQ indicating full inhibition of soluble guanylyl cyclase (E_{max} −7 ± 3 vs. 54 ± 9% relaxation. $P < 0.001$). (F) CGRP-induced relaxations during K^+-induced contractions were not altered in the presence of ODQ. Data are expressed as % K^+_{max} or as % reduction of the pre-existing contraction and are shown as mean ± SEM ($n = 4–6$). $^{**}P < 0.01$, $^{***}P < 0.001$ versus control.

contractile responses to 10 μM NA (NA_{max}) averaged 3.52 ± 0.35 $N \cdot m^{-1}$. Relaxing responses to ACh (10 μM) during this contraction averaged 97 ± 5%, indicating intact endothelium (Hilgers and De Mey, 2009). Before initiation of the experimental protocol, sensory motor nerves were desensitized with CAPS as described earlier (De Mey *et al.*, 2008) to prevent influences of endogenously released neuropeptides like CGRP. Next, one of the inhibitors was added to the organ chambers, allowed to incubate for 20 min and remained present throughout the protocol. The following protocol was performed: arteries were contracted four times with K^+ (initially a concentration–response curve, CRC; 11.6–40 mM) was generated; thereafter, arteries were contracted with 40 mM K^+ and relaxing responses to (i) SNP (0.01–10 μM), (ii) ISO (0.01–10 μM), (iii) FORS (0.01–10 μM) or (iv) CGRP (0.1–100 nM) were determined. Thereafter, contractile responses to ET-1 (0.25–32 nM) were assessed, and arteries were exposed to CGRP (0.1–100 nM). Once the maximal relaxation had stabilized, both ET-1 and CGRP were removed from the organ chamber, and wall tension was monitored for an additional 8 min to assess the anti-endothelinergic effect of CGRP as described earlier (Figure 1A and Meens *et al.*, 2010). In parallel to these experiments, relevant time controls were performed.

Contribution of Gβγ or PI3 to CGRP-induced relaxing and anti-ET-1 effects. MRA and pharmacological interventions: (i) gallein [Gβγ inhibitor; 1–100 μM (Lehmann *et al.*, 2008)]; (ii) fluorescein [gallein-like compound that does not bind Gβγ; 100 μM (Lehmann *et al.*, 2008)]; (iii) M119 [Gβγ inhibitor; 1–100 μM (Bonacci *et al.*, 2006)]; and (iv) wortmannin [PI3K inhibitor; 20 nM or 0.1 μM (Powis *et al.*, 1994)] were used to

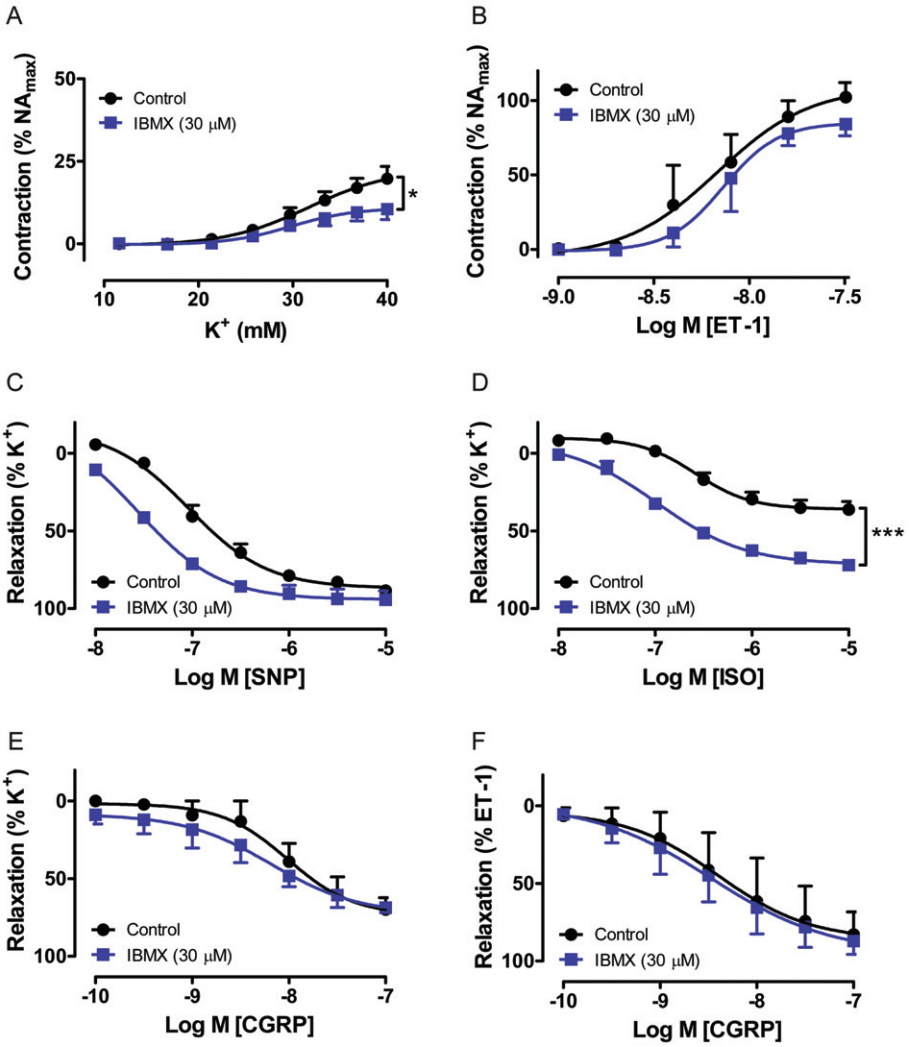

Figure 2

In rat MRA, inhibition of PDEs increases relaxations induced by (i) SNP or (ii) ISO but does not increase relaxing and anti-endothelinergic effects of CGRP. (A) K^+-induced contractions were increased in the presence of IBMX (E_{max} 10 ± 3 vs. 20 ± 3%.of NA_{max}. $P < 0.05$). (B) ET-1-induced contractions were not altered in the presence of IBMX. (C) During 40 mM K^+-induced contractions, SNP induced relaxations more potently in the presence of IBMX (EC_{50} 27 ± 0.2 vs. 92 ± 0.09 nM. $P < 0.05$). (D) During 40 mM K^+-induced contractions, ISO induced relaxations more potently and with bigger amplitude in the presence of IBMX (EC_{50} 0.10 ± 0.03 vs. 0.26 ± 0.1 μM. $P < 0.05$. E_{max} 72 ± 2 vs. 36 ± 5% relaxation. $P < 0.001$). (E) During 40 mM K^+-induced contractions, CGRP-induced relaxations were not altered by IBMX. (F) During 32 nM ET-1-induced contractions, CGRP-induced relaxations were not altered by IBMX. Data are expressed as % NA_{max} or as % reduction of the pre-existing contraction and are shown as mean ± SEM ($n = 6$). *$P < 0.05$, ***$P < 0.001$ versus control.

study the contribution of Gβγ to CGRP-mediated effects. These inhibitors were used in similar protocols as described above.

Cell culture

CHO cells stably expressing human ET_A or both human receptor activity modifying protein 1 (RAMP1) and human calcitonin receptor-like receptor (CLR) (Euroscreen, Brussels, Belgium) were grown at 37°C with 5% CO_2 in Ham-F12 culture medium supplemented with 10% fetal calf serum, 400 μg·mL^{-1} G418, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin and 2.5 μg·mL^{-1} fungizone (all from Alldrich, Bornem, Belgium). Mesenteric artery smooth muscle cells were isolated and subsequently cultured from rat mesenteric arteries as previously described (Hendriks-Balk *et al.*, 2008).

Radioligand binding

Competition binding experiments were performed in duplicate in 96-well plates (Master block, Greiner, 786201) containing binding buffer [(50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 0.5% protease-free BSA, protease inhibitor cocktail (Complete Mini, EDTA-free, Roche, Basel, Switzerland, 1836170), isolated CHO cell membranes (1 μg protein per well), 0.5 nM [^{125}CGRP] (Perkin Elmer NEX354; Perkin Elmer, Groningen, the Netherlands)]. Radioligand binding was assessed at 25°C in the absence and presence of αCGRP

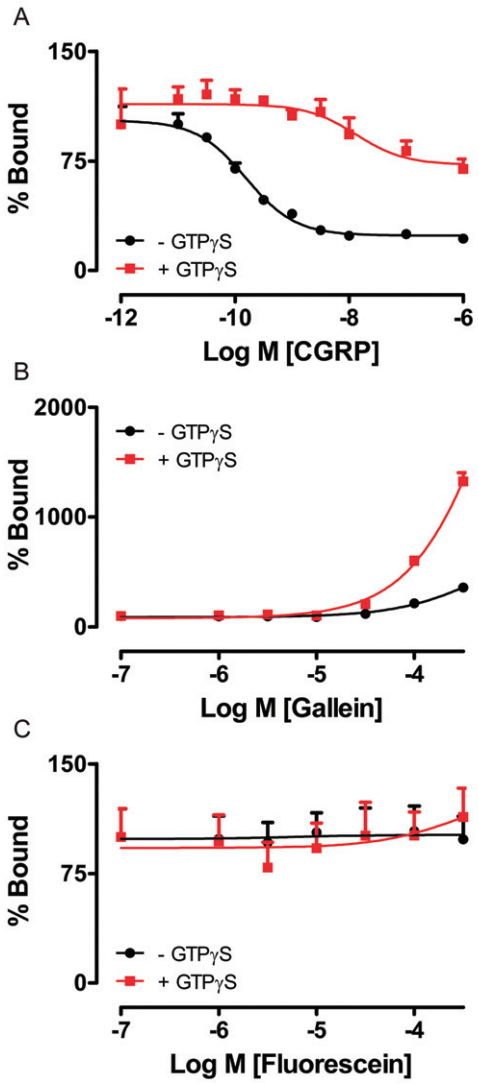

Figure 3

The presence of gallein acutely increases the binding of [^{125}I]-CGRP to membrane fragments from CHO cells expressing human CGRP receptors. (A) Displacement of 0.5 nM [^{125}I]-CGRP by unlabelled CGRP in the absence and presence of 10 μM GTPγS. (B) Gallein markedly increased the binding of 0.5 nM [^{125}I]-CGRP in the absence and presence (red line) of 10 μM GTPγS. (C) Fluorescein did not affect binding of 0.5 nM [^{125}I]-CGRP in the absence and presence of 10 μM GTPγS. Data are shown as mean ± SEM and are the means of three experiments in duplicate.

(0.0003–1000 nM), gallein (0.1–300 μM) or fluorescein (0.1–300 μM). Saturation binding experiments with [^{125}I]-CGRP (0.0001–5 nM) were performed in a similar setting in the absence and presence of gallein (100 μM). The competition and saturation binding experiments were performed in the absence and presence of GTPγS (10 μM).

cAMP measurement

Mesenteric artery smooth muscle cells (passages p2, p3) were plated in 96-well plates and grown till confluency. After being deprived of serum overnight, cells were washed with buffer

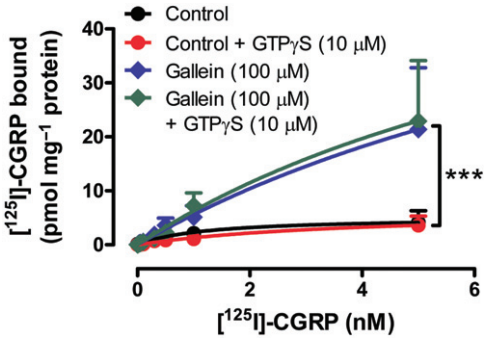

Figure 4

The presence of gallein increases binding of [^{125I}]-CGRP. Saturation binding experiment performed (i) in the absence of GTPγS or gallein (control), (ii) in the presence of GTPγS (10 μM), (iii) in the presence of gallein (100 μM) and (iv) in the presence of gallein (100 μM) and GTPγS (10 μM). Data are shown as mean ± SEM and are the means of three experiments in duplicate. ***$P < 0.001$ control versus gallein.

[HBSS, 0.05% BSA (fatty-acid free) and 5 mM HEPES] and subsequently pre-incubated for 15 min in the presence or absence of gallein (100 μM) or fluorescein (100 μM). The cells were subsequently stimulated for 15 min at 37°C in buffer containing a high concentration of IBMX (0.5 mM) and the indicated ligands in the presence or absence of gallein or fluorescein. After removal of the stimulation mixture, the cells were lysed with 50 μL 0.5% Triton-X100 in buffer with 0.5 mM IBMX. Detection of the cAMP accumulated during stimulation was performed using the LANCE™ cAMP 384 kit (Perkin Elmer) according to the manufacturer's protocol in a total volume of 20 μL; 10 μL of the lysate was added to the 384-well optiplate in triplicate. Measurements were performed 3 h after adding detection buffer and antibody mixture using a Wallac 1420 Victor^2 (Perkin Elmer).

Statistical analyses and nomenclature

Contractile responses are expressed as percentage of either K^+_{max} or NA_{max}. Relaxing responses are expressed as percentage reduction of the level of pre-contraction. Radioligand binding is expressed as either percentage of initial binding or as fmol·mg^{-1} protein. CGRP-induced cAMP production is expressed relative to maximal CGRP-induced cAMP accumulation in the absence of gallein. Curves were fitted using Graphpad Prism 5.0 (Graphpad, La Jolla, CA, USA). All data are shown as mean ± SEM. Statistical significance was assessed using either one-way ANOVA (comparison of EC_{50} and E_{max}), two-way ANOVA (comparison of CRCs). Bonferroni's *post hoc* test was used to compare multiple groups. A $P < 0.05$ was considered statistically significant. For nomenclature of drugs and molecular targets, the BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2011) was used.

Results

Arterial effects of CGRP are cyclic nucleotide-independent

To evaluate the anti-endothelinergic effect of CGRP, rat isolated MRAs were contracted with ET-1 and subsequently

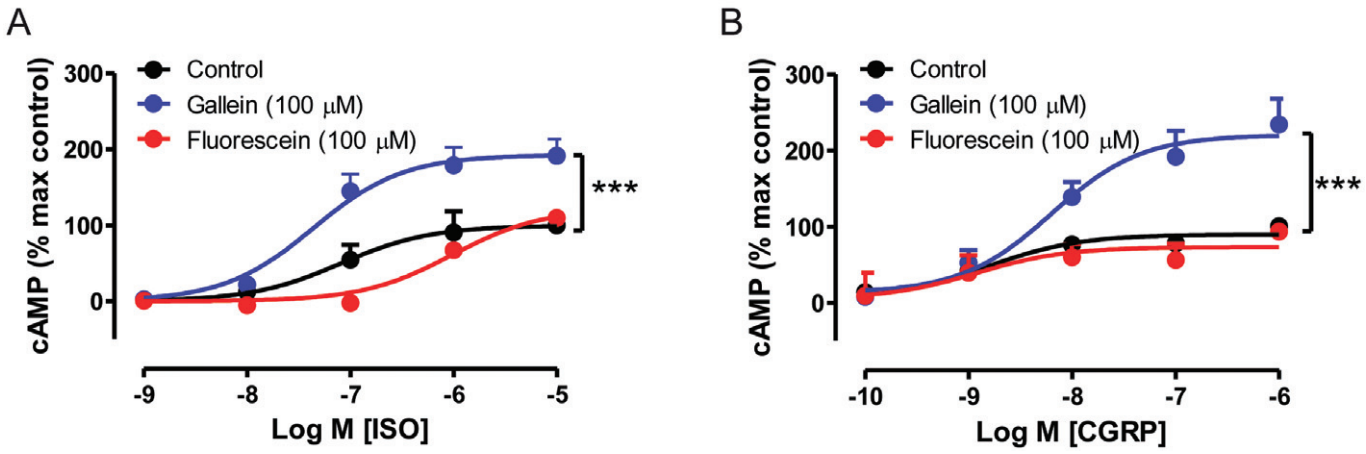

Figure 5

The presence of gallein increases cAMP production in mesenteric artery smooth muscle cells. (A) The presence of gallein but not fluorescein increases ISO-induced cAMP production (E_{max} 192 ± 22 vs. 100 ± 3%. $P < 0.001$). (B) The presence of gallein but not fluorescein increases CGRP-induced cAMP production (E_{max} 216 ± 33 vs. 100 ± 2%. $P < 0.001$). Data are expressed as % of maximal agonist-induced cAMP accumulation in the absence of gallein/fluorescein and are shown as mean ± SEM ($n = 6/7$). ***$P < 0.001$ versus control.

exposed to CGRP. Thereafter, both peptides were removed from the organ baths, and the anti-endothelinergic effect of CGRP was determined 8 min later (see Figure 1A for a schematic overview of the protocol). ET-1 potently stimulated strong contractions (Figure 1B). The maximal response to ET-1 was poorly reversible (Figure 1C). That is, more than 80% of the response initiated by ET-1 persisted at 8 min after washout of the peptide (Figure 1C), while responses to other contractile stimuli (e.g. K^+ and NA) were readily reversed. Administration of CGRP relaxed contractile responses to ET-1 and terminated the long-lasting contractile effect initiated by ET-1 (Figure 1C). The latter effect is selective for CGRP because other vasodilator agents relax this ET_A receptor mediated response reversibly (Meens *et al.*, 2010). It results from CGRP receptor mediated dissociation of ET-1/ET_A receptor complexes on the VSMC (Meens *et al.*, 2010).

The role of cyclic nucleotides in the vasorelaxing and anti-endothelinergic effects of CGRP was evaluated with pharmacological tools. Effects of putative inhibitors of AC (SQ 22536 and 2′5′-dideoxyadenosine) or of PKA (H-89 or KT5720) were inconclusive. They did not modify relaxing responses to β-adrenoceptor-mediated and direct stimulation of AC, by ISO and FORS, respectively, or displayed non-selective effects such as marked reduction of arterial contractile responses to K^+ (data not shown). ODQ (inhibitor of soluble guanylyl cyclase) increased contractile responses to K^+ (Figure 1D) and blocked relaxing responses to the NO donor SNP (Figure 1E). ODQ did not, however, modify the sensitivity and maximal contractile responses to ET-1. In addition, ODQ did not alter relaxing responses to CGRP during K^+-induced contractions (Figure 1F), and it did not prevent the termination by CGRP of contractions initiated by ET-1 (Figure 1C). A comparatively low concentration of the PDE inhibitor IBMX (30 μM) reduced maximal contractile responses to K^+ (Figure 2A) but did not modify sensitivity or maximal contractile responses to ET-1 (Figure 2B). It increased arterial sensitivity to the relaxing effects of SNP (Figure 2C) and of the β-adrenoceptor agonist ISO (Figure 2D) during K^+-induced contractions. Moreover, the maximal relaxing effect of ISO was increased in the presence of IBMX (Figure 2D). However, IBMX did not increase responses to CGRP during either K^+- or ET-1-induced contractions (Figure 2E,F). These findings and earlier observations that ISO and FORS can only reversibly relax ET-1-induced contractions (Meens *et al.*, 2009; 2010) suggest that the arterial effects of CGRP are cyclic nucleotide-independent.

Gβγ inhibition increases binding of [^{125}I]-CGRP and CGRP-induced cAMP production

In view of the finding that the arterial effects of CGRP are not dependent on cyclic nucleotides, we considered the involvement of Gβγ instead of Gα. For this purpose, we used low molecular weight inhibitors of Gβγ that were evaluated with respect to receptor-binding, cAMP production and ultimately arterial reactivity. In competition binding experiments performed on membranes of CHO cells expressing human CGRP receptors, CGRP displaced [^{125}I]-CGRP from CGRP receptors (Figure 3A). In the presence of GTPγS, which reduced basal binding of [^{125}I]-CGRP, the effect of CGRP was similar (Figure 3A). In contrast, irrespective of the presence or absence of GTPγS, the presence of gallein [Gβγ inhibitor (Lehmann *et al.*, 2008)] markedly increased radioligand binding (Figure 3B). Fluorescein [a gallein-like compound that does not bind Gβγ (Lehmann *et al.*, 2008)] did not have an effect (Figure 3C) in the presence or absence of GTPγS. In saturation binding experiments, the presence of GTPγS did not alter apparent B_{max} (4173 ± 2134 vs. 3626 ± 1695 fmol·mg^{-1} protein, control vs. GTPγS, respectively) (Figure 4), but tended to increase K_D (1.2 ± 0.7 vs. 3.9 ± 3.2 nM, control vs. GTPγS). Irrespective of the presence or absence of GTPγS, gallein tended to increase K_D (1.2 ± 0.7 vs. 11.94 ± 24.4 nM, control vs. gallein and 3.9 ± 3.2 vs. 9.96 ± 16.18 nM, control vs. gallein + GTPγS, respectively) and

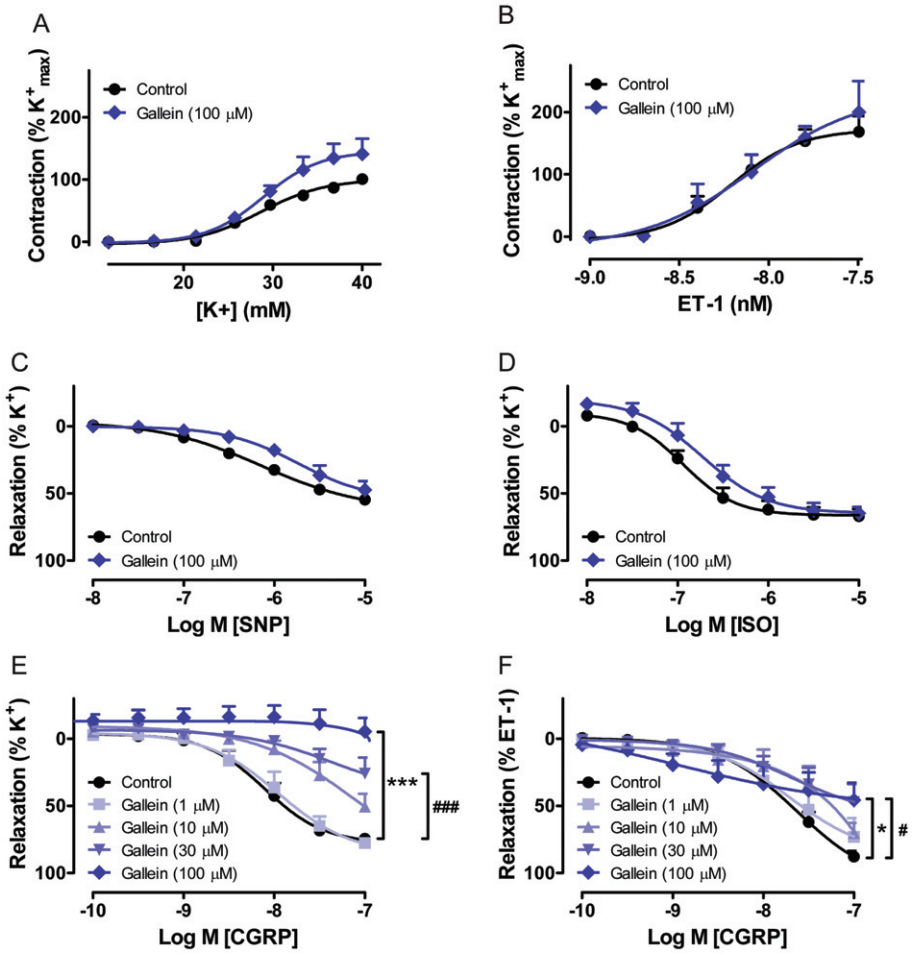

Figure 6

In rat MRA, an inhibitor of Gβγ subunits reduces the relaxing effects of CGRP. (A) K^+-induced contractions were not altered by the presence of gallein. (B) ET-1-induced contractions were not affected by the presence of gallein. (C) During 40 mM K^+-induced contractions, SNP-induced relaxations were not altered by the presence of gallein. (D) During 40 mM K^+-induced contractions, ISO-induced relaxations were not affected by the presence of gallein. (E) During 40 mM K^+-induced contractions, the presence of gallein concentration-dependently inhibited CGRP-induced relaxations [E_{max} 26 ± 12 (in the presence of 30 μM gallein) or –6 ± 10 (in the presence of 100 μM gallein) vs. 74 ± 3 (control) % relaxation; $P < 0.001$ for both conditions]. (F) During 32 nM ET-1-induced contractions, the presence of gallein concentration-dependently inhibited CGRP-induced relaxations [E_{max} 47 ± 13 (in the presence of 30 μM gallein) or 45 ± 13 (in the presence of 100 μM gallein) vs. 88 ± 4 (control) % relaxation; $P < 0.05$ for both conditions]. Data are expressed as % K^+_{max} or as % reduction of the pre-existing contraction and are shown as mean ± SEM ($n = 5/6$). *$P < 0.05$ 30 μM gallein versus control; ***$P < 0.05$ 100 μM gallein versus control; $^{\#}P < 0.05$ 100 μM gallein versus control; $^{\#\#\#}P < 0.05$ 30 μM gallein versus control.

caused a marked acute increase in the binding of [^{125}I]-CGRP to membrane fragments of CHO cells expressing CGRP receptors (apparent B_{max}; 4173 ± 2134 vs. 21401 ± 11383 fmol·mg^{-1} protein, control vs. gallein and 3626 ± 1695 vs. 22905 ± 11218, control + GTPγS vs. gallein + GTPγS, respectively) (Figure 4).

Gβγ has been shown to inhibit or activate AC (Bayewitch *et al.*, 1998a,b). Therefore, we evaluated whether Gβγ inhibition by gallein modified receptor-mediated cAMP production. In line with earlier findings in cardiomyocytes (Casey *et al.*, 2010), gallein tended to increase basal cAMP production in absence of stimuli (data not shown) and significantly increased ISO-induced cAMP production in mesenteric VSMC (Figure 5A), while fluorescein did not have an effect. Moreover, in the presence of gallein but not in the presence of fluorescein, CGRP-induced cAMP production by rat cultured mesenteric VSM cells was significantly enhanced (Figure 5B).

Involvement of Gβγ in arterial effects of CGRP

Although CGRP-induced activation of CGRP receptors caused cAMP production in rat cultured mesenteric VSMC, cyclic nucleotides do not seem to be involved in either CGRP-induced vasorelaxation or in the anti-endothelinergic effects of CGRP. This suggests that these CGRP-induced effects involve Gβγ. Gallein did not modify the sensitivity or maximal contractile responses of rat MRA to K^+ (Figure 6A) or ET-1 (Figure 6B), or affect the relaxing responses to SNP (Figure 6C) or ISO (Figure 6D) during K^+-induced contractions. In contrast, independent of the contractile stimulus,

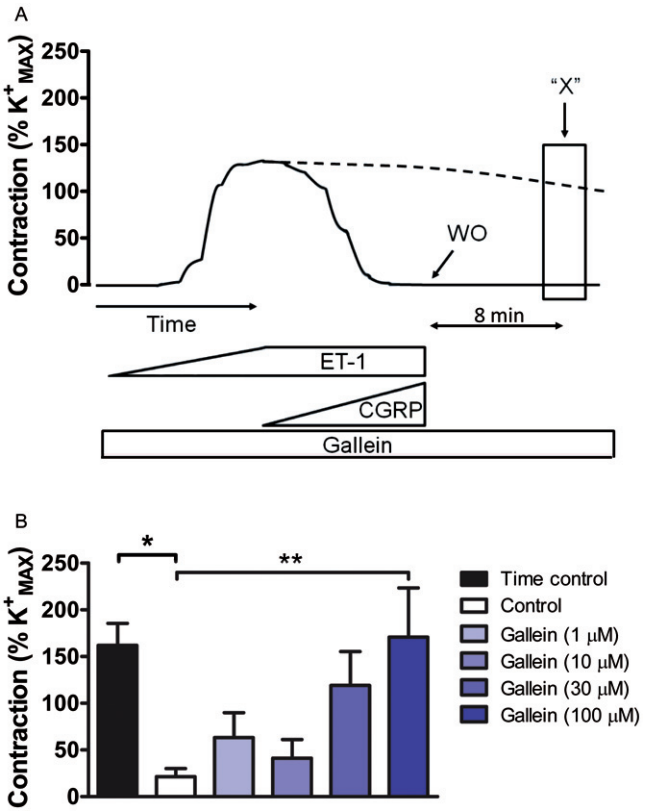

Figure 7

The presence of gallein concentration-dependently inhibits CGRP-induced reduction of long-lasting ET-1-induced contractions. (A) Schematic trace illustrating the experimental protocol. WO: washout; removal of all vasoactive peptides. Data were collected at 'X'. (B) Average wall tension at 'X' for time control, control and for experiments performed in presence of increasing concentrations of gallein [162 ± 23 (time control) or 170 ± 53 (100 μM gallein) vs. 21 ± 8% (control) of K^+_{max} respectively]. Data are expressed as % K^+_{max} and are shown as mean ± SEM (n = 5/6). *P < 0.05, **P < 0.01 versus control.

CGRP-induced relaxations were concentration-dependently inhibited by gallein (Figure 6E,F). Additionally, gallein concentration-dependently inhibited CGRP-induced reduction of long-lasting arterial contractions initiated by ET-1 (Figure 7). Fluorescein did not have an effect on any of the contractile or relaxing responses investigated (Figure 8). The presence of M119 [gallein-like Gβγ inhibitor (Bonacci *et al.*, 2006)] also concentration-dependently decreased relaxing responses induced by CGRP during K^+-induced contraction (Figure 9). The presence of wortmannin tended to reduce the sensitivity to the relaxing effects of CGRP in arteries made to contract by ET-1 (Figure S2). The effect of the inhibitor of PI3K, which is a candidate effector protein of Gβγ (Vanhaesebroeck *et al.*, 1997), was, however, not statistically significant.

Discussion and conclusions

The novel findings of this work (summarized in Figure 10) are that (i) CGRP receptor activation causes cyclic nucleotide-independent relaxation of VSMC via Gβγ, and (ii) CGRP receptor stimulation terminates arterial effects of ET-1 via Gβγ. This is, to the best of our knowledge, the first indication of Gβγ-mediated effects in blood vessels in general and by CGRP receptor activation in particular.

Contribution of cyclic nucleotides to CGRP receptor functions

In the vasculature, CGRP receptor activation causes relaxation of VSM (Brain and Grant, 2004; Meens *et al.*, 2009; 2010). This has been linked to Gα-mediated pathways like activation of AC and opening of K_{ATP} channels (Brain and Grant, 2004). In addition, CGRP receptor activation promotes dissociation of established ET-1/ET_A complexes and thus terminates ET-1-induced signalling in rat mesenteric arteries, i.e. CGRP-/ET_A receptor cross-talk (Meens *et al.*, 2010). The CGRP-induced dissociation of ET-1 from ET_A receptors is (i) not mimicked by vasodilators that act through activation of AC, release of NO or opening of K_{ATP} channels (Meens *et al.*, 2010), and (ii) can also be observed in a variety of other rat arteries and is sufficiently widespread to affect systemic effects of ET-1 in intact rats (Meens *et al.*, 2011). This indicates that the CGRP receptor-induced dissociation of ET-1 from ET_A receptors is widely distributed over the arterial tree and suggests that it is not mediated by Gαs. In addition, these observations indicate that the relaxation induced by CGRP receptor activation and the dissociation of ET-1 from its receptor may be initiated by different molecular mechanisms. In this study, the presence of IBMX did not increase, and the presence of ODQ did not reduce, responses to CGRP in rat isolated MRA, although inhibitors of PDE or soluble guanylyl cyclase changed the responses to K^+, SNP and ISO as expected (Hilgers and De Mey, 2009; Meens *et al.*, 2009; 2010). Thus, arterial relaxing and anti-ET-1 effects of CGRP receptor activation seem to be independent of cyclic nucleotides. We therefore investigated the role of Gβγ subunits of GTP binding regulatory proteins (Gβγ) in the functions of the CGRP receptor.

Contribution of Gβγ to CGRP receptor functions in isolated arteries

Inhibition of Gβγ subunits using two recently described Gβγ inhibitors (Bonacci *et al.*, 2006; Lehmann *et al.*, 2008) did not reduce but rather increased binding of [^{125}I]-CGRP. The exact molecular mechanism by which gallein increased agonist binding remains to be established, but there are several candidates. (i) CLR- and RAMP1-homodimers exist in the plasmalemma (Heroux *et al.*, 2007). Gβγ may be involved in stabilization of these homodimers. In such a scenario, inhibition of Gβγ would result in increased formation of RAMP1/CLR heterodimers which would increase the B_{max} for [^{125}I]-CGRP. (ii) Subsets of the total CGRP receptor population can exist in either a high or a low-affinity state, depending on pre-coupling to Gαβγ (Maton *et al.*, 1988; Wimalawansa and MacIntyre, 1988; Chatterjee and Fisher, 1991; van Rossum *et al.*, 1993; Schindler and Doods, 2002; Gales *et al.*, 2006). Therefore, as gallein displayed its effects in the presence of GTPγS (Figure 3), Gβγ can bind directly to the parathyroid hormone 1 receptor (Mahon *et al.*, 2006), a related class B 7TM receptor, and mathematical models predict a negative

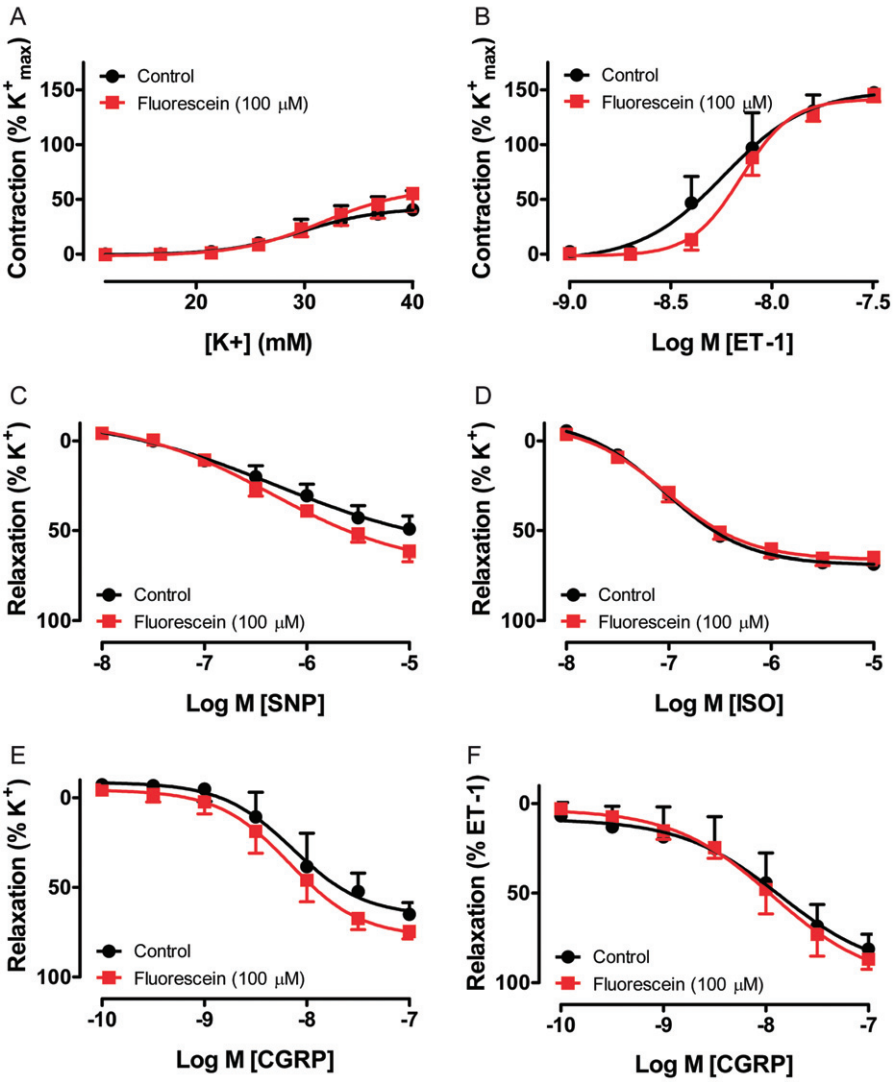

Figure 8

The presence of fluorescein (100 μM), a gallein-like molecule that does not bind Gβγ subunits, does not affect vasomotor function. (A) K^+-induced contractions in the presence or absence of 100 μM fluorescein. (B) ET-1-induced contractions in the presence or absence of fluorescein. (C) Relaxations induced by SNP during 40 mM K^+-induced contractions in the presence or absence of fluorescein. (D) Relaxations induced by ISO during 40 mM K^+-induced contractions in the presence or absence of fluorescein. (E) Relaxations induced by CGRP during 40 mM K^+-induced contractions in the presence or absence of fluorescein. (F) Relaxations induced by CGRP during 32 nM ET-1-induced contractions in the presence or absence of fluorescein. Data are expressed as % K^+_{max} or as % reduction of the pre-existing contraction and are shown as mean ± SEM (n = 6).

effect of Gβγ on receptor ligand binding affinity (Onaran *et al.*, 1993). Gβγ may directly retain CGRP receptors in a low-affinity state that we could not have observed in our experiments due to a limited amount of radioligand. In addition to increasing [^{125}I]-CGRP binding, gallein increased cAMP production induced by CGRP and ISO in cultured VSMCs, in line with recent findings by others in cardiomyocytes (Casey *et al.*, 2010). However, perhaps due to the abundant expression of PDEs, this did not seem to affect the vasorelaxing effects of ISO or CGRP and the anti-endothelinergic effects of CGRP receptor activation. Therefore, we investigated the contribution of Gβγ to CGRP receptor-induced effects in isolated arteries. Both the vasorelaxing and the anti-ET-1 effects of exogenous CGRP were selectively and concentration-dependently inhibited by the Gβγ inhibitors gallein and M119. Thus, similar to other 7TM receptors (Smrcka, 2008), CGRP receptors cause intracellular signalling and relaxation of VSM via Gβγ. Previously, Gβγ have been shown to activate or inhibit various effector proteins such as phospholipases, AC, K^+ channels, G-protein receptor kinases and PI3K (Sunahara *et al.*, 1996; Schneider *et al.*, 1997; Vanhaesebroeck *et al.*, 1997; Bonacci *et al.*, 2006). The effector protein(s) involved in the arterial effects of CGRP remain to be directly demonstrated, but at least AC and K_{ATP} do not seem to be involved (Meens *et al.*, 2009; 2010 and Figure 2 of this study). PI3K and phospholipases are pobably

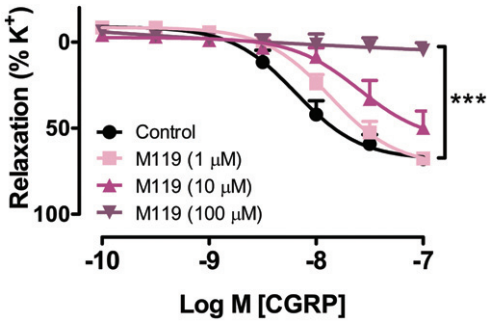

Figure 9

The presence of M119, a gallein-like inhibitor of Gβγ subunits, concentration-dependently inhibits CGRP-induced relaxations during K^+-induced contractions (E_{max} 5 ± 4 vs. 67 ± 3% relaxation in the presence and absence of 100 μM M119, respectively; $P < 0.001$). Data are expressed as % reduction of the pre-existing contraction and are shown as mean ± SEM (n = 4–8). ***$P < 0.001$.

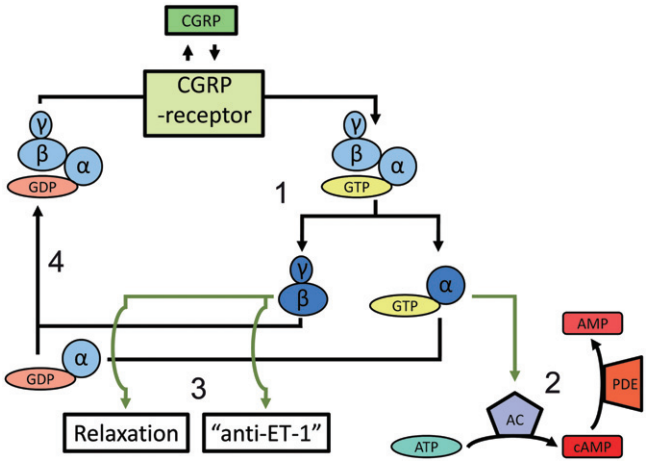

Figure 10

Scheme illustrating interactions of Gα and Gβγ with arterial smooth muscle CGRP receptors and downstream targets. (1) Activated CGRP receptors function as guanine nucleotide exchange factor and cause conversion of GDP bound to Gα to GTP. Consequently, the heterotrimeric Gαβγ dissociates into Gα and Gβγ, which can both interact with intracellular targets. (2) Gα activates AC, which produces cAMP, but, possibly due to abundantly expressed PDEs, this does not seem to be involved in the relaxation of VSM in our setting. (3) In contrast, activated Gβγ causes both the relaxing and the 'anti-endothelinergic' effects induced by activation of CGRP receptors. (4) Ultimately, the intrinsic GTPase activity of Gα converts GTP into GDP allowing Gαβγ to reassemble.

not involved because (i) the presence of wortmannin did not affect the vascular effects of CGRP, and (ii) activation of these proteins has mostly been linked to intracellular pathways that enhance, rather than inhibit, vasoconstriction (Somlyo and Somlyo, 2003; Yin and Janmey, 2003). Thus, the exact intracellular mechanism involved in the cross-talk between CGRP and ET_A receptors remains to be unraveled, but was at least found to involve Gβγ. Cross-talk between various 7TM receptors has been proposed by mathematical modelling (Quitterer and Lohse, 1999; Flaherty *et al.*, 2008; Cervantes *et al.*, 2010; Tubio *et al.*, 2010). It can involve 'G-protein hijacking' (Tubio *et al.*, 2010), cross-talk via arrestin (Cervantes *et al.*, 2010) and formation of receptor heterodimers or oligomers (Prezeau *et al.*, 2010). In addition, Gβγ involved in receptor cross-talk have been identified before they are, for example, implicated in stimulating cross-talk between Gα(i)- and Gα(q)-coupled receptors (Quitterer and Lohse, 1999). However, many, if not all, of these molecular mechanisms suggested to be involved in 7TM receptor cross-talk to date can potentially also affect the contractile apparatus of VSMC, and our results do not provide information regarding possible Gβγ effector proteins. Therefore, at present we cannot address the amount of convergence between the signalling pathways involved in the relaxing and anti-endothelinergic effects of CGRP receptor stimulation. In a previous study, we used fluorescently labelled ET-1 to directly monitor the dissociation of ET-1/ET_A receptor complexes in intact isolated arteries (Meens *et al.*, 2010). Unfortunately, the dye-like properties of gallein, M119 and fluorescein prevent the inclusion of these compounds in such molecular imaging experiments.

In conclusion, our data indicate that CGRP receptor activation causes cAMP production but the relaxation of rat MRA induced by activation of this receptor involves Gβγ and is not dependent on cAMP . Moreover, in rat MRA, CGRP receptors terminate the effects of ET-1 via Gβγ, which also reduces CGRP binding to CGRP receptors (Figure 10). In the future, these findings may lead to new drugs for both CGRP- and ET-1-related diseases. Orthosteric Gβγ-biased (Zheng *et al.*, 2010) CGRP receptor agonists could be used to terminate the effects of ET-1 without causing side effects due to Gαs-mediated effects. Small molecular weight Gβγ inhibitors, which have already shown beneficial effects in animal studies focusing on inflammation and heart failure (Lehmann *et al.*, 2008; Casey *et al.*, 2010), could be used for treatment of diseases characterized by either an excess of Gβγ-mediated effects of CGRP or a defect in Gαs-mediated signalling by the peptide.

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Conflict of interest

None.

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Supporting information

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

Figure S1 Normalization according to D_{opt} or to $0.9D_{100}$ results in similar resting wall tension (A) and in similar contractile responses to K^+ (B) or ET-1 (C).
Figure S2 The presence of wortmannin, a selective PI3K inhibitor, does not affect CGRP-induced relaxations during K^+- (A) or ET-1 (B)-induced contractions.

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