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RESEARCH ARTICLE

Intracellular pathways of calcitonin gene-related peptide-induced relaxation of human coronary arteries: A key role for Gβγ subunit instead of cAMP

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Abstract

Background and Purpose: Calcitonin gene-related peptide (CGRP) is a potent vasodilator. While its signalling is assumed to be mediated via increases in cAMP, this study focused on elucidating the actual intracellular signalling pathways involved in CGRP-induced relaxation of human isolated coronary arteries (HCA).

Experimental Approach: HCA were obtained from heart valve donors (27 M, 25 F, age 54 ± 2 years). Concentration–response curves to human α-CGRP or forskolin were constructed in HCA segments, incubated with different inhibitors of intracellular signalling pathways, and intracellular cAMP levels were measured with and without stimulation.

Results: Adenylyl cyclase (AC) inhibitors SQ22536 + DDA and MDL-12330A, and PKA inhibitors Rp-8-Br-cAMPs and H89, did not inhibit CGRP-induced relaxation of HCA, nor did the guanylyl cyclase inhibitor ODQ, PKG inhibitor KT5823, EPAC1/2 inhibitor ESI09, potassium channel blockers TRAM-34 + apamin, iberiotoxin or glibenclamide, or the $G\alpha_q$ inhibitor YM-254890. Phosphodiesterase inhibitors induced a concentration-dependent decrease in the response to KCl but did not potentiate relaxation to CGRP. Relaxation to forskolin was not blocked by PKA or AC inhibitors, although AC inhibitors significantly inhibited the increase in cAMP. Inhibition of Gβγ subunits using gallein significantly inhibited the relaxation to CGRP in human coronary arteries.

Conclusion: While CGRP signalling is generally assumed to act via cAMP, the CGRP-induced vasodilation in HCA was not inhibited by targeting this intracellular signalling pathway at different levels. Instead, inhibition of Gβγ subunits did inhibit the

Abbreviations: CGRP, calcitonin gene-related peptide; CLR, calcitonin-like receptor; CTR, calcitonin receptor; EPAC, exchange protein directly activated by cAMP; RAMP, receptor activity modifying protein.

relaxation to CGRP, suggesting a different mechanism of CGRP-induced relaxation than generally believed.

KEYWORDS
cAMP, CGRP, GPCR, signalling, vasodilation

1 | INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide and potent vasodilator. It exists in two isoforms, **α-CGRP** and **β-CGRP**, which differ by three amino acids in humans. In sensory nerves, α-CGRP is predominantly present, whereas in the enteric nervous system β-CGRP is more abundant (Mulderry et al., 1988). The isoform α-CGRP is involved in the regulation of the cardiovascular system (Smillie et al., 2014) and was the main focus of the current study. Under healthy, physiological circumstances, CGRP is not primarily involved in regulation of vascular tone (Smillie & Brain, 2011). However, in a pathophysiological state, such as during hypertension or ischemia, CGRP can act as a safety molecule by inducing vasodilation and increasing the blood flow to organs and tissues (MaassenVanDenBrink et al., 2016; Smillie & Brain, 2011).

In addition to its effects on the cardiovascular system, CGRP is involved in the pathophysiology of migraine. During an attack, the trigeminovascular system is activated and CGRP is released, causing a relaxation of blood vessels and subsequent activation of nociceptors, resulting in pain (Noseda & Burstein, 2013). Moreover, infusion of CGRP in migraine patients can induce a migraine-like headache (Hansen et al., 2010). Novel antimigraine medication aims to block either CGRP or its receptor, using small-molecule CGRP receptor antagonists (gepants; e.g., **atogepant**, **rimegepant**, **ubrogepant** and **zavegepant**) or monoclonal antibodies targeting CGRP (**eptinezumab**, **fremanezumab**, **galcanezumab**) or its receptor (**erenumab**) (de Vries et al., 2020). In human isolated arteries, gepants or the monoclonal antibody erenumab potently block the relaxation to CGRP (de Vries et al., 2023; Rubio-Beltrán, Labastida-Ramírez, et al., 2019). However, CGRP (receptor) blockade does not effectively reduce migraine in all patients.

CGRP binds to the canonical **CGRP receptor**, which consists of a seven-transmembrane protein called calcitonin-like receptor (**CLR**) coupled to receptor activity modifying protein 1 (**RAMP1**) and receptor component protein (RCP) (Hay et al., 2018). CLR is a G protein-coupled receptor (**GPCR**), and binding of an agonist to the extracellular domain of the receptor leads to activation of G proteins by exchange of **GDP** for **GTP** (Weis & Kobilka, 2018). Subsequently, the α subunit of the G protein bound to GTP can dissociate from the other subunits β and γ and can activate different intracellular signalling pathways. When $G\alpha_s$ is activated, this leads to subsequent activation of adenylyl cyclase (**AC**) which converts **ATP** into cyclic 3′-5′ adenosine monophosphate (**cAMP**), an important second messenger molecule that is involved in many intracellular processes (Brain & Grant, 2004).

From previous literature, it is known that the binding of CGRP to its receptor leads to an increase in intracellular cAMP in vascular smooth muscle cells, as well as other cell types (e.g., transfected Cos 7 cells, endothelial cells) (Hay et al., 2018; Hirata et al., 1988); accordingly, relaxation of blood vessels is assumed to be mediated by cAMP. In this study, we focused on elucidating the exact signalling pathways downstream of the CGRP receptor that are involved in CGRP-induced relaxation of human isolated coronary arteries, by blocking the intracellular signalling at different levels (Figure 1). The current research aims to enhance the understanding of the exact mechanism of action of the potent vasodilator CGRP, and it could potentially lead to new targets for more effective antimigraine therapy.

What is already known

- CGRP receptor activation results in increases in intracellular cAMP levels and relaxation of human arteries.
- CGRP (receptor) blockade is used as a novel target for the treatment of migraine.

What does this study add

- CGRP-induced vasodilation of human coronary arteries is not inhibited by targeting the cAMP-dependent pathway.
- Inhibition of Gβγ subunits results in inhibition of CGRP-induced relaxation of human isolated coronary arteries.

What is the clinical significance

- Unravelling exact intracellular mechanisms allows development of novel antimigraine medication devoid of cardiovascular side effects.

2 | METHODS

2.1 | Human tissue collection

Human hearts were provided by ETB-BISLIFE (Heart Valve Department, Beverwijk, The Netherlands), after removal of the aortic and pulmonary valves for homograft valve transplantation. Donor

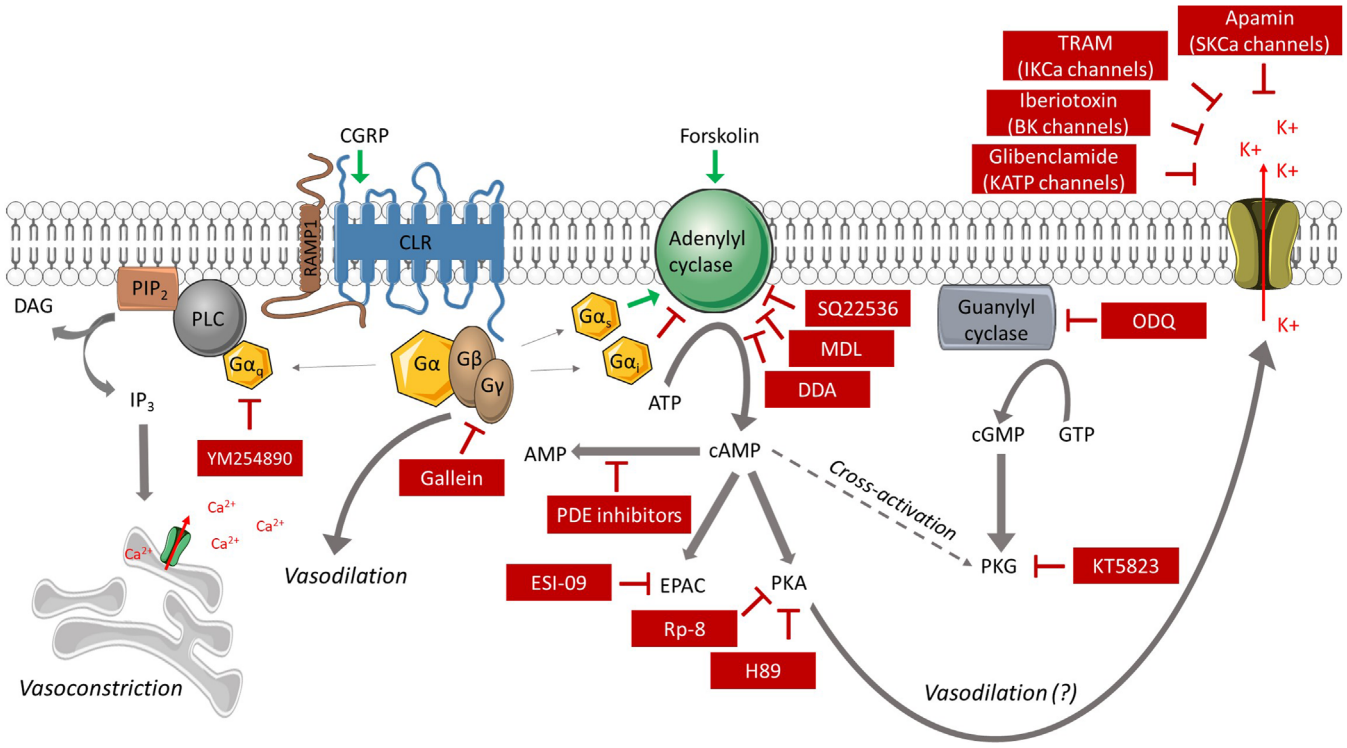

FIGURE 1 Suggested intracellular signalling pathways activated after binding of CGRP to the canonical CGRP receptor resulting in vasodilation or vasoconstriction and suggested inhibitors that could interfere with this signalling. The GPCR of the canonical CGRP receptor consists of RAMP1 and CLR subunits. Binding of CGRP to this receptor could lead to activation of $G\alpha_s$, $G\alpha_q$ and $G\alpha_i$ subunits, which each activate their own signalling cascade.

screening and acceptance was performed by the Dutch Transplant Foundation (Leiden, The Netherlands). After circulatory arrest, the hearts were harvested and stored in a sterile organ protecting solution kept at 4°C. Human coronary arteries were isolated from 27 male (age 54 ± 2 years) and 25 female (age 55 ± 3 years) post-mortem heart valve donors, with permission to use for research. Both distal human coronary arteries (0.3- to 1.5-mm internal diameter) and proximal coronary arteries (3- to 4-mm internal diameter) were obtained and stored at 4°C in carbonated and oxygenated Krebs solution composed of 118-mM NaCl, 4.7-mM KCl, 2.5-mM $CaCl_2$, 1.2-mM $MgSO_4$, 1.2-mM KH_2PO_4, 25-mM $NaHCO_3$ and 8.3-mM glucose (pH = 7.4).

2.2 | Organ bath experiments with human coronary arteries

For functional experiments with the distal coronary arteries, 2-mm segments were mounted in a Mulvany myograph system (Danish Myo Technology, Aarhus, Denmark) using Ø 40-μm stainless-steel wires. In some segments, the endothelium was mechanically removed using a human hair before mounting into the Mulvany myograph system. The organ baths were filled with carbonated and oxygenated Krebs solution and kept at 37°C during the experiment. Vessel segments were allowed to equilibrate for 30 min before normalization, in which the vessel segment was stretched to a tension normalized to 0.9 times the estimated diameter at 100-mmHg transmural pressure (Mulvany & Halpern, 1977). The LabChart data acquisition system (AD instruments Ltd, Oxford, UK) was used to record the tension during the experiment. The vessel segments were contracted using 30-mM KCl, washed with new Krebs solution twice, and subsequently exposed to 100-mM KCl, followed by two additional washing steps. Next, vessel segments were incubated for 30 min with or without different inhibitors of intracellular signalling pathways. The concentrations of the inhibitors used were based on previous studies in which they effectively reduced relaxation or affected intracellular signalling pathways. The inhibitors used were the protein kinase A (**PKA**) inhibitors Rp-8-Br-cAMPs (100 μM) (Ono et al., 2014) or **H89** (1 μM) (Boittin et al., 2003), the AC inhibitors SQ22536 (100 μM) (Tanaka et al., 2003; Turcato & Clapp, 1999; Yamaki et al., 2001) plus **2′,5′-dideoxyadenosine** (DDA, 100 μM) (Dong et al., 1998) or **MDL-12330A** (100 nM) (Granata et al., 2007; Tota et al., 2012), the Exchange Protein directly Activated by cAMP 1/2 (**EPAC1** / **Epac2**) inhibitor **ESI09** (20 μM, 10 μM, 1 μM), the guanylyl cyclase (**GC**) inhibitor **ODQ** (10 μM) (Batenburg et al., 2004; Bautista Niño et al., 2015), the **PKG** inhibitor KT5823 (1 μM) (Li et al., 2015) or the potassium (K^+) channel blockers **TRAM-34** (10 μM) plus **apamin** (100 nM) (Golshiri et al., 2020), **iberiotoxin** (100 nM) (Dong et al., 1998) or **glibenclamide** (10 μM or 100 μM) (Bruch et al., 1997). $G\alpha_q$ signalling was inhibited using **YM-254890** (100 nM, 1 μM) (Clark et al., 2021)

and Gβγ signalling was inhibited using gallein (1 μM, 10 μM, 100 μM, 1 mM) (Meens et al., 2012). To investigate whether phosphodiesterase (**PDE**) inhibitors could potentiate the relaxation to CGRP, the HCA segments were incubated with phosphodiesterase 1 inhibitor **vinpocetine** (1 μM or 10 μM) (Bautista Niño et al., 2015), phosphodiesterase 3 inhibitor **cilostazol** (100 nM, 1 μM or 10 μM) (Liu et al., 2010) or phosphodiesterase 5 inhibitor **sildenafil** (100 nM) (Bautista Niño et al., 2015). PDE1, PDE3 and PDE5 were selected for the current study as they all have a major role in arterial smooth muscle (Omori & Kotera, 2007). After 15 min of incubation with the inhibitors, the vessel segments were precontracted using 30-mM KCl, and after another 15 min human αCGRP (0.01 nM—1 μM, half logarithmic steps) or **forskolin** (0.1 nM—10 μM, half logarithmic steps) was added in increasing concentrations. Next, the vessel segments were washed, precontracted using **U46619** (10 nM—100 nM) followed by addition of the endothelium-dependent vasodilator **substance P** (10 nM—100 nM) to verify the quality of the endothelium, and whether mechanical removal of endothelium was successful in the respective condition.

Functional experiments with the proximal coronary arteries were performed using larger organ baths. The arteries were cut into segments of 4–5 mm and mounted on stainless steel hooks. The vessel segments were stretched to 15 mN (Gupta et al., 2006). Next, the vessels were contracted using 30-mM KCl twice and 100-mM KCl, followed by two washing steps after every contraction. Vessel segments were incubated with AC inhibitors SQ22536 (100 μM) or MDL-12330A (100 nM), the PKA inhibitor H89 (1 μM) or vehicle for 15 min, before precontraction using 30-mM KCl. After another 15 min, increasing concentrations of CGRP (0.1 nM, 10 nM, 1 μM) or forskolin (1 nM, 100 nM, 10 μM) were added to the segments in the presence or absence of PKA or AC inhibitors. Finally, the quality of endothelium was investigated using U46619 and substance P, similar to the distal coronary artery segments.

2.3 | Intracellular cAMP measurements

For measurements of intracellular cAMP levels, vessel segments were incubated with or without SQ22536 (100 μM) or DDA (100 μM) for 30 min in Krebs solution containing **isobutylmethylxanthine** (IBMX, 100 μM), followed by stimulation with CGRP (100 nM) or forskolin (10 μM) for 5 min. Next, vessel segments were snap frozen using liquid nitrogen and stored at −80°C until analysis using the Direct cAMP ELISA kit (Enzo Life Sciences, Farmingdale, USA), according to manufacturer's instructions.

2.4 | Data analysis

The functional responses in human isolated coronary arteries were analysed using Prism 8 (GraphPad Software, San Diego, CA, USA). Nonlinear regression analysis was used to construct sigmoidal curves and data are presented as mean ± SEM. Control experiments, and experiments with inhibitors, were performed in segments from the same human donor to obtain paired results. For each inhibitor, experiments were performed in five to eight different donors. The response to KCl, pEC_{50} values of individual curves and cAMP levels were compared between conditions with and without inhibitors using a paired *t*-test for a comparison of two conditions within tissue from a single donor, or by one-way ANOVA with each row representing matched data from the same donor for comparing multiple conditions. If the ANOVA results were significant, groups were compared with the control condition using Dunnett's multiple comparisons test. The significance threshold for all statistical tests was set at $P < 0.05$; significant results are indicated by a single asterisk (*) in the figures. Data and statistical analyses comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis (Curtis et al., 2022).

2.5 | Materials

Human αCGRP was obtained from PolyPeptide Group (Baar, Switzerland). Forskolin, ESI09, cilostazol, YM-254890 and gallein were purchased from Tocris (Bristol, United Kingdom). SQ22536, MDL-12330A, IBMX, Rp-8-Br-M-cAMPs, substance P, U46619, TRAM-34, apamin, KT5823, ODQ, vinpocetine, sildenafil, glibenclamide, iberiotoxin, L-NAME and H89 were purchased from Sigma-Aldrich (Saint Louis, USA). DDA was obtained from MedChemExpress (Monmouth Junction, USA).

2.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

3.1 | CGRP-induced relaxation of human coronary arteries is not affected by inhibitors of AC, PKA, EPAC, $G\alpha_q$, ODQ, PKG or various potassium channels

CGRP potently relaxed human isolated coronary arteries precontracted with KCl (pEC_{50}: 8.55 ± 0.08). AC inhibitors SQ22536 + DDA or MDL-12330A did not affect the concentration–response curve to CGRP in these human isolated coronary arteries (Figure 2a,b). When targeting PKA, which is activated downstream of the CGRP receptor by cAMP (Miyoshi & Nakaya, 1995), the concentration–response curve to CGRP was not affected with the PKA inhibitors Rp-8-Br-MB-cAMPs or H89 (Figure 2c,d). Moreover, the EPAC1/2 inhibitor ESI09 had no effect on the relaxation to CGRP at the concentration used in the current study (1 μM) (Figure 2e). When higher concentrations of ESI09 (10 μM, 20 μM) were used, the vessel segments could no longer be precontracted using KCl, and therefore functional

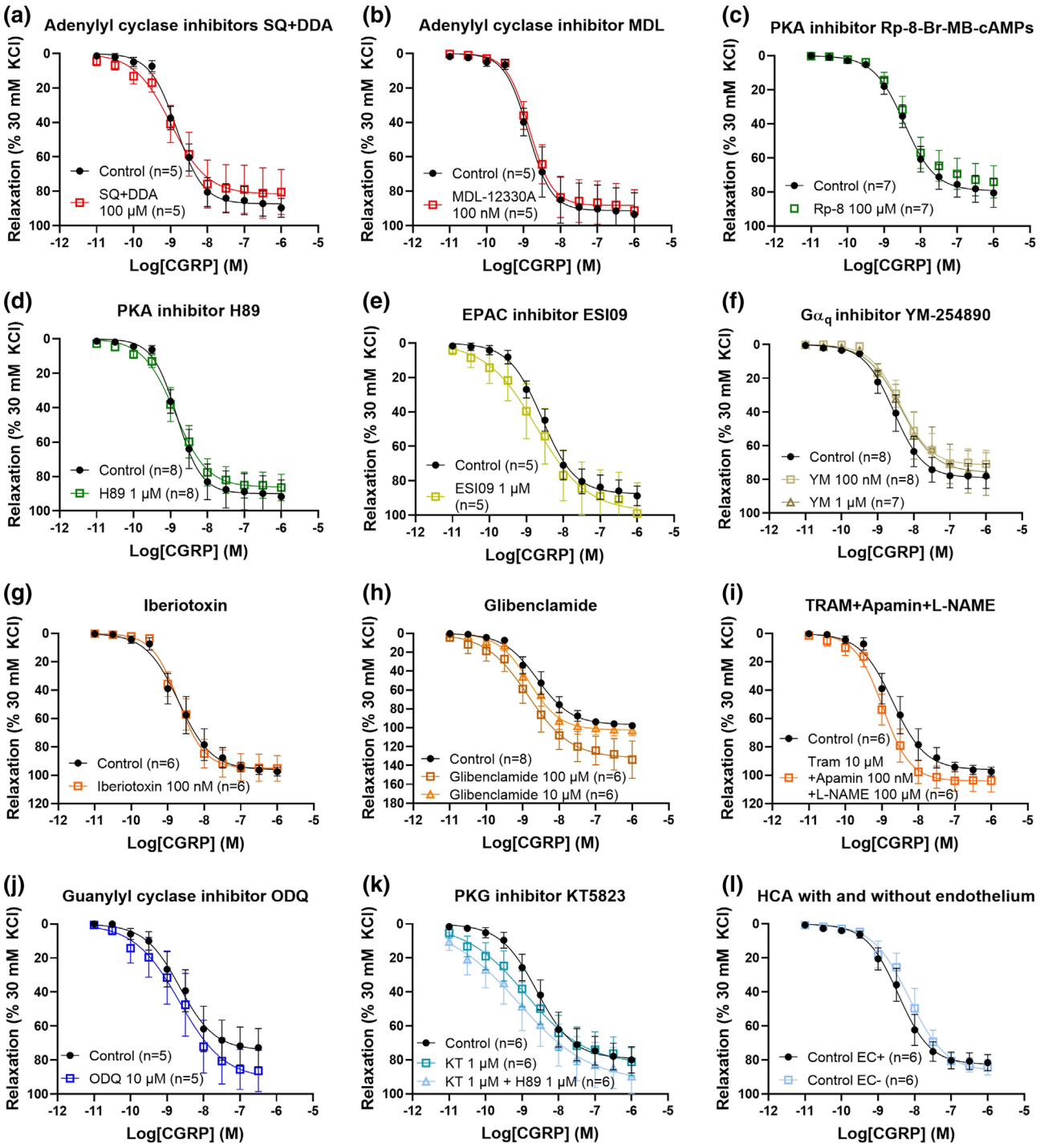

FIGURE 2 The effect of different inhibitors on concentration–response curves to human αCGRP (10 pM–1 μM) in human isolated coronary arteries. The concentration response curve to CGRP was not affected by the PKA inhibitors Rp-8-Br-MB-cAMPs (Rp-8) (a) or H89 (b), nor the AC inhibitors SQ22536 + DDA (c) or MDL-12330A (d). The EPAC inhibitor ESI09 (e) or $G\alpha_q$ inhibitor YM-254890 (f) also did not affect the relaxation to CGRP. Potassium channel blockers iberiotoxin (g) or glibenclamide (h) did not affect the concentration–response curve to CGRP. Moreover, the combination of two potassium blockers, TRAM-34 plus apamin, together with an inhibitor of endothelial nitric oxide synthase (L-NAME) did not affect the concentration response curve to CGRP (i). Inhibition of GC using ODQ (j), or inhibition of PKG using KT5823 (k) did not affect the concentration–response curve to CGRP, nor did mechanical removal of the endothelium (l).

experiments were not possible. To determine whether $G\alpha_q$ signalling was involved, the vessel segments were incubated with the $G\alpha_q$ inhibitor YM-254890. However, no shift in the concentration response curve to CGRP could be observed (Figure 2f). Interestingly, the $G\alpha_q$ inhibitor completely abolished the endothelium-dependent vasorelaxation induced by substance P, even after removing the YM-254890

compound by washing the vessel segment twice (Figure S1A). Next, we investigated the involvement of K^+ channels in CGRP-induced relaxation. The large-, intermediate- and small-conductance Ca^{2+}-activated K^+ channels were targeted using iberiotoxin, TRAM-34 and apamin respectively. ATP-sensitive K^+ channels were blocked using glibenclamide. None of these K^+ channel blockers affected the relaxation to CGRP (Figure 2g–i). Inhibition of GC using ODQ (Figure 2j) or inhibition of PKG using KT5823 (Figure 2k) did not affect the concentration–response curve to CGRP, nor did mechanical removal of the endothelium (Figure 2l), after which the average response to substance P expressed as percentage of precontraction to U46619 decreased from 83 ± 8 to 6 ± 1, indicating successful denudation of the arteries (Figure S1B). The response to KCl was not affected by removal of endothelium (7 ± 1 mN and 6 ± 1 mN for EC+ and EC−, respectively) (Figure S1C).

3.2 | Phosphodiesterase inhibitors do not potentiate the relaxation to CGRP in human coronary arteries

Phosphodiesterases catalyse hydrolysis of cAMP to **5′AMP** and/or **cGMP** to **5′GMP**, thereby lowering intracellular cAMP and cGMP levels, respectively (Conti & Beavo, 2007). Here, specific phosphodiesterases were inhibited, to assess whether this could potentiate the relaxation to CGRP. Neither the phosphodiesterase 1 inhibitor vinpocetine, the phosphodiesterase 3A inhibitor cilostazol or the phosphodiesterase 5 inhibitor sildenafil could potentiate the relaxation to CGRP (Figure 3a–c). However, phosphodiesterase inhibitors vinpocetine and cilostazol decreased the contractile response to KCl, which is used for the precontracting the arteries before the concentration–response curve to CGRP. The response is expressed as

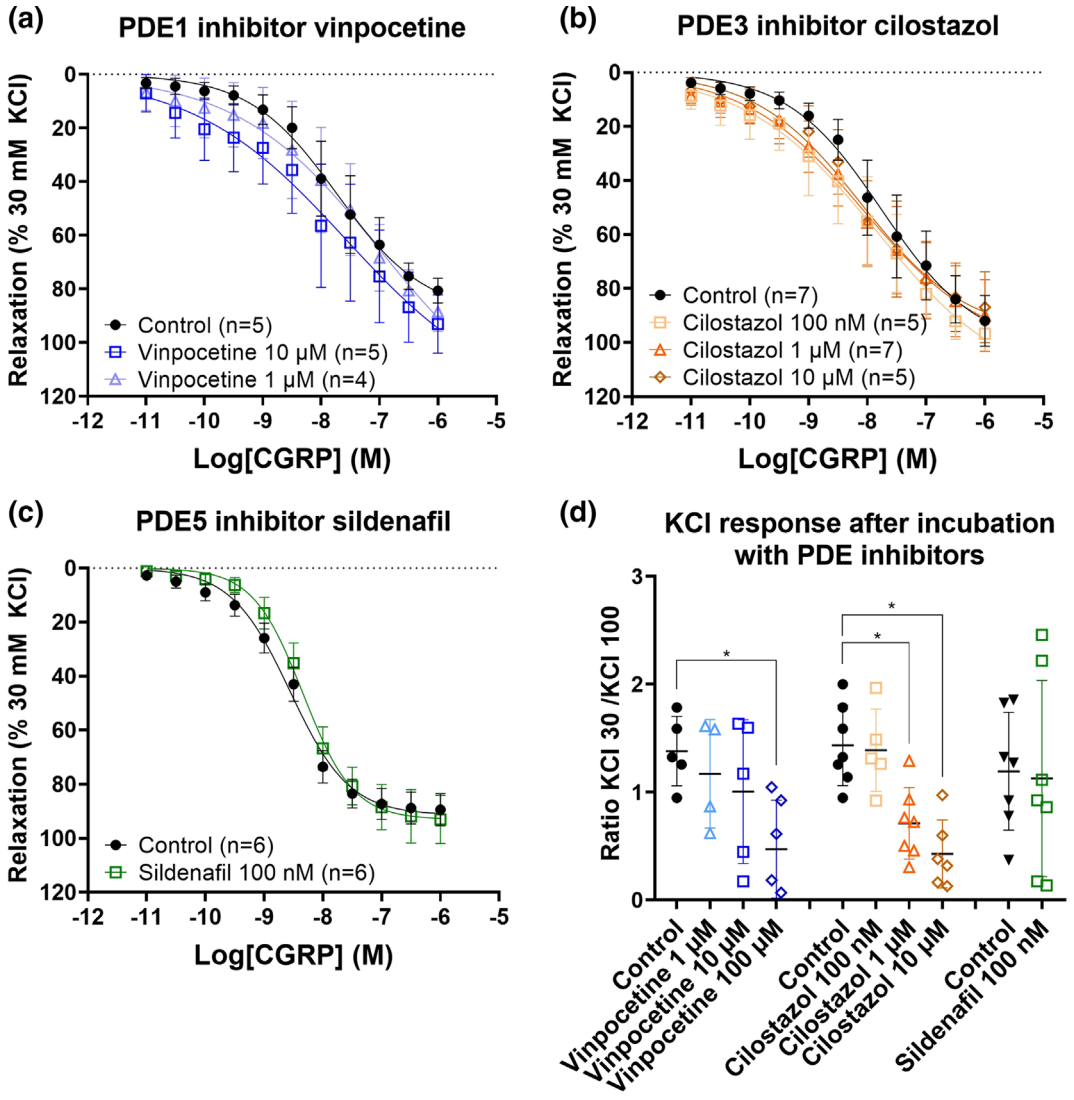

FIGURE 3 The effect of phosphodiesterase inhibitors on CGRP-induced vasorelaxation of human isolated coronary arteries. Phosphodiesterase 1 inhibitor vinpocetine (a), phosphodiesterase 3A inhibitor cilostazol (b) and phosphodiesterase 5 inhibitor sildenafil (c) did not affect the concentration response curve to CGRP. Increasing concentrations of phosphodiesterases vinpocetine and cilostazol decrease the preconstriction using 30-mM KCl compared with the KCl 100-mM response of the same vessel segment (d).

the precontraction induced by 30-mM KCl after incubation with phosphodiesterase inhibitors, relative to the response to 100-mM KCl in the same vessel segment before addition of phosphodiesterase inhibitors (Figure 3d).

3.3 | Forskolin-induced relaxation of human coronary arteries is not inhibited by PKA or AC inhibitors

Forskolin directly activates AC and was able to induce relaxation of human isolated coronary artery segments (pEC_{50}: 6.98 ± 0.50). However, the PKA inhibitors Rp-8-Br-MB-cAMPs or H89 (Figure 4a,b), and the AC inhibitors SQ22536 + DDA or MDL-12330A (Figure 4c,d) could not attenuate the relaxation.

3.4 | In proximal coronary arteries PKA and AC inhibition has no effect on CGRP- or forskolin-induced relaxation

To investigate whether the responses to CGRP and forskolin, and its intracellular signalling, vary between distal coronary artery segments and more proximal larger coronary arteries, the effect of PKA inhibition and AC inhibition was assessed in proximal coronary arteries as well. CGRP was significantly less potent in proximal coronary arteries compared with distal coronary arteries (pEC_{50}: 7.15 ± 0.38 and 8.55 ± 0.08, respectively) and had a smaller maximum response (E_{max}: 20.96 ± 5.66 and 87.78 ± 3.88 respectively). However, the AC inhibitors SQ22536 and MDL-12330A, and the PKA inhibitor H89, did not attenuate the relaxation to CGRP in proximal coronary arteries (Figure 5a), similar to the observations in distal coronary arteries. Similarly, forskolin was significantly less potent in proximal coronary arteries compared with distal coronary arteries (pEC_{50}: 6.07 ± 0.25 and 6.98 ± 0.50, respectively) and showed a smaller maximum response (E_{max}: 56.69 ± 9.74 and 82.22 ± 12.76 respectively), while the AC inhibitors and PKA inhibitor did not attenuate the relaxation to forskolin (Figure 5b).

3.5 | AC inhibitors affect intracellular cAMP levels in human coronary arteries

The intracellular cAMP levels were measured after stimulation of human isolated coronary arteries with CGRP or forskolin in the absence or presence of different inhibitors, to verify whether the inhibitors are effective in our system. CGRP (100 nM) and

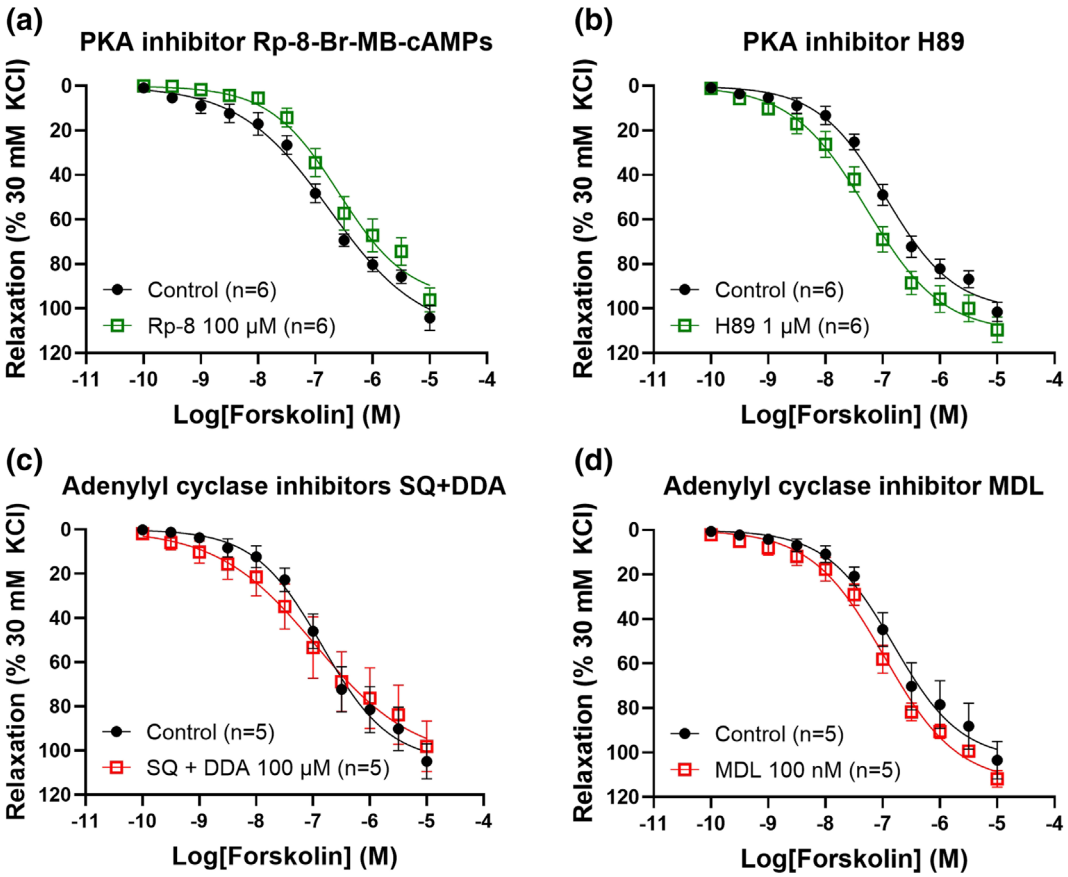

FIGURE 4 The effect of AC or PKA inhibitors on forskolin-induced relaxation of human isolated coronary arteries. The concentration response curve to forskolin was not affected by PKA inhibitors Rp-8-Br-MB-cAMPs (Rp-8) (a) or H89 (b), nor the AC inhibitors SQ22536 (SQ) + DDA (c) or MDL-12330A (MDL) (d).

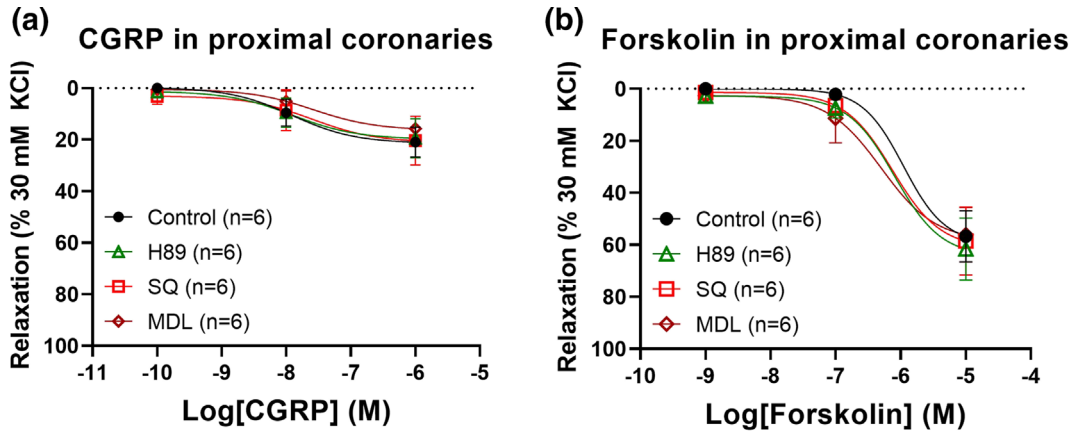

FIGURE 5 The effect of AC inhibitors or a PKA inhibitor in proximal coronary arteries. The concentration response curve to CGRP (a) or forskolin (b) was not affected by AC inhibitors SQ22536 (SQ) or MDL-12330A (MDL), or PKA inhibitor H89.

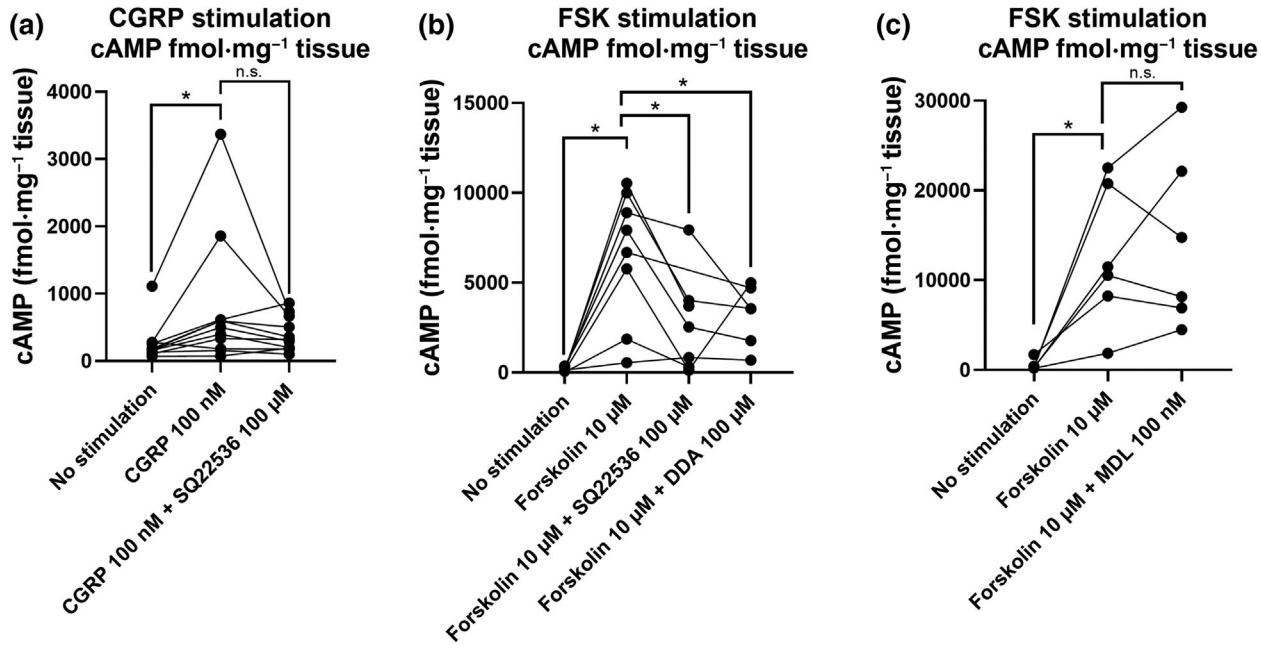

FIGURE 6 cAMP measurements in human isolated coronary arteries. Arteries were incubated with AC inhibitors SQ22536 (100 µM), DDA (100 µM) or MDL-12330A (100 nM) for 30 min and stimulated with (a) CGRP (100 nM, n = 11), (b) FSK (10 µM, n = 8), (c) FSK (10 µM, n = 6).

forskolin (10 µM) both significantly increased the intracellular cAMP levels compared with no stimulation (Figure 6a,b), while SQ22536 and DDA significantly inhibited this increase in cAMP after stimulation with forskolin (Figure 6b). For stimulation with 100-nM CGRP, the inhibition caused by SQ22536 (n = 11) did not reach significance, although a clear trend was observed (Figure 6a). For DDA, the same trend could be observed (CGRP 1585 ± 670 fmol·mg^{-1} tissue cAMP, CGRP + DDA 1062 ± 296 fmol·mg^{-1} tissue in n = 4 paired experiments). However, the increase in cAMP induced by 100-nM CGRP was much smaller compared with the increase after stimulation with 10-µM forskolin (approximately eightfold difference) and increasing the CGRP concentration of the stimulation from 100 nM to 1 µM did not lead to a larger increase in cAMP levels (789 ± 296 and 402 ± 77 fmol·mg^{-1} tissue, respectively, n = 11). Moreover, while 10-µM forskolin increased cAMP levels to a great extent (6524 ± 1295 fmol·mg^{-1} tissue) compared with no stimulation (196 ± 38 fmol·mg^{-1} tissue), a 10-fold lower concentration of forskolin (1 µM) did not induce a significant increase in cAMP levels compared with no stimulation. MDL-12330A at a concentration of 100 nM was not able to decrease cAMP levels (Figure 6c).

3.6 | Gβγ inhibitor gallein inhibits CGRP-induced relaxation of human coronary arteries

Finally, the effect of the Gβγ inhibitor gallein was assessed on CGRP-induced relaxation. Gallein dose-dependently shifted the concentration–response curve to the right (Figure 7a). The

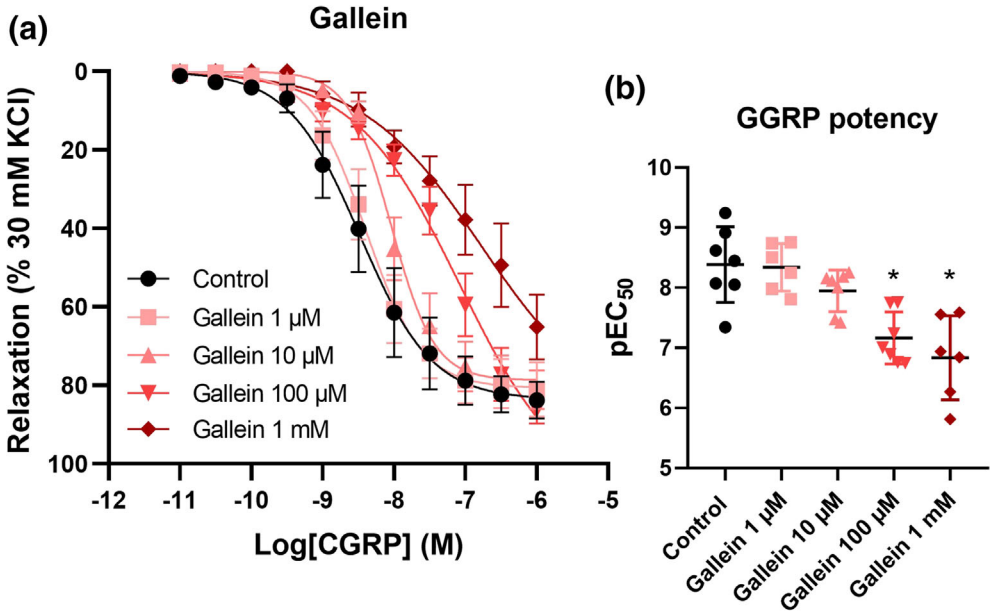

FIGURE 7 Inhibition of CGRP-induced relaxation of human coronary arteries using the Gβγ subunit inhibitor gallein. (a) Gallein significantly shifts the concentration–response curve to CGRP (n = 7). (b) pEC_{50} for the concentration–response curve to CGRP in the presence or absence of gallein (n = 7).

concentrations of 100 µM and 1 mM significantly increased the EC_{50} (pEC_{50}: 7.16 ± 0.16 and 6.84 ± 0.29, respectively) (Figure 7b). Moreover, the Gβγ inhibitor gallein was combined with the adenylyl cyclase inhibitor SQ22536. However, 100 µM of SQ22536 did not induce a further shift on top of 100-µM gallein (Figure S2).

4 | DISCUSSION

The current study investigates the intracellular signalling of CGRP-induced relaxation of human coronary arteries. In accordance with our earlier studies (Gupta et al., 2007, 2006), we found that CGRP-induced vasodilation was mediated via a direct effect on smooth muscle cells in the arterial wall, because mechanical removal of the endothelium did not affect the response to CGRP. Previous research has shown that CGRP-induced relaxation can be inhibited using small molecule CGRP receptor antagonists or a monoclonal antibody against the CGRP receptor in human isolated coronary arteries (de Vries et al., 2023; Rubio-Beltrán, Labastida-Ramírez, et al., 2019), confirming that CGRP receptor activation induces relaxation of blood vessels. Likewise, it has been shown that binding of CGRP to its receptor leads to an increase in intracellular cAMP levels (Hay et al., 2018; Hirata et al., 1988), which was confirmed in the current study. This increase in cAMP is assumed to be involved in the relaxation of blood vessels (Murray, 1990). Moreover, stimulation with forskolin, which directly activates AC, leads to an increase in cAMP levels and potently relaxes blood vessels. However, in the current study, three different types of AC inhibitors could not attenuate the relaxation induced by CGRP or forskolin, suggesting that the increase in cAMP might not be responsible for the relaxation. In humans, AC exists in nine membrane-bound isoforms or can be present in a soluble form in the cytoplasm (Bitterman et al., 2013). In the current study, three different AC inhibitors were used, of which SQ22536 can most selectively inhibit isoform 5 and 6, DDA specifically binds transmembrane isoforms of AC, while MDL-12,330A does not discriminate between membrane-bound AC and soluble AC (Bitterman et al., 2013; Brand et al., 2013), yet none of these inhibitors affected the relaxation to CGRP or forskolin.

AC inhibitors SQ22536 and DDA were tested with regard to their ability to reduce cAMP production after stimulation with forskolin and CGRP. Both inhibitors significantly decreased cAMP production after stimulation with 10-µM forskolin, showing that they are effective in these human tissues and in the current experimental setup. For stimulation with 100-nM CGRP, the inhibition caused by SQ22536 and DDA did not reach significance. However, the overall increase in cAMP levels after stimulation with 100-nM CGRP was much smaller compared with stimulation with 10-µM forskolin. Possibly, higher numbers of experiments would have been necessary to reach significant inhibition for this minor increase in cAMP. Further increasing the concentration of CGRP used for stimulation of the tissue to 1 µM did not result in higher levels of cAMP. Moreover, the fact that much higher levels of cAMP can be reached after stimulation with forskolin, while the relaxation is similar as to CGRP, could also gave an indication that cAMP levels might not be the main inducing factor of relaxation in these tissues. It is important to note that in our system, 100 nM of MDL-12330A cannot effectively reduce the cAMP levels in human coronary arteries, while higher concentrations of MDL-12330A do not allow precontraction of the artery. Therefore, this compound cannot be used to study the role of AC inhibitors in CGRP- or forskolin-induced relaxation in our myograph system.

These findings that AC inhibitors do not affect CGRP- or forskolin-induced relaxation in human coronary arteries, while they do affect cAMP production, are similar to what has been observed previously in ovine pulmonary veins (Gao & Raj, 2001), in which the AC inhibitors SQ22536 and **W-7** did not inhibit forskolin-induced relaxation, although they affected cAMP elevation. Furthermore, cAMP

levels only seemed to robustly increase at a concentration of forskolin above 300 nM, while at this concentration the vessel tension had already decreased for 75%, suggesting that the relaxation already occurs at a concentration of forskolin that does not increase intracellular cAMP levels substantially (Gao & Raj, 2001). This is similar to the current study, in which 1-μM forskolin did not induce a significant increase in cAMP levels, while an average relaxation of 82% was already reached at this concentration. Moreover, a study in uterine arteries from pregnant women showed that SQ22536 did not attenuate the relaxation to CGRP, although it inhibited relaxation to other peptides (Chauhan et al., 2022).

Inhibition of Epac or PKA, which are both downstream of cAMP in intracellular signalling, did not affect the relaxation to CGRP in human coronary arteries. PKA activates different potassium channels at the plasma membrane, which in turn leads to a hyperpolarization of the cell and subsequent relaxation of the smooth muscle cells in the arterial wall (Jackson, 2017). However, inhibition of different types of potassium channels, including large-, intermediate- and small-conductance Ca^{2+}-activated K^+ channels and ATP-sensitive K^+ channels, did not affect the relaxation to CGRP. Interestingly, it was shown that this same ATP-sensitive K^+ blocker glibenclamide was unable to block CGRP-induced headache (Coskun et al., 2021) or **levcromakalim**-induced headache (Al-Karagholi et al., 2020) in healthy volunteers, although the binding of glibenclamide to the **Kir6.1 / SUR2B** subunits of the K_{ATP} channels in the clinical studies might have been suboptimal at the 10-mg dose, with only low target engagement of glibenclamide (Christophersen & Dyhring, 2023). Moreover, signalling via GC and PKG or $G\alpha_q$ signalling is not involved in the relaxation to CGRP. However, the $G\alpha_q$ inhibitor YM-254890 completely abolished the endothelium-dependent vasorelaxation induced by substance P, indicating that this compound did elicit an effect in the human isolated coronary arteries, even if it did not inhibit CGRP-induced relaxation. Substance P acts on the **neurokinin 1 (NK_1) receptor** in endothelial cells, which subsequently leads to activation of $G\alpha_q$ and $G\alpha_s$ signalling (Thom et al., 2021), which is effectively blocked in our system by the $G\alpha_q$ inhibitor YM-254890.

The relaxation to CGRP was not potentiated using different phosphodiesterase inhibitors, which are involved in the hydrolyses of cAMP to 5′AMP, or cGMP to 5′GMP, thereby decreasing the intracellular cAMP and cGMP levels. However, the phosphodiesterase inhibitors vinpocetine and cilostazol showed a concentration-dependent effect on precontraction of the vessels with KCl, suggesting that they can elicit an effect in these human isolated coronary arteries, albeit not on CGRP-induced relaxation. Together with the effective inhibition of cAMP production by AC inhibitors and the inhibition of endothelium-dependent relaxation with YM-254890, the phosphodiesterase effect on KCl response serves as a positive control that these inhibitors are effective in our model system.

The results in the current study suggest that increases in cAMP do not necessarily have to result in relaxation of arteries, which is in line with a previous study (Vegesna & Diamond, 1986) that showed a simultaneous increase in cAMP and relaxation in bovine coronary arteries after stimulation with **prostacyclin** (PGI_2) whereas, in rabbit aorta, the increase in cAMP was accompanied by a contraction. Therefore, the relation between increased cAMP levels and relaxation might not be causal. Over the years, multiple reports have shown that the increase in cAMP does not always coincide with the relaxation (Peters & Michel, 2003). Cholera toxin, **iloprost**, **beraprost** and the β-adrenoceptor agonist **isoprenaline** are all known to increase intracellular cAMP levels and can induce relaxation of arteries (Tanaka et al., 2003; Turcato & Clapp, 1999; Yamaki et al., 2001). However, successful inhibition of cAMP increases using the AC inhibitor SQ22536 does not attenuate relaxation to cholera toxin or prostacyclin analogues iloprost or beraprost in guinea pig aorta or tracheal smooth muscle (Tanaka et al., 2003; Turcato & Clapp, 1999; Yamaki et al., 2001). Moreover, AC inhibition did not affect isoprenaline-induced relaxation of rat abdominal aorta (Matsushita et al., 2006). When comparing the relaxation to **vasoactive intestinal polypeptide** (VIP) and isoprenaline in rat aorta, it was discovered that although stimulation with VIP resulted in a much larger increase of cAMP levels compared with isoprenaline, the relaxant effect of isoprenaline was much more pronounced, suggesting that a direct correlation between the total tissue cAMP level and relaxation was absent (Schoeffter & Stoclet, 1985). It is important to note that cAMP levels can vary over different cellular compartments. It does not freely diffuse throughout the cell and can exert distinct effects based on its localization (Johnstone et al., 2018). Therefore, total cAMP levels do not have to directly represent the levels in a certain functional compartment of the cell.

The findings in the current study on CGRP signalling are in accordance with the results of a study using rat mesenteric resistance arteries, in which the authors conclude that CGRP-induced relaxation is mediated via Gβγ signalling instead of activation of $G\alpha_s$ and subsequent increase in cAMP (Meens et al., 2012). They show that inhibition of AC, GC, PKA or phosphodiesterase did not affect CGRP-induced relaxation, while inhibition of Gβγ using gallein could decrease the relaxant response to CGRP. Moreover, gallein increased the cAMP production after stimulation with CGRP, confirming that cAMP levels are not directly coupled to CGRP-induced vasorelaxation. Based on the findings of this study, the Gβγ inhibitor gallein was tested in the human isolated coronary arteries in our myograph system and was shown to significantly inhibit the relaxation to CGRP, albeit less potently than in rat mesenteric arteries (Meens et al., 2012). Because the relaxation to CGRP was not inhibited completely, the Gβγ inhibitor gallein was combined with the adenylyl cyclase inhibitor SQ22536. However, this did not induce a further shift on top of gallein. The observed inhibition induced by gallein further strengthens the idea that CGRP-induced relaxation is not mediated via cAMP. However, cAMP is often measured as the main second messenger of CGRP receptor activation (Hay et al., 2018), and cAMP production can be inhibited by CGRP receptor blockade (Manoukian et al., 2019). While a significant increase in cAMP after stimulation with CGRP was observed in the current study, the main functional outcome of interest, in this case vasorelaxation, is not related to total cAMP levels. Therefore, the results of the current study emphasize the importance of measuring relevant outcomes.

Moreover, it has been shown that activation of the CGRP receptor results in activation of multiple intracellular pathways in endothelial cells next to an increase in cAMP, including **NO** production, Ca^{2+} mobilization, **pERK1** / **pERK2** activation, and increased cell proliferation (Clark et al., 2021).

Gβγ subunits likely induce relaxation via an interaction with **K_V7.4 channels** (Stott et al., 2015). Patch-clamp experiments showed that Gβγ subunits are necessary for K_V7.4 channel responses to membrane voltage and that gallein and other Gβγ subunit inhibitors can disrupt the colocalization of Gβγ and K_V7.4. Moreover, gallein could prevent isoprenaline-induced relaxation in rat renal arteries (Stott et al., 2015). Furthermore, K_V7.4 / **Kv7.5** heteromers were shown to be involved in CGRP-induced relaxation of rat middle cerebral arteries (Chadha et al., 2014). Interestingly, K_V7 channels are affected during hypertension in different types of rat arteries, including aorta, coronary and mesenteric arteries, but not in the middle cerebral artery (Chadha et al., 2014; Jepps et al., 2011). Further research should determine whether the K_V7.4 channels are involved in the CGRP-induced relaxation of human coronary arteries, as well as other human vascular beds.

The finding that cAMP levels increase after activation of the CGRP receptor is likely mediated via activation of $G\alpha_s$ subunits, while the CGRP-induced relaxation of human coronary arteries now seems to be mediated via Gβγ. The rise in cAMP could be a coincidental occurrence, because CGRP receptor activation leads to both activation of Gα and Gβγ, resulting in a simultaneous rise in cAMP together with relaxation, while the two are not necessarily causal. The current study shows that total cAMP levels do not directly correlate with relaxation, because AC inhibitors can decrease cAMP levels while they do not affect relaxation. However, we cannot exclude that local cAMP levels are involved in inducing relaxation of human coronary arteries.

For the relaxation experiments performed in the current study, human coronary artery segments were precontracted using KCl. Extracellular KCl results in depolarization of the cell membrane of vascular smooth muscle cells, which results in vasoconstriction via activation of voltage-gated ion channels. In case the vasodilation mechanism of a certain agonist acts via these voltage-gated ion channels, the effect might be affected by the precontraction with KCl (Wenceslau et al., 2021). Moreover, high KCl concentrations could interfere with endothelium-dependent vasodilation by affecting hyperpolarization. Therefore, we cannot categorically exclude that the present experiments are affected by the choice of KCl as contractile agent. However, due to unstable precontraction induced by other agents, we were obliged to use KCl in these human arteries to be able to construct a reliable concentration–response curve to CGRP. However, some preliminary data (not shown) gave us no indication that the inhibitors would respond differently after precontraction with U46619. In the current experiments, removal of endothelium did not affect the relaxation response to CGRP. However, CGRP-induced relaxation was shown to be endothelium-dependent in rodent arteries precontracted with **noradrenaline** (Wang et al., 1991). Potentially, using KCl as precontraction eliminates the role of endothelium in CGRP-induced relaxation. Therefore, the results in the current manuscript describe endothelium-independent mechanisms of CGRP-induced relaxation. However, even without contribution of endothelium to the vasodilation caused by CGRP, relaxation reached approximately 88% of precontraction, suggesting that the endothelial contribution, if present in these arteries, could only have minor additional effects on top of the relaxation that is currently observed.

The results in the current study show that the response to CGRP is diminished in proximal coronary arteries compared with distal coronary arteries, which in line with previous findings from our lab (Rubio-Beltrán, Chan, et al., 2019; Rubio-Beltrán, Labastida-Ramírez, et al., 2019). The smaller the artery, the more it contributes to overall resistance and to regulating blood flow. Future studies should investigate whether the CGRP-induced relaxation mechanisms are similar in human cranial arteries, which are in the same potency range for CGRP as the distal coronary arteries (Rubio-Beltrán, Chan, et al., 2019).

The observations in the current manuscript are relevant in the context of migraine treatment. Previously, infusion of CGRP was shown to induce migraine-like attacks in migraine patients (Hansen et al., 2010). Interestingly, it was recently shown that migraine can be provoked by targeting cAMP signalling directly, without activation of the CGRP receptor (Do et al., 2023). Combined with the data from the current manuscript, this could suggest that CGRP-mediated cAMP signalling is involved in migraine attacks, whereas the cAMP component is less relevant for the CGRP-induced relaxation of blood vessels. Considering potential cardiovascular side effects of blocking CGRP (MaassenVanDenBrink et al., 2016), including worsening of ischaemic outcome in a stroke mouse model after blocking CGRP receptors (Mulder et al., 2020), this new knowledge about intracellular signalling pathways that differ between CGRP-induced migraine attacks and CGRP-induced relaxation could be used for development of new and safe antimigraine drugs, absent of any potential cardiovascular side effects.

5 | CONCLUSIONS

While CGRP signalling is generally assumed to act via cAMP, the CGRP-induced vasodilation in HCA could not be inhibited by targeting this intracellular signalling pathway at different levels. Moreover, inhibition of GC, PKG or $G\alpha_q$ also did not affect relaxations to CGRP. In contrast, inhibition of Gβγ subunits did block CGRP-induced relaxation of human coronary arteries, suggesting that, in contrast to common belief, CGRP-induced vasodilation of human coronary arteries is not mediated via cAMP. cAMP levels do increase after activation of the CGRP receptor, but this increase in cAMP is not coupled to the functional relaxation of arteries. It is important to further identify the intracellular signalling cascade after binding of CGRP to its receptor in human arteries of several vascular beds. Further knowledge on the intracellular signalling cascade of CGRP would ultimately allow novel antimigraine medication to target specific parts of the intracellular signalling pathway that reduces migraine, while possibly limiting (cardiovascular) side effects.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

AUTHOR CONTRIBUTIONS

T. de Vries: Conceptualization (lead); formal analysis (lead); investigation (lead); visualization (lead); writing—original draft (lead). **S. Labruijere:** Investigation (supporting); writing—review and editing (supporting). **E. Rivera-Mancilla:** Investigation (supporting); writing—review and editing (supporting). **I. M. Garrelds:** Investigation (supporting). **R. de Vries:** Investigation (supporting). **D. Schutter:** Investigation (supporting); writing—review and editing (supporting). **A. van den Bogaerdt:** Resources (lead); writing—review and editing (supporting). **D. R. Poyner:** Conceptualization (supporting); writing—review and editing (supporting). **G. Ladds:** Conceptualization (supporting); writing—review and editing (supporting). **A. H. J. Danser:** Supervision (supporting); writing—review and editing (supporting). **A. MaassenVanDenBrink:** Conceptualization (equal); funding acquisition (lead); supervision (lead); writing—review and editing (lead).

CONFLICT OF INTEREST STATEMENT

TdV, SL, ERM, IMG, RdV, DS, GL, AvdB, DRP, and AHJD declare no conflicts of interest. AMvdB received personal fees (fees as advisor or speaker, consultancy, any other) from Allergan-Abbvie, Lilly, Novartis and Teva. She received research support from Novartis, Satsuma and Tonix, as well as independent research support from the Dutch Research Council and the Netherlands Organisation for Health Research and Development.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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REFERENCES

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Davies, J. A., Abbracchio, M. P., Alexander, W., Al-hosaini, K., Bäck, M., Barnes, N. M., Bathgate, R., … Ye, R. D. (2021). The Concise Guide to PHARMACOLOGY 2021/22: G protein-coupled receptors. *British Journal of Pharmacology*, *178*(S1), S27–S156. https://doi.org/10.1111/bph.15538

Al-Karagholi, M. A., Ghanizada, H., Kokoti, L., Paulsen, J. S., Hansen, J. M., & Ashina, M. (2020). Effect of K (ATP) channel blocker glibenclamide on levcromakalim-induced headache. *Cephalalgia*, *40*(10), 1045–1054. https://doi.org/10.1177/0333102420949863

Batenburg, W. W., Popp, R., Fleming, I., de Vries, R., Garrelds, I. M., Saxena, P. R., & Danser, A. H. (2004). Bradykinin-induced relaxation of coronary microarteries: S-nitrosothiols as EDHF? *British Journal of Pharmacology*, *142*(1), 125–135. https://doi.org/10.1038/sj.bjp.0705747

Bautista Niño, P. K., Durik, M., Danser, A. H., de Vries, R., Musterd-Bhaggoe, U. M., Meima, M. E., Kavousi, M., Ghanbari, M., Hoeijmakers, J. H., O'Donnell, C. J., Franceschini, N., Janssen, G. M., De Mey, J. G., Liu, Y., Shanahan, C. M., Franco, O. H., Dehghan, A., & Roks, A. J. (2015). Phosphodiesterase 1 regulation is a key mechanism in vascular aging. *Clinical Science (London, England)*, *129*(12), 1061–1075. https://doi.org/10.1042/CS20140753

Bitterman, J. L., Ramos-Espiritu, L., Diaz, A., Levin, L. R., & Buck, J. (2013). Pharmacological distinction between soluble and transmembrane adenylyl cyclases. *The Journal of Pharmacology and Experimental Therapeutics*, *347*(3), 589–598. https://doi.org/10.1124/jpet.113.208496

Boittin, F. X., Dipp, M., Kinnear, N. P., Galione, A., & Evans, A. M. (2003). Vasodilation by the calcium-mobilizing messenger cyclic ADP-ribose. *The Journal of Biological Chemistry*, *278*(11), 9602–9608. https://doi.org/10.1074/jbc.M204891200

Brain, S. D., & Grant, A. D. (2004). Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiological Reviews*, *84*(3), 903–934. https://doi.org/10.1152/physrev.00037.2003

Brand, C. S., Hocker, H. J., Gorfe, A. A., Cavasotto, C. N., & Dessauer, C. W. (2013). Isoform selectivity of adenylyl cyclase inhibitors: Characterization of known and novel compounds. *The Journal of Pharmacology and Experimental Therapeutics*, *347*(2), 265–275. https://doi.org/10.1124/jpet.113.208157

Bruch, L., Bychkov, R., Kästner, A., Bülow, T., Ried, C., Gollasch, M., Baumann, G., Luft, F. C., & Haller, H. (1997). Pituitary adenylate-cyclase-activating peptides relax human coronary arteries by activating K (ATP) and K (Ca) channels in smooth muscle cells. *Journal of Vascular Research*, *34*(1), 11–18. https://doi.org/10.1159/000159197

Chadha, P. S., Jepps, T. A., Carr, G., Stott, J. B., Zhu, H. L., Cole, W. C., & Greenwood, I. A. (2014). Contribution of kv7.4/kv7.5 heteromers to intrinsic and calcitonin gene-related peptide-induced cerebral reactivity. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *34*(4), 887–893. https://doi.org/10.1161/ATVBAHA.114.303405

Chauhan, M., Betancourt, A., Balakrishnan, M., Mishra, A., Espinosa, J., Shamshirsaz, A. A., Fox, K., Belfort, M., & Yallampalli, C. (2022). Calcitonin gene related peptide, adrenomedullin, and adrenomedullin 2 function in uterine artery during human pregnancy. *Endocrinology*, *163*(1), 1–14. https://doi.org/10.1210/endocr/bqab204

Christophersen, P., & Dyhring, T. (2023). The shortcoming of using glibenclamide in exploratory clinical headache provocation studies. *Cephalalgia*, *43*(12), 1–5. https://doi.org/10.1177/03331024231219475

Clark, A. J., Mullooly, N., Safitri, D., Harris, M., de Vries, T., MaassenVanDenBrink, A., Poyner, D. R., Gianni, D., Wigglesworth, M., & Ladds, G. (2021). CGRP, adrenomedullin and adrenomedullin 2 display endogenous GPCR agonist bias in primary human cardiovascular cells. *Communications Biology*, *4*(1), 776–787. https://doi.org/10.1038/s42003-021-02293-w

Conti, M., & Beavo, J. (2007). Biochemistry and physiology of cyclic nucleotide phosphodiesterases: Essential components in cyclic nucleotide signaling. *Annual Review of Biochemistry*, *76*, 481–511. https://doi.org/10.1146/annurev.biochem.76.060305.150444

Coskun, H., Elbahi, F. A., Al-Karagholi, M. A., Ghanizada, H., Sheykhzade, M., & Ashina, M. (2021). The effect of K (ATP) channel blocker glibenclamide on CGRP-induced headache and hemodynamic in healthy volunteers. *Frontiers in Physiology*, *12*, 1–10. https://doi.org/10.3389/fphys.2021.652136

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Patel, H. H., Sobey, C. G.,

Stanford, S. C., Stanley, P., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., & Ahluwalia, A. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*(15), 3907–3913.

de Vries, T., Boucherie, D. M., Van den Bogaerdt, A., Danser, A. H. J., & MaassenVanDenBrink, A. (2023). Blocking the CGRP receptor: Differences across human vascular beds. *Pharmaceuticals*, *16*(8), 1075–1085. https://doi.org/10.3390/ph16081075

de Vries, T., Villalón, C. M., & MaassenVanDenBrink, A. (2020). Pharmacological treatment of migraine: CGRP and 5-HT beyond the triptans. *Pharmacology & Therapeutics*, *211*(107528), 1–13. https://doi.org/10.1016/j.pharmthera.2020.107528

Do, T. P., Deligianni, C., Amirguliyev, S., Snellman, J., Lopez Lopez, C., Al-Karagholi, M. A., Guo, S., & Ashina, M. (2023). Second messenger signaling bypasses CGRP receptor blockade to provoke migraine attacks in humans. *Brain*, *146*(12), 5224–5234. https://doi.org/10.1093/brain/awad1261

Dong, H., Waldron, G. J., Cole, W. C., & Triggle, C. R. (1998). Roles of calcium-activated and voltage-gated delayed rectifier potassium channels in endothelium-dependent vasorelaxation of the rabbit middle cerebral artery. *British Journal of Pharmacology*, *123*(5), 821–832. https://doi.org/10.1038/sj.bjp.0701680

Gao, Y., & Raj, J. U. (2001). SQ22536 and W-7 inhibit forskolin-induced cAMP elevation but not relaxation in newborn ovine pulmonary veins. *European Journal of Pharmacology*, *418*(1–2), 111–116. https://doi.org/10.1016/S0014-2999(01)00929-3

Golshiri, K., Ataei Ataabadi, E., Brandt, R., van der Pluijm, I., de Vries, R., Danser, J., & Roks, A. (2020). Chronic sildenafil treatment improves vasomotor function in a mouse model of accelerated aging. *International Journal of Molecular Sciences*, *21*(13), 4667. https://doi.org/10.3390/ijms21134667

Granata, R., Settanni, F., Biancone, L., Trovato, L., Nano, R., Bertuzzi, F., Destefanis, S., Annunziata, M., Martinetti, M., Catapano, F., Ghè, C., Isgaard, J., Papotti, M., Ghigo, E., & Muccioli, G. (2007). Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: Involvement of 3′,5′-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidyl inositol 3-kinase/Akt signaling. *Endocrinology*, *148*(2), 512–529. https://doi.org/10.1210/en.2006-0266

Gupta, S., Lozano-Cuenca, J., Villalón, C. M., de Vries, R., Garrelds, I. M., Avezaat, C. J., van Kats, J. P., Saxena, P. R., & MaassenVanDenBrink, A. (2007). Pharmacological characterisation of capsaicin-induced relaxations in human and porcine isolated arteries. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *375*(1), 29–38. https://doi.org/10.1007/s00210-007-0137-y

Gupta, S., Mehrotra, S., Villalón, C. M., Garrelds, I. M., de Vries, R., van Kats, J. P., Sharma, H. S., Saxena, P. R., & Maassenvandenbrink, A. (2006). Characterisation of CGRP receptors in human and porcine isolated coronary arteries: Evidence for CGRP receptor heterogeneity. *European Journal of Pharmacology*, *530*(1–2), 107–116. https://doi.org/10.1016/j.ejphar.2005.11.020

Hansen, J. M., Hauge, A. W., Olesen, J., & Ashina, M. (2010). Calcitonin gene-related peptide triggers migraine-like attacks in patients with migraine with aura. *Cephalalgia*, *30*(10), 1179–1186. https://doi.org/10.1177/0333102410368444

Hay, D. L., Garelja, M. L., Poyner, D. R., & Walker, C. S. (2018). Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. *British Journal of Pharmacology*, *175*(1), 3–17. https://doi.org/10.1111/bph.14075

Hirata, Y., Takagi, Y., Takata, S., Fukuda, Y., Yoshimi, H., & Fujita, T. (1988). Calcitonin gene-related peptide receptor in cultured vascular smooth muscle and endothelial cells. *Biochemical and Biophysical Research Communications*, *151*(3), 1113–1121. https://doi.org/10.1016/S0006-291X(88)80481-9

Jackson, W. F. (2017). Potassium channels in regulation of vascular smooth muscle contraction and growth. *Advances in Pharmacology*, *78*, 89–144. https://doi.org/10.1016/bs.apha.2016.07.001

Jepps, T. A., Chadha, P. S., Davis, A. J., Harhun, M. I., Cockerill, G. W., Olesen, S. P., Hansen, R. S., & Greenwood, I. A. (2011). Downregulation of Kv7.4 channel activity in primary and secondary hypertension. *Circulation*, *124*(5), 602–611. https://doi.org/10.1161/CIRCULATIONAHA.111.032136

Johnstone, T. B., Agarwal, S. R., Harvey, R. D., & Ostrom, R. S. (2018). cAMP signaling compartmentation: Adenylyl cyclases as anchors of dynamic signaling complexes. *Molecular Pharmacology*, *93*(4), 270–276. https://doi.org/10.1124/mol.117.110825

Li, R. W., Yang, C., Chan, S. W., Hoi, M. P., Lee, S. M., Kwan, Y. W., & Leung, G. P. (2015). Relaxation effect of abacavir on rat basilar arteries. *PLoS ONE*, *10*(4), 1–18. https://doi.org/10.1371/journal.pone.0123043

Liu, C. Q., Wong, S. L., Leung, F. P., Tian, X. Y., Lau, C. W., Lu, L., Yao, X., Chen, Z. Y., Yao, T., & Huang, Y. (2010). Prostanoid TP receptor-mediated impairment of cyclic AMP-dependent vasorelaxation is reversed by phosphodiesterase inhibitors. *European Journal of Pharmacology*, *632*(1–3), 45–51. https://doi.org/10.1016/j.ejphar.2010.01.008

MaassenVanDenBrink, A., Meijer, J., Villalon, C. M., & Ferrari, M. D. (2016). Wiping out CGRP: Potential cardiovascular risks. *Trends in Pharmacological Sciences*, *37*(9), 779–788. https://doi.org/10.1016/j.tips.2016.06.002

Manoukian, R., Sun, H., Miller, S., Shi, D., Chan, B., & Xu, C. (2019). Effects of monoclonal antagonist antibodies on calcitonin gene-related peptide receptor function and trafficking. *The Journal of Headache and Pain*, *20*(44), 44. https://doi.org/10.1186/s10194-019-0992-1

Matsushita, M., Tanaka, Y., & Koike, K. (2006). Studies on the mechanisms underlying beta-adrenoceptor-mediated relaxation of rat abdominal aorta. *Journal of Smooth Muscle Research*, *42*(6), 217–225. https://doi.org/10.1540/jsmr.42.217

Meens, M. J., Mattheij, N. J., van Loenen, P. B., Spijkers, L. J., Lemkens, P., Nelissen, J., Compeer, M. G., Alewijnse, A. E., & De Mey, J. G. (2012). G-protein βγ subunits in vasorelaxing and anti-endothelinergic effects of calcitonin gene-related peptide. *British Journal of Pharmacology*, *166*(1), 297–308. https://doi.org/10.1111/j.1476-5381.2011.01774.x

Miyoshi, H., & Nakaya, Y. (1995). Calcitonin gene-related peptide activates the K+ channels of vascular smooth muscle cells via adenylate cyclase. *Basic Research in Cardiology*, *90*(4), 332–336. https://doi.org/10.1007/BF00797911

Mulder, I. A., Li, M., de Vries, T., Qin, T., Yanagisawa, T., Sugimoto, K., van den Bogaerdt, A., Danser, A. H. J., Wermer, M. J. H., van den Maagdenberg, A., MaassenVanDenBrink, A., Ferrari, M. D., & Ayata, C. (2020). Anti-migraine calcitonin gene-related peptide receptor antagonists worsen cerebral ischemic outcome in mice. *Annals of Neurology*, *88*(4), 771–784. https://doi.org/10.1002/ana.25831

Mulderry, P. K., Ghatei, M. A., Spokes, R. A., Jones, P. M., Pierson, A. M., Hamid, Q. A., Kanse, S., Amara, S. G., Burrin, J. M., Legon, S., et al. (1988). Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. *Neuroscience*, *25*(1), 195–205. https://doi.org/10.1016/0306-4522(88)90018-8

Mulvany, M. J., & Halpern, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circulation Research*, *41*(1), 19–26. https://doi.org/10.1161/01.RES.41.1.19

Murray, K. J. (1990). Cyclic AMP and mechanisms of vasodilation. *Pharmacology & Therapeutics*, *47*(3), 329–345. https://doi.org/10.1016/0163-7258(90)90060-F

Noseda, R., & Burstein, R. (2013). Migraine pathophysiology: Anatomy of the trigeminovascular pathway and associated neurological symptoms, CSD, sensitization and modulation of pain. *Pain*, *154*(2013), 44–53.

Omori, K., & Kotera, J. (2007). Overview of PDEs and their regulation. *Circulation Research*, *100*(3), 309–327. https://doi.org/10.1161/01.RES.0000256354.95791.f1

Ono, S., Nagaoka, T., Omae, T., Tanano, I., Kamiya, T., Otani, S., Ishibazawa, A., & Yoshida, A. (2014). Beraprost sodium, a stable prostacyclin analogue, elicits dilation of isolated porcine retinal arterioles: Roles of eNOS and potassium channels. *Investigative Ophthalmology & Visual Science*, *55*(9), 5752–5759. https://doi.org/10.1167/iovs.14-14902

Peters, S. L., & Michel, M. C. (2003). cAMP-independent relaxation of smooth muscle cells via Gs-coupled receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *368*(5), 329–330. https://doi.org/10.1007/s00210-003-0816-2

Rubio-Beltrán, E., Chan, K. Y., Danser, A. H. J., MaassenVanDenBrink, A., & Edvinsson, L. (2019). Characterisation of the calcitonin gene-related peptide receptor antagonists ubrogepant and atogepant in human isolated coronary, cerebral and middle meningeal arteries. *Cephalalgia*, *40*(4), 357–366. https://doi.org/10.1177/0333102419884943

Rubio-Beltrán, E., Labastida-Ramírez, A., Haanes, K. A., van den Bogaerdt, A., Bogers, A. J., Dirven, C., Danser, A. J., Xu, C., Snellman, J., & MaassenVanDenBrink, A. (2019). Characterisation of vasodilatory responses in the presence of the CGRP receptor antibody erenumab in human isolated arteries. *Cephalalgia*, *39*(14), 1735–1744. https://doi.org/10.1177/0333102419863027

Schoeffter, P., & Stoclet, J. C. (1985). Effect of vasoactive intestinal polypeptide (VIP) on cyclic AMP level and relaxation in rat isolated aorta. *European Journal of Pharmacology*, *109*(2), 275–279. https://doi.org/10.1016/0014-2999(85)90430-3

Smillie, S. J., & Brain, S. D. (2011). Calcitonin gene-related peptide (CGRP) and its role in hypertension. *Neuropeptides*, *45*(2), 93–104. https://doi.org/10.1016/j.npep.2010.12.002

Smillie, S. J., King, R., Kodji, X., Outzen, E., Pozsgai, G., Fernandes, E., Marshall, N., de Winter, P., Heads, R. J., Dessapt-Baradez, C., Gnudi, L., Sams, A., Shah, A. M., Siow, R. C., & Brain, S. D. (2014). An ongoing role of α-calcitonin gene-related peptide as part of a protective network against hypertension, vascular hypertrophy, and oxidative stress. *Hypertension*, *63*(5), 1056–1062. https://doi.org/10.1161/HYPERTENSIONAHA.113.02517

Stott, J. B., Povstyan, O. V., Carr, G., Barrese, V., & Greenwood, I. A. (2015). G-protein βγ subunits are positive regulators of Kv7.4 and native vascular Kv7 channel activity. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(20), 6497–6502. https://doi.org/10.1073/pnas.1418605112

Tanaka, Y., Yamashita, Y., Yamaki, F., Horinouchi, T., Shigenobu, K., & Koike, K. (2003). Evidence for a significant role of a Gs-triggered mechanism unrelated to the activation of adenylyl cyclase in the cyclic AMP-independent relaxant response of guinea-pig tracheal smooth muscle. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *368*(5), 437–441. https://doi.org/10.1007/s00210-003-0809-1

Thom, C., Ehrenmann, J., Vacca, S., Waltenspühl, Y., Schöppe, J., Medalia, O., & Plückthun, A. (2021). Structures of neurokinin 1 receptor in complex with G(q) and G(s) proteins reveal substance P binding mode and unique activation features. *Science Advances*, *7*(50), 1–14. https://doi.org/10.1126/sciadv.abk2872

Tota, B., Gentile, S., Pasqua, T., Bassino, E., Koshimizu, H., Cawley, N. X., Cerra, M. C., Loh, Y. P., & Angelone, T. (2012). The novel chromogranin A-derived serpinin and pyroglutaminated serpinin peptides are positive cardiac β-adrenergic-like inotropes. *The FASEB Journal*, *26*(7), 2888–2898. https://doi.org/10.1096/fj.11-201111

Turcato, S., & Clapp, L. H. (1999). Effects of the adenylyl cyclase inhibitor SQ22536 on iloprost-induced vasorelaxation and cyclic AMP elevation in isolated guinea-pig aorta. *British Journal of Pharmacology*, *126*(4), 845–847. https://doi.org/10.1038/sj.bjp.0702383

Vegesna, R. V., & Diamond, J. (1986). Elevation of cyclic AMP by prostacyclin is accompanied by relaxation of bovine coronary arteries and contraction of rabbit aortic rings. *European Journal of Pharmacology*, *128*(1–2), 25–31. https://doi.org/10.1016/0014-2999(86)90553-4

Wang, X., Han, C., & Fiscus, R. R. (1991). Calcitonin gene-related peptide (CGRP) causes endothelium-dependent cyclic AMP, cyclic GMP and vasorelaxant responses in rat abdominal aorta. *Neuropeptides*, *20*(2), 115–124. https://doi.org/10.1016/0143-4179(91)90061-M

Weis, W. I., & Kobilka, B. K. (2018). The molecular basis of G protein-coupled receptor activation. *Annual Review of Biochemistry*, *87*, 897–919. https://doi.org/10.1146/annurev-biochem-060614-033910

Wenceslau, C. F., McCarthy, C. G., Earley, S., England, S. K., Filosa, J. A., Goulopoulou, S., Gutterman, D. D., Isakson, B. E., Kanagy, N. L., Martinez-Lemus, L. A., Sonkusare, S. K., Thakore, P., Trask, A. J., Watts, S. W., & Webb, R. C. (2021). Guidelines for the measurement of vascular function and structure in isolated arteries and veins. *American Journal of Physiology. Heart and Circulatory Physiology*, *321*(1), 77–111. https://doi.org/10.1152/ajpheart.01021.2020

Yamaki, F., Kaga, M., Horinouchi, T., Tanaka, H., Koike, K., Shigenobu, K., Toro, L., & Tanaka, Y. (2001). MaxiK channel-mediated relaxation of guinea-pig aorta following stimulation of IP receptor with beraprost via cyclic AMP-dependent and -independent mechanisms. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *364*(6), 538–550. https://doi.org/10.1007/s002100100485

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