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RESEARCH ARTICLE

Gβγ subunit activation of K_V7 and BK_{Ca} channels underlies calcitonin gene-related peptide vasorelaxation in myogenic rat coronary resistance arteries

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Abstract

Background and Purpose: CGRP is a potent, clinically relevant coronary vasodilator known to play a role in cardioprotection. Here, we investigated the precise intracellular signalling pathways leading to vasodilation in small coronary arteries.

Experimental Approach: This study used *ex vivo* myography and intracellular recording techniques to investigate α-CGRP-induced vasorelaxation and vascular smooth muscle cell (VSMC) hyperpolarization in myogenically active rat isolated coronary arteries.

Key Results: CGRP-induced vasorelaxation was not dependent on the endothelium, but relied heavily on K^+ channel activation. Immunohistochemistry indicated K_V7 and BK_{Ca} channel expression in VSMC and endothelial cells. A combination of the K_V7 channel inhibitor, linopirdine and the BK_{Ca} channel inhibitor, paxilline, significantly attenuated CGRP-induced vasorelaxation in endothelium-intact or denuded arteries. Electrophysiology confirmed that CGRP caused hyperpolarization and showed this was prevented by linopirdine and paxilline, also demonstrating a role for K_V7 and BK_{Ca} channels in suppressing depolarizing smooth muscle spikes. These spikes were also suppressed by NO• and HNO donors, resulting in hyperpolarization. Gβγ subunit inhibition with gallein markedly right-shifted CGRP-induced vasorelaxation in both endothelium-intact and denuded coronary arteries.

Conclusion and Implications: α-CGRP stimulates robust vasorelaxation in the coronary microvasculature that relies on Gβγ subunit-activated VSMC hyperpolarization involving K_V7 and BK_{Ca} channels. These data enhance understanding of coronary microvascular physiology and inform the design of future therapeutic strategies targeting coronary vascular dysfunction.

KEYWORDS
CGRP, coronary artery, Gβγ subunit, membrane potential, myogenic tone, tension

Abbreviations: E_m, membrane potential; IPA-NO, isopropylamine NONOate; SNAP, (*S*)-nitroso-*N*-acetylpenicillamine; VSMC, vascular smooth muscle cell.

Lucy A. Donovan and JinHeng Lin should be considered joint first author.

1 | INTRODUCTION

It is vital for the heart to dynamically adjust blood distribution in response to the metabolic demands of the myocardium. This is achieved through the modulation of coronary artery tone, with the greatest changes in vascular resistance occurring in the microvasculature. There is growing evidence that impairment of the vasodilatory capacity of these small vessels occurs in most forms of cardiovascular disease (Buono et al., 2021); therefore, it is crucial to define how vasodilators regulate coronary vascular resistance. CGRP is the most potent endogenous vasodilator first reported to cause vasodilation of microvessels in the seminal study by Brain et al. (1985). **α-CGRP** is a 37-amino acid neuropeptide produced via alternative splicing of the *CALCA/CALC1* gene transcripts (Amara et al., 1982). Another structurally similar isoform, **β-CGRP**, is encoded by a separate gene, *CALCB/CALC2* (Amara et al., 1985). Despite these two distinct isoforms sharing >90% homology and having similar biological roles, β-CGRP expression is approximately a third that of α-CGRP in the rat heart (Mulderry et al., 1988). Therefore, the α-CGRP isoform is the primary focus in this study.

CGRP is primarily stored and released from perivascular sensory nerves across various vascular beds, including coronary (Gulbenkian et al., 1993; Lundberg et al., 1985; Wharton et al., 1988), mesenteric (De Mey et al., 2008; Kawasaki et al., 1988; Le et al., 2020) and cerebral (Uddman et al., 1985, 1986) arteries. **CGRP receptor** comprises the **calcitonin receptor-like receptor**, a GPCR, together with **receptor activity-modifying protein 1 (RAMP1)** and the receptor component protein (Hay et al., 2018). CGRP receptor expression is widespread throughout the vasculature, with calcitonin receptor-like receptor and RAMP1 identified in both the vascular smooth muscle cells (VSMCs) and ECs of rat and murine mesenteric arteries (Boerman & Segal, 2016; Sheykhzade et al., 2017) and human epicardial and intramyocardial coronary arteries (Chan et al., 2010; Hagner et al., 2001). However, expression is likely heterogeneous depending on vascular bed and vessel calibre.

CGRP receptors are generally considered to be -coupled and operate through activation of the **cAMP-PKA** pathway (Aiyar et al., 1999; Russell et al., 2014). PKA phosphorylates downstream targets leading to reduced intracellular Ca^{2+}, decreased myosin light chain kinase affinity for Ca^{2+}-calmodulin and activation of K^+ channels (Argunhan & Brain, 2022; Sohn et al., 2020). In this way, CGRP causes hyperpolarization and relaxation of the smooth muscle and, ultimately, vasodilation. Many studies have implicated **K_{ir}6.1** (K_{ATP}) channels in CGRP-induced vasodilation (Kitazono et al., 1993; Nelson et al., 1990; Wellman et al., 1998), although a role for K_{Ca} channels (Sheykhzade & Berg Nyborg, 2001) and **K_V7 channels** (Chadha et al., 2014; Stott et al., 2018) has also been demonstrated. Recent studies suggest a role for the Gβγ subunit in CGRP-induced vasodilation (de Vries et al., 2024; Meens et al., 2012; Stott et al., 2018), but the precise signalling pathway is unclear. Despite clear direct actions in VSMCs, CGRP may also mediate endothelium-dependent vasodilation, possibly through **NO** release (Argunhan & Brain, 2022; Russell et al., 2014).

What is already known

- CGRP is a potent vasodilator known to act via the $G\alpha_s$ or Gβγ pathway to stimulate hyperpolarization.
- Elevated plasma levels of CGRP are associated with cardioprotection.

What does this study add

- First demonstration that CGRP acts via the Gβγ subunit to hyperpolarize and relax coronary arteries.
- Both K_V7 and BK_{Ca} channels are responsible for vascular smooth muscle cell (VSMC) hyperpolarization.

What is the clinical significance

- CGRP contributes to improved coronary blood flow by hyperpolarizing resistance arteries.
- Disruption of the vasorelaxation pathways might contribute to coronary microvascular dysfunction, which precedes coronary artery disease.

CGRP has significant pathophysiological functions alongside its physiological effects. As well as being an effective therapeutic target in migraine treatment (Cohen et al., 2022), it is now understood that CGRP confers cardioprotection in several diseases, including ischaemia, hypertension and heart failure (Kumar et al., 2019). However, despite this clinical relevance, there is still uncertainty surrounding the precise signalling pathways involved. Moreover, research in the coronary microvasculature remains limited, particularly lacking data where myogenic tone provides a baseline level of contraction and where hyperpolarization is assessed alongside vasodilation. By utilizing a combination of *ex vivo* techniques, we aimed to explore the intracellular signalling mechanisms that contribute to CGRP-induced vasorelaxation from myogenic tone in rat intramuscular coronary septal arteries.

2 | METHODS

2.1 | Animals and tissue preparation

The use of wild-type male Wistar Han and Wistar rats (200–300 g; Charles River Laboratories, UK or Germany) in the present study was approved by the University of Oxford ethical committee. These studies comply with the latest ARRIVE guidelines (Percie du Sert et al., 2020a, 2020b) and updated recommendations from the *British*

Journal of Pharmacology (Lilley et al., 2020). Animals were housed in individually ventilated cages under a 12:12 h light:dark cycle at 20–22°C. Standard chow and water were available *ad libitum*. Following delivery, rats were housed for at least 10 days to allow for acclimatization. Rats were killed in accordance with UK legislation as specified by Schedule 1 of the Animals (Scientific Procedures) Act 1986 by increasing CO_2 concentration for 3 min, confirmed with cervical dislocation. The heart was then placed in ice-cold Krebs-buffered solution. The wall of the left atrium and right ventricle was partially removed to allow three to four proximal segments of the intraventricular septal artery (diameter ~150–350 μm) to be isolated from the adhering connective tissue and cardiomyocytes.

2.2 | Solutions

During tissue collection, dissection and myography experiments, Krebs-buffered solution was used containing (in mM): 121 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4(7H_2O)$, 1.18 KH_2PO_4, 11.6 glucose and 1.25 $CaCl_2$, and gassed with 21% O_2, 5% CO_2 and balance N_2. Drug and compound stock solutions were diluted in Krebs-buffered solution as possible. Where DMSO was required, the total amount in the myography chambers never exceeded 0.3%, which the laboratory has shown is the maximum amount that can be used before directly stimulating EC Ca^{2+} (unpublished). Arteries were allowed to recover for at least 15 min between concentration response curves. All inhibitors (receptor antagonists and channel/pathway blockers) were incubated with the tissue for at least 15 min prior to experimentation and until myogenic tone had plateaued where relevant; these agents were considered irreversible and readded following washout.

2.3 | Wire myography

Isolated artery segments (~ 1–2 mm) were mounted luminally using two 25 μm diameter gold-plated tungsten wires in the 5-ml chamber of a Mulvany–Halpern wire myograph (Danish Myo Technology Organ Bath Model 700MO or 610M) in Krebs-buffered solution gassed with 21% O_2, 5% CO_2 and balance N_2. With the aid of a microscope, the length of arteries was then measured (mm; for subsequent calculation of $mN{\cdot}mm^{-1}$). The artery was allowed to equilibrate for 30 min while the chamber was warmed to 37°C. Changes in tension (mN) were recorded at 10 Hz using a PowerLab/4SP data acquisition system (ADInstruments, New Zealand) and LabChart software (v8.1.17, AD Instruments). The arteries were normalized to a resting tension equivalent to that generated at 90% of the diameter of the vessel at 80 mmHg. Arteries were allowed to equilibrate at their optimal resting tensions for 60 min, during which time the ability to generate myogenic tone was established.

Artery viability was assessed by the development of spontaneous myogenic tone followed by endothelium-dependent relaxation to **acetylcholine (ACh**; 10–300 nM). Only arteries that developed >0.5 $mN{\cdot}mm^{-1}$ tone above baseline (no tone) were considered to develop sufficient myogenic tone for further experimentation. If sufficient myogenic tone was not obtained, artery contractility was assessed by constriction to **phenylephrine** (1–5 μM). Only arteries that sustained robust contraction to either stretch or phenylephrine and >90% relaxation to 300-nM ACh were considered viable. To study vasorelaxation, arteries were not precontracted with phenylephrine, but often arteries developed myogenic tone in the presence of blockers and were therefore used. For endothelium-denuded experiments, ECs were removed with a hair and successful removal confirmed by <10% relaxation to 1-μM ACh.

The relaxation to the lower concentrations of CGRP was gradual, so the protocol was developed to expose each cumulative concentration of CGRP for 2 min, at which point the tension was recorded for analysis. For each relaxation response, the developed tone (myogenic; or in the presence of phenylephrine for ACh only) was the level of tone immediately prior to the addition of the test agent, and the minimum tension was achieved with 1-μM **nifedipine** at the end of the experiment. Vasorelaxation (%) = ((Developed tone − tone after agonist)/(Developed tone − minimum tension)) × 100. Each data point represents the mean tension over a ~10-s period.

2.4 | Electrophysiology

Isolated coronary septal arteries (~200–250 μm) were mounted in a 10-ml wire myograph chamber, as described above, for simultaneous measurement of VSMC membrane potential (E_m) and isometric tension, as previously described (Smith et al., 2020). After normalization and viability test, E_m and tension were recorded through a preamplifier (Neurolog system, Digitimer Ltd.) linked to a MacLab data acquisition system (model 4e, AD Instruments) and LabChart software (v8.1.17 AD Instruments). Individual VSMCs were impaled with sharp glass microelectrodes (backfilled with 2-M KCl; tip resistances circa 60 MΩ), observed as a rapid deflection towards the membrane potential during myogenic tone, near −40 mV.

Values for E_m were presented consistent with previous analysis (Ng et al., 2024; Smith et al., 2020). In general, for E_m and tension in the absence and presence of treatments, values are the simultaneously acquired average E_m and tension over a 10- to 20-s period. Since depolarizing spikes were often observed, values for the high and low peaks (amplitude of spikes) were also averaged and reported alongside the overall mean E_m.

2.5 | Immunohistochemistry

The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). Arteries mounted in the wire myograph were checked for EC and VSMC function. Subsequently they were fixed in situ by adding 4% paraformaldehyde aqueous solution to the chamber for 60 min at room temperature, before being washed with PBS. Fixed arteries were cut open laterally and removed from the wire myograph.

Arteries were then incubated for 90 min with blocking buffer (1% BSA, 0.5% Triton X-100, 0.05% Tween 20 in PBS) and then with the primary antibodies overnight at 4°C. The following day, arteries were washed in PBS and incubated with the secondary antibodies and Hoechst 33342 for 2 h at room temperature, before being washed in PBS again. After staining, the arteries were carefully opened and placed flat on glass slide sized coverslips in mounting medium (VECTASHIELD, H-1000; Vector Laboratories Inc., USA), with the EC layer facing up. Small round coverslips were gently lowered on top and sealed (CoverGrip, 23005; Biotium).

Arteries were imaged using a laser scanning confocal microscope (FV1200; Olympus) using a 40× water immersion objective (1.15 NA, 1024 × 1024 pixels; Olympus). Sequential z-stacks were acquired at 0.5-µm intervals using Fluoview Software (FV10-ASW 3.0; Olympus) and reconstructed in Imaris Software (v8.0.2; Bitplane).

2.6 | Data and statistical analysis

All myography and electrophysiology data were analysed identically with methods determined prior to data collection; therefore, data randomization and blinding were not necessary in these current investigations. Minimum group sizes were determined through a priori power calculations, performed with G*Power software (v3.1.9.6, open source; Faul et al., 2007) and were increased to at least $n = 5$ if calculated group sizes were smaller than five. Sample size was calculated at $\alpha = 0.05$, power = 0.8, 15% SD and effect size estimated as ability to detect 50% difference from control. Group sizes were designed to be equal, with any extra biological replicates being added if excess arteries were available on the day, prioritizing same-day control experiments.

Randomization and blinding were not performed as they were impractical and would require additional personnel. Different pharmacological agents had to be clearly labelled and had different dilution factors for final concentrations. Furthermore, lack of randomization and blinding was unlikely to skew the results as multiple arteries from one animal were used for different treatment protocols.

The data and statistical analysis comply with the BJP recommendations on experimental design and analysis (Curtis et al., 2025). All data are summarized as the mean ± SEM of *n* arteries, where *n* denotes biological replicates. All datasets have sample sizes greater than or equal to 5, unless specified. All datasets included in statistical analyses met the minimum sample size requirement ($n \geq 5$ biological replicates). For experiments where this threshold was not met, results are described as 'exploratory' and no inferential statistics were applied. Concentration-response curves were fitted using variable slope nonlinear regression. Statistical analysis was carried out in GraphPad Prism (v9.4.0, GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant; the type of analysis for each dataset is indicated within the respective figure legend. All dataset comparisons are unpaired, unless specified. Parametric analysis was only performed if at least half of the datasets passed the Shapiro–Wilk normality test. Post-hoc tests were run only if *F* achieved $P < 0.05$ and there was no significant variance inhomogeneity.

2.7 | Materials

The following were purchased from Tocris (UK), rat CGRP (1161), gallein (3090), BIBN 4096 (olcegepant; 4561), levcromakalim (1378), (*S*)-nitroso-*N*-acetylpenicillamine (SNAP; 0598), linopirdine (1999), SQ 22536 (1435) and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; 0880). Acetylcholine (ACh; A6625), glibenclamide (G0639), paxilline (P2928), isoprenaline (I5627), N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; N5751), nifedipine (N7634), phenylephrine (PE; P6126), and retigabine (90221) were purchased from Sigma (UK). Isopropylamine NONOate (IPA-NO) was a gift from K. M. Miranda (University of Arizona).

Details of other materials and suppliers are provided in specific sections.

Antibodies and Hoechst were diluted in blocking buffer. Primary antibodies were as follows: rabbit polyclonal anti-mouse **K_V7.4** (3.2 µg·ml^{-1}; aa 594–607; APC-164, Alomone Labs; RRID:AB_2341042), rabbit polyclonal anti-mouse **K_V7.5** (3.2 µg·ml^{-1}; aa 856–869; APC-155, Alomone Labs; RRID:AB_2341038), rabbit polyclonal anti-mouse **K_{Ca}1.1** (BK_{Ca}) (3 µg·ml^{-1}; aa 1097–1196; APC-021, Alomone Labs; RRID:AB_2313725), mouse monoclonal anti-human ZO-1 (2 µg·ml^{-1}; aa 334–634; 33-9100, Thermofisher Scientific; RRID:AB_2533147). Secondary antibodies were as follows: Alexa Fluor 488 goat anti-rabbit IgG (2 µg·ml^{-1}; A11034, Thermofisher Scientific), Alexa Fluor 633 goat anti-mouse IgG (2 µg·ml^{-1}; A21126, Thermofisher Scientific). Nuclei were stained with Hoechst 33342 (1 µg·ml^{-1}; H3570, Thermofisher Scientific).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2025/2026 (Alexander, Davenport, et al., 2025; Alexander, Fabbro, et al., 2025; Alexander, Striessnig, et al., 2025).

3 | RESULTS

3.1 | CGRP causes potent vasorelaxation in rat septal arteries

Cumulative CGRP concentration-response curves (1 pM–100 nM) were constructed in rat isolated septal arteries against myogenic tone (control; 1.4 ± 0.2 mN·mm^{-1}). CGRP stimulated potent, concentration-dependent vasorelaxation (Log $EC_{50} = -9.29 \pm 0.13$; Figure 1a). The curve was not affected by either Wistar rat strain or pre-tone (Figure S1). The CGRP receptor antagonist, **BIBN 4096 (olcegepant**; 10 nM–1 µM), inhibited CGRP-induced vasorelaxation in a concentration-dependent manner, with the response to 100-nM CGRP being reduced from 96.8% ± 1.1% to 90.3% ± 1.6%, 56.0% ± 8.6% and 5.5% ± 1.4% at 10-nM, 100-nM and 1-µM BIBN 4096, respectively (Figure 1b).

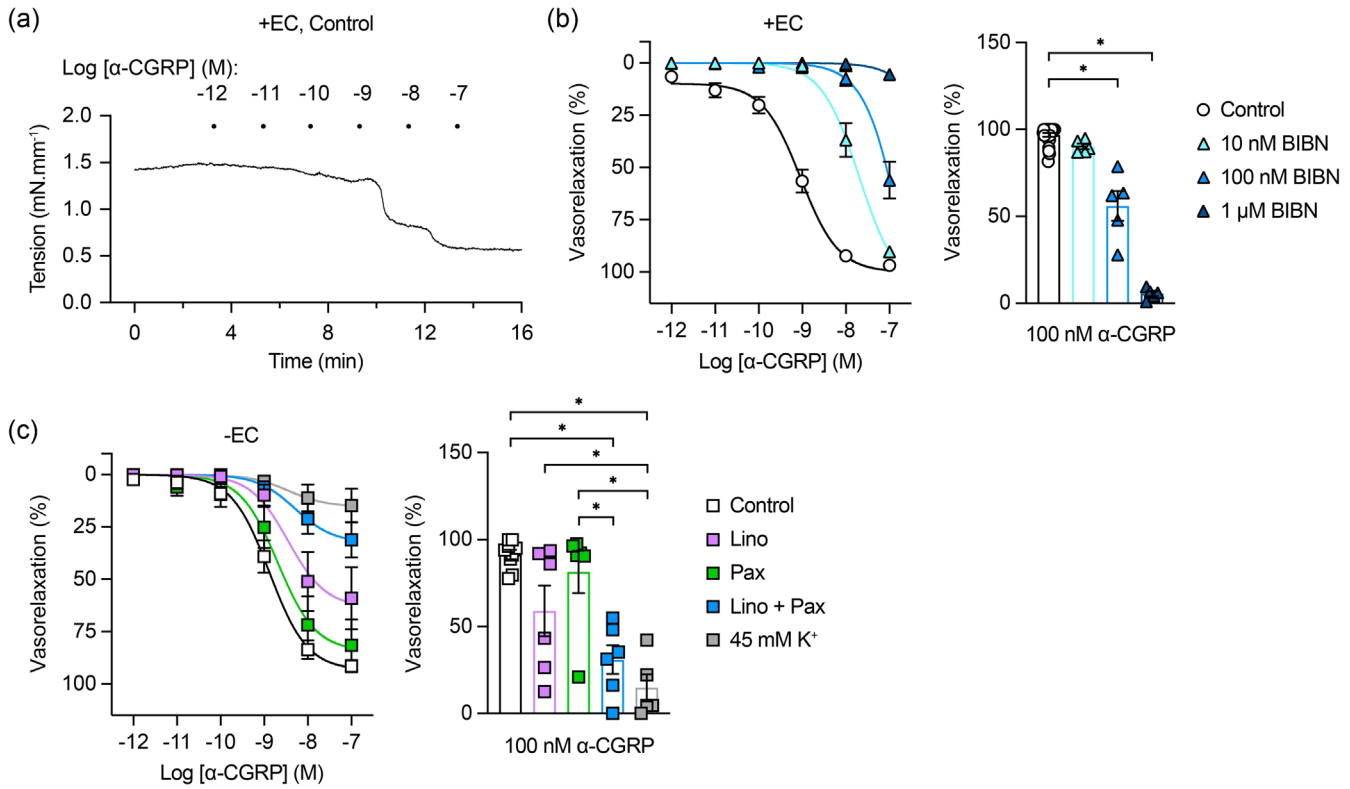

FIGURE 1 CGRP relaxes rat septal arteries through vascular smooth muscle K^+ channels. (a) Representative wire myograph trace of vasorelaxation stimulated by rat α-CGRP (1 pM–100 nM) from myogenic tone. (b) Concentration–response curves (left) of CGRP-induced vasorelaxation in endothelium-intact (+EC) arteries without pharmacological intervention (control; $n = 24$) or in the presence of BIBN 4096 (10 nM–1 μM, 30 min; $n = 5$ each), and summary of vasorelaxation in response to 100-nM CGRP (right). (c) Concentration–response curves (left) of CGRP-induced vasorelaxation in endothelium-denuded (−EC) arteries without pharmacological intervention ($n = 9$), or in the presence of 45-mM extracellular K^+ ($n = 5$), or preincubated with linopirdine (1 μM, 30 min; $n = 6$), paxilline (1 μM, 30 min; $n = 6$) or both ($n = 6$). One-way ANOVA, with Holm–Sidak multiple comparisons test, was performed for (b) and (c). *$P < 0.05$.

3.2 | Vasorelaxation to CGRP was largely dependent on opening K^+ channels in the VSMCs

To define VSMC signalling pathways, the septal artery endothelium was denuded (−EC; Figure S1b). In −EC arteries, the control response to 100-nM CGRP (91.4% ± 2.7%) was not affected compared with +EC arteries, but there was a significant rightward shift in the concentration response curve (to Log $EC_{50} = -8.78 \pm 0.12$). In −EC arteries, incubation with isotonic 45-mM K^+ Krebs-buffered solution significantly inhibited the response to CGRP, reducing vasorelaxation at 100-nM CGRP from to 14.8% ± 7.9% (Figure 1c). This indicated a major role for K^+ channels in CGRP-vasorelaxation.

3.3 | Vascular smooth muscle K_V7 and BK_{Ca} channels independently play predominant roles CGRP vasorelaxation of denuded septal arteries

To further define the K^+ channels underpinning CGRP-mediated vasorelaxation, arteries were incubated with the K_V7 channel inhibitor, linopirdine. In order to determine the **linopirdine** concentration required to block K_V7 channels without affecting K_{ATP} channels, the inhibitory effect of linopirdine on vasorelaxation to the K_V7 channel activator, retigabine (10 μM), was compared with that by the K_{ATP} channel activator, levcromakalim (1 μM). It was found that 1-μM linopirdine blocked vasorelaxation to **retigabine** (from 73.7% ± 15.0%, to 8.8% ± 2.3%; exploratory data), while vasorelaxation to levcromakalim was unaffected (Figure S2). In contrast, 10-μM linopirdine reduced hyperpolarization and vasorelaxation to levcromakalim (Figure S2b; exploratory data); therefore, 1-μM linopirdine was used throughout the current study. In −EC arteries, linopirdine reduced 100-nM CGRP-induced vasorelaxation from 91.6% ± 2.8% to 59.0% ± 14.7% (Figure 1c), but the difference was not statistically significant. Similarly, significant attenuation was not observed in the presence of BK_{Ca} inhibitor, **paxilline** (1 μM; 81.5% ± 12.2%, Figure 1c). However, the combined application of linopirdine and paxilline significantly inhibited vasorelaxation at 100-nM CGRP, leaving only a small residual relaxation (31.0% ± 8.3%, Figure 1c).

Several studies have reported a central role for K_{ATP} channels in CGRP-induced vasorelaxation (Kitazono et al., 1993; Nelson et al., 1990; Pinkney et al., 2017; Sakai & Saito, 1998). This possibility was investigated with **glibenclamide** (5 μM), which abolished levcromakalim-induced hyperpolarization and vasorelaxation (Figure S2; exploratory data). Glibenclamide, either alone (93.9%

± 3.3% vs. control 91.4% ± 2.7%) or in combination with linopirdine and paxilline (29.4% ± 6.0% vs. lino + pax only 31.0% ± 8.3%), did not affect vasorelaxation to 100-nM CGRP in denuded septal arteries, an observation consistent with +EC arteries (Figure S2d).

3.4 | K_V7 channels and BK_{Ca} channels expression on the ECs and VSMCs of rat septal arteries

Immunohistochemistry indicated expression of $K_V7.4$ and $K_V7.5$ channels in the VSMCs of rat septal arteries (Figure 2), two of the possible channels sensitive to linopirdine (Sogaard et al., 2001), and for the BK_{Ca} channel (α-subunit, $K_{Ca}1.1$), which is sensitive to paxilline (Sanchez & McManus, 1996). Interestingly, K_V and BK_{Ca} channel labelling was also evident in ECs, the former as found in rat mesenteric arteries (Baldwin et al., 2020). In both cases, the labelling was punctate and distributed within the cell, consistent with both intracellular (including perinuclear) and membrane distribution.

3.5 | Nitric oxide contributes to endothelium-dependent relaxation independently of K^+ channel activation

Removing the influence of the endothelium revealed a small EC-dependent component to CGRP-induced vasorelaxation, hence this was probed further. The effect of the **endothelial NOS (eNOS)** blocker, **L-NAME** (100 μM) was not evident against the maximum vasorelaxation to 100-nM CGRP (88.9% ± 3.8% in the presence of L-NAME; Figure 3a); however, L-NAME did significantly right-shift the CGRP concentration response curve (to Log EC_{50} = −8.35 ± 0.17; Figure 3b), similar to that observed in denuded arteries. Raised K^+ significantly inhibited vasorelaxation to 100-nM CGRP in +EC arteries (to 28.6% ± 4.3%; Figure 3a) and the addition of L-NAME further attenuated the CGRP response (13.0% ± 5.4%; Figure 3a), similar to denuded arteries. The residual relaxation was not sensitive to inhibition of **soluble guanylate cyclase** with **ODQ** (10 μM; 16.3% ± 4.6%; Figure 3a) or the further addition of the **adenylate cyclase** inhibitor SQ 22536 (100 μM) (8.4% ± 4.1%; Figure 3a).

As found in denuded arteries, neither linopirdine (96.7% ± 2.7%; Figure 3c) nor paxilline (85.2% ± 5.2%; Figure 3c) alone altered vasorelaxation to 100-nM CGRP in arteries with an intact endothelium. Furthermore, the concentration-dependent vasorelaxation to CGRP was not significantly altered compared with control (Log EC_{50}: linopirdine = −8.70 ± 0.03, paxilline = −9.14 ± 0.11). In contrast, following NO synthesis blockade with L-NAME, significant inhibition was observed with each of linopirdine, (53.5% ± 8.9%; Figure 3d) and paxilline (47.5% ± 15.1%; Figure 3c) compared with L-NAME alone (88.9% ± 3.8%; Figure 3d), suggesting that parallel NO and K^+ channel vasorelaxation pathways operate in the absence of L-NAME. The combination of linopirdine and paxilline mimicked the inhibitory effect of 45-mM K^+ on CGRP-induced vasorelaxation when added either without L-NAME (39.8% ± 10.7%; Figure 3c), or combined with L-NAME (18.9% ± 9.7%; Figure 3d).

3.6 | K_V7 channels, BK_{Ca} channels and NO contribute to CGRP-induced hyperpolarization in septal arteries

To further elucidate K^+ channel involvement in the CGRP response in septal arteries, the VSMC E_m was measured using sharp microelectrodes while recording isometric tension. There was no significant change in the mean resting E_m in the presence of L-NAME, paxilline or linopirdine, either alone or in combination (Figure 4a). Depolarizing spikes were relatively small or absent under control conditions

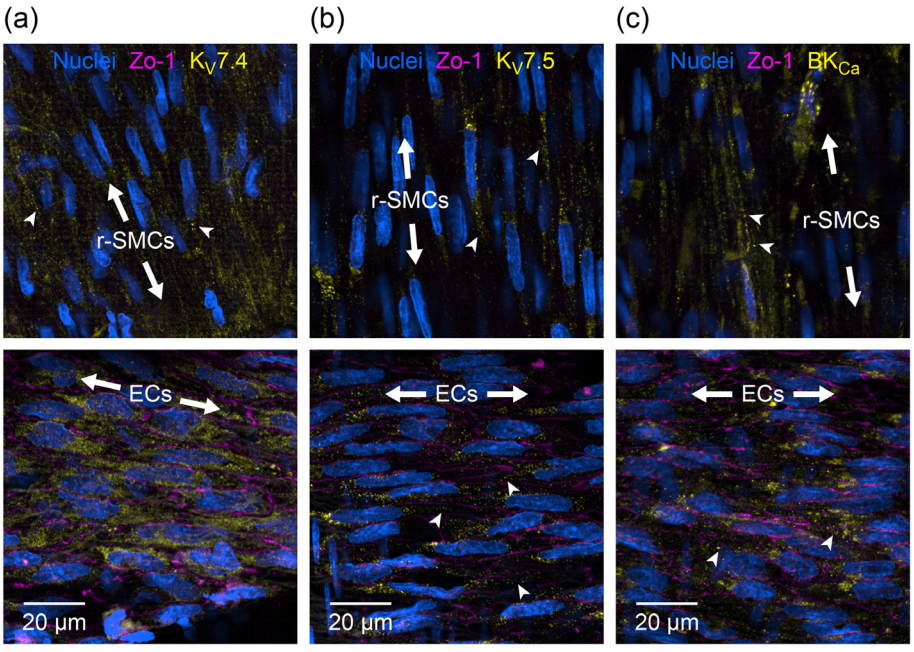

FIGURE 2 Protein expression of various isoforms of K_V7 channels, and BK_{Ca} channels, in rat septal arteries. Representative immunohistochemistry labelling for $K_V7.4$ (a; $n = 5$), $K_V7.5$ (b; $n = 6$) and BK_{Ca} (c; $n = 9$) channels in the smooth muscle cells and endothelial cells (yellow). Zo-1, a tight junction protein expressed on endothelial cells, was labelled in magenta. Nuclei were labelled in blue.

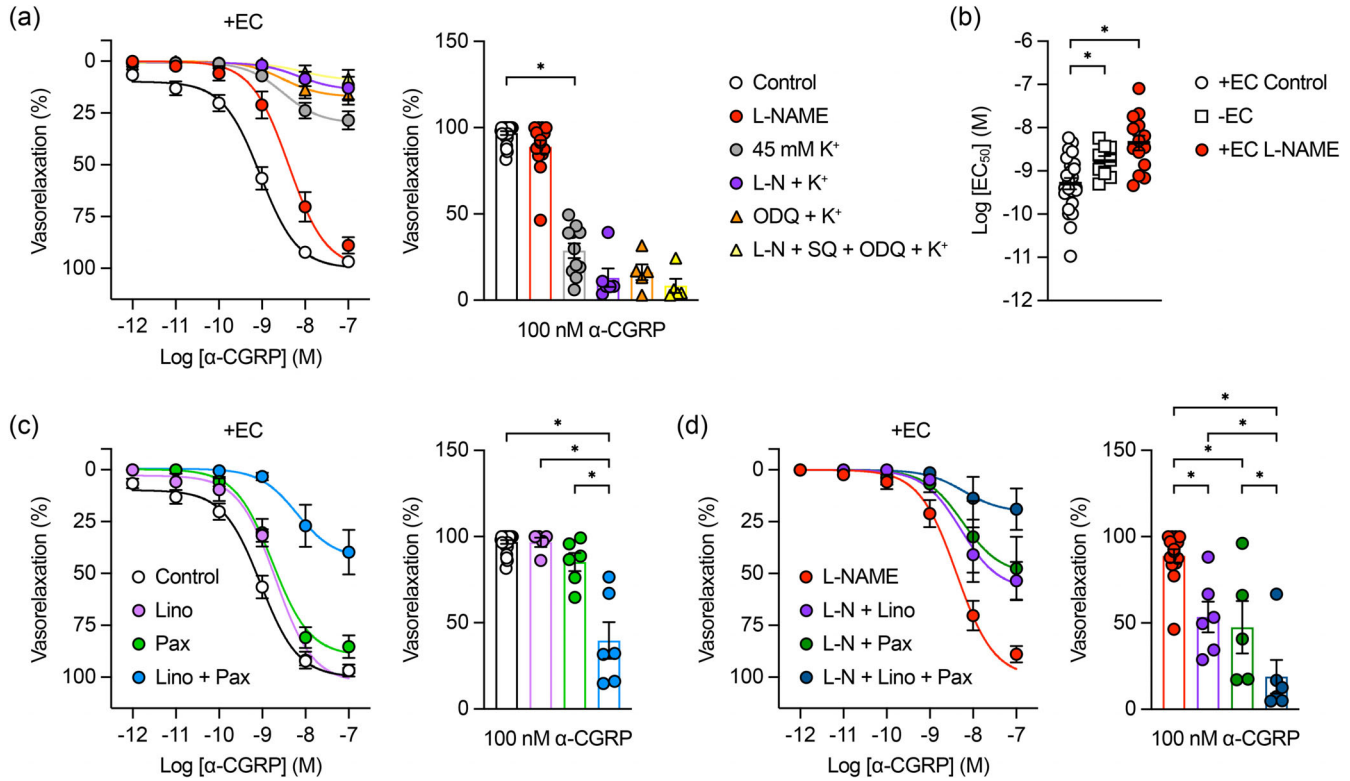

FIGURE 3 Nitric oxide and K^+ channels independently drive vasorelaxation to CGRP in rat septal arteries. (a) Concentration–response curves (left) of CGRP-induced vasorelaxation in endothelium-intact (+EC) arteries with ($n = 14$) or without ($n = 24$) preincubation with L-NAME (100 μM, 30 min), or in the presence of 45-mM extracellular K^+, with ($n = 6$) or without ($n = 11$) preincubation with L-NAME, or ODQ (10 μM, 30 min; $n = 5$), or L-NAME + ODQ + SQ 22536 (100 μM, 30 min; $n = 5$), and summary of vasorelaxation in response to 100-nM CGRP (right). (b) Summary of Log [EC_{50}] of CGRP concentration-response curves from endothelium-intact (+EC) arteries with and without L-NAME and in endothelium denuded (−EC) arteries. (c) Concentration–response curves and summary of CGRP-induced vasorelaxation in septal arteries without pharmacological intervention, or preincubated with linopirdine ($n = 5$), paxilline ($n = 6$) or both ($n = 6$). (d) Same experiments as (c) but in the presence of L-NAME ($n = 14, 6, 5, 6$). Kruskal–Wallis test, with Dunn's multiple comparisons test, was performed for (a). One-way ANOVA, with Holm–Sidak multiple comparisons test, was performed for (b), (c) and (d). *$P < 0.05$.

(Figures 4a,b and S3b). L-NAME alone variably increased the amplitude of depolarizing spikes, as reported previously (Ng et al., 2024; Smith et al., 2020). However, the combined presence of paxilline and linopirdine (with and without L-NAME) caused significantly greater amplitudes, but not frequencies, of depolarizing spikes associated with an increase in myogenic tone (Figures 4a–c and S3a,b). These data support a feedback role for different K^+ channels in suppressing depolarizing spikes and stabilizing myogenic tone.

The addition of CGRP resulted in the hyperpolarization of VSMCs in endothelium-intact arteries in a concentration-dependent manner (100-nM CGRP: $\Delta E_m = -20.6 \pm 3.0$ mV; Figure 4b,d). The Log EC_{50} for hyperpolarization (-8.73 ± 0.15) was right shifted compared with the simultaneously obtained values for vasorelaxation (Log $EC_{50} = -9.41 \pm 0.12$), reflecting continued hyperpolarization beyond the point of maximal vasorelaxation (Figure 4b). The hyperpolarization and vasorelaxation were significantly inhibited by the combination of paxilline and linopirdine (100-nM CGRP: $\Delta E_m = -8.1 \pm 6.1$ mV; Figure 4c,d). In the absence of L-NAME, linopirdine or paxilline alone failed to inhibit CGRP-induced hyperpolarization and vasorelaxation (100-nM CGRP: linopirdine $\Delta E_m = -20.5 \pm 4.8$ mV, paxilline $\Delta E_m = -26.4 \pm 2.7$ mV; Figure 4d). In the presence of L-NAME, while the Log EC_{50} for hyperpolarization (-8.78 ± 0.21) was not different to control, the Log EC_{50} for vasorelaxation was right shifted compared with control (Log $EC_{50} = -8.70 \pm 0.12$), consistent with the tension alone experiments. In the combined presence of L-NAME and paxilline (100-nM CGRP: $\Delta E_m = -5.6 \pm 2.0$ mV), or L-NAME and linopirdine (100-nM CGRP: $\Delta E_m = -1.1 \pm 2.2$ mV), hyperpolarization to 100-nM CGRP was virtually abolished compared with L-NAME alone (100-nM CGRP: $\Delta E_m = -12.8 \pm 2.6$ mV; Figure 4e), although vasorelaxation was less affected (Figure 4e). The combination of L-NAME, linopirdine and paxilline abolished CGRP-induced hyperpolarization and vasorelaxation (100-nM CGRP: $\Delta E_m = 0.9 \pm 1.6$ mV; Figure 4e).

The close inspection of the effect of CGRP against depolarizing spikes shows that, as the VSMCs repolarize, the amplitude of depolarizing spikes is reduced until they are effectively abolished (Figures 4b and S3b). This inhibitory effect was less with linopirdine and paxilline present (Figures 4c and S3a,b), but spikes could still be suppressed by ACh (Figures 4c and S3a). These data align well with development of spikes at membrane potentials more depolarized than ∼−41 mV (Ng et al., 2024; Smith et al., 2020).

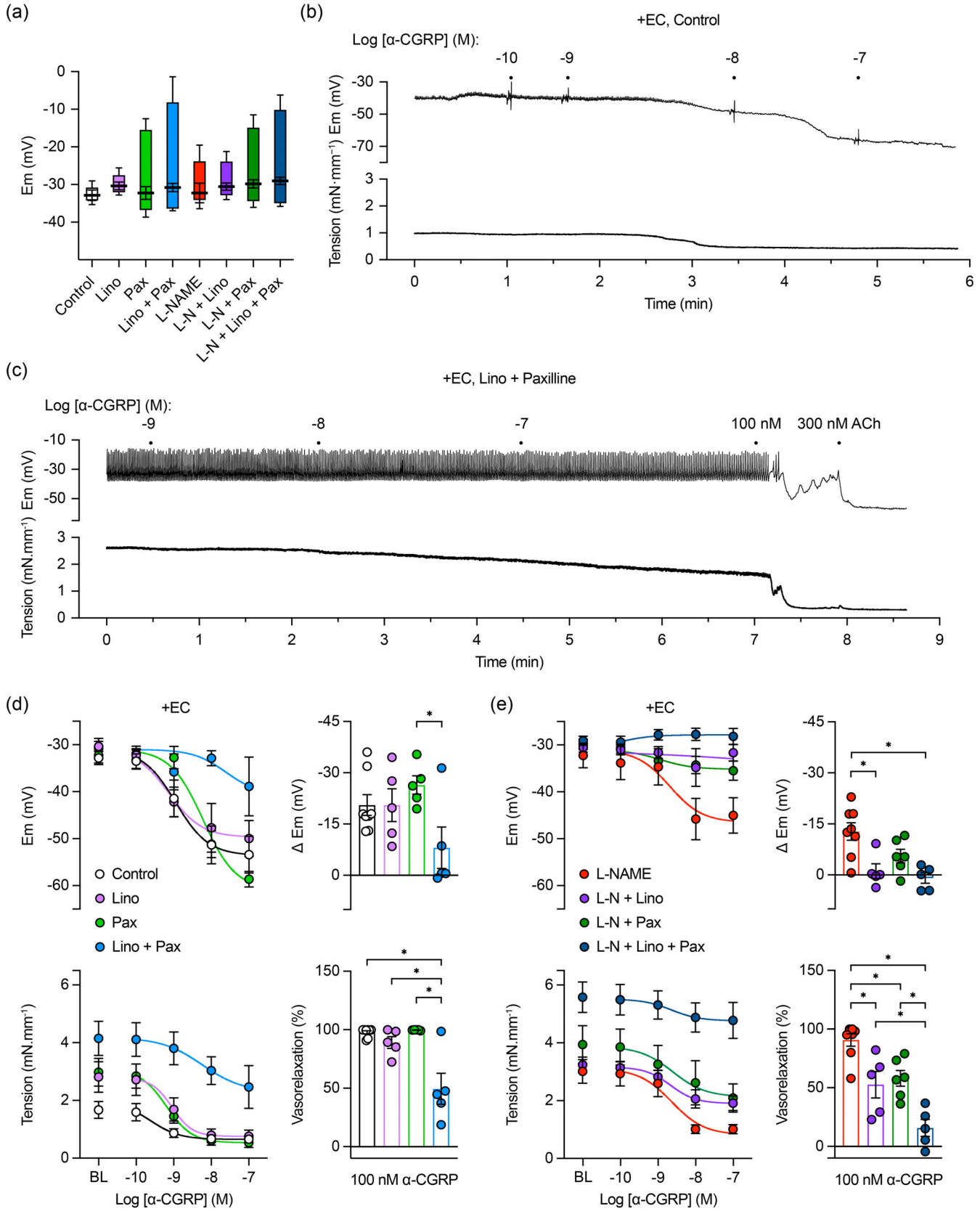

FIGURE 4 Legend on next page.

3.7 | Exogenous NO hyperpolarizes rat septal arterial VSMCs

The possibility that the release of NO could, in itself, contribute to the CGRP-mediated hyperpolarization was investigated by adding NO donors while recording membrane potential. To establish which form of NO, when released, might contribute to CGRP-induced hyperpolarization, we exogenously added the NO donors isopropylamine NONOate (IPA-NO; HNO donor) and SNAP (NO• donor) (Pinkney et al., 2017). Previous studies have implicated HNO as an endothelium-derived hyperpolarizing factor (Andrews et al., 2009), as well as being a K_V channel activator (Irvine et al., 2003). Therefore, we hypothesized that HNO released in response to CGRP might contribute to hyperpolarization and vasorelaxation. We found that in rat septal arteries preincubated with L-NAME, IPA-NO (10 nM–10 μM) caused a modest hyperpolarization (10-μM IPA-NO: $\Delta E_m = -9.8 \pm 1.3$ mV at 10 μM; Figure 5a,c), which was similar to that stimulated by SNAP (10 nM–10 μM) (10 μM SNAP: $\Delta E_m = -12.3 \pm 2.9$ mV; Figure 5b,c). Additionally, 50% ($n = 6$ out of 12) of these arteries developed action potential spikes, and both NO donors were able to suppress the depolarizing spikes, from 5.0 ± 1.6 mV to 0.38 ± 0.32 mV at 1 μM and 0 mV at 10 μM (both donor responses pooled; Figure 5d). These data do not distinguish between an ability of the NO donors to suppress depolarizing spikes by targeting voltage-gated calcium channels per se, or by hyperpolarization preventing the initiation of spikes.

3.8 | CGRP vasorelaxation is partially mediated by the Gβγ subunit

The Gβγ subunit has been implicated in CGRP-mediated vasorelaxation in other vascular beds and species (Meens et al., 2012; Stott et al., 2018). The Gβγ subunit inhibitor, gallein (100 μM), which we recently showed does not affect depolarization-induced vasoconstriction, G_q-coupled constriction and Ca^{2+} events, or myogenic tone in septal arteries (Lin et al., 2023), was used to study the role of the Gβγ subunit in CGRP signalling. The Gβγ subunit can inhibit or activate isoforms of adenylate cyclase (Bayewitch, Avidor-Reiss, et al., 1998; Bayewitch, Avidor-Reiss, et al., 1998) so the effect of gallein against the β-adrenoceptor agonist **isoprenaline** (0.1 nM–10 μM) was assessed for comparison. No change in isoprenaline-induced vasorelaxation occurred in the presence of gallein (Log $EC_{50} = -7.60 \pm 0.12$) compared with control (Log $EC_{50} = -7.62 \pm 0.11$; Figure S4).

In marked contrast, endothelium-intact septal arteries, gallein significantly inhibited CGRP-induced vasorelaxation at low concentrations of CGRP (10-nM CGRP: with gallein 6.9% ± 3.7% vs control 92.3% ± 1.9%, Figure 6a), but near maximal vasorelaxation was still obtained with 100-nM CGRP (80.7% ± 8.1% with gallein present). This gallein-insensitive component at 100-nM CGRP was significantly inhibited by linopirdine (to 36.0% ± 8.9%), but not by paxilline (79.9% ± 9.6%) although more inhibition was obtained when paxilline was combined with linopirdine (to 23.7% ± 8.7%; Figure 6a). This suggests that the residual relaxation to 100-nM CGRP is mediated predominantly via K_V7.4/5 channels. The application of gallein and L-NAME significantly attenuated CGRP-induced vasorelaxation compared with gallein alone (43.1% ± 15.7%; Figure 6a), which is also consistent with the possible involvement of NO on K_V7.4/5 channels.

In denuded arteries, gallein similarly right-shifted the CGRP concentration-response curve, with relaxation to 10-nM CGRP reduced from control −EC of 83.6% ± 4.3% to 15.1% ± 6.4% in the presence of gallein (Figure 6b); however, a sharp gallein-insensitive relaxation remained with 100-nM CGRP (64.3% ± 11.0%). The addition of either paxilline (49.2% ± 11.5%) or linopirdine (31.4% ± 10.4%) in addition to gallein did not significantly inhibit the response to 100-nM CGRP further, but the combination of all three inhibitors significantly reduced CGRP-induced vasorelaxation to 24.5% ± 7.3% (Figure 6b). These data suggest that CGRP is activating a Gβγ-mediated signalling pathway that is independent from the $G\alpha_s$-mediated pathway that is usually associated with isoprenaline signalling.

4 | DISCUSSION

The present study investigated the signalling pathways responsible for CGRP-induced vasorelaxation in rat intramuscular coronary septal arteries. This is the first functional study to examine the effects of CGRP on isolated small coronary arteries that are myogenically contracted. Our findings clearly show that low concentrations of CGRP rely on the Gβγ-subunit of the BIBN-sensitive CGRP receptor to stimulate vasorelaxation. Two forms of K^+ channels play a major role in this pathway: K_V7 and BK_{Ca} channels, which are sensitive to linopirdine and paxilline, respectively. CGRP-induced vasorelaxation is shown to be associated with VSMC hyperpolarization, an action that suppresses depolarizing spikes. Furthermore, while vasorelaxation to CGRP is not dependent on the endothelium, we provide evidence that NO can contribute to the CGRP response and stimulate

FIGURE 4 Nitric oxide and K^+ channels contribute to CGRP-induced hyperpolarization of rat septal arterial smooth muscle cells. (a) The mean baseline membrane potential (E_m, 10–20 s average, thick black lines) of arterial smooth muscle cells without pharmacological intervention, or in the presence of linopirdine, paxilline and L-NAME, in different combinations. The mean high and low peak E_m values during depolarizing spikes are represented by the top and bottom of each box. See Figure S3 for further analysis and simultaneously recorded mean myogenic tone (ΔTension, $mN \cdot mm^{-1}$). (b,c) Representative trace of simultaneous measurements of E_m and isometric tension in response to CGRP under control conditions (b) or in the presence of linopirdine and paxilline (c). (d,e) Concentration–response curves (left) and summary (right) of CGRP-induced changes in E_m and vasorelaxation in endothelium-intact arteries, with or without linopirdine, paxilline or both ([d], without L-NAME ($n = 8, 5, 5, 5$); [e], in the presence of L-NAME ($n = 8, 5, 6, 5$). One-way ANOVA was performed for (a), without post hoc analysis as ANOVA was not significant. One-way ANOVA, with Holm–Sidak multiple comparisons test, was performed for (d,e). *$P < 0.05$.

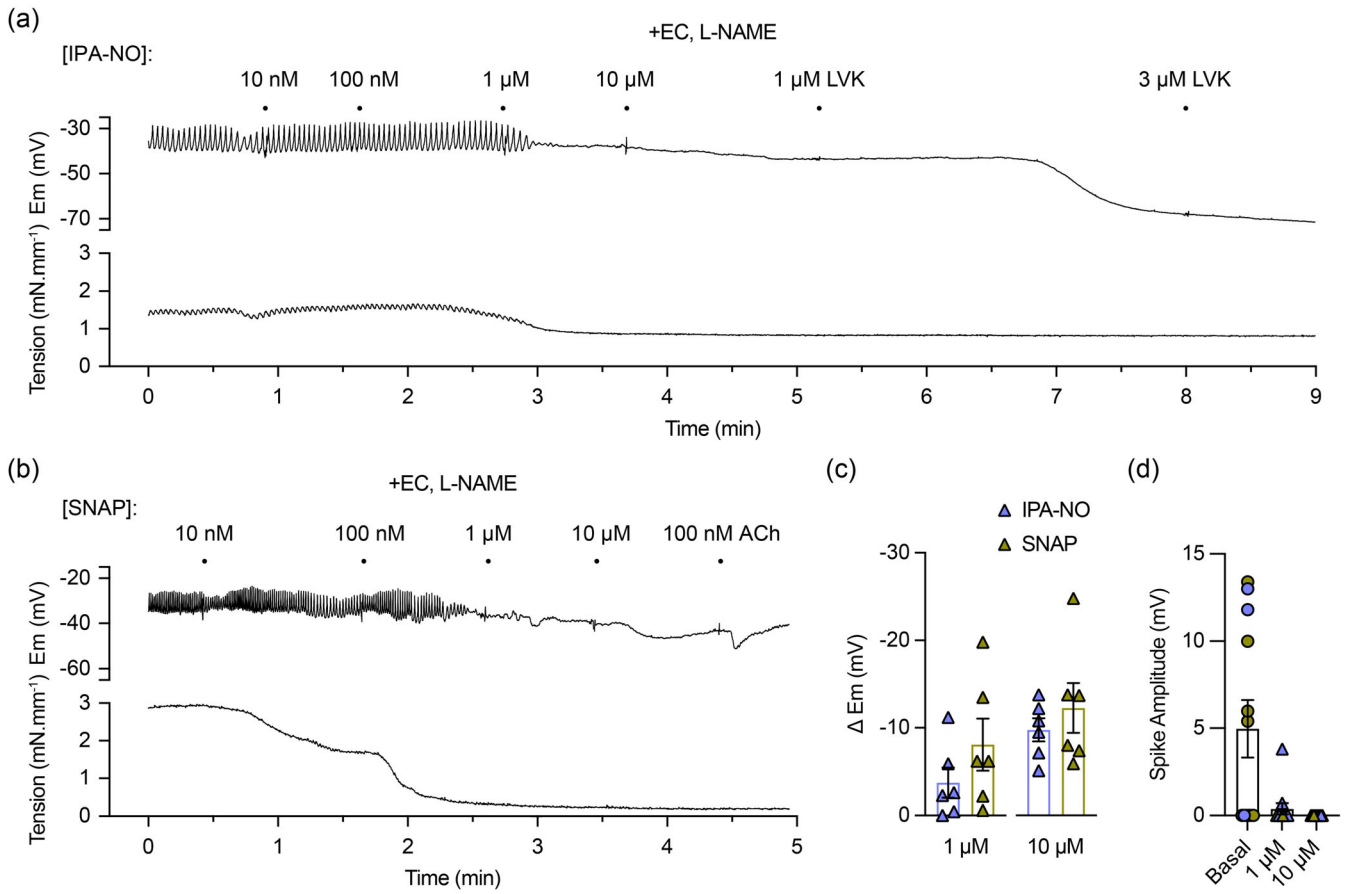

FIGURE 5 Nitric oxide directly hyperpolarizes rat septal arterial smooth muscle cells. (a,b) Representative trace of E_m and isometric tension in response to 10-nM to 10-μM isopropylamine NONOate (IPA-NO) (a) or SNAP (b) in endothelium-intact septal arteries preincubated with L-NAME. (c) Summary of changes in E_m in response to 1- and 10-μM IPA-NO ($n = 6$) or SNAP ($n = 6$). (d) Summary of action potential spike amplitude in the presence of L-NAME only (basal), and following stimulation with 1- and 10-μM IPA-NO or SNAP ($n = 12$, both donors pooled). Mixed effects analysis, with Holm–Sidak multiple comparisons test, was performed for (c). Friedman test, with Dunn's multiple comparisons test, was performed for (d). *$P < 0.05$.

hyperpolarization itself. As such, CGRP may activate both EC and VSMC K^+ channels.

In other studies of coronary artery vasorelaxation to CGRP, raised K^+ was used to pre-constrict arteries as it induces a robust, reversible and relatively stable level of vasoconstriction. Shoji et al. (1987) and Kageyama et al. (1993) found pre-constriction with 50-mM K^+ and 30-mM K^+, respectively, prevented vasorelaxation to CGRP in porcine coronary arteries. The same effect was recorded in the presence of 45-mM K^+ in the current study. These observations are not surprising as K^+ channels have been widely implicated in the CGRP response in other vascular beds. However, increasing external [K^+] inhibits efflux of K^+, making it impossible to distinguish the input and importance of different forms of K^+ channel, a limitation acknowledged by de Vries et al. (2024). These findings emphasize that the pre-constricting agent used must be taken into careful consideration when conducting such experiments. Using our methodology, the septal arteries routinely develop spontaneous myogenic tone; this not only provides a more accurate representation of the physiological state of the arteries *in vivo* but also removes the need for a pre-constrictor that may interfere with CGRP-induced vasorelaxation.

Our data confirm CGRP as a potent vasodilator, and for the first time in rat septal coronary arteries, acting via the CGRP receptor. Although it is widely accepted that K^+ channels are involved in CGRP-induced vasorelaxation, it seems there is heterogeneity in the type of contributory K^+ channels between vascular beds and species. The present study demonstrated that K_{ATP} channel inhibition had no effect on CGRP-mediated vasorelaxation, consistent with other studies using coronary arteries (Kageyama et al., 1993; Prieto et al., 1991). Nevertheless, a role for K_{ATP} channels in CGRP-mediated responses might occur under ischaemic conditions when cytosolic ATP levels decrease, as reported in porcine isolated coronary VSMCs (Wellman et al., 1998). This possibility is clearly of physiological relevance and requires further investigation. Although inhibition of K_V7 or BK_{Ca} channels individually failed to affect CGRP vasorelaxation, we demonstrated that blocking both channel types markedly attenuated the CGRP response and abolished hyperpolarization.

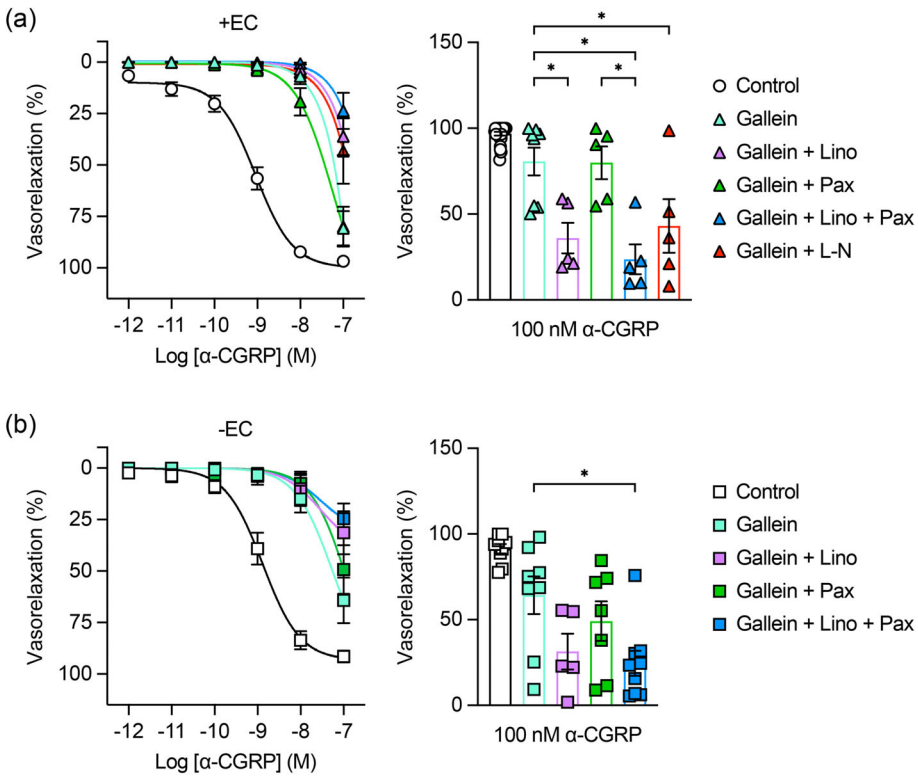

FIGURE 6 CGRP vasorelaxation is partially mediated by the Gβγ subunit. Concentration–response curves (left) and summary (right) of CGRP-induced vasorelaxation in endothelium-intact (+EC, [a]; $n = 24, 8, 5, 5, 5, 5$) or -denuded (−EC, [b]; $n = 9, 8, 5, 7, 9$) arteries without pharmacological intervention (control), or in the presence of gallein (100 μM, 30 min) with or without linopirdine, paxilline or both, or L-NAME. One-way ANOVA, with Holm–Sidak multiple comparisons test, was performed for (a) and (b). *$P < 0.05$.

The current study also aimed to examine the role of the endothelium in CGRP-induced vasorelaxation, and its relative importance compared with the endothelium-independent component. There is a lack of consensus in the literature as to whether there is an endothelium-dependent component to CGRP-mediated vasorelaxation; endothelium removal abolishes the CGRP response in rat aorta (Brain et al., 1985; Gray & Marshall, 1992) and mesenteric arteries (Iwatani et al., 2008), but has no effect in cat cerebral (Edvinsson et al., 1985) or porcine coronary arteries (Yoshimoto et al., 1998). Interestingly, Prieto et al. (1991) found that removing the endothelium attenuated CGRP-induced vasorelaxation in rat proximal epicardial coronary arteries, but not in distal myocardial coronary arteries. It appears, therefore, that the contribution of the endothelium is variable depending on coronary artery size. The present findings demonstrate that, although either removal of the endothelium or addition of L-NAME alone does indeed right shift the CGRP response curve in these arteries, the primary mechanism is direct stimulation of VSMC CGRP receptors. Nevertheless, this endothelium/NO component of CGRP-induced vasorelaxation should still be considered important and warrants further study.

Simultaneous recording of membrane potential and tension also demonstrated that both K_V7 and BK_{Ca} channels are involved in CGRP-induced hyperpolarization as well as vasorelaxation. These experiments also showed a component linked to NO, as inhibition of NOS attenuated CGRP-induced hyperpolarization and abolished the residual hyperpolarization in the presence of linopirdine and paxilline. HNO does contribute as an endothelium-derived relaxing and hyperpolarizing factor in resistance vessels (Andrews et al., 2009) and a study by Favaloro and Kemp-Harper (2007) also demonstrated a vasodilatory effect of HNO in rat coronary vessels which was attenuated with CGRP 8–37, thus providing a possible link to CGRP release. Furthermore, Irvine et al. (2003) proposed that HNO activates K_V channels, showing that a voltage-dependent K^+ channel inhibitor, **4-aminopyridine**, reduced vasorelaxation to the HNO donor, Angeli's salt, in rat mesenteric arteries. Our electrophysiology experiments demonstrated that 10-μM IPA-NO causes ~10-mV hyperpolarization in rat septal arteries and suppresses depolarizing spikes. A very similar profile was observed with the NO• donor SNAP, which at 10 μM caused ~12-mV hyperpolarization and abolished depolarizing spikes. Further investigations are necessary to establish the relationship between NO and hyperpolarization as this may be important in understanding not only the CGRP signalling pathways but other coronary vasodilators. Importantly, it is clear that NO should be considered an endothelium-derived hyperpolarizing factor in coronary arteries, alongside endothelium-dependent hyperpolarization via EC K^+ channels and the passage of hyperpolarizing current to the adjacent VSMCs, which relies on the myoendothelial gap junctions present in rat septal arteries (Smith et al., 2008). A clear demonstration of an endothelial action of CGRP would require freshly isolated septal artery ECs, either dispersed or as EC tubes for electrophysiological recordings. While Boerman and Segal (2016) showed CGRP hyperpolarized ECs of mouse mesenteric arteries, the isolation of EC tubes or even single ECs has not yet been established using rat septal arteries and is beyond the scope of this study. Our study does indicate that CGRP releases NO, presumably from the endothelium, and that $K_V7.4$ and $K_V7.5$ channels and the α-subunit of the BK_{Ca} channel are

expressed in the ECs of the rat septal arteries which will inform future studies. Demonstrating whether the apparent EC BK_{Ca} channels are functional alone or in combination with the β-subunit will be important. In general terms, our data highlight both K_V7 and BK_{Ca} channels, and suggest that both forms of voltage-dependent K^+ channel must be considered in the modulation of VSMC membrane potential.

It also remains unclear how activation of the Gβγ subunit of the CGRP receptor leads to the opening of K_V7 and BK_{Ca} channels in VSMCs, and potentially in ECs. This mechanism warrants further investigation, for example using proximity ligation assays. Potential avenues to explore regarding $K_V7.4$ channels include findings from rat renal arteries demonstrating that the Gβγ subunit regulates $K_V7.4$ channel activity following stimulation with isoprenaline, seemingly via an interaction with A-kinase anchoring protein (Stott et al., 2018; Stott & Greenwood, 2024). It would appear, however, that the rat septal arteries may form a different category of protein interactions wherein the vasorelaxation response to CGRP is gallein-sensitive but not sensitive to inhibition of PKA, a combination of signalling not observed in rat mesenteric and renal arteries during stimulation with isoprenaline (Stott et al., 2016, 2018). For example, **exchange protein directly activated by cAMP (Epac)** may interact with small GTPases (Ras-related proteins, Raps) to activate $K_V7.4$ channels. Alternatively, and perhaps likely, there may be no role for adenylate cyclase. To establish this will require multiple approaches combined with pharmacological tools. The presence of $K_V7.4$ channels, either alone or as heteromers with $K_V7.5$, means that their regulation by the Gβγ subunit and other factors (e.g. KCNE4) is complex but highly relevant (reviewed by Stott & Greenwood, 2024). Notably, regulation of BK_{Ca} by any receptor-associated Gβγ subunit has not been previously reported. There is however evidence that direct activation of the Epac pathway can increase the open probability of VSMC ryanodine receptors (Humphries et al., 2017; Lezoualc'h et al., 2016), which in rat septal arteries could lead to activation of BK_{Ca} channels. The gallein-insensitive component of the CGRP response was partly sensitive to NOS inhibition; therefore, it is possible that there is a parallel pathway contributing to CGRP-induced vasorelaxation involving NO release and K_V7 channel activation as this residual component was also sensitive to linopirdine. There is precedence for this suggestion as the NO-cGMP-PKG signalling pathway can activate K_V7 channels (Mondéjar-Parreño et al., 2019; Stott et al., 2015). The nature of the interaction between the CGRP receptor Gβγ subunit and eNOS remains unclear and will require investigation using gallein-sensitive concentrations of CGRP.

Since only male rats were used in the present study, to limit intersex variability, the conclusions generated from our study may not apply to females. Both pregnancy and female sex steroid hormones have been shown to enhance the vasodilation response to CGRP (Gangula et al., 1999, 2001, 2002), with evidence reporting an increase in CGRP receptor density (Yallampalli et al., 2004) and CGRP mRNA levels in the dorsal root ganglia during pregnancy or to oestradiol and **progesterone** treatment (Gangula et al., 2000). Gangula et al. (2001) demonstrated that the coronary vasculature was more sensitive to CGRP during pregnancy and in ovariectomized rats treated with oestradiol and progesterone. Interestingly, both migraine and CMVD are more prevalent in women; therefore, this may reflect sex differences in CGRP signalling.

The major findings of this study in intramuscular coronary septal arteries during myogenic tone are that CGRP stimulates hyperpolarization and vasorelaxation via activation of the CGRP receptor, with a central role for the Gβγ subunit in mediating these effects. Both K_V7 and BK_{Ca} channels are activated in this pathway, and there is also evidence for a parallel contribution from NO. Future studies will aim to establish the regulation of these K^+ channels by the Gβγ subunit and the precise role of the endothelium in these responses. Our data demonstrate CGRP as a potent vasodilator in small coronary arteries and provide new insight into the cellular mechanisms that allow it to suppress coronary vasoreactivity.

AUTHOR CONTRIBUTIONS

Lucy Donovan A: Conceptualization; formal analysis; writing—original draft; investigation; writing—review and editing. **JinHeng Lin:** Conceptualization; formal analysis; investigation; supervision; writing—review and editing; validation; writing—original draft; visualization. **Milind Khashu:** Investigation. **Christopher Garland J:** Formal analysis; investigation; writing—review and editing; funding acquisition. **Kim Dora A:** Conceptualization; formal analysis; visualization; investigation; supervision; project administration; writing—review and editing; funding acquisition; validation; writing—original draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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.

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, Immunoblotting and Immunochemistry and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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