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RESEARCH PAPER

Protein kinase A facilitates relaxation of mouse ileum via phosphorylation of neuronal nitric oxide synthase

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Background and Purpose: The enteric neurotransmitter nitric oxide (NO) regulates gastrointestinal motility by relaxing smooth muscle. Pharmacological cAMP induction also relaxes gastrointestinal smooth muscle, but it is uncertain whether cAMP augments or suppresses enteric NO signalling. In other organ systems, cAMP can increase neuronal NO production by stimulating protein kinase A (PKA) to phosphorylate neuronal NOS (nNOS) Serine-1412 (S1412). We hypothesized that cAMP also increases nNOS S1412 phosphorylation by PKA in enteric neurons to augment nitrergic relaxation of mouse ileum.

Experimental Approach: We measured contractile force and nNOS S1412 phosphorylation in ileal rings suspended in an organ bath. We used forskolin to induce cAMP-dependent relaxation of wild type, nNOS^{S1412A} knock-in and nNOSα-null ileal rings in the presence or absence of PKA, protein kinase B (Akt) and NOS inhibitors.

Key Results: Forskolin stimulated phosphorylation of nNOS S1412 in mouse ileum. Forskolin relaxed nNOSα-null and nNOS^{S1412A} ileal rings less than wild-type ileal rings. PKA inhibition blocked forskolin-induced nNOS phosphorylation and attenuated relaxation of wild type but not nNOS^{S1412A} ileum. Akt inhibition did not alter nNOS phosphorylation with forskolin but did attenuate relaxation of wild type and nNOS^{S1412A}. NOS inhibition with L-NAME eliminated the effects of PKA and Akt inhibitors on relaxation.

Conclusion and Implications: PKA phosphorylation of nNOS S1412 augments forskolin-induced nitrergic ileal relaxation. The relationship between cAMP/PKA and NO is therefore synergistic in enteric nitrergic neurons. Because NO regulates gut motility, selective modulation of enteric neuronal cAMP synthesis may be useful for the treatment of gastrointestinal motility disorders.

Abbreviations: 1400W, *N*-([3-(aminomethyl)-phenyl]-methyl)-ethanimidamide HCl; CaM, calmodulin; CFKB, calcium-free Krebs buffer; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; eNOS KO nNOS^{S1412A}, mice doubly homozygous for the nNOS^{S1412A} mutation and eNOS knockout; eNOS KO, mice lacking eNOS; eNOS, endothelial NOS; EPAC, exchange factor activated by cAMP; FSK, forskolin; GI, gastrointestinal; H-89, *N*-[2-(*p*-Br-cinnamylamino)-ethyl]-5-isoquinolinesulfonamide HCl; IJP, inhibitory junction potential; KB, Krebs buffer; L-NAME, L-Nitro-L-arginine methyl ester; Myr-PKI, myristoylated PKA inhibitor peptide, residues 14–22; NANT*N*-[(4S)-4-amino-5-[(2-aminoethyl)-amino]-pentyl]-*N*′-nitroguanidinetris trifluoroacetate; nNOS, neuronal NOS; nNOS^{S1412A}, mice with knock-in mutation of nNOS serine-1412 to alanine; nNOSα KO, mice lacking the first exon of nNOS; ODQ, 1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one; pS1412, nNOS phosphorylated at serine-1412; S1179, serine-1179 of mouse eNOS; S1412, serine-1412 of mouse nNOS; sGC, soluble GC; TTX, tetrodotoxin; WT, wild type.

1 | INTRODUCTION

Nitric oxide (NO) is a NANC neurotransmitter that relaxes smooth muscle via the soluble guanylate cyclase (sGC)-**cGMP-PKG** pathway in myocytes (D. D. Guerra & Hurt, 2019; Lefebvre, Smits, & Timmermans, 1995). The primary NO source in the gastrointestinal (GI) tract is **neuronal NOS** (nNOS), expressed in inhibitory myenteric neurons (Mang, Truempler, Erbelding, & Kilbinger, 2002). Neuronal depolarization increases NO synthesis by raising intracellular [Ca^{2+}], which stimulates nNOS via Ca^{2+}/**calmodulin** (CaM; Thatte, He, & Goyal, 2009). nNOS Serine-1412 (S1412) phosphorylation by **PKA** promotes nitrergic relaxation of penile smooth muscle (Hurt et al., 2012). We recently showed that low frequency depolarization relaxes mouse ileum via **Akt** phosphorylation of nNOS S1412 (D.D. Guerra et al., 2019); this may explain why NO responses are tuned to depolarization frequency in the gut (Spencer et al., 2018) and why nitrergic inhibitory junction potentials (IJPs) have a longer duration than action potentials (Hurt et al., 2012; Keef et al., 2013).

cAMP is a second messenger for **β-adrenoceptors** and **vasoactive intestinal peptide** receptors that relax GI smooth muscle by stimulating PKA phosphorylation of myocyte **K^+ channels** (Koh, Sanders, & Carl, 1996; Smith et al., 1993). cAMP pathways are less clear in enteric neurons. The adenylate cyclase (AC) activator **forskolin** (FSK) depolarizes afferent neurons in guinea pig small intestine myenteric plexus (Nemeth, Palmer, Wood, & Zafirov, 1986), and selective neuronal expression of a dominant negative **PKA regulatory subunit** causes intestinal pseudo-obstruction in mice (Howe et al., 2006). These studies suggest that neuronal cAMP/PKA may regulate GI motility.

Interactions between cAMP and NO pathways are not clearly defined in the gut. A cAMP analogue relaxes ileum from wild type (WT) and protein kinase G type 1 (PKG1)-deficient mice with equal efficacy (Bonnevier, Fässler, Somlyo, Somlyo, & Arner, 2004), and forskolin does not depolarize guinea pig myenteric plexus efferent motor neurons (Nemeth et al., 1986). However, pharmacological cAMP elevation blocks nitrergic IJPs in mucosa-denuded mouse colon (Hwang et al., 2008), suggesting cAMP- and NO-relaxation pathways are antagonistic or independent. Other studies have drawn contrary conclusions. For example forskolin sensitizes rat ileum to NO donor relaxation (Ekblad & Sundler, 1997), and pharmacological inhibition of cAMP and cGMP additively impairs mouse ileal relaxation by **relaxin** (Idrizaj, Garella, Francini, Squecco, & Baccari, 2018). These findings imply that cAMP and NO pathways can interact. Prior work has not demonstrated a mechanism by which physiological cAMP–NO synergy promotes GI smooth muscle relaxation, and the cell type (e.g. myocytes, neurons and endothelium) has not been identified.

Forskolin relaxation of human corpus cavernosum requires cGMP (Uckert et al., 2004). cAMP–NO synergy may occur in neurons because forskolin relaxes rat corpus cavernosum by activating PKA to phosphorylate cavernosal nerve nNOS S1412 (Hurt et al., 2012). We reasoned that cAMP–NO synergy in enteric neurons might also promote GI smooth muscle relaxation. Using kinase and NO pathway inhibitors and non-phosphorylatable $nNOS^{S1412A}$ knock-in mice, we assessed forskolin relaxation of ileal rings. We found that PKA phosphorylation of nNOS S1412 mediates a significant portion of forskolin relaxation of mouse ileum, suggesting that enteric neuronal cAMP promotes nitrergic GI relaxation. Our findings reconcile prior conflicting reports on the relationship between cAMP/PKA and NO-cGMP-PKG signalling in forskolin relaxation of GI smooth muscle.

What is already known

- nNOS and cAMP each regulate gastrointestinal motility, and PKA activates nNOS in other organ systems.
- cAMP/PKA is reported to both facilitate and antagonize nitrergic gastrointestinal relaxation.

What does this study add

- Forskolin stimulates PKA via cAMP to phosphorylate and activate nNOS in mouse ileum.
- In enteric neurons, forskolin-stimulated NO signalling is partly mediated by nNOS Serine-1412 phosphorylation.

What is the clinical significance

- Enteric neuronal PKA may influence gastrointestinal motility and transit rates.
- Selective stimulation of cAMP pathways in enteric nitrergic neurons may improve gastrointestinal dysmotility symptoms.

2 | METHODS

2.1 | Animals

2.1.1 | Ethics statement and husbandry

The University of Colorado Institutional Animal Care and Use Committee approved all animal procedures (IACUC protocol 90). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*. All animals were male C57Bl/6 mice (*Mus musculus*) and maintained by Office of Laboratory Animal Research (OLAR) veterinary technicians at 20°C in a 12-hr day/night cycle vivarium with ad libitum standard rodent chow (Teklad 8640). Mice were housed two to five animals per cage in Allentown 75 JAG modular ventilated cages with hardwood bedding. To maintain hybrid vigour, we backcrossed mutant males to WT C57Bl/6 females and genotyped pups at postnatal day 9 via tail snip. All mutant mice were F5 generation or later. Mice were 10–40 weeks of age (median 16 weeks) and weighed 29–43 g

(median 33 g) at euthanasia via CO_2 asphyxiation and cervical dislocation.

2.1.2 | Mouse strains

Non-phosphorylatable nNOS knock-in mutant (nNOS^{S1412A}): The nNOS^{S1412A} knock-in mouse is homozygous for the nNOS^{S1412A} allele, in which the nNOS Serine-1412 phosphorylation site is replaced with non-phosphorylatable alanine. *nNOS knockout (nNOSα KO):* The nNOSα null strain (Jackson Laboratory 002986) is homozygous for the *NOS1^{tm1Plh}* allele, which lacks the membrane localizing PDZ domain (amino acids 1–159). These mice show ~90% reduced nNOS activity, but nNOSβ is still expressed throughout the cytoplasm. **Endothelial NOS** *knockout (eNOS KO):* The eNOS null strain (Jackson Laboratory 002684) is homozygous for the *NOS3^{tm1Unc}* allele, which abolishes eNOS enzymatic activity. *nNOS^{S1412A}/eNOS KO double mutants:* Mice doubly homozygous for the nNOS^{S1412A} knock-in and eNOS knockout mutations were obtained by nNOS^{S1412A} × eNOS KO crosses. *WT:* WT mice were obtained from Charles River laboratories or by heterozygous nNOS^{S1412A} crosses.

2.2 | Study design

2.2.1 | Groups

For all forskolin experiments, we defined control and treatment groups as follows:

1. Chemical treatments of one genotype: Controls received vehicle, 0.1% (*v*/v) DMSO or water. The treatment group received one drug in the same volume of DMSO or water. For L-NAME experiments, controls received L-NAME and vehicle, while treatment groups received L-NAME and a drug in the same volume as vehicle.
2. Comparison by genotype: The control group was WT or eNOS KO. The treatment groups were nNOS^{S1412A}, nNOSα KO or eNOS KO/nNOS^{S1412A}.
3. Immunoblots of nNOS S1412 phosphorylation: The control group received 0.1% (v/v) DMSO vehicle. The treatment group received 30- or 300-nM forskolin. If the experiment used kinase inhibitors, controls received Akt or PKA inhibitor *and* vehicle, while treatment groups received the same volume of Akt or PKA inhibitor *and* 300-nM forskolin.

2.2.2 | Sample sizes and randomization

For all experiments, "*n*" is the number of individual ileal rings obtained from two or more mice. In our prior study (D.D. Guerra et al., 2019), nNOS^{S1412A} mutants exhibited impaired EFS relaxation, with relative effect sizes and SDs of ~1.5 and ~30% of the mean respectively. Assuming similar parameters for forskolin ileal relaxation and using power = 0.8 and α = 0.05, we calculated a sample size of at least n = 5. Considering previous pharmacological studies, we planned to use N = 6–15 (Mo, Michel, Lee, Kaumann, & Molenaar, 2017; Shuttleworth, Sanders, & Keef, 1993; Zavala-Mendoza, Grasa, Zavala-Sánchez, Pérez-Gutiérrez, & Murillo, 2016). Deviation from equal allocation occurred when some rings were unresponsive or failed to respond to **substance P** and were therefore discarded prior to the experiment. The number of vehicle samples exceeds that of specific chemical treatments because controls accompanied each chemical treatment (Motulsky & Michel, 2018) and identically treated rings from different experiments and mice were combined for analysis. We divided each mouse ileum into rings that were randomly placed into separate organ bath chambers and assigned to control or treatment conditions. We were unable to perform blinding because ear tags identified strain and because many chemical treatments predictably altered ileal tension.

2.3 | Ileal ring preparation and organ bath pharmacology

We removed mesentery from the distal 8 cm of small intestine 1 cm proximal to the ileocecal valve and placed the ileum in ice-cold 95% O_2/5% CO_2 (95/5)-perfused Ca^{2+}-free Krebs buffer (CFKB; 118-mM NaCl +4.7-mM KCl + 1.2-mM $MgSO_4$ heptahydrate + 25-mM $NaHCO_3$ + 1.2-mM KH_2PO_4 + 11 mM **glucose** + 5-mM HEPES + 50-μM EDTA + 100-μM EGTA). We flushed the lumen with CFKB using an 18-gauge 1.8 × 40 mm blunt tip syringe, replaced buffer with fresh CFKB, and cut the ileum into 0.5-cm circular segments with a clean razor blade. We performed all organ bath experiments at 37°C using tandem Radnoti 159920-X1/10 systems and 95/5 oxygenated Krebs buffer (KB; CFKB + 3.3-mM $CaCl_2$, without EGTA). To maintain NANC conditions (Shuttleworth et al., 1993), we added **atropine**, **propranolol**, **phentolamine** (all 1 μM) and **indomethacin** (10 μM). Ileal rings were positioned in organ bath chambers between supports and stationary mounts (Radnoti 158817 and 160152-14) and MLT0201/RAD force transducers (AD instruments). Powerlab 16/35 and LabChart7.0 (AD Instruments) enabled detection and recording of ileal tensile force.

Ileal rings were equilibrated for 15 min under NANC conditions before adding vehicle or inhibitors. To inhibit PKA, we used 1-μM **H-89**, 25-μM **Rp-cAMPs**, or 10-μM Myr-PKI. To inhibit Akt, we used 10-μM **MK-2206**. To inhibit sGC and PKG, we used 5-μM **ODQ** or 5-μM Rp-8-Br-cGMPS respectively. cPTIO (300 μM) was used to scavenge NO. To inhibit all NOS activity non-selectively, we used 1-mM L-**NAME**. To inhibit nNOS selectively over eNOS, we used 100-μM NANT (*N*-[(4S)-4-amino-5-[(2-aminoethyl)-amino]-pentyl]-*N*′-nitroguanidinetris trifluoroacetate) or 50-μM **1400W**. L-**Arginine** (2 mM) was used to reverse L-NAME effects. To inhibit neuronal depolarization we used 10-μM **TTX**. Prior publications indicate that these concentrations of H-89, Myr-PKI, MK-2206, ODQ, cPTIO, L-NAME, 1400W and TTX are selective (Denisova et al., 2014; Kaya

et al., 2012; Lefebvre et al., 1995; Mang et al., 2002; May et al., 2019; Paisley & Martin, 1996; Tajima et al., 2012).

After 15 min of vehicle or inhibitor treatment, we added 1-μM substance P (Briejer, Akkermans, Meulemans, Lefebvre, & Schuurkes, 1993) to ensure tissue viability and promote consistent contractility during the entire concentration–response curve. We discarded rings that did not respond to substance P; 5 min after substance P and with stable regular baseline contractility, we cumulatively increased [forskolin] every 2 min to 0.1-, 1.1-, 11.1-, 44.1-, 144-, 477- and 1480-nM forskolin. To evaluate relaxation with the stable cAMP analogue Sp-cAMPs, we added 25-μM Sp-cAMPs or water 8 min after substance P.

Control experiments with WT ileal rings (Figure S2) revealed no loss of tensile force at 0.1-nM forskolin relative to vehicle (0.01% (*v*/v) DMSO). Therefore, we normalized mean tensile force at each [forskolin] to mean tensile force after 2-min incubation with 0.1-nM forskolin. Mean tensile force is the average phasic contraction force relative to baseline and was calculated by dividing the AUC by the time interval.

2.4 | Estimating the proportion of nitrergic forskolin relaxation

To estimate the contribution of nitrergic/NOS sources to forskolin relaxation, we compared mean tensile force at multiple [forskolin] for control (WT or vehicle) and experimental groups (mutant strains or pharmacological treatments). The groups were WT treated with vehicle (Figure 4c), L-NAME (Figure 1c), NANT (Figure 4c) or H-89 (Figure 1b); $nNOS^{S1412A}$ treated with vehicle (Figure 3c), L-NAME (Fig., 3D) or H-89 (Figure 3c); and eNOS KO treated with vehicle (Figure 4a) or L-NAME (Figure 4a). Our procedure precluded variance calculation and was therefore a non-statistical exploratory analysis giving a quantitative estimate of the contribution of NO synthesis or nNOS S1412 phosphorylation to forskolin relaxation. We defined relaxation as the inverse of mean tensile force (i.e. 100—mean tensile force). To calculate total percent relaxation due to treatment (Figure S7), we subtracted experimental relaxation from control relaxation. To calculate relative relaxation (i.e. proportion of relaxation at a given forskolin concentration due to treatment; Figure S8), we divided the difference in control and experimental relaxation by control relaxation and expressed as a new percentage. Values less than or equal to zero were expressed as zero.

2.5 | Tissue collection, protein extraction, and immunoblotting

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). To evaluate nNOS S1412 phosphorylation, we added vehicle (0.1% DMSO), 30- or 300-nM forskolin to ileal rings in organ baths 5 min after substance P. To assess PKA and Akt inhibition, we added H-89, Rp-cAMPs or MK-2206 to organ baths 15 min prior to substance P. Ileal rings were vitrified in liquid nitrogen 2 min after FORSKOLIN or vehicle treatment and homogenized in 25-mM Tris–HCl (pH 7.5) + 1-mM EGTA + 1-mM DTT + 0.4% (v/v) Triton X-100 + 1× protease and phosphatase inhibitors. Homogenates were centrifuged at 16,000× *g*, and supernatant protein concentration was determined by the ThermoFisher Pierce 660-nm assay. Supernatants were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies to nNOS (ImmunoStar, RRID AB_572255; 1:500) or phospho-nNOS S1412 (pS1412; Abcam, RRID 304964; 1:250) as described previously (Hurt et al., 2012). We quantified protein band fluorescence with Li-Cor Odyssey Image Studio 5.2. We expressed nNOS pS1412 relative to total nNOS in the same sample and normalized to the mean pS1412/total nNOS ratio for vehicle treatments without inhibitors.

2.6 | Statistical tests

We calculated statistics using GraphPad Prism 7.04 with groups of $N \geq 6$ and significance set at $P < .05$ (denoted by *). "N" refers to independent biological samples. We plotted normalized tensile force versus log [forskolin] and fit data with log [Inhibitor]—normalized response least squares sigmoidal regression models with variable Hill slopes to determine relative IC_{50} values for forskolin relaxation. We used normalized data to compare IC_{50} values, an accepted procedure to correct for initial variation (i.e., variable contractility due to differences in ileal ring size and positioning; Sebaugh, 2011). Sigmoidal curves were compared via extra sum of squares *F* tests to determine if one curve adequately explained all data. All regressions exhibited $P > .05$ for the replicates test (Table S1), suggesting goodness of fit. To determine if forskolin promoted nNOS S1412 phosphorylation and if Sp-cAMPs relaxed WT and $nNOS^{S1412A}$ rings, we used Kruskal–Wallis non-parametric ANOVA. We conducted Dunn's post hoc tests only if ANOVA 'F' values coincided with $p<0.05$ and variance inhomogeneity was not significant. All samples subjected to statistical analysis contained a minimum of n = 5 ileal rings, where n = biologically independent values. To determine if Akt or PKA inhibitors affected forskolin-induced nNOS S1412 phosphorylation, we performed unpaired, two-tailed non-parametric Mann–Whitney tests for each drug treatment. To estimate fold-relaxation of a treatment relative to a control group, we divided the IC_{50} of the treatment group by the IC_{50} of the control group. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.7 | Materials

Forskolin, 1,9-dideoxyforskolin, dithiothreitol (DTT), protease and phosphatase inhibitors, NANC inhibitors, substance P, L-Nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one (ODQ), tetrodotoxin (TTX) and all bulk reagents were from Millipore-Sigma (Burlington, USA). The 100× protease inhibitor cocktail comprised 104-mM AEBSF + 80-μM

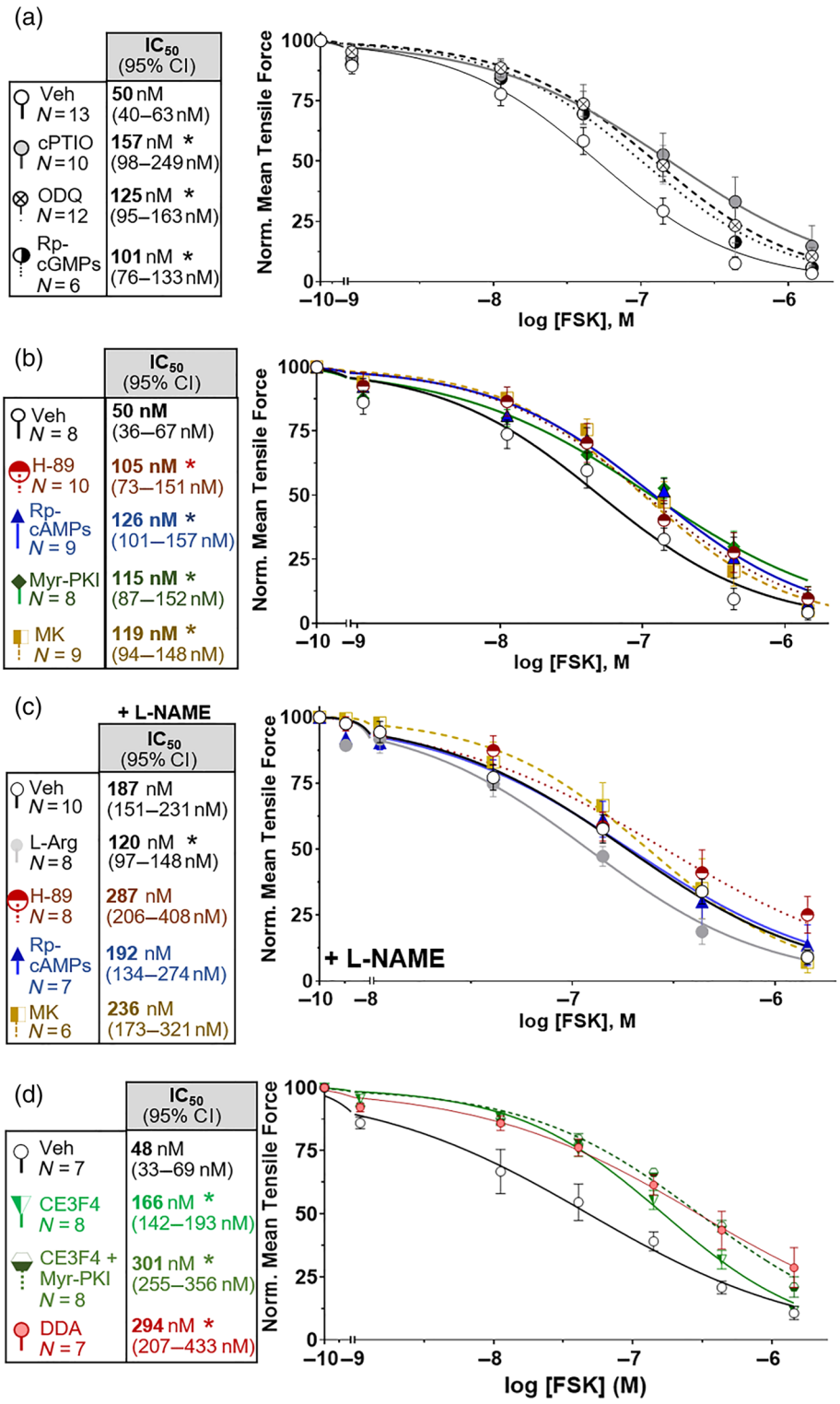

FIGURE 1 PKA and Akt inhibitors attenuate nitrergic forskolin (FSK) relaxation of ileum. (a) The NO signalling inhibitors cPTIO (300 μM), ODQ (5 μM), and Rp-cGMPs (5 μM) attenuate FSK relaxation. (b) PKA inhibitors (H-89, 1 μM; Myr-PKI, 10 μM; and Rp-cAMPs, 25 μM) and the Akt inhibitor MK-2206 (MK; 10 μM) attenuate FSK relaxation. (c) Akt and PKA inhibitors do not further attenuate FSK relaxation under NOS blockade with L-NAME (1 mM), but the NOS substrate L-Arginine (L-Arg; 2 mM) partially restores FSK relaxation. (d) The EPAC inhibitor CE3F4 (10 μM) attenuates FSK relaxation independently of PKA, and adenylate cyclase blockade with dideoxyadenosine (DDA; 20 μM) attenuates relaxation as much as combined PKA and EPAC inhibition. FSK IC_{50} values are bold, and 95% confidence intervals are in parentheses. $^{*}P < .05$ versus Veh IC_{50}. Veh: DMSO = control. Error bars: SEM. *n*, number of ileal rings

aprotinin + 4-mM bestatin + 1.4-mM E-64 + 2-mM leupeptin + 1.5-mM pepstatin A. The 100× phosphatase inhibitor cocktail was a proprietary mixture of Na_3VO_4, Na_2MoO_4, tartaric acid and imidazole. Myristoylated PKA inhibitor residues 14–22 (Myr-PKI), and PVDF membranes were from ThermoFisher (Waltham, USA). *N*-[2-(*p*-Br-cinnamylamino)-ethyl]-5-isoquinolinesulfonamide HCl (H-89), *N*-([3-(aminomethyl)-phenyl]-methyl)-ethanimidamide HCl (1400W) and 2-(4-carboxyphenyl)-4,-4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were from Cayman Chemical (Ann Arbor, USA). MK-2206 was from Apex Bio (Boston, USA). Rp- and Sp-cAMPs, Rp-8-Br-cGMPs and *N*-[(4S)-4-amino-5-[(2-aminoethyl)-amino]-pentyl]-*N*′-nitroguanidine tris (trifluoroacetate; NANT) were from Santa Cruz Biotech (Dallas, USA).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos, et al., 2019; Alexander, Fabbro, et al., 2019; Alexander, Kelly, et al., 2019).

3 | RESULTS

3.1 | PKA, Akt and NOS facilitate forskolin relaxation of mouse ileum

NO and cAMP can each promote GI relaxation in rodents, but reports vary on whether cAMP facilitates or antagonizes nitrergic relaxation in the gut (Bonnevier et al., 2004; Hwang et al., 2008). To assess if cAMP acts via NO, we treated ileal rings from WT mice with forskolin in the presence of NO signalling pathway inhibitors and measured circular smooth muscle relaxation. Forskolin selectively activates AC over sGC (Allerston, von Delft, & Gileadi, 2013). An NO scavenger (cPTIO), a sGC inhibitor (ODQ) and a PKG inhibitor (Rp-8-Br-cGMPs) increased the IC_{50} of forskolin 2- to 3-fold over vehicle (Figures 1a and S1), indicating that the NO-cGMP-PKG pathway facilitates a portion of forskolin relaxation. While forskolin promoted ileal relaxation, vehicle DMSO and the inactive analogue 1,9-dideoxyforskolin did not (Figure S2).

Next, we examined which forskolin-induced relaxation mechanisms require NO. Downstream targets of cAMP include the protein kinases PKA and Akt (Garcia-Morales, Luaces-Regueira, & Campos-Toimil, 2017), both of which activate nNOS via S1412 phosphorylation in other organ systems (Hurt et al., 2012; Rameau et al., 2007). When we inhibited PKA with competitive (H-89 and Myr-PKI) and allosteric (Rp-cAMPs) antagonists, we observed a 2- to 2.5-fold increase in the IC_{50} of forskolin (Figure 1b). The NOS inhibitor L-NAME increased the IC_{50} of forskolin 3.7-fold over vehicle treatment, and PKA inhibitors failed to shift the IC_{50} further than L-NAME alone. However, the NOS substrate L-arginine partly reversed the L-NAME attenuation of forskolin relaxation, confirming that L-NAME inhibition was related to NO (Figure 1c). Although we cannot rule out incomplete PKA inhibition, similar IC_{50} values with multiple PKA inhibitors imply that NO facilitates most of the PKA-dependent relaxation. We also tested whether forskolin relaxation requires Akt. The Akt inhibitor MK-2206 attenuated forskolin relaxation to the same extent as PKA inhibitors, and MK-2206 did not shift the IC_{50} further than L-NAME alone (Figure 1b–c), indicating that cAMP targets other than PKA may be involved. Because exchange factor activated by cAMP (EPAC) mediates cAMP-dependent Akt activation (Garcia-Morales et al., 2017), we tested the EPAC inhibitor CE3F4. We found that CE3F4 alone or in combination with Myr-PKI increased the forskolin IC_{50} 3.4-fold and 6.3-fold respectively (Figure 1d). The AC inhibitor dideoxyadenosine attenuated relaxation as much as combined PKA and EPAC inhibition, but complete relaxation still occurred (Figure 1d), indicating that some forskolin relaxation is cAMP-independent. Together, these data suggest that forskolin stimulates relaxation via distinct PKA and Akt pathways, both of which are

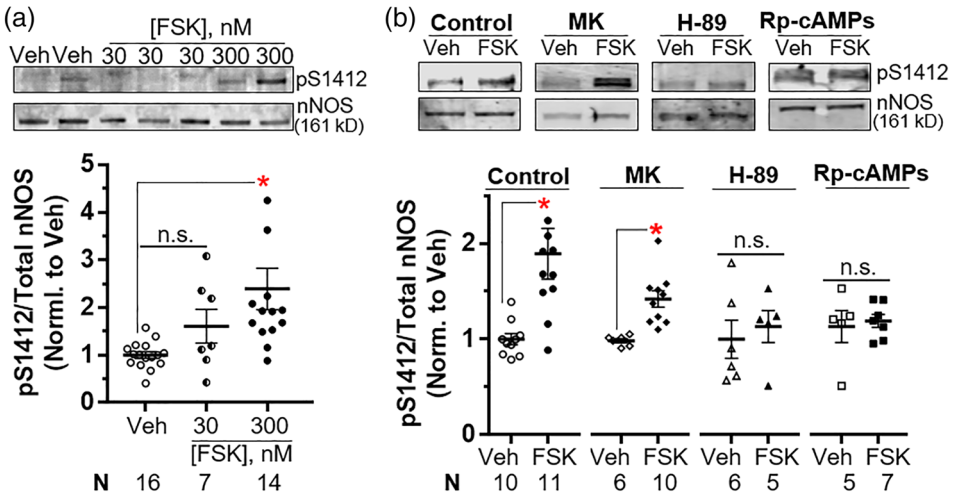

FIGURE 2 Forskolin (FSK) promotes nNOS S1412 phosphorylation by PKA in the ileum. (a) FSK enhances phosphorylation of nNOS at S1412 (pS1412). (b) PKA (H-89 and Rp-cAMPs), but not Akt (MK), inhibition blocks FSK-enhanced phosphorylation of nNOS S1412. Representative immunoblots and quantifications are shown on the top and bottom panels respectively. Quantification of pS1412/total nNOS is normalized to Veh (a) or Veh for each treatment (b). Symbols represent individual ileal samples, and bars are means ± SEM. FSK: 300 nM. $^{*}P < .05$ versus Veh by Kruskal–Wallis and Dunn's post hoc tests (a) or versus Veh for each treatment by Mann–Whitney tests (b). n.s., not significant

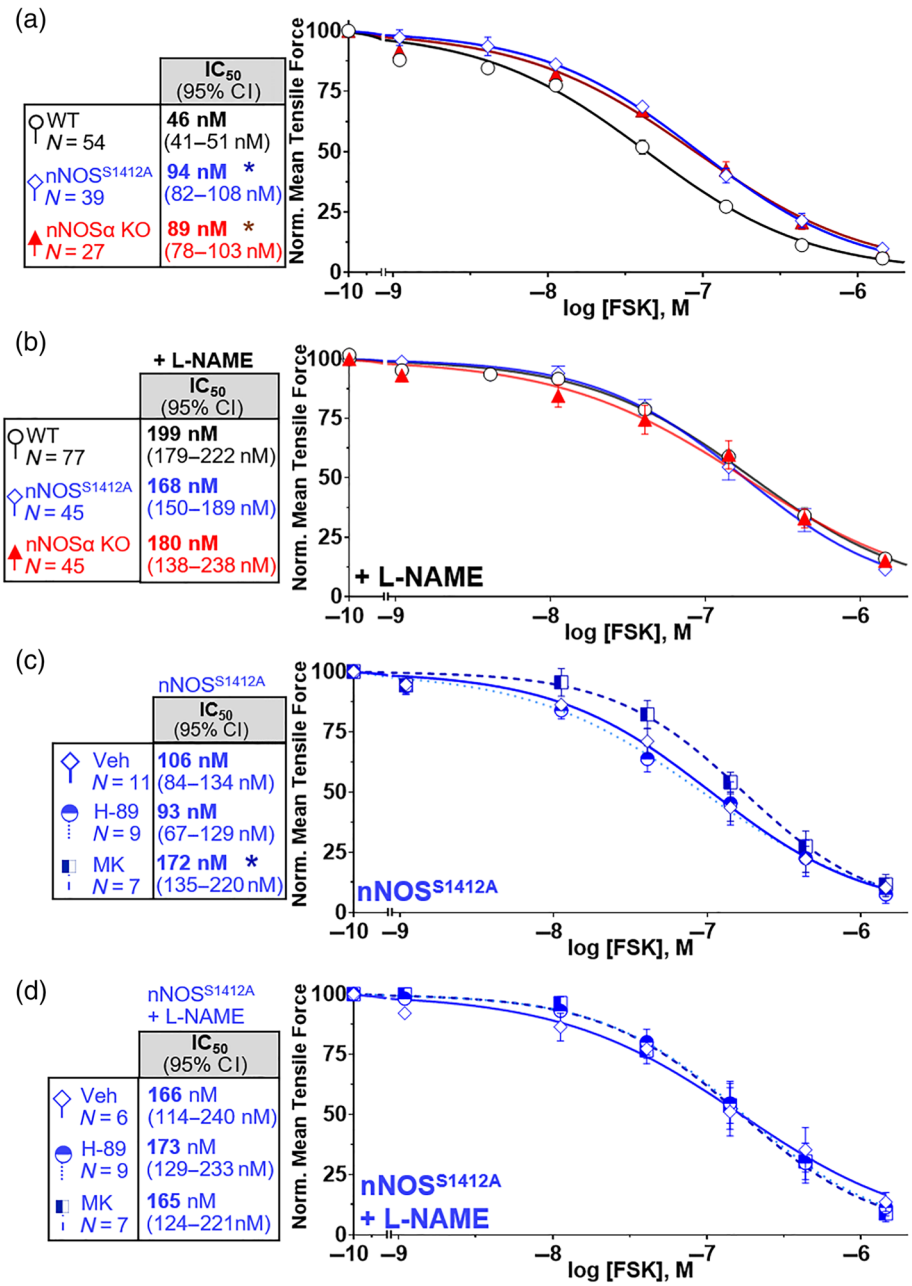

FIGURE 3 nNOS Serine-1412 potentiates forskolin (FSK) ileal relaxation. (a) FSK relaxation is reduced in both nNOS^{S1412A} and nNOSα-null (nNOSα KO) compared to wild-type (WT) mice. (b) WT, nNOS^{S1412A} and nNOSα KO ilea exhibit the same sensitivity to FSK under NOS blockade with L-NAME. (c) Akt (MK), but not PKA (H-89), inhibition further attenuates nNOS^{S1412A} ileal relaxation by FSK. D. Under NOS blockade with L-NAME, neither PKA (H-89) nor Akt (MK) inhibitors further attenuate FSK relaxation. $^{*}P < .05$ versus WT IC_{50} (a) or Veh IC_{50} (c)

cAMP-dependent. Forskolin induction of NO synthesis could involve one or both of these kinases.

3.2 | Forskolin stimulates PKA phosphorylation of nNOS Serine-1412

Because PKA and Akt phosphorylate nNOS S1412 in rat pelvic ganglia and hippocampal neurons (Hurt et al., 2012; Rameau et al., 2007), we examined whether forskolin induces PKA and/or Akt to phosphorylate nNOS S1412 in the ileum. Compared with vehicle treatment, 300-nM forskolin increased nNOS-S1412 phosphorylation twofold (Figure 2a). While the PKA inhibitors H-89 and Rp-cAMPs blocked forskolin-stimulated phosphorylation of nNOS S1412, the Akt inhibitor MK-2206 did not (Figure 2b). Thus, PKA and Akt both facilitate relaxation, but forskolin only induces PKA to phosphorylate nNOS S1412 in ileal neurons.

3.3 | A component of forskolin relaxation requires nNOS Serine-1412

Forskolin relaxation coincides with nNOS S1412 phosphorylation, so we wondered if nNOS S1412 phosphorylation facilitates forskolin relaxation. We previously developed a nNOS^{S1412A} knock-in mouse and determined that mutation of serine-1412 to alanine attenuates electrical field stimulation-induced ileal relaxation under NANC conditions. The nNOS^{S1412A} mutation increased the IC_{50} of forskolin by

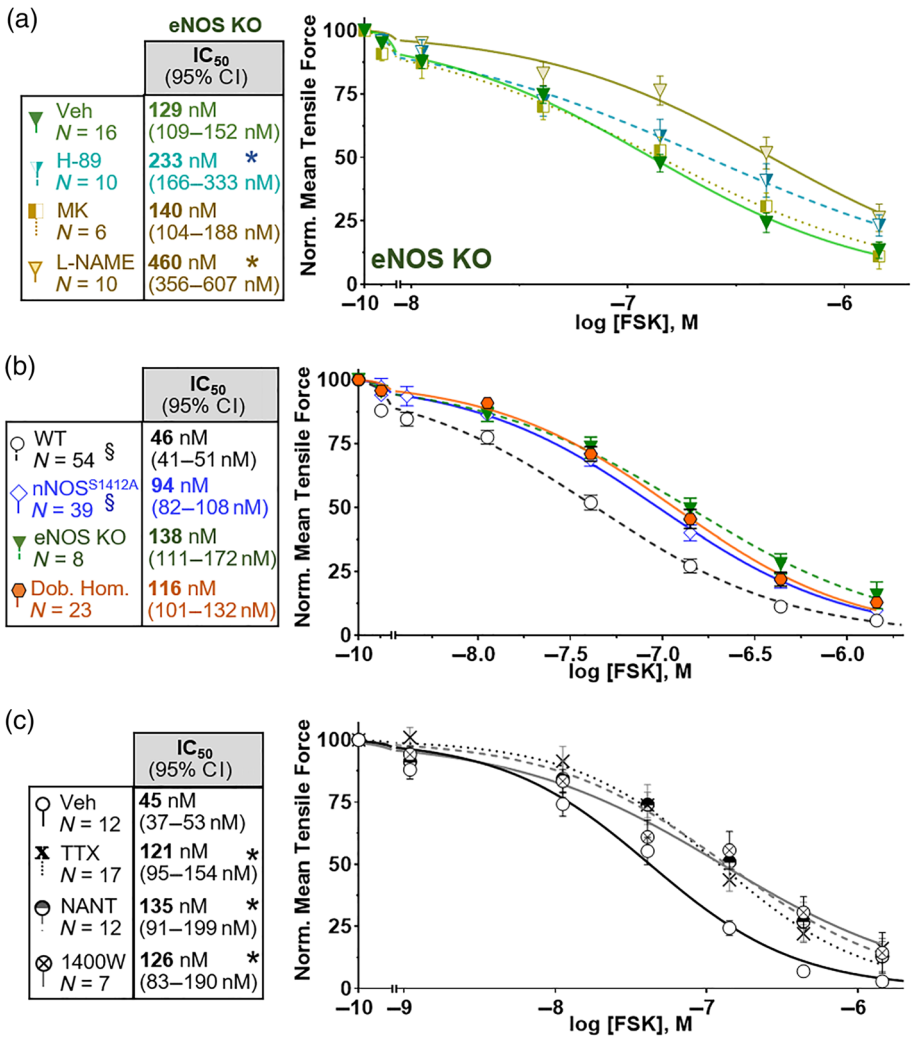

FIGURE 4 NO-dependent forskolin (FSK) relaxation primarily requires nNOS. (a) FSK relaxation of endothelial NOS-null (eNOS KO) ilea is sensitive to PKA (H-89) and NOS (L-NAME) inhibition, but not Akt inhibition (MK). (b) FSK relaxation of the eNOS KO nNOS^{S1412A} double homozygote (Dob. Hom.) is indistinguishable from the eNOS KO and nNOS^{S1412A} single mutants. (c) Inhibition of neuronal depolarization (TTX; 10 μM) or nNOS (100 μM NANT or 50 μM 1400W) blocks most NOS-dependent FSK relaxation. $^{*}P < .05$ versus Veh IC_{50} (a,c). §Datasets from Figure 3a repeated to illustrate non-additivity of eNOS KO and nNOS^{S1412A} mutations

2.1-fold compared to WT (Figures 3a and S3). The IC_{50} increased by 1.8-fold more under NOS blockade with L-NAME (Figure 3b). Because L-NAME blocks all NOS activity (Toque et al., 2013), this indicates that nNOS S1412 phosphorylation facilitates about half of NOS-dependent forskolin relaxation. The nNOS^{S1412A} mutation reduced ileal forskolin sensitivity to a similar extent as loss of nNOSα (nNOSα KO; Figure 3a). nNOSα KO mice lack the nNOS membrane-anchoring domain and exhibit only 5–10% of WT nNOS activity under physiological conditions (Huang, Dawson, Bredt, Snyder, & Fishman, 1993; Hurt et al., 2006). Interestingly, ileal rings from WT, nNOS^{S1412A} and nNOSα KO mice exhibited indistinguishable forskolin IC_{50} values under NOS blockade with L-NAME (Figure 3b). Thus, both membrane localization and S1412 phosphorylation likely facilitate nNOS-dependent forskolin relaxation.

To confirm that forskolin promotes PKA phosphorylation of nNOS S1412, we treated WT and nNOS^{S1412A} ileal rings with the direct PKA activator Sp-cAMPs. Compared with WT, nNOS^{S1412A} relaxed significantly less with Sp-cAMPs, and L-NAME blocked WT relaxation (Figure S4). We observed that L-NAME increased the basal tone of WT rings, but not of nNOS^{S1412A} rings, although subsequent substance P treatment abolished the L-NAME effect (Figure S4). Next, we measured forskolin relaxation of nNOS^{S1412A} ileal rings in the presence of H-89 or MK-2206. While MK-2206 increased the forskolin IC_{50} 1.6-fold, H-89 had no effect (Figure 3c). Thus, the nNOS^{S1412A} mutation blocks PKA-dependent forskolin relaxation but not the Akt-dependent effect. H-89 and MK-2206 also failed to increase the forskolin IC_{50} in nNOS^{S1412A} more than L-NAME alone (Figure 3d). Together, these data imply that forskolin stimulates PKA to promote ileal relaxation via nNOS S1412 phosphorylation.

3.4 | Nitrergic forskolin relaxation is primarily nNOS-dependent

In the presence of L-NAME, the Akt inhibitor MK-2206 did not increase the forskolin IC_{50} for WT rings (Figure 1c), suggesting that Akt-dependent relaxation requires NO synthesis. However, MK-2206 induced a similar approximately twofold increase in forskolin IC_{50} for both WT and nNOS^{S1412A} ileal rings, so Akt could mediate relaxation independently of nNOS S1412. Inducible NOS (iNOS) is not typically expressed in small intestine in the absence of inflammation (Cui et al., 1997), and histology showed no inflammatory infiltrate in WT or

nNOS^{S1412A} ilea (D.D. Guerra et al., 2019). The GI microvasculature expresses eNOS (Palatka et al., 2005), and Akt can phosphorylate serine 1179 in eNOS to increase Ca^{2+}-independent NO synthesis (Dimmeler et al., 1999; Fulton et al., 1999; Hurt et al., 2002). We found that eNOS-deficient (eNOS KO) rings were sensitive to the PKA inhibitor H-89 (1.8-fold forskolin IC_{50} increase), but MK-2206 had no effect (Figures 4a and S5). This is consistent with eNOS mediating Akt-dependent forskolin relaxation. Therefore, we assessed the eNOS contribution to overall nitrergic forskolin relaxation by treating eNOS KO rings with L-NAME. We observed an increase in the forskolin IC_{50} of 3.5-fold (Figure 4a). This mirrors attenuation of WT relaxation by L-NAME (Figure 3a–b) and shows that NO synthesis contributes similarly to forskolin relaxation in WT and eNOS KO ilea. Additionally, forskolin relaxation was similar for nNOS^{S1412A} single mutants and eNOS KO/nNOS^{S1412A} double mutants. Compared with WT, nNOS^{S1412A} and eNOS KO/nNOS^{S1412A} rings exhibited 2.1- and 2.5-fold higher forskolin IC_{50} values (Figure 4b). L-NAME increased the IC_{50} 1.6-fold for eNOS KO/nNOS^{S1412A} rings (Figure S6), comparable to the 1.8-fold increase for nNOS^{S1412A} rings, indicating that nitrergic forskolin relaxation is similar in both mutants. These data are consistent with a small role for Akt-mediated eNOS activation in NOS-dependent forskolin relaxation and affirms that eNOS is not the major source of nitrergic relaxation in the gut (Mang et al., 2002).

Forskolin can directly and indirectly promote K^+ channel closure and depolarization of enteric neurons (Hoshi, Garber, & Aldrich, 1988; Nemeth et al., 1986). Therefore, to estimate the contributions of neuronal depolarization and nNOS activity to forskolin-dependent relaxation, we measured forskolin relaxation of WT ilea in the presence of the neuronal Na^+ channel blocker TTX or the nNOS selective inhibitors NANT and 1400W (Boer et al., 2000; Hah, Roman, Martasek, & Silverman, 2001). TTX increased the forskolin IC_{50} 2.7-fold for WT ileal rings (Figure 4c), indicating that neuronal depolarization partially mediates forskolin-stimulated relaxation. Inhibition of nNOS with NANT and 1400W increased the IC_{50} 2.8- to 3-fold (Figure 4c). These values were slightly less than the 3.7-fold increase in IC_{50} obtained via blockade of all NOS activity with L-NAME (Figure 3b). To estimate

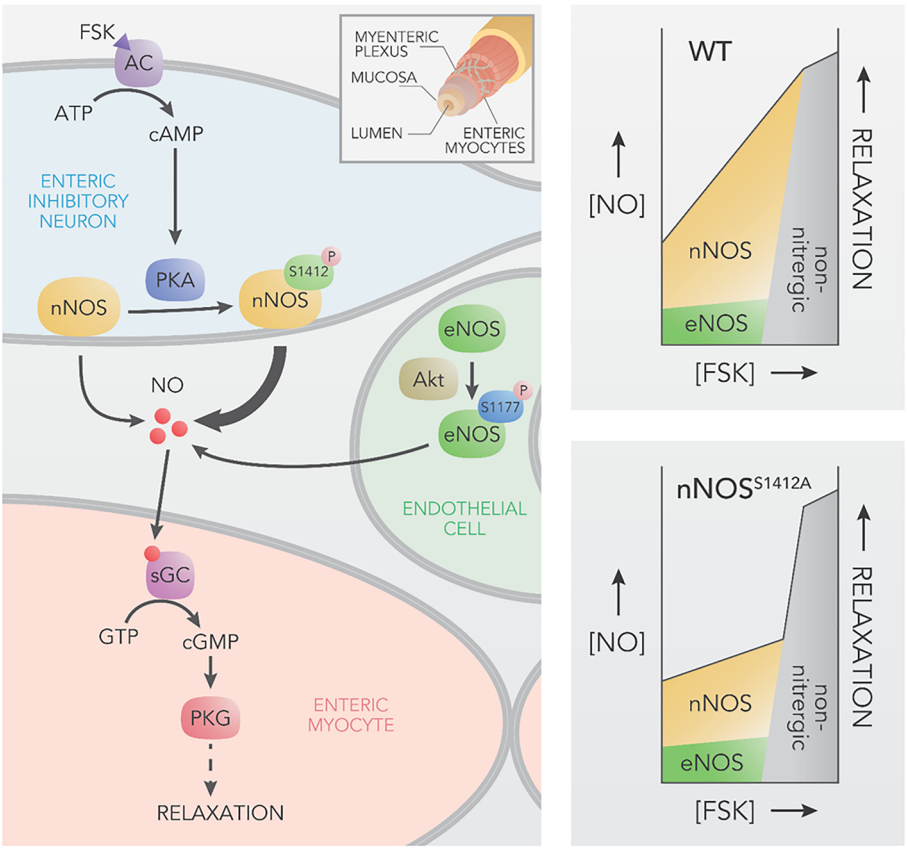

FIGURE 5 nNOS S1412 phosphorylation augments cAMP-dependent enteric relaxation. Forskolin (FSK) relaxes GI smooth muscle via nitrergic (NO-dependent) and non-nitrergic mechanisms. Left. A model for nitrergic FSK relaxation. FSK stimulates AC to synthesize cAMP, which activates PKA phosphorylation of nNOS S1412 in myenteric inhibitory neurons. Phosphorylation enhances nNOS activity, thereby increasing NO-cGMP-dependent relaxation in myocytes. Akt phosphorylation of eNOS facilitates basal NO release, but eNOS stimulation plays a small role in nitrergic FSK relaxation. Inset shows gut layers. Right. Contribution of nNOS, eNOS and other factors in FSK relaxation. Top. At low to moderate [FSK], nNOS activation is the primary relaxation mechanism in WT animals with a small contribution from eNOS. At high [FSK], relaxation is primarily non-nitrergic and may involve AC-independent effects of FSK. Bottom. nNOS^{S1412A} mutation abolishes most nitrergic FSK relaxation, but non-phosphorylation dependent nNOS activation and possibly eNOS can still mediate nitrergic relaxation at low to moderate [FSK]

the nitrergic component of forskolin relaxation, we calculated differences in ileal relaxation upon drug treatment or genetic knock-in mutation (nNOS^{S1412A}) compared with vehicle or WT controls. We estimated that nNOS mediates about 20% total relaxation at moderate forskolin concentrations (44–477 nM), of which two-thirds is due to nNOS S1412 (Figure S7). Thus, nNOS facilitates most nitrergic forskolin relaxation (via nNOS S1412 phosphorylation) with minimal additional contribution from eNOS. While low forskolin concentrations (1–44 nM) stimulate very slight absolute relaxation, the relative contribution of nNOS is 50–80% (Figure S8). At the highest forskolin concentration (1,440 nM), forskolin relaxation is large and predominantly NOS- and PKA-independent (Figures S7 and S8).

4 | DISCUSSION AND CONCLUSIONS

Our primary finding in this study is that cAMP increases nitrergic relaxation of the ileum by activating PKA in enteric neurons to phosphorylate nNOS S1412 (Figure 5). This complements our prior report that low frequency electrical field stimulation promotes nitrergic ileal relaxation via nNOS S1412 phosphorylation (D.D. Guerra et al., 2019) and shows that two different kinases can augment nNOS inhibitory signals in the gut. Our findings further suggest that exchange factor activated by cAMP (EPAC) may facilitate PKA-independent Akt activation of eNOS, but knockout animal experiments indicate that eNOS contributes only a minor fraction of forskolin relaxation. Other non-PKA targets of cAMP may also be involved. Nonetheless, this and our previous study demonstrate that distinct stimuli can activate Akt or PKA to phosphorylate nNOS S1412 and alter ileal tone. This broadens potential therapeutic targets for nitrergic GI dysmotility. nNOS mutant mouse strains allowed us to isolate the neuronal component of the cAMP–NO interaction because only neurons express nNOS (Huber, Saur, Kurjak, Schusdziarra, & Allescher, 1998; Nasser, Ho, & Sharkey, 2006). However, bath application of forskolin elevates cAMP in all cells expressing AC, and we found that most forskolin relaxation was both non-nitrergic and PKA-independent at concentrations over 400-nM forskolin (Figures S7 and S8).

Prior disagreements concerning cAMP and nitrergic relaxation may derive from pharmacological effects in multiple GI cellular compartments. Forskolin stimulates myocyte AC in addition to neuronal AC. NO promotes GI myocyte relaxation via PKG. PKG activation of K^+ channels decreases Ca^{2+} entry, and PKG activation of myosin light chain phosphatase decreases Ca^{2+} sensitivity of the myocyte contractile apparatus (Khromov et al., 2006; Koh et al., 2001). While cAMP can activate transient **BK_{Ca}** current in myocytes, prolonged PKA stimulation blocks K^+ current, opens L-type Ca^{2+} **channels**, and thereby attenuates nitrergic IJPs (Hwang et al., 2008). The strengths of our current study include using multiple NO signalling, NO synthesis and PKA inhibitors to test forskolin relaxation with several mutant NOS mouse strains. Our approach allowed us to estimate that a substantial portion of forskolin relaxation is via nNOS S1412 phosphorylation. The twofold increase in forskolin IC_{50} due to nNOS^{S1412A} mutation may reflect simultaneous cAMP elevation in ileal neurons (IJP inducing) and myocytes (IJP inhibitory at low to moderate cAMP levels). Additionally, because AC can activate CNS excitatory neurons (Zhang et al., 2008), perhaps it can also facilitate enteric excitatory pathways. AC can be activated in nitrergic neurons by **β_2-adrenoceptors** and **5-HT$_4$ receptors** that co-localize with nNOS in myenteric nerve cells (M. T. Liu, Kuan, Wang, Hen, & Gershon, 2009; Nasser et al., 2006), suggesting a means of endogenous nNOS S1412 phospho-regulation in the gut. In our studies, we used substance P to maintain reliable contractions during the concentration–response curve. Substance P stimulates guinea pig ileum NO synthesis and up-regulates cAMP in astrocytoma cells (Fowler & Brannstrom, 1994; Garcia-Villar, Dupuis, Martinolle, Fioramonti, & Bueno, 1996). Thus, substance P may influence cAMP-dependent nNOS S1412 phosphorylation. However, basal nNOS S1412 phosphorylation in vehicle-treated ileal rings is minimal despite substance P (D.D. Guerra et al., 2019), and we employed substance P in every treatment. Studies using inducible neuron- and smooth muscle-specific PKA mutant mice, selective AC agonists and additional contractile stimulants could confirm compartment specific cAMP signalling. The interactions between cyclic nucleotides and their degrading **phosphodiesterases** (PDEs) may also be cell-specific. NO elevates cGMP that can inhibit cAMP-specific **PDE3** (D.D. Guerra, Bok, & Hurt, 2020; Murthy, Zhou, & Makhlouf, 2002). nNOS S1412 phosphorylation could therefore enhance cGMP attenuation of PDE3 activity in myocytes.

The functional phenotype of nNOSα KO ileum affirms a role for nNOS S1412 phosphorylation in facilitating forskolin relaxation. Classically, NO synthesis increases after neuronal depolarization elevates cytosolic Ca^{2+}, which binds calmodulin (CaM) and stimulates nNOS (Thatte et al., 2009). The nNOSα KO mutation abolishes 90% of physiological nNOS activity by eliminating membrane-associated nNOSα (Huang et al., 1993). Although nNOSα KO ileum was less sensitive than WT to forskolin, L-NAME further attenuated nNOSα KO relaxation, indicating that forskolin still induces NOS activity in nNOSα KO ileum, probably via alternatively spliced nNOSβ (Huber et al., 1998; Hurt et al., 2006). Our data are consistent with S1412 phosphorylation of nNOSβ mediating some NOS-dependent forskolin relaxation in nNOSα KO ileum. GI motility does not require eNOS in mice (Mang et al., 2002), but we found that eNOS ablation influences forskolin relaxation. While cAMP activates eNOS by phosphorylating S1179 in endothelial cells (Boo et al., 2002), nNOS is the primary locus of cAMP–NO synergy in GI relaxation. L-NAME induces similar fold changes in forskolin IC_{50} for WT and eNOS KO ilea, and eNOS KO and nNOS^{S1412A} mutations are non-additive. Endogenous cAMP may promote an EPAC-Akt-eNOS pathway that contributes to ileal relaxation, but forskolin does not significantly enhance eNOS activity (Figure 5). Alternatively, the eNOS contribution to forskolin relaxation could be a procedural artefact because our *ex vivo* model lacks the potent NO scavenging of circulating red blood cells (Wennmalm, Benthin, & Petersson, 1992). Recent work suggests that red blood cells do not scavenge all eNOS-derived NO, particularly in microvasculature (Cortese-Krott & Kelm, 2014). Like eNOS, iNOS is probably not involved in forskolin ileal relaxation. 1400W has greater

affinity for iNOS than nNOS (Boer et al., 2000), but NANT and 1400W shifted the forskolin IC_{50} to the same extent, and rat small intestine does not express iNOS in the absence of inflammation (Cui et al., 1997).

Our findings are relevant to the pathophysiology of GI motility disorders. Slow transit constipation, gastroparesis and oesophageal achalasia are associated with diminished or excess nitrergic neurotransmission (Grover et al., 2011; X. Liu, Liu, Xu, Liu, & Sun, 2015; Shteyer et al., 2015). Although non-selective AC activation (e.g. forskolin) may not be clinically useful, selective modulation of enteric neuronal AC could be a therapeutic strategy to enhance nitrergic relaxation. Human myenteric neurons express AC-coupled A_2 **purinoceptors** (Christofi, 2008). Purinergic IJPs precede nitrergic IJPs during GI smooth muscle relaxation (Christofi, 2008; Keef et al., 2013), and **A_{2A} purinoceptor**-dependent colon relaxation requires NO synthesis (Blandizzi et al., 2006). Selective A_2 agonists may modulate nNOS S1412 phosphorylation. Another potential AC-activator is **pituitary AC activating polypeptide** (PACAP), which co-localizes with nNOS in myenteric neurons and regulates nNOS membrane association (Hannibal, Ekblad, Mulder, Sundler, & Fahrenkrug, 1998; Ohnishi et al., 2008).

In summary, we present pharmacological and genetic evidence that enteric neuronal cAMP and nNOS synergistically regulate GI muscle tone. PKA mediates a component of forskolin relaxation via phosphorylation of nNOS S1412, which does not preclude a role for Akt in nNOS-mediated GI relaxation by other stimuli such as neuronal depolarization (D.D. Guerra et al., 2019). GI motility disorders often feature altered nNOS activity (Grover et al., 2011; X. Liu et al., 2015; Shteyer et al., 2015), and rodent models of GI dysmotility feature PKA hyperactivity and hypoactivity (Howe et al., 2006; Yu et al., 2017). Thus, selective modulation of AC-coupled receptors on enteric nitrergic neurons may be clinically useful. Along with the GI tract and the cavernosal nerve (Hurt et al., 2012), PKA phosphorylation of nNOS S1412 could be important in other areas of neuronal communication, such as regulation of synaptic strength (Bhattacharyya, Biou, Xu, Schluter, & Malenka, 2009; Colledge et al., 2000). The role of PKA in central and peripheral nitrergic neurotransmission deserves renewed attention.

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AUTHOR CONTRIBUTIONS

D.D.G., R.A.L., and K.J.H. designed the research. D.D.G. and R.B. conducted the experiments. All authors analysed the data. D.D.G. and K.J.H. wrote the manuscript.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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

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SUPPORTING INFORMATION

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