Nitric-Oxide Synthase Knockout Modulates Ca^{2+}-Sensing Receptor Expression and Signaling in Mouse Mesenteric Arteries

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ABSTRACT

Extracellular calcium (Ca^{2+}_e)-induced relaxation of isolated, phenylephrine (PE)-contracted mesenteric arteries is dependent on an intact perivascular sensory nerve network that expresses the Ca^{2+}-sensing receptor (CaSR). Activation of the receptor stimulates an endocannabinoid vasodilator pathway, which is dependent on cytochrome P450 and phospholipase A_2 but largely independent of the endothelium. In the present study, we determined the role of nitric oxide (NO) in perivascular nerve CaSR-mediated relaxation of PE-contracted mesenteric resistance arteries isolated from mice. Using automated wire myography, we studied the effects of NO synthase (NOS) gene knockout ($NOS^{-/-}$) and pharmacologic inhibition of NOS on Ca^{2+}_e-induced relaxation of PE-contracted arteries. Endothelial NOS knockout ($eNOS^{-/-}$) upregulates but neuronal NOS knockout ($nNOS^{-/-}$) downregulates CaSR expression. $NOS^{-/-}$ reduced maximum Ca^{2+}_e-induced relaxation with no change in EC_{50} values, with $eNOS^{-/-}$ having the largest effect. The responses of vessels to calindol and Calhex 231 indicate that the CaSR mediates relaxation. L-N^5-(1-iminoethyl)-ornithine reduced Ca^{2+}_e-induced relaxation of PE-contracted arteries from C57BL/6 control mice by ≈38% but had a smaller effect in vessels from $eNOS^{-/-}$ mice. 7-Nitroindazole had no significant effect on relaxation of arteries from $NOS^{-/-}$ mice, but both N^G-nitro-L-arginine methylester and N^G-monomethyl-L-arginine significantly reduced the relaxation maxima in all groups. Interestingly, the nNOS-selective inhibitor S-methyl-L-thiocitrulline significantly increased the EC_{50} value by ≈60% in tissues from C57BL/6 mice but reduced the maximum response by ≈80% in those from $nNOS^{-/-}$ mice. Ca^{2+}-activated big potassium channels play a major role in the process, as demonstrated by the effect of iberiotoxin. We conclude that CaSR signaling in mesenteric arteries stimulates eNOS and NO production that regulates Ca^{2+}_e-induced relaxation.

Introduction

The mesenteric bed is innervated by sensory nerve fibers that have their cell bodies located in the dorsal root ganglia. The perivascular nerve (PvN) of mesenteric arteries expresses a Ca^{2+}-sensing receptor (CaSR) that is functionally different from the parathyroid gland receptor (Awumey et al., 2007, 2008). Extracellular calcium (Ca^{2+}_e)-induced relaxation of isolated mesenteric arteries from rats is dependent on an intact PvN network and mediated by a hyperpolarizing endocannabinoid vasodilator transmitter (Ishioka and Bukoski, 1999). The relaxation occurs through pathways dependent on cytochrome P450 (P450) and cytosolic phospholipase A_2

(Awumey et al., 2008). Specifically, our studies indicate that vasodilators resulting from the metabolism of 2-arachidonoylglycerol, which is catalyzed by P450, trigger relaxation by activating Ca^{2+}-activated big potassium (BK) channels. A component (≈20%) of the relaxation, however, is dependent on the endothelium, suggesting that endothelium-derived relaxation factors such as nitric oxide (NO) may also play a role.

A number of studies have shown that CaSR is also expressed in vascular endothelium (Berra Romani et al., 2009) and smooth muscle cells (Wonneberger et al., 2000; Molostvov et al., 2008; Alam et al., 2009), suggesting that CaSR-mediated relaxation of mesenteric arteries may involve multiple components within the vasculature. Thus, activation of the PvN CaSR may be coupled to increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and activation of neuronal and/or endothelial NO synthase (nNOS and eNOS, respectively) and synthesis of NO. The NO can then diffuse into the vascular

ABBREVIATIONS: BK, Ca^{2+}-activated big potassium (channel); Ca^{2+}_e, extracellular calcium; $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CaSR, Ca^{2+}-sensing receptor; CB_1, cannabinoid receptor type 1; eNOS, endothelial nitric-oxide synthase; IbTX, iberiotoxin; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methylester; L-NMMA, N^G-monomethyl-L-arginine; 7-NI, 7-nitroindazole; L-NIO, L-N^5-(1-iminoethyl)-ornithine; nNOS, neuronal nitric-oxide synthase; NO, nitric oxide; NOS, nitric-oxide synthase; P450, cytochrome P450; PE, phenylephrine; PSS, physiological salt solution; PvN, perivascular nerve; S-MeTC, S-methyl-L-thiocitrulline.

smooth muscle to contribute to or modulate relaxation. NO is important in the maintenance of vascular tone, and genetic deletions of NOS isoforms are used to determine the importance of these isoforms to specific processes. The main source of NO in the vasculature is the microvascular endothelium, from where it can contribute to cGMP activation in vascular smooth muscle cells and thus influence vascular reactivity (McCarron et al., 2006; Berra Romani et al., 2009). However, NO can also be produced from nNOS activation in the peripheral nerve terminals on blood vessels (Toda and Okamura, 2011). In the present study, we tested the hypothesis that NOS knockout modulates Ca^{2+}_{e}-induced relaxation of mesenteric arteries as a compensatory mechanism to counteract the effects of reduced NO synthesis and to confirm that the relaxation is mediated by the CaSR. We employed $nNOS^{-/-}$ and $eNOS^{-/-}$ mice; the BK channel blocker iberiotoxin (IbTX); and the NOS inhibitors N^{G}-nitro-L-arginine methylester (L-NAME; nonselective), N^{G}-mono-methyl-L-arginine (L-NMMA; competitive, nonselective) L-N^{5}-(1-iminoethyl)-ornithine (L-NIO; nonselective), 7-nitroindazole (7-NI; highly selective for the neuronal enzyme), and S-methyl-L-thiocitrulline (S-MeTC; selective for rat nNOS) to determine the role of nNOS and eNOS in Ca^{2+}_{e}-induced vascular relaxation.

Materials and Methods

Animals. All procedures using animals were carried out according to protocols approved by the North Carolina Central University Institutional Animal Care and Use Committee.

Chemicals. L-NAME, L-NMMA, L-NIO, 7-NI, and S-MeTC were obtained from Calbiochem (La Jolla, CA). Phenylephrine (PE) and IbTX were from Sigma-Aldrich (St. Louis, MO). Affinity-purified rabbit polyclonal antibodies raised against synthetic peptides corresponding, to human nNOS (Cat. #4234), to residues around Ser600 of human eNOS (Cat. #9572) and Ser100 of human iNOS (Cat. # 2877) were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal CaSR antibodies, raised against a synthetic polypeptide corresponding to the N terminus of rat CaSR, were from Pierce Biotechnology (Rockford, IL). Calindol, Calhex 231, and glyceraldehyde-3-phosphate dehydrogenase antibody (sc-25778) raised against the full-length glyceraldehyde-3-phosphate dehydrogenase of human origin were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals used were of the purest grade available commercially.

Expression Analysis of NOS Isoforms and the CaSR in Mesenteric Arteries. Total proteins were extracted from mesenteric arcades isolated from C57BL/6, $nNOS^{-/-}$, and $eNOS^{-/-}$ mice and analyzed for NOS isoforms and CaSR protein by Western blotting. Briefly, mesenteric arcades were isolated from anesthetized animals, trimmed of fat, and stored at $-80°C$ until used. Tissues were homogenized in ice-cold Tris buffer (10 mM Tris, pH 7.5; 0.25 M sucrose and 3 mM $MgCl_2$) with freshly added Halt Protease Inhibitor cocktail (Pierce Biotechnology). Homogenates were centrifuged at 800g for 10 minutes and the supernatant fractions analyzed by electrophoretic separation on 8% sodium dodecyl sulfate polyacrylamide gel, transferred onto polyvinylidene difluoride (Immuno-Blot polyvinylidene difluoride; Bio-Rad, Hercules, CA) membranes, and blotted with polyclonal NOS (nNOS, eNOS, and iNOS) or CaSR antibodies. The blots were then incubated with horseradish peroxidase–conjugated anti-rabbit IgG and visualized using the enhanced chemiluminescence method.

Vessel Isolation. Mesenteric arteries were dissected from mice under deep anesthesia with isoflurane and sacrificed by open-chest cardiac puncture as previously described (Bridges et al., 2011). Briefly, the small intestine and all vessels feeding it were removed in

block and placed in physiological salt solution (PSS) (mM: NaCl, 115; KCl, 4.7; $MgSO_47H_2O$, 1.4; $NaHCO_3$, 5; KH_2PO_4, 1.2; Na_2HPO_4, 1.1; $CaCl_2$, 1.0; HEPES, 20; and glucose, 5; pH 7.4). Branch I and II mesenteric arteries were carefully dissected from the surrounding fat and mesenterium, taking care to leave a portion of the omental membrane attached to the vessel. A 40-μm-diameter stainless steel wire was inserted into the lumen to remove blood and to serve as a handle for moving the vessel segment between solutions.

Wire Myography. Isometric force generation in isolated arteries was determined as previously described (Awumey et al., 2008; Bridges et al., 2011). Segments (2 mm long) of mesenteric branch arteries were mounted in a small-vessel Mulvany-Halpern 510A Auto Dual Wire Myograph (DMT-USA, Marietta, GA) by means of tungsten-free stainless steel wires inserted through the lumen and maintained in PSS with 1 mM $CaCl_2$ and 100 μM ascorbic acid. When gassed with a mixture of 95% air and 5% CO_2, this solution has a pH of 7.4. After 30-minute equilibration at 37°C, vessels were "normalized" by stepwise stretching and challenged with 5 μM PE until reproducible responses were observed. Relaxation was assessed by adding cumulative concentrations of test compounds to vessels that were precontracted to maximal tone. When inhibitors were used, tissues were preincubated with the compounds for 20 minutes before relaxation assays.

Statistical Analysis. Concentration-response curves were analyzed with SigmaPlot 11.0 statistics programs from Systat Software (Point Richmond, CA). Comparisons between groups and within groups were carried out by one-way analysis of variance and differences with $P < 0.05$ were considered significant. EC_{50} values were determined by fitting data to a standard four-parameter logistic function in the Pharmacology menu of the program.

Results

Modulation of CaSR Expression in Mesenteric Arcade by NOS Knockout. To confirm the knockout of NOS genes in mice used in the present studies and to determine the expression of the CaSR in mesenteric arteries, we analyzed total tissue proteins by Western blotting with NOS and CaSR antibodies. Figure 1A shows that nNOS was highly expressed at similar levels in mesenteric arteries from $eNOS^{-/-}$ and C57BL/6 mice but significantly reduced in tissues from $nNOS^{-/-}$ mice, and eNOS was highly expressed at similar levels in $nNOS^{-/-}$ and C57BL/6 but significantly reduced in $eNOS^{-/-}$ animals (Fig. 1B). Residual eNOS and nNOS were detected in $eNOS^{-/-}$ and $nNOS^{-/-}$, respectively. No detectable iNOS expression was observed in these tissues (unpublished data). Analysis of CaSR expression showed that nNOS knockout animals (eNOS expression) have lower and eNOS knockout animals (nNOS expression) have higher CaSR expression relative to the C57BL/6 animals in mesenteric arteries (Fig. 1C).

Normalization of Mounted Mesenteric Arteries. Figure 2A shows changes in vessel tension during "normalization" of a mounted mesenteric artery segment from a C57BL/6 mouse. The vessel was stretched stepwise following equilibration in PSS medium until a passive tension developed (Angus and Wright, 2000). Figure 2B shows the normalized tensions in isolated mesenteric arteries from control and NOS knockout mice, and Fig. 2C shows the developed tensions in these tissues following the application of 5 μM PE. Initial PE tensions were similar in tissues from control and $nNOS^{-/-}$ mice but lower in those from $eNOS^{-/-}$ mice. The PE tensions did not change significantly in the presence of NOS inhibitors.
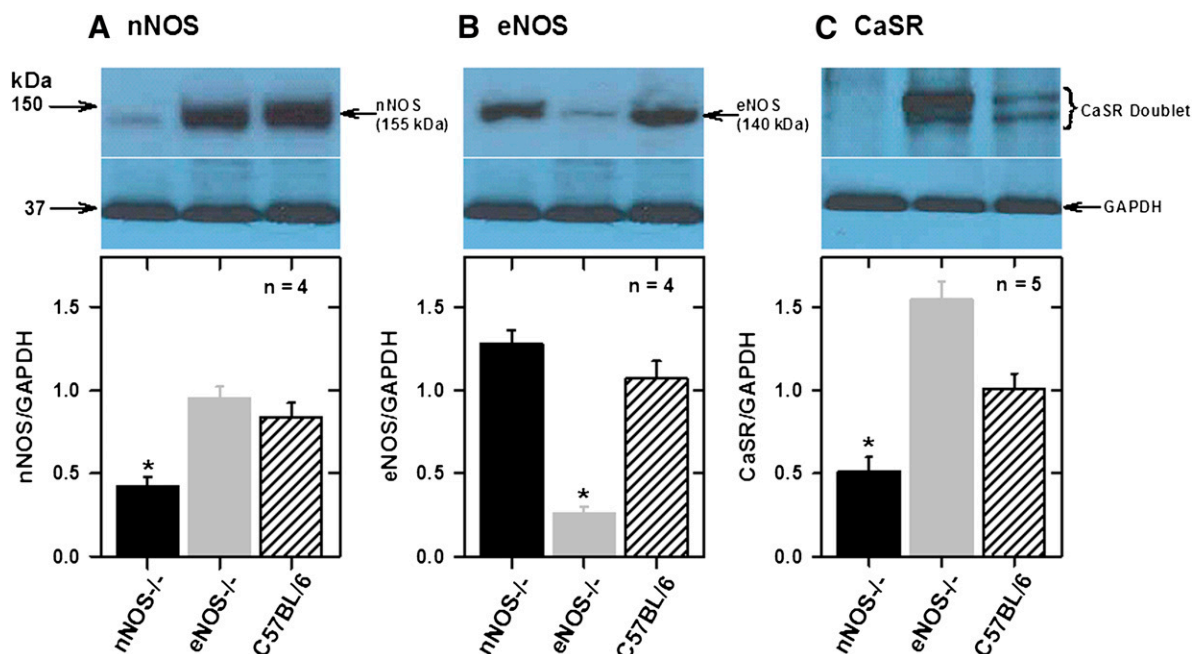
Fig. 1. Analysis of NOS isoforms and CaSR expressions in mesenteric arcades from C57BL/6 (control) and NOS$^{-/-}$ mice. Western blot analysis of nNOS (A), eNOS (B), and CaSR (C) in tissues from control and NOS$^{-/-}$ mice. Bar charts show individual density values of protein bands expressed as ratios of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. nNOS knockout downregulates but eNOS knockout upregulates CaSR expression. *$P < 0.001$ vs. other groups; one-way analysis of variance (ANOVA).

Mediation of Ca^{2+}-Induced Relaxation of Mesenteric Arteries by CaSR. To confirm that Ca^{2+}-induced relaxation of PE-contracted mesenteric arteries was mediated by the CaSR, we also determined responses to calindol (a CaSR agonist) and Calhex 231 (a CaSR antagonist), which act at the allosteric site of the receptor. Relaxations to both compounds were dependent on the concentration, and in all cases, Calindol (1 and 10 μM) relaxed the precontracted arteries by 75 and 90%, respectively (Fig. 3, A–D). On the other hand, 1 and 10 μM Calhex 231 caused only 45 and 70% relaxation, respectively, in tissues from control C57BL/6 mice (Fig. 3D). Ca^{2+}-induced relaxation was similar in tissues from control and nNOS$^{-/-}$ mice and attenuated in those from eNOS$^{-/-}$ mice (Fig. 3E).

Effects of NOS and BK Inhibitors on Tension Changes in Mesenteric Arteries. Cumulative applications of Ca$^{2+}_e$ to PE-contracted mesenteric arteries from C57B/6 and NOS$^{-/-}$ mice induced relaxation; however, NOS knockout reduced maximum responses by about 25 and 8%, respectively, in tissues from eNOS$^{-/-}$ and nNOS$^{-/-}$ animals with no change in the EC$_{50}$ values (Fig. 4). Deletion of eNOS reduced Ca$^{2+}_e$-induced relaxation maximum significantly compared with C57BL/6 ($P < 0.05$).

To determine the relative roles of neuronal and endothelial NO in Ca$^{2+}_e$-induced relaxation resulting from acute blockade, we examined the effect of several NOS inhibitors on relaxation of isolated, PE-contracted mesenteric arteries.
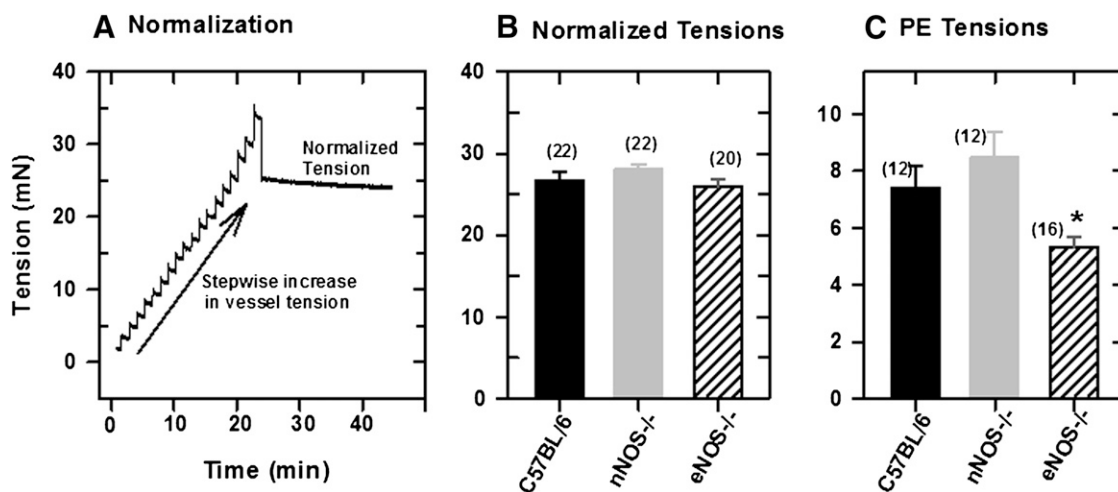

Fig. 2. Normalization of a mesenteric artery segment mounted in a wire myograph in PSS with 1 mM CaCl$_2$. (A) Normalization: stepwise increase in vessel tension. (B) Normalized tensions in mesenteric artery segments mounted in the myograph chamber. (C) Tensions in normalized vessel segments following applications of 5 μM PE. Values plotted are means ($\pm$ S.E.M.). Differences in normalized tensions are statistically significant (*$P = 0.05$; one-way analysis of variance).
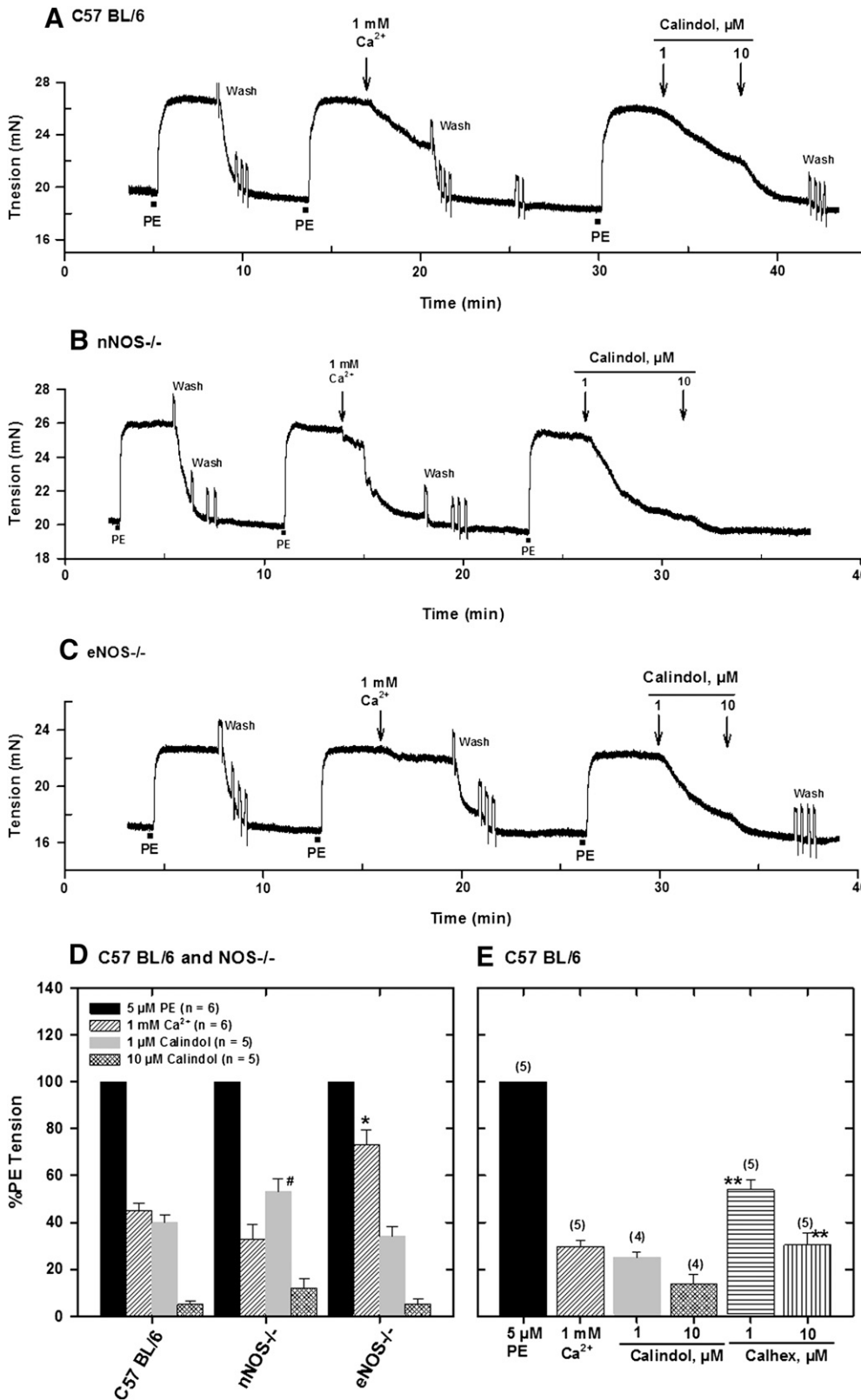
Fig. 3. Effect of Ca^{2+}_e and the CaSR agonist calindol on relaxation of PE-contracted mesenteric arteries from C57BL/6 (A), nNOS$^{-/-}$ (B), and eNOS$^{-/-}$ (C) mice mounted in PSS with 1 mM CaCl$_2$. Responses of precontracted arteries to 1 mM Ca^{2+} and calindol (1 and 10 μM) are shown. (D) Relaxations of precontracted arteries to Ca^{2+} and calindol. (E) Relaxations of isolated, precontracted arteries from C57BL/6 mice to Ca^{2+}, calindol, and the calcilytic Calhex 231. Values plotted are means ($\pm$ S.E.) of 4–6 animals. *$P < 0.05$ vs. C57BL/6 and nNOS$^{-/-}$; #$P < 0.05$ vs. C57BL/6 and eNOS$^{-/-}$; **$P < 0.05$ vs. calindol responses (analysis of variance).

Generally, the NOS inhibitors L-NIO, L-NAME, L-NMMA, and S-MeTC had less effect on Ca^{2+}_e-induced relaxations of arteries from eNOS$^{-/-}$ than nNOS$^{-/-}$ mice. Figure 5A shows a typical force tracing in an artery segment from eNOS$^{-/-}$ mice in the absence and presence of 10 μM L-NIO, a potent and nonselective NOS inhibitor (nNOS K_i, 1.7 μM in rat; eNOS K_i, 3.9 μM in bovine; 5 times more potent inhibitor of eNOS, IC$_{50}$ = 500 nM, than L-NAME and L-NMMA). After equilibration of tissue for 30 minutes at 37°C with constant aeration, 5 μM PE was added to contract the vessel repeatedly

A Force tracings

B Ca²⁺ Response
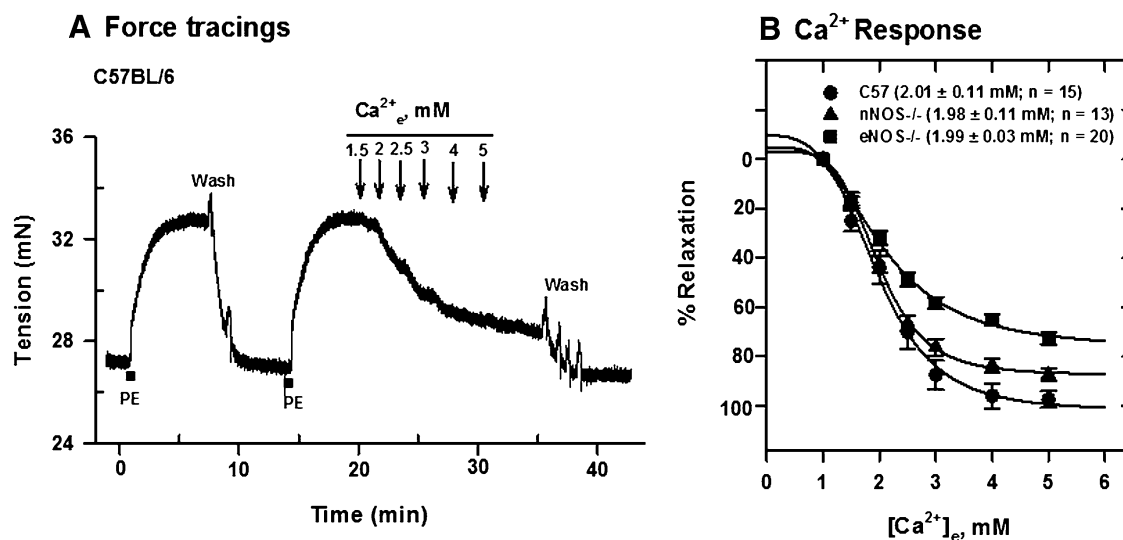
Fig. 4. Ca^{2+}_e-induced relaxation of PE-contracted mesenteric arteries from C57BL/6, nNOS$^{-/-}$, and eNOS$^{-/-}$ mice mounted in PSS containing 1 mM CaCl$_2$. (A) Tension changes in an artery segment from C57BL/6 mouse following contraction with 5 μM PE and cumulative additions of Ca^{2+}_e. (B) $[Ca^{2+}]_e$-response curves generated from force tracing data obtained in tissues from C57BL/6, nNOS$^{-/-}$, and eNOS$^{-/-}$ mice. Values plotted are means ($\pm$ S.E.M.). NOS knockout reduced the maximum on the Ca^{2+}-relaxation curve significantly ($P < 0.05$; one-way analysis of variance), with no change in EC$_{50}$ values.

until a constant tension developed. The tissue was then relaxed with cumulative additions of Ca^{2+}_e and Ca^{2+}_e concentration ($[Ca^{2+}]_e$)-response curves constructed as shown in Fig. 5, B–D. Ca^{2+}_e-induced relaxations were substantially reduced in arteries from C57BL/6 and nNOS$^{-/-}$ mice in the presence of the inhibitor compared with tissues from eNOS$^{-/-}$ mice. The $[Ca^{2+}]_e$-response curve was substantially shifted to the right in the presence of the inhibitor in tissues from control C57BL/6 mice. The EC$_{50}$ for relaxation increased from 2.0 $\pm$ 0.1 mM to 3.4 $\pm$ 0.3 mM in the presence of the inhibitor in mesenteric arteries from C57BL/6 mice. L-NIO reduced the maximum response substantially ($\approx$70%) in mesenteric arteries from nNOS$^{-/-}$ mice without changing the EC$_{50}$. The inhibition pattern in control animals appears to be a composite of that observed in tissues from nNOS$^{-/-}$ and eNOS$^{-/-}$ mice. Similar procedures of contraction with 5 μM PE and relaxation with cumulative additions of Ca^{2+}_e were carried out with other NOS inhibitors and $[Ca^{2+}]_e$-response curves constructed. L-NAME and L-NMMA, competitive inhibitors of NOS, reduced Ca^{2+}_e-induced relaxation in similar fashion in C57BL/6 (Fig. 6A) and nNOS$^{-/-}$ (Fig. 6B) mice, leaving an insensitive component, which was much larger in tissues from eNOS$^{-/-}$ mice (Fig. 6C). Furthermore, the reversible and competitive NOS inhibitor 7-NI had no inhibitory effect in tissues from NOS$^{-/-}$ animals but apparently reduced the maximum response in tissues from C57BL/6 mice (Fig. 7A). S-MeTC, a NOS inhibitor with a greater selectivity for rat nNOS, shifted the $[Ca^{2+}]_e$-response curves to the right in tissues from C57BL/6 control and eNOS$^{-/-}$ mice (Fig. 7, A and C). This shift was more remarkable in C57BL/6 mice. Interestingly, S-MeTC almost abolished Ca^{2+}_e-induced relaxation of tissues from nNOS$^{-/-}$ mice (Fig. 7B). The EC$_{50}$ for Ca^{2+} relaxation was 1.7 $\pm$ 0.2 mM and the maximum response was reduced by $\approx$80%. Table 1 shows the EC$_{50}$ data in tissues from C57BL/6, nNOS$^{-/-}$, and eNOS$^{-/-}$ mice.

The BK channel blocker IbTX (100 nM) shifted the $[Ca^{2+}]_e$-response curves to the right in tissues from all animals (Fig. 8). A larger effect was seen in tissues from eNOS$^{-/-}$ mice,

which show a higher level of CaSR expression. The order of IbTX potency in mesenteric arteries was eNOS$^{-/-}$ > nNOS$^{-/-}$ > C57BL/6. The EC$_{50}$ values determined from nonlinear fitting of relaxation data to a four-parameter sigmoid curve were 1.9 $\pm$ 0.2 mM, 3.2 $\pm$ 0.3 mM, and 4.1 $\pm$ 0.6 mM for C57BL/6, nNOS$^{-/-}$, and eNOS$^{-/-}$, respectively (Fig. 9).

Discussion

Ca^{2+}_e-induced relaxation of isolated, PE-contracted rat mesenteric arteries is dependent on an intact sensory network (Bukoski et al., 1997; Mupanomunda et al., 1998; Wang and Bukoski, 1998, 1999; Mupanomunda et al., 1999; Bukoski, 2001). Endothelium denudation reduced relaxation, but a major component was due to endocannabinoids and their metabolites because cannabinoid receptor type 1 (CB$_1$) antagonism blocked these responses (Awumey et al., 2008). However, endothelial factors, such as NO, may also play a role in this process. We carried out studies with isolated arteries from NOS$^{-/-}$ mice to determine the role of eNOS and nNOS in Ca^{2+}_e-induced relaxation of PE-constricted arteries.

CaSR Expression and Activation, NOS Knockout, and Pharmacological Inhibition of NOS on Ca²⁺-Induced Relaxation. Our results show that eNOS$^{-/-}$ upregulates but nNOS$^{-/-}$ downregulates CaSR expression in mesenteric arteries. The expression of the receptor doublet indicates the presence of mature (complex carbohydrate) and immature (high mannose) glycosylated forms of the receptor (Bai et al., 1996, 1998; Wang et al., 2003). Generally, three different protein bands between 100 and 160 kDa, representing different monomeric forms of the receptor, are observed in Western blot analysis. In this study, the 130- to 140-kDa and 150- to 160-kDa proteins correspond to the immature and mature forms, respectively. The former is expressed in the endoplasmic reticulum (Huang and Miller, 2007) and the latter on the cell surface (Bai et al., 1998; Brown and MacLeod, 2001). Basal tensions in tissues from control and NOS$^{-/-}$ mice were similar, but PE tensions were reduced in
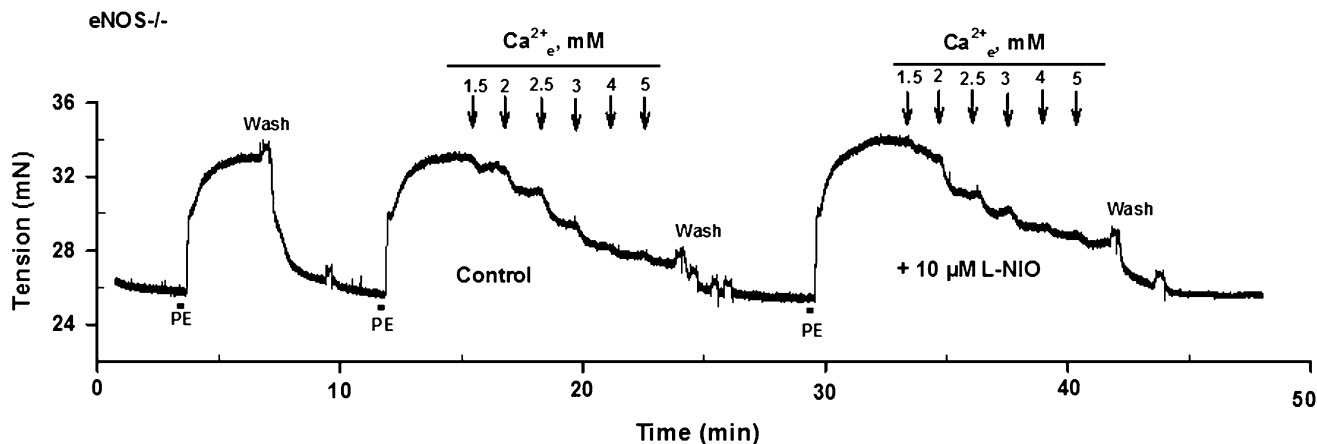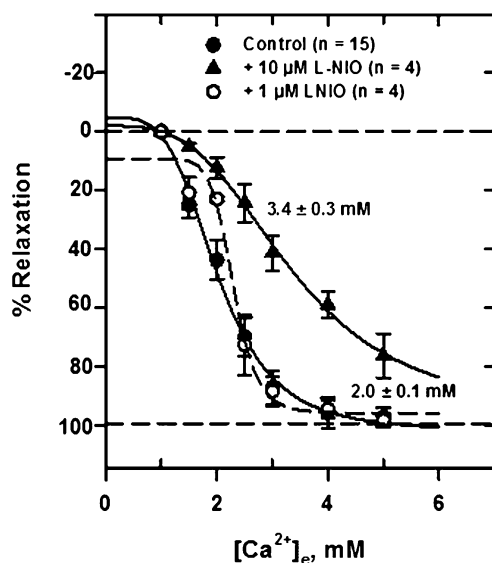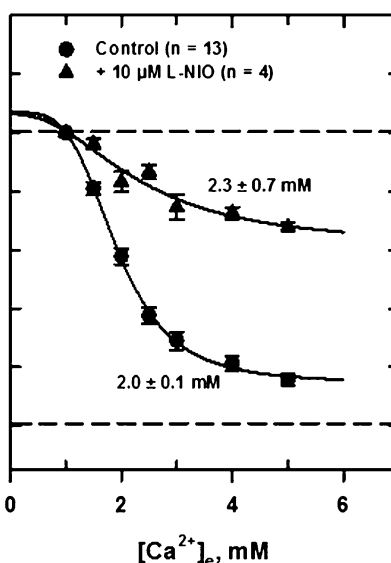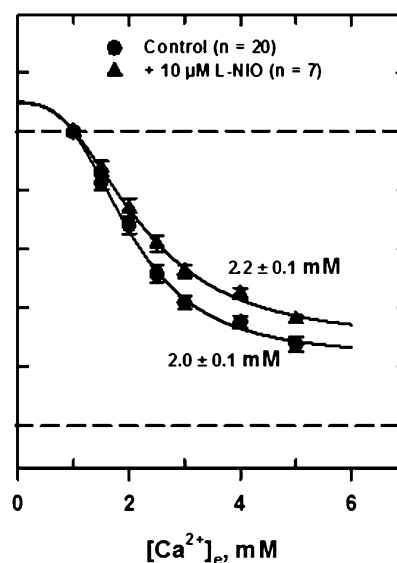
A Force tracings

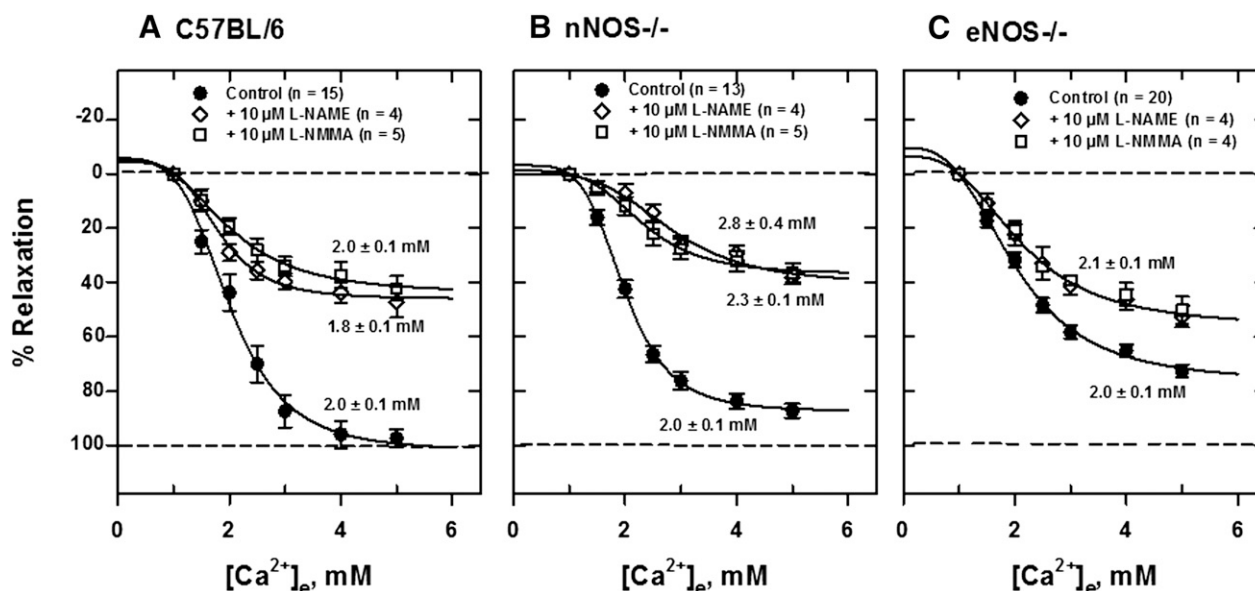

Fig. 5. Inhibition of Ca^{2+}_e-induced relaxation of PE-contracted mesenteric arteries by L-NIO. (A) Tracings of tension changes in a mesenteric artery segment from an eNOS$^{-/-}$ mouse following contraction with 5 μM PE and cumulative additions of Ca^{2+}_e. (B–D) Inhibition of Ca^{2+}_e-induced relaxation by 10 μM L-NIO. [Ca^{2+}]$_e$-response curves were generated from tracing data obtained in tissues from C57BL/6 (B), nNOS$^{-/-}$ (C), and eNOS$^{-/-}$ (D) mice mounted in PSS with 1 mM CaCl$_2$. L-NIO had a larger effect in tissues from C57BL/6 and nNOS$^{-/-}$ mice than those from eNOS$^{-/-}$ mice.

tissues from eNOS$^{-/-}$ mice, probably as a result of high levels of endocannabinoid-derived vasodilators produced in the vessels via the CaSR signaling pathway in the presence of low NO release. Endothelial NO is important in the regulation of vascular tone (Pilette et al., 1996; Sogni et al., 1996); therefore, the reduced PE tension in eNOS$^{-/-}$ arteries may be attributable to stimulation of the CaSR-cannabinoid pathway to generate P450-derived vasodilators in addition to NO from residual eNOS activity. Hyporesponsiveness to vasoconstrictors in mesenteric resistance arteries is due to high NO production, which counteracts the effects of vasoconstrictive agents, and targeted disruption of the eNOS gene has been shown to produce higher myogenic tone (Scotland et al., 2001, 2005). Ca^{2+}_e-induced relaxation of isolated, PE-contracted rat mesenteric arteries is mediated by endocannabinoids (Awumey et al., 2008). Other studies have shown that CB$_1$ receptor antagonism inhibits Ca^{2+}_e-induced relaxation in CB$_1$ receptor–deficient mice, indicating

that direct activation of this receptor is not involved (Bukoski et al., 2002). Thus, in tissues from eNOS$^{-/-}$ mice, the reduction in PE tension in the presence of reduced NO synthesis may be due to activation of the CaSR-endocannabinoid metabolic pathway and generation of vasodilators that act directly on smooth muscle cells to cause hyperpolarization and relaxation. The present data also confirm the CaSR as the mediator of the Ca^{2+}_e relaxation responses, as calindol, a CaSR agonist, enhanced while Calhex 231, a calcilytic at the receptor, reduced relaxation of PE-contracted arteries in a concentration-dependent manner. Both compounds bind to the allosteric site of the CaSR to increase or reduce Ca^{2+} sensitivity (Kessler et al., 2004; Ray et al., 2005; Weston et al., 2005; Ciceri et al., 2012).

A comparison of Ca^{2+} responses of arteries from nNOS$^{-/-}$ and eNOS$^{-/-}$ mice with that from C57BL/6 mice indicates that NOS knockout reduced Ca^{2+}_e-induced relaxation maxima, with eNOS gene deletion having a larger effect than nNOS gene deletion, but the EC$_{50}$ for Ca^{2+} relaxation

A C57BL/6   **B** nNOS-/-   **C** eNOS-/-

Fig. 6. Inhibition of Ca^{2+}_e-induced relaxation of PE-contracted mesenteric arteries by L-NAME and L-NMMA. $[Ca^{2+}]_e$-response curves were generated from tracing data obtained in tissues from C57BL/6 (A), $nNOS^{-/-}$ (B), and $eNOS^{-/-}$ (C) mice mounted in PSS with 1 mM $CaCl_2$. A larger component of the relaxation in tissues from $eNOS^{-/-}$ was insensitive to inhibition by both compounds.

remained the same. $NOS^{-/-}$ had no effect on the potency of Ca^{2+} but reduced its efficacy, indicating a functional change in the CaSR-mediated response system and suggesting a contributory role for eNOS-derived NO. Furthermore, non-selective, acute inhibition of NOS reduced Ca^{2+} relaxation maxima in control and $nNOS^{-/-}$ mice but not in $eNOS^{-/-}$ mice, indicating that endothelium-derived NO modulates the response. The effects of the NOS inhibitors L-NIO, L-NAME, L-NMMA, and S-MeTC on Ca^{2+}_e-induced relaxation of PE-contracted arteries were similar. The data support our

earlier findings that the overall Ca^{2+}_e-induced relaxation was largely due to nonendothelial factors (Awumey et al., 2008), but eNOS-derived NO may have a regulatory role. The inhibitory effects of L-NAME and L-NMMA in tissues from C57BL/6 and $nNOS^{-/-}$ mice are consistent with a primary action of Ca^{2+}_e on eNOS either as a result of activation of the CaSR or following influx through Ca^{2+} entry pathways to increase $[Ca^{2+}]_i$ and NO synthesis. This conclusion is consistent with our earlier finding using rats in which endocannabinoids were reported to mediate a large component of

A C57BL/6   **B** nNOS-/-   **C** eNOS-/-
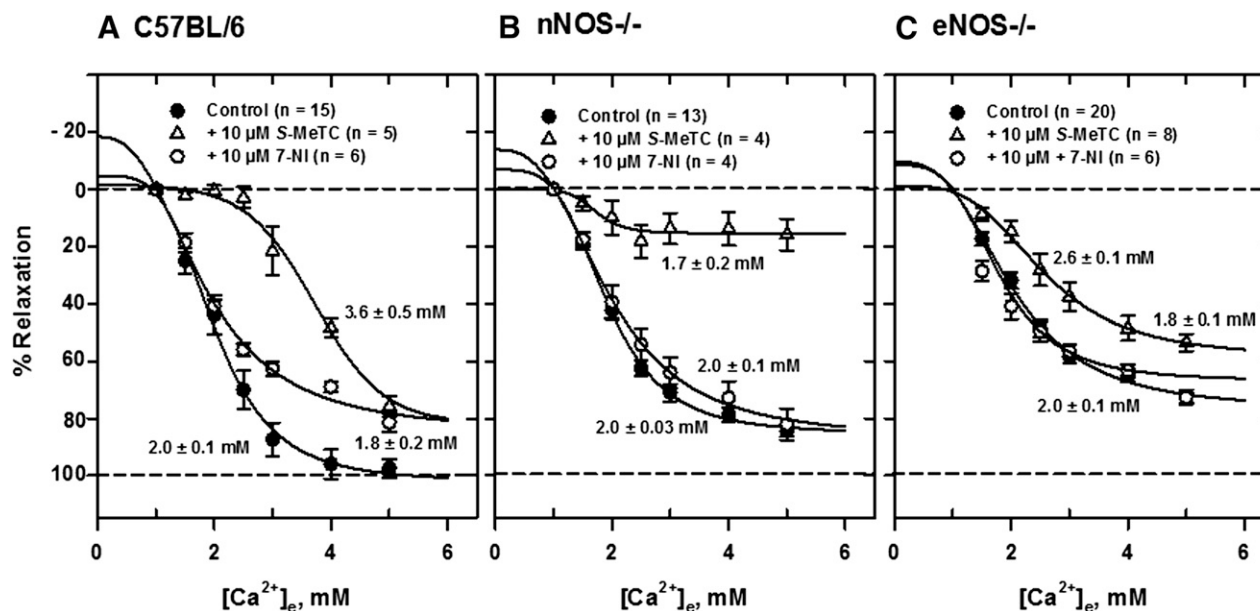
Fig. 7. Inhibition of Ca^{2+}_e-induced relaxation of PE-contracted mesenteric arteries by S-MeTC and 7-NI. $[Ca^{2+}]_e$-response curves were generated from tracing data obtained in tissues from C57BL/6 (A), $nNOS^{-/-}$ (B), and $eNOS^{-/-}$ (C) mice mounted in PSS with 1 mM $CaCl_2$. The EC_{50} for Ca^{2+}_e-induced relaxation of tissues from C57BL/6 mice was substantially reduced by S-MeTC compared with control (P < 0.05). The EC_{50} in $NOS^{-/-}$ remained the same but the maximum responses were reduced by about 80% in $nNOS^{-/-}$ and 10% in $eNOS^{-/-}$. 7-NI had no effect in tissues from $NOS^{-/-}$ mice.

TABLE 1
EC_{50} values for Ca^{2+}_e-induced relaxation of PE-contracted mesenteric arteries in the absence (Control) and presence of IbTX (100 nM) or of NOS inhibitors (10 μM each)
Values are means ($\pm$ S.E.M.) derived from data fitted to a standard four-parameter sigmoid curve in the pharmacology menu of the SigmaPlot 11.0 program. The number of animals for each group is indicated.

Group	EC$_{50}$ Values in Presence of:						
	Control	IbTX	L-NIO	L-NAME	L-NMMA	S-MeTC	7-NI
	mM						
C57BL/6	2.0 ± 0.1 (n = 15)	1.9 ± 0.2 (n = 7)	3.4 ± 0.3* (n = 4)	2.0 ± 0.1 (n = 5)	1.8 ± 0.1 (n = 5)	3.6 ± 0.5* (n = 5)	1.8 ± 0.2 (n = 6)
nNOS$^{-/-}$	2.0 ± 0.1 (n = 13)	3.2 ± 0.3¶ (n = 5)	2.3 ± 0.7¶ (n = 4)	2.8 ± 0.4 (n = 4)	2.3 ± 0.1 (n = 4)	1.7 ± 0.2¶ (n = 4)	2.0 ± 0.1 (n = 4)
eNOS$^{-/-}$	2.0 ± 0.1 (n = 20)	4.4 ± 0.8¶ (n = 5)	2.2 ± 0.1¶ (n = 7)	2.1 ± 0.1 (n = 4)	2.1 ± 0.1 (n = 4)	2.6 ± 0.1¶ (n = 8)	1.8 ± 0.1 (n = 6)

* $P < 0.05$ vs. Control; ¶$P < 0.05$ vs. C57BL/6.

the response to CaSR activation (Awumey et al., 2008). Endothelial and smooth muscle cells have also been reported to express the CaSR (Weston et al., 2005; Berra Romani et al., 2009); therefore, it is possible that activation of the receptor by Ca^{2+}_e stimulates both NO and endocannabinoid synthesis. Such a mechanism will be unique because it is manifested only after PE contraction of vessels. Thus, under conditions of increased tone, small changes in interstitial fluid Ca^{2+} concentration can activate the CaSR signaling pathway that leads to vasodilator synthesis and release from the nerve network (Mupanomunda et al., 1999; Palmer et al., 2003). Based on the current data and earlier findings (Awumey et al., 2008), it is reasonable to conclude that vasodilator release occurs in the nerve network of mesenteric arteries and acts to hyperpolarize the adjacent smooth muscle cells to cause relaxation. It therefore appears that NO from eNOS modulates the relaxation response but does not account for the majority of CaSR dilator activity.

Cellular Mechanisms Involved in NO Modulation of CaSR Expression and Signaling. The current findings suggest that NO from eNOS exerts a negative-feedback effect on the CaSR signaling. Such a mechanism will be physiologically important in regulating vasodilation and blood pressure. This mechanism is consistent with the finding that 7-NI, a primary inhibitor of nNOS, has no inhibitory effect on Ca^{2+}_e-induced relaxation of PE-contracted arteries from nNOS$^{-/-}$ and eNOS$^{-/-}$ mice. 7-NI significantly reduced Ca^{2+}_e relaxation maxima in tissues from C57BL/6 mice and had no effect in tissues from nNOS$^{-/-}$ mice and a small effect in eNOS$^{-/-}$ mice. The major relaxing factors in the vasculature are endocannabinoids, NO, prostacyclin, and endothelium-derived hyperpolarizing factors such as epoxyeicosatrienoic acid (Feletou and Vanhoutte, 2006). eNOS, nNOS, and iNOS compensate for each other; therefore, one has to be cautious in interpreting the inhibition data. The effect of residual NO production can complicate the interpretation of the effects on Ca^{2+}_e-induced relaxation. However, iNOS is not involved, as we did not detect its expression in the mesentery. In nNOS$^{-/-}$ mice, the main source of NO is from the endothelium; therefore, the reduced relaxation maxima without a change in the EC_{50} indicates a negative effect of endothelial NO on Ca^{2+}_e-induced relaxation, suggesting eNOS
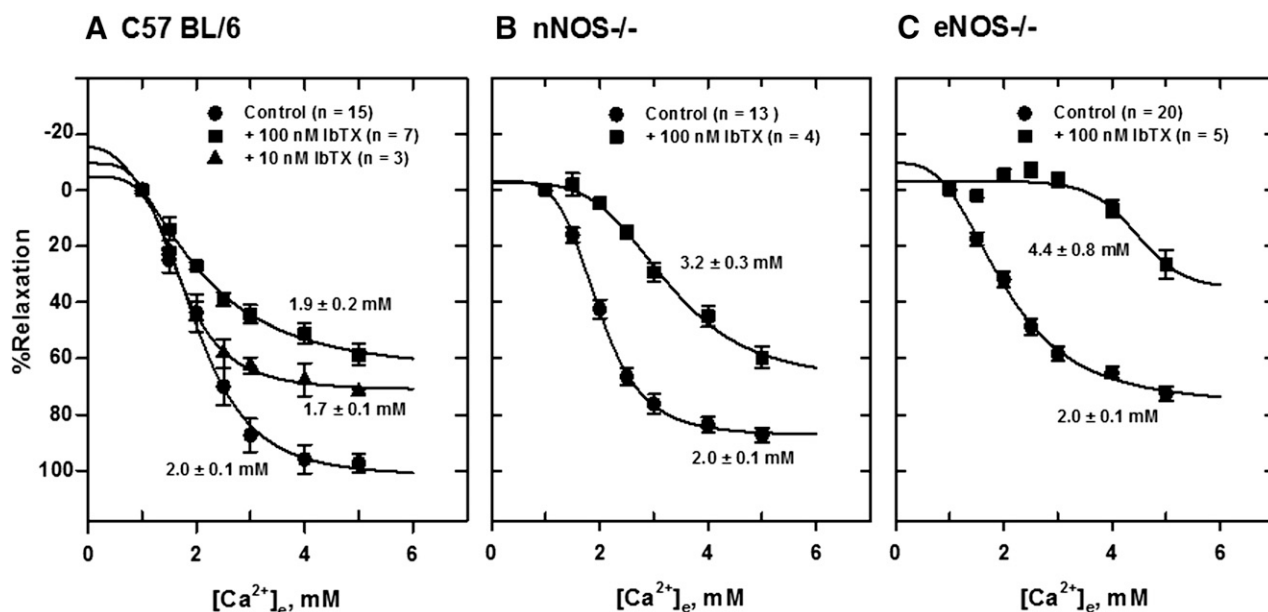
Fig. 8. Inhibition of Ca^{2+}-induced relaxation of PE-contracted mesenteric arteries by 100 nM IbTX. [Ca^{2+}]$_e$-response curves were generated from tension data obtained in tissues from C57BL/6 (A), nNOS$^{-/-}$ (B), and eNOS$^{-/-}$ (C) mice mounted in PSS with 1 mM CaCl$_2$. IbTX (100 nM) shifted the [Ca^{2+}]$_e$-response curves to the right. A larger effect of IbTX was observed in tissues from eNOS$^{-/-}$ mice. The EC_{50} values for Ca^{2+}_e-induced relaxation were significantly higher in tissues from nNOS$^{-/-}$ and eNOS$^{-/-}$ mice.
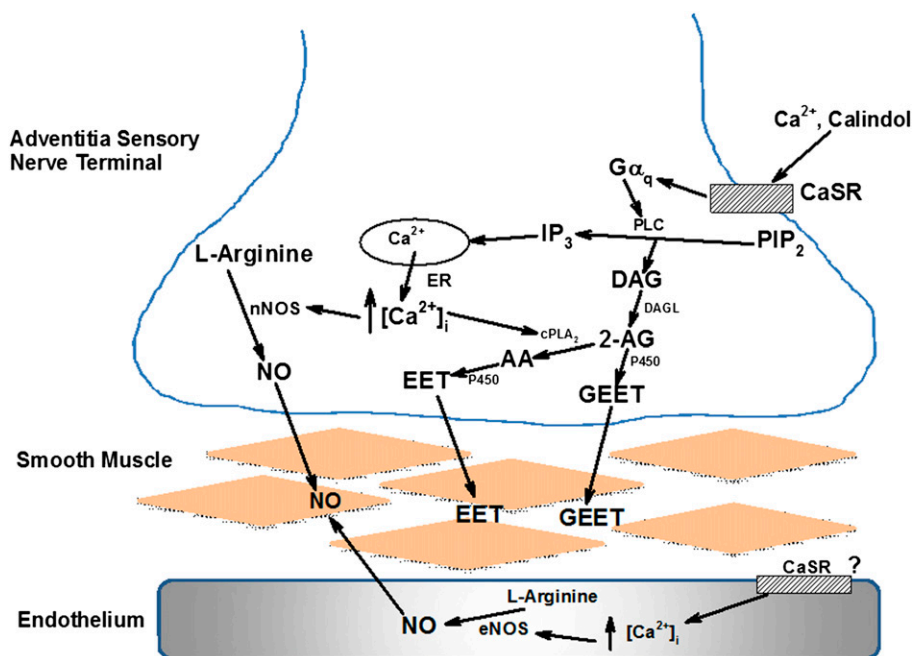
Fig. 9. Proposed model for the PvN CaSR-mediated vasodilator release. Activation of the G-protein-coupled CaSR by agonist leads to hydrolysis of phosphatidylinositol 1,2-diphosphate (PIP_2) by phospholipase C (PLC). to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 releases Ca^{2+} from endoplasmic reticulum (ER) to activate Ca^{2+}-sensitive nNOS to increase NO synthesis in the nerve terminal. Ca^{2+} release also activates cytosolic phospholipase A_2 ($cPLA_2$), which can metabolize the endocannabinoid 2-arachidonoylglycerol (2-AG) [from DAG following metabolism by diacylglycerol lipase (DAGL)] to arachidonic acid (AA). P450 can then metabolize 2-AG and AA to the vasodilators glycerated epoxyeicosatrienoic acid (GEET) and epoxyeicosatrienoic acid (EET), respectively. GEET and EET can then cross the vascular smooth muscle cell (VSMC) plasma membrane to activate BK channels and cause relaxation.

activation. This is further supported by the data obtained with S-MeTC, which indicate a much larger effect of the drug on eNOS compared with nNOS. S-MeTC is considered to be an nNOS-selective inhibitor (Seddon et al., 2009).

In smooth muscle cells, the BK channel contributes to vascular tone by facilitating feedback regulation against an increase in $[Ca^{2+}]_i$, membrane depolarization, and vasoconstriction, thus promoting a K^+ outward current leading to membrane hyperpolarization (Zhao et al., 2007). The current findings suggest that NO from eNOS may interact with PvN CaSR signaling that leads to BK channel activation to reduce hyperpolarization of smooth muscle cells and cause relaxation. The effects of IbTX on relaxation in tissues from eNOS$^{-/-}$ mice indicate that NO from nNOS had no effect on BK channel activity, allowing full manifestation of the effect of the inhibitor. Previous studies from our group showed that tetraethylammonium and IbTX (BK inhibitors) blocked Ca^{2+}_e relaxation of PE-contracted rat mesenteric arteries but apamin and charybdotoxin (small K_{Ca} inhibitors), glinbenclamide (K_{ATP} inhibitor), and 4-aminopyridine (voltage-dependent K^+ channel inhibitor) had no effect (Ishioka and Bukoski, 1999). NO regulates the activity of various K^+ channels, but the hyperpolarizing effects of NO on vascular smooth muscle cells substantially contribute to their relaxation (Vanhoutte et al., 2005; Toda and Okamura, 2011). The larger reduction in the Ca^{2+} response maxima in tissues from eNOS$^{-/-}$ mice was probably due to inefficiency in the CaSR signaling mechanism, as a larger level of receptor expression was observed. It appears that higher CaSR expression in eNOS$^{-/-}$ mice is necessary to compensate for the reduction in NO from the endothelium.

Our studies suggest that in the absence of eNOS, CaSR activation provides a mechanism for relaxation that reduces the increased tone associated with reduced NO production from eNOS. Thus, CaSR in the mesentery may provide a new pathway for vasodilator generation to protect vascular integrity. The findings are novel and the proposed mechanism

(Fig. 9) may be important in endothelial dysfunction. Because CaSR activation leads to release of P450-derived metabolites of 2-arachidonoylglycerol that act at the BK channel (Awumey et al., 2008), the interaction of NO and the receptor may be at the BK channel in smooth muscle cells.

In conclusion, we have identified a novel CaSR signaling mechanism in mesenteric arteries that may serve a protective cardiovascular function and can be a target for the development of new vasodilator compounds.

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