Protein Kinase C Downregulation Enhanced Extracellular Ca^{2+} -Induced Relaxation of Isolated Mesenteric Arteries from Aged Dahl Salt-Sensitive Rats[§]

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ABSTRACT

The Ca^{2+} -sensing receptor (CaSR) detects small changes in extracellular calcium (Ca_e^{2+}) concentration ($[\text{Ca}^{2+}]_e$) and transduces the signal into modulation of various signaling pathways. Ca^{2+} -induced relaxation of isolated phenylephrine-contracted mesenteric arteries is mediated by the CaSR of the perivascular nerve. Elucidation of the regulatory mechanisms involved in vascular CaSR signaling may provide insights into the physiologic functions of the receptor and identify targets for the development of new treatments for cardiovascular pathologies such as hypertension. Protein kinase C α (PKC α) is a critical regulator of multiple signaling pathways and can phosphorylate the CaSR leading to receptor desensitization. In this study, we used automated wire myography to investigate the effects of CaSR mutation and small-interfering RNA downregulation of PKC α on CaSR-mediated relaxation of phenylephrine-contracted mesenteric arteries from aged Dahl salt-sensitive (SS) rats on a low-salt diet. The data showed minimal relaxation responses of arteries to Ca_e^{2+} in wild-type (SS) and CaSR mutant (SS-CaSR^{em1Mcw1}) rats. Mutation of the CaSR gene had no significant effect on relaxation. PKC α expression was similar in wild-type and mutant rats, and small-interfering RNA downregulation of PKC α and/or inhibition of PKC with the Ca^{2+} -sensitive Gö 6976 resulted in a >80% increase in relaxation. Significant differences in EC_{50} values were observed

between treated and untreated controls ($P < 0.05$ analysis of variance). The results indicate that PKC α plays an important role in the regulation of CaSR-mediated relaxation of mesenteric arteries, and its downregulation or pharmacological inhibition may lead to an increased Ca^{2+} sensitivity of the receptor and reversal of age-related changes in vascular tone.

SIGNIFICANCE STATEMENT

G protein-coupled CaSR signaling leads to the regulation of vascular tone and may, therefore, play a vital role in blood pressure regulation. The receptor has several PKC phosphorylation sites in the C-terminal intracellular tail that mediate desensitization. We have previously shown that activation of the CaSR in neuronal cells leads to PKC phosphorylation, indicating that protein kinase C is an important regulator of CaSR function. Therefore, PKC in the CaSR signaling pathway in mesenteric arteries is a potential target for the development of new therapeutic approaches to treat hypertension and age-related vascular dysfunction. The present studies show that small-interfering RNA downregulation of PKC α and pharmacological inhibition of PKC enhanced CaSR-mediated relaxation of phenylephrine-contracted mesenteric arteries from aged Dahl salt-sensitive rats.

Introduction

An important component of many physiologic functions, such as contraction of skeletal and smooth muscle cells, neuronal transmission, hormone secretion, and regulation of gene control, is the precise control of intracellular Ca^{2+} . Availability of Ca_i^{2+} and availability in organs that

participate in cardiovascular control is a crucial factor in the maintenance and regulation of blood pressure (Hatton and McCarron, 1994; McCarron and Reusser, 1999). Small changes in extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_e$) induce changes in parathyroid hormone secretion through activation of the Ca^{2+} -sensing receptor (CaSR) in parathyroid glands to cause subsequent calcium absorption or elimination (Hannan et al., 2016). The CaSR has also been shown to regulate Ca_i^{2+} levels independently of parathyroid hormone activity (Kos et al., 2003; Kantham et al., 2009). The CaSR plays an important role in blood vessel tone and blood pressure control as it is involved in mediating

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ABBREVIATIONS: ANOVA, analysis of variance; CaSR, Ca^{2+} -sensing receptor; Ca_e^{2+} , extracellular Ca^{2+} ; $[\text{Ca}^{2+}]_e$, extracellular Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; EET, epoxyeicosatrienoic acid; GEET, glycerated epoxyeicosatrienoic acid; Gö 6976, 5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile; GRK, GPCR kinase; PE, phenylephrine; PKC, protein kinase C; PSS, physiologic salt solution; siRNA, small-interfering RNA; SS, salt sensitive.

Ca^{2+} -induced vascular reactivity (Schepelmann et al., 2016). Previous studies from our laboratory indicated that a mechanism by which CaSR may regulate blood pressure involves Ca^{2+} -induced activation of perivascular sensory nerve CaSR signaling in the mesenteric artery network that leads to activation of the endocannabinoid system and subsequent vascular relaxation (Bukoski, 2001; Bukoski et al., 2001; Awumey et al., 2008). This transduction of changes in $[\text{Ca}^{2+}]_e$ into changes in cell signaling makes the CaSR a promising target for therapeutic drug design. Elucidation of the regulatory mechanisms of CaSR-mediated signal transduction may not only provide insights into the physiology of the receptor but may also help identify its critical downstream signaling proteins as potential drug targets for treating diseases associated with poor blood pressure regulation.

The CaSR, unlike other G protein-coupled receptors (GPCRs), has a large N-terminal extracellular domain, with glycosylation sites, that binds Ca^{2+} with low affinity (Pin et al., 2005); a seven-transmembrane, hydrophobic domain that binds allosteric modulators; as well as a C-terminal intracellular tail containing multiple phosphorylation sites (Ruat et al., 1995; Ferry et al., 2000). The CaSR couples to several $\text{G}\alpha$ subunits leading to mediation of $\text{G}\beta\gamma$ signaling (Neves et al., 2002) and regulation of numerous second messengers such as inositol-1,4,5-trisphosphate, Ca_i^{2+} , cAMP, and phosphatidic acid (Ward, 2004). We have previously shown that activation of the CaSR leads to $\text{G}\alpha_q$ coupling and generation of inositol 1,4,5-trisphosphate and diacylglycerol that is metabolized to arachidonic acid and subsequent generation of epoxyeicosatrienoic acids (EETs) and glycated epoxyeicosatrienoic acids (GEETs) that cause vascular relaxation (Awumey et al., 2008).

Agonist-induced activation of CaSR is typically followed by initiation of signaling cascades that result in rapid signal reduction, leading to functional desensitization of the receptor. Receptor phosphorylation by GPCR kinases (GRKs) and/or second messenger-dependent protein kinases such as protein kinase C (PKC) is an early step in functional desensitization (Conigrave and Ward, 2013). GRKs can also mediate signal attenuation by direct binding to the activated form of $\text{G}\alpha$ proteins, independently of receptor phosphorylation (Penela et al., 2003), and binding of β -arrestins to uncouple from G proteins (Hannan et al., 2018; Luttrell et al., 2018). An association of the receptor- β -arrestin complex with clathrin also initiates receptor internalization that contributes to functional desensitization (Lefkowitz and Shenoy, 2005). We have previously reported that activation of PKC α and phosphorylation plays an important role in suppressing CaSR-mediated Ca_i^{2+} mobilization and in reduced receptor activity (Sesay et al., 2015). Furthermore, PKC α may indirectly mediate CaSR functional desensitization by modulating the activity of GRK2 (Krasel et al., 2001). In the current study, we investigated the role of PKC α in Ca_e^{2+} -induced activation of the CaSR and relaxation of mesenteric arteries from Dahl salt-sensitive hypertensive rats. Experiments were conducted with isolated arteries from aged wild-type salt-sensitive (SS) and mutant SS-Casr^{em1Mewi} (with only one functional Casr allele) rats that were maintained on a low-salt (0.1% NaCl) diet.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM; cat. no. 11960-044), heat inactivated fetal bovine serum (cat. no. 1614-071), penicillin/streptomycin (cat. no. 15070-063), Opti-MEM I reduced serum medium (cat. no. 31985-062), and Halt Protease and Phosphatase Inhibitor Cocktail (cat. no. 78425) were obtained from Invitrogen (Carlsbad, CA). Gö 6976 (cat. no. 365253) was obtained from EMD biosciences (La Jolla, CA). PKC α small-interfering RNA (siRNA; cat. no. sc-108089), siRNA transfection reagent (cat. no. sc-29528), siRNA transfection medium (cat. no. sc-36868) as well as mouse monoclonal PKC α (H-7) antibody specific for an epitope mapping between amino acids 645–672 at the C-terminus of PKC α of human origin (cat. no. sc-8393), mouse monoclonal CaSR (6D4) antibody raised against amino acids 15–29 of CaSR of rat origin (cat. no. sc-47741), mouse monoclonal GAPDH (6C5) antibody raised against GAPDH purified from muscle of rabbit origin (cat. no. sc-32233), and m-IgG κ BP-HRP (cat. no. sc-516102), a purified recombinant mouse IgG κ light-chain binding protein conjugated to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals. The animal studies have been carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the U.S. National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of North Carolina Central University. Dahl SS wild-type and mutants (SS-Casr^{em1Mewi}) rats were maintained in the Animal Research Center at the Julius L. Chambers Biomedical/Biotechnology Research Institute at constant temperature and humidity and provided with 0.1% NaCl (cat. no. TD.7034) rodent chow (Envigo Tekland Diets, Madison, WI) and water ad libitum. Animals used were approximately 8 months old and of weight, 450–500 g (Fig. 1).

Generation of Mutant SS-Casr^{em1Mewi} Rats. The SS-Casr^{em1Mewi} mutant rat was generated from SS/JrHsdMewi parental strain (ID 61499) in collaboration with the Gene Editing Rat Resource Center at the Medical College of Wisconsin (Milwaukee, WI) in Round 2 of a competitive process in March 2014. The mutant rats were produced by injecting a clustered regulatory interspaced short palindromic repeat (CRISPR) targeting the Casr (RGD Gene ID: 2277) sequence, GTACTTCCGCTGGAAGTGGG, into SS/JrHsdMewi rat embryos. The mutation region (RGSC 5.0/rns; chromosome 11: 70,329,456–70,329,457) is a 1-bp insertion resulting in a frame-shift in exon 4 of the Casr. The target site and the insertion of a T is shown below:

GTACTTCCGCTGGAAGTGGG Casr CRISPR target (PAM in *italics*).

GTACTTCCGCTGGAAGTGGG Casr-m1 1-bp insertion mutation in exon 4 results in predicted frame-shift.

Founder animals were genotyped by the Cel-1 assay and confirmed by Sanger sequencing. The founders were backcrossed to the parental strain, and subsequent litters were identified by fluorescent genotyping. We received heterozygous pairs for breeding on April 4, 2015 and obtained offspring that were used to establish a colony for our studies.

Protein Extraction and Western Blot Analysis. Total proteins were extracted from mesenteric arteries isolated from SS and SS-Casr^{em1Mewi} mutant rats, under isoflurane anesthesia, and analyzed for expression of PKC α and CaSR proteins by Western blotting. After trimming fat from the arteries, tissues were pooled from three animals each to obtain enough protein for assay. Proteins were also isolated from younger animals (<3 months old) for analysis to determine if there were any changes in the expression levels of the CaSR and PKC α owing to aging. Isolated arteries were also incubated with PKC α siRNA or with DMEM alone (control) for 24 hours and stored at -80°C until used for

Fig. 1. Average ages and body weights of animals. (A) Average ages of SS and SS-Casr^{em1Mewi} rats. (B) Average body weights of SS and SS-Casr^{em1Mewi} rats. Data are means ($\pm$ S.E.M.) of 10 SS and 12 SS-Casr^{em1Mewi} animals.

protein extraction. Tissues were homogenized in ice-cold Tris buffer (10 mM Tris, pH 7.5, 0.25 M sucrose and 3 mM MgCl₂) with freshly added Halt Protease and Phosphatase Inhibitor Cocktail. Homogenates were centrifuged at 800g for 10 minutes, and the supernatant fractions separated using SDS-PAGE. Separated proteins were transferred onto polyvinylidene fluoride membranes, blocked with nonfat milk (Biorad, Hercules, CA), and then blotted with antibodies followed by incubation with m-IgG κ BP-HRP (1:5000 dilution) and visualization by enhanced chemiluminescence (ECL). Densitometry analysis of protein bands was done using Licor Image Studio software.

Vessel Isolation. Mesenteric arteries were dissected from rats under deep anesthesia with isoflurane and sacrificed by open-chest cardiac puncture as previously described (Awumey et al., 2008; Bridges et al., 2011). The small intestine and all the associated vessels were removed in block and placed in cold physiologic salt solution (PSS; millimolars: 115 NaCl, 4.7 KCl, 1.4 MgSO₄·7H₂O, 5 NaHCO₃, 1.1 Na₂HPO₄, 1.2 KH₂PO₄, 1.0 CaCl₂, 20 HEPES, and 5 glucose; pH 7.4). Branches I and II mesenteric arteries were carefully dissected from the surrounding fat leaving a portion of the omental membrane attached to the vessel. Isolated vessels were then cut into 2-mm segments for wire myography.

siRNA Downregulation of PKC α in Mesenteric Arteries. To induce PKC α knockdown, isolated mesenteric arteries were incubated with PKC α siRNA for 24 hours. A solution containing 130 nM PKC α siRNA was mixed with transfection reagent in transfection medium and incubated at room temperature for 30 minutes to allow complex formation. Mesenteric arteries were then incubated at 37°C in 5% CO₂ for 6 hours, after which media was replaced with fresh DMEM containing 10% fetal bovine serum and antibiotics, and the incubation continued 24 hours before use in protein extraction or assay of reactivity to Ca²⁺ by wire myography.

Wire Myography. Isometric force generation in mesenteric artery segments was determined as previously described (Awumey et al., 2008; Bridges et al., 2011). Stainless steel wires (40- μ m in diameter) were inserted through the lumens of 2-mm long artery segments and mounted in a small-vessel Mulvany-Halpern 510A Auto Dual Wire Myograph (DMT-USA, Marietta, GA). Segments were maintained in PSS containing 1 mM CaCl₂ and 100 μ M ascorbic acid and gassed with a mixture of 95% air and 5% CO₂. After a 30-minute equilibration at 37°C, vessels were normalized by stepwise stretching using pre-established normalization protocols. The parameters used for normalization were as follows: target transmural pressure, 13.3 kPa (100 mm Hg); time, 60 seconds; IC1/IC100 = 0.9 (IC100 – internal circumference corresponding to target pressure, IC1 – internal

circumference of normalized vessel); eyepiece calibration, 2* Δ (mm/division). The normalization procedure defines the lumen diameter (d_{100}) that the artery would have in vivo when relaxed and under a transmural pressure of 100 mm Hg (Halpern et al., 1978; Angus and Wright, 2000). The mounted vessels were then challenged with 5 μ M phenylephrine (PE) until reproducible contractions were observed. Active force development of $\geq$ 10 mN was viewed as ideal for the experiment to proceed. Relaxation was assessed by addition of cumulative concentrations of Ca²⁺ to precontracted vessels. When an inhibitor was used, vessels were preincubated with the PKC inhibitor Gö 6976 for 5 minutes prior to addition of PE and was present throughout the relaxation assays.

Statistical Analysis. [Ca²⁺]_e-response curves and bar graphs were analyzed using Sigma Plot 14 software (SYSTAT, Port Richmond, CA). EC₅₀ values were determined by fitting data to sigmoid curves in the program. Comparisons between groups were determined by one-way analysis of variance (ANOVA) with a difference of $P < 0.05$ considered significant. Power of performed test with $\alpha = 0.050:1.000$.

Results

Genotyping of Mutant SS-Casr^{em1Mewi} Rats. Male and female SS-Casr^{em1Mewi} rats, from our colony, were randomly bred on a 0.3% NaCl diet (TD.95027; Tekland), and DNA extracts from tail samples of offspring were genotyped using a Custom TaqMan SNP Genotyping Assay, on a fee-for-service basis by the Molecular Genetics Core of the Duke Molecular Physiology Institute (Durham, NC). The probe sequences and the reporter dyes (in brackets) used are as follows:

Reporter Sequence 1- CCACCCAAGTTCCAG (VIC dye).

Reporter Sequence 2- CCACCCAGTTCCAG (FAM dye).

Most of the offspring from the breeding were wild-type SS (D/D) and heterozygous SS-Casr^{em1Mewi} mutant (I/D) animals, confirming a single-allele mutation in the Casr (Supplemental Fig. 1). Thus, the breeding protocol we established has allowed us to maintain a colony of heterozygous breeder pairs in sufficient numbers to generate wild-type and age-matched heterozygous mutants in adequate numbers continuously for our studies. Figure 1 shows the ages and weights of SS wild-type and SS-Casr^{em1Mewi} heterozygous animals used in the present

Fig. 2. Analysis of expression of CaSR and PKC α in control and PKC α siRNA-treated mesenteric arteries from SS and SS-Casr^{em1M^{ewi}} rats. Animals were maintained on 0.1% NaCl diet for 8 months and protein extracts from control siRNA-treated mesenteric arteries were analyzed with monoclonal antibodies. CaSR, PKC α and GAPDH bands were analyzed from the separated proteins in the same samples. After separation, proteins were transferred onto membranes and cut into three strips for blotting with monoclonal antibodies to the CaSR (150 kDa), PKC α (80 kDa) and GAPDH (37 kDa). (A) (i) Representative CaSR Western blot, and (ii) bar chart showing densitometric analysis of protein bands from four different Western blot experiments as in (i). Values plotted are means ($\pm$ S.E.M.) of four experiments. (B) (i) Representative PKC α Western blot, and (ii) bar charts showing densitometric analysis of protein bands from four different Western blot experiments as in (i). Values plotted are means ($\pm$ S.E.M.) Protein bands were normalized to GAPDH as the loading standard. (* $P < 0.05$ vs. control; ANOVA followed by multiple comparisons using the Holm-Sidak method).

studies. The ages of the animals used were about 8 to 9 months but the weights were similar.

Effects of CaSR and PKC α Downregulation on Protein Expression. To confirm knockdown of CaSR and PKC α expression, proteins extracted from isolated mesenteric arteries (control and those incubated with PKC α siRNA) were analyzed by Western blotting with CaSR and PKC α antibodies. The results showed significant reduction in the expression of the CaSR in the mesenteric arteries from SS-Casr^{em1M^{ewi}} rats compared with the SS (Fig. 2A), and downregulation of expression of PKC α protein following PKC α siRNA treatment of the vessels (Fig. 2B). Western blotting also showed that there were no significant differences in CaSR and PKC α expression between young (<3 months old) rats compared with the aged rats used in this study (Fig. 3).

Normalization of Mounted Mesenteric Arteries. It is important to set a baseline condition in myography studies of isolated vessels in order to acquire reproducible results. To do this, vessels equilibrated in PSS were stretched in a stepwise manner with increasing tension over time until they developed passive tension (Angus and Wright, 2000). Normalization of isolated mesenteric artery segments from SS and SS-Casr^{em1M^{ewi}} rats were similar (see Supplemental Data). PKC α downregulation did not have any effect on the slopes of the normalization curves. Figure 4 shows resting tensions, after normalization, and tensions resulting from treatment of vessels with 5 μ M PE, in SS and SS-Casr^{em1M^{ewi}}, respectively.

There were no significant differences in resting tensions and PE tensions resulting from either CaSR or PKC α downregulation. No significant changes in PE tensions resulted from treatment of mounted vessels with the PKC inhibitor Gö 6976.

Effects of siRNA Downregulation of PKC α and Pharmacological Inhibition of PKC in Mesenteric Arteries Increased Ca²⁺-Induced Relaxation. Arteries from SS and SS-Casr^{em1M^{ewi}} animals that had PKC α downregulated or PKC inhibited showed significant differences in responses to cumulative addition of Ca²⁺ compared with controls (Fig. 5). Tension changes in these vessels indicate that the nontransfected (control) PE-contracted tissues showed minimal response to Ca_e²⁺, with relaxation starting after addition of high [Ca_e²⁺]_e (Fig. 5, A and C). However, pretreatment of vessels with 5 μ M Gö 6976 resulted in increased responses to Ca_e²⁺. These increased responses were also observed in PKC α siRNA-treated vessels, which showed responses to much lower [Ca_e²⁺]_e compared with the nontransfected tissues (Fig. 5, B and D). This effect was enhanced in tissues in which PKC α was downregulated and treated with 5 μ M Gö 6976. The [Ca_e²⁺]_e-response curves from both sets of tissues also indicate the effect of PKC α knockdown and/or PKC inhibition on relaxation response (Fig. 6, A and B). Response curves in PKC α -downregulated arteries were shifted to the left compared with the nontransfected tissues, with combined downregulation and inhibition shifting the response curves further to the left. EC₅₀

Fig. 3. CaSR and PKC α expression in mesenteric arteries from old ($\approx$ 8 months) and young ($\leq$ 3 months) SS and SS-Casr^{em1Mcowi} rats. Protein extracts from mesenteric arteries were analyzed with monoclonal antibodies to compare expression levels in young and old rats. CaSR, PKC α , and GAPDH bands were analyzed from the separated proteins in the same samples. After separation, proteins were transferred onto membranes and the latter cut into three strips for blotting with monoclonal antibodies to the CaSR (150 kDa), PKC α (80 kDa), and GAPDH (37 kDa). (A) (i) Representative CaSR Western blot; (ii) bar charts showing densitometric analysis of protein bands from four separate experiments as in (i). Values plotted are means ($\pm$ S.E.M.); (B) (i) Western blot of PKC α ; (ii) bar charts showing densitometric analysis of protein bands from four separate experiments as in (i). Values plotted are means ($\pm$ S.E.M.). Protein bands were normalized to GAPDH as the loading standard. (* P < 0.05 vs. control; ANOVA followed by multiple comparisons using the Holm-Sidak method).

values for the Ca²⁺ relaxation responses were derived by fitting sigmoid curves to the data (Fig. 6C). No significant differences in vessel responses to Ca_e²⁺ were observed between the SS and SS-Casr^{em1Mcowi} tissues regardless of treatment.

Discussion

The [Ca²⁺]_e is maintained within a narrow range (1.1–1.4 mM) in humans (Hurwitz, 1996), therefore the CaSR must be able to detect small changes in this concentration for receptor activation to be possible. In fact, the CaSR has been shown to detect small changes in Ca_e²⁺ (Brown and MacLeod, 2001) and small increases in interstitial fluid Ca²⁺ ([Ca²⁺]_{ISF}) that are within the physiologic range (Breitwieser, 2008). In situ microdialysis studies that measured [Ca²⁺]_{ISF} in the duodenal submucosa and kidney cortex, two tissues that are essential for the regulation of peripheral resistance and BP, showed dynamic changes as a function of the gut lumen [Ca²⁺]. Increasing gut [Ca²⁺] from 0 to 6 mM increased the [Ca²⁺]_{ISF} from 1.1 to 1.9 mM (Mupanomunda et al., 1998, 1999, 2000), which is in the range observed to activate the perivascular CaSR expressed in human embryonic kidney (HEK) cells (Awumey et al., 2007), and relaxed isolated mesenteric arteries (Awumey et al., 2008). We have also shown that the CaSR detects decreases in [Ca²⁺]_e and an increase in the action potential duration through

activation of the nonselective cation channel, which in turn attenuates the impact on release probability at neocortical terminals (Chen et al., 2010). The latter suggests that the CaSR provides presynaptic feedback to alter brain excitability in response to Ca_e²⁺. We also found that in HEK-CaSR.EGFP cells expressing the dorsal root ganglia CaSR-EGFP fusion protein, desensitization of the receptor occurs with repeated, prolonged stimulation (Awumey et al., 2007), further suggesting that an understanding of the regulation of this receptor will clarify its role under both normal and hypertensive conditions. Studies have shown that small changes in [Ca²⁺]_{ISF} are enough to activate the CaSR and contribute to vasodilator synthesis under normal conditions (Mupanomunda et al., 1999, 2000; Bukoski, 2001). Other studies have also revealed that a high-salt diet reduced renal [Ca²⁺]_{ISF} and increases systolic BP in Dahl salt-sensitive rats (Palmer et al., 2003; Eley et al., 2008).

The mesenteric arterial network contributes significantly to total peripheral resistance, as it receives about 25% of cardiac output, and so this system is very important for overall regulation of blood pressure (Christensen and Mulvany, 1993). CaSR, and its downstream signaling protein complements, has been shown to play crucial roles in this system and as such is involved in blood pressure regulation (Bukoski et al., 1997; Bukoski, 1998; Wang and Bukoski, 1998; Awumey et al., 2008). Given the key role played by the mesenteric artery CaSR in controlling Ca_i²⁺, the regulation

In this study we examined the role played by PKC α in decreasing CaSR-mediated relaxation of mesenteric arteries, as well as investigating the effects of reduced expression of CaSR. We performed these studies using isolated arteries from wild-type male SS and SS-Casr^{em1Mewi} mutant rats, which have only one functional Casr allele. It is well established that aging results in myriad changes

To accurately and reproducibly conduct mechanical studies on isolated vessels, it is very important to normalize mounted vessel segments to establish standard baseline tension and determine tissue viability. Furthermore, stepwise stretching of the vessel, until a lumen diameter is defined that is comparable to transmural pressure *in vivo*, is important, as this determines vessel activity and sensitivity to various treatments. Our results show

Fig. 5. Effect of downregulation of CaSR and PKC α , and pharmacological inhibition of PKC on Ca²⁺-induced relaxation of PE-contracted mesenteric arteries. Force tracings showing the effect of cumulative additions of Ca_e²⁺ to PE-contracted mesenteric arteries, from SS and SS-Casr^{em1Mewi} rats, in the absence or presence of 5 μ M Gö 6976 on relaxation. (A) SS control; (B) SS + PKC α siRNA; (C) SS-Casr^{em1Mewi} control; (D) SS-Casr^{em1Mewi} + PKC α siRNA. PKC α siRNA downregulation and PKC inhibition by Gö 6976 resulted in increased relaxation responses to cumulative additions of Ca_e²⁺.

that siRNA downregulation of PKC α did not affect basal tensions of both SS and SS-Casr^{em1Mewi} vessels. There was also no effect of PKC α downregulation on PE tension levels. This is consistent with previous studies showing that PKC α is not directly involved in α_1 -adrenergic-mediated vasoconstriction (Ohanian et al., 1996). It should also be noted that even though there was reduced expression of CaSR in the arteries from the SS-Casr^{em1Mewi} animals, this did not result in any significant difference in basal or PE tensions.

As we have previously established, the Ca_e²⁺ relaxation response in mesenteric arteries is a result of CaSR activity (Awumey et al., 2008, 2013). In these studies, we showed that Ca_e²⁺ relaxation of precontracted arteries involved CaSR-mediated increase in intracellular [Ca²⁺] that leads to the generation of cytochrome P450-derived metabolites of the endocannabinoids 2-arachidonoylglycerol and anandamide. Furthermore, endothelial nitric oxide synthase knockout in mice upregulates CaSR expression in mesenteric arteries.

Fig. 6. Effects of downregulation of CaSR and PKC α and inhibition of PKC on $[Ca^{2+}]_e$ -response curves derived from force tracings in Fig. 5. $[Ca^{2+}]_e$ -response curves were generated from tension data obtained in arteries from (A) SS and (B) SS-Casr^{em1Mewi} rats. (C) Bar charts showing EC₅₀ values determined from response data fitted to sigmoid curves *Significantly different from treated groups; **Significantly different from PKC α siRNA-treated and Go 6976-treated groups ($P < 0.05$; ANOVA followed by multiple comparisons using the Holm-Sidak method).

The observed effect of downregulation of PKC α and/or pharmacological inhibition of PKC on mesenteric artery relaxation confirms that PKC α plays an important role in the regulation of CaSR activity, similar to our observation in a previous study (Sesay et al., 2015). It has also been suggested previously that receptor phosphorylation by PKC regulates attenuation of CaSR signaling via a mechanism involving β -arrestin binding to PKC-phosphorylated CaSR and subsequent desensitization to Ca^{2+} (Lorenz et al., 2007). Our results indicate that PKC α mediates relaxation of phenylephrine-contracted mesenteric arteries in response to cumulative addition of Ca_e^{2+} . Both siRNA downregulation of PKC α and pharmacological inhibition of PKC resulted in significant increases in Ca_e^{2+} -induced relaxation of isolated, PE-constricted mesenteric arteries, and, in effect, reversal of the effects of aging on vasodilation in these vessels. PKC α downregulation resulted in increased responsiveness of arteries to Ca_e^{2+} as illustrated by the reduction in EC₅₀ values. The proposed mechanism

for such a process is outlined in Fig. 7. It should also be noted that there were no significant differences in responses to Ca_e^{2+} between mesenteric arteries from SS and SS-Casr^{em1Mewi} animals (EC₅₀ values were similar), indicating that changes in receptor function are the key factor. The current study only involved the use of animals on a low-salt diet, and a future direction would be to investigate whether CaSR plays a more significant role in Ca_e^{2+} -induced vasodilation when there is exposure to a high-salt diet by comparing responses in tissues from SS and SS-Casr^{em1Mewi} rats.

In conclusion, our results confirm that PKC α plays an important regulatory role on CaSR signaling in the context of Ca_e^{2+} -induced vasodilation. Reduction in PKC α activity along with increased exposure to Ca^{2+} could help counteract some of the reduction in vasodilation that occurs because of aging, suggesting a possible direction for combined diet and drug therapeutic studies for the management of hypertension and its related cardiovascular pathologies in aging.

Fig. 7. Proposed mechanism of CaSR-mediated signaling and mesenteric artery relaxation. Mechanism of CaSR-mediated vasodilation and its regulation by PKC. This signaling pathway uses published data from our laboratory (Awumey et al., 2008) and the present studies that focused on PKC and Ca^{2+} . Cytochrome P450 metabolites EET and GEET play a role in CaSR-mediated mesenteric artery relaxation.

Authorship Contributions

Participated in research design: Odotola, Awumey.

Performed data analysis: Odotola, Bridges, Awumey.

Wrote or contributed to the writing of the manuscript: Odotola, Awumey.

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