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RESEARCH PAPER

Endosomal proteolysis regulates calcitonin gene-related peptide responses in mesenteric arteries

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BACKGROUND AND PURPOSE

Calcitonin gene-related peptide (CGRP) is a potent vasodilator, implicated in the pathogenesis of migraine. CGRP activates a receptor complex comprising, calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1). *In vitro* studies indicate recycling of CLR•RAMP1 is regulated by degradation of CGRP in early endosomes by endothelin-converting enzyme-1 (ECE-1). However, it is not known if ECE-1 regulates the resensitization of CGRP-induced responses in functional arterial tissue.

EXPERIMENTAL APPROACH

CLR, ECE-1a-d and RAMP1 expression in rat mesenteric artery smooth muscle cells (RMA-SMCs) and mesenteric arteries was analysed by RT-PCR and by immunofluorescence and confocal microscopy. CGRP-induced signalling in cells was examined by measuring cAMP production and ERK activation. CGRP-induced relaxation of arteries was measured by isometric wire myography. ECE-1 was inhibited using the specific inhibitor, SM-19712.

KEY RESULTS

RMA-SMCs and arteries contained mRNA for CLR, ECE-1a-d and RAMP1. ECE-1 was present in early endosomes of RMA-SMCs and in the smooth muscle layer of arteries. CGRP induced endothelium-independent relaxation of arteries. ECE-1 inhibition had no effect on initial CGRP-induced responses but reduced cAMP generation in RMA-SMCs and vasodilation in mesenteric arteries responses to subsequent CGRP challenges.

CONCLUSIONS AND IMPLICATIONS

ECE-1 regulated the resensitization of responses to CGRP in RMA-SMCs and mesenteric arteries. CGRP-induced relaxation did not involve endothelium-derived pathways. This is the first report of ECE-1 regulating CGRP responses in SMCs and arteries. ECE-1 inhibitors may attenuate an important vasodilatory pathway, implicated in primary headaches and may represent a new therapeutic approach for the treatment of migraine.

Abbreviations

CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; ECE-1, endothelin-converting enzyme-1; EEA1, early endosomal antigen 1; RAMP, receptor activity-modifying protein; RMA-SMC, rat mesenteric artery smooth muscle cell

Introduction

Calcitonin gene-related peptide (CGRP) belongs to the calcitonin family of peptides, which also includes adrenomedullin, calcitonin, intermedin (adrenomedullin 2) and amylin. CGRP is widely distributed in sensory nerves throughout the central and peripheral nervous system (Brain and Grant, 2004). CGRP is a potent vasodilator, increases blood flow and promotes mast cell degranulation, angiogenesis and endothelial cell proliferation (Brain *et al.*, 1985; Haegerstrand *et al.*, 1990; Reynier-Rebuffel *et al.*, 1994; Ohno *et al.*, 2008). In contrast, CGRP has anti-proliferative effects in SMCs (Li *et al.*, 1997; Chattergoon *et al.*, 2005). The relaxing effects of CGRP on the vasculature can be mediated by activating either endothelial cells or smooth muscle cells. For example, CGRP relaxes human radial, coronary, gastric and cerebral arteries via an endothelium-dependent pathway (Thom *et al.*, 1987) whereas, in human and porcine coronary arteries, CGRP acts solely on smooth muscle cells receptors (Shoji *et al.*, 1987). In addition to vasodilatation CGRP has been shown to be beneficial in protecting against myocardial ischaemia (Wu *et al.*, 2001) and heart and kidney damage caused by hypertension (Supowit *et al.*, 2005).

Noxious stimuli and trauma can lead to release of CGRP and another neuropeptide, substance P from primary sensory neurons. Centrally, release of CGRP and substance P facilitates nociceptive transmission; and in the periphery they mediate neurogenic inflammation, which is characterized by neutrophil infiltration, oedema and vasodilatation (McDonald, 1988). These processes are important characteristics of many human diseases including asthma, arthritis and inflammatory bowel diseases.

CGRP has been implicated in the pathogenesis of migraine as infusion of CGRP caused delayed headaches in patients suffering from primary headache diseases (Lassen *et al.*, 2002), and increased levels of CGRP have been detected in the serum and saliva of patients during migraine attacks (Goadsby *et al.*, 1990; Bellamy *et al.*, 2006; Cady *et al.*, 2009). Current migraine treatments include triptans (serotonin 5-HT_{1B} and 5-HT_{1D} receptor agonists), which constrict intracranial blood vessels and reduce release of neuropeptides such as CGRP. However, this treatment is not effective in all migraine patients. Therefore, other drug targets are needed for the treatment of migraine. There are now a number of non-peptidic CGRP receptor antagonists, which are showing promise for the treatment of migraine in clinical trials. The first described non-peptidic antagonist was olcegepant (formerly BIBN4096BS) (Doods *et al.*, 2000), followed by telcagepant (formerly MK-0974) (Paone *et al.*, 2007). Both olcegepant and telcagepant have both been shown to have clinical efficacy in the acute treatment of migraine (Olesen *et al.*, 2004; Ho *et al.*, 2008a,b; Connor *et al.*, 2009; 2011), but trials have now been discontinued.

The receptor for CGRP is an unusual heterodimeric receptor complex comprising the GPCR, calcitonin receptor-like receptor (CLR) and a single transmembrane protein, RAMP1 (McLatchie *et al.*, 1998; receptor nomenclature follows Alexander *et al.*, 2011).). A third protein called CGRP-receptor component protein is required for efficient CGRP-induced signalling (Evans *et al.*, 2000). There are three RAMP family members, which can all heterodimerize with CLR (McLatchie *et al.*, 1998). CLR•RAMP2 and CLR•RAMP3 are normally referred to as high-affinity adrenomedullin receptors. However, high concentrations of CGRP and intermedin can also activate these receptors (Hong *et al.*, 2012). CLR•RAMP1 undergoes agonist-dependent internalization, a process that requires interaction with β-arrestins (Hilairet *et al.*, 2001). CLR•RAMP1 and CGRP traffic together to early endosomes, but the duration of the stimulus determines the post-endocytic sorting of CLR•RAMP1 (Cottrell *et al.*, 2005; 2007). The recycling of CLR•RAMP1 is regulated by the metallopeptidase endothelin-converting enzyme-1 (ECE-1), which degrades CGRP, promoting the release of β-arrestins to allow CLR•RAMP1 to recycle to the cell surface, mediating resensitization (Padilla *et al.*, 2007).

In the current study, we examined the role of ECE-1 in regulating CLR•RAMP1 resensitization in rat mesenteric artery smooth muscle cells (RMA-SMCs) and in controlling resensitization of the vasodilatory effects of CGRP in intact mesenteric arteries. Our aims were to (1) determine whether ECE-1 and CLR•RAMP1 are co-expressed in vascular SMCs of resistance-sized arteries, (2) investigate whether ECE-1 controls resensitization of CGRP signalling in vascular SMCs and (3) determine if ECE-1 controls resensitization of the vasodilatory effects of CGRP. Our results show that ECE-1 is expressed in arteries important for the regulation of blood pressure, and its activity promotes resensitization of CGRP-induced cAMP generation in RMA-SMCs and vasodilation in rat mesenteric resistance arteries.

Methods

Animals

All animal care and experimental procedures complied with the UK Animals (Scientific Procedures) Act (1986) and institutional guidelines for animal welfare. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 27 animals were used in the experiments described here. Male Wistar rats (200–300 g; University of Bath Animal Facility) were killed by cervical dislocation and the mesenteric vascular bed was excised and immediately placed in ice-cold Krebs solution containing (mM) NaCl, 118.0; $NaCO_3$, 24; KCl, 3.6; KH_2PO_4, 1.2; $MgSO_4{\cdot}7H_2O$, 1.2; glucose, 11.0; $CaCl_2$, 2.5.

Primary cell culture

RMA-SMCs were obtained using an explant method. Briefly, the mesenteric vascular tree was pinned out and mesenteric arteries (third order) were cut longitudinally and pieces (1 mm) placed in DMEM containing 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, penicillin–streptomycin and fungizone. After 7–10 days, migrated cells were pooled and maintained in medium (as above). Cells were used for experiments between passages 2 and 4. All cells were routinely grown in 95% air, 5% CO_2 at 37°C.

Immunofluorescence

RMA-SMCs were washed in 100 mM PBS, pH 7.4, and fixed in PBS containing 4% paraformaldehyde, pH 7.4 (20 min, 4°C) or in methanol (100%, –20°C, 10 min). Cells were washed

with PBS containing 0.1% saponin and 2% normal horse serum (30 min, room temperature). Proteins were localized using the primary antibodies: ECE-1 (1:200), α-smooth muscle actin (1:200) and early endosome antigen-1 (EEA1; 1:500) (overnight, 4°C). Cells were washed, incubated with appropriate secondary antibodies (1:500, 2 h, room temperature) and mounted in Vectashield (Vector Laboratories Ltd, Peterborough, UK).

To obtain whole mount preparations, arteries were prepared using a perfusion fix protocol; the mesenteric vascular bed was excised and perfused with Krebs solution for 5 min as previously described (McNeish *et al.*, 2002). Arteries were then perfusion fixed for 10 min with 100 mM PBS, pH 7.4 containing 1% paraformaldehyde, 10 μM sodium azide and 10 mM betaine. Arteries (third order) were excised, cut longitudinally and pinned flat on Sylgard plates and fixed (100 mM PBS, pH 7.4, 4% paraformaldehyde, 2 h, 4°C). Artery whole mounts were washed in PBS containing 10% normal horse serum and 0.3% Triton-X-100 (1 h, room temperature), and proteins were localized using the primary antibodies: ECE-1 (1:200) and PECAM-1 (1:200) (48 h, 4°C). Whole mounts were washed (1 h, room temperature), incubated with appropriate secondary antibodies (1:500, 2 h, room temperature) and mounted in ProLong® Gold (Invitrogen, Paisley, UK). To obtain sections, rat mesenteric arteries (third order) were embedded in OCT and sectioned at 10 μm and fixed in 100 mM PBS, pH 7.4 containing 4% paraformaldehyde (30 min, 4°C). Sections were washed in PBS containing 10% normal horse serum and 0.3% Triton-X-100 (1 h, room temperature), and proteins were localized using the primary antibodies: ECE-1 (1:200), CLR (1:2000), RAMP1 (1:1000) and α-smooth muscle actin (1:200) (overnight, 4°C). Sections were washed (1 h, room temperature), incubated with appropriate secondary antibodies (1:500, 2 h, room temperature) and mounted in ProLong® Gold (Invitrogen).

Confocal microscopy

For epifluorescence microscopy, cells were observed using a Leica DMI4000B microscope using NPlanL 20×/0.35 objective with a Leica DFC420C camera (Milton Keynes, UK). For confocal microscopy, cells, whole mounts and sections were observed with a Zeiss laser-scanning confocal microscope (LSM Meta 510) using EC Plan-Neofluar 20x/0.5, EC Plan-Neofluar 40x/1.3 Oil DIC and Plan-Apochromat 63×/1.4 Oil DIC objectives. Images were collected at a zoom of 1–2 and an iris of <3 μm, and at least five optical sections were taken at intervals of 0.5 μm. Single sections are shown. Images were processed using ImageJ (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA) and Adobe Photoshop software (San Jose, CA, USA).

Isolated artery tension recording

Mesenteric arteries (2 mm segments, third order) were mounted in a Mulvany–Halpern myograph (model 400A, Danish Myo Technology, Aarhus N, Aarhus, Denmark) in Krebs solution, gassed with 95% O_2, 5% CO_2 and maintained at 37°C. After equilibration for 20 min, vessels were normalized to a tension equivalent to that generated at 0.9 times the diameter of the vessel at 80 mm Hg. Smooth muscle tension was recorded with an isometric force transducer and Powerlab software AD Instruments (Bella Vista, New South Wales, Australia). Vessel viability was assessed by significant constriction (>10 mN) to 3 μM phenylephrine and a relaxation of >95% of the phenylephrine tone by acetylcholine (1 μM). To measure responses to CGRP, mesenteric arteries were first contracted by exposing to an increasing concentration of phenylephrine until maximal constriction was achieved. A concentration of phenylephrine producing approximately 70% of this contraction was used in all further studies. Arteries were then exposed to increasing concentrations of CGRP (1 pM–3 nM) and repeated in the presence of a combination of L-NAME (100 μM, NOS inhibitor), indomethacin (10 μM, non-selective inhibitor of COX-1 and –2), apamin [50 nM, selective small conductance Ca^{2+}-activated K^+-channel (SK_{Ca}) inhibitor] and TRAM34 [10 μM, intermediate K_{Ca} (IK_{Ca}) inhibitor] to assess the role of the endothelium in CGRP-induced relaxation. To assess the role of ECE-1 on the resensitization of responses to CGRP, phenylephrine-constricted arteries were relaxed with CGRP (1 pM–3 nM), washed, constricted again and a CGRP challenge repeated after 5 and 30 min. Following a 1 h rest period, arteries were incubated with SM-19712 (10 μM, 30 min), and the challenges with CGRP were repeated. Time controls were performed to ensure that responses to CGRP were maintained in the absence of SM-19712.

Drug treatments

Confluent wells (12-well plates) of RMA-SMCs were used for experimentation. To measure CGRP-induced cAMP generation, RMA-SMCs were serum-starved in HBSS (2 h), incubated with vehicle (control) or SM-19712, challenged with vehicle (control) or CGRP (10 nM), washed, incubated in CGRP-free medium (30 min) and cAMP generation to a subsequent challenge of CGRP (10 nM) recorded. To inhibit PDE activity, RMA-SMCs were incubated with IBMX (500 μM), 15 min prior to the second challenge with CGRP (10 nM). Controls included appropriate vehicle and SM-19712 was present throughout the experimental time course. To measure CGRP-induced ERK activation, RMA-SMCs were serum-starved in DMEM containing 0.1% BSA (16 h), prior to stimulation with CGRP (10 nM).

Measurement of cAMP

cAMP generation in response to CGRP in RMA-SMCs was measured using a Cyclic AMP XP™ Assay Kit according to the manufacturer's guidelines (New England Biolabs, Hitchin, UK).

SDS-PAGE and Western blotting

Cell lysates (10 μg) were prepared as described (Cottrell *et al.*, 2009). Proteins were separated by SDS-PAGE (12% acrylamide) and transferred to PVDF membranes. Membranes were incubated with antibodies to phosphorylated ERK (pERK1/2; 1:1000) and ERK2 (1:10 000) overnight at 4°C. Membranes were treated with secondary antibodies coupled to HRP (1:10 000), and immunoreactive proteins were visualized by enhanced chemiluminescence and quantified using an ImageQuant RT ECL machine (GE Healthcare, Little Chalfont, UK) with ImageQuantTL software.

Reverse transcription-PCR

RNA from intact rat mesenteric arteries (third order) and RMA-SMCs was isolated using Trizol (Invitrogen) and was

reverse-transcribed using standard protocols with random hexamers and TaqMan reverse transcription reagents (Applied Biosystems, Carlsbad, CA). Subsequent PCR reactions used primers specific for rat CLR, RAMP1 and ECE-1 isoforms (Supporting Information Table S1). Control reactions omitted reverse transcriptase. PCR products were separated by electrophoresis, stained with ethidium bromide and sequenced to confirm identity.

Antibody preadsorption

Membranes were prepared from HEK cells transfected with CLR•RAMP1, ECE-1c-GFP or empty vector (control), were prepared as described (Cottrell *et al.*, 2009). CLR (1:2000), RAMP1 (1:1000) and ECE-1 antibody (1:200) were rotated with membrane proteins (5 mg mL^{-1}) in PBS containing 10% normal horse serum, 0.3% Triton X-100 (overnight, 4°C) before use.

Data analysis

Results are expressed as mean ± SE of $n \geq 3$ experiments and were compared using one-way ANOVA with Tukey's post test or Student's t-test, with $P < 0.05$ (*) considered to be significant. Sigmoidal concentration–response curves were generated in Prism (GraphPad, San Diego, CA) using a four-parameter logistic equation fitted to the Hill equation (with variable slope). Immunofluorescence images and Western blots represent $n \geq 3$ experiments.

Materials

Sources of antibodies and reagents: rabbit anti-rat CLR (RK11) and anti-rat RAMP1 (9891) were a gift from Nigel W Bunnett (Monash University, Victoria, Australia; Cottrell *et al.*, 2005); goat anti-human ECE-1 (R&D Systems, Abingdon, UK); mouse anti-EEA1, BD Transduction Laboratories, Oxford, UK); α-smooth muscle actin (smooth muscle cell marker; A5228; Sigma-Aldrich Company Ltd, Dorset, UK); mouse anti-rat platelet endothelial cell adhesion molecule-1 (endothelial cell marker; PECAM-1/CD31, TLD-3A12, Millipore, Watford, UK); mouse anti-pERK1/2 (E-4) and rabbit anti-ERK2 (C-14) (Insight Biotechnology Ltd, Wembley, UK); donkey anti-mouse, goat or rabbit IgG coupled to fluorescein isothiocyanate, Rhodamine Red-X or Cy5 (Stratech Scientific Limited, Newmarket, UK); rat α-CGRP (Bachem, Weil am Rhein, Germany), apamin (Latoxan, Valence, France); SM-19712 (4-chloro-*N*-[[(4-cyano-3-methyl-1-phenyl-1*H*-pyrazol-5-yl)amino]carbonyl] benzene sulphonamide sodium salt, acetylcholine, L-NAME (N^G-nitro-L-arginine methyl ester), TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole), indomethacin and IBMX (3-isobutyl-1-methylxanthine) (Sigma-Aldrich Company Ltd). Other reagents were from Sigma-Aldrich Company Ltd.

Results

CGRP causes vasodilatation of rat mesenteric arteries via an endothelium-independent mechanism

CGRP is a potent vasodilatory peptide in rat mesenteric resistance arteries (Kawasaki *et al.*, 1988). In order to examine if ECE-1 regulates CGRP-induced relaxation of rat mesenteric arteries, we first determined the site of action of CGRP. We hypothesized that CGRP would act on SMCs directly and relaxation would occur via an endothelium-independent mechanism. In vehicle-treated arteries, CGRP induced concentration-dependent relaxation (CGRP pEC_{50}, 10.49 ± 0.07; Supplemental Figure S1). Arteries were then challenged with CGRP in the presence of inhibitors of endothelium-derived relaxation pathways. Incubation with L-NAME (NOS inhibitor) and indomethacin (non-selective inhibitor of COX-1 and -2) or a combination of L-NAME, indomethacin, apamin [selective small conductance Ca^{2+}-activated K^+-channel (SK_{Ca}) inhibitor] and TRAM34 [intermediate K_{Ca} (IK_{Ca}) inhibitor] had no effect on CGRP-induced relaxation (CGRP pEC_{50}, 10.46 ± 0.07 and 10.45 ± 0.04, respectively; Supporting Information Figure S1).

RMA-SMCs express mRNA for CLR, RAMP1 and ECE-1 isoforms and ECE-1 is localized to early endosomes in RMA-SMCs

To assess if ECE-1 was appropriately localized to regulate CLR•RAMP1, we first characterized cultured RMA-SMCs. Our cultured cells were all positive for the SMC marker, α-smooth muscle actin (Figure 1A). Next, we examined expression of CLR, RAMP1 and all four ECE-1 isoforms in RMA-SMCs by RT-PCR. We amplified mRNA of expected sizes for CLR, RAMP1 and each of ECE-1 isoforms and confirmed identity by sequencing (Figure 1B). We have previously shown that CGRP internalizes with CLR•RAMP1 and is degraded by ECE-1 in the early endosomes of HEK-CLR•RAMP1 cells (Cottrell *et al.*, 2005; Padilla *et al.*, 2007). To determine if ECE-1 was appropriately localized to perform this function in RMA-SMCs, we used immunofluorescence and confocal microscopy with antibodies to ECE-1 and a marker for early endosomes, EEA1. Immunoreactive ECE-1 was detected in intracellular vesicles in α-smooth muscle actin-positive cells and co-localized with EEA1 (Figure 1C). Thus, ECE-1 is present in the early endosomes of RMA-SMCs where it may degrade CGRP to regulate recycling of CLR•RAMP1.

RMA-SMCs express a functional CGRP receptor that is regulated by ECE-1

To determine whether the CLR and RAMP1 expressed in RMA-SMC form a functional CGRP receptor, we examined if CGRP (10 pM–1 μM) induced generation of cAMP. CGRP induced a concentration-dependent increase in cAMP generation (Figure 2A). To further determine the functionality of the CGRP receptor in RMA-SMCs, we examined CGRP-induced ERK activation. RMA-SMCs were challenged with CGRP (10 nM, 0–10 min), and levels of pERK1/2 and total ERK2 were determined by Western blotting. CGRP induced an increase in levels of pERK1 and ERK2 at both time points examined (ERK1, 2 ± 0.4-fold, 2 min, 1.2 ± 0.1-fold, ERK2, 1.9 ± 0.6-fold, 2 min, 1.3 ± 0.1-fold; 10 min), indicating that RMA-SMCs express a receptor through which CGRP can activate ERK1 and ERK2 (Figure 2B).

ECE-1 regulates resensitization of CGRP-induced cAMP generation RMA-SMCs

To determine if ECE-1 regulates resensitization of CGRP responses in RMA-SMCs, we examined the effect of the spe-

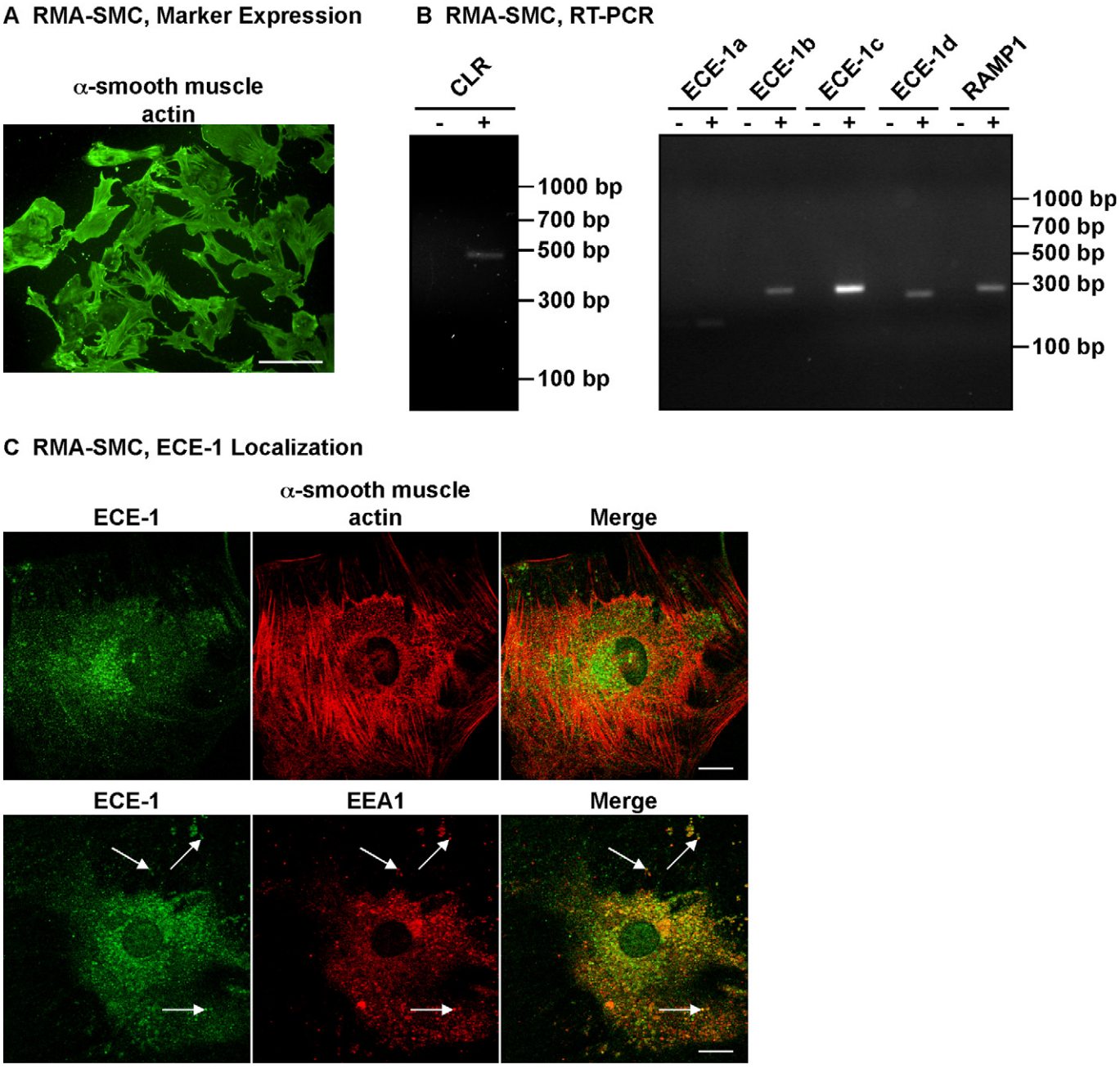


Figure 1

RMA-SMCs express CLR, RAMP1 and ECE-1 isoforms. (A) Expression of immunoreactive α-smooth muscle actin in cultures from rat mesenteric resistance arteries, indicating cultured cells are SMCs. Scale bar, 100 μm. (B) RT-PCR amplification of mRNA for CLR (442 bp), ECE-1a (177 bp), ECE-1b (289 bp), ECE-1c (288 bp), ECE-1d (255 bp) and RAMP1 (277 bp) in RMA-SMCs. bp, base pairs. (C) Immunoreactive ECE-1 was detected in vesicles in α-smooth muscle actin-positive cells. ECE-1 co-localizes with a marker for early endosomes, the early endosome antigen-1 (EEA1) in RMA-SMCs. Scale bar, 10 μm; $n = 3$.

cific ECE-1 inhibitor, SM-19712 (Umekawa *et al.*, 2000) on resensitization of CGRP-induced cAMP generation. RMA-SMCs were incubated with vehicle (control) or SM-19712 and then challenged with vehicle or CGRP (10 min) to desensitize CLR•RAMP1. Cells were washed, incubated in CGRP-free medium (30 min) to allow resensitization to proceed and then challenged with CGRP. cAMP generation to the second challenge of CGRP was then determined for each group. In vehicle-treated cells, resensitization was 70 ± 12% (compared to unstimulated vehicle control [100%], $n = 4$) (Figure 2C). In contrast, in SM-19712-treated cells, resensitization was strongly inhibited (35 ± 10% compared with unstimulated SM-19712 control [100%], $n = 4$) (Figure 2C).

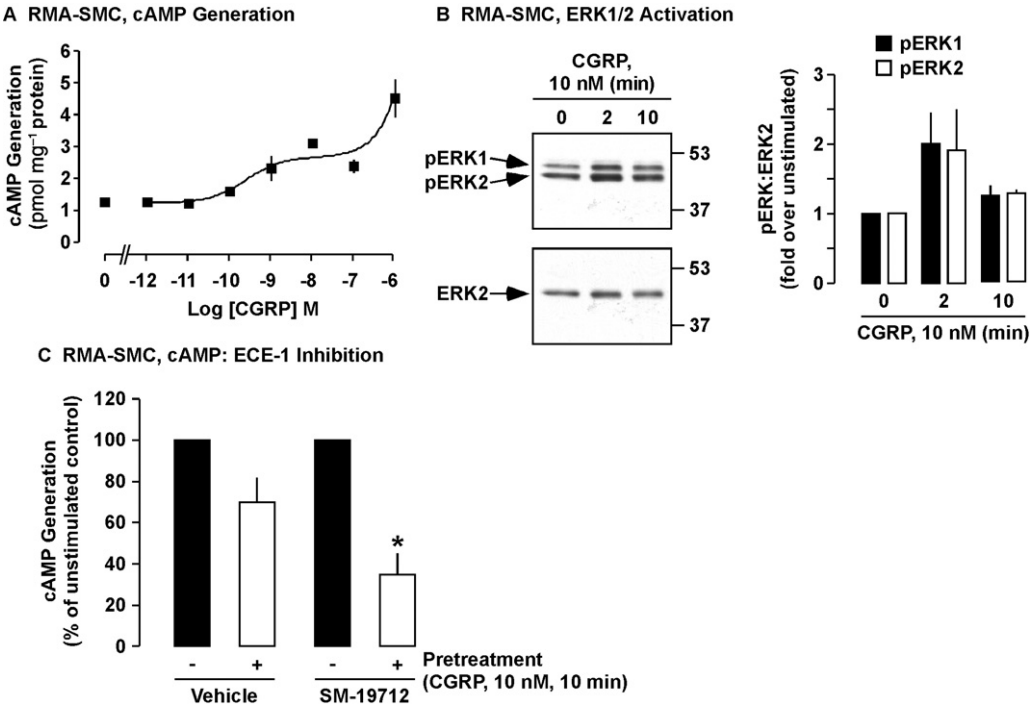


Figure 2

ECE-1 regulates resensitization of CGRP-induced cAMP generation in RMA-SMCs. (A) Concentration–response analysis of CGRP-induced cAMP generation in RMA-SMCs. CGRP induced a biphasic response indicating the presence of more than one receptor type in RMA-SMCs. $n = 3$ wells. (B) Western blot analysis of CGRP-induced phosphorylation of ERK in RMA-SMCs. CGRP induced a prompt increase in levels of phosphorylated ERK1 and ERK2 (pERK1/2) at 2 and 10 min. $n = 3$ (C) RMA-SMCs were incubated with vehicle (control) or SM-19712, stimulated with vehicle (control) or CGRP (10 nM, 10 min), washed, incubated in CGRP-free medium (30 min) and cAMP accumulation to a second challenge of CGRP (10 nM) recorded. SM-19712 reduced resensitization of CGRP-induced cAMP generation (vehicle, 70 ± 12% and SM-19712, 35 ± 10%). $n = 4$, $^*P < 0.05$.

CLR, RAMP1 and ECE-1 are localized to the smooth muscle layer of rat mesenteric arteries

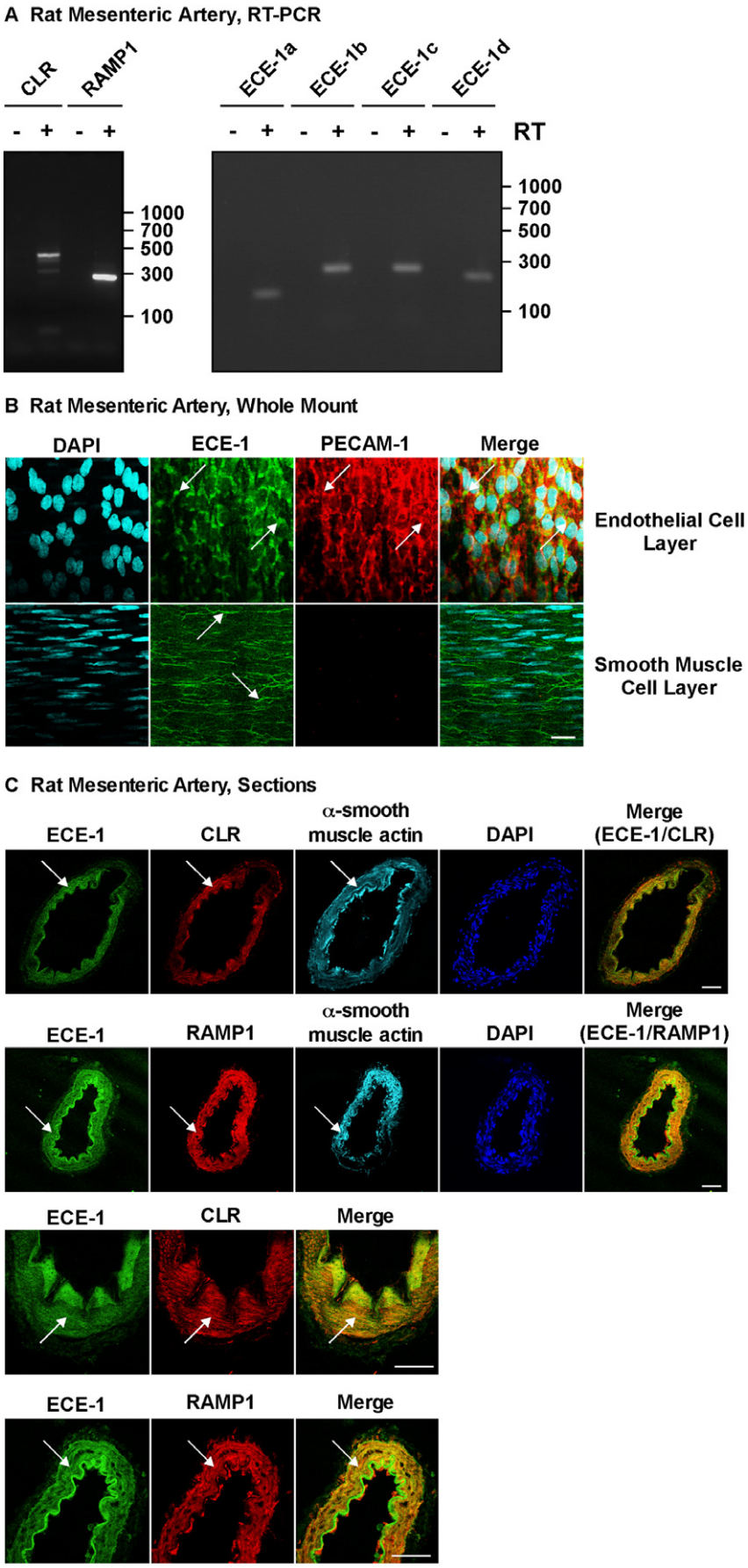
We examined expression of CLR, RAMP1 and all four ECE-1 isoforms in intact rat primary mesenteric arteries by RT-PCR. We amplified mRNA of expected sizes for CLR, RAMP1 and each of ECE-1 isoforms and confirmed identity by sequencing (Figure 3A). Examination of ECE-1 expression at the protein level was determined by staining whole mounts of rat mesenteric arteries. Immunoreactive ECE-1 was detected in the PECAM-1-positive (endothelial) layer and PECAM-1-negative (smooth muscle) layers of arteries (Figure 3B). We also examined expression of CLR, RAMP1 and ECE-1 expression in sections of artery. We observed immunoreactive CLR, RAMP1 and ECE-1 in the smooth muscle layer (Figure 3C). Preadsorption of the ECE-1, CLR and RAMP1 antibodies with membranes from HEK-ECE-1c-GFP cells (ECE-1) or HEK-CLR•RAMP1 (CLR and RAMP1) abolished staining compared to membranes from cells expressing empty vector (Supporting Information Figure S2), confirming the specificity of antibodies for detecting CLR, RAMP1 and ECE-1. Thus, ECE-1 is appropriately localized to regulate CGRP-induced responses in rat mesenteric arteries.

ECE-1 regulates the resensitization of CGRP-induced vasodilatation in rat mesenteric arteries

To investigate if ECE-1 regulates the resensitization of CGRP-induced responses in intact tissues, we examined the effect of SM-19712 on CGRP-induced relaxation of rat mesenteric arteries. Arteries were incubated with vehicle (control), challenged with increasing concentrations of CGRP, washed and challenged again with CGRP, 5 and 30 min later. This process

Figure 3

Rat mesenteric arteries express ECE-1 and the CGRP receptor components, CLR and RAMP1. (A) RT-PCR amplification of mRNA for CLR (442 bp), RAMP1 (277 bp), ECE-1a (177 bp), ECE-1b (289 bp), ECE-1c (288 bp), ECE-1d (255 bp) in rat mesenteric arteries. (B) Localization of nuclei (DAPI), ECE-1 and PECAM-1 in whole mounts of rat mesenteric artery. Immunoreactive ECE-1 was detected in the endothelial and SMC layers of mesenteric arteries. PECAM-1-immunoreactivity was only detected in the endothelial cell layer. Scale bar, 20 μm. (C) Immunoreactive CLR, RAMP1 and ECE-1 was detected in the smooth muscle layer of sections of mesenteric arteries. Nuclei are shown by DAPI staining. Scale bar, 50 μm; $n = 3$.


A Rat Mesenteric Artery, RT-PCR
CLR
RAMP1
ECE-1a
ECE-1b
ECE-1c
ECE-1d
- + - + - + - + - + RT
1000
700
500
300
100
B Rat Mesenteric Artery, Whole Mount
DAPI
ECE-1
PECAM-1
Merge
Endothelial Cell Layer
Smooth Muscle Cell Layer
C Rat Mesenteric Artery, Sections
ECE-1
CLR
α-smooth muscle actin
DAPI
Merge (ECE-1/CLR)
ECE-1
RAMP1
α-smooth muscle actin
DAPI
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CLR
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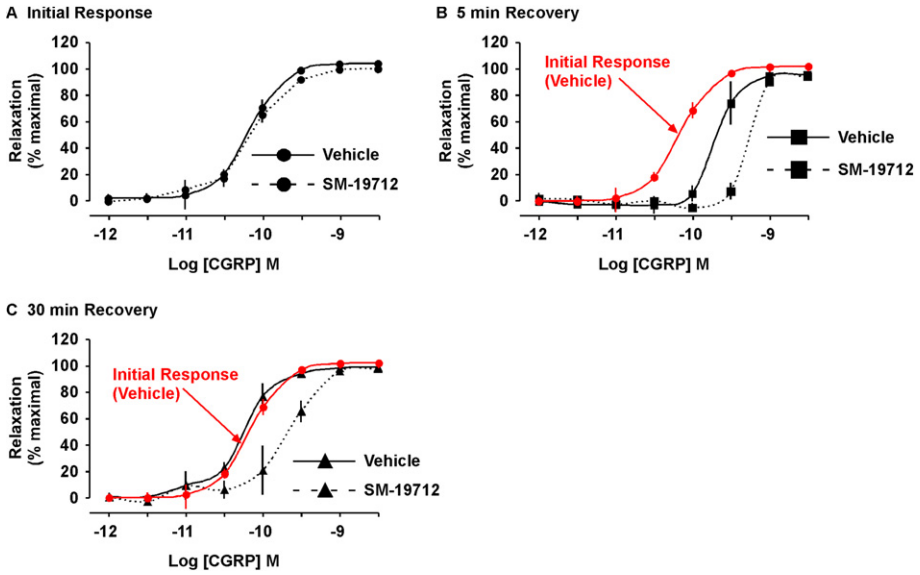

Figure 4

Effect of the ECE-1 inhibitor SM-19712 on CGRP-induced relaxation in rat mesenteric arteries. Arteries were incubated with vehicle (control), contracted with phenylephrine (0.3–10 μM), exposed to increasing concentrations of CGRP (1 pM–3 nM), washed, contracted with phenylephrine and exposed again to increasing concentrations of CGRP (1 pM–3 nM). Arteries were then incubated with SM-19712 (10 μM) and the concentration-response curves repeated. (A) Incubation of arteries with SM-19712 had no effect on initial responses to CGRP. (B, C) SM-19712 shifted the pEC_{50} to a second challenge of CGRP at both 5 and 30 min after the initial challenge. $n = 4$ arteries for each group.

Table 1

Effect of ECE-1 inhibition with SM-19712 on CGRP-induced relaxation in rat mesenteric arteries

	Vehicle	SM-19712
Initial response	10.16 ± 0.05	10.13 ± 0.04
5 min recovery	9.67 ± 0.06*	9.29 ± 0.06*
30 min recovery	10.25 ± 0.04	9.65 ± 0.09*

Data in the Table are pEC_{50} values for initial responses and subsequent challenges, 5 and 30 min after initial challenge in the presence of vehicle (control) or SM-19712. Arteries were incubated with vehicle (control), contracted with phenylephrine (0.3–10 μM), exposed to increasing concentrations of CGRP (1 pM–3 nM), washed, contracted with phenylephrine and exposed again to increasing concentrations of CGRP (1 pM–3 nM). Arteries were then incubated with SM-19712 (ECE-1 inhibitor, 10 μM), and the concentration-response curves were repeated.. *$P \leq 0.005$ compared with vehicle- and SM-19712-treated arteries (initial response). $n = 4$ arteries for each group.

was then repeated in the same arteries following incubation with SM-19712. The initial challenge with CGRP induced a concentration-dependent relaxation that was identical in the presence of vehicle and SM-19712 (CGRP pEC_{50}, 10.16 ± 0.05, vehicle; 10.13 ± 0.04, SM-19712; $n = 4$ for each group) (Figure 4A, Table 1). When challenged 5 min later, the potency of CGRP in both following both vehicle and SM-19712 treatment was reduced, indicating desensitization of CGRP receptors (CGRP pEC_{50}, 9.67 ± 0.06, vehicle; 9.29 ± 0.06, SM-19712; $n = 4$ for each group) (Figure 4B, Table 1). When challenged 30 min later, CGRP induced relaxation of vehicle-treated arteries with a similar potency to initial responses, indicating CGRP receptors had resensitized (CGRP pEC_{50}, 10.25 ± 0.04; $n = 4$) (Figure 4C, Table 1). In contrast, following treatment with SM-19712, the pEC_{50} to the second challenge of CGRP was still reduced compared to initial responses, indicating that CGRP receptors were still desensitized (CGRP pEC_{50}, 9.65 ± 0.09; $n = 4$) (Figure 4C, Table 1).

Discussion and conclusions

In this study, we demonstrate for the first time that ECE-1 regulates the resensitization of CGRP responses in SMCs and regulates CGRP-induced relaxation of mesenteric resistance arteries. CLR and RAMP1 are only co-expressed in the smooth muscle layer of mesenteric arteries, a finding that mimics the expression pattern observed in cranial arteries (Lennerz *et al.*, 2008). Therefore, we used mesenteric resistance arteries not only as a suitable model to study CGRP receptor regulation in relation to migraine (without the technical difficulties associated with accessing cranial arteries) but also because of their importance in regulating blood pressure. Thus, given the important role of CGRP in pain transmission and regulation of vascular tone, our finding that ECE-1 regulates CGRP-induced responses in arteries may be an important development in the search for alternative strategies to treat migraine.

We found that RMA-SMCs and mesenteric arteries contain mRNA for CLR, RAMP1 and all four isoforms of

ECE-1. Furthermore, we observed ECE-1-immunoreactivity in the early endosomes of RMA-SMCs and in the smooth muscle layer of mesenteric arteries. Thus, ECE-1 is expressed in RMA-SMCs, which also express mRNA transcripts for CLR and RAMP1. Therefore, if CGRP internalizes to endosomes with CLR•RAMP1, as observed in transfected HEK cells (Cottrell *et al.*, 2007; Padilla *et al.*, 2007), ECE-1 would be appropriately localized to degrade CGRP. In support of this finding, ECE-1-immunoreactivity has been detected in many types of human SMCs (Barnes and Turner, 1999; Granchi *et al.*, 2002; Jackson *et al.*, 2006). In human umbilical artery SMCs, ECE-1 was present in punctate perinuclear vesicles, similar to the early endosomal vesicles observed in our experiments (Barnes and Turner, 1999). ECE-1 protein has also been detected in human temporal arteries (Lozano *et al.*, 2010), although the location of ECE-1 in these vessels remains undetermined. Thus, we are the first to show the endosomal location of ECE-1 in SMCs, where it may degrade internalized CGRP and perhaps other neuropeptides, to regulate GPCR recycling and resensitization.

Our experiments indicated that RMA-SMCs express a functional CGRP receptor. CGRP induced a concentration-dependent increase in levels of cAMP and phosphorylation of ERK1 and ERK2. In support of this observation, CGRP has been shown to induce accumulation of cAMP and/or phosphorylation of ERKs in many cell types, including smooth muscle, endothelial and epithelial cells (Kubota *et al.*, 1985; Hirata *et al.*, 1988; Van Valen *et al.*, 1990; Kawase *et al.*, 1999; Yu *et al.*, 2006). One interpretation of our cAMP data is that the responses to CGRP yielded a typical biphasic curve, with the first increase (CGRP, 0.1–10 nM) reflecting activation of CLR•RAMP1. The latter increase (CGRP, 1 μM) probably reflecting the ability of CGRP to activate adrenomedullin receptors at high concentrations (Roh *et al.*, 2004). However, further experimentation would be required to confirm the biphasic nature of the CGRP response in RMA-SMCs. This biphasic phenomenon of the CGRP response was not observed in our artery relaxation studies as 100% relaxation was achieved before using micromolar concentrations of CGRP.

SM-19712 is a highly selective and potent inhibitor of ECE-1 that prevents the conversion of big endothelin-1 to endothelin-1 by solubilized rat lung microsomes (IC_{50}, 42 nM) and by cultured porcine aortic endothelial cells (IC_{50}, 31 μM). However, unlike other ECE-1 inhibitors such as phosphoramidon, which can inhibit metallopeptidases such as neprilysin (neutral endopeptidase 24.11) and angiotensin I-converting enzyme (Kukkola *et al.*, 1995) that may play a role in the metabolism of CGRP (Tramontana *et al.*, 1991; Kramer *et al.*, 2006), SM-19712 (10–100 μM) has no effect on these peptidases (Umekawa *et al.*, 2000). Further selectivity was demonstrated by the lack of effect on agonist binding at numerous GPCRs, including the angiotensin II (type I and II) receptors, endothelin-1 (A and B) receptors, neuropeptide Y_2 receptor and vasoactive intestinal peptide receptor (Umekawa *et al.*, 2000). ECE-1 regulated CGRP receptor resensitization in RMA-SMCs, as SM-19712 inhibited the resensitization of CGRP-induced cAMP generation. This result was expected as ECE-1 regulates CGRP-induced Ca^{2+} signalling in cell lines. In HEK cells and a neuroblastoma cell line (SK-N-MC) that naturally expresses CLR and RAMP1 (Van Valen *et al.*, 1990), ECE-1 knockdown and the ECE-1 inhibitors, SM-19712 and PD069185 (Ahn *et al.*, 1998) reduce resensitization of CGRP-induced Ca^{2+} signalling (McLatchie *et al.*, 1998; Padilla *et al.*, 2007). Conversely, ECE-1 overexpression promoted resensitization of CGRP-induced Ca^{2+} signalling (Padilla *et al.*, 2007).

Our results strongly suggest CGRP-induced relaxation is solely dependent on activation of CGRP receptors on SMCs, as inhibitors of NO generation (L-NAME), prostaglandin production (indomethacin) and the endothelium-derived hyperpolarizing factor response [block of SK_{Ca} (apamin) and IK_{Ca} channels (TRAM34)] had no effect on CGRP-induced relaxation of these arteries. This finding is in agreement with a previous study showing that CGRP induces relaxation of rat mesenteric arteries via a mechanism that depends minimally on the endothelium and K^+-channel opening (Lei *et al.*, 1994) and supported by the co-localization of CLR and RAMP1 only in the smooth muscle layer of mesenteric arteries (Cottrell *et al.*, 2005). Interestingly, in rat dura mater and human cranial vessels, CLR and RAMP1 have also been localized to the smooth muscle layers (Oliver *et al.*, 2002; Edvinsson *et al.*, 2010), suggesting that CGRP may regulate brain blood flow via a similar mechanism to the one existing in the mesentery. Thus, the mesenteric artery may represent a useful model to study CGRP receptor regulation in relation to brain disorders.

We further characterized the role of ECE-1 in regulation of CGRP responses by examining the effects of SM-19712 on CGRP-induced relaxation of isolated rat mesenteric arteries. ECE-1 inhibition had no effect on the pEC_{50} for initial challenges with CGRP, which is unsurprising, as ECE-1 does not affect $[Ca^{2+}]_i$ mobilization to initial challenges of CGRP (Padilla *et al.*, 2007). However, the potency of CGRP (5 min later) was reduced in vehicle- and SM-19712-treated arteries, indicating CGRP receptor desensitization. Similarly, tachyphylaxis to CGRP has been observed in other studies using rat mesenteric arteries (Han *et al.*, 1990) and rat intramural coronary arteries (Sheykhzade and Berg Nyborg, 2004). Although the pEC_{50} following SM-19712-treatment reflects a greater degree of desensitization, we hypothesize that this difference in potency is due to rapid recycling of CGRP receptors in the control group and not a greater desensitization/activation of CGRP receptors following SM-19712-treatment. Indeed, at 30 min in arteries treated with vehicle, the pEC_{50} for CGRP-induced relaxation had returned to initial values, indicating CGRP receptor resensitization. In contrast, ECE-1 inhibition prevented CGRP receptor resensitization at 30 min. The kinetics of resensitization we observed in arteries, were much faster than those observed in HEK-CLR RAMP1 cells (Padilla *et al.*, 2007). However, experimental conditions (CGRP concentration and time of exposure) were vastly different in the two experiments, which may account for this discrepancy. Furthermore, it may also reflect differences in levels of ECE-1 expression. Previous studies have shown that ECE-1 knockdown or overexpression can alter the kinetics of resensitization of ECE-1-regulated GPCRs (Padilla *et al.*, 2007). If this ECE-1-dependent mechanism operates in the cerebral sites implicated in migraine remains to be determined. However, as CLR and RAMP1 are expressed in the smooth muscle, but not the endothelial layer of the cerebral vasculature (Oliver *et al.*, 2002), it would seem likely, provided that ECE-1 is also expressed in this location. The degranulation of mast cells, which leads to the release of

histamine, has also been suggested to play an important role in the pathophysiology of migraine (Sicuteri, 1963; Levy, 2009). It is well established that CGRP can trigger mast cell degranulation (Piotrowski and Foreman, 1986), but whether ECE-1 regulates the recycling and resensitization of CGRP receptors in mast cells has yet to be determined. However, if these cells do express ECE-1, it is likely that mast cell CGRP receptors would also be regulated by the same mechanism.

It has been postulated that certain migraineurs may be sensitized to the effects of CGRP, by the up-regulation of RAMP1 (Zhang *et al.*, 2007). Indeed, mice overexpressing human RAMP1 demonstrate an increased sensitivity to CGRP and exhibit light-aversive behaviour similar to photophobia in migraine patients (Recober *et al.*, 2009). Another mechanism that may cause CGRP sensitization could be decreased times for CGRP receptor resensitization. Patients with increased ECE-1 expression would have much reduced receptor recycling times (due to enhanced degradation of CGRP in endosomes) and thus be able to respond more quickly to subsequent releases of CGRP. Whether ECE-1 expression is altered in migraineurs is not yet known, but it is an enticing possibility. In support of this notion, it has been reported that many migraineurs have elevated levels of endothelin-1 (a vasoactive peptide generated by the proteolytic activity of ECE-1) (Farkkila *et al.*, 1992). This lead to the hypothesis that antagonism of endothelin A receptors would be an effective treatment for migraine. However, although bosentan, a selective endothelin A receptor, antagonist was effective at reducing neurogenic plasma extravasation in rats, it was ineffective as a treatment in humans for migraine (May *et al.*, 1996).

The mechanism by which ECE-1 promotes recycling of GPCRs from endosomes back to the cell surface to mediate resensitization of signalling appears to be common for receptors with peptide ligands that are substrates for ECE-1 only at endosomal pH and that exhibit a sustained interaction with β-arrestins (Padilla *et al.*, 2007; Roosterman *et al.*, 2007; 2008). In the acidified environment of endosomes, ECE-1 degrades the peptide ligands to inactive metabolites disrupting the peptide–receptor–β-arrestin complex, allowing the receptor to recycle to the cell-surface. However, it remains to be determined whether this mechanism also controls CGRP-induced endosomal-based signalling, as it does for substance P-induced ERK activation from the neurokinin-1 receptor (Cottrell *et al.*, 2009).

In conclusion, we report that vascular ECE-1 activity promotes the resensitization of CGRP-induced vasodilatation of mesenteric resistance arteries, by promoting resensitization of CLR•RAMP1. CGRP receptor blockade is a current strategy for the treatment of migraine. An advantage that ECE-1 inhibition would have over CGRP receptor antagonism would be the preservation of the acute effects of CGRP. For example, this would maintain the ability of CGRP to play a protective role during ischaemia caused by stroke but would decrease responses to a more sustained release of CGRP, which may occur during a migraine attack. However, additional experimentation is required, as inhibition of ECE-1 would reduce endothelin-1 production, which in itself may cause vascular side effects. In conclusion, we believe inhibitors of endosomal ECE-1, by attenuating resensitization of CGRP receptors, may represent an alternative strategy for the treatment of migraine.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effect of endothelial-derived pathway inhibitors on CGRP-induced relaxation in rat mesenteric arteries. Arteries were incubated with vehicle (control), L-NAME and indomethacin or L-NAME, indomethacin, apamin and TRAM34, contracted with phenylephrine (0.3–10 μM) and exposed to increasing concentrations of CGRP. (A) Incubation with L-NAME, indomethacin or L-NAME, indomethacin, apamin and TRAM34 had no effect on CGRP-induced relaxation. $n = 4$ arteries for each group.

Figure S2 Effect of blocking proteins on detection of CLR, RAMP1 and ECE-1 in rat mesenteric arteries. Antibodies to ECE-1, CLR and RAMP1 were preincubated with HEK cell membranes transfected with empty vector (control), ECE-1c-GFP or CLR•RAMP1 as indicated. Incubation of antibodies with membranes expressing blocking proteins reduced or abolished immunoreactive signals. Staining conducted with antibodies incubated with control membranes was unaffected. Scale bar, 20 μm.

Table S1 Sequences of RT-PCR primers.