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Chemerin-induced arterial contraction is G_i - and calcium-dependent

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Abstract

Chemerin is an adipokine associated with increased blood pressure, and may link obesity with hypertension. We tested the hypothesis that chemerin-induced contraction of the vasculature occurs via calcium flux in smooth muscle cells. Isometric contraction of rat aortic rings was performed in parallel with calcium kinetics of rat aortic smooth muscle cells to assess the possible signaling pathway. Chemerin-9 (nonapeptide of the chemerin S^{157} isoform) caused a concentration-dependent contraction of isolated aorta (EC_{50} 100 nM) and elicited a concentration-dependent intracellular calcium response (EC_{50} 10 nM). Pertussis toxin (G_i inhibitor), verapamil (L-type Ca^{2+} channel inhibitor), PP1 (Src inhibitor), and Y27632 (Rho kinase inhibitor) reduced both calcium influx and isometric contraction to chemerin-9 but PD098059 (Erk MAPK inhibitor) and U73122 (PLC inhibitor) had little to no effect on either measure of chemerin signaling. Although our primary aim was to examine chemerin signaling, we also highlight differences in the mechanisms of chemerin-9 and recombinant chemerin S^{157} . These data support a chemerin-induced contractile mechanism in vascular smooth muscle that functions through G_i proteins to activate L-type Ca^{2+} channels, Src, and Rho kinase. There is mounting evidence linking chemerin to hypertension and this mechanism brings us closer to targeting chemerin as a form of therapy.

Keywords

Chemerin; Smooth muscle; Signaling

1. Introduction

Obesity and hypertension are pathologies that continue to become more prevalent around the world, particularly in adolescent populations [1]. Chemerin is a relatively novel protein with the potential to connect these two diseases. Identified as an adipokine [2], serum chemerin concentrations have been positively correlated with increased levels of human white adipose tissue [3], increased body mass index [4, 5], obesity [4], and even childhood obesity [6]. Loss of white adipose tissue through exercise or bariatric surgery reverses the levels of circulating chemerin [7]. Additionally, the active form of chemerin (chemerin S^{157}) is positively associated with blood pressure in both humans [8] and mice [9].

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Chemerin is produced by hepatocytes and adipocytes [2] and may be converted to an active isoform before it leaves the cell [10]. There are currently three receptors known to bind chemerin: CCRL2 [11], GPR1 [12] and the chemerin receptor [13]. CCRL2 binds chemerin without transducing a signal [11]. GPR1 is able to actively transduce a signal through G_i proteins, RhoA, and MAPK pathways [14]. Our laboratory was the first to demonstrate that chemerin-9 (a nonapeptide derived from the C-terminus of the S¹⁵⁷ isoform [15]) directly caused isometric contraction of aorta through the chemerin receptor [16, 17]. Although the chemerin receptor has been known by several names in the literature (CMKLR1, ChemR23 or DEZ), the currently accepted name for the receptor, as given by *IUPHAR/BPS*, is the “chemerin receptor” [18]. Although GPR1 and CCRL2 can bind chemerin, the term “chemerin receptor” describes a specific protein that is separate from GPR1 or CCRL2.

When chemerin signals through the chemerin receptor, it recruits a wide range of second messengers in a cell-specific manner: ERK stimulates chemotaxis in immune cells [19], p38 and Akt stimulate angiogenesis in endothelial cells [20], and PKC can trigger internalization of the receptor [21]. Our lab has shown that chemerin-9-induced isometric contraction is potentiated by phenylephrine and prostaglandins via a calcium-dependent mechanism. Additionally, chemerin-9 also directly simulated chemerin receptor-dependent contraction of the rat aorta when the endothelium was removed [16]. This broad heterogeneity of potential signaling mechanisms led us to ask the mechanistic question of how chemerin brings about smooth muscle contraction in a calcium-dependent manner that could contribute to the elevated total peripheral resistance commonly found in hypertension.

Calcium signaling is an essential part of smooth muscle contraction. After a flux of calcium into the cytoplasm, it binds and activates calmodulin which activates myosin light chain kinase (MLCK) to phosphorylate myosin heads, promote cross-bridging with actin, and allow contraction [22]. Although calcium is not the direct activator of myosin-actin cross-bridging, it is still a necessary and easily quantifiable step.

With the hypothesis that chemerin causes constriction of vascular smooth muscle through the chemerin receptor in a calcium-dependent manner, we first set out to characterize the pathways that are essential to support contraction of isolated aorta. We started with pharmacological inhibitors that target processes known to be important to smooth muscle signaling: verapamil for the L-type calcium channel [23], PP1 for Src [24], Y27632 for Rho kinase [25], PD098059 for Erk MAPK [17], and U73122 for PLC [26]. Because of the previous G_i protein link to the chemerin receptor [27], PTX was also tested. In whole tissue, there are different cell types that can communicate to influence the concerted action that results in contraction. Because endothelium-denuded aorta showed the greatest response to chemerin [16, 17], we designed parallel studies to investigate how the smooth muscle cells in the tissue are responsible for the physiological effects of chemerin on aortic constriction. The same inhibitors that produced significant reductions of contraction in aortic rings were tested in rat aortic smooth muscle cultures using a calcium fluorophore in a real-time calcium flux detection assay. By comparing the results from these two approaches, we identified which pathways in the smooth muscle are responsible for a chemerin-induced contraction of the vasculature.

2. Methods

2.1. Animal use

All procedures that involved animals were performed in accordance with the institutional guidelines and animal use committee of *Michigan State University* and the *NIH Guidelines on Use of Lab Animals*. Animals were maintained on a 12/12 light/dark cycle at a temperature of 22–25°C. Normal male Sprague-Dawley rats (225–300 g; Charles River Laboratories, Inc., Portage, MI, USA) were used. Prior to all dissection, rats were anesthetized with Fatal Plus® (60–80 mg/kg, i.p.).

2.2. Chemicals

Chemerin-9 was purchased from GenScript (#RP20248, Piscataway, NJ, USA), recombinant chemerin from BioVision (#4002, San Francisco, CA, USA), and both solubilized in deionized water. Pertussis toxin (#P7208), angiotensin II (#A9525), acetylcholine (#A6625), clonidine (#C7897) and phenylephrine (#P6126) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Verapamil (#0654), Y27632 (#1254), PD098059 (#1213), and PP1 (#1397) were purchased from Tocris Bioscience (R & D, Minneapolis, MN, USA). U73122 (#70740) was purchased from Cayman Chemical (Ann Arbor, MI, USA). CCX832 was a gift from Chemocentryx (Mountain View, CA, USA).

2.3. Isometric contraction

Aortic rings [cleaned of perivascular adipose tissue (as an endogenous chemerin source), and endothelium-denuded] were mounted in tissue baths for isometric tension recordings using Grass transducers (FT03) and PowerLab data acquisitions running Chart 7.0 (ADInstruments, Colorado Springs, CO, USA). The endothelium was removed so as to focus on a vascular smooth muscle response. Baths contained standard physiological salt solution (PSS) [mM: NaCl (130.00); KCl (4.70); KH₂PO₄ (1.18); MgSO₄·7H₂O (1.17); CaCl₂·2H₂O (1.60); NaHCO₃ (14.90); dextrose (5.50); and CaNa₂EDTA (0.03), pH 7.2], warmed to 37 °C and aerated (95% O₂/CO₂). Rings were placed under optimum resting tension (4 g) and equilibrated for 1 h, with washing, before exposure to compounds. Administration of an initial concentration of 10 µM phenylephrine (PE) was used to test arterial viability and the absence of the endothelium was verified by a lack of acetylcholine (1 µM)-induced relaxation of a half-maximal PE-induced contraction; this was <10% in all tissues included for analysis.

Tissues were then washed out and incubated with either vehicle (water, 0.1% ethanol, 0.1% DMSO, or 0.01% DMSO) or one of the following inhibitors for 1 h: L-type calcium channel inhibitor verapamil, Rho kinase inhibitor Y27632, PLC inhibitor U73122, Erk MAPK inhibitor PD098059, Src inhibitor PP1 or CCX832. Following this incubation, cumulative response curves were generated to the agonist chemerin-9 (10^{-10} – 3×10^{-6} M). Three different isolated tissue bath systems with four individual organ baths were used to generate these curves (no system or bath dependence of results), vehicle or inhibitors were randomized but incubated with tissues from the same animal when possible, and tissues were exposed to only one vehicle or inhibitor. In other experiments, chemerin-9 (1 µM) was

incubated with tissues for 0 or 5 min in the isolated tissue bath, and tissues were frozen in liquid nitrogen at this point in contraction for western blot analyses.

For the study of pertussis toxin (PTX) in isometric contraction, perivascular adipose tissue and endothelium were removed from paired aortic rings before incubating them on a rotator overnight at 37 °C in Complete Medium (described in Cell culture below) with either PTX (1000 ng/mL) or vehicle (water). Tissues were washed in PSS, placed in a tissue bath (conditions described above), and pulled to a resting tension of 4 g. After the 10 μ M PE challenge and 1 μ M acetylcholine test to confirm endothelial removal, tissues were challenged with a cumulative response curve to chemerin-9 and clonidine (α_2 adrenergic agonist). The order of agonists, baths, and force transducers was randomized.

2.4. Cell culture

Aorta was removed and cleaned of fat and endothelium in a sterile environment with phosphate buffered saline (PBS) containing 2% penicillin-streptomycin (P/S) (#15140122, Gibco/Thermo Fisher, Waltham, MA, USA). Sections were placed lumen side-down on a p60 dish and allowed to grow in Dulbecco's Modified Eagle's Medium (DMEM, Gibco/Thermo Fisher) with 45% Fetal Bovine Serum (FBS; #16000044, Gibco/Thermo Fisher), 1% P/S, and 1% glutamine (#25030081, Gibco/Thermo Fisher). Once confluent, cells were passed and allowed to grow in Complete Medium (DMEM, 10% FBS, 1% P/S, and 1% glutamate). Smooth muscle was confirmed by an immunocytochemistry stain with FITC-conjugated smooth muscle alpha-actin (#F3777, Sigma Chemical; method described below in Immunocytochemistry). Cells were harvested for use in all experiments between passage 2 and passage 5.

2.5. Calcium kinetics

Initial studies titrated the concentration of smooth muscle cells between 5000 and 20,000 cells/well to determine the lowest seed density with discernable signal in a 384-well plate. We recognize that in vivo smooth muscle cells are tightly packed and denser than what we have titrated, but this titration was necessary to maintain the high sensitivity of the instrument and is standard practice in the field. Once optimized, smooth muscle cells were suspended in Complete Medium and allowed to incubate (37 °C and 5% CO₂) in a 384-well plate overnight at a density of 10,000 cells/well. If applicable, PTX (500 ng/mL) was added to the media for an overnight incubation. At the t-60 minute time point before agonist injection, excess media were washed off and replaced by calcium dye (Fluo-4 NW, #F36206, Thermo Fisher). Calcium dye buffer contains 98% HBSS [#14025092, Thermo Fisher; mM: CaCl₂ (1.26), MgCl₂-6H₂O (0.49), MgSO₄-7H₂O (0.41), KCl (5.33), KH₂PO₄ (0.44), NaHCO₃ (4.17), NaCl (137.93), Na₂HPO₄ (0.34), dextrose (5.56)] and 2% HEPES (#15630106, Thermo Fisher). The plate was incubated at 37 °C for 30 min, followed by 30 min at room temperature. Inhibitors were manually added along with the calcium dye at the t-60 minute time point. Calcium dye (and inhibitor, if applicable) was not washed off. The assay plate was loaded directly into the FDSS/ μ cell (Hamamatsu Photonics, Japan) along with separate compound plates (one containing chemerin receptor antagonist for a t-170 second injection and one for an agonist t = 0 injection). Recording began at the t-3 minute time point and both the t-170 second and t = 0 injections were made directly by the FDSS/ μ

cell. For wells that received a manual t-60 minute injection, there was no volume added at the t-170 second injection. Recording occurred every second starting at t-3 min continuing through t + 5 min. A sub-baseline value was taken at t + 2 s (after injection of the agonist). Reported values are in the format of “max-min” where “max” equals the maximum value occurring after t + 2 s and “min” equals the t + 2 second sub-baseline. Each biological replicate was analyzed in triplicate and the N values represent the total number of calcium curves analyzed.

2.6. Immunocytochemistry

Cells were suspended in Complete Medium and allowed to adhere to a coverslip overnight, washed with PBS, then fixed with chilled 1:1 ace-tone-methanol. If applicable, cells were washed with PBS and incubated for 10 min with wheat-germ agglutinin tagged with an Alexa Fluor 555 (W32464, Thermo Fisher). Cells were washed with PBS and blocked for 1 h (horse serum, #S-2000, Vector Laboratories, Burlingame, CA, USA) at room temperature followed by an incubation with a FITC-conjugated alpha smooth muscle actin (anti-mouse, 1:500 concentration), GPR1 antibody (anti-mouse, clone 43.28.4, gift from Dr. Brian Zabel, 1:100 concentration), or chemerin receptor antibody (anti-mouse, #398769, Santa Cruz Biotechnology, Dallas, TX, USA, 1:50 concentration) for 1 h at 37 °C. After a wash with PBS, a FITC secondary (Alexa Fluor 488, #A11029, Thermo Fisher, 1:1000 concentration) was applied for 30 min at room temperature (not needed for FITC-conjugated alpha smooth muscle actin) followed by a final wash with PBS. Prolong Gold with DAPI (#P36935, Gibco/Thermo Fisher) was added before sealing the coverslip to a slide with nail polish. Imaging and normalization for autofluorescence were done using the Olympus FluoView 1000 Confocal Laser Scanning Microscope at Michigan State University Center for Advanced Microscopy. Minimum detection thresholds were set using the no-primary control. Images were taken every 0.2 µm for the entire depth of the cell (about 4–5 µm). Using DAPI to match depth, images were taken from the same level and paired with the no-primary control. To help visualize the signal, all channels were enhanced equally on both primary and no-primary controls using Adobe Photoshop CS6.

2.7. Western blot analyses

For Erk MAPK westerns, protein was isolated from previously frozen vessels (see Isometric contraction) homogenized in the presence of tyrosine, threonine and serine phosphatase inhibitors (10 mg/mL leupeptin, 10 mg/mL aprotinin, and 10 mM PMSF) and western blotting procedures were performed on equivalent amounts of total protein per lane, as measured by a bicinchoninic acid kit (Sigma Chemical Co, St. Louis, MO USA). The positive control was calyculin treated Jurkat cells (Cell Signaling, MA, USA). Nitrocellulose membranes were blocked for 3 h in 5% bovine serum albumin [4 °C, TBS-0.1% Tween +0.025% Na₃]. Primary antibody for Erk (Cell Signaling #4696S, 1:1000 in 5% milk; Boston, MA, USA) or phosphoErk (Millipore #05-797R, 1:1000 in 5% milk; Billerica, MA, USA) was incubated with blots overnight at 4 °C with rocking. Blots were washed (3 times, 10 min each) in TBS-0.1% Tween, then incubated with IRDye 680LT goat anti-mouse (Li-Cor #926-68020, 1:1000 in Li-Cor Blocking Buffer; Lincoln, Nebraska, USA) for testing Erk or IRDye 800CW goat anti-rabbit (Li-Cor #926-32211, 1:1000 in Li-Cor Blocking Buffer) for testing phosphoErk. The blots were visualized using the Odyssey Infrared

Imaging System and the Odyssey FC Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). Blots were reprobed for alpha-actin (#113200, EMD Millipore, Billerica, MA, USA) as a loading control.

For the chemerin receptor, GPR1 and calponin-1, protein was isolated from smooth muscle cells isolated as described in Section 2.4. Cells were placed in RIPA buffer (R3792, Teknova, Hollister, CA USA; with added 10 mg/mL leupeptin, 10 mg/mL aprotinin, and 10 mM PMSF) and rocked at 4 °C for 1 h. The positive control for GPR1 was rat adrenal gland and for the chemerin receptor was rat aorta. Both were isolated in-lab and homogenized by liquid nitrogen and mortar/pestle. Protein was measured by a bicinchoninic acid kit (Sigma Chemical Co). Standard western blotting procedures were performed on positive controls loaded at 75 µg of protein/lane and rat aortic smooth muscle cell lysates at 25 µg of protein/lane. This difference was necessary because of the concentrated nature of the smooth muscle cell lysate. For this reason, the quantity of signal from smooth muscle cell lysates was not compared to their positive controls. Nitrocellulose membranes were blocked with 4% chick egg ovalbumin (A5378, Sigma Chemical). Primary antibody for the chemerin receptor (1:100, anti-mouse, #398769, Santa Cruz Biotechnology), GPR1 (1:250, anti-mouse, clone 43.28.4, gift from Dr. Brian Zabel), calponin-1 (anti-mouse, 1:2000, #MA5-11620, Thermo Fisher), and beta actin (anti-goat, 1:500, #ab8229, Abcam, Cambridge, MA USA) were added one at a time with individual overnight incubations at 4 °C. While GPR1 and the chemerin receptor were not visualized on the same blot, the remainder of the bands were separated by molecular weight and wavelength of the secondary antibodies. Blots were washed (3 times, 10 min each) in TBS-0.1% Tween, then incubated with IRDye 680LT donkey anti-goat (Li-Cor #926-68024, 1:1000 in Li-Cor Blocking Buffer) for testing beta actin or IRDye 800CW goat anti-mouse (Li-Cor #926-32210, 1:1000 in Li-Cor Blocking Buffer) for testing the chemerin receptor, GPR1, or calponin-1. The blots were visualized using the Odyssey FC Infrared Imaging System. Calponin-1 was used as a loading control for quantification of the smooth muscle cell lysates. Beta actin was used to visualize the presence of protein from the rat adrenal gland (does not contain a substantial amount calponin-1).

2.8. Data analysis and statistical procedures

Contractility results are reported as means $\pm$ SEM. Contraction from baseline tone is reported as a percentage of the initial contraction to PE. All EC₅₀, E_{max}, pA₂, curve fitting, t-test and analysis of variance (ANOVA) tests were performed using GraphPad Prism 6.0 (La Jolla, CA, USA). For western analyses of Erk MAPK, each lane shown represents a separate animal, and densitometry was performed using NIH Image J (v 1.45) for the number of animals indicated in parentheses. Changes in ERK or pERK are represented as fold-change from vehicle by dividing chemerin-9-incubated densitometry by vehicle-incubated densitometry. pERK values were not divided by ERK values in an effort to reduce bias in their interpretation. For western analyses of GPR1, the chemerin receptor, and calponin-1, Li-Cor Image Studio (v 5.2.5) was used. For two group statistical comparisons, the appropriate Student's t-test was used. For one-way ANOVA, a Tukey's post hoc test was used and for two-way ANOVA, a Bonferroni correction was made. A p value $\leq$ 0.05 was considered statistically significant.

3. Results

3.1. Chemerin causes a contraction and calcium flux that is G_i-dependent

Chemerin-9 (highlighted from the full S¹⁶³ isoform, Fig. 1A), a nonapeptide derived from the C-terminus of the recombinant chemerin S¹⁵⁷ isoform (all experiments referencing recombinant chemerin were performed with the S¹⁵⁷ isoform) [15], contracted the isolated rat aorta (Fig. 1B). Smooth muscle cells explanted from aorta were processed for a molecular interrogation of muscle activation. The presence of aortic smooth muscle cells in culture was confirmed by alpha actin staining (Fig. 1C).

These cells were used in calcium kinetics experiments where the procedure for the addition of inhibitor/antagonist and agonist to the cells is graphically outlined in Fig. 1D. In these cells, chemerin-9 and recombinant chemerin S¹⁵⁷ increased cytosolic calcium in a concentration-dependent manner (Fig. 1E). Calcium response with recombinant chemerin was not assessed at the same concentration range used for chemerin-9 because we were limited in our supply of recombinant chemerin, and a maximal response was already observed at 100 nM. These basic measures of chemerin-9 and recombinant chemerin-induced calcium flux in the smooth muscle cells are also used in later figures to make comparisons with different conditions. The E_{max} (top value of the curve) of recombinant chemerin (0.17±0.02 arbitrary units) was smaller than chemerin-9 (0.25 ± 0.01 arbitrary units) by 40% (significant with a $p < 0.05$) but their potencies were not significantly different ($-\log EC_{50}[M]$: 8.1 ± 0.7 and 8.0 ± 0.2 respectively; Fig. 1E). The calcium response stimulated by both agonists was abolished by pertussis toxin (PTX) (Fig. 2A and B). The viability of cells after incubation with PTX was confirmed by observing a normal positive calcium elevation to angiotensin II in cells with and without PTX (Fig. 2C). PTX, importantly, also reduced chemerin-9-dependent contraction of an aorta in an isolated tissue bath (Fig. 2D). Tissue viability after overnight PTX incubations was confirmed by a phenylephrine contraction (used to normalize the data; 1920 ± 240 mg force and 1330 ± 230 mg force for vehicle and PTX treatment, respectively; mean ± SEM, not statistically significant $p = 0.114$). Clonidine (α₂ adrenergic agonist and G_i-coupled) was used as a positive control for PTX (Fig. 2E). This indicates that the receptor type(s) that chemerin binds to in the rat aorta are G_i-coupled.

3.2. Chemerin signaling in smooth muscle most likely acts through the chemerin receptor

CCX832 is a selective antagonist for the chemerin receptor that does not show affinity for GPR1 or CCRL2 [16]. As replicated from previous studies [16], 100 nM CCX832 reduced chemerin-9-dependent contraction of the aorta (Fig. 3A). Compared to Fig. 2D, the E_{max} of chemerin-9 and vehicle was higher but not to a statistically significant degree. This difference can be explained by the incubation time needed to create a proper control for the PTX experiments (24 h) versus the fresh tissue shown here. Calcium flux in smooth muscle cells also exhibited a concentration-dependent reduction by CCX832. A Schild analysis of chemerin-9 with CCX832 gave a pA₂ of 8.2 and a clear concentration-dependent, competitive relationship with chemerin-9 (Fig. 3B; significance was specifically calculated between chemerin-9 + vehicle and chemerin-9 + 1 μM CCX832 to make comparisons with recombinant chemerin). However, while 1 μM CCX832 produced a 30-fold shift of EC₅₀

with chemerin-9 and significantly inhibited 1, 10, and 100 nM chemerin-9-dependent calcium flux, it did not shift recombinant chemerin-dependent calcium flux (Fig. 3C).

Immunocytochemistry with confocal microscopy examined smooth muscle cells for GPR1 and chemerin receptor, the two possible receptors that could be responsible for chemerin-9 signal transduction. The rat adrenal and rat brain were used as positive controls for GPR1 and chemerin receptor, respectively. These slides were co-stained with wheat-germ agglutinin to provide context to the cell (Fig. 4A; because cells were permeabilized, most glycoproteins including those in the endoplasmic reticulum and Golgi were also stained). Use of the antibodies and positive controls for the chemerin receptor and GPR1 in immunohistochemistry has been validated by previously published reports [16, 28]. GPR1 was not present in smooth muscle cells while the chemerin receptor was present and localized to the perimeter of the cell.

To support the relative amount of signal from GPR1 and the chemerin receptor seen in Fig. 4A, western blots were performed probing for the chemerin receptor, GPR1, and calponin-1 (Fig. 4B). The presence of calponin-1 confirmed our isolation of smooth muscle cells from the rat aorta. In our western blots, we see expression of the chemerin receptor and no expression of GPR1 which supports the conclusions drawn by the immunocytochemistry – the chemerin receptor predominates while GPR1 is virtually absent.

3.3. Chemerin-9 activation of smooth muscle cells lacks Erk MAPK signaling

Because of a previous report citing a Erk MAPK mechanism in chemerin-dependent vascular contraction [17], we assessed PD098059 as an inhibitor of this contraction. In the hands of multiple researchers, 1 μ M PD098059 did not inhibit chemerin-9 contraction in rat aorta (Fig. 5A). Levels of phosphorylated Erk were measured both with and without incubation with 1 μ M chemerin in the tissue bath (Fig. 5B and C). In tissues receiving a bolus of chemerin-9, the contraction achieved was plateaued at a 5-minute time point and was $40.0 \pm 5.22\%$ PE (10 μ M) initial contraction. These tissues were frozen and homogenized for analysis in western blots. Each lane represents tissue from one rat, with the blot showing two independent experiments for 0 min and 5 min chemerin exposure where both 0 and 5 min samples were taken from the same rat (internal control). Consistent with the lack of effect of PD098059 on chemerin-9-induced contraction, western analyses supported no statistically significant increase in Erk MAPK phosphorylation in tissues contracted with chemerin-9 [pErk MAPK = 1.12 ± 30.14 (44 kDa) and 1.11 ± 0.29 (42 kDa) fold-increase with chemerin-9 contraction compared to vehicle, $p > 0.05$].

3.4. Calcium flux with chemerin-9 exhibits the same dependence on signaling elements as physiologic contraction

The pathway of chemerin signaling was further investigated by measuring cumulative response curves in isometric contractility and calcium flux. For both, the near-maximal chemerin-induced response with inhibitor was normalized to the near-maximal chemerin-induced response with the vehicle of the inhibitor and is represented as a percent of vehicle response (Fig. 6A and B). Values of isometric contraction were measured at a chemerin concentration of 1 μ M (arrows in 6A) while calcium flux was measured at 100 nM (arrows

in 6B) due to a 10-fold shift in the EC₅₀ values of the different methods (100 nM and 10 nM, respectively). Isometric contraction was significantly inhibited by verapamil (L-type calcium channel), Y27632 (Rho kinase), and PP1 (Src) (Fig. 6C). U73122 also had significant inhibition of contraction but not to the magnitude of the other inhibitors. The calcium profile with a 1-hour incubation models the inhibitor incubation in the isometric experiments. At this 1-hour incubation, verapamil, Y27632, and PP1 all caused significant inhibition of calcium flux stimulated by chemerin-9 while U73122 did not (Fig. 6D). A graphical representation of the predicted signaling pathway is represented in Fig. 7.

4. Discussion

4.1. Chemerin-induced contraction is dependent on calcium flux

Establishing a clear path by which chemerin-9 causes smooth muscle contraction is essential to define the action of this isoform as opposed to other potentially anti-inflammatory isoforms [29], but also to define chemerin's role in smooth muscle (and hypertension) as opposed to its role elsewhere in the body (e.g. tumor angiogenesis, immune cell migration, and adipogenesis). We confirmed the contraction of aorta to chemerin-9 (Fig. 1B), discovered that the smooth muscle cells initiated a calcium flux in response to chemerin-9/recombinant chemerin (Fig. 1E) and localized the chemerin receptor to the perimeter of the cell (Fig. 4A). All together, these data build on previous work [16] to support the connection between the physiological contraction and the smooth muscle cell through the chemerin receptor.

4.2. Receptors mediating chemerin's action in smooth muscle cells

Macrophages, dendritic cells [27], and artificial systems like the HEK293A cell line [14] show chemerin-related G_i mechanisms involved in cell migration and transcription (respectively). However, ours is the first report to show this mechanism in smooth muscle cells. Of all the inhibitors tested, PTX was the only one to abolish calcium flux at all concentrations of agonist (Fig. 2A and B). Importantly, chemerin-9-induced contraction of the aorta was affected by PTX, but the effects were not as pronounced as those in the isolated cells (Fig. 2D and E) and required a higher concentration (1000 ng/mL versus 500 ng/mL in the cellular assay). This difference can be explained by needing higher concentration of PTX to penetrate the tissue. These results suggest that all other tested elements of the signaling cascade leading to calcium mobilization must funnel through this heterotrimeric G protein. The lack of complete inhibition of aortic contraction by PTX (Fig. 2D) indicates there may still be other G_i-independent mechanisms involved in chemerin-dependent contraction, but they are likely calcium-independent. The data from PTX combined with immunocytochemistry (Fig. 4A) and CCX832 (Fig. 3A and B) point to the chemerin receptor being G_i-coupled and the primary functional receptor in these cells. Additionally, there is recent data suggesting that the receptor GPR1 does not significantly activate G-proteins and may act like a decoy receptor [30]. This is consistent with our data and further solidifies the chemerin receptor's role as the principle receptor for chemerin in vascular smooth muscle cells.

4.3. Chemerin signaling relies on various second messengers

Because of the direct importance calcium has on the activation of myosin light chain, the study of real-time calcium mobilization in cells can give us insight into the mechanisms necessary for smooth muscle contraction. The similarities between the qualitative outcome of isometric contraction and calcium flux implies that all three inhibitors block contraction by modulating calcium levels.

4.3.1. Chemerin and L-type calcium channels—L-type calcium channels are voltage gated and regulate extracellular calcium entry. As mentioned previously, this calcium flux is essential to smooth muscle contraction so it is logical that these channels would be involved. It has been implied before (by use of nifedipine and KCl-potentiated chemerin-9 isometric contraction) that the chemerin receptor and chemerin signaling are linked to L-type voltage gated calcium channels [16], but this is the first study to show that direct stimulation by chemerin-9 is dependent on these channels. Although others suggest that calcium is vital to the chemerin signaling of immune cells [27, 31], hematopoietic [32] and mesenchymal stem cells [33], we are the first to link chemerin to the smooth muscle cell and to the L-type voltage gated calcium channel in any system. In both rat myocytes and vascular smooth muscle cells, L-type calcium channels have been linked to G_i-protein mechanisms [34, 35], lending support to the finding that extracellular calcium is essential to chemerin-induced vasoconstriction.

4.3.2. Chemerin and Rho kinase—Rho kinase (ROCK) inhibits myosin light chain phosphatase (MLCP) and promotes vascular contraction [36]. Because this effect is downstream of calcium flux but upstream of smooth muscle contraction, inhibition of isometric contraction by ROCK inhibitor Y27632 can be explained by conventional mechanisms. However, the inhibition of calcium flux by Y27632 cannot be explained in the same way. Others have demonstrated that chemerin can signal through ROCK but these studies were performed in HEK293A and lymphocytes [14]. There is a preponderance of evidence to support that Y27632 inhibits KCl-induced contraction (voltage activated) of smooth muscle while leaving KCl-induced calcium flux unchanged [37–41]. This supports the previous mechanism that Y27632 does not block voltage-gated calcium channels but can block contraction by inhibiting phosphorylation of MLCP. In human purified protein activity assays with 10 μ M Y27632, there were negligible off-target effects on other kinases [42]. We are confident that Y27632 is selective for ROCK but we are not the only ones to report that it has the ability to reduce intracellular calcium levels [43, 44]. Most interestingly, a recent paper assessed the ability for Y27632 to inhibit the contractility and calcium mobilization in rat aortic and mesenteric arteries stimulated by norepinephrine, AIF₄⁻ (G protein activator), and KCl (voltage activator) [45]. In this study, Y27632 inhibited both contractility and calcium release stimulated by norepinephrine and AIF₄⁻ but only inhibited contractility and not calcium flux in rat aorta stimulated by KCl. After additional testing, they concluded that ROCK was stimulated by G proteins to directly interact with extracellular cationic channels not stimulated by voltage, not involved in regulating membrane potential, and not associated with potassium. Their hypothesis led them to suspect TRPC6 channels as the target of ROCK. Independent from these studies, TRPC6 channels are associated with G protein agonists, smooth muscle contraction, and positively

correlated with hypertension [46]. Although ROCK is commonly associated with $G_{12/13}$ [47, 48], there is evidence in vascular endothelial cells that supports a link between G_i and ROCK [49]. Taken together, it is possible that chemerin-induced contraction could be mediated through ROCK activation of non-voltage dependent ion channels, like TRPC6. Future studies will need to directly assess the involvement of TRPC6 in a chemerin-induced response.

4.3.3. Chemerin and Src—GPCRs can influence Src to alter respiratory smooth muscle in a calcium-dependent manner [50, 51]. In both myometrial and vascular smooth muscle, Src has been linked specifically to the G_i protein [52, 53]. One study showed that in vascular smooth muscle, PP1 did not change calcium flux induced by sphingosylphosphorylcholine or KCl. However, PP1 still reduced smooth muscle contraction by indirect inhibition of ROCK [54]. To our knowledge, there are no previous reports that link chemerin signaling to Src. If Src is an upstream modulator of Rho, the previous evidence on ROCK and its actions on TRPC6 could explain our observed results. A recent report on Src stated that its influence is not on ROCK but rather on the guanine nucleotide exchange factor (RhoGEF) [55]. Independently from ROCK, Src is required for the activity of TRP channels [56] and can phosphorylate L-type calcium channels to enhance their activity [57]. The results in this report agree with the notion that Src could have influence over ROCK's new mechanism proposed above (through a RhoGEF) but does not rule out Src's direct action at a calcium channel.

4.3.4. Chemerin and PLC—The evidence in our study as a whole does not support the role of PLC in chemerin-dependent vascular contraction. Although PLC has been associated with smooth muscle signaling pathways [26], it has not yet been directly linked to chemerin signaling. There was a minor inhibition of isometric contraction but the lack of significant inhibition of calcium flux suggests that this is not a major pathway activated by chemerin in a rat aorta. The primary mechanism for PLC is to activate IP_3 -mediated in-tracellular calcium channels so if calcium flux is not changing, altogether it is not a major participant.

4.3.5. Chemerin and Erk MAPK—A previous report suggested that chemerin potentiates phenylephrine and endothelin-1-induced contraction through an Erk MAPK pathway [17]. A recent report also noted that the binding of chemerin-9 to the chemerin receptor activated Erk MAPK [30]. Beyond this, Erk MAPK is extensively associated with the actions of chemerin in chemotaxis [19], angiogenesis [58], and adipogenesis [10]. We tested the Erk MAPK pathway in smooth muscle with PD098059 against direct chemerin-induced contraction. When we failed to observe a reduction in isometric contraction with PD098059, we tested the phosphorylation of Erk MAPK. A five-minute treatment of 1 μ M chemerin-9 (the same concentration that produced a near-maximal chemerin-9-dependent contraction of rat aorta) did not change the level of phosphorylation of Erk MAPK. It is possible that Erk MAPK could be involved in the signaling pathways of phenylephrine or endothelin-1, but it is clear in this report that Erk MAPK does not have any role in the contraction of the aorta by direct stimulation of chemerin-9.

4.4. Chemerin-9 as a model for recombinant chemerin?

From a practical standpoint, the existence of short peptides (like chemerin-9) is crucial to the feasibility of in vitro studies involving expensive recombinant proteins. Chemerin-9 was confirmed to bind the chemerin receptor with the same affinity as recombinant chemerin [15]. However, the effects of chemerin-9 differ from the recombinant version in a number of ways. Chemerin-9 activated smooth muscle calcium flux with a higher $E_{\max}$ than recombinant chemerin but both had similar EC_{50} . Both peptides appear to act on the same receptor, as shown by PTX inhibition, but the lack of inhibition of recombinant chemerin by CCX832 reveals that these analogs are not binding the chemerin receptor in the same manner. It is possible that the two agonists could be binding different sites on the receptor. The chemerin receptor can bind a number of different ligands to initiate different outcomes: the active chemerin S^{157} typically triggers inflammatory responses [27, 59] while other ligands like chemerin-15 (analogous to mouse chemerin A^{154}) [29] or lipid resolvin E1 [60] are anti-inflammatory. These outcomes, presumably mediated by the same receptor, suggest that the chemerin receptor may act in a biased fashion. This is the first report the authors are aware of that implicates biased agonism as accounting for the disparity between the actions of chemerin-9 and recombinant chemerin.

4.5. Limitations

We recognize limitations of the present study. Although recombinant chemerin was used in these calcium flux studies, it was not feasible to use in an isolated tissue bath. Additionally, as mentioned previously in Section 3.1, this limited use of recombinant chemerin restricted the breadth of the concentration-dependent response curves.

Calcium is not the final step in smooth muscle contraction and cannot inform us about any effects intracellular signals may have on the myosin light chain itself. In this way, ROCK or Src may have actions outside of what was investigated here. Future studies may examine myosin activation as the ultimate step in muscle contraction to uncover calcium-independent mechanisms.

One inherent limitation of analyzing these cells for calcium flux is that the instrument used requires we titrate the cell count to be as low as possible. This maintains the sensitivity of the instrument and also allows us to run a host of experiments on cells at the same passage. The downfall is its ability to replicate the in vivo environment of a multilayered, tightly packed structure as in the aorta. Since the results in the isometric contractility reflect the results in the calcium flux, it is unlikely the difference in cell density played a significantly limiting role in our research. It should be taken into account for future experiments.

The contraction of vessels to chemerin-9 was also variable. In Fig. 3A, the contraction of the aorta to 3 μ M chemerin-9 was about 60% while in Fig. 5A the contraction to the same concentration of chemerin-9 was about 80%. These measures were taken at different times, with different sets of animals (although all from the same source) and is consistent with the variability of this adipokine. The possible correlation between an increase in maximal isometric contraction and an increase in DMSO concentration is not consistent with the current biological mechanism of DMSO and is not a factor contributing to the variability.

While the focus of this research is the smooth muscle of the vasculature, we recognize that the endothelium is also an important component to the vascular system. Because the vasculature did not contract to chemerin in the presence of endothelium [16], we did not initially consider the endothelium to play an important role in chemerin signal transduction thus, it was not the focus of this current study. However, determining the role of the endothelium and a comparison to the now-defined role of smooth muscle would be an important next step.

The animal model was a major consideration when formulating the experiments in this paper. We have previously reported that the mouse as a model for vascular actions of chemerin should be avoided because this aorta lacks a functional response to chemerin-9. By contrast, arteries from the rat as a model for chemerin in the human do contract to chemerin-9 [16]. This limits the technologies available to us that may otherwise be used if we were using the mouse. For example, Cre-Lox and other transgenic techniques are more difficult to perform in the rat.

5. Conclusions

Contraction of vascular smooth muscle by chemerin is dependent on G_i proteins, L-type Ca^{2+} channels, Src, and Rho kinase that all work by altering calcium flux. Erk MAPK and PLC are not involved in chemerin-dependent calcium flux and contraction. With a host of literature describing the possibility of chemerin's involvement in hypertension [3–9], further research is needed to connect this knowledge with an actual treatment. This specific pathway helps to characterize a once unknown mechanism by which chemerin may contribute to obesity-related hypertension. Understanding these mechanisms is necessary to develop treatments against novel targets like chemerin. It also highlights a new role for Src and Rho kinase as well as possible benefits and pitfalls for future use of the nonapeptide, chemerin-9.

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Abbreviations

Akt	protein kinase B
ANG II	angiotensin II
CaM	calmodulin
CCRL2	chemokine (CC motif) receptor-like 2
CMKLR1	chemokine-like receptor 1
Erk MAPK	extracellular signal-regulated kinase mitogen activated protein kinase
GAP	GTPase activating protein

GEF	guanine nucleotide exchange factor
GPR1	G protein-coupled receptor 1
MLC	myosin light-chain
MLCK	myosin light-chain kinase
MLCP	myosin light-chain phosphatase
PD098059	2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one
PE	phenylephrine
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
ROCK	Rho kinase
TRPC	transient receptor potential cation channel
WGA	wheat-germ agglutinin
Y27632	4-[(1R)-1-aminoethyl]-N-pyridin-4-ylcyclohexane-1-carboxamide

References

1. Kelly RK, Magnussen CG, Sabin MA, Cheung M, Juonala M. Development of hypertension in overweight adolescents: a review, *Adolesc. Health Med. Ther.* 2015; 6:171–187. <http://dx.doi.org/10.2147/AHMT.S55837>.
2. Bozaoglu K, Bolton K, McMillan J, Zimmet P, Jowett J, Collier G, et al. Chemerin is a novel adipokine associated with obesity and metabolic syndrome. *Endocrinology.* 2007; 148:4687–4694. <http://dx.doi.org/10.1210/en.2007-0175>. [PubMed: 17640997]
3. Shin HY, Lee DC, Chu SH, Jeon JY, Lee MK, Im JA, et al. Chemerin levels are positively correlated with abdominal visceral fat accumulation. *Clin. Endocrinol.* 2012; 77:47–50. <http://dx.doi.org/10.1111/j.1365-2265.2011.04217.x>.
4. Sell H, Divoux A, Poitou C, Basdevant A, Bouillot JL, Bedossa P, et al. Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric surgery. *J. Clin. Endocrinol. Metab.* 2010; 95:2892–2896. <http://dx.doi.org/10.1210/jc.2009-2374>. [PubMed: 20375212]
5. Landgraf K, Friebe D, Ullrich T, Kratzsch J, Dittrich K, Herberth G, et al. Chemerin as a mediator between obesity and vascular inflammation in children. *J. Clin. Endocrinol. Metab.* 2012; 97:E556–E564. <http://dx.doi.org/10.1210/jc.2011-2937>. [PubMed: 22438234]
6. Schipper HS, Nuboer R, Prop S, van den Ham HJ, de Boer FK, Kesmir C, et al. Systemic inflammation in childhood obesity: circulating inflammatory mediators and activated CD14++ monocytes. *Diabetologia.* 2012; 55:2800–2810. <http://dx.doi.org/10.1007/s00125-012-2641-y>. [PubMed: 22806355]
7. Chakaroun R, Raschpichler M, Kloting N, Oberbach A, Flehmig G, Kern M, et al. Effects of weight loss and exercise on chemerin serum concentrations and adipose tissue expression in human obesity. *Metabolism.* 2012; 61:706–714. <http://dx.doi.org/10.1016/j.metabol.2011.10.008>. [PubMed: 22136911]

8. Dong B, Ji W, Zhang Y. Elevated serum chemerin levels are associated with the presence of coronary artery disease in patients with metabolic syndrome. *Intern. Med.* 2011; 50:1093–1097. <http://dx.doi.org/10.2169/internalmedicine.50.5025>. [PubMed: 21576834]
9. Kunimoto H, Kazama K, Takai M, Oda M, Okada M, Yamawaki H. Chemerin promotes the proliferation and migration of vascular smooth muscle and increases mouse blood pressure. *Am. J. Physiol. Heart Circ. Physiol.* 2015; 309:H1017–H1028. <http://dx.doi.org/10.1152/ajpheart.00820.2014>. [PubMed: 26254337]
10. Goralski KB, McCarthy TC, Hanniman EA, Zabel BA, Butcher EC, Parlee SD, et al. Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *J. Biol. Chem.* 2007; 282:28175–28188. <http://dx.doi.org/10.1074/jbc.M700793200>. [PubMed: 17635925]
11. Zabel BA, Nakae S, Zuniga L, Kim JY, Ohyama T, Alt C, et al. Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis. *J. Exp. Med.* 2008; 205:2207–2220. <http://dx.doi.org/10.1084/jem.20080300>. [PubMed: 18794339]
12. Barnea G, Strapps W, Herrada G, Berman Y, Ong J, Kloss B, et al. The genetic design of signaling cascades to record receptor activation. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105:64–69. <http://dx.doi.org/10.1073/pnas.0710487105>. [PubMed: 18165312]
13. Gantz I, Konda Y, Yang YK, Miller DE, Dierick HA, Yamada T. Molecular cloning of a novel receptor (CMKLR1) with homology to the chemotactic factor receptors. *Cytogenet. Cell Genet.* 1996; 74:286–290. [PubMed: 8976386]
14. Rourke JL, Dranse HJ, Sinal CJ. CMKLR1 and GPR1 mediate chemerin signaling through the RhoA/ROCK pathway. *Mol. Cell. Endocrinol.* 2015; 417:36–51. <http://dx.doi.org/10.1016/j.mce.2015.09.002>. [PubMed: 26363224]
15. Wittamer V, Gregoire F, Robberecht P, Vassart G, Communi D, Parmentier M. The C-terminal nonapeptide of mature chemerin activates the chemerin receptor with low nanomolar potency. *J. Biol. Chem.* 2004; 279:9956–9962. <http://dx.doi.org/10.1074/jbc.M313016200>. [PubMed: 14701797]
16. Watts SW, Dorrance AM, Penfold ME, Rourke JL, Sinal CJ, Seitz B, et al. Chemerin connects fat to arterial contraction. *Arterioscler. Thromb. Vasc. Biol.* 2013; 33:1320–1328. <http://dx.doi.org/10.1161/ATVBAHA.113.301476>. [PubMed: 23559624]
17. Lobato NS, Neves KB, Filgueira FP, Fortes ZB, Carvalho MHC, Webb RC, et al. The adipokine chemerin augments vascular reactivity to contractile stimuli via activation of the MEK-ERK1/2 pathway. *Life Sci.* 2012; 91:600–606. <http://dx.doi.org/10.1016/j.lfs.2012.04.013>. [PubMed: 22521290]
18. Monaghan, AE. [accessed June 8, 2016] Chemerin Receptor: Chemerin Receptor, IUPHAR/BPS Guid. to Pharmacol. <http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=792016>
19. Albanesi C, Scarponi C, Pallotta S, Daniele R, Bosio D, Madonna S, et al. Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J. Exp. Med.* 2009; 206:249–258. <http://dx.doi.org/10.1084/jem.20080129>. [PubMed: 19114666]
20. Kaur J, Adya R, Tan BK, Chen J, Randeve HS. Identification of chemerin receptor (ChemR23) in human endothelial cells: chemerin-induced endothelial angiogenesis. *Biochem. Biophys. Res. Commun.* 2010; 391:1762–1768. <http://dx.doi.org/10.1016/j.bbrc.2009.12.150>. [PubMed: 20044979]
21. Zhou JX, Liao D, Zhang S, Cheng N, He HQ, Ye RD. Chemerin C9 peptide induces receptor internalization through a clathrin-independent pathway. *Acta Pharmacol. Sin.* 2014; 35:653–663. <http://dx.doi.org/10.1038/aps.2013.198>. [PubMed: 24658352]
22. Hill-Eubanks DC, Werner ME, Heppner TJ, Nelson MT. Calcium signaling in smooth muscle. *Cold Spring Harb. Perspect. Biol.* 2011; 3:a004549. <http://dx.doi.org/10.1101/cshperspect.a004549>. [PubMed: 21709182]
23. Jackson WF. Ion channels and vascular tone. *Hypertension.* 2000; 35:173–178. <http://dx.doi.org/10.1161/01.HYP.35.1.173>. [PubMed: 10642294]

24. Touyz RM, Wu XH, He G, Park JB, Chen X, Vacher J, et al. Role of c-Src in the regulation of vascular contraction and Ca²⁺ signaling by angiotensin II in human vascular smooth muscle cells. *J. Hypertens.* 2001; 19:441–449. [PubMed: 11288814]
25. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature.* 1997; 389:990–994. <http://dx.doi.org/10.1038/40187>. [PubMed: 9353125]
26. Ureña J, del Valle-Rodríguez A, López-Barneo J. Metabotropic Ca²⁺ channel-induced calcium release in vascular smooth muscle. *Cell Calcium.* 2007; 42:513–520. <http://dx.doi.org/10.1016/j.ceca.2007.04.010>. [PubMed: 17559931]
27. Wittamer V, Franssen JD, Vulcano M, Mirjolet JF, Le Poul E, Migeotte I, et al. Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *J. Exp. Med.* 2003; 198:977–985. <http://dx.doi.org/10.1084/jem.20030382>. [PubMed: 14530373]
28. Moore A, Zabel BA, Burnett R, Watts SW. Short communication chemerin peptide releases catecholamines from rat adrenal medulla 1. *Pharmacologica.* 2016; 7:290–295. <http://dx.doi.org/10.5567/pharmacologia.2016.290.295>.
29. Cash JL, Hart R, Russ A, Dixon JP, Colledge WH, Doran J, et al. Synthetic chemerin-derived peptides suppress inflammation through ChemR23. *J. Exp. Med.* 2008; 205:767–775. <http://dx.doi.org/10.1084/jem.20071601>. [PubMed: 18391062]
30. De Henau O, Degroot G-N, Imbault V, Robert V, De Poorter C, Mcheik S, et al. Signaling properties of chemerin receptors CMKLR1, GPR1 and CCRL2. *PLoS One.* 2016; 11:e0164179. <http://dx.doi.org/10.1371/journal.pone.0164179>. [PubMed: 27716822]
31. Zabel BA, Ohyama T, Zuniga L, Kim J-Y, Johnston B, Allen SJ, et al. Chemokine-like receptor 1 expression by macrophages in vivo: regulation by TGF- β and TLR ligands. *Exp. Hematol.* 2006; 34:1106–1114. <http://dx.doi.org/10.1016/j.exphem.2006.03.011>. [PubMed: 16863918]
32. Muruganandan S, Dranse HJ, Rourke JL, McMullen NM, Sinal CJ. Chemerin neutralization blocks hematopoietic stem cell osteoclastogenesis. *Stem Cells.* 2013; 31:2172–2182. <http://dx.doi.org/10.1002/stem.1450>. [PubMed: 23766088]
33. Kumar JD, Holmberg C, Balabanova S, Borysova L, Burdyga T, Beynon R, et al. Mesenchymal stem cells exhibit regulated exocytosis in response to chemerin and IGF. *PLoS One.* 2015; 10:e0141331. <http://dx.doi.org/10.1371/journal.pone.0141331>. [PubMed: 26513261]
34. Zhang Y, Ying J, Jiang D, Chang Z, Li H, Zhang G, et al. Urotensin-II receptor stimulation of cardiac L-type Ca²⁺ channels requires the $\beta\gamma$ subunits of Gi/o-protein and phosphatidylinositol 3-kinase-dependent protein kinase C β 1 isoform. *J. Biol. Chem.* 2015; 290:8644–8655. <http://dx.doi.org/10.1074/jbc.M114.615021>. [PubMed: 25678708]
35. Fujii K, Machida T, Iizuka K, Hirafuji M. Sphingosine 1-phosphate increases an in-tracellular Ca(2+) concentration via S1P3 receptor in cultured vascular smooth muscle cells. *J. Pharm. Pharmacol.* 2014; 66:802–810. <http://dx.doi.org/10.1111/jphp.12214>. [PubMed: 24450400]
36. Loirand G, Pacaud P. Involvement of Rho GTPases and their regulators in the pathogenesis of hypertension. *Small GTPases.* 2014; 5:1–10. <http://dx.doi.org/10.4161/sgtp.28846>.
37. Sakamoto K, Hori M, Izumi M, Oka T, Kohama K, Ozaki H, et al. Inhibition of high K⁺-induced contraction by the ROCKs inhibitor Y-27632 in vascular smooth muscle: possible involvement of ROCKs in a signal transduction pathway. *J. Pharmacol. Sci.* 2003; 92:56–69. [PubMed: 12832856]
38. Urban NH, Berg KM, Ratz PH. K⁺ depolarization induces RhoA kinase translocation to caveolae and Ca²⁺ sensitization of arterial muscle. *Am. J. Phys. Cell Physiol.* 2003; 285:C1377–C1385. <http://dx.doi.org/10.1152/ajpcell.00501.2002>.
39. Mita M, Yanagihara H, Hishinuma S, Saito M, Walsh MP. Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. *Biochem. J.* 2002; 364:431–440. <http://dx.doi.org/10.1042/BJ20020191>. [PubMed: 12023886]
40. Kupittayanant S, Burdyga T, Wray S. The effects of inhibiting Rho-associated kinase with Y-27632 on force and intracellular calcium in human myometrium. *Pflugers Arch. - Eur. J. Physiol.* 2001; 443:112–114. <http://dx.doi.org/10.1007/s004240100668>. [PubMed: 11692274]

41. Janssen LJ, Tazzeo T, Zuo J, Pertens E, Keshavjee S. KCl evokes contraction of airway smooth muscle via activation of RhoA and Rho-kinase. *Am. J. Phys. Lung Cell. Mol. Phys.* 2004; 287:L852–L858. <http://dx.doi.org/10.1152/ajplung.00130.2004>.
42. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 2000; 351:95–105. [PubMed: 10998351]
43. Ratz PH, Miner AS. Role of protein kinase C ζ and calcium entry in KCl-induced vascular smooth muscle calcium sensitization and feedback control of cellular calcium levels. *J. Pharmacol. Exp. Ther.* 2009; 328:399–408. <http://dx.doi.org/10.1124/jpet.108.142422>. [PubMed: 19011165]
44. Shabir S, Borisova L, Wray S, Burdya T. Rho-kinase inhibition and electromechanical coupling in rat and guinea-pig ureter smooth muscle: Ca²⁺-dependent and -independent mechanisms. *J. Physiol.* 2004; 560:839–855. <http://dx.doi.org/10.1113/jphysiol.2004.070615>. [PubMed: 15331677]
45. Ghisda P, Vandenberg G, Morel N. Rho-dependent kinase is involved in agonist-activated calcium entry in rat arteries. *J. Physiol.* 2003; 551:855–867. <http://dx.doi.org/10.1113/jphysiol.2003.047050>. [PubMed: 12853654]
46. Abramowitz J, Birnbaumer L. Physiology and pathophysiology of canonical transient receptor potential channels. *FASEB J.* 2009; 23:297–328. <http://dx.doi.org/10.1096/fj.08-119495>. [PubMed: 18940894]
47. Kedziora KM, Leyton-Puig D, Argenzio E, Boumeester AJ, van Butselar B, Yin T, et al. Rapid remodeling of invadosomes by Gi-coupled receptors: dissecting the role of Rho GTPases. *J. Biol. Chem.* 2016; 291:4323–4333. <http://dx.doi.org/10.1074/jbc.M115.695940>. [PubMed: 26740622]
48. Rouach N, Pébay A, Mème W, Cordier J, Ezan P, Etienne E, et al. S1P inhibits gap junctions in astrocytes: involvement of G and Rho GTPase/ROCK. *Eur. J. Neurosci.* 2006; 23:1453–1464. <http://dx.doi.org/10.1111/j.1460-9568.2006.04671.x>. [PubMed: 16553609]
49. Kurt AH, Tiftik RN, Un I, Ulker S, Buyukafsar K. G protein-coupled estrogen receptor1 (GPER1) may mediate Rho-kinase (ROCK-2) up-regulation in coronary endothelial cells. *Endocr. Regul.* 2013; 47:75–84. [PubMed: 23641788]
50. Shaifta Y, Irechukwu N, Prieto-Lloret J, MacKay CE, Marchon KA, Ward JPT, et al. Divergent modulation of Rho-kinase and Ca²⁺ influx pathways by Src family kinases and focal adhesion kinase in airway smooth muscle. *Br. J. Pharmacol.* 2015; 172:5265–5280. <http://dx.doi.org/10.1111/bph.13313>. [PubMed: 26294392]
51. Katsumoto TR, Kudo M, Chen C, Sundaram A, Callahan EC, Zhu JW, et al. The phosphatase CD148 promotes airway hyperresponsiveness through SRC family kinases. *J. Clin. Invest.* 2013; 123:2037–2048. <http://dx.doi.org/10.1172/JCI66397>. [PubMed: 23543053]
52. Brighton PJ, McDonald J, Taylor AH, Challiss RAJ, Lambert DG, Konje JC, et al. Characterization of anandamide-stimulated cannabinoid receptor signaling in human ULTR myometrial smooth muscle cells. *Mol. Endocrinol.* 2009; 23:1415–1427. <http://dx.doi.org/10.1210/me.2009-0097>. [PubMed: 19477951]
53. Tanimoto T, Lungu AO, Berk BC. Sphingosine 1-phosphate transactivates the platelet-derived growth factor beta receptor and epidermal growth factor receptor in vascular smooth muscle cells. *Circ. Res.* 2004; 94:1050–1058. <http://dx.doi.org/10.1161/01.RES.0000126404.41421.BE>. [PubMed: 15044318]
54. Nakao F. Involvement of Src family protein tyrosine kinases in Ca²⁺ sensitization of coronary artery contraction mediated by a sphingosylphosphorylcholine-Rho-kinase pathway. *Circ. Res.* 2002; 91:953–960. <http://dx.doi.org/10.1161/01.RES.0000042702.04920.BF>. [PubMed: 12433841]
55. MacKay CE, Knock GA. Control of vascular smooth muscle function by Src-family kinases and reactive oxygen species in health and disease. *J. Physiol.* 2015; 593:3815–3828. <http://dx.doi.org/10.1113/jphysiol.2014.285304>. [PubMed: 25384773]
56. Vazquez G, Wedel BJ, Kawasaki BT, Bird GSJ, Putney JW. Obligatory role of Src kinase in the signaling mechanism for TRPC3 cation channels. *J. Biol. Chem.* 2004; 279:40521–40528. <http://dx.doi.org/10.1074/jbc.M405280200>. [PubMed: 15271991]
57. Gui P, Chao J-T, Wu X, Yang Y, Davis GE, Davis MJ. Coordinated regulation of vascular Ca²⁺ and K⁺ channels by integrin signaling. *Adv. Exp. Med. Biol.* 2010; 674:69–79. [PubMed: 20549941]

58. Bozaoglu K, Curran JE, Stocker CJ, Zaibi MS, Segal D, Konstantopoulos N, et al. Chemerin, a novel adipokine in the regulation of angiogenesis. *J. Clin. Endocrinol. Metab.* 2010; 95:2476–2485. <http://dx.doi.org/10.1210/jc.2010-0042>. [PubMed: 20237162]
59. Wittamer V, Bondue B, Guillaubert A, Vassart G, Parmentier M, Communi D. Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. *J. Immunol.* 2005; 175:487–493. <http://dx.doi.org/10.4049/jimmunol.175.1.487>. [PubMed: 15972683]
60. Arita M, Bianchini F, Aliberti J, Sher A, Chiang N, Hong S, et al. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *J. Exp. Med.* 2005; 201:713–722. <http://dx.doi.org/10.1084/jem.20042031>. [PubMed: 15753205]

Fig. 1. Chemerin stimulates rat aorta and smooth muscle cells. A) The amino acid sequence for human chemerin S¹⁶³ after cleavage of the preprochemerin fragment (aa 1–20). The sequence for the chemerin-9 peptide is surrounded by a black box. B) A representative tracing of a chemerin-9 concentration-response curve in rat thoracic aorta without endothelium measuring isometric contraction. A phenylephrine (PE) contraction used to normalize results is shown in the upper-left corner. C) The presence of isolated smooth muscle cells was confirmed by immunofluorescence with smooth muscle alpha-actin. This image is representative of four independent isolations. White bars represent 50 μ m and arrows point to areas of interest. D) A representative tracing of a calcium mobilization response. The process by which these cells were assayed is graphically derived from the

Methods. E) Smooth muscle cells display a concentration-dependent calcium response to chemerin. Magnitude of the calcium peak is measured by taking a “max – min” value of the originally normalized “ratio” tracing. Points/bars represent mean $\pm$ SEM for the number of replicates performed. * represents a $p < 0.05$.

Fig. 2.

Chemerin responses are blocked by PTX Concentration-response curves measuring calcium peaks of chemerin-9 (A, best fit line shown; portions reproduced from Fig. 1E) and recombinant chemerin (B; portions reproduced from Fig. 1E) when treated with and without pertussis toxin (PTX, 500 ng/mL). Magnitude of the calcium peak is measured by taking a “max – min” value of the originally normalized “ratio” tracing. C) Separate wells in the same plate were treated with angiotensin II (ANG II, 1 μ M) with and without PTX (500 ng/mL). This was used to confirm cell viability after treatment with PTX. D) Chemerin-9-dependent isometric contraction was inhibited by PTX (1000 ng/mL). E) Inhibition of a clonidine-dependent contraction (α_2 -adrenergic agonist) by PTX confirms that the PTX reached the desired target (G_i protein). Points/bars represent mean $\pm$ SEM for the number of replicates performed. * represents a $p < 0.05$ when compared to the vehicle/agonist of the same concentration.

Fig. 3.

Chemerin acts through the chemerin receptor. A) A cumulative response curve measuring isometric contraction with and without CCX832 (100 nM). This molecule antagonized the receptor for chemerin-9 and inhibited chemerin-9-dependent contraction. B) A CCX832 concentration-dependent shift of chemerin-9 calcium flux shows a concentration-dependent and competitive relationship between chemerin-9 and CCX832 (portions reproduced from Fig. 1E). * represents a $p < 0.05$ for chemerin-9 versus chemerin-9 + CCX832 1 μ M only (for comparisons to Fig. 3C). C) Recombinant chemerin with CCX832 (1 μ M) did not change calcium flux (portions reproduced from Fig. 1E). Points represent mean $\pm$ SEM for the number of replicates performed. Magnitude of the calcium peak is measured by taking a “max – min” value of the originally normalized “ratio” tracing.

Fig. 4. The chemerin receptor predominates at the cell perimeter over GPR1. A) Immunocytochemistry imaged using confocal microscopy. The chemerin receptor and GPR1 are imaged in green (FITC), wheat-germ agglutinin (WGA) is in red (TRITC), and the nucleus is stained blue (DAPI). Only one slice is shown but from the same depth in each cell. These images are representative of four independent isolations. No-primary cells/tissue controls received secondary antibody but no primary antibody. Positive controls for both GPR1 (rat adrenal) and chemerin receptor (blood vessel in rat brain) are also shown. Arrows represent points of interest. White bars represent 10 μ m in the samples and 50 μ m in the positive controls (images were taken at different magnifications). B) Representative images of the chemerin receptor, GPR1, calponin-1, and beta actin expressed in rat aortic smooth

muscle cells. Each lane of the rat aortic smooth muscle cell cultures represents an isolation from a different animal ($N = 5$). Positive controls (rat aorta for the chemerin receptor and rat adrenal for GPR1) are also shown.

Fig. 5.

Chemerin does not signal through Erk MAPK. A) Isometric contractility measurements with PD098059 (Erk MAPK inhibitor) and chemerin-9. B) Biochemical analysis of chemerin-9 induced activation of Erk MAPK in the isolated male thoracic aorta. Representative blot of pErk MAPK and Erk MAPK in aorta contracted at time 0 (vehicle) or 5 min after exposure to chemerin-9 (1 μ M). Each lane represents a separate rat aorta; two 0 and two 5 min samples are shown on this blot. The positive control is calyculin treated Jurkat cells. C) After densitometric analyses where signals were normalized to α -actin as a loading control, chemerin-9 incubated values were divided by vehicle treated values to give a fold-change from vehicle. Points/bars represent means $\pm$ SEM. N = 10 for each experimental condition (Erk vehicle, Erk chemerin-9, pErk vehicle, pErk chemerin-9). Points that remain above the dotted line (representing the signal given by vehicle treatment) are considered to have produced a statistically significant change in signal wither chemerin-9 compared to vehicle ($p < 0.05$), however, no significance was observed.

Fig. 6. Contraction and calcium are mediated by L-type calcium channels, ROCK, and Src. Representative concentration response curves are shown for verapamil in isometric contraction (A) and calcium flux (B). Arrows represent the values used to make the bars in the subsequent graphs. Although full concentration-response curves to chemerin-9 were performed for each inhibitor, concentrations shown in subsequent graphs are near maximal concentrations for chemerin in their respective approaches (the EC₅₀ for isometric contraction is 100 nM while EC₅₀ for calcium flux is 10 nM). Values indicate the percent of total contraction (1 μ M chemerin-9; C) or calcium peak (100 nM chemerin; D) when inhibitor is present. All values are represented as mean $\pm$ SEM. If the error bar does not cross the dotted line (representing 100% agonist response), the inhibitor produced a significant reduction of the agonist-induced contraction or calcium flux. *represents $p < 0.05$ and **represents $p < 0.01$. N values are as follows: B) chemerin-9 + vehicle = 24,

chemerin-9 + inhibitor = 12; C) verapamil (vehicle = 6, treatment = 4), Y27632 (vehicle = 4, treatment = 6), U73122 (vehicle = 5, treatment = 7), PP1 (vehicle = 4, treatment = 4); D) chemerin-9 + vehicle = 24, chemerin-9 + inhibitor = 24.

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Fig. 7. Schematic figure. Depiction of the potential interaction of chemerin-9 with the chemerin receptor and signaling pathways identified within the work of this manuscript. Dashed lines represent predictions.