The effect of substance P and its common in vivo-formed metabolites on MRGPRX2 and human mast cell activation

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Abstract
The tachykinin neuropeptide substance P (SP) is the canonical agonist peptide for the neurokinin 1 receptor (NK1R). More recently, it has also been shown to activate the Mas-related G protein-coupled receptor X2 (MRGPRX2) receptor on mast cells (MCs), triggering degranulation and release of inflammatory mediators. SP undergoes rapid C-terminal truncation in vivo by a number of proteases to generate the metabolites SP(1–9)-COOH and in particular SP(1–7)-COOH. While the C terminal of SP is critical for NK1R activation, studies have shown that the peptide polycationic N terminus is key for MRGPRX2 and mast cell activation. The study thus aimed to determine if the C-terminally truncated metabolites of SP, SP(1–9)-COOH, and SP(1–7)-COOH retained stimulatory activity at MRGPRX2. SP, SP(1–9)-COOH, and SP(1–7)-COOH were synthesized and tested on HEK293 cells expressing NK1R or MRGPRX2, and LAD2 human mast cells, to determine the activity of SP and its metabolites in Ca2+ mobilization, degranulation, and cytokine assays. As expected from prior studies, both C-terminally truncated SP metabolites had essentially no activity at NK1R, even at very high concentrations. In contrast, the in vivo metabolite of SP, SP(1–9)-COOH retained ability to activate MRGPRX2 across all parameters tested, albeit with reduced potency compared to intact SP. SP(1–7)-COOH did not produce any significant MRGPRX2 activation. Our results suggest that the SP metabolite, SP(1–9)-COOH, may play a regulatory role through the activation of MRGPRX2. However, given the relatively low potency of both SP and SP(1–9)-COOH at MRGPRX2, additional work is needed to better understand the biological importance of this expanded SP/MRGPRX2 pathway.

KEYWORDS
mast cells, MRGPRX2, neuroinflammation, NK1R, substance P

Abbreviations: DMF, dimethylformamide; MCs, mast cells; MRGPRX2, Mas-related G protein-coupled receptor X2; NK1R, neurokinin 1 receptor; SP, substance P; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid.

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1 | INTRODUCTION

Substance P (SP), a member of the tachykinin family of neuropeptides, is found in the brain, peripheral primary sensory neurons, the gastrointestinal tract and also diverse immune cells. Upon release, SP commonly binds to its preferred neurokinin-1 receptor (NK1R) to initiate cellular signaling pathways that are involved in peripheral neurogenic inflammation, gastrointestinal motility, and numerous central nervous system processes including pain, itch, sleep, learning and memory, and mood.

Mast cells (MCs) are often found in close proximity to sensory neurons and are best known for their central role in IgE-dependent allergic inflammatory disease. However, human mast cells can also be activated via IgE-independent pathways, including through the Mas-related G protein-coupled receptor X2 (MRGPRX2). MRGPRX2 is not uniformly expressed across all mast cell populations, being particularly enriched on mast cells localized to the skin and associated adipose tissue.

MRGPRX2 is activated by a diverse group of commonly polycationic ligands, including certain antimicrobial peptides (e.g., LL-37), some peptidergic and non-peptidergic drugs and also by SP. SP binding to MRGPRX2 mediates MC degranulation with secretion of inflammatory mediators including histamine and proteases, triggering allergic inflammatory responses. Emerging data suggest that this pathway is an important process in SP-mediated pain and itch responses and in conditions such as chronic urticaria and asthma.

SP is an undecapeptide with sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-CONH2. The C terminus of SP is known to be essential for recognition by tachykinin receptors, and recent structural studies of SP bound to NK1R have shown that C-terminal interactions deep within NK1R are critical for receptor activation. In contrast, in studies conducted prior to the identification of MRGPRX2, the polycationic N-terminal region has been shown to be important for mast cell activation.

In vivo, SP is known to undergo rapid metabolism and enzymatic conversion to smaller peptide fragments. Perfusion studies have shown that SP (1–9) and SP (1–7) are major endogenous C-terminal metabolites of SP. It has been suggested that SP (1–9) is formed by the actions of a matrix metalloproteinase, while SP (1–7) is a cleavage product derived through the activity of various enzymes, including endopeptidase 24.11, angiotensin-converting enzyme, and other SP-hydrolyzing enzymes.

While these C-terminally cleaved metabolites of SP are inactive at NK1R, both have shown biological activity. In particular, SP (1–7) has been shown to modulate nociception in a number of in vivo studies. This suggests that an alternative receptor target exists for these metabolites. While a role for MRGPRX2 in mediating these actions of SP metabolites has not been explicitly investigated, based on the retention of the polycationic N terminus in SP (1–9) and SP (1–7), in this study, we examined if these endogenous SP metabolites retained agonist activity at MRGPRX2. These results might then extend and diversify the possible in vivo actions of released SP.

2 | METHODS

2.1 Peptide synthesis

All peptides were assembled via 9-fluorenlymethoxy carbonyl (Fmoc) solid-phase peptide synthesis (SPPS) on a CEM Liberty microwave peptide synthesizer (CEM, Matthews, NC, USA). Rink amide resin was used for C-terminally amidated peptides, and SP(1–9)-COOH and SP (1–7)-COOH were synthesized on the corresponding glycin and phenylalanine Wang resins. Fmoc deprotection was performed using 20% piperidine v/v in dimethylformamide (DMF) and amino acids were coupled using O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphat (HCTU) and N,N-dilpropylethylamine. Peptide cleavage from the resin and simultaneous global deprotection was achieved with 90:5:5 trifluoroacetic acid (TFA)/trisopropylsilane/H2O (90 min). The TFA solution was then filtered into a 50-ml polypropylene tube and concentrated under a stream of nitrogen gas to reduce the volume by ~90% and then precipitated with cold diethyl ether. Tubes were then centrifuged and the supernatant decanted to yield the crude peptide (white solid). The peptides were subsequently purified to >95% using a reverse-phase (RP)-HPLC fitted with an Eclipse XDB-C8 4.6 Å, 5 μm column on an Agilent 1200 system (Agilent). The peptides were eluted using a gradient of 10%–50% acetonitrile (ACN) in 0.08% aqueous TFA over 60 min. The purified peptides were lyophilized using a Virtis freeze dryer (SP Industries).

Peptide characterization (see Figure S1) was conducted on an Agilent 1260 Infinity II system LC/MS (Agilent). The photodiode array detector (215 nm unless otherwise stated) was coupled directly to an electrospray ionization source and a single quadrupole mass analyzer (Agilent 6120). Standard RP-HPLC analysis was carried out at 40°C using an InfinityLab Poroshell 120 EC-C8 3.0 × 50 mm 2.7 μm column (Agilent). Peptides were eluted from the column with a gradient of 0%–60% ACN in 0.05% aqueous TFA over 9 min at a flow rate of 0.5 ml/min. Mass spectra were obtained in the positive mode with a scan range of 2–2000 m/z. Buffer A was 0.05% v/v TFA in milli Q water, and buffer B was 0.05% v/v TFA in acetonitrile.

In preliminary syntheses, the most prominent impurity noted by mass spectrometry was an Arg1 deletion. To minimize this, the coupling time of Arg was increased and double coupling implemented before moving on to the final deprotection step. All five peptides were obtained with excellent purity (>95%) following RP-HPLC (Figure S1; Table S1). Peptides were solubilized (10 mM stock solutions) in sterile Milli-Q® water containing BSA (0.1%) and aliquoted and stored at −20°C until use.

2.2 Cell culture

Laboratory of allergic disease 2 (LAD2) cells were cultured in complete Stem-pro34 medium (ThermoFisher), supplemented with recombinant human stem cell factor (100 ng/ml, Peprotech),
GlutaMAX™ (2 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml) (all from ThermoFisher). The cells were maintained in a humidified incubator (5% CO₂ at 37°C). Before use, LAD2 cells were sensitized with conditioned medium from a human 4-hydroxy-5-iodo-3-nitrophenyl acetyl (NIP)-specific IgE secreting cell line JW8 (1 μg/ml) for 48 h. MRGPRX2 knockdown (KD) LAD2 cells,²⁴ cultured as above, were also used in the study.

Human embryonic kidney-293 cells (HEK-293, negative for mycoplasma contamination), stably transfected with the human NK₂ receptor (HEK-NK₂R),²⁵ were maintained in Iscove’s modified Dulbecco's media (IMDM) supplemented with fetal bovine serum (10%; FBS; Corning®). GlutaMAX™ (2 mM), penicillin (50 Units/ml), streptomycin (50 μg/ml), and hygromycin (100 μg/ml) (all from ThermoFisher).

MRGPRX2 expressing HEK-293 cells (HEK-MRGPRX2) were generated through transient co-transfection with a human MRGPRX2 construct (Genscript) and human Gα₁₅ (cDNA Resource Center) to enhance GPCR coupling for detection of Ca²⁺ mobilization.²⁶ Briefly, HEK-293 cells were seeded in six-well plates at an approximate density of 8 × 10⁵ cells/well in transfection medium (IMDM supplemented with FBS [1%; Corning®]) and GlutaMax™ and cultured overnight. Cells were then transfected with plasmid DNA (1.25 μg) using lipofectamine 3000 (ThermoFisher Scientific) according to the manufacturer’s directions. MOCK-transfected cells, which were exposed to the transfection reagent without plasmid DNA, were also generated. Five hours post-transfection, the medium was replaced with fresh medium. Transiently transfected cells were generally harvested 24–48 h later for assays. Flow cytometry analysis, using an anti-human-MRGPRX2 antibody (clone K125H4, Biolegend) was used to confirm surface receptor expression (data not shown).

### 2.3 | Measurement of LAD2 cell degranulation

LAD2 cell degranulation was quantified through the measurement of β-hexosaminidase release. Briefly, IgE-sensitized LAD2 cells were washed in HANKS buffer ( Hank’s balanced salt solution with NaHCO₃ (0.14%), HEPES (10 mM), glucose (5.5 mM), BSA (0.1%), MgSO₄ (0.73 mM), CaCl₂ (1.8 mM); pH 7.4) and then resuspended in the same buffer. Cells (35 000 cells/well) were plated into 96-well round-bottom cell culture plates (Costar®) and activated with a range of concentrations of Sp and its metabolites, SP(1–9)-COOH SP(1–7)-COOH, for 30 min, at 37°C in an oscillating incubator. Control stimuli included compound 48/80 (C48/80; Sigma Aldrich) for the MRGPRX2 pathway and NIP-conjugated BSA (NIP-BSA; Biosearch Technologies) for the IgE-dependent pathway. Triton-X-100 (0.1%; Sigma Aldrich) was used to quantify total cellular β-hexosaminidase.

The cell plate was then centrifuged (120g, 5 min) and supernatant (25 μl) transferred into a fresh 96-well flat-bottom culture plate (Costar®). The β-hexosaminidase substrate p-nitrophenyl N-acetyl-β-D-glucosaminide (p-NAG; 75 μl; 4 mM in 40 mM phosphate/citrate buffer; pH = 4.5; Glycosynth) was added to the plate and incubated at 37°C in an oscillating incubator for 90 min, protected from light. The reaction was quenched and developed by adding glycine (100 μl/well, 0.4 mM, pH 10.7) and the plate measured at 405 nm in a microplate reader (Multiskan Ascent, Thermo Fisher). β-hexosaminidase levels were expressed as the percentage of the total cellular pool of enzymes determined by cellular lysis with Triton-X-100 (0.1%). Spontaneous release of β-hexosaminidase (that occurring in the absence of any stimulus) was subtracted from all results.

### 2.4 | Measurement of intracellular Ca²⁺ mobilization in LAD2 and HEK cells using Fura2

LAD2 cells (IgE-sensitized), HEK-NK₂R or HEK-MRGPRX2 cells, were washed with calcium buffer (HANKS buffer as above but with 1 mM probenecid) then loaded with Fura-2 AM (2 μM for LAD2 cells; 4 μM for HEK-NK₂R; Abcam) and incubated for 60–90 min at 37°C, in an oscillating incubator protected from light. Fura-2 AM-loaded cells were washed and then plated into flat-bottom 96-well cell culture plates (Costar®) at a density of 100 000 cells/well (HEK-NK₂R or HEK-MRGPRX2 cells) or 60 000 cells/well (LAD2 cells).

Stimuli (as above) were prepared separately in a round-bottom 96-well cell culture plate (Costar®). The protease-activated receptor-2 (PAR2) agonist SLIGRLI (Auspep) was used as a stimulus for the endogenously expressed PAR2 receptor on HEK cells³⁷ and the calcium ionophore ionomycin (0.5 μM; Abcam) used as a nonreceptor-driven mobilizer of Ca²⁺.

Ratiometric measurement of intracellular Ca²⁺ mobilization was achieved using a FlexStation 3® (Molecular Devices) at 37°C with excitation wavelengths at 340/380 nm and emission wavelength at 510 nm with typical runs of 4 min (HEK-NK₂R or HEK-MRGPRX2 cells) or 6 min (LAD2 cells), and stimuli injected after a 30-s baseline recording. In some assays, the NK₁R antagonist GR205171 (vofopitant; 1 μM; ABX Gmbh) or DMSO vehicle control was used. This was added to the cells following a 30-s baseline recording and incubated for 2 min before stimulus addition. Real-time data were displayed and interpreted using Softmax® Pro Version 7.0 (Molecular Devices).

### 2.5 | CCL2 release by LAD2 mast cells

IgE-sensitized LAD2 cells (3.5 × 10⁵ cells/ml) in IMDM as above but with BSA (0.1%; Bovogen Biologicals) replacing the FBS were transferred to a 24-well plate (Corning®) and activated with various stimuli for 24 h, at 37°C, in a humidified incubator (5% CO₂). Samples were transferred to 1.5-ml tubes and centrifuged (120g; 5 min) to collect supernatant and CCL2 levels measured using an ELISA according to the manufacturer’s protocol (OptEIA; BD Biosciences). Absorbance values were read at 450 nm using a microplate reader (Multiskan Ascent, Thermo Fisher), and CCL2 concentrations extrapolated from a standard curve.
**FIGURE 1** The C-terminally truncated SP metabolite SP(1–9)-COOH has ablated NK1R activation but retains MRGPRX2 activation. HEK293-NK1R cells and HEK293-MRGPRX2 cells were loaded with FURA-2 and stimulated with a range of concentrations of peptides SP, SP(1–9)-COOH, and SP(1–7)-COOH. Peak calcium response ratios were plotted, or time courses generated. The parent peptide SP exhibited concentration-dependent calcium mobilization response at both NK1R (A, B) and MRGPRX2 (C, D). The endogenous metabolites SP(1–9)-COOH and SP(1–7)-COOH showed no response at NK1R while only the former retained activity at MRGPRX2 (C, E, F). Responses were MRGPRX2-dependent as mock-transfected cells showed no response to SP and its metabolites (D, E, F). Data shown are means ± SEM, n = 7–9. Dotted lines represent the time of administration of peptides at t = 30s.
2.6 | Statistical analysis and data presentation

Results were analyzed and graphically presented using Prism 8 (version 8.0 for MAC OS; Graphpad Prism Software Inc.). Results are shown as mean±standard error of mean (SEM) of n independent experiments. Statistical significance was determined using either a paired t-test or a two-way ANOVA with Bonferroni multiple comparison post hoc test with significance level shown in relevant figure legends.

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,28 and are permanently archived in the Concise Guide to Pharmacology 2021/22 GPCR section.29

3 | RESULTS

3.1 | NK1R and MRGPRX2 activation by SP and its C-terminal truncated metabolites in transfected HEK293 cells

Synthesized SP peptides were analyzed for their ability to activate NK1R and MRGPRX2 using transfected HEK293 cells and fluorimetric measurement of Ca2+ mobilization using Fura2 dye. As expected, SP produced a concentration-dependent calcium mobilization response in HEK-NK1R (Figure 1A,B) with an EC50 value of 0.4 nM (Table 1). SP(1–9)-COOH and SP(1–7)-COOH failed to induce significant activation of NK1R in this assay even at high concentrations (Figure 1A,B). This supports previous reports of the critical role of the SP C terminus in NK1R activation.1

In transiently transfected HEK-MRGPRX2 cells, SP stimulated Ca2+ mobilization in a concentration-dependent manner (Figure 1C) albeit at much higher concentrations relative to NK1R. SP(1–9)-COOH, but not SP(1–7)-COOH, also stimulated MRGPRX2 but with reduced potency compared to SP (Figure 1C). To confirm this response was dependent on MRGPRX2, mock-transfected cells were also tested and showed no response to these SP peptides (Figure 1D–F).

3.2 | MRGPRX2 activation by SP and truncated metabolites in LAD2 human mast cells

Studies on transfected HEK293 cells were extended to the human mast cell line LAD2, which natively expresses MRGPRX2. In these cells, MRGPRX2 activity was assessed by Ca2+ mobilization and functional degranulation, measured by β-hexosaminidase release. Results generated were consistent with findings in transfected HEK293 cells. SP triggered both a Ca2+ mobilization and subsequent degranulation in LAD2 cells with EC50 values of 1.8 μM and 5.9 μM, respectively (Figure 2A,B). SP(1–9)-COOH also stimulated Ca2+ mobilization and degranulation in the LAD2 mast cells, with 10-fold lower potency compared to SP (Figure 2A,B; Table 1). SP(1–7)-COOH again failed to produce cellular activation (Figure 2A,B).

3.3 | SP(1–9)-COOH-mediated mast cell degranulation is MRGPRX2 dependent

To determine the MRGPRX2 dependence of SP and metabolite responses in LAD2 cells, we next used MRGPRX2 knockout LAD2 cells, generated via a CRISPR-Cas9 approach.34 Both Ca2+ mobilization (Figure 3A) and degranulation assays (Figure 3B) in MRGPRX2 knockout LAD2 cells had significantly diminished responses to SP, SP(1–9)-COOH, and the control stimulus C48/80. In contrast, responses initiated by the IgE-dependent pathway were unaffected by reduced MRGPRX2 expression.

Previous studies have indicated that NK1R expressed on MCs partially mediates responses to SP.22,40 To further negate a possible role for the NK1R in SP-mediated LAD2 activation, we used the potent, selective NK1R antagonist GR205171.41 Pretreatment of cells with this antagonist (100nM) effectively blocked SP responses in HEK-NK1R cells (Figure 4A), but had no effect on SP, SP(1–9)-COOH, or C48/80-induced Ca2+ mobilization in LAD2 cells (Figure 4B,C). These combined results demonstrate that SP and SP(1–9)-COOH trigger the responses measured in LAD2 mast cells through MRGPRX2 activation.

### Table 1 Summary of the SP peptide potency (EC50 values) across Ca2+ mobilization, degranulation, and CCL2-release assays

| PEPTIDE | HEK-NK1R Ca2+ mobilization (μM) | LAD2 Ca2+ mobilization (μM) | LAD2 degranulation (μM) | LAD2 CCL2 release (μM) |
|---------|-------------------------------|-----------------------------|------------------------|----------------------|
| SP      | 0.0004                        | 1.2                         | 5.9                    | 1.8                  |
| SP(1–9)-COOH | N/A                          | 11                          | 43.3                   | 11.8                 |
| SP(1–9)-CONH2 | 7.3                          | 3.5                         | 18.5                   | N/A                  |
| SP(1–7)-COOH | N/A                          | N/A                         | N/A                    | N/A                  |
| SP(1–7)-CONH2 | 1.6                          | 38.7                        | N/A                    | N/A                  |

Note: EC50 values were determined using PRISM with nonlinear four-parameter fitting to acquired data. N/A—not measurable across concentrations tested.
3.4 | SP, SP(1–9)-COOH but not SP(1–7)-COOH trigger CCL2 release from LAD2 human mast cells

As some stimuli at MRGPRX2 are known to stimulate cytokine release in the absence of degranulation, we next investigated if SP and its metabolites could promote CCL2 release from LAD2 mast cells. Both SP (EC₅₀ = 1.8 μM) and SP(1–9)-COOH (EC₅₀ = 12 μM) produced concentration-dependent CCL2 cytokine release from LAD2 cells (Figure 5). However, SP(1–7)-COOH-mediated CCL2 cytokine release was minimal and comparable to spontaneous release,
further emphasizing the lack of MRGPRX2 activation by this shorter SP metabolite.

3.5 Introduction of a C-terminal NH$_2$ enhances SP metabolite activity at MRGPRX2

With previous studies indicating that the C-terminal amine of SP is critical for NK$_1$R activation$^{21}$ and positive charges in a range MRGPRX2 ligands being generally associated with enhanced MRGPRX2 activation, we also synthesized SP metabolites that contained a C-terminal NH$_2$. SP(1–9)-NH$_2$ had greater potency at MRGPRX2 compared to the carboxylated form (measured through both Ca$^{2+}$ mobilization and degranulation) albeit with reduced potency relative to the full-length SP peptide (Figure 6A,B). Interestingly, the NH$_2$ modified variant of SP (1–7) was now capable of stimulating moderate levels of Ca$^{2+}$ mobilization and a weak degranulation response (Figure 6A,B).

4 DISCUSSION

The C-terminal truncated SP metabolites have previously been shown to have minimal activity at NK$_1$R. However, their ability to activate MRGPRX2, another SP receptor, has not been directly examined. Given that the cationic residues of SP that reside in N-terminal region are considered critical for MRGPRX2 activation, we examined if the C-terminal metabolites of SP retained activity at MRGPRX2 and would thus represent an alternative pathway for pathophysiological regulation by SP. Here, we have identified that the endogenous SP metabolite SP(1–9)-COOH retains the ability to
activate MRGPRX2, albeit with lower potency compared to the intact parental peptide. However, the shorter and more investigated endogenous peptide SP(1–7)-COOH failed to significantly activate MRGPRX2 even at high concentrations.

It has long been known that SP can activate mast cells\(^42,43\) and more recent studies have identified that this occurs through MRGPRX2, a receptor activated by diverse, commonly polycationic ligands.\(^{14–16}\) SP levels have been found to be elevated in certain inflammatory conditions,\(^{19,44}\) and more recently, MRGPRX2 expression was also shown to be elevated in chronic spontaneous urticaria and allergic asthma.\(^{16,45}\) The MRGPRX2-SP axis has also been recently highlighted as an important mechanism in inflammatory pain.\(^{18}\) Thus, there is significant current interest in MRGPRX2 as a therapeutic target for inflammatory disease.

Proteolytic cleavage of SP resulting in the production of the metabolites SP(1–9)-COOH and SP(1–7)-COOH has been previously shown to effectively prevent SP activation of the NK\(_{1}\)R.\(^{1,25}\) Our study similarly confirmed this effect even when these metabolites were used at high concentrations. Thus, C-terminal metabolites of SP, in particular SP(1–7)-COOH, have been suggested to exert their biological effects through alternative, but currently unidentified, receptor sites.\(^{29–32}\)

In the present study, we identified that SP(1–9)-COOH does indeed act as an agonist at MRGPRX2, mobilizing Ca\(^{2+}\) in transfected cells as well as inducing degranulation and cytokine release in the human mast cell line LAD2. However, potency across all measures was reduced compared to SP. SP(1–7)-COOH lacked MRGPRX2 agonist activity across all our assays.

Recent publication of the cryo-EM structure of MRGPRX2, ligand, and G protein complexes has provided greater clarity on how MRGPRX2 is activated by diverse agonists.\(^{46,47}\) In one study, SP was used as the agonist and while only the first three to four residues of the peptide were directly visualized in the structure, this confirmed the importance of polar interactions between the cationic SP N terminus and MRGPRX2 acidic residues E164 and D184.\(^{46}\) However, earlier studies conducted prior to identification of MRGPRX2,\(^{22,23}\) and the present study, demonstrate that additional structural elements in SP are also necessary for effective MRGPRX2 activation. Our data, comparing SP(1–9)-COOH with SP(1–7)-COOH, show that the removal of Phe8 and Gly9 results in a complete loss of SP ability to activate MRGPRX2. This suggests the presence of an important additional binding site in the receptor. It was noteworthy that retention of the native C-terminal amide group enhanced the potency of both SP (1–9) and SP (1–7) suggesting a role for this charged group in MRGPRX2 activation, as with NK\(_{1}\)R.\(^{48}\) Moreover, the importance of hydrophobic interactions within SP for MRGPRX2 activation is
highlighted by work demonstrating conjugation of SP (1–4) to doc-decylamine produces a more potent mast cell activator than the native agonist. A recent study, however, has shown that loss of the C-terminal amide group in SP leads to evidence of misfolding in the peptide. Thus, possible effects of C-terminal truncation on the tertiary structure of SP need to be considered in interpreting our data. More expansive MRGPRX2–ligand structural work would greatly assist in identifying the SP-binding site(s) and may reveal additional and targetable binding pockets in MRGPRX2. The shallowness and relative superficial nature of peptide ligand binding to MRGPRX2 has, however, been identified as being technically challenging for clear structural resolution.

Numerous studies report that SP(1–7)-COOH has a range of biological actions most notably as an anti-nociceptive. These studies proposed that SP(1–7)-COOH binds specific but as yet unidentified sites that are distinct from neurokinin receptors. Our study would suggest that these in vivo effects of SP(1–7)-COOH are unlikely to be mediated by MRGPRX2 agonism, and thus, alternative explanations are necessary. One such mechanism might be possible antagonism of the actions of SP, or other agonists, at receptor sites. In pilot studies (results not shown), we have not been able to demonstrate any antagonism by SP(1–7)-COOH of SP activation of MRGPRX2, suggesting that the metabolite has low binding affinity for MRGPRX2. Specific binding analysis is, however, necessary to definitively show this. The C-terminal amidated SP (1–7) had somewhat enhanced activity over SP(1–7)-COOH as has been reported by others in a different system. That SP(1–7)-NH₂ had the ability to mobilize Ca²⁺ in human LAD2 mast cells but not induce degranulation positions it as a potential tool compound to examine possible mechanisms of biased agonism at MRGPRX2. This segregation of activity at MRGPRX2 is rare, but importantly, it is also shown for some small molecule drug activators of MRGPRX2 including the neuromuscular blocking agent rocuronium which also shows a mobilization of Ca²⁺ without triggering concomitant degranulation in LAD2 cells.

In summary, we have validated and extended the important role of the C terminus of SP not only for NK₃ receptor activation but also at MRGPRX2. We also demonstrate that the SP metabolite SP(1–9)-COOH retains activity at MRGPRX2, which suggests that it might play a regulatory role in vivo through this pathway, which given the emerging role of MRGPRX2 in a variety of diseases, may contribute to inflammation and pain. Given the relatively low potency of both SP and, in particular, SP(1–9)-COOH in activating MRGPRX2, additional work is needed to reveal the clinical importance of this expanded SP-MRGPRX2 axis. Newly identified selective MRGPRX2 inverse agonists will assist with this.

AUTHOR CONTRIBUTIONS
GAM: Designed and planned the study. LH: conducted experiments and collected the majority of data. NAF, JD, CL, NV, JAK, SEN: generated key tools and reagents and provided expertise on their use. LH, GAM: wrote the draft manuscript. NV, JAK, SEN: provided critical insight into the generated data and revised the draft manuscript. All authors approved the final version of the manuscript.

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DISCLOSURE
All authors have no relevant conflicts of interest to declare.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT
All studies used established cell lines as experimental models. As such, no ethics approval (human or animal) was necessary in completing this study.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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