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**Protease-activated receptor-2 peptides activate
neurokinin-1 receptors in the mouse isolated
trachea**

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PAR₂, protease-activated receptor-2;

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Abstract

Protective roles for protease-activated receptor-2 (PAR₂) in the airways including activation of epithelial chloride (Cl⁻) secretion are based on the use of presumably PAR₂-selective peptide agonists. To determine if PAR₂ peptide-activated Cl⁻ secretion from mouse tracheal epithelium is dependent on PAR₂, changes in ion conductance across the epithelium (increase in short circuit current, I_{SC}) to PAR₂ peptides were measured in Ussing chambers under voltage clamp. Also, epithelium- and endothelium-dependent relaxations to these peptides were measured in two established PAR₂ bioassays, isolated ring segments of mouse trachea and rat thoracic aorta respectively. Apical application of the PAR₂ peptide SLIGRL, caused increases in I_{SC} which were inhibited by three structurally different neurokinin receptor-1 (NK₁R) antagonists and inhibitors of Cl⁻ channels, but not by capsaicin, the calcitonin gene-related peptide (CGRP) receptor antagonist, CGRP₈₋₃₇, or the non-selective COX-inhibitor, indomethacin. Only high concentrations of trypsin caused an increase in I_{SC}, but did not affect the responses to SLIGRL. Relaxations to SLIGRL in the trachea and aorta were unaffected by the NK₁R antagonist SR140333, but were abolished by trypsin desensitisation. The rank order of potency for a range of peptides in the trachea I_{SC} assay was 2-furoyl-LIGRL>SLCGRL>SLIGRL=SLIGRT>LSIGRL compared with 2-furoyl-LIGRL>SLIGRL>SLIGRT>SLCGRL (LSIGRL inactive) in the aorta relaxation assay. In the mouse trachea, PAR₂ peptides activate both epithelial NK₁R coupled to Cl⁻ secretion and PAR₂ coupled to PGE₂-mediated smooth muscle relaxation. Such a potential lack of specificity of these commonly used peptides needs to be considered when determining roles for PAR₂ in airway function in health and disease.

Introduction

Mouse and human airway epithelial cells express protease-activated receptor-2 (PAR₂) (Cocks et al., 1999; Asokanathan et al., 2002; De Campo and Henry, 2005), which when activated by either trypsin or the mouse PAR₂ peptide SLIGRL, release prostaglandin E₂ (PGE₂) (Lan et al., 2001; Asokanathan et al., 2002; De Campo and Henry, 2005) to cause airway smooth muscle relaxation (Cocks et al., 1999; Chow et al., 2000; Lan et al., 2001; De Campo and Henry, 2005) and inhibition of immune cell trafficking (Moffatt et al., 2002; De Campo and Henry, 2005). However, some effects of PAR₂ peptides have been claimed to be independent of PAR₂ activation (Moffatt, 2004), including mediator release from mast cells (Stenton et al., 2002) and contractions in endothelium-intact umbilical vein tissue (Saifeddine et al., 1998). In mice, which are commonly used to model airway inflammatory disease, precise roles for PAR₂ in airway physiology and pathology remain controversial. While some studies have shown clear anti-inflammatory protective effects, including a range of PGE₂-dependent mechanisms (Cocks et al., 1999; Cocks and Moffatt, 2000; De Campo and Henry, 2005), others have reported pro-inflammatory actions of PAR₂ in the airways (Schmidlin et al., 2002; Ebeling et al., 2005; Su et al., 2005). One of the main roles for epithelial PAR₂ appears to be activation of mucosal chloride (Cl⁻) secretion. This has been shown to occur in dog pancreatic duct epithelial cells (Nguyen et al., 1999), rat jejunum (Vergnolle et al., 1998), porcine ileum (Green et al., 2000), mouse distal colon (Cuffe et al., 2002) and monolayers of human cultured bronchial epithelial cells (Danahay et al., 2001). Such an increase in Cl⁻ secretion would maintain the protective layer underneath the mucous and as such aid in the efficient removal of inhaled antigens and has been proposed as a possible protective role of epithelial PAR₂ (Cocks and Moffatt, 2000). In the mouse trachea,

Kunzelmann et al (2005) reported that PAR₂ located on the basolateral surface of epithelial cells couple to basolateral K⁺ channels and mucosal Cl⁻ channels to mediate net Cl⁻ secretion into the lumen of the airways. They also suggested that these basolateral PAR₂, as well as apical PAR₂ are coupled to the production of PGE₂ to mediate smooth muscle relaxation. As in our previous study (Cocks et al., 1999), Kunzelmann et al (2005) similarly reported that PAR₂ are predominantly expressed on the apical surface of mouse tracheal epithelial cells. Activation of these apically-expressed PAR₂, however, with either trypsin or SLIGRL, did not have any effect on ion conductance (Kunzelmann et al., 2005). In the present study we found an increase in Cl⁻ conductance to SLIGRL in the mouse trachea that was not PGE₂-dependent and was not mimicked by trypsin or affected by prior exposure to high concentrations of trypsin. Also, the supposedly inactive PAR₂ peptide L₁SIGRL (Lindner et al., 2000; Fiorucci et al., 2001; Stenton et al., 2002) caused a similar increase in Cl⁻ conductance to SLIGRL. Furthermore, the increase in Cl⁻ conductance across the epithelium in response to SLIGRL was blocked by three structurally different NK₁R antagonists, SR140333, GR205171 and GR82334. Thus, results from our study indicate that PAR₂-like peptides directly activate apical NK₁R coupled to Cl⁻ secretion and as such may need to be considered when claiming roles for PAR₂, particularly in inflammatory diseases of the airways.

Methods

Materials. The following drugs were used: 4,4-diisothio-cyanostilbene-2,2-disulfonic acid (DIDS), acetylcholine chloride, adenosine 5' triphosphate trisodium salt (ATP), capsaicin, carbamylcholine chloride (carbachol), phenylephrine hydrochloride, substance P, thrombin (all Sigma); carboxyl-terminally amidated peptides (custom synthesised, single letter amino acid code) 2-furoyl-LIGRL (Mimotopes, Clayton, Victoria, Australia), LSIGRL, SLIGRL, SLIGRT, SLCGRL, SLSGRT and calcium gene-related peptide (CGRP) receptor antagonist CGRP₈₋₃₇ (Auspep, Parkville, Victoria, Australia); glibenclamide and nifedipine (Biomol); bovine trypsin (Worthington Biochemical Corporation, Lakewood, New Jersey, U.S.A.); GR82334 (TOCRIS Bioscience, Missouri, U.S.A.); GR205171 (gift from GlaxoSmithKline, Middlesex, U.K. to JB Furness) and SR140333 (gift from Sanofi Recherche, Montpellier, France to JN O'Neil). All stock solutions of drugs were made in distilled water with the exception of indomethacin, which was dissolved in 0.1 M Na₂CO₃ and nifedipine and SR140333, which were dissolved in ethanol to stock concentrations of 1 mM and then further diluted in distilled water.

Epithelial ion conductance studies: Ussing chamber assay. Male Balb/C mice (30 g) were killed by CO₂ asphyxiation, their tracheae rapidly dissected, cut into sheets via an incision through the dorsal part of the tracheal cartilage rings and mounted between two plastic sheets with an aperture of 0.03 cm². The preparations were then mounted in Lucite Ussing chambers, allowing separate perfusion of each side of the preparations with Krebs solution (composition (mM): Na⁺ 143.1, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, Cl⁻ 127.8, HCO₃⁻ 25.0, SO₄²⁻ 1.2, H₂PO₄⁻ 1.2 and glucose 11.0) heated and continuously bubbled with 95% O₂/5% CO₂ via gas lift to maintain pH

7.4 and temperature at 37°C. Electrodes were placed in each side of the Ussing chambers and connected to an amplifier (DVC 1000, World Precision Instruments, Sarasota, Florida, U.S.A.) The preparations were then clamped at a potential difference of zero volts to record short circuit current (I_{SC}), a measure of the net ionic flux across the preparation. Once mounted, the preparation was allowed to equilibrate (> 20 min) until I_{SC} was stable and any antagonists to be used were added for at least 15 min prior to challenge with agonists.

Mouse tracheal and rat aorta smooth muscle contractility studies. Mouse tracheae (isolated as described above) and thoracic aortae from male Sprague-Dawley rats (200 – 300 g) were cut into 3 mm rings and mounted on stainless steel hooks in Krebs-filled organ baths maintained at 37°C and continuously bubbled with 95% O₂/5% CO₂. One hook was attached to a micrometre-driven anchor in the bath, whilst the other hook was attached to a force transducer for isometric recording of aortic and trachealis muscle tone. After a 30 min equilibration period, 0.5 and 1.0 g tension were applied to the trachea and aorta respectively and the preparations then allowed to return to a steady plateau (> 20 min) before the maximal contractile capacity of the preparation (F_{max}) was determined by contracting the trachea with 10 μ M acetylcholine and the aorta with high K⁺ (120 mM) isotonic Krebs solution (KKS). After F_{max} had been obtained, the preparations were washed repeatedly and allowed to equilibrate at baseline passive tension for a further 30 min in the presence of 0.3 μ M nifedipine to reduce spontaneous phasic contractile activity. The preparations were then contracted with titrated concentrations of carbachol (trachea) or phenylephrine (aorta) until a stable, active tension equivalent to approximately 40 – 50% F_{max} was reached and allowed to stabilise before cumulative additions of agonists were added to the bath.

Intracellular Ca^{2+} measurement in A549 cells. Ca^{2+} fluorescence was measured with a FLUOstar® plate reader (BMG LabTechnologies, Mornington, Victoria, Australia). A549 human epithelial cells (ATCC, Manassas, Virginia, U.S.A.) were grown to ~80% confluency and harvested with non-enzymatic cell dissociation solution (Sigma, St. Louis, Missouri, U.S.A.) and rinsed with DMEM with HEPES (Gibco, Grand Island, New York, U.S.A.). Pelleted cells were resuspended in a Krebs-HEPES buffer with 1 mM Probenecid (Sigma) at pH 7.4. Cells were then loaded with 2 μM Fura-2AM (Molecular Probes, Eugene, Oregon, U.S.A.) with 0.01% Pluronic F127 to facilitate more even dispersion of the dye, for 30 min at 37°C and 5% CO_2 . Following this incubation, cells were rinsed twice and resuspended in the Krebs-HEPES buffer and left for 20 min at room temperature for de-esterification. For experiments testing responses in the presence of any antagonists, cells were pre-incubated with the specific antagonist at room temperature for the final 10 min of de-esterification. At the completion of this procedure, all cells were placed on ice until use, to reduce leakage of dye. Cells were plated into black 96-well plates (BMG LabTechnologies) at a density of 2×10^6 cells. ml^{-1} . Microplates were then inserted into the pre-incubated plate reader (37°C). Estimation of $[\text{Ca}^{2+}]_i$ was achieved by exposure of the cells to rapidly alternating 340 and 380 nm wavelengths and measuring the resulting 510 nm emission from the Fura-2AM. After a 30 s baseline reading, test compounds were injected *via* the plate reader's pre-fitted injectors, into each specified well.

Statistics. All responses are expressed as the mean \pm standard error of the mean (\pm SEM). I_{SC} responses are expressed as a ratio of the surface area of the preparation ($\mu\text{A}.\text{cm}^{-2}$; assuming no edge damage). In the tracheal and aortic smooth muscle contractility studies, relaxant responses are expressed as a percentage of carbachol- or phenylephrine-induced tone. Where possible,

pEC₅₀ values were determined by non-linear regression analysis using GraphPad Prism 4.0a (GraphPad Software, San Diego, California, U.S.A.). Statistical comparisons were performed with either paired or non-paired Student's t-tests or analysis of variance (ANOVA) as appropriate (Snedecor and Cochran, 1989).

Results

Tension and short circuit current (I_{SC}) responses to trypsin and PAR peptides. In isometric tension studies of mouse airways, trypsin ($0.01 - 3 \text{ U.ml}^{-1}$) caused concentration-dependent relaxations of mouse trachea (Figure 1). Trypsin is known to cleave the N-terminus of PAR receptors, creating tethered agonist ligands that activate PARs, leading to tracheal relaxation (Cocks et al., 1999; Chow et al., 2000; Lan et al., 2001). Similar relaxant responses produced by the PAR₂ peptide agonist, the isolated N-terminal hexapeptide of rodent PAR₂, SLIGRL ($0.1 - 30 \mu\text{M}$), were prevented if the preparation was initially desensitized to trypsin (3 U.ml^{-1} , Figure 1). However, in the Ussing chamber assay, using an identical protocol, trypsin failed to either elicit a response or prevent I_{SC} responses to SLIGRL (Figure 1) when added to either side of the preparation at concentrations ten-fold in excess of those required for relaxation. SLIGRL was only active if applied to the mucosal surface of the preparations (Figure 2). A hundred-fold higher concentration of trypsin (100 U.ml^{-1}) caused a small, slowly developing increase in I_{SC} (Figure 1), again only if applied to the mucosal surface (Figure 2). However, at the plateau of this response, addition of SLIGRL elicited a further, sharp increase in I_{SC} that was not different in magnitude to the response to SLIGRL alone, suggesting that trypsin and SLIGRL do not act via the same mechanism (Figure 1).

Epithelium-dependent smooth muscle relaxations to trypsin and SLIGRL in the tracheal mechanical assay (Figures 1 & 4) were within the previously reported range (Cocks et al., 1999) with EC_{50} of $0.5 \pm 0.1 \text{ U.ml}^{-1}$ and pEC_{50} of 5.6 ± 0.1 respectively. Two novel PAR₂ peptide agonists, SLIGRT (pEC_{50} 5.9 ± 0.2) and SLCGRL (pEC_{50} 5.3 ± 0.2), were approximately 6- and

20-fold less potent respectively than SLIGRL ($pEC_{50} 6.4 \pm 0.1$) in causing endothelium-dependent relaxation of the rat aorta preparation (Figure 3), which has previously been shown to relax to SLIGRL via PAR_2 in an endothelium-dependent manner (Hwa et al., 1996; Saifeddine et al., 1998). Also we confirmed that a recently reported stable PAR_2 peptidomimetic agonist, 2-furoyl-LIGRL, was more potent ($pEC_{50} 7.3 \pm 0.1$) than SLIGRL in this assay (Kawabata et al., 2004). By contrast another novel PAR_2 peptide agonist SLSGRT and the commonly used partial scramble peptide for SLIGRL, LSIGRL (Lindner et al., 2000; Fiorucci et al., 2001; Stenton et al., 2002), were both inactive in the aorta up to 100 μM (Figure 3). Thus, an estimated rank order of potency for these peptides in the rat aortic endothelium-dependent relaxation assay was 2-furoyl-LIGRL > SLIGRL > SLIGRT > SLCGRL with LSIGRL and SLSGRT inactive. Also in the aorta, responses to 2-furoyl-LIGRL, SLIGRL, SLIGRT and SLCGRL were abolished if preparations were desensitised to trypsin ($3 U \cdot ml^{-1}$; data not shown), suggesting that all peptides activate PAR_2 . In the Ussing chamber mouse tracheal assay, however, SLCGRL, SLIGRT and LSIGRL, but not SLSGRT, induced I_{SC} increases over similar concentration ranges as SLIGRL. 2-furoyl-LIGRL, however, appeared to be more potent than any of the other active peptides, but with a maximum increase in I_{SC} of only $9.0 \pm 3.0 \mu A \cdot cm^{-2}$ at a concentration of 3 μM (Figure 3). Therefore, an apparent rank order of potency of the peptides was 2-furoyl > SLCGRL > SLIGRT = SLIGRL > LSIGRL and SLSGRT inactive (Figure 3), with 2-furoyl-LIGRL appearing to act as a partial agonist.

Involvement of Cl^- conductance and prostanoids in I_{SC} responses to SLIGRL. Replacement of Cl^- with gluconate abolished I_{SC} responses to SLIGRL (Figure 4). Also, glibenclamide (100 μM) and DIDS (4,4-diisothio-cyanostilbene-2,2-disulfonic acid, 100 μM), inhibitors of cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+} -activated Cl^- channels

respectively, inhibited responses to SLIGRL, each by approximately 50% and the combination of glibenclamide and DIDS further attenuated responses to approximately 80% of control (Figure 4). Indomethacin (3 μM) had no significant effect on the I_{SC} response curve to SLIGRL (Figure 5).

Effects of substance P, capsaicin, tachykinin and CGRP antagonists. Substance P caused increases in I_{SC} in the mouse trachea over two concentration ranges. The threshold concentration of the first range varied from 0.01 fM to 1 fM with the maximum occurring at 0.1-1.0 pM. Threshold concentration for the second phase of the I_{SC} curve to substance P usually occurred at around 10 pM and was maximum at approximately 1 nM (Figure 5). The selective NK_1R antagonist, SR140333 (100 nM) abolished the first phase of the substance P curve and caused an estimated approximate 1000-fold shift to the right in the second component (Figure 5). SR140333 (100 nM) had no effect on the increase in I_{SC} to a sub-maximum concentration (10 μM) of ATP ($97 \pm 18 \mu\text{A}\cdot\text{cm}^{-2}$ control; $95 \pm 18 \mu\text{A}\cdot\text{cm}^{-2}$ SR140333, $n = 4$). Two further NK_1R -selective antagonists, GR205171 (10 nM) and GR82334 (0.1 μM) similarly blocked the I_{SC} curve to SLIGRL (Figure 5).

Capsaicin (10 μM) added to both sides of the Ussing tracheal preparation caused an increase in I_{SC} of $32.9 \pm 7.3 \mu\text{A}\cdot\text{cm}^{-2}$ ($n = 4$) which declined to baseline levels after 30 min, at which time it had no inhibitory effect on the I_{SC} curve to SLIGRL, if anything it appeared to be potentiated although not significantly (Figure 5). Also, addition of the CGRP antagonist, CGRP₈₋₃₇ (100 μM) for 20 min to both sides of the preparation had no effect on the increase in I_{SC} to luminal SLIGRL (Figure 4). Both substance P and SLIGRL failed to cause any increase in I_{SC} when added to the

contraluminal surface of the tracheal preparation (data not shown) and treatment of the luminal side with the mast cell degranulating agent, Compound 48/80 (10 μ M for 20 min) had no effect itself nor inhibited increases in I_{SC} to SLIGRL (data not shown). Surprisingly, perhaps, indomethacin (3 μ M) caused a significant, approximate 10-fold rightwards shift in the concentration-dependent I_{SC} curve to substance P (Figure 5). As for SLIGRL (see above and Figure 4), similar indomethacin treatment failed to have any effect on the increases in I_{SC} to ATP (data not shown).

In tension assays in the mouse trachea, the NK_1R antagonist SR140333 (100 nM) had no inhibitory effect on the relaxation curve to SLIGRL, whereas the biphasic response curve to substance P was abolished by SR140333 (Figure 6). Additionally, in rat thoracic aorta, where substance P failed to cause any mechanical responses (either endothelium-dependent relaxation or contraction; data not shown), SR140333 had no effect on the relaxation curve to SLIGRL and LSIGRL was inactive (Figure 6).

Ca²⁺ measurement in A549 cells. Substance P (100 nM) caused little if any increase in intracellular Ca²⁺ in A549 cells and the response to SLIGRL was unaffected by SR140333, but was inhibited following desensitisation with trypsin (Figure 7), confirming the above tension data in the rat thoracic aorta. Clearly SR140333 has no inhibitory effect at PAR₂.

Discussion

The results of this study indicate that the supposed selective PAR₂ peptide agonist, SLIGRL, is an agonist of NK₁R located on the apical surface of epithelial cells of the mouse trachea.

Therefore, given the accepted role of substance P and NK₁R in asthma (Barnes, 1998), any claims for roles of PAR₂ in inflammatory airway disease that are based solely on data from experiments using SLIGRL or other similar PAR₂-activating peptides, may need to be reconsidered in light of our study.

Unlike Kunzelmann et al (2005), we found no change in I_{SC} to the PAR₂ activators trypsin and SLIGRL when they were applied to the basolateral surface of mouse tracheal epithelial cells.

Also, contrary to our findings here, Kunzelmann et al (2005) found no response to apically-applied trypsin or SLIGRL, even though, as we reported earlier (Cocks et al., 1999), they found PAR₂ was predominantly expressed on the apical surface of mouse tracheal epithelial cells. Both the inability of Kunzelmann et al (2005) to demonstrate responses to apically-applied SLIGRL and our inability to similarly show responses to basolaterally-applied SLIGRL, most likely reflect a lack of NK₁R expressed on the mucosal surface of airway epithelial cells in the strain of mice used by Kunzelmann et al (2005) and the inability of basolateral PAR₂ to couple to Cl⁻ secretion in the strain of mouse (Balb/C) used in our study.

The increase in I_{SC} to SLIGRL in the trachea was most likely due to NK₁R-mediated stimulation of luminal Cl⁻ secretion as it was inhibited by removal of Cl⁻ from the bathing Krebs solution, by

inhibitors of cystic fibrosis transmembrane conductance regulator (CFTR; glibenclamide; (Schultz et al., 1999)) and Ca²⁺-activated (DIDS; (Kidd and Thorn, 2000)) Cl⁻ channels and by a potent and selective NK₁R antagonist, SR140333 (Emonds-Alt et al., 1993; Moriarty et al., 2001). Also, the increase in I_{SC} to SLIGRL only occurred when the peptide was applied to the luminal surface of the trachea and was unaffected by indomethacin. Together, these results indicate that SLIGRL causes an increase in luminal Cl⁻ secretion in the mouse trachea *via* activation of NK₁R located on the apical surfaces of epithelial cells and that the resultant increase in I_{SC} is not due to concomitant PAR₂-dependent abluminal PGE₂ release (Asokanathan et al., 2002) which has been shown previously to mediate epithelium-dependent relaxation in this tissue (Cocks et al., 1999; Lan et al., 2001; De Campo and Henry, 2005).

Fortner *et al* (2001b) have claimed that substance P causes similar increases in luminal Cl⁻ conductance in the mouse airway, but that this activity is linked in some way to smooth muscle relaxation, which the same group showed was due to concomitant abluminal release of PGE₂ from the epithelium (Fortner et al., 2001). A possible simple explanation for this rather confusing concept is that some of the PGE₂ released contraluminally into the wall of the airway activates basolateral EP receptors to increase luminal ion conductance. This idea is supported by our current finding that part of the increase in ion conductance to luminal substance P was inhibited by indomethacin, which abolished the concomitant relaxation and which, like that to ATP, is epithelium-dependent (Kao et al., 1999). The lack of effect of indomethacin on increases in I_{SC} to SLIGRL observed here is most likely due to the relatively weak efficacy of SLIGRL at luminal NK₁R such that levels of PGE₂ released abluminally are insufficient to stimulate basolateral epithelial EP receptors to increase I_{SC}. Similarly, the failure of indomethacin to block relatively large increases in I_{SC} to ATP indicates poor coupling of the purinoceptor involved in PGE₂

synthesis. This possibility is supported by ATP only being able to cause weak epithelium-dependent relaxations in the mouse trachea which, like those for SLIGRL and substance P, are abolished by indomethacin (Moffatt and Cocks, unpublished data).

Since SLIGRL, 2-furoyl-LIGRL, LSIGRL and SLCGRL are all cationic with arginine at position 5, it is possible that these and other peptides increase I_{SC} indirectly *via* non-receptor dependent degranulation of mast cells (Stenton et al., 2002). That the response is blocked by SR140333, however, still implies a role for NK_1R , which is possible as rat colonic mast cells have been shown to release substantial amounts of substance P in response to anti-IgE which was inhibited by SR140333 (Moriarty et al., 2001). Such a non-specific action on mast cells is unlikely to explain the current findings, however, for two reasons. First, we found that the mast cell degranulating agent compound 48/80, failed to cause an increase in I_{SC} or affect subsequent I_{SC} responses to SLIGRL (Abey and Cocks, unpublished data). The second reason why mast cells are unlikely to be involved in the increase in I_{SC} to PAR_2 peptides is that capsaicin, which failed to affect I_{SC} increases to SLIGRL in the present study, has been shown to deplete mast cells of substance P (Moriarty et al., 2001), most likely *via* activation of vanilloid VR1 receptors (Biro et al., 1998). Furthermore, another novel R^5 cationic peptide, SLSGRT, failed to cause either an increase in I_{SC} in the mouse trachea or smooth muscle relaxation in both the mouse trachea and rat thoracic aorta. That single amino acid substitutions can markedly modify the binding characteristics of these peptides further argues against a non-specific action of these molecules such as their gross charge.

Substance P and capsaicin both caused increases in I_{SC} in the mouse trachea. Therefore, it is possible that the similar responses to PAR_2 peptides in this preparation were due to their ability to

cause release of substance P from sensory nerves, either directly through activation of PAR₂ on sensory nerves (Fiorucci et al., 2001; Kawabata et al., 2001; Su et al., 2005) or indirectly *via* a mechanism similar to that reported for mast cells (Stenton et al., 2002). This seems unlikely, however, since sensory neuropeptide depletion with prolonged exposure to capsaicin had no inhibitory effect on the I_{SC} response to SLIGRL. Also, any role for sensory nerves in the I_{SC} response to SLIGRL was further ruled out by the lack of effect of an antagonist (CGRP₈₋₃₇) of the other major sensory neurotransmitter in the airways, CGRP (Barnes, 1998). Rather, combined with the different rank order of potencies of 2-furoyl-LIGRL, SLIGRL, LSIGRL, SLIGRT and SLCGRL for ion secretion compared with that for endothelium-dependent nitric oxide production in the rat aorta (Hwa et al., 1996), the lack of activity of LSIGRL in both the mouse tracheal and rat aortic smooth muscle assays and the selectivity of SR140333 for substance P in the I_{SC} and airway relaxation assays, our data suggest that SLIGRL directly activates NK₁R located on the epithelium.

The biphasic concentration-I_{SC} curve to substance P may reflect two molecular isoforms of NK₁R that have been shown in molecular recombination studies to be determined by the type of G-protein each NK₁R form couples to (Holst et al., 2001). Also, the high potency of substance P in this assay may reflect high affinity with both isoforms of the receptor. If all of the PAR₂ peptides activated the same isoform of NK₁R to increase I_{SC}, then some structure-activity can be deduced. Thus, their rough rank order of potency (2-furoyl-LIGRL > SLCGRL > SLIGRL = SLIGRT > LSIGRL with SLSGRL inactive) indicates that reversal of the first two amino acids diminishes NK₁R activity, S³ for I³ abolishes it, C³ for I³ substitution appears to increase it, T⁶ for L⁶ substitution has little effect and substitution of 2-furoyl for S¹ increases potency, but markedly reduces efficacy. This apparent weak partial agonist activity of 2-furoyl-LIGRL at NK₁R in the

mouse trachea as compared with its potent, full agonist profile at PAR₂ in the rat aorta makes it an attractive alternative to SLIGRL for further research into the biology of PAR₂, particularly where complicating effects of NK₁R are possible.

The results presented here allow a closer examination of some recent studies where PAR₂ has been suggested to mediate effects in the airways, but where the pharmacological data present are not always consistent with classic PAR₂ pharmacology (Moffatt, 2004). In some studies, the non-specific mechanism of action may account for the findings rather than PAR₂ *per se*. For example, high micromolar concentrations of PAR₂-activating peptides have been shown to increase I_{SC} in human airway epithelial cells (Danahay et al., 2001), act as a mitogen for airway smooth muscle (Chambers et al., 2000) and activate eosinophils (Miike et al., 2001). Although trypsin may also elicit a response in these studies, it is often not practically possible to perform cross desensitisation studies to establish that trypsin and peptide activators act *via* a common mechanism. Furthermore, concentrations of trypsin that are supramaximal for PAR₂ activation, as we found here in the I_{SC} response to trypsin, are often required to elicit a response. Therefore, in the absence of PAR₂ antagonists and the ability to cross-desensitise with trypsin, PAR₂ knockout animals provide the only certain information regarding the role of this receptor in airway inflammation. Thus, Schmidlin *et al* (2002) showed that the early, but not late, phase of immune cell trafficking into the airway lumen after ovalbumin (OVA) challenge in OVA-sensitised wild type mice was reduced and increased in PAR₂ knockout and PAR₂ over-expressing transgenic mice respectively, suggesting that PAR₂ was indeed involved in this early phase allergic response in the airways. However, unexplained effects of PAR₂ deletion on the production of IgE in response to sensitisation (Schmidlin et al., 2002) may account for this difference.

In conclusion, roles for PAR₂ in airway barrier defence, inflammation and other processes will only be unequivocally resolved with the development of more selective PAR₂ ligands (agonists and antagonists). Although reported PAR₂-binding peptides are typically selective for PAR₂ over PAR₁, they may not be selective for PAR₂ over other GPCRs (e.g. NK₁) and as such, receptor specificity beyond simple PAR₂ subtypes clearly needs to be considered when drawing conclusions as to the roles of PAR₂ in airway inflammation.

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Footnotes

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Figure Legends

Figure 1. Comparison of the effects of the PAR₂ agonists trypsin and SLIGRL in the mouse trachea in smooth muscle relaxation (a, b) and ion conductance (c, d, e) assays. (a) Cumulative concentration-relaxation curves for trypsin in the trachea. Responses (mean ± SEM, n = 6) are expressed as percentage relaxations of approximately 40% maximum carbachol contractions. (b) Sample trace in the smooth muscle relaxation assay demonstrating that desensitisation to trypsin abolishes the response to SLIGRL (for controls see Figure 5). (c) Sample trace in the ion conductance assay demonstrating the lack of effect of trypsin applied to the mucosal surface of the mouse trachea on short circuit current (I_{SC}). Although the same protocol in (b) was followed in (c), there is no desensitising effect of trypsin on the responsiveness of the preparation to SLIGRL added to the same side of the preparation. (d) A high concentration of trypsin (100 U.ml⁻¹), caused a slowly developing increase in I_{SC} , however subsequent addition of SLIGRL (30 μM) caused a further rapid increase in I_{SC} . After washout and 20 min re-equilibration (indicated by the break in the trace), the recovery response to SLIGRL was not statistically different in magnitude to that elicited in the presence of trypsin (e).

Figure 2. Comparison of the effects of trypsin (100 U.ml⁻¹) and SLIGRL (30 μM) on short circuit current (I_{SC}) in the mouse trachea ion conductance assay when added to either the mucosal or basal surface of the preparation.

Figure 3. A comparison of the effects of SLIGRL and modified PAR₂ peptides in the rat aorta smooth muscle relaxation assay (a) and the mouse tracheal ion conductance assay (b). Responses in (a) are expressed as percentage relaxations of approximately 40% maximum phenylephrine

contractions. The number of experiments conducted for each peptide in the rat thoracic aorta and Ussing chamber assays were; SLSGRT (◆, n = 10, n = 7 respectively), LSIGRL (■, n = 3, n = 4 respectively), SLCGRL (○, n = 8, n = 7 respectively), SLIGRT (▲, n = 13, n = 6 respectively), SLIGRL (▼, n = 9, n = 13 respectively) and 2-furoyl-LIGRL (●, n = 6, n = 5 respectively). All data points are presented as means ± SEM.

Figure 4. The effects of Cl⁻ free bathing solution (n = 4) and the Cl⁻ channel inhibitors glibenclamide (G, n = 4), 4,4-diisothio-cyanostilbene-2,2-disulfonic acid (D, n = 3) alone and in combination with glibenclamide (G + D, n = 4) on SLIGRL (30 μM) induced increases in short circuit current (I_{SC}) in the mouse trachea. Responses (means ± SEM) are presented as a percentage inhibition of the increase in I_{SC} of SLIGRL (30 μM). Determination of a 95% confidence interval for the mean of each treatment revealed significance from zero indicated by (*).

Figure 5. The effects of the NK₁ antagonists SR140333, GR205171 and GR82334 (●, 0.1 μM; △, 10 nM; and □, 0.1 μM respectively), indomethacin (▲, 0.3 μM), capsaicin (◆, 10 μM) and CGRP antagonist CGRP₈₋₃₇ (▼, 3 μM) on increases in short circuit current (I_{SC}) in the mouse trachea to cumulative concentrations of substance P (a), SLIGRL (b) and LSIGRL (c). Results are expressed as increases in I_{SC} (μA.cm⁻²) from basal and data points are means ± SEM. (*) indicates statistical significance at specific concentrations using one-way ANOVA and unpaired t-tests (p < 0.05). Numbers of experiments conducted were; controls (■, n ≥ 4), SR140333 (n = 4), indomethacin (n ≥ 4), capsaicin (n = 3) and CGRP₈₋₃₇ (n = 3).

Figure 6. The effect of SR140333 (◆, 0.1 μM) on relaxations to SLIGRL (■) or substance P (▲) in smooth muscle relaxation assays of mouse trachea (a, b) and rat aorta (c). LSIGRL (●) was inactive in both preparations. Responses are expressed as percentage relaxations of approximately 40% maximum carbachol (a, b) and phenylephrine (c) contractions. Data points are means ± SEM. Number of experiments for control and SR140333 curves were; (a) n = 6, (b) n = 14 and (c) n = 3. Number of experiments for LSIGRL and substance P were n = 3 (a and c respectively).

Figure 7. The effects of SR140333 (SR, 0.1 μM) and trypsin (0.1 U.ml⁻¹) desensitisation on SLIGRL (10 μM) induced increases in free intracellular calcium (as measured by changes in the emission wavelength ratio 340/380 nm of Fura-2) in A549 cells. Also shown is the near zero average response to substance P (SP, 100 nM). (*) indicates statistical significance using a one-way ANOVA. All experiments were performed in triplicate and data is presented as mean ± SEM.

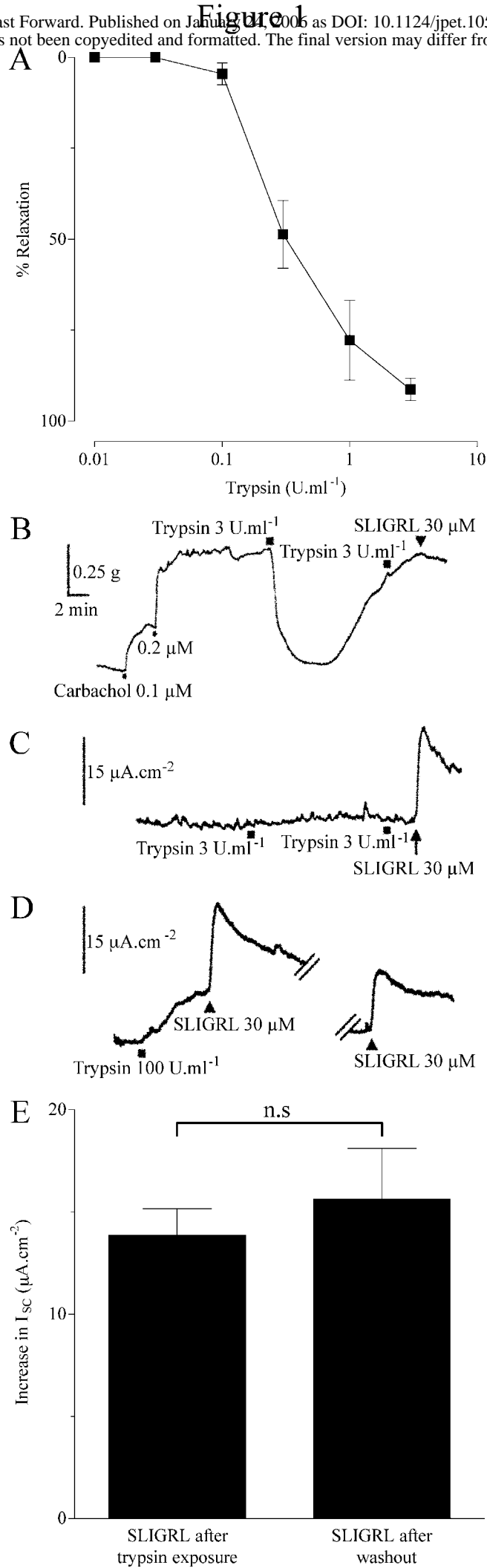


Figure 2

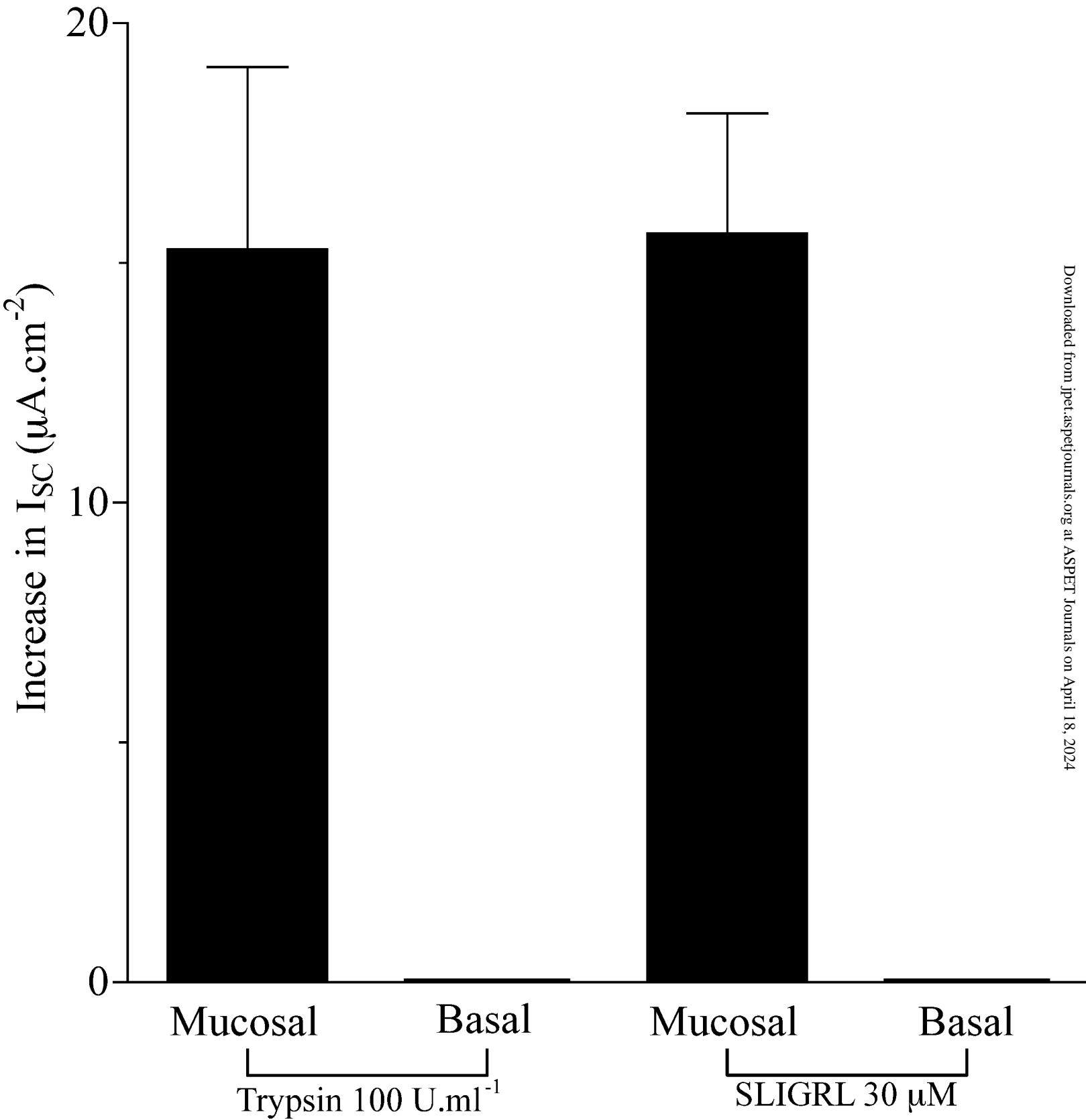


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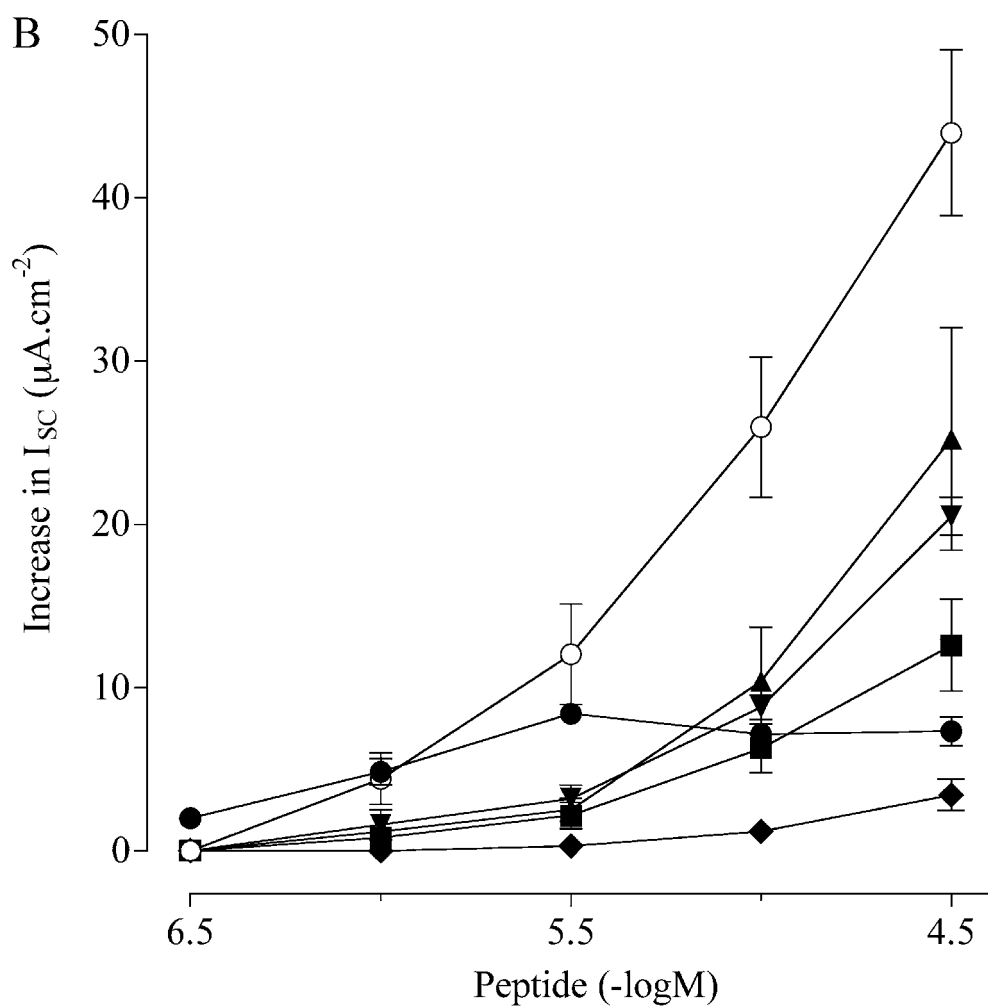
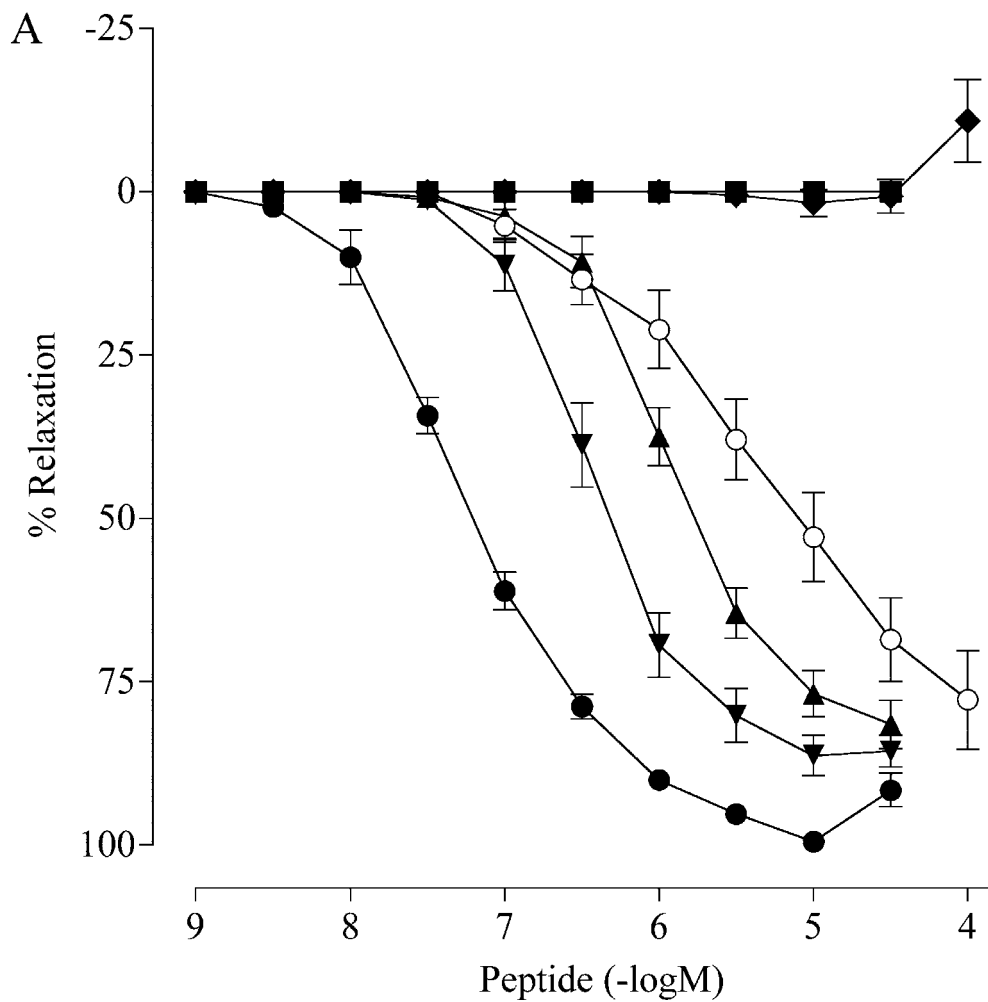


Figure 4

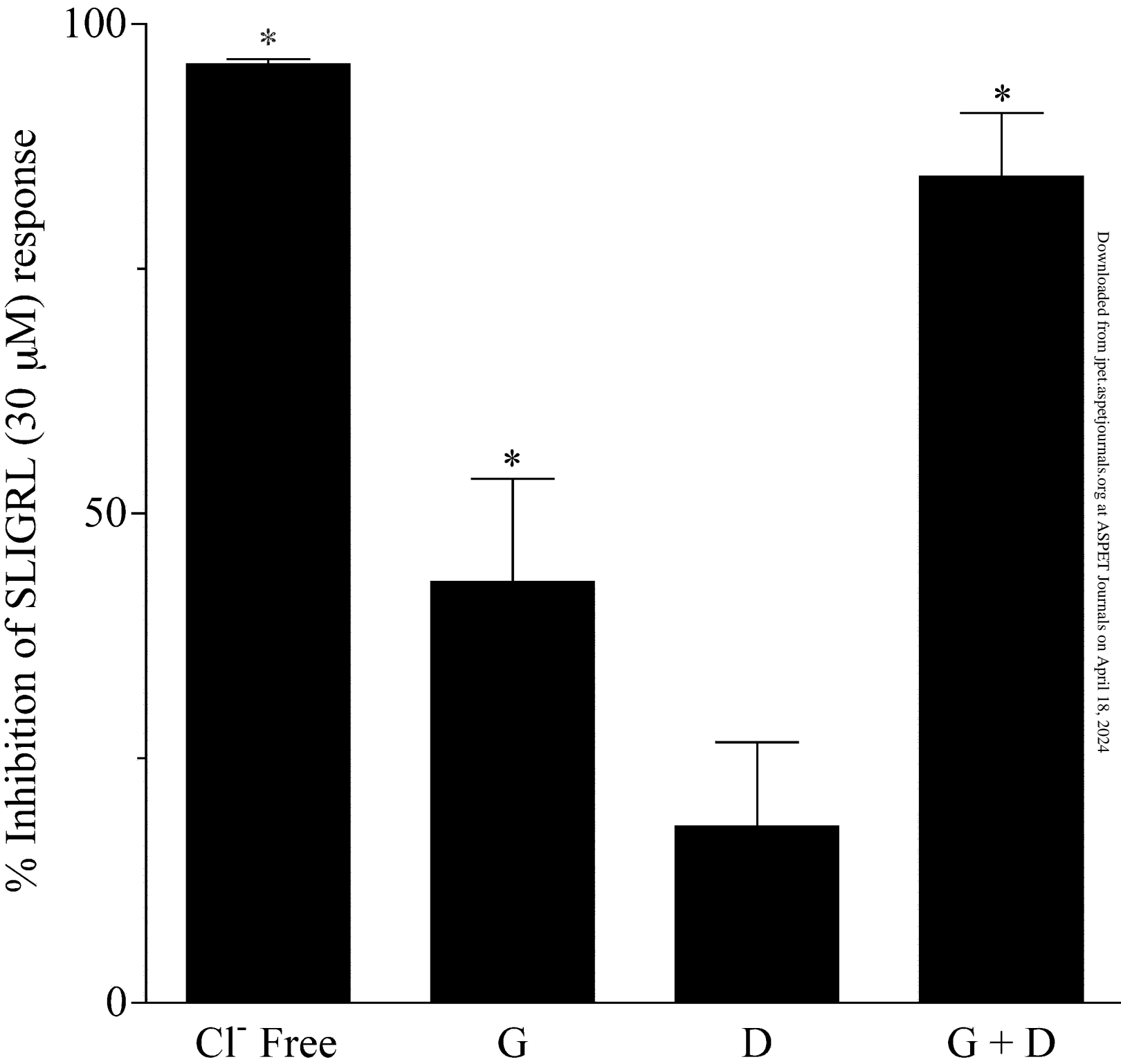


Figure 5

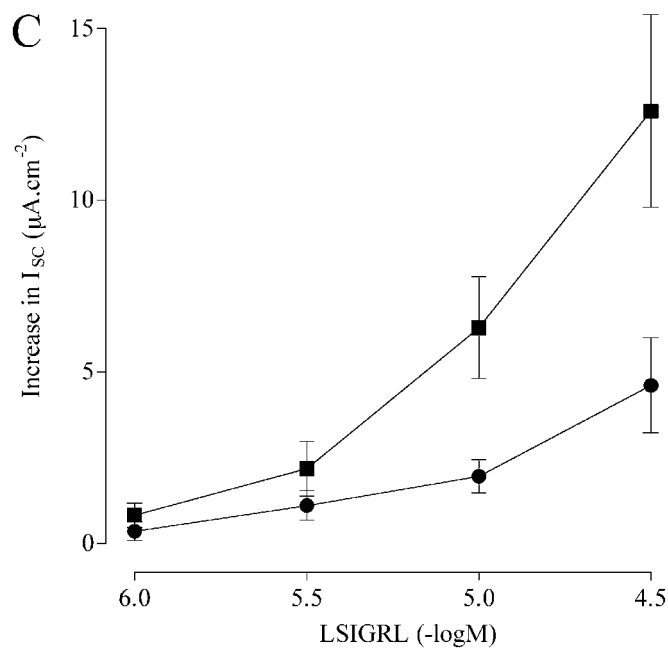
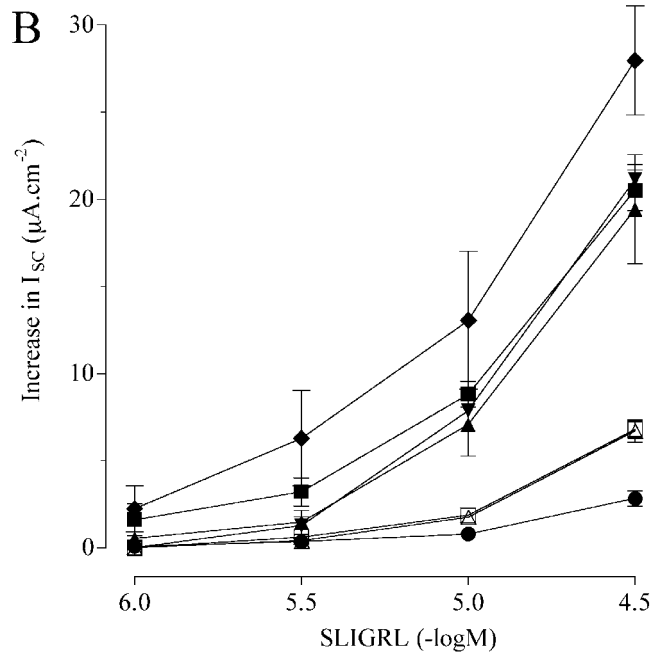
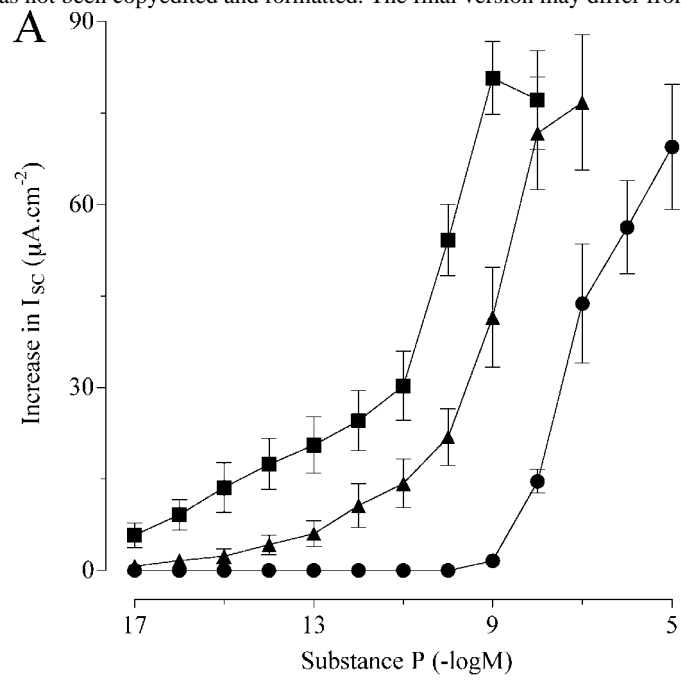


Figure 6

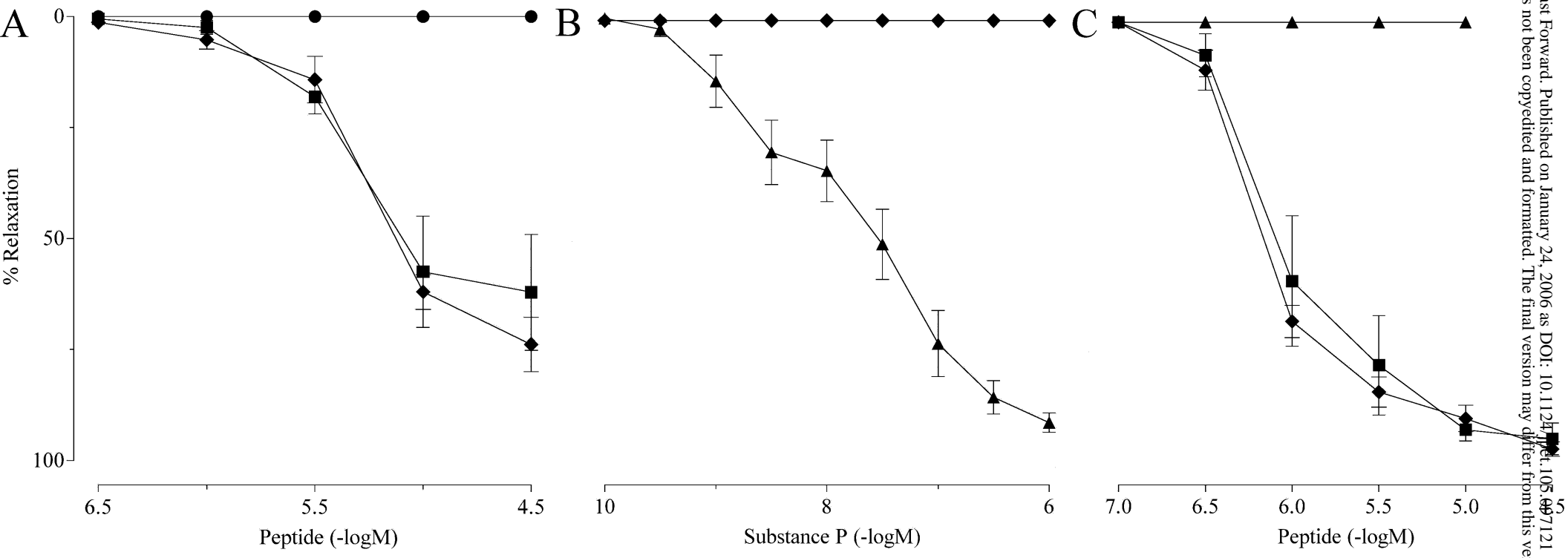


Figure 7

