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Hepta and octapeptide agonists of protease-activated receptor 2

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Abstract: Protease-activated receptor 2 (PAR_2) is a G protein-coupled cell surface receptor for trypsin-like enzymes. Proteolytic cleavage at a specific site in the extracellular *N*-terminus exposes a receptor-activating sequence, the 'tethered ligand', which binds intramolecularly to initiate receptor signalling. Peptide or small molecule agonists for PAR_2, devoid of the non-specific and proteolytic effects of enzyme activators, may be promising therapeutic agents for proliferative and inflammatory diseases reportedly mediated by PAR_2. Synthetic hexapeptides that correspond to the native tethered ligand of human or rodent PAR_2 (SLIGKV and SLIGRL, respectively) can activate the receptor independently of proteolytic cleavage; however, known peptide agonists have much lower potency compared to protease-mediated activation. Here, we investigated the agonist activity of 94 hepta and octapeptide derivatives of the human and rodent PAR_2-tethered ligand sequences in human airway epithelial (A549) cells which endogenously express PAR_2. Thirty synthetic peptides were found to be as potent as or more potent than SLIGRL on the basis of intracellular Ca^{2+} responses. The more active peptide agonists were also examined for agonist cross-reactivity at PAR_1 in Chinese Hamster Ovary (CHO) cells that endogenously express functional PAR_1 but not PAR_2. Two potent and PAR_2-selective agonists were further examined for their capacity to relax phenylephrine-contracted rat aortic rings. Our findings reveal an important role for carboxyl extensions to native PAR_2 activating peptides in potentiating agonist activity. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: PAR_2; protease-activated receptor; agonist; inflammation; cancer; GPCR; A549 cells; calcium fluorescence; SLIGRL; SLIGKV; structure-activity relationship

INTRODUCTION

PAR_2 is the second of four members of the protease-activated receptor (PAR) family of enzyme-activated receptors. Activation of PAR_2 has been implicated in both protective [1–3] and pro-inflammatory responses [4–7] suggesting that both agonists and antagonists of PAR_2 may exert potentially valuable therapeutic effects under specific conditions [1,2,8,9].

Via a mechanism thought to be unique to PARs, PAR_2 is 'self-activated' by its own extracellular *N*-terminal tethered ligand which is unmasked after proteolytic cleavage by trypsin or trypsin-like serine proteases (e.g. mast cell tryptase, acrosin, Factor Xa) [10]. Although there are no known endogenous non-enzymatic peptide/protein agonists of PAR_2, the receptor can be activated by hexapeptides that mimic the tethered ligand sequence of the rodent (SLIGRL) or human (SLIGKV) receptor. These hexapeptides, which are usually carboxyl-terminal amidated to reduce degradation by carboxypeptidases (i.e. SLIGRL-NH_2), are valuable pharmacological tools for studying PAR_2-mediated responses and also offer a starting point for the design of more potent and selective PAR_2 ligands. In one of the few reported sequence–activity studies of peptide agonists for PAR_2 [11], Maryanoff and coworkers investigated the potency at human PAR_2 of 100 synthetic hexapeptide analogues of SLIGKV comprising entirely natural amino acids. Unfortunately, none of these peptides had significantly higher potency than the native human or rodent tethered ligand sequences [11]. Other groups have focussed on modifying the *N*-terminus to increase agonist potency [12], notably resulting in 2-furoyl-LIGRL [13].

This paper reports the use of both natural and unnatural amino acids in seven- and eight-residue carboxyl-terminal-amidated peptides that substantially enhance agonist activity relative to the native peptide sequences, SLIGKV and SLIGRL. Activation of the endogenously expressed human PAR_2 receptor in A549 cells by hepta and octapeptide derivatives of SLIGKV/SLIGRL, in which natural and unnatural amino acids are appended to the *C*-terminus or incorporated within the sequence, was measured by fluorescence arising from agonist-stimulated intracellular calcium release. The PAR_2 selectivity over the widely co-expressed protease-activated receptor 1 (PAR_1) was determined for the more potent agonists in Chinese Hamster Ovary (CHO) cells which respond robustly to the synthetic PAR_1 tethered ligand peptide TFLLR, but not to PAR_2 activating peptides.

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MATERIALS AND METHODS

Peptide Synthesis

Rink amide MBHA resin, Fmoc-L-amino acids and all unnatural amino acids were obtained from Novabiochem (Melbourne, Australia). HBTU was obtained from Richelieu Biotechnologies (Quebec, Canada). All other reagents were of peptide synthesis grade from Auspep (Melbourne, Australia). Peptides were synthesized on a 0.075-mmol scale using standard Fmoc conditions on Rink Amide MBHA resin (peptides **1–94**). Fmoc-protected amino acids were activated using 4 equiv. of HBTU and 8 equiv. of DIPEA (diisopropylethylamine) and coupled (4 equiv., 1.5 h). Fmoc-deprotection was achieved by 2×2 min treatments with excess 1 : 1 piperidine/DMF. Coupling yields were monitored by quantitative ninhydrin assay; double couplings were used when yields were <99.8%. Peptides were cleaved from the resin with simultaneous removal of the protecting groups using 95% trifluoroacetic acid (TFA), 2.5% H_2O, 2.5% TIPS (25 ml of solution per 1 g of peptide resin) for 2 h at room temperature. TFA solutions were filtered, concentrated *in vacuo* and diluted with 1 : 1 CH_3CN/water (0.1% TFA). Peptides were purified by semi-preparative RP-HPLC using a Waters Delta 600 chromatography system fitted with a Waters 486 tunable absorbance detector with detection at 219 and 254 nm. Eluants were 0.1% TFA in water (A) and 0.1%TFA in 9 : 1 CH_3CN/H_2O (B) with a gradient of 0–100% B over 30 min on a Vydac C_{18} 250 × 22 mm (300 Å) column at 15 ml/min. Peptide identity was established by mass spectrometry on a Waters micromass ZQ spectrometer using ESI. Purity was assessed by analytical RP-HPLC using the same gradient on a Vydac C_{18} 250 × 4.6 mm (300 Å) column at 1.0 ml/min.

Intracellular Ca^{2+} Measurement in Human Airway Epithelial (A549) Cells and CHO Cells

Adherent A549 or CHO cells (<passage 30) were grown in 75 cm^2 flasks to 80% confluence in Dulbecco's modified Eagle medium (DMEM) containing 5% v/v heat-inactivated fetal bovine serum (JRH Bioscience, USA), 20 mM HEPES and 100 μg/mL penicillin and streptomycin. On the day of each experiment, cells were harvested using 8 mL of non-enzymatic cell dissociation solution (Sigma Chemical Company, USA) and washed with 12 mL of DMEM containing 20 mM HEPES before centrifugation (500 g, 5 min). The supernatant was discarded and the pellet resuspended in 5 mL of assay buffer (Krebs-HEPES buffer, in mM; 118 NaCl, 4.6 KCl, 10 HEPES, 1.8 $CaCl_2$, 1.2 $MgCl_2 \cdot 6H_2O$, 24.9 $NaHCO_3$, 1.0 KH_2PO_4, 11.1 dextrose, pH 7.4) containing 1 mM probenecid and 0.1% w/v bovine serum albumin (BSA). Cells were centrifuged as before and the pellet resuspended in 5 mL of Krebs-HEPES buffer containing 2 μM FURA 2-AM. The light-protected cells were incubated for 30 min at 37 °C, centrifuged (400*g*, 3 min), washed once with 5 mL of assay buffer and resuspended in 5 mL of assay buffer.

Cells were allowed to sit for 20 min at room temperature prior to the measurement of intracellular Ca^{2+} accumulation. A549 cells used for the determination of PAR_2 activation by novel peptides underwent PAR_1 desensitisation by pre-treatment for 10 min with thrombin (1 U/mL, 10 min, 37 °C). Cells were held on ice for up to 45 min prior to measurement of intracellular Ca^{2+} fluorescence.

Intracellular fluorescence measurements were performed using a FluoSTAR OPTIMA fluorometer. For each determination, 180 μl of cells ($2–4 \times 10^6$ cells/mL) were added to the well of a 96-well plate maintained at a constant temperature (37 °C). Each sample was exposed to a single concentration of agonist (10 μM) and measurements were conducted in triplicate. In this assay, SLIGRL has an EC_{50} of approximately 10 μM, and to maximise the detection of significantly higher potency agonists from a single point while maintaining an appreciable Ca^{2+} response level, novel peptides were also initially assayed at this concentration. Cells were subjected to excitation at two wavelengths, 340 and 380 nm, and the emitted light was collected at the photomultiplier through a 500-nm filter. The ratio of the fluorescence due to excitation at 340 nm to that at 380 nm ($R_{340/380}$) was calculated by the FluoSTAR software.

Rat Aorta Smooth Muscle Contractility Studies

Thoracic aortae from male Sprague-Dawley rats (200–300g) were cut into 3-mm-wide rings and mounted on stainless steel hooks in Krebs' solution-filled organ baths maintained at 37 °C and continuously bubbled with 95% O_2/5% CO_2. One hook was attached to a micrometer-driven anchor in the bath, whereas the other hook was attached to a force transducer for isometric recording of aortic muscle tone. After a 30 min equilibration period, 1.0 g of tension was applied to the aorta and the preparation was 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 aorta with high K^+ (120 mM) isotonic Krebs' solution. 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 μM nifedipine to reduce spontaneous phasic contractile activity. The preparations were then contracted with titrated concentrations of phenylephrine until stable, active tension equivalent to approximately 40–50% F_{max} was reached and allowed to stabilize before cumulative additions of agonists were added to the bath.

Data Analysis

Fluorescence measurements were taken for 30 s to ensure a stable baseline prior to the automated injection of PAR_2-activating peptides, with the resultant intracellular Ca^{2+} response being measured for a further 90 s. Peak fluorescence responses were calculated by subtracting the mean, pre-injection baseline fluorescence from the peak response following agonist stimulation. To normalise inter-assay variability, the intracellular Ca^{2+} responses to novel peptides are presented as a percentage of the response to SLIGRL on a given day. Statistical analysis was performed using PRISM 4.0 for Mac (GraphPad Software, USA) and all data are presented as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Variations to H-SLIGKVXaa-NH_2

On the basis of the sequence for the human PAR_2-activating hexapeptide (H-SLIGKV-NH_2, compound **1**)

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capped by an amide at the *C*-terminus, we created a 16-membered heptapeptide library H-SLIGKVXaa-NH_2 (**2**–**17**, Figure 1) in which the seventh residue (Xaa) was variable at the *C*-terminus (Table 1 for non-standard amino acid abbreviations). Novel peptides were screened against A549 cells that had been pre-treated with thrombin (1 U/mL, 10 min, 37 °C) to desensitise PAR_1-mediated responses. Fourteen compounds showed greater or comparable potency to SLIGKV (Figure 1) at the single concentration tested (10 μM). For comparison, the rat sequence SLIGRL (**18**) was also assayed at 10 μM. The fact that SLIGRL was more potent ($pEC_{50} = 4.84 \pm 0.09$, $n = 4$) than the human sequence, SLIGKV ($pEC_{50} = 4.37 \pm 0.14$; $p < 0.05$), at human PAR_2, was confirmed from full concentration–response curves in A549 cells (data not shown) and is consistent with previous reports [12–14].

Compounds **5** (Xaa = homophenylalanine), **7** (*p*-nitrophenylalanine), **9** (*m*-iodophenylalanine), **11** (1-naphthylalanine) and **13** (leucine) were either as potent as or more potent than **18**. These results suggest that a seventh residue at the *C*-terminus can enhance the potency of the hexapeptide agonist SLIGKV, and that an aromatic ring or steric bulk can be accommodated at this position by PAR_2. These compounds were also selective for PAR_2 over PAR_1 in wild-type CHO cells (Figure 4). CHO cells endogenously express PAR_1 and respond robustly to the PAR_1-selective peptide, TFLLR, but not to the PAR_2-activating peptide, SLIGRL, or the inactive (reverse) PAR_2 peptide, LRGILS (Devlin and Cocks, unpublished data).

On the basis of an increased potency of selected heptapeptide derivatives of SLIGKV over the 'parent' hexapeptide, together with the higher potency of SLIGRL over the human sequence SLIGKV, we then investigated the agonist activity of heptapeptides derived from SLIGRL.

Table 1 Abbreviations for non-standard amino acids

1-Nal	1-Napthylalanine
2Ahta	2-Aminoheptanoic acid
Chg	Cyclopentylalanine
Har	Homoarginine
Hle	Homoleucine
Hph	Homophenylalanine
Hse	Homoserine
Hty	Homotyrosine
*m*IPhe	*meta*-Iodophenylalanine
Nva	Norvaline
*p*ClPhe	*para*-Chlorophenylalanine
*p*FPhe	*para*-Fluorophenylalanine
*p*MePhe	*para*-Methylphenylalanine
*p*NPhe	*para*-Nitrophenylalanine
tBuGly	Tertiarybutylglycine

Variations to SLIGRLXaa-NH_2

A 12-membered heptapeptide library H-SLIGRLXaa-NH_2 (**19**–**30**, Figure 2) was created in which the seventh residue (Xaa) at the amidated *C*-terminus was varied. Three heptapeptides were more potent agonists than SLIGRL (**22**, Xaa = Cha; **24**, Xaa = 1-Nal; **25**, Xaa = Leu) and substantially more potent than SLIGKV. In addition to screening this library for PAR_2 activity, peptides **27**–**30** were also screened for PAR_1 activity in CHO cells (Figure 4). Compounds **29** and **30** displayed PAR_1 activity (103 ± 7 and 109 ± 7% respectively, relative to 10 μM TFLLR) and featured

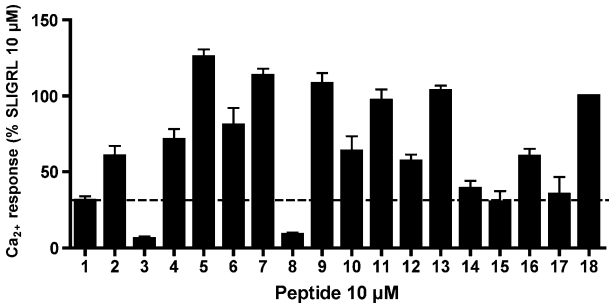

Figure 1 PAR_2-mediated intracellular calcium response to 10 μM *C*-terminal amidated heptapeptides (**2–17**) based on amino acid additions to the carboxyl terminus of SLIGKV (i.e. H-SLIGKV-Xaa-NH_2) in human airway epithelial (A549) cells *in vitro*. Amino acid additions (Xaa) are: **2** = Tyr; **3** = Tyr (and tBuGly at position 2); **4** = Phe; **5** = Hph; **6** = Cha; **7** = *p*NPhe; **8** = *p*MePhe; **9** = *m*IPhe; **10** = Trp; **11** = 1-Nal; **12** = Arg; **13** = Leu; **14** = Ala; **15** = Glu; **16** = Lys; **17** = Gln. All bars represent mean ± SEM of a single experiment conducted in triplicate, except for **1** (H-SLIGKV-NH_2; $n = 4$) and **18** (H-SLIGRL-NH_2; $n = 3$) conducted in triplicate. The dashed line shows the activity of the parent peptide, H-SLIGKV-NH_2.

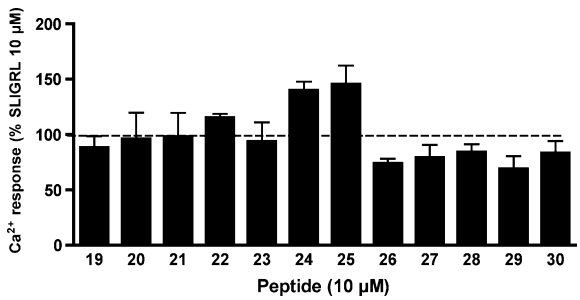

Figure 2 PAR_2-mediated intracellular calcium response to 10 μM *C*-terminal amidated peptides **19–30** based on amino acid additions to the carboxyl terminal of SLIGRL (i.e. H-SLIGRL-Xaa-NH_2) in human airway epithelial (A549) cells *in vitro*. Amino acid additions (Xaa) are in **19** = Tyr; **20** = Hty; **21** = hPhe; **22** = Cha; **23** = *p*NPhe; **24** = 1-Nal; **25** = Leu; **26** = Nva; **27** = *p*FPhe; **28** = *p*ClPhe, while multiple substitutions are in **29** = H-S-*p*FPhe-Cha-AR-2Ahta-*p*NPhe-NH_2 and **30** = H-S-*p*FPhe-Cha-AR-2Ahta-NH_2. All bars represent mean ± SEM of a single experiment conducted in triplicate. The dashed line shows the activity of the parent peptide, H-SLIGRL-NH_2.

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what we found to be a PAR_1-conferring residue (*p*-fluorophenylalanine) at position 2 adjacent to serine, while compound **27** featured the *p*-fluorophenylalanine at position 7 at the *C*-terminus. Thus, while **27** was selective for PAR_2 over PAR_1, **29** and **30** were dual PAR_1/PAR_2 agonists.

Encouraged by the enhanced activity of heptapeptides **22**, **24** and **25**, a further 56 heptapeptides were synthesised (**31–78, 87–94**) and assayed for PAR_2 agonist activity in A549 cells (Figure 3, Table 2). Heptapeptides that gave higher intracellular calcium responses than SLIGRL were **50** (H-SL-Nle-GRLL-NH_2), **55** (H-SLIARLL-NH_2), **65** (H-SLIG-Har-LL-NH_2), **70** (H-SLIG-*p*NPhe-LL-NH_2), **73** (H-SLIGR-Nle-L-NH_2), **74** (H-SLIGRWL-NH_2), **88** (H-SLIGR-*p*NPhe-L-NH_2), **89** (H-SLIGR-Cit-L-NH_2), **90** (H-SLIGR-Har-L-NH_2), **91** (H-SLIGR-1-Nal-L-NH_2) and **92** (H-SLIGRLI-NH_2), while **41** (H-SLIG-Cha-LL-NH_2), **57** (H-SLI-Nle-RLI-NH_2), **71** (H-SLIGRAL-NH_2) and **72** (H-SLIGRIL-NH_2) were equipotent with H-SLIGRL-NH_2. A selection of both active (**55, 65, 70, 89–92**) and inactive (**54, 60, 94**) heptapeptide agonists was tested in wild-type CHO cells (Figure 4), but none exhibited PAR_1 agonist activity. Taken together, these results support the conclusion that a hydrophobic residue appended to the *C*-terminus of SLIGRL significantly increases PAR_2 agonist potency, whereas substitution of leucine for *p*-fluorophenylalanine at the second position confers PAR_1 agonist activity.

To examine whether an eight-residue peptide would influence agonist activity, several octapeptides were

Table 2 PAR_2 agonist activity of 10 μM *C*-amidated peptides H-[**31–94**]-NH_2 measured as intracellular calcium release in human airway epithelial (A549) cells. Activity has been normalised to the percentage of the response to 10 μM H-SLIGRL-NH_2 (100%) on a given experimental day. All activity data is presented as the mean ± SEM of a single experiment conducted in triplicate

Peptide	Sequence	Activity[a]	Peptide	Sequence	Activity[a]
31	Hse L I G R L L	37 ± 5	**63**	S L I G K L L	37 ± 12
32	β-Ala L I G R L L	9 ± 3	**64**	S L I G Orn L L	82 ± 14
33	Aib L I G R L L	24 ± 3	**65**	S L I G Har L L	136 ± 24
34	T L I G R L L	16 ± 4	**66**	S L I G Cit L L	14 ± 5
35	C L I G R L L	25 ± 7	**67**	S L I G E L L	28 ± 2
36	S L I E R L L	9 ± 3	**68**	S L I G Q L L	31 ± 4
37	S L I Q R L L	6 ± 1	**69**	S L I G 1-Nal L L	52 ± 4
38	S L I R R L L	N.T.	**70**	S L I G *p*NPhe L L	140 ± 22
39	S L I K R L L	14 ± 3	**71**	S L I G R A L	102 ± 7
40	S L I G W L L	70 ± 20	**72**	S L I G R I L	92 ± 18
41	S L I G Cha L L	109 ± 7	**73**	S L I G R Nle L	119 ± 12
42	S Nva I G R L L	40 ± 10	**74**	S L I G R W L	122 ± 18
43	S L *p*FPhe G R L L	32 ± 11	**75**	S L I G R Aib L	17 ± 6
44	S L *p*NPhe G R L L	8 ± 2	**76**	S L I G R Cha L	72 ± 2
45	S L W G R L L	15 ± 7	**77**	S L I G R Q L	59 ± 17
46	S L 1-Nal G R L L	4 ± 1	**78**	S L I G R E L	58 ± 12
47	S L Chg G R L L	76 ± 19	**79**	S L I G R L L L	153 ± 18
48	S L Cha G R L L	82 ± 10	**80**	S L I G R L L Cha	206 ± 30
49	S L Nva G R L L	49 ± 11	**81**	S L I G R L L W	157 ± 26
50	S L Nle G R L L	123 ± 9	**82**	S L I G R L L R	110 ± 8
51	S L L G R L L	59 ± 5	**83**	S L I G R L L E	150 ± 22
52	S L K G R L L	4 ± 1	**84**	S L I G R L L Q	160 ± 13
53	S L E G R L L	13 ± 1	**85**	O S L I G R L L	21 ± 6
54	S L Q G R L L	11 ± 5	**86**	β-Ala S L I G R L L	4 ± 1
55	S L I A R L L	199 ± 13	**87**	S L I G R R L	84 ± 5
56	S L I Nva R L L	28 ± 7	**88**	S L I G R *p*NPhe L	146 ± 7
57	S L I Nle R L I	103 ± 7	**89**	S L I G R Cit L	142 ± 26
58	S L I Aib R L L	8 ± 2	**90**	S L I G R Har L	185 ± 7
59	S L I β-Ala R L L	12 ± 1	**91**	S L I G R 1-Nal L	166 ± 32
60	S L I Chg R L L	6 ± 1	**92**	S L I G R L I	208 ± 21
61	S L I Cha R L L	80 ± 11	**93**	Abu L I G R L L	36 ± 9
62	S L I W R L L	25 ± 4	**94**	S Abu I G R L L	29 ± 1

[a] Percentage of intracellular Ca^{2+} response from A549 cells relative to 100% by SLIGRL.
N.T. not tested.

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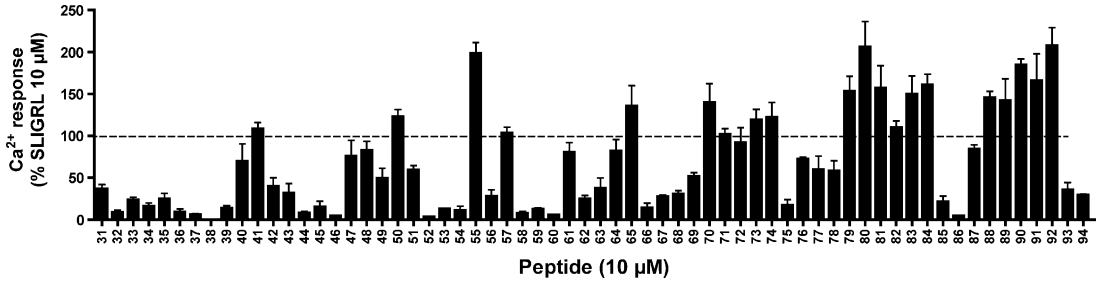

Figure 3 PAR_2-mediated intracellular calcium response to 10 μM *C*-terminal amidated peptides (**31–94**) derived from H-SLIGRL-NH_2 in A549 cells (except **38** which was not tested). Heptapeptides (**31–78** and **87–94**) that displayed greater potency than H-SLIGRL-NH_2 were **50** (H-SL-Nle-GRLL-NH_2), **55** (H-SLIARLL-NH_2), **65** (H-SLIG-Har-LL-NH_2), **70** (H-SLIG-*p*NPhe-LL-NH_2), **73** (H-SLIGR-Nle-L-NH_2), **74** (H-SLIGRWL-NH_2), **88** (H-SLIGR-*p*NPhe-L-NH_2), **89** (H-SLIGR-Cit-L-NH_2), **90** (H-SLIGR-Har-L-NH_2), **91** (H-SLIGR-1Nal-L-NH_2) and **92** (H-SLIGRLI-NH_2), while **41** (H-SLIG-Cha-LL-NH_2), **57** (H-SLI-Nle-RLI-NH_2), **71** (H-SLIGRAL-NH_2) and **72** (H-SLIGRIL-NH_2) had activity comparable to H-SLIGRL-NH_2. Octapeptides (**79–86**) that gave appreciably higher activity than SLIGRL were **79** (H-SLIGRLLL-NH_2), **80** (H-SLIGRLL-Cha-NH_2), **81** (H-SLIGRLLW-NH_2), **83** (H-SLIGRLLE-NH_2) and **84** (H-SLIGRLLQ-NH_2). All bars represent mean ± SEM of a single experiment conducted in triplicate. The dashed line shows the activity of the parent peptide, H-SLIGRL-NH_2.

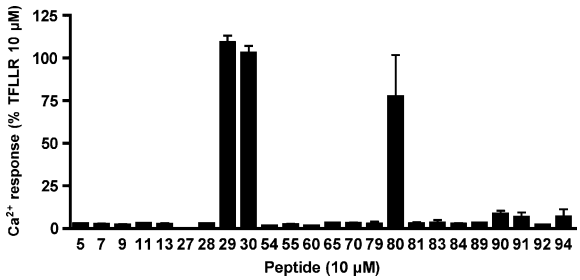

Figure 4 The PAR_1 activity, determined in Chinese Hamster Ovary (CHO) cells, of *C*-terminal amidated hepta and octapeptides: **5** = H-SLIGKV-Hph-NH_2; **7** = H-SLIGKV-*p*NPhe-NH_2; **9** = H-SLIGKV-*m*IPhe-NH_2; **11** = H-SLIGKV-1-Nal-NH_2; **13** = H-SLIGKVL-NH_2; **27** = H-SLIGRL-*p*FPhe-NH_2; **28** = H-SLIGRL-*p*ClPhe-NH_2; **29** = H-S-*p*FPhe-Cha-AR-2Ahta-*p*NPhe-NH_2; **30** = H-S-*p*FPhe-Cha-AR-2Ahta-NH_2; **54** (H-SLQGRLL-NH_2), **55** (H-SLIARLL-NH_2), **60** (H-SLI-Chg-RLL-NH_2) **65** (H-SLIG-Har-LL-NH_2), **70** (H-SLIG-*p*NPhe-LL-NH_2), **89** (H-SLIGR-Cit-L-NH_2), **90** (H-SLIGR-Har-L-NH_2), **91** (H-SLIGR-1Nal-L-NH_2), **92** (H-SLIGRLI-NH_2) and **94** (H-S-Abu-IGRLL-NH_2) and octapeptides **79** (H-SLIGRLLL-NH_2), **80** (H-SLIGRLL-Cha-NH_2), **81** (H-SLIGRLLW-NH_2), **83** (H-SLIGRLLE-NH_2) and **84** (H-SLIGRLLQ-NH_2). Bars represent the mean ± SEM of one experiment conducted in triplicate.

prepared (**79–86**) and tested for induction of intracellular calcium release in A549 cells. Compounds **79** (H-SLIGRLLL-NH_2), **80** (H-SLIGRLL-Cha-NH_2), **81** (H-SLIGRLLW-NH_2), **83** (H-SLIGRLLE-NH_2) and **84** (H-SLIGRLLQ-NH_2) had appreciably greater agonist activity than H-SLIGRL-NH_2, suggesting that two bulky hydrophobic residues at the *C*-terminus of H-SLIGRL-NH_2 do confer enhanced agonist activity. As a measure of selectivity, five of these compounds (**79–81, 83, 84**) were also further tested for PAR_1 activity in CHO cells (Figure 4), with **80** exhibiting significant PAR_1 agonist activity. In contrast to compounds **29** and **30** in which PAR_1 activity was conferred by the substitution of *p*-fluorophenylalanine at position 2, compound **80** indicates that PAR_1 activity can also be conferred by certain carboxyl terminal residues, in this case cyclohexylalanine. Taken together, these findings suggest as yet unexplored structure–activity requirements that may prove helpful in the development of selective PAR_2-directed drugs.

Agonist Activity in Rat Aorta

Two novel peptides (**55** and **92**) that displayed enhanced agonist activity compared with SLIGRL in A549 cells were further evaluated in a secondary, *in vitro* PAR_2 assay. Relaxation of phenylephrine-contracted rat aortic rings has been shown to be a sensitive and reliable measure of PAR_2 activity [12–14]. Relaxation responses to **55** (H-SLIARLL-NH_2) and **92** (H-SLIGRLI-NH_2) were compared with the relaxation induced by H-SLIGRL-NH_2 (Figure 5). Compound **92**

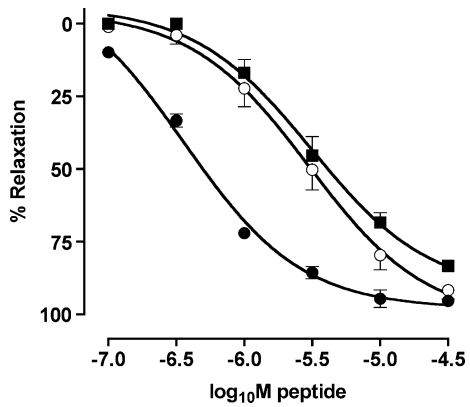

Figure 5 Relaxation of phenylephrine-contracted, rat-isolated thoracic aortic rings by PAR_2-activating heptapeptides **55** (open circles) and **92** (closed circles) compared with hexapeptide H-SLIGRL-NH_2 (squares). All symbols represent the mean ± SEM of three tissues. Error bars that are not shown are contained within the symbol.

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($pEC_{50} = 6.4 \pm 0.1$, $n = 3$) was approximately 10-fold more potent than either SLIGRL ($pEC_{50} = 5.5 \pm 0.1$, $n = 3$) or compound **55** ($pEC_{50} = 5.5 \pm 0.1$, $n = 3$). Although **55** appeared to have significantly higher potency than SLIGRL in the A549 cells, enhanced activity was not detected in the rat aorta, a finding which may represent differences in agonist potency at human *versus* rodent PAR_2.

CONCLUSIONS

The results of this study indicate the potential of carboxyl terminal extensions to H-SLIGRL-NH_2 to increase the potency of PAR_2 agonists. Specifically, extensions using amino acids that are either hydrophobic or introduce additional steric bulk at the *C*-terminal leucine and beyond appear to be effective in enhancing agonist potency, without compromising selectivity for PAR_2 over PAR_1. These findings suggest that hydrophobic residues at the *N*-terminus (Ser1-Leu2) and the *C*-terminus (Leu6-Leu7) influence PAR_2 binding. The new information may provide valuable clues for the development of potent and selective PAR_2-directed drug leads [12], as well as for the generation of highly sought after high-affinity PAR_2 ligands suitable for use in radioligand binding and *in vivo* studies. Further work is required to profile other types of carboxyl terminal modifications for their potentiation of agonist affinity, selectivity and efficacy, as are molecular studies to define the key interactions of these additional residues with amino acids at or near the presently unknown orthosteric ligand-binding site in PAR_2.

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