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Binding of a highly potent protease-activated receptor-2 (PAR2) activating peptide, [^{3}H]2-furoyl-LIGRL-NH_2, to human PAR2

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1 To determine the binding characteristics of a highly potent agonist for protease-activated receptor-2 (PAR2), 2-furoyl-Leu-Ile-Gly-Arg-Leu-amide (2-furoyl-LIGRL-NH_2), whole-cell binding assays were performed utilising a radioactive ligand, [^{3}H]2-furoyl-LIGRL-NH_2.

2 Specific binding of [^{3}H]2-furoyl-LIGRL-NH_2 was observed in NCTC2544 cells, dependent upon PAR2 expression, and competitively displaced by the addition of unlabeled PAR2 agonists. Scatchard analysis of specific saturation binding suggested a single binding site, with K_d of 122 ± 26.1 nM and a corresponding B_{max} of 180 ± 6 f mol in 3.0×10^5 cells.

3 The relative binding affinities of a series of modified PAR2 agonist peptides obtained from competition studies paralleled their relative EC_{50} values for Ca^{2+} mobilisation assays, indicating improved binding affinities by substitution with 2-furoyl at the N-terminus serine.

4 Pretreatment of cells with trypsin reduced specific binding of [^{3}H]2-furoyl-LIGRL-NH_2, demonstrating direct competition between the synthetic agonist peptide and the proteolytically revealed tethered ligand for the binding site of the receptor.

5 In HCT-15 cells endogenously expressing PAR2, the binding of [^{3}H]2-furoyl-LIGRL-NH_2 was displaced by addition of unlabeled ligands, Ser-Leu-Ile-Gly-Lys-Val (SLIGKV-OH) or 2-furoyl-LIGRL-NH_2. The relative binding affinity of 2-furoyl-LIGRL-NH_2 to SLIGKV-OH was comparable to its relative EC_{50} value for Ca^{2+} mobilisation assays.

6 The binding assay was successfully performed in monolayers of PAR2 expressing NCTC2544 and human umbilical vein endothelial cells (HUVEC), in 96- and 24-well plate formats, respectively.

7 These studies indicate that [^{3}H]2-furoyl-LIGRL-NH_2 binds to human PAR2 at its ligand-binding site. The use of this radioligand will be valuable for characterising chemicals that interact to PAR2.
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Abbreviations: 2-furoyl-LIGKV-NH_2, 2-furoyl-Leu-Ile-Gly-Lys-Val-amide; 2-furoyl-LIGKV-OH, 2-furoyl-Leu-Ile-Gly-Lys-Val; 2-furoyl-LIGRL-NH_2, 2-furoyl-Leu-Ile-Gly-Arg-Leu-amide; 2-furoyl-LIGRL-OH, 2-furoyl-Leu-Ile-Gly-Arg-Leu; HUVEC, human umbilical vein endothelial cells; LRGILS-NH_2, Leu-Arg-Gly-Ile-Leu-Ser-amide; PAR, protease-activated receptor; SAR, structure–activity relationship; SLIGKV-NH_2, Ser-Leu-Ile-Gly-Lys-Val-amide; SLIGKV-OH, Ser-Leu-Ile-Gly-Lys-Val; SLIGRL-NH_2, Ser-Leu-Ile-Gly-Arg-Leu-amide; SLIGRL-OH, Ser-Leu-Ile-Gly-Arg-Leu; TFLLR-NH_2, Thr-Phe-Leu-Leu-Arg-amide; *tc*-LIGRLO-NH_2, *trans*-cinnamoyl-Leu-Ile-Gly-Arg-Leu-amide

Introduction

Protease-activated receptor-2 (PAR2) is one of a four family subgroup of G-protein-coupled receptors (GPCRs), called PARs. They are distinguished from other GPCRs through their unique proteolytic mechanism of activation. For PAR2, activating proteases, such as trypsin, tryptase and coagulation factors VIIa and Xa (Nystedt *et al*., 1994; Molino *et al*., 1997; Camerer *et al*., 2000; Kawabata *et al*., 2001c), cleave a specific extracellular amino-terminal domain of the receptor to reveal a 'tethered ligand', SLIGKV- and SLIGRL- for human and mouse/rat PAR2, respectively, which subsequently interacts with the activation domain of the receptor, initiating intracellular signaling pathways (Dery *et al*., 1998; Macfarlane *et al*., 2001). The synthetic agonist peptides mimicking the tethered ligand of PAR2, Ser-Leu-Ile-Gly-Lys-Val (SLIGKV-OH), Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL-OH) (Nystedt *et al*., 1995a, b) and their amidated forms Ser-Leu-Ile-Gly-Lys-Val-amide (SLIGKV-NH_2) Ser-Leu-Ile-Gly-Arg-Leu-amide (SLIGRL-NH_2) (Bohm *et al*., 1996; Hollenberg *et al*., 1996) have also been demonstrated being able to activate the receptor without enzymatic cleavage, therefore, have been utilised as biological tools to examine physiological functions of PAR2.

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A number of studies have demonstrated a wide range of important physiological roles for PAR2. In particular, the proinflammatory roles of PAR2 are highlighted in several systems, that is, PAR2 activating peptide elicits inflammatory responses in the rat hind paw (Kawabata *et al*., 1998; Vergnolle *et al*., 1999), mouse knee joint (Ferrell *et al*., 2003) and mouse colon (Cenac *et al*., 2002). A critical role of PAR2 has also been demonstrated in skin (Kawagoe *et al*., 2002) and neurogenic inflammation (Ricciardolo *et al*., 2000; Steinhoff *et al*., 2000). On the other hand, PAR2 also plays protective roles in several biological systems, such as the lung (Cocks *et al*., 1999; Lan *et al*., 2000; 2001) and gastrointestinal tract (Kawabata *et al*., 2001b; Kawao *et al*., 2002). Given the potential roles of PAR2 in these conditions, either PAR2 agonists or antagonists might be therapeutically appropriate in a given disease state.

Although currently no clear information is available on PAR2 selective antagonists, our group identified a series of modified peptides, substituted with 2-furoyl on the N-terminal serine residue of native PAR2-activating peptides, as potent agonists of PAR2 both *in vitro* and *in vivo* systems (Ferrell *et al*., 2003; Kawabata *et al*., 2004). The most potent agonist, 2-furoyl-Leu-Ile-Gly-Arg-Leu-amide (2-furoyl-LIGRL-NH_2), has been shown to be approximately 100 times more potent compared to SLIGKV-OH in cellular Ca^{2+} mobilisation assays. The increased resistance to a peptide-metabolising enzyme, aminopeptidase, was a striking feature of the furoylated agonists, resulting in a dramatic increase in PAR2 activating potency *in vivo* (Kawabata *et al*., 2004). Consistent with an independent report (McGuire *et al*., 2004), furoylated peptides are likely to be the most potent agonists currently available for PAR2. In order to evaluate the precise structure–activity relationships (SARs) of agonists/antagonists, the receptor–ligand binding studies are essential. Previously, Al-Ani *et al*. (1999) demonstrated the PAR2 binding assay using tritiated *trans*-cinnamoyl (tc)-LIGRLO-NH_2 on recombinant rat PAR2. However, the binding assay only utilised rat PAR2 expressed in KNRK cells and ligand-binding studies using human PAR2 (hPAR2) have remained uncharacterised.

In the present study, we have established a PAR2 binding assay utilising a radiolabeled highly potent PAR2-activating peptide, [^{3}H]2-furoyl-LIGRL-NH_2, and characterised its binding profile in hPAR2 expressing cells. The SARs of a series of PAR2 agonist peptides were evaluated as compared with their potencies for Ca^{2+} mobilisation. We also examined the effect of trypsin to present the direct competition between [^{3}H]2-furoyl-LIGRL-NH_2 and proteolytically revealed tethered ligand at the receptor-binding site.

Methods

Activating peptides for PAR-1 and PAR2 and other chemicals

The PAR2-activating peptides used were: SLIGKV-OH, SLIGRL-OH, SLIGKV-NH_2, SLIGRL-NH_2. Modified PAR2 activating peptides with 2-furoyl substitutions at the N-terminus serine have recently been described (Kawabata *et al*., 2004): 2-furoyl-Leu-Ile-Gly-Lys-Val (2-furoyl-LIGKV-OH), 2-furoyl-Leu-Ile-Gly-Arg-Leu (2-furoyl-LIGRL-OH), 2-furoyl-Leu-Ile-Gly-Lys-Val-amide (2-furoyl-LIGKV-NH_2), 2-furoyl-LIGRL-NH_2. Another modified PAR2 activating peptide, *trans*-cinnamoyl-Leu-Ile-Gly-Arg-Leu-Orn-amide (*tc*-LIGRLO-NH_2), was described by Hollenberg *et al*. (1997). The PAR-1-activating peptide Thr-Phe-Leu-Leu-Arg-amide (TFLLR-NH_2) (Hollenberg *et al*., 1997) was also used in some experiments. The reverse sequence peptide of murine PAR2 agonist, Leu-Arg-Gly-Ile-Leu-Ser-amide (LRGILS-NH_2), was used as a negative control peptide. All peptides were synthesised and purified (>95%) by high-performance liquid chromatography (HPLC), and the structures were confirmed by mass spectrometry (MS). Trypsin from bovine pancreas (9300 U mg^{-1}), A23187 and probenecid were obtained from Sigma-Aldrich Co. (MO, U.S.A.). Dibutyl phthalate and dinonyl phthalate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Soybean trypsin inhibitor (SBTI, >7000 BAEE U mg^{-1}) was obtained from Invitrogen (CA, U.S.A.).

Preparation of [^{3}H]2-furoyl-LIGRL-NH_2 for ligand-binding studies

The 2-furoyl-LIGRL-NH_2 was synthesised at Kowa Tokyo New Drug Research Laboratories (Tokyo, Japan) and the radiolabel peptide was custom prepared at Amersham Biosciences (Buckinghamshire, U.K.). The [^{3}H]2-furoyl-LIGRL-NH_2 (Figure 1) was prepared by tritiation of Dehydro-Leu-2-furoyl-LIGRL-NH_2 with tritium gas in the presence of 10% Pd/$CaCO_3$ in DMF. After tritiation, the [^{3}H]2-furoyl-LIGRL-NH_2 was purified by HPLC and prepared as ethanol: water (1 : 1, v v^{-1}) solution at a concentration of 9.2 μM (37 MBq ml^{-1}). The radiochemical purity of [^{3}H]2-furoyl-LIGRL-NH_2 was 99.4% with a specific activity of 4.03 TBq mmol^{-1}. The aliquots were kept at -20°C and used in the binding assay within 6 months.

Cell culture

The human skin epithelial cell line NCTC2544 expressing human PAR2, designated as NCTC2544-PAR2 cells, was described previously (Kanke *et al*., 2001). Control NCTC2544 cells (WT-NCTC2544 cells) were grown in Medium 199 with Earle's salts (Sigma-Aldrich) containing 10% (v v^{-1}) fetal calf serum (FCS), sodium bicarbonate (50 mM), L-glutamine (2 mM), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). NCTC2544-PAR2 cells were cultured in complete medium containing geneticin (400 μg ml^{-1}) to maintain selection pressure. The human colorectal adenocarcinoma cell line HCT-15 obtained from American Type Tissue Culture Collection (MD, U.S.A.) was maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10%

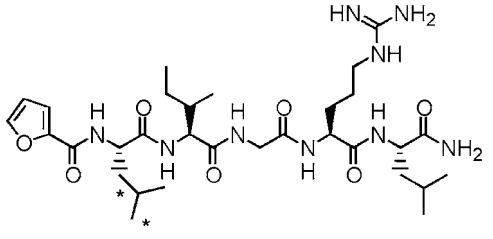

Figure 1 Structure of [^{3}H]2-furoyl-LIGRL-NH_2. *, tritiated position.

(v v^{-1}) FCS, sodium bicarbonate (50 mM), L-glutamine (2 mM), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). Human umbilical vein endothelial cells (HUVEC) obtained from Cambrex (Walkersville, MD, U.S.A.) were cultured in the complete endothelial cell growth medium (EGM, Cambrex) containing bovine brain extract (BBE) (12 μg ml^{-1}), human endothelial growth factor (hEGF) (10 ng ml^{-1}), hydrocortisone (1 μg ml^{-1}), FCS (2%, v v^{-1}), gentamicin (50 μg ml^{-1}) and amphotericin-B (50 ng ml^{-1}). All cells were grown at 37°C in an incubator with saturated humidity and 5% CO_2, and NCTC2544 cells and HCT-15 cells were passaged using Versene (0.53 mM EDTA in PBS) to avoid trypsin exposure. HUVEC were passaged with trypsin, and passages at 4–8 were used for the experiments.

Ligand-binding assay in cell suspension

Ligand-binding assay was performed with a modified method of *trans*-cinnamoyl-LIGRLO-NH_2 to rat PAR2 (Al-Ani *et al*., 1999). Cell suspension was prepared by dissociation of cells from flasks using Versene, harvested by centrifugation at 1500 r.p.m. at 25°C for 3 min and resuspended in the serum-free medium containing 0.1% (w v^{-1}) BSA and 0.1% (w v^{-1}) NaN_3. The cell suspension (0.2 ml) was incubated at 25°C along with [^{3}H]2-furoyl-LIGRL-NH_2 in either absence or presence of unlabeled ligands. After incubation, the cell suspension was transferred to a microcentrifuge tube (total volume 0.4 ml, Assist, Tokyo, Japan) onto 0.1 ml of a mixture of dinonyl phthalate : dibutyl phthalate (4 : 6 (v v^{-1})), and the cell-bound radioactivity was pelleted by centrifugation (15,000 r.p.m.) for 5 min at room temperature. The cell pellet was cut from the bottom of the tube and solubilised overnight using 0.5 ml Soluene-350 (Perkin-Elmer, MA, U.S.A.), and cell-bound radioactivity was measured by scintillation counting (efficiency, about 65%, Tri-Carb 2700TR, Perkin-Elmer) following addition of 4 ml scintillation reagent (Hionic-Fluor, Perkin-Elmer).

To initially characterise specific binding, a suspension (0.2 ml, 3.0×10^5 cells) of either NCTC2544-PAR2 or WT-NCTC2544 cells was incubated at 25°C for 60 min along with [^{3}H]2-furoyl-LIGRL-NH_2 (9.2 nM, 1 μCi ml^{-1}) in either absence or presence of unlabeled ligands, 2-furoyl-LIGRL-NH_2 (0.1–100 μM) or SLIGKV-OH (10–1000 μM). Saturation binding studies were carried out by incubating NCTC2544PAR2 cells (0.2 ml, 3.0×10^5 cells) at 25°C for 60 min along with a range of concentrations of [^{3}H]2-furoyl-LIGRL-NH_2 (2.3–92 nM). The amount of specific binding was calculated by subtraction from the total amount of the radioligand bound in the absence of competing ligands, nonspecific binding in the presence of an excess (100 μM) of unlabeled 2-furoyl-LIGR-L-NH_2. Competition studies with various PAR2 agonist peptides were performed by incubating cells with [^{3}H]2-furoyl-LIGRL-NH_2 (9.2 nM, 1 μCi ml^{-1}) and a range of concentrations of test compounds. The displacement curve for each peptide was constructed by measuring the percentage of the specific [^{3}H]2-furoyl-LIGRL-NH_2 binding (% specific binding) in the presence of each peptide concentration, relative to the maximum specific binding in the absence of unlabeled 2-furoyl-LIGRL-NH_2.

In the studies of trypsin pretreatment, NCTC2544-PAR2 cells in suspension were incubated with a range of concentrations of trypsin (0.1–10 nM) for 15 min at 25°C. Subsequently, SBTI was added at a final concentration of 10 μg ml^{-1} prior to the addition of radioligand. Specific binding of [^{3}H]2-furoyl-LIGRL-NH_2 was examined after incubation of further 60 min at 25°C. Nonspecific binding was determined using 100 μM unlabeled 2-furoyl-LIGRL-NH_2. In an independent experiment, the same concentration of SBTI was added prior to the trypsin (10 nM) treatment (SBTI + Trypsin) to confirm the inactivation of trypsin by SBTI.

In HCT-15 cells, cell suspension (0.2 ml, 1.0×10^6 cells) was incubated with a concentration of [^{3}H]2-furoyl-LIGRL-NH_2 (46 nM, 5 μCi ml^{-1}) at 25°C for 60 min in either absence or presence of increasing concentrations of unlabeled competing peptides. The displacement curve for each peptide was constructed in the same manner to NCTC2544-PAR2 cells, defining the nonspecific binding in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH_2.

Ligand-binding assay in monolayer cells

NCTC2544-PAR2 cells were dissociated using Versene and seeded in 96-well culture plates at 5.0×10^4 cells well^{-1} 18 h prior to the assay. After overnight culture, the medium was discarded and cells were incubated in 100 μl binding medium containing 0.1% (w v^{-1}) BSA and 0.1% (w v^{-1}) NaN_3 at 25°C for 60 min along with [^{3}H]2-furoyl-LIGRL-NH_2 (46 nM, 5 μCi ml^{-1}) in either absence or presence of various concentrations of unlabeled competing peptides. After 60 min incubation, the cells were washed twice with ice-cold binding medium and solubilised with 100 μl lysis buffer containing 0.2 M NaOH and 1% (w v^{-1}) SDS for 5 min on a plate shaker with gentle agitation. The cell lysates were transferred to scintillation vials and subjected to scintillation counting following the addition of 4 ml scintillation reagent. Nonspecific binding was determined using 100 μM unlabeled 2-furoyl-LIGRL-NH_2.

Similarly, but with a larger scale of 24-well culture plates, HUVEC were seeded at 1.0×10^5 cells well^{-1}. After 48 h culture, the medium was discarded and cells were incubated in 300 μl binding medium containing 0.1% (w v^{-1}) BSA and 0.1% (w v^{-1}) NaN_3 at 25°C for 60 min along with [^{3}H]2-furoyl-LIGRL-NH_2 (46 nM, 5 μCi ml^{-1}) in either absence or presence (100 μM) of unlabeled 2-furoyl-LIGRL-NH_2. After 60 min incubation, cells were washed twice with ice-cold binding medium, solubilised and subjected to scintillation counting.

Measurement of Ca^{2+} mobilisation

Trypsin-stimulated intracellular calcium mobilisation was measured as described previously (Kawabata *et al*., 2004) using 96-well scanning fluorometer, FlexStation (Molecular Devices, CA, U.S.A.). NCTC2544-PAR2 cells (3.0×10^4 cells well^{-1}) were seeded in black-wall clear-bottom 96-well plates (Corning Inc., NY, U.S.A.) 24 h prior to the assay and grown to reach confluent. Cells were washed once with SF medium and replaced with 80 μl of SF medium. Subsequently, 80 μl of calcium assay dye solution (FlexStation Calcium Plus Assay kit, Molecular Devices) dissolved in Hank's balanced salt solution (HBSS; pH 7.4) containing 5 mM probenecid was added and incubated for 60 min at 37°C. Then cells were stimulated with 20 μl of various concentrations of trypsin prepared in HBSS and fluorescence change was measured at 25°C (excitation 485 nm and emission 525 nm). Agonist-induced calcium responses (max–min) were expressed as

percentage of the reference calcium response induced by calcium ionophore, A23187 (10 μM). The half-maximal effective concentration values (EC_{50}) were estimated from the concentration–response curve.

Data analysis

Radioligand binding data was analysed by nonlinear regression analysis using GraphPad Prism 4 (GraphPad Software, CA, U.S.A.). Specific saturation-binding data were analysed to provide estimates of K_d and B_{max} values by equation outlined by Cheng & Prusoff (1973). The displacement curves by a series of peptides were assumed to fit to a one-site model to determine IC_{50} values. The inhibitor constant, K_i of each peptide was then derived from the IC_{50} and the K_d obtained from the saturation binding; $K_i = IC_{50}$ $[1 + (\text{radioligand})\ K_d^{-1}]^{-1}$. The binding competition curve with unlabeled 2-furoyl-LIGRL-NH$_2$ was also used to calculate K_d and B_{max} by fitting to homologous competition analysis.

For calcium mobilisation studies, the peak fluorescence change was plotted *versus* the concentration of trypsin and the concentration–response curve fitted using a four-parameter logistic equation to determine the EC_{50} value.

Results

Binding of [^{3}H]2-furoyl-LIGRL-NH$_2$ in wild-type- and PAR2 expressing NCTC2544 cells

In NCTC2544-PAR2 cells, total [^{3}H]2-furoyl-LIGRL-NH$_2$ binding was 972 ± 88 c.p.m. in 3.0×10^5 cells (Figure 2, control, filled column). By addition of unlabeled 2-furoyl-LIGRL-NH$_2$ (0.1–100 μM) or SLIGKV-OH (10–1000 μM), the binding of [^{3}H]2-furoyl-LIGRL-NH$_2$ was reduced in a concentration-dependent manner. On the other hand, total [^{3}H]2-furoyl-LIGRL-NH$_2$ binding in WT-NCTC2544 cells (Figure 2, open columns) was approximately 20% of that observed in NCTC2544-PAR2 cells equating to the nonspecific values observed in the PAR2 expressing cell line. In addition, binding was only marginally reduced by addition of unlabeled ligands. Nonspecific binding defined in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH$_2$ in NCTC2544-PAR2 cells and WT-NCTC2544 cells were 22 and 78% of the total binding, respectively.

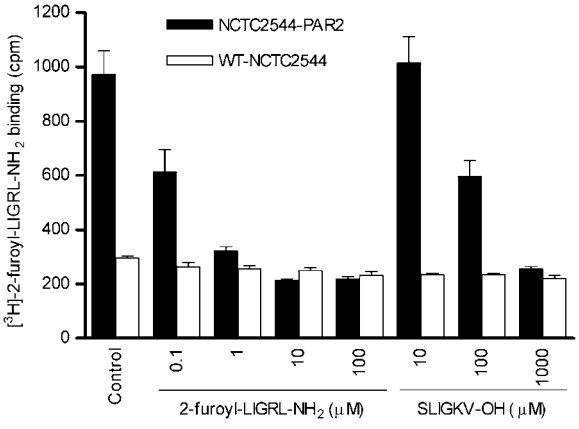

Figure 2 [^{3}H]2-furoyl-LIGRL-NH$_2$ binding in NCTC2544 cells. NCTC2544-PAR2 cells (filled columns) or WT-NCTC2544 cells (open columns) were incubated with [^{3}H]2-furoyl-LIGRL-NH$_2$ (9.2 nM, 1 μCi ml^{-1}) either in the absence (Control) or presence of unlabeled ligand, 2-furoyl-LIGRL-NH$_2$ (0.1–100 μM) or SLIGKV-OH (10–1000 μM) at 25°C for 60 min. [^{3}H]2-furoyl-LIGRL-NH$_2$ binding was assayed as described in Methods. Data are expressed as the mean ± s.e.m. of three independent experiments.

Saturation binding of [^{3}H]2-furoyl-LIGRL-NH$_2$ in NCTC2544-PAR2 cells

The total binding of [^{3}H]2-furoyl-LIGRL-NH$_2$ to NCTC2544-PAR2 cells observed to be saturable at over a concentration range of 2.3–92 nM while the nonspecific binding appeared to be proportional to the radioligand concentration (Figure 3a). The specific binding data fitted to a one-binding site model, yielding K_d of 122 ± 26 nM and corresponding B_{max} of 180 ± 6 f mol in 3.0×10^5 cells (Figure 3b).

Competition study of PAR2-agonist peptides

All PAR2-activating peptides exhibited concentration-dependent displacement on [^{3}H]2-furoyl-LIGRL-NH$_2$ binding to

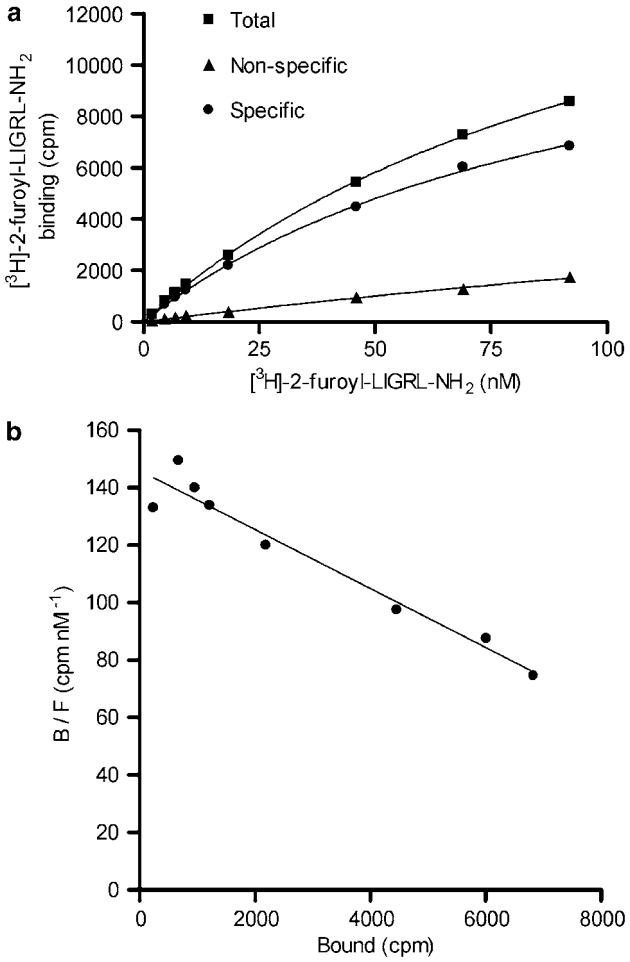

Figure 3 Saturation binding (a) and the Scatchard analysis (b) of [^{3}H]2-furoyl-LIGRL-NH$_2$ binding to NCTC2544-PAR2 cells. The amount of specific binding was calculated by subtracting nonspecific binding in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH$_2$ from the total amount of radioligand bound. A representative result from three independent experiments is shown.

NCTC2544-PAR2 cells. The order of the affinity of the peptides was: 2-furoyl-LIGRL-NH$_2$ > 2-furoyl-LIGKV-NH$_2$ > 2-furoyl-LIGRL-OH > 2-furoyl-LIGKV-OH > SLIGRL-NH$_2$ > SLIGKV-NH$_2$ > SLIGRL-OH > SLIGKV-OH (Figure 4). The calculated K_i value from the displacement curve of each agonist and its relative binding affinity to the original peptide, SLIGKV-OH, are shown in Table 1 and compared with its EC_{50} value for the Ca^{2+} assay in the same cells as shown previously (Kawabata *et al*., 2004). There was minimal binding competition for [^{3}H]2-furoyl-LIGRL-NH$_2$ binding by the inactive reverse PAR2 agonist peptide LRGILS-NH$_2$. PAR-1 selective agonist peptide, TFLLRN-NH$_2$, reduced the [^{3}H]2-furoyl-LIGRL-NH$_2$ binding only at high concentrations and its estimated K_i value was higher than 1 mM. A modified PAR2 agonist peptide, *tc*-LIGRLO-NH$_2$, presented the competition for [^{3}H]2-furoyl-LIGRL-NH$_2$ binding to human PAR2, providing a similar K_i (14.6 μM) to that of SLIGRL-OH ($K_i = 15.5$ μM).

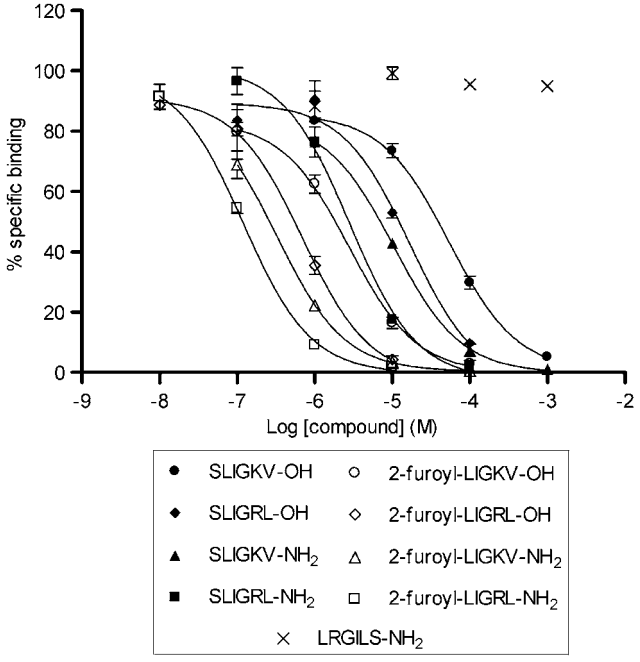

Figure 4 Displacement of [^{3}H]2-furoyl-LIGRL-NH$_2$ binding by various agonist peptides in NCTC2544-PAR2 cells. NCTC2544-PAR2 cells in suspension (0.2 ml final volume) were incubated with [^{3}H]2-furoyl-LIGRL-NH$_2$ (9.2 nM, 1 μCi ml^{-1}) at 25°C for 60 min in either absence or presence of increasing concentrations of unlabeled competing peptide. Nonspecific binding was determined in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH$_2$. Binding competition curves for unlabeled peptides were presented by the percentage of radioligand-binding (% specific binding) at each peptide concentration relative to the maximum specific binding. Representative results from a single set of experiment are shown as the mean ± s.e.m. ($n = 3$). The experiments were replicated three times with similar results.

The binding competition curve with unlabeled 2-furoyl-LIGRL-NH$_2$ fitted to homologous competition analysis, yielding a similar K_d (112 nM) to that of saturation binding.

[^{3}H]2-furoyl-LIGRL-NH$_2$ binding in HCT-15 cells

In addition to assessing exogenously expressed PAR2 in NCTC2544 cells, the ability of [^{3}H]2-furoyl-LIGRL-NH$_2$ binding to native human PAR2 was examined using human colon adenocarcinoma cell line, HCT-15, which expresses PAR2 endogenously (Kawabata *et al*., 2004). When 46 nM (5 μCi ml^{-1}) radioligand was used for the binding, the total binding of [^{3}H]2-furoyl-LIGRL-NH$_2$ was 7765.4 ± 130.0 c.p.m. for 1.0×10^6 cells. The binding of [^{3}H]2-furoyl-LIGRL-NH$_2$ to HCT-15 cells was displaced by addition of either SLIGKV-OH (Figure 5; filled circle) or 2-furoyl-LIGRL-NH$_2$ (Figure 5; open square) in a concentration-dependent manner. The nonspecific binding in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH$_2$ (3520 ± 136.9 c.p.m. in 1.0×10^6 cells) was approximately 45% of the total binding. The IC_{50} values of SLIGKV-OH and 2-furoyl-LIGRL-NH$_2$ were 171 and 1.10 μM, respectively. The relative binding affinity (155) of 2-furoyl-LIGRL-NH$_2$ to SLIGKV-OH was comparable to the relative potency for Ca^{2+} mobilisation (90.1) in HCT-15 cells (Table 2).

Effect of trypsin pre-treatment on [^{3}H]2-furoyl-LIGRL-NH$_2$ binding

Trypsin activated PAR2 to induce cytosolic Ca^{2+} mobilisation in NCTC2544-PAR2 cells, yielding an EC_{50} value of 0.07 nM (Figure 6a). At 1–10 nM, the trypsin-induced Ca^{2+} response reached the submaximal levels (Figure 6b). Preincubation of cells with trypsin (0.1, 1 and 10 nM) for 15 min at 25°C prior to the addition of radioligand reduced the [^{3}H]2-furoyl-LIGRL-NH$_2$ binding to cells in a concentration-dependent manner by 0, 54 and 96%, respectively (Figure 7, filled columns). In order to protect radioligand from degradation by trypsin during the

Table 1 Comparison of PAR2 agonist peptides on binding competition and potency on Ca^{2+} mobilisation in NCTC2544PAR2 cells

	Binding assay			*Ca^{2+} mobilisation*	
	IC_{50} (μM)	(K_i) (μM)	*Relative to SLIGKV-OH*	*EC_{50}* (μM)	*Relative to SLIGKV-OH*
SLIGKV-OH	54.1	(50.3)	*1*	0.54	*1*
SLIGRL-OH	16.7	(15.5)	3.25	0.20	2.70
SLIGKV-NH$_2$	10.4	(9.64)	5.24	0.075	7.20
SLIGRL-NH$_2$	2.80	(2.61)	19.3	0.046	11.7
2-furoyl-LIGKV-OH	2.77	(2.57)	19.6	0.067	8.06
2-furoyl-LIGRL-OH	0.695	(0.646)	77.9	0.024	22.5
2-furoyl-LIGKV-NH$_2$	0.326	(0.303)	166	0.0076	71.1
2-furoyl-LIGRL-NH$_2$	0.120	(0.112)	449	0.0050	108
tc-LIGRLO-NH$_2$	15.7	(14.6)	3.45	ND	ND
TFLLR-NH$_2$	1266	(1177)	0.0428	ND	ND

ND = not determined.
'*1*'s represent the relative value to their own values.

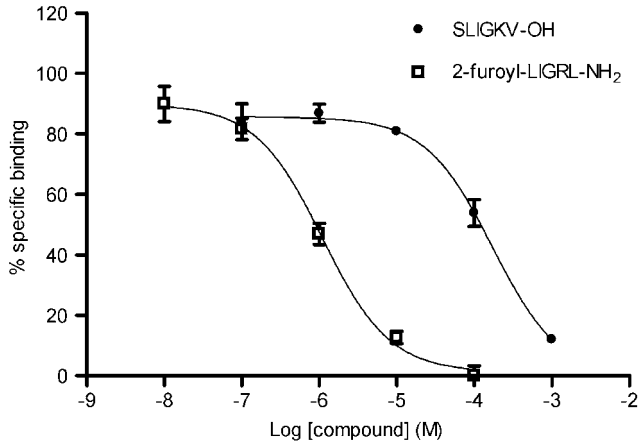

Figure 5 Displacement of [^{3}H]2-furoyl-LIGRL-NH_2 binding by agonist peptides in HCT-15 cells. HCT-15 cells in suspension (0.2 ml final volume) were incubated with [^{3}H]2-furoyl-LIGRL-NH_2 (46 nM, 5 μCi ml^{-1}) at 25°C for 60 min in either absence or presence of increasing concentrations of unlabeled competing peptide. Non-specific binding was determined in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH_2. Binding competition curves for unlabeled peptides were presented by the percentage of radioligand-binding (% specific binding) at each peptide concentration relative to the maximum specific binding. Data are expressed as the mean ± s.e.m. of three independent experiments.

Table 2 Comparison of PAR2 agonist peptides on binding competition and potency on Ca^{2+} mobilisation in HCT-15 cells

	Binding assay		*Ca^{2+} mobilisation*	
	IC_{50} (μM)	*Relative to SLIGKV-OH*	*EC_{50}* (μM)	*Relative to SLIGKV-OH*
SLIGKV-OH	171	*1*	22.8	*1*
2-furoyl-LIGRL-NH_2	1.10	150	0.253	90.1

'*1*'s represent the relative value to their own values.

binding period, SBTI (10 μg ml^{-1}) was added following the each trypsin treatment. When SBTI was added prior to the trypsin (10 nM) treatment, no inhibition was observed in the specific binding of [^{3}H]2-furoyl-LIGRL-NH_2 to the NCTC2544-PAR2 cells (Figure 7, shaded column).

PAR2 binding assay in monolayer cells

Specific binding of [^{3}H]2-furoyl-LIGRL-NH_2 was also observed in monolayer NCTC2544PAR2 cells in 96-well format (Figure 8). When cells were incubated with 46 nM (5 μCi ml^{-1}) [^{3}H]2-furoyl-LIGRL-NH_2 for 60 min, total binding of 1780 ± 181 c.p.m. ($n = 3$) was observed, while the nonspecific binding in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH_2 was detected at 313 ± 101 c.p.m. ($n = 3$), which was 18% of the total binding. A concentration-dependent binding competition was observed by addition of unlabeled 2-furoyl-LIGRL-NH_2 and SLIGKV-OH, yielding similar K_i of 0.119 and 35.6 μM, respectively, to the cell suspension assays.

Similarly, in the monolayer of HUVEC, total [^{3}H]2-furoyl-LIGRL-NH_2 was 1525 ± 242 c.p.m. ($n = 4$), while nonspecific binding in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH_2 was detected at 666 ± 44 c.p.m. ($n = 4$), 44% of the total binding.

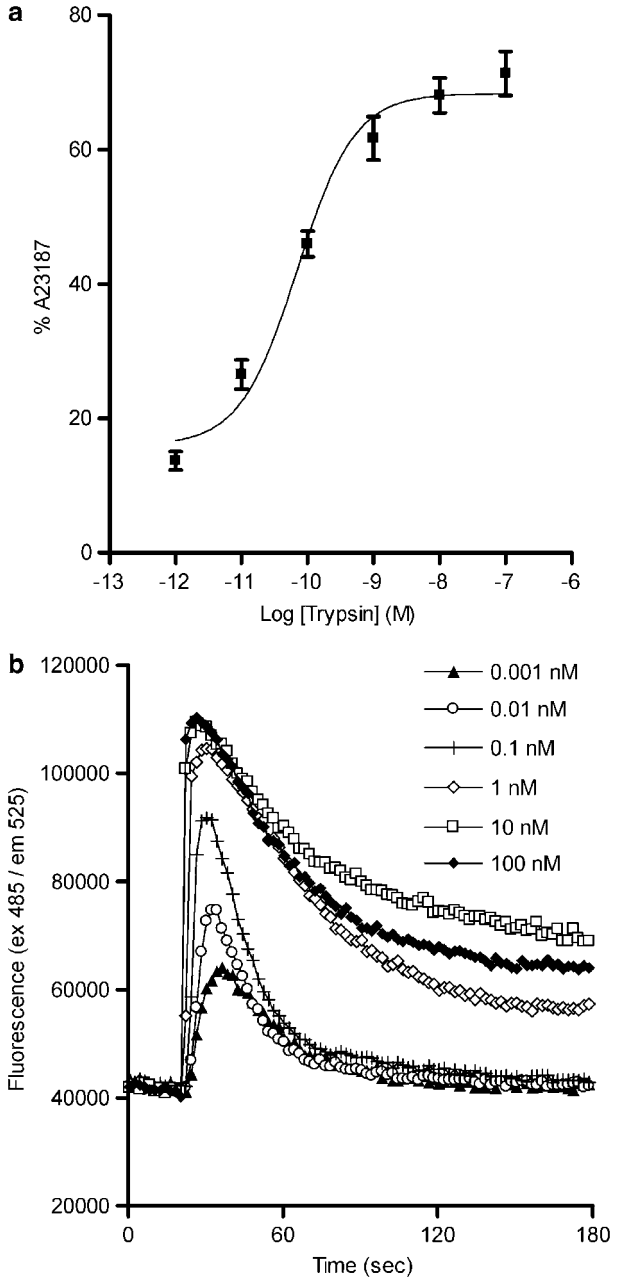

Figure 6 Trypsin-induced Ca^{2+} mobilization in NCTC2544-PAR2 cells. NCTC2544-PAR2 cells in 96-well plates loaded with fluorescent Ca^{2+} indicator dye were challenged with various concentrations of trypsin. Peak fluorescence changes induced by trypsin with different concentrations were normalised by the maximal response mediated by A23187 (10 μM) (a). Data are expressed as the mean ± s.e.m. of three independent experiments. Representative traces of fluorescence changes induced by trypsin (0.001–100 nM) are presented (b).

Discussion

In the present study, we characterised the binding of a novel highly potent radioactive PAR2 ligand, [^{3}H]2-furoyl-LIGRL-NH_2, to human PAR2 in whole-cell systems. Studies using the

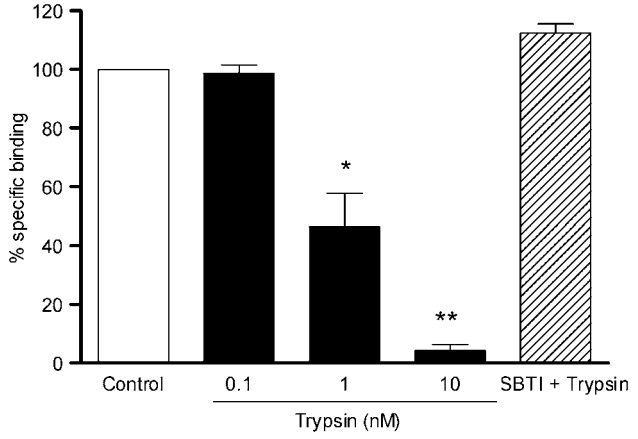

Figure 7 The effect of trypsin treatment on [^{3}H]2-furoyl-LIGRL-NH_2 binding in NCTC2544 cells. The NCTC2544-PAR2 cells in suspension (0.2 ml final volume) were treated with various concentrations of trypsin as indicated for 15 min at 25°C (filled columns). SBTI (10 μg ml^{-1}) was added prior to the addition of [^{3}H]2-furoyl-LIGRL-NH_2 (1 μCi ml^{-1}) and radioactivity bound was determined after 60 min incubation at 25°C. In the parallel experiments, SBTI was added prior to the trypsin (10 nM) treatment (shaded column). Data are expressed as the mean ± s.e.m. of three independent experiments. *$P<0.05$, **$P<0.01$ compared with the control value (open column) untreated with trypsin.

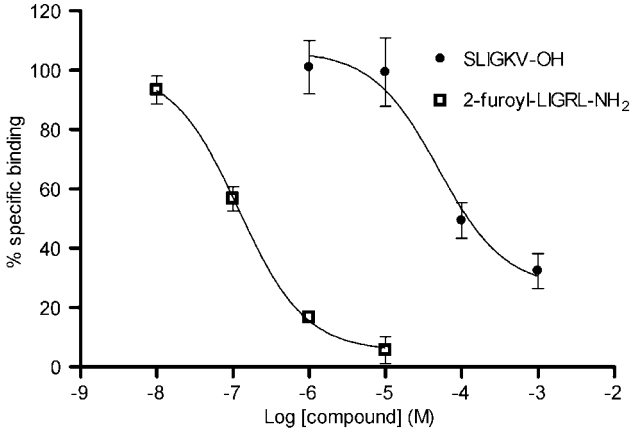

Figure 8 Displacement of [^{3}H]2-furoyl-LIGRL-NH_2 binding by agonist peptides in monolayer NCTC2544PAR2 cell. The monolayer NCTC2544-PAR2 cells in 96-well plate (5×10^4 cells well^{-1}) were incubated with 46 nM [^{3}H]2-furoyl-LIGRL-NH_2 (5 μCi ml^{-1}) at 25°C for 60 min in either absence or presence of increasing concentrations of agonist peptides, SLIGKV-OH or 2-furoyl-LIGRL-NH_2. Nonspecific binding was determined in the presence of 100 μM unlabeled 2-furoyl-LIGRL-NH_2. Competitive effects were represented by the percentage of radioligand-binding (% specific binding) at each peptide concentration relative to the maximum specific binding. Data are expressed as the mean ± s.e.m. of three independent experiments.

WT-NCTC2544 and NCTC2544-PAR2 cells demonstrated the specific binding of [^{3}H]2-furoyl-LIGRL-NH_2 to human PAR2. The specificity of the [^{3}H]2-furoyl-LIGRL-NH_2 binding for PAR2 was further confirmed in the competition studies, that PAR2 activating peptides displaced the radioligand binding parallel to their agonist potency, while an inactive peptide with a reversed sequence of mouse PAR2 activating peptide, LRGILS-NH_2, did not cause displacement for the binding of [^{3}H]2-furoyl-LIGRL-NH_2 at concentrations up to 1 mM.

Saturation studies indicated a single-site for binding of [^{3}H]2-furoyl-LIGRL-NH_2 to human PAR2 expressed in NCTC2544 cells, with K_d value of 122 ± 26 nM and corresponding B_{max} value of 180 ± 6 f mol in 3.0×10^5 cells. This implies approximately 36,000 binding sites exist in a single NCTC2544-PAR2 cell. The Scatchard analysis of the saturation binding reasonably fitted ($r\sim0.998$) to a single-site binding, which suggested the affinity of the receptor in the assay system constant. However, full saturation was not obtained probably due to the relative low affinity of the ligand; nevertheless, the estimated K_d value compared well with that obtained in homologous competition analysis ($K_d = 112$ nM).

The competition binding studies provided constant and reliable results, and demonstrated clear SARs. (1) The mouse/rat agonist peptide (SLIGRL) sequence exhibited a higher affinity than a human sequence (SLIGKV). (2) C-terminal amidation of the peptides (−NH_2) enhanced the binding affinity compared to the free carboxyl peptides (−OH). (3) The replacement with 2-furoyl for N-terminus Ser resulted in a remarkable increase in binding affinity. All these effects were additive, suggesting independent contribution of either N-terminal or C-terminal modification to enhancement of PAR2 binding affinity. In addition to the 2-furoyl-peptides, *tc*-LIGRLO-NH_2 exhibited the competitive effects on [^{3}H]2-furoyl-LIGRL-NH_2 binding in this assay system with a K_i similar to that of SLIGRL-OH. When compared with 2-furoyl-LIGRL-NH_2, the binding affinity of *tc*-LIGRLO-NH_2 was approximately 30 times lower. Therefore, 2-furoyl-LIGRL-NH_2 binds to human PAR2 with the highest affinity among the peptide mimetic PAR2 agonists discovered so far. Currently, little is known in terms of the molecular interactions to explain high-affinity binding of 2-furoyl peptides to the receptor. In the study on SARs of PAR-1-activating peptides, substitution of the N-terminal Ser with a neutral hydrophobic N-acyl group (e.g. *trans*-cinnamoyl group) provided peptides with partial agonist or antagonist activity for PAR-1 (Bernatowicz *et al*., 1996). In contrast, the similar modification of PAR2 agonist peptides resulted in enhanced agonistic activity, indicating different chemical properties required between ligands acting on PAR-1 and PAR2. More precise structural studies using co-crystallisation of ligand-receptor complex or 3-D structural analysis is required for complete understanding of the ligand–receptor interaction.

In addition to PAR2 transfected NCTC2544 cells, the specific binding of [^{3}H]2-furoyl-LIGRL-NH_2 was observed in human colon adenocarcinoma HCT-15 cells, which endogenously express PAR2. The competition studies of unlabeled 2-furoyl-LIGRL-NH_2 indicated approximately 10-times higher IC_{50} value for HCT-15 cells compared to PAR2 expressed NCTC2544 cells. Although the K_d from homologous competition can be ambiguous, as is often affected by the assay conditions, this may represent different affinities between the endogenous and overexpressed receptors, for example different levels of glycosylation of the receptor (Compton *et al*., 2002) or single-nucleotide polymorphisms (SNPs) (Compton *et al*., 2000) could influence the sensitivity of the endogenous receptor. However, these possibilities are speculative and require further elucidation. Despite the difference in the IC_{50} range, the relative affinity of 2-furoyl-LIGRL-NH_2 to SLIGKV-OH was in parallel with the relative EC_{50} for Ca^{2+} mobilisation assays, suggesting that the higher agonist

potency of 2-furoyl-LIGRL-NH_2 reflected the higher affinity of the ligand.

The evidence that [^{3}H]2-furoyl-LIGRL-NH_2 bound to PAR2 at its ligand-binding site was demonstrated in studies of trypsin pretreatment in NCTC2544-PAR2 cells. Although in PAR systems, synthetic agonist peptides have long been used for activation of the receptor, the exact interaction between the synthetic peptides and the receptor activation domain has not been fully understood. An example was in a previous PAR-1 binding study, which demonstrated that thrombin treated human platelets exhibited lower binding ability to the PAR-1 ligand (Ahn *et al*., 1997). In our study, the trypsin-generated N-terminal tethered ligand competed with the synthetic PAR2 agonist peptide. Regarding PAR2, to our knowledge, this is the first direct evidence for competitive interaction between the synthetic peptide and the N-terminal-connected intermolecular ligand at the ligand-binding site. Furthermore, taking the number of the receptors expressed on the cell membrane into consideration, the concentration of the trypsin-generated tethered ligand available to compete with the radioligand should be much smaller than the inhibitory concentration range for a synthetic ligand such as SLIGKV-OH, suggesting a much higher affinity of the N-terminal-tethered ligand compared to the synthetic peptide free in the solution.

Finally, the receptor-binding assay in NCTC2544-PAR2 cells was successfully miniaturised into 96-well microplate format. Despite the relatively low affinity ($K_d \sim 100$ nM) of [^{3}H]2-furoyl-LIGRL-NH_2, the specific binding could be detected in the monolayer assays including several washing procedures. The advantage of this assay system is not only the convenience and higher throughput of the assay but to be applicable to other adhesive cells such as HUVEC, difficult to be dissociated unless using trypsin. Indeed, preliminary experiments used the HUVEC monolayer system to explore the possibility that the PAR-1 peptide derived from thrombin cleavage of PAR-1 could interact with PAR2 (O'Brien *et al*., 2000). However, we found that thrombin could not displace [^{3}H]2-furoyl-LIGRL-NH_2 binding (results not shown), suggesting that this might not be the case. Nevertheless, investigation of other concepts relating to PAR2 function including upregulation of the receptor and dimerisation will be facilitated using this ligand.

Given the important physiological roles played by PAR2 in association with various disease states, PAR2 could be a novel target for drug development, and both agonists and antagonists could be therapeutically beneficial. Therefore, the future development of selective agonist/antagonist for PAR2 is highly prospective. A number of studies have reported functional analysis of PAR2 activation in cell signaling events (Belham *et al*., 1996; Kawabata *et al*., 1999; Seatter *et al*., 2004), *in vitro* tissue contraction assays (Hollenberg *et al*., 1996; 1997) and *in vivo* responses (Kawabata *et al*., 2000; 2001a), which are useful for identification of chemicals acting on PAR2-mediated events. However, caution should be required in respect whether the chemicals are working on the receptor directly or on the intermediate molecules to modulate responses mediated by the receptor. Therefore, the availability of this radioligand will be valuable for characterising chemicals that interact to PAR2 and the SAR on a series of agonist peptides will be an important information for future development of PAR2 agonists/antagonists.

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