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Lanthanide labeling of a potent protease activated receptor-2 agonist for time-resolved fluorescence analysis

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Abstract

Protease activated receptor-2 (PAR_2) is one of four G-protein coupled receptors (GPCRs) that can be activated by exogenous or endogenous proteases, which cleave the extracellular amino-terminus to expose a tethered ligand and subsequent G-protein signaling. Alternatively, PAR_2 can be activated by peptide or peptidomimetic ligands derived from the sequence of the natural tethered ligand. Screening of novel ligands that directly bind to PAR_2 to agonize or antagonize the receptor has been hindered by the lack of a sensitive, high-throughput, affinity binding assay. In this report we describe the synthesis and use of a modified PAR_2 peptidomimetic agonist, 2-furoyl-LIGRLO-(diethylenetriaminepentaacetic acid)-NH_2 (2-f-LIGRLO-dtpa), designed for lanthanide-based time resolved fluorescence screening. We first demonstrate that 2-f-LIGRLO-dtpa is a potent and specific PAR_2 agonist across a full spectrum of *in vitro* assays. We then show that 2-f-LIGRLO-dtpa can be utilized in an affinity binding assay to evaluate the ligand-receptor interactions between known high potency peptidomimetic agonists (2-furoyl-LIGRLO-NH_2, 2-f-LIGRLO; 2-aminothiazol-4-yl-LIGRL-NH_2, 2-at-LIGRL and; 6-aminonicotinyl-LIGRL-NH_2, 6-an-LIGRL) and PAR_2. A separate N-terminal peptidomimetic modification (3-indoleacetyl-LIGRL-NH_2, 3-ia-LIGRL) that does not activate PAR_2 signaling was used as a negative control. All three peptidomimetic agonists demonstrated sigmoidal competitive binding curves, with the more potent agonists (2-f-LIGRLO and 2-at-LIGRL) displaying increased competition. In contrast, the control peptide (3-ia-LIGRL) displayed limited competition for PAR_2 binding. In summary, we have developed a Europium-containing PAR_2 agonist that can be used in a highly sensitive affinity binding assay to screen novel PAR_2 ligands in a high-throughput format. This ligand can serve as a critical tool in the screening and development of PAR_2 ligands.

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SUPPORTING INFORMATION AVAILABLE:

Detailed Experimental Procedures

Three figures: **S1**: Synthesis scheme for 2-f-LIGRLO-dtpa; **S2**: HPLC example of 2-f-LIGRLO-dtpa; **S3**: ESI-MS example of 2-f-LIGRLO-dtpa

One video file: Ca^{2+} response to 2-f-LIGRLO-dtpa in model epithelial cells

This information is available free of charge via the Internet at http://pubs.acs.org/.

INTRODUCTION

PAR_2 is one of a four-member family of GPCRs that are activated in response to numerous different proteases[1–5]. PAR_2 is expressed in a variety of cell types and associated with a number of diseases including arthritis, allergic asthma, cancer, and acute and chronic pain. Similar to the other PARs, proteolytic cleavage of the extracellular N-terminus exposes a tethered ligand sequence. This ligand intramolecularly binds to the receptor to initiate G-protein dependent and independent intracellular signaling pathways[1,5,6]. Exposure of the primary PAR_2 protease cleavage site reveals a tethered ligand (peptide sequence ending in SLIGRL in rodent and SLIGKV in humans) that elicits two dominant intracellular signaling events: an increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) and increased phosphorylation of mitogen activated protein kinases (MAPKs). Interestingly, alternative protease cleavage sites and subsequent ligands can induce biased MAPK signaling over Ca^{2+} signaling, and potentially, alternative physiological responses initiated at PAR[25–8]. All of the possible signaling and physiological outcomes that follow PAR_2 activation share in common a ligand-receptor interaction. While this ligand-receptor interaction is important to initiate signaling, the interaction itself remains ill-defined.

Small activating peptides or peptidomimetics that mimic the binding properties of the natural tethered ligand can activate PAR_2 specifically and potently in the absence of proteolytic cleavage[1,3,5,9]. Such ligands have the specific advantage of eliminating the potential off-target effects of proteases that limit the use of proteases and protease inhibitors in evaluating PAR_2 effects *in vitro* and *in vivo*. The continued screening and development of novel ligands that specifically bind PAR_2 to agonize or antagonize the receptor can serve as probes to better characterize downstream signaling pathways of PAR_2, its physiological and pathophysiological roles, and serve as templates for drug design. We recently developed a high-throughput *in vitro* screening assay that includes physiological and signaling pathway responses to PAR_2 ligands[9]. This method helped to establish full characterization of known and novel PAR_2 ligands; however, it lacked a biochemical approach to evaluate direct interactions between novel ligands and PAR_2.

Published methods for evaluating PAR_2 ligand-receptor interactions include the use of peptidomimetic analogs (*trans*-cinnamoyl-LIGRLO-NH_2, 2-furoyl-LIGRL-NH_2 and 2-furoyl-LIGRLO-NH_2) modified by radiolabels or traditional fluorescence[10–13]. These studies demonstrated binding curves and evaluated PAR_2 ligand binding (e.g., IC_{50}s[10,13], K_is[11–13], relative K_is (compared to SLIGKV-OH)[12,13] or relative IC_{50}s (compared to SLIGRL-NH_2)[10,11] following competitive binding assays in tissue culture plates and/or suspended cultures. While these assays showed the utility of modifying known peptidomimetic ligands to develop competitive binding assays, a distinct disadvantage of these ligands is their limited adaptability to high-throughput analyses, thus compromising their use for PAR_2 ligand discovery. The recent development of lanthanide series luminescence/fluorescence assays allows for fluorescence-detection based competitive binding assays *in vitro* using a multi-well plate format, as well as the potential to develop similar probes for *in vivo* PAR_2 studies[14,15]

Lanthanides (e.g., Terbium, Tb; Dysprosium, Dy; Europium, Eu; Samarium, Sm) exhibit the unique characteristics of a low detection limit (10^{-12}–10^{-15} M), narrow emission bands (10–20 nm), large Stokes shift (greater than 200 nm), and long-lived fluorescence (milliseconds for Eu)[16–18]. Prolonged fluorescence enables the use of time-resolved fluorescence (TRF) measurements[18], greatly improving the signal to noise ratio[19] and thus, increases fluorescent measurement sensitivity. We wanted to take advantage of the physical properties of lanthanides and used a modified Dissociation Enhanced Lanthanide Fluoroimmunoassay (DELFIA; Wallac/PerkinElmer Life Sciences) to carry out competitive binding assays. This

technique has proven to be successful in screening (e.g. ligand binding assays), biodistribution and biomedical applications and is the most successful lanthanide-based immunoassay reported to date[14,17,18,20].

In this study we sought to develop a potent, PAR_2-specific ligand, whose characteristics allow for a functional high-throughput screening assay to characterize ligand-receptor interactions. We synthesized a derivative of the highly potent 2-f-LIGRLO modified with diethylenetriaminepentaacetic acid (dtpa), which serves as the chelate for a Europium (III) to be used in a time-resolved fluorescence assay[14,17,18]. The resulting compound, 2-f-LIGRLO-dtpa was first characterized for its ability to function as a full and specific PAR_2 agonist using both *in vitro* physiological and cellular signaling assays. We next showed that 2-f-LIGRLO-dtpa can be used in a competitive binding assay with potent, full PAR_2-specific agonists (2-f-LIGRLO, 2-at-LIGRL, and 6-an-LIGRL), and the inactive peptidomimetic 3-ia-LIGRL. We propose that 2-f-LIGRLO-dtpa can be used to evaluate PAR_2 ligand-receptor interactions to both screen novel compounds and to better understand the role of ligand-receptor interactions in the signaling pathway-specific activation of PAR_2.

EXPERIMENTAL PROCEDURES

Materials

Materials and chemicals were of molecular biology grade or higher. Sources are listed in the Supplemental Material.

PAR_2 ligand synthesis

Peptides and peptidomimetics (2-f-LIGRLO, 2-at-LIGRL, 6-an-LIGRL and 3-ia-LIGRL) were prepared as previously described[21]. The lanthanide-labeled peptidomimetic, 2-f-LIGRLO-dtpa was made by coupling dtpa to the ε-amino group of the ornithine in the parent PAR_2 ligand, 2-f-LIGRLO (Figure 1) followed by the chelation of a Europium (III) ion via a synthesis scheme previously described[18]. Specific details on syntheses and purity are provided in the Supplemental Material.

Epithelial cell culture and cellular signaling measurements

The 16HBE14o- human bronchial epithelial cell line was used for physiological and second messenger screening assays and in competitive binding experiments were grown as previously described[9]. PAR_2 and vector control-transfected Kirsten virus-transformed normal rat kidney (kNRK-PAR_2 and kNRK, respectively) cells were used to test specificity of the 2-f-LIGRLO-dtpa ligand. Cell growth and specifics on physiological and signaling analyses are described in the Supplemental Material.

Competitive binding assay

Black 96-well plates with white, opaque wells were initially incubated with a matrix coating solution (MCS consisting of 88% LHC basal medium, 10% bovine serum albumin (BSA), 1% bovine collagen type I and 1% human fibronectin for two h at 37°C. MCS was removed and plates allowed to dry for at least one h. 16HBE14o- cells were plated on at a density of 6.0×10^4 cells per well and grown at 37°C, 5% CO_2 to confluence (4 – 5). On the day of the experiment, growth media was aspirated using a multi-well vacuum manifold (Corning Inc, Corning, NY) and cells were washed with 200 μl of pre-warmed (37°C) Hanks' balanced saline solution additionally buffered with 25 mM HEPES, pH 7.4 (HBSS) using a multi-channel pipet. HBSS was replaced with 100 μl of a 2% BSA in HBSS solution and incubated for 75 min to block non-specific interactions. All peptide competition solutions were made at 2× final concentration in a deep well mixing plate. Competition solutions consisted of a fixed concentration of 2-f-LIGRLO-dtpa (final concentration: 300 nM) and

concentration response concentrations of peptidomimetics (final concentrations: 1 nM to 600 μM in log or ½ log steps). 100 μl of competition solutions were added to each well in the 96-well plate (final volume of 200 μl) and incubated with the BSA-blocked live cells for 45 min at 25°C. Competition solutions were carefully removed, and cells were washed three times with a wash solution (Base medium: HBSS supplemented with 20 μM EDTA, 0.01% Tween, and 0.2% BSA). The wash solution was removed and replaced with 100 μl of DELFIA enhancement solution (Perkin Elmer; Waltham, MA) for 40 – 45 min at room temp and fluorescence determined as previously described[17,18]. Briefly, 96-well plates were read on a Wallac VICTOR$_3$ instrument (Perkin Elmer; Waltham, MA) using standard Eu time-resolved fluorescence (TRF) measurement: excitation light (340 nm) followed by a 400 μsec delay before a 400 μsec emission collection (615 nm). The total measurement cycle was set to 1000 μsec per well.

Statistics

All statistical analyses were evaluated with GraphPad software (San Diego, CA). Multivariate comparisons were done with a one-way ANOVA with Tukey's multiple comparison post-test. Pair-wise comparisons were done with a 2-tailed Student's *t* test. Relative IC$_{50}$s were calculated using a one-site fit logIC50 non-linear regression curve-fit analysis. A value of $p < 0.05$ was used to establish a significant difference between samples. Data in figures are graphed as ± SEM unless otherwise noted.

RESULTS

Evaluation of 2-f-LIGRLO-dtpa as a full PAR$_2$ agonist

We used a high PAR$_2$-expressing human bronchial epithelial cell line (16HBE14o-)[22] to evaluate the ability of 2-f-LIGRLO-dtpa to activate PAR$_2$ with a three-tiered screening assay that included *in vitro* physiological, MAPK signaling and Ca^{2+} signaling assays[9]. To examine physiological activation of 16HBE14o- cells by 2-f-LIGRLO-dtpa, we used the xCELLigence Real Time Cell Analyzer (Roche; RTCA). Cells were exposed to a concentration response (0.05 μM–2.5 μM) of either the parent compound, 2-f-LIGRLO, or 2-f-LIGRLO-dtpa and examined for the ability of each compound to activate PAR$_2$ (Figure 2). We found 2-f-LIGRLO-dtpa stimulated a maximum Cell Index at a concentration of 2.5 μM. The calculated EC$_{50}$ of 2-f-LIGRLO-dtpa (388 nM; 95% CI, 336 – 448 nM) was slightly less potent than 2-f-LIGRLO (130 nM; 95% CI, 107 – 159 nM), and similar to another highly potent peptidomimetic PAR$_2$ agonist, 6-an-LIGRL[9].

We next evaluated the ability of 2-f-LIGRLO-dtpa to induce MAPK signaling pathways. 16HBE14o- cells were exposed to a concentration response (1 – 10 μM) of 2-f-LIGRLO-dtpa for 5 min, and examined for activation of MAPK signaling using immunoblot of pERK/tERK (Figure 3). Exposure of 2.5 μM 2-f-LIGRLO-dtpa to 16HBE14o- cells induced a significant increase in pERK/tERK when compared with control. Increasing concentrations up to 10 μM 2-f-LIGRLO-dtpa induced higher pERK/tERK values. In the same assay, 2-f-LIGRLO induced a significant increase of pERK/tERK at 1 μM exposure, and reached a maximum by 5 μM exposure. Although 2-f-LIGRLO-dtpa was slightly less potent than 2-f-LIGRLO, it was comparable to alternative full peptidomimetic agonists 2-at-LIGRL and 6-an-LIGRL[9].

In a complementary signaling assay to evaluate PAR$_2$ activation by 2-f-LIGRLO-dtpa, we examined the activation of Ca^{2+} signaling in 16HBE14o- cells using digital imaging microscopy[9,21]. Upon addition of 5 μM 2-f-LIGRLO-dtpa, an initial increase in $[Ca^{2+}]_i$ was observed within 30 sec, with a peak response observed between 45 and 60 sec (Figure 4; supplemental video). The majority of cells in the field of view responded within the 3 min

experiment. An advantage of this imaging method is the detection of $[Ca^{2+}]_i$ changes in individual cells and thus, an increased sensitivity in the PAR_2 response. A concentration response curve (0.5 μM – 25 μM 2-f-LIGRLO-dtpa) is shown in Figure 4B. The EC_{50} for 2-f-LIGRLO-dtpa (1.34 μM; 95% CI, 0.85–2.11 μM), was slightly less efficacious than that reported for 2-f-LIGRLO but more efficacious than another high potency PAR_2 specific agonist, 2-at-LIGRL[9,21]. In summary, 2-f-LIGRLO-dtpa displays signaling responses across a variety of assays that are similar to other high potency, full PAR_2 agonists.

Specificity of 2-f-LIGRLO-dtpa to PAR_2

To test the specificity of 2-f-LIGRLO-dtpa, we used Kirsten virus-transformed normal rat kidney cells, a low-PAR_2 expressing epithelial cell line. We compared the activation of Ca^{2+} signaling in PAR_2-transfected kNRK cells (kNRK-PAR_2) to those transfected with a control vector (kNRK) (Figure 5). Exposure of kNRK-PAR_2 cells to 5 μM or 2.5 μM 2-f-LIGRLO-dtpa resulted in Ca^{2+} signaling in a high percentage of cells (90.1% ± 4.8 and 55.1% ± 11.5, respectively). Similar experiments using kNRK cells resulted in limited Ca^{2+} signaling (2.98% ± 0.09 and 0.42% ± 0.42, respectively). These results indicate our novel 2-f-LIGRLO-dtpa ligand requires PAR_2 for initiating cellular signaling and thus, is a specific agonist to PAR_2.

PAR_2 competitive binding assay using 2-f-LIGRLO-dtpa

The primary advantage of 2-f-LIGRLO-dtpa compared to other PAR_2 ligands is its use to evaluate novel PAR_2 ligand-receptor interactions in live cells using time-resolved fluorescence. We used 2-f-LIGRLO-dtpa in a modified Dissociation Enhanced Lanthanide Fluoroimmunoassay (DELFIA) based Europium-DTPA competitive binding assay, similar to the methods previously used[17,18], to evaluate the ligand-receptor interaction between novel ligands and PAR_2. We competed 2-f-LIGRLO-dtpa (300 nM) with its parent compound, 2-f-LIGRLO, and a recently described highly potent and specific PAR_2 peptidomimetic agonist, 2-at-LIGRL[9,21]. Incubation with concentration responses of 2-f-LIGRLO or 2-at-LIGRL resulted in sigmoidal competitive binding curves (Figure 6), with apparent IC_{50}s of 3.29 μM (95% CI: 2.51 – 4.30 μM) and 2.06 μM (95% CI: 1.61 – 2.65 μM) respectively. Similar experiments conducted in the absence of cells did not result in competitive binding, although the highly fluorescent 2-f-LIGRLO-dtpa did display significant signal (15.1 % ± 4.4 of the total signal measured), indicative of an interaction with the reflective plate surface. Because of this background, we are only reporting apparent IC_{50}s for competitive binding. To fully demonstrate the utility of this assay, we tested a less potent ligand, 6-an-LIGRL, and a similar peptidomimetic, 3-ia-LIGRL, which does not activate PAR_2[21]. 6-an-LIGRL displayed a typical sigmoidal binding curve that was significantly shifted to the right of the 2-f-LIGRLO and 2-at-LIGRL curves with an apparent IC_{50} of 18.54 μM (95% CI, 14.11 – 24.36 μM; Figure 6), indicative of reduced competition for binding at PAR_2. In contrast, 3-ia-LIGRL did not compete for PAR_2 binding (Figure 6). We conclude that our novel PAR_2 agonist, 2-f-LIGRLO-dtpa, can be utilized in a unique, sensitive, high-throughput screening assay to examine PAR_2 ligand binding.

DISCUSSION

In this report we introduce the Europium (III) containing compound, 2-f-LIGRLO-dtpa, as a potent and specific PAR_2 agonist and demonstrate its use as a probe in a novel, radioligand-free, competitive binding assay to evaluate ligand-receptor interactions at PAR_2. In the live-cell affinity binding experiments using three known full agonists (2-f-LIGRLO, 2-at-LIGRL and 6-an-LIGRL) and a control peptidomimetic (3-ia-LIGRL) a rank order of apparent IC_{50}s was obtained (2-f-LIGRL ≈ 2-at-LIGRL > 6-an-LIGRL >>> 3-ia-LIGRL). This rank order potency closely matches the rank order potency of these compounds tested across

physiological assays[9,21]. These data strongly suggest that differences in the described ligand potency is directly related to their avidity for PAR_2 and highlight the importance of evaluating PAR_2 binding.

The development and use of PAR_2-specific activating peptides is an increasingly prevalent method to study downstream signaling pathways of PAR_2, the physiological and pathophysiological roles of PAR_2, and provides an avenue for the discovery of therapeutics targeting PAR_2-associated pathologies. Although many methods have been employed for the development and screening of ligands, the current screening of such ligands that directly bind PAR_2 has been limited by the availability of a high-throughput competitive binding assay. Because we had previously used lanthanide-labeled ligand targeting to evaluate a variety of GPCRs[15,17,20], we hypothesized that lanthanide-labeled PAR_2 peptidomimetic agonists would be viable candidates for the protease activated receptor family of GPCRs. The lanthanide containing 2-f-LIGRLO-dpta compound reported herein was synthesized by coupling a diethylenetriaminepentacetic acid (dtpa) molecule, with its inherent ability to effectively chelate a Europium (III), to the ornithine residue of the potent PAR_2 agonist 2-f-LIGRLO. The ornithine had previously been used to attach fluorescent markers without greatly affecting biological activity or receptor binding ability of the PAR_2 parent peptidomimetic (e.g., Alexa Fluor 594 or rhodamine labeled 2-f-LIGRLO[10,23]). Because of the highly charged and relatively large chelate, we found it necessary to verify 2-f-LIGRLO-dtpa maintained the ability to interact with PAR_2 through a series of *in vitro* assays designed to screen and assess the structure-activity relationship of novel PAR_2 ligands[9]. In the *in vitro* physiological response assay (RTCA), 2-f-LIGRLO-dtpa displayed full agonist properties at both acute (e.g., 10 min) and long-term (e.g., 4 h) time points (Figure 2). Similarly, 2-f-LIGRLO-dtpa acted as a full agonist in cell signaling assays (Figures 3 and 4). A final test for use of 2-f-LIGRLO-dtpa was to determine specificity for PAR_2, which was accomplished using a kNRK cell model (Figure 5). From these assays, it was determined that 2-f-LIGRLO-dtpa acts as a full and specific agonist to PAR_2 with slightly reduced potency when compared with its parent 2-f-LIGRLO compound. As such, it can be considered a suitable probe for competitive binding experiments.

Competitive binding assays for PAR_2 have been reported with both tritiated and fluorescent compounds directed at PAR_2[10–13]. Radioactive tritiated compounds (e.g.,[^{3}H]propionyl-2-furoyl-LIGRLO-NH_2) serve as sensitive binding probes, however use of radiolabeled compounds limits ability for adaptation to high-throughput analysis. In contrast, our modified DELFIA competitive binding assay allows for rapid assessment of multiple ligands in one 96-well test plate while providing sensitivity consistent with radioligand binding experiments[17,20,24]. Studies using modified ligands with Alexa series dyes have also been used in competitive binding experiments with PAR_2[10]. The relatively small Stokes shift (27 nm) which increases sampling error due to scattering of the excitation light by instrument optics, and significant background fluorescence from sample vessels, matrix or cells during measurement of Alexa Fluor 594 reduces measurement sensitivity and presents a disadvantage in using this dye in high-throughput screening. Further disadvantages include the inherent aqueous quenching and short-lived fluorescence that also reduce signal to noise ratio[14]. We overcome these limitations by utilizing a modified DELFIA technique combined with the luminescence properties of lanthanide series ions (e.g., Eu). The Eu present in our compound has relatively narrow emission bands and a Stokes shift of ~275 nm, much larger than any available fluorescent dye. These properties significantly reduce noise contributions due to spectral overlap between excitation and emission spectra. Furthermore, our use of the dtpa chelate saturates the lanthanide coordinating sphere and the micellar environment present during fluorescence measurement protect the Eu ion from aqueous quenching[14,20]. The long-lived luminescence of the Eu that allows for a delay between excitation and

emission measurement readings prevents inherent short-lived background fluorescence from contributing to measurements, and thus, increases signal to noise fidelity.

The availability of a rapid competitive binding assay described herein can now be used to discern relative ability of a ligand to interact at a site that results in full activation of PAR_2 signaling. This will be a critical assessment tool since the evaluation of ligand-receptor interactions is a fundamental screening assay in drug development. Combining this assay with structure-activity relationship data obtained from our *in vitro* physiological and cell signaling screening assay (e.g.,[9]) provides a potent selection process to identify novel drug candidates to the most common activation site of PAR_2. It should be noted that PAR_2, like other G-protein coupled receptors (GPCRs), can activate alternative signaling pathways[4,6,25–27]; however, mechanisms for such diverse activation of GPCRs and PARs remain ill-defined. The development of highly sensitive and high-throughput competitive binding assays as described herein or to potential alternative ligand binding sites on PAR_2, will help us to better understand these novel signaling mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS USED

16HBE14o-	Human bronchial epithelial cell line
2-f-LIGRLO-dtpa	2-furoyl-LIGRLO-(diethylenetriaminepentaacetic acid)-NH_2
2-f-LIGRLO	2-furoyl-LIGRLO-NH_2
2-at-LIGRL	2-aminothiazol-4-yl-LIGRL-NH_2
6-an-LIGRL	6-aminonicotinyl-LIGRL-NH_2
3-ia-LIGRL	3-indoleacetyl-LIGRL-NH_2
$[Ca^{2+}]_i$	calcium ion concentration
Aloc	allyloxycarbonyl
BSA	Bovine serum albumin
***t*Bu**	tert-butyl
DCM	dichloromethane
DIC	N,N’-diisopropylcarbodiimide
DIEA	diisopropylethylamine
DELFIA	Dissociation Enhanced Lanthanide Fluoroimmunoassay
DMEM	Dulbecco’s Modified Eagle Medium

DMF	N,N'-dimethylformamide
DMSO	dimethylsulfoxide
dtpa	diethylenetriamine-N^1,N^2,N^3,N^4-pentaacetic acid
Eu	Europium (III)
E-plate	96-well tissue culture plate lined with gold electrodes used in RTCA
TRF	Time-resolved fluorescenceESI-MS Electrospray ionization - mass spectrometry
Fmoc	(9H-fluoren-9-ylmethoxy)carbonyl
FT-ICR	Fourier Transform - Ion Cyclotron Resonance
GPCR	G-protein coupled receptor
HBSS	Hanks' balanced salt solution additionally buffered with 25 mM HEPES, pH 7.4
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate
HOBt	N-hydroxybenzotriazole
HOCt	6-chloro-1H-hydroxybenzotriazole
kNRK	Kirsten virus-transformed normal rat kidney cells transfected with a vector control
kNRK-PAR_2	Kirsten virus-transformed normal rat kidney cells transfected with PAR_2
LHC	Lechner and LaVeck basal medium
MALDI	Matrix Assisted Laser Desorption Ionization - Time of Flight
MAPK	mitogen activated protein kinase
MCS	matrix coating solution
MEM	Minimal Essential Medium with Earle's salts
PAR_2	Protease activated receptor-2
Pbf	2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl
$Pd(TPP)_4$	Palladium (0) tetrakis triphenylphoshine
RP-HPLC	reverse-phase high performance liquid chromatography
RTCA	xCELLigence real time cell analyzer (Roche)
SPE	solid-phase extraction
SPPS	solid-phase peptide synthesis
TEAA	triethylammonium acetate
THF	tetrahydrofuran
TFA	trifluoroacetic acid
TRF	time-resolved fluorescence

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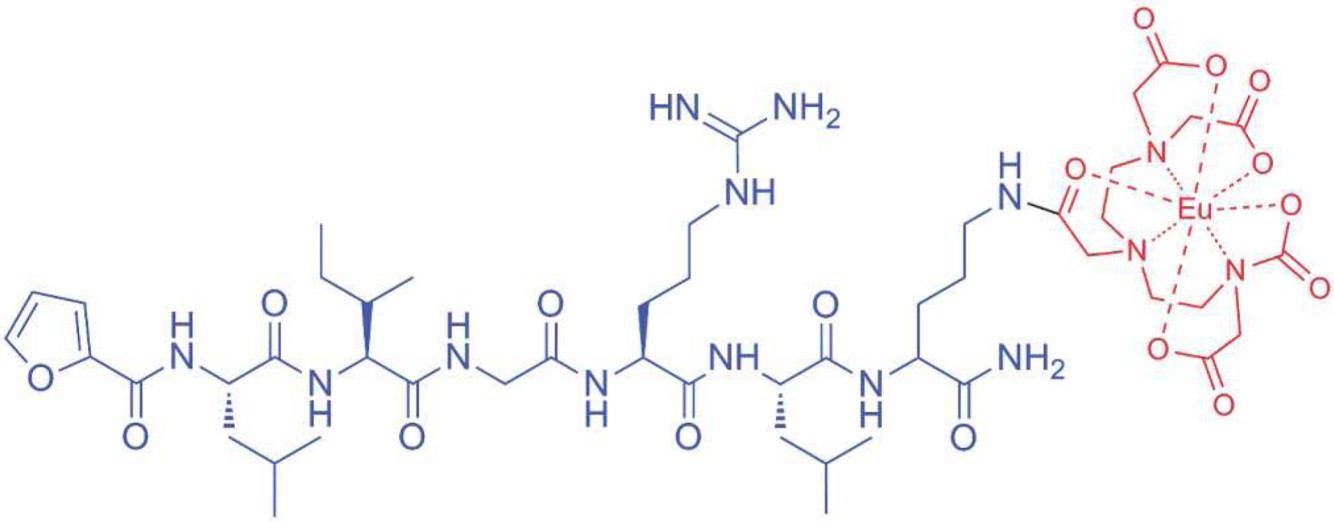

Figure 1. Structure of 2-furoyl-LIGRLO-dtpa-NH_2 (2-f-LIGRLO-dtpa)

A) Chemical structure of the parent PAR_2 hexapeptidomimetic 2-furoyl-LIGRLO-NH_2 is shown in blue. The diethylenetriaminepentaacetic acid (dtpa) chelating a Europium (III) and conjugated with the parent compound via the ε ornithine is shown in red.

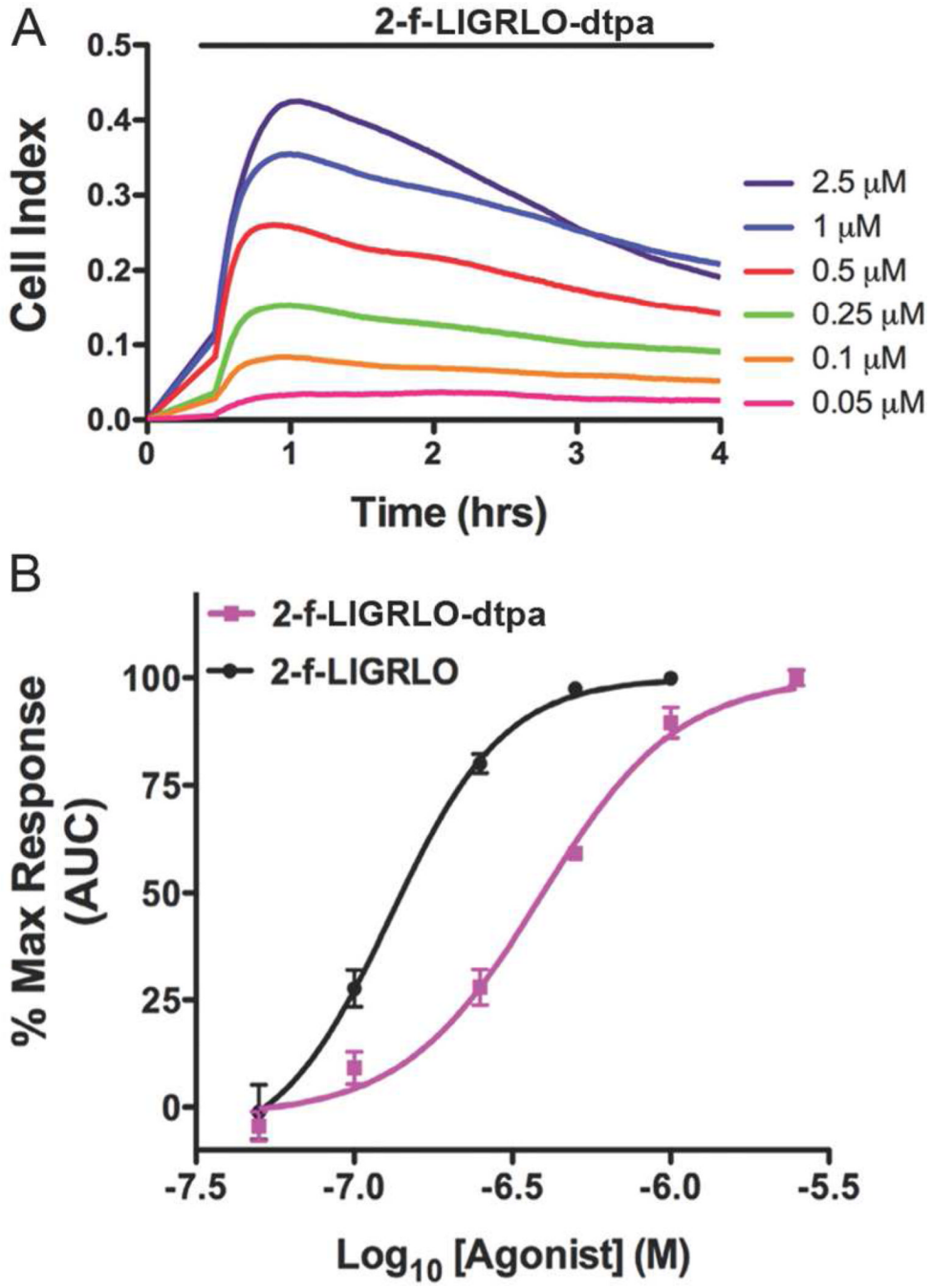

Figure 2. 2-f-LIGRLO-dtpa stimulates a physiological response in model epithelial cells

(A) 16HBE14o- cells were plated onto E-plates, exposed to a concentration response (50 nM – 2.5 μM) of 2-f-LIGRLO-dtpa and Cell Index was measured every 15 s for 4 h. Cells showed a concentration-dependent physiological response over the 4 h experiment. (B) A concentration response curve was constructed for 2-f-LIGRLO-dtpa and the high potency agonist 2-f-LIGRLO from the area under the curve of individual experiments. EC_{50} for 2-f-LIGRLO-dtpa (388 nM) is consistent with a potent full agonist, albeit slightly reduced from 2-f-LIGRLO (130 nM)[9]. Traces in (A) and curves in (B) are mean responses from 4 experiments, error bars are eliminated in (A) for clarity.

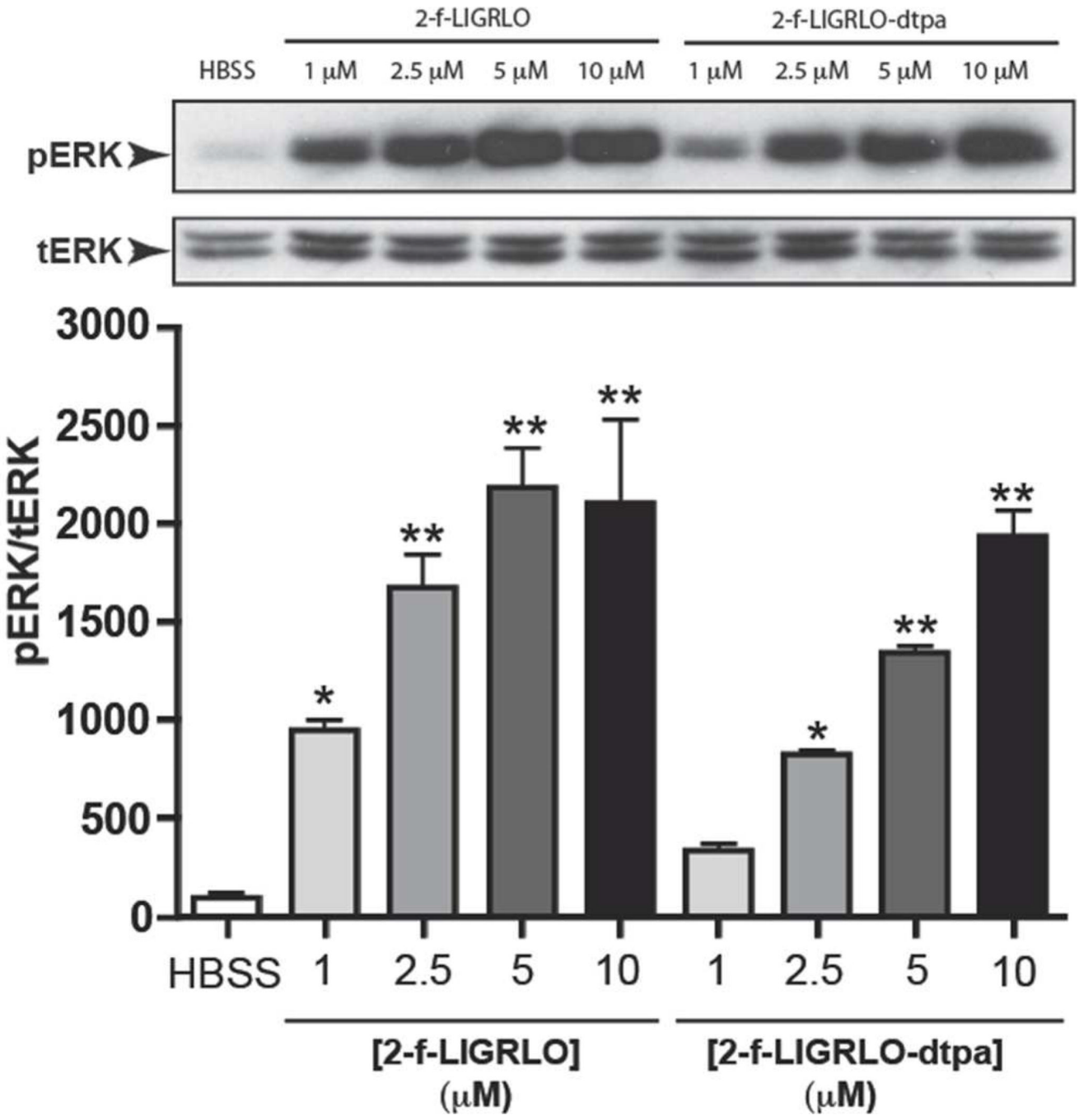

Figure 3. 2-f-LIGRLO-dtpa stimulates MAPK signaling in model epithelial cells

16HBE14o- cells were exposed to a concentration response of either 2-f-LIGRLO or 2-f-LIGRLO-dtpa for 5 min. Representative immunoblots show increased ERK1/2 phosphorylation (pERK) compared to total ERK1/2 (tERK) beginning at 1 μM agonist concentration and maintained at concentrations up to 10 μM for each ligand. Densitometric quantification (n=4) demonstrates significant increase of pERK at 1 μM and peak activation by 5 μM for 2-f-LIGRLO, while 2-f-LIGRLO-dtpa was slightly less potent, with a significant increase in pERK at 2.5 μM and a peak response by 10 μM. Significant difference from matching HBSS control are shown: "*", $p < 0.05$; "**", $p < 0.01$.

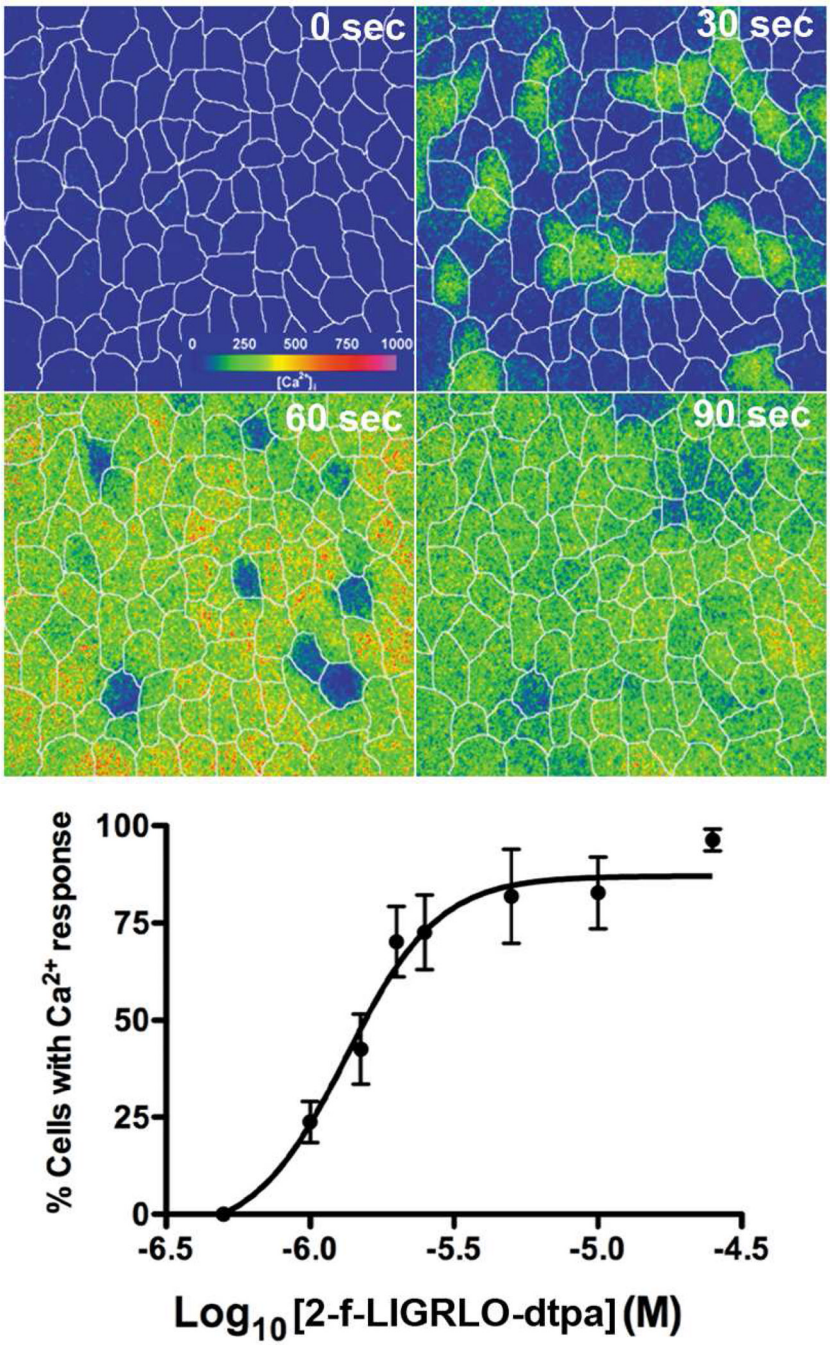

Figure 4. 2-f-LIGRLO-dtpa stimulates Ca^{2+} signaling in model epithelial cells

16HBE14o-cells were exposed to 2-f-LIGRLO-dtpa and $[Ca^{2+}]_i$ was monitored over time. (A) Individual panels depict $[Ca^{2+}]_i$ in all cells within a field of view at listed times (sec) following application of 5 μM 2-f-LIGRLO-dtpa at time 0. White lines approximate individual cell borders in each image and estimated $[Ca^{2+}]_i$ is depicted in the color bar shown in upper left panel. 2-f-LIGRLO-dtpa stimulates a rapid increase in $[Ca^{2+}]_i$ that reaches peak changes by 45 – 60 sec in most cells and begins to return towards base line by 90 sec. (B) Percentage of cells in the field of view that show $[Ca^{2+}]_i$ responses within the 3 min experiment are graphed as a concentration response to 2-f-LIGRLO-dtpa. The calculated EC_{50} of 2-f-LIGRLO-dtpa for a Ca^{2+} response is 1.34 μM, comparable to known

full PAR_2 agonists[9]. A minimum of 3 and maximum of 9 experiments are plotted for each concentration shown in (B).

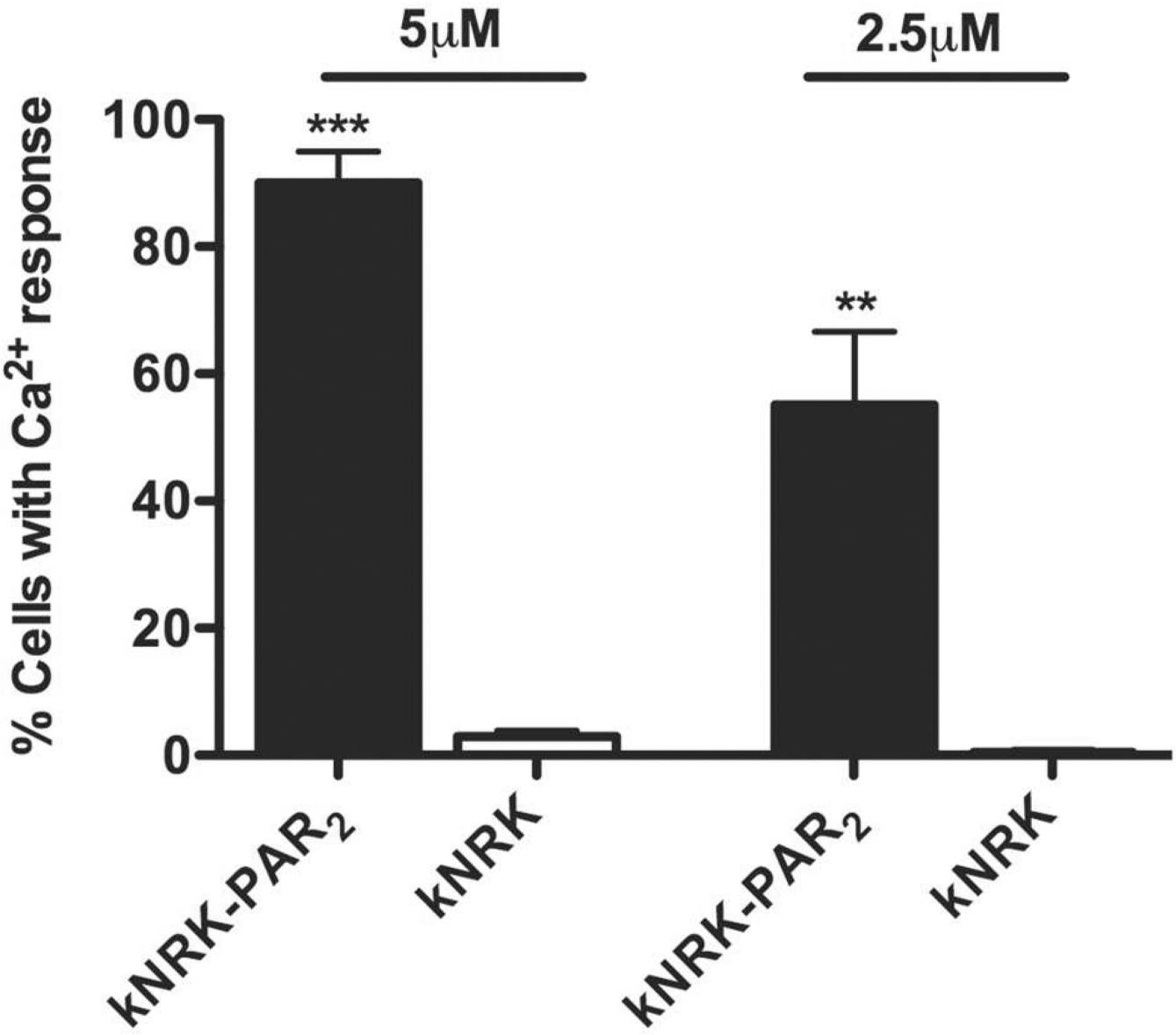

Figure 5. 2-f-LIGRLO-dtpa requires PAR_2 expression to induce Ca^{2+} signaling

PAR_2-transfected (kNRK-PAR_2) or control vector-transfected (kNRK) rat kidney cells were exposed to 2-f-LIGRLO-dtpa and evaluated for Ca^{2+} response as described in Fig. 4. Addition of 5 or 2.5 μM 2-f-LIGRLO-dtpa resulted in a robust Ca^{2+} response in PAR_2 expressing cells that was largely absent in control cells. "**" denotes $p < 0.01$ and "***" denotes $p < 0.001$ when compared to matched control; $n \geq 4$ for each experiment.

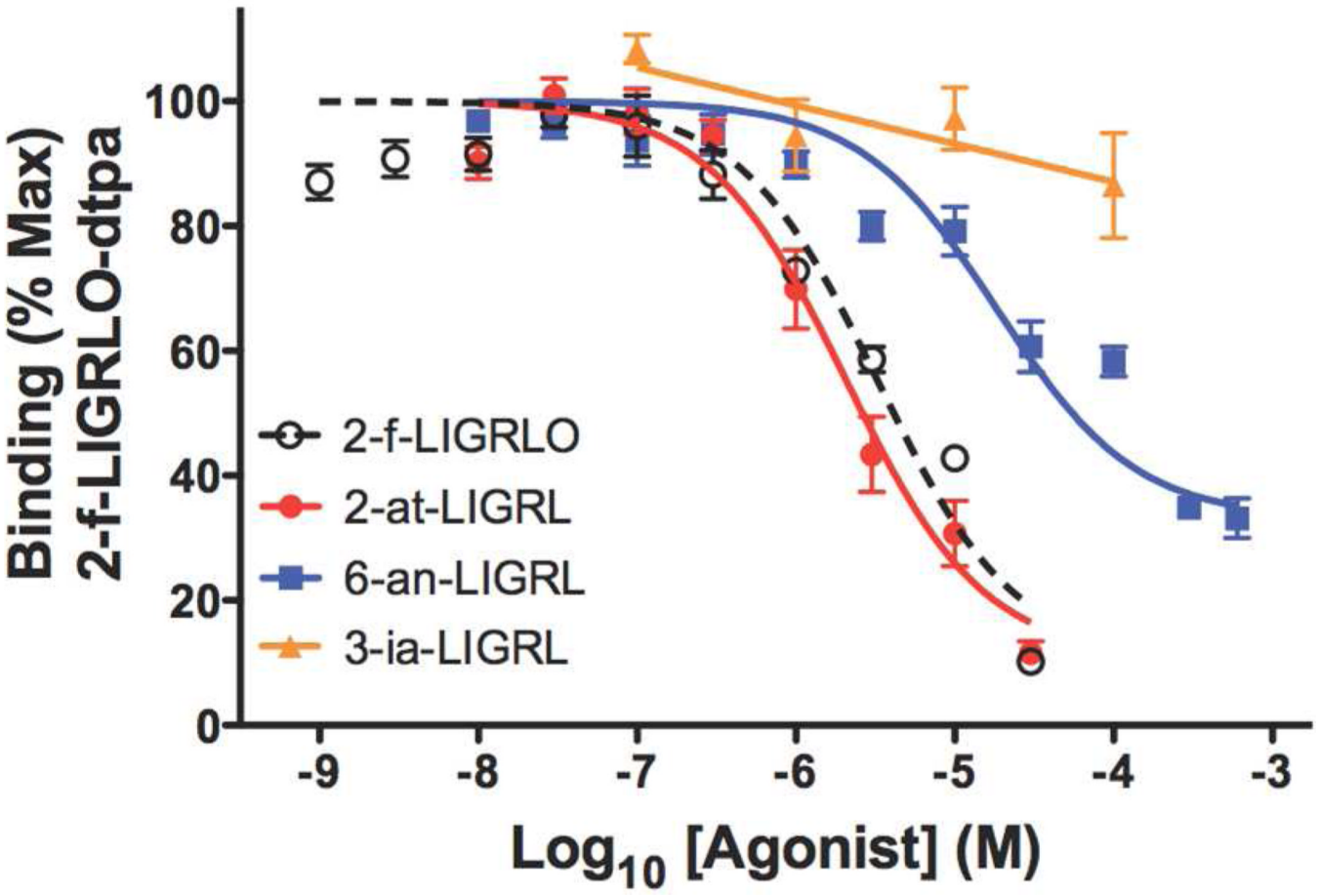

Figure 6. Competitive binding of 2-f-LIGRLO-dtpa with peptidomimetic PAR_2 agonists and controls

16HBE14o- cells plated onto 96-well plates were simultaneously exposed to 300 nM 2-f-LIGRLO-dtpa and various concentrations of known agonists 2-f-LIGRLO, 2-at-LIGRL, 6-an-LIGRL or a control peptidomimetic, 3-ia-LIGRL and assessed for competitive binding. Binding is expressed as a percentage of the maximal net Eu fluorescence measured from 2-f-LIGRLO-dtpa (300 nM) binding (% Max). The two most potent agonists, 2-f-LIGRLO and 2-at-LIGRL displayed sigmoidal curves consistent with high competition for PAR_2 binding. The slightly less potent full agonist, 6-an-LIGRL, displayed a right-shifted sigmoidal binding curve, indicative of reduced ability to compete against 2-f-LIGRLO-dtpa for receptor binding. The control peptidomimetic, which does not initiate PAR_2 signaling, displayed limited binding, and was best fit with a linear function. Apparent IC_{50} values for agonists showing competitive binding for PAR_2 are as follows: 2-f-LIGRLO: 3.29 μM; 2-at-LIGRL, 2.06 μM and; 6-an-LIGRL, 18.54 μM. Data of all agonists are from $n \geq 3$ samples at each concentration of all agonists in each multi-well experiment, and from at least two independent multi-well experiments. Control peptidomimetic (3-ia-LIGRL) data is from one multi-well experiment.