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Supporting Information

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Peptide Modifications Differentially Alter G Protein-Coupled Receptor Internalization and Signaling Bias**

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Supporting Information

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1. Supplementary Results

Supplementary Figures:

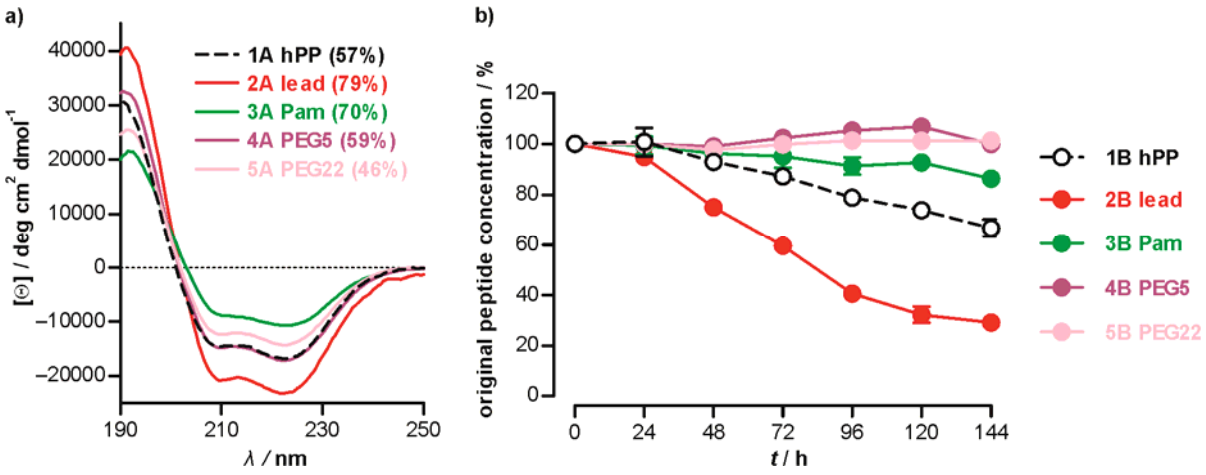


Figure S1: Conformational and stability analysis of [K^{22},Q^{34}]hPP-based peptides. a) CD spectroscopy was performed with 10 µM peptide solutions in 10 mM phosphate buffer (pH 7.1). Percentages of α-helical content of PP shown in parentheses were calculated by *http://dichroweb.cryst.bbk.ac.uk*. b) Metabolic stability of peptide analogues in human blood plasma. Peptide conjugates, N-terminally modified with TAMRA, were incubated in blood plasma over a 144 h period at 37 °C. At indicated time points, samples were taken, processed and analyzed by RP-HPLC (reversed-phase high-performance liquid chromatography) with fluorescence detection. At each time point, peptide-specific fluorescence was quantified and calculated as a fraction of control at 0 h. Means ± S.E.M. of two independent experiments are shown.

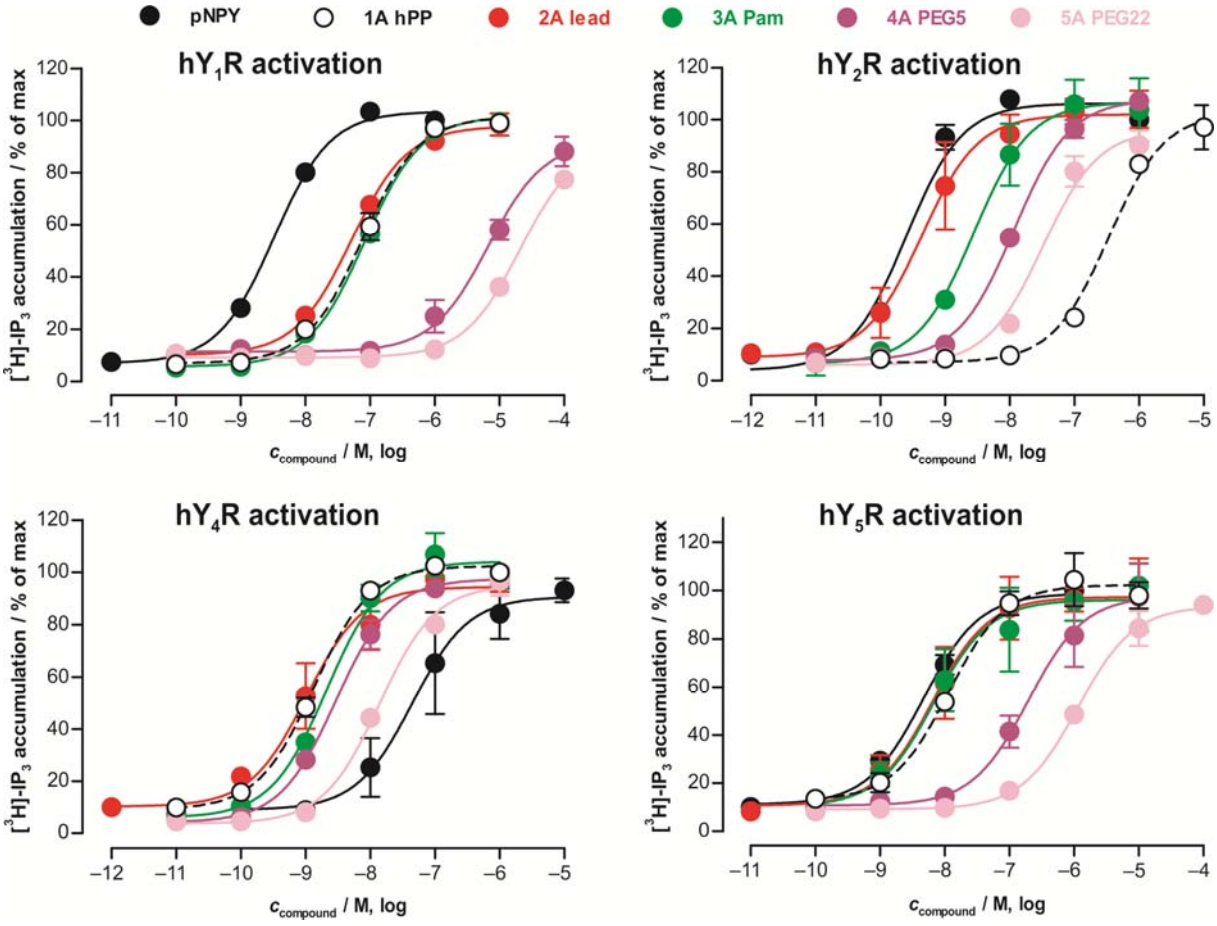


Figure S2: Stimulation of the four hYR subtypes by [K^{22},Q^{34}]hPP analogues. Inositol phosphate accumulation was measured in COS-7 (African green monkey kidney) cells stably expressing indicated hYR and a chimeric $G_{i/q}$ protein. Native ligands are pNPY for hY_1R, hY_2R, hY_5R, and hPP for hY_4R. Mean curves from at least two independent experiments with S.E.M. are shown.

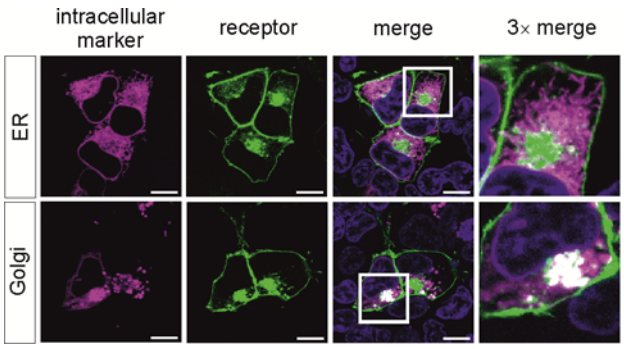


Figure S3: Localization of hY_4R in intracellular compartments. Co-localization in transiently transfected, unstimulated HEK293 (human embryonic kidney) cells. White areas in the merged channel indicate co-localization of hY_4R (green) and markers of endoplasmatic reticulum (ER, magenta; first panel) or Golgi apparatus (Golgi, magenta; second panel). Scale bar: 10 µm; white boxes in merged pictures represent magnified detail.

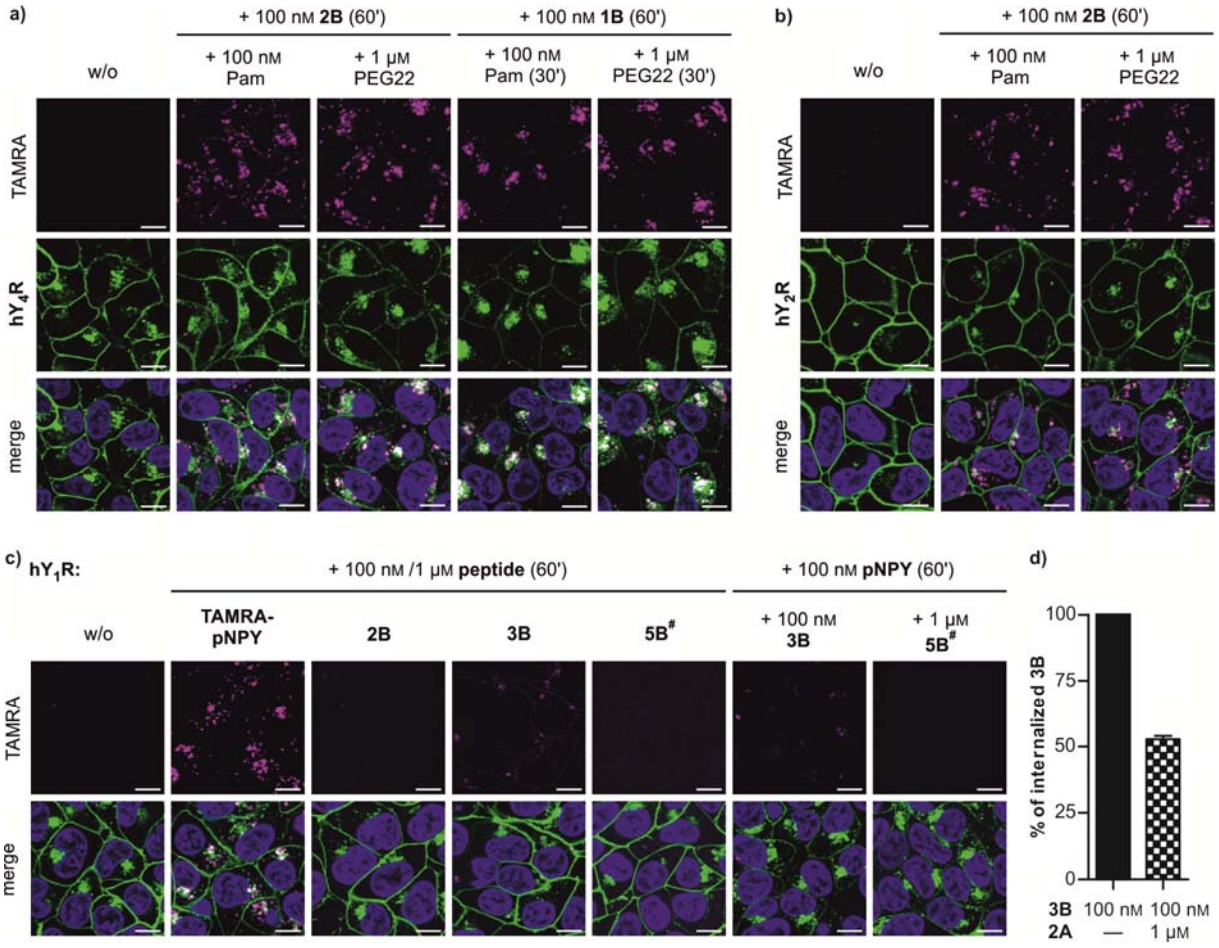


Figure S4: Internalization of [K^{22},Q^{34}]hPP compounds in HEK293 cells expressing different hYR types to verify the effect of covalent peptide modifications at hY_4R (a) and hY_2R (b) and potency-related, receptor-specific internalization (c). a) Uptake of TAMRA-labeled lead peptide (**2B**) after 60 min in presence of unconjugated, soluble Pam (second column) or PEG22 (third column) in HEK293-hY_4R-EYFP cells. 30 min pre-incubation with unconjugated Pam (fourth column) or PEG22 (fifth column) show also unimpaired TAMRA-hPP (**1B**) internalization after 60 min. b) Internalization of TAMRA-labeled lead peptide (**2B**) after 60 min in presence of unconjugated, soluble Pam (second column) or PEG22 (third column) in HEK293-HA-hY_2R-EYFP cells. a, b) First columns display fluorescence of TAMRA (upper panel) and hYR (second panel) prior to stimulation (w/o). Bottom panels show the overlay of peptide (magenta) and receptor (green). c) Intracellular uptake of TAMRA-modified peptide analogues (**TAMRA-pNPY**, **2B**, **3B**, **5B**$^{\#}$) in HEK293-hY_1R-EYFP cells. Internalization before (first column) and after agonist exposure for 60 min is shown. Last two columns comprise co-stimulation experiments illustrating that **pNPY** induces strong hY_1R internalization after 60 min (receptor panel) without uptake of modified TAMRA-peptides **3B** and **5B**$^{\#}$ (peptide panel). White areas in the merged images indicate co-localization of receptor (green) and TAMRA-modified peptides (magenta). Agonist concentration was 100 nM for all compounds except for **5**$^{\#}$ which was 1 µM. Scale bar: 10 µm. d) Uptake of **3B** in HEK293-hY_4R-EYFP cells was competed by 10-fold excess of **2A**.

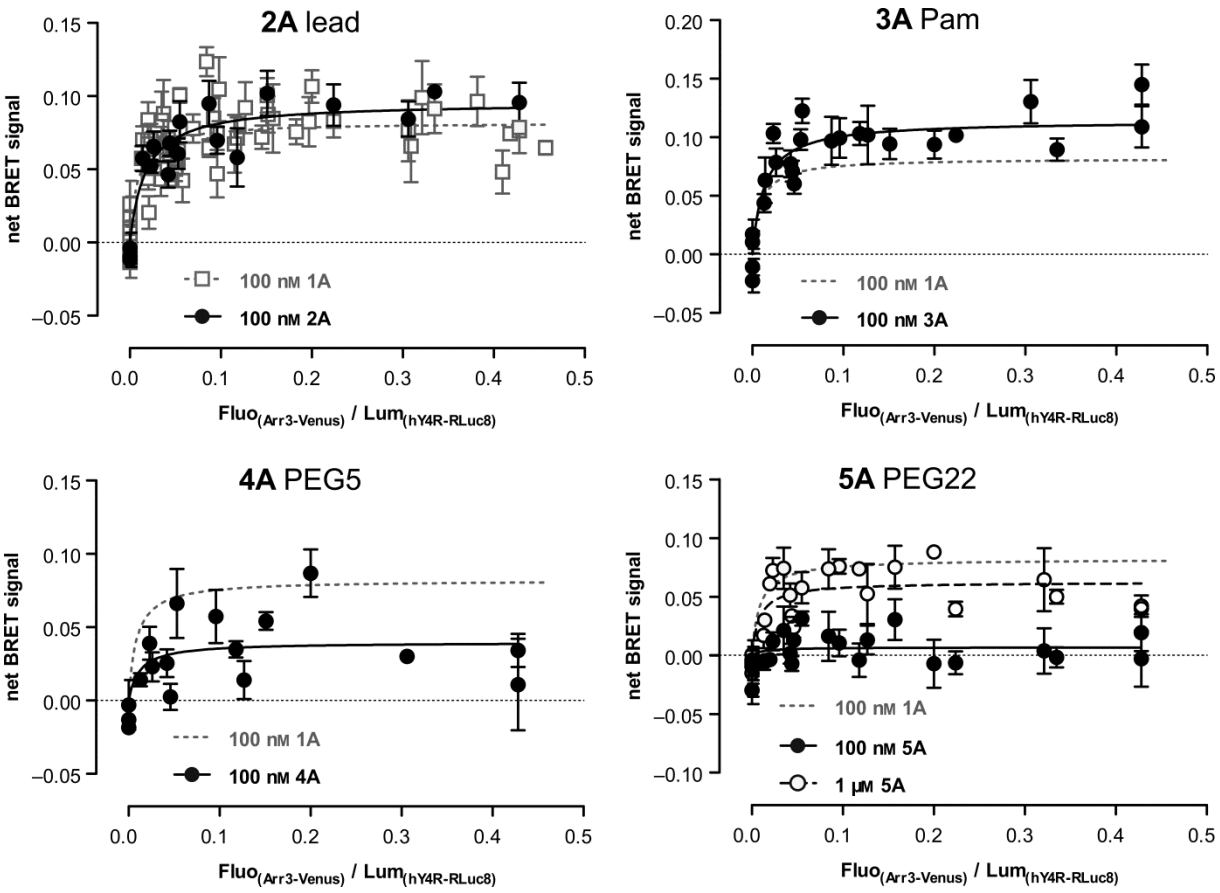
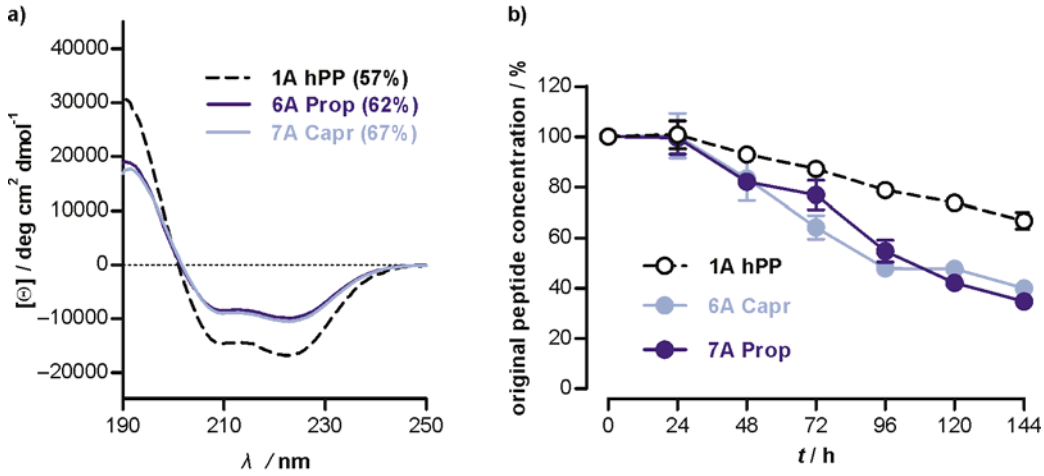


Figure S5: Arr3 recruitment induced by [K^{22},Q^{34}]hPP analogues, as determined by BRET assay. Graphs show averaged values ± S.E.M. of at least three independently performed experiments and corresponding saturation curve. The curve for each compound is shown in black. For comparison, the binding curve of hPP is shown as a dashed grey line.



c)

Cpd.	Activation				Binding
	hY_1R	hY_2R	hY_4R	hY_5R	hY_4R
	EC_{50}/nM (pEC_{50} ± S.E.M.)	EC_{50}/nM (pEC_{50} ± S.E.M.)	EC_{50}/nM (pEC_{50} ± S.E.M.)	EC_{50}/nM (pEC_{50} ± S.E.M.)	IC_{50}/nM (pIC_{50} ± S.E.M.)
6A	577.8 (6.2 ± 0.1)	3.5 (8.5 ± 0.1)	0.8 (9.1 ± 0.2)	14.3 (7.8 ± 0.1)	3.0 (8.5 ± 0.1)
7A	770.8 (6.1 ± 0.1)	2.2 (8.7 ± 0.1)	0.8 (9.1 ± 0.1)	49.2 (7.3 ± 0.2)	2.7 (8.6 ± 0.2)

Figure S6: Characterization of modified peptides bearing propionic (Prop) or caprylic (Capr) acids at position 22. a) CD spectroscopy and b) metabolic stability were performed as described in Figure S1. c) Potency of analogues was calculated from concentration-response curves as mentioned in Figure S2.

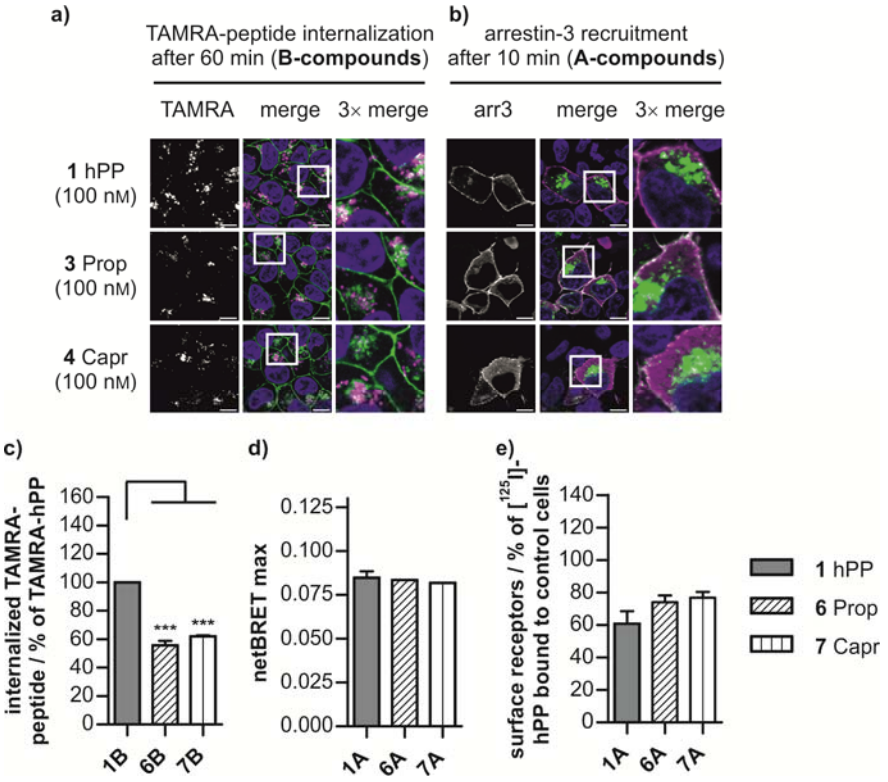


Figure S7: Influence of modification with shorter fatty acids on hY_4R internalization and arr3 recruitment. a) Life-cell imaging of TAMRA-peptide uptake and b) arr3 redistribution as described in the main text. c) Quantification of peptide uptake, d) arr3 recruitment and e) decline in cell surface receptors upon stimulation with 100 nM **6A** and **7A** as described in the main article. While peptide uptake was significantly reduced, arr3 recruitment and receptor internalization was not changed with respect to the native hPP.

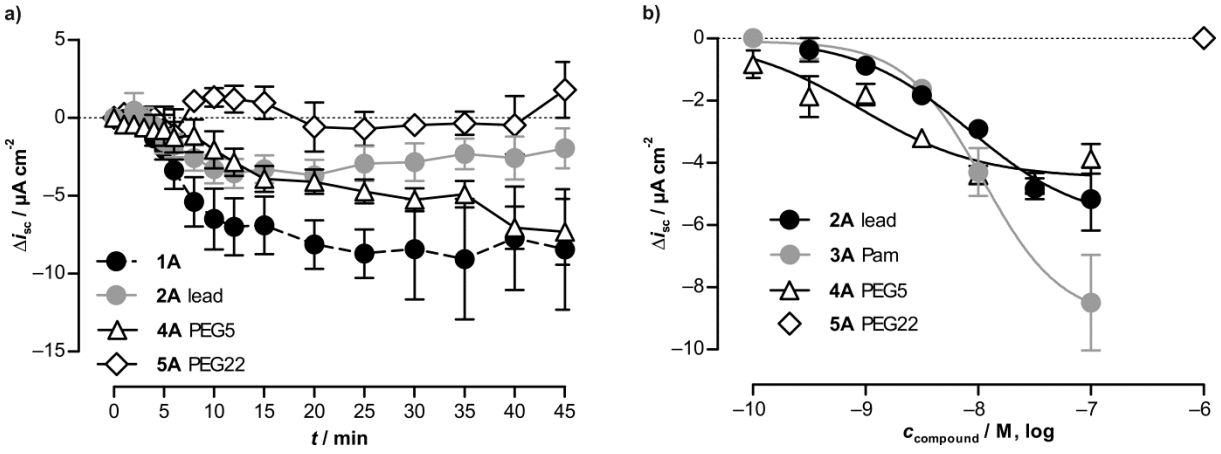


Figure S8: a) Time-dependent responses to [K^{22},Q^{34}]hPP compounds in human colon mucosa. Reductions in I_{sc} in response to 100 nM hPP (**1A**) or modified peptides (**2A**, **4A**, **5A**) are shown for a period of 45 min. Each value is the mean ± S.E.M. (n≥3). b) Concentration-response curves obtained after 5 min (**2A**) or 45 min (**3A, 4A, 5A**) in human Col-24 epithelial layers (EC_{50} in nM: **2A**: 8.4, **3A**: 11.0, **4A**: 0.9, **5A**: inactive).

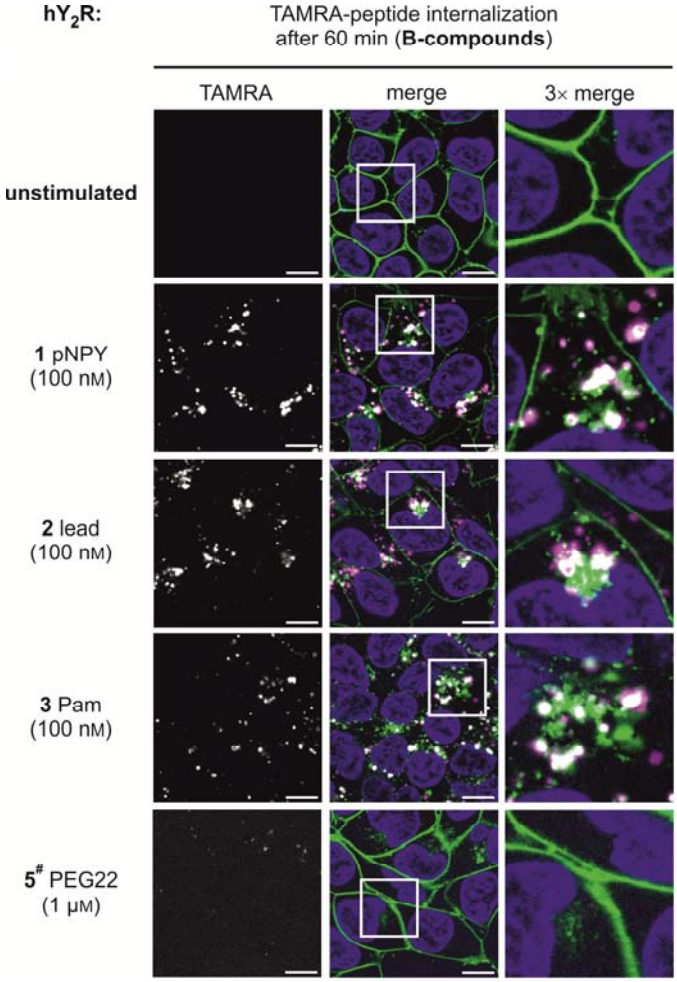


Figure S9: Internalization of [K^{22},Q^{34}]hPP analogues in HEK293 cells stably expressing hY_2R. Intracellular uptake of TAMRA-modified peptide analogues before (upper panel) and after agonist exposure for 60 min is shown. White areas in the merged channel demonstrate co-localization of receptor (green) and peptides (magenta). Agonist concentration was 100 nM for all compounds except for **5**$^{\#}$, which was 1 µM. Scale bar: 10 µm, white boxes in merged pictures indicate magnified detail.

Supplementary Tables:

Table S1: Analytical data of synthesized [K^{22},Q^{34}]hPP analogues.

	Compound	$M_{calc.}$ / Da	$M_{obs.}$ / Da[a]	Elution in % of B in A[b]		Purity / %	Yield / %	
				C_{18} 90Å[c]	C_{18} 300Å[d]		raw	purified
	pNPY	4251.1	4252.1	44.1	46.3	>99	44.3	19.2
1A	hPP	4179.1	4180.0	44.3	46.0	>99	41.3	17.9
2A	[K^{22},Q^{34}]hPP	4268.1	4269.0	46.8	48.7	>96	61.4	17.6
3A	[K^{22}(E-Pam),Q^{34}]hPP	4634.4	4635.4	55.6	61.5	>99	46.1	9.7
4A	[K^{22}(PEG5),Q^{34}]hPP	9268	9230	47.2	47.4	>88	59.8	9.7
5A	[K^{22}(PEG22),Q^{34}]hPP	26268	26106	51.3	49.7	>90	70.2	7.0
6A	[K^{22}(E-Prop),Q^{34}]hPP	4452.2	4453.2	45.4	47.1	>99	50.3	12.4
7A	[K^{22}(E-Capr),Q^{34}]hPP	4522.3	4523.3	47.5	49.6	>99	44.0	9.5

[a] Peptides were identified by MALDI-MS (matrix-assisted laser desorption/ionization-mass spectrometry), observed masses ($M_{obs.}$) correspond to molecule ion peaks ($[M+H]^+$). [b] RP-HPLC was performed for peptide characterization. Concentrations of eluent B (0.08% (*v/v*) TFA in acetonitrile) in A (0.1% (*v/v*) TFA in water) at the point of peptide elution from different analytical HPLC columns are shown. Purity was determined by using these two different columns: [c]: Jupiter 4u Proteo 90 Å, C18 (4 µm), Phenomenex, 250 × 4.6 mm; [d]: Jupiter 5u Proteo 300 Å, C18 (5 µm), Phenomenex, 250 × 4.6 mm.

Table S2: Analytical characterization of synthesized TAMRA-[K^{22},Q^{34}]hPP compounds.

	Compound	$M_{calc.}$ / Da	$M_{obs.}$ / Da[a]	Elution in % of B in A[b]		Purity / %	Yield / %	
				C_{18} 90Å[c]	Polym. 200Å[d]		raw	purified
	TAMRA-pNPY	4663.3	4664.5	45.8	42.1	>99	64.0	6.8
1B	TAMRA-hPP	4591.2	4592.3	46.9	45.5	>98	44.3	12.2
2B	TAMRA-[K^{22},Q^{34}]hPP	4679.3	4680.3	46.2	43.3	>84	40.2	12.7
3B	TAMRA-[K^{22}(E-Pam),Q^{34}]hPP	5046.6	5047.5	60.4	53.1	>84	34.6	11.6
4B	TAMRA-[K^{22}(PEG5),Q^{34}]hPP	9679	9604	48.7	45.0	>91	72.3	19.3
5B	TAMRA-[K^{22}(PEG22),Q^{34}]hPP	26679	26609	51.0	46.9	>99	67.1	24.6
6B	TAMRA-[K^{22}(E-Prop),Q^{34}]hPP	4864.4	4865.3	50.6	45.3	>92	33.8	11.1
7B	TAMRA-[K^{22}(E-Capr),Q^{34}]hPP	4934.5	4935.5	52.7	46.9	>88	35.6	13.4

[a] Peptides were identified by MALDI-MS, $M_{obs.}$ corresponds to $[M+H]^+$. [b] RP-HPLC was performed for peptide characterization. Concentrations of eluent B in A at the point of peptide eluation from different analytical HPLC columns are shown. Purity was determined by using these two different columns: [c]: Jupiter 4u Proteo 90 Å, C18 (4 µm), Phenomenex, 250 × 4.6 mm; [d]: VariTide RPC 200 Å, 6 µm, Varian, 250 × 4.6 mm.

2. Experimental Section

Peptide synthesis:

All peptides were prepared by a combination of automated and manual solid-phase peptide synthesis (SPPS; 15 µmol scale) applying orthogonal Fmoc (9-fluorenylmethoxy-carbonyl)/*t*Bu protecting group strategy. Activation of the N^{α}-Fmoc-amino acids (Iris Biotech) carboxylic groups was performed with HOBt (1-hydroxybenzotriazole; Novabiochem) or Oxyma Pure (Iris Biotech)/DIC (*N,N′*-diisopropylcarbodiimide; Iris Biotech) on a Rink amide AM (Iris Biotech) or NovaSyn TGR R resin (Novabiochem) to achieve an amidated C-terminus. For robot-assisted SPPS, 8 eq Fmoc-protected amino acid was activated in situ with equimolar amounts of OxymaPure (2 min pre-incubation on resin) and DIC and reacted twice in DMF (*N,N*-dimethylformamide; Biosolve; 2 × 30 min). Automated Fmoc deprotection was carried with 40% (*v/v*) piperidine (Sigma-Aldrich) in DMF for 3 min and 20% (*v/v*) piperidine in DMF for 10 min. Chemical stabilization building blocks were introduced into [Q^{34}]hPP with the aid of the N^{ε}-group of lysine at position 22 (compound set **A** and **B**) or position 13 (compound set **C** and **D**). Synthesis and analytical characterization of **C**- and **D**-compounds was described in Bellmann-Sickert et al.,[1] whereas **A**- and **B**-compounds were prepared with minor modifications.

For lipidated peptides, the sequence was synthesized automatically until amino acid position 23 with a glutamine at position 34. After that, manual coupling of 5 eq Dde-L-Lys(Fmoc)-OH for 3 h using HOBt/DIC (5 eq each) in DMF was performed, followed by Fmoc removal with 10% DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene; Aldrich)/piperidine (*v/v*, 2 min and 10 min) in DMF and introduction of Fmoc-L-Glu-O*t*Bu (5 eq of amino acid, HOBt and DIC) in DMF with its carboxy-side chain to the N^{ε}-group of lysine at position 22. The N-terminal Fmoc group of this glutamate linker was cleaved with 10% DBU/piperidine (*v/v*) in DMF and 5 eq fatty acid-coupling (propionic, caprylic (octanoic), or palmitic (hexadecanoic) acid: Sigma-Aldrich) for 3 h with HOBt/DIC (5 eq) in DMF or NMP (*N*-methyl-2-pyrrolidone; Biosolve) was applied. After Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) removal by hydrazine (Sigma-Aldrich) treatment,[1-2] peptide elongation with an automated multiple synthesizer followed. Finally, the Fmoc group of the N-terminal amino acid was cleaved and the peptide was either cleaved from resin or incubated with 3 eq 6-TAMRA (6-carboxytetramethylrhodamine; emp Biotech) employing equimolar amounts of HATU (O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate; Novabiochem) and DIPEA (*N,N*-diisopropylethylamine; Sigma-Aldrich) in DMF for 3 h. After fluorophore coupling, deprotection of side chains and cleavage from resin was performed with a mixture of TFA (trifluoroacetic acid; Sigma-Aldrich), EDT (1,2-ethanedithiol; Sigma) and TA (thioanisole; Fluka) (90:7:3, *v/v/v*) for 3 h. All peptides were additionally incubated with TFA/TMSBr (Bromotrimethylsilane; Fluka)/EDT (97.2:1.6:1.2, *v/v/v*) for 30 min in order to reduce partially oxidized methionine residues. Purification of crude products was performed by preparative RP-HPLC on a Phenomenex column (Jupiter 10u Proteo 90 Å, C_{18}, 7.78 µm, 250 × 21.2 mm) applying linear gradients dependent on the peptide hydrophobicity with a flow rate of 10 ml min^{-1} (eluents: water with 0.1% (*v/v*) TFA and ACN (acetonitrile; VWR)

with 0.08% (*v/v*) TFA). Compounds were identified with MALDI MS and purity was confirmed by analytical RP-HPLC using different column systems (Jupiter 4u Proteo 90 Å (Phenomenex), C_{18}, 4 µm, 250 × 4.6 mm; Jupiter 5u Proteo 300 Å (Phenomenex), C_{18}, 5 µm, 250 × 4.6 mm; VariTide RPC 200 Å (Varian), polymer, 6 µm, 250 × 4.6 mm). Analytical data of all **A**- and **B**-compounds are summarized in Table S1 and S2.

Synthesis of PEGylated peptide conjugates was performed by automated multiple SPPS with a lysine at position 22 and glutamine at position 34, followed by Fmoc removal with 40% (*v/v*) piperidine in DMF and N-terminal coupling of 5 eq of the photocleavable Nvoc (6-nitroveratryloxycarbonyl; Aldrich)-group utilizing 5 eq HOBt and 10 eq DIPEA in the dark according to Koglin et al.[2] N-terminal 6-TAMRA labeling was performed as reported for lipidated peptides. Peptides were subsequently cleaved from resin, reduced and purified preparatively following the recently described protocols.[3] PEGylation with OMe-PEG5- or OMe-PEG22-NHS (monomethoxy-polyethylene glycol-*N*-hydroxysuccinimide with poly-disperse PEG of 5 (PEG5) or 22 kDa (PEG22); Iris Biotech) was carried out in solution by diluting peptide in DMF and incubating 2 eq PEG5 or PEG22, DMAP (4-dimethylaminopyridine; Aldrich) and DIC in DMF overnight in the dark. Then, peptides were precipitated by addition of 10 ml ice-cold diethylether. After 10 × washing, Nvoc cleavage was performed for non-fluorescently labeled peptides by irradiation with UV-light (366 nm) for 3 h[2] and finally, peptides were purified preparatively and analyzed as described above.

Circular dichroism spectroscopy:

CD measurements were performed with 10 µM peptide conjugate solutions in 10 mM sodium phosphate buffer (Sigma-Aldrich, Merck) at pH 7.1 applying a Jasco-715 spectropolarimeter with a constant nitrogen stream. Spectra in the far UV region between 190 nm and 250 nm were measured in a cuvette of 2 mm path length and following parameters: sensitivity of 100 mdeg, data pitch of 0.5 nm, continuous scanning mode, 50 nm min^{-1} scanning speed, 4 s response, 2 nm bandwidth, temperature of 25 °C and 6 accumulations. The compound concentration was calculated from aromatic absorption (tyrosine residues) of peptides in aqueous solution using the molar extinction coefficient at 280 nm (ε = 5960 M^{-1} cm^{-1}). Baseline-corrected averaged spectra were converted into mean residue molar ellipticity [Θ] given in deg cm^2 $dmol^{-1}$ by the formula: $[\Theta] = (\Theta \times M) / (10 \times c \times l \times n)$, where Θ is the ellipticity in mdeg, M is the compound molar mass in g mol^{-1}, c is the concentration in mg ml^{-1}, l is the pathlength of the cuvette in cm and n is the number of peptide bond residues.[4] Secondary structure estimation was performed by *http://dichroweb.cryst.bbk.ac.uk/html/home.shtml* applying K2D. CD measurements were independently performed twice.

Stability studies in human blood plasma:

Proteolytic degradation of fluorescently modified peptide analogues was examined by incubation with human plasma, as described previously with slight modifications.[1] Prior to incubation, 15 nmol TAMRA-peptide was freshly reduced with TFA/TMSBr/EDT (92:4:4,

v/v/v) for 30 min, precipitated from 1 ml ice-cold ether for 20 min, washed three times with ether and dried in vacuo. Then, peptide aliquots were diluted to 10 µM in human plasma and incubated at 37 °C with shaking (500 rpm). Samples were taken at indicated time points (0, 24, 48, 72, 96, 120 and 144 h) and proteins were precipitated with equal volumes of ACN/EtOH (Applichem) (1:1, *v/v*) at -20 °C for at least 3 h. After centrifugation, supernatants were processed in Costar® Spin-X® tubes (Corning; 0.22 µm membrane pore size) for HPLC analysis at a VariTide column with fluorescence detection (λ_{exc}: 525 nm, λ_{em}: 572 nm). Degradation curves were independently determined twice.

Plasmids:

Coding sequences of hY_1R, hY_2R, hY_4R and hY_5R were C-terminally fused to EYFP (enhanced yellow fluorescent protein) and cloned into the pVitro2-hygro-mcs (Invivogen) and the pEYFP-N1 expression vectors (Clontech), as described.[5] For the HA-(hemagglutinin)-tagged hY_2R, nine residues (YPYDVPDYA) were added after the initiation codon. The pcDNA3 vector with hY_4R C-terminally fused to the *Renilla* luciferase variant 8 (RLuc8) was used. The hY_2R sequence was C-terminally fused to mCherry and cloned into the pVitro2-hygro-mcs vector. Bovine arr3 was N-terminally tagged with Venus and cloned into the pcDNA3 vector for BRET studies, as described.[6] For live-cell imaging, bovine arr3 was cloned into mCherry-NE/S vector.[6] $G\alpha_{\Delta 6qi4myr}$, generated as described,[7] was kindly provided by E. Kostenis. Vectors encoding DsRed-ER and DsRed-monomer-Golgi marker were purchased from Clontech. All constructs were verified by sequencing.

Cell culture:

All cell lines were grown in a humidified atmosphere at 37 °C and 5% CO_2 to confluence prior to use.

Stable cell lines. HEK293-hY_1R-EYFP, HEK293-HA-hY_2R-EYFP, HEK293-hY_4R-EYFP cell lines were generated using linearized $hY_{1/2/4}$-EYFP-encoding pVitro2-hygro-mcs vectors as described,[5b] and maintained in DMEM (Dulbecco's modified Eagle's medium) with 4.5 g l^{-1} glucose and L-glutamine and Ham' F-12 (1:1, *v/v*; PAA) supplied with 15% (*v/v*) heat-inactivated FCS (fetal calf serum; PAA) and 100 µg ml^{-1} hygromycin B (Invivogen). COS-7 cells were co-transfected with 2 µg of each linearized $hY_{1/2/4/5}$-EYFP-pVitro2-hygro-mcs vector and 2 µg of linearized $G_{\Delta 6qi4myr}$-pVitro2-neo-mcs using 12 µl Metafectene® transfection reagent (Biontex) according to manufacturer's protocol. Three days after transfection, selection was started using 1.5 mg ml^{-1} G418-sulfate (Amresco) and 146 µg ml^{-1} hygromycin B. Cell lines were raised from single colonies. Cells were maintained in DMEM with 4.5 g l^{-1} glucose containing L-glutamine and 10% (*v/v*) heat-inactivated FCS, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (Invitrogen), 1.5 mg ml^{-1} G418-sulfate and 146 µg ml^{-1} hygromycin B. All HEK and COS cell lines were tested with respect to receptor binding and activation using competition binding and inositol phosphate accumulation assays, respectively.

Other cell lines HEK293 cells were maintained in DMEM with 4.5 g l^{-1} glucose and L-glutamine and Ham's F-12 (1:1, *v/v*) supplemented with 15% (*v/v*) heat-inactivated FCS.

Colony-24 (Col-24; a human colonic adenocarcinoma cell line that constitutively expresses hY_4R)[8] cells were grown in DMEM with 4.5 g l^{-1} glucose and L-glutamine (PAA) supplemented with 10% (*v/v*) FCS, 1.2 µg ml^{-1} amphotericin B (Sigma) and 100 µg ml^{-1} kanamycin (Sigma) in cell culture flasks or on collagen-coated Millipore filters, as described in detail previously.[9]

Receptor activation:

For signal transduction (inositol phosphate accumulation) assays, stably transfected COS-7 cells were seeded into 48-well plates (70,000 cells per well) and grown, labeled and stimulated following a recently published protocol.[3] Briefly, after 24 h cells were labeled with 2 µCi ml^{-1} myo-[2-^{3}H]-inositol (Perkin-Elmer) in culture medium without penicillin and streptomycin for at least 16 h. Then cells were washed and incubated with peptides at concentrations 10^{-4}-10^{-12} M (depending on potency) in DMEM with 4.5 g l^{-1} glucose and L-glutamine containing 10 mM LiCl (Sigma) for 1 h at 37 °C. Radioactive phosphoinositides were isolated according to Hofmann et al.[3] and Findeisen et al.,[10] and measured by a scintillation counter. EC_{50} and pEC_{50} values were calculated from concentration-response curves using non-linear regression (*GraphPad Prism 5.0*). Assays were performed in duplicate in at least two independent experiments.

Receptor binding:

For competitive binding experiments, 50,000 HEK293 cells stably expressing EYFP-tagged hY_4R (HEK293-hY_4R-EYFP) were resuspended in MEM (minimal essential medium without L-glutamine; PAA) supplemented with 5 mM pefabloc SC (Fluka) and 1% (*v/v*) BSA (bovine serum albumin fraction V; PAA). Peptide solutions were prepared in water with 1% (*v/v*) BSA to obtain final concentration of 10^{-5}-10^{-12} M (depending on affinity). [^{125}I]-hPP (Perkin-Elmer), diluted in water containing 1% (*v/v*) BSA, was added at final concentration of 60 pM (calculation based on specific radioactivity; corresponds to K_d).[11] Cells were incubated with radioligand, and with or without cold ligands for 60 min at room temperature by shaking. After centrifugation at 4 °C, cell pellets were washed three times with ice-cold PBS (phosphate buffered saline; PAA), resuspended in 100 µl PBS, mixed with scintillation cocktail (Perkin-Elmer) and the radioactivity was measured in a scintillation counter. IC_{50} and pIC_{50} values were calculated from concentration-dependent displacement of [^{125}I]-hPP (*GraphPad Prism 5.0*). For each ligand, at least two independent experiments were performed in triplicate for total and non-specific binding.

Remaining surface receptors after internalization were measured by specific radioligand binding. HEK293-hY_4R-EYFP cells were seeded into poly-D-lysine (in PBS; Sigma) pre-coated 48-well plates (200,000 cells per well) and after 24 h, cells were starved for 30 min with Opti-MEM reduced serum medium (Gibco) at 37 °C. Cells were pre-stimulated with 100 nM of all hPP analogues for 60 min at 37 °C in Opti-MEM containing 6 µg ml^{-1} BFA (brefeldin A dissolved in dimethylsulfoxide; Santa Cruz) and 100 µg ml^{-1} CHX (cycloheximide dissolved in EtOH; Calbiochem). After three washes to remove non-

internalized peptide from the cell surface (2 × acidic wash with 50 mM glycine (Serva) and 100 mMNaCl (Sigma-Aldrich) at pH 3.1, 1 × HBSS (Hank's balanced salt solution; PAA)), cells were placed on ice. Cell surface binding was measured with 60 pM [^{125}I]-hPP for 2 h on ice in DMEM supplemented with 4.5 g l^{-1} glucose and L-glutamine, 5 mM pefabloc SC, 1% (*v/v*) BSA, 6 µg ml^{-1} BFA and 100 µg ml^{-1} CHX. Non-specific binding was determined in presence of 100 nM cold hPP. After incubation, cells were washed three times with ice cold PBS to remove unbound radioligand, lyzed with 0.2 M NaOH (Grüssing), mixed with scintillation cocktail and radioactivity was measured in a scintillation counter. Specific binding was determined in triplicate in four independently performed experiments.

BRET assay:

BRET assay was used to measure the arr3-hY_4R-interaction induced by individual hPP derivatives. HEK293 cells were seeded into a 25 cm^2 flask and transiently co-transfected with hY_4R-Luc (130 ng) and increasing amounts of Venus-arr3 (0-8 µg) using Metafectene® transfection reagent according to manufacturer's protocol. 24 h post-transfection, cells were re-seeded into poly-D-lysine-coated black (for fluorescence) or white (for luminescence) clear-bottom 96-well plates using phenolred-free HEK293 medium (Invitrogen). 48 h post-transfection, the medium was replaced with HBSS buffer supplemented with 25 mM HEPES (4-(2-hydroxethyl)-1-piperazineethanesulfonic acid; Merck), pH 7.3. Venus fluorescence was determined at 488 nm excitation and 530 nm emission wavelength. For BRET, cells were stimulated with 100 nM to 1 µM of respective hPP compounds or vehicle (unstimulated cells) for 8 min. Coelenterazine h (DiscoveRx) was added to a final concentration of 4.2 µM and incubated for further 2 min. After 10 min of stimulation, fluorescence and luminescence were measured at 37 °C with a Tecan infinite M 200 reader using filter set Blue1 (luminescence 370-480 nm) and Green1 (fluorescence 520-570 nm). NetBRET was determined by subtracting BRET signals of unstimulated cells from stimulated samples. The relative change in netBRET was plotted as a function of Venus fluorescence, which was normalized by the basal luminescence of hY_4R-Luc to account for variability in cell number and transfection efficiency. Resulting hyperbolic curves were obtained by non-linear regression. All experiments were performed in duplicate, at least three times.

Live-cell fluorescence microscopy:

HEK293-hY_4R-EYFP cells were grown on µ-slide 8 wells (ibidi) to full confluence overnight. Empty HEK293 cells were grown on µ-slide 8 wells to 70% confluence overnight and subsequently transfected using Lipofectamine® 2000 (Invitrogen), as recommended by the manufacturer. For single transfection, 1 µg receptor DNA and 1 µl Lipofectamine® 2000 per well was used. For co-transfection, cells were incubated with 0.9 µg receptor DNA and 0.1 µg of arr3-mCherry-N1 using 1 µl Lipofectamine® 2000 per well.
Fluorescence microscopy was performed with a Zeiss Axio Observer.Z1 inverted microscope equipped with an ApoTome Imaging System and a Heating Insert P Lab-Tek S1 unit, as described.[5b] To visualize fluorescence-labeled peptide internalization as well as arr3 recruitment in living cells, cells were starved for 30 min at 37 °C with Opti-MEM containing

Hoechst33342 (Sigma) for nuclei staining. In order to observe intracellular uptake of TAMRA- or CF-compounds, stable HEK293-hY_4R-EYFP or transiently transfected HEK293 cells were stimulated with 100 nM or 1 µM modified peptide analogues, or a combination for competition experiments, for 60 min. For TAMRA-peptides, cells were washed to remove non-internalized peptide (1 × HBSS for 2 min at 37 °C, 1 × acidic wash for 30 s at RT, 2 × HBSS for 2 min at 37 °C, 1 × Opti-MEM; for **5B**: 1 × HBSS for 2 min at 37 °C, 2 × 1 µM palmitic acid (Sigma-Aldrich)-solution (in HBSS) for 2 min at 37 °C, 1 × HBSS for 2 min at 37 °C, 1 × Opti-MEM). For CF-peptides, cells were washed twice with acidic wash and once with HBSS. Afterwards, images were taken with the same settings and light exposure times. For quantification of the HEK293-hY_4R-EYFP, the same procedure was performed for HEK293 cells not expressing the hY_4R. Intracellular raw intensity density of four independent full images (image section: 1300 × 1030 pixel) per peptide was measured by *ImageJ* for TAMRA-peptides. Corresponding background (HEK293 cells treated in the same manner as HEK293-hY_4R-EYFP cells) was subtracted and referred to % of mean of TAMRA-hPP. Due to the transient transfection of HEK293 cells, CF-peptide uptake was quantified by measuring the fluorescence intensity within one cell relative to the cell area. At least five cells were measured in three independently performed experiments. Arr3 recruitment was visualized with transiently transfected HEK293 cells which were stimulated for 10 min with 100 nM or 1 µM peptide solution at 37 °C.

For Col-24-hY_4R-EYFP imaging, cells (600,000 cell per well) were transiently transfected with 1 µg hY_4R-EYFP-pVitro2 in suspension and seeded into µ-slide 8 wells pre-coated with 25 µg ml^{-1} fibronectin (Sigma-Aldrich; in PBS). Internalization of TAMRA-compounds was measured as described above. Quantification was performed by fluorescence intensity measurement of at least two cells per image for each peptide. Corresponding background (untreated Col-24 cells) was subtracted and referred to % of mean of TAMRA-hPP (**1B**), which was set to 100%. If not described otherwise, all imaging experiments were independently performed twice.

Additional live-cell images were obtained as follows. HEK293 cells were co-transfected with 250 ng Golgi-dsRed-N1 or 250 ng ER-dsRed-N1 and 500 ng hY_4R-EYFP-pVitro2 in order to observe the localization of hY_4R in intracellular compartments. Fluorescence microscopy was used as described without peptide stimulation. TAMRA-peptide internalization at HEK293 cells stably expressing the hY_2R (HEK293-HA-hY_2R-EYFP cells) was carried out as specified without peptide removal after 60 min. To ensure that chemical modifications have to be covalently attached to the peptide, non-modified peptide analogues together with unattached Pam and PEG moieties were pre-incubated at room temperature for 30 min. Cells were then stimulated with these solutions as described above.

Electrogenic ion transport measurement in Col-24 epithelial monolayers:

Col-24 epithelial layers were grown on individual filters and mounted between the hemi-chambers of an Ussing chamber with an exposed area of 0.2 cm^2 for the measurement of vectorial ion transport. Reservoirs bathing either surface of these polarized monolayers contained oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer (in mM: NaCl 118, KCl 4.7,

$NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, D-glucose 11.1; pH 7.4), and were maintained at 37 ºC. Preparations were voltage-clamped at 0 mV and resulting I_{sc} was measured continuously as described.[8-9] After 15-20 min equilibration, Col-24 layers were pre-treated with the secretagogue vasoactive intestinal polypeptide (VIP, 30 nM, all drug additions were basolateral) and once the VIP-elevated I_{sc} had stabilized, single concentrations of hPP or analogues were added and their responses monitored for up to 45 min. Peak changes in I_{sc} were calculated and expressed as μA cm^{-2}.
To assess the desensitization of the hY_4R response, two sequential additions of a near-maximal concentration of hPP (10 nM, 25 min apart, controls) or addition of the agonist of choice followed 25 min later by hPP (10 nM) were used. Peak reductions in I_{sc} to hPP after the different initial agonist additions were pooled and the means ± S.E.M. are shown. Concentration-response curves were analyzed by non-linear regression to obtain mean EC_{50} values (*GraphPad Prism 5.0*).

Electrogenic ion transport measurement in human colon mucosae:

Macroscopically normal human colon specimens were obtained from consenting patients undergoing bowel resection surgery for primary intestinal carcinoma, as described previously.[8] The study was approved by Guy's and St. Thomas' NHS Foundation Trust (REC ref: 12/LO/0069). Tissue was bathed in Krebs-Henseleit buffer and transported to the lab where mucosa was separated from the overlying muscle layers by blunt dissection. Mucosal preparations were mounted in Ussing chambers with an exposed area of 0.64 cm^2 or 0.14 cm^2. The activity of analogues was investigated on basal I_{sc} levels, which were sufficiently high in human colon mucosa for clear inhibitory responses to be measured (as seen previously).[8]

Statistical analysis:

Nonlinear regression, sigmoidal concentration-response fitting as well as calculations of means, S.E.M. and statistical analyses were performed using the *GraphPad Prism 5.0* software. Significances were calculated by ANOVA and Dunnett's post-hoc test or unpaired *t*-test with * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

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