Supplementary Information

Parathyroid hormone senses extracellular calcium to modulate endocrine signaling upon binding to the family B GPCR parathyroid hormone 1 receptor

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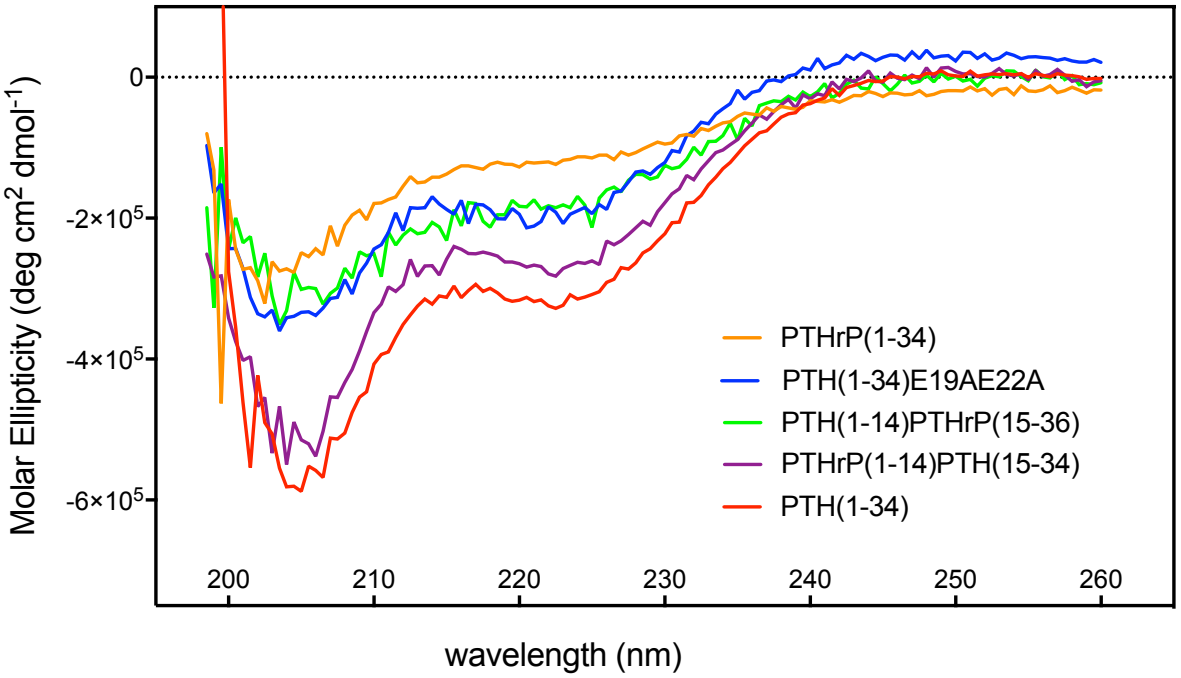
Supplementary Data

Circular dichroism spectroscopy shows the tested ligands have defined secondary structures. To measure if the chimeric and mutant ligands affected the peptide's secondary structure, we collected circular dichroism (CD) spectra of each of the five tested ligands (Figure S1). The observed spectra show expected characteristics of defined secondary structures. The CD spectra of the chimera, PTHrP(1-14)PTH(15-34), which does not bind to PTH1R-ND, shows similar helical content to PTH(1-34). In addition, the spectra of PTH(1-14)PTHrP(15-36) shows similar negative peaks as the PTHrP(1-34) spectra. Thus, the CD spectra show the chimeric and mutant ligands have a defined secondary structure that is influenced primarily by the identity of the C-terminal residues.



SI Figure S1: Chimeric and mutant peptides maintain their secondary structures

Chimeric and mutant ligands are able to activate PTH1R in mammalian cells. In order to test the bioactivity of all peptides under study, cell-based cAMP accumulation assays were performed. Upon ligand binding, PTH1R undergoes a conformational change that activates G proteins. The activated G protein then initiates a signaling cascade that causes cAMP production. Thus, cAMP production was measured in stable HEK293S cells overexpressing PTH1R. All of the peptides were able to activate PTH1R (SI Figure S2). Of the tested ligands, PTH(1-34) has the best potency with an EC_{50} = 0.3 ± 0.1 nM. PTH(1-14)PTHrP(15-36) and PTH(1-34)E19AE22A share similar potency values with EC_{50} = 1.7 ± 0.9 nM and EC_{50} = 3.7 ± 1.0 nM, respectively. PTHrP(1-36) and PTHrP(1-14)PTH(15-34), which shows no binding in our fluorescence anisotropy assay, both activate PTH1R at least 10 time worse, with EC_{50} = 9.4 ± 4.3 nM and EC_{50} = 12.0 ± 4.0 nM, respectively.

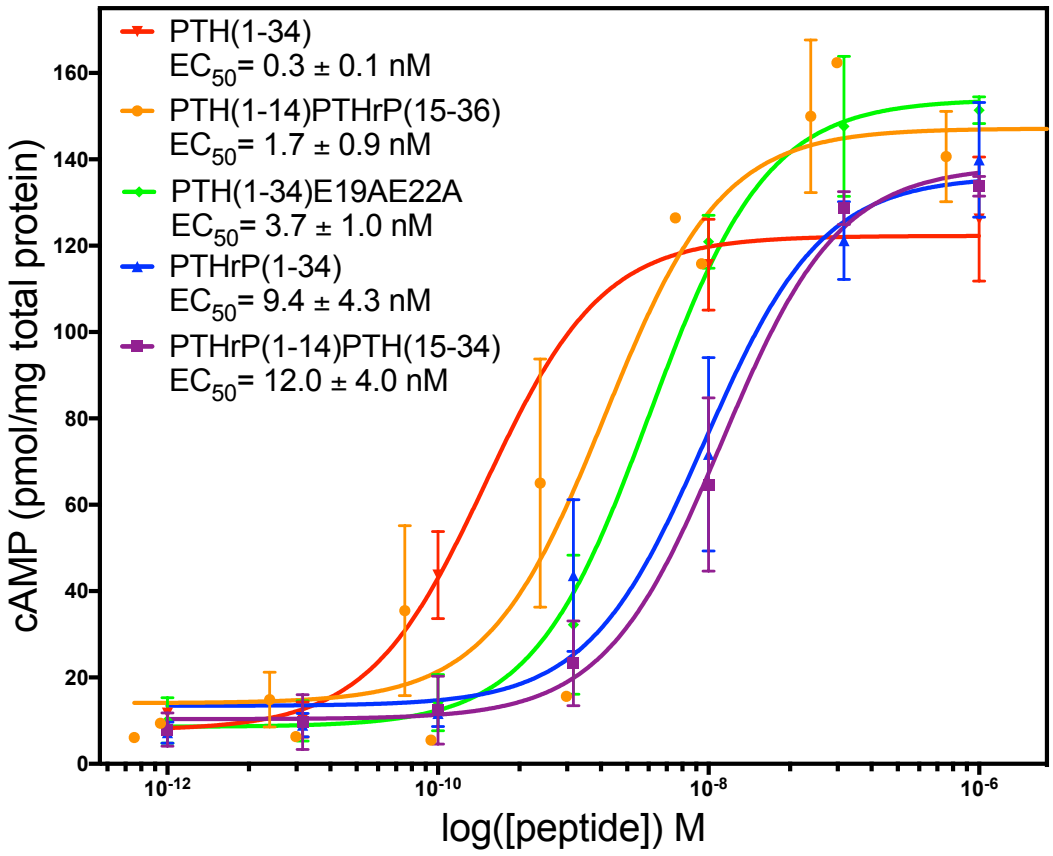
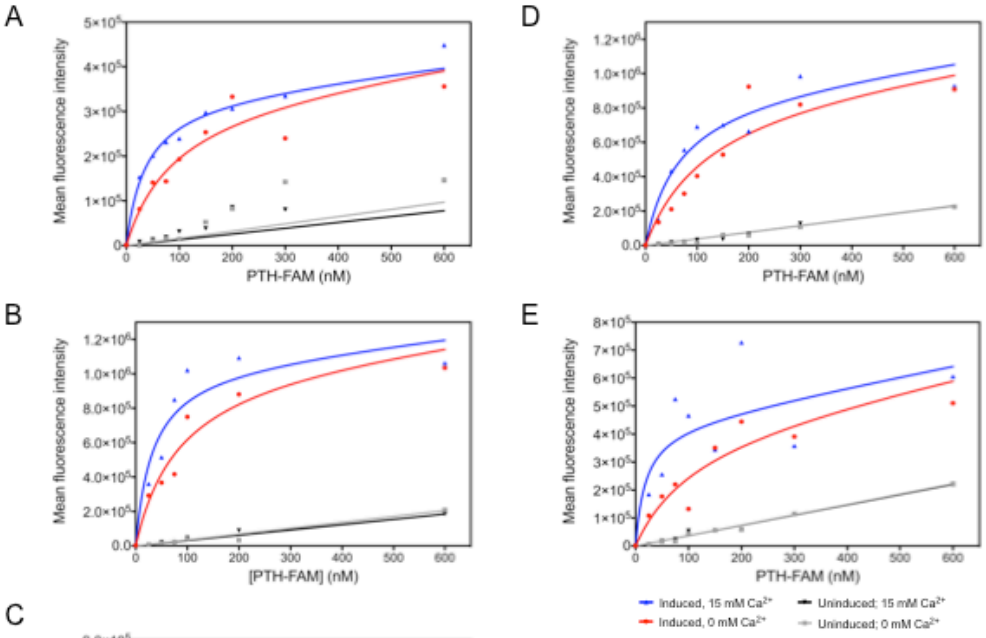


Figure S2: Chimeric and mutant peptides activate PTH1R to produce cAMP. HEK293S cells stably expressing PTH1R produce cAMP upon stimulation for 20 minutes with the mutant and chimeric ligands. Each point is an average of 3 different experiments with the error bars showing SEM.

Flow cytometry titrations. Titration curves were collected over multiple trials and each curve fitted to determine the average K_D (Figure S3). Fitting parameters and results are discussed in the main text. Each trial qualitatively shows that 15 mM Ca^{2+} decreases the K_D of PTH(1-34) for PTH1R expressed in HEK293S cells.



Trial	K_D 0 mM Ca^{2+} (nM)	K_D 15 mM Ca^{2+} (nM)
A	91 ± 35	31 ± 21
B	117 ± 27	64 ± 17
C	209 ± 52	25 ± 11
D	83 ± 18	33 ± 8
E	112 ± 44	19 ± 13
average	122 ± 50	34 ± 17

Figure S3: Flow Cytometry titrations of HEK293S cells. Increasing concentrations of PTH(1-34)-FAM were titrated with HEK293S cells induced to overexpress PTH1R in the presence (blue) and absence (red) of 15 mM Ca^{2+}. As a control PTH(1-34)-FAM was titrated with uninduced HEK293S cells in the presence (black) and absence (gray) of 15 mM Ca^{2+}. Each panel represents a single trial using the same batch of HEK293S cell culture performed on the same day. The fitted K_D values for each trial are shown in the table.

Glucagon-like peptide 1 receptor (GLP1R) purification and ligand binding. GLP1R is a family B GPCR expressed in pancreatic beta cells that is a validated drug target for type 2 diabetes.[1] GLP1R binds glucagon-like peptide 1 (GLP-1), which can be secreted as GLP-1 (7-37).[2] In addition, the 39 amino acid peptide exendin-4 (Ex-4) has been developed as a GLP1R agonist for diabetes treatment.[3] To determine if the Ca^{2+} sensing ability of PTH1R applies to other family B GPCRs, we purified GLP1R in nanodiscs for fluorescence anisotropy assays to test ligand binding in the presence and absence of 15 mM Ca^{2+}. Figure S4 shows the binding of GLP-1 and Ex-4 to GLP1R in nanodiscs. The addition of 15 mM Ca^{2+} does not significantly

affect the binding affinity of both GLP-1 and Ex-4. These results highlight the uniqueness of the built in Ca^{2+} sensing ability of PTH(1-34).

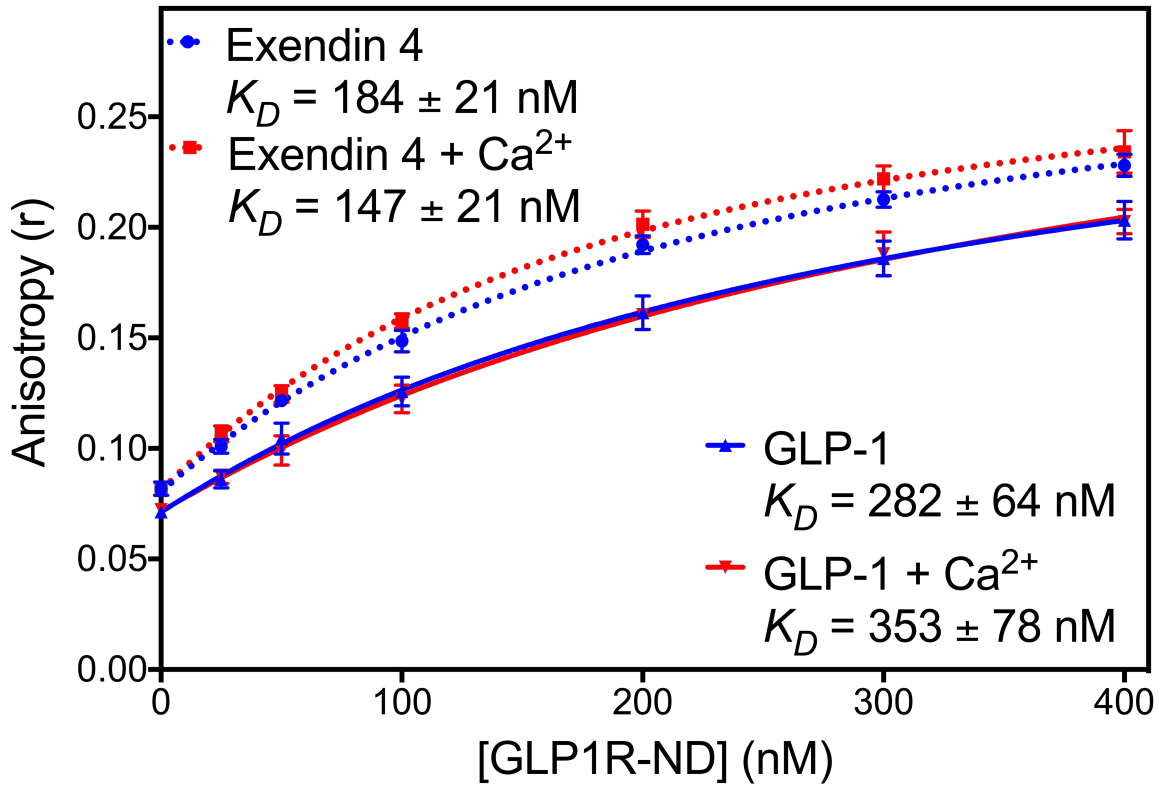


Figure S4: Ligand binding to GLP1R. Titration of 50 nM GLP-1 (solid lines) and 50 nM Exendin-4 (dotted lines) with GLP1R in nanodiscs (GLP1R-ND) in the presence (red) and absence (blue) of 15 mM Ca^{2+} show Ca^{2+} does not affect ligand binding to GLP1R. Fitted K_D values are indicated in the legend. Data shown are the average of 3 experiments.

Observation of Ca2+-dependence binding of PTH(1-34) to PTH(1-34) in the absence of lipid bilayer. We purified PTH1R in detergent micelles for fluorescence anisotropy experiments. The receptor still shows calcium dependent binding using the fluorescence anisotropy assay (Figure S5). The titration data does not reach saturation, suggesting that the PTH(1-34) binds with lower affinity to PTH1R purified in detergent micelles. Thus, PTH1R appears to be less stable in detergent micelles compared to the nanodiscs. Because these titrations do no reach saturation, we are unable to determine the K_D values. However, we observe an increase in ligand binding in the presence of 15 mM Ca^{2+}. This result suggests the lipid bilayer is not required for the Ca^{2+} sensing ability of PTH. The result also highlights the importance of nanodisc purification to stabilize PTH1R."

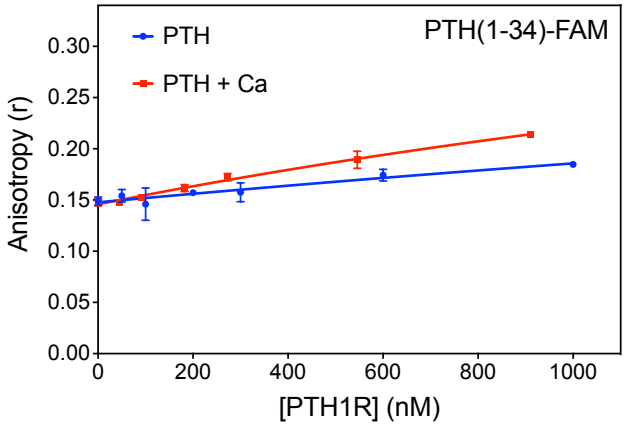


Figure S5: PTH(1-34) binding to PTH1R stabilized in detergent micelles. PTH(1-34) binds to PTH1R in solublization buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 4 mM EDTA, 10% glycerol, 1% n-Dodecyl-β-D-maltoside (DDM)) with (red) and without (blue) 15 mM Ca^{2+}. Each curve shows 3 replicates, with error bars showing the standard deviation.

Appendix Methods

***Circular Dichroism Spectroscopy*.** Tested peptides were prepared in anisotropy buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 3mM $MgCl_2$, and 100 μM EDTA). Ellipticity was measured using a Jasco-J810 spectrophotometer in a quartz cuvette with a 2-mm path length. Each spectrum was repeated 2 times and averaged with a step size of 0.5 nm and a bandwidth of 1.0 nm. Molar ellipticity was calculated from millidegrees using the equation: Molar ellipticity = M*100/(path length*concentration).

Cell-based cAMP study. Tetracycline-inducible stable HEK293S cells expressing PTH1R were grown and induced in 48-well plates. To determine the efficacy of the mutant peptide ligands the cells were washed with binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5% FBS and 5% heat-inactivated FBS) then treated with 120 μL of cAMP assay buffer (DMEM with 1 mM IBMX, 1 mg/mL BSA, 35mM HEPES pH 7.4) followed by adding 60 μL binding buffer containing peptides (concentrations indicated). Cells were stimulated with the peptide for 20 minutes at 37 °C then lysed with lysis buffer (0.1M HCl, 0.5% Triton X-100) and the cAMP produced determined using the direct cAMP ELISA assay. Each point was measured in duplicate in one experiment and each curve represents an average of three experiments.

***Detergent purification of PTH1R*.** PTH1R was overexpressed in HEK293S *GnTI*$^-$ cell lines as previously described.[4] For purification, 10-cm plates of stable cells lines were induced with 2 μg/μL tetracycline and 0.55 mg/mL sodium butyrate for 48 hrs. The cells were harvested in 1X PBS with protease inhibitor tablets (Roche Complete, EDTA free), centrifuged at 500 x g for 5 min and the cell pellet resuspended in 1 mL/plate of solubilization buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM EDTA, 10% glycerol and 0.5% n-Dodecyl-β-D-maltoside (DDM, ACROS Organics)) for 3 hrs at 4°C. Solubilized membranes were centrifuged at ~110,000 x g to precipitate cell debris. PTH1R detergent micelles were purified using a single step affinity purification against the 1D4 epitope.

Nanodisc Purification of GLP1R. GLP1R was purified in nanodiscs as described previously.[3] After nanodisc purification GLP1R was used for fluorescence anisotropy assays, as described in the main text. GLP-1(7-37) was labeled with 5(6)-carboxyfluorescein (FAM) at position E21K and Ex-4 was labeled with FAM at L21K. Both FAM labeled peptides were obtained from the Keck Biotechnology Resource Laboratory at Yale University.

References

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[2] Wang, X., Liu, H., Chen, J., Li, Y., and Qu, S. (2015) Multiple factors related to the secretion of glucagon-like peptide-1, *Int. J. Endocrinol. 2015*.

[3] Cai, Y., Liu, Y., Culhane, K. J., DeVree, B. T., Yang, Y., Sunahara, R. K., and Yan, E. C. (2017) Purification of family BG protein-coupled receptors using nanodiscs: Application to human glucagon-like peptide-1 receptor, *PloS One 12*, e0179568.

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