SUPPLEMENTAL DATA FIGURE LEGENDS:

Supplemental Figure 1:

Effect of CaSR epitope tagging on receptor pharmacology. Ca$^{2+}$\textsubscript{o}-mediated Ca$^{2+}$\textsubscript{i} release was determined in the absence or presence of (A) 1µM NPS-2143 or (B) 0.3µM cinacalcet. Circles represent non-tagged CaSR, and triangles represent c-myc-CaSR, stably expressed in HEK293-TREx cells induced by overnight incubation with 100ng/mL tetracycline. Results were normalized to the response to 0.1µM ionomycin and expressed as a % of the maximal DMSO control response. Each data point represents the mean ± SEM of 3 individual experiments performed in duplicate. Curves through the points represent the best fit of Eqn 1 to the data. Assays were performed in a buffer containing 0.1 mM Ca$^{2+}$. Student t-test comparisons between pEC$_{50}$, n$_{H}$ and E$_{max}$ parameters from the c-myc-CaSR and CaSR data above revealed that the c-myc tag did not significantly alter the pharmacology of the receptor.

Supplemental Figure 2:

CaSR-mediated specificity. The response of HEK293-TREx c-myc-CaSR cells to Ca$^{2+}$\textsubscript{o} (circles, 12-18h 100 ng/mL tetracycline-induced expression) is distinct from responses in untransfected HEK293-TREx cells (triangles) in both Ca$^{2+}$\textsubscript{i} mobilisation (A) and pERK1/2 assays (B). Untransfected HEK293-TREx cells (C) do not show PM ruffling in buffer (0.1mM Ca$^{2+}$\textsubscript{o}) or to the addition of 0.7 mM (submaximal) Ca$^{2+}$\textsubscript{o} in either the absence or presence of 100 nM cinacalcet, in contrast to CaSR transfected cells (main text Figures 3, 4). Ca$^{2+}$\textsubscript{i} release was normalised to response of HEK293-TREx c-myc-CaSR cells (12-18h 100 ng/mL tetracycline treated) to the addition of 10$^{-2.5}$ M Ca$^{2+}$\textsubscript{o}, whilst pERK1/2 results were normalised to percent response to 3% FBS. All assays were conducted in the presence of 0.1 mM Ca$^{2+}$ assay buffer (not included in the axis but accounted for in regression). Data points in A and B are mean±SEM from 2-6 experiments performed in duplicate and non-linear regression was performed with Eqn 1. Images in C were
obtained at 20X objective and are representative of 3 experiments performed in duplicate; Green, F-actin stain (Alexa Fluor 568 phalloidin), Blue, nuclei stain (Hoechst 33342) and white scale bar, 20 µm.

**Supplemental Figure 3:**

**Effect of allosteric modulator incubation time on (A, B) maximal efficacy ($E_{\text{max}}$) and (C, D) potency of Ca$^{2+}$.** Concentration-response (C/R) curves to Ca$^{2+}$ in the Ca$^{2+}$i mobilization assay were constructed in the absence or presence of (A, C) 1µM cinacalcet or (B, D) 3µM NPS-2143, and the data subjected to nonlinear regression. The modulators were co-added or pre-incubated for the indicated time periods. For panels A and B, the results are expressed as the % response of the $E_{\text{max}}$ of the DMSO control curve. Each line represents the mean ($E_{\text{max}}$) ± SEM of 3-7 individual experiments (data points) performed in duplicate. Statistical analysis performed by one-way ANOVA with Dunnet’s post-test **p<0.01, ***p<0.0001 compared to DMSO control. Panels C and D show the complete C/R profile for two treatment conditions. Note that for, NPS-2143 in particular, preincubation is required to reveal equilibrium-state changes in Ca$^{2+}$o potency.

**Supplemental Figure 4:**

**Theoretical effects of ambient agonist on allosteric receptor modulation.** An operational model of allostery/agonism that we have previously applied to the CaSR (35) was modified to include a fixed concentration of agonist, and utilized to simulate agonist concentration-response (C/R) curves in the absence (black) or presence (colored) of increasing allosteric modulator concentrations, both in the absence (A, B) or presence of fixed ambient agonist in the media (C, 0.1mM; D, 0.2mM). In this model, $E_m$ is the maximum possible cellular response, [A] and [B] are the concentrations of orthosteric and allosteric ligands, respectively, $K_A$ and $K_B$ are the equilibrium dissociation constant of the orthosteric and allosteric ligands, respectively, $\tau_A$ and $\tau_B$ are operational measures of orthosteric and allosteric ligand efficacy, respectively, n is a transducer slope that links agonist
occupancy to observed response, \( \alpha \) is the binding cooperativity parameter between the orthosteric and allosteric ligand, and \( \beta \) denotes the allosteric effect of the modulator on the efficacy of the orthosteric agonist. Parameters used for the simulations were as follows: \( K_A = 10^{-3} \) M, \( K_B = 10^{-6} \) M, \( \tau_A = 3, \tau_B = 0, n = 5.5, E_m = 100, \alpha = 1, \beta = 3.16 \) for A and C, \( \beta = 0.16 \) for B and D. Theoretical concentrations of modulator were increased from \( 10^{-7} \) M (yellow) to \( 10^{-5} \) M (purple) in 0.5 log unit increments. Panel (E) shows the effect on the Hill slope (nH) and panel (F) shows the effect on agonist potency (pEC\(_{50}\)) under the simulated conditions. Closed symbols = absence of ambient agonist; open symbols, presence of ambient agonist; circles = positive modulator; triangles = negative modulator.

Supplemental Figure 5:

**Effect of increasing the background divalent cation concentration on allosteric modulation at the CaSR.** \( \text{Ca}^{2+}_o \)-mediated \( \text{Ca}^{2+}_i \) release was determined in the presence of 0.1mM CaCl\(_2\) (circles) or 1mM CaCl\(_2\) (triangles). HEK293-TREx cmyc CaSR cells were incubated with either concentration of background CaCl\(_2\) for 20 min in the absence (closed symbols) or presence (open symbols) of 0.1µM cinacalcet, immediately prior to \( \text{Ca}^{2+}_o \) C/R curve determination. Results were normalized to the response to 0.1µM ionomycin and expressed as a % of the maximal DMSO control response of each buffer condition. Each data point represents the mean ± SEM of 3 individual experiments performed in duplicate. Curves through the points represent the best fit of **Eqn 1** to the data. The potency of \( \text{Ca}^{2+}_o \) was significantly reduced (pEC\(_{50}\): 3.20±0.02 reduced to 2.76±0.01) in the presence of higher background divalent cation concentrations most likely due to desensitization of the receptor population. However, the Hill slope was significantly more shallow in the presence of modulator, the effect being more pronounced in the 1mM CaCl\(_2\) buffer (12.69±2.27 in absence of cinacalcet; 8.44±0.28 in presence of cinacalcet) than in the 0.1 mM CaCl\(_2\) buffer (4.39±0.63 in absence of cinacalcet to 3.41±0.84 in presence of cinacalcet).
Supplemental Fig 1

(A) Graph showing calcium release (Ca\textsuperscript{2+} release) against calcium concentration ([Ca\textsuperscript{2+}] mM). The graph includes data for DMSO (CaSR) and DMSO (cmycCaSR).

(B) Graph showing calcium release (Ca\textsuperscript{2+} release) against calcium concentration ([Ca\textsuperscript{2+}] mM). The graph includes data for modulator (CaSR) and modulator (cmycCaSR).
Supplemental Fig 2

A

$\text{Ca}^{2+}$ release

B

pERK1/2 stimulation

C

<table>
<thead>
<tr>
<th>Buffer</th>
<th>0.7mM $\text{Ca}^{2+}_o$</th>
<th>0.7mM $\text{Ca}^{2+}_o$ + 100nM cinacalcet</th>
</tr>
</thead>
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[Images of graphs and cell images]
\[ E = \frac{E_m \left( \tau_A [A+C](K_B + \alpha \beta [B]) + \tau_B [B] K_A \right)^n}{\left( [A+C] K_B + K_A K_B + [B] K_A + \alpha [A+C][B] \right)^n + \left( \tau_A [A+C](K_B + \alpha \beta [B]) + \tau_B [B] K_A \right)^n} \]