

Illuminating the allosteric modulation of the calcium-sensing receptor

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Many membrane receptors are regulated by nutrients. However, how these nutrients control a single receptor remains unknown, even in the case of the well-studied calcium-sensing receptor CaSR, which is regulated by multiple factors, including ions and amino acids. Here, we developed an innovative cell-free Förster resonance energy transfer (FRET)-based conformational CaSR biosensor to clarify the main conformational changes associated with activation. By allowing a perfect control of ambient nutrients, this assay revealed that Ca^{2+} alone fully stabilizes the active conformation, while amino acids behave as pure positive allosteric modulators. Based on the identification of Ca^{2+} activation sites, we propose a molecular basis for how these different ligands cooperate to control CaSR activation. Our results provide important information on CaSR function and improve our understanding of the effects of genetic mutations responsible for human diseases. They also provide insights into how a receptor can integrate signals from various nutrients to better adapt to the cell response.

G protein-coupled receptor | allosteric modulator | calcium | amino acids | nutrient sensing

Cells have to constantly adapt to their environment and, as such, sense through specific receptors, a large number of nutrients, such as ions, L-amino acids (L-AAAs), glucose, various metabolites, and lipids (1). Despite the importance of such processes, how one receptor senses various nutrients remains elusive, as it is difficult to control nutrient concentrations around living cells.

G protein-coupled receptors (GPCRs) form the largest family of membrane receptors and major drug targets (2). Many of them are activated or modulated directly by nutrients (3, 4). Among GPCRs, the calcium-sensing receptor (CaSR) is a prototypical nutrients sensory receptor activated or modulated by both calcium (5) and amino acids (6), but also by endogenous and exogenous compounds such as different cations, polyamines, polypeptides, and aminoglycoside antibiotics (7, 8) (Fig. 1A).

CaSR is essential in the parathyroid gland for maintaining extracellular calcium homeostasis (5). It is also expressed in other tissues such as bone, gut, kidney, and brain (5, 9), where it has additional effects. Many genetic mutations that lead to loss or gain of function of the CaSR, have been identified in patients with metabolic syndromes (9, 10). In addition, CaSR autoantibodies that modify the signaling properties of the receptor have been identified in rare diseases (11). Finally, CaSR is the target of three commercial drugs acting as a positive allosteric modulator (PAM). Etelcalcetide (12, 13) targets the extracellular domain, while cinacalcet (14, 15) and evocalcet (16) bind to the transmembrane domain.

CaSR belongs to class C GPCRs, most of which are activated by L-AAAs or derivatives that bind to the conserved extracellular Venus flytrap (VFT) binding domain, such as the mGlu, GABA_B, GPCR6A, and umami taste receptors, as well as the fish olfactory

receptors and some pheromone receptors (17). These receptors form obligatory dimers, providing a unique mode of activation compared to GPCRs from other classes. Like most class C GPCRs, each subunit of the CaSR homodimer is composed of an extracellular domain (ECD), made up of a VFT and a cysteine-rich domain (CRD) connected to a heptahelical transmembrane (TM) domain, responsible for G protein coupling (Fig. 1A). Numerous studies have been performed with the aim to understand the molecular basis of calcium ion and amino acid actions at the CaSR (18). Recent crystal structures of the isolated ECD have been solved in the absence or presence of calcium and amino acids by two different groups (19, 20) (Fig. 1B), but how the binding of these nutrients in this domain triggers receptor activation remains elusive.

These solved CaSR structures led to the proposed rearrangement of the ECD dimer upon activation (19). This rearrangement is limited to the closure of both VFTs upon Ca^{2+} binding, without a major reorientation of the VFTs (Fig. 1B). This is in contrast to what has been observed with the closely related mGlu receptors, for which the closure of the VFTs results in a major reorientation of the VFTs (21–24) (Fig. 1B). However, this CaSR model appears similar to what was observed with the distantly related GABA_B heterodimer (25, 26) (Fig. 1B), but this needs to be firmly documented with the full-length CaSR.

Significance

G protein-coupled receptors regulate several physiological processes, many of which sense nutrients. The mode of action of these nutrients remains elusive, as it is difficult to control nutrient concentrations around living cells. The calcium-sensing receptor is regulated by multiple factors, including ions and amino acids, to control calcium homeostasis; and genetic mutations are responsible for human diseases. Here, we investigated this receptor in a perfect control of ambient nutrients. Based on the identification of calcium ion activation sites, we propose a molecular basis for how calcium and amino acids cooperate to control receptor activation. This leads to a model for the mechanism of activation of this receptor and its modulation by multiple ligands and genetic mutations.

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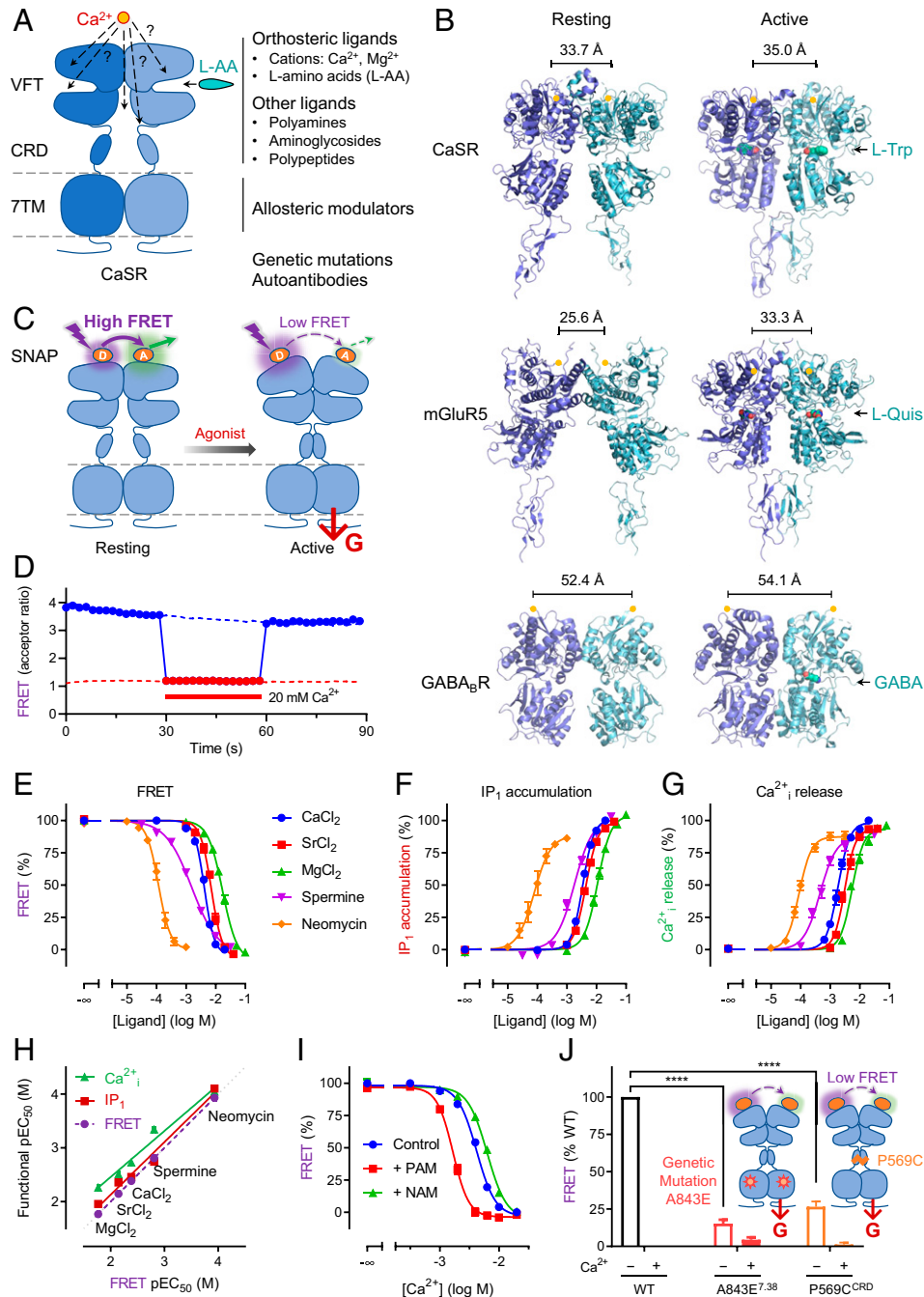


Fig. 1. A conformational FRET-based sensor to investigate the structural dynamics of the CaSR ECD. (A) Orthosteric binding sites for calcium remain unclear and controversial. Many genetic mutations have been found in patients with calcemic disorders, and they are located in the different regions of the receptor. Besides, autoantibodies have been identified in rare autoimmune diseases. (B) Crystal structures of the extracellular domain of class C GPCR dimers in the resting forms (*Left*, CaSR PDB 5K5T biological assembly 2, mGluR5 PDB 6N52, and GABA_BR PDB 4MQE) and in the active forms in the presence of L-Trp, L-quisqualate, or GABA (*Right*, CaSR PDB 5K5S, mGluR5 PDB 6N51, and GABA_BR PDB 4MS3, respectively). The residues used to measure distances [D23 for CaSR, R26 for mGluR5, R50 for GABA_{B1b} (*Right*) and S53 for GABA_{B2} (*Left*), respectively] are highlighted as orange spheres. (C) Cartoon illustrating the full-length SNAP-CaSR labeled with the Lumi4-Tb donor and the green fluorescent acceptor, with high FRET signal in the absence of agonist and a lower FRET signal in the presence of agonist. (D) FRET signal between the two VFTs after cell surface labeling of SNAP-CaSR-expressing cells with fluorophores as indicated in C, in the presence of a saturating concentration of calcium (20 mM CaCl₂, injection at t = 30 s) and after calcium removal (t = 60 to 90 s). Data are mean ± SEM of a typical experiment performed in replicates. The control with 20 mM CaCl₂ (dotted red line) or buffer alone (dotted blue line) are shown for the same period of time (0 to 90 s). (E–H) Correlation (H) between the potencies (pEC₅₀) of different agonists on CaSR determined by FRET sensor assay (E), inositol monophosphate (IP₁) accumulation assay (F), and intracellular calcium (Ca²⁺) release assay (G). Data in E–G are mean ± SEM of at least three independent experiments performed in triplicates and normalized to the maximum response of CaCl₂. Data in H are mean ± SEM from the fitted curves for each individual experiment in E–G. (I) FRET signal measured for CaCl₂ in the presence of either PAM (10 μM NPS R-568) or NAM (10 μM NPS 2143). Data are mean ± SEM of at least three independent experiments performed in triplicates and normalized to the maximum response of the control. (J) FRET signal measured for the indicated mutants in the absence or presence of 20 mM CaCl₂. Data are mean ± SEM of at least three independent experiments performed in triplicates and normalized to WT. Data are analyzed using two-way ANOVA with Tukey's multiple comparisons test to determine significance, with ****P ≤ 0.0001.

CaSR structures also revealed in much detail the L-AA binding mode, but the Ca^{2+} sites involved in receptor activation remain unclear, especially due to the limited resolution of the structures. The two studies reporting CaSR structures eventually did not propose the same Ca^{2+} sites (*SI Appendix, Fig. S1 A and B*). The molecular basis for L-AA activity was also difficult to characterize in functional studies using cell-based assays, due to the ambient concentrations of ions and other nutrients including L-AAAs that are difficult to control tightly.

In this study, we have developed an innovative assay to clarify the conformational changes occurring during CaSR activation, and to investigate how nutrients control this activation. We have set up a time-resolved Förster resonance energy transfer (TR-FRET) conformational CaSR biosensor that enables work in cell-free conditions, where the nutrient concentrations can be well controlled (*SI Appendix, Fig. S2*). Using this assay, we demonstrate that calcium ions are sufficient to stabilize the active state of the receptor, while L-AAAs have no effect on their own but enhance the effect of Ca^{2+} then acting as pure PAMs. Moreover, we show that chloride ions identified in the CaSR structures also act as PAMs, potentiating the effect of Ca^{2+} . We propose a model with two separate binding sites for calcium ions in each CaSR VFT, allowing an indirect interaction of both Ca^{2+} ions with the L-AA.

Results

Development of a FRET-Based Conformational Sensor for the CaSR. In order to better understand the mode of action of various ligands of the CaSR, we first aimed to clarify the conformational changes associated with its activation. We developed a TR-FRET-based conformational biosensor using an approach previously reported to examine conformational changes in mGlu and GABA_B receptors (21, 24). The CaSR subunit was fused at its N terminus with a SNAP-tag that can be covalently labeled with fluorophores using specific cell-impermeant substrates (27) (Fig. 1C). In the absence of agonists, a high signal is measured between the two SNAP-tags of the CaSR dimer (Fig. 1D). Upon calcium addition, a large and fast decrease in FRET signal was observed, and the high FRET signal was restored with a calcium-free buffer (Fig. 1D). Accordingly, the fluorescence decay of the donor, measured through the fluorescence decay of the sensitized acceptor, becomes slower in the presence of calcium (*SI Appendix, Fig. S3A*). It revealed a lower FRET efficiency between the donor and acceptor due to a larger distance between them in the presence of an agonist. Finally, since the TR-FRET signal is mostly dependent on the distance between the donor and the acceptor, the strong decrease of FRET revealed a large increase in distance between the two N-terminal SNAP-tags during activation. A similar increase of distance between the two N-terminal SNAP-tags was observed in FRET-based conformational mGluR and GABA_B receptors (21, 24), but not in the GABA_B receptor (28).

To prove that the change in TR-FRET signal reflects receptor activation, we analyzed the effect of different CaSR agonists. They all consistently induced a decrease in FRET efficiency with potencies (fitted pEC_{50}) in the range of those measured using cellular functional assays: the inositol phosphate (IP_1) accumulation and intracellular calcium (Ca^{2+}_i) release assays (Fig. 1E–H and *SI Appendix, Fig. S3 B–D*). Such movement of the ECDs should also be affected by allosteric modulators acting in the 7TM domain (21) (*SI Appendix, Fig. S3E*). As expected, two commercial allosteric modulators of the CaSR, NPS R-568 (29) and NPS 2143 (30), which are positive and negative allosteric modulators, respectively, were found to influence the agonist-induced changes in TR-FRET. While NPS 2143 decreased agonist potencies, NPS R-568 enhanced agonist potencies in the TR-FRET assay (Fig. 1J). These data are perfectly in line with the results obtained from the IP_1 functional assay (*SI Appendix, Fig. S3F*). Finally, we show that G protein coupling favors the

rearrangement of the ECDs, consistent with the allosteric effect of the G protein that favors the active state of GPCRs (31). Accordingly, overexpression of the $\text{G}\alpha_q$ subunit, known to couple to the CaSR (8), reduced basal FRET signal (*SI Appendix, Fig. S3J*). Of note, nonhydrolyzable $\text{GTP}\gamma\text{S}$ that uncouples G proteins to GPCRs (31) had no effect (*SI Appendix, Fig. S3J*), most probably due to the low abundance of Gq in the membrane preparations used in this assay, as the $\text{G}\alpha_q$ type of G proteins is not myristoylated (32).

Altogether, our data show that the CaSR dimer undergoes a large conformational change of the VFTs during activation, compatible with a relative reorientation of VFT dimer. This conformational change can be used to monitor the activation of the receptor and to analyze the effects of ligands including allosteric modulators.

FRET Sensor to Detect the CaSR Conformation Stabilized by Mutations.

To further validate our sensor, we introduced mutations in regions of the receptor known to control its active state. We show that the A843E^{7,38} genetic and gain-of-function mutation (33) in the transmembrane domain 7 (TM7) which induced autosomal dominant hypocalcemia, stabilized the active state of the ECD as revealed by the low FRET signal (Fig. 1J), without changing the cell surface expression of the receptor (*SI Appendix, Fig. S3G*). This is consistent with the reported constitutive activity of this mutant (33) (*SI Appendix, Fig. S3H*).

The mechanism of activation of the CaSR remains largely unknown (8). Crystal structures of the isolated CaSR ECD revealed that the lower lobes of the VFT come closer in the presence of agonists, leading to a close contact between the CRDs (19). To mimic activation-induced proximity of CRDs, we created a disulfide cross-linking of the two CRDs, as reported for mGlu2 receptor (34). The mutation of Pro569 into Cys led to a largely reduced FRET signal (Fig. 1J) despite a normal cell surface expression (*SI Appendix, Fig. S3G*). This mutation also induced strong constitutive activity (*SI Appendix, Fig. S3H*), consistent with the stabilization of the receptor in an active state, likely resulting from the cross-linking of the two CRDs. These data are consistent with Pro569 being at the CRD interface in the active ECD structure of the CaSR (19). Interestingly, Ca^{2+} can still further decrease the FRET signal (Fig. 1J), an effect that may be due to a fraction of receptors not being cross-linked, or alternatively, that cross-linking did not stabilize the receptor in its fully active state.

FRET Sensor Unravels the Rearrangement of the CaSR Dimer Interface during Activation.

We used the FRET sensor to investigate the changes in the 7TM dimer interface during the activation of the CaSR by a disulfide cross-linking approach, as recently reported for the mGlu (22, 35) and the GABA_B (36) receptors (Fig. 2A and B). We speculated that locking the active state 7TM interface should result in a lower basal FRET, while locking the inactive interface should limit the Ca^{2+} -induced FRET change (Fig. 2C). We screened positions in TM4, TM5, TM6, and TM7, as these transmembrane helices were involved in the interface of other class C receptors (22, 35, 36). After copper phenanthroline (CuP) treatment to favor disulfide cross-linking between the introduced Cys residues, we observed a low FRET signal relative to control for six mutants in TM6 (I822C^{6,54}, P823C^{6,55}, A824C^{6,56}, A826C^{6,58}, S827C^{6,59}, and T828C^{6,60}) (Fig. 2C), but not for other positions in TM6 and in the other TMs (Fig. 2C and *SI Appendix, Fig. S4A and B*). In contrast, for some mutants in TM4 and TM5 (V740C^{4,48}, I741C^{4,49}, Y744C^{4,52}, T745C^{4,53}, and M771C^{5,38}), CuP treatment resulted in a decrease in the Ca^{2+} effect, suggesting that these mutations prevent the full rearrangement of the ECD dimer induced by the agonist, or alternatively, that a fraction of the receptors can no longer reach an active state (Fig. 2C). No such effect on the Ca^{2+} -induced signal was observed with other cysteine mutants in these TMs (*SI Appendix, Fig. S4A*), as well as in TM6

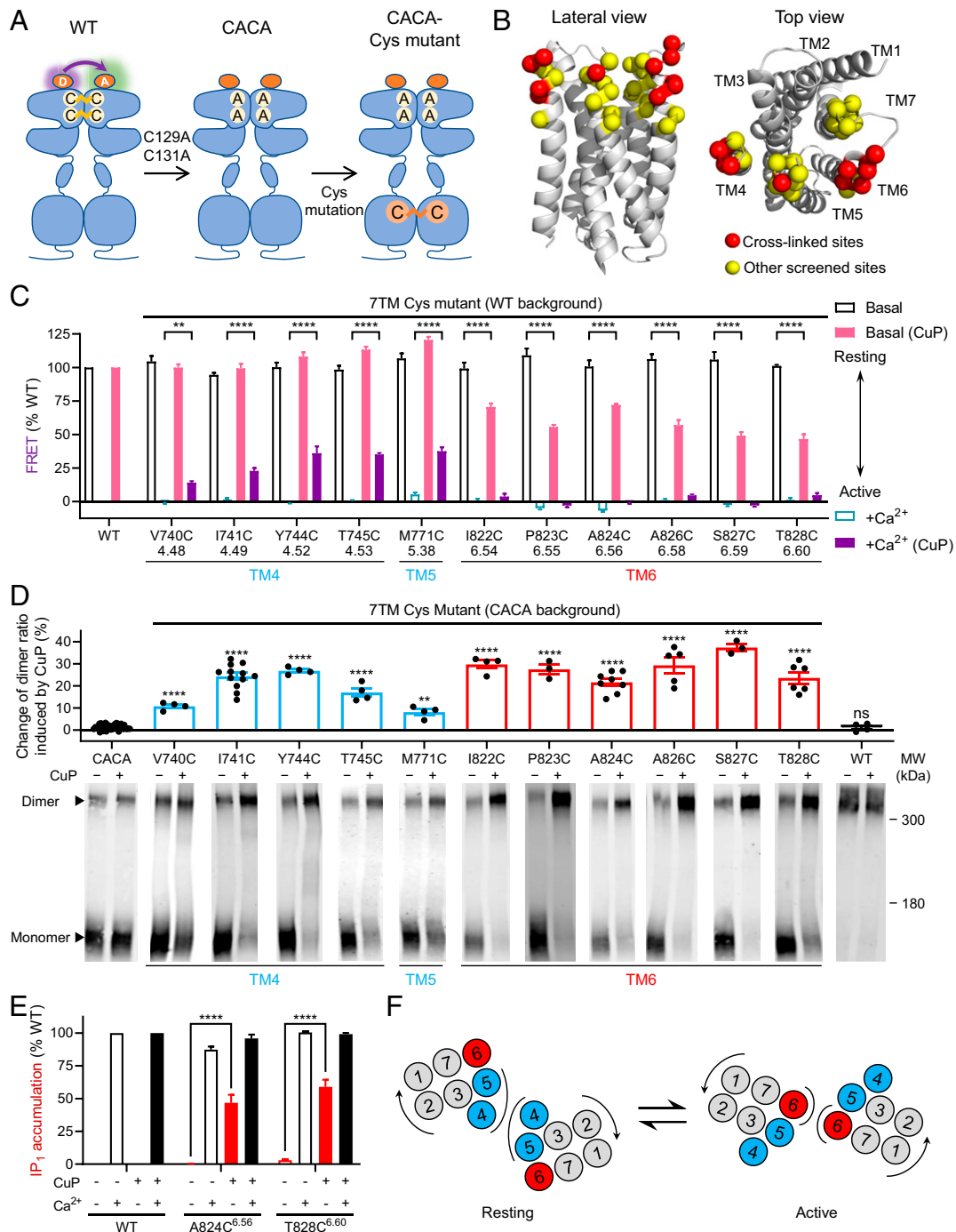


Fig. 2. The 7TM interface rearrangement is revealed by cysteine cross-linking and FRET. (A) Schematic representation of CaSR WT, mutant C129A-C131A (CACA, to remove the endogenous disulfide bonds between the two VFTs of CaSR dimer) and 7TM cysteine mutants with CACA background. (B) A 3D model of the CaSR 7TM in lateral and top view. Residues substituted by cysteine are highlighted as yellow spheres (α carbon), and the well cross-linked ones are highlighted in red. (C) Cysteine cross-linked mutants screened by TR-FRET in the absence or presence of 20 mM CaCl_2 with and without CuP treatment. Data are mean $\pm$ SEM of at least three independent experiments performed in triplicates and normalized to WT (data with and without CuP are normalized separately). Data are analyzed using two-way ANOVA with Tukey's multiple comparisons test to determine significance within each mutant group, with $****P \leq 0.0001$ and $**P \leq 0.01$. (D) Analysis of cell surface CaSR subunits of the cysteine mutants (CACA as control) in SDS/PAGE experiments under non-reducing conditions, after treatment (+) or without treatment (–) with CuP. Changes of dimer ratio induced by CuP treatment for WT, the CACA control, and every indicated mutant are quantified and shown. Quantitative data are mean $\pm$ SEM of at least four independent experiments ($n = 4$ to 12) while the blot for each mutant is representative of one of these experiments. Data are analyzed using one-way ANOVA with Dunnett's multiple comparisons test to determine significance (compared with CACA control), with $****P \leq 0.0001$, $**P \leq 0.01$, and $ns P > 0.05$. (E) IP₁ accumulation for WT and the indicated TM6 mutants after treatment (+) or without treatment (–) with CuP, in the absence or presence of 20 mM CaCl_2 . Data are mean $\pm$ SEM of at least three independent experiments performed in triplicates and normalized to WT (data with or without CuP are normalized separately). Two-way ANOVA with Tukey's multiple comparisons test within each mutant group, with $****P \leq 0.0001$. (F) Model highlighting the TMs involved in the dimerization of CaSR in the resting state and in the active state.

and TM7 (Fig. 2C and *SI Appendix, Fig. S4B*). This indicates that TM4s and TM5s form the resting interface of the CaSR. All mutants were well expressed on the cell surface compared to the wild-type (WT) receptor (*SI Appendix, Fig. S4 C and D*).

We have verified that both resting and active interfaces were cross-linked by blot analysis of the SNAP-CaSR subunits (Fig. 2D). Cell surface SNAP-tagged CaSR constructs were covalently labeled with the cell-impermeant fluorescent substrate SNAP-Surface 649, and its signal was used as the readout of the blots, as previously reported (35). The CaSR WT dimer is covalently linked by disulfide bridges between the two VFTs, involving two conserved cysteines (Cys129 and Cys131), but that are not required for receptor assembly and activity (37, 38). First, we mutated these cysteines into alanine in a construct named CaSR^{CACA} (Fig. 2A). As expected, this construct migrated mostly as a monomer in nonreducing conditions, in contrast to WT CaSR that migrated as a dimer (Fig. 2D). We then analyzed whether the Cys residues introduced in the TM could cross-link the CaSR^{CACA}. The efficiency of cross-linking between the two subunits induced by CuP was quantified by the change in the dimer signal to the total quantity of the CaSR subunit detected on the blots (Fig. 2D). The results revealed efficient cross-linking when Cys was introduced in TM4, 5, or 6. As a negative control, no such cross-linking was observed with the mutant V737C^{4,45} (*SI Appendix, Fig. S4E*), a site in TM4, which cannot be cross-linked in agreement with the FRET results (*SI Appendix, Fig. S4A*). These data show that TM4, 5, and 6 likely constitute the CaSR dimer interface. Finally, we have confirmed that the TM6 dimer interface corresponds to the active state, as stabilizing this interface with a cysteine cross-linking at residues Ala824^{6,56} or Thr828^{6,60} leads to a constitutively active receptor (Fig. 2E).

Altogether, our results revealed a relative rearrangement between the two 7TMs during activation. While TM4-5 of each subunit faces each other in the inactive state, a TM6-TM6 contact occurs in the active state of the dimer (Fig. 2F and *SI Appendix, Fig. S4F*). This movement is then similar to that proposed for other class C GPCRs (22, 35, 36) and is consistent with the inactive and active-like structures of mGlu5 (22) and the inactive and active states of GABA_B (25, 26).

Ambient L-Amino Acids Are Pure PAMs of CaSR. Some L-AAs, specifically aromatic ones, are known to regulate CaSR activity (6). The binding site of the L-AAs in the VFT domain was found to be equivalent to the glutamate binding site in mGluRs, as revealed by site-directed mutagenesis (39, 40) and the crystal structures of the CaSR VFT dimer (19, 20). However, the exact role of L-AAs in the activation process of the CaSR remains elusive, as the CaSR function has always been studied in cellular assays, and thus in the presence of unknown concentrations of L-AAs (41). Probably due to these ambient L-AAs, L-Phe produced a modest, but statistically significant PAM effect measured either with functional assays (*SI Appendix, Fig. S5 A and B*), or with our FRET sensor (*SI Appendix, Fig. S5C*) in living cells.

To be able to perfectly control the ambient L-AA concentration, we established a cell-free assay for the CaSR based on membranes containing our TR-FRET-based sensor. The sensor was functional (Fig. 3 A–C), although agonist potencies were slightly better in living cells than in membranes (Fig. 3E). This may potentially result from a loss of bound L-AAs in the CaSR during membrane preparations.

To completely remove any L-AAs, membranes were dialyzed, and under such conditions, Ca²⁺ potency was significantly lower, but it induced a full rearrangement of the ECD, indicating that L-AAs are not required for Ca²⁺ to fully activate CaSR (Fig. 3 D and E). As expected, the addition of L-AAs such as L-Phe restored the high CaCl₂ potency measured in cells (Fig. 3 D and E). As a control, D-Phe had no effect on both nondialyzed and dialyzed membranes (*SI Appendix, Fig. S5 D and E*), consistent

with previous studies (6). As an additional control, NPS R-568 induced similar potency changes of CaCl₂ in nondialyzed and dialyzed membranes consistent, with the absence of the ambient NPS R-568-like compound (*SI Appendix, Fig. S5 D and E*).

We then determined the effect of all 20 natural L-AAs at 10 mM (except L-Tyr at 1 mM) on these dialyzed membranes (Fig. 3 F and G and *SI Appendix, Fig. S6*). In the absence of calcium, most L-AAs did not induce a significant decrease in FRET, except for L-Cys (Fig. 3F and *SI Appendix, Fig. S6*). We could not exclude that slight basal FRET change in the presence of L-Cys as well as L-Leu and L-Met, might be due to fluorescent quenching or some effects unrelated to receptor conformation change (Fig. 3F). This shows that L-AAs have no intrinsic capacity to stabilize the active state of the CaSR in the absence of calcium. However, most L-AAs had a significant PAM effect at 10 mM, as revealed by the increased calcium potency, except L-Leu, L-Asp, L-Lys, and L-Pro (Fig. 3G). The aromatic L-AAs have the strongest PAM effects, as previously reported (6). The potency of each L-AA was determined in the presence of 5 mM CaCl₂ (Fig. 3H and *SI Appendix, Fig. S7*), at which concentration L-AAs showed the maximum PAM effects. Aromatic L-AAs are the most potent, with EC₅₀ values ranging from less than 100 μM (L-Trp and L-Tyr) to less than 1 mM (L-Phe and L-His). Of note, the L-Trp derivative TNCA (L-1,2,3,4-tetrahydronorharman-3-carboxylic acid) observed in the CaSR structure (20) displayed the highest potency among all L-AAs tested (Fig. 3 G and H and *SI Appendix, Fig. S5F*). This high potency (less than 100 nM) likely explains why this compound was copurified with the CaSR from HEK293 cells (20). Other nonaromatic L-AAs displayed potencies of ~1 mM (L-Ala, L-Gln, L-Glu, L-Ile, L-Ser, L-Thr, and L-Val) to more than 10 mM (*SI Appendix, Fig. S7*).

A Genetic Mutation Shows a Stronger PAM Effect of L-AAs. Our FRET biosensor enables the precise investigation of the molecular basis of genetic mutations in the action of L-AAs on CaSR. We studied the genetic mutation E297D, responsible for the gain of function in autosomal dominant hypocalcemia (42). Glu297 is part of the L-AA binding site in the crystal structures (19, 20) and was proposed to be involved in Ca²⁺ binding (42–44). In the cellular format, Ca²⁺ had a higher potency for the mutant compared to WT (*SI Appendix, Fig. S8A*), that was lost in nondialyzed membranes, where its potency is similar to the WT receptor (*SI Appendix, Fig. S8C*). The simplest explanation is that the E297D mutant has a higher affinity for some L-AAs, such that the endogenous L-AA concentration in the vicinity of the living cells is sufficient to almost maximally increase Ca²⁺ potency. This ambient L-AA concentration is expected to be lower in the membrane preparation, leading to a loss of this effect on the E297D mutant, making it similar to the WT receptor. In agreement with this proposal, the addition of L-Phe in the assay restored the high Ca²⁺ potency (*SI Appendix, Fig. S8C*). This is further demonstrated after membrane dialysis, where the E297D mutant behaves as the WT receptor (*SI Appendix, Fig. S8B*). These data suggested that this gain-of-function mutation results from a more efficacious PAM effect of L-AAs.

Chloride Ions Are PAMs at CaSR. The structure of CaSR ECD in the active state (Protein Data Bank [PDB] 5FBK) (20) revealed three possible chloride binding sites (sites b, c, and g; Fig. 4A and *SI Appendix, Fig. S1A*). As Cl[−] ions were recently found to allosterically regulate most mGluRs (41, 45, 46), we investigated whether they could have any effect on the CaSR. The TR-FRET assay was performed in a gluconate buffer containing no chloride ions (0 mM Cl[−] buffer, Cl[−] being replaced by gluconate), gluconate having no effect on CaSR activity (*SI Appendix, Fig. S9A*). The main interest of gluconate, a small molecule, is to keep the negative charge of chloride but to be sure it cannot mimic a chloride effect by binding to the chloride site, due to its large

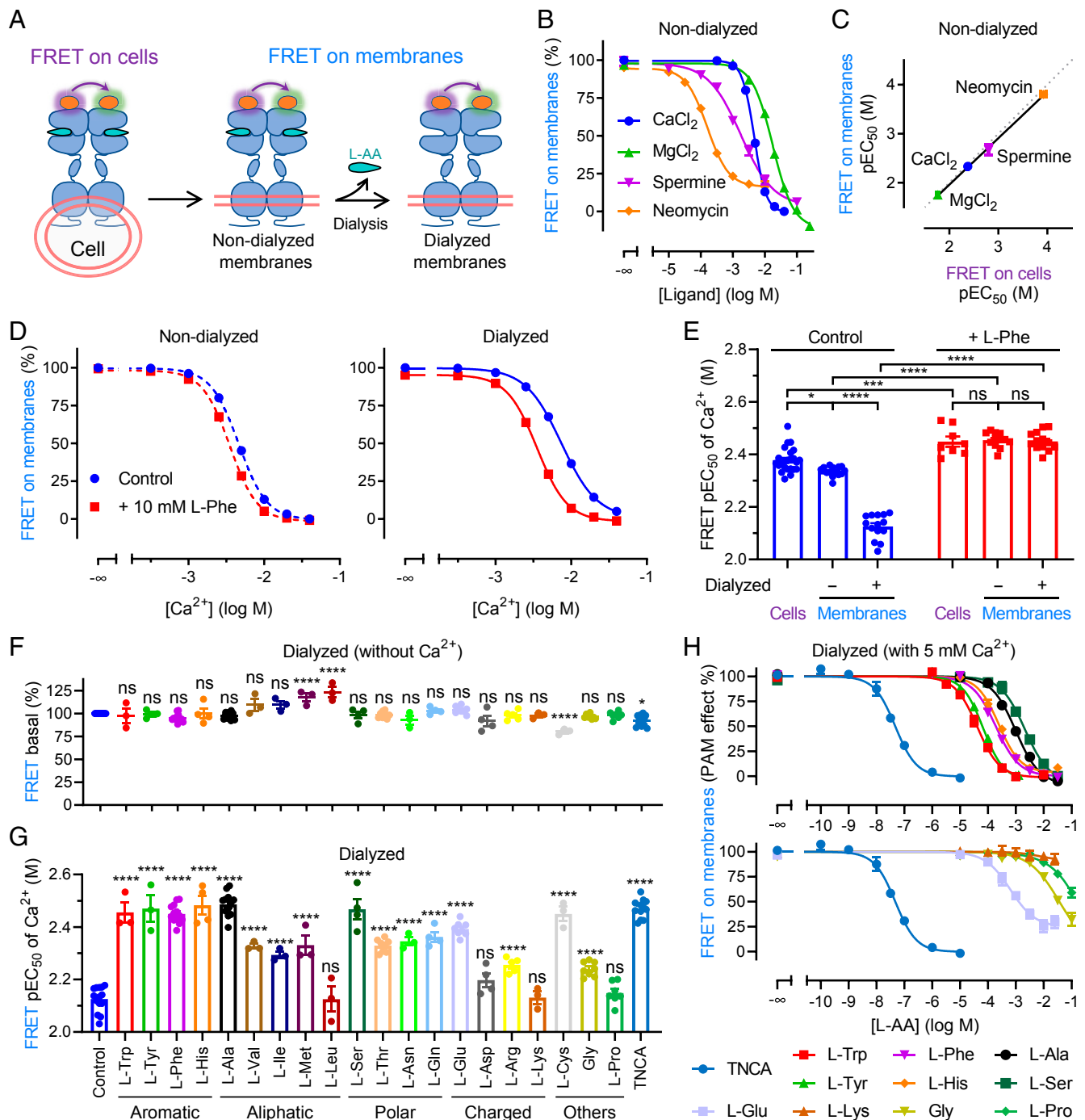


Fig. 3. Allosteric modulation by L-AA is clarified with a cell-free assay. (A) Cartoon illustrating the development of FRET-based CaSR biosensor in a cell-free assay based on cellular membrane preparations as indicated. (B) FRET signal measured on nondialyzed membranes with the indicated ligands. Data are mean $\pm$ SEM of at least three independent experiments performed in triplicates and normalized to the maximum response of CaCl₂. (C) Correlation between the FRET potencies (pEC₅₀) of the indicated agonists determined on cells and nondialyzed membranes. (D) FRET measurement performed with CaCl₂ on nondialyzed and dialyzed membranes in the absence or presence of 10 mM L-Phe. Data are mean $\pm$ SEM of at least three independent experiments performed in triplicates and normalized to the basal of control and the maximum response of L-Phe. (E) FRET potencies (pEC₅₀) of calcium on cells and on nondialyzed (–) or dialyzed (+) membranes in the absence or presence of 10 mM L-Phe. Data are mean $\pm$ SEM from at least eight independent experiments ($n = 8$ to 24). Two-way ANOVA with Tukey's multiple comparisons, with **** $P \leq 0.0001$, *** $P \leq 0.001$, * $P \leq 0.05$, and ns $P > 0.05$. (F) Basal FRET in the presence of the indicated L-AA at 10 mM (for Tyr-1 mM is used due to low solubility) or 10 μ M TNCA. Data are mean $\pm$ SEM of at least three independent experiments ($n = 3$ to 13) performed in triplicates and normalized to the control. One-way ANOVA with Dunnett's multiple comparisons test (compared with control) with **** $P \leq 0.0001$, * $P \leq 0.05$, and ns $P > 0.05$. (G) FRET potencies (pEC₅₀) of calcium on dialyzed membranes in the presence of the indicated amino acid (same concentrations as in F). Data are mean $\pm$ SEM from at least three independent experiments ($n = 3$ to 13). One-way ANOVA with Dunnett's multiple comparisons test (compared with control) with **** $P \leq 0.0001$ and ns $P > 0.05$. (H) FRET signal change induced by the indicated L-AA performed on dialyzed membranes in the presence of 5 mM CaCl₂. Data are mean $\pm$ SEM of at least three independent experiments performed in triplicates. Data are normalized to the basal conditions (5 mM CaCl₂ without L-AA) and the maximum response induced by L-AA.

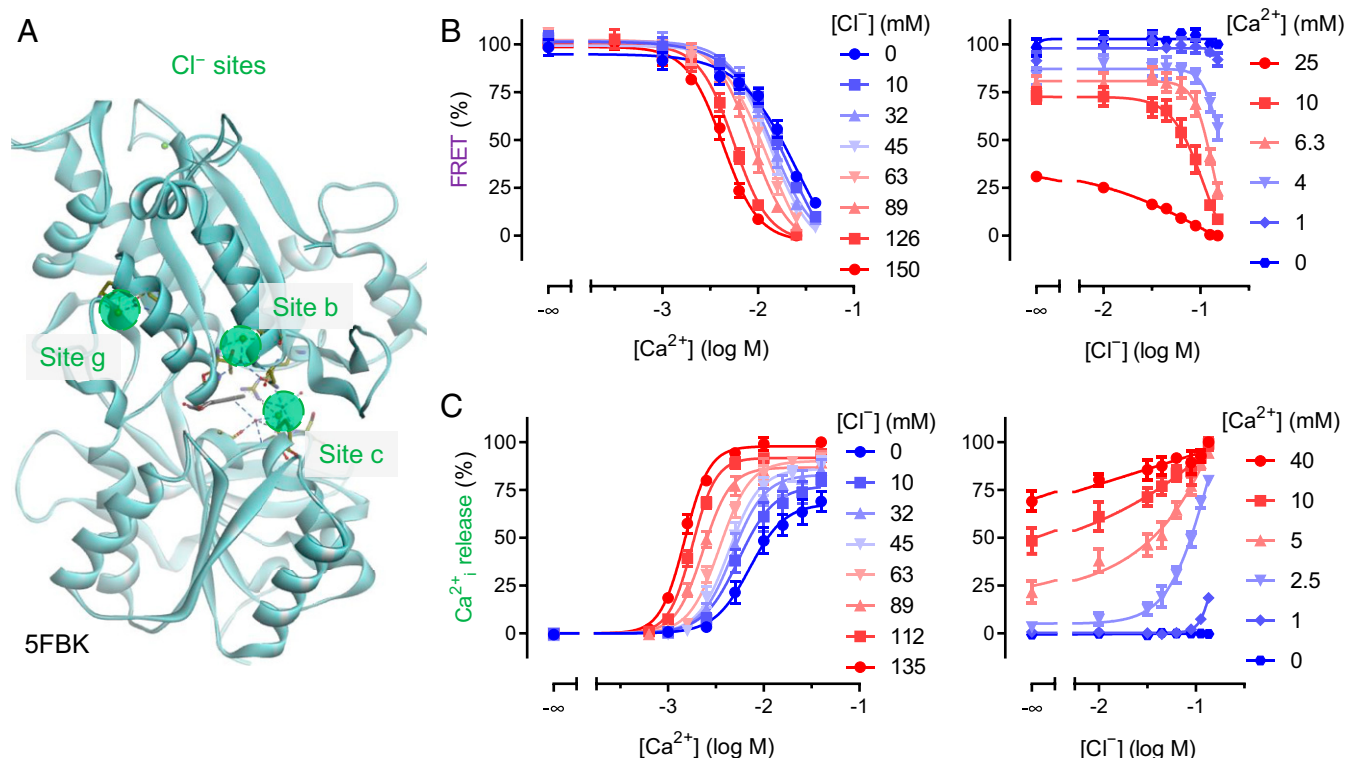


Fig. 4. Allosteric modulation induced by chloride ions in CaSR VFT. (A) Three chloride binding sites were reported in the CaSR structure (as shown in PDB 5FBK, sites *b*, *c*, and *g* refer to *SI Appendix*, Fig. S1). (B and C) FRET measurement (B) and Ca^{2+} release (C) for calcium gluconate on CaSR performed in a buffer with the indicated concentrations of chloride ions. Either Ca^{2+} concentration (Left) or Cl^- concentration (in buffer, Right) is used as the x axis. Data are mean $\pm$ SEM of three independent experiments performed in triplicates and normalized to the maximum response in the buffer with the highest Cl^- concentration.

size. Calcium gluconate induced a large decrease in FRET, indicating that Ca^{2+} induced rearrangement of the CaSR ECD in the absence of Cl^- , although with a lower potency (Fig. 4B and *SI Appendix*, Fig. S9B). Chloride ions enhanced the Ca^{2+} effect by largely increasing its potency, while having no effect on their own, revealing a pure PAM action of Cl^- up to its physiological concentration (larger than 100 mM) (Fig. 4B). Similar results were obtained in Ca^{2+} release assays (Fig. 4C).

We then investigated the functional effect of these three potential chloride binding sites on CaSR activity by site-directed mutagenesis (*SI Appendix*, Fig. S9 C–E). Sites *b* and *c* are proposed to be Ca^{2+} binding sites in another active conformation of CaSR (PDB 5K5S) (*SI Appendix*, Fig. S1). Site *b* was also proposed to bind calcium in the resting state (PDB 5K5T). Site *b* is the most conserved Cl^- binding site in mGluRs (46) and other class C GPCRs such as the taste receptors T1Rs (PDB 5X2P) (47). Mutation of the most conserved residues of this site (*SI Appendix*, Fig. S10A), T100A, strongly impaired the activation of the receptor by calcium. In contrast, the mutation T100E did not impair the activity of the receptor, consistent with the distal carboxylate group of Glu100, mimicking a chloride ion (46) (*SI Appendix*, Fig. S9C). These two mutations produced similar effects to the same mutations in the mGlu4 receptor (46), suggesting that this site could be a chloride binding site important for receptor function. In contrast, site *c* is composed of non-conserved residues (Arg66, Ser302, and Ser303) and their mutations do not strongly impair receptor activity (*SI Appendix*, Fig. S9D), suggesting that this site is not important for calcium effects on CaSR, but it could be a site responsible for PAM action of Cl^- . Finally, site *g* exists only in this structure, but it is most probably a structural binding site for chloride, and as such was not studied further except through a mutation that has no effect (*SI Appendix*, Fig. S9E).

Functional Calcium Binding Sites Near L-AAs at CaSR. As already mentioned, several Ca^{2+} binding sites have been proposed based on the crystal structures of the CaSR dimer (19, 20), but none of them have been firmly validated, and discrepancies exist between the two studies (*SI Appendix*, Fig. S1).

The main Ca^{2+} site was proposed to be located between the lower lobes (lobe 2) in the VFT dimer interface, and other cations (magnesium, gadolinium) have been proposed to also bind at this interface (see sites *d* through *f*) (Fig. 5A and *SI Appendix*, Fig. S1) (19, 20, 48). It is a region composed of highly negatively charged residues with numerous Glu and Asp residues (Fig. 5A and *SI Appendix*, Figs. S10B and S11A) and has been reported to stabilize the active state of other class C GPCRs by binding cations that neutralize these negative repulsive electrostatic charges (49). To investigate the importance of this interface for calcium activation, we have mutated all negatively charged residues including residues proposed to bind calcium, magnesium, or even gadolinium in the CaSR structures (Fig. 5A and B and *SI Appendix*, Fig. S11 B–E). These single and multiple mutants were well activated by calcium, suggesting that the activating binding site for calcium is not at this interface.

Among the other monatomic ion binding sites found in the crystal structures (*SI Appendix*, Fig. S1 A and B), sites *b* and *c* have low probability to bind functional Ca^{2+} , as they likely bind Cl^- , as shown above. In addition, site *a* has also low probability to bind cations important for CaSR activation, since it is formed by the polypeptide backbone (*SI Appendix*, Fig. S11 F and G) and it is a conserved structural binding site for cations as reported in mGluRs (50) and a fish taste T1R2/T1R3 receptor (47) (*SI Appendix*, Fig. S11H).

Since L-AAs have strong PAM activity, and calcium was proposed to bind near the L-AA binding pocket in the receptor (20, 42, 43), we then explored this possibility for the Ca^{2+} binding site

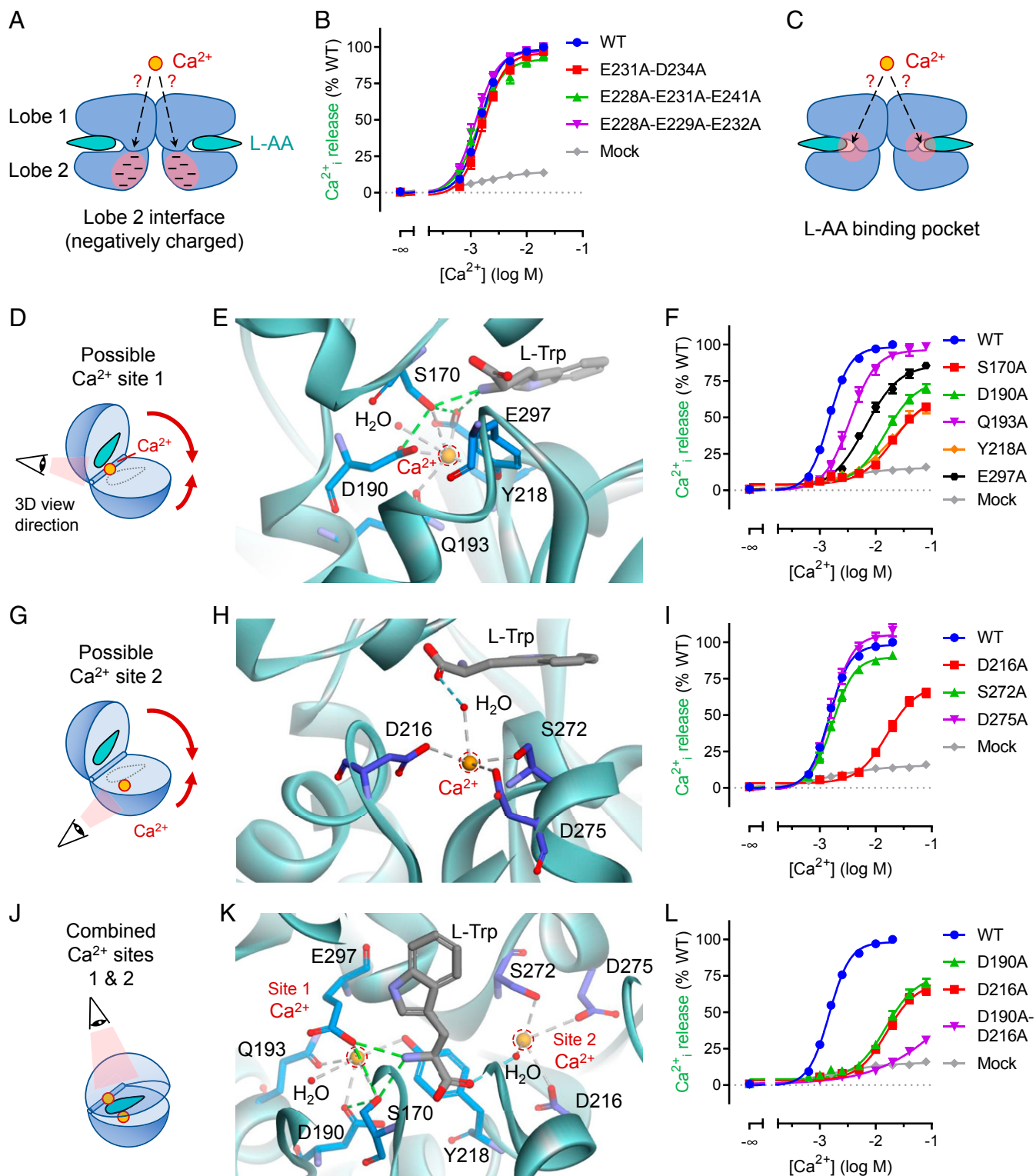


Fig. 5. Binding of two calcium ions nearby L-AA stabilizes the active state. (A) Cartoon illustrating the possible calcium binding at the lobe 2 interface which was previously proposed to be important for receptor activation. (B) Ca²⁺ release for the indicated mutants in this lobe 2 interface. (C) Possible Ca²⁺ binding in the L-AA binding pocket. (D–F) Proposed Ca²⁺ binding site 1 in the VFT hinge as illustrated by the cartoon (D), the 3D model of this site based on the crystal structure of the VFT (PDB 5K5S) (E), and Ca²⁺ release data for the indicated mutant in this site (F). Ca²⁺ is proposed to be bound to S170, D190, Q193, Y218, E297, and one water molecule found in the crystal structure. (G–I) Similar analysis for the proposed calcium binding site 2 in the VFT adjacent to L-AA, and Ca²⁺ release data for the indicated mutants. Ca²⁺ is proposed to be bound to the lobe 2 residues D216, S272, D275, and one water molecule found in the crystal structure (bridging this Ca²⁺ and bound L-Trp). (J–L) Combination of the two functional calcium binding sites 1 and 2 adjacent to the bound L-AA, top view of the L-AA surrounded by the two Ca²⁺ and Ca²⁺ release data for the indicated mutants. In the 3D model, interactions are shown as dashed lines (green for H-bonds, gray for metal bonds). Data in B, F, I, and L are mean ± SEM of at least three independent experiments performed in triplicates and normalized to WT. Mock indicates mock transfected cells with empty pRK5 vector.

(Fig. 5C). The first possible site (site 1, Fig. 5D and E) is located close to the hinge of the VFT. At this site, previous computational studies described a calcium ion binding to the conserved network of residues found in class C GPCRs activated by amino acids (e.g., mGluRs, DmXR, T1Rs, OR5.24, and GPRC6A) (42, 48). This network makes a signature motif that interacts with the amino acid moiety of the various agonists (51) (*SI Appendix, Fig. S10A*). However, none of the CaSR VFT crystal structures displayed an ion at such a position. Instead, a water molecule is reported in the two crystal structures with sufficient resolution (5FBK and 5K5S) (19, 20). Changing this water molecule to a calcium ion and minimizing the binding residues, revealed the interactions previously computed (Ser170, Asp190, Gln193, Tyr218, and Glu297; Fig. 5E) (42, 48). Indeed, the electronic density radii of a calcium ion and oxygen of a water molecule are close (<http://abulafia.mt.ic.ac.uk/shannon/radius.php>). Accordingly, we have validated this site 1 by site-directed mutagenesis and functional assays. Among the five mutants of the residues predicted to bind calcium, three (S170A, D190A, and Y218A) largely impaired the Ca^{2+} effect (Fig. 5F), consistent with Silve et al. (42). Interestingly, the Hill number of Ca^{2+} on these mutants (S170A, $n_H = 1.44 \pm 0.18$; D190A, $n_H = 1.57 \pm 0.19$; and Y218A, $n_H = 1.09 \pm 0.19$) was much lower than that on the WT ($n_H = 2.33 \pm 0.07$). This suggests that one calcium ion binds to site 1. Due to the dimeric nature of the CaSR, there are two sites 1 per receptor, which may explain why the n_H of the mutant is still higher than 1. Among the residues binding the calcium ion, Asp190 and Glu297 can establish strong ionic interactions because of their negatively charged side chains. Additionally, Asp190 is found in the hinge of the VFT where the closing movement is initiated; this supports its major role. The mutations S170 or Y218 to Ala had even a stronger effect but it is difficult to provide an explanation, as all four residues are well-conserved in class C VFTs (51).

The highly cooperative nature of Ca^{2+} activation ($n_H > 2$) suggests the existence of at least a second site that is important for receptor activation (Fig. 5G). Zhang et al. suggested that the electron density surrounded by Asp216, Ser272, Asp275, and a coordinating water molecule could be a magnesium ion (Mg^{2+}) (20). They opted for a highly ordered water molecule instead of a metal cation. A similar water molecule is found in all three structures of the closed CaSR VFT (PDB 5FBK, 5FBH, and 5K5S) (19, 20). We propose that a calcium ion may be found in place of this water molecule, although this water molecule could be in dynamic equilibrium with Ca^{2+} . Binding of calcium would provide a better stabilization of the closed VFT than a water molecule, since the Ca^{2+} ion can make up to eight bounds and a water molecule only four bounds. Thus, we implemented the change in the three-dimensional (3D) structure 5FBK and similarly in 5K5S. We observed interactions between calcium and Asp216, Ser272, Asp275, and a coordinating water molecule (Fig. 5H). In order to validate our model, we mutated these three residues to alanine at site 2. Mutations D216A strongly impaired the activation of the receptor by calcium (Fig. 5I), and the Hill number is decreased ($n_H = 1.66 \pm 0.25$). This supports the idea that one calcium ion could bind at site 2. Finally, when site 1 and 2 mutations were combined in the double mutant D190A-D216A (Fig. 5J–L and *SI Appendix, Fig. S12A*), the receptor activation by Ca^{2+} was strongly impaired, in agreement with the additivity of the effect of these single mutants (Fig. 5L). We have verified that all of the mutants were correctly expressed at the cell surface by ELISA (*SI Appendix, Fig. S12D*). Notably, we did not use the FRET CaSR biosensor to analyze calcium binding, as the mutations already impaired the conformational equilibrium of ECD. Indeed, we have shown that these mutations reduced the basal FRET signal of the CaSR (*SI Appendix, Fig. S12B*), indicating they favor the rearrangement of the VFT dimer toward an active-like conformation. We excluded that these mutations impaired receptor folding since

they did not abolish agonist-induced CaSR rearrangement in the TR-FRET assay (*SI Appendix, Fig. S12B*) and allosteric modulation by the NPS R-568 (*SI Appendix, Fig. S12C*). In addition, mutations did not impair cell surface expression of the CaSR (*SI Appendix, Fig. S12D*). Thus we proposed that these mutants are relevant to draw our conclusions on the calcium binding sites.

Altogether, our results suggest that there are two calcium binding sites in the L-AA binding pocket that are important for the activation of CaSR (Fig. 6A and B). Eventually, both sites 1 and 2 were validated at the website CheckMyMetal, the metal binding site validation server (52, 53). Both calcium ions are on either side of the L-AA and interact indirectly with it (Fig. 5E, H, and K). Ca^{2+} at site 1 interacts with L-AA through Ser170, Glu297, and Tyr218, while at site 2, calcium interacts with L-AA through one water molecule. Consistent with the possible physiological role of these Ca^{2+} /L-AA interacting sites, a mixture of L-AAs at concentrations found in fasting human brain plasma nicely potentiates Ca^{2+} -mediated responses in a physiological concentration range (Fig. 6C); but increasing the L-AA mix concentration, as observed after a protein-rich meal (54, 55), can further increase the effect of the physiological Ca^{2+} concentration (Fig. 6D).

Discussion

CaSR is a prototypical nutrient receptor regulated by various signaling compounds, including ions (Ca^{2+} and Mg^{2+} as activators, while SO_4^{2-} and PO_4^{3-} act as negative allosteric modulators [NAMs]) (19, 56), L-AAs, and polyamines like spermine (57). Structural studies also revealed that CaSR can bind Cl^- . In the present study, we investigated how such structurally different compounds regulate this receptor. We first analyzed the conformational changes associated with receptor activation at the level of the VFT, CRD, and 7TM domains using a FRET-based approach together with mutagenesis studies. This allowed us to validate a CaSR biosensor that was used to examine the action of various ligands in a cell-free and nutrient-controlled environment. We show that Ca^{2+} alone can fully stabilize the active state of the CaSR by inducing VFT closure, and we identified two important sites for this effect. In contrast, most L-AAs have no effect on their own, but enhance Ca^{2+} potency at this receptor, acting then as pure PAMs, rather than coagonists. We also revealed that chloride ions also act as PAMs of CaSR.

We show that SNAP-tag fusion at the N terminus of the CaSR subunits allowed a direct analysis of the conformational change occurring during receptor activation. This can be recorded through TR-FRET measurements after covalent labeling of the subunits with SNAP substrates carrying compatible fluorophores. These data reveal a similar change in the VFT orientation of the CaSR compared to mGluRs upon activation (58) (Fig. 6E). This is in contrast to the proposed structures for the resting and active states of the CaSR (19), as the relative orientation of the VFTs is similar in both proposed states (Fig. 1B). Our data suggest a different conformation for the resting CaSR, likely closer to that observed with the resting mGluRs such as the full-length mGlu5 structure (22). The CaSR active form predicted a close contact between the CRDs (19) that we validated by cross-linking experiments (Fig. 6E). Moreover, the sensor was also helpful in identifying positions in the 7TM domain that can be cross-linked to lock the receptor either in the resting high FRET state or in the active low FRET state. Such analysis suggests a similar movement of the 7TM domains as previously reported for mGluR2 (35) or observed in the mGluR5 cryogenic electron microscopy-EM structures (22). Indeed, the TM4 and TM5 of 7TM domains face each other in the resting state, while TM6s appear to be in close contact in the active state (Fig. 6E). Whether the 7TM domains contact each other in the inactive CaSR state is still questionable, as this is not the case in the resting mGlu5 structure (22), although TM4 and TM5 are indeed facing each other, allowing cross-linking to occur upon CuP treatment (35).

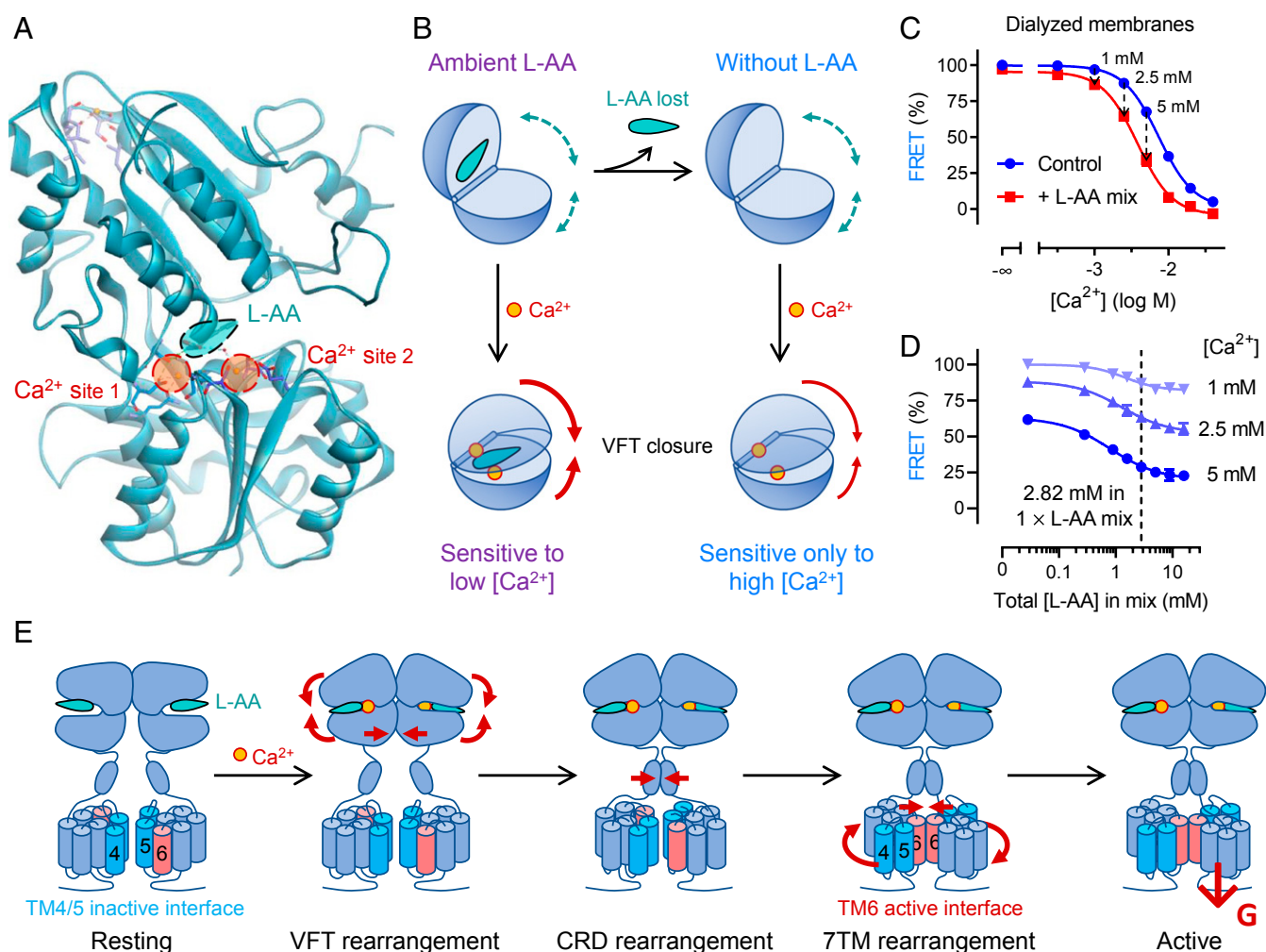


Fig. 6. Model for the activation of CaSR. (A) A 3D model of two possible functional calcium sites 1 and 2 near the bound L-AA based on the crystal structure of the VFT (PDB 5K55). (B) VFT close state is proposed to be stabilized by calcium ions in the presence of ambient L-AA (cell-based conditions), but also by calcium ions alone in the absence of L-AA (cell-free conditions) during activation. The ambient L-AAs bound to CaSR VFT contribute to the high calcium potency and enable the receptor to sense low concentrations of calcium ions. But this high sensitivity to calcium is reduced when L-AA is lost. (C) FRET measurement performed with CaCl_2 on dialyzed membranes in the absence or presence of one-fold L-AA mixture containing L-AA concentrations mimicking the human fasting plasma. Data are mean $\pm$ SEM of at least three independent experiments performed in triplicates and normalized to the basal of control and the maximum response of L-AA mixture. (D) FRET signal change induced by different folds of the L-AA mixture (C) performed in dialyzed membranes in the presence of CaCl_2 . Data are mean $\pm$ SEM of at least three independent experiments performed in triplicates. Data are normalized to the basal (without Ca^{2+} or L-AA) and the maximum response induced by Ca^{2+} in the presence of L-AA mixture. The vertical dotted line represents the related total concentration 2.82 mM of L-AAs used in one-fold L-AA mixture. (E) Molecular mechanism of activation of the CaSR upon L-AA and calcium binding. Binding of calcium ions in the VFT binding pocket most probably occupied by L-AA in physiological conditions, is expected to stabilize VFT closure and their relative rearrangement. Then it would induce CRD interactions and 7TM interface reorientation through allosteric propagation of the conformation changes. In the active state, TM6s will be at the dimer interface, a conformation required to stabilize at least one of the 7TMs in the active state for G protein activation.

The use of the CaSR sensor allowed us to study its activation under controlled conditions, in the absence of Ca^{2+} or in the absence of L-AAs. This allowed us to demonstrate that Ca^{2+} alone was able to fully activate the receptor, while L-AAs at a concentration of 10 mM cannot, indicating that L-AAs are pure PAMs. This is in contrast to many class C GPCRs, including mGluRs, fish olfactory receptors such as zOlfCc1 (59) and OR5.24 (60), GPRC6A (61), the murine pheromone receptor mVmn2r1 (59), or the umami taste receptor (47) (including rat/mouse receptors) that can be directly activated by the L-AAs. This is even more surprising when one considers that the amino acid binding mode is very similar in all these receptors and involves the same residues interacting with the α -amino and α -carboxylic groups (51). However, we found that most L-AAs except for Leu, Asp, Lys, and Pro (at 10 mM), potentiated the effect of Ca^{2+} by increasing its potency, such that the active L-AA

appears able to activate CaSR in the presence of low concentrations of Ca^{2+} . This raises the question of whether some other class C GPCRs activated by L-AAs also require Ca^{2+} for activation. Indeed, many of these receptors are also regulated by Ca^{2+} (59, 61–63). Finally, why some L-AAs do not show a PAM effect on CaSR is speculative, it might be due to steric hindrance in the VFT that prevents some L-AAs to bind, such as their binding could be restored only when the VFT is mutated (64).

Structural studies revealed that chloride ions bound to the CaSR VFT at three sites. Our data show that, as observed with mGluRs, Cl^- at physiological concentrations is a PAM of the CaSR, an effect that involves two binding sites corresponding to those identified in mGluRs (41, 46), further illustrating the similarity between these receptors. However, such an effect of chloride ions is unlikely to have any physiological effect, as their PAM effect is saturated at

their physiological concentration, and it is not expected that plasma Cl^- concentrations will change sufficiently to affect CaSR activity. Although it is speculative, we propose that site *c* might be responsible, at least in part, for the PAM effect of Cl^- , by stabilizing the closed form of the VFT as L-AAs do, even though the effect of mutations in this site is weak. Indeed, site *c* involves residues from both lobe 1 (upper lobe) and lobe 2 (lower lobe) of the VFT and then Cl^- binding at this site could stabilize the closed state of the VFT required for class C receptor activation (58). Binding of Cl^- to site *b* has most probably a structural function, since this chloride is highly conserved in class C GPCRs (46) and it involves only residues of lobe 1. Thus it is difficult to interpret how this site could be associated with a PAM effect. Finally, we cannot exclude that additional chloride binding sites in CaSR ECD, but not yet identified, can play a role in the PAM effect of Cl^- .

Although calcium ions appear to be the main and only direct activator of CaSR, it was surprising that its binding sites remained elusive, despite the solved structures of the CaSR ECD by two groups (19, 20). Surprisingly, mutation of the Ca^{2+} sites based on these structures did not affect Ca^{2+} activation of the receptor, indicating that even though Ca^{2+} may bind at those sites, it does not affect the activity of the CaSR. Another possible Ca^{2+} site was previously predicted by modeling and docking studies in the L-AA binding pocket (42, 43, 65), called site 1. The second site in this VFT binding pocket, site 2, was proposed to bind Mg^{2+} by Zhang et al. (20). Our data clearly suggest that site 1 and site 2 are responsible for CaSR activation by Ca^{2+} . Mutating one of these sites resulted in a decreased Ca^{2+} effect, and most importantly, in a decreased n_H , from 2.3 for the WT to ~ 1.7 or below for the mutants, consistent with a decrease in the number of activating Ca^{2+} sites. The fact that n_H remains significantly higher than 1 is also consistent with the dimeric nature of CaSR with a possible positive cooperativity between the subunits, as observed for the activation of mGluRs by glutamate (66). Notably, mutating both Ca^{2+} sites in each subunit results in a drastic decrease in Ca^{2+} activation, demonstrating the essential role of these two sites in CaSR activation. The low potency of Ca^{2+} for these sites, and the difficulty in assigning ions to specific densities in crystal structure (67), may explain why these two important sites have been missed when analyzing CaSR ECD structures.

Together, these data assist us to propose a model for how Ca^{2+} and L-AAs regulate CaSR activity. Ca^{2+} at site 1 interacts with residues close to the hinge region of the VFT, and contact residues from both lobes, thus, likely stabilizing the close active state of the VFT. Site 2 only involves residues from lobe 2 such that it is more difficult to imagine how Ca^{2+} binding at this site may be sufficient to activate the receptor, as observed when site 1 is mutated. It is possible that Ca^{2+} neutralizes negative charges, such as that carried by D216, allowing the closure of the VFT, or that networks involving water molecules participate in stabilizing the closed VFT. Since L-AAs do not activate CaSR on their own, it is questionable whether they bind to the VFT in the absence of Ca^{2+} .

However, because of the number of interactions they make with lobe 1 residues, we think they do. It is possible that they cannot stabilize the closed active VFT because of the repulsion of lobe 2, due to the negative charge of D216. Only when this charge is neutralized with Ca^{2+} bound at site 2 can the VFT get close. This residue may be responsible for the activation difference between the CaSR and other class C receptors that are activated by L-AAs.

Taken together, our data clarify the mode of action of Ca^{2+} and L-AAs on CaSR. The question of course remains, however, whether the L-AA PAM effect is the result of the evolution of this receptor from L-AA-activated class C GPCRs such as the mGlu, the fish L-AA olfactory, or even the umami taste receptor, or whether such a L-AA effect on CaSR is of physiological importance. Indeed, our well-controlled assay clearly confirms and further elucidates that a L-AA mix, corresponding to that found in fasting human plasma, can potentiate the effect of physiological concentrations of Ca^{2+} on the CaSR. Notably, increasing this L-AA mix concentration, as observed after a protein-rich meal, further potentiates the Ca^{2+} response. It is therefore likely that the increase in L-AA plasma concentration after a meal (up to 30 mM) enhances the Ca^{2+} effect, leading to a decreased PTH secretion (6). Indeed, PTH plasma concentration is reduced after meals (54, 55). Whether this is only due to the allosteric control of CaSR by L-AAs or to other processes remains to be examined, but it is likely that this, at least, plays a role. In conclusion, our data illustrate how a receptor could integrate the information coming from various structurally different nutrients to generate an optimized cellular response.

Materials and Methods

Information on materials, transfection, cell surface quantification, inositol phosphate accumulation, Ca^{2+} release, cross-linking, molecular modeling, curve fitting, and data analysis is provided in [SI Appendix](#).

FRET Measurements on Cells and Membranes. Labeling and FRET measurements on cells were performed as described previously (21, 24). Membranes were prepared and dialyzed after labeling on cells. See [SI Appendix](#) for more details.

Data Availability. All data and associated protocols for this study are available in the main paper and [SI Appendix](#). Materials may be requested to P.R.

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