Modeling and Mutagenesis of the Binding Site of Calhex 231, a Novel Negative Allosteric Modulator of the Extracellular Ca\(^{2+}\)-sensing Receptor*§

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A model of the Ca\(^{2+}\)-sensing receptor (CaSR) seven transmembrane domains was constructed based on the crystal structure of bovine rhodopsin. This model was used for docking (1S,2S,1'R)-N\(^1\)-(4-chlorobenzoyl)-N\(^2\)-(1-naphthyl)ethyl-1,2-diaminocyclohexane (Calhex 231), a novel potent negative allosteric modulator that blocks (IC\(_{50}\) = 0.39 \(\mu\)M) increases in \(^{3}H\)inositol phosphates elicited by activating the human wild-type CaSR transiently expressed in HEK293 cells. Two receptor mutations, F684A and E387A, caused a loss of the ability of Calhex 231 to inhibit Ca\(^{2+}\)-induced accumulation of \(^{3}H\)inositol phosphates. Three other mutations, F688A, W818A, and I841A, produced a marked increase in the IC\(_{50}\) of Calhex 231 for the Ca\(^{2+}\) response, whereas L776A and F821A led to a decrease in the IC\(_{50}\). Our data validate the proposed model for the allosteric interaction of Calhex 231 with the seven transmembrane domains of the CaSR. Interestingly, the residues at the same positions have been shown to delimit the antagonist-binding cavity of many diverse G-protein-coupled receptors. This study furthermore suggests that the crystal structure of bovine rhodopsin exhibits sufficient mimicry to the ground state of a very divergent class 3 receptor to predict the interaction of antagonists with the heptahelical bundle of diverse G-protein-coupled receptors.

The extracellular Ca\(^{2+}\)-sensing receptor (CaSR) plays an essential role in the regulation of Ca\(^{2+}\) homeostasis. Located at the cell surface of the parathyroid cell, the CaSR is stimulated by serum Ca\(^{2+}\) and controls parathyroid hormone release (1). Initially cloned from bovine parathyroid (2), the CaSR has been isolated from various species and tissues (3–6). CaSR activation results in calcitonin secretion in the thyroid and Ca\(^{2+}\) reabsorption in the kidney. The CaSR on nerve terminals may regulate neurotransmitter release (3, 7), and its presence on oligodendrocyte cells suggests that it participates in the complex processes of myelination (8, 9). Its physiological importance is further illustrated in several disorders linked to Ca\(^{2+}\) homeostasis resulting in gain- or loss-of-function mutations (10).

The CaSR belongs to G-protein-coupled receptor (GPCR) class 3, which comprises eight metabotropic glutamate receptors and γ-aminobutyric acid type B, vomeronasal, pheromone, and taste receptors. These GPCRs possess an unusual long bilobed amino-terminal extracellular domain resembling bacterial periplasmic binding protein implicated in nutrient transport and postulated to contain the ligand-binding sites of these receptors (11, 12). The CaSR is activated by Ca\(^{2+}\) and Mg\(^{2+}\) present in the extracellular fluids and by charged molecules, including spermine, spermidine, β-amyloid peptides, and several antibiotics (2, 4, 8, 13–15). Recently, low molecular mass synthetic molecules activating the CaSR have been identified, and their pharmacological properties with respect to cloned CaSR have been reported (16–19). It has been proposed that these molecules, named calcilytics, interact allosterically within the seven transmembrane domains to potentiate the effect of Ca\(^{2+}\) (20–22). On the other hand, compounds that inhibit the effect of Ca\(^{2+}\) on the CaSR are called calcilytics (23).

Controlling transient parathyroid hormone release by blocking the parathyroid CaSR with such molecules has been hypothesized to produce anabolic effects in bone and represents a major therapeutic interest in the treatment of osteoporosis (23). Moreover, such compounds might be useful for studying the roles played by the CaSR in tissues under physiological and pathological states. NPS 2143 was the first negative allosteric modulator acting on the CaSR whose properties have been investigated both in vitro and in vivo (24, 25). We have recently synthesized and evaluated the in vitro pharmacological properties of a novel structurally different series of calcilytics acting on the cloned rat CaSR (26). We now report the calcilytic properties of (1S,2S,1'R)-N\(^1\)-(4-chlorobenzoyl)-N\(^2\)-(1-naphthyl)ethyl-1,2-diaminocyclohexane (Calhex 231) (Fig. 1), and its effects on the parathyroid CaSR with such molecules (23).

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§ The on-line version of this article (available at http://www.jbc.org) contains Supplemental Table 2 and Supplemental Refs. 1–54.

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¶ The abbreviations used are: CaSR, Ca\(^{2+}\)-sensing receptor; GPCR, G-protein-coupled receptor; WT, wild-type; TM, transmembrane; IP, inositol phosphate; GST, glutathione S-transferase; ECL2, extracellular loop 2.

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which belongs to this family of molecules, and the characterization of its potency toward the human CaSR transiently expressed in HEK293 cells. Calhex 231 shows in vitro potency comparable to NPS 2143 in inhibiting Ca\(^{2+}\)-induced activation of the human CaSR (25). We have developed a three-dimensional model of the transmembrane domains of the human CaSR based on the crystal structure of bovine rhodopsin (27). This model has allowed us to dock Calhex 231 into a hydrophobic cavity centered on Glu-837 (position 7.39 according to the numbering of Ballesteros et al. (28)) with two adjacent hydrophobic pockets. We used site-directed mutagenesis of amino acid residues likely involved in the recognition of Calhex 231 to demonstrate the validity of this model and to propose a possible binding mode of this negative allosteric CaSR modulator within the seven transmembrane domains.

**EXPERIMENTAL PROCEDURES**

**Preparation of Calhex 231—**Calhex 231 was prepared as its hydrochloride salt. Briefly, cyclohexene in acetonitrile was treated with (N-p-nitrobenzenesulfonyl)iminio phenyldiazene in the presence of a catalytic quantity of copper(II) triflate to give the aziridine (\(\geq 7\%\)-(4-nitrobenzenesulfonyl)-7-azabicyclo[4.1.0]heptane). The latter was reacted in triethylamine-containing tetrahydrofuran with (R)-1-(1-naphthyl)ethylamine to afford the aziridine ring-opened product N\(^{-}\)-(4-nitrobenzenesulfonyl)-N\(^{1}\)-(1-naphthyl)ethyl-1,2-diaminocyclohexane. Removal of the p-nitrobenzenesulfonyl group by the action of thiophenol/potassium carbonate in acetonitrile/Me\(_2\)SO followed by acylation of the resulting free amine with 4-chlorobenzoyl chloride provided (1S,2S,1R)-N\(^{-}\)-(4-chlorobenzoyl)-N\(^{1}\)-(1-naphthyl)ethyl-1,2-diaminocyclohexane, i.e. Calhex 231, as the slower moving of two components on silica gel. The absolute configuration of Calhex 231 was deduced by x-ray crystallography. Details of the synthesis and structural characterization of Calhex 231 will be published elsewhere.

**Site-directed Mutagenesis—**To mutate amino acids possibly involved in the binding site of Calhex 231, the coding region of the human wild-type (WT) CaSR, kindly provided by Prof. M. Freichel (6), was first cloned in the HindIII-XbaI sites in a modified pUC18 plasmid in which SacI and SmaI restriction sites were removed (pUCmCaSR). A SacI-cloned in the HindIII-XbaI sites in a modified pUC18 plasmid in which the coding region of the human CaSR (25) was first manually docked into the TM cavity by anchoring its seven transmembrane domains fixed. A slow pairwise alignment using the BIOPOLYMER module of SYBYL (Tripos Associates, Inc., St. Louis, MO) was then replaced in the pUCmCaSR plasmid to obtain the final mutant CaSR. Finally, WT and mutant CaSR coding regions were subcloned into HindIII-XbaI sites of the pcDNA3 expression vector (Invitrogen). All point mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) with specific oligonucleotides (Eurobio, Les Ulis, France) to convert residues to alanine. Sequencing was performed on both strands in pBluescript SK+ (Eurobio, Les Ulis, France) to convert residues to alanine.

**Preparation of Starting Protein Coordinates—**The three-dimensional model of the human CaSR was constructed using a previously described procedure (34). Briefly, starting from the x-ray structure of bovine rhodopsin (Protein Data Bank code 1R88), a first model of the seven transmembrane domains was obtained by mutating the side chains of the amino acids in rhodopsin. Standard geometries for the mutated side chains were given by the BIOPOLYMER module of SYBYL (Tripos Associates, Inc., St. Louis, MO). Whenever possible, the side chain torsional angles were kept to the values occurring in bovine rhodopsin. Otherwise, a short screening of side chain angles was performed to remove sterical clashes between the mutated side chain and the other amino acid residues. The third intracellular loop between TM5 and TM6, which shows a high degree of variability, was not included in any of the models. This loop is believed for most GPCRs to be far away from the TM-bonding cavity (35). We therefore assume that omitting this loop should not influence our docking results. The observed insertions/deletions in the loops of the CaSR were achieved through a simple knowledge-based loop search procedure as previously reported (34). Special caution had to be given to extract the TM loop from TM1 to TM3, which has been described in bovine rhodopsin to fold back over the heptahedral bundle (27) and therefore limits the size of the active site. Two models of this loop were proposed. The first one assumes a rhodopsin-dependent fold-invariant which was obtained by direct threading to the rhodopsin template. In this model, one residue was inserted eight positions before Cys-765, and nine amino acids were deleted after His-766. The second model assumes a rhodopsin-independent fold and was obtained by searching for two loops of 12 and 11 residues linking Ser-750 to Thr-764 and His-766 to Ser-768, respectively. After the heavy atoms were modeled, all hydrogens were added, and the protein coordinates were then minimized with AMBER6 using the AMBER95 force field as previously described (34).

**Modeling the Calhex 231-bound CaSR—**To obtain a ligand-bound model of the CaSR, the above-described coordinates were refined as previously described (34) to enlarge the binding cavity. Briefly, Calhex 231 was first manually docked into the TM cavity using its protonable nitrogen atom to the only negatively charged residue of the binding site (Glu-837) and fitting the shape of the two aromatic moieties.
into the two proximal hydrophobic pockets. The bulky naphthalene group was docked into the bigger of the two pockets (pocket A: Pro-682, Phe-688, Val-689, Tyr-744, Pro-748, Leu-766, Trp-818, Phe-821, and Ala-840), whereas the substituted phenyl moiety was located into the smaller of the two pockets (pocket B: Phe-612, Ala-615, Leu-616, Ser-665, Phe-668, Ile-669, Phe-684, Val-838, and Ile-841). After parameterization of Calhex 231 for the AMBER6 force field using a previously reported protocol (39), the resulting protein-ligand complex was then refined by minimization using the above-described AMBER parameters. Removing the ligand atoms from the minimized complex finally yielded one set of coordinates for the Calhex 231-bound receptor model.

Automated Docking of Calhex 231—To verify that the above-described coordinates were not biased by the manual docking procedure, the Sulfex docking program (37) was used to automatically dock Calhex 231. An idealized active-site ligand or protomol (38) was first generated from 33 consensus positions (34) supposed to map the TM cavity of most GPCRs. This protomol consists of the preferred locations of various molecular probes (CH₃, C=O, N–H), which are then used by the docking engine to search for the best three-dimensional morphological similarity between the protomol and the ligand to dock. A protocol, thresh value of 0.5 and a proto_bloat value of 0 were used to generate a compact protomol. A Tripos mol2 file of Calhex 231, obtained from a two-dimensional sketch as previously reported (34), was docked into the TM cavity using standard parameters of Sulfex used in the “whole” docking approach (37). The best 30 solutions were finally stored in mol2 format.

RESULTS

Potency of Calhex 231—In a recent preliminary report, we described the synthesis and characterization of a novel series of molecules displaying calcilytic properties toward the rat CaSR (26). We have now synthesized Calhex 231, which belongs to this family of molecules, and we have investigated its potency toward the human CaSR by measuring its effects on Ca²⁺-induced accumulation of [³H]IP, a well-characterized response linked to CaSR activation (2, 13, 39). Increasing the concentration of extracellular Ca²⁺ from 0.3 to 10 mM caused a 10-fold increase in [³H]IP accumulation in HEK293 cells transiently transfected with the human WT CaSR, whereas we did not detect a significant increase in [³H]IP accumulation in cells transiently transfected with an empty control plasmid (Fig. 2A) (data not shown). Analysis of the dose-response curve led to an EC₅₀ for Ca²⁺ of 3.4 ± 0.1 mM (mean ± S.E., n = 10). These data fit well with the affinity for Ca²⁺-mediated increases in IP accumulation previously determined for the human CaSR (39). Preincubation of HEK293 cells expressing the human WT CaSR with Calhex 231 caused a concentration-dependent inhibition of the IP response to 10 mM Ca²⁺ (Fig. 2B). Analysis of the dose-response curve led to an IC₅₀ for Calhex 231 of 0.39 ± 0.08 μM (mean ± S.E., n = 7). These data indicate that Calhex 231 is a potent calcilytic of the human CaSR transiently expressed in HEK293 cells.

Molecular Modeling of the Seven Transmembrane Domains of the Human CaSR—To elucidate the binding mode of Calhex 231, we postulated that the ligand-binding pocket could be localized within the seven transmembrane domains of the CaSR. First, we developed a model of the human CaSR based on the x-ray structure of bovine rhodopsin (27), which was used as a template to model the seven transmembrane domains of the CaSR. Using the in-house developed GPCRmod program, we could unambiguously assign the positions of the seven helices using GPCR-dependent TM-specific amino acid fingerprints (Fig. 3A) and thread CaSR three-dimensional coordinates onto those of bovine rhodopsin. Two models were generated differing only in the fold of ECL2 (Fig. 3, B and C). The first one assumes a conserved folding of ECL2 over the seven-TM bundle as in bovine rhodopsin. The rationale for this first choice was the presence of two conserved cysteine residues at position 677 in TM3 and position 765 in ECL2 of the CaSR, which are also present in bovine rhodopsin, to form a disulfide bridge. The second model of ECL2 was derived independently from the rhodopsin structure, as it is questionable whether the particular fold of ECL2 in rhodopsin is a common feature of most GPCRs (Fig. 3C).

As previously reported (34), the TM cavity was enlarged to accommodate a ligand for automated docking. This procedure requires the manual positioning of Calhex 231. As there is only a single accessible negatively charged residue in the TM cavity (Glu-837) available to neutralize the positively charged secondary amine of the ligand, anchoring Calhex 231 into the TM cavity was straightforward (see “Experimental Procedures”). After energy refinement of the receptor-ligand complex and subsequent expansion of the binding cavity, the CaSR forms a well-defined hydrophobic cavity centered on Glu-837 located in the seven transmembrane domains with the two adjacent hydrophobic pockets A and B. The bigger pocket A is delineated by hydrophobic side chains between TM3, TM5, and TM6, whereas the smaller pocket B is located between TM1, TM2, TM3, and TM7.

Hypothesized Binding Mode of Calhex 231—Automated docking of Calhex 231 with the recently described Sulfex docking program (37) disclosed a preferred binding mode (Fig. 4, A and B) in which both nitrogen atoms are H-bonded to Glu-837.
FIG. 3. Amino acid sequence alignment of the seven transmembrane domains of five GPCRs and close-up of the ECL2 alignment of the human CaSR with bovine rhodopsin. A, alignment of the human CaSR, metabotropic glutamate receptor-1 (MGR1), γ-aminobutyric receptor-1 (GBR1), the β2-adrenoreceptor (B2AR), and bovine rhodopsin (OPSD) was performed by GPCRalign (D. Rognan, C. Bissantz, and...
The close proximity of the protonated secondary amine to the negatively charged Glu-837 side chain indicates a likely ionic interaction between both moieties. The naphthyl moiety is embedded in pocket A and interacts with neighboring hydrophobic side chains (Pro-682, Phe-688, Val-689, Tyr-744, Pro-748, Leu-776, Trp-818, and Phe-821). The p-chlorophenyl group is buried in the additional pocket B (Phe-612, Ala-615, Leu-616, Phe-668, Ile-669, Phe-684, Val-838, and Ile-841). Interestingly, the cyclohexyl scaffold is proposed to be located in a small hydrophobic niche delimited by Pro-682, Phe-684, and Gly-685. The important methyl group at the chiral carbon atom directly faces the Phe-821 aromatic ring. The proposed interaction model suggests a tight binding of Calhex 231, as 85% of it overall surface (573 of 674 Å²) is buried upon binding to the TM cavity.

**Generation of Point Mutations and Characterization of the Mutant CaSR**—We have mutated into alanine Thr-764 and His-766 located in ECL2 as well as seven other amino acid residues located in TM3 and TM5–7 by site-directed mutagenesis to investigate their possible interactions with Calhex 231. These mutants and the WT receptor were transiently transfected into HEK293 cells. We then analyzed their ability to respond to Ca²⁺ by measuring [³H]IP accumulation and their expression by Western blotting using a specific rabbit antiserum (141Ab) developed against the carboxyl-terminal tail of the human CaSR.

This antiserum was generated against a 330-amino acid polypeptide starting from amino acid 747 of the CaSR and fused to GST. The 141Ab antiserum was first evaluated by Western blot analysis against the human WT receptor. Under reducing conditions, two polypeptides migrating with a mobility corresponding to relative molecular masses of 150 and 130 kDa were identified in membrane preparations from cells transfected with the WT receptor, whereas these signals were absent in mock cell preparations, thereby indicating their specificity corresponding to relative molecular masses of 150 and 130 kDa.

**DISCUSSION**

In this study, we have reported the characterization of Calhex 231, a novel negative allosteric modulator of the human CaSR. We used molecular modeling approaches, mutagenesis, and functional activity (phospholipase C) to identify for the first time residues involved in the binding pocket of a negative modulator of the CaSR. The amino-terminal domain of the CaSR is thought to contain the Ca²⁺-binding sites and has been submitted to extensive mutation and deletion studies, which have given insight on the mechanism of CaSR activation (41–43). However, little is known about the binding sites of positive or negative allosteric modulators of the CaSR. An amino acid residue (Glu-837) located in TM7 has been reported to interact

A. Logean, submitted for publication. Residues in boldface are typical fingerprints (31) from either class 1 (β₂-adrenergic receptor and bovine rhodopsin) or class 3 (CaSR, metabotropic glutamate receptor-1, and γ-aminobutyric receptor-1) GPCRs. Boxes correspond to mutation effects described herein. The residue numbering of Ballesteros et al. (28) is indicated above the proposed sequence alignment. B, close-up of the sequence alignment of ECL2 of the human CaSR with bovine rhodopsin. Residues neighboring the conserved cysteine residue involved in a disulfide bridge with the third transmembrane domain are boxed. C, two putative models of ECL2 of the CaSR obtained either by direct threading to the rhodopsin x-ray structure (in green) or by a loop search procedure (in cyan). The SCG residues of bovine rhodopsin (in white) facing retinal (27) are displayed as sticks with the following color coding: carbon atom, white; oxygen atom, red; nitrogen atom, blue; and sulfur atom, yellow. The retinal structure and location of TM4 and TM5 are shown. Arrows indicate the path of the main chain.
with the calcimimetic NPS R-568 (21), whose pharmacological properties with respect to cloned CaSR have been previously reported (16, 18). At the present time, the sites of interaction with the CaSR of NPS 2143, the first and sole calcilytic whose pharmacokinetic properties have been reported in vitro and in vivo (24, 25), have not yet been described. We recently identified a novel class of molecules inhibiting the effect of Ca\(^{2+}\)/H\(_{11001}\) on the cloned rat CaSR expressed in Chinese ovary cells (26). We have now synthesized Calhex 231, which belongs to this family of molecules, and have shown that it behaves as a potent and high affinity negative allosteric modulator of the human CaSR. Although the CaSR and rhodopsin display little amino acid identity, we have generated a three-dimensional model of the seven transmembrane domains of the CaSR that has allowed us to identify putative residues implicated in the recognition of Calhex 231. We submitted nine of these residues to mutations and found that seven of them affect the binding affinity of Calhex 231 as measured by inhibition of Ca\(^{2+}\)/H\(_{11001}\)-induced IP accumulation, a well characterized functional response linked to CaSR activation in these cells (39), thus confirming that Calhex 231 is a negative allosteric modulator of the CaSR.

Indeed, the binding cavity of Calhex 231, as disclosed by this study, shares numerous similarities with antagonist-binding cavities of other GPCRs (Table II and Supplemental Table 2). Our current data demonstrate that three residues, Phe-6843.32, Phe-6883.36, and Glu-8377.39, occupying central positions in the

![Fig. 4. Molecular modeling of the human CaSR complexed with Calhex 231. Shown is the proposed interaction model between Calhex 231 and the CaSR binding cavity. TM helices are displayed as yellow ribbons. Calhex 231 and important CaSR heavy atoms are indicated by sticks using the following color coding: carbon atom of Calhex 231, cyan; carbon atom of the CaSR, white; oxygen atom, red; nitrogen atom, blue; and chloride atom, green. Important side chain positions of the CaSR are labeled at the C-α atom. Intermolecular hydrogen bonds between Glu-837 and the two nitrogen atoms of Calhex 231 are represented by dotted yellow lines. Mutations discussed herein are displayed by yellow labels. A, front view; B, top view from the extracellular side. Calhex 231 is embedded in pockets A and B.](http://www.jbc.org/)

![Fig. 5. Expression of the CaSR mutants. Immunoblot analysis of whole cell lysates (4 μg of proteins) from HEK293 cells transiently transfected with an empty vector (MOCK) or with a vector containing the WT CaSR or the indicated mutant CaSRs was performed by SDS-PAGE as described under “Experimental Procedures.” CaSR proteins were detected using the specific rabbit 141Ab serum directed against the carboxyl-terminal region of the human CaSR. The position of the molecular mass markers is shown on the left. Arrowheads on the right indicate the molecular masses (in kDa) of two major bands corresponding to the WT and mutant receptors.](http://www.jbc.org/)

![Fig. 6. Effect of some CaSR mutations on Ca\(^{2+}\)-induced accumulation of [\(^3\)H]IP after transient transfection in HEK293 cells. Shown are concentration-response curves of Ca\(^{2+}\)-induced IP stimulation (expressed as % of maximal response observed with 10 mM Ca\(^{2+}\)) in HEK293 cells transfected with WT or mutant receptors as indicated in A and B. The cells were transfected with the adequate vector, and the IP response to Ca\(^{2+}\) was performed as described under “Experimental Procedures.” Data are means ± S.E. of triplicates from a typical experiment representative of three to five experiments.](http://www.jbc.org/)

Mapping the Binding Pocket of a Calcilytic
**Summary of the effects of various CaSR mutations on the properties of Ca\(^{2+}\) and Calex 231 on the IP response**

Concentration-response curves for Ca\(^{2+}\) and Calex 231 were generated as described in the legend to Figs. 6 and 7. EC\(_{50}\) values and maximal stimulation for Ca\(^{2+}\) compared with a maximal Ca\(^{2+}\) response of the WT CaSR and IC\(_{50}\) values for Calex 231 were calculated. Data shown are means ± S.E. from 3–10 independent experiments.

| Receptor | Position | Maximal WT response | Ca\(^{2+}\) EC\(_{50}\) | Calex 231 IC\(_{50}\) |
|----------|----------|---------------------|----------------------|----------------------|
| WT       |          | 100 ± 4             | 3.4 ± 0.1            | 0.39 ± 0.08          |
| F684A    | TM3      | 50 ± 4\(^{a}\)      | 5.9 ± 0.4\(^{a}\)    | >10\(^{-5}\)         |
| F688A    | TM3      | 50 ± 3\(^{a}\)      | 5.9 ± 0.2\(^{a}\)    | 3.20 ± 0.98\(^{a}\)  |
| T764A    | ECL2     | 120 ± 9             | 3.0 ± 0.3            | 0.28 ± 0.05          |
| H766A    | ECL2     | 102 ± 8             | 3.2 ± 0.3            | 0.64 ± 0.03          |
| L776A    | TM5      | 121 ± 9             | 2.2 ± 0.3            | 0.07 ± 0.03\(^{b}\)  |
| W818A    | TM6      | 72 ± 4\(^{a}\)      | 3.4 ± 0.2            | 3.30 ± 0.50\(^{b}\)  |
| F821A    | TM6      | 112 ± 10            | 2.6 ± 0.2            | 0.06 ± 0.01\(^{b}\)  |
| E837A    | TM7      | 72 ± 5\(^{a}\)      | 3.8 ± 0.2            | >10\(^{-5}\)         |
| I841A    | TM7      | 98 ± 6              | 2.9 ± 0.2            | 2.71 ± 0.10\(^{a}\)  |

\(^{a}\) P < 0.001 compared with the WT receptor.
\(^{b}\) P < 0.01 compared with the WT receptor.

**TABLE II**

**GPCRs that share with the CaSR the same TM positions for delimiting the antagonist-binding cavity**

| Position\(^{a}\) | Receptors\(^{b}\) |
|------------------|------------------|
| 3.32             | Monoamine receptors, OPSD |
| 3.36             | A1AR, CRK5, D2DR, D3DR, MGR5, NK1R, OPSD, OXYS, TSHR |
| 5.42             | AG2R, CRK5, monoamine receptors, OPSD |
| 6.48             | A3AR, ACM1, ACM2, AG2R, BRB2, D2DR, GASR, GRHR, OPRD, OPRX, OPSD, MGR1, TRFR, V1AR |
| 6.51             | 5H4, A1AR, ACM1, AG2R, BRB2, D2DR, GASR, GRHR, NK1R, BK1R, NTR1, NY2R, OPSD, V1AR |
| 7.39             | 5H1D, A1AR, AA2A, ACM1, B2AR, C5a, CCKR, CR2, CRK5, D2DR, D3DR, GASR, MGR1, OPRD, OPRX, OPSD, P2YR, P2R, US28, V1AR |
| 7.43             | A1AR, AA2A, AA3R, ACM1, AG2R, BRB2, GRFR, NTR1, OPRD, OPRM, OPSD |

\(^{a}\) Numbering of Ballestero et al. (28).
\(^{b}\) Swiss Protein Data Bank identification: monoamine receptors (acetylcholine, adrenergic, dopamine, histamine, serotonin, octopamine, and trace amine receptors); OPSD, bovine rhodopsin; A1AR, a-adrenoceptor type 1a; CRK5, C-C chemokine receptor-5; D2DR, dopamine D2 receptor; D3DR, dopamine D3 receptor; MGR5, metabotropic glutamate receptor-5; NK1R, neurokinin type 1 receptor; OXYS, oxytocin receptor; TSHR, thyrotropin-stimulating hormone receptor; AG2R, angiotensin type 2 receptor; AA3R, adenosine type 3 receptor; ACM1, muscarinic m1; ACM2, muscarinic m2; BRB2, bradykinin receptor type 2; GASR, cholecystokinin receptor type B; GRHR, gonadotropin-releasing hormone receptor; OPRD, opioid receptor-δ; OPRX, nociceptin receptor; MGR1, metabotropic glutamate receptor-1; TRFR, thyrotropin-releasing hormone receptor; V1AR, vasopressin type 1a receptor 5H4, serotonin 5-HT4; A1AB, adrenoreceptor type 1B; NK2R, neurokinin type 2 receptor; NTR1, neurotensin receptor-1; NY2R, neuromedin Y type 2 receptor; 5H1D, serotonin 5-HT1D; AA2A, adenosine type 2 receptor; B2AR, β-adrenoceptor type 2; C5a, C5a anaphylatoxin chemotactic receptor; CCKR, cholecystokinin receptor type A; CRK2, C-C chemokine receptor-2; P2YR, purinergic P2Y1 receptor; P2R, prostacyclin receptor, US28 type 1 receptor, human cytomegalovirus US28 receptor; A1AR, adenosine type 1 receptor; GRFR, bombesin receptor; OPRM, opioid receptor-μ.

**FIG. 7.** Effect of CaSR mutations on inhibition of Ca\(^{2+}\)-stimulated increases in IP by Calex 231. Shown is the concentration-dependent inhibition of Ca\(^{2+}\)-stimulated (10 mM) increases in the IP response by Calex 231 in HEK293 cells expressing the WT or mutant receptors as indicated in A and B. The cells were transfected with the adequate vector, and the IP response to Ca\(^{2+}\) was performed as described under “Experimental Procedures.” After the prelabeling and washing procedures, cells were incubated with 10 mM Ca\(^{2+}\) alone or in the presence of increasing concentrations of Calex 231 for 30 min. Data are expressed as % of maximal IP response observed with 10 mM Ca\(^{2+}\) and are means ± S.E. of replicates from a typical experiment representative of three to five experiments.

seven-TM bundle (Fig. 3A), are in direct contact with Calex 231. These three positions are well known to map the competitive antagonist-binding cavity of many GPCRs (Table II). For example, position 3.32 is the principal anchoring residue (Asp\(^{3.32}\)) of all monoamine receptors (44). Position 3.36 has also been shown to play a pivotal role in antagonist binding to several different GPCRs. Last, Glu-837\(^{39}\) is a key residue of various unrelated GPCRs for anchoring competitive antagonists and is implicated in the recognition of a reference calcimimetic in the CaSR (21). Our results concerning the E837A mutant are in agreement with those obtained by Hu et al. (21) showing that this mutant is expressed at levels comparable to those of the WT receptor when transfected in HEK293 cells, and its sensitivity to Ca\(^{2+}\) is not altered despite a lower maximal response. Interestingly, mutating a glutamic acid at this position in the few GPCRs in which it is conserved (most chemokine receptors and interleukin-8 receptors) leads to the same detrimental effect on ligand binding (45–47). The fourth
important CaSR residue (Trp-816\textsuperscript{5,48}) delineated by our study is conserved in 70% of all GPCRs of classes I–III. Its hydrophobic side chain is believed to lock the GPCR in a ground state and to interact with most antagonists (Table II). In the hypothesized model, Trp-816\textsuperscript{5,48} is surrounded by two other aromatic residues (Phe-821\textsuperscript{6,51} and Phe-868\textsuperscript{3,36}), forming an aromatic cluster around Glu-837\textsuperscript{3,37} and preventing rotation of TM6, which seems mandatory for activation of many GPCRs (48).

A significant decrease in the effect of Calhex 231 was observed after mutation of Ile-841\textsuperscript{7,43}. In our model, Ile-841\textsuperscript{7,43} is a serine that has been shown to directly interact with receptor mutants with significantly enhanced Calhex 231 changes allowing better accommodation of the bulky naphthalene group of Calhex 231.

...concerning transfection and mutagenesis. The mutation of two positions (Leu-776\textsuperscript{5,42} and Phe-821\textsuperscript{6,51}) led as well as peptide receptor antagonists (Table II). Surprisingly, position has already been shown to directly contact adenosine but also the transmembrane domains of the very divergent ECL2 structure remain. In opposition to retinal, the two residues (Trp-818) and 7.39 (Glu-837) also play a key role in recognizing ticitic approach for treating osteoporosis (23), it is of major interest in summary, we have...
Modeling and Mutagenesis of the Binding Site of Calhex 231, a Novel Negative Allosteric Modulator of the Extracellular Ca\(^{2+}\)-sensing Receptor

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