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Positive and Negative Allosteric Modulators of the Ca^{2+}-sensing Receptor Interact within Overlapping but Not Identical Binding Sites in the Transmembrane Domain*

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A three-dimensional model of the human extracellular Ca^{2+}-sensing receptor (CaSR) has been used to identify specific residues implicated in the recognition of two negative allosteric CaSR modulators of different chemical structure, NPS 2143 and Calhex 231. To demonstrate the involvement of these residues, we have analyzed dose-inhibition response curves for the effect of these calcilytics on Ca^{2+}-induced [^{3}H]inositol phosphate accumulation for the selected CaSR mutants transiently expressed in HEK293 cells. These mutants were further used for investigating the binding pocket of two chemically unrelated positive allosteric CaSR modulators, NPS R-568 and (*R*)-2-[1-(1-naphthyl)ethylaminomethyl]-1*H*-indole (Calindol), a novel potent calcimimetic that stimulates (EC_{50} = 0.31 μM) increases in [^{3}H]inositol phosphate levels elicited by activating the wild-type CaSR by 2 mM Ca^{2+}. Our data validate the involvement of Trp-818$^{6.48}$, Phe-821$^{6.51}$, Glu-837$^{7.39}$, and Ile-841$^{7.43}$ located in transmembranes (TM) 6 and TM7, in the binding pocket for both calcimimetics and calcilytics, despite important differences observed between each family of compounds. The TMs involved in the recognition of both calcilytics include residues located in TM3 (Arg-680$^{3.28}$, Phe-684$^{3.32}$, and Phe-688$^{3.36}$). However, our study indicates subtle differences between the binding of these two compounds. Importantly, the observation that some mutations that have no effect on calcimimetics recognition but which affect the binding of calcilytics in TM3 and TM5, suggests that the binding pocket of positive and negative allosteric modulators is partially overlapping but not identical. Our CaSR model should facilitate the development of novel drugs of this important therapeutic target and the identification of the molecular determinants involved in the binding of allosteric modulators of class 3 G-protein-coupled receptors.

Cloning the extracellular Ca^{2+}-sensing receptor (CaSR)[1] from bovine parathyroid has shed light on the molecular mechanisms implicated in the regulation of parathyroid hormone (PTH) secretion (1). The role of CaSR in calcitonin secretion in the thyroid and ion maintenance in the kidney has also been investigated and its presence has been detected in various tissues such as the intestine, the lung, as well as in bone (2) where it has been proposed as a molecular target for the actions of strontium ranelate, a candidate drug for the treatment of female osteoporosis (3, 4). Its expression in oligodendrocyte cells during development (5, 6) and its presence on nerve terminals suggest additional roles for this receptor (7, 8).

The CaSR belongs to family 3 of G-protein-coupled receptors (GPCRs) characterized by a long bilobed amino-terminal tail proposed to contain the ligand binding sites. This family includes metabotropic glutamate receptors (mGluRs), γ-aminobutyric acid, taste, and putative pheromone receptors (9, 10). Positive allosteric modulators of the CaSR, also called calcimimetics, have been characterized (11–14). One of these, NPS R-568, a phenylalkylamine (Fig. 1), has been proposed to selectively activate the parathyroid CaSR resulting in an inhibition of PTH secretion both *in vitro* and *in vivo* (15). The therapeutic potential of NPS R-568 and its derivatives has been demonstrated in patients with primary hyperparathyroidism as well as in patients exhibiting secondary hyperparathyroidism linked to renal disease. NPS R-568 has been shown to interact with residues located in the extracellular region delimited by the seven transmembrane domains (TMs) (16–19).

The negative allosteric modulator NPS 2143 (Fig. 1) was recently introduced as the first negative allosteric modulator of the CaSR. This compound, also called a calcilytic, inhibits the biological effects elicited by Ca^{2+} or by a calcimimetic acting on the CaSR but does not affect the responses elicited by the activation of several other GPCRs. When applied to bovine parathyroid cells in culture, NPS 2143 stimulated the secretion of PTH (20). After *in vivo* injection of this molecule, a rapid and sustained increase of plasma PTH was observed in the rat, and long term treatment of ovariectomized rats, an animal model of osteoporosis, was followed by a large increase in bone turnover (20, 21). These data suggest that NPS 2143, and calcilytics in general, might be useful for regulating the plasma PTH level, thereby representing an interesting pharmacological target for drug development. The identification and characterization of calcimimetics and calcilytics as well as their associated molecular mechanisms of action are therefore important goals.

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[1] The abbreviations used are: CaSR, calcium-sensing receptor; GPCR, G-protein-coupled receptor; PTH, parathyroid hormone; TM, transmembrane domain; mGluR, metabotropic glutamate receptor; IP, inositol phosphates; WT, wild-type.

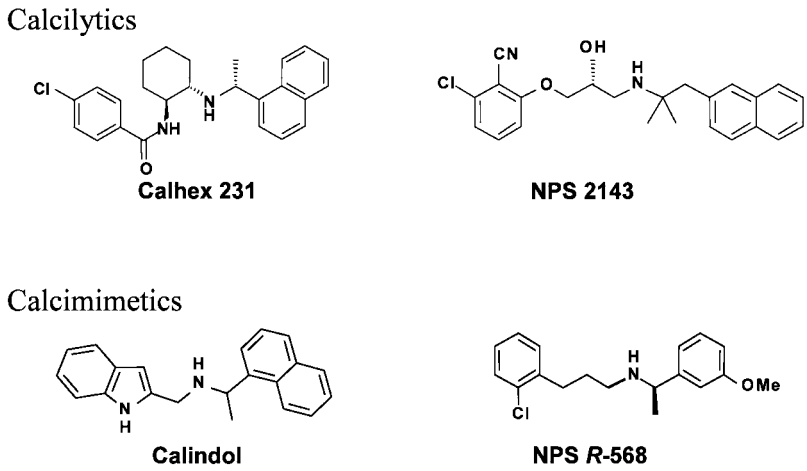

FIG. 1. **Chemical structure of calcilytics and calcimimetics.** Calcilytics: Calhex 231, (1*S*,2*S*,1′*R*)-N^1-(4-chlorobenzoyl)-*N*^{2}-[1-(1-naphthyl)ethyl]-1,2-diaminocyclohexane and NPS 2143, *N*-[(*R*)-2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl]-1,1-dimethyl-2-(2-naphthyl)ethylamine. Calcimimetics: Calindol, (*R*)-2-[1-(1-naphthyl)ethylaminomethyl]-1*H*-indole and NPS R-568, (*R*)-*N*-(3-methoxy-α-phenylethyl)-3-(2-cholorophenyl)-1-propylamine.

In this report, we have identified the binding site(s) of NPS 2143 in the human CaSR using a three-dimensional model of this receptor based on the x-ray structure of bovine rhodopsin (22) that we have recently reported (23). Comparison of the ligand binding pocket of both NPS 2143 and Calhex 231 (Fig. 1), a structurally different negative allosteric modulator of the CaSR that we have recently characterized (23), led us to demonstrate that these two calcilytics interact with overlapping binding sites in the TMs. Moreover, we report the calcimimetic properties of (*R*)-2-[1-(1-naphthyl)ethylaminomethyl]-1*H*-indole (Calindol) (Fig. 1), which also belongs to a novel structurally different series of calcimimetics (24). We have, furthermore, examined if the amino acids involved in the recognition of the calcilytics Calhex 231 and NPS 2143 are also implicated in the binding of the calcimimetics Calindol and NPS R-568.

Our data suggest that calcilytics and calcimimetics interact with several identical residues only within the sixth and seventh TMs. These studies further validate our CaSR model based on the crystal structure of bovine rhodopsin and provide a rational framework for the development of more selective and potent allosteric modulators of the CaSR.

MATERIALS AND METHODS

Preparation of Calindol—Calindol was prepared as its hydrochloride salt. Briefly, indole-2-carboxylic acid in chloroform was treated for 16 h at room temperature with excess thionyl chloride in the presence of catalytic *N*,*N*-dimethylformamide. The reaction of the resulting acid chloride with (*R*)-(+)-1-(1-naphthyl)ethylamine and triethylamine afforded (*R*)-[1-(1-naphthyl)ethyl]-1*H*-indole-2-carboxamide. Reduction of the latter with lithium aluminum hydride-aluminum chloride in refluxing tetrahydrofuran for 24 h gave (*R*)-2-[1-(1-naphthyl)ethylaminomethyl]-1*H*-indole, *i.e.* Calindol. Details of the synthesis and structural characterization of Calindol will be published elsewhere. NPS 2143, *N*-[(R)-2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl]-1,1-dimethyl-2-(2-naphthyl)ethylamine, was prepared as described (25). NPS R-568, (R)-*N*-(3-methoxy-α-phenylethyl)-3-(2-chlorophenyl)-1-propylamine, was synthesized as its hydrochloride salt as described (5).

Plasmids—The human wild-type (WT) CaSR cDNA (provided by Prof. M. Freichel), and the mutant CaSRs T764A, H766A, F684A, F688A, L776A, W818A, F821A, I841A, E837A, have been reported (23). R680A mutant was prepared as the other CaSR mutants previously described (23), using the appropriate oligonucleotides (Eurobio, Les Ulis, France) to convert arginine to alanine (sequences of oligonucleotides are available on request). Sequencing of the resulting mutated DNA was performed as described (23). EC_{50} of R680A for Ca^{2+} (3.4 ± 0.1 mM, mean ± S.E., $n = 2$) was determined by measuring accumulation of [^{3}H]inositol phosphates ([^{3}H]IP) by increasing concentrations of Ca^{2+} after transient expression of the R680A mutant in HEK293 cells (see below). Expression of the CaSR mutant R680A was investigated by Western blot analysis using the 141Ab antiserum directed against the human CaSR as described (23) and was comparable with the expression of the WT CaSR (data not shown).

Cell Culture and Transfections—HEK293 cells (Eurobio, Les Ulis, France) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum (Invitrogen) and were transiently transfected by electroporation (270 volts, 975 microfarads) using a gene pulser apparatus (Bio-Rad). Briefly, 4 μg of pCDNA$_3$ plasmid containing WT or mutated human CaSR DNA were supplemented with 6 μg of pRK5 plasmid and were used to transfect 10^7 cells in a total volume of 300 μl of electroporation buffer (K_2HPO_4, 50 mM; CH_3COOK, 20 mM; KOH, 20 mM; $MgSO_4$, 26.6 mM; pH 7.4). After electroporation, cells were resuspended in culture medium and distributed on a 24-well plate coated with 100 μg/ml rat tail collagen (BD Biosciences) for [^{3}H]IP analysis.

[^{3}H]IP Formation—Cells were labeled by 0.5 μCi/well of *myo*-[^{3}H]inositol (Amersham Biosciences) for 20 h in their growth medium and measurement of [^{3}H]IP accumulation was performed as described (26). The activities of WT and mutant CaSRs were determined in response to NPS 2143 or Calhex 231 in the presence of 10 mM Ca^{2+}. Data are expressed as the mean ± S.E. of triplicate determinations and are representative of one of three to 10 independent experiments. IC_{50} values for NPS 2143 and Calhex 231 were calculated using GraphPad prism 2.01 (GraphPad Prism Software Inc., San Diego, CA).

Graphs of concentration dependence for the [^{3}H]IP response were obtained by increasing the concentration of Ca^{2+} with or without 1 μM Calindol or NPS R-568 and were constructed for the WT and mutant receptors using GraphPad prism 2.01 software. The effect of the R680A mutant was determined in the presence of 1 μM NPS R-568 or Calindol at 3.4 mM Ca^{2+} concentration corresponding to its EC_{50}. Significance was assayed by the Excel 98 Student's *t* test (Microsoft, Seattle, WA). Each value on a curve is the mean of duplicate determinations normalized to the maximal activation of each receptor obtained at 10 mM Ca^{2+}. The graphs presented correspond to two to five independent experiments.

Automated Docking of NPS 2143—The Surflex docking program (27) was used to automatically dock NPS 2143 to the previously described three-dimensional model of the CaSR (23). An idealized active site ligand or protomol (28) was first generated from 33 consensus positions (29) supposed to map the TM cavity of most GPCRs. This protomol consists of the preferred locations of various molecular probes (CH_4, C = O, N-H) that 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 proto_thresh value of 0.5 and a proto_bloat value of 0 were used to generate a compact protomol. A TRIPOS mol2file (TRIPOS Associates Inc., St. Louis, MO) of the antagonist, obtained from a two-dimensional sketch as previously reported (29) was docked into the TM cavity using standard parameters of Surflex used in the "whole" docking approach (27). The best 30 solutions were finally stored in mol2 format.

RESULTS

Hypothesized Binding Mode of NPS 2143—We have recently developed a model of the human CaSR based on the crystal structure of bovine rhodopsin that has allowed us to delineate the putative TMs of the CaSR (Ref. 23 and Fig. 2). We then used this model to dock the novel calcilytic Calhex 231 (Fig. 1) into a hydrophobic cavity centered on Glu-837 and formed by two pockets (23). This observation prompted us to use the same

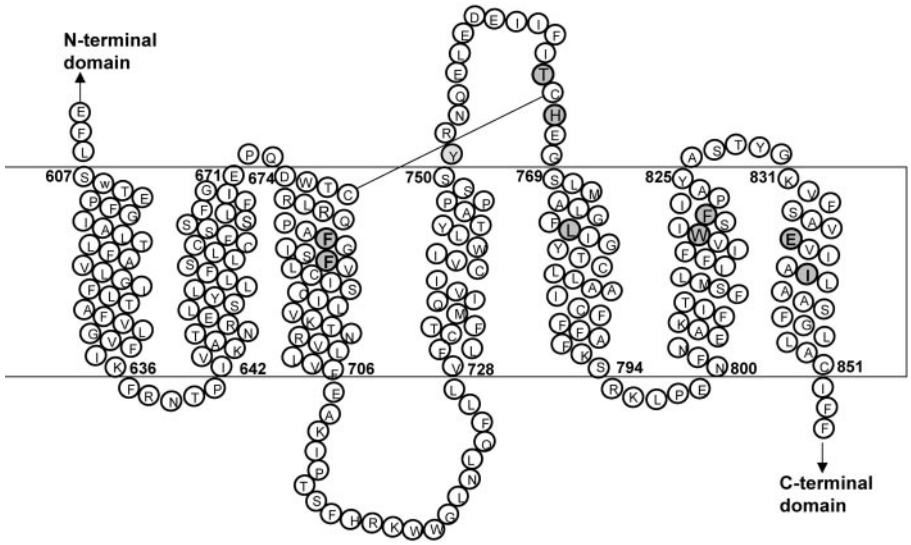

FIG. 2. **Schematic representation of the human CaSR and location of the mutated amino acids.** The schematic representation of the TM7 domain of the human CaSR is based on a previously described model constructed from the crystal structure of bovine rhodopsin (23). Amino acids are represented by a *white circle*. Residues assayed by site-directed mutagenesis are in *gray circles*. *Numbers* indicate amino acid positions within the putative transmembrane domains.

model to analyze the potential sites of interaction of NPS 2143, a structurally different calcilytic (Fig. 1). Automated docking of NPS 2143 using the recently described Surflex docking engine (27) disclosed a preferred binding mode (Fig. 3, *A* and *B*) in which both the amine and the hydroxyl functions are H-bonded to Glu-837. The close proximity of the protonated secondary amine to the negatively charged Glu-837 side chain indicates a likely ionic interaction between both moieties. An additional H-bond between the Arg-680 side chain and the ether oxygen of NPS 2143 contributes to anchoring the calcilytic in the binding cavity. It should be noted that the guanidine moiety of Arg-680 does not directly interact with the hydroxyl group of NPS 2143, although a water-mediated H-bond would be topologically possible. Furthermore, the naphthalene moiety of the calcilytic 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), whereas the disubstituted phenyl ring is buried in the additional pocket B (Phe-612, Ala-615, Leu-616, Phe-668, Ile-669, Arg-680, Phe-684, Val-838, and Ile-841). The *gem*-dimethyl group directly faces the Phe-684 aromatic ring. The proposed interaction model suggests a tight binding of NPS 2143 because 68% of the overall surface of the antagonist (457 of 667 Å^2) is buried upon binding to the TM cavity.

Functional Analysis of CaSR Mutants for NPS 2143 Inhibition of Ca^{2+}-promoted Increases of IP Response—We first determined the potency of NPS 2143 in inhibiting the increase of IP response induced by 10 mM Ca^{2+} in HEK293 cells transiently expressing the human WT CaSR. Incubation of these cells with NPS 2143 caused a concentration-dependent inhibition of the IP response to 10 mM Ca^{2+} (Fig. 4, *A* and *B*). Analysis of the dose-response curve led to an IC_{50} for NPS 2143 of 0.35 ± 0.08 μM (mean ± S.E., $n = 10$), which is comparable with that of Calhex 231 (IC_{50} = 0.39 ± 0.08 μM, Table I) for the inhibition of IP response elicited by Ca^{2+} under similar experimental conditions.

We then examined the ability of NPS 2143 to block the Ca^{2+}-induced [^{3}H]IP accumulation in HEK293 cells expressing the mutant CaSRs previously used by us for identifying the Calhex 231 binding site, as well as to that harboring the R680A mutation that we have now characterized (see "Materials and Methods"). We have previously shown by Western blot analysis using 141Ab antiserum directed against the human CaSR, that the expression pattern of WT and the CaSR mutants transfected in HEK293 cells was comparable (see "Materials and Methods" and Ref. 23). The potencies of NPS 2143 and Calhex 231 in blocking the IP response elicited by Ca^{2+} on these receptors are reported in Table I.

The dose-response curves of NPS 2143 for the three mutants F684A,F688A located in TM3, and E837A located in TM7, respectively, were profoundly affected as shown in Fig. 4, *A* and *B*. Thus, NPS 2143 lost its ability to block the Ca^{2+}-induced IP response in CaSR having the point mutation F684A,F688A (<30% inhibition by 10 μM NPS 2143), as well as E837A (<20% inhibition by 10 μM NPS 2143). These data indicate that these residues play a key role in NPS 2143 recognition. Analysis of the dose-response of the R680A mutant revealed a ~12-fold increase of the IC_{50} value for NPS 2143 thereby demonstrating that Arg-680, which is located next to the two crucial phenylalanines Phe-684 and Phe-688 in TM3, also participates in the binding of NPS 2143 (Table I). However, this mutation led to a significant decrease in the IC_{50} of Calhex 231 in inhibiting the Ca^{2+}-induced IP response (IC_{50} = 0.12 ± 0.02 μM) compared with the WT receptor (IC_{50} = 0.39 ± 0.08 μM, $p < 0.05$) (Table I). Analysis of the dose response of the I841A mutant indicated a ~12-fold increase of the IC_{50} value for NPS 2143, which demonstrates that Ile-841, located next to Glu-837 in TM7, is also implicated in the binding of NPS 2143. The last mutations studied, T764A and H766A, corresponding to two residues located in the extracellular loop 2 that could potentially block access of compounds to the TM region, as well as L776A located in TM5, and W818A,F821A both located in TM6, did not significantly affect the ability of NPS 2143 to block the Ca^{2+}-induced IP response in transfected cells (Table I).

Potency of Calindol—In a recent preliminary report, we described the synthesis and characterization of a novel series of molecules displaying calcimimetic properties toward the rat CaSR (24). We have now synthesized and investigated the potency of Calindol (Fig. 1), which belongs to this family of molecules, toward the human CaSR. In the presence of 2 mM Ca^{2+}, increasing concentrations of Calindol led to a dose-dependent increase of the IP response in transfected cells, which was similar to that obtained with a reference calcimimetic NPS R-568 (11–13), in parallel experiments performed under the same experimental conditions (Fig. 5). Analysis of the dose-response curves gave an EC_{50} of 0.31 ± 0.05 and 0.50 ± 0.05 μM (mean ± S.E., $n = 5$) for Calindol and NPS R-568, respectively. In the presence of 1 μM Calindol or NPS R-568, the concentration-response curve for Ca^{2+} was significantly left-shifted (EC_{50} for Ca^{2+} = 3.4 ± 0.1 mM; EC_{50} for Ca^{2+} + 1 μM Calindol or NPS R-568 = 1.6 ± 0.3 mM) and the maximal responses were significantly increased (Fig. 6, Table II). These results show

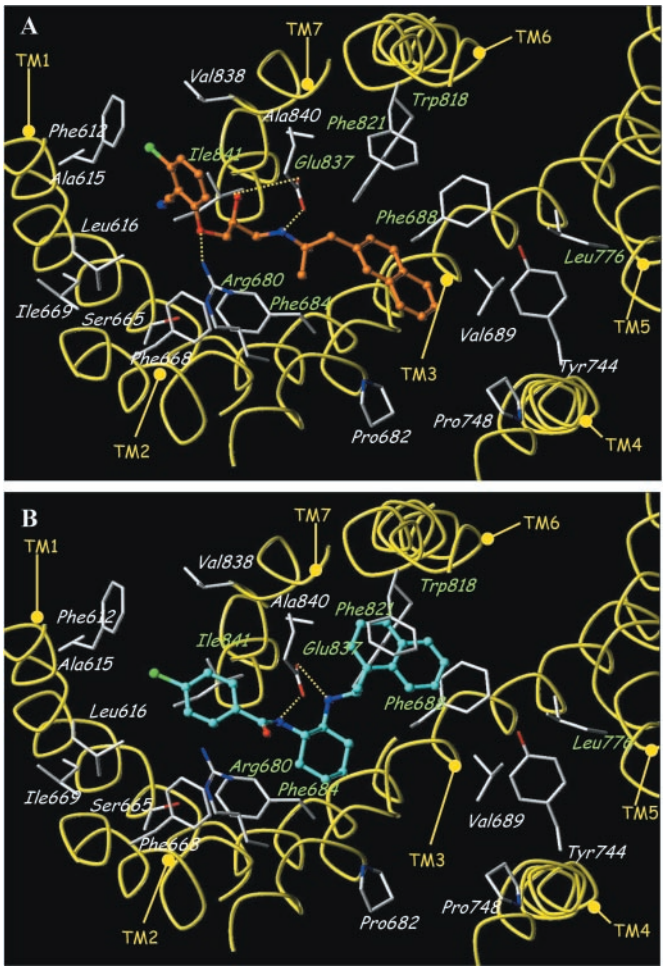

FIG. 3. **Proposed models of the human CaSR complexed with NPS 2143 and Calhex 231.** *A*, proposed interaction model between NPS 2143 and the CaSR binding cavity. TM helices are displayed as *yellow ribbons*. NPS 2143 as well as important CaSR heavy atoms are indicated by *sticks* using the following color coding: carbon atom of NPS 2143, *orange*; carbon atom of the CaSR, white; oxygen atom, *red*; nitrogen atom, *blue*; chloride atom, *green*. Important side chain positions of the CaSR are labeled at the Cα atom. Intermolecular hydrogen bonds between CaSR and NPS 2143 are represented by *dotted yellow lines*. Mutations discussed herein are displayed by *green labels*. *B*, comparison of NPS 2143 and Calhex 231 binding pockets. Carbon atoms of Calhex 231 are displayed in *cyan*. Whereas the substituted phenyl side chain of NPS 2143 and Calhex 231 largely occupy pocket B in a similar manner, the naphthalene moiety of both molecules markedly differ in their orientation in pocket A, facing Phe-688 located in TM3 for NPS 2143 and facing Trp-818 and Phe-F821 in TM6 for Calhex 231. *A* and *B*, top view from the extracellular side. Transmembrane helices (TM) are numbered from 1 to 7.

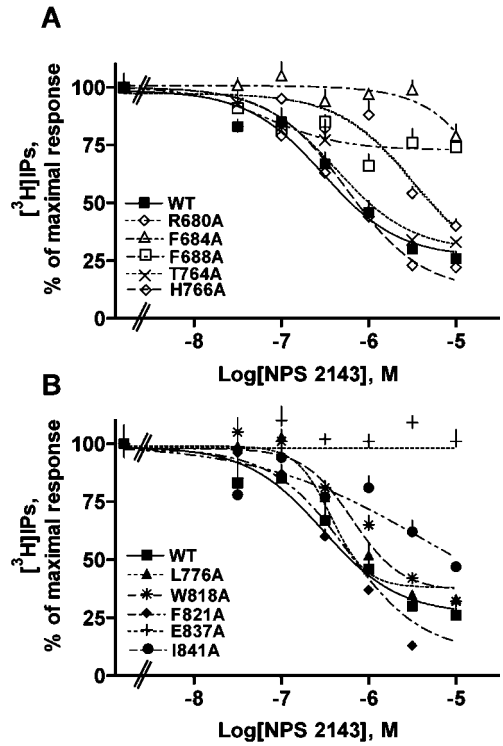

FIG. 4. **Effect of CaSR mutations on inhibition of Ca^{2+}-stimulated increases of IP by NPS 2143.** Concentration-dependent inhibition of Ca^{2+}-stimulated increases of IP response by NPS 2143 in HEK293 cells expressing the WT or mutated human CaSRs as indicated in *A* and *B*. The cells were transfected with the adequate vector and the IP response to Ca^{2+} performed as described under "Materials and Methods." After prelabeling and washing procedures, cells were incubated with 10 mM Ca^{2+} alone or in the presence of increasing concentrations of NPS 2143 for 30 min. Data are expressed as % of maximal IP response observed with 10 mM Ca^{2+}, which represented between 4 and 8 times the IP basal level observed in the presence of 0.3 mM Ca^{2+}, and are mean ± S.E. of triplicates from a typical experiment representative of three to five experiments.

TABLE I
Summary of the effects of various CaSR mutations on the properties of NPS 2143 and Calhex 231

Data of EC_{50} for Ca^{2+} and IC_{50} for Calhex 231 are from Ref. 23, and have been generated as described under "Materials and Methods" for the Arg-680 mutant, those for NPS 2143 were calculated from experiments similar to those described in the legend to Fig. 4 and are mean ± S.E. from three to ten independent experiments performed in triplicate.

Receptor	Position	IC_{50} ± S.E.	
		Calhex 231	NPS 2143
		μM	
WT		0.39 ± 0.08	0.35 ± 0.08
R680A	TM3	0.12 ± 0.02[a]	4.1 ± 0.8[a]
F684A	TM3	$>10^{-5}$	$>10^{-5}$
F688A	TM3	3.20 ± 0.98[b]	$>10^{-5}$
T764A	ECL2	0.28 ± 0.05	0.44 ± 0.06
H766A	ECL2	0.64 ± 0.03	0.54 ± 0.14
L776A	TM5	0.07 ± 0.03[a]	0.37 ± 0.06
W818A	TM6	3.30 ± 0.50[b]	0.65 ± 0.09
F821A	TM6	0.06 ± 0.01[a]	0.57 ± 0.14
E837A	TM7	$>10^{-5}$	$>10^{-5}$
I841A	TM7	2.71 ± 0.10[b]	4.17 ± 0.52[b]

[a] $p < 0.01$ with the WT receptor.
[b] $p < 0.001$ with the WT receptor.

that Calindol and NPS R-568 display similar pharmacological properties toward the human CaSR and that both compounds enhance the affinity of Ca^{2+} for its receptor. These data also suggest that these molecules are allosteric modulators, and possibly interact at the level of the TM for CaSR. We therefore examined whether amino acid residues involved in the recognition of the calcilytics are also implicated in the recognition of these two calcimimetics.

Functional Analysis of Calindol and NPS R-568 for Stimulation of Ca^{2+}-promoted Increases of IP Response in CaSR Mutants—CaSRs harboring the F684A, F688A, L776A, W818A, F821A, E837A, and I841A mutants were transiently transfected into HEK293 cells and dose-response curves for Ca^{2+} ranging from 0.3 to 10 mM alone or in the presence of 1 μM Calindol or NPS R-568 were constructed (Fig. 6, Table II). The marked shift in the EC_{50} for Ca^{2+} observed for the WT CaSR corresponding to the potentiation of the Ca^{2+} effect by the calcimimetics was completely abolished in the presence of Calindol or NPS R-568 in cells transfected with the mutants bearing E837A or I841A mutations. It was also completely abrogated in the presence of Calindol but not of NPS R-568 when the W818A mutant was tested, and in the presence of NPS

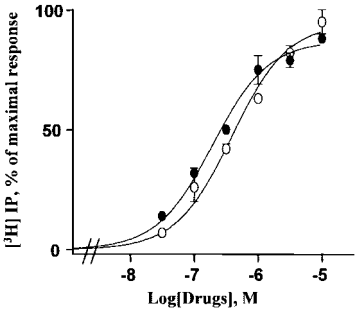

FIG. 5. **Effect of Calindol and NPS R-568 in stimulating accumulation of [^{3}H]IP by the human CaSR transiently transfected in HEK293 cells.** Concentration dependence for Calindol and NPS R-568 (*open* and *closed circles*, respectively) activation of IP response in HEK293 cells expressing the WT CaSR in the presence of 2 mM Ca^{2+}. Data are expressed as % of maximal IP response observed with 10 mM Ca^{2+} from a typical experiment representative of three to five experiments and are mean ± S.E. of triplicates.

R-568 but not of Calindol when the F821A mutant was analyzed. For all other mutants tested, we observed a decrease in the EC_{50} for Ca^{2+} in the presence of either Calindol or NPS R-568 (Fig. 6, Table II). For example, the mutant receptors bearing the F684A or F688A mutations exhibited a marked decrease of their EC_{50} for Ca^{2+}, being shifted from 5.9 mM for Ca^{2+} alone to 2–3 mM in the presence of calcimimetics. Interestingly, the mutant receptor bearing the L776A mutation, which shows an increased sensitivity to Ca^{2+} (EC_{50} = 2.2 mM) compared with the WT CaSR (EC_{50} = 3.4 mM, Fig. 6, Table I) (23), displayed a further shift to the left of the Ca^{2+} dose-response curve in the presence of either calcimimetic (EC_{50} ~ 0.7–0.8 mM). The effect of both calcimimetics (1 μM) was investigated on the IP response induced by Ca^{2+} (3.4 mM) in cells transfected with the mutant receptor harboring the R680A mutation or the WT CaSR. However, the R680A mutation did not affect the Ca^{2+} responses in the presence of Calindol and NPS R-568 compared with the WT receptor (data not shown).

A substantial reduction in the maximal receptor response to Ca^{2+} was observed for the I841A mutant in the presence of both calcimimetics (~40%) and a more modest reduction (~10–20%) was observed only in the presence of Calindol for the E837A and F684A mutants. The W818A mutant displayed a marked increase of the maximal response to Ca^{2+} (28–35%) in the presence of both calcimimetics (Fig. 6, Table II).

DISCUSSION

Negative allosteric modulation has been reported for many GPCRs including mGluRs that are structurally related to the CaSR (9, 10). However, despite the obvious therapeutic interest in identifying such molecules for the CaSR (15), only a limited number of structurally different positive and negative allosteric CaSR modulators has been described. To date, the phenylalkylamine NPS R-568 and its derivatives such as Cinacalcet (30) belong to the first family of calcimimetics to have been evaluated as candidate drugs. In this study, we have characterized a CaSR positive allosteric modulator, Calindol, which belongs to a novel chemically different family of molecules. Moreover, we have identified and compared the sites of interaction of both Calindol and NPS R-568 with the CaSR. As well, prior to this study, the binding sites of NPS 2143, the first and sole calcilytic whose pharmacokinetic properties had been reported *in vitro* and *in vivo* (20, 21), were not known. We have now characterized several crucial residues involved in its recognition and compared its sites of interaction to those of Calhex 231, a negative allosteric modulator of the CaSR that we have recently described (23). This work has allowed us to identify the presence of a positive and a negative allosteric binding site located at the level of the transmembrane domains of the CaSR and to demonstrate that these sites are overlapping but not identical. Moreover, we show important differences in the binding of the two families of calcimimetics and calcilytics that should allow the development of compounds of higher selectivity and affinity.

We have recently synthesized Calhex 231 and characterized its antagonist properties toward the human CaSR and proposed a model of its allosteric interaction with the TM region using a series of CaSR mutants (23). One of the major findings of our present study concerns the identification of five residues Arg-680$^{3.28}$, Phe-684$^{3.32}$, Phe-688$^{3.36}$, Glu-837$^{7.39}$, and Ile-841$^{7.43}$ implicated in the recognition of both NPS 2143 and Calhex 231, two structurally different negative allosteric modulators. Moreover, our data allow delineation of ligand binding pockets for both molecules that are largely overlapping, and are located within the bundle formed by the TMs (Fig. 3*B*).

The proposed binding mode of NPS 2143 to the TM cavity of the CaSR exhibits similar features to that previously reported for Calhex 231 and clearly defines the TM region as the primary determinant for its sites of interaction with the receptor. Both compounds are primarily anchored through an H-bond assisted salt bridge to a negatively charged amino acid (Glu-837) located in TM7. Two adjacent hydrophobic pockets are used to locate the two aromatic groups of both compounds, with the bulkier naphthalene moiety anchored to the largest (pocket A) of the two subsites (Fig. 3*B*). Last, the *gem*-dimethyl moiety of NPS 2143 mainly overlaps with the cyclohexyl ring of Calhex 231 and faces the aromatic ring of Phe-684. However, despite these similarities, significant differences are observed that may explain the herein described different affinity profile of both compounds for selected CaSR mutants (Table I). NPS 2143 is proposed to directly H-bond to the Arg-680 side chain as fully supported by the present study that unambiguously demonstrates that R680A mutation unfavorably affects NPS 2143 binding (Table I). However, the latter amino acid mutation led to a receptor mutant with significantly enhanced Calhex 231 antagonist activity, which is difficult to predict from our model but that we have already observed for L776A and F821A mutants (23). Furthermore, the naphthalene moiety of Calhex 231 is very close to Trp-818, a residue of TM6 demonstrated to be crucial for binding this compound (23), whereas the corresponding naphthalene ring of NPS 2143 is more oriented toward the TM3 residues of pocket A (Phe-688, Val-689). Thus, the W818A mutation is much more detrimental to Calhex 231 binding than to that of NPS 2143. Conversely, NPS 2143 is in closer proximity than Calhex 231 to TM3 amino acids (Phe-688, Val-689) explaining the completely abolished binding of NPS 2143 to the F688A CaSR mutant (Table I and Fig. 4*A*). Slight differences are also observed in the positioning of the other substituted phenyl ring of both compounds in pocket B (Fig. 3*B*) that may explain dissimilar loss of affinity resulting from the Ile-841 mutation (Table I). Interestingly, the negatively charged Glu-837 side chain presumably interacts with the protonated secondary amine present in both calcilytics that indicates that this residue plays a key role in calcilytic recognition (Fig. 3*B*). In agreement with our published model and current data (Figs. 3 and 4 and Table I), Miedlich *et al.* (19) have recently reported that F668A, a mutation at a residue located in pocket B of our model and proposed to interact with Calhex 231 (23), as well as R680A, F684A, and E837A mutations, are accompanied by attenuated IP responses to NPS 2143 after transfection of the human CaSR mutants in HEK293 cells.

These data demonstrate that an allosteric binding pocket located within the seven TMs of the CaSR, a member of class 3

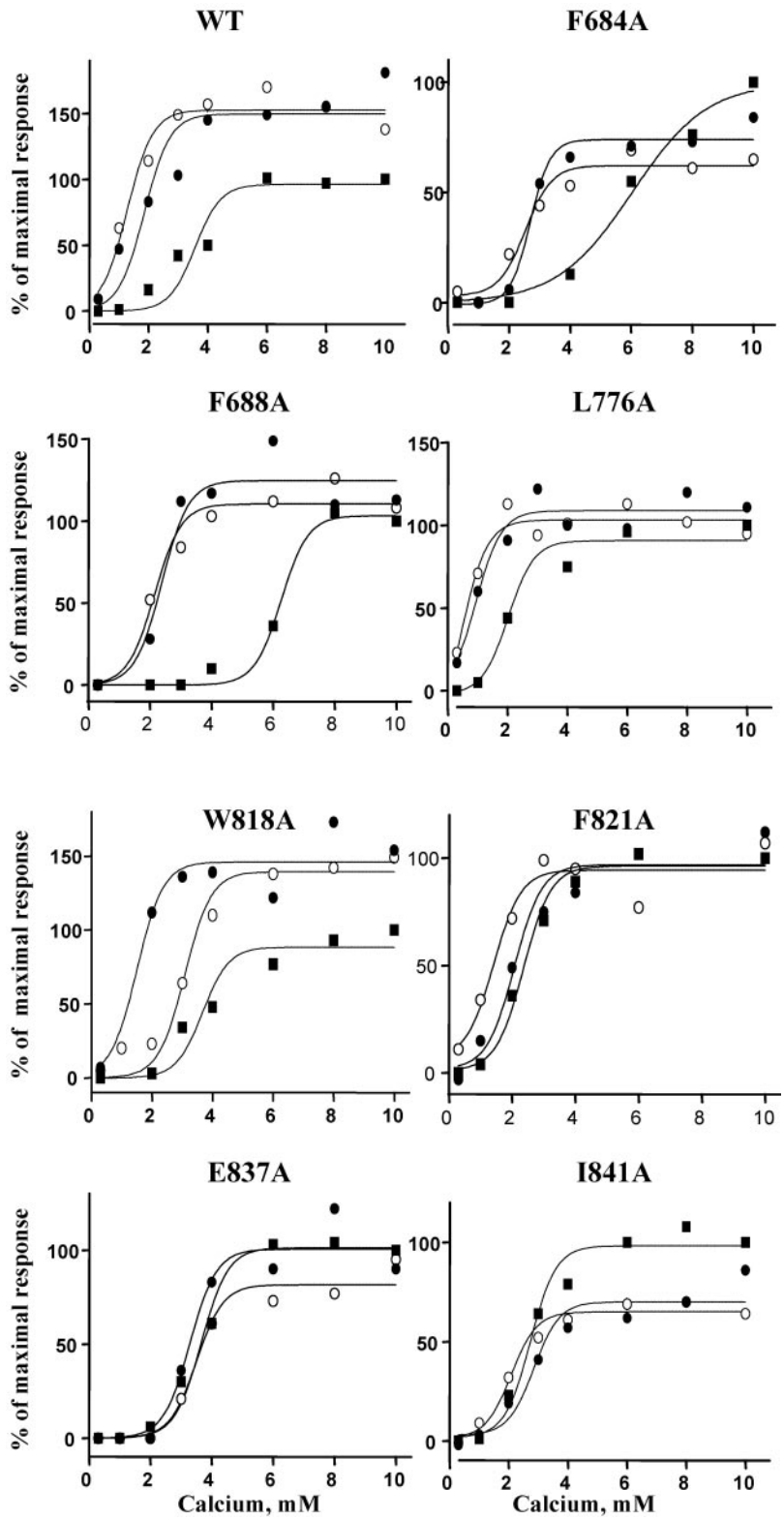

FIG. 6. **Effect of CaSR mutations in stimulating Ca^{2+}-induced accumulation of IP by Calindol and NPS R-568.** HEK293 cells were transiently transfected as described under "Materials and Methods" with the WT or appropriate mutant receptors, and were stimulated by increasing concentrations of Ca^{2+} alone (*filled squares*), or in the presence of 1 μM Calindol or NPS R-568 (*open* and *closed circles*, respectively). Data are expressed as % of maximal IP response of each mutant observed with 10 mM Ca^{2+} and are means of duplicates from a typical experiment representative of three to five experiments.

GPCRs, can be modeled using the crystal structure of bovine rhodopsin, a member of class 1 GPCRs. Like other members of this class, activation of the CaSR is believed to occur upon ligand binding to sites located within the extracellular domain constituted by its long amino-terminal tail (1, 7) called a Venus flytrap module (31). The subsequent conformational change that presumably occurs throughout the cysteine-rich region affects the seven TM regions leading to receptor activation. Interestingly, three acidic residues located in the extracellular loop 2 of the CaSR have been demonstrated to maintain an inactive conformation of the seven TM regions, further highlighting the complex processes of CaSR activation (17). However, the precise molecular mechanisms involved in such activation are not known. The negative allosteric modulators NPS 2143 and Calhex 231 can either block direct contact of the Venus flytrap module with the seven TM region of the receptor that functions as a dimer, or can prevent the switch between the inactive and active conformation of the TM region, thus preventing further rotation of TM6 required for activation of many GPCRs (32).

TABLE II

Summary of the effects of various CaSR mutations on the properties of Ca^{2+}, Calindol, and NPS R-568

Effect of CaSR mutations on maximal [^{3}H]IP accumulation by 10 mM Ca^{2+} and EC_{50} of Ca^{2+} in the presence or absence of 1 μM Calindol or NPS R-568. Concentration response curves for Ca^{2+}, Calindol, and NPS R-568 were generated as described in the legend to Fig. 6. EC_{50} values and maximal stimulation for Ca^{2+} in the absence or presence of 1 μM Calindol or NPS R-568 are compared with a maximal Ca^{2+} response of the WT CaSR. Data shown are mean ± S.E. from three to ten independent experiments.

Receptor	Position	Maximal response			Ca^{2+}, EC_{50}		
		Control	Calindol	NPS R-568	Control	Calindol	NPS R-568
		%			mM		
WT		100 ± 4	125 ± 11[a]	135 ± 7[b]	3.4 ± 0.1	1.6 ± 0.3[b]	1.6 ± 0.3[b]
F684A	TM3	50 ± 4	42 ± 2[a]	46 ± 2	5.9 ± 0.4	2.5 ± 0.1[b]	2.9 ± 0.2[b]
F688A	TM3	50 ± 3	45 ± 4	60 ± 2	5.9 ± 0.2	2.1 ± 0.1[b]	2.5 ± 0.2[b]
L776A	TM5	121 ± 9	116 ± 5	113 ± 5	2.2 ± 0.3	0.7 ± 0.2[b]	0.8 ± 0.1[b]
W818A	TM6	72 ± 4	92 ± 2[b]	97 ± 5[b]	3.4 ± 0.2	3.1 ± 0.1	1.7 ± 0.2[a]
F821A	TM6	112 ± 10	112 ± 6	125 ± 4	2.6 ± 0.2	1.4 ± 0.3[a]	2.6 ± 0.3
E837A	TM7	72 ± 5	65 ± 3[a]	73 ± 5	3.8 ± 0.2	3.6 ± 0.1	3.0 ± 0.3
I841A	TM7	98 ± 6	60 ± 2[b]	59 ± 4[b]	2.9 ± 0.2	2.3 ± 0.2	2.7 ± 0.2

[a] $p < 0.01$ compared to the WT receptor.
[b] $p < 0.001$ compared to the WT receptor.

No attempt was made to model the CaSR in its activated form bound to Calindol or NPS R-568, because of the lack of an adequate three-dimensional template (33). However, our data emphasizes the crucial role of Glu-837 in anchoring NPS R-568, as previously observed (17, 19), and of Calindol, presumably through a salt bridge with the protonated secondary amine of the two compounds as we previously proposed for the calcilytics. Our study also underscores the role of Ile-841 in anchoring the two calcimimetics as indicated by the lack of Ca^{2+} potentiation of IP response by both compounds in the mutants. We were also able to demonstrate the non-involvement of Arg-680, Phe-684, and Phe-688 located in TM3, and Leu-776 located in TM5, previously implicated in calcilytics recognition, in anchoring both calcimimetics because we observed a marked left-shift of the dose-response curve to Ca^{2+} in the presence of 1 μM Calindol or NPS R-568 (Table II, Fig. 6). A previous report has shown that a F684A mutation exhibits normal activation by Ca^{2+} and a profound reduction in the maximal response to Ca^{2+}, but attenuated responses to NPS R-568, both with respect to IP formation and to mobilization of intracellular Ca^{2+} after transfection of the CaSR mutant in HEK293 cells (19). However, our data indicate that the F684A mutant displays a reduced affinity for Ca^{2+} (EC_{50} = 5.9 mM) as deduced from dose-response curve analysis of Ca^{2+}-induced IP formation as well as a decrease in the maximal response to Ca^{2+} (Table II and Ref. 23), and shows a left-shift of the IP response in the presence of both calcimimetics (EC_{50} for Ca^{2+} = 5.9 mM, and EC_{50} for Ca^{2+} + 1 μM Calindol or NPS R-568 = 2.5–2.9 mM) (Table II and Fig. 6). It should be noted that the apparent affinity of NPS R-568 and also of Calindol, varies with the concentration of extracellular Ca^{2+} and therefore is directly linked to the EC_{50} of Ca^{2+} for the receptor mutant. The discrepancies between the two studies of R680A might be attributable to differences in experimental conditions and EC_{50} determination.

The current data then suggest that some common features previously reported to participate in the activation of class 1 rhodopsin-like receptors might be conserved in the molecular activation of class 3 GPCRs. Hence, the mutation of two residues (Trp-818 and Phe-821), rather conserved among GPCRs and known to lock class 1 GPCRs in an inactive ground state (32), affects the ability of both calcimimetics to potentiate Ca^{2+} binding. Whereas NPS R-568 is proposed to mainly interact with Phe-821, Calindol interacts only with Trp-818. Thus, one of these two key residues could be free for inducing, upon binding of one of the latter two compounds, the conformational switch triggering receptor activation.

Hence, the mutation of Trp-818 and Phe-821 in TM6, Glu-837 and Ile-841 in TM7, affecting both calcimimetics and calcilytics recognition, indicates an overlapping binding pocket for both positive and negative allosteric modulators. However, the complete lack of effect of some mutations on calcimimetics binding but which affect the recognition of calcilytics in TM3 and TM5 (despite the proposed involvement of Phe-668 located in TM3 in the binding of NPS R-568 (19)) suggests rather different modes of interaction between these two classes of ligands. Other amino acids are presumably involved in the recognition of calcimimetics. Recently, Ser-688 and Gly-689 in TM4 and Asn-735 in TM5 have been demonstrated to be involved in the binding of LY487379, a selective positive allosteric modulator at human mGluR2 (34). Interestingly, these residues do not overlap with those implicated in the binding pocket of the noncompetitive mGluR1 receptor antagonist 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo(d)azepin-3-yl)-1,6-dihydropyrimidine-5-carbonitrile (EM-TBPC), which involved multiple residues located in TM5, TM6, and TM7 (35).

In summary, our present study delineates important residues implicated in the recognition of four CaSR allosteric modulators of different chemical structures and should be of substantial help to further map the region of the CaSR, as well as that of other members of class 3 GPCRs, that are critical for modulation by both agonists and antagonists. We can therefore anticipate that our current model of the CaSR should facilitate the development of novel positive and negative allosteric modulators displaying improved affinity and selectivity and acting within the seven TM region of the CaSR, an attractive drug target.

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