Delineating a calcimimetic and a calcilytic binding pockets

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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Running title: Delineating a calcimimetic and a calcilytic binding pockets

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ABSTRACT

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 phosphates 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 of (\(R\))-2-[1-(1-naphthyl)ethylaminomethyl]-1H-indole (Calindol), a novel potent calcimimetic that stimulates (EC\(_{50} = 0.31 \ \mu\text{M}\)) 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 W818\(^{6.48}\), F821\(^{6.51}\), E837\(^{7.39}\) and I841\(^{7.43}\) located in TM6 and TM7, in the binding pocket for both calcimimetics and calcilytics, despite important differences observed between each family of compounds. The transmembrane domains (TMs) involved in the recognition of both calcilytics include residues located in TM3 (R680\(^{3.28}\), F684\(^{3.32}\), F688\(^{3.36}\)). However, our study indicates subtle differences between the binding of these two compounds. Importantly, the observation that some mutations which 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 GPCRs.
INTRODUCTION

Cloning the extracellular Ca\(^{2+}\)-sensing receptor (CaSR) from bovine parathyroid has shed light on the molecular mechanisms implicated in the regulation of parathyroid hormone (PTH) secretion (1). The CaSR’s role 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 aminoterminal tail proposed to contain the ligand binding sites. This family includes metabotropic glutamate receptors (mGluRs), GABA\(_B\) , 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 parathyroid hormone (PTH) secretion both in vitro and in vivo (15). The therapeutic potential of NPS R-568 and of 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
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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 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 an important goals.

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]-1H-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.
MATERIAL AND METHODS

Compounds

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]-1H-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]-1H-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 Pr. 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, means ± S.E.M., n = 2) was determined by measuring accumulation of $[^3]$Hinositol phosphates ($[^3]$HIP) 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
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CaSR as described (23) and was comparable to 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 (DMEM) 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 pCDNA3 plasmid containing WT or mutated human CaSR DNA were supplemented with 6 µg of pRK5 plasmid and were used to transfect 10⁷ cells in a total volume of 300 µL of electroporation buffer (K₂HPO₄, 50 mM; CH₃COOK, 20 mM; KOH, 20 mM; MgSO₄, 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 of rat tail collagen (Becton Dickinson, Meylan, France) for [³H]IP analysis.

[³H]IP formation

Cells were labeled by 0.5 µCi/well of myo-[³H]inositol (Amersham) for 20 h in their growth medium and measurement of [³H]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 Ca²⁺. Data are expressed as the mean ± S.E.M of triplicate determinations and are representative of one out of 3 to 10 independent experiments. IC₅₀ values for NPS 2143 and Calhex 231 were calculated using GraphPad prism 2.01 (GraphPad Prism Software Inc., San Diego, USA).

Graphs of concentration dependence for [³H]IP response were obtained by increasing the concentration of Ca²⁺ with or without 1 µM of Calindol or NPS R-568 and were constructed for the WT and mutant receptors using GraphPad prism 2.01 software. The effect
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**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 (CH₄, 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 Assoc. 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 which has allowed us to delineate the putative TM5 of the CaSR (23) and Fig. 2). We then used this model to dock the novel calcilytic Calhex 231 (Fig. 1) into a
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hydrophobic cavity centered on Glu837 and formed by two pockets (23). This observation prompted us to use the same 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 (Figs. 3A, B) in which both the amine and the hydroxyl functions are H-bonded to Glu837. The close proximity of the protonated secondary amine to the negatively-charged Glu837 side chain indicates a likely ionic interaction between both moieties. An additional H-bond between Arg680 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 Arg680 does not directly interact with the hydroxyl group of NPS 2143, although a water-mediated H-bond would be topologically possible. Furthermore, the calcilytic’s naphthalene moiety is embedded in pocket A and interacts with neighboring hydrophobic side chains (Pro682, Phe688, Val689, Tyr744, Pro748, Leu776, Trp818, Phe821) while the disubstituted phenyl ring is buried in the additional pocket B (Phe612, Ala615, Leu616, Phe668, Ile669, Arg680, Phe684, Val838, Ile841). The gem-dimethyl group directly faces the Phe684 aromatic ring. The proposed interaction model suggests a tight binding of NPS 2143 since 68 % of the overall surface of the antagonist (457 out of 667 Å²) is buried upon binding to the TM cavity.

Functional analysis of CaSR mutants for NPS 2143 inhibition of Ca²⁺-promoted increases of IP response

We first determined the potency of NPS 2143 in inhibiting the increase of IP response induced by 10 mM Ca²⁺ 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 Ca²⁺ (Figs. 4A, B). Analysis of the dose-response curve led to an IC₅₀ for NPS 2143 of 0.35 ± 0.08 µM (mean ± S.E.M., n = 10) which is comparable to that of Calhex
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231 (IC$_{50}$ = 0.39 ± 0.08 µM, Table 1) 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 $[^{3}\text{H}]$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 Material 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 Material and Methods and reference 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 1.

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 Figs. 4A and B. Thus, NPS 2143 lost its ability to block the Ca$^{2+}$-induced IP response in CaSR having the point mutation F684A and 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 R680, which is located next to the two crucial phenylalanines F684 and F688 in TM3, also participates in the binding of NPS 2143 (Table 1). 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 to the WT receptor (IC$_{50}$ = 0.39 ± 0.08 µM, p < 0.05) (Table 1). 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 I841, located next to E837 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 which could potentially block access of
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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 1).

**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 μM and of 0.50 ± 0.05 μM (mean ±
S.E.M., 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 2). These results show 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 CaSR’s
TM. 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, 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 of Calindol or of NPS R-568 were constructed (Fig. 6, Table 2). 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 the E837A or the I841A mutation. 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 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 2). For example, the mutant receptors bearing the F684A or F688A mutation 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 to the WT CaSR (EC_{50}=3.4 mM, Fig. 6, Table 1) (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 presence of Calindol and NPS R-568 compared to 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 (~
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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 and Table 2).

**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 \textit{in vitro} and \textit{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 which 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 seven TM
Delineating a calcimimetic and a calcilytic binding pockets region using a series of CaSR mutants (23). One of the major findings of our present study concerns the identification of five residues Arg680\textsuperscript{3.28}, Phe684\textsuperscript{3.32}, Phe688\textsuperscript{3.36}, Glu837\textsuperscript{7.39} and Ile841\textsuperscript{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 which are largely overlapping, and are located within the bundle formed by the seven TMs (Fig. 3B).

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 seven 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 (E837) 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. 3B). Lastly, the gem-dimethyl moiety of NPS 2143 mainly overlaps with the cyclohexyl ring of Calhex 231 and faces the aromatic ring of F684. 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 1). NPS 2143 is proposed to directly H-bond to the Arg680 side chain as fully supported by the present study which unambiguously demonstrates that R680A mutation unfavorably affects NPS 2143 binding (Table 1). 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 W818, a residue of TM6 demonstrated to be crucial for binding this compound (23), while the corresponding naphthalene ring of NPS 2143 is more oriented towards the TM3 residues of pocket A (F688, V689). Thus, the W818A mutation is much more detrimental to
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Calhex 231 binding than to that of NPS 2143. Conversely, NPS 2143 is in closer proximity than Calhex 231 to TM3 amino acids (F688, V689) explaining the completely abolished binding of NPS 2143 to the F688A CaSR mutant (Table 1 and Fig. 4A). Slight differences are also observed in the positioning of the other substituted phenyl ring of both compounds in pocket B (Fig. 3B) that may explain dissimilar loss of affinity resulting from the I841 mutation (Table 1). Interestingly, the negatively-charged E837 side chain presumably interacts with the protonated secondary amine present in both calcilytics which indicates that this residue plays a key role in calcilytic recognition (Fig. 3B). In agreement with our published model and current data (Fig. 3, 4 and Table 1), 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 TM of the CaSR, a member of class 3 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 aminoterminal tail (1,7) called a Venus flytrap module (VFTM) (31). The subsequent conformational change which presumably occurs throughout the cysteine-rich region affects the seven TM region 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 region, 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 VFTM with the seven TM
region of the receptor which functions as a dimer, or can prevent the switch between the inactive and active conformation of the seven TM region, thus preventing further rotation of TM6 required for activation of many GPCRs (32).

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 3-D template (33). However, our data emphasizes the crucial role of E837 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 I841 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 R680, F684, F688 located in TM3 and L776 located in TM5, previously implicated in calcilytics recognition, in anchoring both calcimimetics since we observed a marked left-shift of the dose response curve to Ca\(^{2+}\) in presence of 1 µM Calindol or NPS R-568 (Table 2 and 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 2 and (23)), and shows a left-shift of the IP response in 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 2 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
Delineating a calcimimetic and a calcilytic binding pockets between the two studies of R680A might be attributable to differences in experimental conditions and EC₅₀ 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 (Trp818, Phe821), 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 Ca²⁺ binding. Whereas NPS R-568 is proposed to mainly interact with Phe821, Calindol interacts only with Trp818. 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 Trp818 and Phe821 in TM6, Glu837 and Ile841 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 F668 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, Ser688 and Gly689 in TM4 and Asn735 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-dihydro-pyrimidine-5-carbonitrile (EM-TBPC) which involved multiple residues located in TM5, TM6 and TM7 (35).
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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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Footnote:
The abbreviations used are: CaSR, Calcium sensing receptor; ECL2, extracellular loop 2;
GABA<sub>B</sub>, gamma amino butyric acid; GPCR, G-protein coupled receptor; PTH, parathyroid
hormone; TM, transmembrane domain; mGluR, metabotropic glutamate receptor; 3-D, three
dimensional; IP, inositol phosphates; WT, wild-type.
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LEGEND OF THE FIGURES

Fig. 1. Chemical structure of calcilytics and calcimimetics

Calcilytics: Calhex 231, (1S,2S,1’R)-N1-(4-chlorobenzoyl)-N2-[1-(1-naphthyl)ethyl]-1,2-diaminocyclohexane, 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]-1H-indole, NPS R-568, (R)-N-(3-methoxy-α-phenylethyl)-3-(2-cholorophenyl)-1-propylamine.

Fig. 2. Schematic representation of the human CaSR and location of the mutated amino acids.

The schematic representation of the seven transmembrane 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 grey circles. Numbers indicate amino acid position within the putative transmembrane domains.

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 pocket. 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 F688 located in TM3 for NPS 2143 and facing W818 and F821 in TM6 for Calhex 231. (A, B) Top view from the extracellular side. Transmembrane helices (TM) are numbered from I to VII.

**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 in “Material 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 to 8 times the IP basal level observed in the presence of 0.3 mM Ca\(^{2+}\), and are means ± S.E.M. of triplicates from a typical experiment representative of 3-5 experiments.

**Fig. 5. Effect of Calindol and NPS R-568 in stimulating accumulation of \[^{3}\text{H}\]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 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 3-5 experiments and are means ± S.E.M. of triplicates.
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 in “Material and Methods” with the WT or appropriate mutant receptors, and were stimulated by increasing concentrations of Ca\(^{2+}\) alone (filled squares), or in presence of 1 \(\mu\)M of 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 3-5 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 (23), and have been generated as described in “Material and Methods” for the R680 mutant, those for NPS 2143 were calculated from experiments similar to those described in Fig. 4. and are means ± S.E.M. from 3-10 independent experiments performed in triplicate. Level of significance: *p<0.01; **p<0.001 with the WT receptor.

Table II. Summary of the effects of various CaSR mutations on the properties of \(\text{Ca}^{2+}\), 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 \(\mu\)M Calindol or NPS R-568. Concentration response curves for Ca\(^{2+}\), Calindol, and NPS R-568 were generated as described in the legend of Fig. 6. EC\(_{50}\) values and maximal stimulation for Ca\(^{2+}\) in absence or in presence of 1 \(\mu\)M Calindol or NPS R-568 are compared with a maximal Ca\(^{2+}\) response of the WT CaSR. Data shown are
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means ± S.E.M from 3 to 10 independent experiments. Level of significance: * p<0.01; ** p<0.001 compared to the WT receptor.
Table 1

| Receptor Position | IC$_{50}$ ± S.E.M., µM |
|-------------------|-------------------------|
|                   | Calhex 231 | NPS 2143 |
| WT                | 0.39 ± 0.08 | 0.35 ± 0.08 |
| R680A TM3         | 0.12 ± 0.02* | 4.1 ± 0.8* |
| F684A TM3         | >10$^{-5}$ | >10$^{-5}$ |
| F688A TM3         | 3.20 ± 0.98** | >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* | 0.37 ± 0.06 |
| W818A TM6         | 3.30 ± 0.50** | 0.65 ± 0.09 |
| F821A TM6         | 0.06 ± 0.01* | 0.57 ± 0.14 |
| E837A TM7         | >10$^{-5}$ | >10$^{-5}$ |
| I841A TM7         | 2.71 ± 0.10** | 4.17 ± 0.52** |
## Table 2

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

- Calhex 231
- NPS 2143

Calcimimetics

- Calindol
- NPS R-568

FIG. 1
Delineating a calcimimetic and a calcilytic binding pockets

FIG. 2
Fig. 3.
Delineating a calcimimetic and a calcilytic binding pockets

FIG. 4
Delineating a calcimimetic and a calcilytic binding pockets

FIG. 5
Delineating a calcimimetic and a calcilytic binding pockets

FIG. 6
Positive and negative allosteric modulators of the Ca2+ -sensing receptor interact within overlapping but not identical binding sites in the transmembrane domain
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