Calindol, a Positive Allosteric Modulator of the Human Ca\(^{2+}\) Receptor, Activates an Extracellular Ligand-binding Domain-deleted Rhodopsin-like Seven-transmembrane Structure in the Absence of Ca\(^{2+}\) *

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The extracellular calcium-sensing human Ca\(^{2+}\) receptor (hCaR), 2 a member of the family-3 G-protein-coupled receptors (GPCR) possesses a large amino-terminal extracellular ligand-binding domain (ECD) in addition to a seven-transmembrane helical domain (7TMD) characteristic of all GPCRs. Two calcimimetic allosteric modulators, NPS R-568 and Calindol ((R)-2-[1-(1-naphthyl)ethyl]aminomethylindole), that bind the 7TMD of the hCaR have been reported to potentiate Ca\(^{2+}\) activation without independently activating the wild type receptor. Because agonists activate rhodopsin-like family-1 GPCRs by binding within the 7TMD, we examined the ability of Calindol, a novel chemically distinct calcimimetic, to activate a Ca\(^{2+}\) receptor construct (T903-Rhoc) in which the ECD and carboxy-terminal tail have been deleted to produce a rhodopsin-like 7TMD. Here we report that although Calindol has little or no agonist activity in the absence of extracellular Ca\(^{2+}\) for the ECD-containing wild type or carboxy-terminal deleted receptors, it acts as a strong agonist of the T903-Rhoc. In addition, Ca\(^{2+}\) alone displays little or no agonist activity for the hCaR 7TMD, but potentiates the activation by Calindol. We confirm that the activation of T903-Rhoc by Calindol is truly Ca\(^{2+}\) independent using in vitro reconstitution with purified G\(_4\). These findings demonstrate distinct allosteric linkages between Ca\(^{2+}\) site(s) in the ECD and the 7TMD and the 7TMD site(s) for calcimimetics.

The human Ca\(^{2+}\) receptor (hCaR) is the unique cation-sensing G-protein-coupled receptor (GPCR). In parathyroid cells, hCaR responds to changes in extracellular Ca\(^{2+}\) to regulate parathyroid hormone secretion (1). The hCaR is more widely expressed, found in especially high abundance in the central nervous system, than would be expected for the sole function of serum calcium homeostasis via parathyroid hormone secretion. Although Ca\(^{2+}\) is the endogenous ligand for the Ca\(^{2+}\) receptor, this cation-sensing receptor is also responsive in vitro to several di- and trivalent cations (Mg\(^{2+}\), Gd\(^{3+}\)) and organic cationic compounds, such as spermine, polyamine, amylid β-peptide, and poly-L-arginine (2). Moreover, two structurally distinct phenylalkylamine compounds lacking polycationic structures, NPS R-568 and Calindol ((R)-2-[1-(1-naphthyl)ethyl]aminomethylindole), have been identified as positive allosteric modulators (calcimimetics), which do not activate the receptor, but potentiate the action of Ca\(^{2+}\) and other polycationic agonists at the hCaR (3–5). In addition, two structurally distinct negative allosteric modulators or calcilytics, NPS 2143 and Calhex 231 ((1S,2S,1’R)-N\(^-\)-(4-chlorobenzoyl)-N\(^-\)([1-(1-naphthyl)ethyl]-1,2-diaminocyclohexane), have been identified and recently characterized (6–8).

The family-3 GPCRs of which hCaR is a member include receptors for the excitatory and inhibitory neurotransmitters glutamate and γ-amino butyric acid (GABA) as well as receptors for sweet tastants and pheromones (9, 10). For the family-3 GPCR structures the primary ligand-binding domain is located within a large (~600 residues) amino-terminal extracellular domain (ECD) that shares structural similarity to the bilobed “Venus flytrap motif” (VFTM) of bacterial periplasmic binding proteins. In some family-3 GPCRs including the hCaR, the VFTM is followed by a 9-cysteine containing peptide “Cys-rich” domain (CRD) that is connected to the first transmembrane α helix of the seven-transmembrane helical domain (7TMD) by a 14-amino acid long sequence. Unlike GPCRs in the largest family (rhodopsin-like or family-1 GPCR), in which ligands bind within the 7TMD, both chimeric receptor analysis of several subtypes of mGluRs, hCaR, and GABA\(_{\beta}\) receptor and direct binding assays with purified ECDs from mGluRs and GABA\(_{\beta}\) receptors have shown that the VFTM within the ECD of these family-3 receptors is responsible for ligand recognition and activation of these receptors (11–14). The crystal structures solved for the mGluR1 ECD reveal three different crystal structures all in disulfide-linked homodimeric complexes; one structure contains bound glutamate, whereas two are unliganded. The ECD monomers obtain either an “open” or “closed” conformation and glutamate is bound to the closed form (15). How the changes in the orientation of VFTM within the ECD dimer transmit the activating transition through the 7TMD to intracellularly bound G-protein remains unsolved.

Different functional studies have shown that the hCaR is activated by Ca\(^{2+}\) ions with a Hill coefficient greater than 3 and multiple Ca\(^{2+}\) ions are assumed to interact at several sites in both the ECD and 7TMD (14, 16). Whereas the specific residues of contact for the Ca\(^{2+}\)-binding sites within the ECD and 7TMD are unknown, data obtained by mutagenesis and chimeric receptor constructions indicate that the binding sites for the calcimimetic and calcilytic allosteric compounds identified thus far...
for the hCaR (NPS-R568, Calindol, NPS 2143, and Calhex 231) reside within a crevice formed by transmembrane helices TM3, TM5, TM6, and TM7 of the 7TMD (4, 8, 17). This crevice seems to be conserved and corresponds to the ligand-binding site for rhodopsin-like receptors. Because of the low sequence similarity between the 7TMD of family-3 GPCRs and rhodopsin-like family-1 receptors and because family-3 receptors utilize a quite different mode of orthosteric ligand binding to an ECD structure not present within family-1 GPCRs, different molecular determinants may control the conformational changes for activation of the 7TMD of these receptors. To gain insight into the molecular determinants controlling the activation process of the hCaR 7TMD and to understand the mechanisms of allosteric regulation by calcimimetic compounds on this domain, we have examined the regulation of signaling by these calcimimetic compounds on intact and ECD-deleted constructs of the hCaR. We report here that the allosteric activating ligands act as agonists of the hCaR 7TMD in the absence of the Ca$^{2+}$-sensing ECD. Furthermore, in the 7TMD it is possible that a divergent cation site for Ca$^{2+}$ allosterically regulates the activation by calcimimetics.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis and Human Ca$^{2+}$ Receptor Constructs—*Cloning of wild type hCaR in pCR3.1 mammalian expression vector and construction of the T903 carboxyl-terminal tail truncation mutant have been described previously (18). In the T903 construct, truncation of the carboxy-terminal tail of the hCaR was achieved by introducing stop codons at amino acids 903 and 904 of the hCaR thus lacking the last 174-amino acid long carboxyl-terminal residues. In this study, we added 20 amino acid residues (MNGTEGPNFYVPSNKTGVV) corresponding to the amino terminus of bovine rhodopsin after the amino terminus signal peptide between residues Tyr20 and Gly21 of the wild type hCaR (epitope-tagged receptor named as r-hCaR) and T903 (epitope-tagged receptor named as r-T903) receptor constructs using a mutagenesis strategy known as QuikChange™ (Stratagene) site-directed mutagenesis as described in Ray et al. (18). The rhodopsin amino terminus tag has been shown to enhance proper processing and cell surface expression of several GPCRs (19, 20). A rhodopsin epitope-tagged ECD truncation mutant initially referred to as Rhoc-C-CaR (21) and later a similar construct designated by us as T903-Rhoc was constructed and described previously (16). T903-Rhoc construct is devoid of the 1–598 aminoterminal ECD but included 20 amino acids of the amino terminus 19-20 peptide between residues Tyr20 and Gly21 of the wild type hCaR (epitope-tagged receptor named as r-hCaR) and T903 (epitope-tagged receptor named as r-T903) receptor constructs using a mutagenesis strategy known as QuikChange™ (Stratagene) site-directed mutagenesis as described in Ray et al. (18). The rhodopsin amino terminus tag has been shown to enhance proper processing and cell surface expression of several GPCRs (19, 20). A rhodopsin epitope-tagged ECD truncation mutant initially referred to as Rhoc-C-CaR (21) and later a similar construct designated by us as T903-Rhoc was constructed and described previously (16). T903-Rhoc construct is devoid of the 1–598 amino-terminal ECD but included 20 amino acids of the amino terminus 19-20 peptide between residues Tyr20 and Gly21 of the wild type hCaR (epitope-tagged receptor named as r-hCaR) and T903 (epitope-tagged receptor named as r-T903) receptor constructs using a mutagenesis strategy known as QuikChange™ (Stratagene) site-directed mutagenesis as described in Ray et al. (18).

**Preparation of P2 Membranes and Urea Treatment**—Membranes were prepared from the T903Rhoc2.1 cells, a HEK-293 cell line expressing a stably transfected T903-Rhoc receptor. T903-Rhoc receptor-enriched membranes were obtained as a P2 fraction from these cells. Cells were washed twice with 10 ml of Ca$^{2+}$-free PBS at room temperature and incubated at 4°C for 15 min in 5 ml of Solution A (10 μM MOPS, pH 7.4, 1 mM EGTA) fortified with 100 μM 4-(2-aminoethyl)benzenesulfonyl fluoride-HCl (an inhibitor of proteases). The swollen cells were harvested by scraping and homogenized in a Dounce homogenizer and the nuclei and cell debris were removed by centrifugation at 750 × g for 10 min at 4°C. The postnuclear membrane fraction (P2) was collected from the supernatant by centrifugation at 75,000 × g for 30 min at 4°C. P2 membranes were extracted three times by incubation for 30 min on ice in a solution of 7 M urea dissolved in Solution A. The final pellet was washed once with Solution A without urea and resuspended in Solution A.
A supplemented with 12% sucrose, and aliquots were frozen and stored at −80 °C.

Purification of G Proteins—Squid transducin, a member of the Gq family was purified from squid photoreceptors as described by Hartman and Northup (22). Gβγ subunits were purified from bovine retina using previously published protocols (23).

Functional Reconstitution and GDP/GTPγS Exchange Assay—T903-Rhoc receptor catalyzed binding of GTPγS by squid Goq was determined by modification of the method described for bovine rhodopsin-transducin (24). P2 membranes containing the T903-Rhoc receptors were mixed with G-protein subunits on ice in a total volume of 30 μl. An addition of 20 μl contained a final concentration of 50 μM MOPS, pH 7.5, 100 μM NaCl, 1 μM EGTA, 3 μM MgSO4, 0.1 mM diithiothreitol, 3 mg/ml bovine serum albumin, 2.5 μM GDP, and GTPγS (about 4 × 10^5 cpm) when required Calindol was added to the reaction. Reactions were incubated at 30 °C and terminated by the addition of ice-cold Solution F, followed by filtration over nitrocellulose membranes on a vacuum manifold. Filters were washed 4 times with Solution F and dried, and the radioactivity was quantitated by liquid scintillation counter.

Synthesis of Calindol and Calhex 231—Details of synthesis and structural characterization of Calindol and Calhex 231 were described and published previously (4, 5, 7, 8). The structure and absolute configuration of Calhex 231 was confirmed by x-ray crystallography.

RESULTS

Expression of Rhodopsin Epitope-tagged Wild-type, Carboxyl-tail Deleted, and ECD-deleted hCaR—To investigate the pharmacological properties of hCaR 7TMD, three constructs were generated with rhodopsin epitope tags at the amino terminus (Fig. 1A). The first one, a wild type hCaR with the rhodopsin epitope tag at the amino terminus was created and named r-hCaR. This epitope tag attached at the amino terminus enabled us to monitor total expression as well as cell surface expression levels. The second construct, r-T903, lacked the 174-amino acids of the carboxyl-terminal tail sequences but contained the entire ECD structure. The r-T903 was tagged at the amino terminus with the same rhodopsin epitope tag at the same position as the r-hCaR construct just after the signal peptide. The third construct, T903-Rhoc, as described earlier (16), contained similarly truncated carboxyl terminus as the r-T903 but also lacked most of the ECD (VFTM and CRD) sequences of the hCaR.

Initial experiments for constructs transiently expressed in HEK-293 cells showed that both r-hCaR and r-T903 express efficiently at the cell surface as judged by immunoblotting with the monoclonal antibody B6-30N (Fig. 1B, lanes 1 and 2). Under reducing conditions, B6-30N antibody detected two major monomeric bands in r-hCaR receptor expressing cells of ~150- and 130-kDa mass similar to the pattern of expression found for wild type hCaR. We and others have previously reported that the 150-kDa band contains hCaR forms expressed at the cell surface, modified with complex carbohydrates by N-glycosylation of several Asn residues in the ECD (25). The 130-kDa band contains high mannose-modified forms, trapped intracellularly and sensitive to endoglycosidase-H digestion (25). Similarly, the rhodopsin epitope tag antibody also detected two bands of r-T903 receptor on the immunoblot. The doublet bands of r-T903 were faster migrating corresponding to 130- and 120-kDa masses. These mobility differences are expected because of differences in length generated by truncation in the r-T903 protein. Interestingly, as seen previously also for the non-epitope-tagged T903 mutant receptor (18), we observed higher overall expression as well as higher cell surface expression of the r-T903 receptor compared with the epitope-tagged wild type r-hCaR receptor. In contrast, the B6-30N monoclonal antibody detected a single broad band corresponding to 40 kDa mass for both transiently expressed T903-Rhoc receptor and for stably expressed T903-Rhoc receptor in HEK-293 cells (T903Rhoc2.1 clonal cell line, Fig. 1B, lanes 4 and 5). Bovine rhodopsin protein ran as a positive control on this immunoblot displayed a very similar mobility (Fig. 1B, lane 3). T903-Rhoc receptor expresses in the T903-Rhoc2.1 stable cell line much more abundantly than transiently expressed T903-Rhoc receptor in HEK-293 cells. Also, because deletion of the ECD contributed to lack of N-glycosylation modification on the T903-Rhoc receptor, no doublet bands were observed for this mutant receptor on the immunoblot.

To provide a more certain quantitation of cell surface expression, we performed an intact cell immunoassay with B6-30N antibody to determine the cell surface expression levels of these receptors. Initial experiments showed that compared with both r-hCaR and r-T903, T903-Rhoc cell surface expression was 60–80% lower for receptors...
Transiently expressed in HEK-293 cells. Thus, to conduct functional studies with more closely matched cell surface-expressed T903-Rhoc receptor, we used a previously characterized stable HEK-293 cell line expressing the T903-Rhoc mutant receptor (T903Rhoc2.1 stable line), which showed relatively high levels of expression on immunoblot (Fig. 1B, lane 5). Cell surface expression levels of r-hCaR, r-T903, and T903-Rhoc expressed in the T903Rhoc2.1 stable cell line were then quantified by using intact cell immunoassay. Because both r-hCaR and r-T903 receptors showed higher levels of cell surface expression in transiently expressed cells when optimal (8 μg) cDNA amounts were used for transfection, plasmid DNA amounts of these constructs were varied to 6 μg/flask for r-hCaR and 5 μg/flask for r-T903 to achieve comparable cell surface receptor levels as the T903Rhoc2.1 cells expressing T903-Rhoc receptor for 1 × 10^6 cells as shown in Fig. 1C.

**Allosteric Properties of Calindol on the Human Ca^{2+} Receptor**—Two highly specific but structurally distinct positive allosteric modulators of the hCaR, NPS R-568 and Calindol, have recently been identified (3, 4). By themselves these compounds display little or no agonist activity but have been shown to potentiate the activation by Ca^{2+}. Whereas the primary Ca^{2+} binding site(s) are contained within the ECD, the binding sites of both the compounds have been reported to overlap within the 7TMD (4, 17).

We have investigated the allosteric properties of Calindol for the r-hCaR and r-T903 receptors expressed transiently in HEK-293 cells at defined cell surface levels by PI assay (Fig. 2, A and B). In the presence of 1 μM Calindol, the saturation curves for Ca^{2+} for both r-hCaR and r-T903 were significantly left-shifted with calculated EC_{50} values for Ca^{2+} of 1.98 ± 0.24 (n = 3) and 1.64 ± 0.30 mM (n = 3), respectively.
compared with the EC$_{50}$ values of 3.0 ± 0.26 (n = 4) and 2.57 ± 0.45 mM (n = 3) for Ca$^{2+}$ in the absence of Calindol, respectively. In addition, the maximal response amplitude in the presence of Calindol showed a significant increase for r-hCaR (r-hCaR 100% ± 6 without Calindol and with Calindol 120% ± 10) but not for r-T903. We next determined the EC$_{50}$ values of Calindol for these receptors in the presence of 2 mM Ca$^{2+}$ (Fig. 2, C and D). Analysis of the Calindol saturation data yielded EC$_{50}$ values of 0.95 ± 0.36 (n = 3) and 1.24 ± 0.27 µM (n = 3) for Calindol on r-hCaR and r-T903 receptors, respectively, in good agreement with reported the EC$_{50}$ value of 0.31 ± 0.05 µM on wild type hCaR (4). These results confirm that Calindol is a positive allosteric modulator of the hCaR.

Additional experiments were performed to determine whether Calindol could activate the epitope-tagged wild type hCaR and T903 receptors in the absence of Ca$^{2+}$. Application of 2 µM Calindol alone produced no or very little increase in IP formation when extracellular Ca$^{2+}$ present in the media was washed out before the assay (Fig. 2, E and F). However, at higher concentrations of Calindol (30 µM), increased IP formation was observed in HEK-293 cells expressing r-hCaR and r-T903 receptors with about 2- and 4-fold increases in IP formation from the basal levels, respectively. Co-application of 2 µM Calindol with 4 mM Ca$^{2+}$ resulted in a dramatically enhanced IP formation. From these experiments, it seems that at higher concentrations Calindol alone may have a weak activity at least in cells expressing r-T903 receptor, suggesting that it could act as a partial agonist of this hCaR structure. Although it is possible that the Ca$^{2+}$-independent activation by the r-T903 receptor may be because of a very high level expression of this receptor structure as seen in Fig. 1. Overall, these results show that Calindol and Ca$^{2+}$ display quite similar affinities for activation of the epitope-tagged wild type hCaR and epitope-tagged T903 receptor as reported earlier for the non-tagged wild type hCaR (4).

Calindol and Ca$^{2+}$ Synergizes the Activation of T903-Rhoc—The ECD-truncated 7TMD structure of some family-3 GPCRs like mGluRs and GABAA receptors have not been reported to possess ligand-binding sites for either glutamate or GABA, but the 7TMD of the hCaR seems to contain one or more cation-binding site(s) within the transmembrane domain (14, 16). We have shown previously that by itself Ca$^{2+}$ does not activate the T903-Rhoc structure efficiently but either with NPS R-568 or poly-Arg shows strong synergistic potentiation of Ca$^{2+}$-activation of the T903-Rhoc receptor (16).

Because Calindol is structurally distinct from NPS-R568, we tested whether similar Ca$^{2+}$-mediated synergistic activation of the T903-Rhoc can be achieved in the presence of Calindol. Fig. 3A shows the PI hydrolysis responses of T903Rhoc2.1 cells expressing the T903-Rhoc receptor. Whereas Ca$^{2+}$ and Calindol individually at the concentrations tested showed very weak agonist activity on T903-Rhoc receptor, co-addition of 2 or 10 mM Ca$^{2+}$ with 2 µM Calindol resulted in significant increases in IP formation. The Ca$^{2+}$-saturation curve in the presence of 2 µM Calindol on the T903-Rhoc receptor was found to be very similar to what we have observed previously in the presence of NPS R-568 (16) with a calculated EC$_{50}$ value for Ca$^{2+}$ of 0.96 ± 0.25 mM (n = 3) in the presence of 2 µM Calindol (Fig. 3B). This observation is consistent with our previous hypothesis that the 7TMD of hCaR may contain one or more Ca$^{2+}$-binding site(s) and binding of Calindol along with Ca$^{2+}$ synergistically potentiates the T903-Rhoc receptor response.

Ca$^{2+}$-independent Activation of ECD-deleted T903-Rhoc by Calindol—Because Calindol and Ca$^{2+}$ synergizes activation of the ECD-truncated T903-Rhoc receptor, we examined the affinity of Calindol for the T903-Rhoc receptor in the presence and absence of Ca$^{2+}$. Fig. 4A presents results for these experiments performed in intact T903Rhoc2.1 cells by measuring IP response. Calindol in the presence of 2 mM Ca$^{2+}$ led to a concentration-dependent increase of the IP formation in T903Rhoc2.1 cells and analysis of the dose-response curves gave an EC$_{50}$ of 7.2 ± 0.25 µM (n = 3) for Calindol. Surprisingly, when Ca$^{2+}$ present in cell culture media was depleted by washing the cells in Ca$^{2+}$-free solution and the PI assay was performed in the absence of Ca$^{2+}$, Calindol still showed strong agonist activity on the T903-Rhoc receptor. The Ca$^{2+}$-independent activating effect of Calindol for T903-Rhoc receptor expressing T903Rhoc2.1 cells saturates with an EC$_{50}$ value of 8.3 ± 0.15 µM (n = 4). The dose-response curves also showed that the presence of Ca$^{2+}$-left-shifted the Calindol dose-response curve and significantly increased the maximal response amplitude at saturating concentrations of Calindol. Also, Calindol showed about 7-8-fold lower efficacy for T903-Rhoc receptor in the presence or absence of Ca$^{2+}$ compared with Calindol affinities (EC$_{50}$ values of 0.95 ± 0.36 and 1.24 ± 0.27 µM, respectively) on the epitope-tagged wild type hCaR and T903.
receptor in the presence of 2 mM Ca$^{2+}$. To assess the cellular basis for the Calindol-stimulated PI hydrolysis, we have compared T903Rhoc2.1 cells with vector-transfected HEK-293 cells, the active R-enantiomer, and less active S-enantiomer of Calindol and the negative allosteric modulator Calhex 231. Fig. 4B shows that whereas 10 and 30 μM Calindol stimulated IP accumulation in T903Rhoc2.1 cells by ~8–12-fold, up to 100 μM S-enantiomer of Calindol induced only 2–3-fold increases. 30 μM R-Enantiomer of Calindol elicited no change in IP accumulation in vector-transfected HEK-293 cells from the basal level (data not shown). As a further test of the Calindol selectivity of T903-Rhoc, we investigated the activity of the negative allosteric modulator Calhex 231, which has been shown to bind to an overlapping site with that for Calindol within the 7TMD of the wild type hCaR (4, 8). As seen in Fig. 4C, Calhex 231 antagonized the activation of T903-Rhoc by 10 μM Calindol although incompletely at 100 μM with about 70% inhibition and indicates a complex interaction between Calindol and Calhex 231 binding sites. Because of technical limitation in the maximum concentration of Calhex 231 we can apply to the cells, we could not distinguish between competitive and non-competitive mechanisms in these experiments. Taken together, these data demonstrate that Calindol is a true agonist of the T903-Rhoc and can activate this receptor structure in the absence of extracellular Ca$^{2+}$ in T903Rhoc2.1 cells. Like Calindol, allosteric activator NPS R-568 also displayed similar Ca$^{2+}$-independent activation of the T903-Rhoc (data not shown). Also, it should be noted that the T903-Rhoc receptor transiently expressed in HEK-293 cells displayed only a 3–4-fold increase in IP formation in the presence of 30 μM Calindol similar to that seen for r-T903 (data not shown). This is considerably smaller than that for the response in the T903Rhoc2.1 stable cell line. Based on immunoblotting and cell surface immunoassay data (Fig. 1, B and C), we suspect that this is probably because of a significantly lower level of cell surface expression of the T903-Rhoc receptor transiently expressed in HEK-293 cells compared with the higher level of expression in the T903Rhoc2.1 cells.

Next, to unambiguously test for Ca$^{2+}$-independent activation of the 7TMD of hCaR by Calindol and to examine coupling efficiency of ECD-truncated hCaR with the Gq protein, we tested Calindol on the T903Rhoc2.1 cell line expressing the T903-Rhoc mutant receptor using an in vitro reconstitution GTPγS binding assay in which we completely eliminated Ca$^{2+}$ from all solutions. To perform this in vitro reconstitution assay, T903Rhoc2.1 cells were first washed thoroughly
with PBS buffer containing no Ca\(^{2+}\), P2 membrane fractions of the T903Rhoc2.1 cells were prepared without Ca\(^{2+}\) and with 1 mM EGTA in the solutions, and all assay solutions used thereafter contained no Ca\(^{2+}\). As shown in Fig. 5, under conditions with no Ca\(^{2+}\) present, Calindol activates the ECD-truncated 7TMD structure of the hCaR with a K\(_A\) value of 1.23 ± 0.5 μM (n = 3). Taken together, these findings confirm that the 7TMD of the hCaR contains a Calindol binding site and activation of hCaR 7TMD by Calindol binding may be independent of the ECD (VFTM and CRD) and extracellular calcium.

**DISCUSSION**

Whereas most previous reports have examined the calcimimetic compounds NPS R-568 and Calindol as synergists for Ca\(^{2+}\) activation of the hCaR, in the present study we demonstrate that Calindol and NPS R-568 (not shown) activate the hCaR 7TMD in the absence of calcium. Whereas our results with the intact hCaR confirm that these compounds are synergistic with Ca\(^{2+}\), the deletion of the ECD of hCaR allows these calcimimetic agents to be independent agonists of the 7TMD at a site distinct from the orthosteric ligand-binding site located in the VFTM. This finding is in agreement with the proposed binding site of the NPS R-568, Calindol, NPS 2143, and Calhex 231 within the hCaR 7TMD (4, 8, 17). Molecular modeling and site-directed mutagenesis suggest this site to be amino acid residues homologous to the retinal binding pocket in bovine rhodopsin (26). Similarly, both positive and negative allosteric compounds such as CPCCOEt, MPEP, R01-6128, R67-7476 identified for mGluR1, mGluR2, and mGluR5 also show binding pockets within the 7TMD with important residues located in TM4 and TM5 (27, 28). Recently, 3,3’-difluorobenzaldehyde (DFB) and CGP7930, two positive allosteric modulators of mGluR5 and GABA\(_B\) receptors have been reported similarly to activate directly the 7TMD of the respective receptor (29, 30).

Here we confirm that the positive allosteric modulator of hCaR, Calindol has very little agonist activity on the wild type hCaR or T903 receptor containing the ECD in the absence of calcium even at higher concentrations reported in this paper. This is consistent with previous observations for NPS R-568 that this compound in the absence of Ca\(^{2+}\) does not significantly activate the intact hCaR (3), and reinforces the idea that these calcimimetic compounds are allosteric modulators of the Ca\(^{2+}\) activation of the hCaR. Based upon these observations, it can be proposed that these compounds do not activate the 7TMD per se but stabilize a conformation of the 7TMD that in turn facilitates the active closed state of the VFTM, thus increasing Ca\(^{2+}\) affinity. An alternative proposal is that the positive allosteric modulators act by increasing the allosteric coupling between the active closed ECD and the 7TMD, rather than by directly activating the 7TMD. Neither of these hypotheses are consistent with Calindol activation of the 7TMD in the absence of calcium. It would appear that in the intact hCaR structure the presence of the ECD prevents Calindol from activating the 7TMD. We previously suggested a strong allosteric coupling between the ECD and 7TMD, probably conveyed through interactions with the exo-loops, as a basis for Ca\(^{2+}\) binding in the ECD allosterically activating the 7TMD for G-protein coupling (31). The data presented in this report are consistent with such a model as we find reciprocal enhancement of affinities of Ca\(^{2+}\) and Calindol for activation of PI for the wild type hCaR or T903 receptors, with little or no influence of Ca\(^{2+}\) for Calindol affinity for activation of ECD-deleted T903-Rhoc. Whereas the Ca\(^{2+}\) concentrations eliciting enhancement of Calindol activation are consistent with the EC\(_{50}\) for Ca\(^{2+}\) activation of the intact hCaR, a definitive statement of the mechanism(s) underlying these phenomena cannot be provided in the absence of radioligand binding assays for the interactions of Calindol and Ca\(^{2+}\). From these data, we suspect Calindol affinity in the 7TMD of hCaR may be at least partially regulated allosterically by other domains such as the ECD or that a specific conformational state of the 7TMD is necessary for Calindol interaction. Our data also suggest that the ECD and 7TMD Ca\(^{2+}\) sites of the hCaR display distinct allosteric interactions with the calcimimetic site(s) of the 7TMD.

The residues comprising the binding site(s) of hCaR for extracellular Ca\(^{2+}\) remain undetermined. Chimeric receptors in which the ECD of the hCaR is fused to mGluR1 7TMD yield functional receptors responsive to Ca\(^{2+}\), indicating a Ca\(^{2+}\)-binding site(s) within the ECD that can activate the chimeric receptor (14). However, we and others (16, 21, 32) have reported that, whereas Ca\(^{2+}\) alone produces very little or no activation of the ECD-truncated T903-Rhoc construct, in the presence of NPS R-568 and as shown in this study, in the presence of Calindol, Ca\(^{2+}\) dramatically stimulates the 7TMD hCaR construct. These results are consistent with our previous hypothesis that the 7TMD of the hCaR contains an activating cation-binding site(s) (16). In contrast to the full agonist activity of Ca\(^{2+}\) for the full-length receptor, Ca\(^{2+}\) does not act as an independent activating ligand for the ECD-deleted 7TMD structure. Rather, Ca\(^{2+}\) appears to act allosterically to enhance Calindol activation of the T903-Rhoc receptor, producing no activation alone even at a concentration of 10 mM. The physiologic relevance of this allosteric cationic activation mechanism is currently unknown. Whether Calindol-mediated activation of the T903-Rhoc receptor enhances entry of extracellular Ca\(^{2+}\) through Ca\(^{2+}\) channels to elicit increased IP formation cannot be ruled out by our data. If indeed a Ca\(^{2+}\)-binding site(s) exists in the 7TMD of hCaR, one possibility for the hCaR activation mechanism is that conformational changes induced in the ECD (VFTM) upon Ca\(^{2+}\) binding are transmitted to the 7TMD exposing the 7TMD Ca\(^{2+}\) binding site(s). Multiple Ca\(^{2+}\) binding in the ECD as well as the 7TMD thus may explain the highly cooperative activation mechanism unique to Ca\(^{2+}\) receptor. It is also possible that a 7TMD Ca\(^{2+}\) site is conserved among the family-3 GPCRs, which participates in an analogous allosteric regulation of the ECD-7TMD transmission of activation in these receptors. Reports of enhanced responses of both mGluR1 and GABA\(_B\) receptors to their cognate ligands in the presence of extracellular Ca\(^{2+}\) ions support the latter hypothesis. However, the proposed Ca\(^{2+}\) binding sites for these receptors are in the ECD (33, 34). Further
The 7TMD is in an inactive state (VFTM0) in the absence of bound Ca2+. We hypothesize the initial Ca2+ binding to the VFTM, inducing a closed state of the VFTM and exposing the 7TMD allosteric site(s) for Ca2+. Higher Ca2+ concentrations, via sequential binding of multiple Ca2+ ions on the VFTM and 7TMD, produce the maximally active conformation (VFTM*). The positive allosteric modulator, Calindol, is proposed to stabilize an intermediate active state (7TMD*) from which the fully active state can be produced by the 7TMD binding Ca2+. The allosteric interactions are proposed to be similar, but differ for the ECD-deleted 7TMD receptor in that the basal state for the 7TMD construct differs from that for the wild type structure that we indicate by 7TMD0. The only allosteric interactions available are confined to the 7TMD. Because of a decreased barrier the resting conformation of the 7TMD0 (VFTM0) and different active conformational states (7TMD*) may require ligand binding or a combination of Ca2+ ions. The maximally active state 7TMD* allows the positive allosteric modulator Calindol to act as an agonist. The activated VFTM and calcimimetics may bind and stabilize this active 7TMD dimeric state necessary for G-protein activation. Future biochemical and structural studies are clearly required to test the validity of these proposed mechanisms.

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