Evidence for Distinct Cation and Calcimimetic Compound (NPS 568) Recognition Domains in the Transmembrane Regions of the Human Ca^{2+} Receptor*

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The Ca^{2+} receptor, a member of the family 3 of G protein-coupled receptors (GPCR), responds not only to its primary physiological ligand Ca^{2+} but also to other di- and trivalent metals (Mg^{2+}, Gd^{3+}) and the organic polycations spermine and poly-L-Arginine. As has been found for other family 3 GPCRs, the large amino-terminal extracellular domain (ECD) of the Ca^{2+} receptor is the primary Ca^{2+} binding domain. To examine how the signal is propagated from the ECD to the seven-transmembrane core domain (7TM) we constructed a Ca^{2+} receptor mutant (T903-Rhoc) lacking the entire ECD but containing the 7TM. We have found that this structure initiates signaling in human embryonic kidney (HEK) 293 cells stably expressing the construct. One or more cation recognition sites are also located within the 7TM. Not only Ca^{2+}, but also several other Ca^{2+}-receptor-specific agonists, Mg^{2+}, Gd^{3+}, spermine, and poly-L-Arginine, can activate T903-Rhoc truncated receptor-activated phosphoinositide hydrolysis in HEK 293 cells. The phenylalkylamine compound, NPS 568, identified as a positive allosteric modulator of the Ca^{2+} receptor can selectively potentiate the actions of Ca^{2+} and other polycationic agonists on the T903-Rhoc receptor. Similarly, organic polycations synergistically activate T903-Rhoc with di- and trivalent metals. Alanine substitution of all the acidic residues in the second extracellular loop of the T903-Rhoc receptor significantly impairs activation by metal ions and organic polycations in the presence of NPS 568 but not the synergistic activation of Ca^{2+} with poly-L-Arginine. These data indicate that although the ECD has been thought to be the main determinant for Ca^{2+} recognition, the 7TM core of the Ca^{2+} receptor contains activating site(s) recognizing Ca^{2+} and Gd^{3+} as well as the allosteric modulators NPS 568 and organic polycations that may play important roles in the regulation of receptor activation.

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‡ The abbreviations used are: GPCR, G-protein-coupled receptor; GABA_{B}, γ-aminobutyric acid receptor, type B; 7TM, seven-transmembrane segments; ECD, amino-terminal extracellular domain; mGluR, metabotropic glutamate receptor; hCaR, human calcium sensing receptor; Exo-loop2, second extracellular loop; PI, phosphoinositide; HEK, human embryonic kidney; IP, inositol phosphates; PIPES, 1,4-piperazinediethanesulfonic acid.

Systemic Ca^{2+} homeostasis is regulated largely by parathyroid hormone that acts on the kidney and bone to increase the level of plasma calcium. Parathyroid hormone secretion is regulated by the Ca^{2+} receptor, a novel cation-sensing G-protein-coupled receptor (GPCR) in parathyroid cells, which responds to changes in extracellular Ca^{2+} concentrations (1). 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+}), certain cationic compounds (neomycin), and organic polycationic compounds (spermine, polyamine, amyloid β-peptide, poly-L-Arginine) (2). Moreover, a small phenylalkylamine compound, NPS 568, has been identified as a positive allosteric modulator, which potentiates the actions of Ca^{2+} and other polycationic agonists at the Ca^{2+} receptor yet lacks a polycationic structure (3).

The Ca^{2+} receptor belongs to a structurally unique GPCR family (family 3) that includes Ca^{2+} receptor, eight subtypes of metabotropic glutamate receptors (mGluR1–8), several putative pheromone receptors expressed in the rodent vomeronasal organ (V2Rs), three sweet taste receptors (T1R1–3), and GABA_{B} receptors (4). The family 3 GPCRs are characterized by a very large (~600 residues) autonomously folded extracellular amino-terminal domain (ECD) that shares structural similarity to the bilobed structure of bacterial periplasmic binding proteins (4, 5). Unlike GPCRs in the largest family (rhodopsin family or family 1) with ligand binding within the seven-transmembrane (7TM) core domain, both chimera receptor analysis and direct binding assays with purified ECDs of several subtypes of mGluRs and GABA_{B} receptor have suggested that the ECD of these family 3 receptors is responsible for ligand recognition (6–8). Further, the crystal structures of the autonomously expressed ECD of mGluR1a in a complex with glutamate and in two unliganded forms show that each monomeric ECD contains a single bound glutamate (9). The mechanism by which the binding of small ligands like glutamate and γ-aminobutyric acid to the ECD leads to conformational changes in the transmembrane domains and G protein activation has yet to be determined. It has been suggested that the family 3 GPCR structures evolved by the fusion of a periplasmic binding protein (the ECD) to a family 1 receptor (10). This proposal implies that although the family 3 GPCR structures contain unique amino-terminal ligand recognition domains they will have conserved a mechanism of activation of the 7TM domain in common with family 1 receptors.

The structural determinants for agonist binding and activation of the human Ca^{2+} receptor (hCaR) are presently unknown. Chimeric receptors with the hCaR ECD-linked to the mGluR1 7TM domain confer intracellular phosphoinositide (PI) hydrolysis responsiveness to extracellular Ca^{2+} to cells expressing the constructs (11, 12). These results clearly identify the ECD as containing a Ca^{2+} recognition site(s). However,
the existence of additional cation-binding site(s) in the 7TM domain of the hCaR has not been excluded by these results. Two laboratories have reported inconclusive or conflicting results suggesting the existence of either a Ca$^{2+}$- or a Gd$^{3+}$-binding site in the 7TM core domain of the hCaR (13, 14). Available data also indicate that the binding site for the synergistic phenylalkylamine NPS 568 resides in the transmembrane domain rather than the ECD of the hCaR (14). Interestingly, although the hCaR lacks the well characterized Ca$^{2+}$-binding domain rather than the ECD of the hCaR (14). Available data also indicate that the binding site for the synergistic phenylalkylamine NPS 568 resides in the transmembrane domain rather than the ECD of the hCaR (13, 14). The initial 20 amino acid residues (MNGTEGPNFYVPF) of the amino terminus of bovine rhodopsin (22). A clonal HEK 293 cell line T903-Rhoc2.1 stably expressing T903-Rhoc was expanded in culture for functional studies.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and hCaR Constructs**—Site-directed mutagenesis was performed on the hCaR cDNA cloned in the pCR3.1 expression vector using a commercial kit (QuickChange™, Stratagene) as described by Ray et al. (17). The first 20 amino acid residues (MNGTEGPNFYVPF-SNKTGVV) from the amino terminus of bovine rhodopsin were added to the amino terminus at residue 599 of a truncated hCaR construct (T903) with a stop codon at position 903 as described earlier (17). The rhodopsin amino terminus tag has been shown to enhance proper processing and cell surface expression of several GPCRs (18, 19). The resultant construct, designated as T903-Rhoc, replaced residues 1–599 of the amino-terminal ECD of the hCaR with the initial 20 amino acid residues of bovine rhodopsin followed by the amino acid residues 599–903 of the hCaR (see Fig. 1). As a strategy to ensure a high level of cell surface receptor expression, we generated T903-Rhoc mutant on the T903 carboxy-terminal truncated mutant background with the carboxy-terminal I77 amino acid residues deleted because the T903 carboxy-terminal truncated receptor has shown a higher level of cell surface expression but very similar phosphoinositide hydrolysis response to Ca$^{2+}$-equivalent to that of the wild-type hCaR (17). This construction was identical to the Rho-C-hCaR previously reported (20). A second construct, designated T903-Rhoc/E/D5A, contains alanine substitution for all 5 acidic residues located in the Exo-loop2 of the T903-Rhoc receptor. Residues Glu-75, Glu-75, Asp-75, Glu-75, and Glu-75 were collected into a single amino acid change using QuickChange™ site-directed mutagenesis kit. Mutations were confirmed by using a dRhodamine terminator cycle sequencing reaction kit and ABI prism 377 DNA sequencer (Applied Biosystems).

**Generation of HEK 293 Cells Stably Expressing T903-Rhoc Mutant Receptor**—HEK 293 cells were cultured using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml penicillin, and 100 mg/ml streptomycin. A plasmid encoding T903-Rhoc under control of the cytomegalovirus promoter in the eukaryotic expression vector pCR 3.1 (Invitrogen) was transfected into HEK 293 cells using LipofectAMINE (Invitrogen) as previously described (21). Geneticin (G418, 800 mg/ml) selection of transfecteds was initiated 2 days after transfection. Individual clones were isolated 3 weeks later, and the two highest expressing T903-Rhoc cell lines were selected on the basis of immunostaining and whole cell surface enzyme-linked immunoassays using the monoclonal antibody (B6–30N) recognizing the amino terminus of bovine rhodopsin (22). A clonal HEK 293 cell line T903-Rhoc2.1 stably expressing T903-Rhoc was expanded in culture for functional studies.

**PI Hydrolysis Assay**—The PI hydrolysis assay has been described previously (17, 21). Briefly, confluent cells in 24-well plates were replenished in medium containing 3.0 μCi/ml myo[3H]inositol (PerkinElmer Life Sciences) in complete Dulbecco’s modified Eagle’s medium for overnight (16 h), followed by 30 min of incubation with Solution A (120 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl$_2$, 20 mM LiCl, and 25 mM PIPES, pH 7.2) devoid of Ca$^{2+}$. After removal of Solution A, cells were incubated for an additional 1 h with test agents dissolved in Solution A. The reactions were terminated by the addition of 1 ml of HCl/methanol (1:1000, v/v) per well. Total inositol phosphates were purified by chromatography on Dowex 1-X8 columns.

**Molecular Cloning**—Plasmid DNA harboring T903-Rhoc and T903-RhocE/D5A cDNAs in pCR3.1 vector was introduced into HEK 293 cells by the LipofectAMINE transfection (Invitrogen) method. PI hydrolysis assays were performed 48 h after transfection.

**RESULTS**

**Characterization of HEK 293 Cell Lines Stably Expressing the T903-Rhoc Truncated Receptor**—To investigate the pharmacological properties of the 7TM core domain of the hCaR, site-directed mutagenesis was performed to construct an ECD truncated hCaR with a rhodopsin epitope tag, resulting in a mutant receptor T903-Rhoc with 20 amino acids of the amino terminus of rhodopsin and the amino acid residues 599–903 of the hCaR. Fig. 1 depicts schematically the structure of T903-Rhoc receptor with all amino acid residues present in this 7TM core domain of the hCaR. Forty-six clonal lines were selected by G418 resistance after transfection with the T903-Rhoc construct. Twelve of the surviving clones expressed the T903-Rhoc mutant identified by positive immunoreactivity with the monoclonal anti-rhodopsin antibody B6–30N as visualized by ECL described (17).

**Materials**—NPS 568, (R)-N-(3-methoxy-a-phenylethyl)-3-(2'-chlorophenyl)-1-(propylamine hydrochloride), was generously provided by Drs. Allen Spiegel and Kenneth Jacobson, NIDDK. Poly-L-arginine (M$_r$, 100,000) and spermine were obtained from Sigma. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was obtained from Transduction Laboratories, and the ECL visualization kit was from Pierce. The hybridoma clone B6–30N was generously provided by Dr. Paul Har (University of Florida).
Ca\textsuperscript{2+}-binding Site in Transmembrane Regions of Ca\textsuperscript{2+} Receptor

HEK 293 Cells Expressing T903-Rhoc Mutant Receptor Show Cation-induced IP Formation—The experiments presented in Fig. 2 demonstrate that our ECD-deleted hCaR construct T903-Rhoc can be activated by many of the agonists regulating activity of the wild-type receptor. As shown in Fig. 2A, with no added Ca\textsuperscript{2+} present in the assay, several known agonists of the wild-type hCaR including Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Gd\textsuperscript{3+}, and cationic molecules, poly-l-Arg and spermine, when tested individually showed very weak stimulation of PI hydrolysis in HEK 293 cells expressing T903-Rhoc. We consistently observed that 1 \mu M NPS 568, 2 mM Ca\textsuperscript{2+}, and 250 nM poly-l-Arg alone stimulated a small but significant increase in IP accumulation over basal values. However, co-addition of all of the cationic agonists with 1 \mu M NPS 568 resulted in varied but significant increases in IP accumulation in these cells, establishing that the T903-Rhoc structure contains activating ligand-binding sites for all of these agents. These results differ from data obtained for the full-length wild-type hCaR structure. As opposed to the T903-Rhoc, HEK 293 cells transfected with the wild-type hCaR respond fully to cations in the absence of NPS 568 and in the absence of added Ca\textsuperscript{2+} show no increase in the IP hydrolysis response over basal when exposed to 1 \mu M NPS 568 alone (data not shown). Activation by NPS 568 is obtained only in the presence of Ca\textsuperscript{2+} as has been reported previously (3).

Synergistic activation of the T903-Rhoc was not limited to the phenylalkylamine NPS 568. Co-addition of 250 nM poly-l-Arg with Ca\textsuperscript{2+} generated an equivalent increase in PI hydrolysis as seen with NPS 568 (Fig. 2B). Synergy by poly-l-Arg distinguishes among metal ion species. Whereas Ca\textsuperscript{2+} and Gd\textsuperscript{3+} produce equivalent activation of PI hydrolysis in the presence of NPS 568, the Gd\textsuperscript{3+} response is significantly lower than Ca\textsuperscript{2+} with poly-l-Arg (Fig. 2B). This response seems to be specific for a polycation as no significant synergism of Ca\textsuperscript{2+} was found for poly-l-Glu. All these agonist effects were specific for T903-Rhoc expressing HEK 293 cells. Neither HEK 293 cells transiently transfected with vector alone nor an HEK 293 cell line stably expressing an equivalent ECD truncated mGluR1 7TM core domain showed any response to the optimal concentrations of these agents alone or in combination (data not shown).

Fig. 3 presents the results of experiments characterizing the affinity of the metal ion activation of the 7TM core structure. As shown in Fig. 3A, in the presence of 1 \mu M NPS 568 a significant increase in IP formation occurs below 0.5 mM Ca\textsuperscript{2+}. From these saturation data we calculate an EC\textsubscript{50} value of 1.8 \pm 0.4 mM for Ca\textsuperscript{2+} for activation of T903-Rhoc reflecting a greater sensitivity of the ECD-deleted receptor to Ca\textsuperscript{2+} than found for the wild-type structure. Further, although the activation of wild-type hCaR by Ca\textsuperscript{2+} is highly cooperative with apparent Hill coefficients of 3–4, the Ca\textsuperscript{2+} saturation of T903-Rhoc is well fit by a simple, one-site binding model (dashed line in Fig. 3A). Similarly, as shown in Fig. 3B, in the presence of 1 \mu M NPS 568 a significant increase in IP formation occurs between 25 and 100 \mu M Gd\textsuperscript{3+}. Neither the efficacy nor the apparent affinity of Gd\textsuperscript{3+} for the T903-Rhoc is well defined because concentrations above 0.5 mM showed a profound inhibition of IP accumulation (Fig. 3B). This observation is consistent with other reports that at millimolar concentration, this lanthanide inhibited IP formation, and thus Gd\textsuperscript{3+} cannot act as full functional agonist for the Ca\textsuperscript{2+} receptor (23). Taken together, these findings are consistent with the hypothesis that the 7TM core domain of the hCaR contains one or more Ca\textsuperscript{2+}-binding sites. In addition, these results indicate that other cationic molecules such as Mg\textsuperscript{2+}, Gd\textsuperscript{3+}, and the organic polycations poly-l-Arg...
and spermine can interact with the same or overlapping binding domains within the 7TM domain and can activate the ECD truncated T903-Rhoc receptor.

**Regulation of Asp/Glu → Ala Exo-loop2 Mutant T903-Rhoc Receptor (T903-RhocE/D5A) by Cations and NPS 568**—Within the 7TM core domain, the Exo-loop2 contains a cluster of acidic residues that may form a binding site for Ca\(^{2+}\) and other cationic agonists. Based on this hypothesis, we constructed a mutant, T903-RhocE/D5A, in which all five negatively charged (Asp and Glu) residues located within the second extracellular loop domain of the T903-Rhoc (Glu-755, Glu-757, Asp-758, Glu-760, Glu-767) were collectively mutated into alanine (see Fig. 1). Both the T903-Rhoc and T903-RhocE/D5A mutant receptor expression constructs were transiently transfected in HEK 293 cells, and the PI hydrolysis assay was performed 48 h after transfection. As shown in Fig. 4, the T903-RhocE/D5A mutant receptor did not show significant synergistic response to Ca\(^{2+}\), Gd\(^{3+}\), or poly-l-Arg in the presence of 1 mM NPS-568 as compared with the T903-Rhoc. However, the T903-RhocE/D5A receptor-like T903-Rhoc demonstrates a significant synergistic response in the presence of poly-l-Arg. As shown in Fig. 4, the T903-Rhoc receptor was myo\(^{3H}\)inositol-labeled, and intact cell PI hydrolysis was assessed for the combination of poly-L-Arg and Ca\(^{2+}\) similar to that for the T903-Rhoc receptor. These findings confirm that mutation of Exo-loop2 acidic residues impairs the cationic response of the T903-Rhoc receptor in the presence of one allosteric modifier NPS 568. However, in the presence of poly-l-Arg, the synergistic Ca\(^{2+}\) response of this mutant receptor remained largely intact.

**DISCUSSION**

Signaling by the 7TM Domain of the hCaR—Like many family 3 GPCRs including mGluR1, mGluR2, and GABA<sub>B</sub> receptor, agonist binding seems to occur primarily within the large ECD of the hCaR rather than in the pocket defined by the 7TM helices as is characteristic of most classical rhodopsin-like (family 1) GPCRs (5). As reported by several laboratories, a chimera with the entire hCaR ECD linked to the mGluR1 7TM confers PI hydrolysis responsiveness to extracellular Ca\(^{2+}\) (11, 12). This has been taken as evidence that the hCaR ligand specificity resides in the ECD and not in the 7TM domain. Two prior studies have investigated the signaling properties of hCaR constructs with deleted amino-terminal ECDs as controls for chimeric ECD constructs mapping the site(s) for Ca\(^{2+}\) recognition within the hCaR (13, 14). A deletion construct of this receptor (ΔNtCaR), which lacked virtually the entire ECD, was unable to respond to Ca\(^{2+}\) but responded to Gd\(^{3+}\) when expressed in Xenopus oocytes (13). In contrast, Hauache et al. (14)
Hydrolysis stimulation, and in the presence of poly-L-Arg, Ca\(^{2+}\) exhibited significant PI hydrolysis response. In the presence of added individually none of the cationic agonists or NPS 568. These cation sites and the site for the compound, NPS 568 (1\(\mu\)M) as a co-agonist. HEK 293 cells transiently transfected with the T903-Rhoc receptor or T903-Rhoc/Exo loop2 to respond functionally to Ca\(^{2+}\) in the presence of 4 mM Ca\(^{2+}\) and Gd\(^{3+}\) (0.5 mM) was investigated in the presence of a phenylalkylamine compound, NPS 568 (1 \(\mu\)M), identified as a positive allosteric modulator of the Ca\(^{2+}\) receptor. Similarly, Ca\(^{2+}\) response of this mutant receptor was evaluated in the presence of poly-L-Arg (250 nM) as a co-agonist. HEK 293 cells transiently transfected with the T903-Rhoc receptor or T903-Rhoc/Exo loop2 mutant receptors were evaluated 48 h after transfection. Transfected cells were myo-[\(^{3}\)H]inositol-labeled, and the intact cell PI hydrolysis was determined in the presence of 4 mM Ca\(^{2+}\), 0.5 mM Gd\(^{3+}\), 250 nM poly-L-Arg, and 1 \(\mu\)M NPS 568 and the combination of NPS 568 with Ca\(^{2+}\), Gd\(^{3+}\) or poly-L-Arg and poly-L-Arg with Ca\(^{2+}\). Values presented are the means \(\pm\) S.E. of data obtained for triplicate determinations. The results are representative of those obtained in three independent experiments.

Recently reported the 7TM domain of the hCaR as the site of action of NPS 568 compound as several hCaR mutants lacking all or part of the ECD were partially responsive to Ca\(^{2+}\) in the presence of NPS 568 in HEK 293 cells. Both of these studies raised the possibility that the 7TM core structure of the hCaR is sufficient for ligand-regulated activation of signaling as for a family 1 GPCR. Our data with T903-Rhoc confirm the signaling properties of the 7TM core domain and are the first demonstration that this structure has preserved a complex set of allosteric interactions autonomous of the amino-terminal ECD. These data lend credence to the proposal that the family 3 receptor participates directly in the binding of NPS 568 or indirectly in the allosteric interaction of the NPS 568 site with the Ca\(^{2+}\) site.

**Both the ECD and 7TM Domain of the hCaR Contain Cation Recognition Sites**—Our data with T903-Rhoc clearly demonstrate Ca\(^{2+}\) recognition by the 7TM domain of the hCaR that is only revealed in the presence of an allosteric regulator such as NPS 568 and poly-L-Arg. These data lead us to conclude that the hCaR 7TM core contains at least three distinct activation sites: a Ca\(^{2+}\) and Gd\(^{3+}\) recognition site, a site for organic polycations (poly-L-Arg and spermine), and a site for the phenylalkylamine NPS 568. These cation sites and the site for the NPS 568 in the 7TM core are allosterically interacting. When added individually none of the cationic agonists or NPS 568 exhibited significant PI hydrolysis response. In the presence of NPS 568, cationic agonists exhibited varied but significant PI hydrolysis stimulation, and in the presence of poly-L-Arg, Ca\(^{2+}\) activated the T903-Rhoc receptor similar to the activation with NPS 568. These results indicate that NPS 568 or poly-L-Arg binds to an allosteric site(s) in the transmembrane region and synergistically potentiates cationic response of the T903-Rhoc receptor’s 7TM domain.

For a number of proteins that bind the Ca\(^{2+}\) ion, the side chains of Asp and Glu have been shown to participate in the stabilization hydrogen bond networks (24). Moreover, residues in the second extracellular loop of several GPCRs including the CCK5, adenosine, and gonadotropin receptors, have been shown to be important for high affinity agonist binding and activation of the receptors (25, 26). Here we found that the mutant T903-Rhoc/Exo loop2 Asp and Glu residues changed to Ala did not respond to Gd\(^{3+}\) or poly-L-Arg in the absence or presence of NPS 568, and Ca\(^{2+}\) elicited a significantly lower maximal IP response compared with the T903-Rhoc receptor. Although this observation is consistent with the idea that some of the Asp and Glu residues in the Exo-loop2 may be directly involved in cation binding, the synergistic response of T903-Rhoc/Exo loop2 to Ca\(^{2+}\) and poly-L-Arg demonstrates that this mutant retains both a Ca\(^{2+}\) and an organic polycation recognition that stimulate the receptor equivalently to the parent T903-Rhoc. An alternative explanation for this result is that the Exo-loop 2 acidic residues either participate directly in the binding of NPS 568 or indirectly in the allosteric interaction of the NPS 568 site with the Ca\(^{2+}\) site.

**Implications of the Existence of Cation Recognition Site(s) in the 7TM Domain of the Ca\(^{2+}\) Receptor**—In this study we identify NPS 568 and organic cations as allosteric modulators of Ca\(^{2+}\) and other cationic agonists at sites within the 7TM of the hCaR using the ECD truncated hCaR (T903-Rhoc). These findings pose intriguing questions. What is the significance of a cation-binding site within the hCaR 7TM domain and does this cation-binding site have any functional importance in the context of the native receptor? Among all GPCRs, the hCaR is the only receptor known so far that binds several cations and cationic compounds and exhibits a cooperative activation process (1). This cooperativity suggests the possible existence of multiple interacting cation-binding sites; we suggest that these include sites in the ECD as well as in the 7TM domain of the hCaR. The mechanism by which the binding of Ca\(^{2+}\) to the ECD leads to conformational changes in the 7TM domain within the native hCaR has yet to be determined. A diverse spectrum of binding and activation motifs is recognized for the several varieties of GPCR structure (27). It is commonly thought for family 3 receptors that conformational changes induced in the ECD by ligand binding are transmitted to the 7TM domain by structural contacts between the ECD and Exo-loops connecting the 7TM domains. In the hCaR this allosteric interaction of the ECD with the Exo-loops may expose
the 7TM domain Ca$^{2+}$-binding site(s) accounting for the cooperative activation properties of the receptor. In this context, our results with the Exo-loop2 mutant T903-Rhoc/D5A might be interpreted to reveal an essential linkage in such an allostERIC transition because this mutation has abrogated all of the synergistic interactions by the NPS 568 compound. Although the basic mechanism of the ECD conformational change and its interaction with the 7TM core domain seem to be critical for the activation of hCaR, mGluR1, and other receptors in this family (5), there is little precedent for the molecular properties unique to the hCaR. Highly cooperative activation and allostERIC activation by two different agonists is seemingly unique to hCaR and has not been observed even for the related mGluR receptor family (1). Our results led us to propose distinct ligand recognition sites both in the ECD and the 7TM core of the hCaR that are allosterically cooperative. It may be that such distinct ligand recognition within the 7TM cores of other family 3 GPCR structures will be revealed by compounds analogous to the NPS 568 (5), there is little precedent for the molecular properties unique to the hCaR. Highly cooperative activation and allostERIC activation by two different agonists is seemingly unique to hCaR and has not been observed even for the related mGluR receptor family (1). Our results led us to propose distinct ligand recognition sites both in the ECD and the 7TM core of the hCaR that are allosterically cooperative. It may be that such distinct ligand recognition within the 7TM cores of other family 3 GPCR structures will be revealed by compounds analogous to the NPS 568. Future biochemical and structural studies are needed to test the generality of this proposed hypothesis.

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