A Missense Mutation in the Seven-transmembrane Domain of the Human Ca\textsuperscript{2+} Receptor Converts a Negative Allosteric Modulator into a Positive Allosteric Modulator*\textsuperscript{S}

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G protein-coupled receptors (GPCRs) are the most common targets of drug action. Allosteric modulators bind to the seven-transmembrane domain of family 3 GPCRs and offer enhanced selectivity over orthosteric ligands that bind to the large extracellular N terminus. We characterize a novel negative allosteric modulator of the human Ca\textsuperscript{2+} receptor, Compound 1, that retains activity against the E837A mutant that lacks a response to previously described positive and negative modulators. A related compound, JKJ05, acts as a negative allosteric modulator on the wild type receptor but as a positive modulator on the E837A mutant receptor. This positive modulation critically depends on the primary amine in JKJ05, which appears to interact with acidic residue Glu\textsuperscript{837} in our model of the seven-transmembrane domain of the receptor. Our results suggest the need for identification of possible genetic variation in the allosteric site of therapeutically targeted GPCRs.

Family 3 of the GPCR\textsuperscript{2} superfamily has recently become a focus for the discovery of new allosteric modulators with therapeutic potential (1). This family includes the Ca\textsuperscript{2+} receptor (CaR) (2) and receptors for neurotransmitters such as eight subtypes of metabotropic glutamate receptors (mGluR) and two subtypes of \gamma-amino butyric acid type B receptors. They have a characteristic Venus flytrap-like structure (3) in their N-terminal extracellular domain, which constitutes the orthosteric site for binding of endogenous agonists (Fig. 1A). A crystalline structure of homodimeric mGluR1 Venus flytrap-like domain in agonist-bound form verified this (3). Exogenous allosteric modulators bind to sites in the seven transmembrane-spanning domains of the receptors topographically distinct from the orthosteric sites (Fig. 1A). Allosteric modulators offer advantages over classic orthosteric ligands as therapeutic agents, including the potential for greater GPCR subtype selectivity and safety.

A number of allosteric modulators have been identified targeting specifically the CaR, which plays a central role in the regulation of calcium homeostasis (4, 5). Positive allosteric modulators of the CaR, such as NPS R-568 (Fig. 1B), increase CaR activation, thereby decreasing secretion of parathyroid hormone, and thus may be useful in the treatment of primary and secondary hyperparathyroidism. Cinacalcet is the first-in-class GPCR allosteric modulator approved by the U. S. Food and Drug Administration recently for treatment of secondary hyperparathyroidism in patients with chronic kidney disease on dialysis and for treatment of hypercalcemia in patients with parathyroid cancer. Negative allosteric modulators of the CaR, such as NPS 2143 (Fig. 1B), decrease receptor activation, thereby stimulating endogenous parathyroid hormone secretion. This potentially offers a novel method for treatment of osteoporosis.

NPS R-568, cinacalcet, NPS 2143, and several other allosteric modulators of the CaR are structurally related phenylalkylamines with a positively charged central amino group (Fig. 1B). We and others recently reported that the residue Glu\textsuperscript{837} in transmembrane helix 7 of the CaR is crucial for action of those phenylalkylamines tested (6–8). Another mutation, I841A, has also been reported to abolish responsiveness to positive and negative allosteric modulators (8). It is speculated that allosteric binding sites of these phenylalkylamines partially overlap and that a critical salt bridge might form between the negatively charged Glu\textsuperscript{837} and the positively charged central amine in these compounds.

Recently, a novel negative allosteric modulator, Compound 1, was described (9). It is structurally distinct from those phenylalkylamines (Fig. 1B), and in a competition assay, it did not displace a radiolabeled analogue of NPS 2143 bound to the receptor, suggesting that its unique allosteric site is distinct from those of phenylalkylamines (9). In the present work, we synthesized and studied Compound 1 ana-
logues and examined the effects of alanine substitutions at some key residues of the CaR, such as Glu$^{767}$, Glu$^{837}$, and Ile$^{841}$, on allosteric modulation of the receptor by these compounds.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of the hCaR—The full-length human CaR (hCaR) cDNA cloned in the pCR3.1 expression
hCaR Mutation Converts a Negative into a Positive Modulator

vector was described previously (6). Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA), according to the manufacturer’s instructions. Parental hCaR cDNA in pCR3.1 vector was amplified using pfu Turbo DNA polymerase with mutagenic oligonucleotide primers (sequences available on request) for 16 cycles in a DNA thermal cycler (PerkinElmer Life Sciences). After digestion of the parental DNA with DpnI for 1 h, the amplified DNA with incorporated nucleotide substitution was transformed into Escherichia coli (DH-5α strain). The sequence of mutant receptors was confirmed by automated DNA sequencing using a dRhodamine terminator cycle sequencing kit and ABI PRISM-373A DNA sequencer (Applied Biosystems, Foster City, CA).

Transient Transfection of Wild Type and Mutant Receptors in HEK-293 Cells—Transfections were performed using 12 μg of plasmid DNA for each transfection in a 75-cm² flask of HEK-293 cells. DNA was diluted in serum-free DMEM (BioFluids Inc., Rockville, MD) and mixed with diluted Lipofectamine (Invitrogen), and then the mixture was incubated at room temperature for 5 h. After 5 h of incubation, 15 ml of complete DMEM containing 10% fetal bovine serum (BioFluids Inc., Rockville, MD) and diluted Lipofectamine (2.5 ml) into toluene (2.5 ml), and the resulting mixture was stirred for 48 h at 90 °C. The solution was added with known literature methods (20) in toluene (2.5 ml), and the resulting mixture was stirred for 48 h at 90 °C. The solution was removed under reduced pressure. The resulting crude product was purified by column chromatography (1:1 MeOH:EtOAc) to afford JKJ-05 as a colorless solid (90%).

For the synthesis of JKJ05-Ac, pyridine (11.0 μl, 0.14 mmol), 4-dimethylaminopurine (0.5 mg, 0.0045 mmol), and Ac2O (13.2 μl, 0.14 mmol) were added to a solution of JKJ-05 (25 mg, 0.045 mmol) in CH2Cl2 (2 ml) and stirred for 1 h. Solvent was removed under reduced pressure, and the resulting crude product was purified by column chromatography (1:1 EtOAc:hexanes) to afford JKJ05-Ac as a colorless solid; 20 mg, 74%. 1H NMR (CDCl3, 300 MHz) δ 1.83 (s, 3H), 2.72–3.40 (m, 2H), 3.62 (q, J HH1 = 5.4 Hz, 2H), 3.80 (s, 3H), 3.814 (s, 6H), 3.88 (t, J HH1 = 5.4 Hz, 2H), 4.28–4.41 (m, 2H), 4.49–4.60 (m, 1H), 5.85 (t, J HH1 = 5.4 Hz, 1H), 6.05 (d, J HH1 = 8.1 Hz, 1H), 6.74 (dd, J HH1 = 8.7, 1.2 Hz, 2H), 6.81 (d, J HH1 = 8.7 Hz, 1H), 6.83 (s, 2H), 6.94 (tt, J HH1 = 7.5, 1.2 Hz, 1H), 7.18–7.29 (m, 7H), 7.96 (d, J HH1 = 8.7 Hz, 1H); (time-of-flight mass spectrometry) m/z 558.2629 (M+H+). (calculated for C32H36N2O6+ 558.2604.)

RESULTS

Negative Allosteric Modulation of the Wild Type and E837A Mutant CaR by Compound 1—Recently, a novel negative allosteric modulator, Compound 1, was identified, and this modulator bears little structural similarity with the phenylalkylamine class in that it lacks a central positively charged amino group (9). To compare the effects of this novel compound with NPS 2143 on the wild type (WT) and mutant CaRs, we measured PI hydrolysis as a function of extracellular Ca2+ concentrations of Ca2+ in 1× PI buffer with or without treatment of compounds. We and others reported earlier that mutant CaRs with E837A or I841A mis-sense mutations were well expressed and functional (6–8). In the present study, we found that, similar to NPS 2143, Compound 1 decreased sensitivity to Ca2+ by the WT with an IC50 of 0.51 μM but did not reduce the Ca2+ sensitivity of the I841A mutant CaR. Interestingly, unlike NPS 2143, Compound 1 also right-shifted the response to Ca2+ by the E837A mutant CaR with an IC50 of 0.23 μM (Fig. 2, A and B, data for determination of IC50 not shown). These results indicate that residue Glu837, which is crucial for allosteric modulation by phenylalkylamines, is not crucial for allosteric modulation by Compound 1. This is consistent with the report that Compound 1 did not compete for receptor binding of a radiolabeled analogue of NPS 2143, suggesting that its unique allosteric site is distinct from those of...
phenylalkylamines (9). Interestingly, residue Ile841 seems to be crucial for allosteric modulation by both Compound 1 and phenylalkylamines.

JKJ05, an Analogue of Compound 1, Is Also a Negative Allosteric Modulator of the Wild Type CaR—To test whether modification of Compound 1 by isomerization to create a positively charged primary amino group alters its negative allosteric modulator action, we synthesized an analogue of Compound 1, JKJ05 (Fig. 1B). The synthetic preparation of JKJ05 involved the treatment of the previously described intermediate B with (S)-(−)-2-amino-3-phenyl-propanol in the presence of 1.5 equivalence of potassium tert-butoxide in toluene at 90 °C (Fig. 1C). This permitted the hydroxyl group to serve as the primary nucleophile rather than the amine. Our in vitro functional assay showed that JKJ05 is also a negative allosteric modulator for the WT CaR, albeit a less potent one than Compound 1 (Fig. 3A). We tested a range of concentrations of JKJ05 up to 100 μM in the PI hydrolysis assay at 4 mM [Ca²⁺] réseau and found that the effect of JKJ05 reached a maximum at 50 μM with an IC₅₀ of 6.8 μM (Fig. 3C). Similar to Compound 1, JKJ05 exhibited a minimum inhibitory effect on the response to calcium by the I841A mutant CaR (Fig. 2B), indicating that allosteric modulation by all these compounds critically depends on residue Ile841.

E837A Mutation in the CaR Converts JKJ05 into a Positive Allosteric Modulator—We also tested the effects of JKJ05 on the E837A mutant CaR. Surprisingly, JKJ05 significantly increased sensitivity of the E837A mutant to Ca²⁺ with an EC₅₀ of 2.5 μM (Fig. 3, B and C) rather than inhibiting response to Ca²⁺ by the receptor. E837A mutation abolished allosteric modulator action by phenylalkylamines tested, whereas this same mutation evidently does not eliminate binding of JKJ05 to the receptor, suggesting that JKJ05, similar to Compound 1, might not share a binding pocket with NPS 2143. On the other hand, different allosteric actions associated with Compound 1 and its analogue JKJ05 indicate distinct interactions between functional group(s) in JKJ05 and surrounding residues(s) in the CaR that did not occur with Compound 1.

FIGURE 2. Concentration dependence for [Ca²⁺]ᵣ stimulation of PI hydrolysis of CaR in transiently transfected HEK-293 cells expressing WT hCaR, E837A, or I841A mutant receptor with or without treatment of 1 μM NPS 2143, 1 μM Compound 1, or 10 μM JKJ05. Transfection and the PI assay were performed as described under “Experimental Procedures.” Results of PI assay are expressed as the percentage of maximal response (WT hCaR at 8 mM Ca²⁺ in A and I841A at 30 mM Ca²⁺ in B without compound treatment).

FIGURE 3. A and B, concentration dependence for [Ca²⁺]ᵣ stimulation of PI hydrolysis of CaR in transiently transfected HEK-293 cells expressing WT hCaR or E837A mutant receptor with or without treatment of 1 μM Compound 1, 10 μM JKJ05, or 10 μM JKJ05-Ac. Transfection and the PI assay were performed as described under “Experimental Procedures.” The results of the PI assay are expressed as the percentage of maximal response (WT at 30 mM Ca²⁺ in A and E837A at 30 mM Ca²⁺ in B without compound treatment). C, concentration dependence for JKJ05 inhibition or stimulation of PI hydrolysis at 4 mM [Ca²⁺]ᵣ in transiently transfected HEK-293 cells expressing WT or E837A mutant CaR. The results of the PI assay are expressed as percentages of maximal response (WT at 0 μM JKJ05). The results are the means ± S.E. of quadruple determinations.
Positive Modulation of E837A Mutant Car by JKJ05 Critically Depends on the Primary Amino Group in the Compound—To verify whether positive modulation of the E837A mutant Car by compound JKJ05 requires the positively charged primary amine in the compound, we synthesized JKJ05-Ac, an acetylated derivative of JKJ05, via treatment with acetic anhydride, pyridine, and a catalytic amount of dimethylaminopurine (Fig. 1, B and C). We found that JKJ05-Ac remained a weak negative modulator of the wild type Car, comparable with JKJ05 (Fig. 3A). However, the augmentation of E837A mutant receptor sensitivity to Ca\(^{2+}\) by compound JKJ05 was abolished by acetylation of this compound (Fig. 3B). Thus, JKJ05-Ac could be considered as a “silent” allosteric modulator of the E837A mutant Car, defined as not altering the response of an orthosteric ligand but binding to an allosteric site of the GPCR.

Molecular Modeling of the Car 7TM Domain and Docking of Compound 1 and JKJ05—In an effort to understand the mechanism of allosteric modulation exerted by Compound 1 and its analogues, we constructed a homology model of the Car 7TM domain based on the crystal structure of bovine rhodopsin (10) and performed docking experiments with these compounds (see supplemental materials). No external bias was given on the precise location of the potential binding site. Instead, the docking region initially was loosely defined as the whole upper half of the helical bundle.

Compound 1 and related analogues could potentially bind to two adjacent pockets (Fig. 4), which we designate as P1 (enclosed within TM3, TM4, TM5, TM6, and the second extracellular loop (EL2)) and P2 (enclosed within TM1, TM2, TM3, TM6, TM7, and EL2) (Fig. 4). It has been proposed that NPS 2143 and related phenylalkylamines bind to the P2 pocket, with their positively charged amino group engaged in an electrostatic interaction with the side chain of Glu\(^{837}\) (7.39) (6–8). As Compound 1 did not compete with a NPS 2143 analogue for binding to the receptor (9), we speculate that Compound 1 and its analogues JKJ05 and JKJ05-Ac bind to the P1 pocket. Our result that the E837A mutation in the P2 pocket blocked negative modulation by NPS 2143 but not by Compound 1 is consistent with our above modeling hypothesis.

Our model suggests that the striking positive modulation of the E837A mutant Car by JKJ05 might result from the combined effects of three distinct factors (Fig. 5): 1) the alanine substitution of residue Glu\(^{837}\) (7.39), which altered the ground state interactions of this residue; 2) the formation of a salt bridge between the positively charged primary amino group of JKJ05 and the acidic residue Glu\(^{767}\) residing in EL2; and 3) the van der Waals interactions of the benzyl moiety adjacent to the amino group of JKJ05 with a critical cluster of hydrophobic amino acids located in the upper half of TM6 (Fig. 5). This TM6 region, Trp\(^{818}\) (6.48)–Tyr\(^{825}\) (6.55), is part of a “hot spot” of the Car receptor active site (7). Our model predicts that the formation of a salt bridge between the positively charged amino group of JKJ05 and residue Glu\(^{767}\) (exo-loop 2), we constructed an E767A/E837A double mutant Car and compared the effects of Compound 1, JKJ05, and JKJ05-Ac on this mutant. We reasoned that upon disruption of the ground state interactions of both residue Glu\(^{837}\) (again by alanine substitution) and Glu\(^{767}\) (this time by alanine substitution to prevent an electrostatic interaction with the charged amino group of JKJ05), a certain van der Waals interaction between compound JKJ05 (and even JKJ05-Ac) and the critical hydrophobic cluster could activate the receptor.

Both JKJ05 and JKJ05-Ac Are Positive Allosteric Modulators of E767A/E837A Double Mutant Car—To test our model and the hypothesized interaction between the primary amino group of JKJ05 and residue Glu\(^{767}\) (exo-loop 2), we constructed an E767A/E837A double mutant Car and compared the effects of Compound 1, JKJ05, and JKJ05-Ac on this mutant. We reasoned that upon disruption of the ground state interactions of both residue Glu\(^{837}\) (again by alanine substitution) and Glu\(^{767}\) (this time by alanine substitution to prevent an electrostatic interaction with the charged amino group of JKJ05), a certain van der Waals interaction between compound JKJ05 (and even JKJ05-Ac) and the critical hydrophobic cluster could activate the receptor.
Our in vitro assay shows that the function of this double mutant was impaired as seen in a decreased maximal response to extracellular calcium. However, consistent with our hypothesis, both JKJ05 and JKJ05-Ac, which interact with the critical hydrophobic cluster in a similar way, dramatically increased the calcium response of the receptor (Fig. 6). Conversely, Compound 1 interacted with the hydrophobic cluster in a different way (Fig. 5), due to its different chemical structure, leading to the slightly right-shifted calcium response of the receptor. These results support our model of the CaR 7TM and speculations on the mechanism of action of JKJ05.

**DISCUSSION**

GPCRs are the most common targets of drug action. Family 3 of the GPCR superfamily has recently become a focus for the discovery of new allosteric modulators with therapeutic potential. Allosteric modulators offer advantages over classic orthosteric ligands as therapeutic agents, including the potential for greater GPCR subtype selectivity and safety. Novel allosteric modulators of the CaR are being vigorously explored in an effort to identify potential drugs for treatment of disorders of calcium metabolism.

In addition to their therapeutic potential, allosteric modulators of the CaR offer unique insights into the mechanisms of receptor activation. How the signal of conformational changes in orthosteric sites upon ligand binding is transmitted to the 7TM, leading to receptor activation, is a major unanswered question. It is speculated that movements of the helices within 7TM and/or between two 7TMs in dimeric CaRs are ultimately responsible for receptor activation and G protein-coupling. Allosteric modulators bound to the allosteric sites in the 7TM domain of the CaR likely facilitate (positive modulators) or impede (negative modulators) these movements.

We published earlier that residue Glu837 in TM7 is crucial for positive allosteric modulation by NPS R-568 (6), and later, it was reported that this same residue is also crucial for allosteric modulation by other phenylalkylamines (8). It is speculated that a critical salt bridge is formed between the positively charged central group of these phenylalkylamines and the acidic side chain of residue Glu837. Here we report that Compound 1, a novel negative allo-
mutant CaR by JKJ05 and positive modulation of the E767A/E837A double mutant by JKJ05 and JKJ05-Ac might be due to enhanced TM6 rotation by these compounds interacting with the TM6 hot spot comprising residues Trp618–Tyr825.

Our model offers an interpretation of our findings, and at this stage, we cannot exclude other possible mechanisms. Further studies are necessary to understand why positive modulation of the CaR by JKJ05 and JKJ05-Ac critically depends on alteration of the ground state interactions of Glu767 and Glu837 by either alanine substitution or a salt bridge formation. Moreover, residue Ile841 seems to be crucial for action of all allosteric modulators of the CaR tested so far, including previously published phenylalkylamines and Compound 1 and its analogues. The exact role this residue plays in receptor activation remains to be elucidated.

We emphasize that a single missense mutation, E837A, converts a negative allosteric modulator (JKJ05) of the CaR into a positive modulator. To our knowledge, this is the first report of an allosteric modulator whose action, negative versus positive modulation, critically depends on a single residue in a GPCR. There have been reports that mutations in the orthosteric site of GPCRs converted antagonists to agonists (16, 17). We now show that such a phenomenon can also happen at the allosteric binding site of the CaR, suggesting the need for caution in therapeutic application of allosteric modulator-based agents and the need for identification of possible genetic variation in the allosteric site of therapeutically targeted GPCRs.

Genetic variants of many GPCRs have been reported (see Ref. 18 for a review), and the CaR genetic locus appears to harbor one of the largest numbers (~100) of variants with over 20 variations identified in the 7TM domain alone (19). Incorporation of studies of genetic variants into strategies for GPCR drug discovery and clinical drug testing has an important potential to improve efficacy and decrease toxicity of drugs (18). Given the risk of “paradoxical” effects if the receptor targeted carries an unsuspected missense mutation in the 7TM, further delineation of residues comprising allosteric binding sites, elucidation of allosteric modulation mechanism, and appreciation of potential sequence variation in the 7TM of the receptor among populations to be treated appear warranted.

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