Rescue of Calcium-sensing Receptor Mutants by Allosteric Modulators Reveals a Conformational Checkpoint in Receptor Biogenesis*

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The calcium-sensing receptor (CaR), a member of G protein-coupled receptor family C, regulates systemic calcium homeostasis by activating Gq- and Gi-linked signaling in the parathyroid, kidney, and intestine. CaR is ubiquitinated by the E3 ligase dorfin and degraded via the endoplasmic reticulum-associated degradation pathway (Huang, Y., Niwa, J., Sobue, G., and Breitwieser, G. E. (2006) J. Biol. Chem. 281, 11610–11617). Here we provide evidence for a conformational or functional checkpoint in CaR biogenesis using two complementary approaches. First we characterized the sensitivity of loss- or gain-of-function CaR mutants to proteasome inhibition by MG132. The stabilization of loss-of-function mutants and insensitivity of gain-of-function mutants to MG132 suggests that receptor sensitivity to calcium influences susceptibility to proteasomal degradation. Second, we used the allosteric activator NPS R-568 and antagonist NPS 2143 to promote the active and inactive conformations of wild type CaR, respectively. Overnight culture in NPS R-568 increased expression of CaR, whereas NPS 2143 had the opposite effect. NPS R-568 and NPS 2143 differentially regulated maturation and cell surface expression of wild type CaR, directly affecting maximal signaling responses. NPS R-568 rescued expression of loss-of-function CaR mutants, increasing plasma membrane expression and ERK1/2 phosphorylation in response to 5 mM Ca2+. Disorders of calcium homeostasis caused by CaR mutations may therefore result from altered receptor biogenesis independent of receptor function, i.e. a protein folding disorder. The allosteric modulators NPS R-568 and NPS 2143 not only alter CaR sensitivity to calcium and hence signaling but also modulate receptor expression.

Calcium-sensing receptors (CaR)‡ are expressed in tissues critical to organ-ismal Ca2+ homeostasis, including the parathyroid gland, kidney, intestine, and bone (2). CaR is a G protein-coupled receptor of family C, which translates changes in plasma Ca2+ concentration into reciprocal changes in parathyroid hormone secretion from the parathyroid glands (2). Circulating parathyroid hormone in turn regulates renal Ca2+ excretion, intestinal uptake, and the balance of bone mineralization and resorption (2). Calcium handling disorders result from mutations in CaR. Familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT) result from loss-of-function mutations in CaR, leading to generalized resistance to extracellular calcium and an attendant increase in circulating levels of parathyroid hormone (3, 4). Autosomal dominant hypocalcemia (ADH) results from gain-of-function mutations in CaR, leading to hyper-responsiveness to extracellular calcium and decreased parathyroid hormone secretion (3, 4). Autoimmune responses directed against the extracellular domain of CaR can lead to acquired hyperparathyroidism, and autoimmune disorders with symptoms of FHH have been described (3). Finally, severe hyperparathyroidism can result from chronic dialysis of patients with end-stage renal disease (3).

The importance of CaR in regulation of mineral ion metabolism has led to a search for pharmacological modulators. Ligands that bind at the extracellular, agonist-binding domain and activate CaR include Ca2+‡, inorganic di- and trivalent cations plus organic polycations (5). CaR activation is also enhanced by amino acids including phenylalanine (5) or the peptide glutathione (6), which bind within the extracellular domain and synergize with extracellular Ca2+. Polycationic aminoglycoside antibiotics including neomycin, gentamycin, and tobramycin activate CaR in the presence of Ca2+‡ (5, 7), and may contribute to nephrotoxicity in some patients treated for Gram-negative infections (8). Finally, phenylalkylamine derivatives represent a group of clinically important allosteric drugs for treatment of disorders of bone and mineral metabolism, including osteoporosis and hyperparathyroidism (9). Allosteric agonists, including first generation NPS R-568, NPS R-467, AMG 073, and the first-in-class cinacalcet, licensed in Europe and United States for treatment of hyperparathyroidism in end-stage renal disease (10), increase CaR activation in the presence of Ca2+. Allosteric antagonists, including NPS 2143 and Calhex Helix; WT, wild type; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium.

* This work was supported by National Institutes of Health GM077563, the Weis Center for Research, and Syracuse University. The costs of publication of this article were defrayed in part by the payment of page charges. This work must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: CaR, calcium-sensing receptor; FHH, familial hypocalciuric hypercalcemia; ADH, autosomal dominant hypocalcemia; NSHPT, neonatal severe hyperparathyroidism; ECD, extracellular domain; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; WT, wild type; ER, extracellular receptor; TM, transmembrane domain; TMH, transmembrane helix; WT, wild type; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium.
231, antagonize the stimulatory effects of Ca\(^{2+}\) and are in clinical trials as a treatment for osteoporosis (11). These allosteric modulators bind CaR within the transmembrane heptahelical domain and either increase (calcimimetics) or decrease (calcilytics) the apparent affinity of CaR for Ca\(^{2+}\) (12, 13).

CaR undergoes dorfin-mediated ubiquitination and degradation via ERAD (endoplasmic reticulum-associated degradation) during biogenesis (1), but little is known about the quality control checkpoints. Pharmacological chaperones, including hydrophobic agonists and antagonists, can assist in G protein-coupled receptor folding, resulting in increased expression and plasma membrane localization of wild type (WT) or mutant receptors (14–16). In this report, we characterize a potential conformational or functional checkpoint affecting CaR stability during biogenesis: gain-of-function mutations or the allosteric agonist NPS R-568 stabilize CaR and increase CaR abundance, whereas loss-of-function mutations or the allosteric antagonist NPS 2143 destabilize CaR and increase degradation by ERAD. Treatment of loss-of-function mutants with NPS R-568 increases processing of the 130-kDa form to the mature 150-kDa form, resulting in increased plasma membrane localization and robust signaling in response to 5 mM Ca\(^{2+}\), suggesting that some CaR mutations cause a protein folding/processing defect (17, 18) that can be ameliorated by pharmacochaperones (18). These results suggest that long-term treatment with calcimimetics and calcilytics can regulate turnover of CaR, providing unique possibilities for interventions in Ca\(^{2+}\) handling diseases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human CaR, NPS R-568 ((R)-N-(3-methoxy-α-phenylethyl)-3-(2’-chlorophenyl)-1-propylamine hydrochloride), and NPS 2143 ((R)-N-(3-methoxy-α-phenylethyl)-3-(2’-chlorophenyl)-1-propylamine hydrochloride) were kind gifts from K. Seuwen (Novartis Pharma, AG).

**Plasmid Construction**—CaR with an amino-terminal FLAG epitope (FLAG-CaR) was generated as described (19). Point mutations in FLAG-CaR were generated by inverse PCR mutagenesis (20) with Pfu Ultra High Fidelity DNA polymerase (Stratagene) and verified by dideoxy-DNA sequencing (Weis Center Sequencing Facility). Primer sequences are available upon request.

**Cell Transfection**—HEK293 cells (American Tissue Culture Collection) were transiently transfected with Novafector (Venn Nova LLC) and cultured as described (1). Prior to assay, transfected cells were treated for 12 h with drugs including 10 μM MG132, 10 μM NPS R-568, or 10 μM NPS 2143, solubilized in 1% Me\(_2\)SO. 1% Me\(_2\)SO was also compared with no drug treatment, because Me\(_2\)SO has been shown to act as a chemical chaperone in some systems (18).

**Immunoprecipitation, SDS-PAGE, and Western Blotting**—Transiently transfected HEK293 cells were lysed, proteins were immunoprecipitated with anti-FLAG antibody (Sigma) and resolved by SDS-PAGE as described (1). Nitrocellulose membranes were probed with anti-CaR polyclonal antibody (LRG, 1:1000), and visualized by enhanced chemiluminescence (Super West Pico Chemiluminescent Substrate, Pierce), and quantitated as described (1).

**Enzyme-linked Immunoabsorption Assay (ELISA)**—Transiently transfected HEK293 cells in 96-well poly-L-lysine-coated plates were exposed to drugs as indicated in the figure legends, fixed, and developed with anti-FLAG horseradish peroxidase antibody (1:1000 dilution, 3 h) as described (21). Untransfected HEK293 cells were used to estimate background.

**Assay of Extracellular Signal-regulated Kinase (ERK1/2) Activation**—Transiently transfected HEK293 cells were incubated overnight in serum-free 0.5 mM Ca\(^{2+}\) DMEM plus drugs, as indicated in the figure legends, exposed to 0.5 or 5 mM Ca\(^{2+}\)

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**FIGURE 1. CaR functional mutants have differential sensitivity to the proteasomal inhibitor MG132. A.** HEK293 cells were transiently transfected with FLAG-CaR (WT), loss-of-function mutantFLAG-CaR(R795W), or gain-of-function mutant FLAG-CaR(A843E) cDNA and incubated with Me\(_2\)SO (−) or 10 μM MG132 (+) for 12 h prior to lysis. Lysates were immunoprecipitated (IP) with anti-FLAG antibody and Western blots probed with anti-CaR LRG antibody. B, HEK293 cells transfected with FLAG-CaR (WT) or loss-of-function mutants FLAG-CaR(R66C), FLAG-CaR(R185Q), FLAG-CaR(R680C), or FLAG-CaR(V817I) were treated and analyzed as described in A. C, HEK293 cells transfected with WT FLAG-CaR or gain-of-function mutants FLAG-CaR(F128L), FLAG-CaR(E191K), FLAG-CaR(Q681H), or FLAG-CaR(F788C) were treated and analyzed as in A. D and E, CaR protein in the presence of Me\(_2\)SO (black bars) or MG132 (gray bars) was quantified and normalized to the amount of WT CaR in Me\(_2\)SO. Graphs represent averaged results of six independent experiments (*, p < 0.05), for loss-of-function CaR mutants (D) or gain-of-function CaR mutants (E). IB, immunoblot.
Conformational Checkpoint in CaR Biogenesis

TABLE 1
Summary of the properties of functional CaR mutants in the present study

| Receptor mutant | Mutation location (41) | Functional phenotype | Associated human disease | Expression studies, response to Ca\textsuperscript{2+}o |
|-----------------|------------------------|----------------------|--------------------------|---------------------------------------------------|
| R66C            | ECD                    | Loss-of-function     | FHH/NSHPT                | No response (30)                                  |
| F128L           | ECD                    | Gain-of-function     | ADH                      | [EC\textsubscript{50}] (33)                       |
| R185Q           | ECD                    | Loss-of-function     | ADH                      | [EC\textsubscript{50}] (33)                       |
| E191K           | ECD                    | Gain-of-function     | ADH                      | [EC\textsubscript{50}] (33)                       |
| R680C           | TMH3                   | Loss-of-function     | FHH                      | [EC\textsubscript{50}] (33)                       |
| Q681H           | TMH3                   | Gain-of-function     | ADH                      | [EC\textsubscript{50}] (33)                       |
| F788C           | TMH5                   | Gain-of-function     | ADH                      | [EC\textsubscript{50}] (33)                       |
| R795W           | IC3                    | Loss-of-function     | FHH                      | [EC\textsubscript{50}] (33)                       |
| V817I           | TMH6                   | Loss-of-function     | ADH                      | [EC\textsubscript{50}] (33)                       |
| A843E           | TMH7                   | Gain-of-function     | ADH                      | [EC\textsubscript{50}] (33)                       |

* ND, not determined.

(10 min, 37 °C). ERK1/2 phosphorylation was analyzed as described (19).

RESULTS

CaR Mutants Have Differential Sensitivity to Proteasomal Degradation—CaR undergoes dorfin-mediated ubiquitination and degradation via the ERAD pathway (1). The molecular mechanisms underlying differential sorting of CaR to the Golgi complex and plasma membrane or ERAD pathway are unknown. To determine whether there is a conformational or functional checkpoint in CaR biogenesis, we used the sensitivity of CaR mutants to the proteasomal inhibitor MG132 as an indicator of degradation via ERAD (1). HEK293 cells transiently transfected with equivalent amounts of cDNA for FLAG-tagged WT CaR, the loss-of-function mutant R795W (22), or the gain-of-function mutant A843E (22), were treated without or with 10 μM MG132 for 12 h. Western blots of immunoprecipitated CaR revealed that MG132 increased the amount of WT CaR (173.8 ± 10.2% relative to Me\textsubscript{2}SO) (Fig. 1, A and D). MG132 similarly increased the amount of the loss-of-function mutant R795W (from 80.2 ± 7.0% (control) to 134.9 ± 6.0% (MG132)), but had no effect on the gain-of-function mutant A843E (117.5 ± 2.9% (control) versus 119.1 ± 2.7% (MG132)) (Fig. 1, A, D, and E). Mutant CaR abundance in the absence or presence of MG132 were normalized to the abundance of WT CaR in the absence of MG132 to illustrate baseline expression of the mutants relative to WT CaR and the effect of treatment with MG132. WT CaR and mutants were seen as doublets with molecular masses of 130/150 kDa in the monomeric and corresponding dimeric forms (Fig. 1A), indicating that all undergo glycosylation and dimerization (23). To determine whether the differential sensitivity to degradation is generalizable to additional CaR mutants, the effect of MG132 was quantified for loss-of-function CaR mutants identified in patients with FHH or NSHPT, having decreased sensitivity to extracellular Ca\textsuperscript{2+} (R66C, R185Q, R680C, and V817I) and gain-of-function CaR mutants from patients with ADH having increased sensitivity to extracellular Ca\textsuperscript{2+} (F128L, E191K, Q681H, and F788C) (Table 1) (22). All loss-of-function mutants exhibited decreased expression relative to WT CaR (summarized in Fig. 1D, black bars), and MG132 significantly increased the amount of each loss-of-function mutant (R66C, 44.7 ± 4.9% (control) to 98.1 ± 7.9% (MG132); R185Q, 62.4 ± 7.6% (control) to 114.6 ± 11.0% (MG132); R680C, 40.0 ± 8.0% (control) to 96.4 ± 5.5% (MG132); and V817I, 85.7 ± 13.0% (control) to 121.3 ± 10.0% (MG132)) (Fig. 1, B and D). Thus, for all loss-of-function mutants examined, treatment with MG132 increased abundance of the mutants to a level equal to or greater than the amount of WT CaR under control conditions. Treatment with MG132 also increased maturation of CaR loss-of-function mutants, as reflected in the abundance of the mature 150-kDa and corresponding dimeric forms.

In contrast, all gain-of-function mutants exhibited baseline abundance greater than WT CaR (summarized in Fig. 1E, black bars), and MG132 did not alter the abundance of gain-of-function mutants (normalized to the abundance of WT CaR in the absence of MG132: F128L, 148.5 ± 5.7 (control) to 152.8 ± 5.6% (MG132); E191K, 147.9 ± 5.7% (control) to 156.2 ± 3.7% (MG132); Q681H, 113.8 ± 9.3% (control) to 117.0 ± 9.1% (MG132); and F788C, 123.4 ± 8.2% (control) to 126.3 ± 6.0% (MG132)) (Fig. 1, C and E). These results demonstrate that gain-of-function CaR mutants are more stable than loss-of-function mutants, i.e. less subject to ERAD. The two classes of mutants are defined functionally by their decreased (loss-of-function) or increased (gain-of-function) signaling responses to extracellular Ca\textsuperscript{2+}. The free Ca\textsuperscript{2+} concentration in the ER is estimated to be in the range of 1–400 μM, depending upon the filling state of the stores (24), and glutathione (oxidized > reduced forms) is also present (25). The free concentrations of Ca\textsuperscript{2+} and glutathione (oxidized > reduced forms) is also present (25). The free concentrations of Ca\textsuperscript{2+} plus glutathione are sufficient to activate CaR (EC\textsubscript{50} for Ca\textsuperscript{2+} = 3 mM (3–5), but maximal activation of CaR is observed with 0.5 mM Ca\textsuperscript{2+} plus 10 μM GSH or GSSG (6)). We therefore hypothesize that there is a conformational or functional checkpoint in the ER quality control of CaR biogenesis, i.e. loss-of-function mutants that predominantly exist in inactive conformation(s) are less stable than gain-of-function mutants that can achieve active conformation(s) in the ER.

Allosteric Modulators Differentially Regulate the Stability of WT CaR—Mutations may alter CaR stability by a variety of potential mechanisms, so we sought to confirm the hypothesis using WT CaR. The allosteric modulators NPS R-568 and NPS 2143 are able to alter CaR activation in the presence of constant Ca\textsuperscript{2+} concentrations (reviewed in Ref. 26), so we determined
Allosteric Modulation of CaR Mutant Stability—To determine whether NPS R-568 was able to rescue the phenotype of the loss-of-function CaR mutant R795W, cells expressing WT CaR, R795W, or A843E were treated with NPS R-568 for 12 h, followed by immunoprecipitation and Western blotting. NPS R-568 increased WT CaR and R795W abundance, but had no effect on the gain-of-function mutant A843E (Fig. 3A). Conversely, NPS 2143 treatment decreased the amounts of WT CaR and the gain-of-function mutant A843E, but had no effect on the loss-of-function mutant R795W (Fig. 3B). These results suggest that allosteric modulators are able to stabilize distinct conformations of CaR mutants thereby affecting receptor stability. Importantly, NPS R-568 increased the protein amounts of all loss-of-function mutants (R66C, R186Q, R680C, and V817I, Fig. 4A), whereas it had no effect on levels of gain-of-function mutants (Fig. 4C). Stabilization of loss-of-function mutants led to the appearance (R680C or V817I) in glycosylated forms (Fig. 4A). In contrast, NPS 2143 decreased the amount of all gain-of-function mutants (Fig. 4D), whereas it had no effect on the loss-of-function mutants (Fig. 4B). Our results demonstrate that calcimimetic NPS R-568 can stabilize loss-of-function mutant CaR abundance.

Whether chronic treatment with these allosteric modulators could affect the abundance of WT CaR. NPS R-568 is an allosteric agonist that binds the transmembrane domain and acts synergistically with Ca\(^{2+}\) to activate CaR (12), whereas the allosteric antagonist NPS 2143 acts at the same site to antagonize Ca\(^{2+}\)-dependent CaR activation (12). HEK293 cells transiently expressing WT FLAG-CaR were incubated without drugs or with Me\(_2\)SO, MG132, NPS R-568, or NPS 2143 for 12 h, and subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting. Me\(_2\)SO (1%), the solvent for MG132 and the NPS compounds, had no effect on CaR abundance. NPS R-568 doubled the amount of CaR protein (200.5 ± 19.1%), comparable with the increase observed upon addition of MG132 (163.6 ± 12.1%), whereas NPS 2143 decreased CaR abundance (72.6 ± 4.6%) (Fig. 2A). To determine whether the effects of NPS R-568 and NPS 2143 on CaR abundance were the result of drug-mediated alterations in CaR susceptibility to ERAD, the effect of MG132 on CaR abundance in the presence of NPS R-568 or NPS 2143 was examined. MG132 had no effect on the amounts of WT FLAG-CaR in cells treated with NPS R-568 (101.2 ± 1.9% relative to control), whereas MG132 caused a significant increase in WT FLAG-CaR in cells treated with NPS 2143 (194.1 ± 4.9% relative to control) (Fig. 2B). The effects of the allosteric modulators were the result of specific binding to the transmembrane domain of CaR, because neither modulator affected the abundance of the CaR mutant E837I, which disrupts the allosteric binding site for both NPS R-568 and NPS 2143 (Fig. 2C) (12). These results imply that WT CaR is more sensitive to ERAD when inactive conformations are favored by either mutation (Fig. 1) or drugs (Fig. 2), and conversely, is stabilized by mutations or drugs that favor active conformation(s).
CaR mutants and increase the amount of mature receptor, whereas the calcilytic NPS 2143 destabilizes gain-of-function CaR mutants.

**Allosteric Modulators Influence CaR Cell Surface Expression and Function**—The maturely glycosylated 150-kDa form of CaR has been shown to be localized to the plasma membrane (27, 28), and thus allosteric modulator-mediated changes in the abundance of mature CaR should be reflected in surface localization and signaling. ELISA was used to evaluate the effects of allosteric modulators on CaR cell surface expression. HEK293 cells expressing WT FLAG-CaR were treated with Me2SO, MG132, NPS R-568, or NPS 2143 for 12 h prior to fixation with 4% paraformaldehyde (4 °C). MG132 (120.9 ± 4.9%) and NPS R-568 (151.4 ± 2.4%) increased plasma membrane-localized CaR, whereas NPS 2143 significantly decreased plasma membrane CaR (77.1 ± 5.9%) (Fig. 5B). These results are consistent with the effects of MG132, NPS R-568, and NPS 2143 on the amount of the 150-kDa form of CaR (Fig. 2A). Functional studies confirmed the increased plasma membrane localization of CaR. HEK293 cells expressing WT FLAG-CaR were incubated in DMEM containing 0.5 mM Ca2+ supplemented with Me2SO, MG132, NPS R-568, or NPS 2143 for 12 h prior to exposure to 5 mM Ca2+ for 10 min (37 °C). Under control conditions (Me2SO), 5 mM Ca2+ increased ERK1/2 phosphorylation (Fig. 5, A and C). After 12 h treatment with MG132, CaR activation resulted in slightly higher ERK1/2 phosphorylation compared to control conditions.
Conformational Checkpoint in CaR Biogenesis

FIGURE 5. Allosteric modulators differentially regulate cell surface expression and function of CaR. A, FLAG-CaR cDNA transfected HEK293 cells were incubated overnight in serum-free DMEM containing 0.5 mM Ca\textsuperscript{2+}, plus 

Me\textsubscript{2}SO, MG132 (10 \muM), NPS R-568 (10 \muM), or NPS 2143 (10 \muM) for 12 h prior to exposure to 0.5 or 5 mM Ca\textsuperscript{2+} for 10 min (37 °C). Phosphorylated (upper panel) and total ERK1/2 (lower panel) were identified by immunoblotting cell lysate with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies, respectively. B, FLAG-CaR expressing HEK293 cells in 96-well plates were incubated with Me\textsubscript{2}SO, MG132 (10 \muM), NPS R-568 (10 \muM), or NPS 2143 (10 \muM) for 12 h, 

and fixed with 4% paraformaldehyde (4 °C), and the ELISA experiment was processed as described under “Experimental Procedures.” Graph represents averaged results of six independent experiments (*, p < 0.05). C, experiments as in A were quantified, and the graph indicates Ca\textsuperscript{2+}-stimulated ERK1/2 phosphorylation (band intensity at 5 mM Ca\textsuperscript{2+} minus 0.5 mM Ca\textsuperscript{2+}) normalized to that observed in Me\textsubscript{2}SO (average of six independent experiments, *p < 0.05). D, to determine the effects of NPS 2143 on gain-of-function mutants surface expression, HEK293 cells expressing FLAG-CaR (WT), FLAG-CaR(R185Q), FLAG-CaR(R66C), FLAG-CaR(R185Q), FLAG-CaR(R680C), or FLAG-CaR(V817I) in 96-well plates were incubated with Me\textsubscript{2}SO or NPS R-568 (10 \muM) for 12 h, and fixed with 4% paraformaldehyde (4 °C), and as processed in B. The cell surface expression level of each mutant in the presence of either Me\textsubscript{2}SO (black bars) or NPS 2143 (gray bars) was normalized to the cell surface expression of FLAG-CaR (WT) in the presence of Me\textsubscript{2}SO. Graph represents averaged results of three independent experiments (*, p < 0.05). E, to determine the effects of NPS R-568 on loss-of-function mutant surface expression, HEK293 cells expressing FLAG-CaR (WT), FLAG-CaR(R795W), FLAG-CaR(R680C), or FLAG-CaR(R185Q) in 96-well plates were incubated with Me\textsubscript{2}SO or NPS R-568 (10 \muM) for 12 h, and fixed with 4% paraformaldehyde (4 °C), and as processed in B. The cell surface expression level of each mutant in the presence of either Me\textsubscript{2}SO (black bars) or NPS R-568 (gray bars) was normalized to the cell surface expression of FLAG-CaR (WT) in the presence of Me\textsubscript{2}SO. Graph represents averaged results of three independent experiments (*, p < 0.05). with Me\textsubscript{2}SO alone (117.8 ± 13.2%, Fig. 5, A and C), whereas overnight treatment with NPS R-568 caused a significant increase in CaR-mediated ERK1/2 phosphorylation (172.6 ± 21.6%). NPS 2143 decreased CaR-mediated ERK1/2 phosphorylation (50.64 ± 10.2%, Fig. 5, A and C). Acute exposure to allosteric modulators can directly affect CaR-mediated ERK1/2 phosphorylation (29). In these studies, we used a saturating concentration of Ca\textsuperscript{2+} (5 mM) to activate CaR to minimize acute modulation of CaR signaling by allosteric modulators. To confirm that acute exposure to the allosteric modulators had minimal effects on ERK1/2 phosphorylation under these conditions, we measured the response to 5 mM Ca\textsuperscript{2+} plus drugs (10 min, 37 °C) in cells cultured overnight in 0.5 mM Ca\textsuperscript{2+} serum-free media. When compared with responses in the presence of Me\textsubscript{2}SO, neither MG132 (10 \muM) nor NPS R-568 (10 \muM) altered ERK1/2 phosphorylation (102.8 ± 3.5 (n = 6) or 103.6 ± 3.0% (n = 6), respectively), whereas NPS 2143 (10 \muM) decreased ERK1/2 phosphorylation (78.2 ± 6.5% (n = 6)). In addition, acute incubation with allosteric modulators (10 min, 37 °C) had no effect on cell surface CaR abundance (data not shown). These results confirm that the dominant consequence of chronic (overnight) exposure to MG132 or allosteric modulators NPS R-568 and NPS 2143 is altered in plasma membrane CaR levels, which is directly reflected in CaR-mediated signaling. We next evaluated the effects of allosteric modulators on cell surface expression of CaR mutants. NPS 2143 decreased the cell surface expression of all gain-of-function mutants, consistent with the decrease in total protein expression (Fig. 4D), whereas NPS R-568 increased cell surface expression of all loss-of-function mutants except R66C (Fig. 5, D and E). Interestingly, NPS R-568 led to the appearance of the 150-kDa form (Fig. 4A) and significant cell surface expression of the loss-of-function mutant R680C, comparable with WT CaR in the absence of NPS R-568 (117.0 ± 11.4%, Fig. 5E). Functional studies were used to investigate whether NPS R-568-mediated rescue of plasma membrane expression of the loss-of-function mutants also rescued function. HEK293 cells expressing WT FLAG-CaR or loss-of-function mutants were incubated in DMEM containing 0.5 mM Ca\textsuperscript{2+} for 12 h, and fixed with 4% paraformaldehyde (4 °C), and the ELISA experiment was processed as described under “Experimental Procedures.” Graph represents averaged results of six independent experiments (*, p < 0.05).
or maturation problem during biogenesis, and not the result of an inability to transduce a signal at the plasma membrane upon binding of extracellular Ca\textsuperscript{2+}. Functional rescue was also observed for mutants R795W, R185Q, and V817I, which exhibited an increase in function proportional to the increase in plasma membrane expression, but not for R66C. Overall, these results suggest that some CaR loss-of-function mutants can be rescued by drugs that increase surface expression, whereas other may be insensitive to such rescue.

**DISCUSSION**

We recently identified the E3 ubiquitin ligase dorfin as a binding partner for the intracellular carboxyl terminus of CaR and demonstrated that dorfin participates in ER quality control during CaR biogenesis by catalyzing CaR ubiquitination, leading to proteasomal degradation (1). The checkpoints in the ER quality control mechanism for CaR remain to be investigated. Studies on CaR mutants that eliminate critical glycosylation sites or cysteine residues suggest that there are multiple structural checkpoints during the biogenesis of CaR, i.e. receptors with impaired glycosylation or disulfide bond formation are retained in the ER, resulting in reduced cell surface expression and/or total protein abundance (23, 30, 31). Biochemical studies on loss- or gain-of-function CaR mutants identified in patients suggest that signaling changes may result from differences in cell surface expression (30, 32, 33). In the present study, we asked whether there is a conformational or functional checkpoint in the biogenesis of CaR using several complementary strategies. First, we used a set of loss- and gain-of-function CaR mutants with documented alterations in the apparent affinity for extracellular Ca\textsuperscript{2+} as estimated from their ability to activate cell signaling (Table 1). We reasoned that these mutants would be differentially affected by the Ca\textsuperscript{2+}/glutathione concentrations in the ER stores, potentially affecting their stability, i.e. susceptibility to proteasomal degradation. Five loss-of-function mutations (R66C, R185Q, R680C, R795W, and V817I) and five gain-of-function mutations (F128L, E191K, Q681H, F788C, and A843E) that are located in different domains of the receptor (22) were tested. R66C, R185Q, F128L, and E191K are in the extracellular domain, R680C and Q681H are in transmembrane helix (TMH) 3, R795W is in the third intracellular loop, F788C and V817I are in TMH 6, and A843E is in TMH 7. Despite the disparate locations of these mutations, the abundance of all loss-of-function mutants was significantly increased by MG132, suggesting that proteasomal degradation regulates their turnover, presumably via ERAD (1). In contrast, all gain-of-function mutants were insensitive to MG132 treatment, implying higher stability of these mutants. The loss- and gain-of-function mutants studied were defined functionally, both in patients and in heterologous expression systems, by their differential sensitivity to extracellular Ca\textsuperscript{2+} (Table 1), i.e. the concentration of extracellular Ca\textsuperscript{2+} required to activate cell signaling. The luminal Ca\textsuperscript{2+} and/or glutathione concentrations in the ER are sufficient to promote WT CaR activation (EC\textsubscript{50} \approx 3 mM (3–5) or 0.5 mM Ca\textsuperscript{2+} plus 10 \mu M GSH/GSSG yields maximal activation (6)), but may be insufficient to activate loss-of-function mutants that have reduced sensitivity to Ca\textsuperscript{2+}. Therefore, these results support the hypothesis that CaR conformation or activation serves as a checkpoint in the biosynthetic process: receptors with “active” conformation(s) are more stable than those with “inactive” conformation(s), resulting in differential sensitivity to ERAD.

As a second, complementary approach to determine whether a conformational or functional checkpoint regulated CaR biogenesis, we altered the activation state of WT CaR at ambient ER lumenal Ca\textsuperscript{2+} and glutathione concentrations with the allosteric modulators NPS R-568 and NPS 2143 (34). If a conformational or functional checkpoint regulates CaR stability, increasing or decreasing the apparent affinity of WT CaR for extracellular Ca\textsuperscript{2+} with NPS R-568 or NPS 2143, respectively, should modulate WT CaR abundance. Indeed, prolonged (12 h) incubation with NPS R-568 increased the amount of CaR, whereas NPS 2143 decreased the amount of CaR, strongly suggesting that conformation affects CaR stability. Whereas both MG132 and NPS R-568 increase the abundance of WT CaR and loss-of-function CaR mutants, they do so by distinct but related mechanisms. MG132 blocks degradation of ubiquitinated CaR (1) and thus WT CaR or loss-of-function mutants targeted for degradation are stabilized by MG132. NPS R-568, by favoring active conformational states, reduces WT CaR or loss-of-function CaR mutant ubiquitination, and hence degradation. Support for these related mechanisms comes from the differential sensitivity of WT CaR to MG132 in the presence of the modulators: NPS R-568 renders CaR insensitive to MG132, whereas the amount of CaR is increased by MG132 treatment in the presence of NPS 2143. Overall, these results strongly suggest that CaR conformation defines CaR stability by serving as a checkpoint during receptor biogenesis, and provide a potential mechanism for CaR protein up-regulation in parathyroid glands of rats treated chronically with NPS R-568 (26, 35).

The current studies made use of the well characterized CaR allosteric modulators, NPS R-568 and NPS 2143. NPS R-568
has been extensively studied \textit{in vitro}, in rats, and in clinical studies of patients with primary or secondary hyperparathyroidism, or with parathyroid carcinomas (recently reviewed in Ref. 26). The binding site for NPS R-568, which overlaps that of NPS 2143, has been identified within the transmembrane domain of human CaR (12). Whereas it remains to be determined whether other allosteric modulators of CaR also have effects on CaR stability, it is likely because they are hydrophobic (providing access to intracellular compartments), they have some contact residues within the binding site in common, particularly Glu-837 (Calhex 231 (36) and Calindol (37)), and they modulate the CaR conformational state at constant Ca\textsuperscript{2+} concentration. Elucidation of the long-term effects of allosteric modulators on the protein stability of CaR has important pharmacological and clinical implications. Whereas allosteric modulators potentiate or antagonize CaR activity by binding to plasma membrane-localized mature receptors (12, 13, 34), the current results strongly suggest that these compounds can bind to immature forms of CaR and affect their stability. Chemical chaperones, including Me\textsubscript{2}SO, glycerol, 4-phenylbutyric acid, and trimethylamine N-oxide, and more specific pharmacological chaperones, including substrate analogs, agonists, and antagonists, have been shown to promote folding of receptors, ion channels, and enzymes (18). G protein-coupled receptors, including the V2 vasopressin receptor, the gonadotropin-releasing hormone receptor, and the \delta-oid receptor, are stabilized by binding of either agonists or antagonists (18), suggesting that ligand binding to the receptor is sufficient for chaperone-mediated rescue, perhaps by stabilizing the hydrophobic core of the transmembrane domain (15). Distinct from these reports, CaR stabilization is not conferred merely by ligand binding, because NPS R-568 and NPS 2143 have overlapping binding sites within the transmembrane domain of CaR, anchored by Glu-837 at the start of TMH 7 (12). Rather, stabilization of WT CaR or mutants requires binding of the allosteric agonist NPS R-568, which favors active receptor conformations, particularly in the presence of ER luminal Ca\textsuperscript{2+} and glutathione, whereas receptor destabilization and degradation is promoted by the allosteric antagonist NPS 2143. The allosteric modulators of CaR affect stability exclusively by inducing conformational changes in the receptor, because NPS 2143 does not further decrease the stability of loss-of-function mutants and NPS R-568 cannot further stabilize gain-of-function mutants, and a CaR mutant that cannot bind the allosteric modulators is unaffected by drug treatment. There is precedence for a functional checkpoint in ER quality control for inotropic glutamate receptors: mutations that eliminate binding to glutamate or kainate prevent trafficking to the plasma membrane, although protein abundance was not affected (38). The metabotropic glutamate receptor type 2 (mGluR2) has also been shown to bind drugs in intracellular compartments, inducing Ca\textsuperscript{2+} signals in the nucleoplasm that can be blocked by hydrophobic receptor antagonists (39). The mechanisms responsible for conformation-sensitive degradation of the CaR during biogenesis remain to be explored. We have proposed a model in which CaR conformation defines stability during biogenesis, but we cannot directly assess CaR conformation under these conditions. Rather, we infer that either mutation or drugs favor certain receptor conformations, and that this leads to either distinct protein-protein interactions or formation of nascent signaling complexes that may shield CaR from dorfim-mediated ubiquitination (1) or assist in forward trafficking of the receptor. Recent evidence suggests that assembly of G protein-coupled receptor signaling complexes may occur in the ER (40), and such complexes may function in some signaling capacity (39). Whether CaR transduces signals within intracellular compartments remains to be determined. We have previously shown that filamin A can be coimmunoprecipitated with CaR in the presence of activating concentrations of extracellular Ca\textsuperscript{2+}, and that filamin A stabilizes CaR protein expression (19). It remains to be determined whether CaR and filamin A interact at the ER when CaR achieves an active conformation thereby stabilizing CaR against ERAD.

The rescue of loss-of-function mutants, particularly R680C, by NPS R-568, restoring both plasma membrane expression and attendant function, suggests that a class of CaR mutations produce a protein folding disorder (18) that can be ameliorated by the allosteric agonist. Four of the five loss-of-function mutants (R185Q, R680C, R795W, and V817I) were similarly rescued, exhibiting both increased plasma membrane expression and function. Notably, R66C was not, although treatment with MG132 or NPS R-568 increased the abundance of this mutant, there was no attendant increase in the 150-kDa mature form or function (Figs. 1, B and D, 5E, and 6). Interestingly, this mutant was able to dimerize (Fig. 1B and Ref. 30) and has been previously shown to accumulate in a perinuclear ER compartment in HEK293 cells (30). The CaR(R66C) mutant therefore undergoes dimerization at the ER, but is unable to successfully navigate the subsequent conformational checkpoint, even in the presence of NPS R-568. Characterization of additional CaR loss-of-function mutants should allow identification of critical checkpoints and a determination of their order of engagement during CaR biogenesis. Overall, our results support the existence of a conformational or functional checkpoint in the biogenesis of CaR and suggest that some CaR mutations can produce a protein folding disorder that can be rescued by NPS R-568. Furthermore, the present results suggest that allosteric modulators of CaR may differentially regulate the function of plasma membrane-localized CaR by regulating receptor turnover.

Acknowledgments—We thank Dr. Klaus Seuwen (Novartis Pharma, AG) for providing human CaR, NPS R-568, and NPS 2143 and Drs. Larry Rothblum and Gina Visser Smit for helpful comments.

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