The extracellular calcium (Ca\(^{2+}\))-sensing receptor (CaR) can be potentiated by allosteric activators including calcimimetics and L-amino acids. In this study, we found that many mutations had differential effects on the functional modulation of the CaR by these two allosteric activators, supporting the idea that these modulators act through distinct sites. 10 mM L-phenylalanine and 1 \(\mu\)M NPS R-467, submaximal doses of the two agents, each elicited similar modulation of R185Q. However, there are different relative potencies for these two modulators with some receptors being more responsive to L-phenylalanine and others being more responsive to NPS R-467. The responsiveness of the CaR to Ca\(^{2+}\) appears to be essential to observe the potentiating action of L-phenylalanine but not of NPS R-467 on the receptor. NPS R-467 reduces the Hill coefficients of the wild-type as well as mutant receptors, suggesting that engagement of all Ca\(^{2+}\) binding sites is not required when the receptor is activated by NPS R-467. In contrast, L-phenylalanine has little effect on the Hill coefficients of mutant receptors. The two-site model is further supported by the observation that these two classes of modulators exert a synergistic effect on CaRs with inactivating mutations that are responsive to both modulators.

The extracellular calcium (Ca\(^{2+}\))-sensing receptor (CaR) plays a key role in mineral ion homeostasis by sensing small perturbations in the level of Ca\(^{2+}\) and modulating the functions of the parathyroid and the kidney so as to restore Ca\(^{2+}\) to its normal level. The CaR has been identified as an important therapeutic target for disorders of systemic Ca\(^{2+}\) homeostasis such as primary and secondary hyperparathyroidism. Earlier studies have documented that the CaR can be activated allosterically by L-amino acids and the phenylalkylamine calcimimetics in the presence of Ca\(^{2+}\), at millimolar levels. In turn, these allosteric modulators stereoselectively enhance the sensitivity of the CaR to its polycationic agonists such as Ca\(^{2+}\) and spermine. It has been documented that calcimimetics act through the transmembrane domain of the receptor (3).

The CaR is a G protein-coupled receptor in the same subfamily C as the metabotropic glutamate receptors (mGluRs). The CaR can be activated allosterically by L-amino acids and the phenylalkylamine calcimimetics in the presence of Ca\(^{2+}\), at millimolar levels. In turn, these allosteric modulators stereoselectively enhance the sensitivity of the CaR to its polycationic agonists such as Ca\(^{2+}\) and spermine. It has been documented that calcimimetics act through the transmembrane domain of the receptor (3).

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0.1% dextrose and then washed once with a bath solution (20 mM HEPES, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 0.1% dextrose, and 0.1% bovine serum albumin) at 37 °C for 20 min. The coverslips were then placed diagonally in a thermostated cuvette containing the bath solution using a modification of the technique employed previously in this laboratory (7). The L-phenylalalanine and/or NPS R-467 were added into the bath solution and preincubated for 50 s. Concentrated Ca²⁺ was added stepwise to an experimental solution containing 0.5 mM Ca²⁺ to achieve the following concentrations: 1.5, 2.5, 3.5, 4.5, 5.5, 10, 15, 20, 30, 40, and 50 mM. Excitation monochrometers were centered at 340 and 380 nm, and emission light was collected at 510 ± 40 nm through a wide band emission filter. The 340/380 excitation ratio of emitted light was used to estimate Ca²⁺, as described previously (7).

Statistics—The activities of the wild-type and mutant CaRs were determined in response to increasing concentrations of Ca²⁺, in the presence or absence of modulators. The mean responses at various concentrations of Ca²⁺ were calculated from individual experiments and were expressed with the standard error of the mean (± S.E.) as the index of dispersion. A comparison of responses of various receptors at 50 mM Ca²⁺ was performed using ANOVA or Duncan’s multiple comparison test (p ≤ 0.05) (8). Each of the experiments presented in the results was performed at least three times. To calculate the EC₅₀, the maximal response, and the Hill coefficient, we used the MacFitCurve program and the equation (X) = α[X₁(1 + X₂)] where “α” is a variant as a function of X, “PXY” is the response at a given concentration with agonist (X), “α” is maximal response, “α” is EC₅₀, and “h” is Hill coefficient to fit the experimental data.

RESULTS

We compared the modulatory effects of a phenylalkylamine calcimimetic and a l-amino acid on the wild-type and various mutant CaRs. Our initial studies examined the effects of 1 μM NPS R-467 and 10 mM phenylalanine on the wild-type receptor. NPS R-467 decreased the EC₅₀ value of the wild-type CaR by 39%, whereas l-phenylalanine decreased it by 16% (Fig. 1A and Table I). However, NPS R-467 markedly decreased the maximal responses elicited by high concentrations of Ca²⁺ (Table I). Thus, the extent of receptor activation in the presence of 1 μM NPS R-467 or 10 mM l-phenylalanine was similar at physiological concentrations of Ca²⁺. Unlike phenylalanine, NPS R-467 also significantly reduced the Hill coefficient of the wild-type receptor (Table I), suggesting that the requirement for Ca²⁺ binding is different for phenylalanine and NPS R-467.

Similar to phenylalanine as demonstrated by Zhang et al. (9), NPS R-467 significantly increased the Ca²⁺-elicited maximal responses of several mutant receptors. For instance, NPS R-467 and l-phenylalanine each increased the maximal response of R185Q to a similar extent (~1.4-fold (Fig. 1B). NPS R-467 and l-phenylalanine also substantially decreased the EC₅₀ value of R185Q to a greater extent than that seen for the wild-type CaR. There appears to be a limit to the positive activators in the presence of either l-phenylalanine or NPS R-467, the EC₅₀ values for these three mutant receptors were also significantly lowered (Table I). Therefore, the responsiveness of the mutant receptors to l-phenylalanine and NPS R-467 were not uniformly affected by mutations, strongly suggesting that these two modulators act through distinct binding sites.

The mutant receptors G143E, R795W, S169A/S170A/S171A and also showed no potentiation by l-phenylalanine. In contrast, NPS R-467 enhanced the responses of each of these mutant receptors to Ca²⁺ (Fig. 4). G143E is a special case where there was no activation by Ca²⁺ unless NPS R-467 was also present, indicating that the responsiveness of the CaR to agonist stimulation is not essential for its modulation by NPS R-467 and that these two allosteric activators act on the receptor through different mechanisms.

Heterodimeric receptors were also affected differently by
**Table I**

EC$_{50}$ values and Hill coefficients predicted using Hill equation for the wild-type and mutant CaRs

| Mutants | Response$^a$ at 50 mM Ca$^{2+}$ | EC$_{50}$ [Ca$^{2+}$]$^b$ | Hill coefficient$^b$ |
|---------|----------------|----------------|-------------------|
|         | Control | 1 µM R-467 | Control | 10 mM Phe | 1 µM R-467 | Control | 10 mM Phe | 1 µM R-467 |
| WT      | 100 ± 2.9 (21) | 101.9 ± 4.6 (12) | 82.1 ± 5.1 (10)* | 2.9 ± 0.1 | 2.6 ± 0.1 | 1.9 ± 0.1 | 2.99 ± 0.08 | 2.74 ± 0.09 | 2.38 ± 0.26 |
| Y218S   | 20.5 ± 2.2 (5)  | 47.1 ± 6.7 (7)* | 60.4 ± 6.2 (5)* | 31.7 ± 2.7 | 27.9 ± 0.9 | 23.1 ± 0.6 | 2.81 ± 0.08 | 1.50 ± 0.03 |
| E297K   | 12.7 ± 1.4 (3)  | 45.1 ± 4.6 (3)* | 28.4 ± 2.1 (5)* | 30.1 ± 4.4 | 22.4 ± 0.6 | 22.2 ± 0.8 | — | — | — |
| R185Q   | 51.2 ± 7.0 (3)  | 71.8 ± 9.6 (3)  | 69.7 ± 1.5 (3)  | 20.5 ± 0.4 | 12.3 ± 0.3 | 10.8 ± 0.3 | 2.69 ± 0.12 | 2.69 ± 0.12 | — |
| S147A   | 45.4 ± 1.8 (7)  | 81.6 ± 1.2 (5)* | 57.1 ± 2.6 (5)* | 18.0 ± 0.4 | 10.0 ± 0.2 | 9.7 ± 0.3 | 2.74 ± 0.12 | 2.77 ± 0.12 | 2.48 ± 0.14 |
| S170A   | 34.6 ± 3.3 (5)  | 54.9 ± 6.3 (6)* | 83.6 ± 7.1 (3)* | 23.1 ± 0.5 | 23.3 ± 0.8 | 15.7 ± 0.1 | 2.73 ± 0.11 | 2.84 ± 0.17 | 2.20 ± 0.02 |
| D190A   | 42.2 ± 7.5 (4)  | 71.8 ± 9.4 (4)* | 55.8 ± 9.3 (5)  | 22.1 ± 0.4 | 13.9 ± 0.3 | 12.7 ± 0.5 | 3.11 ± 0.12 | 2.78 ± 0.13 | 2.17 ± 0.12 |
| G143E   | No response   | No response   | 26.3 ± 2.3 (3)  | — | — | — | — | — | — |
| R795W   | 13.8 ± 2.0 (3)  | 17.0 ± 1.7 (5)  | 24.5 ± 2.5 (5)* | 11.0 ± 0.7 | 11.2 ± 0.6 | 5.4 ± 0.2 | 2.69 ± 0.36 | 2.83 ± 0.32 | 2.60 ± 0.21 |
| Triple  | 14.7 ± 2.6 (6)  | 11.4 ± 4.3 (7)  | 60.1 ± 4.4 (3)* | 17.9 ± 1.0 | 17.0 ± 0.5 | 16.1 ± 0.3 | 2.54 ± 0.36 | 1.87 ± 0.20 | 2.20 ± 0.07 |
| G143E&A877Stop$^d$ | 57.8 ± 1.8 (6)  | 66.8 ± 3.4 (6)* | 70.1 ± 3.5 (5)* | 20.9 ± 0.8 | 19.0 ± 0.5 | 8.1 ± 0.3 | 2.56 ± 0.16 | 2.78 ± 0.15 | 1.79 ± 0.07 |
| Triple&A877Stop$^d$ | 67.2 ± 8.0 (3)  | 67.5 ± 3.7 (3)  | 72.4 ± 3.4 (3)  | 16.7 ± 0.3 | 15.5 ± 0.4 | 6.9 ± 0.2 | 2.13 ± 0.06 | 2.23 ± 0.08 | 1.88 ± 0.07 |
| A877Stop | No response   | No response   | No response   | — | — | — | — | — | — |

$^a$ Values are means ± S.E., which are normalized to the response of the wild-type receptor at 50 mM Ca$^{2+}$. The number of the experiments is indicated in the parentheses. $^b$ values are significant different from the values of controls ($p \leq 0.05$).

$^c$ EC$_{50}$[Ca$^{2+}$], and Hill coefficient are fit parameters using the MacCurveFit program.

$^d$ Co-transfection of two mutant CaRs. For instance, G143E&A877Stop represents co-transfection of mutant receptors G143E and A877Stop.

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**Figure 2**

**A. S170A**

**B. Y218S**

**Y218A and Y218F Mutations in CaR Mutants**

These two types of mutants were in the same cases. For instance, Y218A and Y218F mutations were analyzed in S170A and S171A, respectively.

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**Synergistic Activation of the CaR**

S.E., which are normalized to the response of the wild-type receptor at 50 mM Ca$^{2+}$. The number of the experiments is indicated in the parentheses.
The EC50 was reduced by nearly half (18–11 mM) in the presence of either 30 μM NPS R-467 or 30 mM L-phenylalanine, whereas the presence of both agents resulted in a further reduction in the EC50 to nearly 25% of its original level (18–5 mM). As shown in Fig. 6, 30 μM NPS R-467 and 30 mM L-phenylalanine were the maximal doses for these modulators; thus, doubling the concentration of either modulator had no further effect on receptor activity. Consistent with the lack of L-phenylalanine modulation of the mutant CaRs, G143E and S169A/S170A/S171A, synergistic effects were not observed (Fig. 6B). The EC50 was reduced by nearly half (18–11 mM) in the presence of either 30 μM NPS R-467 or 30 mM L-phenylalanine, whereas the presence of both agents resulted in a further reduction in the EC50 to nearly 25% of its original level (18–5 mM). As shown in Fig. 6, 30 μM NPS R-467 and 30 mM L-phenylalanine were the maximal doses for these modulators; thus, doubling the concentration of either modulator had no further effect on receptor activity. Consistent with the lack of L-phenylalanine modulation of the mutant CaRs, G143E and S169A/S170A/S171A, synergistic effects were not observed.
with the mutant receptors G143E (Fig. 6C) and S169A/S170A/S171A (data not shown).

**DISCUSSION**

In this study, we compared the modulatory effects of NPS R-467 and L-phenylalanine, two allosteric activators of the CaR. Both compounds were found to potentiate the activation of the wild-type receptor as well as many mutant receptors with inactivating mutations. With the wild-type receptor, the primary effect observed with these modulators is a decrease in the EC₅₀ for activation of the CaR by Ca²⁺. Most mutant receptors also showed a decrease in EC₅₀. In addition, many of these receptors demonstrated increases in their maximal responses to agonist activation, which was not found in the wild-type CaR. Interestingly, NPS R-467 consistently decreased the EC₅₀ for the mutant receptors, whereas L-phenylalanine often showed little or no effect on EC₅₀ despite producing significant

**FIG. 5.** Allosteric modulation of heterodimeric receptors formed in cells co-transfected with G143E and A877Stop or S169A/S170A/S171A and A877Stop. In HEK293 cells, G143E (A) or S169A/S170A/S171A (B) was co-transfected with A877Stop. Ca²⁺-evoked Ca²⁺ responses were measured as described in Fig. 1. Before the additions of Ca²⁺, the cells were not exposed (circle), exposed to 1 μM NPS R-467 (square), or exposed to 10 mM Phe (triangle). Responses are normalized to the maximal cumulative Ca²⁺ responses of the wild-type receptor in untreated cells. Each point is the mean value of the number of measurements indicated in Table I. Standard errors of the mean (±S.E.) are indicated with vertical bars through each point. Some error bars are smaller than the symbol. The data were fitted using MacCurveFit as described under "Experimental Procedures."

**FIG. 6.** Synergistic effects of NPS R-467 and L-phenylalanine on the wild-type and mutant receptors. HEK293 cells were transfected with the wild type (A), mutant receptor E297K (B), or G143E (C). Ca²⁺-evoked Ca²⁺ responses were measured in the presence of NPS R-467 (square) or L-phenylalanine (triangle) or the presence (filled circle) or absence (open circle) of both modulators. The open and filled squares represent two concentrations of NPS R-467, 30 and 60 μM, respectively. The open and filled triangles represent two concentrations of L-phenylalanine, 30 and 60 mM, respectively. All of the responses are normalized to the maximal cumulative Ca²⁺ responses of the wild-type receptor in non-treated cells. Each point is the mean value of 3–15 measurements. Means ± S.E. are indicated with vertical bars through each point. Some error bars are smaller than the symbol. The data were fitted using MacCurveFit as described under “Experimental Procedures.”
increases in the maximal responses of many mutant receptors. Another difference between NPS R-467 and L-phenylalanine is that NPS R-467 significantly decreased the maximal response of the wild-type receptor, whereas L-phenylalanine had no effect on this parameter in the wild-type receptor. The presence of the physiological polycationic CaR agonist Ca$^{2+}$ is essential for the actions of both NPS R-467 and L-phenylalanine on the wild-type and mutant receptors.

We chose 1 μM NPS R-467 and 10 mM L-phenylalanine as our experimental doses for the two modulators because these concentrations gave submaximal but substantial effects on the wild-type CaR. Using these two doses, we found that the efficacies of the two modulators were quite different depending on the mutant CaR that was tested. In most cases, NPS R-467 gave more pronounced changes in maximal response and EC$_{50}$ than did L-phenylalanine. In fact, there were several mutant CaRs that were responsive to NPS R-467 but not to L-phenylalanine. The most obvious exception to this observation is the wild-type CaR, which shows an unexplained decline in its maximal activity in the presence of NPS R-467. In addition, there were also several mutants that appeared to be more responsive to L-phenylalanine such as S147A and E297K. No mutant receptors were found to be responsive to L-phenylalanine but were found to be insensitive to NPS R-467. This difference in the efficacies of NPS R-467 and L-phenylalanine with mutant receptors suggests separate sites of action for these two allosteric modulators. Corroborating this point was the synergistic behavior of NPS R-467 and L-phenylalanine. Each modulator had additional potentiating effects on some mutant receptors in the presence of a maximal concentration of the other modulator.

NPS R-467 can be effective with mutant CaRs that are not normally responsive to Ca$^{2+}$, For example, the responsiveness of G143E is restored to 26% of that of the wild-type receptor in the presence of 1 μM NPS R-467, whereas the mutant receptor is completely inactive in the absence of NPS R-467 (see Fig. 4). In contrast, L-phenylalanine appears to need some degree of Ca$^{2+}$ responsiveness to positively modulate the activity of CaR as suggested by the lack of positive modulation of G143E and G549R by L-phenylalanine. The difference may be attributed to the mechanism of action of NPS R-467, which may allow it to magnify subthreshold activation of the CaR.

L-Phenylalanine had little effect on Hill coefficients of the wild-type and mutant CaRs, whereas NPS R-467 often had a negative impact on the Hill coefficients of the wild-type and some mutant receptors (Table I). These findings suggest that the action of NPS R-467 appears to be less dependent on the Ca$^{2+}$ binding sites. It is probable that the interaction of the CaR with NPS R-467 increases the affinity of the receptor for its cognate G proteins or enhances signal transduction from the head of the CaR to its intracellular domains. When taken together with previous studies on the probable binding sites of NPS R-467 and L-phenylalanine, our data are consistent with the findings that NPS R-467 acts at the transmembrane region of the CaR (3).

In conclusion, these two types of allosteric modulators act on the receptor not only through distinct sites but also through distinct mechanisms. Our findings provide a solid base for developing novel therapeutic agents that interact with the CaR with high affinities through the amino acid binding site. In addition, the synergistic effect of these two types of allosteric modulators on the CaR potentially provides a new therapeutic approach to the management of severe hypercalcemia associated with various types of hyperparathyroidism.

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