The Venus Fly Trap Domain of the Extracellular Ca\textsuperscript{2+}-sensing Receptor Is Required for L-Amino Acid Sensing

Hee-Chang Mun\textsuperscript{‡,}, Alison H. Franks\textsuperscript{‡,}, Emma L. Culverston\textsuperscript{‡,}, Karen Krupcho\textsuperscript{‡,}, Edward F. Nemeth\textsuperscript{‡}, and Arthur D. Conigrave\textsuperscript{‡‡}

From the \textsuperscript{‡}School of Molecular and Microbial Biosciences, University of Sydney, NSW 2006, Australia and \textsuperscript{‡‡}NPS Pharmaceuticals, Salt Lake City, Utah 84108

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We previously demonstrated that the human calcium-sensing receptor (CaR)\textsuperscript{1} is allosterically activated by L-amino acids (Conigrave, A. D., Quinn, S. J., and Brown, E. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4814–4819). However, the domain-based location of amino acid binding has been uncertain. We now show that the Venus Fly Trap (VFT) domain of CaR, but none of its other major domains, is required for amino acid sensing. Several constructs were informative when expressed in HEK293 cells. First, the wild-type CaR exhibited allosteric activation by L-amino acids as previously observed. Second, two CaR-mGlu chimeric receptor constructs that retained the VFT domain of CaR, one containing the extracellular Cys-rich region of CaR and the other containing the Cys-rich region of the rat metabotropic glutamate type-1 (mGlu-1) receptor, together with the rat mGlu-1 transmembrane region and C-terminal tail, retained amino acid sensing. Third, a CaR lacking residues 1–599 of the N-terminal extracellular head but retaining an intact CaR transmembrane region and a functional but truncated C terminus (headless-T903 CaR) failed to respond to L-amino acids but retained responsiveness to the type-II calcimimetic NPS R-467. Finally, a T903 CaR control that retained an intact N terminus also retained L-amino acid sensing. Taken together, the data indicate that the VFT domain of CaR is necessary for L-amino acid sensing and are consistent with the hypothesis that the VFT domain is the site of L-amino acid binding. The findings support the concept that the mGlu-1 amino acid binding site for L-glutamate is conserved as an L-amino acid binding site in its homolog, the CaR.

The extracellular Ca\textsuperscript{2+}-sensing receptor (CaR)\textsuperscript{1} plays a key role in the regulation of whole body calcium metabolism. In keeping with this, the CaR-null mouse exhibits loss of feedback control of parathyroid hormone secretion, hyperparathyroidism, and a metabolic bone disease (2). Furthermore, inactivating and activating mutations of the receptor in humans have been shown to induce various disorders of calcium metabolism (for review, see Ref. 3).

Although the CaR is a key molecular regulator of whole body metabolism, it presents something of a conundrum. It is widely expressed in mammalian tissues, including tissues such as the brain, that are not clearly involved in calcium metabolism. Furthermore, its closest relatives in molecular terms are members of sub-group C of the G protein-coupled receptors, receptors for specific amino acids such as L-glutamate (mGlu) and glutamate analogs, e.g. GABA. The large extracellular heads of these receptors are related to nutrient-sensing, bacterial periplasmic-binding proteins (4). The finding that the CaR is allosterically activated by a broad spectrum of L-amino acids, including aromatics such as L-Phe and L-Trp and aliphatic and polar amino acids such as L-Ala and L-Ser, has the effect of drawing it closer functionally to other members of subgroup C (1). However, the site of amino acid binding has been unclear. A site-directed mutagenesis study implicated a role for a triple serine motif (Ser-169/Ser-170/Ser-171) in the VFT domain (5). Furthermore, in an analysis of CaR mutants, differential effects of the type-II calcimimetic NPS R-467, whose binding site lies in the transmembrane region of the receptor, and the aromatic amino acid L-Phe indicated that their binding sites were distinct (6).

In the current study, we have evaluated the domain-based requirements for amino acid sensing using chimeric receptors devised by domain swapping between two receptor homologs, the human CaR, which responds to Ca\textsuperscript{2+} and aromatic and aliphatic amino acids, and the rat mGlu-1, which responds to L-glutamate. Functional analysis of three key chimeric receptor constructs (listed as VFT/Cys-rich region/transmembrane domain+C-tail), including CaR/CaR/mGlu-1, CaR/mGlu-1/mGlu-1, and T903 CaR constructs together with two controls, the wild-type CaR and T903 CaR, indicates that the VFT domain alone is required for amino acid sensing. Thus, the VFT domain of CaR is the likely site of L-amino acid binding.

EXPERIMENTAL PROCEDURES

Materials—pcDNA3.1(+)(Invitrogen) containing the wild-type human calcium-sensing receptor (cassette version, Ref. 7, was the kind gift of Dr. Mei Bai and Professor Edward Brown (Endocrine-Hypertension Division, Brigham and Womens Hospital, Boston, MA). pBluescript containing the wild-type rat mGluR1 receptor used in the synthesis of the hCaR/mGlu-1/mGlu-1 construct was generously provided by Professor S. Nakanishi (Kyoto University, Japan). DNA ligase and restriction enzymes BspE1, NotI, SpeI, XbaI, XhoI, and ApaI were obtained from New England Biolabs. PCR reagents were obtained from Invitrogen. Site-directed mutagenesis was performed using QuikChange\textsuperscript{TM} kits (Stratagene, La Jolla, CA). pGEM T-Easy (Promega) was used to clone the "headless" PCR product.

Construction of hCaR, Rat mGlu-1 Chimeric Receptors—The two chimeric constructs studied (in the format VFT domain/Cys-rich region/transmembrane region+C-tail) were CaR/CaR/mGlu-1 and CaR/mGlu-1/mGlu-1. A CaR/CaR/mGlu-1 chimera encoded a protein containing the VFT and Cys-rich regions of hCaR (residues 1–589) fused to the transmembrane region plus cytoplasmic tail of the rat mGlu-1 receptor. The hCaR/mGlu-1/mGlu-1 construct was generously provided by Professor S. Nakanishi (Kyoto University, Japan). DNA ligase and restriction enzymes BspE1, NotI, SpeI, XbaI, XhoI, and ApaI were obtained from New England Biolabs. PCR reagents were obtained from Invitrogen. Site-directed mutagenesis was performed using QuikChange\textsuperscript{TM} kits (Stratagene, La Jolla, CA). pGEM T-Easy (Promega) was used to clone the "headless" PCR product.
dues 579–1199). Its construction has previously been described in detail (8).

CaR/mGlut-1/mGlut-1 was designed to encode a protein that contained the CaR VFT domain (residues 1–540) with the rat mGlut-1 Cys-rich region, transmembrane region, and cytoplasmic tail (residues 524–1199). Site-directed mutagenesis was performed to introduce BspE1 restriction enzyme sites at residues 522 of rmGlutR1α and 541 of CaR/CaR/mGlut-1. A second BspE1 site at amino acid 877 of the mGlut-1 sequence was removed by site-directed mutagenesis without disturbing the encoded peptide sequence prior to performing the above digestion and ligation reactions. The DNA fragments containing the bases encoding residues 1–540 of hCaR and 523–1199 of rmGlutR1α, together with an interlinking single Gly residue, were then purified and ligated into pcDNA3.1 (+) to create CaR/mGlut-1/mGlut-1. This junction was confirmed by double-stranded DNA sequencing (Australian Genome Research Facility, Brisbane, Qld, Australia).

Headless CaR Construct—A headless CaR deletion mutant elsewhere referred to as Rho-C-CaR (9–11) or T903-Rhoc (12) containing 20 amino acids of the N terminus of rhodopsin and amino acids 600–903 of the hCaR was constructed in pcDNA3.1 using PCR as described previously (9, 11). Briefly, the sense primer was 100 nucleotides long, corresponding to the nucleotide sequence of the N-terminal 20-amino acid signal peptide of bovine rhodopsin and nucleotide positions 1797–1832 of the hCaR (GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ accession number GenBank™ 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transferred to 24-well plates and grown for a further 24 h. Selection of stable transformants was then carried out in the presence of 50–100 
μg/ml hygromycin (Invitrogen) or 100–400 μg/ml geneticin (Invitrogen). Individual resistant clones were isolated 3 weeks later, screened by aequorin luminescence for activity, and subsequently studied by microfluorimetry using Fura-2.

Detection of Changes in Cytoplasmic-free Ca\textsuperscript{2+} Concentration—The aequorin luminescence screening assay used to identify CaR-expressing HEK293 cell clones for further analysis was performed as described previously (13). For analysis of amino acid- and type-II calcimimetic-dependent activation of the CaR and CaR chimeras, changes in cytoplasmic-free Ca\textsuperscript{2+} concentration were determined by microfluorimetry after loading with Fura-2/AM. HEK293 cells that had been transfected with the wild-type CaR or one of the chimeric CaR constructs were cultured on glass coverslips in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum in 6-well plates and loaded with Fura-2/AM (5 
μM, 2 h; 37 °C) in physiological saline solution containing 125 mM NaCl, 4.0 mM KCl, 1.0 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 20 mM HEPES (NaOH, pH 7.4), 0.1% D-glucose, bovine serum albumin 1 mg/ml. After 2 h the Fura-2-containing solution was removed, and the cells were resuspended in physiological saline solution for 30 min at 37 °C. Fura-2-loaded cells were transferred into a superfusion chamber and placed in the light path of a Nikon Diaphot microscope as described previously (14). The control superfusion solution had the following composition: 125 mM NaCl, 4.0 mM KCl, 0.2 or 0.5 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 20 mM HEPES (NaOH, pH 7.4). Excitation at alternating wavelengths (340 and 380 nm), detection of fluorescent light (F; peak 510 nm) and its digitized recording using Acqknowledge software for Macintosh was performed as described previously (14). Data for cytoplasmic-free Ca\textsuperscript{2+} concentrations were expressed either as uncorrected mobilization data or concentrations in a microfluorimetry apparatus as described under “Experimental Procedures.” The values (means ± S.E.) are returned parameters from analysis of Ca\textsuperscript{2+} concentration dependence curves using MacCurveFit 1.5 for Macintosh.

**Table I**

| Amino acid | Wild-type CaR | CaR/CaR/Glu | CaR/Glu/Glu |
|------------|---------------|-------------|-------------|
| Nil (Control) | 4.9 ± 0.2 (10) | 3.8 ± 0.3 (10) | 6.4 ± 0.3 (9) |
| l-Phe | 3.6 ± 0.3 (4) | 2.9 ± 0.3 (4) | 2.8 ± 0.3 (4) |
| l-His | 3.6 ± 0.1 (3) | 3.0 ± 0.1 (4) | 2.7 ± 0.5 (4) |
| l-Ala | 3.2 ± 0.3 (4) | 3.2 ± 0.5 (4) | 2.6 ± 0.4 (6) |
| l-Trp | 4.2 ± 0.1 (3) | 3.2 ± 0.1 (3) | 3.7 ± 0.2 (4) |
| l-Glu | 4.7 ± 0.8 (3) | 3.5 ± 0.5 (4) | 3.9 ± 0.7 (4) |
| l-Arg | 5.0 ± 0.6 (4) | 3.9 ± 0.4 (4) | 5.8 ± 0.5 (4) |
| l-Leu | 5.4 ± 0.5 (3) | 4.2 ± 0.3 (5) | 7.7 ± 0.5 (5) |
| d-Phe | 4.6 ± 0.3 (3) | 3.6 ± 0.3 (3) | 6.9 ± 0.8 (3) |
| d-Ala | 4.7 ± 0.3 (3) | 4.0 ± 0.4 (3) | 6.2 ± 0.5 (3) |

**RESULTS**

**Effects of L-Amino Acids and NPS R-467 on CaR-expressing HEK293 Cells**—Two lines of HEK293 cells that stably expressed the wild-type human calcium-sensing receptor were studied. After Fura-2 loading, cells from both lines exhibited extracellular Ca\textsuperscript{2+}-dependent intracellular Ca\textsuperscript{2+} mobilization that was allosterically activated by either L-amino acids or the type-II calcimimetic NPS R-467 (Fig. 1 and Table I). Exposure of Fura-2-loaded CaR-expressing HEK293 cells to both L-Phe (10 mM) and R-467 (5 μM) at high concentrations resulted in a small additional enhancement of the response (Fig. 1C). Characterization of the effects of various L- and D-amino acids was consistent with previous data (Table I) (1).

**Effects of L-Amino Acids and NPS R-467 on CaRmGlu-1 Chimeric Receptors Composed of mGlu-1 Transmembrane Domains**—Two chimeric receptor constructs that were composed...
FIG. 3. Effects of L-Phe and NPS R-467 on a headless CaR construct. HEK293 cells were transiently transfected with the empty pcDNA 3.1 vector alone (A), the wild-type CaR (B), the wild-type CaR construct. HEK293 cells were transiently transfected with the empty pcDNA 3.1 vector alone (Fig. 3A). The wild-type CaR construct in which 599 of the 612 amino acid residues of the N-terminal extracellular domain (i.e., VFT and Cys-rich domains) were replaced with a much shorter N terminus containing the bovine rhodopsin signal peptide sequence. In addition, the C terminus of this headless construct was truncated after residue 903 to promote surface expression. This construct and a corresponding wild-type receptor construct that was also truncated after residue 903 were synthesized in the current study and used to further evaluate the hypothesis that the VFT domain of CaR is required for l-amino acid sensing.

In transiently transfected HEK293 cells, cells transfected with vector alone exhibited a small concentration-dependent response to elevated Ca^{2+} that was sensitive to neither 10 mM L-Phe nor 5 μM R-467 (Fig. 3A). The maximal response in cells transfected with vector alone and in untransfected cells (not shown) was ~15–20% of that observed in cells transfected with the wild-type CaR (Fig. 3B). The wild-type CaR, the CaR truncated after residue 903 (Fig. 3C, T903 CaR), and the headless-T903 CaR (Fig. 3D) all exhibited Ca^{2+}-dependent increases in cytoplasmic-free Ca^{2+} concentration when compared with cells transfected with vector alone (Fig. 3A).

The phenylalkylamine type-II calcimimetic, R-467, potentiated the responses of all three constructs, enhancing the receptors’ sensitivities to Ca^{2+}. The finding that R-467 retains activity in the headless receptor is consistent with the idea that the transmembrane domain region of CaR contains binding sites for type-II calcimimetics (10) as well as Ca^{2+} (12). However, R-467 exhibited differential potencies on the control and headless constructs. Whereas 1 and 5 μM R-467 activated the wild-type CaR and T903 CaR, only 5 μM R-467 activated the headless-T903 CaR (Fig. 3D), indicating loss of potency.

The headless-T903 CaR, unlike T903 CaR or the wild-type CaR, was insensitive to L-Phe at 10 mM (Fig. 3, B–D, and Table I) and up to 100 mM (not shown). In addition, L-Phe failed to promote the responses of the headless-T903 CaR to either 1 or 5 μM R-467 (Fig. 3D). Thus, the headless CaR retained sensi-
tivity to R-467, albeit with moderately reduced potency, but was completely resistant to L-Phe at concentrations 100–1000 times its normal plasma concentration (1).

**DISCUSSION**

The current work has demonstrated that the VFT domain of CaR alone is required for L-amino acid sensing. Only the headless-T903 CaR exhibited absolute resistance to L-Phe at concentrations 100–1000-fold those found in plasma (1). Furthermore, the selectivity of CaR for aromatic (L-Phe, L-His, L-Trp) and aliphatic (L-Ala), but not branched chain or charged amino acids (L-Leu, L-Arg), was unaffected by replacement of the extracellular Cys-rich region of CaR or its transmembrane region and C-terminal tail with the corresponding domains from the rat mGlu-1 receptor. The data clearly distinguish the domains that are required for sensing by L-amino acids and phenylalkylamine type-II calcimimetics, whose binding site has been localized previously to the transmembrane region (9–12).

The finding that the VFT domain, and none of the other domains, is required for L-amino acid sensing does not, in itself, establish that amino acids bind there. Because the CaR forms functional homodimers (15), however, it is most likely that the L-amino acid binding site is located in the CaR's own VFT domain and not on a currently unrecognized accessory subunit. In addition, amino acid binding in the VFT domain provides a potential explanation for the observation that the L-amino acid and type-II calcimimetic binding sites are distinct and interacting (6) and that alanine substitutions of the triple serine motif, Ser-169/Ser-170/Ser-171, in the VFT domain disable amino acid sensing (5). Confirmation requires x-ray crystal analysis as for the rat mGlu-1 VFT domain (16, 17) or competitive binding analysis as for basic amino acid binding to the VFT domain of the goldfish 5.24 odorant receptor (18).

The headless-T903 CaR used in the current study has been shown previously to retain sensitivity to Ca\(^{2+}\), in the presence of the phenylalkylamine R-568 (9, 10). In the current study, similar behavior was observed with another CaR-active phenylalkylamine analog, R-467. However, using Fura-2 to monitor changes in cytoplasmic-free Ca\(^{2+}\) concentration, the headless-T903 CaR was sensitive to elevations in extracellular Ca\(^{2+}\) concentration even in the absence of R-467 (Fig. 3D). This effect does not appear to represent receptor-independent Ca\(^{2+}\) influx because the Fura-2 excitation ratio in headless-T903 CaR-transfected cells was approximately twice that observed in untransfected cells or in cells transfected with vector alone (Fig. 3A). It is not clear whether, in the absence of R-467, the headless-T903 CaR mediated Ca\(^{2+}\)-induced Ca\(^{2+}\) influx and/or Ca\(^{2+}\)-induced release of Ca\(^{2+}\) from intracellular stores. In favor of the idea that it mediates Ca\(^{2+}\)-induced Ca\(^{2+}\) influx, previous analyses of inositol phosphate production have shown that in the absence of R-568 the impact of the headless-T903 CaR on phosphatidylinositol-phospholipase C activity is small (10–12). Somewhat to our surprise, there was an apparently reduced potency in the presence of the type-II calcimimetic R-467 with respect to the headless-T903 CaR when compared with its wild-type and T903 controls because 5 \(\mu\)M, but not 1 \(\mu\)M, R-467 promoted the response of the headless receptor. R-467 is an allosteric activator that is inactive in the absence of extracellular Ca\(^{2+}\) ions. The reduction in R-467 potency may have arisen from a resistance to Ca\(^{2+}\)-dependent activation or from a partial rearrangement of the transmembrane helices that form the phenylalkylamine binding site (19).

The locations of the binding sites for extracellular Ca\(^{2+}\) ions remain unresolved. Studies on headless receptors indicate that sensing of Ca\(^{2+}\) and polyvalent cations can be mediated by the transmembrane region alone (11, 12). This conclusion is supported by the analysis of the headless receptor in the current study (Fig. 3). On the other hand, studies on chimeric receptors in which the transmembrane region of CaR is replaced by the mGlu-1 transmembrane region (8, 10) indicate that Ca\(^{2+}\)-sensing is retained. The conclusion that the CaR transmembrane region is not necessary for Ca\(^{2+}\) sensing is also supported by the current study (Fig. 2 and Table I). According to one interpretation of these findings, Ca\(^{2+}\)-binding is mediated by both the VFT domain of CaR and its transmembrane region. Alternatively, Ca\(^{2+}\)-binding may be restricted to the transmembrane regions of both the CaR and mGlu-1 receptors. This idea is consistent with evidence for Ca\(^{2+}\) sensing by mGlu-1 (20). Clearly, further work is required to resolve this issue.

HEK293 cells that stably expressed the CaR were more sensitive to L-Phe than cells that expressed it transiently. This behavior was observed in the absence of R-467 but was apparently more marked in its presence (compare Figs. 1C and 3B). For example, in the presence of 5 \(\mu\)M R-467, 10 mM L-Phe lowered the EC\(_{50}\) for Ca\(^{2+}\) in HEK293 cells that stably expressed the wild-type CaR from around 1.3 to 0.6 mM (Fig. 1C). In the presence of 5 \(\mu\)M R-467, however, 10 mM L-Phe lowered the EC\(_{50}\) for Ca\(^{2+}\) by only 0.1–0.4 mM in cells that transiently expressed the wild-type or T903 CaR (Fig. 3, B and C, and Table II). The origin of the difference in sensitivity to L-Phe in stably and transiently expressing cells is not clear. However, it may reflect differences in receptor expression, surface localization, covalent modification, or the intracellular environment, e.g. arising from CaR-dependent gene transcription.

Interestingly, extracellular Ca\(^{2+}\) is a full agonist of the CaR, whereas L-amino acids and the type-II calcimimetic NPS R-467 are allosteric activators. Thus, the activating effects of L-amino acids and type-II calcimimetics require the presence of a threshold concentration of Ca\(^{2+}\). Glutamate, which binds to the VFT domain, is generally considered a full agonist rather than an allosteric activator of mGluRs. Furthermore, the interaction between glutamate and Ca\(^{2+}\) on mGlu-1 may be indirect.
Additional information on the nature of the activation mechanism for these receptors is clearly required. In the case of the CaR, for example, it will be necessary to determine whether L-amino acids promote Ca\(^{2+}\) binding or the sensitivity of the signaling mechanism.

In conclusion, this study has indicated that the Ca\(^{2+}\)-sensing receptor’s VFT domain, but none of its other domains, is necessary for L-amino acid sensing and, by analogy with other sub-group C receptors, the findings suggest that the VFT domain is the likely site of amino acid binding.

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