Allosteric activation of the extracellular Ca^{2+} -sensing receptor by L-amino acids enhances ERK1/2 phosphorylation

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The calcium-sensing receptor (CaR) mediates feedback control of Ca^{2+}_o (extracellular Ca^{2+}) concentration. Although the mechanisms are not fully understood, the CaR couples to several important intracellular signalling enzymes, including PI-PLC (phosphoinositide-specific phospholipase C), leading to Ca^{2+}_i (intracellular Ca^{2+}) mobilization, and ERK1/2 (extracellular-signal-regulated kinase 1/2). In addition to Ca^{2+}_o , the CaR is activated allosterically by several subclasses of L-amino acids, including the aromatics L-phenylalanine and L-tryptophan. These amino acids enhance the Ca^{2+}_o -sensitivity of Ca^{2+}_i mobilization in CaR-expressing HEK-293 (human embryonic kidney) cells and normal human parathyroid cells. Furthermore, on a background of a physiological fasting serum L-amino acid mixture, they induce a small, but physiologically significant, enhancement of Ca^{2+}_o -dependent suppression of PTH (parathyroid hormone) secretion. The impact of amino acids on CaR-stimulated ERK1/2, however, has not been determined. In the present study, we examined the effects of L-amino acids on Ca^{2+}_o -stimulated ERK1/2 phosphorylation as determined by Western blotting and a newly developed quantitative assay (SureFire). L-Amino acids induced a

small, but significant, enhancement of Ca^{2+}_o -stimulated ERK1/2. In CaR-expressing HEK-293 cells, 10 mM L-phenylalanine lowered the EC_{50} for Ca^{2+}_o from approx. 2.3 to 2.0 mM in the Western blot assay and from 3.4 to 2.9 mM in the SureFire assay. The effect was stereoselective ($L > D$), and another aromatic amino acid, L-tryptophan, was also effective. The effects of amino acids were investigated further in HEK-293 cells that expressed the CaR mutant S169T. L-Phenylalanine normalized the EC_{50} for Ca^{2+}_o -stimulated Ca^{2+}_i mobilization from approx. 12 mM to 5.0 mM and ERK1/2 phosphorylation from approx. 4.6 mM to 2.6 mM. Taken together, the data indicate that L-phenylalanine and other amino acids enhance the Ca^{2+}_o -sensitivity of CaR-stimulated ERK1/2 phosphorylation; however, the effect is comparatively small and operates in the form of a fine-tuning mechanism.

Key words: allosteric activator, L-amino acid, calcium-sensing receptor, extracellular calcium, extracellular-signal-regulated kinase 1/2 (ERK1/2), mitogen-activated protein kinase (MAPK).

INTRODUCTION

The calcium-sensing receptor (CaR) is a multi-metabolic sensor belonging to Class 3 (Family C) of the G-protein-coupled receptor superfamily (for reviews, see [1–3]). Metabolic modalities that are detected by the receptor include changes in extracellular Ca^{2+} and Mg^{2+} concentration, ionic strength [4], pH [5] and the concentrations of amino acids [6]. In addition, the CaR exhibits an allosteric activator site for phenylalkylamine type-II calcimimetics [7] and an allosteric inhibitory site for so-called calcilytics [8].

Analysis of receptor chimaeras composed of the hCaR (human CaR) and its homologue, rmGlu-1 (rat metabotropic glutamate receptor type-1) indicate that different classes of activators bind at distinct sites on the CaR. These include a positive-charge-sensitive site for Ca^{2+} and polycations in the transmembrane region [9,10] and, possibly, the VFT (Venus Fly Trap) domain [11–13], a stereoselective binding site for L-amino acids in the VFT domain [12] and a stereoselective binding site for phenylalkylamine type-II calcimimetics in a pocket defined, in part, by transmembrane helices VI and VII [10,14]. Ca^{2+}_o (extracellular Ca^{2+}) and polycations are receptor agonists, and exposure of the CaR to these activators stimulates signal transduction pathways that lead

to the activation of various intracellular enzymes, including PI-PLC (phosphoinositide-specific phospholipase C) and ERK1/2 (extracellular-signal-regulated kinase 1/2) (for reviews, see [2,15]). Amino acids or other type-II calcimimetics, on the other hand, activate the CaR only in the presence of a threshold concentration of a receptor agonist such as Ca^{2+}_o .

Recent evidence suggests that different CaR activators may not be equally effective with respect to all signalling pathways. In HEK-293 (human embryonic kidney) cells expressing CaR, for example, amino acids that activate CaR markedly enhance the Ca^{2+}_o -sensitivity of Ca^{2+}_i (intracellular Ca^{2+}) mobilization [6] by, at least in part, stimulating Ca^{2+}_i oscillations [16,17]. However, amino acids appear to have little or no effect on Ca^{2+}_o -dependent activation of PI-PLC [18], and it is currently not known whether amino acids also activate other key signalling enzymes that are coupled to the CaR, such as ERK1/2, and, if so, whether the impact of amino acids is substantial, as in the case of Ca^{2+}_i oscillations, or relatively small, i.e. akin to fine-tuning the system. Consistent with this latter concept, elevated concentrations of L-amino acids above a physiological fasting serum amino acid mixture lower the IC_{50} for Ca^{2+}_o -regulated PTH (parathyroid hormone) secretion from approx. 1.15 mM to approx. 1.05 mM [19]. In the present study, we have examined whether CaR-active amino

Abbreviations used: Ca^{2+}_i , intracellular Ca^{2+} ; Ca^{2+}_o , extracellular Ca^{2+} ; CaR, calcium-sensing receptor; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ERK, extracellular-signal-regulated kinase; fura 2/AM, fura 2 acetoxymethyl ester; HEK-293, human embryonic kidney; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI-PLC, phosphoinositide-specific phospholipase C; PSS, physiological saline solution; PTH, parathyroid hormone; rmGlu-1, rat metabotropic glutamate receptor type-1; VFT, Venus Fly Trap.

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acids, including L-phenylalanine and L-tryptophan, activate CaR-dependent ERK1/2. We conclude that L-phenylalanine and L-tryptophan have a small but significant effect on CaR-stimulated ERK1/2 activity as determined by Western blotting or by a new AlphaScreen™-based quantitative method (SureFire) for the MEK [MAPK (mitogen-activated protein kinase)/ERK kinase]-activated doubly phosphorylated Thr(OPO₃²⁻)-X-Tyr(OPO₃²⁻) form. Estimates of the EC₅₀ values for Ca²⁺_o were found to be more accurate using this latter method because it did not saturate at moderate Ca²⁺_o concentrations (approx. 3.0–4.0 mM). In addition, we identified a CaR mutant (S169T), which, in assays of Ca²⁺_i mobilization, exhibited markedly impaired Ca²⁺_o-sensitivity that was significantly restored by L-phenylalanine. In assays of CaR-activated ERK1/2 phosphorylation, however, S169T exhibited normal or near-normal sensitivity to Ca²⁺_o together with enhanced sensitivity to L-phenylalanine, when compared with wild-type.

We conclude that the Ca²⁺_o-sensitivity of wild-type CaR-stimulated ERK1/2 is enhanced by L-amino acids and that the maximum level of increased Ca²⁺_o-sensitivity is small, consistent with the operation of a 'fine-tuning' control mechanism. Such a mechanism would appear to be appropriate for a homeostatic system that is tightly regulated by small changes in Ca²⁺_o concentration. The results indicate that different CaR activators initiate distinct signalling responses and, furthermore, that distinct sensing domains in the VFT and transmembrane domains couple differentially to distinct signalling pathways.

EXPERIMENTAL

Stable expression of wild-type and S169T mutant receptor in HEK-293 cells and cell culture techniques

HEK-293 cells (A.T.C.C., Manassas, VA, U.S.A.) were maintained in 25 cm² culture flasks and transfected with 8 µg of wild-type human CaR (GenBank® U20759) (a gift from Dr Mei Bai and Professor Edward Brown, Brigham and Women's Hospital, Boston, MA, U.S.A.) or S169T mutant CaR using Lipofectamine™ 2000 according to the manufacturer's instructions (Invitrogen). After 24 h, cells were 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) as appropriate. Individual resistant clones were isolated 3 weeks later and screened by aequorin luminescence for the presence of Ca²⁺_o-sensitive activity. The aequorin assay was performed as described previously by transient transfection of HEK-293 cells with the apoaequorin gene and acute incubation with the cell-permeable coenzyme coelenterazine-*cp* [12,20]. HEK-293 cells were cultured in DMEM (Dulbecco's Modified Eagle's medium) (Invitrogen) containing 1.8 mM Ca²⁺_o supplemented with 10% (v/v) fetal bovine serum (Invitrogen).

Analysis of CaR-dependent activation of ERK1/2

Two methods were used to quantify CaR-dependent activation of ERK1/2 after cultured cells had been exposed to PSSs (physiological saline solutions) containing various Ca²⁺_o concentrations in the absence or presence of L-amino acids. In the first method, lysates were subjected to SDS/PAGE and Western blotting for the doubly phosphorylated and total forms of ERK1/2. The total ERK1/2 assay behaved linearly over a wide range of protein loading conditions. Under the conditions used to detect the doubly phosphorylated form of ERK1/2, however, the assay was approximately linear in the Ca²⁺_o concentration range 0.5–3 mM, but saturation was observed at higher Ca²⁺_o concentrations.

In the second method, doubly phosphorylated ERK1/2 was quantified using the SureFire cellular ERK assay (TGR Bio-Sciences), which utilizes the AlphaScreen™ detection system (PerkinElmer). Unlike the Western blot assay, the SureFire cellular ERK assay was found to behave linearly with sample dilution over the complete range of Ca²⁺_o concentrations tested (0.5–10 mM).

Preparation of cultured cells for analysis of ERK1/2

For analysis by either Western blotting or the SureFire cellular ERK1/2 assay, confluent cell monolayers in 24-well plates were serum-starved overnight in 1 ml volumes of DMEM containing 1.5 mM Ca²⁺_o supplemented with 0.2% BSA. Cells were pre-incubated for 30 min at 37 °C in PSS (125 mM NaCl, 4 mM KCl, 20 mM Hepes, 0.1% D-glucose, 0.8 mM NaH₂PO₄ and 1 mM MgCl₂, pH 7.45) containing 0.1% BSA and 1.25 mM Ca²⁺_o. In some experiments, the pre-incubation Ca²⁺_o concentration was 0.5 or 1.8 mM. The cells were then exposed for 10 min at 37 °C to BSA-free PSS containing various concentrations of Ca²⁺_o in the absence or presence of other activators. In preliminary experiments, 10 min was found to yield a maximum level of Ca²⁺_o-activated ERK1/2 phosphorylation, using the Western blot assay similar to that described previously [21]. Similarly, 10 min was found to yield a maximum level of Ca²⁺_o-activated ERK1/2 phosphorylation, using the SureFire ERK assay.

Western blot analysis of the doubly phosphorylated and total forms of ERK1/2

Reactions were terminated by washing with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.4). LB (Luria–Bertani) medium, pH 7.4, contained 150 mM NaCl, 20 mM Tris/HCl, 25 mM NaF, 2.0 mM EDTA, 1.0 mM DTT (dithiothreitol), 50 mM β-glycerophosphate, 2.0 mM sodium vanadate as well as 1% (w/v) Triton X-100, 10% glycerol, and a protease inhibitor cocktail tablet (Roche Diagnostics). The plates were transferred to solid CO₂ and stored at –80 °C or thawed immediately on ice. The contents of all wells were then recovered and transferred to microfuge tubes. After centrifugation at 10 000 g for 2 min, the supernatants were recovered and first assayed for protein concentration using the DC Protein Assay microplate protocol (Bio-Rad). Equivalents (20 µg) of each protein sample were then resuspended in a volumetric ratio of 4:1 in 5× gel-loading buffer (final composition: 0.06 M Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.1% Bromophenol Blue), and then loaded for SDS/PAGE. Samples and a standard biotinylated protein ladder (Cell Signaling Technology) were resolved through duplicate 4% stacking and 10% resolving gels in electrophoresis tank buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) at room temperature (23 °C). The gels were then rinsed briefly in Towbin transfer buffer (25 mM Tris/HCl, 192 mM glycine and 20% methanol, pH 8.3) before electrotransfer on to nitrocellulose membranes (70 mA, 90 min). The membranes were incubated in blocking buffer (5% dried skimmed milk powder in PBS containing Tween 20) overnight at 4 °C with continuous agitation, then washed three times for 5 min in TBST (Tris-buffered saline containing Tween 20: 0.13 M NaCl, 10 mM Tris/HCl, pH 7.5, and 0.1% Tween 20) before overnight incubation in primary antibody solution at 4 °C. One membrane of each duplicate pair was incubated in either blocking solution or, in later experiments, 5% BSA in TBST that contained anti-(phospho-p44/42 MAPK) antibody (1:1000 dilution) (Cell Signaling Technology). The other membrane was incubated in blocking buffer that contained anti-ERK1/2 antibody (diluted 1:5000) (Promega) that recognizes ERK1/2 independent of its phosphorylation state and then washed. All membranes

were incubated overnight at 4°C with blocking buffer containing a mixture of two secondary antibodies: a goat anti-rabbit HRP (horseradish peroxidase)-conjugated antibody (diluted 1:1250) (Promega), and, for the detection of the protein markers, an anti-biotin HRP-conjugated antibody (diluted 1:1250) (Cell Signaling Technology). The membranes were washed then developed in a dark room using ECL[®] (enhanced chemiluminescence) Plus[™] Western Blotting Detection Reagents (Amersham Biosciences). Images were digitized using the Bio-Rad Gel Doc 2000 documentation system, and the bands were quantified using ImageQuant 5.1 software (Molecular Dynamics). For each data set, the maximum level of phosphorylation for control cells was estimated using the Hill equation as described below. All of the data for control and experimental samples were then expressed as percentages of this maximum control value.

Analysis of phosphorylated ERK1/2 using the SureFire cellular ERK1/2 assay

The SureFire AlphaScreen-based phosphorylated ERK1/2 (pERK1/2) assay is based on a two-antibody system directed to the doubly phosphorylated epitope and a second invariant epitope. Co-incubation with AlphaScreen General IgG (Protein A) kit AlphaScreen beads (PerkinElmer) permits selective proximity-based detection of pERK1/2, since only this form of the protein binds both antibodies and thus cross-links the two interacting forms of AlphaScreen beads. The assay was linear over a wide range of pERK1/2 concentrations. Since the total ERK1/2 level is not measured by the assay, the level of total sample protein was measured to ensure that comparable levels of total ERK1/2 were present in all samples.

All cellular reactions were terminated by the removal of PSS, followed immediately by the addition of 0.1 ml of SureFire lysis buffer. Plates were gently agitated at room temperature for 5–10 min. Aliquots of 75 µl of the lysates were then transferred to 96-well plates and stored at –70°C. For analysis, the plates were thawed at room temperature, and 40 µl of each sample was then transferred to a separate plate, and 10 µl of SureFire activation buffer was added. After mixing for 1 min, 5 µl aliquots (corresponding to approx. 40–100 µg of protein) were transferred to the wells of a PerkinElmer 384-well Proxiplate. Aliquots of 6 µl of the antibody-containing reaction buffer plus AlphaScreen beads were then added. The plates were gently agitated for 1–2 min, then incubated in the dark for 2 h. The plates were read using an Envision AlphaScreen plate reader (PerkinElmer). For each data set, the maximum level of phosphorylation for control cells was estimated by curve-fitting as described below. All of the data for control and experimental samples were expressed as percentages of the maximum control value.

Detection of changes in cytoplasmic free Ca²⁺ concentration

Changes in cytoplasmic free Ca²⁺ concentration were determined by microfluorimetry after loading with fura 2/AM (fura 2 acetoxymethyl ester). HEK-293 cells that had been transfected with the wild-type CaR or S169T mutant CaR were cultured on glass coverslips in DMEM containing 10% (v/v) fetal bovine serum in six-well plates and loaded with 5 µM fura 2/AM for 2 h at 37°C in PSS that contained 125 mM NaCl, 4.0 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 0.1% D-glucose, 20 mM Hepes (pH 7.4) and 1 mg/ml BSA. After 2 h the fura 2-containing solution was removed, and the cells were resuspended in PSS 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. The control superfusion solution was BSA-free PSS containing 0.5 mM Ca²⁺. Excitation at alternating

wavelengths (340 and 380 nm), detection of fluorescent light (peak 510 nm) and its digitized recording were performed as described previously [22]. Fluorescence ratio data (F_{340}/F_{380}) were converted into cytoplasmic free Ca²⁺ concentrations using a calibration procedure [22].

Analysis of wild-type and mutant CaR surface expression in HEK-293 cells by Western blotting

HEK-293 cells were cultured in 24-well plates and transiently transfected with a FLAG-tagged CaR construct using Lipofectamine[™] 2000 (Invitrogen) as described above. Proteins on the surface of intact cells were labelled with sulfo-*N*-hydroxysuccinimido-biotin (Pierce) before lysis with a solution that contained 150 mM NaCl, 20 mM Tris/HCl, 25 mM NaF, 2 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM DTT, 50 mM β-glycerophosphate and 100 mM iodoacetamide (all Sigma) and protease inhibitor cocktail. The FLAG-tagged CaR was immunoprecipitated with anti-FLAG M2 monoclonal antibody (Sigma) according to the manufacturer's instructions. The immunopurified protein samples were then eluted in SDS sample buffer (60 mM Tris/HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.1% Bromophenol Blue) heated to 65°C for 30 min and subjected to SDS/PAGE (4% stacking gel, 6% resolving gel). Equivalence of protein loading was checked using the DC protein assay. The proteins were then transferred to nitrocellulose membranes by Western blotting as described above. Biotin-labelled cell-surface CaR proteins were then detected using an avidin–HRP conjugate (Bio-Rad) followed by ECL[®] Plus detection reagents and Kodak GBX developer and fixer reagents (Cedex).

Curve-fitting and statistical analysis

Quantitative analysis of pERK1/2 and Ca²⁺_i mobilization was performed as described above. The results obtained for each Ca²⁺_o concentration (in the absence or presence of activators) were expressed relative to the maximum level calculated for the control data set by curve-fitting techniques. Concentration–response data were fitted to the following form of the Hill equation using MacCurveFit 1.5 for Macintosh:

$$R = b + (a - b) \cdot C^h / (e^h + C^h)$$

where R is response, a is maximum response, b is basal response, C is Ca²⁺_o concentration (in mM), e is EC₅₀ (the concentration of Ca²⁺ that induces a half-maximal response) and h is Hill coefficient.

The data are routinely presented as means ± S.E.M. (number of observations). Statistical comparisons of curve-fit parameters were performed using the F -test as described previously [23], and statistical comparisons between data sets were performed using the paired or unpaired Student's t test.

RESULTS

Ca²⁺_o-dependent activation of ERK1/2 in CaR-expressing HEK-293 cells and the effect of the type-II calcimimetic NPS R467

Samples prepared from CaR-expressing and control HEK-293 cells were applied to the same SDS/PAGE gels, and the Western blots prepared from the gels were probed for total ERK1/2 as well as its active MEK-phosphorylated form. As described previously using the Western blot method [21], Ca²⁺_o maximally stimulated ERK1/2 phosphorylation 10–15-fold in CaR-expressing HEK-293 cells, but not in control HEK-293 cells that did not express the CaR (results not shown). Using the SureFire cellular ERK assay, Ca²⁺_o maximally stimulated ERK1/2 phosphorylation approx. 15–20-fold in CaR-expressing HEK-293 cells.

Figure 1 Activation of Ca²⁺_o-stimulated ERK1/2 by calcimimetic NPS R467 in CaR-expressing HEK-293 cells

HEK-293 cells that stably expressed the human wild-type CaR were incubated in PSS that contained various Ca²⁺_o concentrations in the absence or presence of 1 μM NPS R467. The incubations were stopped by the addition of ice-cold PBS and the cells were lysed. In (A) and (B), the samples were processed by SDS/PAGE and Western blotting, and the blots were developed for total and phosphorylated ERK1/2 and then quantified as described in the Experimental section. The data were then expressed as a percentage of the maximum level of control phosphorylation for each experiment. (A) A representative Western blot for total (upper panel) and phosphorylated (lower panel) ERK1/2. Molecular masses are indicated in kDa. (B) Ca²⁺_o concentration–response data showing the effect of 1 μM NPS R467 (R-467) on Ca²⁺_o-stimulated ERK1/2 phosphorylation detected by Western blotting. In (C), the samples were processed for SureFire cellular ERK1/2 analysis as described in the Experimental section. ○, Control; ▲, NPS R467.

The phenylalkylamine type-II calcimimetic NPS R467 enhanced the Ca²⁺_o-sensitivity of ERK1/2 activation in CaR-expressing HEK-293 cells as revealed by both Western blotting (Figures 1A and 1B) as described previously [21] and the SureFire assays (Figure 1C). Using the Western blot method, in the absence of NPS R467, the EC₅₀ for Ca²⁺_o was 2.6 ± 0.3 mM. However, in the presence of 1 μM NPS R467, the EC₅₀ for Ca²⁺_o fell to 1.4 ± 0.2 mM and the Hill coefficient fell from 3.6 ± 1.2 to 2.1 ± 0.4 ($n = 5$). Using the SureFire assay, in the absence of NPS R467, the EC₅₀ for Ca²⁺_o was 2.9 ± 0.1 mM. However, in the presence of 1 μM NPS R467, the EC₅₀ for Ca²⁺_o fell to 1.6 ± 0.1 mM and the Hill coefficient appeared to fall from 7.2 ± 0.7 to 5.5 ± 1.0 ($n = 4$; Figure 1C). NPS R467 had no apparent effect on the maximal level of ERK1/2 phosphorylation whether assayed using the Western blot or SureFire methods (Figures 1B and 1C).

Effect of L-phenylalanine on Ca²⁺_o-stimulated ERK1/2 activity in CaR-expressing HEK-293 cells

At a Ca²⁺_o concentration of 2.0 mM, 10 mM L-phenylalanine enhanced ERK1/2 phosphorylation in HEK-293 cells expressing CaR, as detected by Western blotting (Figure 2). Similar data were obtained in two additional experiments. The impact of L-phenyl-

Figure 2 Effects of L-phenylalanine on Ca²⁺_o-stimulated ERK1/2 phosphorylation in CaR-expressing HEK-293 cells

CaR-expressing HEK-293 cells were incubated in the presence of 2 mM Ca²⁺_o in the absence (–) or presence (+) of 10 mM L-phenylalanine for 10 min. The incubations were then stopped by exposure to ice-cold PBS, and the cells were lysed and processed by SDS/PAGE and Western blotting. Films were developed by ECL[®] as described in the Experimental section.

alanine on the Ca²⁺_o-sensitivity of ERK1/2 activation was analysed initially using the Western blot assay under three different pre-incubation Ca²⁺_o concentrations: 0.5, 1.25 and 1.8 mM ($n = 10$, 10 and 5 respectively). In all three cases, similar results were obtained and L-phenylalanine induced a statistically significant enhancement of the Ca²⁺_o-sensitivity of ERK1/2 activation. Representative blots for total and MEK-phosphorylated ERK1/2 are

Figure 3 Effects of L-phenylalanine on the Ca²⁺_o-sensitivity of ERK1/2 phosphorylation in CaR-expressing HEK-293 cells

Wild-type CaR-expressing HEK-293 cells were incubated (**A** and **B**) in the presence of various Ca²⁺ concentrations (0.5–6.0 mM) in the absence or presence of 10 mM L-phenylalanine for 10 min. The incubations were stopped by exposure to ice-cold PBS, and the cells were then lysed and processed by SDS/PAGE and Western blotting as described in the Experimental section. All Ca²⁺_o-concentration-dependent data were expressed as a percentage of the maximum level of phosphorylation obtained for each control data set. (**A**) Representative blot demonstrating a small enhancement of the Ca²⁺-sensitivity of ERK1/2 phosphorylation by 10 mM L-phenylalanine in the region of the EC₅₀ for Ca²⁺_o (approx. 2.0 mM). (**B**) Ca²⁺-concentration-dependent activation of ERK1/2 phosphorylation in the absence or presence of 10 mM L-phenylalanine from a total of 25 experiments. (**C**) CaR-expressing HEK-293 cells were incubated in the presence of various Ca²⁺ concentrations between 0.5 and 10 mM and then processed for the SureFire ERK1/2 assay as described in the Experimental section ($n = 14$). ○, Control; ▲, 10 mM L-phenylalanine.

shown in Figure 3(A). The data were combined and subjected to curve-fitting analysis. In the absence of the L-phenylalanine, the EC₅₀ for Ca²⁺_o was 2.29 ± 0.08 mM; in the presence of L-phenylalanine, however, there was a small, but consistent, fall in the EC₅₀ for Ca²⁺_o to 1.96 ± 0.07 mM ($n = 25$; Figure 3B). Statistical comparison of the curves using the *F*-test as described by Meddings et al. [23] demonstrated that the difference between the EC₅₀ values was highly significant ($F[1,171] = 18.0$; $P < 0.0001$). No significant differences were observed, however, between the minimum and maximum values or the Hill coefficients. Thus, in the absence of L-phenylalanine, the Hill coefficient was 4.7 ± 0.8 and, in its presence, the Hill coefficient was 4.6 ± 0.7 .

Since, as described in the Experimental section, the Western blot assay appeared to underestimate the EC₅₀ values for Ca²⁺_o owing to saturation of the assay at Ca²⁺_o concentrations above 3 mM, the experiments were repeated using the SureFire cellular ERK1/2 assay, which exhibited linearity over a wide range of protein loading conditions and Ca²⁺_o concentrations. In the absence of L-phenylalanine, the EC₅₀ for Ca²⁺_o was 3.4 ± 0.1 mM; in the presence of L-phenylalanine, however, there was a small, but consistent, fall in the EC₅₀ for Ca²⁺_o to 2.9 ± 0.1 mM ($n = 14$; Figure 3C). Statistical comparison of the curves using the *F*-test demonstrated that the difference between the EC₅₀ values was highly significant ($F[1,94] = 41.9$; $P < 0.001$). Although no changes in the Hill coefficients were observed, there appeared to be a small decrease in the maximal response. Thus, under standard conditions, in the absence

and presence of L-phenylalanine, the maximal responses were $98.4 \pm 2.6\%$ and $88.9 \pm 2.5\%$ respectively.

Concentration response and stereoselectivity of the effects of L-phenylalanine and the effects of other amino acids

The effect of L-phenylalanine was concentration-dependent (Figure 4A). After pre-incubation under standard conditions followed by incubation at a Ca²⁺_o concentration of 2.0 mM, L-phenylalanine elevated CaR-stimulated ERK1/2 phosphorylation maximally by approx. 50% with respect to the control (results not shown). Under these conditions, the effect of L-phenylalanine was maximal at approx. 10 mM (Figure 4A). Previous analyses have indicated that the CaR is activated stereoselectively by aromatic, but not branched-chain, L-amino acids [6,19]. Therefore the effects of the following amino acids were tested: L-phenylalanine, D-phenylalanine, L-tryptophan, L-leucine and D-leucine under standard conditions. Using the Western blot assay, L-phenylalanine and L-tryptophan were effective, but D-phenylalanine, L-leucine and D-leucine were not (Figures 4B and 4C).

Effect of L-phenylalanine on HEK-293 cells that stably express the CaR mutant S169T

It has been reported previously that, in assays of Ca²⁺_i mobilization, several mutations of the CaR's VFT domain exhibit impaired Ca²⁺_o-sensitivity that is substantially restored in the presence of CaR-active amino acids [24]. We identified S169T as

Figure 4 Concentration-dependence and stereoselectivity of L-phenylalanine and effects of various other amino acids

HEK-293 cells that stably expressed the wild-type CaR were pre-incubated for 30 min and then exposed to (A) various concentrations of Ca²⁺ (0.5, 2.0 and 6.0 mM) and L-phenylalanine (0–30 mM) as shown, (B) various concentrations of Ca²⁺ in the presence of 10 mM L-phenylalanine or D-phenylalanine and (C) various concentrations of Ca²⁺ (0.5, 2.0 and 6.0 mM) in the absence or presence of various amino acids as shown. The samples were processed by SDS/PAGE and Western blotting as described in the Experimental section. In (A), lanes between the L-phenylalanine concentration–response data (lanes 1–4) and low- and high-Ca²⁺ controls (shown as lanes 5 and 6 from the left) have been deleted from the image. Molecular masses are indicated in kDa.

an example of this phenomenon. The wild-type and S169T CaR exhibited similar levels of surface expression (Figure 5). CaR-dependent mobilization of Ca²⁺_i was analysed in fura 2-loaded HEK-293 cells that stably expressed either wild-type (Figure 6A) or S169T (Figure 6B) CaR. As described previously, 10 mM L-phenylalanine enhanced the Ca²⁺_o-sensitivity of Ca²⁺_i mobilization in wild-type CaR-expressing cells (Figure 6A). In the absence of L-phenylalanine, the EC₅₀ for Ca²⁺_o was 5.2 ± 0.4 mM ($n = 9$); however, in the presence of 10 mM L-phenylalanine, the EC₅₀ for Ca²⁺_o was 3.4 ± 0.3 mM ($n = 9$). Compared with wild-type under control conditions, S169T-expressing cells exhibited impaired Ca²⁺_o-sensitivity, which took the form of an increase in EC₅₀ for Ca²⁺_o (Figure 6B). In the absence of L-phenylalanine, the EC₅₀ for Ca²⁺_o was 11.6 ± 2.7 mM ($n = 6$). In the presence of 10 mM L-phenylalanine, however, S169T-expressing cells exhibited near-normal Ca²⁺_o-sensitivity (EC₅₀ for Ca²⁺_o = 5.0 ± 0.7 mM; $n = 6$) (Figure 6B).

Figure 5 Comparison of surface expression between the wild-type CaR and CaR mutant S169T

HEK-293 cells were transiently transfected with FLAG-tagged wild-type or S169T mutant CaRs as described in the Experimental section. The cells were then labelled with sulfo-*N*-hydroxy-succinimido-biotin and lysed in the presence of 100 mM iodoacetamide. CaR proteins were immunoprecipitated using anti-FLAG antibody, suspended in SDS/PAGE sample buffer and assayed for protein content. Equivalent amounts of protein were then processed by SDS/PAGE. Western blotting and detection of biotin-labelled proteins was performed using avidin–HRP and ECL[®] as described in the Experimental section. The arrows indicate the molecular masses of markers in kDa. The lower-molecular-mass form (approx. 160 kDa) is likely to represent monomers of the mature CaR [32]. The higher-molecular-mass species is presumed to correspond to homodimers.

We next tested the effect of the CaR-active amino acid L-phenylalanine on Ca²⁺-stimulated ERK1/2 phosphorylation in S169T-expressing HEK-293 cells using the SureFire cellular ERK1/2 assay. A preliminary comparison of the maximum responses for HEK-293 cells expressing the wild-type and S169T CaR revealed that they were similar under conditions of comparable protein loading. S169T-expressing cells exhibited Ca²⁺_o-stimulated ERK1/2 that had a mildly right-shifted Ca²⁺_o-sensitivity in the absence of L-phenylalanine that was clearly enhanced in the presence of L-phenylalanine (Figure 6C). Thus, in the absence of L-phenylalanine, the EC₅₀ for Ca²⁺_o was 4.6 ± 0.8 mM; however, in the presence of 10 mM L-phenylalanine, the EC₅₀ for Ca²⁺_o fell to 2.6 ± 0.2 mM ($n = 6$). Statistical analysis using the *F*-test indicated that the difference was highly significant ($F[1,44] = 114$; $P < 0.001$). L-Phenylalanine had no effect on the maximum response. The data indicate that L-phenylalanine substantially restored Ca²⁺_o-sensitivity to HEK-293 cells that stably express S169T CaR in the case of both Ca²⁺_i mobilization and ERK1/2 phosphorylation. However, Ca²⁺_i mobilization exhibited greater sensitivity to L-phenylalanine than ERK1/2 phosphorylation for both the wild-type CaR and the S169T mutant.

DISCUSSION

The results demonstrate that certain amino acids, including L-phenylalanine and L-tryptophan, allosterically activate Ca²⁺_o-stimulated ERK1/2 phosphorylation in CaR-expressing HEK-293 cells whether assayed using the standard Western blot approach or the SureFire cellular pERK1/2 assay [25]. The principal effect was on the Ca²⁺_o-sensitivity of the response, and no effect was observed at Ca²⁺_o concentrations below a threshold of approx. 1.0 mM (see Figure 3B). This behaviour resembles that previously observed for the effects of amino acids on Ca²⁺_o-dependent Ca²⁺_i mobilization in CaR-expressing HEK-293 cells [6]. In addition, as previously observed in assays of Ca²⁺_i mobilization in CaR-expressing HEK-293 cells [6] and normal human parathyroid cells [19], the effect was stereoselective (*L* > *D*) and selective for the aromatic amino acids L-phenylalanine and L-tryptophan rather than the branched-chain amino acid, L-leucine. The stereoselective and amino acid-selective nature of the effects of L-phenylalanine and L-tryptophan on Ca²⁺_o-induced ERK1/2 phosphorylation excludes the possibility that changes in osmolality or ionic strength might have been responsible [4].

Although the basic conclusions obtained using the Western blot and SureFire ERK1/2 assays were similar, the estimates of

Figure 6 Effects of L-phenylalanine on Ca^{2+}_i mobilization in HEK-293 cells expressing the wild-type CaR and on Ca^{2+}_i mobilization and ERK1/2 phosphorylation in HEK-293 cells expressing S169T

HEK-293 cells that stably expressed the wild-type CaR (A) or S169T mutant CaR (B) were loaded with fura 2 and investigated for Ca^{2+}_o - and L-phenylalanine-stimulated Ca^{2+}_i mobilization by microfluorimetry. In (A) and (B), the receptor response is expressed in the form of a calibrated cytoplasmic free Ca^{2+} concentration (in nM). Compared with the wild-type CaR, S169T exhibited markedly impaired sensitivity to Ca^{2+}_o that was restored to near-normal Ca^{2+}_o -sensitivity by the addition of 10 mM L-phenylalanine. (C) Ca^{2+}_o concentration–response data for the activation of ERK1/2 as determined by the SureFire assay in the absence and presence of L-phenylalanine in HEK-293 cells that stably expressed the S169T mutant. The data in (A) were obtained in nine experiments. The data in (B) and (C) were obtained in six experiments. ○, Control; ▲, 10 mM L-phenylalanine.

the curve-fitting parameters obtained were different, with values of EC_{50} for Ca^{2+}_o being consistently higher in the case of the SureFire assay, owing to saturation of the Western blotting assay at Ca^{2+}_o concentrations above 3 mM. The SureFire assay, on the other hand, behaved linearly upon dilution over the entire Ca^{2+}_o concentration range tested. Thus the estimates of the curve-fitting parameters derived from the SureFire ERK1/2 assay, were clearly superior to those obtained by Western blotting.

Unlike the effect of CaR-active amino acids on Ca^{2+}_i mobilization, the effects of L-phenylalanine and L-tryptophan on Ca^{2+}_o -stimulated ERK1/2 were relatively small. Thus, in the presence of a maximally effective concentration of L-phenylalanine (10 mM), the EC_{50} for Ca^{2+}_o -stimulated ERK1/2 phosphorylation fell by 0.3 mM as determined by Western blotting and by 0.5 mM (Figure 3) using the SureFire cellular ERK1/2 assay. In contrast, the phenylalkylamine type-II calcimimetic NPS R467 markedly enhanced Ca^{2+}_o -sensitivity, lowering the EC_{50} for Ca^{2+}_o for the wild-type CaR by approx. 1.2–1.3 mM in both assays (Figure 1). The difference in the impact of L-phenylalanine, which binds in the VFT domain, and NPS R467, which binds in the transmembrane domain, suggests that there is differential coupling of these domains to the ERK1/2 pathway.

To investigate further the effect of amino acids on CaR-dependent ERK1/2 phosphorylation, we examined the impact of L-phenylalanine on a mutant form of the CaR, S169T, that was also stably expressed in HEK-293 cells. Ser¹⁶⁹ lies in the CaR's VFT domain and has been identified previously as a residue that contributes to Ca^{2+}_o - and amino-acid-sensing [24]. Under conditions of transient expression in HEK-293 cells, the wild-type CaR and S169T mutant exhibited similar levels of surface expression (Figure 5). In assays of Ca^{2+}_i mobilization, however, the S169T mutant, like several other previously reported mutants of the CaR's VFT domain, including S147A, Y218A and E297K [24], exhibited markedly reduced sensitivity to Ca^{2+}_o that was substantially restored by L-phenylalanine (Figure 6B). When studied using the SureFire Cellular ERK1/2 assay in the absence of L-phenylalanine, however, S169T exhibited a more modest impairment of Ca^{2+}_o -sensitivity. Thus, in the absence of L-phenylalanine, the EC_{50} for Ca^{2+}_o in S169T-expressing cells (4.6 mM) was approx. 1.0 mM greater than in wild-type CaR-expressing cells (3.4 mM; compare Figures 3C and 6C). Surprisingly, however, 10 mM L-phenylalanine restored the Ca^{2+}_o -sensitivity of S169T-dependent ERK1/2 activation to normal. Thus, the impact of L-phenylalanine on Ca^{2+}_o -induced activation of ERK1/2 was substantially greater for S169T than for the wild-type receptor.

Ser¹⁶⁹ is located in a region that in homology-based models is predicted to form the interface between the two lobes of the human CaR's VFT domain (see, e.g., [24]). This interface in rmGlu-1, which is a known CaR homologue, is the site of ligand-dependent, as well as ligand-independent, domain closure essential for receptor activation [26,27]. Furthermore, the key rmGlu-1 residue Thr¹⁸⁸ in this region aligns to hCaR residue Ser¹⁷⁰, i.e. immediately adjacent to Ser¹⁶⁹. Previous site-directed mutagenesis studies of this region in the CaR have led to the conclusions that it is critical for Ca^{2+}_o -stimulated [28] and L-amino-acid-stimulated receptor function [24]. Surprisingly, loss of the hydroxy side chain at position 169, i.e. S169A, was shown previously to have little or no impact on Ca^{2+}_o -sensitivity [24], whereas, in the present study, S169T clearly impaired Ca^{2+}_o -dependent receptor activation. These findings appear to indicate that Ser¹⁶⁹ is located at an extremely sensitive site in lobe 1 of the VFT domain, but that its side chain is not required for domain closure or receptor activation. On the other hand, a small increase in the size of its side chain (as a result of a serine-to-threonine mutation) would appear to interfere with domain closure and receptor activation, perhaps by

disrupting the hydrogen-bond network based on Ser¹⁷⁰. The finding that amino acids can restore normal or near-normal function suggests that a bound amino acid molecule can stabilize the local hydrogen-bond networks that form across the lobe-I-lobe-II interface.

Analysis of amino-acid- and Ca²⁺_o-induced Ca²⁺_i mobilization in CaR-expressing HEK-293 cells has led to evidence that distinct pathways are involved [16,18]. In particular, the pathway responsible for L-amino acid-induced enhancement of Ca²⁺_o-induced Ca²⁺_i mobilization appears to be independent of changes in InsP₃ levels and requires the selective activation of the small G-protein rho, changes in actin polymerization state and activation of the plasma membrane Ca²⁺ channel TRPC1 (transient receptor potential canonical 1) [18,29]. The present study demonstrates that the impact of amino acids on the CaR is not restricted to Ca²⁺_i mobilization, but extends, albeit more subtly, to other key signalling pathways.

Although the effect of amino acids on ERK1/2 activation induced by the wild-type CaR was small, such an effect is potentially significant for endocrine cells that are responsible for regulating Ca²⁺_o concentration, because the normal concentration range for Ca²⁺_o is extremely tight, i.e. between 1.1 and 1.3 mM. Recent analysis indicates, for example, that physiologically relevant increases in the concentrations of CaR-active amino acids suppress PTH secretion by approx. 30–40% in the physiological Ca²⁺_o concentration range, but lower the IC₅₀ for Ca²⁺_o by only 0.1 mM, from approx. 1.15 mM to 1.05 mM [19]. Thus the effects of L-phenylalanine and L-tryptophan on Ca²⁺_o-stimulated ERK1/2 in CaR-expressing HEK-293 cells are comparable with the effects of these amino acids on PTH secretion from normal human parathyroid cells. Viewed in this way, the effect of amino acids on CaR-stimulated ERK1/2 phosphorylation is not only physiologically significant, but also appropriate for a system that is markedly perturbed by a change in Ca²⁺_o concentration as small as 0.1 mM.

It was reported previously that PD98059, a specific inhibitor of MEK, the bifunctional protein kinase activator of ERK1/2, eliminates high-Ca²⁺_o-induced suppression of PTH secretion from human parathyroid cells [30]. These results indicate that the activation of ERK1/2 is a key step in the mechanism that underlies the CaR-dependent regulation of PTH secretion. Thus ERK1/2 could also contribute to the mechanism by which amino acids inhibit human PTH secretion [19] and, perhaps more generally, to other instances where variations in dietary protein intake provoke changes in calcium metabolism (reviewed in [31]). Furthermore, CaR-dependent activation of ERK1/2 could provide a general mechanism by which amino acids regulate gene expression via transcription factors such as Elk-1.

In conclusion, L-amino acids allosterically activate Ca²⁺_o-stimulated ERK1/2 in CaR-expressing HEK-293 cells; however, this effect is substantially smaller than the effect of CaR-active amino acids on Ca²⁺_i mobilization. Amino acid activation appears to provide a mechanism by which variations in dietary protein intake or, more generally, amino acid metabolism could induce fine adjustments of the cellular signalling responses to Ca²⁺_o.

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