Allosteric Modulation of the Calcium-sensing Receptor by γ-Glutamyl Peptides

INHIBITION OF PTH SECRETION, SUPPRESSION OF INTRACELLULAR cAMP LEVELS, AND A COMMON MECHANISM OF ACTION WITH L-AMINO ACIDS

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The extracellular calcium-sensing receptor (CaR) is a multi-modal class C G-protein coupled receptor that is widely distributed in mammalian tissues (1). It controls the plasma Ca\(^{2+}\) ion concentration by mediating negative feedback regulation of parathyroid hormone secretion as well as Ca\(^{2+}\) reabsorption in the distal nephron (review: (2)). Analysis of the receptor’s physiological significance has been based on its tissue distribution, analyses of its actions in cultured cells and tissues, and demonstrations of the impact of global knock-outs (3) and, more recently, conditional knock-outs in endocrine and mesenchymal tissues (4). These studies demonstrate that the CaR is a pluripotent sensor, regulator and modulator. In addition to regulating whole body calcium metabolism, it senses nutrient signals in the gastrointestinal tract (reviews: Refs. 5, 6), modulates synaptic transmission (7) and cellular differentiation in mesenchymal tissues (4), as well as engraftment of stem cells in specific niches (8), and organogenesis e.g. in the lung (9) and central nervous system (10, 11). Accompanying this remarkable plasticity, the CaR, like several other class C GPCRs, binds multiple physiologically relevant ligands to control intracellular signaling pathways (Reviews: Refs. 12, 13).

In addition to sensing multivalent cations including Ca\(^{2+}\) and Mg\(^{2+}\), the CaR is modulated by various L-amino acids (review: Ref. 1). Based on chimeric receptor and mutational analyses, L-amino acids bind in the receptor N-terminal Venus Fly Trap (VFT) domain (14) and the effects of L-amino acids are selectively impaired by a double mutant (T145A/S170T), which exhibits normal Ca\(^{2+}\) sensing (15).

Comparative molecular modeling of the mGlu-1 and CaR VFT domain ligand binding surfaces indicates that, whereas the amino acid side-chain binding region is tightly defined by a cluster of positively charged residues in mGlu-1 and other mGlus (16, 17) it is relatively unrestricted in the CaR and closely related class C GPCRs including GPRC6A and T1R1 (18). The larger side-chain binding surfaces in this subgroup of receptors appear to explain, at least in part, their notable promiscuity for various sub-classes of amino acids (reviews: Refs. 19, 20) and has led to the successful prediction that small peptides that retain α-amino and α-carboxylate functional groups at their N termini, including the tri-peptide glutathione would bind and activate the CaR expressed in HEK-293 cells (21). Building on this observation, γ-glutamyl peptides were recently found to be potent taste enhancers (22) raising the possibility that they may be physiologically im-
portant receptor modulators in various tissues in which the CaR is expressed.

To further evaluate the physiological significance of these observations we have now tested the effects of the γ-glutamyl-tripeptides γ-Glu-Cys-Gly (glutathione), S-methylglutathione and S-propylglutathione, and dipeptides γ-Glu-Ala and γ-Glu-Cys, on extracellular Ca\(^{2+}\)-induced intracellular Ca\(^{2+}\) mobilization and suppression of cAMP levels in CaR-expressing HEK-293 cells. We then extended the investigation to consider the effects of these peptides on intracellular Ca\(^{2+}\) mobilization and PTH secretion in normal human parathyroid cells, and whether a double mutant T145A/S170T that selectively impairs amino acid-dependent activation of the CaR (15) might also impair the actions of γ-glutamyl peptides. The results indicate that various γ-glutamyl peptides including glutathione and its metabolite S-methylglutathione are potent positive allosteric modulators of the CaR that promote Ca\(^{2+}\) mobilization and lower intracellular cAMP levels in CaR-expressing HEK-293 cells and promote Ca\(^{2+}\) mobilization and suppress PTH secretion in normal human parathyroid cells. Results of a comparative analysis of HEK-293 cells that express either the wild-type or double mutant T145A/S170T CaR indicate that, distinct from Ca\(^{2+}\)-o and phenyl-alkylamines, γ-glutamyl peptides and L-amino acids modulate the receptor via a common mechanism linking binding of L-amino acids or γ-glutamyl peptides at closely related sites in the VFT domain to a characteristic change in receptor conformation that couples to the modulation of cytoplasmic Ca\(^{2+}\) and cAMP levels.

**EXPERIMENTAL PROCEDURES**

**Cell Culture of Control and Stably Transfected HEK-293 cells**—Human embryonic kidney (HEK)-293 cells stably expressing the CaR (HEK-CaR cells), untransfected HEK-293 cells, and HEK-293 cells that stably expressed a double mutant form of the CaR (HEK-CaR-T145A/S170T cells) were thawed from frozen stocks and cultured in DMEM (Invitrogen)/10% fetal bovine serum as described previously (15). In general, cultured cells were studied between passage numbers 4 and 20. For microfluorimetry experiments, HEK-293 cells were transferred onto sterilized 15-mm diameter coverslips in 24-well plates and cultured for a further 24–48 h.

**Preparation of Human Parathyroid Cells**—Samples from normal human parathyroid auto-transplants were obtained during thyroid surgery at the Royal North Shore Hospital and North Shore Private Hospital, St Leonards, New South Wales and the Mater Misericordiae Hospital, North Sydney, New South Wales, Australia. All procedures were performed under guidelines established by the relevant human research ethics committees, and all patients provided written informed consent for the use of the tissue for experimental purposes.

Parathyroid tissue was transported to the laboratory in MEM that contained 1.25 mM CaCl\(_2\). Upon arrival in the laboratory, it was either used immediately or, more typically, stored overnight in MEM at 4 °C as described previously (23). Prior to collagenase digestion, parathyroid tissue retains viability under these conditions (24). For digestion, it was transferred to MEM that contained 1 mg/ml collagenase and 0.1 mg/ml DNase I. After brief oxygenation it was incubated at 37 °C for 20 min. The enzyme suspension was then decanted and the parathyroid tissue was transferred into 5 ml of MEM that contained 1 mg/ml of bovine serum albumin (BSA). The tissue was then subjected to trituration (10–15 times) through the tip of a disposable 5-ml syringe (no needle attached). The cloudy suspension containing clumped parathyroid cells was then passed through a 200-μm pore size nylon filter, sedimented (70 × g, 2.5 min), and the cell pellet gently resuspended then washed twice with 5 ml of BSA-containing MEM. It was finally resuspended in 2 ml of bovine serum albumin-containing MEM. Remaining pieces of undigested parathyroid tissue were then incubated for a further 20 min at 37 °C in fresh medium containing collagenase and DNase I. The enzyme suspension was once more decanted, and the parathyroid tissue subjected to trituration and centrifugal isolation as above.

**Amino acid and Peptide-containing Solutions**—Stock amino acid-containing solutions were routinely made up in physiological saline at 50 or 100 mM and stored at −20 °C then diluted in amino acid-free physiological saline as required. Dipeptide and tripeptide containing solutions, on the other hand, were made up fresh in physiological saline and used on the day of experiment. The control physiological saline solution (PSS) used in all microfluorimetry experiments had the following composition: 125 mM NaCl, 4.0 mM KCl, 0.5 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 20 mM HEPES (NaOH), 0.1% d-glucose (pH 7.4).

**Microfluorimetry of HEK-293 Cells and Human Parathyroid Cells for Determination of Intracellular Ca\(^{2+}\) Concentration**—HEK-293 cells that had been cultured on coverslips in 24-well plates were loaded with 5 μM fura-2-AM in PSS that contained 1 mg/ml bovine serum albumin at 37 °C for 2 h in the dark. This solution was then removed and replaced by fresh albumin-containing PSS and the cells were incubated for a further 30 min to promote the conversion of fura-2 to its fully ionized, Ca\(^{2+}\)-binding form.

Suspensions of normal human parathyroid cells, which had been prepared by collagenase digestion, were loaded with 1 μM fura-2-AM for 20 min at 37 °C in MEM containing bovine serum albumin (1 mg/ml). The cells were sedimented (70 × g, 2.5 min) and then resuspended in albumin-free physiological saline solution.

Fura-2-loaded HEK-293 cells that had been cultured on coverslips or fura-2 loaded parathyroid cells, which readily attach to glass surfaces, were transferred into a superfusion chamber, placed in the light path of a Zeiss Axiovert fluorescence microscope (63× objective) and perfused with inorganic phosphate-free physiological saline solutions that were modified to contain various concentrations of Ca\(^{2+}\), L-Phe, and γ-glutamyl di- and tri-peptides. A Lambda DG-4 150 watt Xenon light source (Sutter) was programmed to alternate between two excitation wavelengths (340 and 380 nm) at a frequency of 1 s\(^{-1}\). Areas of interest were selected and digital images were then captured and downloaded using a high resolution AxioCam camera controlled by Stallion SB.4.1.0 PC software (Zeiss, Australia). Fura-2-loaded cells were imaged at 510 nm. For concentration-response analysis, fura-2 ratio

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data were integrated and then either plotted directly or expressed as a ratio of a control response (e.g. L-Phe or R-467).

Detection of Changes in Intracellular CaM Levels in Car-expressing HEK-293 Cells—HEK-CaR, HEK-CaR-T145A/S170T, or control HEK-293 cells were seeded onto 15-mm coverslips in 24-well plates for 24 h and then transfected for 48 h with pcDNA3.1 containing the CaM bio-sensor CFPnd-EPac1-cpVenus (EPac1; the generous gift of Dr Kees Jalink, Netherlands Cancer Institute) using Lipofectamine-2000 according to the manufacturer’s instructions (Invitrogen). The media were replaced with PSS containing 0.5 mM Ca2⁺ for 30 min at 37 °C, and coverslips were then transferred to a chamber placed in the light path of a Zeiss Axiocover epi-fluorescence microscope (63× objective) and perfused with PSS that contained various concentrations of Ca²⁺. L-Phe, γ-glutamyl di- or tri-peptides, or the type-II calcimimetic NPS R-467 as required. Epac1-transfected cells were excited continuously with light centered on a wavelength of 436 nm using the Lambda DG-4 light source and emitted light corresponding to an initial control period were subtracted to yield activator- and concentration-dependent receptor-response values. The resulting data were plotted unmodified or expressed as ratios of control responses. For γ-glutamyl peptide responses, either 10 mM L-Phe or 5 μM NPS R-467 were used as controls. Experimental data were plotted using DeltaGraph 5.0 or GraphPad Prism 5.0b and curve-fitting was performed using the Hill equation: \[ r = b + (a-b) \frac{C^n}{(e^{n+C})} \] where \( r \) = response, \( a \) = maximum response, \( b \) = basal response, \( C \) = extracellular Ca²⁺ concentration (in mM), \( e = EC_{50} \) (the Ca²⁺ concentration that induced a half-maximal response) and \( n = \text{Hill coefficient} \). The data were expressed routinely as means ± S.E. (number of experiments). Statistical comparisons between treatments within PTH secretion data sets were performed using Student’s paired \( t \) test (two-tailed) in SPSS Statistics 17.0. Statistical comparisons between the effects of positive allosteric modulators on pEC\(_{50}\) values, maximum responses, or specific data points in Ca²⁺ concentration-response curves were performed using Student’s unpaired \( t \) test (two-tailed) in SPSS. Statistically significant comparisons were accepted at \( p < 0.05 \).

RESULTS

Effects of γ-Glutamyl Peptides on Ca²⁺,o-dependent Intracellular Ca²⁺ Mobilization in Car-expressing HEK-293 Cells—No effects of γ-glutamyl peptides were observed in untransfected HEK-293 cells (not shown). At extracellular Ca²⁺ (Ca²⁺o) concentrations below 1.0 mM, the amino acid L-Phe (10 mM) and the tripeptide glutathione and two analogs S-methylglutathione and γ-Glu-Ala had no effect on intracellular Ca²⁺ (Ca²⁺i) mobilization in HEK-CaR cells. For example, in the presence of 0.5 mM Ca²⁺o none of the peptides was effective at concentrations up to 100 μM (not shown). At Ca²⁺o concentrations above 1.0 mM, however, L-Phe and glutathione, acutely and reversibly stimulated oscillations in the intracellular Ca²⁺ concentration in HEK-CaR cells and a Ca²⁺o concentration of 2.5 mM was selected for experiments comparing the efficacies and potencies of γ-glutamyl peptides including the dipeptides γ-Glu-Ala and γ-Glu-Cys, and tripeptides, glutathione and S-methylglutathione (Fig. 1A and Table 1).

All four γ-glutamyl peptides were effective positive allosteric modulators of Ca²⁺i mobilization in the presence of 2.5 mM Ca²⁺o. The order of potency for Ca²⁺i mobilization was as follows (at Ca²⁺o = 2.5 mM; Fig. 1, B and C and Table 1): S-methylglutathione > glutathione > γ-Glu-Ala > L-Phe. In separate experiments, similar potencies were observed for γ-Glu-Cys and γ-Glu-Ala (Table 1). Co-administration of the
TABLE 1

| γ-Glutamyl peptide | EC50 for peptide |
|---------------------|------------------|
| S-Methylglutathione  | 1.7 ± 0.5 (n = 4) |
| Glutathione          | 3.9 ± 0.7 (n = 4) |
| γ-Glu-Cys            | 4.7 ± 0.9 (n = 3) |
| γ-Glu-Ala            | 4.8 ± 0.7 (n = 3) |

recognize CaR-active amino acid, L-Phe and high potency γ-glutamyl peptide S-methylglutathione, at maximal concentrations i.e. 10 mM and 10 μM respectively, induced no additional effect when compared with either L-Phe or S-methylglutathione alone (not shown). On the other hand, at a Ca2+ concentration of 2.5 mM, maximally effective concentrations of S-methylglutathione and NPS R-467 exhibited significant positive interactions when compared with the effects of either compound alone (Fig. 2).

Effects of γ-Glutamyl Peptides on Ca2+−dependent Intracellular Ca2+ Mobilization in Normal Human Parathyroid Cells—Of the amino acids and peptides that had been identified as positive allosteric modulators of Ca2+ mobilization in HEK-CaR cells, L-Phe, γ-Glu-Ala, glutathione, and S-methylglutathione, together with another glutathione analog, S-propylglutathione were selected for further studies of intracellular Ca2+ mobilization in fura-2 loaded normal human parathyroid cells. At Ca2+ concentrations at or below 0.5 mM, none of the compounds was effective. At Ca2+ concentrations greater than 1.0 mM, however, all of the compounds acutely mobilized intracellular Ca2+ (Fig. 3A). At a Ca2+ concentration of 1.5 mM, the concentration-response relations indicated that the γ-glutamyl peptides were effective in the micromolar concentration range with S-methylglutathione exhibiting a near maximal effect at around 2–5 μM (Fig. 3, B and C). The order of potency for intracellular Ca2+ mobilization was as follows: S-methylglutathione > glutathione > γ-Glu-Ala > S-propylglutathione (Fig. 3C and Table 2).

Effects of γ-Glutamyl Peptides on PTH Secretion from Normal Human Parathyroid Cells—We next tested the effects of γ-glutamyl peptides including γ-Glu-Ala, glutathione and S-methylglutathione (Fig. 4) on PTH secretion from perfused normal human parathyroid cells. All three peptides induced acute and reversible suppression of PTH secretion at a Ca2+ concentration of 1.0 mM (Fig. 4A) and exhibited concentration-dependent inhibition of secretion (Fig. 4B; p < 0.01 for the effect of 20 μM S-methylglutathione with respect to the pre- and post controls). For S-methylglutathione, the IC50 value was 4.8 ± 0.3 μM (n = 3; Fig. 4, B and C). For γ-Glu-Ala, the IC50 value was ~30 μM (supplemental Fig. S1). The results indicate that γ-glutamyl peptides and S-methylglutathione concentrations in individual experiments and correcting for the baseline response (n = 4 for SMG; n = 5 for GSH; n = 3 for γ-Glu-Ala). The symbols are as follows: circles, γ-Glu-Ala; triangles, S-methylglutathione; squares, glutathione. The data were corrected for baseline.
thione, in particular, are potent suppressors of PTH secretion raising the possibility that glutathione or an extracellular metabolite may act physiologically to modulate PTH secretion. In addition, S-methylglutathione or a structural analog may be of value in the treatment of various forms of hyperparathyroidism.

Impact of the Double Mutant T145A/S170T on Ca\(^{2+}\) Mobilization Responses in CaR-expressing HEK-293 Cells to L-Phe and S-Methylglutathione—Chimeric receptor analysis previously localized the CaR \(\alpha\)-amino acid (14) and glutathione (21) binding sites to the VFT domain and we previously identified T145A/S170T as a double mutant form of the VFT domain with near-normal Ca\(^{2+}\)\(\alpha\)-sensitivity but markedly impaired \(\alpha\)-amino acid sensing (15). In the current study, we first confirmed that this mutant exhibited normal or near-normal Ca\(^{2+}\)\(\alpha\)-sensitivity in the absence of L-Phe or S-methylglutathione (Fig. 5, A and B). In the presence of 10 mM L-Phe, HEK-293 cells expressing the wild-type CaR exhibited markedly enhanced Ca\(^{2+}\)\(\alpha\)-sensitivity (Fig. 5C) whereas HEK-293 cells expressing the T145A/S170T double mutant CaR exhibited no change in Ca\(^{2+}\)\(\alpha\)-sensitivity (Fig. 5D) as reported previously (15).

We next tested the impacts of the potent \(\gamma\)-glutamyl peptide, S-methylglutathione on the Ca\(^{2+}\)\(\alpha\)-sensitivity of CaR mobilization in wild-type CaR and T145A/S170T CaR-expressing cells. In HEK-CaR cells, 10 \(\mu\)M S-methylglutathione induced a marked increase in Ca\(^{2+}\)\(\alpha\) potency (Fig. 5E) but had little or no effect on Ca\(^{2+}\)\(\alpha\) potency in HEK-293 cells express-
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TABLE 2
Potencies of γ-glutamyl peptides for Ca\(^{2+}\), mobilization in normal human parathyroid cells

| γ-Glutamyl peptide | EC\(_{50}\) for peptide (μM) |
|--------------------|-----------------------------|
| S-Methylglutathione | 0.38 ± 0.09 (n = 5)          |
| Glutathione         | 5.4 ± 1.0 (n = 5)            |
| γ-Glu-Ala           | 13.9 ± 5.7 (n = 6)           |
| S-Propylglutathione | 221 ± 107 (n = 4)            |

FIGURE 3. Impacts of various γ-glutamyl peptides on intracellular Ca\(^{2+}\) mobilization in normal human parathyroid cells. Normal human parathyroid cells were loaded with fura-2AM and transferred into the light-path of a microfluorescence apparatus for analysis of intracellular Ca\(^{2+}\) mobilization. A, effects of various γ-glutamyl-peptides (all 5 μM) as well as 5 mM L-Phe in the presence of a submaximal Ca\(^{2+}\) concentration, 1.5 mM. Multiple single cell time-courses (n = 10) are shown from a representative experiment. B, effect of stepwise increases in SMG concentration on Ca\(^{2+}\) in 9 single cells from a representative experiment; L-Phe was included as a control. C, concentration-response curves for various γ-glutamyl peptides derived from experiments similar to that shown in B. The number of independent experiments was as follows: glutathione (n = 5); γ-Glu-Ala (n = 6); SMG (n = 5); and S-propyl-glutathione (SPG; n = 4). The symbols are as follows: open circles, γ-Glu-Ala; open triangles, glutathione; open squares, SMG; filled squares, SPG.

FIGURE 4. Effects of γ-glutamyl peptides on PTH secretion from normal human parathyroid cells. Normal human parathyroid cells were loaded into a perifusion column for the analysis of acute changes in PTH secretion as described under “Experimental Procedures.” A, representative time course showing the acute effects of γ-Glu-Ala and SMG (both 20 μM) as well as L-Phe (3 mM) on PTH secretion; similar results were obtained in two other experiments. B, effect of stepwise increases in SMG concentration (0.5–20 μM) on PTH secretion. The data are means ± S.E. from three experiments. Student’s t test analysis of comparisons between pre- and post-controls and 20 μM SMG were significant in both cases (p < 0.01; p < 0.02). C, concentration-response curve demonstrating the inhibitory effect of SMG on PTH secretion in the presence of 1.0 mM Ca\(^{2+}\); the data were derived from the time-course experiments shown in B above (n = 3).

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Ca\(^{2+}\)\textsubscript{o} were as follows: 3.9 ± 0.2 mM (n = 4) for HEK-293 cells that stably expressed the wild-type CaR and 3.6 ± 0.3 mM (n = 4) for HEK-293 cells that stably expressed the T145A/S170T double mutant CaR, respectively. Although the wild-type CaR exhibited markedly enhanced Ca\(^{2+}\)\textsubscript{o} sensitivity in the presence of 10 mM l-Phe (EC\(_{50}\) for Ca\(^{2+}\)\textsubscript{o} = 1.7 ± 0.3 mM; n = 4) or 10 \(\mu\)M S-methylglutathione (EC\(_{50}\) for Ca\(^{2+}\)\textsubscript{o} = 2.4 ± 0.3 mM; n = 4), the double mutant exhibited markedly impaired responses to both modulators (see Fig. 6, A and B). Thus, the EC\(_{50}\) values for Ca\(^{2+}\)\textsubscript{o} (in mM) were, respectively, 3.6 ± 0.2 (n = 7), 3.4 ± 0.2 (n = 7), and 3.5 ± 0.2 (n = 7) in the absence of modulators or in the presence of 10 mM l-Phe or 10 \(\mu\)M S-methylglutathione (p = 0.78 for l-Phe and p = 0.82 for S-methylglutathione with respect to control).

To further investigate the impacts of l-Phe and S-methylglutathione on the CaR double mutant T145A/S170T, we examined the effects of stepwise increases in the concentrations of l-Phe and S-methylglutathione in the presence of a submaximal Ca\(^{2+}\)\textsubscript{o} concentration, 2.5 mM using 5 \(\mu\)M NPS R-467 as a positive control (Fig. 7). In the case of the wild-type CaR, both l-Phe and S-methylglutathione induced robust concentration-dependent increases in Ca\(^{2+}\), mobilization in the presence of 2.5 mM Ca\(^{2+}\)\textsubscript{o} (Fig. 7, A and C). In the case of the CaR double mutant T145A/S170T, however, the effects of

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**FIGURE 5. Sensitivity of double mutant CaR (T145A/S170T) to modulation of Ca\(^{2+}\)\textsubscript{o}-dependent Ca\(^{2+}\)\textsubscript{i} mobilization in HEK-293 cells by l-Phe or S-methylglutathione.** HEK-293 cells that stably expressed either the wild-type CaR (left panels: A, C, E) or the double mutant T145A/S170T (right panels: B, D, F) were loaded with fura-2AM as described under “Experimental Procedures” and then exposed to stepwise changes in Ca\(^{2+}\)\textsubscript{o} concentration in the absence of modulators (panels A and B) or the presence of 10 mM l-Phe (panels C and D) or 10 \(\mu\)M SMG (panels E and F). The data shown were obtained in single representative experiments (8–10 cells per experiment; 4–6 experiments per panel).
Ca2⁺-dependent CaR activation and signaling

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FIGURE 6. Behavior of double mutant (T145A/S170T) CaR in Ca2⁺o concentration-response analyses in the absence or presence of L-Phe or S-methylglutathione. Ca2⁺o concentration-response relations were derived from data obtained in experiments similar to, and including, those shown in Fig. 5. In A, the effects of 10 mM L-Phe (open triangles; n = 4) and 10 μM SMG (open squares; n = 4) are compared with control (open circles; n = 4) for HEK-293 cells that stably expressed the wild-type CaR. The differences between L-Phe or SMG and control were statistically significant at the following submaximal Ca2⁺o concentrations: 2.0 mM (p < 0.05; p = 0.05); 3.0 mM (p < 0.01; p < 0.01); and 4.0 mM (p < 0.01; p < 0.01). In B, the effects of 10 mM L-Phe (filled triangles; n = 4) and 10 μM S-methylglutathione (filled squares; n = 4) are compared with control (filled circles; n = 4) for HEK-293 cells that stably expressed the CaR double mutant T145A/S170T.

both L-Phe and S-methylglutathione were attenuated (Fig. 7, B and D). Concentration-response relations for L-Phe and S-methylglutathione derived from experiments including those shown in Fig. 7 demonstrated marked impairment of Ca2⁺i mobilization responses in HEK-293 cells expressing the T145A/S170T double mutant when compared with wild-type (Fig. 8) and similar results were obtained whether the data were expressed as integrated fluorescence ratio responses (Fig. 8) or, alternatively, as fractional responses with respect to a control, 5 μM R-467 (not shown).

Interestingly, the double mutant exhibited markedly suppressed maximum responses to L-Phe and S-methylglutathione but appeared to have little or no effect on the potencies of the modulators (Fig. 7, B and D; Fig. 8). These results suggest that the double mutant has little or no effect on modulator binding but instead impairs the coupling between Ca2⁺o and positive allosteric modulators such as L-Phe and glutathione analogs that bind in the VFT domain.

Impacts of L-Phe and S-Methylglutathione on Ca2⁺o-dependent cAMP Suppression in HEK-293 Cells That Stably Express the Wild-type CaR or T145A/S170T Double Mutant—Finally, we investigated the impact of L-Phe, S-methylglutathione and elevated Ca2⁺o on intracellular cAMP levels in HEK-CaR cells that were transfected with the cAMP reporter CFPnd-EPac1-cpVenus since the CaR is known to suppress cAMP levels in a Gᵢ and Ca2⁺,o-dependent manner (27). cAMP levels were first elevated by exposure to 5 μM forskolin and the cells were then exposed to one or more CaR activators in the continuing presence of forskolin. Elevated Ca2⁺o induced a concentration-dependent inhibition of cAMP levels that appeared to reach a maximum at around 6 mM (Fig. 9A). Restoring Ca2⁺o to the baseline level (0.5 mM) led to a prompt recovery in intracellular cAMP levels, which took the form of an overshoot following exposure to high Ca2⁺o concentration (≥ 5 mM) (Fig. 9A), consistent with the phenomenon of sensitization or superactivation of adenylyl cyclase (review: Ref. 28). No effects of elevated Ca2⁺o, L-Phe, or S-methylglutathione were observed in control HEK-293 cells.

Consistent with their behaviors as positive allosteric modulators, L-Phe and S-methylglutathione were without effect on intracellular cAMP levels in HEK-CaR cells at a Ca2⁺o concentration of 0.5 mM (not shown). In the presence of submaximal Ca2⁺o concentrations (1.5 or 2.5 mM), however, 10 mM L-Phe and 10 μM S-methylglutathione acutely and reversibly suppressed cAMP levels (Fig. 9B), and the phenylalkylamine type-II calcimimetic NPS R-467 (0.2–5 μM) had a similar effect (not shown). We next tested the effects of L-Phe, S-methylglutathione, and elevated Ca2⁺o on intracellular cAMP levels in HEK-293 cells that stably expressed the T145A/S170T CaR double mutant. The effects of 10 mM L-Phe and 10 μM S-methylglutathione were markedly attenuated, however, the effects of 5 mM Ca2⁺o (Fig. 9C) and 5 μM R-467 (not shown) were unaffected.

DISCUSSION

The CaR has several binding sites for Ca2⁺ ions and other multivalent cations in its VFT (29) and heptahelical (14, 30) domains. These so-called type-I (cationic) calcimimetics are generally regarded as full agonists of the receptor since they activate multiple downstream signaling pathways linked to the activation of Gᵢ₁₁, Gᵢₒ, or G₁₂/₁₃ (reviews: Refs. 12, 13, 31). Thus, their binding site(s) define the location(s) of the receptor orthosteric site(s). In addition, the CaR binds so-called type-II calcimimetics, which are typically uncharged organic compounds that act as positive allosteric modulators. Two main sites for the binding of positive allosteric modulators have been identified: one in the heptahelical domain for type-II calcimimetics such as the phenylalkylamines NPS R-467 and cinacalcet (30, 32); and one or more in the VFT domain for L-amino acids such as L-Phe or L-Trp (14) and glutathione and analogs (21). Previous work suggested that the L-amino acid binding site is selectively disabled by the double mutant T145A/S170T (15).

In the current study, we provide further evidence of the efficacy of γ-glutamyl peptides as positive allosteric modulators of Ca2⁺o-induced Ca2⁺ mobilization in CaR-expressing HEK-293 cells (Fig. 1 and Table 1) and demonstrate their efficacy in normal human parathyroid cells (Fig. 3 and Table 2). In addition, we found that two glutathione analogs, S-methylglutathione and S-propylglutathione and two other γ-glut-
tamyl peptides, γ-Glu-Ala (Figs. 1 and 3; Tables 1 and 2) and γ-Glu-Cys (Table 1) are positive modulators of CaR function, and establish that these compounds are effective inhibitors of PTH secretion (Fig. 4; supplemental Fig. S1). Of the compounds tested, S-methylglutathione exhibited the highest potency activation of Ca\(^{2+}\)\(_i\) mobilization in human parathyroid cells (EC\(_{50}\) 0.5 μM at Ca\(^{2+}\)\(_o\) 1.5 mM; Fig. 3C) and inhibition of PTH secretion (IC\(_{50}\) 5 μM at Ca\(^{2+}\)\(_o\) 1.0 mM; Fig. 4C). The results suggest that extracellular glutathione or one of its metabolites, including perhaps, the natural metabolite S-methylglutathione, may modulate CaR function in a physiologically relevant manner. The results also identify lead compounds from which new groups of high potency activators or inhibitors of the CaR may emerge. The finding that maximally effective concentrations of S-methylglutathione and NPS R-467 exhibit positive interactions on the receptor Ca\(^{2+}\)\(_i\) mobilizing response (Fig. 2) supports this notion and indicates that combination therapy with cinacalcet and an agent that targets the glutathione binding site in the receptor’s VFT domain may be an option in clinical situations in which cinacalcet alone is ineffective.

The CaR negatively regulates cAMP levels in parathyroid cells (review: Ref. 2) and CaR-expressing HEK-293 cells in which both Gi/o and Ca\(^{2+}\)\(_i\)-dependent mechanisms have been identified (27). In the current study, we transiently expressed a FRET-based cAMP sensor, CFPnd-EPac1-cpVenus (33) in CaR-expressing HEK-293 cells to permit detection of changes in intracellular cAMP levels in real-time. As expected, resting levels of cAMP were promptly elevated upon exposure of CaR-expressing HEK-293 cells to forskolin and, in its continuing presence, were suppressed in a concentration-dependent manner upon exposure to elevated Ca\(^{2+}\)\(_o\) (Fig. 9A). In addition, the positive allosteric modulators L-Phe and S-methylglutathione (Fig. 9B), as well as NPS R-467 (not shown) all reversibly suppressed cAMP levels in the presence of 1.5 mM Ca\(^{2+}\)\(_o\). Thus, the positive allosteric modulators of the CaR, L-Phe, and S-methylglutathione both effectively suppressed cAMP levels. It is not yet known whether these effects arose primarily from activation of Gi/o or Ca\(^{2+}\)\(_i\) mobilization. Previous studies have suggested that L-Phe primarily promotes a CaR-dependent pathway linked to Ca\(^{2+}\)\(_i\) mobilization (34, 35) although it also has a small positive modulatory effect on ERK1/2 phosphorylation (36).

Chimeric receptor analysis previously demonstrated that the binding sites for L-amino acids (14) and glutathione (21) both localize to the receptor VFT domain. To investigate whether these two classes of positive allosteric modulator might bind at a common or overlapping site and/or act via a common mechanism, as suggested by molecular modeling analysis (18), we took advantage of a previously identified double mutant of the CaR VFT domain (T145A/S170T) that selectively disables L-amino acid sensing with...
little or no effect on Ca\(^{2+}\)-sensing (15). This mutant disabled the effects of L-Phe as well as S-methylglutathione, on Ca\(^{2+}\) mobilization (Figs. 5–8) as well as suppression of forskolin-elevated cAMP levels in CaR-expressing HEK-293 cells (Fig. 9). However, in the current study, the double mutant appeared to markedly impair efficacy with little or no effect on potency in the cases of both L-Phe and S-methylglutathione (Fig. 8) suggesting that its primary impact was not on ligand binding but on the coupling of the amino acid and \(\gamma\)-glutamyl peptide binding sites to Ca\(^{2+}\)-dependent receptor activation. This suggests that the mutant receptor is unable to sustain a normal conformational response to either amino acid or peptide binding with impacts on both Ca\(^{2+}\) mobilization and cAMP signaling pathways. The impact on other CaR-linked signaling pathways remains to be determined. Although these results do not allow a firm conclusion to be drawn regarding the relationship between the binding sites for L-amino acids and glutathione analogs, molecular modeling suggests that the S-methylglutathione binding site in the CaR (supplemental Fig. S2) is closely related to that previously described for the amino acid L-glutamate in mGlu-1 (16), mGlu-3, and mGlu-7 (17). Thus, L-amino acids and glutathione analogs, distinct from Ca\(^{2+}\), and R-467, act via a common mechanism as revealed by the selective impacts of the T145A/S170T double mutant and are likely to occupy overlapping binding sites in the receptor VFT domain.
Calcium-sensing Receptor Modulation by γ-Glutamyl Peptides

Global and conditional knock-out studies for assessing CaR function have, thus far, identified roles for the CaR in the control of parathyroid hormone secretion (3, 4), renal Ca\(^{2+}\) reabsorption (3), and the formation of cartilage and bone (4). However, the realization that an earlier global knock-out strategy, based on the in-frame deletion of exon-5, was incomplete due to residual receptor function (4), together with studies demonstrating diverse pluripotent roles in development and tissue function (review: Ref. 37) suggest that the CaR in the gut lumen following the ingestion of protein metabolites modulate the CaR by a common mechanism, raises the possibility that glutathione and/or a metabolite such as S-methylglutathione may play a specific role in CaR-dependent neuromodulation.

The CaR belongs to GPCR class C, members of which are typified by N-terminal Venus FlyTrap domains linked to heptahelical domains. In mammalian biology, VFT domains are also found in ionotropic, as well as metabotropic, receptors for glutamate. Interestingly, glutathione and analogs including S-methylglutathione and glutathione sulfonate have been previously shown to modulate NMDA and AMPA receptors (43, 44). In addition, like the CaR, the NMDA receptor binds multivalent cations including neomycin, spermine, and Mg\(^{2+}\) (45). The current work on the physiological impact of γ-glutamyl peptides on CaR function strengthens the notion that the CaR and ionotropic, as well as metabotropic, glutamate receptors share conserved VFT domain nutrient binding surfaces and may thus contribute to coordinate control of CNS function in response to a common signal including, perhaps, extracellular glutathione and/or glutathione analogs. Attention has been drawn previously to potential roles for glutathione and its metabolites in the modulation of CNS function (reviews: Refs. 46, 47).

In conclusion, we have demonstrated that γ-glutamyl peptides including glutathione and its S-alkyl derivatives, S-methylglutathione, and S-propylglutathione, along with the di-peptides γ-Glu-Ala and γ-Glu-Cys are potent positive allosteric modulators of the calcium-sensing receptor that stimulate Ca\(^{2+}\) mobilization in CaR-expressing HEK-293 cells and normal human parathyroid cells and powerfully suppress PTH secretion. In addition, they suppress cAMP levels in forskolin-stimulated CaR-expressing HEK-293 cells transfected with the cAMP sensor CFpnd-EPac1-cpVenus. Finally, unlike responses to elevated Ca\(^{2+}\), or the type-II calcimimetic NPS R-467, both γ-glutamyl peptide-induced Ca\(^{2+}\) mobilization and suppression of cellular cAMP levels were markedly attenuated in the case of the CaR double mutant T145A/S170T indicating that L-amino acids and γ-Glu peptides activate the CaR via a common mechanism.

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