Activation of Family C G-protein-coupled Receptors by the Tripeptide Glutathione

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The Family C G-protein-coupled receptors include the metabotropic glutamate receptors, the γ-aminobutyric acid, type B (GABA_B) receptor, the calcium-sensing receptor (CaSR), which participates in the regulation of calcium homeostasis in the body, and a diverse group of sensory receptors that encompass the amino acid-activated fish 5.24 chemosensory receptor, the mammalian T1R taste receptors, and the V2R pheromone receptors. A common feature of Family C receptors is the presence of an amino acid binding site. In this study, a preliminary in silico analysis of the size and shape of the amino acid binding pocket in selected Family C receptors suggested that some members of this family could accommodate larger ligands such as peptides. Subsequent screening and docking experiments identified GSH as a potential ligand or co-ligand at the fish 5.24 receptor and the rat CaSR. These in silico predictions were confirmed using a [3H]GSH radioligand binding assay and a fluorescence-based functional assay performed on wild-type and chimeric receptors. Glutathione was shown to act as an orthosteric agonist at the 5.24 receptor and as a potent enhancer of calcium-induced activation of the CaSR. Within the mammalian receptors, this effect was specific to the CaSR because GSH neither directly activated nor potentiated other Family C receptors including GPRC6A (the putative mammalian homolog of the fish 5.24 receptor), the metabotropic glutamate receptors, or the GABA_B receptor. Our findings reveal a potential new role for GSH and suggest that this peptide may act as an endogenous modulator of the CaSR in the parathyroid gland where this receptor is known to control the release of parathyroid hormone, and in other tissues such as the brain and gastrointestinal tract where the role of the calcium receptor appears to subserve other, as yet unknown, physiological functions.

Free amino acids act within the large extracellular ligand binding domains of Family C G-protein-coupled receptors as either the direct acting orthosteric agonists or as allosteric modulators. Glutamate, and the glutamate metabolite GABA, are the endogenous ligands at the metabotropic glutamate receptors (mGluRs) and the GABA_B receptor, respectively. In contrast to the restricted activation of the mGluRs and the GABA_B receptor by glutamate and GABA, other Family C receptors can be activated by multiple amino acids. The fish 5.24 chemosensory receptor, which is expressed in several sensory organs and is thought to play a role in feeding and navigation, can be activated by most amino acids (1). A similar, albeit non-identical, spectrum of amino acid activation is also an intrinsic property of GPRC6A, the putative mammalian homolog of the 5.24 receptor (2, 3), and the T1R1/T1R3 heteromeric taste receptor (4).

Amino acids also allosterically regulate the activity of the calcium-sensing receptor (CaSR), another member of the Family C receptors. Although calcium is considered the primary endogenous ligand, calcium-induced responses mediated by the CaSR are enhanced in the presence of amino acids (5). The CaSR possesses an amino acid binding site in the extracellular domain analogous to the glutamate and GABA sites in the mGluR and GABA_B receptors (6, 7). The physiological importance of amino acid modulation of the CaSR has been demonstrated in human parathyroid cells where amino acids have been shown to enhance the CaSR-mediated suppression of parathyroid hormone release (8).

In addition to amino acids and cations, several peptides have been reported to activate or modulate Family C receptors. For example, the endogenous dipeptide N-acetylaspartylglutamate has been reported to activate mGluR3 (9), and β amyloid peptides have been shown to modulate intracellular calcium levels induced by activation of the CaSR (10). However, the β amyloid peptides appear to affect CaSR activity indirectly via stimulation of calcium permeable ion channels (10, 11). The activity of some members of the V2R class of Family C pheromone receptors may also be modulated by peptides. Although most of the endogenous ligands for the V2R receptors are not yet known, Kimoto et al. (12) have demonstrated that peptides secreted from the extraorbital lacrimal gland of mice activate c-fos activity selectively in V2R expressing neurons in the vomeronasal organ where they appear to function as chemical signals mediating social and sexual behaviors. However, whether these effects on V2R-expressing neurons are mediated by direct interactions between the secreted peptides and V2R receptors remains to be established.

Based on these observations indicating that peptides can activate some Family C receptors, we initiated an in silico examination of the amino acid binding pockets of Family C G-protein-coupled receptors. An analysis of the size and shape of the amino acid binding pockets of selected Family C receptors suggested that both of these parameters differed considerably among the receptors investigated and that some of the binding pockets may be large enough to accommodate small peptides. Subsequent screening and docking experiments identified the tripeptide GSH as a potential ligand or co-ligand at the fish 5.24 receptor and the rat CaSR. To investigate these in silico predictions, individual receptor subtypes were expressed in human embryonic kidney 293 (HEK-293) cells and tested for their ability to be activated or potentiated by GSH. Our results demonstrate that GSH acts within the extracellular ligand binding domain of the 5.24 chemosensory receptor and the CaSR.
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EXPERIMENTAL PROCEDURES

Materials—Glutathione and oxidized glutathione (GSSG), S-nitroso-glutathione, γ-Glu-Cys, Cys-Gly, and L-arginine were from Sigma. The purity of the GSH (Ultra pure from Sigma, catalog number G-6529) was confirmed by HPLC analysis and via mass spectrometry. [3H]Glutathione (specific activity 41.5 Ci/mmol) was purchased from PerkinElmer Life Sciences, Inc., Woodbridge, Ontario, Canada. The anti-c-myc mouse monoclonal antibody was obtained from Upstate Inc., Charlottesville, VA (clone 4A6, epitope, MEQKLIIEEDL), and the anti-CaSR mouse monoclonal antibody was from Affinity BioReagents (Golden, CO) (clone 5C10, ADD, epitope, ADDDYGRPIEKFREE-AEERD). All cell culture reagents were from Invitrogen.

Molecular Modeling and Ligand Docking—Homology models of the wild-type 5.24 receptor, CaSR, and GPCR6A were generated using the x-ray crystal structure of the extracellular domain of rat mGlur1 as a template (Protein Data Bank coordinates 1EWK) using version 6.0 of the MODELER program (13) as described by Wang and Hampson (14).

Sybyl 7.0 (Tripos Inc., St. Louis, MO) was used to view, analyze, and manipulate the structure. The most potent amino acid ligand for each receptor (arginine for 5.24 receptor and GPCR6A and phenylalanine for the CaSR) was manually docked into the amino acid binding pockets. These ligands were included in the subsequent molecular mechanics refinement to keep the residues in the binding pockets in appropriate positions, and the force field parameters for the ligands were prepared with the aid of Antechamber module in AMBER 7.0 (15). The refinement of ligand-receptor complexes was performed using AMBER 7.0 in the presence of explicit water as described by Wang and Hampson (14).

In the first step, the solvent was equilibrated for 500 ps with the protein fixed to allow the water molecules filling in the empty space between the ligand and receptor in the binding pocket. Subsequently the whole system was relaxed by another 500 ps of molecular dynamics simulation. Finally, the entire system was energy minimized for 20,000 steps by gradually reducing the restraints on the backbone to zero.

For ligand docking, glutathione peptide and related derivatives were constructed and minimized using Sybyl. Prior to docking, to prevent distortion of the peptide bond and to impart rigidity, the N–C bonds in GSH were converted to double bonds. The ligand molecules were docked into the binding pocket, defined as all residues within 12 angstroms of the ligand, using the docking program FlexX (16) as implemented in Sybyl 7.0. The optimally docked conformer was selected based on the scores calculated from the scoring function in the CSCORE module of Sybyl.

Expression Constructs, Transient Transfections, and Immunoblotting—The rat CaSR cDNA in the pRKS vector was prepared as described by Ruat et al. (17), and the Ca-Glu-Glu CaSR/mGlur1 chimera was generated as outlined by Hu et al. (18). The wild-type fish 5.24 (1), the rat mGlur1, and mGlur2, mGlur4 receptors (19), and the mouse GABA A R1 and R2 subunits and the GPCR6A receptor (20) were subcloned into the pcDNA3.1 expression vector. A truncated fragment of the CaSR (truncated at serine 540) was obtained by PCR using wild-type rat CaSR cDNA as a template. The two primers were as follows: forward, 5′-CGCAAGCTTGAGAGGCGGAAAGCTATGCGAAGCACTTCGGAAGAAGGCAAGATC-3′; reverse, 5′-AGTCCTGATTAGATGAGAAGGAAAAGGAACTCTCTGAGAAGAC-3′. The forward primer contained a HindIII restriction site and 17-bp 5′-untranslated region of CaSR; the reverse primer contained an XbaI site and a stop codon. The PCR amplification product was digested by HindIII/XbaI and ligated into pcDNA3.0 opened by HindIII/XbaI. The truncated goldfish 5.24 receptor cDNA was constructed as previously described (20). The plasmid cDNAs were transiently transfected into HEK-293 cells using the Lipofectamine 2000 reagent (Invitrogen). The cells were cultured in minimal essential medium with 6% fetal bovine serum (Invitrogen). For the truncated secreted receptors, ~16 h after transfection, the cell culture medium was replaced with Opti-MEM (Invitrogen), and the cell culture medium containing the secreted truncated receptors was collected for analysis 4 days post-transfection. The procedures for immunoblotting were carried out as described previously by Yao et al. (21). Immunoblots were labeled with either an anti-c-myc or an anti-CaSR monoclonal antibody (Upstate Inc.).

The 5.24/GPCR6A chimera containing the extracellular domain of 5.24 (1 to serine 493) and the transmembrane and COOH terminus of mouse GPCR6A (lysine 512–928) was constructed as described by Kuang et al. (2). The mouse GPCR6A/5.24 chimeric receptor containing the extracellular domain of GPCR6A (1 to serine 520) and the transmembrane and intracellular domains of 5.24 receptor (lysine 494–877) was constructed by overlapping PCR. The first round of PCR was performed with two pairs of primers (pair I, MPGR6A-Xhol-F, 5′-CCTG GGA TCT GTG CCA AGC TCG AG-3′ and M/5.24-Ser-R, 5′-CGT GTC CGA ACA CAT AGA TAG ATG TTG AAA TTT CCT GAA TTC-3′; pair II, M/5.24-Ser-F, 5′-TTA AGC AAA TTC TAT CTA AGT GTT CCG ACA GCT GTC AAT CAG CAG-3′ and 5.24-NotI-R, 5′-GCA AGC CCC CCG CTG AAA GCA CAT TGT CTT TGG GTA C-3′). The second PCR was performed using MPGR6A-Xhol-F and 5.24-NotI-R. The PCR product was gel-purified, digested with Xhol/NotI, and used to replace the Xhol/NotI fragment in the c-myc-tagged mouse GPCR6A-pcDNA3.0 construct (2). All PCR products were sequenced to ensure that the correct sequence was used in the final expression construct.

Truncated Soluble Protein Preparation and Radioligand Binding Assay—The preparation of soluble truncated 5.24 receptor and truncated CaSR and radioligand binding assay were performed as described previously (20). Briefly, the culture medium for mock, truncated 5.24, or truncated CaSR-transfected cells was collected and centrifuged at 4,800 × g for 15 min at 4 °C. The culture medium was replaced with Opti-MEM. The supernatant was collected at 4 °C in 3 × 1 liter over 24 h in binding buffer (50 mM Tris, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride. The samples were centrifuged at 48,000 × g for 45 min at 4 °C and subjected to radioligand binding or stored at −70 °C. The radioligand binding assay was conducted in a total volume of 250 μl. For the truncated 5.24 receptor, 200 μl of soluble protein was mixed with 20 μl of 50 nM of [3H]GHS (final concentration 50 nM) and 10 nM of unlabeled GSH (final concentration 1 μM). For truncated CaSR, the assay condition was the same as for the truncated 5.24 receptor, except the assay buffer contained 0.75 mM CaCl2. The mixture was incubated for 1 h on ice with shaking; 20 μl of γ-globulin (17 mg/ml) and 200 μl of 30% cold polyethylene glycol were added to each sample. The mixture was incubated on ice for 4 min and centrifuged at 13,200 × g for 4 min. The pellet was washed once with cold 15% polyethylene glycol and solubilized overnight in 500 μl of 1 M NaOH. The samples were counted on a Parkard Tricarb liquid scintillation counter.

Calcium Release Assay—For analysis of the 5.24 receptor, and the 5.24/GPCR6A and GPCR6A/5.24 chimeras, the functional assay was performed as described previously (20). For analysis of the CaSR and the Ca-Glu-Glu CaSR/mGlur1 chimera, the same procedure was used with several modifications. The buffer for Fluo-4 loading and cell washing was a low [Ca2+]2 buffer (20 mM HEPES, pH 7.4, 146 mM NaCl, 5 mM
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KCl, 1 mM MgCl₂, 0.5 mM CaCl₂, 1 mg/ml bovine serum albumin, and 1 mg/ml glucose. For activation of the CaSR and the Ca-Glu-Glu CaSR/mGluR1 chimera, HEK-293 cells were exposed to buffer containing an additional 0.25 mM Ca²⁺ alone (total [Ca²⁺] = 0.75 mM), or 0.25 mM Ca²⁺ plus various concentrations of the test compounds, and the responses were recorded on a FLEXstation scanning benchtop fluorimeter (Molecular Devices Corp.), as described previously by Kuang et al. (20). The GraphPad Prism 3.0 software was used to plot fluorescence intensities and calculate the EC₅₀ values.

RESULTS

A Comparison of the Binding Pockets of Selected Family C G-protein-coupled Receptors—Homology models of the extracellular domains of several subtypes of Family C receptors including the CaSR, the 5.24 receptor, and GPRC6A were constructed using the closed form of the crystal structure of mGluR1 as the template. A comparison of the putative amino acid binding pockets of these receptors is shown in Fig. 1. Both the shape and size of the pockets differ considerably in the four receptors. In terms of size, the rank order from the smallest to the largest was mGluR1 < GPRC6A < 5.24 receptor < CaSR. In silico docking of the preferred ligands for mGluR1 (L-glutamate) and GPRC6A (L-arginine) indicated a relatively tight fit within the binding pockets. In contrast, docking of one of the preferred amino acid ligands at the 5.24 receptor (l-arginine) and the CaSR (L-phenylalanine) indicated that the pockets of these two receptors encompassed additional space not occupied by the bound amino acid. This observation led us to investigate the possibility that additional larger ligands such as peptides may interact at some Family C receptors. An in silico docking analysis of several endogenous peptides suggested that GSH could be accommodated in the 5.24 amino acid binding pocket in a conformation different from that of arginine (Fig. 2).

Activation of the 5.24 Chemosensory Receptor by GSH—To test the ability of GSH to act as an agonist at the fish 5.24 receptor, HEK-293 cells were transiently transfected with a c-myc-tagged expression construct and analyzed in a functional assay 48 h post-transfection. Both the 5.24 receptor and GPRC6A couple to the Gₛ family of G-protein α subunits and activate phospholipase C after agonist stimulation; therefore responses to these receptors can be assessed by measuring the release of intracellular calcium in cells loaded with the calcium-sensitive dye Fluo-4. Arginine, which was used as a positive control, and GSH (Fig. 3A) induced rapid responses in HEK-293 cells expressing the 5.24 receptor; the EC₅₀ values for arginine and GSH at the 5.24 receptor were 3.7 ± 1.9 and 70 ± 21 μM, respectively (Fig. 3B). In addition to GSH (reduced glutathione), the oxidized form of glutathione (GSSG), and two intermediate products of glutathione, γ-Glu-Cys and Cys-Gly, and a modified glutathione analogue, S-nitrosoglutathione were also tested on the 5.24 receptor. Rapid dose-dependent increases in fluorescence were also observed in cells expressing the 5.24 receptor after exposure to γ-Glu-Cys and S-nitrosoglutathione (EC₅₀ values = 98 ± 19 and 52 ± 10 μM, respectively (Fig. 3B). Thus, GSH, γ-Glu-Cys, and S-nitrosoglutathione showed affinities for the 5.24 receptor in the 50–100 μM range that were about 20-fold lower than L-arginine. In contrast, neither GSSG nor the Cys-Gly dipeptide had any effect on the 5.24 receptor.

In contrast to the 5.24 receptor, at concentrations of up to 5 mM, GSH did not activate mouse GPRC6A, the putative mammalian homolog of the 5.24 receptor. To confirm that GSH acts as an agonist at 5.24 but not GPRC6A, and to establish that the effects of GSH on the 5.24 receptor were mediated by determinants in the extracellular domain, we examined two receptor chimeras. A chimera consisting of the extracellular domain of GPRC6A ligated to the transmembrane region and carboxyl terminus of 5.24 was tested with both arginine and GSH. Arginine activated this receptor with an EC₅₀ = 130 ± 18 μM, but no activation was seen with GSH at concentrations of up to 5 mM. The reciprocal chimera 5.24/GPRC6A (2) possessing the extracellular ligand binding domain of the 5.24 receptor ligated to the heptahelical and carboxyl terminus of GPRC6A was also tested. Although GSH had no effect on the full-length
GPRC6A receptor at concentrations up to 5 mM, GSH activated the 5.24/GPRC6A chimera with an EC50 of 32 μM (Fig. 3C). Together, these results demonstrate that GSH acts as an agonist at the 5.24 receptor but not at its putative mammalian homolog, GPRC6A, and that the effects on 5.24 are mediated via the extracellular amino acid binding domain of the receptor.

To directly examine the binding of GSH to the 5.24 receptor, we developed a radioligand binding assay using [3H]GSH. Preliminary experiments conducted on membranes prepared from mock-transfected HEK-293 cells indicated the presence of a high level of [3H]GSH background binding, similar to what we had previously observed in membrane fractions from mock-transfected HEK-293 cells using [L-3H]lysine and [L-3H]arginine (20). To circumvent this problem, we tested the truncated version of the 5.24 receptor in which the extracellular domain is expressed independently of the heptahelical and carboxyl-terminal domains as soluble protein secreted into the culture medium of transfected cells (20). Specific [3H]GSH binding was detected in the cell culture medium from cells transfected with the truncated 5.24 receptor, whereas very little binding was seen in the medium collected from mock-transfected cells (Fig. 3D). Further experiments were conducted on the secreted extracellular domain of the 5.24 receptor. Competition experiments using 35 nM [3H]GSH and various concentrations of unlabeled GSH gave an IC50 value of 48 μM (n = 2).

Since both the radioligand binding experiments on the truncated 5.24 receptor and the 5.24/GPRC6A chimeric receptor results confirmed the postulate that GSH was mediating its effects via the extracellular ligand binding domain, a series of point mutations in the putative ligand binding pocket of the 5.24 receptor were generated to further probe the docking orientation of GSH in the binding site. The 5.24 homology model was used as a guide for choosing candidate residues for mutation, and the mutants were then examined in the calcium release assay. The model indicated that threonine 175 and aspartate 309 in the 5.24 receptor interact with the amino group of GSH and serine 152 make a contact with the carboxyl group of GSH. No responses were observed with the T175A and D309A mutants for L-arginine or GSH at concentrations up to 5 mM (Table 1). The S152A mutant displayed a 26-fold lower affinity for L-arginine than wild-type receptor, while GSH produced only a very small response at 1 mM. These results suggested that the amino and carboxyl groups of glutamate within the GSH mol-

![FIGURE 3. Activation and radioligand binding of GSH and GSH analogues to the 5.24 chemosensory receptor. A, example of fluorescence intensity after exposure of the 5.24 receptor to various concentrations of GSH, B, activation curves for the 5.24 receptor after exposure to various concentrations of L-arginine, GSH, γ-Glu-Cys, and S-nitroso glutathione (GSNO). The EC50 values were 3.7 ± 1.9, 70 ± 21, 98 ± 19, and 52 ± 10 μM for L-arginine, GSH, γ-Glu-Cys, and GSNO, respectively (n = 3–6). C, activation curves for 5.24/GPRC6A chimera receptor. The EC50 values were 3.1 ± 0.6 and 4.7 μM for L-arginine and GSH, respectively (n = 3). No responses to GSH, γ-Glu-Cys, and GSNO were seen in mock-transfected cells at concentration up to 5 mM. D, binding of [3H]GSH in the supernatant fraction from mock-transfected HEK cells and cells expressing the secreted truncated 5.24 receptor. The binding assays were conducted using 50 nM [3H]GSH (mean ± S.E., n = 3). The histogram indicates the relative levels of specific [3H]GSH binding in mock-transfected cells and cells expressing the truncated 5.24 receptor. The ratio of specific counts/min to blank counts/min in the truncated 5.24 receptor was ~80-fold higher than that in mock-transfected cells. The band above the histogram indicates protein expression on an immunoblot using an anti-c-myc monoclonal antibody (estimated relative Mr ~110 for the dimeric form shown).]
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The S111A mutant displayed an affinity for L-arginine and GSH that was 52-fold higher than that on mock (Fig. 5A). Specific [3H]GSH binding was detected in the absence of Ca2+ in assay buffer (50 mM Tris acetate, pH 7.4), whereas in the presence of 0.75 mM Ca2+, specific [3H]GSH binding was detected in culture media collected from cells transfected with the truncated CaSR; the ratio of specific counts/min to blank counts/min on truncated CaSR was 52-fold higher than that on mock (Fig. 5B); unlabeled GSH inhibited [3H]GSH binding with an IC50 = 153 ± 36 nM. Thus, both the [3H]GSH binding experiments, and the results with the Ca-Glu-Glu chimera demonstrated that GSH acts within the extracellular domain of the CaSR.

GSH Does Not Activate Other Family C G-protein-coupled Receptors—In addition to the 5.24 receptor, GPRC6A, and the CaSR, we also tested whether GSH could activate mGluR1, mGluR2, and mGluR4 receptors (19) representing Group I, II, and III mGluRs in the functional fluorescence-based assay. Since mGluR1 couples to phospholipase C, these cells were tested directly, whereas the mGluR2- and mGluR4-expressing cells were co-transfected with the promiscuous G-protein Ga15 to switch the signal transduction from inhibition of cAMP to stimulation of phospholipase C (21–23). Despite activation of all three receptors by L-glutamate, no activation by GSH was observed at concentrations of up to 5 mM nor was any
type for calcium responses, albeit with reduced maximal responses (18). Using the same protocol described above (0.5 + 0.25 mM Ca2+), both GSH and phenylalanine potentiated the calcium-induced responses on the Ca-Glu-Glu receptor; the EC50 values were 0.0048 ± 0.0004 and 108 ± 15 μM for GSH and phenylalanine, respectively (Fig. 5A).

To further confirm that GSH acts via the extracellular region of the CaSR, a truncated form of rat CaSR analogous to the truncated 5.24 receptor was transfected into HEK-293 cells, and [3H]GSH binding was conducted on the cell culture medium containing the secreted truncated CaSR. No specific [3H]GSH binding was detected in the absence of Ca2+ in assay buffer (50 mM Tris acetate, pH 7.4), whereas in the presence of 0.75 mM Ca2+, specific [3H]GSH binding was detected in culture media collected from cells transfected with the truncated CaSR; the ratio of specific counts/min to blank counts/min on truncated CaSR was 52-fold higher than that on mock (Fig. 5B); unlabeled GSH inhibited [3H]GSH binding with an IC50 = 153 ± 36 nM. Thus, both the [3H]GSH binding experiments, and the results with the Ca-Glu-Glu chimera demonstrated that GSH acts within the extracellular domain of the CaSR.

Reduced and Oxidized Glutathione Potentiate the CaSR—The molecular model of the CaSR indicated that the amino acid binding pocket should be sufficiently large to accommodate GSH or GSSG. However, no responses were observed in HEK-293 cells transfected with the CaSR in the calcium release assay after exposure to GSH or GSSG at concentrations up to 5 mM. We hypothesized that GSH (and GSSG) might potentiate the CaSR as observed with large aromatic amino acids such as tryptophan and phenylalanine (5, 8). Aromatic amino acids have been shown to potentiate the CaSR-induced suppression of the secretion of parathyroid hormone from human parathyroid cells, and the threshold for this effect is around 0.5 mM calcium (8). Therefore, in the experiments reported here, submaximal calcium concentrations were used; the cells were preincubated in buffer containing low calcium (0.5 mM) and then exposed to buffer containing an additional 0.25 mM Ca2+ to activate the receptor (under these conditions the range of the increase in fluorescence units above baseline was 2000–4000 units). Upon exposure to 0.25 mM Ca2+ + 10 μM GSH, 10 μM GSSG, or 5 mM phenylalanine, the fluorescence responses were further increased by 2.5-, 1.6-, and 2.9-fold, respectively, compared with the responses produced by 0.25 mM calcium only (Fig. 4A). Additional dose-response experiments were performed using the same submaximal 0.5 + 0.25 mM [Ca2+] protocol; the EC50 values for GSH, GSSG, and phenylalanine under these conditions were 0.083 ± 0.013, 0.33 ± 0.07 and 299 ± 36 μM, respectively (Fig. 4B), indicating that GSH and GSSG are potent potentiators of the calcium induced responses of the CaSR. No significant shift in the EC50 was observed in the presence of GSH; the EC50 value for calcium in the absence of GSH was 1.26 ± 0.05 mM (n = 3), while it was 1.15 mM ± 0.01 mM (n = 4) in the presence of 0.5 or 1 mM GSH (p > 0.05, t test). A small (less than 2-fold) shift to higher affinity was previously observed with the CaSR in the presence of some amino acids (5).

The Effects of GSH on the CaSR Are Mediated via the Extracellular Domain—The effects of GSH were also tested on a chimeric receptor, which contained the extracellular domain of CaSR and the cysteine-rich and transmembrane domains of mGluR1. This chimera, called the Ca-Glu-Glu receptor, has been shown to possess a wild-type CaSR phenotype for calcium responses, albeit with reduced maximal responses (18). Using the same protocol described above (0.5 + 0.25 mM Ca2+), both GSH and phenylalanine potentiated the calcium-induced responses on the Ca-Glu-Glu receptor; the EC50 values were 0.0048 ± 0.0004 and 108 ± 15 μM for GSH and phenylalanine, respectively (Fig. 5A).

To further confirm that GSH acts via the extracellular region of the CaSR, a truncated form of rat CaSR analogous to the truncated 5.24 receptor was transfected into HEK-293 cells, and [3H]GSH binding was conducted on the cell culture medium containing the secreted truncated CaSR. No specific [3H]GSH binding was detected in the absence of Ca2+ in assay buffer (50 mM Tris acetate, pH 7.4), whereas in the presence of 0.75 mM Ca2+, specific [3H]GSH binding was detected in culture media collected from cells transfected with the truncated CaSR; the ratio of specific counts/min to blank counts/min on truncated CaSR was 52-fold higher than that on mock (Fig. 5B); unlabeled GSH inhibited [3H]GSH binding with an IC50 = 153 ± 36 nM. Thus, both the [3H]GSH binding experiments, and the results with the Ca-Glu-Glu chimera demonstrated that GSH acts within the extracellular domain of the CaSR.

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Table 1: EC50 values for wild-type and mutant 5.24 receptors

| Receptor | 1-Arg EC50 (μM) | GSH EC50 (μM) |
|----------|----------------|--------------|
| F5.24-WT | 3.7 ± 1.9 (n > 3) | 70 ± 21 (n > 3) |
| T175A    | NR (n = 2) | NR (n = 2) |
| D309A    | NR (n = 2) | NR (n = 2) |
| S152A    | 98 ± 36 (n = 2) | >1 mM (n = 2) |
| S111A    | 3.3 ± 0.5 (n = 3) | 64 ± 11 (n = 3) |
| K74A     | 2.2 ± 0.7 (n = 2) | >500 (n = 2) |

* NR, no response.

FIGURE 4: Activation of the CaSR by calcium and potentiation of the calcium responses by GSH and GSH analogs. A, relative maximal responses of the CaSR to Ca2+ alone, Ca2+ + GSH, Ca2+ + GSSG, and Ca2+ + phenylalanine. The cells were preincubated in low calcium buffer (0.5 mM calcium) and then exposed to buffer containing an additional 0.25 mM Ca2+ to activate the CaSR or to 0.25 mM Ca2+ + 10 μM GSH, 0.25 mM Ca2+ + 10 μM GSSG, or 0.25 mM Ca2+ + 5 mM phenylalanine. The data (mean ± S.E.; n = 3–6) were normalized as a percentage relative to the response to Ca2+ only (100%). B, dose–response curves for GSH, GSSG, and phenylalanine at the CaSR. The EC50 values were 0.003 ± 0.013, 0.33 ± 0.07, and 299 ± 36 μM for GSH, GSSG, and phenylalanine, respectively (n = 3–6).

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potentiation of the glutamate-induced responses observed (data not shown). Glutathione was also tested on the GABA receptor. For these experiments the GABAA_R1 and R2 subunits were co-transfected together with the chimeric G-protein, Galpha9. Robust activation of the receptor was observed with 10 μM GABA but not with 100 μM GSH; moreover, GSH did not potentiate GABA-induced responses at submaximal concentrations of GABA (data not shown).

**DISCUSSION**

We demonstrate for the first time that GSH acts as both an agonist and potentiator of G-protein-coupled receptors. The peptide is an orthosteric agonist at the fish 5.24 amino acid-activated chemosensory receptor and a potent enhancer of the mammalian CaSR. The effects of GSH appeared to be selective among Family C receptors; highly related receptor and a potent enhancer of the mammalian CaSR. The effects of orthosteric agonist at the fish 5.24 amino acid-activated chemosensory and potentiator of G-protein-coupled receptors. The peptide is an agonist at submaximal concentrations of GABA (data not shown).

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FIGURE 5. A, dose-response curves from the calcium release assay for GSH and phenylalanine at the Ca-Glu-Glu CaSR/mGluR1 chimeric receptor. The EC50 values were 0.0048 ± 0.0004 (n = 5) and 108 ± 15 μM (n = 3) for GSH and phenylalanine respectively. B, binding of [3H]GSH (50 nM) in cell culture media collected from mock-transfected HEK-293 cells and from cells expressing the secreted truncated CaSR. The data are the means ± S.E. of three experiments. The histogram depicts the relative levels of specific [3H]GSH binding in mock-transfected cells and cells expressing the truncated CaSR. The ratio of specific counts/min to blank counts/min in the truncated CaSR was ~50-fold higher than that in mock-transfected cells. The band above the histogram indicates the protein expression level from an immunoblot labeled with an anti-CaSR monoclonal antibody (estimated M, ~90).

The observation that specific [3H]GSH binding was present in the culture medium of HEK-293 cells transfected with a construct encompassing only the extracellular domain of the 5.24 receptor, together with the results of the chimeric 5.24/GPRC6A receptors, demonstrated that GSH binds to a site within the extracellular ligand binding domain and not in the transmembrane heptahelical domain. A more refined analysis entailing the use of homology modeling, in silico peptide docking, and the characterization of a series of point mutations in the binding pocket, provided insight into the possible orientation of GSH in the 5.24 receptor binding pocket.

Amino acid ligands bind to Family C receptors in part via bonding interactions between the free α amino and α carboxyl groups of the ligands and a set of highly conserved residues in the binding pocket. In the structure of GSH, the α carboxyl group (rather than the α amino group) of the glutamate residue is used to form the peptide bond with the adjacent cysteine residue in the γ-Glu-Cys-Gly tripeptide. Thus, the free α amino and α carboxyl groups on the glutamate in GSH allow this residue in the peptide to establish the same bonding interactions with the same set of conserved residues in the binding pocket that mediate the binding of amino acid ligands. However, compared with the preferred 5.24 amino acid ligand, arginine, where the extended side chain likely binds in a linear conformation (20, 25), our docking experiments with GSH suggest that the peptide may bind in a “bent” conformation whereby the terminal glycine residue in GSH protrudes upward into the pocket; the results of the 5.24 binding pocket mutants are compatible with this proposed configuration which is depicted in Fig. 2.

At the CaSR, GSH did not activate the receptor directly but it did potentiate the calcium-induced responses. The effects of GSH were similar to those reported previously for aromatic amino acids; however, GSH appears to be much more potent. Whereas phenylalanine displays effects in the high micromolar to low millimolar range depending upon the amino acid and the calcium concentration used in the assay (5, 8), the effects of GSH on the rat CaSR seen here are in the submicromolar range.

Our results show that GSH also acts within the extracellular domain of the CaSR. The work of Silve et al. (26) indicates that the calcium binding site in the CaSR is located adjacent to and in close proximity to the amino acid binding site in the extracellular domain of the receptor. We propose that the free amino acids, GSH may act in conjunction with divalent cation ligands to promote closure of the extracellular Venus fly trap domain and initiate receptor activation. Interestingly, the oxidized dimeric form of GSH, GSSG, also potentiated the CaSR, albeit with lower potency than GSH. At first glance, the ability of GSSG to bind within the amino acid binding pocket of the CaSR seems surprising, since the large size of the GSSG dimer would seem to preclude binding at this site. However, the docking analysis indicated that the putative amino acid pocket in the CaSR is sufficiently large to accommodate the dimer (see supplemental Fig. S1).

What are the potential biological ramifications of GSH actions on Family C G-protein-coupled receptors? The 5.24 chemosensory receptor is expressed in the olfactory epithelium, gill, lips, and tongue of the fish and is thought to play a role in feeding and navigation (1). Our observations suggest that the 5.24 receptor may also be used by fish to detect GSH released into the aqueous environment from plants or animals. In various cnidarian organisms such as several species of hydra, GSH acts a stimulus for feeding (27–29). We speculate that the cnidarian homolog of the fish 5.24 receptor may mediate this effect. In teleost and elasmobranch fish, the CaSR appears to play a role as a salinity

logical functions and pharmacological properties of related members of a receptor family.

Glutathione was also tested on the GABA_B receptor. For these experiments the GABAA_R1 and R2 subunits were co-transfected together with the chimeric G-protein, Galpha9. Robust activation of the receptor was observed with 10 μM GABA but not with 100 μM GSH; moreover, GSH did not potentiate GABA-induced responses at submaximal concentrations of GABA (data not shown).

**DISCUSSION**

We demonstrate for the first time that GSH acts as both an agonist and potentiator of G-protein-coupled receptors. The peptide is an orthosteric agonist at the fish 5.24 amino acid-activated chemosensory receptor and a potent enhancer of the mammalian CaSR. The effects of GSH appeared to be selective among Family C receptors; highly related receptors including GPRC6A, the putative mammalian homolog of the fish 5.24 receptor, the mGluRs, and the GABAA_R1 receptor were neither directly activated nor potentiated by the peptide. Importantly, this restricted spectrum of GSH activation among Family C receptors was directly activated nor potentiated by the peptide. Importantly, this restricted spectrum of GSH activation among Family C receptors was markedly smaller than that of the CaSR and the 5.24 receptor (Fig. 1). The smaller volumes of the mGluR1 and GPRC6A pockets likely preclude the binding of relatively large molecules such as GSH. It is intriguing that the volumes and GSH activation profiles of the CaSR. The work of Silve et al. (26) indicates that the calcium binding site in the CaSR is located adjacent to and in close proximity to the amino acid binding site in the extracellular domain of the receptor. We propose that the free amino acids, GSH may act in conjunction with divalent cation ligands to promote closure of the extracellular Venus fly trap domain and initiate receptor activation. Interestingly, the oxidized dimeric form of GSH, GSSG, also potentiated the CaSR, albeit with lower potency than GSH. At first glance, the ability of GSSG to bind within the amino acid binding pocket of the CaSR seems surprising, since the large size of the GSSG dimer would seem to preclude binding at this site. However, the docking analysis indicated that the putative amino acid pocket in the CaSR is sufficiently large to accommodate the dimer (see supplemental Fig. S1).

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sensor (30), whereas in humans, a well established role for this receptor is the negative regulation of parathyroid hormone secretion. The CaSR-mediated suppression of parathyroid hormone secretion forms the therapeutic basis for the drug Cinacalcet, a positive allosteric modulator of the CaSR approved for treatment of hyperparathyroidism (31). A key question is whether GSH could in fact modulate CaSR activity in cells, as demonstrated previously by the finding that amino acids potentiate CaSR activity in acutely isolated human parathyroid cells (8). In contrast to the very high intracellular levels of GSH in mammalian cells (0.5–5 mM; Refs. 32–34), the extracellular concentrations of GSH in most tissues and in blood plasma are very low, typically in the range of 2–20 μM (32, 34). Our data suggest that the potency of GSH on the CaSR is within the range that could affect the regulation of CaSR-mediated responses in cells and tissues. Although our estimate of the EC₅₀ value for GSH at the CaSR is in the submicromolar range, suggesting that the site could be saturated in vivo, a number of factors argue against this point. These include the fact that our data were generated using the highly expressed cloned receptor and therefore the estimate of affinity may be different from those in vivo and that the extracellular GSH concentrations in tissues likely fluctuate. Moreover, the putative endogenous levels of some neurotransmitters are near or above the affinity constants calculated for their cognate receptors (e.g. glutamate and the mGluR3 and mGluR4 subtypes of mGluRs). In addition to the parathyroid gland and the brain, the CaSR is also expressed in the gastrointestinal tract where it has been suggested to play a role as a nutrient sensor (35, 36). As food passes through the intestinal tract, cells lining the tract are exposed to widely fluctuating concentrations of amino acids and peptides such as GSH; we propose that GSH might also be an important determinant in regulating CaSR responses in this organ.

Finally, we note that in addition to the well characterized roles of GSH in the body relating to modulation of intracellular redox reactions and the conjugation of xenobiotics, there have also been reports of a GSH determinant in regulating CaSR responses in this organ.

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