Elucidation of a Novel Extracellular Calcium-binding Site on Metabotropic Glutamate Receptor 1α (mGluR1α) That Controls Receptor Activation*§

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Metabotropic glutamate receptor 1α (mGluR1α) exerts important effects on numerous neurological processes. Although mGluR1α is known to respond to extracellular Ca2+ ([Ca2+]o) and the crystal structures of the extracellular domains (ECDs) of several mGluRs have been determined, the calcium-binding site(s) and structural determinants of Ca2+-modulated signaling in the Glu receptor family remain elusive. Here, we identify a novel Ca2+-binding site in the mGluR1α ECD using a recently developed computational algorithm. This predicted site (comprising Asp-318, Glu-325, and Asp-322 and the carboxylate side chain of the receptor agonist, Glu) is situated in the hinge region in the ECD of mGluR1α adjacent to the reported Glu-binding site, with Asp-318 involved in both Glu and calcium binding. Mutagenesis studies indicated that binding of Glu and Ca2+ to their distinct but partially overlapping binding sites synergistically modulated mGluR1α activation of intracellular Ca2+ ([Ca2+]i) signaling. Mutating the Glu-binding site completely abolished Glu signaling while leaving its Ca2+-sensing capability largely intact. Mutating the predicted Ca2+-binding residues abolished or significantly reduced the sensitivity of mGluR1α not only to [Ca2+]o and Gd33+- but also, in some cases, to Glu. The dual activation of mGluR1α by [Ca2+]o and Glu has important implications for the activation of other mGluR subtypes and related receptors. It also opens up new avenues for developing allosteric modulators of mGluR function that target specific human diseases.

Metabotropic glutamate receptors (mGluRs)² have key functions in a variety of different neurological processes, including memory, learning, pain, synaptic plasticity, and the control of the activity of various circuits throughout the brain (1). The mGluRs belong to family C of the large superfamily of G protein-coupled receptors (GPCRs). Family C GPCRs (also referred to as family 3 GPCRs, the nomenclature that will be utilized here) also include the Ca2+-sensing receptor (CaSR), GABA_B receptors, taste receptors, and putative pheromone receptors (2). All members of the family 3 GPCRs share similar domain architecture, including venus flytrap-like extracellular domains (ECD), heptahedral transmembrane domains, and intracellular C-terminal C-tails. The mGluRs fall into three groups and eight subtypes. Group I comprises mGluR1 and mGluR5 (3). mGluR1 is expressed mainly around a core of ionotropic glutamate receptors in the postsynaptic densities of neurons and functions as a disulfide-linked homodimer (4).

Upon activation by its agonists, the intracellular domains of the group I mGluRs associate with the G protein Gq/11 to activate phospholipase C, which subsequently converts phosphatidylinositol bisphosphate (PIP2) to diacylglycerol and inositol trisphosphate (IP3), thereby releasing Ca2+ from the endoplasmic reticulum, as well as activating protein kinase C (PKC) and other downstream effectors (5).

The issues of whether mGluRs respond to extracellular calcium ([Ca2+]o) and how calcium binding modulates the family 3 GPCRs have attracted extensive investigation. On the basis of sequence homology to CaSR, mGluRs were postulated to be capable of responding to [Ca2+]o. [Ca2+]o has been proposed to either activate mGluR1 directly or to act as a positive mGluR1 modulator (6, 7). Kubo * et al. (6, 8) reported that [Ca2+]o, as well as Glu, can trigger intracellular responses elicited by mGluR1, mGluR3, and mGluR5. [Ca2+]o or Gd3+ further stimulate the activity of mGluR1α even after saturation of the Glu response and vice versa (6). In addition, mGluR1α responds to 5 mM [Ca2+]o in Purkinje cells prepared from global mGluR1α knock-out mice in which the receptor has been specifically knocked into Purkinje cells, whereas the Purkinje cells from the mGluR1α global knock-out mice themselves cannot sense [Ca2+]o (9, 10). On the basis of these studies, [Ca2+]o is postulated to mediate postsynaptic efficacy through it action on mGluR1 (11). Moreover, Glu triggers [Ca2+]o oscillations in a manner that is modulated by [Ca2+]o (12), as R, 5-3, 5-Dihy-
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droxylphenylglycine, an agonist of group I mGluRs, generated inward currents that were enhanced by [Ca\(^{2+}\)]\(_o\) (10). In contrast, Nash et al. (13) concluded that mGluR1α is not a calcium-sensing receptor because its response to the agonist L-quisqualate is not sensitive to [Ca\(^{2+}\)]\(_o\). However, the effect of [Ca\(^{2+}\)]\(_o\) on the EC\(_{50}\) for quisqualate was not examined. Any putative Ca\(^{2+}\)-binding sites capable of regulating mGluR signaling remain “invisible” in six crystal structures of the ECD of mGluR1α determined to date (14, 15), as well as the ECDs and cysteine-rich domains of mGluR3 and mGluR7 (15, 16). One Gd\(^{3+}\) ion binds to mGluR1 between the helices of lobes 2 (LB2) at the dimer interface of the ECD, far from the Glu-binding site (14, 17). Removing the Gd\(^{3+}\)-binding residue, E238Q, eliminated sensitivity to Gd\(^{3+}\) but not sensitivity to [Ca\(^{2+}\)]\(_o\) and Glu (17, 18). Two Gd\(^{3+}\) ions visible in the crystal structure were ignored by these authors, although one of them is located near the critical hinge region coordinated by Asp-322, Asp-324, and Asp-325 at the predicted site (20–22). MUG is a graphic geometry-based Ca\(^{2+}\) algorithm recently developed the MUG (multiple geometries) algorithm (23). Two Gd\(^{3+}\) ions visible in the crystal structure were ignored by these authors, although one of them is located near the critical hinge region coordinated by Asp-322, Asp-324, and Asp-325 at the predicted site (20–22). MUG is a graphic geometry-based Ca\(^{2+}\) algorithm recently developed, with the MUG algorithm (23) developed by our laboratory. The Ca–O distance in the software was set to 1.6–3.1 Å with a set average cutoff of 2.4 Å (26, 27), and the O–O distance was set to 6.0 Å (21). Side chain atoms were rotated to accommodate Ca\(^{2+}\)-induced local conformational changes (48). Furthermore, electrostatic surface potential maps were constructed using Delphi (28), and GRASP (29) was then used to render and modify the image. The linear, putative Ca\(^{2+}\)-binding site was added into the scaffold protein CD2 between Ser-52 and Gly-53 with triple Gly linkers at both ends, and the combined grafting model was generated by Modeler 9v4 (30).

**EXPERIMENTAL PROCEDURES**

**Computational Prediction of Ca\(^{2+}\)-binding Sites in mGluR1α and Molecular Modeling**—The three-dimensional coordinates of the crystal structures of the ECD of mGluR1α were obtained from the PDB (PDB entry codes: 1EWT, 1EWK (15), and 1ISR (14)). Hydrogen atoms were added using the Sybyl7.2 package (Tripos Inc., St. Louis, MO). The identification of putative Ca\(^{2+}\)-binding sites in the ECD of mGluR1α was performed using MUG, a graph theory-based algorithm (21) developed by our laboratory. The Ca–O distance in the software was set to 1.6–3.1 Å with a set average cutoff of 2.4 Å (26, 27), and the O–O distance was set to 6.0 Å (21). Side chain atoms were rotated to accommodate Ca\(^{2+}\)-induced local conformational changes (48). Furthermore, electrostatic surface potential maps were constructed using Delphi (28), and GRASP (29) was then used to render and modify the image. The linear, putative Ca\(^{2+}\)-binding site was added into the scaffold protein CD2 between Ser-52 and Gly-53 with triple Gly linkers at both ends, and the combined grafting model was generated by Modeler 9v4 (30).

**Plasmid, Expression, and Purification of Proteins**—The predicted linear Ca\(^{2+}\)-binding site, termed mGluR1-1, resides between Gly-316 and Gly-337 (GSDGWADRDEVIEGYLEVEANGG). This sequence, grafted into CD2 between Ser-52 and Gly-53 in the plasmid pGEX-2T-CD2 (31), was named CD2.D1. The engineered protein was expressed as a GST fusion protein and purified using GS4B resin as described (32). Site-directed mutagenesis was performed using the multisite-directed mutagenesis kit (Stratagene, Cedar Creek, TX).

**Tb\(^{3+}\) Titration and Ca\(^{2+}\) Competition**—In Trp-sensitized Tb\(^{3+}\)-LRET experiments, emission spectra from 500 to 580 nm were recorded with excitation set at 282 nm; slit widths were set at 8 nm for excitation and 12 nm for emission. A glass filter with a cutoff of 320 nm was utilized to circumvent secondary Rayleigh scattering. Tb\(^{3+}\) titration and metal competition assays were performed as described previously (24). 500 mM K\(^{+}\), 10 μM La\(^{3+}\), 10 μM Gd\(^{3+}\), 1 mM Mg\(^{2+}\), and 1 mM Ca\(^{2+}\), respectively, was used to selectively compete with Tb\(^{3+}\). Each experiment was carried out independently in triplicate.

**Quantitatively Determined Membrane Expression of the mGluR1α Mutants Using Flow Cytometry**—PcDNA-mGluR1α (donated by Dr. Randy Hall’s laboratory) contained a FLAG tag at the N terminus of the receptor, and mCherry was genetically fused to the C terminus with a linker, GGNGSG. After 2 days of transient expression of mGluR1α and its mutants (D318I, D322I, E325I, and N335I) in HEK293 cells grown on polylsine-coated dishes, cells were incubated in 1× phosphate-buffered saline (PBS) supplemented with 1/1000 anti-FLAG and 1/100 fetal bovine serum (FBS) at 4 °C. The cells were then washed three times with 1× Tris-buffered saline (TBS) and fixed using 4% formaldehyde at room temperature for 15 min. After being washed three times with 1× TBS, the receptors on the cell sur-
face were then labeled with Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen) for 30 min at 37 °C. The cells were then collected in 1× PBS, and the intensity of green and red fluorescence was measured using LSRFortessa (BD Biosciences). The ratios of green and red fluorescence from mGluR1α and the mutated receptors were normalized to the amount of receptors expressing on the cell surface relative to total receptors (total cellular expression of receptor). Data were collected from three dishes.

**Measurement of [Ca\(^{2+}\)]\(_i\), Responses of mGluR1α and Its Mutants with or without [Ca\(^{2+}\)]\(_o\), or Glu**—Measurement of [Ca\(^{2+}\)]\(_i\), was performed as described (24). In brief, wild type mGluR1α and its mutants (D318I, D322I, D324I, E325I, and E328I) were transiently transfected into HEK293 cells and cultured for 2 additional days. The cells on the coverslips were subsequently loaded using 4 μM Fura-2 AM in 2 ml of physiological saline buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.55 mM MgCl₂, and 1 mM CaCl₂, pH 7.4). The coverslips were mounted in a bathing chamber on the stage of a fluorescence microscope. Fura-2 emission signals from single cells excited at 340 or 380 nm were collected utilizing a Leica DM6000 fluorescence microscope and its liganding groups in 144 calcium-binding proteins was measured as described above during changes in [Ca\(^{2+}\)]\(_i\), and/or Glu.

**Measurement of Intracellular Ca\(^{2+}\) Release Mediated by mGluR1α and Its Mutants in the Presence of Extracellular Gd\(^{3+}\)**—Changes in [Ca\(^{2+}\)]\(_i\), in response to the addition of Gd\(^{3+}\) were determined as just described. Specifically, cells were incubated in incubation buffer (140 mM NaCl, 4 mM KOH, 10 mM HEPES, 1.5 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, pH 7.4) for up to 1.5 h, and Gd\(^{3+}\) (made up in 140 mM NaCl, 4 mM KOH, 10 mM HEPES, and 0.3 mM MgCl₂, pH 7.4) was added at the concentrations described under “Results.” The [Ca\(^{2+}\)]\(_i\), responses of mGluR1α after the introduction of mutations in the Glu-binding site were measured similarly.

**Data Analysis and Curve Fitting**—At each agonist concentration, all of the transfected cells in the microscopic field from three independent experiments were selected for analysis, and at least 60% of the cells displaying normal responses were analyzed. The cells that did not respond to the agonists or displayed a sigmoidal curve with a stable plateau after treatment with high [Ca\(^{2+}\)]\(_i\) were excluded. These latter cells maintained a constant, high plateau of the intracellular Ca\(^{2+}\) concentration, perhaps because the plasma membrane was excessively permeable to Ca\(^{2+}\). To normalize the concentration response curves for the responses to [Ca\(^{2+}\)]\(_o\), the maximal response of wild type mGluR1α to extracellular Glu was set at 100% so that the maximal responses of mutant receptors to [Ca\(^{2+}\)]\(_o\), or Glu were transformed into percentages relative to the response of WT mGluR1α to Glu. Data were fitted using the Hill equation as described previously (23).

**RESULTS**

**Prediction of a Novel Ca\(^{2+}\)-binding Site Adjacent to the Glu-binding Site in the ECD of mGluR1α**—We recently developed the computational algorithm MUG, which predicts Ca\(^{2+}\)-binding sites using graph theory by identifying all possible liganding oxygen clusters and finding maximal cliques. The positions of Ca\(^{2+}\) and its liganding groups in 144 calcium-binding proteins can be predicted with 0.22–0.49 Å accuracy by geometric filters established on the basis of an extensive survey of known Ca\(^{2+}\)-binding sites in the Protein Data Bank (19). To accommodate Ca\(^{2+}\)-induced conformational changes, the side chains of putative Ca\(^{2+}\)-binding ligand residues were subjected to rotation using a rotamer library (MUGSR) (48). Fig. 1 shows one predicted Ca\(^{2+}\)-binding site identified here in the crystal structure of the mGluR1α ECD (PDBe entry code: 1EWK) using the MUG algorithm. Two other predicted sites not included in this report were also revealed by MUG, one of them (site 2) residing in the Mg\(^{2+}\)-binding pocket (Leu-86—Gly-102) inferred from the crystal structure and the other one located within a long loop (Asp-125—Lys-153) that was invisible in the crystal structure because of its high flexibility and was repaired using Modeler (30). The third predicted Ca\(^{2+}\)-binding site (site 3), encompassing Ser-129 to Gly-144, was present within this missing loop.
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The predicted Ca$^{2+}$-binding site studied in detail here comprises the carboxyl side chains of Asp-318 and Glu-325, the main chain carbonyl Asp-322 in a flexible loop of mGlur1α, and the carboxyl side chain of Glu-701 (a ligand for glutamate). This predicted Ca$^{2+}$-binding site is located at the hinge region in the ECD adjacent to the reported Glu-binding site (Arg-74, Ser-165, Thr-188, Asp-208, Tyr-236, Asp-318, and Lys-409) (15, 25), with Asp-318 predicted to be involved in both Glu and Ca$^{2+}$ binding. Thus, Ca$^{2+}$ and Glu, when bound to the receptor, both bind to Asp-318. Asp-318 and Asp-322 can be identified in the Glu-free form (PDB entry code: 1EWT), whereas the direct binding of Ca$^{2+}$ to the carboxyl side chain of the agonist Glu-701 is visualized only in the Glu-loaded form (PDB entry code: 1EWK). Thus, the agonist Glu provides an additional ligand for Ca$^{2+}$ when the former is bound to the receptor, which is very different from intracellular Ca$^{2+}$-binding trigger proteins such as calmodulin that lack any additional chelating groups from molecules other than the residues within the Ca$^{2+}$-binding protein itself, except for water. Fig. 1B shows that the predicted Ca$^{2+}$-binding pocket has a highly negatively charged surface as revealed by Delphi in the structures of three solved forms of the ECD within mGlur1α (Fig. 1B).

Obtaining Site-specific Ca$^{2+}$/Ln$^{3+}$-binding Affinities by a Grafting Approach—To probe the Ca$^{2+}$-binding capability of the predicted Ca$^{2+}$-binding site in mGlur1α, we utilized our grafting approach by inserting the protein sequence encompassing the putative mGlur1α Ca$^{2+}$-binding site into the host protein, CD2.D1 (denoted as CD2-mGlur1α-1). The inserted sequence contains all predicted Ca$^{2+}$-binding residues except Glu-701. This approach had previously enabled us to obtain site-specific Ca$^{2+}$-binding affinities of the EF-hand motifs from calmodulin and linear Ca$^{2+}$ binding sequences, free from the limitations of working with membrane proteins (32, 33). The putative mGlur1α Ca$^{2+}$-binding site was flanked by flexible triple-Gly linkers and inserted between Ser-52 and Gly-53 of CD2.D1 (Fig. 2A) to ensure a native-like conformation and close proximity (<15–20 Å) to Trp-32 in order to enhance the Tb$^{3+}$-LRET signal. Indeed, grafting the putative Ca$^{2+}$-binding loop from mGlur1α did not significantly change the secondary and tertiary structures of CD2, as revealed by circular

![A Novel Extracellular Calcium-binding Site on mGlur1α](image_url)
A Novel Extracellular Calcium-binding Site on mGluR1α

TABLE 1

| Proteins       | Mutations | Dissociation constant (Kd) |
|----------------|-----------|----------------------------|
| CD2.D1-1       | WT        | 49 ± 9 μM (Ca2+, 1.8 ± 0.1 mM) |
| CD2.D1-1       | E331/E333I| 85 ± 18 μM*                 |
| CD2.D1-2       | D324/E325I| 113 ± 5 μM                  |
| CD2.D1-3       | D318/D322I| >4.8 mM*                    |
| CD2.D1-4       | E328/N335I| 299 ± 15 μM*                |

*, p < 0.05.

extracellular, Trp fluorescence, and NMR chemical shifts (supplemental Fig. S1). Fig. 2B shows that Tb3+ (which has the same coordination chemistry as Ca2+) elicits an increase in fluorescence of CD2.D1 at 550 nm when excited at 280 nm due to Tb3+ binding affinity of 49 ± 9 μM (Fig. 2B and Table 1). Ca2+ displaced bound Tb3+ (Fig. 2B), thereby decreasing the Tb3+-LRET signal. CD2.D1 has a Ca2+ dissociation constant of 1.80 ± 0.12 mM determined in this manner (Table 1).

Because mGluR1α is modulated by various polyvalent cations, including Ca2+, Gd3+, Tb3+, La3+, Mn2+, and Mg2+ (6), we tested the metal binding selectivity of CD2.D1 by applying K+, Mg2+, La3+, or Gd3+ to compete with prebound Tb3+. Fig. 2C shows that the luminescence intensity of Tb3+ decreased significantly upon adding trivalent La3+ or Gd3+, indicating that Tb3+ bound to the pocket was replaced by these metal ions. Gd3+ had the strongest capacity to displace Tb3+. Similarly, adding La3+ to CD2.D1 produced a split in the resonance of CD2.D1 at 10 ppm (Fig. 2D). Ca2+ competed more effectively than Mg2+, whereas K+ failed to compete with Tb3+.

Next, we utilized mutagenesis studies to examine the contribution of proposed ligand-binding residues to metal-binding capability. Double substitutions of negatively charged residues by Ile to delete negative charges but preserve bulky side chains of proposed ligand-binding residues to metal-binding pocket, as seen in the mutants D324I/D322I and E325I/D328I/N335I, produced 2.3-, 6.1-, or 1.7-fold changes in the Kd of LRET. CD2.D1 had a Tb3+ LRET signal. CD2.D1 has a Ca2+ binding affinity of 49 ± 9 μM (Fig. 2B and Table 1). Ca2+ displaced bound Tb3+ (Fig. 2B), thereby decreasing the Tb3+-LRET signal. CD2.D1 has a Ca2+ dissociation constant of 1.80 ± 0.12 mM determined in this manner (Table 1).

Molecular Recognition—Intracellular mGluR1α responses were monitored by measuring cerulean fluorescence (Fig. 3A) and its mutants. Emission at 520 – 610 nm indicates the membrane expression levels of mGluR1α and its mutants. Emission at 520 nm (green signal) reflects the membrane expressed receptors, whereas the red signal at 610 nm from mCherry is a measure of total expression of the receptor. NT indicates non-transfected cells, which display no fluorescence. Although E325I displays a relatively lower surface expression, the other mutants have membrane expression level comparable to that of WT mGluR1α (n = 3).

Extracellular Ca2+ Triggers mGluR1α-mediated Intracellular Responses—We next examined the mGluR-mediated intracellular Ca2+ responses in HEK293 cells transfected with mGluR1α-mCherry. The fluorescent protein mCherry was fused to mGluR1α to correlate cellular responses with the expression of mGluR1α. We chose HEK293 cells as a model because this cell line lacks endogenous mGluR1α (34). mGluR1α-mCherry was well expressed and correctly targeted to the cell membrane (Figs. 3 and 4A and supplemental Fig. S2), and Fura-2 was efficiently loaded (Fig. 4B). Single cell, real time imaging was performed using fluorescence microscopy. To minimize receptor desensitization by agonists, the responses to each concentration of added Ca2+ or Gru were examined using separate coverslips.

In the absence of exogenous Glu, the addition of [Ca2+]o at less than 1.8 mM did not induce any [Ca2+]i response in HEK293 cells transfected with WT mGluR1α. Adding ≥3.0 mM Ca2+ to the medium elicited a transient [Ca2+]i increase followed by a long lasting plateau (Fig. 4C); this response was maximal at ≥5.0 mM [Ca2+]o with an EC50 of 3.0 mM. Furthermore, adding 0.5 or 30 μM Glu significantly enhanced the maximal response of mGluR1α to [Ca2+]o by 1.3- and 1.7-fold, respectively (Fig. 5). The EC50 value for [Ca2+]o decreased from 3.0 to 2.8 and 0.1 mM, respectively (Fig. 5 and Table 2).

The response of wild type mGluR1α to Glu was investigated in physiological saline buffer with 1.8 mM Ca2+. Wild type mGluR1α only responded at ≥0.5 μM Glu, and this response was saturated at 30 μM Glu. Fig. 6A shows that at 1.8 mM Ca2+, the addition of >30 μM Glu evoked large [Ca2+]i responses.
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Effects of Mutating Proposed Ca2+-binding Residues on [Ca2+]i, or Glu-evoked [Ca2+]i, Responses—To further understand the potential physiological role of this proposed Ca2+-binding site, we compared the mGluR-mediated [Ca2+]i responses in HEK293 cells transfected with the WT or mutated versions of mGluR1α. Fig. 4C shows that without Glu, substituting the predicted Ca2+-binding residues Asp-318 and Glu-325 with Ile eliminates the transient [Ca2+]i response toward [Ca2+]i. In addition, the mutation D322I also reduced the transient [Ca2+]i response by 16–20% while increasing the EC50 from 3.0 mM [Ca2+]i for the wild type receptor to 4.2 mM for D322I (Fig. 5 and Table 2), although all mutants were expressed at levels comparable with the WT receptor, as assessed by immunofluorescence and flow cytometry (Fig. 3 and supplemental Fig. S2). These results suggest that the predicted Ca2+-binding residues, Asp-318, Glu-325, and Asp-322 (especially the first two), are important for the sensitivity of mGluR1α to modulation by [Ca2+]i. However, S166A maintains Ca2+ sensitivity with a lower maximal response (Fig. 4C), although Ser-166 was previously reported to be a potential Ca2+-binding residue (6).

Effects of Mutating Predicted Ca2+-binding Residues on Glu-potentiated [Ca2+]i, Responses—The Ca2+-binding site identified here is adjacent to the previously defined Glu-binding site (Arg-78, Ser-165, Thr-188, Asp-208, Tyr-236, Asp-318, and Lys-409) (15, 25) (Fig. 1A). Asp-318 is the lone residue used in both the Glu- and Ca2+-binding sites. Figs. 4 and 6 show that the mutation D318I completely eliminates the [Ca2+]i response of mGluR1α to both [Ca2+]i and Glu, even at concentrations of the latter as high as 30 μM. In contrast, the mutant E325I completely abolishes [Ca2+]i-mediated [Ca2+]i responses without the agonist Glu (Figs. 4C and 5 and Table 2) but retains Glu-mediated [Ca2+]i responses (Fig. 6A). However, its EC50 value for Glu-mediated responses is increased by ~18-fold (Fig. 6B and Table 2). These results further confirm that Glu-325 contributes to Ca2+ binding without directly liganding Glu (as shown by earlier studies of the binding site for Glu, which did not identify Glu-325 as a Glu ligand). However, the proximity of the Ca2+- and Glu-binding sites may produce indirect, conformational effects of mutating residue 325 on Glu binding. Furthermore, D322I exhibited a reduction in EC50 for [Ca2+]i by only 33%, consistent with it making a relatively minor contribution as a ligand for Ca2+ binding. In contrast to the marked impact of D318I and E325I on the EC50 for [Ca2+]i, removal of other charged residues, such as D324I and E328I, did not alter either the EC50 (3 and 8% changes, respectively) or the magnitude of the response to [Ca2+]i significantly in the absence of Glu (104 ± 10 and 102 ± 5, respectively, of the control level) (Fig. 5 and Table 2).

Effect of Mutating Glu-binding Site on [Ca2+]i, Responses to Glu and Ca2+—To further explore the synergistic interaction between the predicted Ca2+ and Glu-binding sites, four mutations at Glu ligand residues (S165A, T188A, D208I, and Y236F) were generated. Consistent with studies reported previously (25), T188A and D208I entirely eliminated Glu sensitivity, whereas S165A and Y236F could be activated only by high concentrations (100 μM) of Glu (Fig. 8A). Interestingly, all receptors with mutated Glu-binding ligand residues (exception for

However, when [Ca2+]i was reduced from 1.8 mM to close to zero (no Ca2+ added to the medium), 30 μM Glu still activated mGluR1α (Fig. 5). There was, however, a reduced maximal response (29%) (Fig. 5), possibly because of the depletion of intracellular calcium stores. This result suggests that Glu triggers the activation of mGluR1α and that this effect can be enhanced by [Ca2+]i.

To study the synergy of [Ca2+]i and Glu binding to mGluR1α, the combination indices for quantitative evaluation of synergy were calculated using CalcuSyn (35). With 0.5 or 10 mM [Ca2+]i, the receptor or its mutants. D318I and E325I eliminate the [Ca2+]i response of WT mGluR1α, and that this effect can be enhanced by [Ca2+]i.

**Effects of [Ca2+]i on Glu-induced [Ca2+]i, Release by Wild Type mGluR1α**—To determine the role of [Ca2+]i on Glu-mediated activation of wild type mGluR1α. 5 and 10 mM [Ca2+]i were added to the perfusion system in addition to the 1.8 mM already present in the perfusate. As shown in Fig. 7, compared with the response in 1.8 mM [Ca2+]i, both 5 and 10 mM [Ca2+]i enhanced the responses of WT mGluR1α to Glu by reducing the EC50 values from 1.7 to 0.9 and 0.4 μM, respectively (Table 2), although the maximal responses remained comparable with those at 1.8 mM [Ca2+]i. This indicates that [Ca2+]i potentiates the sensitivity of mGluR1α to Glu and that this effect increases at higher levels of [Ca2+]i.
Asp-318) retained a sensitivity to \([\text{Ca}^{2+}]_o\) (Fig. 8B and Table 3), although their EC\(_{50}\) values were increased compared with that of wild type mGluR1\(\alpha\) (Table 3), again perhaps owing to local conformational effects of mutating the Glu-binding site on \([\text{Ca}^{2+}]_o\) binding. S165A and D208I increased the EC\(_{50}\) of the wild type receptor for \([\text{Ca}^{2+}]_o\) from 3.0 to 8.1 and 4.6 mM, respectively, although their maximal responses were comparable to that of the wild type receptor (Fig. 8B and Table 3). Conversely, T188A and D208I exhibited much reduced maximal responses (26 and 66%, respectively), whereas their EC\(_{50}\) values were comparable with that of the wild type receptor (Table 3). Taken together, these data show that it is possible to generate mGluR1\(\alpha\) variants responding to either Glu or to \([\text{Ca}^{2+}]_o\) alone. Thus mGluR1\(\alpha\) can function as a true \([\text{Ca}^{2+}]_o\)-sensing receptor, as certain mutants, such as S165A and D208I, do not respond to Glu but maintain their \([\text{Ca}^{2+}]_o\)-sensing capability with only a modest increase in the EC\(_{50}\) for \([\text{Ca}^{2+}]_o\).

**Effects of Mutations in Predicted \([\text{Ca}^{2+}]_o\)-binding Site on \([\text{Gd}^{3+}]_o\)-induced \([\text{Ca}^{2+}]_i\) Responses—**

\([\text{Gd}^{3+}]_o\) is also revealed at the hinge region in the Fourier map, where it shares residues Asp-322 and Asp-324 from the loop that contributes to \([\text{Ca}^{2+}]_o\) binding (14). Because of the low resolution of this crystal structure (4 Å), the highly flexible loop that binds \([\text{Gd}^{3+}]_o\) in the crystal structure, and the similarity of the binding geometries of \([\text{Gd}^{3+}]_o\) and \([\text{Ca}^{2+}]_o\), these two cations probably share, at least in part, the same residues. To address this possibility, the responses to \([\text{Gd}^{3+}]_o\) of D318I and E325I were compared with that of the wild type receptor. Consistent with results reported by Abe et al. (17, 36), the dose-response profiles of wild type mGluR1\(\alpha\) display a bell-shaped curve. However, the introduction of the mutation D318I or E325I completely eliminated the receptor sensitivity to \([\text{Gd}^{3+}]_o\) (Fig. 9).

**DISCUSSION**

We utilized the computational algorithm MUG (22) to predict a novel \([\text{Ca}^{2+}]_o\)-binding site in mGluR1\(\alpha\) adjacent to the Glu-binding site shown in Fig. 1. This predicted \([\text{Ca}^{2+}]_o\)-binding site (comprising Asp-318, Glu-325, Asp-322, and the carboxylate side chain of Glu-701) does not completely overlap the Glu-binding site (15, 25). However, both sites include Asp-318, which our data suggest is involved in both Glu and \([\text{Ca}^{2+}]_o\) binding. The metal-binding capability of the predicted \([\text{Ca}^{2+}]_o\)-binding residues in mGluR1\(\alpha\) was verified by a grafting approach. Like wild type mGluR1\(\alpha\), the predicted \([\text{Ca}^{2+}]_o\)-binding site grafted in a scaffold protein (CD2) exhibited metal selectivity for \([\text{Ca}^{2+}]_o\) and its trivalent analogs, \([\text{Gd}^{3+}]_o\), \([\text{Tb}^{3+}]_o\), and \([\text{La}^{3+}]_o\), in contrast to the physiological...
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TABLE 2

| Variants | Glu concentration (μM) | [Ca^{2+}]_i | Maximal response | EC_{50} | Maximal response |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| WT              | 0               | 3.0             | 63 ± 2          | 1.7             | 111 ± 2         |
| 0.5             | 2.8             | 1.7             | 109 ± 7        | 100 ± 3        |
| 30              | 0.1             | 0.5             | 128 ± 6        | 108 ± 1'        |
| D318I           | 0               | None            | No response    | None            | No response     |
| 10              | 4.1             | 1.7             | 86 ± 5         | 0.9'           |
| 30              | 0.5             | 7.2             | 105 ± 3        | 0.4''          |
| E325I           | 0               | None            | No response    | None            | No response     |
| 10              | 4.3             | 2.3             | 89 ± 12        | 146 ± 2        |
| 100             | ND^e             | ND^e             | 117 ± 3        | 117 ± 7        |
| D324I           | 0               | 3.9             | 104 ± 10       | 0.3             |
| 10              | 2.9             | 1.8             | 144 ± 4        | 0.3             |
| 30              | 0.6             | 1.0             | 162 ± 6        | 1.2             |
| E328I           | 0               | 2.5             | 102 ± 5        | 1.2             |
| 3               | 1.6             | 1.5             | 135 ± 13       | 107 ± 12        |
| 30              | 2.2             | 0.3             | 147 ± 3        |                 |

^a The maximal responses are normalized to the maximal response of wild type mGluR1α to Glu.
^b Glu dose response was performed in 1.8 mM Ca^{2+}.
^c Experiments performed in 5 mM Ca^{2+}.
^d Experiments performed in 10 mM Ca^{2+}.
^e ND, not determined.

FIGURE 6. Intracellular Ca^{2+} responses to extracellular Glu in HEK293 cells transfected with WT mGluR1α or its mutants. Three negatively charged residues in the predicted Ca^{2+}-binding pocket (Asp-318, Asp-322, and Glu-325) were mutated into Ile. Along with WT mGluR1α, the mutants were transiently expressed in HEK293 cells. In the presence of 1.8 mM Ca^{2+}, extracellular Glu-induced intracellular Ca^{2+} release was measured by recording emission intensities at 510 nm excited at 340 and 380 nm, respectively. A, responses to Glu of mutations on Ca^{2+}-binding site. Except for mutant D318I, two other mutants, D322I, E325I, and WT mGluR1α display responsiveness to Glu. B, maximal response of WT mGluR1α and its mutants to Glu at a saturating concentration. Each single data point was performed in an individual dish, and the cells expressed mGluR1α showing responses to Glu were selected for analysis (n = 3).

monovalent cation, K^+, which exhibited no measurable affinity for the predicted site. The metal-binding capability of the predicted metal-binding ligand residues in mGluR1α was further verified by replacing negatively charged residues with Ile. The Ca^{2+} binding affinity of mGluR1α determined by the grafting approach (~1.8 mM) is within the physiological concentration of [Ca^{2+}]_o in the nervous system (0.8–1.5 mM) (37), although this Ca^{2+} binding constant may be changed slightly in vivo by the local microenvironment and/or the presence of Glu released by nearby cells.

We further demonstrated that mGluR1α could be activated by either Glu or [Ca^{2+}]_o. Indeed, Glu and [Ca^{2+}]_o act synergistically to elicit the maximal [Ca^{2+}]_i responses observed here (Table 2). Mutating the Glu-binding site, such as T188A and D208I, in mGluR1α (Fig. 8A and Table 3), completely abolished the Glu-signaling capability of the receptor while leaving its Ca^{2+}-sensing capability largely intact, with only modest increases in EC_{50} for [Ca^{2+}]_o. These results suggest that mGluR1α can function as a true [Ca^{2+}]_o-sensing receptor, i.e. exhibiting robust activation by [Ca^{2+}]_o in the absence of added Glu. Although we cannot rule out release of low concentrations of Glu by the HEK293 cells, it should be noted that some mutants of the receptor retained responsiveness to [Ca^{2+}]_o despite complete loss of responsiveness to Glu. Moreover, even the addition of high exogenous concentrations of Glu failed to activate these mutants.

We have also shown that mutating predicted Ca^{2+}-binding residues abolishes or significantly not only reduces [Ca^{2+}]_i sensitivity but also, in some cases, affects Glu-mediated responses. For example, E325I completely abolished [Ca^{2+}]_o-mediated [Ca^{2+}]_i responses in the absence of Glu (Fig. 3C and Table 2). At the same time, this mutant retained sensitivity to Glu, albeit with an ~18-fold reduction in EC_{50} (without any decrease in maximal response) (Fig. 5 and Table 2). Thus, despite its not being a Glu-binding residue, the presence of an intact Ca^{2+}-binding ligand at Glu-325 considerably enhanced the affinity of mGluR1α for Glu. How could this take place? These results, in fact, are consistent with separate but overlapping Ca^{2+}- and Glu-binding sites (Figs. 1 and 10). As noted, positively charged Arg-323 is also very close to Ca^{2+} in the model, but it might not
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This page discusses a novel extracellular calcium-binding site on the mGluR1α receptor. The text explains how mutations in the receptor can affect calcium binding, particularly focusing on the role of Glu-325 as a ligand for binding calcium. The binding of Glu could stabilize the side chain of Glu-325 in a conformation favorable for it to serve as a ligand for calcium binding. Mutations at the Glu-binding site can either enhance or reduce calcium binding, with some mutations eliminating calcium binding altogether. The text also mentions the role of other calcium-binding sites and the synergistic effects of calcium and glutamate agonists.

FIGURE 7. Extracellular Ca$^{2+}$ enhanced mGluR1α to sense extracellular Glu. Responses of WT mGluR1α to extracellular Glu were assessed in buffers containing additional [Ca$^{2+}$]o (1.8, 5, and 10 mM). The maximal responses of the receptor to [Glu]o in 1.8, 5, and 10 mM [Ca$^{2+}$]o are comparable, but the EC$_{50}$ values for the responses in the presence of 5 and 10 mM [Ca$^{2+}$]o are significantly decreased. Clearly, higher [Ca$^{2+}$]o reduces the EC$_{50}$ of the receptor for [Glu]o (n = 3).

FIGURE 8. Receptors with mutations in the Glu-binding pocket can sense Ca$^{2+}$ but not Glu in physiological buffer. Four residues from the reported Glu-binding pocket were selected to mutate into non-polar residues. Intracellular Ca$^{2+}$ levels induced by Fura-2 were recorded using fluorescence microscopy, which detected the ratio of the emission at 510 nm with excitation at 340 and 380 nm. A, T188A and D208I abolish sensitivity to Glu, but S165A and Y236F remain capable of responding to [Ca$^{2+}$]o; the maximal responses of S165A, T188A, and D208I are comparable with that of WT mGluR1α, whereas Y236F decreases the sensitivity of the receptor to [Ca$^{2+}$]o (n = 3).
an electrostatic interaction. As revealed by the grafting approach (Fig. 2A), E325I significantly reduces the Ca$^{2+}$-binding ability in mGluR1$\alpha$, possibly by disturbing the favorable local charge environment.

Fig. 1C shows that Asp-318 in mGluR1$\alpha$, located at the hinge region, is conserved in all members of the three GPCRs, corresponding to Asp-295 of mGLUR2, Asp-301 of mGLUR3, Asp-309 of mGLUR8, and Glu-297 of CaSR (23, 24, 42). Figs. 4 and 6 clearly demonstrate that Asp-318 contributes not only to Ca$^{2+}$-but also Glu-triggered [Ca$^{2+}$], responses. This residue seems to play an essential role in the activation of mGLURs. Consistent with this finding, a D318A mutation was shown previously to reduce receptor expression on the membrane and abolish Glu-triggered [Ca$^{2+}$], and inositol triphosphate responses (25).

Our findings also appear to be applicable to other members of the three GPCRs, especially CaSR. The mutation E297I in CaSR, equivalent to D318I in mGluR1$\alpha$, impairs receptor activation (23, 24, 42). Glu-297 is an important residue in our reported Ca$^{2+}$-binding site in the CaSR hinge region (23, 24); the mutant E297I significantly impairs sensitivity to [Ca$^{2+}$]$_{o}$ with an EC$_{50}$ of 9.6 ± 0.2 mM [Ca$^{2+}$]$_{o}$. Mutations at or around this Ca$^{2+}$-binding site are also associated with clinical syndromes (autosomal dominant hypocalcemia and familial hypocalciuric hypercalcemia) because of either an increase or a decrease in the sensitivity of the respective receptors to [Ca$^{2+}$]$_{o}$. Zhang _et al._ (43) and others (44, 45) have also reported that mutations around this site, Ser-147, Ser-170, Asp-190, Tyr-218, and E297K, impair the activation of human CaSR by [Ca$^{2+}$]$_{o}$. Recently Silve _et al._ (42) have shown that the missense mutations E297K and Y218S significantly reduce the maximal response of the CaSR. Although E297K was considered a key factor in impairing protein folding, thus leading to lower expression on the cell surface and impaired responsiveness to [Ca$^{2+}$]$_{o}$, our unpublished data study show that E297I has a membrane expression level comparable with that of the wild type CaSR. Therefore, the low expression level of E297K could be, at least in part, the result of the substitution of an unfavorable positive charge, which modifies the local charge balance, leading to reduced folding efficiency. Furthermore, our assessment of the surface expression of D318I by flow cytometry showed that it was at the same level as wild type; this echoes the impact of mutating the equivalent residue in CaSR (e.g. Glu-297). It has been postulated that residues Ser-170, Asp-190, Glu-193, Ser-296, and Glu-297 are critical for Ca$^{2+}$ binding to CaSR and functionality of the receptor (42), which is in excellent agreement with our prediction. In addition, CaSR functions primarily as a [Ca$^{2+}$]$_{o}$-sensing receptor but can also integrate information about protein metabolism (i.e. amino acids) with that of divalent cations (e.g. calcium) (46). CaSR displays sensitivity to amino acids, especially phenylalanine and other aromatic amino acids, likely via three serine residues (Ser-169—Ser-171), our unpublished data study show that Ca$^{2+}$ and phe-

**FIGURE 9. Mutations D318I and E325I in the predicted Ca$^{2+}$-binding site abolish [Ca$^{2+}$], responses of the receptor to [Gd$^{3+}$]$_{o}$.** HEK293 cells expressing D318I, E325I, and WT mGluR1$\alpha$ were preincubated in 140 mM NaCl, 4 mM KOH, 10 mM HEPES, 1.5 mM CaCl$_2$, and 10 mM glucose, pH 7.4, for up to 1.5 h before fluorescence microscopy was carried out. Gd$^{3+}$ was added into the loading buffer (140 mM NaCl, 4 mM KOH, 10 mM HEPES, and 0.3 MgCl$_2$, pH 7.4) in the perfusion system. [Ca$^{2+}$]$_{o}$ levels indicated by Fura-2 are presented by the ratio of the fluorescence at 510 nm when excited at 340 and 380 nm as above. The [Ca$^{2+}$]$_{o}$ response of wild type mGluR1$\alpha$ displays a bell-shape curve, but D318I and E325I completely abolish (Ca$^{2+}$), release to [Gd$^{3+}$]$_{o}$ (n = 3).

**TABLE 3**

| Mutants | EC$_{50}$ (mM) | $n_{Hill}$ | Maximal response* |
|---------|----------------|------------|--------------------|
| S165A   | 8.1            | 3.1        | 86 ± 3             |
| T188A   | 3.4            | 2.2        | 74 ± 2             |
| D208I   | 4.6            | 2.3        | 95 ± 3             |
| Y236F   | 3.3            | 10.9       | 33 ± 3             |

*Maximal responses are normalized to the maximal response of wild type mGluR1$\alpha$ to Glu.

As shown in Fig. 1C, the predicted Ca$^{2+}$-binding residue Asp-322 is conserved in group I mGLURs. Glu-325 is highly conserved in group I (mGLUR1 and mGLUR5) and group II (mGLUR2 and mGLUR3) mGLURs (Fig. 1C). Interestingly, mGLUR5 in group I and mGLUR3 in group II sense [Ca$^{2+}$]$_{o}$ at physiological levels, whereas mGLUR2 is activated only when [Ca$^{2+}$]$_{o}$ is more than 10 mM (6). On the basis of our observation that E325I abolished Ca$^{2+}$-induced responses for mGluR1$\alpha$ but retained responsiveness to Glu, we concluded that Glu-325 might be very important for Ca$^{2+}$ binding in the mGLUR family generally. Analysis by the Contacts of Structural Units server indicates that Glu-325 interacts electrostatically with Arg-297 and Arg-323 in the Glu-bound structures of mGluR1$\alpha$ (41), suggesting that Glu-325 stabilizes the local structure through a conserved in group I (mGLUR1 and mGLUR5) and group II (mGLUR2 and mGLUR3) mGLURs (Fig. 1C). Interestingly, mGLUR5 in group I and mGLUR3 in group II sense [Ca$^{2+}$]$_{o}$ at physiological levels, whereas mGLUR2 is activated only when [Ca$^{2+}$]$_{o}$ is more than 10 mM (6). On the basis of our observation that E325I abolished Ca$^{2+}$-induced responses for mGluR1$\alpha$ but retained responsiveness to Glu, we concluded that Glu-325 might be very important for Ca$^{2+}$ binding in the mGLUR family generally. Analysis by the Contacts of Structural Units server indicates that Glu-325 interacts electrostatically with Arg-297 and Arg-323 in the Glu-bound structures of mGluR1$\alpha$ (41), suggesting that Glu-325 stabilizes the local structure through

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**TABLE 3**

| [Ca$^{2+}$], response of mutants in Glu-binding site (n = 3) |
|----------------------------------------------------------|
| Mutants | EC$_{50}$ (mM) | $n_{Hill}$ | Maximal response* |
|---------|----------------|------------|--------------------|
| S165A   | 8.1            | 3.1        | 86 ± 3             |
| T188A   | 3.4            | 2.2        | 74 ± 2             |
| D208I   | 4.6            | 2.3        | 95 ± 3             |
| Y236F   | 3.3            | 10.9       | 33 ± 3             |

*Maximal responses are normalized to the maximal response of wild type mGluR1$\alpha$ to Glu.
nylalanine also synergistically modulate the signaling functions of CaSR.

In summary, we have predicted and confirmed experimentally a calcium-binding site in the extracellular domain of mGluR1. We have also shed new light on the co-activation of mGluR1 by Glu and \([\text{Ca}^{2+}]_o\). These findings provide novel perspectives on mGluR1, which may be viewed as capable of integrating information from two very different types of ligands (an amino acid neurotransmitter and a divalent cation). The levels of \([\text{Ca}^{2+}]_o\) in the brain are highly dynamic (37), and the affinity constants that we have determined in our studies on calcium binding to mGluR1 are well within the dynamic, physiological range of \([\text{Ca}^{2+}]_o\) in the brain. For family 3 GPCRs other than CaSR, the physiological importance of \([\text{Ca}^{2+}]_o\) binding has been uncertain; but the findings reported here may be useful in resolving this mystery by allowing for the development of knock-in mutations to mGluR1, and resultant mouse models, that disrupt the ability of the receptor to bind \([\text{Ca}^{2+}]_o\) while leaving Glu binding intact. Moreover, because many of the key calcium-binding residues defined in our studies are conserved for other family 3 GPCRs, our findings may have relevance for a host of other receptors beyond just mGluR1 (6, 7). Family 3 GPCRs have tremendous potential as therapeutic targets, and therefore the advances described here may facilitate the development of novel family 3 GPCR-targeted drugs for use in the treatment of many different diseases.

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