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Amino-Pyrrolidine Tricarboxylic Acids Give New Insight into Group III Metabotropic Glutamate Receptor Activation Mechanism

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ABSTRACT

Like most class C G-protein-coupled receptors, metabotropic glutamate (mGlu) receptors possess a large extracellular domain where orthosteric ligands bind. Crystal structures revealed that this domain, called Venus FlyTrap (VFT), adopts a closed or open conformation upon agonist or antagonist binding, respectively. We have described amino-pyrrolidine tricarboxylic acids (APTCs), including (2*S*,4*S*)-4-amino-1-[(*E*)-3-carboxyacryloyl]pyrrolidine-2,4-dicarboxylic acid (FP0429), as new selective group III mGlu agonists. Whereas FP0429 is an almost full mGlu4 agonist, it is a weak and partial agonist of the closely related mGlu8 subtype. To get more insight into the activation mechanism of mGlu receptors, we aimed to elucidate why FP0429 behaves differently at these two highly homologous receptors by focusing on two residues within the binding site that differ between mGlu4 and mGlu8. Site-directed mutagenesis of Ser157 and Gly158 of mGlu4 into their mGlu8 homologs (Ala) turned FP0429 into a weak partial agonist. Conversely, introduction of Ser and Gly residues into mGlu8 increased FP0429 efficacy. Docking of FP0429 in mGlu4 VFT 3D model helped us characterize the role of each residue. Indeed, mGlu4 Ser157 seems to have an important role in FP0429 binding, whereas Gly158 may allow a deeper positioning of this agonist in the cavity of lobe I, thereby ensuring optimal interactions with lobe II residues in the fully closed state of the VFT. In contrast, the presence of a methyl group in mGlu8 (Ala instead of Gly) weakens the interactions with the lobe II residues. This probably results in a less stable or a partially closed form of the mGlu8 VFT, leading to partial receptor activation.

In the brain, glutamate (Glu) is the transmitter of most fast excitatory synapses, acting on either ionotropic glutamate or metabotropic glutamate (mGlu) receptors, which belong to class C of the G-protein-coupled receptors (GPCRs). The ligand binding domain (LBD) of mGlu receptors was shown to adopt a so-called Venus FlyTrap (VFT) fold, composed of two lobes interconnected by a flexible hinge (Kunishima et al., 2000). Agonists bind to lobe I and are then trapped in the cleft upon VFT closure, whereas competitive antagonists keep the VFT domain in an open conformation (Bessis et al., 2002; Tsuchiya et al., 2002). Crystal structures (Kunishima et al., 2000; Tsuchiya et al., 2002) and homology models (Bessis et al., 2000; Malherbe et al., 2001; Bertrand et al., 2002; Rosemond et al., 2004) of both open and closed conformations of the VFT domain have been reported for most of the eight cloned mGlu receptor subtypes.

According to their sequence similarity and transduction mechanism, but also their pharmacology, the eight mGlu receptors have been further classified into three groups (Pin and Acher, 2002). Group III mGlu receptors (i.e., mGlu4, -6, -7, and -8 subtypes) are coupled to pertussis toxin-sensitive

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ABBREVIATIONS: mGlu, metabotropic glutamate receptor; GPCR, G-protein-coupled receptor; LBD, ligand binding domain; VFT, Venus FlyTrap; ACPT-I, 1-aminocyclopentane 1,3,4-tricarboxylic acid; APTC, amino-pyrrolidine tricarboxylic acid; FP0429, (2*S*,4*S*)-4-amino-1-[(*E*)-3-carboxyacryloyl]pyrrolidine-2,4-dicarboxylic acid; 3D, three-dimensional; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; wt, wild type; HA, hemagglutinin; AMN082, *N*,*N*′-dibenzhydrylethane-1,2-diamine dihydrochloride.

G_i and/or G_o G-proteins and are activated by group-specific agonists such as L(+)-2-amino-4-phosphonobutyric acid or ACPT-I (Nakanishi, 1992; Acher et al., 1997). Although mGlu6 receptor expression is restricted to the bipolar cells of the retina, subtypes mGlu4, -7, and -8 are expressed throughout the brain on nerve terminals, preferentially on the presynaptic region, where they exert a control on neurotransmitter release (Schoepp, 2001). Localization studies have shown that subtype mGlu7 is located directly in the synaptic cleft, whereas mGlu4 and -8 are mostly present at perisynaptic sites outside the active zone of neurotransmitter release. These diverse localizations may indicate separate roles in regulating Glu release and spillover effects and may also account for the fact that the affinities of group III mGlu receptors for their natural ligand Glu, and many other well characterized agonists, vary over a very large concentration range (Schoepp et al., 1999). The molecular basis for these affinity discrepancies was recently investigated: residues of the LBD, but also additional residues of the N terminus, were identified as determinants of the pharmacological profile of group III receptors (Rosemond et al., 2004). It is noteworthy that of the dozen residues usually considered to constitute the LBD of mGlu receptors, only two differ between the mGlu4 and mGlu8 subtypes. Therefore, very few agonists have been described to have significantly different pharmacological properties on these two receptor subtypes (De Colle et al., 2000). One of the only exceptions is (*S*)-3,4-dicarboxyphenylglycine, showing nanomolar potency toward subtype mGlu8 and only low micromolar potency for mGlu4 (Thomas et al., 2001).

We recently described a new family of molecules, the amino-pyrrolidine tricarboxylic acids (APTCs), whose design was based on medicinal chemistry around group III specific agonist ACPT-I (Acher et al., 1997). The APTC head of series, FP0429, presented the very attractive feature of behaving as an almost full agonist on mGlu4 while being a very weak partial agonist on mGlu8 (Schann et al., 2006). This FP0429 compound therefore appeared as an exciting tool to decipher partial agonism mechanism regarding mGlu receptors. Indeed, concerning partial agonists, no specific studies have yet been reported with class C GPCRs. However, suggestions to explain the mode of action of several partial agonists of receptors holding a similar VFT domain (i.e., the ionotropic glutamate receptors), have been made and recently reviewed (Mayer, 2005; Chen and Wyllie, 2006). Efficacy of activation of these receptors was correlated to either the extent of VFT closure or to the stabilization degree of the VFT closed state.

In the present study, we performed site-directed mutagenesis on the residues differing between mGlu4 and mGlu8 LBD (i.e., Ser157 and Gly158 in mGlu4, which, respectively align with Ala154 and Ala155 in mGlu8) and analyzed the functional responses of the mutant receptors to both Glu and FP0429 by measuring intracellular Ca^{2+} mobilization. We also docked FP0429 in the previously described 3D model of mGlu4 LBD (Bertrand et al., 2002) to draw hypotheses explaining the impact of the presence of different residues in either mGlu4 or mGlu8 on FP0429 binding and subsequent VFT closure. Together, information derived from both mutagenesis study and 3D models converged to demonstrate the importance of Ala154 and Ala155 in preventing FP0429 from fully activating mGlu8, thereby giving insight into mGlu partial agonist activation mechanism by either partial VFT closure or weaker stabilization of the closed VFT domain.

Materials and Methods

Materials. FP0429 was synthesized as described previously (Schann et al., 2006). Glutamate and ACPT-I (1-aminocyclopentane 1,3,4-tricarboxylic acid) were purchased from Sigma-Aldrich (Lyon, France) and Tocris Cookson (Bristol, UK), respectively.

Culture medium, fetal calf serum, and all other products used for cell culture were purchased from PAA Laboratories (Les Mureaux, France). Fluo4-AM was purchased from Invitrogen (Cergy-Pontoise, France).

All reagents for the ELISA (paraformaldehyde, Triton X-100, bovine serum albumin, and Tween 20) were purchased from Sigma-Aldrich (Lyon, France). Anti-HA mouse 4C12 monoclonal antibody was purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated goat anti-mouse secondary antibody and revelation kit Amplex Red ELISA II were purchased from Invitrogen (Cergy-Pontoise, France).

Cell Culture and Transfection. HEK293 cells were cultured in modified Eagle's medium supplemented with 10% fetal calf serum and transfected by electroporation as described previously (Brabet et al., 1998; Frauli et al., 2006). For intracellular Ca^{2+} measurement and ELISA, 5 million cells were transfected with 5 μg of plasmid DNA encoding rat mGlu4 or mGlu8 receptors (either wild type or mutants). The construction of the plasmids expressing wild-type mGlu receptors has been described previously (Joly et al., 1995; Gomeza et al., 1996; Parmentier et al., 1998). Receptors were coupled to the phospholipase C pathway by cotransfection with 2 μg of plasmid DNA encoding the chimeric G_{qi9} protein (Gomeza et al., 1996). For partial/full agonism experiments, 3×10^6 cells were transfected with increasing amounts of mGlu-encoding plasmid DNA, ranking from 0 (Mock) to 5 μg, completed to a total amount of 5 μg of DNA with carrier vector pRK6.

Site-Directed Mutagenesis. Mutagenesis was performed using the Gene Tailor site-directed mutagenesis system from Invitrogen. Polymerase chain reactions were performed using the high-fidelity Pfu DNA Polymerase (Promega, Charbonnières, France) and specific forward primers (MWG Biotech, Ebersberg, Germany) for the different constructions: mGlu4SA (i.e., with Ser157 and Gly158 mutated in SA; tgggtgtcattggagcttcagcgagctccgtctcg), mGlu4AG (mutation of SG in AG; agtggtgggtgtcattggagctgcagggagctccgtctc), mGlu4AA (mutation of SG in AA; tggtgggtgtcattggagctgcagcgagctccgtctcg), mGlu8SA (i.e., with Ala154 and Ala155 mutated in SA; tttctggcgtcataggtgcttcagcaagctctgtg), mGlu8AG (mutation of AA in AG; ctggcgtcataggtgctgcaggaagctctgtgtcc), and mGlu8SG (mutation of AA in SG; tttctggcgtcataggtgcttcaggaagctctgtgtcc). For the three mutant constructions of each mGlu receptor, a single reverse primer was used (mGlu4: agctccaatgacacccaccactcgttcggg; mGlu8: agcacctatgacgccagaaatcttgtcggg).

Intracellular Calcium Measurements. After transfection, cells were seeded in polyornithine-coated, black-walled, clear-bottomed 96-well plates and cultured for 24 h. Cells were washed with freshly prepared buffer (1× Hanks' balanced salt solution supplemented with 20 mM HEPES, 1 mM $MgSO_4$, 3.3 mM Na_2CO_3, 1.3 mM $CaCl_2$, 2.5 mM probenecid, and 0.5% bovine serum albumin) and loaded with 1 μM Fluo-4 AM (a Ca^{2+}-sensitive fluorescent dye) for 90 min at 37°C. After washing, cells were incubated with 50 μl of buffer; 50 μl of 2× drug solution (prepared in buffer) was then added by the fluorescence microplate reader FlexStation II384 (Molecular Devices, Sunnyvale, CA) in each well after 20 s of recording. Fluorescence signals (excitation at 485 nm; emission at 525 nm) were then measured at sampling intervals of 1.5 s for 60 s.

Enzyme-Linked Immunosorbent Assay. The procedure of the ELISA on intact cells was described previously (Goudet et al., 2004). In brief, after 24 h of culture in polyornithine-coated, black-walled, clear-bottomed 96-well plates, electroporated cells were fixed with

4% paraformaldehyde in PBS, then half of the wells were permeabilized using 0.05% Triton X-100 in PBS. Blocking of unspecific binding sites and cell incubations with antibodies (1:1000 dilutions of either primary anti-HA 4C12 or secondary horseradish peroxidase-conjugated anti-mouse antibody) were performed in PBS containing 1% bovine serum albumin. Washing steps after antibody incubations were performed using 0.05% Tween 20 in PBS. Revelation was performed using the Amplex Red ELISA II kit according to the manufacturer's instructions.

Sequence Alignment. For group III mGlu sequence alignment, the following SwissProt or GenBank accession numbers were used: P31423 (rat mGlu4), NM_001013385.1 (mouse mGlu4), Q14833 (human mGlu4), P70579 (rat mGlu8), P47743 (mouse mGlu8), and O00222 (human mGlu8). Full-length sequence alignment was performed using the ClustalW multiple alignment program (Thompson et al., 1994) at http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl.

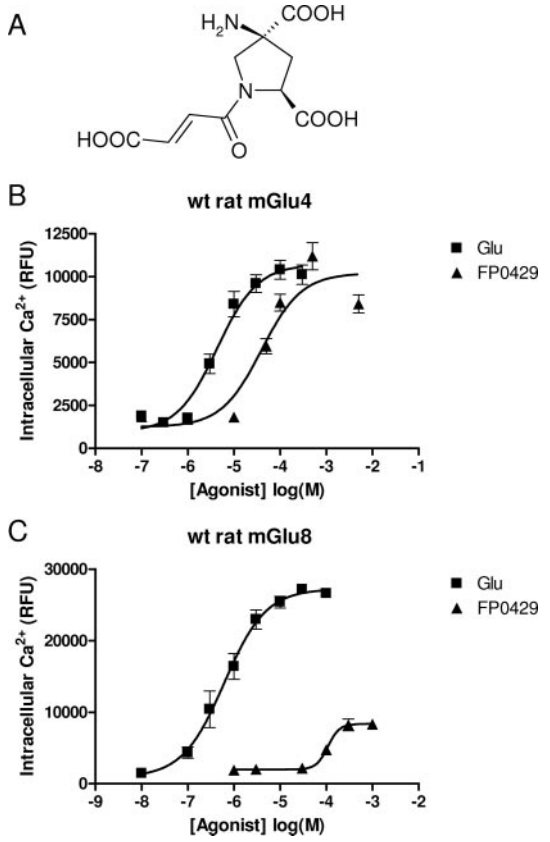

Fig. 1. Comparative effects of FP0429 on wt mGlu4 and mGlu8 receptors. A, structure of group III mGlu specific agonist FP0429. This compound belongs to the recently described family of APTCs, which are products from medicinal chemistry around the group III mGlu agonist ACPT-I. B and C, dose-response curves of Glu and FP0429 on rat wt mGlu4 (B) or mGlu8 (C). HEK 293 cells were electroporated with plasmids encoding rat wt mGlu receptor and a chimeric G protein, allowing receptor coupling to the phospholipase C pathway. Transfected cells were loaded with 1 μM Fluo4-AM, and then monitored for intracellular Ca^{2+} mobilization upon agonist addition using FlexStation II (Molecular Devices). Data are from one experiment representative of at least three independent experiments performed in triplicates, and used to determine mean EC_{50} values (Table 1).

3D Modeling of mGlu Extracellular Domains, Ligand Docking, and Complex Refinement in the Model. Homology modeling of mGlu receptor LBD and molecular docking were performed according to the protocol described by Bessis et al. (2000) and Bertrand et al. (2002). Comparative models were generated using MODELER 7.00 based on the coordinates of mGlu1 receptor LBD (Protein Data Base code 1ewk) (Kunishima et al., 2000). Our previously validated docking protocol (Triballeau et al., 2005) consisted first of the flexible fitting of the ligand within the rigid receptor using the shape-based docking algorithm LigandFit (Venkatachalam et al., 2003). The obtained poses were subsequently scored using the LigScore2 scoring function (Krammer et al., 2005). The best pose was then energy-minimized with CHARMm (Brooks et al., 1983), allowing full flexibility for both ligand and receptor. Solvation effects were explicitly taken into account by embedding the binding cleft in a 25-Å sphere of water molecules (model transferable intermolecular potential three point). The sphere of water was centered on the geometric center of the ligand and restrained using a miscellaneous mean field potential. All calculations were carried out in the Discovery Studio 1.5 environment (Accelrys Inc., San Diego, CA).

Data Analysis. The dose-response or partial/full agonism curves were fitted using the PRISM program (GraphPad Software, San Diego, CA). All data are mean ± S.E.M. from at least three individual experiments performed in triplicate. ANOVA analysis of independent experiments was performed with a general linear model using a Dunnett test (MiniTab14 for Windows; Minitab Inc., State College, PA).

Results

Effects of FP0429 on Wild-Type Group III mGlu Receptors. APTCs were designed on the basis of structural modifications of ACPT-I to obtain a scaffold close to ACPT-I with similar highly polar side chains and amenable to parallel synthesis. A virtual library of 100 APTCs was designed, and 65 compounds were finally synthesized and then tested on all mGlu subtypes (Schann et al., 2006). FP0429 (Fig. 1A) was found to be highly interesting, in that it did not interact with either group I or II mGlu receptors at up to 5 mM but showed agonist activity at group III mGlu receptors.

FP0429 dose-response curves were performed on cells transfected with wild-type (wt) mGlu4 or mGlu8, in comparison with Glu (Fig. 1, B and C, respectively). FP0429 EC_{50} values were comparable for both receptors (48.3 ± 5.2 and 56.2 ± 14.6 μM, respectively; Table 1), whereas a clear difference could be observed in the E_{max} values with respect to the Glu maximal effect. Indeed, FP0429 dose-response amplitude on wt mGlu4 was comparable with that of Glu (Fig. 1B), whereas the compound gave partial responses on wt mGlu8 (Fig. 1C), corresponding only to 36 ± 7% of the Glu maximum, under these conditions (Table 1).

Because this difference in response amplitude could be explained by partial agonism of FP0429 on wt mGlu8, this issue was further examined by testing the effects of maximal

TABLE 1

Mean Glu or FP0429 EC_{50} values and FP0429 E_{max} values (%Glu max) for wt and mutant mGlu4 and mGlu8 receptors

All dose-response curves were fitted using Prism 4. At least three independent experiments performed in triplicates were used to determine mean EC_{50} values and E_{max} values.

Receptor	mGlu4wt	mGlu4SA	mGlu4AG	mGlu4AA	mGlu8wt	mGlu8SA	mGlu8AG	mGlu8SG
Glu EC_{50} (μM)	3.0 ± 0.3	3.8 ± 0.3	3.2 ± 0.4	2.8 ± 0.5	1.3 ± 0.5	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.2
FP0429 EC_{50} (μM)	48.3 ± 5.2	40.1 ± 3.8	48.9 ± 8.3	79.6 ± 14.3	56.2 ± 14.6	47.3 ± 5.1	71.2 ± 14.2	50.8 ± 5.7
FP0429 E_{max} (%Glu max)	75 ± 6	45 ± 4	76 ± 4	52 ± 9	36 ± 7	52 ± 8	70 ± 11	90 ± 16

doses of Glu, FP0429, or ACPT-I on cells transfected with increasing amounts of receptor plasmid DNA. As shown in Fig. 2A, ACPT-I maximal effect on mGlu4 was similar to that generated by Glu, whatever the expression level of cell surface receptor, as quantified using ELISA on intact cells. This demonstrated that ACPT-I was definitely a full agonist at this receptor subtype. It is noteworthy that the maximal effect of FP0429 was always somewhat lower than that obtained with Glu (with values between 70 and 95%), suggesting that this compound is a partial agonist with high efficacy regarding mGlu4 (and as such we decided to call it an "almost full agonist") (Fig. 2A). On the contrary, when a similar experiment was performed on cells expressing various amounts of mGlu8, the maximal effect of FP0429 was always dramatically weaker than that of Glu (Fig. 2B). Indeed, at low receptor expression level, when the presence of reserve receptor is very unlikely, the maximal effect of FP0429 reached only 6 to 10% of that of Glu, demonstrating that this compound was a very weak partial agonist on this receptor subtype (Fig. 2B). Besides, these data also indicate that ACPT-I was a partial agonist on mGlu8, generating responses equal to 37 to 41% of that of Glu at low receptor expression level (Fig. 2B).

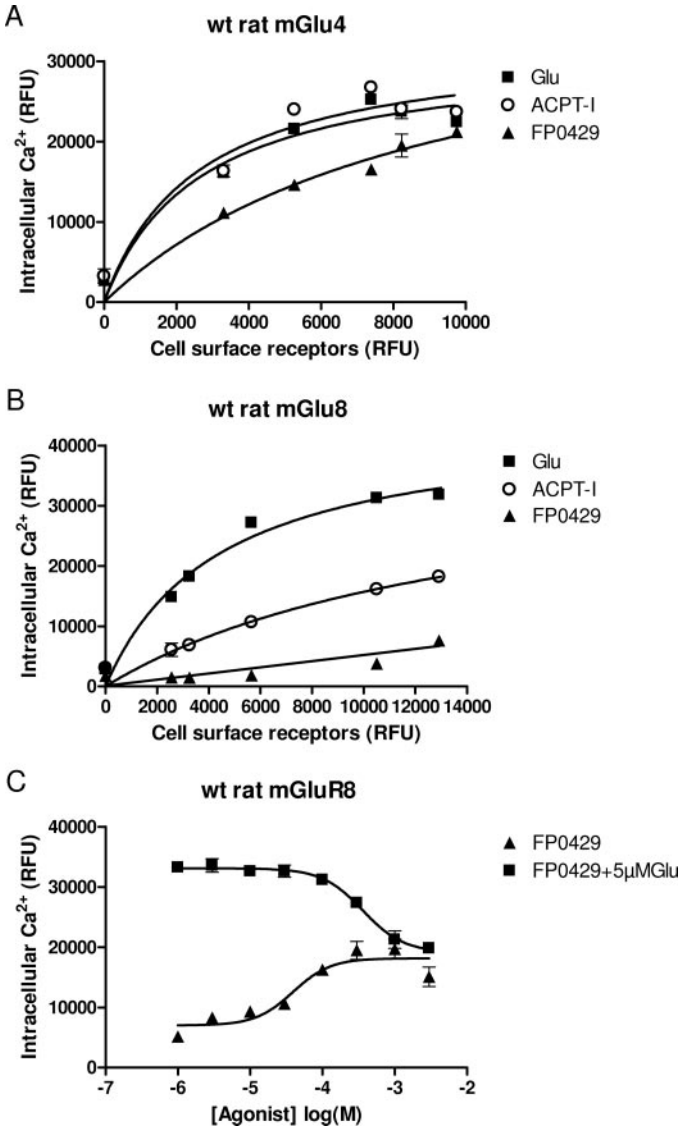

Fig. 2. FP0429 is a partial agonist of wt mGlu8 receptor. A and B, comparison of the functional responses mediated by Glu, ACPT-I, and FP0429 to assess full/partial agonist activity on subtypes mGlu4 (A) and -8 (B). Cells were transfected with increasing amounts of wt mGlu4 (A) or mGlu8 (B) plasmid DNA (encoding HA-tagged receptors) and were then assayed for intracellular Ca^{2+} mobilization with maximal doses of Glu (100 μM), ACPT-I (100 μM), or FP0429 (1 mM). For each transfection, the corresponding expression level of cell surface receptor was quantified by anti-HA ELISA. C, bivalent agonist and antagonist activity of FP0429 regarding wt mGlu8. mGlu8-transfected cells were challenged with FP0429 either alone or in combination with a submaximal Glu dose (5 μM). The K_B value was determined using the equation $K_B = IC_{50}/(1 + L/EC_{50})$, where L is the Glu concentration used in the experiment (5 μM) and EC_{50} the mean Glu EC_{50} on wt mGlu8 (1.3 μM; see Table 1). Data are from one experiment representative of at least three independent experiments performed in triplicates.

The partial agonist activity of FP0429 at mGlu8 was further characterized by testing its bivalent agonist and antagonist activity (Fig. 2C). Indeed, in the presence of a submaximal Glu dose (here, 5 μM), FP0429 behaved as an antagonist, decreasing Glu response down to its own maximum and not to basal level (as a full antagonist would have done). Using the equation $K_B = IC_{50}/(1 + L/EC_{50})$, where L is the Glu concentration in the experiment and EC_{50} is the mean experimental Glu EC_{50} at mGlu8 (see Table 1), the K_B value obtained under these experimental conditions (74.6 μM) was, as expected, close to the FP0429 EC_{50} value for mGlu8 (56.2 $\pm$ 14.6 μM; Table 1).

Potential Molecular Determinants Involved in FP0429 Full or Partial Agonism on Group III mGlu Receptors. Information on the potential molecular determinants involved in this differential agonist activity came from ACPT-I docking in wt mGlu4 ligand binding pocket 3D model (Bessis et al., 2000; Bertrand et al., 2002) (Fig. 3A). In this model, 15 amino acid residues from lobes I and II are involved in ligand binding or surrounding the ligand. Alignments of mouse, rat, and human mGlu4 and -8 sequences demonstrated the perfect conservation of 13 residues among each receptor subtype in these three mammalian species but revealed that 2 residues differed between these two receptor subtypes (Fig. 3B). Indeed, mGlu4 Ser157 and Gly158 align with two Ala residues in the three mammalian mGlu8 sequences (Ala154 and Ala155) (Fig. 3B). Moreover, a homology model of the mGlu4 binding site docked with FP0429 showed a close contact between Gly158 and the fumaryl side chain of FP0429 (see Fig. 6 and *3D Docking of FP0429 in mGlu4 Ligand Binding Site*). We therefore decided to investigate the potential role of these two residues in the differential activity of FP0429 at mGlu4 and mGlu8.

Expression and Function of the Mutated mGlu4 and mGlu8 Receptors. For each of the two residues potentially involved in FP0429 differential agonist efficacy on subtypes mGlu4 and mGlu8, we constructed mutant receptors bearing either a single (mGlu4SA, mGlu4AG, mGlu8SA, and mGlu8AG) or a double mutation [mGlu4AA ("mGlu8-like") and mGlu8SG ("mGlu4-like")]. Because all wt and mutant receptors were tagged with an HA epitope, their expression was checked using anti-HA ELISA. All mGlu4 and mGlu8 mutants were expressed at the same level as their wt counterparts at the cell surface of transfected HEK293 cells (Fig. 4, A and C, for mGlu4 and mGlu8 wt and mutant receptors, respectively). In agreement with this observation, Glu generated similar Ca^{2+} signals to those produced by wt receptors (Fig. 4, B and D for mGlu4 and mGlu8 wt and mutant receptors, respectively).

Impact of the Mutations on Receptor Responses to FP0429. The fact that the mGlu4 and mGlu8 mutant receptors responded to their natural ligand Glu as efficiently as wt receptors was confirmed by dose-response experiments (Fig. 5, A and B). Indeed, these dose dependence curves were identical for both wt and mutated receptors, regarding both signal amplitude (E_{max}) and EC_{50} (Table 1; for mGlu4, compare Figs. 5A and 1B; for mGlu8, compare Figs. 5B and 1C).

It is noteworthy that if Glu responses were not modified after introduction of the mutations, receptor challenged with FP0429 gave, as predicted, different responses. Concerning mGlu4, the SG into SA mutation had no real effect on the EC_{50} value (40.1 ± 3.8 versus 48.3 ± 5.2 μM; no statistically significant difference), but significantly affected on the E_{max} value, which was decreased 1.5-fold, indicating the importance of the Gly158 residue (Fig. 5A; Table 1). The double mutant mGlu4AA displayed not only this decreased E_{max} value but also a lower affinity for FP0429, because its EC_{50} was significantly increased 1.6 fold (79.6 ± 14.3 versus 48.3 ± 5.2 μM; $p < 0.01$; Fig. 5A; Table 1), demonstrating the importance of the Ser157 residue, although the single mutant of this specific residue (mGlu4AG) was found to behave like the wt receptor, concerning both EC_{50} and E_{max} values.

Mutant mGlu8 receptors did not differ much from wt receptor as far as FP0429 EC_{50} was concerned (Fig. 5B; Table 1). As observed for mGlu4, the real impact of the point mutations concerned the E_{max} value, which was found to be strongly increased 1.4-, 1.9-, and 2.5-fold for mGlu8SA, mGlu8AG, and mGlu8SG, respectively. The resulting E_{max} values, expressed as a percentage of the Glu maximal response, reached 52 ± 8, 70 ± 11, and 90 ± 16%, for mGlu8SA, mGlu8AG, and mGlu8SG, respectively, compared with 36 ± 7% for wt mGlu8 at the same receptor expression level (Table 1).

Further experiments were performed with the "mGlu4-like" mGlu8SG receptor; as described for Fig. 2B, a maximal dose of Glu, ACPT-I, or FP0429 was applied on cells transfected with increasing amounts of mGlu8SG plasmid DNA (Fig. 5C). It is noteworthy that both FP0429 and ACPT-I curves were shifted upward and were now almost comparable with the Glu curve. At low cell surface receptor level, the signal values, expressed as a percentage of the Glu maximal response, reached at least 60% for FP0429 and from 75 to 90% for ACPT-I compared with 6 to 10% and 37 to 41% with wt mGlu8, respectively (Fig. 2B). This demonstrates that the

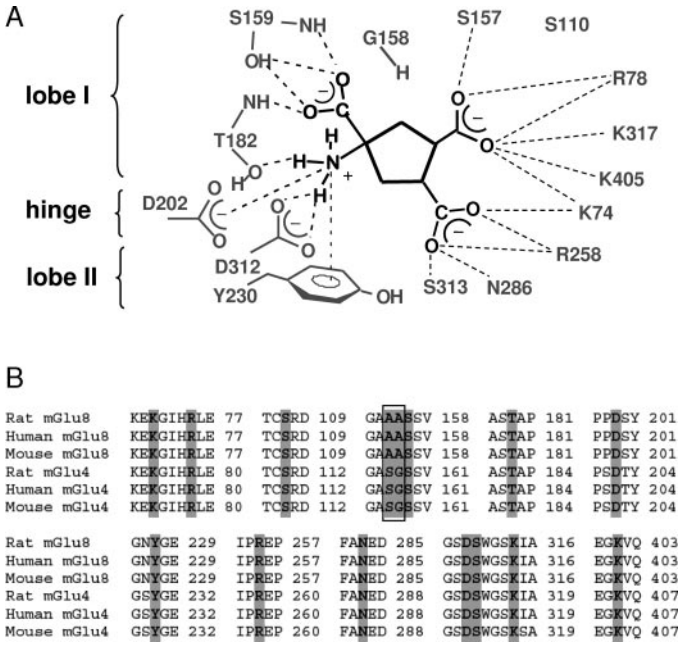

Fig. 3. Identification of the LBD residues potentially involved in FP0429 partial agonist activity at mGlu8. A, residues involved in binding of ACPT-I in mGlu4 LBD. ACPT-I was docked in the 3D homology model of wt mGlu4 (Bertrand et al., 2002), which is based on mGlu1 VFT crystal structure (PDB 1ewk; A). The resulting ionic and H-bond network (black dashed lines) is represented in a two-dimensional scheme, revealing that 15 residues are involved in direct ACPT-I binding or closely surrounding it. B, multiple sequence alignments of wt mGlu4 and mGlu8. Rat, mouse, and human sequences were aligned using the ClustalW algorithm (InfoBiogen). Of the 15 residues directly involved in ACPT-I binding or surrounding it, only two (framed) are not conserved between mGlu4 and mGlu8 and were submitted to site-directed mutagenesis.

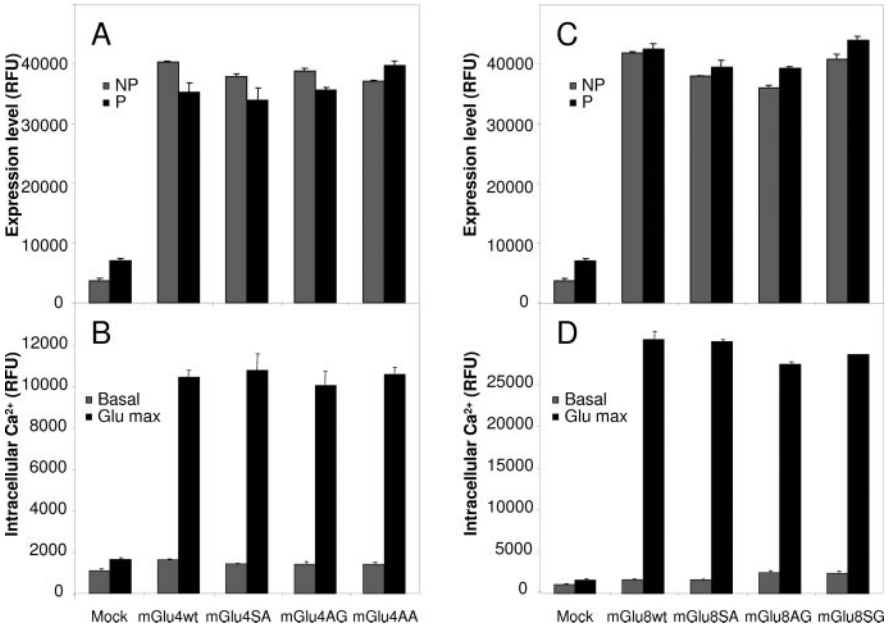

Fig. 4. Expression and function of mGlu4 and mGlu8 mutant receptors. A and C, HEK 293 cells were transiently transfected with plasmids encoding HA-tagged versions of the various mutants constructed for mGlu4 (A) (mGlu4SA, mGlu4AG, mGlu4AA) or mGlu8 (C) (mGlu8SA, mGlu8AG, mGlu8SG). Mutant receptor expression was evaluated against their wt counterparts by anti-HA ELISA, on either nonpermeabilized cells (NP) (to estimate cell surface expression) or Triton-permeabilized cells (P) (to determine total expression). B and D, cells transiently transfected with either mGlu4 (B) or mGlu8 (D) mutants were assayed for Ca^{2+} mobilization upon addition of a maximal Glu dose (100 μM) to assess mutant functionality in comparison with wt receptors. Data are from one experiment representative of at least three independent experiments performed in triplicates.

two residues AA in mGlu8 are responsible for the partial agonist activity of both ACPT-I and FP0429.

3D Docking of FP0429 in mGlu4 Ligand Binding Site. Dockings at mGlu4 binding site revealed that most of the binding features observed for ACPT-I (Bessis et al., 2000; Bertrand et al., 2002) are conserved for its close analog FP0429 (compare Figs. 3A and 6A). The α-amino acid function of both ligands binds similarly to a set of conserved proximal residues that build up a signature motif as described previously (Bertrand et al., 2002; Acher and Bertrand, 2005). The polar distal functions of ACPT-I (3- and 4-CO_2H) and of FP0429 (2-CO_2H, CO of amide, fumarate) interact with the same set of distal residues from the two lobes (except for Ser110). However, the distribution of the hydrogen bonds and ionic interactions seems somewhat different between the two ligands, which may explain the difference in potencies between ACPT-I and FP0429 at mGlu4. It is noteworthy that FP0429 is trapped in this dense network of polar bonds in a specific conformation with no possible flexibility. Thus, the β-ethylenic proton of FP0429 is brought in close vicinity (2.1 Å) of the α-proton of Gly158 (see arrow in Fig. 6A). When this Gly158 proton is replaced by the methyl group of an Ala residue, a steric hindrance would be observed if the polar network were kept as in wt mGlu4. Consequently, to avoid the steric clash with Ala155 of mGlu8, a shift of FP0429 is observed [compare Figs. 6C (mGlu4) and 6D (mGlu8); Fig. 6E] in the homology model of the mGlu8 closed conformation. This shift in the positioning of FP0429 induces a modification of the polar distal bindings (Fig. 6B) while maintaining the proximal binding as in mGlu4. In this mGlu8 model, we assume that the same extent of domain closure is reached as with mGlu4 full agonists. A close inspection of this model reveals that on one hand, binding of FP0429 to lobe I is roughly similar to that of FP0429 bound to mGlu4, and that on the other hand, some polar bindings to lobe II are missing (Asn283 and Arg255 in mGlu8) (Fig. 6, B and D). Moreover, it also shows that less interlobe interactions are present in the mGlu8 model compared with the mGlu4 model docked with FP0429 (not shown in figures). All together, the comparison of the two models attests to a weaker stability of the closed-ligand form of the mGlu8 VFT.

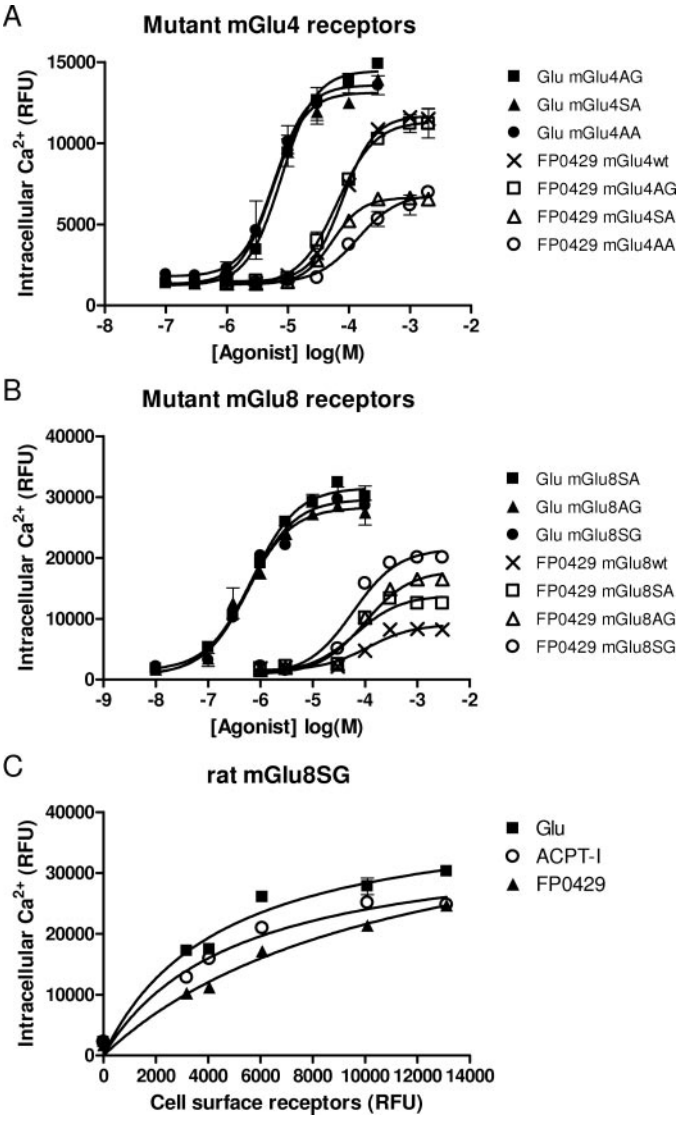

Fig. 5. Impact of the mutations on FP0429 agonist activity. A and B, dose-response curves of Glu and FP0429 on wt and mutant mGlu4 (A) and mGlu8 (B) receptors. C, comparison of the functional responses mediated by Glu, ACPT-I and FP0429 to assess full/partial agonist activity on mutant receptor mGlu8SG ("mGlu4-like"). Cells were transfected with increasing amounts of plasmid DNA encoding HA-tagged mGlu8SG receptor and were then monitored for intracellular Ca^{2+} signals upon application of Glu (100 μM), ACPT-I (100 μM), or FP0429 (1 mM). For each DNA quantity, the corresponding expression level of cell surface receptor was quantified by anti-HA ELISA. Represented data are either averaged data (A) or data from one representative experiment (B and C) of at least three independent experiments performed in triplicate and used to determine mean EC_{50} values (Table 1).

Discussion

The APTC FP0429 was recently described as a specific group III mGlu receptor agonist (Schann et al., 2006). In the present study, we demonstrate the partial agonist activity of FP0429 on mGlu8. Although it displayed a similar potency at both mGlu4 and mGlu8 receptors, FP0429 has a much lower efficacy at the latter subtype versus its related homolog mGlu4. Indeed, FP0429 efficacy at mGlu8 represents only 6 to 10% of that of the full agonist Glu at low receptor expression level. We identified the two residues of the ligand-binding pocket that differ between mGlu8 and mGlu4 and that are responsible for FP0429 differential efficacy. Finally, 3D modeling and docking experiments provided putative explanation for FP0429 partial agonism toward mGlu8 receptor.

The partial agonist activity of FP0429 at mGlu8 was suggested in our previous study by its maximal effect reaching only 36% of that of Glu at high receptor expression (Schann et al., 2006). Here, we confirmed this observation and further demonstrated the partial agonist character of FP0429 by showing that FP0429 was equally potent at activating mGlu8 and at inhibiting the submaximal effect of Glu on this receptor. However, the measured efficacy of a partial agonist depends first on its real agonist efficacy but also on the presence of receptor reserve. Thus, in the presence of a large amount of receptor reserve, a partial agonist can still reach the maximal limit of the response and therefore can behave as a full agonist. As such, to determine the real efficacy of FP0429 at both mGlu4 and mGlu8, its maximal effect was compared with that of Glu at various receptor surface expression levels. Thus, the minimal efficacy of FP0429 is 6 to 10% of that of glutamate on mGlu8, whereas it is more than 70%

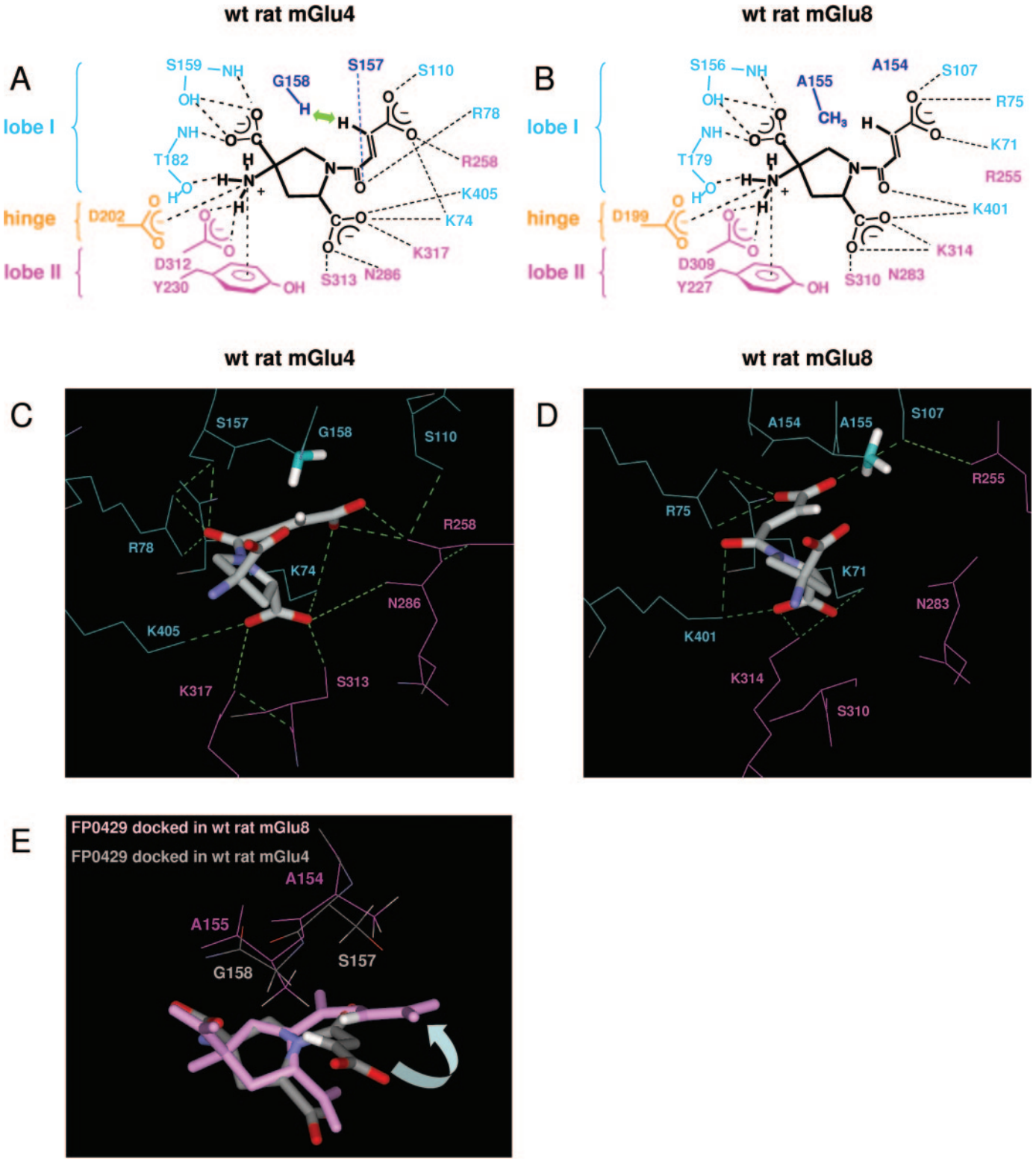

Fig. 6. FP0429 docking in 3D models of mGlu4 and mGlu8 LBD. A and B, residues involved in binding of FP0429 in mGlu4 (A) and mGlu8 (B) LBD 3D models. FP0429 was docked in the mGlu4 and mGlu8 3D homology models, which are based on mGlu1 VFT crystal structure (PDB 1ewk; A). The resulting ionic and H-bond networks (black dashed lines) are represented in a 2D scheme, revealing that the residues involved in direct ligand binding or closely surrounding it slightly vary between wt mGlu4 and mGlu8, possibly explaining the differential agonist activity regarding those two subtypes. Residues from lobe I are cyan; those of lobe II are magenta and hinge in orange. C and D, snapshots of FP0429 docked in wt mGlu4 and mGlu8 LBD 3D models. Only distal residues binding FP429 are displayed. Color code for protein residues are the same as in A and B; for FP429 atoms: carbon, gray; oxygen, red; nitrogen, blue. Polar interactions (hydrogen bonds and ionic interactions) are shown as green dashed lines. E, snapshots of FP0429 docked in wt mGlu4 (gray) or mGlu8 (pink) LBD 3D superimposed models. Residues Ser157/Gly158 (mGlu4) and Ala154/Ala155 (mGlu8) are represented with their side chains. A shift (arrow) in FP0429 positioning can be observed in the presence of the methyl groups of the two Ala residues (mGlu8). Models were superimposed based on the backbone of the residues of the signature motif.

on mGlu4. In fact, because of the strong binding site conservation between wt mGlu4 and -8, most group III ligands described so far usually present comparable activity on both subtypes. This is the case for Glu; linear Glu analogs such as L-serine-*O*-phosphate, L(+)-2-amino-4-phosphonobutyric acid, or 2-methyl-2-amino-4-phosphono-butyric acid; cyclopropyl analogs such as (2*S*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine; or phenylglycines such as (*R*,*S*)-3,5-dihydroxyphenylglycine, α-methyl-4-phosphonophenylglycine, or 4-phosphonophenylglycine (De Colle et al., 2000; Pin and Acher, 2002). Today, only a few ligands may discriminate between group III mGluRs, such as the phenylglycine (*S*)-3,4-dicarboxyphenylglycine, which is 10 to 100 times more potent at mGlu8 than at mGlu4 (Thomas et al., 2001), or the positive allosteric modulators (−)-*N*-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide, AMN082, and mGluR8-B, which are selective for mGlu4 (Maj et al., 2003), mGlu7 (Mitsukawa et al., 2005), and mGlu8 (Wilson et al., 2005), respectively. Thus, by almost fully activating mGlu4 and inhibiting submaximal effects of Glu at mGlu8, FP0429 is a good tool to discriminate between these subtypes of group-III mGluRs in the brain.

A number of recent studies examined the molecular events leading to partial agonist activity at class A GPCRs. These studies are consistent with partial agonists stabilizing a specific conformation of the receptor (Ghanouni et al., 2001; Baneres et al., 2005; Kim et al., 2006; Nikolaev et al., 2006), being able to unlock some but not all the locks that maintain the receptor in its inactive state (Seifert et al., 2001). However, the activation mechanism of class C GPCRs seems more complex, with the agonist binding site located in the VFT, within the large extracellular domain. The high sequence identity between mGlu4 and mGlu8, and the difference of FP0429 efficacy at these two receptors, offer a nice opportunity to identify the molecular determinants responsible for partial activity at class C GPCRs.

We found that two residues that differ between mGlu4 and mGlu8 binding sites are responsible for the differential efficacy of FP0429 at these two receptors. Indeed, replacement of Ser157 and Gly158 of mGlu4 by the mGlu8 residues (Ala in both cases) converted FP0429 into a partial agonist. Conversely, replacement of Ala154 and Ala155 by Ser and Gly in mGlu8 largely increased FP0429 efficacy. Single point mutations also revealed a major role of the Gly158 (mGlu4) and Ala155 (mGlu8), in this phenomenon. It is noteworthy that all mutants of both mGlu4 and mGlu8 responded to glutamate stimulation exactly like their wt counterparts. This was in agreement with the effect of linear glutamate analogs on Ser157 or Gly158 mutated mGlu4 or mGlu8 receptors (Hampson et al., 1999; Rosemond et al., 2002). Thus, even with the introduction of more "bulky" residues (mutation of Gly158 into Ala) in the LBD, the flexibility of Glu (or other linear ligands), which can adopt many conformations, and the potential presence in the pocket of "buffering" water molecules, could explain the absence of impact of the different mutations on Glu-induced functional responses.

To further examine the role of the identified residues in the agonist efficacy of FP0429, docking experiments were conducted in closed form 3D models of mGlu4 and mGlu8 VFTs. FP0429 partial agonist activity on mGlu8 seems to involve residues located away from the hinge and interacting with the distal functions of the ligand, or involved in interlobe contacts. On the mGlu4 model depicted in Fig. 6A, the H atom from Gly158 is in close vicinity (2.1 Å) of the ethylenic H atom from FP0429. We hypothesized that the presence of a bulkier residue in mGlu8 (Ala versus Gly) could induce a steric hindrance, forcing FP0429 to position itself differently at lobe I, creating a shift in the molecule position in mGlu8 versus mGlu4. This results in a different orientation of the side chain of FP0429, and a different position of the side chain of several residues both from lobes I and II. As a consequence, fewer interdomain interactions could be formed, likely resulting in a weaker stabilization of the closed state of the mGlu8 VFT, explaining the low agonist efficacy of this compound at mGlu8. Although AMPA and NMDA receptors are ligand-gated channels, their agonist binding site also consists of a VFT domain. It is noteworthy that the 3D crystal structure of the VFT domain of the NMDA receptor NR1 subunit has been solved with either the full agonist glycine or various partial agonists with increasing size (Inanobe et al., 2005). In all cases, a fully closed state was observed. As proposed here for FP0429 at mGlu8, the partial agonist activity at the NR1 subunit was therefore explained by a lower stabilization of the closed state of the VFT, resulting in that case from an incomplete change in conformation of the second VFT interdomain (Inanobe et al., 2005).

However, our docking experiments were performed in a model of the closed conformation of the mGlu8 VFT. Indeed, only a fully closed and a widely open conformation of the mGlu1 VFT are available as templates, thus it was not possible to dock FP0429 in a partially closed state of the mGlu8 VFT. As such, we cannot exclude the possibility that a different interacting mode of FP0429 in the lobe I of the open form of mGlu8 may generate a hindrance preventing the full closure of the VFT. Such a partially closed state may then be responsible for the low agonist efficacy. This second proposal is consistent with structural data obtained with the GluR2 subunit, because kainate, a partial AMPA receptor agonist, stabilizes the GluR2 VFT in a partially closed form (Armstrong et al., 2003), and the extent of domain closure is related to the agonist efficacy (Jin et al., 2003). Whether this is also the case for mGlu receptor remains to be further studied.

To conclude on the present work, using a newly described group III mGlu agonist, and combined approaches of both site-directed mutagenesis and 3D modeling, we propose a dual mechanism for mGlu receptor partial agonism. The two hypotheses that are envisaged are not mutually exclusive, and consist, upon partial agonist binding, of either a partially closed form of the VFT domain or a less stable fully closed conformation of this domain.

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